



Characterization of recombinant human kidney diamine oxidase and equine plasma amine oxidase  
by Bradley Owen Elmore

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of  
Philosophy in Biochemistry  
Montana State University  
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Abstract:

Human kidney diamine oxidase has been overexpressed as a secreted protein in *Drosophila* S2 cell culture. This represents the first heterologous overexpression and purification of a catalytically active, recombinant, mammalian copper-containing amine oxidase. To date, the direct examination of mammalian copper-containing amine oxidases has been difficult and limited, especially true for the human enzyme. The availability of large quantities of highly purified enzyme makes it now possible to investigate the spectroscopic, mechanistic, functional and structural properties of this human enzyme at the molecular level. Visible absorption, circular dichroism, electron paramagnetic resonance and resonance Raman spectroscopic results are presented. The recombinant enzyme contains the cofactors 2,4,5-trihydroxyphenylalanine quinone (TPQ) and copper at stoichiometries around 1.1 and 1.5 mol per mol homodimer, respectively. In addition, tightly bound and stoichiometric calcium ions were identified and are proposed to occupy the second metal binding site. Detailed kinetic studies indicate the preferred substrates are, in order, histamine, 1-methylhistamine, agmatine and putrescine. Inhibition by pharmaceutical compounds has been examined, and most notably, pentamidine has been demonstrated to be a competitive inhibitor with an inhibition constant in low nanomolar range. Azide is shown to be a competitive inhibitor against both substrate amine and dioxygen. Equine plasma copper-containing amine oxidase has also been purified from the natural source and characterized.

CHARACTERIZATION OF RECOMBINANT HUMAN KIDNEY  
DIAMINE OXIDASE AND EQUINE PLASMA AMINE OXIDASE

by

Bradley Owen Elmore

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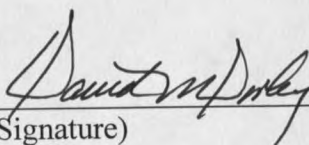
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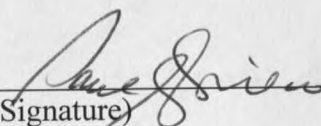
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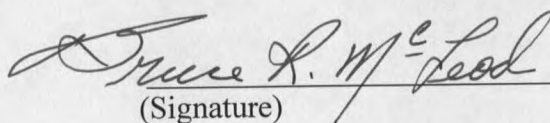
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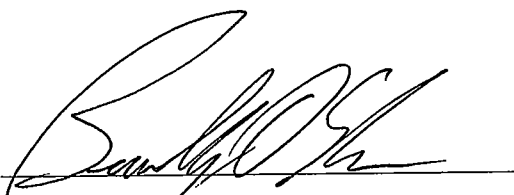
  
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This thesis is dedicated to Nathan and Joshua Elmore,  
who shared in my sacrifice and offered unconditional support, faith and love.

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## ABSTRACT

Human kidney diamine oxidase has been overexpressed as a secreted protein in *Drosophila* S2 cell culture. This represents the first heterologous overexpression and purification of a catalytically active, recombinant, mammalian copper-containing amine oxidase. To date, the direct examination of mammalian copper-containing amine oxidases has been difficult and limited, especially true for the human enzyme. The availability of large quantities of highly purified enzyme makes it now possible to investigate the spectroscopic, mechanistic, functional and structural properties of this human enzyme at the molecular level. Visible absorption, circular dichroism, electron paramagnetic resonance and resonance Raman spectroscopic results are presented. The recombinant enzyme contains the cofactors 2,4,5-trihydroxyphenylalanine quinone (TPQ) and copper at stoichiometries around 1.1 and 1.5 mol per mol homodimer, respectively. In addition, tightly bound and stoichiometric calcium ions were identified and are proposed to occupy the second metal binding site. Detailed kinetic studies indicate the preferred substrates are, in order, histamine, 1-methylhistamine, agmatine and putrescine. Inhibition by pharmaceutical compounds has been examined, and most notably, pentamidine has been demonstrated to be a competitive inhibitor with an inhibition constant in low nanomolar range. Azide is shown to be a competitive inhibitor against both substrate amine and dioxygen. Equine plasma copper-containing amine oxidase has also been purified from the natural source and characterized.

## INTRODUCTION

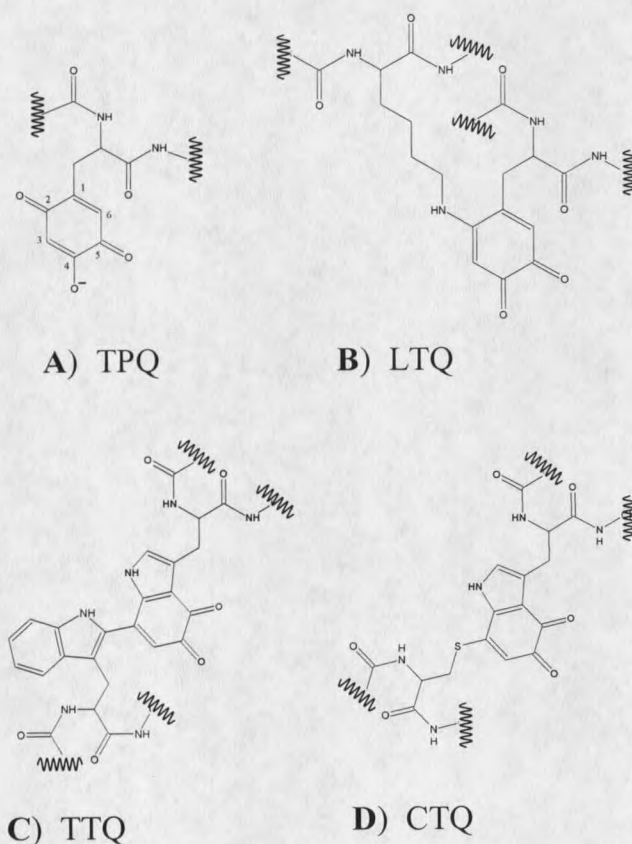
### Post-Translationally Modified Amino Acid Cofactors

Biological systems have developed the means to substantially extend the chemical properties and thus functionality of the twenty common amino acids that make up proteins via chemical modification. A special case is the post-translationally modified amino acids that serve as cofactors in enzyme catalysis and thus supplement the "normal" complement of enzyme cofactors and prosthetic groups, such as NAD(H), flavins, pyridoxal phosphate, Fe-heme groups, metal ions, etc.

This recently discovered group of enzymes requires the post-translational modification of an intrinsic, encoded amino acid residue for catalytic activity. The number of these modified cofactors, currently 19, will likely soon outnumber the naturally occurring amino acids used in protein biosynthesis. This group of enzymes has representatives in every branch of life and includes enzymes of fundamental importance to life on earth. For example, cytochrome c oxidase, the terminal oxidase of the electron transport chain, contains a tyrosine cross-linked to a histidine ligand of Cu<sub>B</sub>. Ribulose-1,5-bisphosphate carboxylase (rubisco), responsible for the first step of photosynthetic CO<sub>2</sub> fixation, contains a carbamylated lysine residue that ligates a magnesium ion. A recent review is by Okeley and Van der Donk [1].

A sub-group of post-translationally modified amino acid cofactors is comprised of those that contain a quinone cofactor (Figure 1). TPQ, 2,4-trihydroxyphenylalanine quinone, is the organic tyrosine derived cofactor in the copper containing amine oxidases

(EC 1.4.3.6). LTQ, lysyl tyrosylquinone, is found in the copper containing lysyl oxidases (EC 1.4.3.13), which oxidize peptidyl lysine residues for cross-linking of elastin and collagen in connective tissue. Tryptophan tyrtrophylquinone (TTQ), an oxidized cross-link between two tryptophans is present in bacterial methylamine dehydrogenase (EC 1.4.99.3). CTQ, cysteine tryptophyl quinone, is a very recent addition to this group and is formed from the oxidation of a cross-linked cysteine and tryptophan in the bacterial quinohemoprotein amine dehydrogenase.



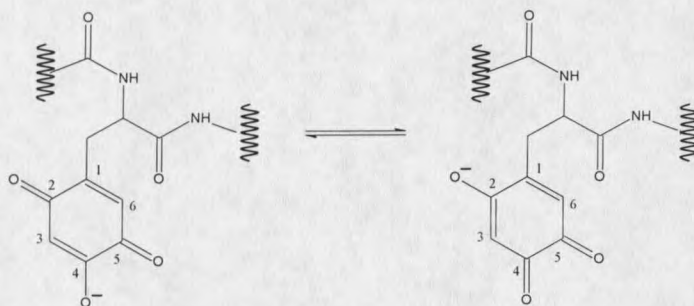
**Figure 1.** Post-translationally modified quinone cofactors; **A)** 2,4-trihydroxyphenylalanine quinone, TPQ; **B)** lysyl tyrosylquinone, LTQ; **C)** tryptophan tyrtrophylquinone, TTQ; **D)** cysteine tryptophyl quinone, CTQ

### Copper Containing Amine Oxidases

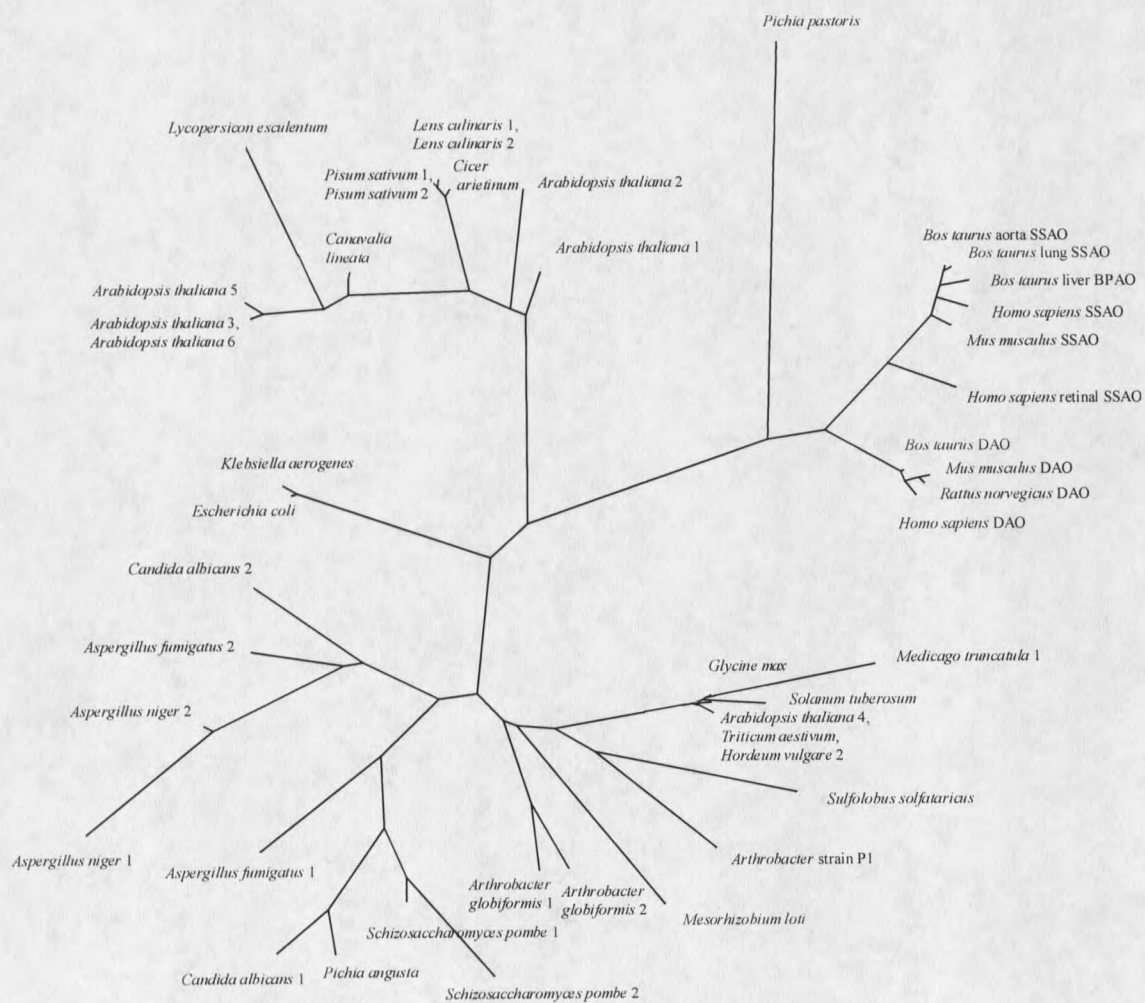
The TPQ containing enzymes, copper containing amine oxidases (CAOs) are the focus of this thesis. There is another distinct class of enzymes referred to as amine oxidase, monoamine oxidase, tyramine oxidase, adrenalin oxidase, or polyamine oxidase. This latter group, EC 1.4.3.4, contains a flavin cofactor and will not be discussed herein.

CAO's are homodimers, generally ranging in size from 140 to 200 kDa, with one active site found in each monomer [2]. Each active site contains two cofactors: 1) a single Type II copper ion, and 2) TPQ. The quinone cofactor is derived from the post-translational modification of an invariant tyrosine residue [3]. TPQ has been shown to be produced in a novel, self-processing reaction requiring only copper and dioxygen [4-7]. CAOs are unique in that they are homodimeric, contain one organic and one inorganic cofactor in each of two active sites, and the organic cofactor is a quinone from the autocatalytic oxidation of an intrinsic tyrosine.

TPQ containing enzymes are pink in color, with a broad absorption feature around 480 nm attributed to the quinone cofactor. In the active enzyme, TPQ is an oxoanion [8]. Resonance Raman spectra indicate the charge is delocalized over the C2 and C4 oxygens (Figure 2) [9]. Differences in the TPQ  $\lambda_{\max}$  values among CAOs from different sources are thought to reflect the degree of charge localization [10].



**Figure 2.** TPQ resonance structures



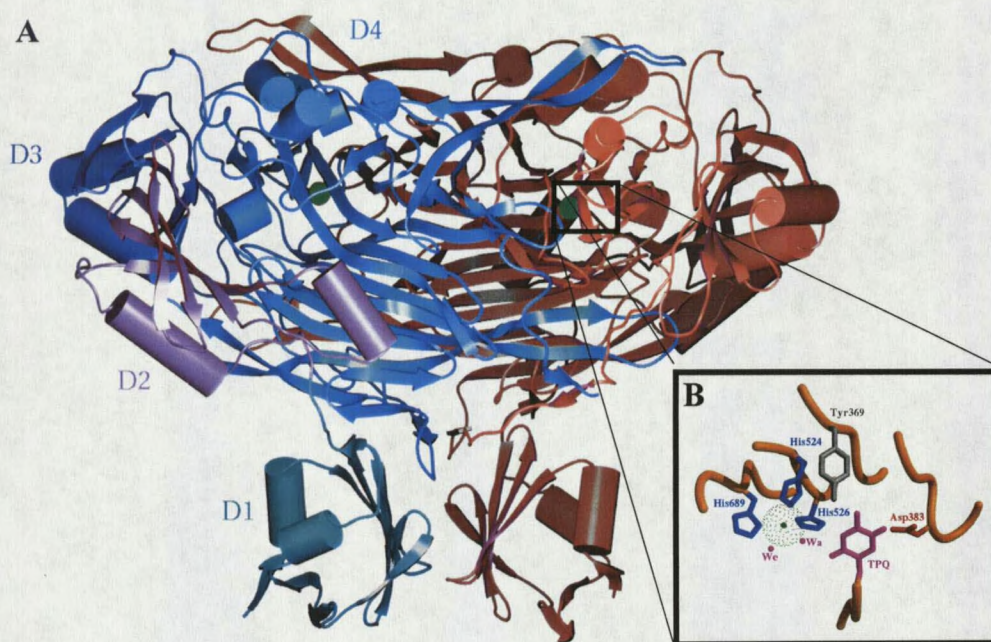
**Figure 3.** Phylogenetic tree for the copper amine oxidases

CAOs are widespread in nature, having been described from gram-positive and gram-negative bacteria, yeast and fungi, plants and animals [2]. Recently the gene encoding a copper amine oxidase has been sequenced from the archaea *Solfolobus solfataricus*. Figure 3 shows a phylogenetic tree constructed for the family of copper containing amine oxidases, see Appendix B for details. An alignment of the amino acid sequences for all CAOs sequenced to date is given in Appendix A.

### Structure

The structures of four copper amine oxidases have been solved by x-ray crystallography: two are bacterial (*Escherichia coli* and *Arthrobacter globiformis*), one is from the yeast *Hansenula polymorpha* (formally classified as *Pichia angusta*), and one is from pea seedling (*Pisum sativum*) [11-14]. Collectively these four enzymes exhibit considerable structural homology, although primary sequence identity is less than 40% between any two. The enzymes are “mushroom” shaped with an extensive intersubunit contact, including a pair of “arms” extending from each subunit to embrace the other. Figure 4 shows the ribbon structure of the *Escherichia coli* copper amine oxidase (ECAO). Each monomer is comprised of four domains. The N-terminal domain (D1) forms the “stalk” portion of the “mushroom” and is not present in all copper amine oxidases. A large (440-amino acid)  $\beta$ -sandwich C-terminal domain contains the active site and forms the majority of the dimer interface. Domains D2 and D3 have remarkably similar folds, an  $\alpha$ -helix and a four-stranded antiparallel  $\beta$ -sheet. A channel for substrate access and product exit lies between domains D3 and D4.

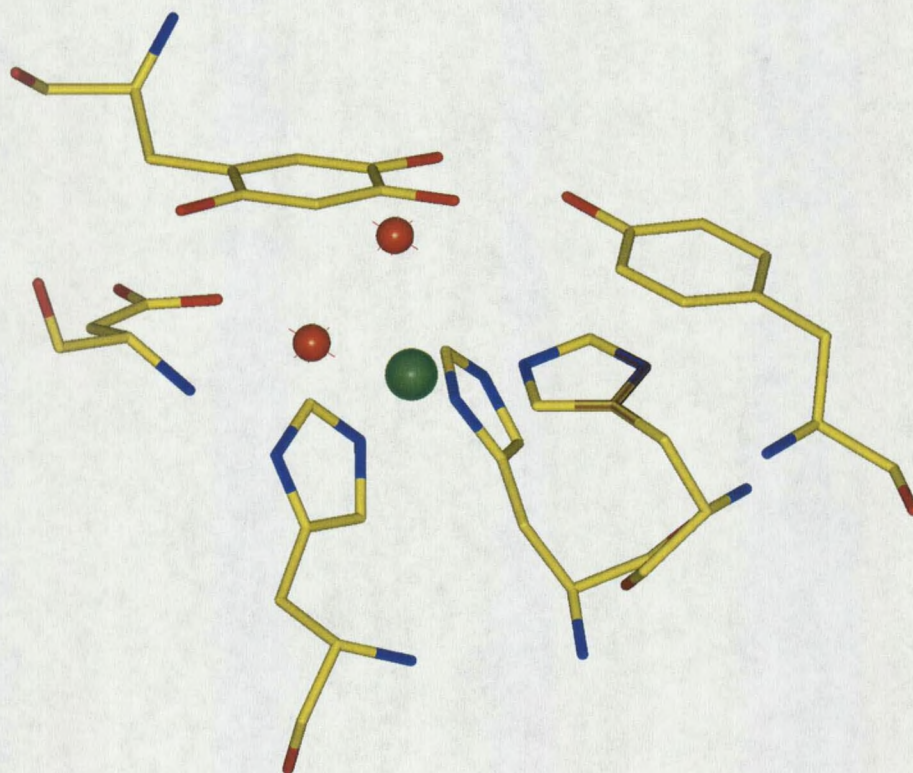




**Figure 4.** Structure of the ECAO homodimer. One monomer is red, and the other monomer has individual domains colored different shades of blue and indicated by D1-D4. Copper ions are shown as green spheres. **B**, Schematic of ECAO active site, showing the copper ion and TPQ cofactors. The water axial and equatorial ligands to the copper ion are labeled Wa and We, respectively. (from Wilmot, C.M., et al. *Science* **1999**, 286, 1724)

The crystallographically determined structures confirm the copper coordination environment as predicted from spectroscopy [15-17]. The active-site copper ion is coordinated in a distorted square pyramidal geometry by three conserved histidine residues and two water molecules, one axial and the other equatorial. Also, the quinone cofactor is in close proximity to the copper ion and can be observed in different conformations, indicative of inherent side-chain flexibility. Figure 5 shows the view of important features from active site of the pea seedling CAO (PSAO).

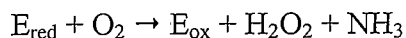




**Figure 5.** The active site of PSAO. The copper ion is represented by a green sphere, water molecules by red spheres. The three histidine and the axial water ligands to copper are shown. TPQ is at the top of the figure, and an invariant tyrosine residue that hydrogen bonds to TPQ is to the right. On the left side is an invariant aspartate that serves as a base during catalysis.

### Mechanism

The copper-containing amine oxidases catalyze the two-electron oxidative deamination of primary amines to the corresponding aldehyde, using dioxygen as the oxidant, with the concomitant production of ammonia and hydrogen peroxide. Catalysis proceeds through a Bi Ter Ping Pong mechanism divided into two half-reactions:

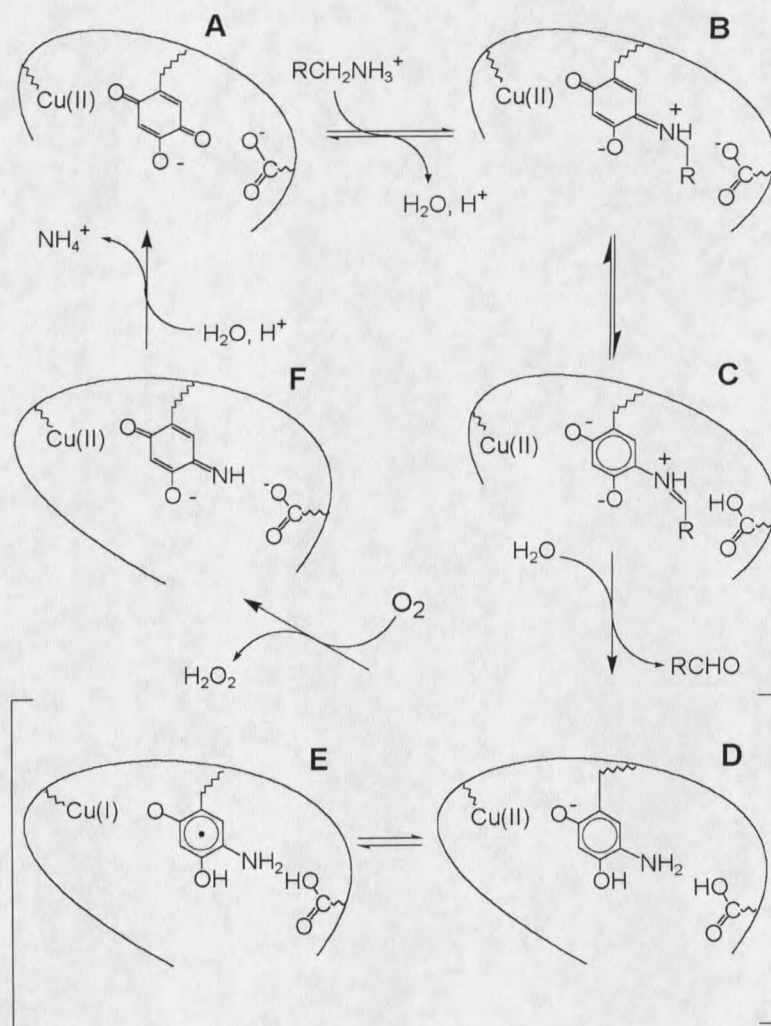


The first half reaction is conventionally termed the “reductive half reaction”.

Substrate amine is oxidized to an aldehyde and the TPQ is reduced by two electrons. The “oxidative half reaction” uses molecular oxygen to regenerate the oxidized form of TPQ. Recent reviews are found in references [10] and [18].

Figure 6 diagrams the enzyme active site and steps in the catalytic cycle. Detailed kinetic studies have elucidated many of the details of the reductive half reaction. Substrate combines with the oxidized, resting form of the enzyme (A, Figure 6) to form a covalent substrate Schiff base adduct at C-5 of TPQ (B, Figure 6). Proton abstraction from the  $\alpha$  carbon of substrate, using a conserved active site aspartate residue, yields a product Schiff base adduct and reduced TPQ (C, Figure 6). Proton abstraction has been shown to at least partially contribute to the rate-determining step in a few mammalian enzymes. Product aldehyde is subsequently released by hydrolysis, leaving the aminoquinol form of TPQ.





**Figure 6.** Proposed reaction mechanism for the oxidation of amines by copper-containing amine oxidases.

The oxidized, resting enzyme (**A**) combines with amine substrate to give a substrate Schiff base (**B**). Proton abstraction by the conserved active-site aspartate from the substrate's  $\alpha$ -carbon results in a product Schiff base and reduced TPQ (**C**). Product aldehyde is released by hydrolysis, leaving the aminoquinol form of the enzyme (**D**). This Cu(II)-aminoquinol is in equilibrium with a Cu(I)-aminoquinone radical form (**E**). The enzyme is then oxidized, with dioxygen serving as the electron acceptor. Oxidation proceeds through a postulated aminoquinone intermediate (**F**), liberates ammonium and regenerating the resting enzyme (**A**).

The oxidative half reaction is less well understood. The substrate-reduced enzyme has been shown to be an equilibrium mixture of the Cu(II)-aminoquinol and Cu(I)-aminosemiquinone radical forms (D, E in Figure 6) under anaerobic conditions, and the Cu(I)-aminosemiquinone species would be expected to readily react with dioxygen [19]. An obligatory mechanistic role for Cu(I) has not been established, and a viable alternative utilizing only the Cu(II) oxidation state has recently been proposed [20]. Dioxygen is reduced to peroxide to give a postulated iminoquinone intermediate (F in Figure 6), and hydrolysis regenerates the oxidized quinone.

The mechanism of dioxygen reduction remains a central question; how does the enzyme activate dioxygen for the two-electron reduction to hydrogen peroxide? The anaerobic substrate-reduced enzyme has been shown by EPR spectroscopy to be an equilibrium mixture of Cu(II)-aminoquinol and Cu(I)-semiquinone radical forms, where Cu(I) would be anticipated to reduce dioxygen [19]. The formation of the Cu(I)-semiquinone has been shown to be kinetically competent in bacterial and plant enzymes [21]. Furthermore, considerable chemical precedence exists for the binding and activation of oxygen by copper(I) ions [22-25].

Recent solvent isotope effect, O-18 isotope effect, and solvent viscosity effect work using bovine serum amine oxidase indicates that the first-electron transfer to dioxygen, and not oxygen binding, is the rate-determining step for the oxidative half-reaction [20]. Because Su and Klinman expected a Cu(I) reduction of dioxygen to be relatively fast, they proposed that dioxygen is prebound, and the rate-limiting, first-electron transfer (to produce superoxide) occurs at a site other than the Cu(I) center. A

hydrophobic pocket in the *Hansenula polymorpha* CAO active site was identified and suggested to be the site of dioxygen binding and initial reduction. The second-electron transfer, resulting in the reduction of superoxide to peroxide, could either occur in the hydrophobic pocket or after superoxide binds to Cu(II). More recent work of Mills and Klinman using the cobalt-substituted *H. polymorpha* enzyme lends support to a nonredox role for copper in TPQ reoxidation [26].

### Mammalian Copper Amine Oxidases

Three general classes of CAOs have been described from mammalian sources; unfortunately, the nomenclature in the literature is frequently confusing. One type of amine oxidase is found tightly associated with tissues, is active against monoamine substrates, and is commonly designated semicarbazide-sensitive amine oxidase (SSAO). It must be noted that all CAOs are inhibited by semicarbazide, so the designation of the tissue-associated amine oxidases as SSAOs is simply a convention. Sequence analysis has recently revealed these tissue-bound enzymes possess a single putative N-terminal transmembrane helix [27]. Another variety of CAO is soluble, found in blood plasma, and is active against a wide range of monoamines, diamines, and aromatic amines. These enzymes are generally termed plasma amine oxidases, serum amine oxidases, or benzylamine oxidases, and are thought to be synthesized in the liver [28]. The third type of mammalian CAO is also soluble, but displays distinct substrate specificity for diamines. This group is therefore termed diamine oxidases (DAOs). These are also distinct in sequence homology from the soluble plasma and the membrane-bound CAOs.

[29,30]. The amino acid alignment for available mammalian copper amine oxidases is given in Appendix A.

Barbry and coworkers identified the first complete nucleotide sequence for a mammalian copper-containing diamine oxidase, that of the human kidney (previously misidentified as a protein associated with the amiloride-sensitive  $\text{Na}^+$  channel) [31]. The translated cDNA sequence encodes a 751 amino acid polypeptide with a predicted 19 amino acid signal sequence for the classical secretory pathway. N-terminal sequencing of human DAO purified from kidney and placenta demonstrated the mature protein lacked these residues, confirming the predicted signal cleavage site. The amino acid sequence contains the copper amine oxidase consensus sequence, T/SXXNYD/EY/N (residues 457-463), in which the first tyrosine residue is modified to TPQ in the mature enzyme. A heparin-binding consensus sequence, RFKRRLPK, was also recognized (residues 569 - 576).

One unanticipated finding in the crystal structures of the *E. coli* and pea seedling CAOs was the identification of an additional metal-binding site in each subunit, modeled as being occupied by a calcium and a manganese ion, respectively. Three aspartate carboxylates, two peptide carbonyl oxygens and one water molecule coordinate this second metal ion. These aspartate ligands are absolutely conserved in all ten sequenced mammalian CAOs, and in more than 50% of all available sequences representing bacteria, fungi, plants and animals. The second-metal site and the inferred location of the heparin binding sequence are both on the solvent exposed upper surface of the "mushroom cap" [14].

### Research Goals

Although considerable details for the copper-containing amine oxidases structure and mechanism have been elucidated, many questions remain, particularly with respect to the mammalian enzymes. One major goal of this research project is to obtain large quantities of highly purified mammalian copper-containing amine oxidases that are amenable to detailed and comprehensive spectroscopic, kinetic, biochemical and structural studies. This work describes the development of a heterologous overexpression system for the recombinant human kidney diamine oxidase (rhKDAO). In addition highly purified equine plasma amine oxidase has been obtained from the natural source.

One outstanding and fundamental aspect of the mammalian copper-containing amine oxidases is that the physiological functions have not yet been unambiguously defined. Examination of the substrate specificities and catalytic kinetics for the recombinant enzyme, using biogenic and exogenous amines, has provided important insight that can be used to infer *in vivo* details. The biogenic amines histamine, 1-methylhistamine, agmatine and putrescine have proven to be excellent substrate for the recombinant enzyme. Investigations into rhKDAO interactions with pharmaceutical compounds has given some striking results that may facilitate the rational design of highly specific diamine oxidase inhibitor and potentially drugs with reduced side effects and toxicity. Furthermore, the overexpression of the recombinant enzyme will facilitate experiments designed to investigate the molecular details of copper amine oxidase



activity. One such experiment, the interaction of the small anion azide, is given here and provides exciting data specific for the controversial oxidative half reaction.

The determination of a mammalian crystal structure from a copper-containing amine oxidase would represent a major breakthrough. Attempts to obtain good quality crystals were performed using the equine plasma amine oxidase. Additional crystallization trials with both of the mammalian enzymes utilized in this work remain a high priority. While a crystal structure is not imminent, when available a structure would provide structural details that are key to understanding features such as the control of substrate and inhibitor recognition and insight into the chemical mechanism.

## EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT HUMAN KIDNEY DIAMINE OXIDASE

### Introduction

The detailed characterization and direct examination of mammalian copper amine oxidases has been greatly hindered by the difficulty in obtaining the quantities of highly purified enzyme required. Purifications from natural sources are long and tedious and give highly variable purities and activities. Many researchers had no choice but to use crude preparations, sometimes tissue homogenates. Prior to the results given herein, there has been no reliable overexpression system for a copper amine oxidase from any multicellular organism.

To further the extent and depth of knowledge about diamine oxidase, an enzyme implicated in fundamental and critically important biological processes, it is vital to have a readily-available source of homogenous protein. Our approach has been to heterologously overexpress the human enzyme using a eukaryotic host, *Drosophila* S2 insect cell culture. This expression system was chosen for its relative ease in culturing, potential for high expression levels, and apparent lack of an endogenous copper-containing amine oxidase. We report herein the heterologous overexpression and purification of human kidney diamine oxidase, the first successful overexpression of any mammalian copper-containing amine oxidase. Furthermore, high levels of expression and a developed purification protocol give the large quantities of highly-purified enzyme required for detailed investigation of molecular properties.

In this chapter, the overexpression system and purification protocol are described. Initial characterization of the recombinant enzyme includes molecular weight determination, cofactor quantification, and measurement of its visible absorption, circular dichroism, electron paramagnetic resonance, and resonance Raman spectra. The nature and quantification of the organic TPQ cofactor and metal analysis are discussed along with elementary biochemical characterization of the recombinant enzyme. Additionally, human tissue-specific expression data is presented.

### Experimental Procedures

#### Construction of cell line

The initial molecular biology, *Drosophila* S2 transfection and expression were performed by Dr. John Bollinger (MSU).

The coding sequence for mature human kidney diamine oxidase was amplified by PCR from a cDNA clone (kindly provided by Dr. Barbry, Institut de Pharmacologie Moléculaire et Cellulaire, France) using Vent DNA Polymerase and appropriate primer adapters. The forward primer (5'-GTGAGATCTCCGGGGACTCTGCCC) replaced the N-terminal codons for glutamic acid and proline of mature kidney diamine oxidase with the codon for arginine and introduced a *Bgl*III site. The reverse primer (5'-CCGGAATTCACGATGCCGGCCCTGGGCTGGGCC) introduced an *Eco*RI site just downstream from the native stop codon. PCR product and the expression vector pMT/BiP/V5-His A (Invitrogen) were digested with *Bgl*III and *Eco*RI, agarose gel

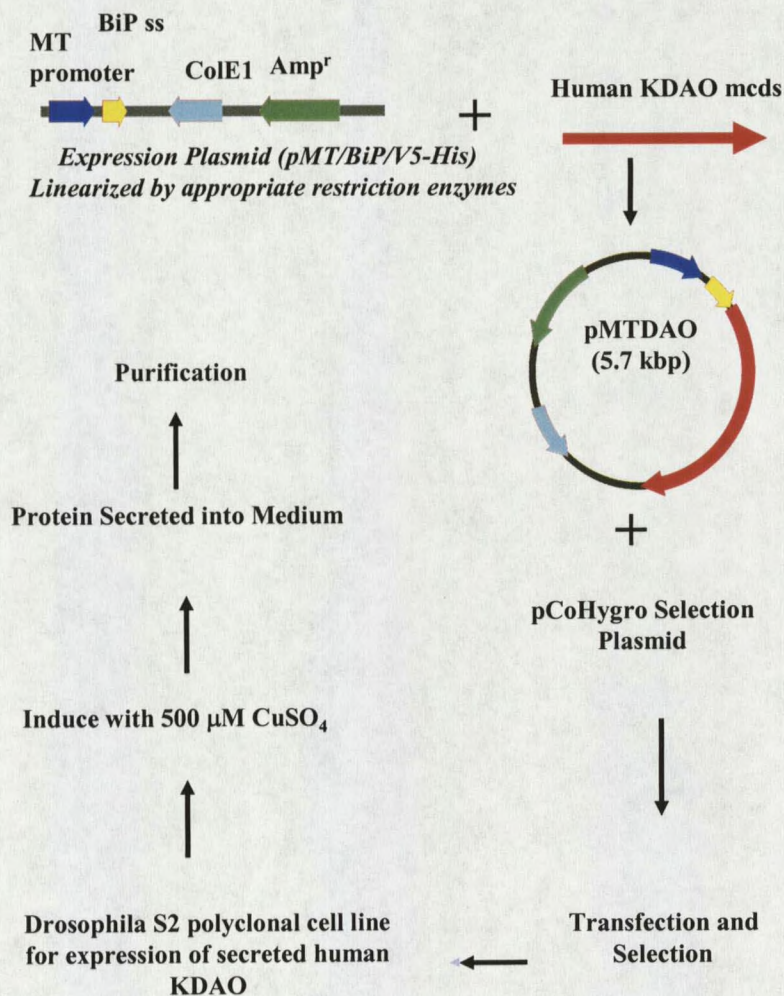
purified, recovered using Prep-A-Gene (Bio-Rad) and ligated with T4 DNA ligase. The 5' end of the resulting construct was confirmed by DNA sequencing (Silver Sequence, Promega) through the fusion site to an internal *Bst*II site, 350 base pairs into the coding sequence. The remainder of the coding sequence was swapped with a *Bst*II and *Eco*RI fragment from the cDNA clone to generate the expression vector pMTDAO. All DNA manipulations used enzymes from New England Biolabs. Constructs were maintained in *E. coli* strain TOP10 and purified using either Perfect Prep (5Prime3Prime) or Quantum Prep (Bio-Rad).

Transfection and cell culturing procedures were those outlined in the *Drosophila* Expression System Manual (Invitrogen), except as noted. Plasmids pMTDAO and the selection vector pCoHYGRO were cotransfected into *Drosophila* Schneider 2 (S2) cells at a ratio of 19:1 ( $\mu$ g) using the Calcium Phosphate Transfection Kit (Invitrogen). Selection for the stably transfected subpopulation used 500  $\mu$ g/mL hygromycin B (Roche Molecular Biochemicals). The resulting polyclonal cell line was adapted to and maintained in a serum-free medium (Ex-Cell 400, JRH Biosciences) supplemented with 300  $\mu$ g/mL hygromycin B at 27 °C.

### Expression

The transfected cell line was expanded from a 5 mL culture in a 25 cm<sup>2</sup> tissue culture flask to a single 130 mL culture in a 250 mL spinner flask. When the spinner culture reached a density of  $1 \times 10^7$  cells/mL, 25 mL of the culture was added to each of four 500 mL baffled shake flasks (Bellco Glass) containing 150 mL serum-free medium.

Flasks were incubated in a gyrotary water bath at 110 rpm and 27 °C for about 24 hours. At a density of  $5 \times 10^6$  cells/mL, expression was induced by addition of copper sulfate to a final concentration of 500  $\mu$ M, and incubation was then continued for 48 hours. Nontransfected *Drosophila* S2 cells and the uninduced expression cell line were used as negative controls.



**Figure 7:** Schematic of the expression of hKDAO in *Drosophila* S2 cells

### Purification

Cultures were harvested and cells spun out by centrifugation for 2 minutes at  $1000 \times g$ . The supernatant was spun for 10 minutes at  $10000 \times g$  to remove particulates and then loaded on a 5mL HiTrap Heparin HP column (Amersham Pharmacia Biotech) using a peristaltic pump. The column was then washed with 100 mM potassium phosphate buffer, pH 7.2, until  $A_{280}$  of the flow through reached zero, and bound protein was eluted with 100 mM potassium phosphate with 1 M sodium chloride, pH 7.2. The eluant was extensively dialyzed against 100 mM potassium phosphate, pH 7.2 and then loaded on Macro-Prep Ceramic Hydroxyapatite (Type I, 40  $\mu$ m particle size, Bio-Rad) in a HR 10/10 column using a FPLC system (Amersham Pharmacia Biotech). Buffers for the ceramic hydroxyapatite column were 100 mM potassium phosphate, pH 7.2 (Buffer A) and 400 mM potassium phosphate, pH 7.2 (Buffer B). Protein fractions were eluted with a two column volume wash at 25% Buffer B, a single column volume wash at 35% Buffer B, and a two column volume linear gradient from 35% to 100% Buffer B. The most active fractions were pooled and concentrated in a 50 mL centrifugal concentrator (Millipore) before being run over a 1.6  $\times$  100 cm Ultrogel AcA 34 (BioSeptra) gel filtration column equilibrated in 100 mM potassium phosphate, pH 7.2. SDS/PAGE and IEF gels were by a PhastSystem (Pharmacia). Substantial absorbance at 280 nm in the culture media necessitated that initial protein concentration be determined by the Bradford protein assay (Bio-Rad) with bovine serum albumin standards. Subsequent protein concentrations were determined spectrophotometrically by absorbance at 280 nm using the predicted extinction coefficient for the mature, recombinant enzyme of 280.5



$\text{mM}^{-1}\text{cm}^{-1}$ . [32] The extinction coefficient at 280 nm was later determined by magnetic circular dichroism and calculated as  $297.6 \text{ mM}^{-1}\text{cm}^{-1}$  (data not shown) [33].

### General Characterization

Amine oxidase activity was measured at 37 °C in a stirred, thermostatted cuvette using a coupled assay with putrescine (dihydrochloride, Sigma) as the substrate. Standard activity assays used 30 U horseradish peroxidase (Sigma), 10 mM putrescine, and 2 mM ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) in 100 mM potassium phosphate, pH 7.2, in a final volume of 2 mL. Reaction progress was followed spectrophotometrically by monitoring the change in absorbance at 414 nm ( $\epsilon = 24.6 \text{ mM}^{-1}\text{cm}^{-1}$ ) [34]. Activities measured by dioxygen depletion using an Instech oxygen electrode and chamber were in close agreement to those measured by the coupled assay (data not shown).

UV and visible absorption data were acquired with either a Hewlett-Packard 8452A or 8453 diode-array spectrophotometer. CD spectra were obtained with a Jasco J-710 spectropolarimeter. Titrations with phenylhydrazine (HCl, Sigma) were used to quantify TPQ in the purified recombinant enzyme [35]. One mL of 10-20  $\mu\text{M}$  protein in 100 mM potassium phosphate buffer was titrated with 2  $\mu\text{L}$  aliquots of fresh, anaerobically-prepared phenylhydrazine ( $\sim 775 \text{ } \mu\text{M}$ ) at room temperature. Spectral changes were monitored and recorded after the absorbance at 445 nm reached a constant value following each addition (10 - 45 minutes). The derivatized enzyme was subsequently concentrated in a Microcon 30 (Millipore) with buffer exchange to remove

unreacted phenylhydrazine. The concentrated phenylhydrazine-derivatized enzyme was then used for resonance Raman spectroscopy on a Spex Triplemate spectrometer with a CCD detector, with excitation by a Coherent argon-ion laser. EPR spectra were recorded on a Bruker 220D SRC interfaced with a personal computer. Simulation of EPR spectra used the EPR XOP for Igor Pro by John Boswell (Oregon Graduate Institute), an adaptation of "Program QPOW by Nilges and Belford [36-38]. Copper, zinc, calcium and magnesium analysis were performed by ICP emission spectroscopy (Little Bear Laboratories, Inc., Golden, CO), or copper analysis by flame atomic absorption spectroscopy using a Buck Scientific Model 210 VGP. Metal-free buffer was prepared by passage over Chelex-100 (Bio-Rad) and by using plasticware treated with 0.1 M EDTA solution. Analytical ultracentrifugation and ES/MS services were kindly provided by Andy Baron and Alison Ashcroft, respectively, at the University of Leeds, Leeds, UK.

#### Tissue-Specific Gene Expression

A Multiple Tissue Expression Array (Clontech) was used to determine the human, tissue-specific expression profile of human diamine oxidase. The manufacturer's instructions were followed for generating  $^{35}\text{P}$ -labelled cDNA probes by random primer labeling, hybridization and autoradiography. This experiment was performed by Dr. John Bollinger (MSU).



## Results

### Expression and purification

The expression vector pMTDAO contains the coding sequence for mature human kidney diamine (hKDAO) oxidase fused to *Drosophila* BiP signal sequence for secretion from *Drosophila* S2 cell culture. Expression is under the control of the *Drosophila* metallothionein promoter and induced by addition of copper sulfate to 500  $\mu$ M. Mature recombinant human kidney diamine oxidase (rhKDAO) primary sequence, as deduced from the DNA sequence, differs from that of the natural protein in only the replacement of the N-terminal glutamic acid and proline with an arginine residue.

Culture medium was harvested 48 hours post-induction at a cell density of  $1.45 \times 10^7$  cells per mL. Cells were greater than 99% viable as determined by trypan blue staining and exhibited normal morphology. No amine oxidase activity was detected in either the uninduced expression cell line or in the parental S2 cell line.

The recombinant enzyme is readily purified by heparin affinity chromatography, ceramic hydroxyapatite chromatography and gel filtration; the summary of a representative purification is given in Table 1. Total enzymatic activity increases during the protocol and likely reflects the loss of an inhibiting substance (of either the amine oxidase or the coupled assay) that is present in the growth medium. The purified protein is estimated at greater than 98% homogenous by SDS/PAGE, and the highest specific activity obtained from any purification was 1.25 I.U./mg.

Purified rhKDAO is stable for several months when stored on ice. However, freezing the enzyme at  $-20^{\circ}\text{C}$  gives a significant drop in specific activity; thawed enzyme has about 40% the specific activity of that before freezing. Thawed protein can recover 85% of the original specific activity after storing on ice for ten days. Bieganski and coworkers have previously noted a 90% loss in activity with natural human diamine oxidase when stored at  $-20^{\circ}\text{C}$  [39].

| Step            | Total Volume (mL) | Total Protein (mg) | Total Activity (I.U.) | Specific Activity (I.U./mg) | Purification Factor |
|-----------------|-------------------|--------------------|-----------------------|-----------------------------|---------------------|
| Culture medium  | 630               | 117                | 7.3                   | 0.063                       | 1                   |
| Hi-Trap Heparin | 13.5              | 41                 | 12.5                  | 0.30                        | 4.8                 |
| Ceramic         | 44                | 14                 | 13.0                  | 0.93                        | 14.8                |
| hydroxyapatite  |                   |                    |                       |                             |                     |
| Ultrogel AcA34  | 0.74              | 12                 | 12.7                  | 1.1                         | 16.7                |

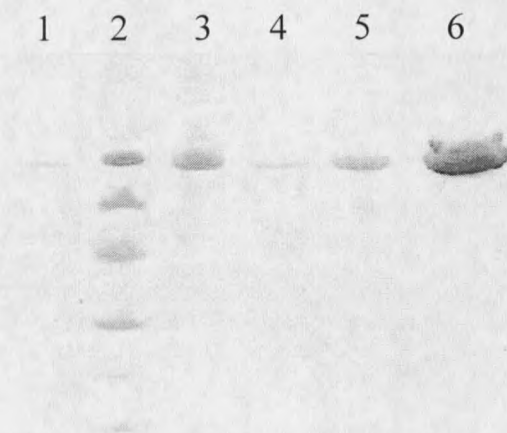
**Table 1.** Purification of recombinant human kidney diamine oxidase

#### Electrophoresis and analytical ultracentrifugation

Isoelectric focusing acrylamide gel electrophoresis gave an estimated pI of 6.2, and only a single protein band was observed. A pI of 6.7 is predicted from the primary sequence of rhKDAO [40]. Previous investigations reported isoelectric points of 6.0 and 7.1 for the natural human DAO [41-43].

SDS/PAGE of the purified enzyme gives a single protein band with an apparent molecular weight of 94 kDa (Figure 8), although the primary sequence of rhKDAO predicts 83.4 kDa for the mature monomer. No corresponding band is detected in

negative controls. The ~10 kDa discrepancy between the predicted and observed molecular weights suggests the expressed protein is substantially glycosylated (vide infra). Literature values for the apparent molecular weight of the natural human DAO have been reported as 70, 90 and 105 kDa by SDS/PAGE [44,45]. As evident in Figure 8, the recombinant protein band represents the major protein in harvested media.



**Figure 8.** SDS/PAGE showing purification of recombinant human kidney diamine oxidase.

- 1) Harvested *Drosophila* S2 media diluted 1:1 with gel loading buffer;
- 2) Molecular weight standards: 94, 67, 43, 30, 20.1 and 14.4 kDa;
- 3) Protein after Hi-Trap Heparin affinity column, 3.6  $\mu$ g;
- 4) Following ceramic hydroxyapatite chromatography;
- 5) Purified protein after gel filtration chromatography, 2.4  $\mu$ g;
- 6) 9.6  $\mu$ g of purified rhKDAO. The distorted band is a consequence of the severe overloading of the gel, however, this lane demonstrates the high degree of purity for the recombinant enzyme.

Several attempts to estimate the native molecular weight by acrylamide gel electrophoresis were unsuccessful. Gel filtration on a calibrated AcA 34 Ultrogel column gave an apparent molecular weight of 113 kDa, unrealistically low for the homodimer (data not shown). Interestingly, Crabbe et al. reported native molecular weights for the purified human placental DAO as 70 kDa by Sephadex G-200 gel filtration and 69.5 kDa by polyacrylamide gel electrophoresis. However, sedimentation equilibrium ultracentrifugation data in the same work indicated a native molecular weight of 235 kDa [44]. Baylin and Margolis reported the native molecular weight of human pregnancy plasma DAO as close to 200 kDa, as determined with Sephadex G-200 [46].

Sedimentation equilibrium ultracentrifugation of rhKDAO gave an average apparent molecular weight of 210 kDa (five runs with two protein concentrations at two speeds, data not shown). Curvature of the  $A_{280}$  versus radius plot suggests the recombinant enzyme is in reversible equilibrium between dimers and higher order complexes. The data could equally fit dimer-tetramer, dimer-hexamer or dimer-octamer models, with an association constant of  $1.2 A_{280}^{-1}$  for the dimer-tetramer model.

Sedimentation velocity ultracentrifugation gave a sedimentation coefficient of 10 S and a diffusion coefficient of 4.4 Ficks (two runs, data not shown). The apparent molecular weight calculated by the Svedberg equation is 200 kDa. Both sedimentation velocity runs indicated a small shoulder of faster moving material, consistent with a very small amount of higher-order protein association.

### Copper, calcium and TPQ

Copper, zinc, calcium and magnesium content in the recombinant enzyme were investigated using either ICP emission spectroscopy or flame atomic absorption. Table 2 shows the results from three different enzyme preparations. The data suggest a mixture of copper and zinc occupying the active sites in the recombinant enzyme; the sum of copper and zinc approaches 2 mol per mol dimeric enzyme. In contrast, purified mammalian copper amine oxidases, as well as those from other organisms, are most often characterized as having two copper ions per dimer [2]. Our results indicate a correlation between copper content and specific activity for the recombinant enzyme; not surprisingly, samples with higher copper content correspond to an increased specific activity.

| SpecificActivity<br>(IU/mg) | Cu                | Zn                | Ca   | Mg   | TPQ  |
|-----------------------------|-------------------|-------------------|------|------|------|
| 0.85                        | 1.03              | 0.79              | 1.84 | 0.91 | 0.72 |
| 1.21                        | 1.42 <sup>a</sup> | N.D. <sup>b</sup> | N.D. | N.D. | 1.04 |
| 1.25                        | 1.48              | 0.40              | 3.20 | 0.0  | 1.08 |

**Table 2.** Metal ion and TPQ stoichiometry for recombinant human kidney diamine oxidase as isolated from three enzyme purifications. Metal ion and TPQ values reported as mol per mole dimeric enzyme.

<sup>a</sup> Value determined by flame atomic absorption spectroscopy; all other metal ion quantifications were by ICP emission spectroscopy.

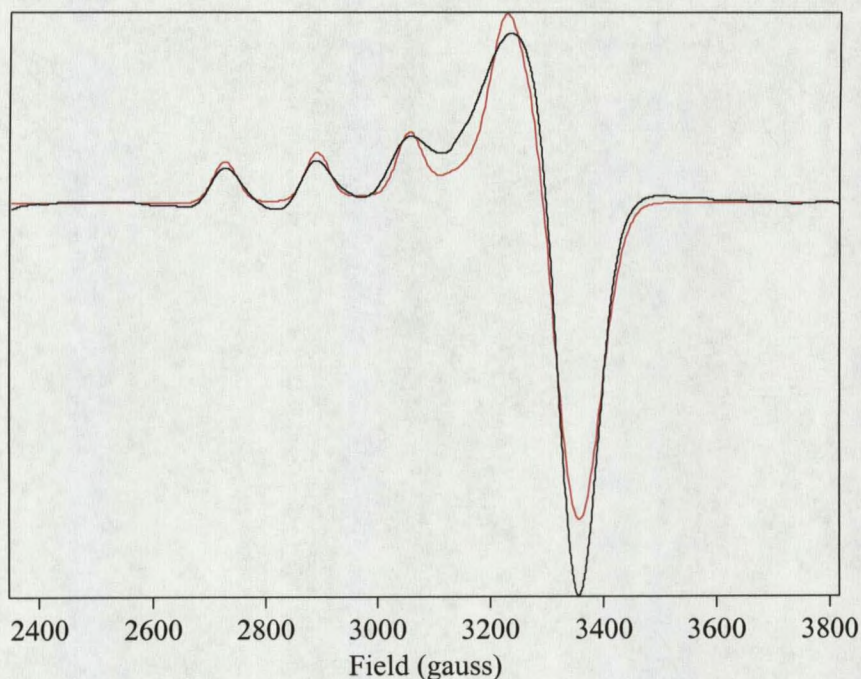
<sup>b</sup> Not determined

Metal analysis and the lack of manganese signal in EPR spectra (Figure 9) strongly suggest calcium occupies the putative second metal site in recombinant human

kidney diamine oxidase. Purified enzyme was dialyzed either against metal-free 100 mM potassium phosphate buffer alone or against three changes of two liters metal-free 100 mM potassium phosphate, 2 mM EDTA, pH 7.2 followed by extensive dialysis against metal-free phosphate buffer. The sample dialyzed against buffer alone was found by ICP emission spectroscopy to contain 2.37 moles calcium and 0.17 moles magnesium per mole of dimeric protein. Whereas the sample treated with EDTA contained 2.16 moles calcium per homodimer, and magnesium levels were below the detection limit of the instrument.

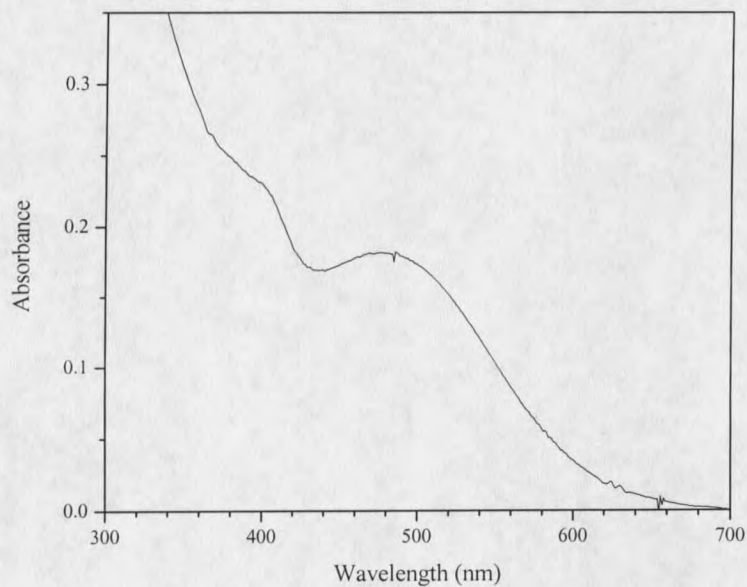
Figure 9 shows the X-band EPR spectra of Cu(II) in purified rhKDAO. The simulated spectrum was kindly provided by Dr. Melanie Rogers (MSU). EPR parameters are derived from simulated spectra ( $g_{\perp} = 2.04$ ,  $g_{\parallel} = 2.265$ ,  $A_{\parallel} = 185$  G) and are consistent with published values for CAOs from various sources [2]. Crabbe et al. reported a  $g_{\perp}$  value of 2.05 from Q-band EPR spectra of purified human placental diamine oxidase, however, the presence of manganese prevented the determination of other copper parameters. Manganese was not removed by passing the protein over a Chelex 100 column, leading those investigators to conclude the human placental diamine oxidase was a Cu(II)-Mn(II) metalloprotein, with an apparent stoichiometry of 2.0 mol copper and 2.4 mol manganese per mol enzyme dimer [43]. The lack of a characteristic six-line Mn(II) signal in our spectra is convincing evidence against any manganese associated with the recombinant enzyme.



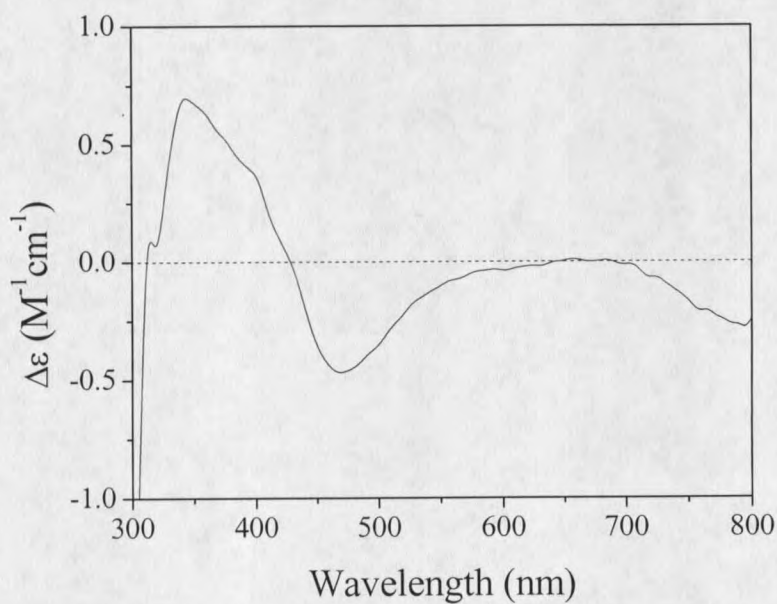


**Figure 9.** X-band EPR of rhKDAO. Enzyme at  $\sim 200 \mu\text{M}$  in 100 m potassium phosphate buffer (upper spectra). The simulated spectra is shown with  $g_{\perp} = 2.04$ ,  $g_{\parallel} = 2.265$  and  $A_{\parallel} = 185 \text{ G}$ . Experimental conditions: 77 K, 9.425 GHz, 0.796 mW, 20 G modulation amplitude.

Purified protein is peach-colored with a broad visible absorption band having a  $\lambda_{\text{max}}$  at 470 nm (Figure 10). This spectral feature is associated with the TPQ and gives copper amine oxidases their recognizable color. The shoulder in the absorption spectra at 400 nm is an unidentified feature, perhaps due to a modified (possibly incompletely processed) form of the cofactor. The CD spectrum shown in Figure 11 exhibits a negative band around 470 nm that is attributed to TPQ and a negative band around 800 nm from a Cu(II) d-d transition. These electronic transitions are consistent with other CAOs [2], and is consistent with a tetragonal Cu(II) complex with N,O donors.



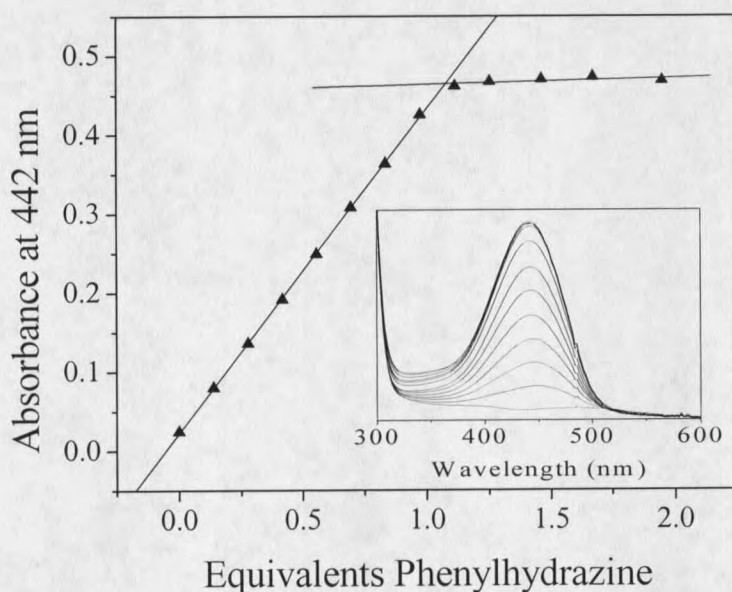
**Figure 10.** Absorption spectra of the purified recombinant human kidney diamine oxidase, 16.4 mg/mL enzyme in 100 mM potassium phosphate buffer, pH 7.2.



**Figure 11.** Circular dichroism spectra of rhKDAO. Experimental conditions: 32  $\mu$ M protein in 100 mM potassium phosphate buffer, pH 7.2, 1 nm resolution, 20 mdeg sensitivity, 1 sec response, 500  $\mu$ m slit, 5 accumulations.

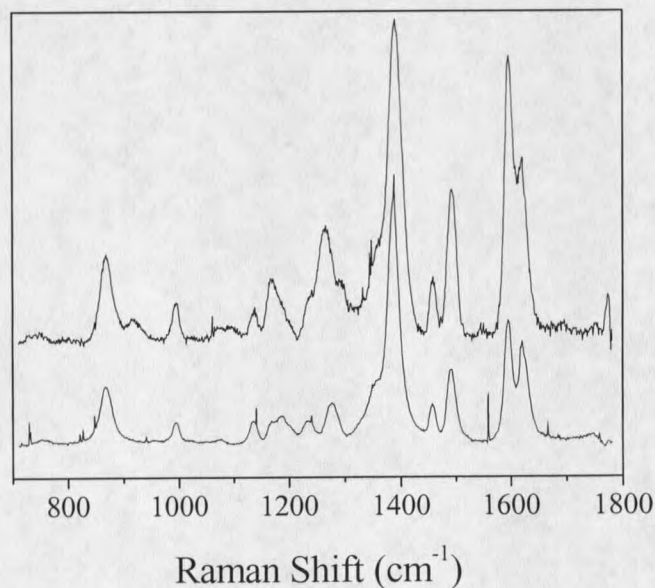


Copper amine oxidases and the carbonyl reagent phenylhydrazine react to give a characteristic, yellow phenylhydrazone adduct that is commonly used to identify and quantify the TPQ cofactor [2]. Recombinant enzyme was titrated with phenylhydrazine and spectra were recorded after each addition when no further change in absorbance at 442 nm was noted. A typical titration demonstrating the formation of the intensely colored covalent adduct is shown Figure 12 and the results of TPQ quantification for three individual enzyme preparations are shown in Table 2. As described above for copper, the quantified organic cofactor correlates with specific activity.



**Figure 12.** Phenylhydrazine titration of TPQ in purified rhKDAO. Plot shows the change in absorbance at 442 nm versus equivalents phenylhydrazine added per enzyme dimer. One mL of enzyme (11.8  $\mu$ M enzyme in 100 mM potassium phosphate buffer, pH 7.2) titrated with 2 830  $\mu$ M phenylhydrazine. The inset shows the increase in absorption of the intensely yellow-colored adduct with successive phenylhydrazine additions.

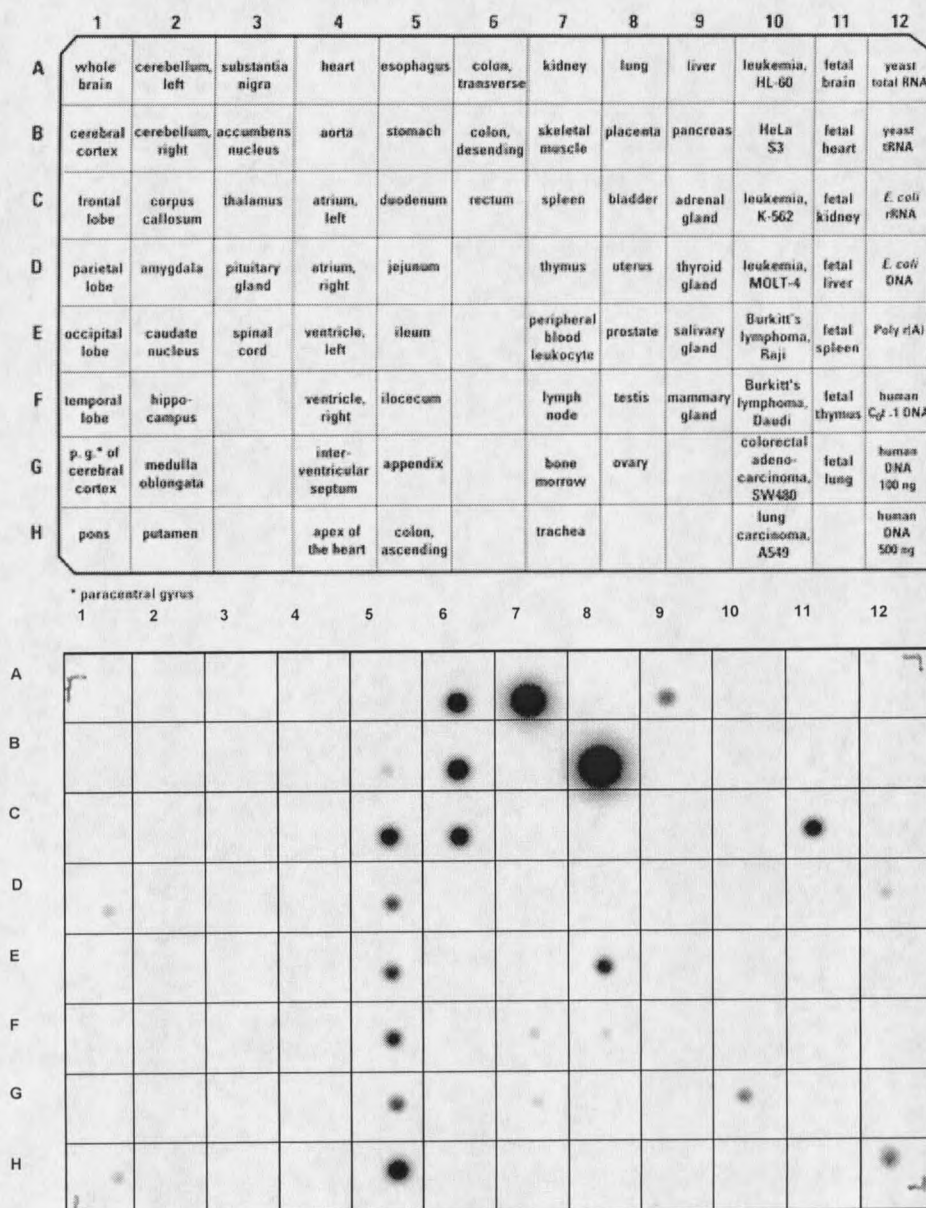
The resonance Raman spectrum of phenylhydrazine-derivatized recombinant enzyme is shown in Figure 13, along with that of the phenylhydrazone of TPQ-hydantoin, a model compound for the cofactor. Protein was concentrated after extensive buffer exchange to remove unreacted phenylhydrazine. The two spectra are essentially identical and thus conclusively identify TPQ as the quinone cofactor in the recombinant enzyme. Neither the visible absorption nor the resonance Raman spectrum of the phenylhydrazine adduct of rhKDAO suggests the visible absorption feature at  $\sim 400$  nm reacts with this carbonyl reagent. Thus this unidentified absorption feature may be a modified form of the TPQ cofactor which is conformationally inaccessible to phenylhydrazine or one which is lacking a reactive carbonyl.



**Figure 13.** Resonance Raman spectra of the phenylhydrazine-derivatized human rhKDAO (upper spectrum). Lower spectrum is the phenylhydrazine-derivatized model compound, TPQ-hydantoin. Experimental conditions: excitation 457.9 nm, power 40 mW, integration 1 min, 10 accumulations.

### Tissue-Specific Expression

A commercially available array of poly A<sup>+</sup> RNA representing 76 different human tissues and cell lines was probed with <sup>35</sup>P-labelled kidney diamine oxidase cDNA probes. The amount of poly A<sup>+</sup> RNA dotted on the nylon membrane were normalized by the manufacturer to give similar signal intensities for eight housekeeping genes and therefore varies from 53-780 ng. Each dot is 1 mm in diameter. Exposed and developed x-ray film is shown in Figure 14. Although the data are not quantifiable, a qualitative interpretation is informative for comparative analysis of tissue-specific gene expression. The highest levels of diamine oxidase gene expression are in placenta, followed by kidney tissue. Exposure at these two mRNA spots has clearly over-saturated the photographic media. Therefore actual mRNA levels may be orders of magnitude greater than that indicated by examination of the film. Gene expression is detectable in stomach tissue and quite significant through the distal tissues of the gastrointestinal tract. Prostate and liver also show fairly strong signals. A positive can be seen with *E. coli* DNA; however, kidney diamine oxidase cDNA and the bacterial genome have no significant sequence homology, and this is assumed to be a false positive. A similar false positive has previously been reported [47]. Low, but detectable, positive results are seen in stomach, bone marrow, lung, pancreas, testis, lymph node and the parietal lobe and pons of human brain. Negative results for controls other than the *E. coli* DNA, as well as for the majority of human tissues and cell lines, suggest that these are genuine positives. However, the weak results for these tissues, as compared to strong signals from other tissues, are such that actual gene expression levels in these tissues are normally quite low.



**Figure 14.** Multiple human tissue expression array with polyA mRNA from 76 tissues and cell lines (Clontech). Probed with randomly primed  $^{32}\text{P}$ -labeled oligonucleotides generated by PCR from a cDNA clone of human kidney diamine oxidase.

### Discussion

The gene encoding human kidney diamine oxidase, under the control of the metallothionein promoter, has been expressed in a *Drosophila* S2 polyclonal cell line. This work describes the first overexpression and purification of a copper-containing amine oxidase from any animal source. Expression levels for the secreted, recombinant enzyme are substantial, approaching 20 mg/L of cell culture. Almost certainly the abundant rhKDAO is a consequence of stably-transfected *Drosophila* S2 cell lines generally containing multicopy genomic inserts of the expression and selection plasmids, often with 500-1000 copies arranged in a head-to-tail fashion [48]. Very recently, Koyanagi and coworkers reported the heterologous overexpression of a copper amine oxidase from a multicellular organism, the plant *P. sativum* [49]. Unfortunately, low expression levels and considerable clonal variation mar this notable accomplishment (K. Tanizawa, pers. comm.). Expression of the *Arabidopsis thaliana* enzyme has previously been described in a baculovirus expression system [50].

Expression in serum-free media avoids introduction of exogenous copper amine oxidases [51,52] as well as the considerable amount of undesired proteins naturally present in fetal calf serum. Furthermore, searches of the *D. melanogaster* genome have not revealed any sequences with significant homology to CAOs, nor, to the best of our knowledge, has a CAO been described from any insect source. However, one member of the copper-containing amine oxidase family, lysyl oxidase (E.C. 1.4.3.13) has been identified in the *Drosophila* genome [53]. Lysyl oxidase contains a lysine-

tyrosylquinone cofactor and catalyzes the oxidative deamination of peptidyl lysine in elastin and collagen—a crucial step for connective tissue cross-linking [54]. Therefore the *Drosophila* Expression System, when used with serum-free media, is an excellent source of secreted human diamine oxidase, with the inherent advantages of low levels of unwanted proteins and apparently no contaminating CAOs, either exogenous or endogenous. *Drosophila* S2 cell have previously been used to express another copper enzyme, human dopamine  $\beta$ -hydroxylase, at protein yields quite similar to those reported in this work [55]. This expression system is thus a promising option for the convenient expression of other eukaryotic metalloenzymes.

Interestingly, in insects histamine is a major neurotransmitter. Histamine is a neurotransmitter in some mechanosensory neurons in *Drosophila*, lobster stomatogastric, cardiac, and olfactory neurons, and has recently been shown to be the major neurotransmitter in arthropod photoreceptors. [56-61]. It will be shown later that histamine is an excellent substrate for human KDAO. The mechanism for histamine catabolism in arthropods remains to be delineated, but a copper amine oxidase does not seem to be involved.

The natural hKDAO heparin-binding site facilitated the development of a rapid purification scheme resulting in the highly efficient recovery of greater than 99% homogenous recombinant human KDAO. The protein yields are impressive, approaching 20 mg/L.

Analytical ultracentrifugation results suggest rhKDAO contains between 20 and 26% glycosylation by weight, a value not unreasonable for the natural mammalian

enzyme. The results given by Crabbe et al. indicate approximately 40% glycosylation for the natural human placental enzyme [43]. Gel filtration by Baylin and Margolis suggest 20% glycosylation for the natural human pregnancy plasma DAO [44]. For comparison, the porcine kidney DAO has been estimated at 20% and 11% carbohydrate by weight [62,63]. Although the data on glycosylation in mammalian DAOs are limited—and it must be noted that differences in glycosylation could very well be tissue-specific—the extent of glycosylation by weight in rhKDAO is within the range of values reported for the natural enzymes.

SDS/PAGE and analytical ultracentrifugation results are consistent with a substantially glycosylated recombinant enzyme, as would be expected for a protein with potential glycosylation sites and processed through the secretory pathway. Electrospray mass spectrometry of the recombinant human kidney protein gave a large  $m/z$  “hump” with no resolvable peaks, indicative of a population of heterogeneously glycosylated proteins (data not shown). Four consensus N-glycosylation sequences (NXS/T) are present in the peptide sequence of human kidney diamine oxidase. N-linked glycosylation in *Drosophila* is much like that in other insect cells, with a high mannose content, although O-linked glycosylation is also possible [64,65]. Glycosylation of the recombinant enzyme is expected to be different from the enzymes processed in mammalian cells, and strategies to reduce the extent of glycosylation in rhKDAO are actively being pursued.

The heterologously expressed enzyme contains substoichiometric copper and displays some variability in copper levels from different purifications. The data shown in



Table 2 indicate copper content ranges from 1 to 1.5 ions per dimer. The recombinant enzyme most likely contains tightly bound zinc in the remaining active sites; copper and zinc together account for nearly full metal occupancy of the two active sites in each protein homodimer. Zinc has previously been described in recombinant *H. polymorpha* CAO overexpressed in *Saccharomyces cerevisiae*, and zinc was determined to have a high affinity for the active site and to compete with copper for binding [66]. The crystal structure of zinc-substituted *H. polymorpha* enzyme has recently been solved and reveals zinc in the active site, coordinated by three histidine residues and a tyrosine residue (copper ligands in the active enzyme and the tyrosine precursor of TPQ) [67]. These residues are remarkably similar in position as those described in the active site of apoCAO from *Arthrobacter globiformis* [13].

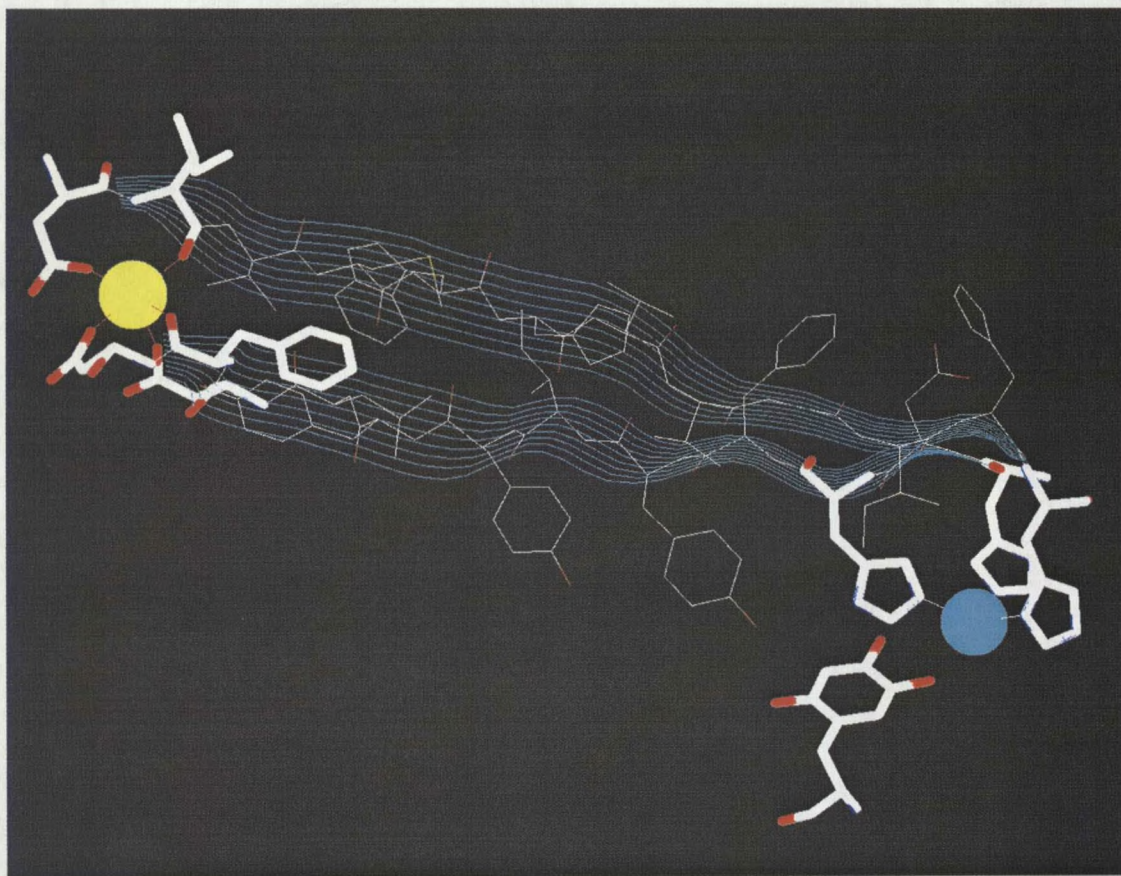
It is proposed that active-site metal incorporation most likely occurs intracellularly during rhKDAO processing in the *Drosophila* secretory pathway, and not following secretion into media loaded with 500  $\mu$ M copper, in which case fully copper-loaded protein could be expected. Rae *et al.* have made a convincing argument for extraordinarily low levels of "free" intracellular copper, less than one per cell, and suggested copper-dependent enzymes require accessory factors (i.e. metallochaperones) [68]. However, their analysis is based upon *cytoplasmic* superoxide dismutase and would not necessarily apply to the contents of membrane bound organelles, such as those of the secretory pathway. Copper most likely enters the *Drosophila* secretory pathway via a P-type ATPase, homologous to the human Menkes disease protein, localized in the trans-Golgi network [69]. To date, no accessory protein for any CAO has been described.

Furthermore, *Drosophila* S2 culture is quite likely a zero background expression host and therefore would not be expected to possess a specific CAO metallochaperone. Copper and zinc content in the overexpressed enzyme may be determined by the total metal ion availability in the secretory pathway and the relative affinity of the active site for these two transition metals. Differences in metal loading between purifications might be the consequence of subtle and as yet unrecognized factors during cell culturing and protein expression.

This study indicates calcium occupies the putative second metal binding site in recombinant human kidney diamine oxidase. The high specificity and affinity of this site for calcium, supported by the resistance to removal by EDTA ( $\log K = 10.61$  at  $25\text{ }^{\circ}\text{C}$  [70]), strongly suggest calcium would be present in the natural enzyme. Stoichiometric calcium is also present in highly purified equine plasma copper amine oxidase (*vide infra*).

Although calcium sites in proteins vary considerably with regard to their coordination structure, the second-metal site in copper amine oxidases is unusual when compared to other structurally defined sites (see reference [71] for a recent review of the diverse calcium binding sites in proteins). Five putative KDAO calcium ligands can be deduced from sequence alignment and the crystal structures of the CAOs from *E. coli* and *P. sativum* [11,12]. Three of these are aspartyl carboxylates and the backbone carbonyl of a leucine in the sequence AspLeuAsp. These residues are located at the C-terminus of one  $\beta$  strand. The other two protein-derived oxygen donors are an aspartyl carboxylate and an adjacent backbone carbonyl (Leu), located at the N-terminus of another  $\beta$  strand.

Interestingly, the opposite ends of these two  $\beta$  strands (7-10 residues away) also provide the active site copper ligands. One coordinating water molecule is resolved in the crystal structures, giving an octahedral coordination geometry with calcium-oxygen distances from 2.2 to 2.5 Å (Figure 15).



**Figure 15.** View of the copper and second metal binding sites from pea seedling amine oxidase. Copper is depicted as a cyan sphere and calcium as a yellow sphere. The protein residues that ligate the metal ions are shown in white with red representing oxygen atoms.

Most calcium ions in biology are heptacoordinate and associated with  $\alpha$  helical and loop regions [72]. The well-known and extensive family of helix-loop-helix (EF hand) calcium sites is typically seven-coordinate with three carboxylates (one of which is



bidentate) two carbonyls, and one water molecule in a pentagonal bipyramid. EF hand ligands are all located in a short sequence at the end of one helix, a small loop, and the beginning of another helix. In contrast, the calcium binding residues in CAOs are at the ends of two  $\beta$  strands separated in sequence by more than 130 intervening amino acids.

Calcium ions are critical for a variety of important biological processes, including some involving extracellular proteins [73-78]. The function of the second metal site in copper-containing amine oxidases remains to be elucidated. However, the bound calcium ion likely serves to stabilize the structure of this extracellular enzyme, although a role in modulating activity is also possible but unlikely given the apparently high affinity for calcium and the separation between the putative Ca site and the active site.

Recombinant human kidney diamine oxidase is conclusively demonstrated to possess the TPQ cofactor. Visible absorption, CD, titration with phenylhydrazine, and resonance Raman of the phenylhydrazine-derivatized enzyme are entirely consistent with previously characterized copper amine oxidases from various sources [2,79]. Similar correlations between specific activity and titratable TPQ have been described by Klinman and coworkers for the bovine plasma amine oxidase and the recombinant yeast methylamine oxidase [35,80]. As shown in Table 2, the titratable TPQ from three different preparations is consistently around 72% of that which could be expected given the copper to protein stoichiometry, assuming that each active site containing copper would readily convert the precursor tyrosine to TPQ. It is presently unclear whether low TPQ-to-copper ratios reflect incomplete organic cofactor biogenesis or enzyme conformations in which the quinone cofactor is inaccessible to phenylhydrazine. Low

and variable TPQ content is not uncommon in CAOs [2]. For example, recombinant yeast methylamine oxidase has been described as containing 2.0 mol copper but only 1.5 mol phenylhydrazine-titratable TPQ per mol of enzyme dimer [80], a TPQ to copper ratio strikingly close to the results presented herein.

### Conclusions

Human kidney diamine oxidase has been heterologously overexpressed in *Drosophila* S2 cell culture. The secreted enzyme is readily purified by three chromatography steps and gives high yields of essentially pure protein. This accomplishment provides the first example of a recombinant copper amine oxidase from any multicellular source. Although the enzyme does not contain a full complement of copper and TPQ, it has proven to be well suited to characterization and analysis.

Spectroscopic and biochemical analysis has clearly established that recombinant hKDAO contains the cofactors copper and TPQ. The visible absorption, CD, EPR and resonance Raman spectra of native rhKDAO as well as the visible and resonance Raman spectra of the phenylhydrazine-derivatized enzyme have been reported and are consistent with the data for copper amine oxidases from other sources.

The recombinant enzyme has only now allowed the detailed characterization of a human copper amine oxidase, a significant and long-awaited development in the field of copper amine oxidase research. Furthermore, biochemical and kinetic analysis, such as

the studies presented in the following chapters, provides key details required to reasonably infer the *in vivo* functions of diamine oxidase in human amine metabolism and homeostasis.

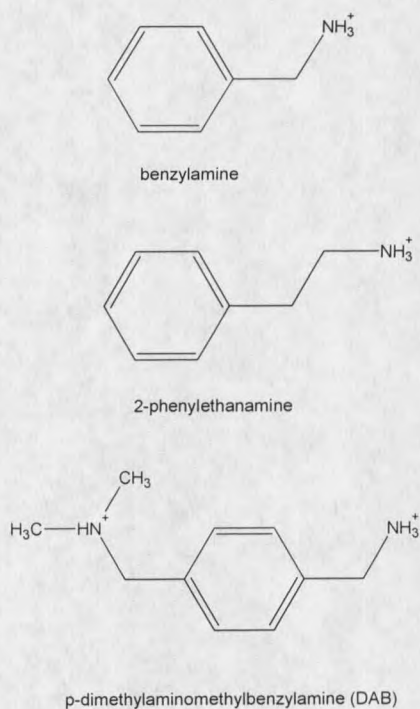
## STEADY STATE KINETICS AND SUBSTRATE PREFERENCE OF rhKDAO

Introduction

A variety of biogenic and exogenous amines have been investigated as potential substrates for the recombinant human kidney diamine oxidase. Steady state kinetic parameters have been determined. These *in vitro* experiments are necessary to delineate the reactivity of rhKDAO and enable comparison with other copper amine oxidases. The quantity and quality of the recombinant protein has made such detailed and extensive studies feasible and substantially adds to the characterization of this human enzyme. Substrate specificity determined *in vitro* allow one to infer the *in vivo* substrates and by extension the possible roles of diamine oxidase in human physiology and its contribution to the complete scheme of amine metabolism and homeostasis.

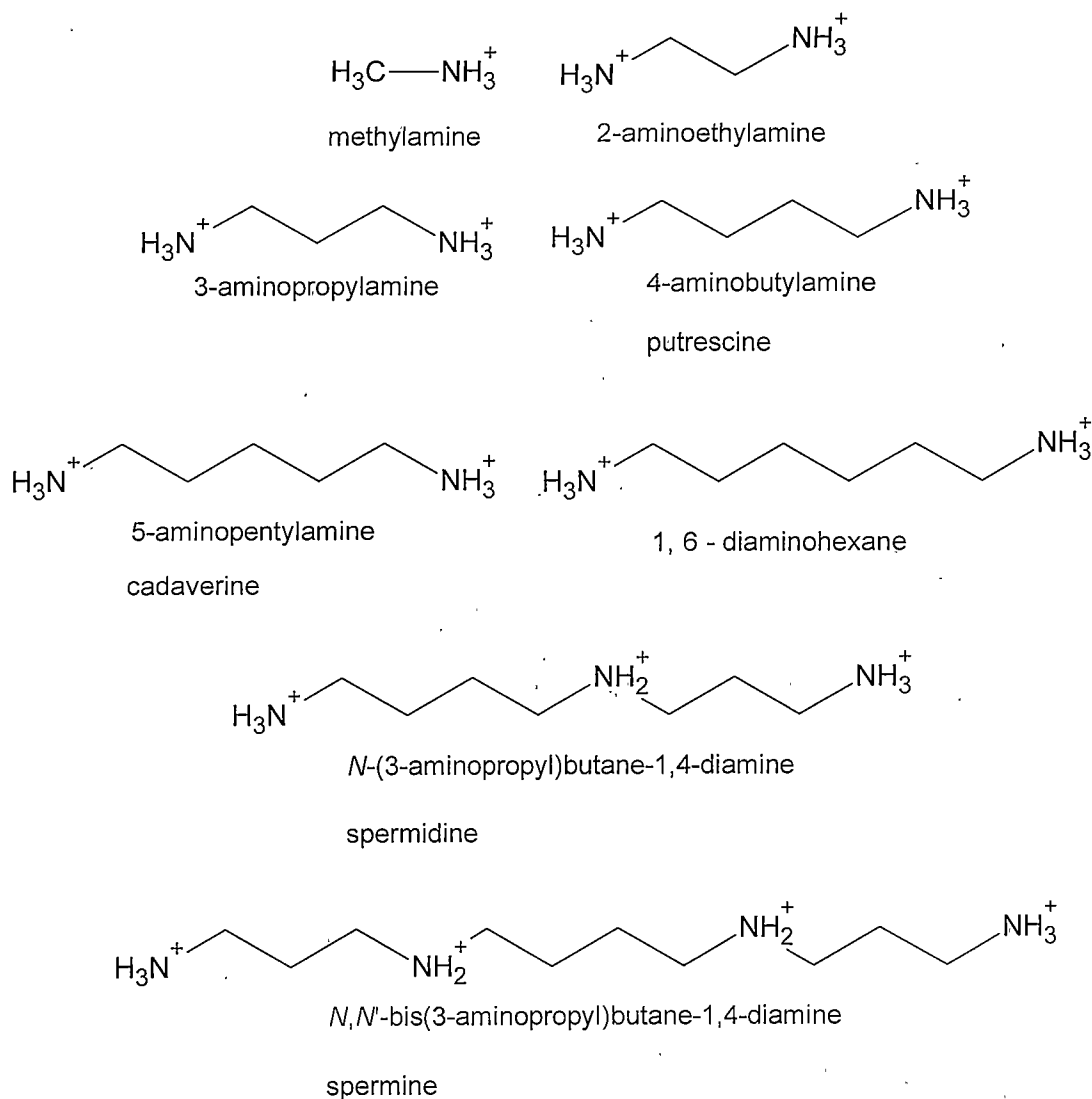
The non physiological and exogenous aromatic amines benzylamine,  $\beta$ -phenethylamine and *p*-dimethylaminomethylbenzylamine (DAB) are commonly used *in vitro* substrates for copper amine oxidases[81,82]. Their corresponding product aldehydes absorb light in the ultraviolet region, enabling spectrophotometric detection and direct enzymatic assays.





**Figure 16.** Structures of exogenous aromatic amine substrates for copper amine oxidases.

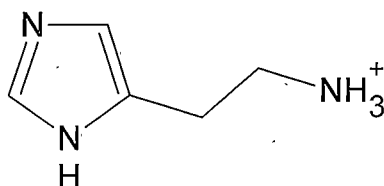
Figure 17 shows the structures of exogenous and endogenous aliphatic diamines and polyamines. Putrescine, cadaverine and the polyamines spermidine and spermine are widespread in biology and have been described as substrates for the copper containing diamine oxidases (*vide infra*).



**Figure 17.** Structures of aliphatic diamines and polyamines

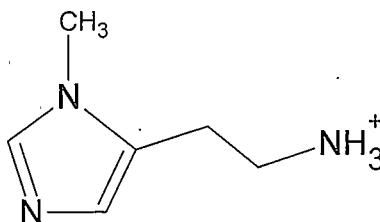
Histamine and 1-methylhistamine are metabolites of the amino acid histidine from decarboxylation and the subsequent methylation catalyzed by the enzymes histidine decarboxylase and histamine methyltransferase. Both are known substrates for mammalian diamine oxidases (*vida infra*). Histamine, in particular, is an extremely

potent biological agent acting as an agonist for G-protein coupled receptors and inducing a range of crucial physiological responses.



2-(1*H*-imidazol-5-yl)ethanamine

histamine

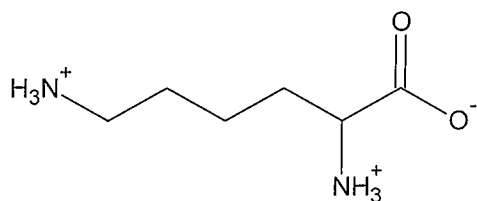


2-(1-methyl-1*H*-imidazol-5-yl)ethanamine

1-methylhistamine

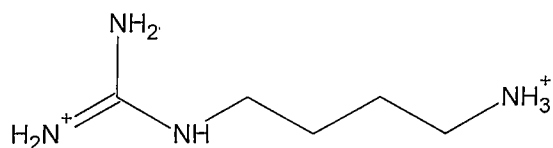
**Figure 18.** Structures of histamine and 1-methylhistamine

Figure 19 shows the structure of the remaining compounds investigated as part of this study. Lysine and two ester derivatives were included in order to determine if this amino acid alone or as part of a peptidyl substrate could be a substrate for human DAO; in other words, to determine if diamine oxidase has a lysyl oxidase function. Metabolic derivatives of the amino acids tryptophan, arginine and tyrosine were also included in this work.

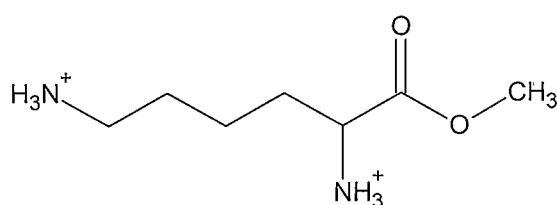


2,6-diaminohexanoate

L-Lysine

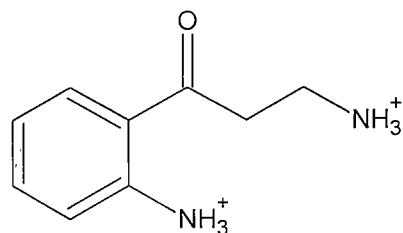
*N*-(4-aminobutyl)guanidine

agmatine



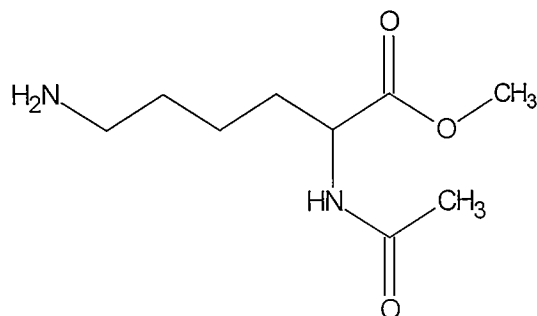
methyl 2,6-diaminohexanoate

L-Lysine methyl ester



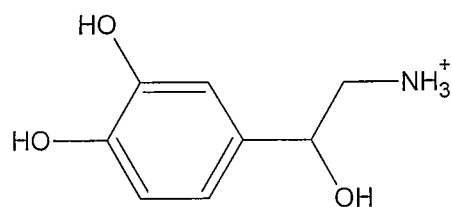
3-amino-1-(2-aminophenyl)propan-1-one

kynuramine



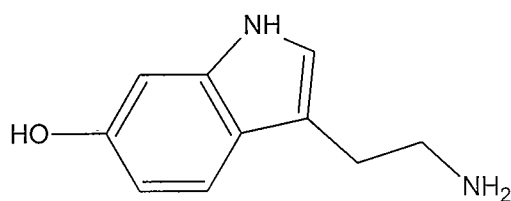
methyl 2-(acetylamino)-6-aminohexanoate

N-Acetyl-L-lysine methyl ester

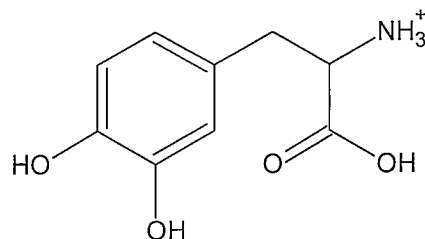


4-(2-amino-1-hydroxyethyl)benzene-1,2-diol

Arterenol (norepinephrine, noradrenaline)

3-(2-aminoethyl)-1*H*-indol-6-ol

5-hydroxytryptamine (serotonin)



2-amino-3-(3,4-dihydroxyphenyl)propanoic acid

3-hydroxytyramine (dopamine)

**Figure 19.** Structures of derivatives of the amino acids lysine, arginine and tyrosine

### Experimental Procedures

$K_M$  and  $k_{cat}$  were determined spectrophotometrically in a 37 °C thermostatted cell holder with magnetic stirring. Ionic strength of the assay buffer, 50 mM HEPES, pH 7.2, was maintained at 150 mM by addition of potassium chloride. Oxidation of *p*-dimethylaminomethylbenzylamine (DAB) and benzylamine were followed by the change in absorbance at 250 nm, using an extinction coefficient of 11 mM<sup>-1</sup>cm<sup>-1</sup> for *p*-dimethylaminomethylbenzaldehyde [82]. Assays with DAB or benzylamine used 890 µL of assay buffer and 10 µL enzyme in a 1 cm pathlength cuvette. This mixture was allowed to equilibrate for a 2-3 minutes in the thermostatted cell holder before rapid addition of 100 µL substrate stock solution. Data acquisition was initiated immediately. All other assays used the coupled assay of Holt et al. [83] and an extinction coefficient for the quinoneimine dye of 6.00 mM<sup>-1</sup>cm<sup>-1</sup> at 498 nm [84]. Chromogen stock solution was prepared to give final concentrations of 1 mM 4-aminoantipyrine and 2 mM vanillic acid. Coupled assays used 880 µL chromogen stock solution, 10 µL (14.5 U) horseradish peroxidase, and 10 µL enzyme. Substrate, horseradish peroxidase and chromogen stock solutions were freshly prepared in the assay buffer, and kept in a 37 °C water bath until use. Recombinant diamine oxidase was kept on ice. Thermal equilibration, substrate addition and data acquisition were as described above. Assays used 10 µL of 4.37 µM recombinant human kidney diamine oxidase, except 4.09 µM enzyme with 1-methylhistamine, and 4.67 µM with putrescine and agmatine. Water bath and thermostatted cell holder temperatures were monitored with an electronic thermocouple.

Initial rates were determined by at least duplicate experiments (most often triplicate) at six or more substrate concentrations with fitting to the Michaelis-Menten equation using Origin 6.0 software (OriginLab Corp.). Those substrates demonstrating substrate inhibition were fit as described below [85]:

(a) Substrates that indicated hyperbolic behavior were fit with the Michaelis-Menten equation:

$$v = \frac{S V_{max}}{S + K_M}$$

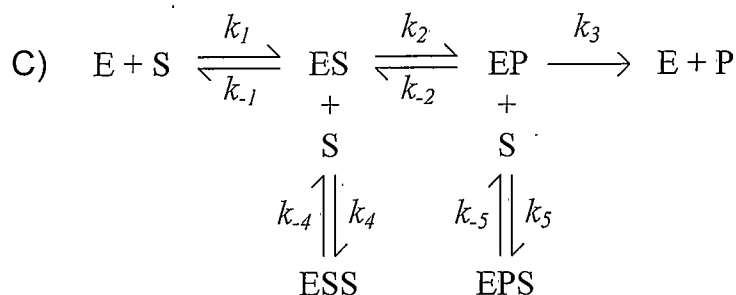
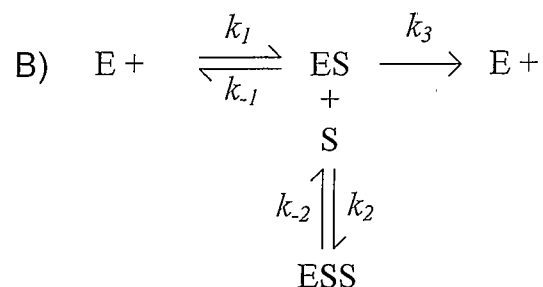
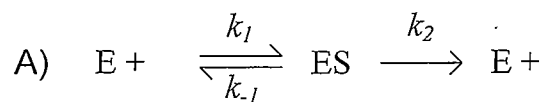
(b) Where required fits used a simple model of substrate inhibition:

$$v = \frac{S V_{max}}{\frac{S^2}{K_i} + K_m + S}$$

(c) In a few cases the fits to data used the following equation, for a model with reversible substrate inhibition at two different forms of the enzyme:

$$v = \frac{S V_{ma}}{K_3 S^2 + S + K_2 S + K_1}$$

Figure 20 shows the schematics for the models that represent the kinetic equations above. Appendix C gives detailed information on the derivation of these equations.



**Figure 20.** Mechanistic models used to derive enzyme rate equations: **A)** Michaelis-Menten model; **B)** Substrate inhibition; **C)** Substrate inhibition at two enzyme states

Substrates that oxidized either extremely slowly or at rates undetectable under these conditions were assayed up to millimolar substrate concentrations. Potential substrates were purchased commercially (highest grade available) and used without further purification, except for DAB, which was synthesized by the method of Bardsley et al. [82].

The pH dependence of Michaelis-Menten parameters for the oxidation of putrescine was determined using the coupled 4-aminoantipyrine/vanillic acid assay as described above. Buffers used were 50 mM MES (pH 5.69 - 6.33), 50 mM HEPES (pH



6.33 - 7.78), and 50 mM CHES (pH 8.12 and 8.85). Ionic strength was adjusted to a final concentration of 150 mM with potassium chloride. After equilibration at 37 °C, the pH was measured with an Orion perpHect LogR meter, model 310, equipped with an Automatic Temperature Compensation Probe. At least two (usually three or four) initial rates were determined at six or more substrate concentrations for each pH value.  $k_{cat}$  values were plotted against proton concentration and fit to the following equation [86]:

$$k_{cat} = (k_{cat})_{max} [H^+] K_{a1} / (K_{a1} K_{a2} + K_{a1} [H^+] + [H^+]^2)$$

Enzyme stability in the pH range used was tested by incubation of enzyme in the appropriate buffers for 15 minutes at 37 °C and then measuring activity. Possible effects of pH on the quinoleimine dye generated during the assay were investigated by titrating hydrogen peroxide into the chromogenic solution plus horseradish peroxidase at appropriate pH values. Neither loss of enzyme activity nor change in quinoleimine dye absorption features was detected in the buffers and pH range investigated.

Heparin effects on enzyme activity were investigated by incubating recombinant kidney diamine oxidase with an approximately four-fold excess of heparin (from porcine intestinal mucosa, average molecular weight of 3,000 Da, Sigma) over dimeric protein for one hour on ice. Initial rate determinations for putrescine oxidation were as described above. The enzyme-heparin mix was stored at 4 °C and assayed for activity with 250 µM putrescine after 24, 48, and 72 hours. All assays used air saturated solutions and dissolved oxygen levels were not varied in these experiments (about 233 µM).

### Results

The data and fits to the data are given in Figures 21 - 34. The results are summarized in Table 3 and are listed in order of the substrate preference of rhKDAO indicated by  $k_{cat}/K_M$ .

The data for histamine turnover clearly indicate partial substrate inhibition. The fit using the model of substrate inhibition required masking of the data points at higher concentrations of histamine, and as shown this model is inadequate for completely describing the data (Figure 21). A model with substrate inhibition at two forms of the enzyme was also fit to the data, and shown in Figure 22. This model also proved inadequate and required masking of the data for the higher concentrations of histamine. Note that the apparent  $K_M$  and  $V_{max}$  values for both of these models are approximately equal and that the fit curves have identical shapes.

It became apparent that no model of partial substrate inhibition would fit all the data points; partial substrate inhibition alone cannot account for the data points at the higher concentrations of histamine. A model that can fit all the data requires an additional mechanism for substrate oxidation, a process that becomes evident at higher substrate concentrations. The data were accordingly fit to the model for substrate inhibition plus an additional Michaelis-Menten model, that is:

$$v = \frac{S V_{max}}{\frac{S^2}{K_i} + K_m + S} + \frac{S V_{max}}{S + K_M}$$

Figure 23 shows that this kinetic model can be used to reasonably fit all the data points.

| Compound                                 | $K_M$<br>( $\mu\text{M}$ ) | $k_{\text{cat}}$<br>( $\text{min}^{-1}$ ) | $k_{\text{cat}}/K_M$<br>( $\mu\text{M}^{-1} \text{min}^{-1}$ ) |
|------------------------------------------|----------------------------|-------------------------------------------|----------------------------------------------------------------|
| Histamine <sup>c</sup>                   | $3.4 \pm 0.08$             | $159 \pm 1.1$                             | 47                                                             |
| 1-Methylhistamine                        | $3.0 \pm 0.1$              | $100 \pm 1.0$                             | 33                                                             |
| Agmatine <sup>c</sup>                    | $13 \pm 1.0$               | $324 \pm 17$                              | 25                                                             |
| Putrescine <sup>c</sup>                  | $17 \pm 0.5$               | $329 \pm 3.1$                             | 20                                                             |
| Cadaverine                               | $22 \pm 0.3$               | $411 \pm 4.1$                             | 19                                                             |
| DAB                                      | $113 \pm 2$                | $532 \pm 5.3$                             | 4.7                                                            |
| 1,3 - Diaminopropane                     | $129 \pm 14$               | $486 \pm 22$                              | 3.8                                                            |
| 1,6 - Diaminohexane                      | $177 \pm 12$               | $324 \pm 9$                               | 1.8                                                            |
| 2-aminoethylamine                        | $626 \pm 11$               | $127 \pm 0.6$                             | 0.2                                                            |
| Spermidine                               | $1092 \pm 8.3$             | $187 \pm 0.5$                             | 0.2                                                            |
| L - Lysine methyl ester                  | $2978 \pm 114$             | $346 \pm 4.6$                             | 0.1                                                            |
| (-) - Arterenol                          | <sup>a</sup>               | +                                         | +                                                              |
| Benzylamine                              | +                          | +                                         | +                                                              |
| 3 - Hydroxytyramine                      | +                          | +                                         | +                                                              |
| Kynuramine                               | +                          | +                                         | +                                                              |
| $\beta$ -Phenethylamine                  | +                          | +                                         | +                                                              |
| Spermine                                 | +                          | +                                         | +                                                              |
| 5 - Hydroxytryptamine                    | <sup>b</sup>               | -                                         | -                                                              |
| L- Lysine                                | -                          | -                                         | -                                                              |
| N $\alpha$ -acetyl-L-lysine methyl ester | -                          | -                                         | -                                                              |
| Methylamine                              | -                          | -                                         | -                                                              |

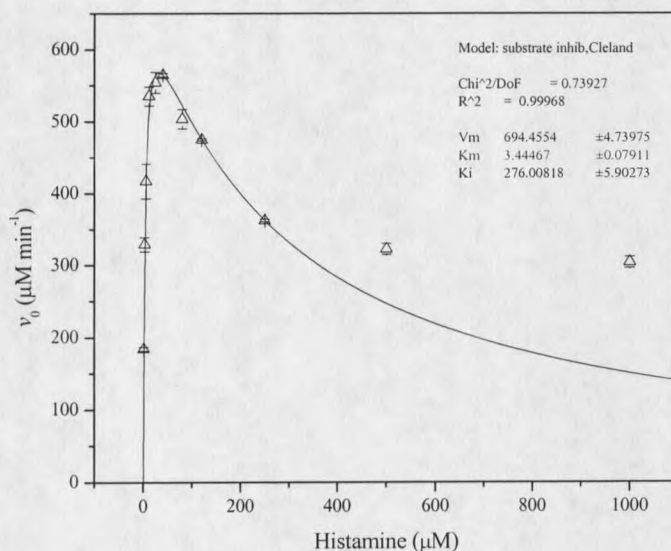
**Table 3.** Steady-state kinetic parameters and substrate specificity ( $k_{\text{cat}}/K_M$ ) for recombinant human kidney diamine oxidase. Errors are reported as standard error at the 95% confidence level. Partial substrate inhibition was observed with histamine, agmatine and putrescine.

<sup>a</sup> The positive symbol (+) denotes substrate oxidation could be detected but at rates too low to determine kinetic parameters.

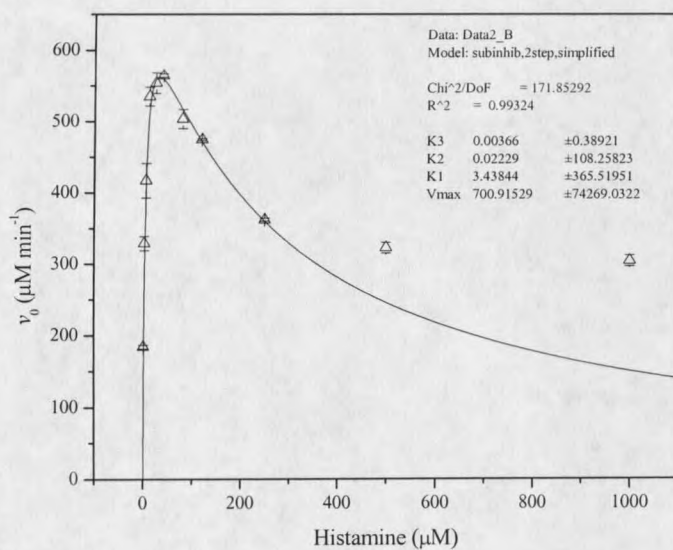
<sup>b</sup> The negative symbol (-) indicates no rate was observed.

<sup>c</sup> Partial substrate inhibition was observed.

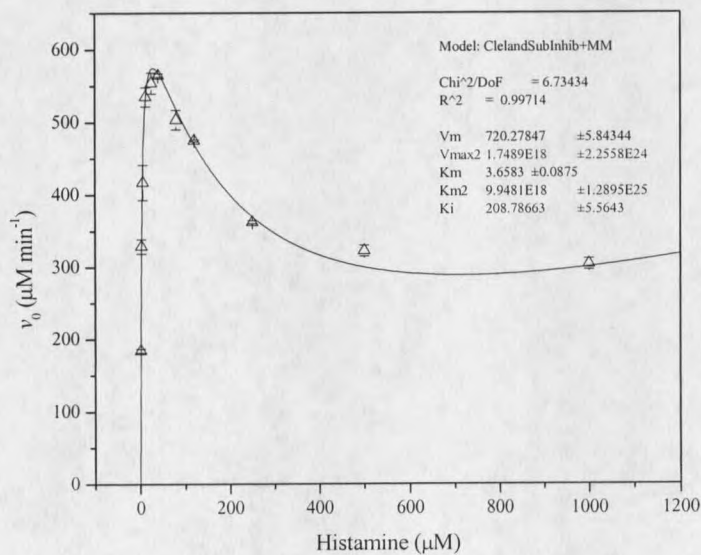
Fits to the more complex model increases the error in each fit parameter. Therefore to derive apparent  $K_M$  and  $V_{max}$  values the data were fit to the substrate inhibition model with the data points at high substrate concentrations disregarded. The data for agmatine oxidation were also interpreted in this manner.



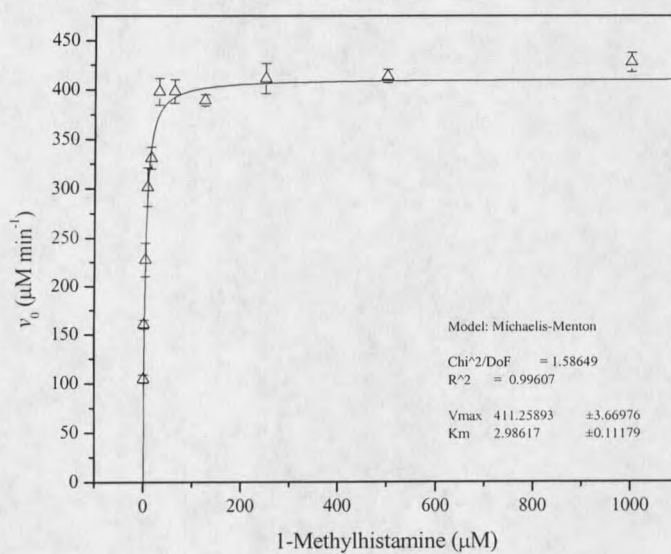
**Figure 21.** Histamine, fit to the substrate inhibition model



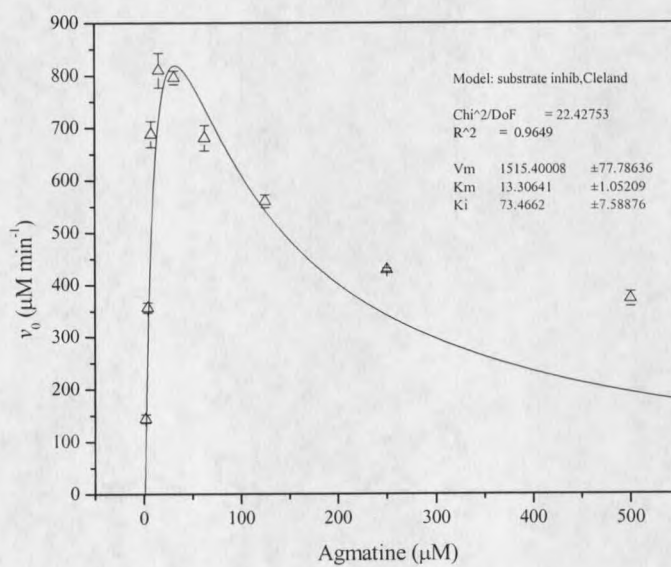
**Figure 22.** Histamine fit to the substrate inhibition at two enzyme forms



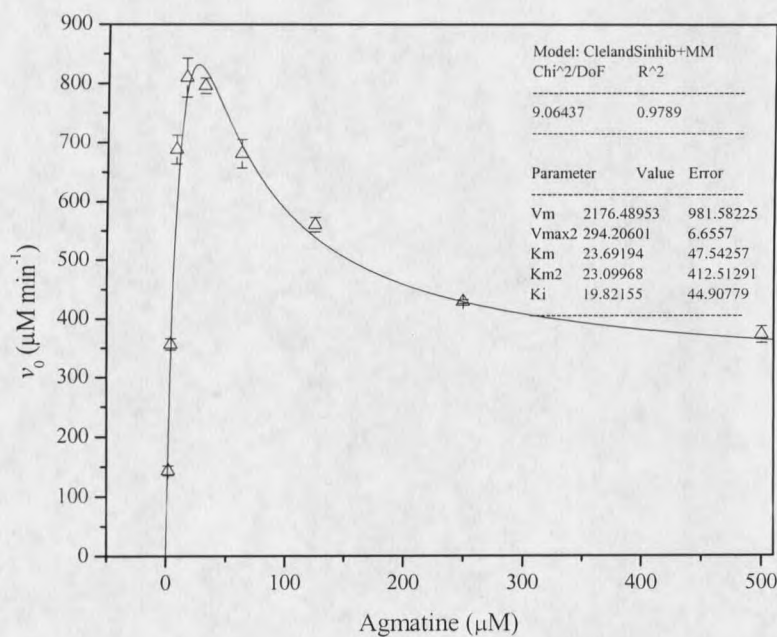
**Figure 23.** Histamine fit to the substrate inhibition model plus an additional Michaelis-Menten model



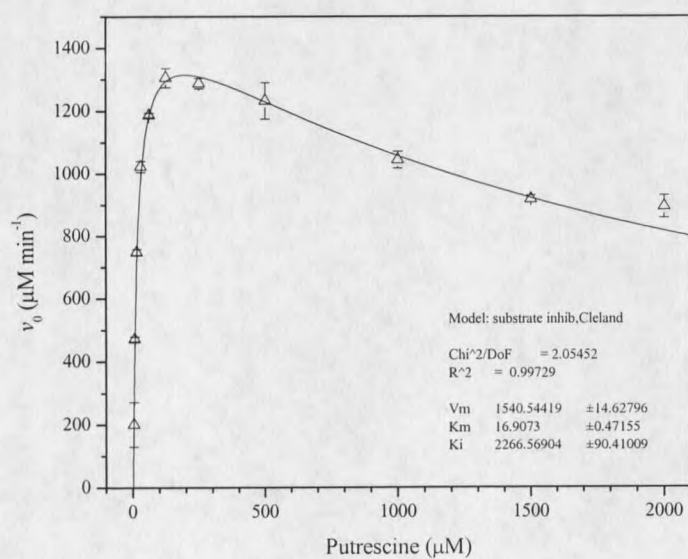
**Figure 24.** 1-methylhistamine fit to the Michaelis-Menten model



**Figure 25.** Agmatine fit to the substrate inhibition model

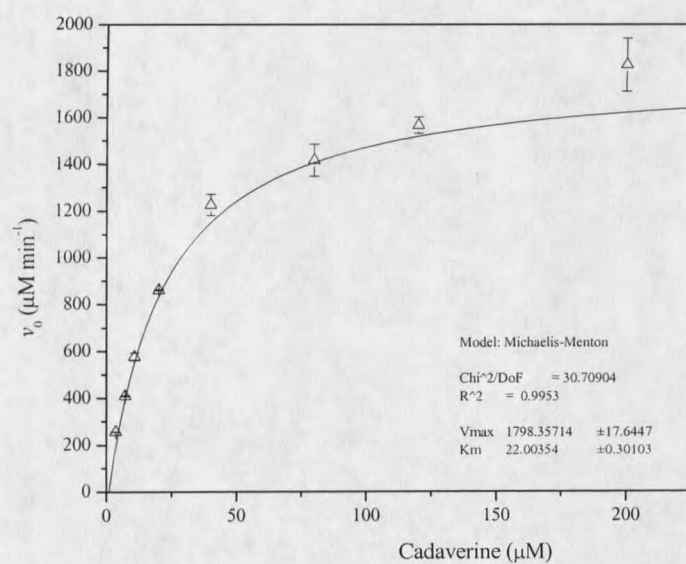


**Figure 26:** Agmatine fit to the substrate inhibition model plus an additional Michaelis-Menten model.

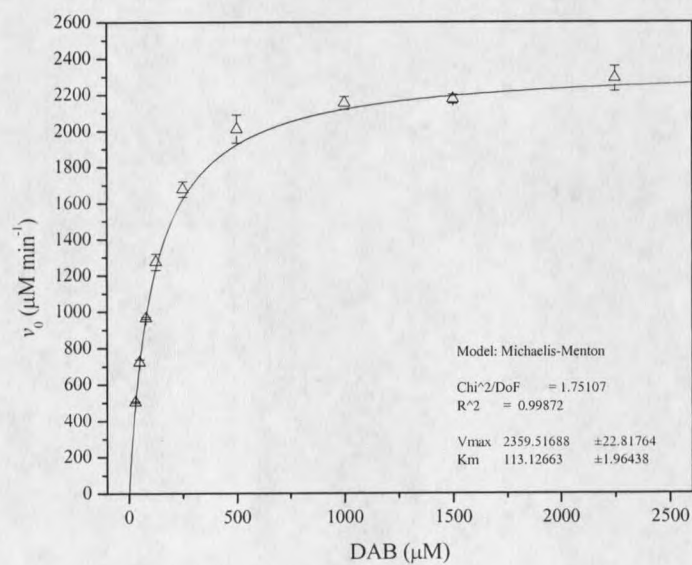


**Figure 27.** Putrescine fit to the substrate inhibition model

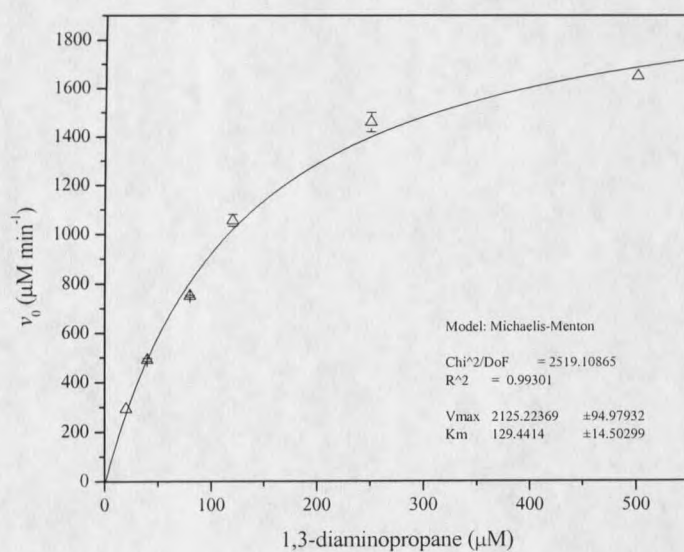




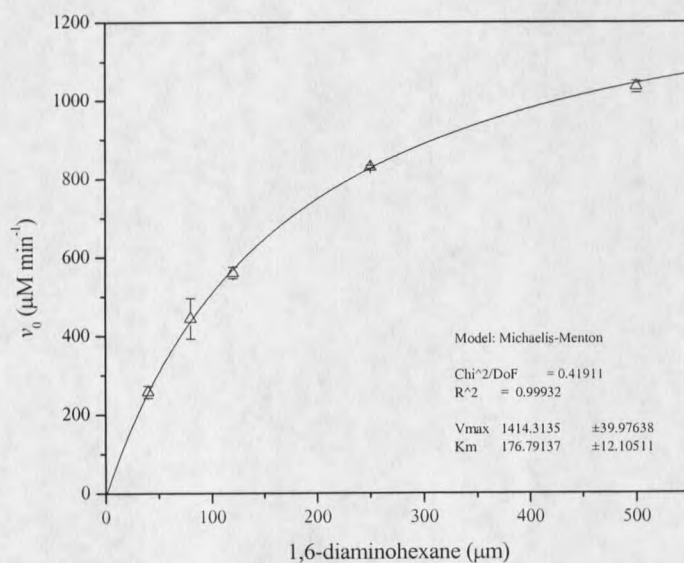
**Figure 28.** Cadaverine fit to the Michaelis-Menten model



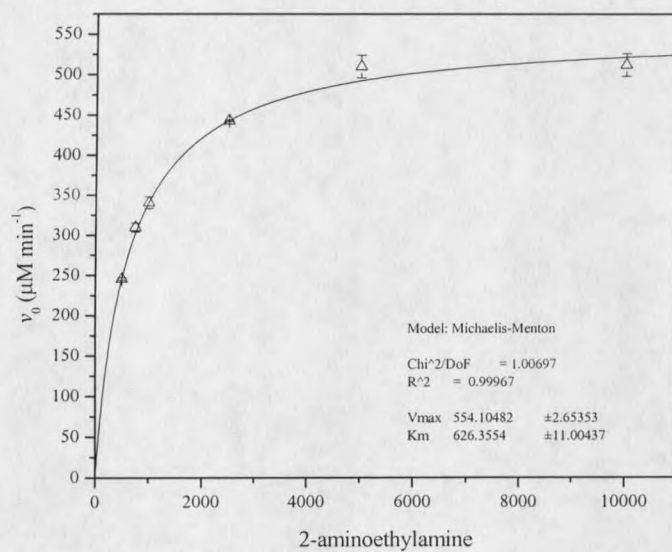
**Figure 29.** DAB fit to the Michaelis-Menten equation



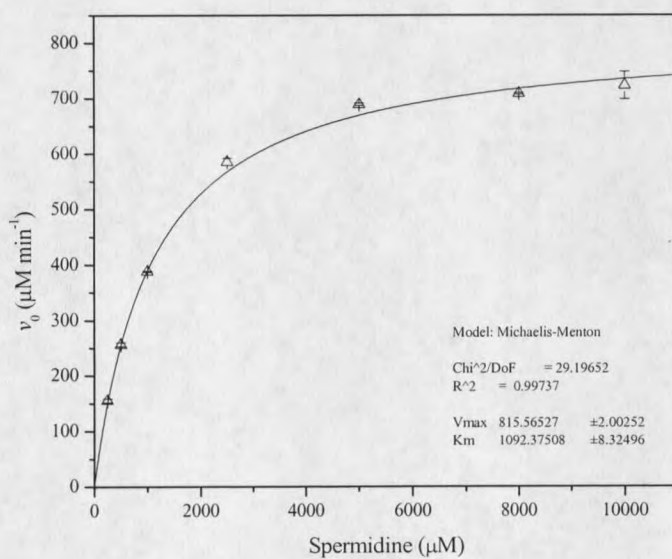
**Figure 30.** 1,3-Diaminopropane fit to the Michaelis-Menten equation



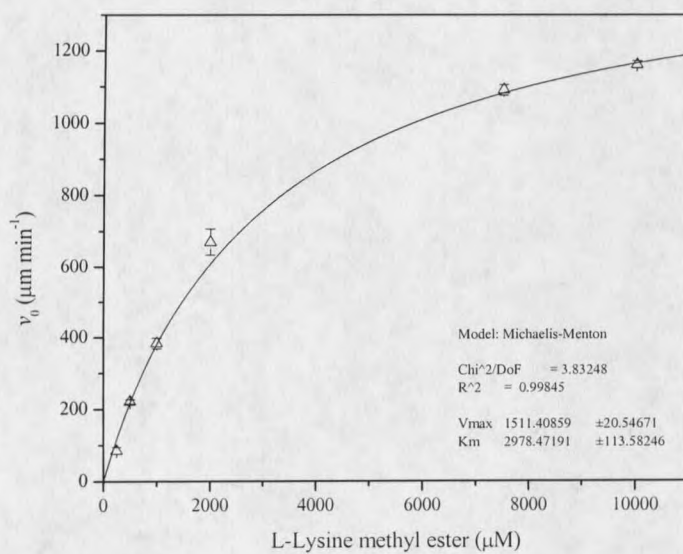
**Figure 31.** 1,6 Diaminohexane fit to the Michaelis-Menten equation



**Figure 32.** 2-Aminoethylamine fit to the Michaelis-Menten equation

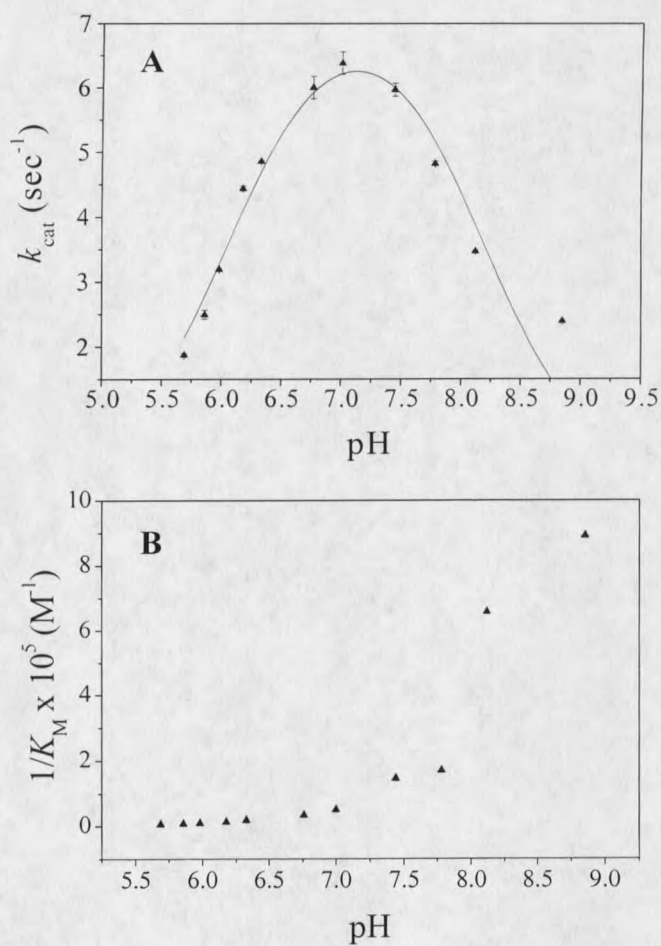


**Figure 33.** Spermine fit to the Michaelis-Menten equation



**Figure 34.** L-Lysine methyl ester fit to the Michaelis-Menten equation

The dependence of the steady state parameters for putrescine oxidation on pH was investigated and the results are shown in Figure 35. The data indicates an optimal pH of about 7. The data for  $k_{\text{cat}}$  against pH give a bell shaped curve and the two inflection points represent apparent pKa values for two ionizable groups.



**Figure 35.** pH dependence of steady state kinetic parameters for putrescine and rhKDAO: **A)**  $k_{\text{cat}}$  versus pH gives two apparent pKa values of 6.0 and 8.2; **B)**  $1/K_{\text{m}}$  versus pH

### Discussion

$K_M$  and  $k_{cat}$  were determined for several aliphatic diamines, the aromatic diamine DAB, and biologically important amines and polyamines. Initial rates were fit using the Michaelis-Menten equation or an equation for substrate inhibition. The steady state parameters were determined under the physiologically approximate conditions of 37 °C, pH 7.2, and an ionic strength of 150 mM.

Recombinant human DAO shows a substrate preference, as indicated by the specificity constant  $k_{cat}/K_M$ , for the diamines histamine (2-[4-imidazolyl]ethylamine) and 1-methylhistamine (1-methyl-4[ $\beta$ -aminoethyl]imidazole), the metabolite of histamine *N*-methyltransferase (E.C.2.1.1.8)). Agmatine, a metabolite of arginine, is next in the series. Note that  $k_{cat}$  for agmatine is three times higher than the turnover number for 1-methylhistamine.

The aliphatic diamines putrescine and cadaverine give  $k_{cat}/K_M$  values close to, but lower than, those of the agmatine. Histamine and 1-methylhistamine exhibit two of the three lowest turnover numbers, but note the turnover constants for all substrates investigated are quite similar and differ by less than a factor of ten. However, the determined  $K_M$  values vary by over three orders of magnitude, with the  $K_M$  for histamine about seven times less than that of any aliphatic diamine. The results of these experiments, namely that histamine and 1-methylhistamine are the preferred substrates, were completely unanticipated given the body of available literature, which inferred that the aliphatic diamines were the preferred substrates of human DAO (*vide infra*).



It must be kept in mind that the recombinant protein used for this work does not contain two copper ions and two organic cofactors per dimer; these experiments used rhKDAO with 1.42 copper ions and 1.04 titratable TPQ per dimer. Therefore the actual substrate turnover numbers for the natural hKDAO could be up to twice the values reported herein.

Earlier literature reported the substrate preference of human DAO solely determined by relative rates of oxidation, a necessary criterion given the limited enzyme available. Misleading conclusions regarding substrate preference were inevitable, given the difficulty in obtaining highly purified mammalian DAO, partial substrate inhibition, and the millimolar substrate concentrations used. Generally, cadaverine was reported as the substrate with highest rate of oxidation, up to nearly 1.5 times the rate for putrescine. Histamine was described as having only 17-50% the relative rate of oxidation as compared to putrescine [41,87,88]. Unfortunately, this has resulted in a predisposition to downplay or discount a possible role for diamine oxidase in the metabolism of histamine in much of the literature.

This work establishes a  $K_M$  for histamine equal to 3.4  $\mu\text{M}$  and a  $k_{\text{cat}}/K_M$  of 47  $\mu\text{M}^{-1} \text{ min}^{-1}$ . Previously reported  $K_M$  values for diamine oxidase purified from human tissues range from 1.5  $\mu\text{M}$  for the enzyme isolated from pregnancy serum to 19  $\mu\text{M}$  for the intestinal DAO [39,89,90]. The apparent  $K_M$  determined for 1-methylhistamine is 3.0  $\mu\text{M}$ , whereas earlier work reported 97  $\mu\text{M}$  [39,90]. It is interesting to note that aliphatic diamines and polyamines would be protonated at physiological pH and therefore be di- or



polycationic. Histamine, however, would only be about 8% present as the doubly charged species at pH 7.2.

The recombinant enzyme exhibits an apparent  $K_M$  for putrescine of 17  $\mu\text{M}$ . Previously reported values from purified human diamine oxidases range from 13  $\mu\text{M}$  to 83  $\mu\text{M}$  [39,88,90]. Hölttä *et al.* reported that human seminal plasma diamine oxidase displays a  $K_M$  of 100  $\mu\text{M}$  for spermine and 560  $\mu\text{M}$  for spermidine [88]. In contrast, we find an apparent  $K_M$  of 1.1 mM for spermidine, and that of spermine could not be determined with our experimental conditions. This discrepancy is perhaps due to contaminating enzymes in the seminal plasma enzyme purification or impurities in commercially available polyamines; in fact, Hölttä and coworkers described contaminating spermidine and putrescine in purchased spermine.

Agmatine, interestingly, was one of the first compounds described by Zeller in 1938 as a substrate for mammalian copper amine oxidases [91], but has received little attention since then. The results presented here are the first reports of steady state kinetic parameters for agmatine using a CAO from a mammalian source. This decarboxylated metabolite or the amino acid arginine has been reported to be oxidized by the pig kidney diamine oxidase, pig kidney diamine oxidase and the amine oxidases from the plants common pea and lentil [92,93].

Partial substrate inhibition was observed with histamine, agmatine and putrescine. Bardsley *et al.* have previously described substrate inhibition with pig kidney DAO and attributed this inhibition as a primary kinetic salt effect, suggesting enzyme and substrate association might be partially rate-limiting [46]. Data for these three substrates were fit

to the classical model for substrate inhibition, in which a second molecule of substrate binds to the enzyme-substrate complex to give a catalytically inactive complex. Histamine concentrations of up to 250  $\mu\text{M}$ , rhKDAO closely fits this model of substrate inhibition. However, at higher substrate concentrations the data deviate from this model. A similar behavior is shown for agmatine, and agmatine data above 125  $\mu\text{M}$  does not fit the model well. As described, the complete data sets can be fit using a scheme that incorporates the model of substrate inhibition and an addition pathway for enzymatic turnover.

A reasonable physical explanation is that there are two forms of rhKDAO in the protein sample, a high activity form and a low activity form that only becomes kinetically apparent at higher concentrations of substrate. A contaminant capable of amine oxidation is extremely unlikely given the source of the enzyme and the high degree of purification. Evidence has been given in the preceding chapter indicating that rhKDAO can aggregate to a small extent. Furthermore, in the final gel filtration step for purification of rhKDAO a small peak is sometimes noted that elutes at a higher molecular weight than the dimer and shows a low specific activity in regards to putrescine oxidation (data not shown). Thus rhKDAO aggregation provides an attractive hypothesis for a low activity form of the enzyme.

The striking difference in substrate kinetic behavior between histamine and 1-methylhistamine is noteworthy. Methylation of the imidazole nitrogen apparently abolishes the substrate inhibition observed with histamine. The  $K_M$  values for these two substrates are essentially identical and indicate that rhKDAO has a high affinity for these

two substrates. The enzyme is evidently unable to bind two of the ring-methylated derivatives, and therefore partial substrate inhibition is not observed.

The pH dependence of  $k_{\text{cat}}$  for putrescine is shown in Figure 35. The plot reveals a bell-shaped curve with apparent  $\text{p}K_{\text{a}}$  values of  $6.04 \pm 0.06$  and  $8.22 \pm 0.09$ . The lower  $\text{p}K_{\text{a}}$  is attributed to the ionization of the active site catalytic base *in the enzyme-substrate complex* (Asp373 in hKDAO). The higher  $\text{p}K_{\text{a}}$  likely represents the ionization of the product Schiff base. Klinman and coworkers have described detailed work on the pH dependence of steady-state parameters for the CAOs from bovine plasma and a yeast [45], and our data are consistent with their interpretation [94]. A plot of  $1/K_{\text{M}}$  versus pH reflects ionization potentials in the free enzyme and free substrate [86]. The reciprocal Michaelis constant for rhKDAO is relatively constant from pH 5.7 to pH 7.0 and increases at higher pH values. These data could not be used to determine apparent  $\text{p}K_{\text{a}}$ s, although the data are in agreement with the interpretation of Klinman and coworkers of the catalytic aspartate residue in the free enzyme with an unusually high apparent  $\text{p}K_{\text{a}}$  ( $\sim 8$ ) and dramatically decreasing by about two orders of magnitude upon substrate binding.

Two recent reports have suggested porcine kidney DAO is inhibited by heparin, either during prolonged binding on a heparin affinity column or through exposing the enzyme to free heparin [95,96]. To investigate the possible effects of heparin on rhKDAO and to determine whether this may be a general phenomenon of mammalian DAOs, the purified recombinant enzyme was incubated with an approximately two-fold excess of heparin over heparin-binding sites.  $K_{\text{M}}$  and  $k_{\text{cat}}$  values were not noticeably different from untreated protein for up to 72 hours (data not shown). Additionally, no

correlation between specific activity and length of time protein was bound to the heparin affinity column during enzyme purification was discerned.

Recombinant human kidney diamine oxidase shows a clear substrate preference for diamines, with the highest specificity constants ( $k_{\text{cat}}/K_M$ ) for histamine and 1-methylhistamine. It is also apparent that substrate specificity depends on the separation between the substrate's two amine groups, as well as other structural features. Although diaminopropane is a good substrate, the polyamine spermine, also with three methylene carbons between the primary amine nitrogen and the proximal secondary amine, is a very poor substrate with an oxidation rate nearly undetectable under the assay conditions. Spermidine, on the other hand, has four methylene carbons between one of the primary amines and the secondary amine and is a fair substrate, but with a specificity constant two orders of magnitude less than that of putrescine. L-L lysine methyl ester is a poor substrate, and no oxidation was observed with either L-lysine or N $\alpha$ -acetyl-L-lysine methyl ester. Therefore, it is extremely unlikely that the DAO catalyzed oxidation of peptidyl lysines would occur to a physiologically relevant extent *in vivo*.

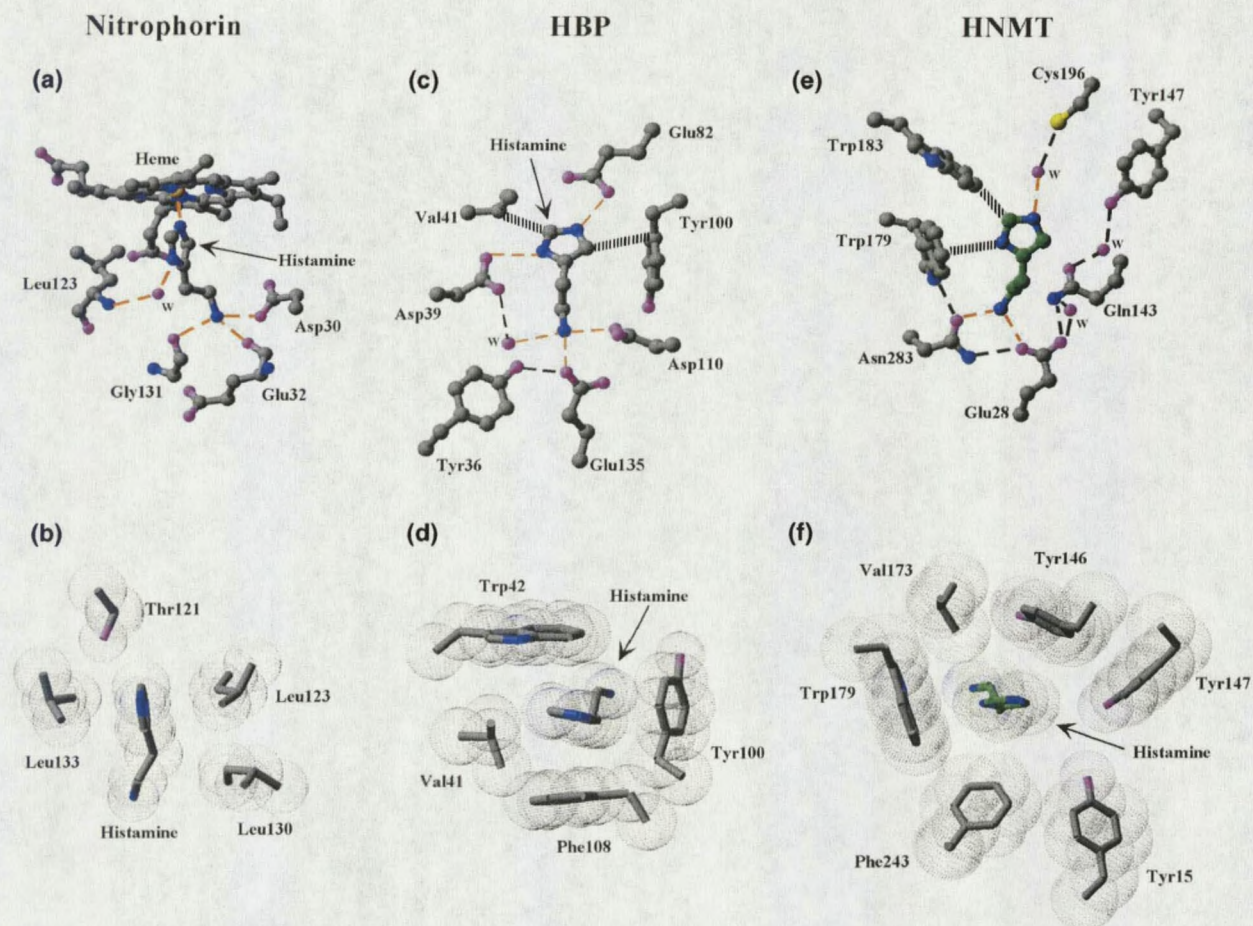
To date, three structures for histamine binding proteins have been solved and may provide insight into histamine binding in rhKDAO. Two are a nitrophorin and a histamine binding protein; both of these are secreted into the host by blood-sucking insects and ticks [97,98]. Once injected into the host, these two proteins serve to sequester histamine and block the host's inflammatory and immune response. The remaining crystal structure is for human histamine methyltransferase [99]; the role of this

particular enzyme in histamine metabolism will be discussed in the following chapter. Figure 36 shows the histamine binding sites for each of these proteins.

The terminal amine of histamine is oxidized and thus would covalently bond with TPQ to give the substrate-Schiff base. The imidazole ring would extend into the substrate channel and the substrate-enzyme interactions here are expected to be critical in substrate recognition. Nitrophorins contain a Fe(III)-heme prosthetic group to which the imidazole ring of histamine ligates, and can be ruled out as a model histamine binding hKDAO. In histamine binding protein both of the imidazole nitrogens are hydrogen bonded to two acidic residues. In contrast, human histamine methyltransferase has no acidic groups in the vicinity of the histamine's imidazole ring, a single water molecule hydrogen bonds to one of the nitrogen atoms. All three structures show the imidazole ring of histamine surrounded by hydrophobic residues.

Recombinant human kidney diamine oxidase can oxidize both histamine and 1-methylhistamine, and the  $K_M$  values for both substrates are  $\sim 3 \mu\text{M}$ . A binding mode with two acidic residues available to form hydrogen bonds can be ruled out. Binding interactions for rhKDAO can be deduced to involve either one acidic residue or none at all. Agmatine is also a very good substrate for the copper amine oxidase with a  $K_M$  value of  $13 \mu\text{M}$ . Under physiological conditions the guanidino group of agmatine would be completely ionized. One can then deduce with confidence that substrate binding "pocket" of human diamine oxidase contains a single acidic residue which would balance the positive charge on substrate and be available for hydrogen bonding.





**Figure 36.** Histamine binding in nitrophorin (a and b), histamine binding protein (c and d), and human histamine methyltransferase (e and f). (Figure from Horton et al. (2001) *Structure* 9,p843)

### Conclusions

Detailed substrate analysis for human kidney diamine oxidase has been presented for a variety of biogenic amines such as aliphatic diamine, polyamines, histamine and 1-methylhistamine, agmatine, and catacholeamines. Studies such as this is critical for any attempts to infer the potential physiological roles of this human enzyme and are only made possible by the availability of the highly expressed and highly purified recombinant protein.

Substrate preference, indicated by the specificity constant  $k_{cat}/K_M$ , has been determined. Histamine and the 1-methylated metabolite are most preferred and exhibit very low  $K_M$  values of about 3  $\mu$ M. Agmatine and the diamine putrescine and cadaverine are also highly preferred substrates.

The low to undetectable enzymatic activity towards spermidine and spermine would suggest that *in vivo* human diamine oxidase would most likely not be involved in the metabolism of these polyamines. Nor does it seem likely this enzyme would oxidize lysine, peptidyl lysine or catacholamine to any significant extent.



## DIAMINE OXIDASE AND HUMAN METABOLISM

### Introduction

Histaminase activity was first reported in 1929, when Charles Best noted the disappearance of histamine (2-[4-imidazolyl]ethylamine) in minced lung, liver and kidney tissues [100], noting the activity exhibited characteristics of an enzymatic system and suggesting the mechanism was likely oxidative. Incidentally, the assay employed by Best measured the blood pressure of anaesthetized cats to quantify histamine; quote "In a fairly sensitive cat it is possible to discriminate between doses of histamine which differ from each other by 15 p.c.". That work is generally accepted as the first description of a copper containing amine oxidase. In 1938, Zeller reported that histaminase also deaminated putrescine, cadaverine, and agmatine and therefore proposed renaming the enzyme diamine oxidase [91]. Since then copper amine oxidases have been described from bacteria, yeast and fungi, plants and multicellular animals. Several subsequent studies suggest cadaverine or putrescine is the preferred substrate for mammalian diamine oxidase [39,41,42,88,101,102].

Seven decades later the recombinant human diamine oxidase is available and allows detailed spectroscopic, biochemical and kinetic experiments. This chapter gives an overview of the metabolism of the preferred *in vitro* substrates of rhDAO, histamine, 1-methylhistamine, agmatine and putrescine.

### Histamine metabolism

Mast cells, enterochromaffin-like cells and nerve cells produce histamine. This potent pharmacological agent has profound biological effects including smooth muscle contraction, vasodilation, stimulation of adenylate cyclase activity in neurons, allergic response, and gastric acid secretion, all of which are mediated by specific G-protein-coupled receptors,  $H_1$ ,  $H_2$  and  $H_3$  [103]. Histamine produced by nervous tissues is a neurotransmitter [104]. Several studies have linked  $H_2$  receptor activity to cell proliferation in gastric carcinoma cell line and cultured melanoma cells [105,106]. One recent report links histamine with inflammation around surgically implanted biomaterials [107].

Circulating histamine is rapidly cleared with a  $t_{1/2}$  of 0.5-2 minutes [108], although no specific, high-affinity histamine transporter has been identified. Histamine degradation occurs through the actions of diamine oxidase and histamine *N*-methyltransferase (E.C.2.1.1.8); ring-methylation followed by side chain oxidation is thought to be the major pathway [51,109]. The product of histamine *N*-methyltransferase, 1-methylhistamine (1-methyl-4[ $\beta$ -aminoethyl]imidazole), is a known *in vitro* substrate for diamine oxidase [39,90,102], as well as a potent inhibitor of histamine *N*-methyltransferase. It follows that diamine oxidase contributes to histamine metabolism through oxidative chemistry and alleviation of product inhibition in histamine *N*-methyltransferase.

Substantial evidence links gastrointestinal histamine to anaphylaxis and allergies, inflammatory diseases including ulcerative colitis and Crohn's disease, and histamine poisoning. Rats orally administered histamine exhibit substantially increased excretion of unmetabolized histamine when treated with diamine oxidase inhibitors, and pigs fed histamine and diamine oxidase inhibitors exhibit severe symptoms of histamine poisoning [110]. One recent study with human subjects describes a correlation between food allergies and low gastrointestinal diamine oxidase activity, suggesting a reduced capacity for histamine oxidation, in addition to the well-known increase in histamine levels, might be a critical factor in the pathology of this condition. These studies suggest histamine *N*-methyltransferase might not completely compensate for diminished diamine oxidase activity in histamine catabolism.

Early investigations into mammalian histamine metabolism often relied on identifying end products from urine and feces following injection of [ $^{14}\text{C}$ ]-labeled histamine [111]. In humans, methylimidazoleacetic acid is usually the major metabolite detected, accounting for 19-53% of recovered radioactivity. A significant quantity, 9-44%, of imidazoleacetic acid is also reported, along with 2-24% 1-ribosylimidazole-4-acetic acid. The immediate aldehyde product of diamine oxidase is undoubtedly further oxidized *in vivo* by an endogenous aldehyde oxidase or dehydrogenase [111,112]. Schayer mentions that injected radioactivity is not fully recovered, and imidazoleacetaldehyde is not identified in urine; therefore it is possible the reactive aldehyde undergoes nonoxidative reactions *in vivo* and is subsequently retained. Oral administration of radiolabelled histamine to one human subject resulted in 64% recovery

of radioactivity, identified as methylimidazoleacetic acid. In contrast, a later work suggests imidazoleacetic acid is the predominant end product of orally-administered histamine in humans [113].

Mammalian diamine oxidases are found in several tissues, with the highest activities reported in placenta, small intestine, and kidney, and also present in liver, lung, fibroblasts, salivary glands, and seminal fluid. Low basal levels of diamine oxidase are present in serum and thought to derive from the gastrointestinal tract [2]. However during pregnancy, diamine oxidase in circulation increases by 500-1000 fold and is likely placental in origin [114]. Intravenous injection of heparin increases circulating diamine oxidase in humans and rodents [115-117]. The intestine is known to be the major source of heparin-induced circulating diamine oxidase activity in the rat [118]. Thus diamine oxidase in plasma represents a rare example of a circulating protein not originating from liver.

Diamine oxidase localization in the intestinal tract mucosa layer, as expected for an enzyme serving a protective function, was first described in 1942 and supported by several later studies [39,119-121]. Enterocytes at the tips of intestinal villa exhibit the highest concentrations of diamine oxidase, and those in the crypt regions apparently have very little of the enzyme present [122-124]. The oxidase has been suggested to be transported from enterocytes to intestinal vascular binding sites, from where it is susceptible to being displaced by heparin and entering circulation [124]. Daniele and Quaroni investigated human intestinal diamine oxidase secretion and cellular localization [125]. Diamine oxidase activity is both constitutively and directionally secreted, likely

via the default secretory pathway in this cell type, and the rate of secretion is rapidly and significantly enhanced upon heparin treatment. Additionally, immunofluorescence staining of human small intestine sections confirms diamine oxidase localization in the basolateral aspects of enterocytes at the apical tips of villi, with no or little staining in crypt regions, lamina propria, underlying muscular layers, blood vessels or serosa. Punctate fluorescence observed in the lamina propria suggests low levels of diamine oxidase associated with microvasculature. Daniele and Quaroni suggest heparin increases release of intestinal diamine oxidase from epithelial cells, and the enzyme could then enter the circulatory system. The cellular and subcellular localization of diamine oxidase in porcine small intestine shows a distribution consistent with its being sequestered in secretory vesicles of enterocytes [126]. Consequently the site or sites of histamine metabolism by intestinal diamine oxidase is unclear; ingested histamine or its methylated form may be metabolized in intracellular compartments, in extracellular surfaces of epithelium, at microvascular structures inside villi, or even in more distal vascular tissue.

Immunohistochemical staining has very recently been used to localize histamine *N*-methyltransferase in guinea pig tissues, and its distribution is quite different from that of diamine oxidase [127]. Intestinal tissue exhibits the highest methyltransferase density in villi epithelial cells, and no obvious difference is noted between apical and crypt regions. This enzyme is also widely distributed in deeper tissue layers. Furthermore, histamine *N*-methyltransferase appears more or less evenly distributed in the cytoplasm of endothelial cells. The methyltransferase, based on location, seem perfectly positioned

for the initial degradation of ingested histamine following cellular uptake. However, the *in vivo* evidence presented above suggests histamine *N*-methyltransferase cannot fully replace diamine oxidase.

The human multiple tissue expression array shown previously suggests transcriptional levels for this copper-containing amine oxidase are very high in kidney tissues. Cellular and subcellular localization of diamine oxidase in porcine kidney is somewhat similar to that in intestinal tissue; both are present in reabsorptive enterocytes and appear to be in vesicular structures closely associated with the basal and lateral cell membrane [126]. The enzyme is confined to proximal tubules in the kidney, where the majority of reabsorption occurs. The physiological role of this enzyme is quite likely for the oxidative degradation of reabsorbed histamine, 1-methylhistamine, and perhaps putrescine, in order to control circulating levels of these bioactive amines. Whether or not this occurs intracellularly or extracellularly in kidney tubules is unknown.

Circulating diamine oxidase levels are normally low in humans, except during pregnancy. Although considerable evidence supports intestinal diamine oxidase actively metabolizing ingested histamine, a role for this enzyme in the distal vasculature is still largely speculative. Baenziger and coworkers, following up on their prior observation of recovery of  $H_1$  receptor activity being dose-dependent on amine oxidase activity present in fetal calf serum, have investigated [ $^3H$ ]histamine degradation through identification and quantification of cell-associated metabolites in tissue cultures of human fibroblasts and vascular endothelial cells [51]. Serum-free cell cultures accumulate 1-methylhistamine, the product of endogenous histamine *N*-methyltransferase.

Methylimidazoleacetic acid was not detected and therefore cell-associated methylhistamine was not available for oxidation by the endogenous mitochondrial flavin-containing monoamine oxidases (E.C. 1.4.3.4), which are known to oxidize methylimidazole *in vitro* and implicated in rat brain *in vivo* oxidation [128,129]. The specific portion of histamine uptake is only 30-50% of total uptake; non-specific means, such as by pinocytosis, are considerable. In the presence of fetal calf serum diamine oxidase, the cells exhibited a 10-fold increase in total histamine uptake, and the cell-associated metabolite was identified as methylimidazoleacetic acid. Furthermore, a significant increase in specific uptake was noted; up to 88% of total uptake was by specific means. Partially purified diamine oxidase increases histamine uptake and accumulation as methylimidazoleacetic acid in a dose-dependent manner, confirming histamine metabolism in cell culture as a function of diamine oxidase activity. The maximal rate of histamine uptake with exogenous human placental diamine oxidase is 2-7 fold greater than with the bovine enzyme. Uptake and accumulation of histamine metabolites was shown to be histamine concentration dependent, saturatable, and to have half-maximal uptake at physiologically relevant concentrations of less than 3  $\mu$ M histamine [130]. Additionally, histamine metabolite uptake is saturatable with respect to diamine oxidase activity. Baenziger and coworkers concluded histamine uptake is directly coupled to its sequential enzymatic metabolism, first ring-methylation by histamine *N*-methyltransferase followed by the optional step of primary amine oxidation by copper-containing diamine oxidase. Both histamine and diamine oxidase are proposed to bind specific and finite cell surface receptors. Evidence for sequential histamine



metabolism is provided by the following: cell associated radiolabelled imidazoleacetic acid was not detected; imidazoleacetic acid is not a substrate for histamine *N*-methyltransferase [131]; and incubation with the histamine *N*-methyltransferase inhibitor SKF91488 (homodimaprit, *S*-[4-(dimethylaminobutyl)]isothiorurea) decreased histamine uptake in a dose-dependent manner and at 50  $\mu$ M totally eliminated the specific component of histamine uptake. It should be noted that although imidazoleacetic acid is not a substrate for histamine *N*-methyltransferase, the direct product of histamine oxidation by diamine oxidase, imidazoleacetaldehyde, has not been investigated as a possible substrate. Furthermore, SKF91488 also inhibits diamine oxidase, although with a 70-fold higher reported  $K_i$  [132,133]. However, these inhibition studies relied upon histamine *N*-methyltransferase from soluble extracts of ammonium sulfate precipitated rat kidney homogenate and diamine oxidase from soluble fraction of rat ileum homogenate.

Additional evidence for concerted histamine metabolism and uptake was provided when Haddock *et al.* reported that neither excessive 1-methylhistamine nor methylimidazoleacetic acid could block endothelial [ $^3$ H]histamine uptake [134]. In the same work, the authors report intracellular accumulation of 1-methylimidazole is unaffected by  $\text{Na}^+$  or  $\text{Cl}^-$  deprivation. In contrast, cell cultures with exogenous diamine oxidase exhibit a strong  $\text{Na}^+$ - and  $\text{Cl}^-$ -dependent accumulation of methylimidazoleacetic acid. Furthermore, experiments with inhibitors indicate the specific degradative uptake pathway is distinct from the well-characterized  $\text{Na}^+$ -dependent or independent chloride-bicarbonate exchanger,  $\text{Na}^+/\text{K}^+$ -ATPase, or  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  ion co-transporter systems.

Baenziger *et al.* further investigated the proposed diamine oxidase-specific cell surface receptor [135]. Results suggest the diamine oxidase receptor is upregulated in cultures grown in low diamine oxidase activity media, and histamine uptake increases about 10-fold over controls, immediately after adding diamine oxidase. Diamine oxidase binding kinetics is apparently biphasic with a rapid initial binding component followed by a slower phase. The cell-associated diamine oxidase is heparin-displaceable and therefore most likely the heparin-binding consensus sequence interacts with cell surface heparan sulfate proteoglycans. Interestingly, bound diamine oxidase in cell cultures at low cell density is wholly displaceable by treatment with heparin, whereas cell culture at high density retains approximately half of cell-associated oxidase activity. Thus high-density cell cultures apparently sequester some portion of diamine oxidase from heparin accessibility, possible via endocytotic uptake. The investigators demonstrated diamine oxidase binding to cells in culture is stable to mild acid treatment and pH levels encountered in early to mid endocytic organelles. Furthermore, cells treated with a protein kinase C activator, known to modulate cell signaling and membrane trafficking pathways, doubled histamine uptake in a cell-density dependent manner.

Copper-containing diamine oxidase very likely plays an important role in histamine metabolism and rapid clearance from the vascular system. Potential sources of this diamine oxidase include endothelial cells of the intestinal tract and neutrophils [136]. Histamine degradation and associated uptake by vascular endothelial cells likely proceeds via either methylation alone or a diamine oxidase rate-amplified pathway. The enhanced rate of histamine uptake is remarkable. For example, Baenziger *et al.* calculated

histamine uptake in endothelial cell culture without an exogenous diamine oxidase as 29,000 molecules/min/cell. In the presence of diamine oxidase the rate can increase to 1.6 million molecules/min/cell. Substrate specificities presented in this work indicate histamine and 1-methylhistamine are more or less equally preferred by human kidney diamine oxidase. This raises questions about an obligatory sequential enzymatic process of histamine methylation followed by oxidation coupled to uptake, or at least indicating further investigation of imidazolacetaldehyde metabolism is warranted.

Modulation of histamine degradation and uptake is apparently sensitive to and regulated by various environmental factors, allowing fine-tuning of the system as required in vasculature microenvironments. Study of this system is complicated by multiple means of histamine uptake; both specific and non-specific pathways are in operation, and components of the specific pathway other than histamine *N*-methyltransferase and diamine oxidase remain to be identified. Furthermore, diamine oxidase may not necessarily bind to only one specific cell surface receptor; the heparin binding site could possibly bind to any available heparan sulfate proteoglycan, further confounding analysis.

Substrate specificity ( $k_{cat}/K_m$ ) presented in this work for histamine and 1-methylhistamine gives apparent  $K_m$  values near 3  $\mu$ M. Partially purified human kidney histamine *N*-methyltransferase has an apparent  $K_m$  for histamine of 13-20  $\mu$ M [137], in close agreement with values reported for the mouse and ox brain enzymes [138,139]. No signal sequence is present in the primary sequences of histamine *N*-methyltransferase and specific staining or SDS/PAGE do not indicate glycosylation [137,139]. Thus there is no

reason to believe histamine *N*-methyltransferase is an extracellular enzyme. In fact, one source of this enzyme is human red blood cells, not plasma [140], and immunohistochemical localization supports an intracellular localization [127]. Therefore it is difficult to reconcile an obligatory sequential pathway of enzymatic histamine degradation coupled to clearance from circulation proceeding via methylation by an apparently intracellular enzyme followed by oxidation by a circulating or cell-surface receptor-bound diamine oxidase. Furthermore, direct oxidation of circulating histamine by diamine oxidase to methylimidazoleacetaldehyde is extremely likely, and the subsequent fate of this metabolite remains to be elucidated.

Histamine present in mast cell granules is complexed to heparin [141], and intravenous injection of heparin is known to release diamine oxidase into circulation [117]. Although direct evidence is lacking, it is possible that histamine from mast cells is linked via the co-release of heparin, which accounts for about 30% of the dry weight of granules, to regulation of diamine oxidase levels in circulation and subsequent availability for binding to distal tissues, thus providing an additional level of fine control in histamine metabolism.

### Agmatine

Agmatine is a recently discovered and an exciting new addition to mammalian biochemical pathways. The decarboxylation of arginine by arginine decarboxylase to agmatine has long been a recognized process in bacteria, plants and invertebrates [142]. In these organisms agmatine is hydrolyzed to putrescine which is then used to synthesize

the polyamines spermidine and spermine. The enzyme responsible for the catalytic hydrolysis is agmatine ueryl hydrolase, common name agmatinase. In bacteria, plants and invertebrates agmatine serves as a precursor for polyamine synthesis; the analogous pathway in mammals uses ornithine decarboxylase to generate putrescine.

Agmatine was not considered to play a role in human metabolism until 1994 when it was conclusively identified from mammalian brain tissues [143]. Since then both arginine decarboxylase and agmatinase have been found in the mammalian brain [144]. Agmatine was also identified into several mammalian tissues [145]. Agmatine has been proposed to be a ligand for imidazoline receptors but its mechanism of action still remains undetermined [146]

In brain tissue agmatine is stored in synaptic vesicles in spatially defined areas, is actively taken up by cells, is released by depolarization and is at concentrations in the same range as common neurotransmitters. Thus agmatine is perhaps a newly discovered neurotransmitter [147]. Furthermore, it induces the release of luteinizing hormone releasing hormone from the hypothalamus and enhances morphine analgesia [148,149].

In peripheral tissues agmatine is thought to modulate the metabolism of insulin and glucose, cause the release of adrenaline and noradrenaline, and block the proliferation of vascular smooth muscle cells [150-152].

Diamine oxidase has been suggested to catabolize agmatine in renal tissues, but in the brain agmatinase is thought to be the means of degradation [153,154]. The expression profile described in this work indicated human diamine oxidase is expressed in certain regions of the human brain, and therefore diamine oxidase may also be

important in agmatine metabolism in this organ. In peripheral tissues agmatine would certainly be a prospective substrate for diamine oxidase, especially in light of the *in vitro* kinetic parameters determined herein.

### Putrescine and Polyamines

Putrescine deamination is the rate-limiting step in catabolism of the polyamines spermidine and spermine [155]. These polyamines are found in millimolar concentrations in cells [156] and are important in cell proliferation and differentiation, likely by affecting transcription and translation [2]. Total cellular polyamine levels are regulated through biosynthesis, uptake, degradation and excretion [157], therefore diamine oxidase may play an important role in regulating cell growth, differentiation, tumor growth and programmed cell death.

Diamine oxidase is considered to be involved in polyamine metabolism and is likely associated with rapidly proliferating cells, potentially influencing such critically important processes as cell division and differentiation [155,158]. The remarkably increased levels of circulating diamine oxidase levels during pregnancy [114] is thought to be placental in origin and to function in preventing fetal putrescine, and perhaps histamine, from entering the maternal circulation [159]. Diamine oxidase expression is inducible by renal and cardiac hypertrophy, in liver after partial hepatectomy, and in response to brain injury. In all these examples increased diamine oxidase activity follows a local increase in putrescine levels [155]. Furthermore, administered putrescine or spermidine in rats causes an increase in diamine oxidase activity, dependent on *de novo*

protein synthesis [160]. The tissue source of diamine oxidase in the brain is uncertain. Our results indicate very little mRNA is present in brain tissues, and that which is present is confined to the pons and parietal lobes. Diamine oxidase gene expression might be upregulated as needed or, alternatively, the enzyme may be delivered to the brain by the vascular system.

Intestinal mucosa tissue undergoes rapid and continual cell proliferation; conditions which would appear to require diamine oxidase for putrescine metabolism and regulation of polyamine catabolism. However, intestinal endothelial cells proliferate in the crypt regions and then migrate towards villa tip. As previously discussed, diamine oxidase is localized almost exclusively on the apical tips of intestinal villi and not in the crypt regions where rapid cell division occurs.

Polyamine levels are quite high in human seminal plasma, approaching 2 mM putrescine, 1 mM spermidine, and 21 mM spermine [161]. In fact, Leeuwenhoek first observed microcrystalline polyamines in seminal fluid during the 17th century, and the common names spermine and spermidine reflect their abundance. Considerable diamine oxidase activity can also be found in seminal plasma [88]. Physiologically relevant diamine oxidase-catalyzed oxidation of putrescine and spermidine in human seminal fluid is strongly supported by the high local concentrations of these amines and the  $K_m$  values reported herein (20  $\mu$ M and 1.1 mM, respectively). Our results suggest spermine oxidation would be minor, consistent with the reported relative polyamine levels in seminal plasma. Our results indicate the prostate gland expresses diamine oxidase mRNA and is therefore the likely source of this enzyme in seminal fluid. Diamine



oxidase is apparently not significantly expressed in testis, a tissue with rapid cell division, again suggesting the oxidase may not be universally required for immediate putrescine degradation in areas of rapid cell multiplication but rather required at a later time.

The biological consequences of polyamine oxidation in semen remain controversial, although the immediate aldehyde products are known cytotoxic agents. Hölttä *et al.* found a negative correlation between diamine oxidase activity and spermatozoa motility, and suggested the products of polyamine oxidation may be responsible for this observation [88]. Other investigators reported decreased spermatozoa motility in the presence of high diamine oxidase activities, although giving conflicting results for the enzyme's relationship to total sperm counts [162] {Crabbe & Kavanagh 1977 #1529}. Interestingly, inhibitors of diamine oxidase have also been suggested to adversely affect spermatozoa motility. Polyamine oxidation in human seminal plasma has been suggested to contribute to cytotoxicity against leukocytes and to result in deleterious *in vivo* immunosuppression. However, the cytotoxic effects required the addition of fetal bovine serum, an exogenous source of diamine oxidase [163,164]. Bartkus and Ablin have subsequently provided evidence and argued the observed cytotoxicity is an artifact [163].

### Conclusions

The available information suggests copper-containing diamine oxidases are quite likely multifunctional enzymes in mammalian species, with specific physiological roles depending on spatial and temporal expression. In general, diamine oxidase appears to

function in the degradation of endogenous and exogenous histamine and putrescine. Further work is required to clarify a potential role in agmatine metabolism. Histamine metabolism and regulation is obviously very complex, with multiple levels of control available. Alternative pathways for histamine degradation are available, as one could expect for the critical process of regulating this potent bioactive agent. Considerable evidence supports a link between diamine oxidase and putrescine, although the details of diamine oxidase involvement in polyamine metabolism are not altogether clear.

## INHIBITION OF rhKDAO BY PHARMACEUTICALS

Introduction

The availability of rhKDAO allows for the first time the detailed study of potential inhibitors against the human enzyme. Especially attractive for kinetic analysis are pharmaceutical compounds in use and compounds that are being developed as potential pharmaceuticals. Prior investigations have been greatly disadvantaged by the difficult, or more likely, impossible task of obtaining human copper amine oxidases in sufficient quantities, and thus other animal species were often used as the enzyme source. Furthermore many investigations relied on "dirty" enzyme, sometimes tissue homogenates, due the difficulty in purification of copper amine oxidases from natural animal sources.

Many compounds currently used in human medicine or in the process of development for potential therapeutic use have been shown to inhibit copper amine oxidases. These compounds include antibiotics against bacteria, fungi and protozoa, and inhibitors of amine metabolism for antiproliferative agents for tumors and leukemia [165-172].

The modulation of polyamine metabolism is an attractive option for control of cell proliferation and tumor growth, and diamine and polyamine derivatives show potential for the development of therapeutics[173-177]. A recent report describes the progress towards the synthesis and development of polyamine analogues that have further been modified so that the compounds are specifically recognized by a trypanosomal transporter [178].

Several diamidines and diguanidine compounds are potent inhibitors of amine oxidases [166,167,179]. Schindler and Bardsley investigated the inhibition of aliphatic guanidinium compounds with chain lengths from two to twelve carbons on human kidney diamine oxidase purified from the natural source. All the guanidinium compounds were demonstrated as potent noncompetitive inhibitors with inhibition constants ranging from 20  $\mu$ M to 150 nM [180]. Bardsley and Ashford later reported porcine KDAO inhibition by aliphatic bisguanidinium compounds with three to eight linking methylenes. Noncompetitive inhibition was described, except for 1,4-butyldiguanidinium, which exhibited competitive inhibition with a  $K_i$  value of 30  $\mu$ M.

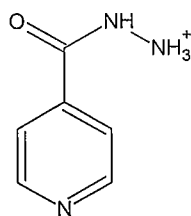
Antagonists of the three subtypes of histamine receptors have found widespread clinical use and continue to be attractive targets for the pharmaceutical research.  $H_1$  receptors are involved in inflammation and allergic responses, thus specific antagonists have considerable therapeutic value and are commonly used (antihistamines) [103,181].  $H_2$  receptors induce gastric acid secretion and selective antagonists are used in the treatment of various types of ulcer [181-183]. Reports suggest that activation of  $H_2$  receptors induce cell proliferation, perhaps via *c-jun* and *c-fos* activation [105,106,184]. Interestingly,  $H_2$  receptor antagonists have also been reported to increase cell proliferation [185]. The  $H_3$  type receptor is found in the brain, and is involved in the regulation of histamine release in brain tissues and regulates inflammation response [186]. Given the catalytic activity of rhKDAO towards histamine, it is not unreasonable to suspect some histamine receptor antagonists, chemical mimics of histamine, may also bind specifically in the active site of diamine oxidase and be potent inhibitors of the enzyme.

Five compounds, described below, that are used pharmaceutically were selected for the initial investigations into their inhibitory effects against recombinant human diamine oxidase.

Isoniazid is an antibacterial, specifically used clinically as an antimycobacteria agent. It is widely prescribed as an orally administered drug used to treat tuberculosis infection and, occasionally, to prevent its reoccurrence. Side effects include liver impairment and peripheral neuropathy [181].

Cases of histamine poisoning induced by consumption of foods high in histamine content, such as bonito fish and cheese, are described in the literature for patients in the course of isoniazid therapy [187,188]. Additionally, in rats the major metabolite of administered radiolabelled histamine has been reported to be imidazoleacetic acid (60% of the recovered radiolabel), and the coadministration of isoniazid substantially increased

the excretion of unmetabolized, radiolabelled histamine [110].



isoniazid

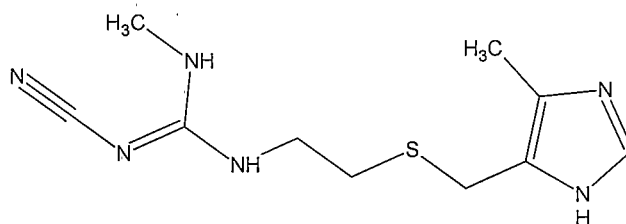
isonicotinohydrazide

This evidence for isoniazid interfering with the first oxidative step of normal rat histamine catabolism, the oxidative deamination by DAO, makes isoniazid an attractive target for rhKDAO inhibition studies.

**Figure 37.** Isoniazid

Cimetidine is a potent histamine  $H_2$  receptor antagonist and a type  $I_1$  imidazoline receptor agonist. This compound is widely used in the treatment of ulcers [189-191]. The active ingredient in the anti-peptic ulcer drug Tagamet is cimetidine.

Binding to  $H_2$  histamine receptors does in itself suggest cimetidine may also compete with histamine for binding on rhKDAO. There are also suggestive indications of hKDAO inhibition in the literature. A 25% decrease in human blood diamine oxidase activity after incubated with cimetidine has been reported [192]. Metaye et al. reported an  $IC_{50}$  of 380 micromolar for cimetidine against diamine oxidase activity in human amniotic fluid [193].

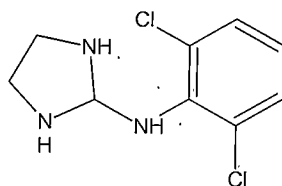


cimetidine

*N'*-cyano-*N*-methyl-*N'*-(2-[[4-methyl-1*H*-imidazol-5-yl)methyl]thio]ethyl)guanidine

**Figure 38.** Structure of cimetidine

Clonidine is an anti-hypertension drug used in the treatment of high blood pressure. Its physiologic action is as an agonist of  $\alpha_2$  and imidazoline receptors [181,194-197]. Clonidine has been reported as a competitive inhibitor of porcine kidney diamine oxidase and of the plant *Lens culinaris* copper amine oxidase, both enzymes give  $K_i$  values of about 600  $\mu M$  [198,199].

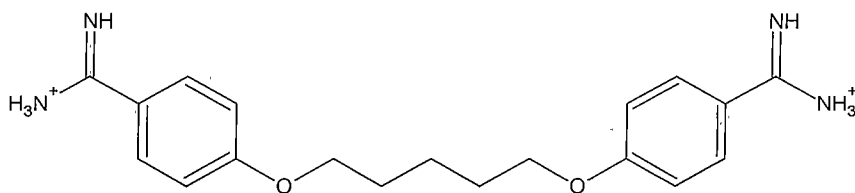


clonidine

*N*-(2,6-dichlorophenyl)imidazolidin-2-amine

**Figure 39:** Structure of clonidine

Pentamidine, an aromatic diamidine, is an anti-protozoal agent effective against *Pneumocystis carinii* and clinically used as pentamidine isethionate in the treatment of *P. carinii* pneumonia (PCP). The mechanism of action is not well understood, but *in vitro* experiments suggest pentamidine interferes with protozoal DNA, RNA, phospholipid and protein synthesis. PCP is a common and very serious complication in persons with compromised immune systems, such as cancer patients, transplant recipients and patients with AIDS [179,181,197,200-202]. Pentamidine is also occasionally used for the treatment of the protozoal infections that cause Leishmaniasis (kala-azar, transmitted by sandflies) and trypanosomiasis (African sleeping sickness, transmitted by the tse-tse fly). Pentamidine has also been characterized as a neuroprotective agent, an inhibitor of brain nitric oxide synthase and a N-methyl-D-aspartate (NMDA) glutamate receptor antagonist [203,204].



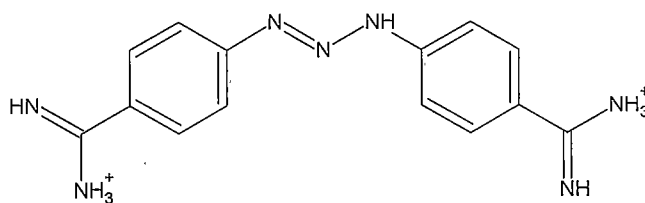
Pentamidine

1, 5 -bis(p-amidinophenoxy)pentane

4-[(5-{4-[amino(imino)methyl]phenoxy}pentyl)oxy]benzenecarboximidamide

**Figure 40.** Structure of pentamidine

Berenil is chemically classified as a diamidine and, along with pentamidine, belongs to the group of benzylamidine derivatives. Berenil is an effective anti-protozoal agent and is used to treat *Trypanosoma* infections. This compound is unique in this work, by reason of being the only compound investigated that is not used in human medicine – its use is found in the treatment of dogs, sheep, horses and cattle as a trypanocide.



Berenil

Diminazene

4-((1E)-3-{4-[amino(imino)methyl]phenyl}triaz-1-enyl)benzenecarboximidamide

**Figure 41.** Structure of berenil



### Experimental Procedures

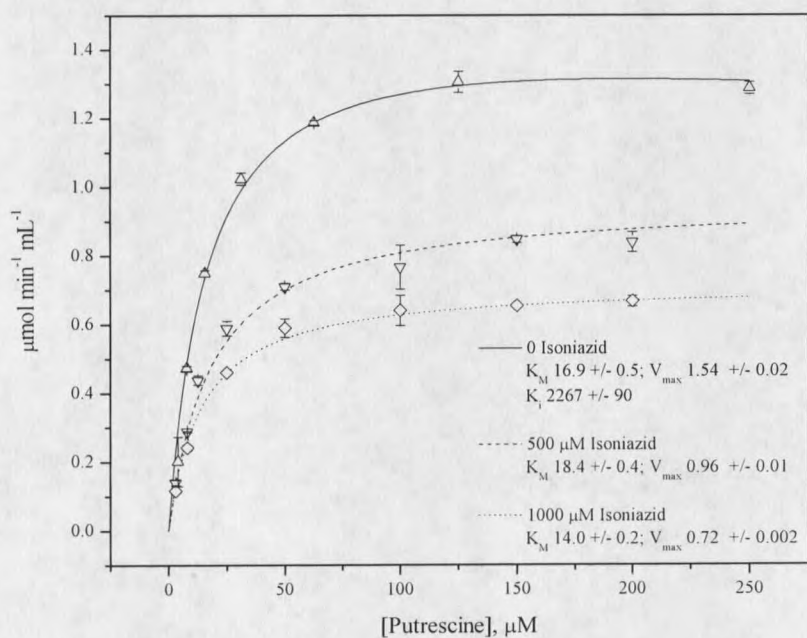
The assay method has previously been described in detail. Briefly, the horseradish peroxidase coupled assay for diamine oxidase was used and the formation of the quinoneimine dye was monitored at 498 nm. The buffer system used was 50 mM HEPES, pH 7.2, with the ionic strength increased to 150 mM by the addition of KCl. Vanillic acid, 4-aminoantipyrine, and the potential inhibitors were incorporated into the buffer, which was freshly prepared before each set of experiments. All potential inhibitor compounds were purchased through Sigma-Aldrich. All assays were at 37 °C and used 10  $\mu$ L of 46.7  $\mu$ M rhKDAO with a specific activity of 0.96 I.U./mg (determined by the standard horseradish peroxidase-ABTS assay previously described). The reaction was initiated by the addition of 100  $\mu$ L of freshly prepared putrescine substrate stock solutions, and the total reaction volume was one mL. None of the compounds tested were turned over by rhKDAO under these conditions.

Data were collected for the first 45 seconds of the reaction and initial rates were determined. All data analysis used Origin 6.0 software (Origin Lab, Corp.). Plots of initial velocity against putrescine concentration were fit to either the Michaelis-Menten equation or the equation for simple, competitive substrate inhibition. The fits to data were then used to generate double reciprocal plots (Lineweaver-Burk plots).

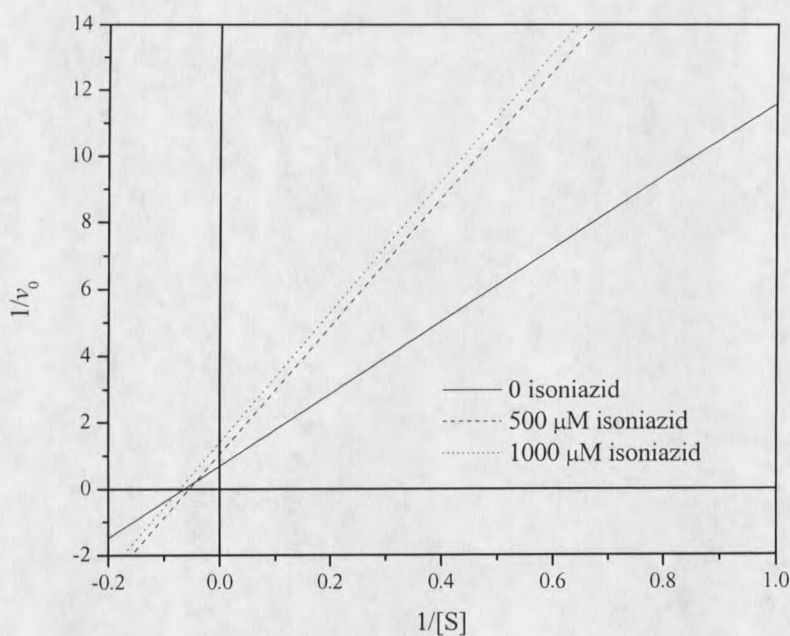
## Results

### Isoniazid.

The data and fit Michaelis-Menten equation are given in Figures 42. Figure 43 shows the double reciprocal plots. The apparent  $K_M$  values are  $18.4 \pm 1.0 \mu\text{M}$  and  $14.0 \pm 0.2 \mu\text{M}$  for 500  $\mu\text{M}$  and 1mM isoniazid, respectively.  $V_{\text{max}}$  values are  $0.96 \pm 0.01 \text{ I.U. mL}^{-1}$  for 500  $\mu\text{M}$  isoniazid and  $0.72 \pm 0.002 \text{ I.U. mL}^{-1}$  for 1 mM isoniazid. The assays were also done in the absence of isoniazid. Parallel lines shown in the Lineweaver-Burk plot are indicative of uncompetitive inhibition [205].



**Figure 42.** Isoniazid inhibition plot of initial reaction velocity against substrate putrescine concentration.

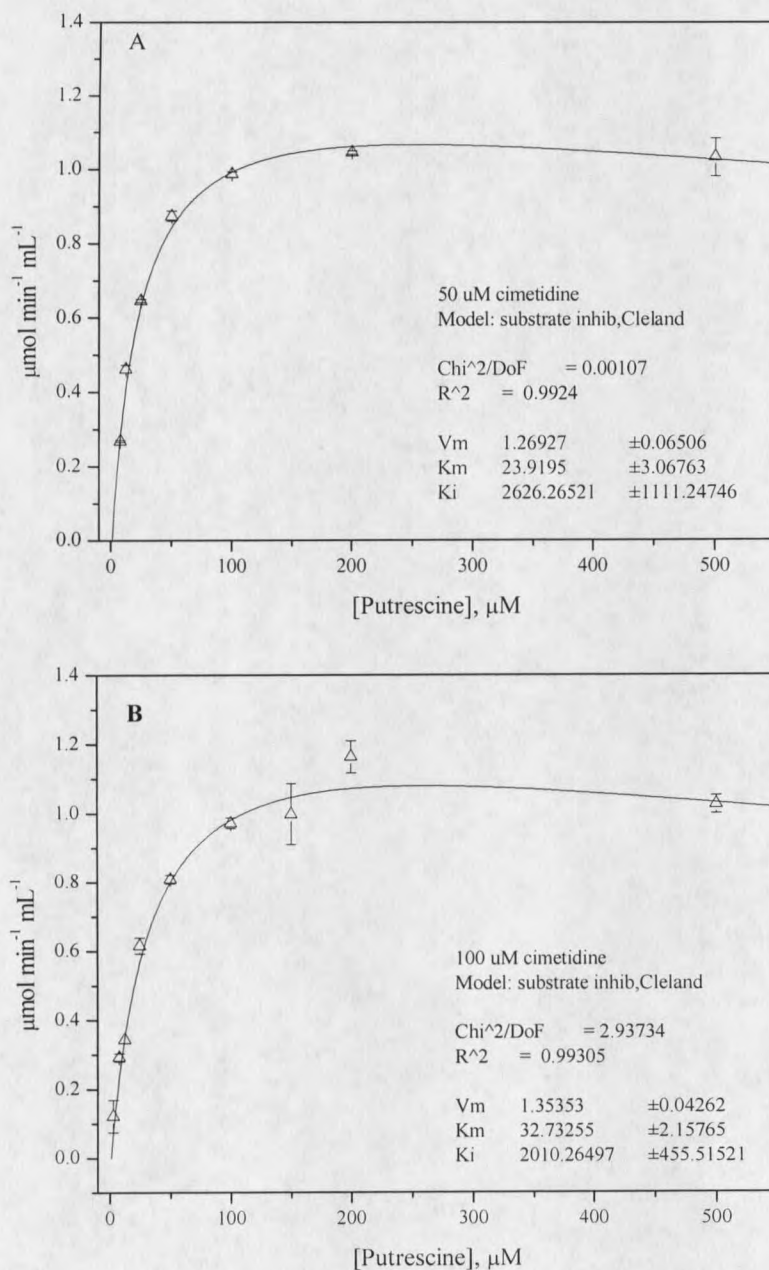


**Figure 43.** Lineweaver-Burk plot for isoniazid inhibition

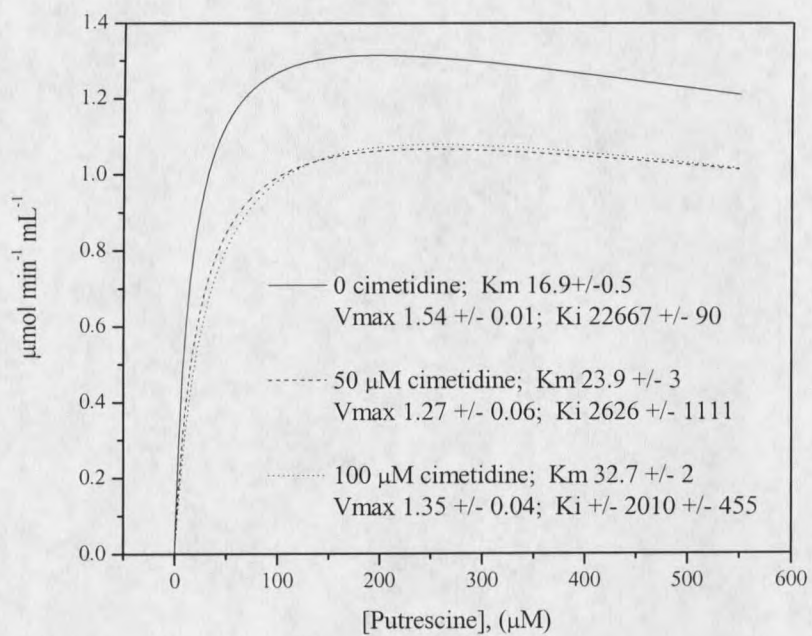
#### Cimetidine.

Data collected with 0, 50 and 100  $\mu\text{M}$  cimetidine are shown in Figures 44 and 45. Putrescine concentrations were up to 500  $\mu\text{M}$  and partial substrate inhibition can be seen. This required the data to be fit to the model for simple substrate inhibition. At 50  $\mu\text{M}$  cimetidine the apparent  $K_M$  value is  $23.9 \pm 3.1 \mu\text{M}$ , and at 100  $\mu\text{M}$  cimetidine the value is  $32.7 \pm 2.2 \mu\text{M}$ . The  $V_{\max}$  values at both inhibitor concentrations are more-or-less equal. The double reciprocal plots (Figure 46) at 0, 50, and 100  $\mu\text{M}$  cimetidine, intersect on the y-axis and are typical of competitive inhibition, that is, the compound competes with substrate for binding in the active site. Plots of apparent  $K_M$  against inhibitor

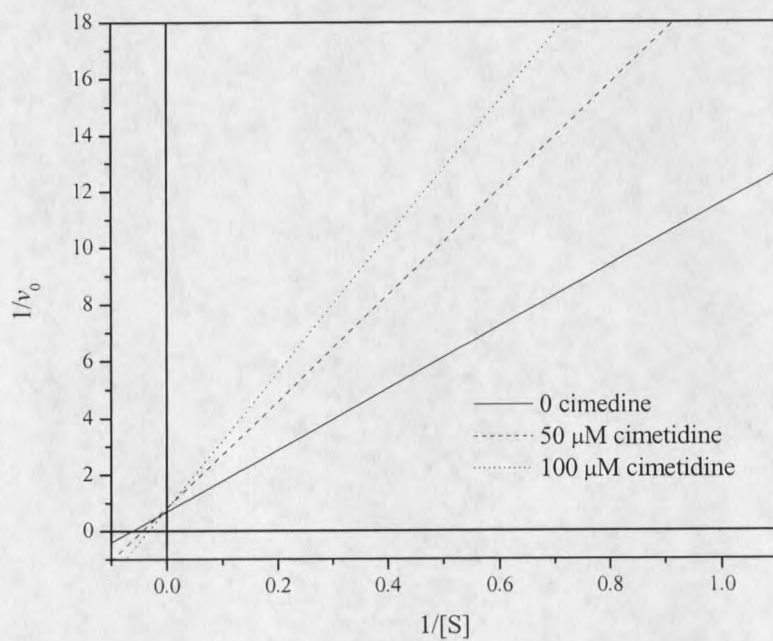
concentration intersect the ordinate at a point equal to  $-K_i$  (Figure 47). For cimetidine the inhibition constant is  $108 \mu\text{M}$ .



**Figure 44.** Cimetidine inhibition plots at **A)** 50 and **B)** 100  $\mu\text{M}$

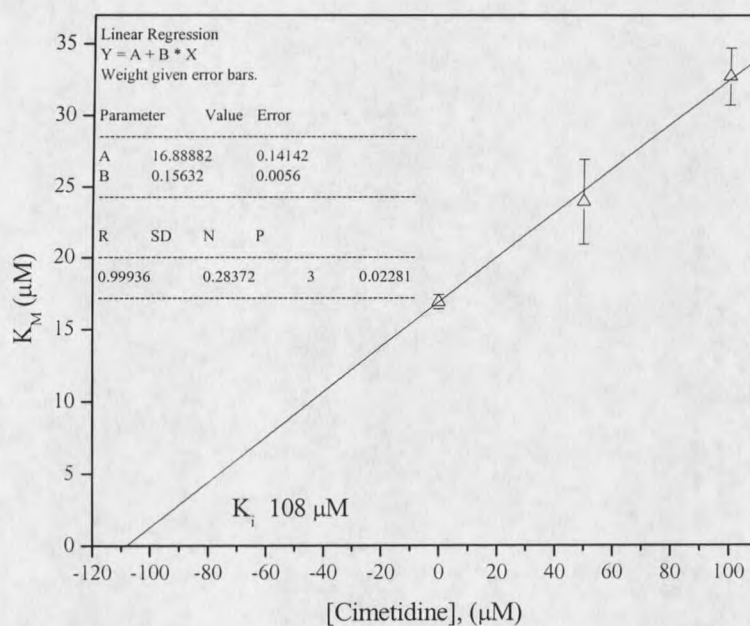


**Figure 45.** Inhibition plot at 0, 50 and 100  $\mu\text{M}$  cimetidine



**Figure 46.** Lineweaver-Burk plot for cimetidine



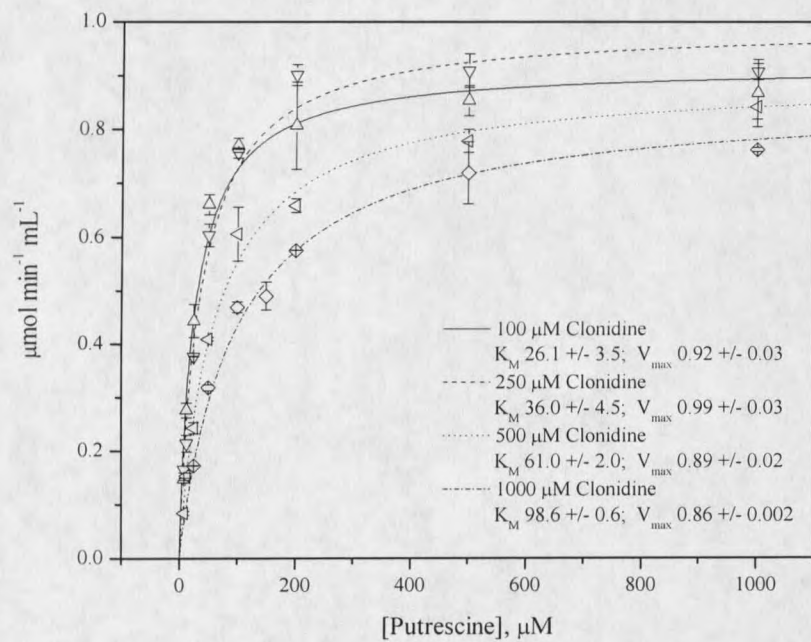


**Figure 47:** Cimetidine  $K_i$  determination

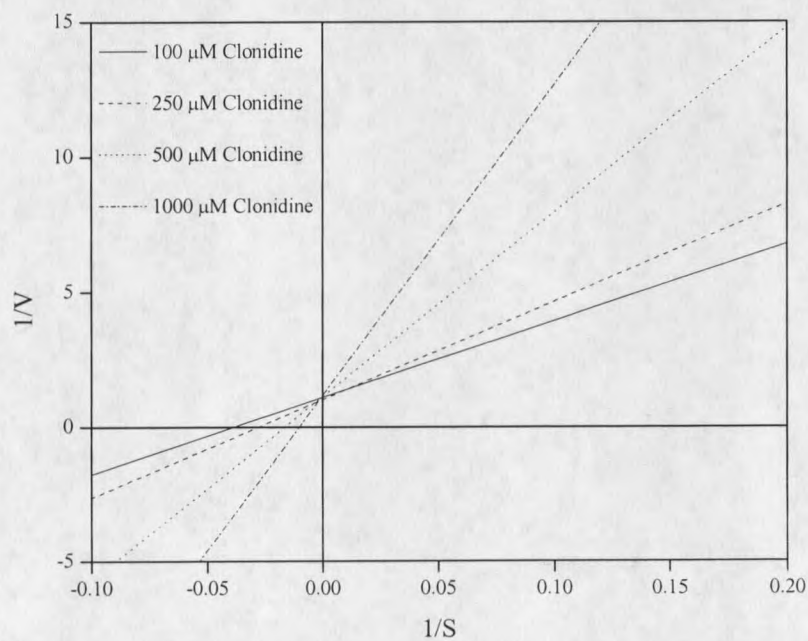
### Clonidine.

Clonidine was examined at 100, 250, 500 and 1000  $\mu\text{M}$ . Competitive inhibition is observed with apparent  $K_M$  values of  $26.1 \pm 3.5$ ,  $36.0 \pm 4.5$ ,  $61.0 \pm 2.0$ , and  $98.6 \pm 0.6$   $\mu\text{M}$ , respectively (Figure 48). Quite surprising is the observation that rhKDAO does not exhibit substrate inhibition with putrescine concentrations up to 1 mM (the data for 250  $\mu\text{M}$  clonidine is ambiguous and can be fit equally well with either the Michaelis-Menten model or the substrate inhibition model). As the data shows,  $V_{\max}$  does vary, from 0.86 to 0.99 I.U. mL, however, if one disregards the  $V_{\max}$  for the 250  $\mu\text{M}$  data then the variation is only from 0.86 to 0.92 I.U./mL.

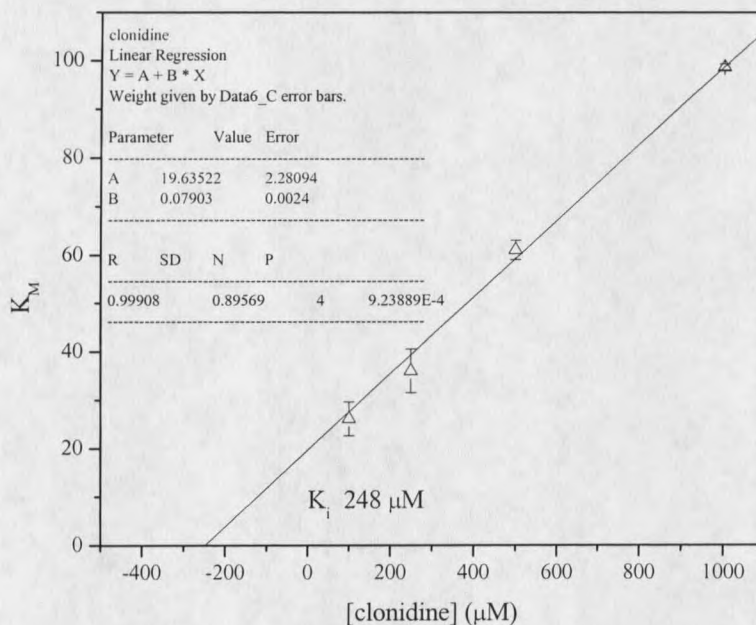
The double reciprocal plot in Figure 49 clearly shows a competitive mode of inhibition. Figure 50 gives an inhibition constant of 248  $\mu\text{M}$ .



**Figure 48.** Inhibition plot for clonidine at 100, 250, 500 and 1000  $\mu\text{M}$ .



**Figure 49.** Lineweaver-Burke plot for clonidine

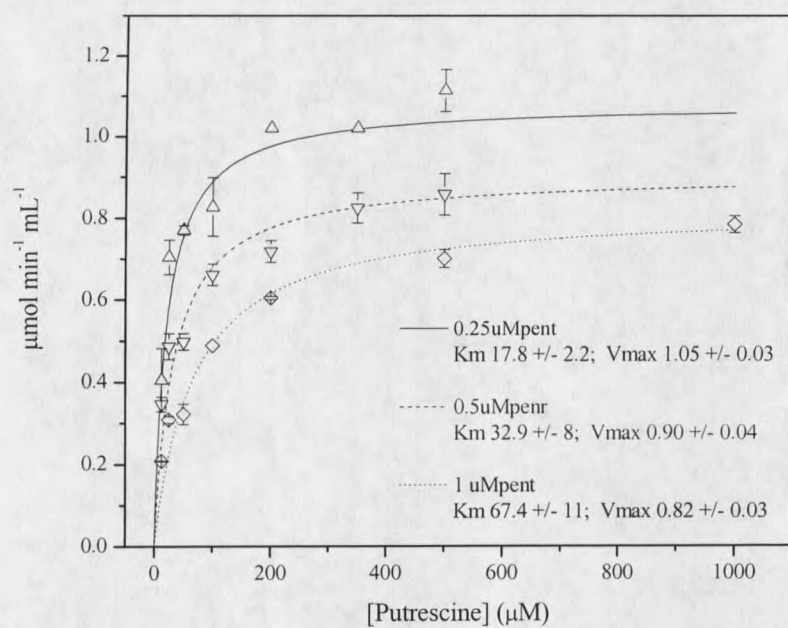


**Figure 50.**  $K_i$  determination for clonidine

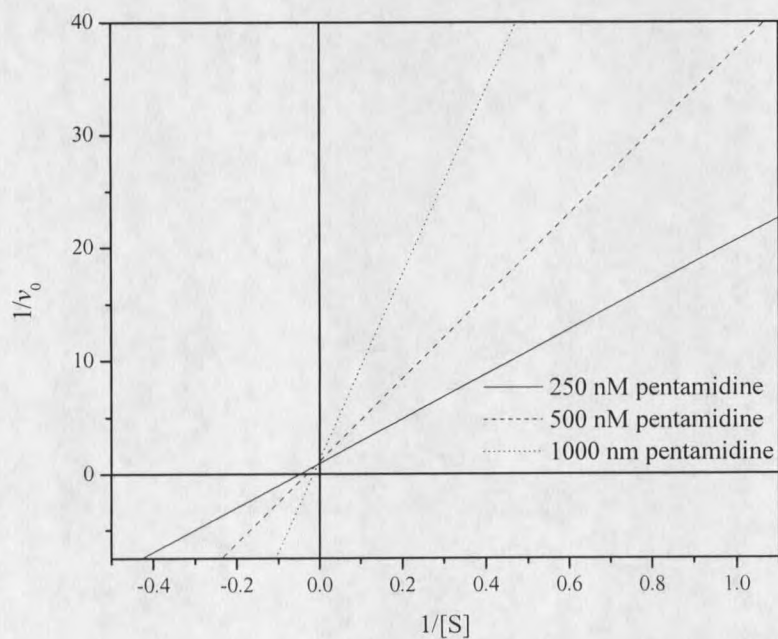
#### Pentamidine.

Pentamidine has proven to be an extremely effective competitive inhibitor for recombinant human kidney diamine oxidase. The data and analysis shown below are for 250 nM, 500 nM and 1  $\mu M$ . Fits to the Michaelis-Menten equations give apparent  $K_M$  values of  $17.8 \pm 2.2$   $\mu M$  for the 250 nM pentamidine data,  $32.9 \pm 8.0$   $\mu M$  for the 500 nM data, and  $67.4 \pm 11$  for the 1  $\mu M$  data. The Lineweaver-Burk plot exhibits competitive inhibition and the determined  $K_i$  is 22 nanomolar.

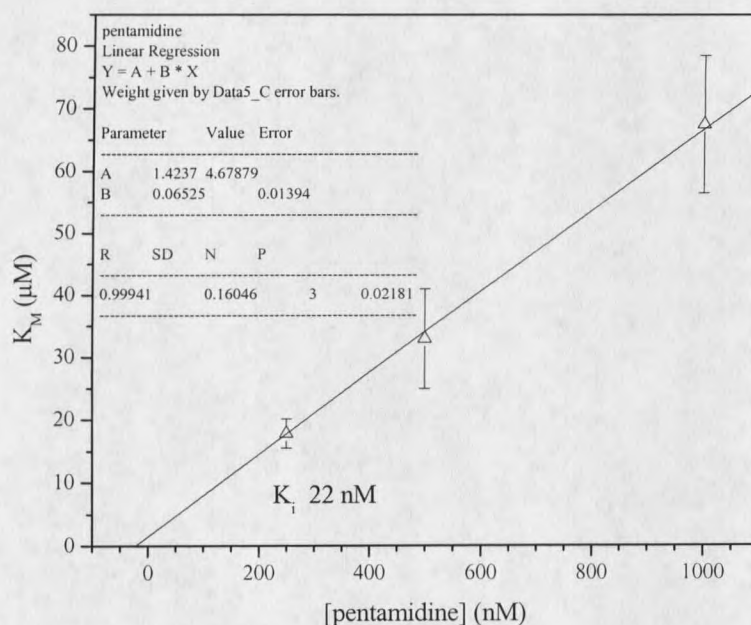




**Figure 51.** Inhibition plot for pentamidine at 250, 500 and 1000 nM.



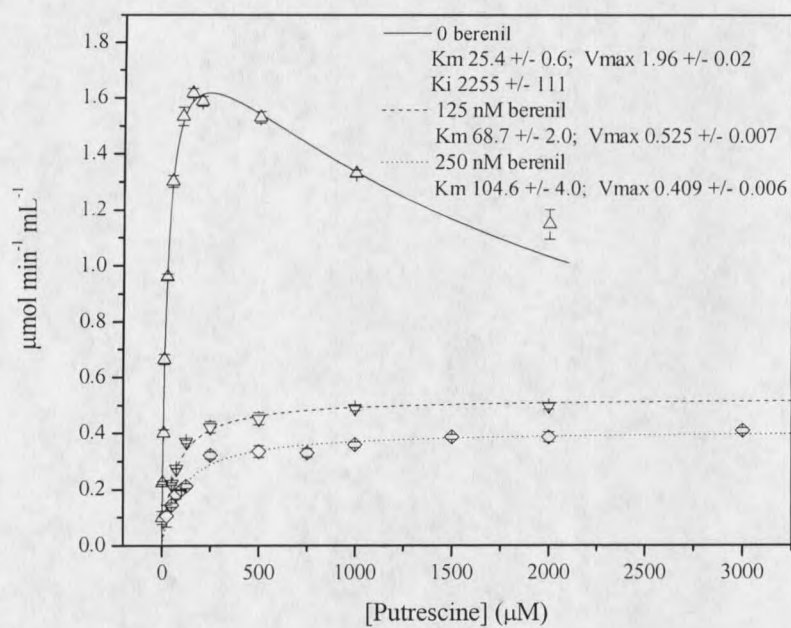
**Figure 52.** Lineweaver-Burk plot for pentamidine



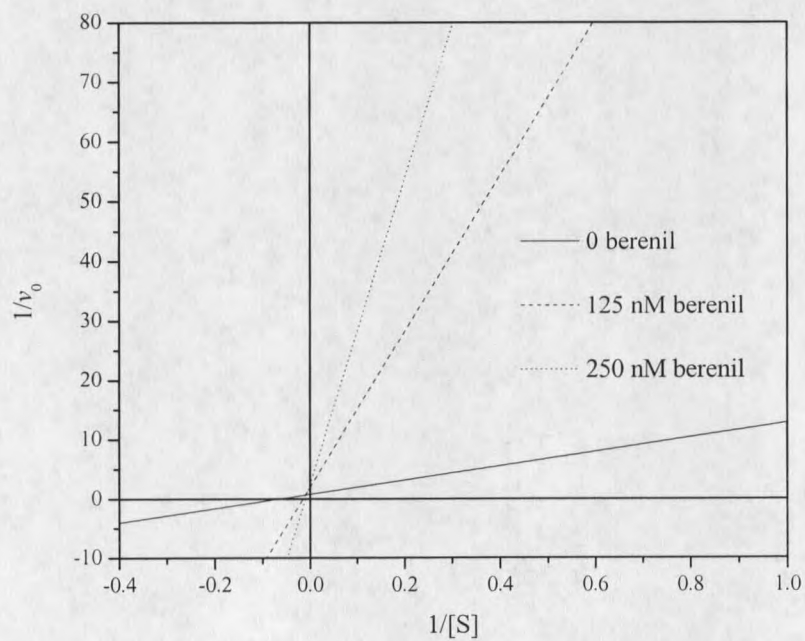
**Figure 53.**  $K_i$  determination for pentamidine.

### Berenil.

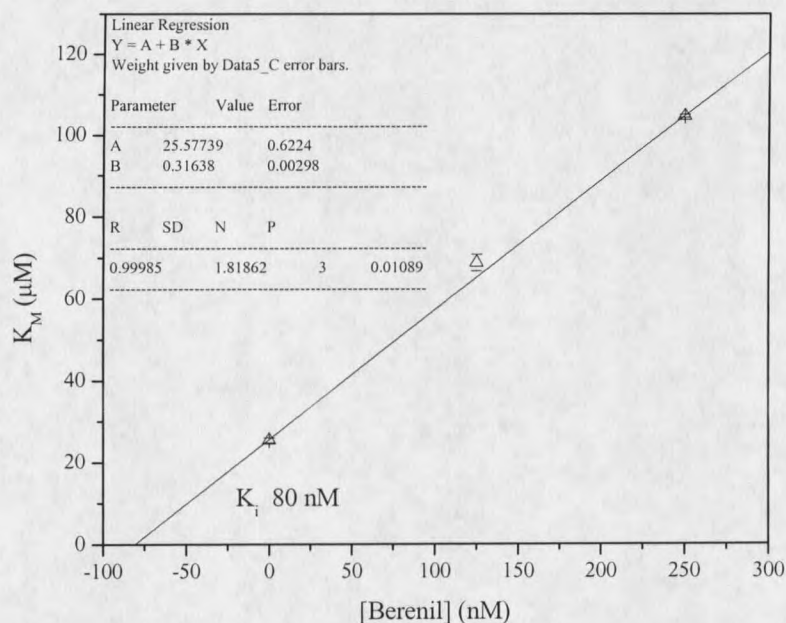
Given the similar benzamidine groups on the ends of berenil and pentamide, it is not surprising to find that berenil is also a very strong competitive inhibitor. Data were collected at 0, 125, and 250 nM berenil and given in Figure 54. Apparent  $K_M$  values are  $68.7 \pm 2.0$  for 125 nM berenil and  $104.6 \pm 4.0$  for 250 nM. The double reciprocal plot show the mode of inhibition is competitive.  $K_i$  has been determined to be 80 nM.



**Figure 54.** Inhibition plot for berenil at 0, 125 and 250 nM.



**Figure 55.** Lineweaver-Burk plot for berenil



**Figure 56.**  $K_i$  determination for berenil

### Discussion

#### Isoniazid.

The apparent  $K_M$  for putrescine oxidation by rhKDAO is 17  $\mu\text{M}$ . In the presence of 500  $\mu\text{M}$  isoniazid the apparent  $K_M = 18.4 \mu\text{M}$ , and with 1 mM isoniazid, the apparent  $K_M = 18.4 \mu\text{M}$ .  $V_{\max}$  values are 0.96 I.U.  $\text{mL}^{-1}$  for 500  $\mu\text{M}$  isoniazid and 0.72 I.U.  $\text{mL}^{-1}$  for 1 mM isoniazid. The Lineweaver-Burke plot at these two inhibitor concentrations give essentially parallel lines, and thus isoniazid could be classified as an uncompetitive inhibitor. However, uncompetitive inhibition is not very reasonable mechanistically.

At lower isoniazid concentrations the inhibition type appears to be mixed inhibition, with changes in both  $K_M$  and  $V_{max}$ . As explained by Cornish Bowden, mixed inhibition is almost always due to a special type of product inhibition where the inhibitor can bind to an alternative form of the enzyme that is generated during the reaction pathway. The Ping Pong mechanism of copper amine oxidases does generate an alternative enzyme form, the reduced aminiquinol form, that could plausibly be the enzyme state to which isoniazid binds. Obviously considerably more data would need to be collected to clarify the mechanism of inhibition, and this may prove to be a rewarding exercise. However, additional data was not collected in this work for the following reasons: 1) the roughly equivalent  $K_M$  values indicate the competitive portion of inhibition is minor and thus uninteresting, and 2) rhKDAO stocks were limited and needed to be preserved for investigation of the other compounds.

Mondovi et al. have reported aromatic hydrazide compounds, similar to isoniazid, act as non-competitive inhibitors of copper amine oxidase, and that prolonged incubation with these compounds irreversibly inhibit amine oxidase activity [206]. In the experiments presented herein, rhKDAO was exposed to isoniazid for only a short period of time, less than one minute, and no suggestion of irreversible inhibition was observed. Additional experiments are required to determine if irreversible inhibition of human diamine oxidase by isoniazid can occur.

### Cimetidine.

The data clearly indicates that cimetidine is a strong competitive inhibitor of rhKDAO. The determined inhibition constant is 108  $\mu\text{M}$ .

The average blood concentration of cimetidine in patients undergoing continual infusion at 37 mg/hr using this drug is 0.9 mg/mL, or 3.6 mM. This commonly used peptic ulcer drug may very well exhibit considerable *in vivo* inhibition of human diamine oxidase. The most common means of treatment is orally. Cimetidine taken in this manner reaches a maximum blood concentration in 45 to 90 minutes and inhibits gastric acid secretion following eight hours. Circulating cimetidine has a half life of about 2 hours, although there is considerable genetic variation [181]. Under these conditions inhibition of diamine oxidase would presumably be fairly short lived.

### Clonidine.

The double reciprocal plot indicates clonidine is a good inhibitor of rhKDAO with a determined  $K_i$  value of 248  $\mu\text{M}$ .

### Pentamidine.

Pentamidine has proven to be an extremely potent competitive inhibitor of recombinant human KDAO. The data above indicates an inhibition constant ( $K_i$ ) of 22 nanomolar. Pig kidney DAO has been reported to be inhibited by pentamidine with a  $K_i$  of 3 micromolar. Other chemically related aromatic diamidines have also been examined with the porcine enzyme; all were found to be reversible competitive inhibitors with inhibition constants in the low micromolar range [207].

Nearly 9% of patients administered pentamidine experience serious adverse side effect that may including hypotension, acute renal failure, leukopenia, hypoglycemia, and thrombocytopenia [181,197]. Fatalities from hypotension, hypoglycemia, acute pancreatitis and cardiac arrhythmias have been reported; in fact a single dosage can result in severe hypotension and death. Moderate side effects are reported in almost 50% of patients, the most common is elevated serum creatinine, diagnostic for renal impairment. More than twenty other diverse side reactions are described.

Plasma pentamidine concentrations in AIDS patients treated for PCP by the intramuscular injection of pentamidine isethionate at 4 mg/kg were steady between 0.3 and 0.5 mg/mL. These plasma levels are readily converted to 0.877 to 1.5  $\mu$ M. This concentration range would certainly inhibit diamine oxidase to the point of complete inactivation and could be expected to severely disrupt normal metabolic pathways involving DAO. Furthermore, the tissue distribution of pentamidine in mice indicates this pharmaceutical accumulates in kidney tissue, a major source of diamine oxidase expression.

As previously discussed, histamine catabolism remains to be completely elucidated, especially with regard to the role and importance of diamine oxidase. A reasonable hypothesis is that during the course of pentamide administration (typically 14 to 21 days) diamine oxidase activity is essentially nil and histamine methyltransferase may not be able to compensate enough to clear circulating histamine. This leads to the question of does an abnormally high level of circulating histamine contribute to, or perhaps even cause, some of the serious side effects that accompany pentamidine

treatment? The involvement of histamine in such fundamental processes as smooth muscle contraction, vasodilation, allergic response and stimulation of adenylate cyclase activity in neurons strongly suggests this may be the case. Intriguing clues can be found in pentamidine's side effects, notably hypotension, diarrhea and rash.

### Berenil.

Structurally similar to pentamidine, berenil also exhibits competitive inhibition with a remarkably low  $K_i$  of 80 nM. Data at additional concentrations are desirable. Note that the apparent  $K_M$  for putrescine used in this analysis (data collected on the same day) is 25  $\mu$ M and is higher than values obtained previously ( $\sim 17 \mu$ M).

Berenil has been investigated as a potential inhibitor of polyamine synthesis and degradation, through its action on diamine oxidase, the first step in putrescine degradation, and S-adenosylmethionine decarboxylase, which prevents spermidine and spermine biosynthesis from putrescine [193]. It has been characterized as a strong uncompetitive inhibitor of diamine oxidase with a  $K_i = 210$  nM, however, this earlier study used a crude source of diamine oxidase, the homogenate of rat small intestine.

### Conclusions

The expression and purification of recombinant human kidney diamine oxidase represents a major advancement in the study of this human enzyme, and for the field of CAO research. The preferred substrates, histamine, putrescine and agmatine, strongly suggest human diamine oxidase acts *in vivo* as a component of fundamental metabolic



pathways and processes, such as cell proliferation, inflammation, allergic response and ischemia. A logical and desirable goal that is only now possible is the pursuit of characterizing inhibition of the human enzyme by various compounds, especially those compounds that have found a use in human medicine. Furthermore, the data presented above differs significantly from the available literature, in describing the types and degrees of inhibition.

It is not unreasonable to speculate that some pharmaceuticals may disrupt the normal functions of human KDAO in the metabolism of biogenic amines, and thus may contribute to or be responsible for deleterious side effects. Inhibition studies may aid in the rational design of highly specific hDAO inhibitors, which perhaps may one day show promise as therapeutic agents, and in the development of compounds with minimal inhibitory effects in order to lessen toxicity. For example, considerable efforts continue towards the development of new and more effective treatments against microbial infections, especially important with the continuing emergence of antibiotic resistant pathogens. Such compounds can now be readily screened *in vitro* for interaction with hDAO.

Particularly noteworthy are the data for the two diamidines, pentamidine and berenil, which show extremely strong competitive inhibition with inhibitor constants in the low nanomolar range. Berenil is often a treatment of last choice due severe and sometimes fatal side effects. It is possible that disruption of normal amine metabolism via hKDAO inhibition may be responsible for or contribute to the serious and sometimes fatal side effects and toxicity associated with diamidine and related guanyl compounds.

On the other hand, disruption of amine metabolism may prove to be a factor in the mechanism of action that makes these compounds effective antibiotics.

## AZIDE INHIBITION OF rhKDAO

Introduction

Copper proteins typically show a broad and varied susceptibility to ligand-substitution chemistry. Spectroscopic and chemical investigations of this chemistry can provide details of the copper coordination, environment and function in copper metalloproteins.

The small anions azide, thiocyanate and cyanide, can bind to and act as effective inhibitors of copper amine oxidase. These ligands substitute for a water molecule at the equatorial position of copper [16,208-216]. The ligands have been described as either mixed or uncompetitive inhibitors.

A major impetus for this investigation of azide inhibition has been the recent report of Klinman and coworkers with the amine oxidase from the yeast, *Hansenula polymorpha*, describing noncompetitive inhibition of azide during the oxidative half reaction [217]. The authors interpret their results to indicate separate binding sites for copper and dioxygen; azide is proposed to undergo ligand substitution chemistry on copper, and dioxygen is proposed to bind in a distinct and proteinaceous site.

The experiments herein are designed to investigate the interaction of azide and rhKDAO in both the reductive and the oxidative half reactions. [218]

### Experimental Methods

All kinetic experiments used 50 mM HEPES, pH 7.2 buffer, and the ionic strength was adjusted by the addition of potassium chloride. Where required sodium azide (Sigma-Aldrich) was added to the buffer and the ionic strength corrected to 250 mM. Kinetic data for putrescine oxidation were collected as previously described using the horseradish peroxidase coupled spectrophotometric assay at 37 °C.

A Clark-type dioxygen electrode and reaction chamber from Instech Laboratories was used for monitoring rates of O<sub>2</sub> consumption. The volume of the reaction chamber is 680 µL and all experiments were at 37 °C. Oxygen concentrations were varied by mixing together air saturated and deoxygenated buffer, which was prepared by sparging with argon gas. Data were collected using an interfaced PC, an analog-to-digital converter and Intake software (Instech Laboratories, MA). Oxygen uptake experiments were initiated by the rapid addition of 2 or 4 µL recombinant human kidney diamine oxidase.

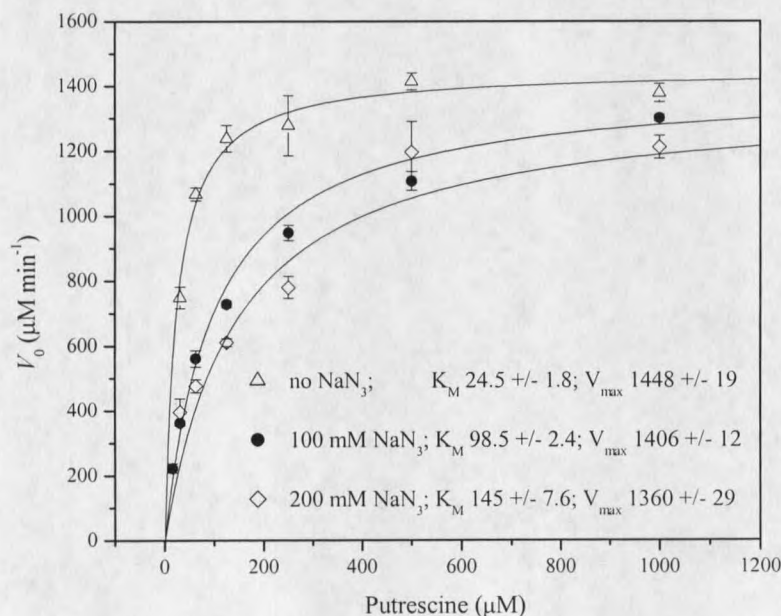
Sodium azide titration of the human enzyme used a 100 µL masked cuvette. To avoid protein precipitation data were collected on a Cary 1E scanning spectrophotometer from 350 to 800 nm. Protein was in 100 mM KP<sub>i</sub>, pH 7.2 and the titration was at room temperature. A stock solution of 250 mM sodium azide was freshly prepared.

### Results

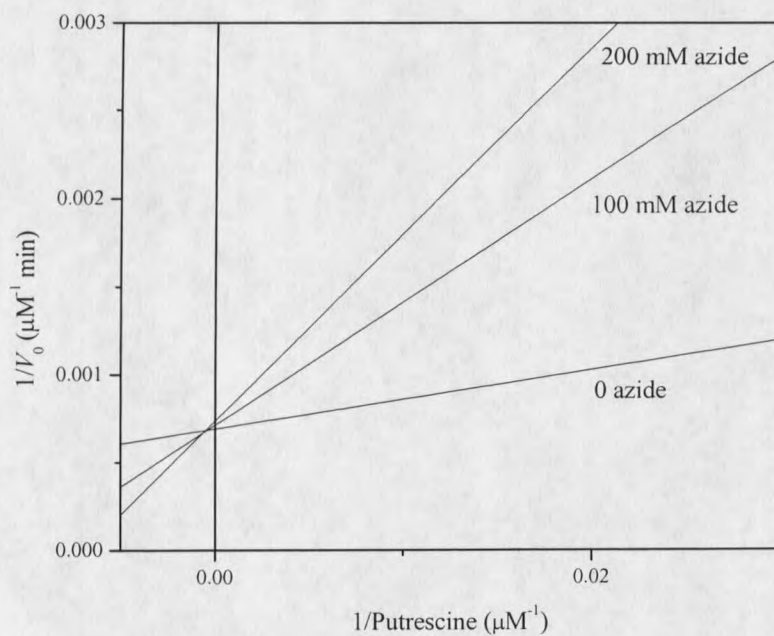
#### Azide and putrescine oxidation.

The effect of azide on the reductive half reaction (the reduction of TPQ and oxidation of putrescine substrate) was investigated in order to determine the appropriate

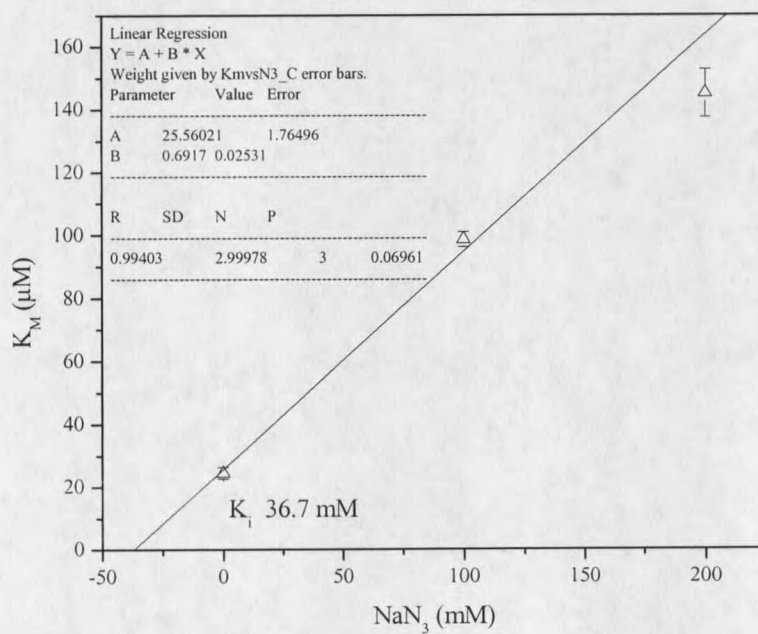
saturating levels of putrescine under the experimental conditions. The buffer's ionic strength was adjusted to 250 mM with KCl for these experiments, necessary to maintain a constant ionic strength while varying the concentration of azide up to 200 mM. Figure 57 shows the data and the fits to the data using the Michaelis-Menten equation. Both  $K_M$  and  $V_{max}$  change with azide concentrations, with the former perturbed to a much greater extent. This indicates mixed inhibition. The Lineweaver-Burk plot in Figure 58 clearly shows the dominant mode of inhibition is competitive, and therefore the noncompetitive contribution can be disregarded.  $K_M$  values determined by the fits to the data are  $24.5 \pm 1.8 \mu\text{M}$  for zero azide,  $98.5 \pm 2.4 \mu\text{M}$  for 100 mM azide, and  $145 \pm 7.6 \mu\text{M}$  for 200 mM azide. Figure 59 shows the graphical determination of  $K_i = 36.7 \text{ mM}$ .



**Figure 57.** Azide inhibition of putrescine oxidation



**Figure 58.** Lineweaver-Burk plot for azide and putrescine oxidation



**Figure 59.** Azide  $K_i$  determination for putrescine oxidation

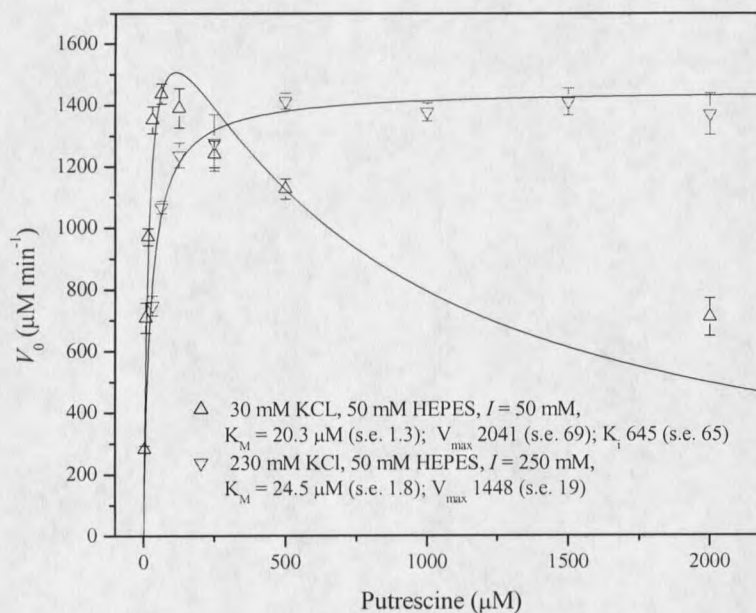
The data indicates azide acts as a competitive inhibitor against putrescine, a result unanticipated from a review of the literature. This triatomic anion of nitrogen is structurally and chemically very different than the preferred substrates of rhKDAO ( $k_{\text{cat}}/K_M$ ) determined in this work. Histamine, 1-methylhistamine, agmatine and putrescine are all at least partially dicationic species under physiological conditions.

Two millimolar putrescine was chosen for the dioxygen uptake experiments to ensure substrate amine saturation for the first half of the Ping Pong reaction mechanism. Data was collected at 2 mM putrescine but is not shown above.

#### Ionic strength and substrate inhibition.

The data in Figure 57 does not show the previously observed pattern of partial substrate inhibition observed in 150 mM ionic strength buffer and putrescine at concentrations above than 250 micromolar. This suggested the experiment shown in Figure 60—an examination into the effect of ionic strength on the partial substrate inhibition of rhKDAO. Buffers at two ionic strengths were used in determining the dependence of initial reaction velocity on substrate concentration, 50 mM HEPES, pH 7.2 supplemented with KCl to either 50 or 250 mM. The results are striking. At the higher concentration of KCl, no substrate inhibition is discernible up to 2 mM putrescine. In contrast, in low ionic strength buffer, substrate inhibition is apparent at putrescine concentrations above 50  $\mu\text{M}$ . The data for physiologically relevant ionic strength, 150 mM, is not shown here but can be found in a previous chapter, and that data appears to be intermediate to the two plots given here. Note that at 50 mM ionic strength, the partial

substrate inhibition model does not fit the data very well. In fact the data are reminiscent of the marked substrate inhibition data exhibited by histamine at 150 mM ionic strength (*vide supra*).



**Figure 60.** Ionic strength effect on the oxidation of putrescine.

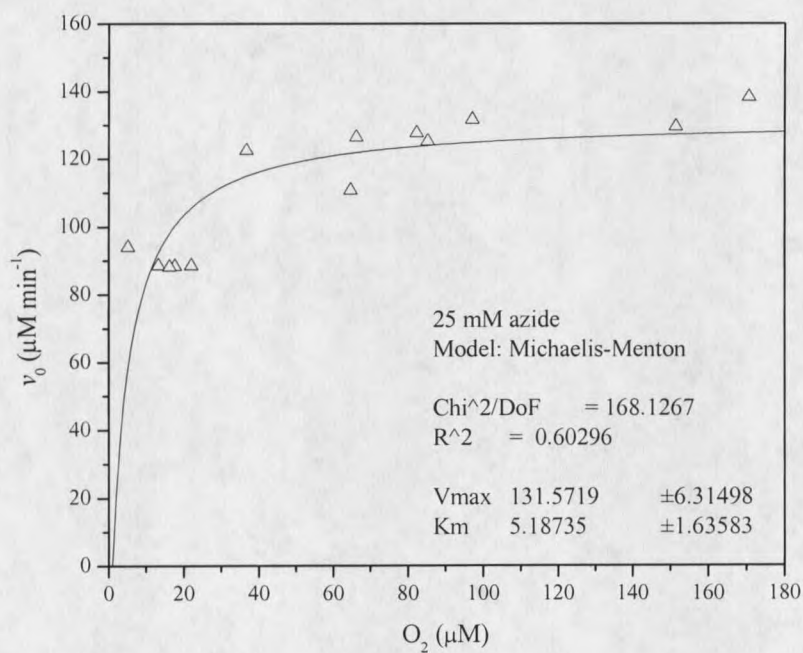
One plausible physical reason for the abrogation of substrate inhibition with increasing ionic strength is that the salt ions may weakly bind to residues involved in substrate binding or in the channel to the active site in a manner that prevent the binding of a second substrate molecule. This would block the formation of a catalytically inactive enzyme-substrate-substrate complex. In other words,  $K^+$  or  $Cl^-$  ions may be competitive inhibitors against the binding of a second putrescine molecule per rhKDAO active site. The data is insufficient to specify which ionic species could be involved at



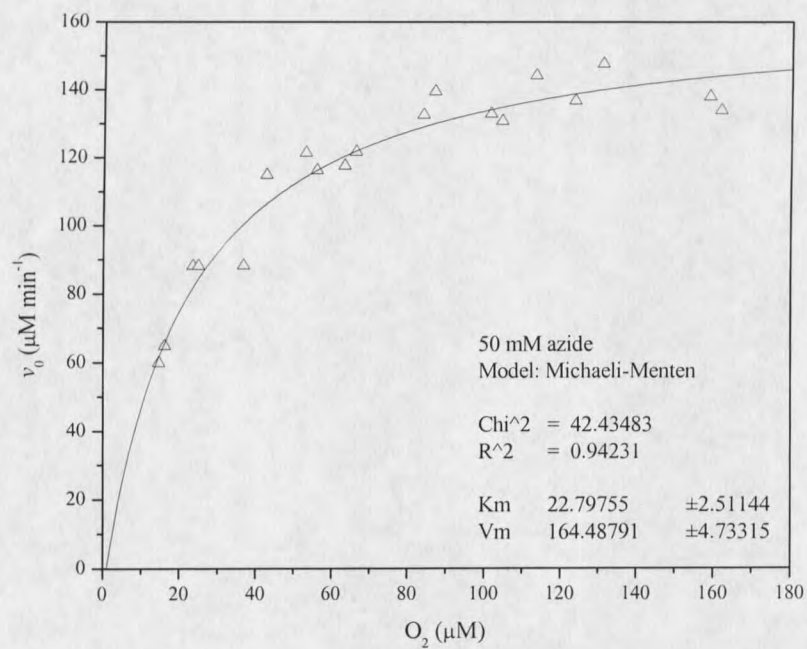
such a proteinaceous site. Further experiments using a variety of salts would perhaps delineate the interactions of various small ions with rhKDAO.

#### Azide inhibition of dioxygen reduction.

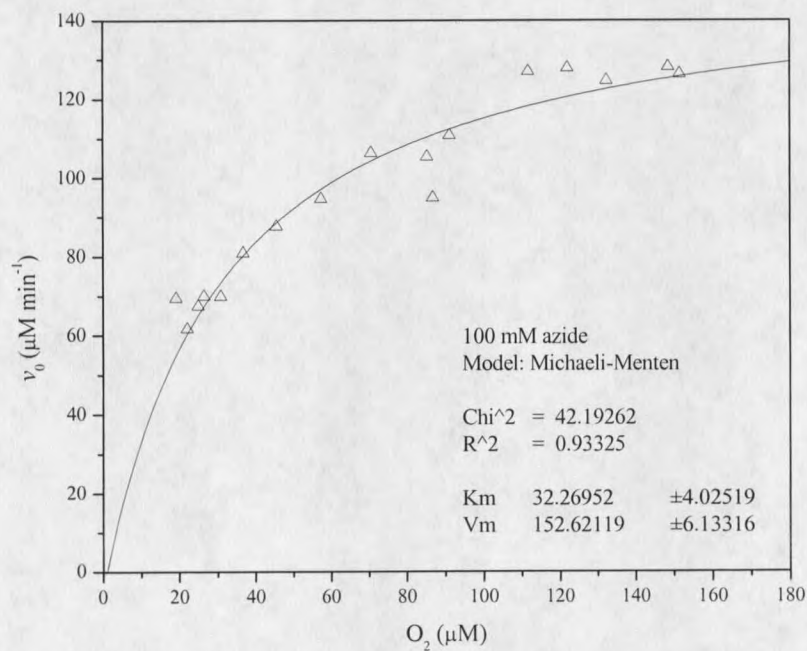
These experiments were performed in 250 mM total ionic strength buffer supplemented with 2 mM putrescine to ensure saturation by the first substrate and thus a non-limiting first half reaction in the Ping Pong mechanism of rhKDAO.



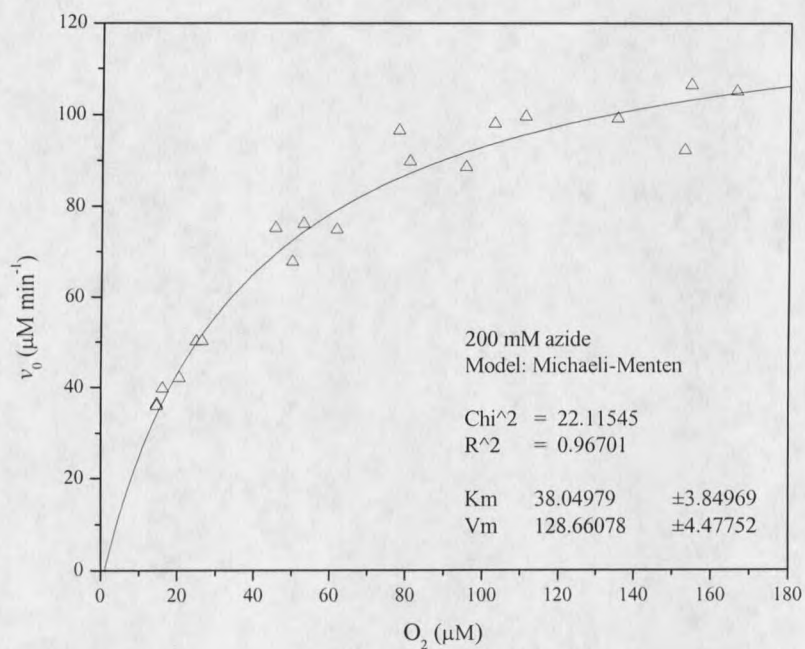
**Figure 61.** Rates of dioxygen consumption at 25 mM azide



**Figure 62.** Rates of dioxxygen consumption at 50 mM azide

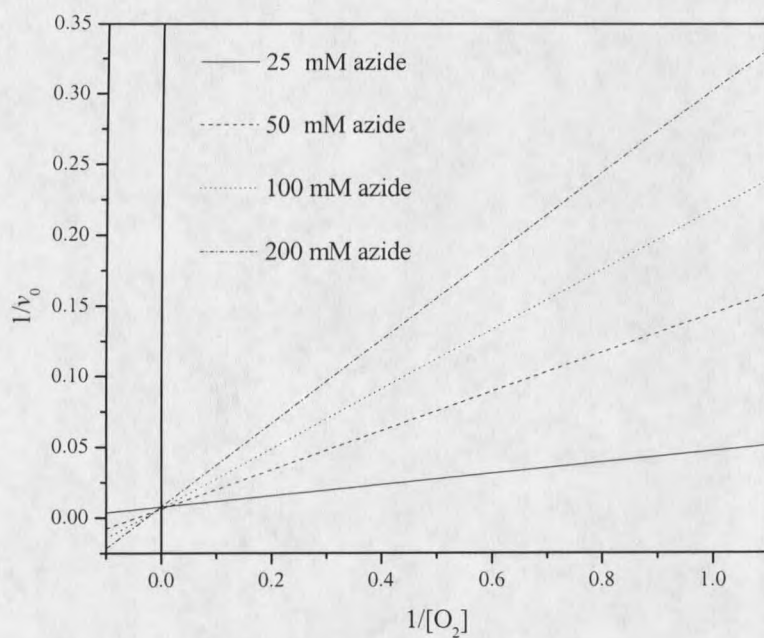


**Figure 63.** Rates of dioxxygen consumption at 100 mM azide

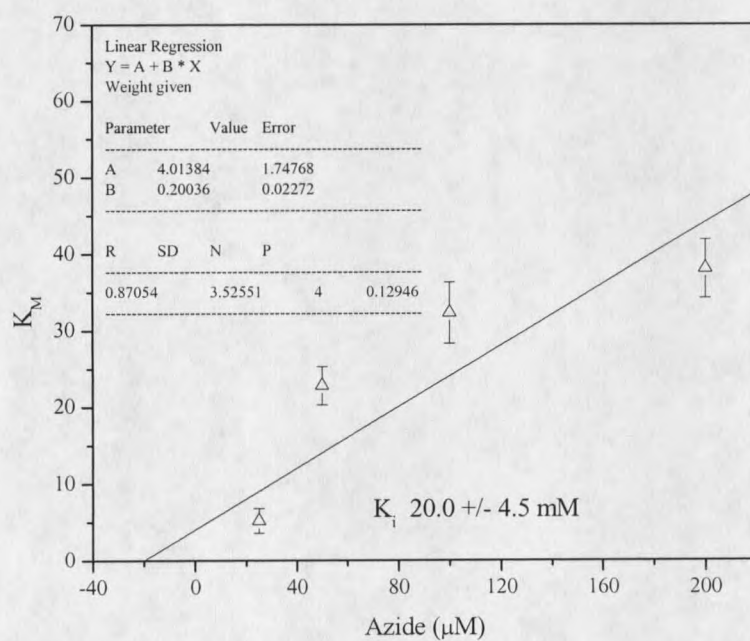


**Figure 64.** Rates of dioxygen consumption at 200 mM azide

Figure 65 shows the double reciprocal plots of the Michaelis-Menten equation fits to the data given above. Azide clearly is a competitive inhibitor against dioxygen in the oxidative half reaction of rhKDAO. The plot of  $K_M$  versus azide concentration is shown in Figure 66 and indicates an inhibition constant of  $20.0 \pm 4.5$  mM.



**Figure 65.** Lineweaver-Burk plot of azide inhibition of dioxygen reduction



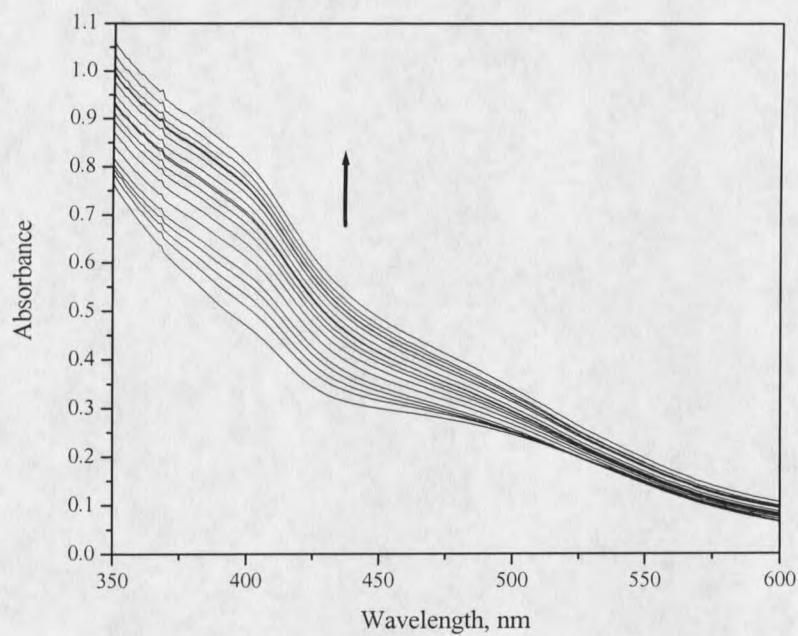
**Figure 66.**  $K_i$  determination for azide inhibition of dioxygen reduction

Recombinant human kidney diamine oxidase was titrated with sodium azide for the purpose of determining the binding constant for azide to the protein bound Cu(II) ions. The equivalence of the copper binding constant and the inhibition constant would provide strong evidence that the nature of competitive inhibition of the oxidative half reaction is a direct consequence of azide binding to Cu(II). Figure 67 shows the spectral changes accompanying the titration and Figure 68 shows the changes in absorbance at 400 nm with increasing azide concentrations.

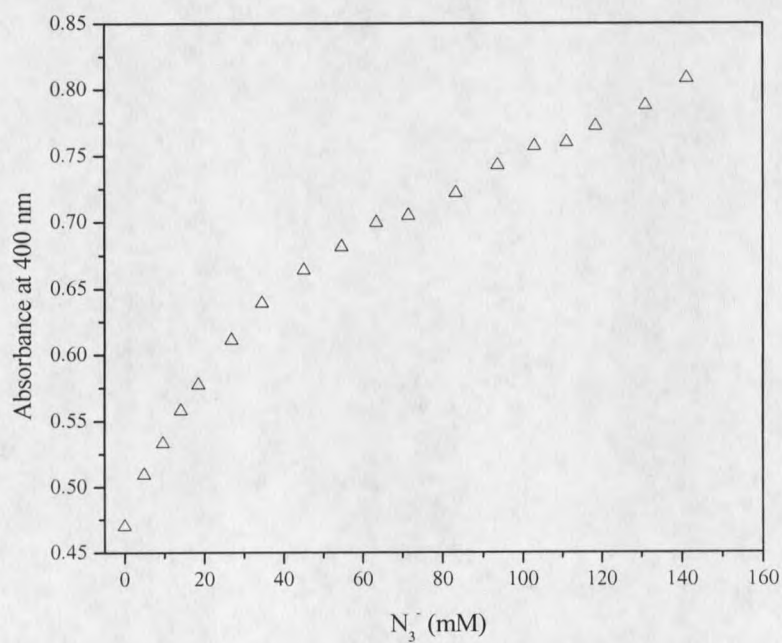
rhKDAO did not give a clearly defined ligand-to-metal charge-transfer band centered around 400 nm as was observed in the bovine plasma amine oxidase and the pig kidney diamine oxidase [212-214], as well as the equine plasma enzyme (data not shown).

The experiment was conducted in 100 mM  $\text{KPi}$ , pH 7.2 at room temperature. Additional experiments using conditions identical to the inhibition data (50 mM HEPES, pH 7.2, ionic strength 150 mM, 37 °C) were confounded by protein precipitation. This experiment is critical and will be repeated upon the availability of a fresh sample of rhKDAO.





**Figure 67.** Azide titration of rhKDAO. The arrow indicates the progression of spectral with increasing azide concentration.



**Figure 68.** Azide titration curve for rhKDAO

### Discussion

The data indicate azide acts as a competitive inhibitor against putrescine oxidation. This type of inhibition for azide in the reductive half reaction has not been previously described. This could suggest that azide binds directly in the substrate binding site. Alternatively, azide binding to copper might perturb the active site in such a way as to disrupt substrate affinity. It can be hypothesized that azide binding to copper at the equatorial site may disrupt the hydrogen bonding network and thereby alter the presentation of TPQ C5 carbonyl to substrate amine. Note that any proposed change in the conformation of TPQ must be at equilibrium with a productive conformation, otherwise noncompetitive inhibition would result. In light of the clear evidence for azide binding to copper and the chemical differences between azide and normal amine substrates, the latter situation is considered more likely.

Azide is also shown to competitively inhibit the oxidative half reaction under conditions of amine substrate saturation. This result is contrary to the noncompetitive inhibition reported by Klinman and coworkers [217], although it is possible that enzymes from different sources have inherently different interactions with azide and oxygen. However, this would suggest that the yeast and human enzymes have evolved separate means of binding dioxygen and thus the reoxidation of TPQ would occur via different mechanisms..

One possible reason for the difference in inhibition types from this study and earlier work could be due to the advances in computer technology that now allow the fast

and easy non-linear curve fitting functions on personal computers. The fits to raw data can then be represented in commonly used and easy to interpret linear graphical representations such as the double reciprocal Lineweaver-Burk plot. The errors inherent to transforming non-linear to linear data before fitting functions are therefore eliminated. It may prove a worthwhile endeavor to reanalyze the previously published data.

The definitive characterization of azide binding to copper in the recombinant human kidney diamine oxidase is required for a complete interpretation of these inhibition results. The preliminary azide titration in this work does suggest an equilibrium binding constant may be determined that would correlate well with the  $K_i$  value for azide inhibition in the oxidative half reaction. Reported disassociation constants are 24 mM for bovine plasma enzyme in phosphate buffer and 83 mM in PIPES, and 55.5 mM for *H. polymorpha* [213,217]. These values suggest that a  $K_i$  of 20 mM is not unreasonable for a copper amine oxidase. Circular dichroism and EPR spectroscopy would also provide much needed information for the characterizing azide binding to copper in rhKDAO..

Note that the data presented here indicate a complex behavior that could not be fit to a single equilibrium constant. There is precedence for this; Dooley and Coté described similar behavior for bovine plasma amine oxidase and azide when titrated in phosphate buffer [212], and, importantly, in 5 mM PIPES with 100 mM NaCl the titration cleanly indicated a single equilibrium constant. Presumably, a titration of rhKDAO with the buffer used for the inhibition studies herein would also give conclusive data.



### Conclusions

Azide is shown to be a competitive inhibitor against rhKDAO for both half reactions in the Ping Pong mechanism of copper amine oxidases. In the reductive half reaction (oxidation of substrate and reduction of TPQ) the inhibition constant has been determined to be 37  $\mu\text{M}$ . Azide has been demonstrated to compete with dioxygen for the oxidative half reaction (reduction of molecular oxygen and reoxidation of TPQ) with an inhibition constant of 20 mM. The data is most consistent with a mechanism involving dioxygen binding directly to copper.

## PURIFICATION AND CHARACTERIZATION OF EQUINE PLASMA AMINE OXIDASE

### Introduction

A major goal of this research into mammalian CAOs has been the purification and characterization of the equine plasma amine oxidase (EPAO). The primary aim of this project is to obtain a highly purified mammalian enzyme suitable for determining the molecular structure via the tools of crystallization and x-ray diffraction. To date, no structure has been determined for a copper amine oxidase from any animal source. A detailed structure would be an extremely important advancement in this field of study. Comparison with the crystal structures from "lower" life forms this may suggest the fine structural details that make mammalian enzymes unique. For example, subtle changes in the active site may lead to a rational proposal for why the Cu(I)-semiquinone species is present at lower concentrations in mammalian enzymes than the others [19], in fact the visible spectrum of anaerobic substrate reduced EPAO (and rhKDAO) do not indicate a semiquinone intermediate (*vide infra*) -- which does *not* mean the semiquinone is not a viable catalytic intermediate, only that the equilibrium constant for the Cu(I)-semiquinone and Cu(II)-aminoquinol couple may not allow the semiquinone to accumulate to levels detectable by visible spectroscopy. The structural basis for substrate specificity and thus potential biological roles is also very poorly understood, especially among the mammalian enzyme. Furthermore, a structure would doubtless open up a plethora of additional questions for further investigation and assist in directed

experimental design using the tools of molecular biology, structural biology, biochemistry and spectroscopy.

Additionally, a quantity of EPAO on hand provides an additional source of enzyme for study and comparative analysis. For example, EPAO from this work is used by Eric Shepard (MSU) in collaboration with Professor Lawrence Sayre (Case Western Reserve University, OH) in the extensive investigation of mechanism-based inhibition of CAOs, and data collected with this equine enzyme has been included in two publications [219,220].

The bovine plasma amine oxidase is the most studied of all mammalian CAOs [15,15,45,212,221-228,228-231,231,232]. In contrast, the equine enzyme has received little attention in the literature. Early investigations for EPAO were performed by Yamada and Yasunobu in the early 1960's and the 1980 Ph.D. thesis of Schallinger [233,234].

More recently Dooley and coworkers reported an improved purification and the characterization of the EPAO [235]. This work established EPAO as a copper containing amine oxidase with two copper ions and two TPQ cofactors per dimer. Room temperature EPR of anaerobically substrate reduced EPAO gave a sharp signal at  $g \approx 2$ , which is attributed to the signal of the topa semiquinone [17,236]. Investigation into the substrates of EPAO is especially noteworthy. EPAO shows considerable enzymatic activity with dopamine and norepinephrine ((-)-arterenol), which is unique among mammalian copper amine oxidases. Histamine, serotonin (5-hydroxytryptamine) and the

polyamine spermine and spermidine were also found to be oxidized although at lower rates.

Although an efficient purification protocol has previously been developed and published by Dooley and coworkers, a completely new protocol is described in this work. This was desired to eliminate the ammonium sulfate fractionation previously used, due to reports that residual ammonium sulfate might interfere with the crystallization and the biochemical properties of amine oxidase (Dooley, D.M., pers. comm.). The purification herein uses a single horse as the source of equine plasma, which should minimize heterogeneity from genetic differences between animals. Additional characterization by visible, EPR and rR spectroscopy and steady state kinetics are given.

### Experimental Procedures

#### Purification.

The following protocol has been developed over ten enzyme purifications. A complete preparation takes approximately 4 weeks.

A single horse, named Sunny (not named after his disposition), was used for all the EPAO preparations. Blood drawings were performed by the owner, Dr. Gordon Hardaway, D.V.M. (Hardaway and Catlin Veterinary Hospital, Belgrade, Montana). Procedures involving the horse were in accordance with the guidelines of the Montana State University Institutional Animal Care and Use Committee and followed the Approved Protocol 1011. No signs of discomfort or pain were noted in the horse.

Equine blood is drawn from the jugular vein using a 10 gauge sterile needle connected to 3/16 inch internal diameter Tygon Clear Laboratory Tubing (Fisher Scientific) and collected in a 10 L Nalgene plastic carboy (Nalge Nunc International) containing 1 L 3.2% trisodium citrate as an anticoagulant. A total of 5 to 10 liters blood has been collected per drawing (over 20 - 40 min), depending on the cooperation of the horse. The carboy is immediately packed in ice. All further purification steps are performed in a cold room at about 14° C.

Blood cells were removed by centrifugation in a GS3 rotor (Sorvall Centrifuges) at 7000 rpm for 10 to 20 minutes at ~ 4° C. The equine plasma was then diluted with an equal amount of cold 10 mM Tris-Cl buffer, pH 8.0, and protease inhibitors were added to the majority of enzyme preparations (Phenylmethanesulfonyl fluoride (PMSF) to 1 mM, Pepstatin A to 100 nM, EDTA Na<sub>2</sub>, to 1 mM). Polyethylene glycol (PEG 8000, Sigma-Aldrich) was slowly added to a final concentration of 12% (w/v) and then stirred overnight (at least 12 hours).

PEG-precipitated proteins are removed by centrifugation for five minutes at 7000 rpm in a GS3 rotor. The supernate is then loaded by gravity onto a 9 × 30 cm column packed with 1 L DEAE-FF anion exchange media (Sigma-Aldrich) pre-equilibrated in 10 mM Tris-Cl, pH 8.0. The column is washed with ≥ 2 L 10 mM Tris-Cl, 50 mM NaCl, pH 8.0 at a flow rate of about 16 mL min<sup>-1</sup>. Protein bound to the column is eluted by a 1.6 L linear gradient of 50 to 300 mM NaCl in 10 mM Tris-Cl, pH 8.0. E PAO elutes just before and along with a dark-blue band attributed to the copper-containing plasma enzyme ceruloplasmin, a blue copper oxidase. All eluant capable of benzylamine

oxidation significantly above that of equine plasma (typically with  $\geq 100$  Spectroscopic Units  $\text{mL}^{-1}$ ) is pooled and dialyzed extensively against 15 mM  $\text{KP}_i$  buffer, pH 6.8.

Anion exchange chromatographies using an FPLC System with a HiLOAD 26/10 Q-Sepharose HP column (Amersham-Pharmacia) comprise the next two steps of the purification scheme. Protein (equilibrated in 15 mM  $\text{KP}_i$ , pH 6.8) is loaded onto the column using a 150 mL Superloop (Amersham-Pharmacia). A maximum of  $1 \times 10^5$  Spectroscopic Units or 2 mg total protein, whichever is greater, is loaded per HiLOAD Q-Sepharose HP run, and multiple runs are typically required. Protein is eluted with a computer-programmed method using a phosphate buffer gradient, where Buffer A is 15 mM  $\text{KP}_i$ , pH 6.8, and Buffer B is 400 mM  $\text{KP}_i$ , pH 6.8. The elution method is as follows: 1) 3 column volume (CV) wash with Buffer A; 2) an 8 CV gradient to 65% Buffer B; and 3) 2.5 CV wash with 100% buffer B. Flow rate is  $6 \text{ mL min}^{-1}$ , and the fraction collector is programmed to collect 9 mL fractions. All fractions with an appropriate specific activity (typically  $\geq 300$  Spectroscopic Units  $\text{mL}^{-1}$ ) are pooled and dialyzed extensively against 15 mM  $\text{KP}_i$ , pH 6.8.

Chromatography using the HiLOAD 26/10 Q-Sepharose HP column is repeated and is nearly identical to the preceding step, except the elution is by a sodium chloride gradient; Buffer B is now 15 mM  $\text{KP}_i$ , 300 mM NaCl, pH 6.8. Again, best results are obtained when limiting the total protein loaded per chromatographic run, and multiple runs are generally necessary. Fractions are assayed and those with the highest specific activities are pooled and concentrated using Millipore Stirred Cells with Amicon YM30

Ultrafiltration Membranes (Amicon Bioseparations). The concentrate is then dialyzed extensively against 5 mM  $\text{KPi}$ , pH 6.8.

Using the FPLC System, the protein is then applied to an HR 10/10 column (1 cm diameter, 10 cm length, Amersham-Pharmacia) packed with Type I Macro-Prep Ceramic Hydroxyapatite, 40  $\mu\text{m}$  particle size (Bio-Rad) and pre-equilibrated in 5 mM  $\text{KPi}$ , pH 6.8. In this low ionic strength buffer, EPAO demonstrates a low binding affinity and is collected in the unbound, flow-through fractions. The majority of contaminating proteins have a higher affinity for the hydroxyapatite matrix and, although not tightly bound to the chromatographic medium, separation from EPAO is possible. Fortunately, undesired proteins can be followed visually by a bright yellow color, presumably from flavin-containing proteins. Multiple loadings of the column are required, and the media is washed clean with 400 mM  $\text{KPi}$ , pH 6.8 and equilibrated in 5 mM  $\text{KPi}$  between runs. The fractions containing EPAO are then concentrated using Millipore Ultrafree Centrifugal Filter Device (Amicon Bioseparations) with a nominal molecular weight limit of 30 kDa.

The purification is completed by gel filtration and buffer exchange using Ultrogel AcA34 (Biosepra) medium packed in a C 16/100 column (1.6 cm diameter, 100 cm length, Amersham-Pharmacia) and equilibrated with 100 mM  $\text{KPi}$ , pH 7.2. Best results are obtain when the protein sample is concentrated between 30 and 40  $\text{mg mL}^{-1}$ , a sample volume of about 1% of the column volume ( $\sim 1\text{mL}$ ) is loaded and the flow rate is approximately  $0.13\text{ mL min}^{-1}$ . Three mL fraction volumes are collected and analyzed by  $A_{280}$  determination, activity assays and SDS/PAGE using a 8-25% gradient gels and Coomassie Brilliant Blue staining. Those fractions with the highest specific activities

and best appearance by SDS/PAGE are pooled. Fractions with lower, but significant specific activities, are also pooled. Both pools of protein are concentrated for a final cleanup on the same column.

#### Analysis of EPAO.

Experimental procedures for the majority of the characterization of the equine enzyme has previously been described in detail in Chapters 1 and 2; only those that are unique to this chapter are described.

Benzylamine oxidation is followed by monitoring the formation of the product benzaldehyde at 250 nm. One spectroscopic unit (S.U.) is defined as giving an  $\Delta A_{250}$  of 0.001 per minute, and 4250 spectroscopic units correspond to 1 International Unit [81]. The standard activity assay uses 3.33 mM benzylamine and a total volume of 3 mL in 100 mM KP<sub>i</sub> buffer at pH 7.2. Steady state kinetics for the oxidation of the exogenous substrate benzylamine were conducted in 50 mM HEPES, pH 7.2 with the ionic strength adjusted to 150 mM by the addition of potassium chloride. Buffer and 10  $\mu$ L of 4.8  $\mu$ M EPAO with a specific activity of 700 S.U./mL were equilibrated in a quartz cuvette with stirring and the reaction was initiated by the addition of 100  $\mu$ L of substrate in buffer. Assays had a final volume of one milliliter and were at 37 °C.



## Results

### EPAO Purification.

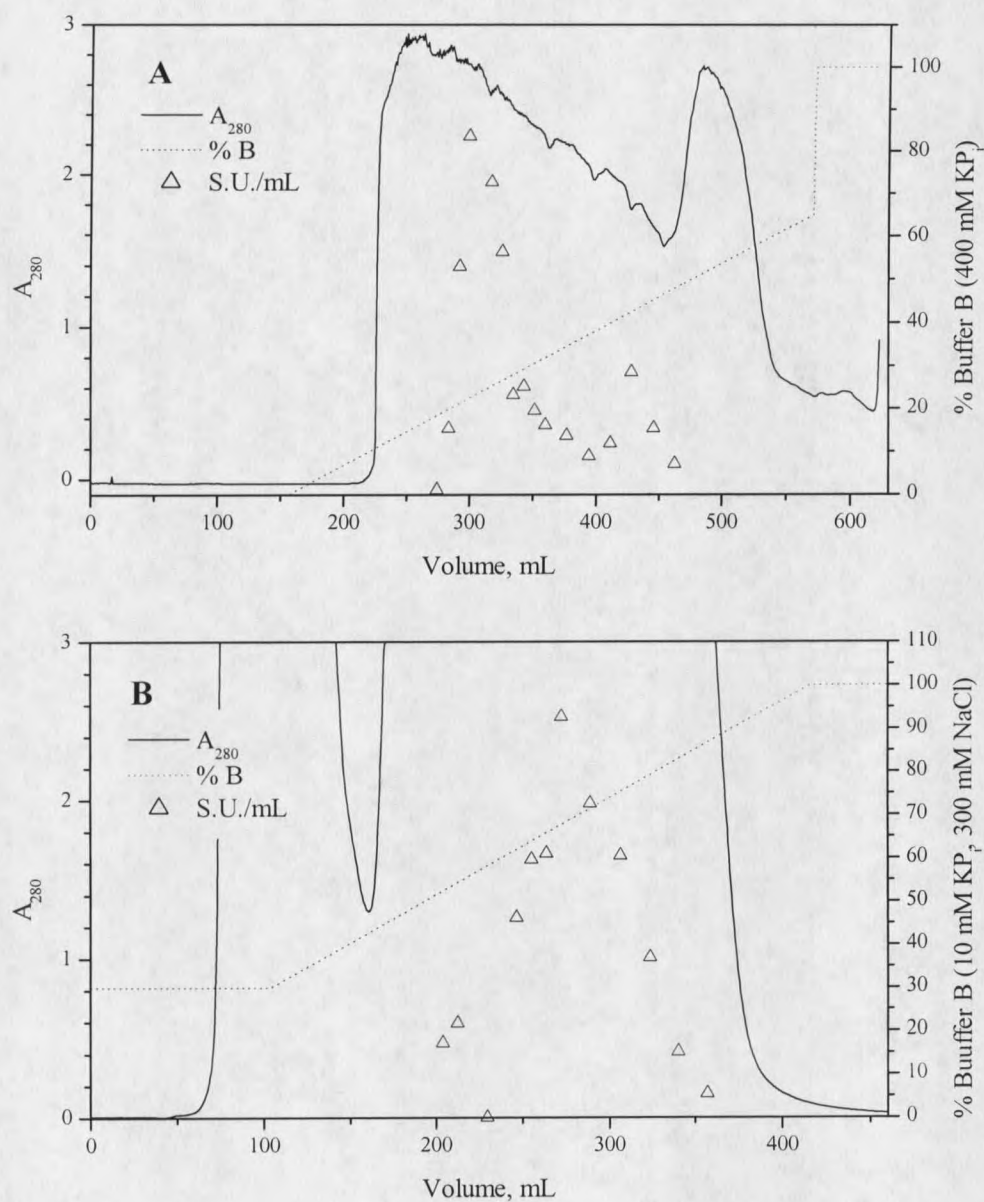
A new purification scheme has been developed for equine plasma amine oxidase, a protocol that specifically does not include ammonium sulfate fractionation. Equine plasma amine oxidase activity is low in freshly collected plasma; the oxidation of benzylamine can be detected spectrophotometrically but quantification of activity is generally not possible. The collected equine plasma is diluted 1:1 with 10 mM Tris-Cl buffer, pH 8.0 and fractionated by polyethylene glycol precipitation. EPAO does not precipitate with the concentrations of PEG used, nor do the majority of proteins present in the plasma. However, this step is crucial in preventing the formation of particulates in the plasma and in eliminating the propensity of the plasma to turn chromatography media into a hard jello-like mass, presumably by removal or inactivation of blood clotting agents. Dilution of the plasma before PEG precipitation was found to appreciably increase the recovery of amine oxidase activity.

The second step in the purification procedure is anion exchange chromatography over DEAE-Fast Flow medium. Loading of this column takes between two and four days, due to the high viscosity of the PEG solution; however PEG is generally considered to act as a protein stabilizer and the extended time EPAO spends with polyethylene glycol has not appeared to be detrimental. This particular step is also the most visually enjoyable part of the preparation because the bland, yellowish plasma partitions into very

colorful layer – deep blue at the top of the column and multiple bands in various shades of red, pink and yellow.

The next steps are two loadings on an anion exchange column and elution under two different buffer condition. Figure 69 shows chromatograms from typical 26/10 Q-Sepharose HP column runs, using a  $KP_i$  gradient for the first run, and a sodium chloride gradient for the second loading.

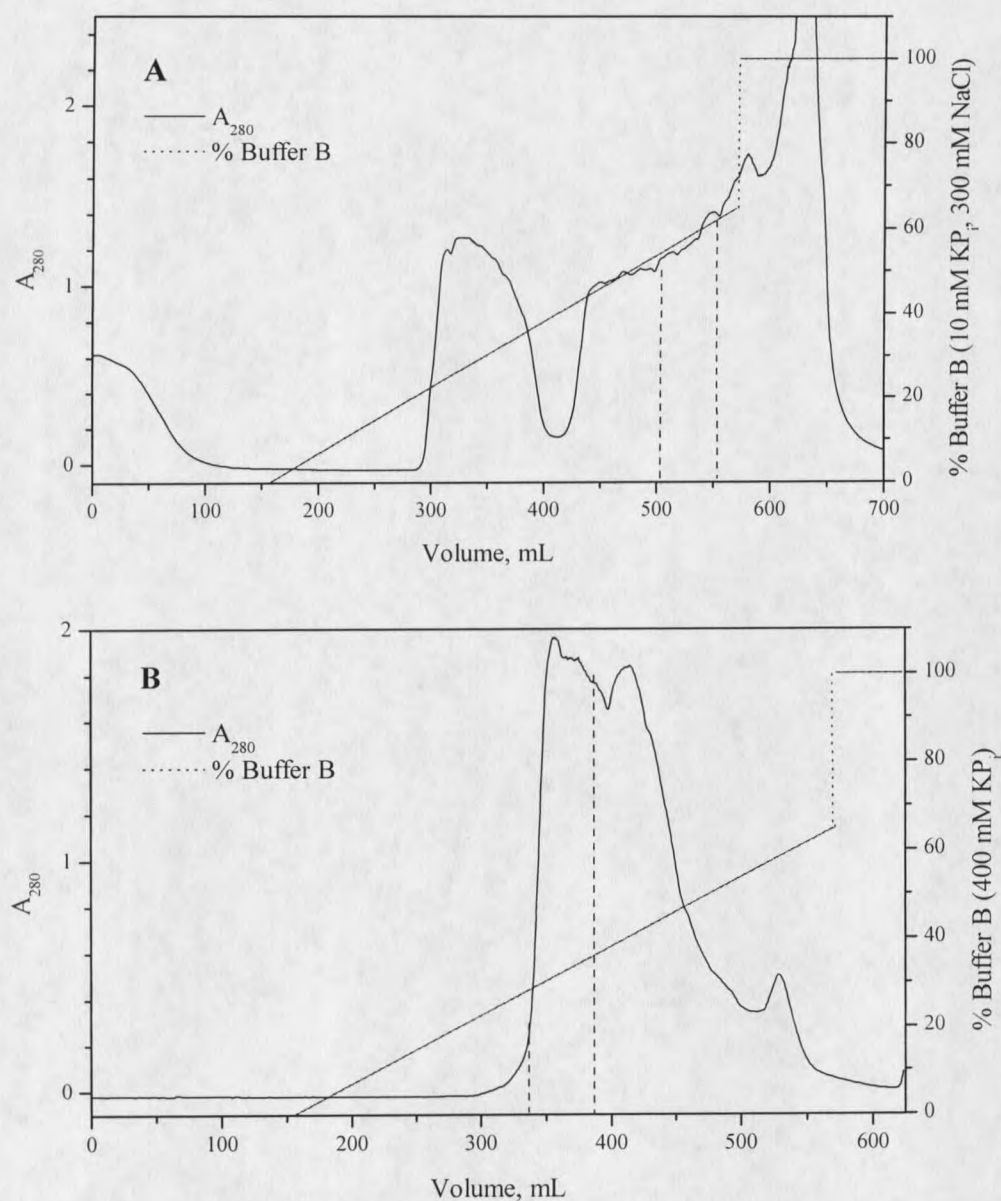
The elution profiles of EPAO from the HiLOAD 26/10 Q-Sepharose HP column can vary considerably among different purifications, and these differences can be more pronounced in either the inorganic phosphate or the sodium chloride elution gradients. For example, in one enzyme purification the EPAO activity may elute in a single, symmetrical peak during the  $KP_i$  gradient. Another batch of collected blood may show EPAO activity eluting in up to three, distinct peaks. Therefore, prudent and appropriate choices must be made for the pooling of chromatographic fractions, based upon the specific activity of each fraction. It is common to have a couple of distinct protein pools with similar specific activities that should be kept separate during subsequent steps of the purification protocol. Additionally, a few preparations used a reversed order of elution, that is the NaCl elution gradient followed by the inorganic phosphate elution (Figure 70). The chromatograms are quite different from the normal protocol, however, this ordering also gives satisfactory results. The  $KP_i$  gradient was selected as the first step because this gradient cleanly separates ceruloplasmin from EPAO, which was particularly troublesome in initial purifications.



**Figure 69.** A nion exchange chromatography with a HiLOAD 26/10 Q-Sepharose HP column.

**A)** First step with a gradient from 10 mM  $KP_i$ , pH6.8 (Buffer A) to 400 mM  $KP_i$ , pH6.8 (Buffer B).

**B)** Second step with a gradient from 10 mM  $KP_i$ , pH6.8 (Buffer A) to 10 mM  $KP_i$ , 300 mM NaCl, pH6.8 (Buffer B).

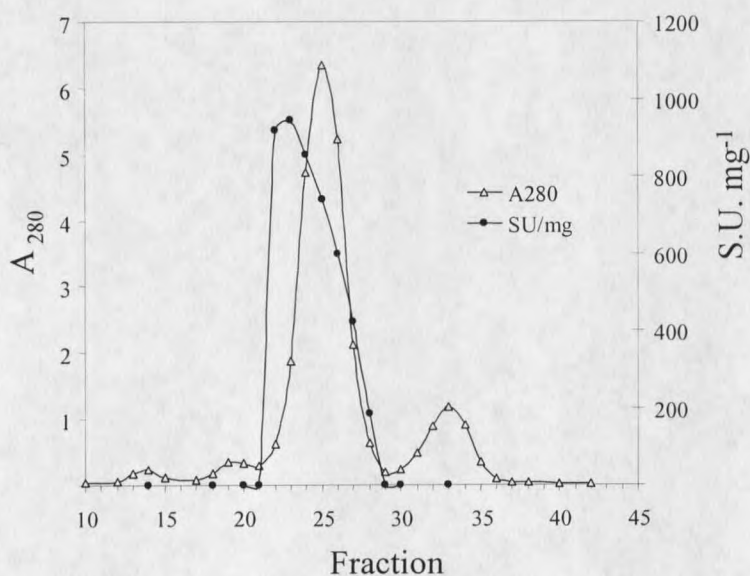


**Figure 70:** Anion exchange chromatography with a HiLOAD 26/10 Q-Sepharose HP column. EPAO activity is demarcated with horizontal dashed lines.

**A)** First step with a gradient from 10 mM  $KP_i$ , pH 6.8 (Buffer A) to 400 mM  $KP_i$ , pH 6.8 (Buffer B).

**B)** Second step with a gradient from 10 mM  $KP_i$ , pH 6.8 (Buffer A) to 10 mM  $KP_i$ , 300 mM NaCl, pH 6.8 (Buffer B).

Gel filtration on a  $1.6 \times 100$  cm Ultrogel Aca34 column is both the penultimate and the ultimate step in the purification protocol. Figure 71 shows a representative chromatogram of the first run over the column. It is evident from the traces of specific activity and absorbance at 280 nm that the highest EPAO activity comes off before the peak in the UV absorbance, indicative of contaminant that nearly co-elutes with EPAO. This suggested contaminant is not resolved by SDS/PAGE (*vide infra*) and does not exhibit noticeable absorption features in the visible portion of the electronic spectrum (data not shown). Fractions from the column are pooled on the basis of their specific activities and subject to additional gel filtration loadings; thus EPAO specific activity (and therefore degree of purity) can be enriched by multiple runs over this column.



**Figure 71.** Chromatogram of gel filtration on a  $1.6 \times 100$  cm Ultrogel Aca34 column

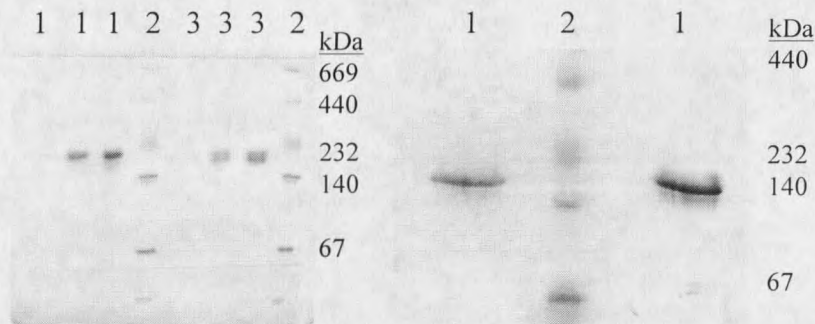
Two EPAO purifications are summarized in Table 4. The highest specific activity obtained was 925 Spectroscopic Units per milligram protein ( $0.22 \text{ I.U. mg}^{-1}$ ).

SDS/PAGE following the steps of the protocol and demonstrating the high degree of purification are given in Figure 72. Calibrated SDS/PAGE gels indicate EPAO has an apparent molecular weight of 96 kDa per monomer (data not shown). Native gels and isoelectric focusing gels are shown in Figures 73 and 74. The apparent molecular weight of the dimer is consistent with the SDS/PAGE monomer. The isoelectric point is low ( $pI \approx 4.5$ ), however, the pea seedling amine oxidase also has a similarly low  $pI$  value [237]. Schallinger reported several bands by isoelectric focusing with a  $pI$  range from 4.7 to 5.7 [234].

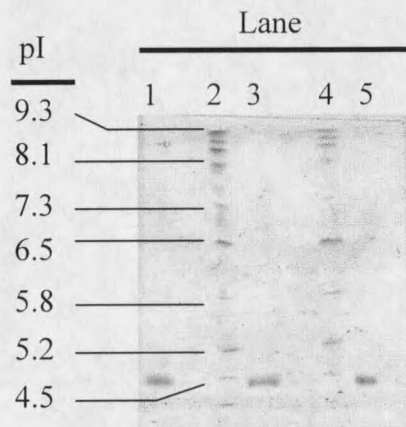


**Figure 72.** SDS/PAGE of the purification for EPAO: 1) Equine plasma 2) PEG precipitation 3) DEAE FF 4) Q Sepharose HP,  $KP_i$  gradient 5) Q Sepharose HP, NaCl gradient 6) Ceramic Hydroxyapatite 7) molecular weight markers (Amersham Pharmacia, molecular weights of markers are indicated) 8) Ultrogel AcA34. Multiple lanes with varying concentrations of EPAO following gel filtration are shown to demonstrate the high purity of the enzyme.





**Figure 73.** Native PAGE. Lanes 1) Purified EPAO 2) Native High Molecular Weight Standards (Amersham-Pharmacia) 3) Post-Ceramic Hydroxyapatite. Molecular weights of standards are indicated. The gel on the right demonstrates the degree of purity.



**Figure 74.** IEF gel. Lanes 1,3,5) EPAO 2,4) IEF standards (Amersham Pharmacia). pI values of standards are indicated.

| Sept. 1998<br>Step                          | Total<br>Volume<br>(mL) | Total<br>Protein<br>(mg) | Total<br>Activity<br>(S.U.) | Specific<br>Activity<br>(S.U./mg) | Purification<br>Factor | Yield |
|---------------------------------------------|-------------------------|--------------------------|-----------------------------|-----------------------------------|------------------------|-------|
| Equine Blood                                | 9000                    | N/D                      | N/D                         | N/D                               | N/A                    | N/A   |
| Equine Plasma                               | 6000                    | $2.0 \times 10^5$        | $2.9 \times 10^5$           | 1.47                              | N/A                    | 100%  |
| PEG                                         | 12800                   | $9.2 \times 10^4$        | N/D                         | N/D                               | N/D                    | N/D   |
| DEAE FF                                     | 620                     | $5.5 \times 10^3$        | $3.2 \times 10^5$           | 49.8                              | 34                     | 110%  |
| Q-Sepharose HP<br>K <sub>p</sub> i Gradient | 191                     | 1175                     | $2.1 \times 10^5$           | 177                               | 120                    | 72%   |
| Q-Sepharose HP<br>NaCl Gradient             | 64                      | 774                      | $1.7 \times 10^5$           | 215                               | 146                    | 59%   |
| Ceramic<br>Hydroxyapatite                   | 8.9                     | 319                      | $1.5 \times 10^5$           | 471                               | 320                    | 52%   |
| Ultrogel AcA34<br>Pool I                    | 3                       | 27                       | $2.5 \times 10^4$           | 925                               | 629                    | 9%    |
| Pool II                                     | 2.4                     | 27                       | $1.9 \times 10^4$           | 706                               | 480                    | 7%    |
| Pool III                                    | 8                       | 80                       | $6.6 \times 10^4$           | 820                               | 558                    | 22%   |
| Oct. 1999<br>Step                           | Total<br>Volume<br>(mL) | Total<br>Protein<br>(mg) | Total<br>Activity<br>(S.U.) | Specific<br>Activity<br>(S.U./mg) | Purification<br>Factor | Yield |
| Equine Blood                                | 9000                    | N/D                      | N/D                         | N/D                               | N/A                    | N/A   |
| Equine Plasma                               | 6300                    | $2.0 \times 10^5$        | $2.7 \times 10^5$           | 1.34                              | N/D                    | 100%  |
| PEG                                         | 13250                   | $8.1 \times 10^4$        | $5.6 \times 10^5$           | 6.94                              | 5.2                    | 204%  |
| DEAE FF                                     | 408                     | 5619                     | $2.9 \times 10^5$           | 52.3                              | 39                     | 107%  |
| Q-Sepharose HP<br>K <sub>p</sub> i Gradient | 142                     | 787                      | $1.3 \times 10^5$           | 168                               | 125                    | 48%   |
| Q-Sepharose HP<br>NaCl Gradient             | 62                      | 532                      | $1.0 \times 10^5$           | 197                               | 147                    | 37%   |
| Ceramic<br>Hydroxyapatite                   | 2.71                    | 114                      | $1.1 \times 10^5$           | 787                               | 587                    | 41%   |
| Ultrogel AcA34<br>Pool I                    | 0.76                    | 21.6                     | $1.71 \times 10^4$          | 791                               | 590                    | 6.3%  |
| Pool II                                     | 1.76                    | 41.1                     | $2.88 \times 10^4$          | 702                               | 524                    | 11%   |
| Pool III                                    | 2.76                    | 37.2                     | $3.14 \times 10^4$          | 844                               | 630                    | 12%   |

Table 4. Summary of two EPAO purifications.



### Copper and Calcium

Divalent metal ion content for purified EPAO was analyzed by ICP emission spectroscopy, flame atomic or absorption spectroscopy. Table 5 shows the results for several enzyme preparations. Copper levels are consistent with two ions per EPAO dimer. Unlike rhKDAO, zinc levels were not above the buffer control samples, or in one case at a low level, and similar results are shown for magnesium. These results were the first to indicate stoichiometric levels of calcium. The presence of calcium in a mammalian CAO, and thus the likely identification of calcium as occupying the second metal binding site, was later supported by analysis of the recombinant human kidney diamine oxidase. However the stoichiometries differ; rhKDAO data suggests two calcium ions per dimer, and the EPAO data is most consistent with three ions per dimer. The extra calcium may reflect adventitious and non-specific binding to the enzyme, although it is also possible that the equine enzyme does have an additional calcium binding site, perhaps at the dimer interface.

The last row in Table 5 shows the ICP-absorption spectroscopy results for two samples from the same purification, one of which was extensively dialyzed against buffer containing 2 mM EDTA followed by dialysis against Chelex-100 treated buffer. The values for Zn and Mg are essentially the same between the sample as purified and the sample treated by dialysis against EDTA. This suggests that the data for these two dications may be an artifact. It is quite interesting that EDTA treatment did remove ~30% of the original calcium, however, calcium is still present at a ratio of 2.6:1 dimer. Thus the calcium ions likely occupy specific and high affinity binding sites in EPAO,

consistent with the results reported above for rhKDAO. Note that calcium and zinc were also detected in the Chelex-100 treated HEPES buffer, at 75 and 17 ppb ( $\mu\text{g mL}^{-1}$ ) respectively. For comparison, 1190 ppb calcium and 172 ppb zinc were determined for EPAO dialyzed against EDTA.

| Specific Activity<br>(SU/mg) | Cu                                   | Zn             | Ca               | Mg  | TPQ                                  |
|------------------------------|--------------------------------------|----------------|------------------|-----|--------------------------------------|
| 700                          | 1.8 <sup>a</sup><br>2.1 <sup>f</sup> | 0 <sup>a</sup> | 2.9 <sup>a</sup> | 0   | 1.3 <sup>c</sup>                     |
| 700                          |                                      |                |                  |     | 1.7                                  |
| 900                          | 2.3 <sup>b</sup>                     |                |                  |     |                                      |
| 925                          | 2.7 <sup>a</sup><br>2.4 <sup>e</sup> |                |                  |     | 1.8 <sup>c</sup><br>1.5 <sup>d</sup> |
| 820 <sup>g</sup>             | 1.6                                  | 0.2            | 4.1              | 0.4 |                                      |
| EDTA treated                 | 1.72                                 | 0.2            | 2.6              | 0.2 |                                      |

**Table 5.** Divalent metal and TPQ quantification for dimeric EPAO

<sup>a</sup> ICP-AES

<sup>b</sup> flame AAS

<sup>c</sup> Phenylhydrazine titration

<sup>d</sup> anaerobic substrate titration, monitoring decrease in absorbance at 466 nm

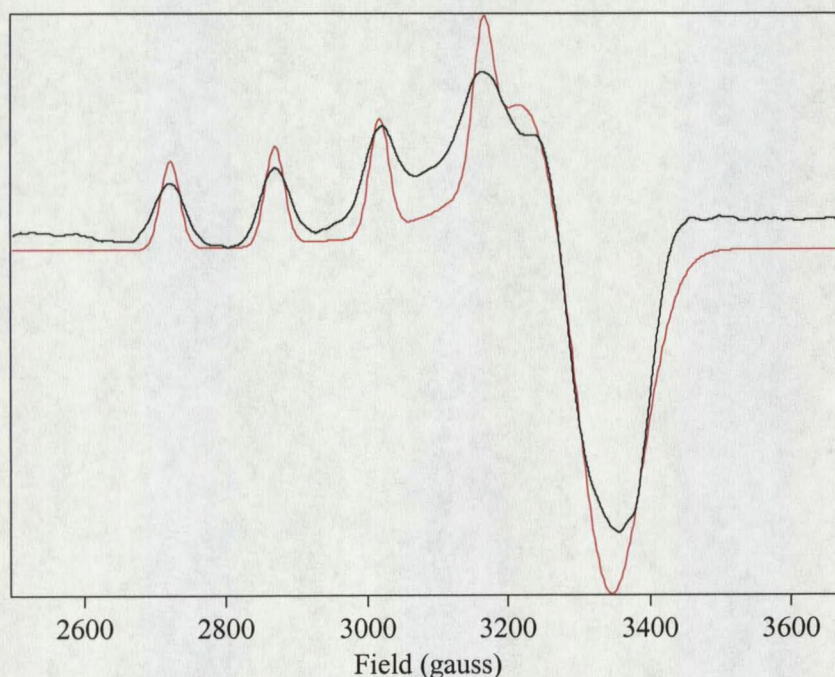
<sup>e</sup> EPAO extensively dialyzed against EDTA containing buffer, followed by Chelex-100 treated buffer.

<sup>f</sup> determined by double integration of EPR signal.

<sup>g</sup> ICP-AAS

Copper was also quantified via EPR (data not shown). Spectra were recorded for 125  $\mu\text{L}$  samples of 53.88  $\mu\text{M}$  EPAO ( $\sim 108 \mu\text{M}$  copper) and 50 and 100  $\mu\text{M}$  copper standard solutions (Cu(II) triethylenetetramine). Conditions were: field center 3100 G, sweep width 1200 G, frequency 9.426 GHz, power 0.399 mW, modulation amplitude 20 G, eight scans at 77 °C. Double integration of the signals indicates 2.1 Cu(II) per homodimer.

Figure 75 shows the X-band EPR spectrum of Cu(II) in purified equine plasma amine oxidase. The simulation was kindly provided by Dr. Melanie Rogers (Montana State University). Parameters derived from the simulated spectra ( $g_{\perp} = 2.045$ ,  $g_{\parallel} = 2.265$ ,  $A_{\parallel} = 169$  G) are consistent with published CAO values from various sources [2]. These are the first reported parameters from a simulated EPAO spectra. Previous values estimated from a spectrum are  $g_{\perp} = 2.06$ ,  $g_{\parallel} = 2.30$ ,  $A_{\parallel} = 150$  G. The spectrum suggests equivalent copper binding sites in the dimer and no other paramagnetic signals were observed.



**Figure 75.** EPR spectrum of 48.6  $\mu$ M EPAO in 100 mM  $\text{KP}_i$ , pH 7.1. The lower spectrum is a simulation with  $g_{\perp} = 2.045$ ,  $g_{\parallel} = 2.265$ , and  $A_{\parallel} = 169$  G. Experimental conditions: frequency 9.425 GHz; power 0.502 mW; modulation amplitude 20 G; 77 K.

### Visible and CD Spectroscopy

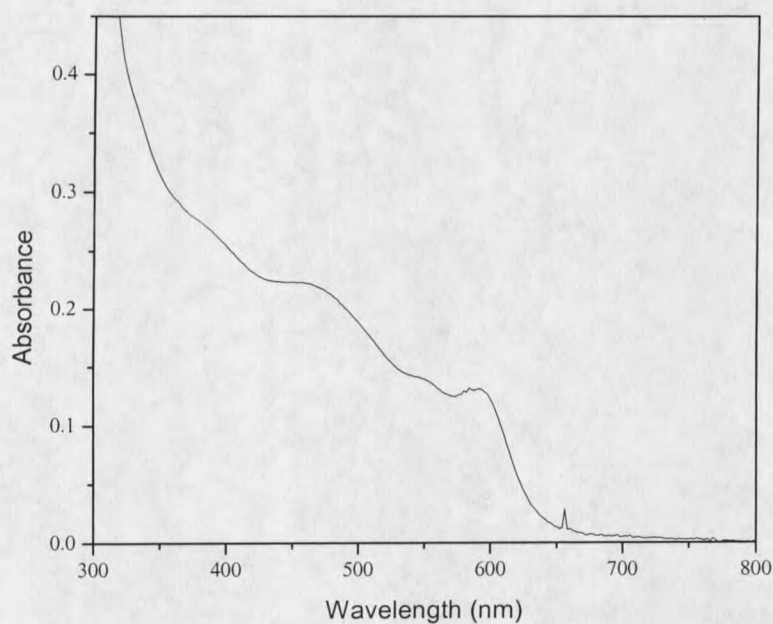
The visible absorption spectrum is shown in Figure 76. The characteristic TPQ band is present and centered at 466 nm. This  $\lambda_{\text{max}}$  is at a higher energy than that reported for other amine oxidases; differences in  $\lambda_{\text{max}}$  values are thought to reflect differences in the degree of charge delocalization in oxidized TPQ [10]. The peak at 592 nm, the shoulder around 550 nm and the shoulder around 380 nm have been reported by other investigators [233-235]. These spectral features have not been assigned. Figure 77 gives the visible spectrum of anaerobic substrate reduced EPAO. As anticipated, the 466 nm band is quenched, but the other spectral features are not affected.

Figure 78 shows the circular dichroism spectra of EPAO. The bands at 800 and 650 nm are assigned to Cu(II) d-d transitions as described in other amine oxidases and the band around 466 nm is attributed to TPQ. [2,238].

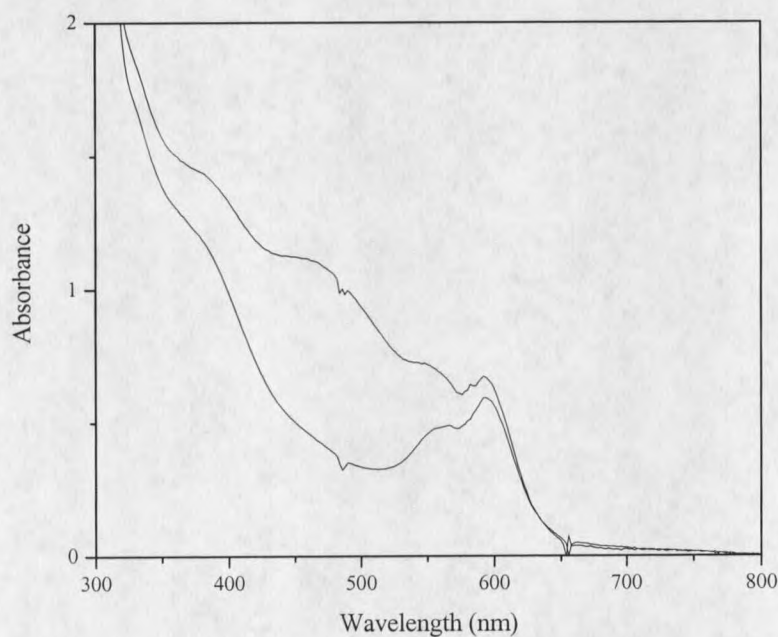
### TPQ quantification.

TPQ can be conveniently quantified by titration with phenylhydrazine, and this technique is described in detail in Chapter 2. Figure 79 shows a representative titration. TPQ quantified from several enzyme preparations are given in Table 5 and values range from 1.3 to 1.8 TPQ moieties per EPAO dimer.

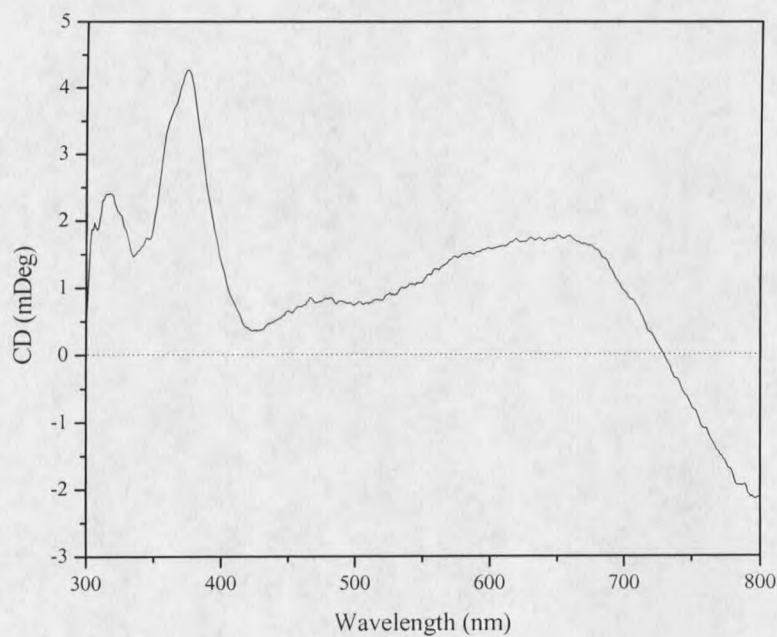




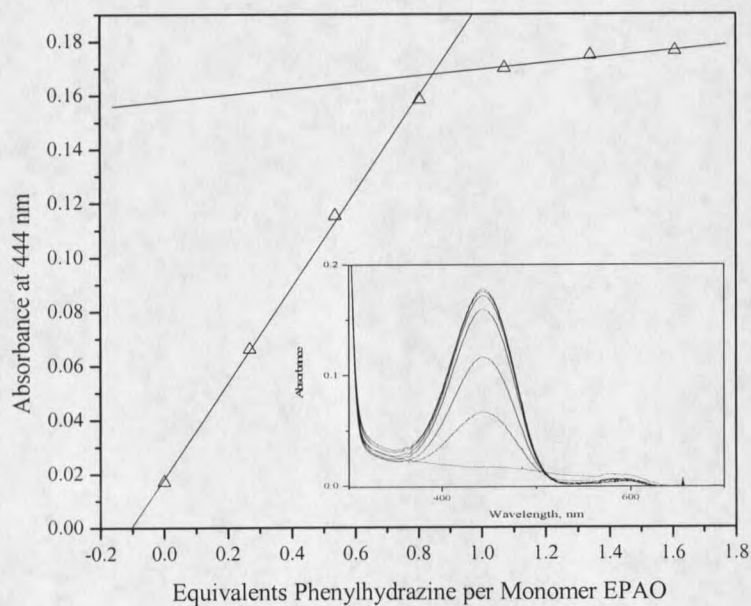
**Figure 76.** Visible absorption spectrum of oxidized form of EPAO (14 mg/mL).



**Figure 77.:** Visible absorption spectrum of the anaerobically substrate reduced equine plasma amine oxidase. The upper spectrum is the oxidized enzyme and is shown for comparison.



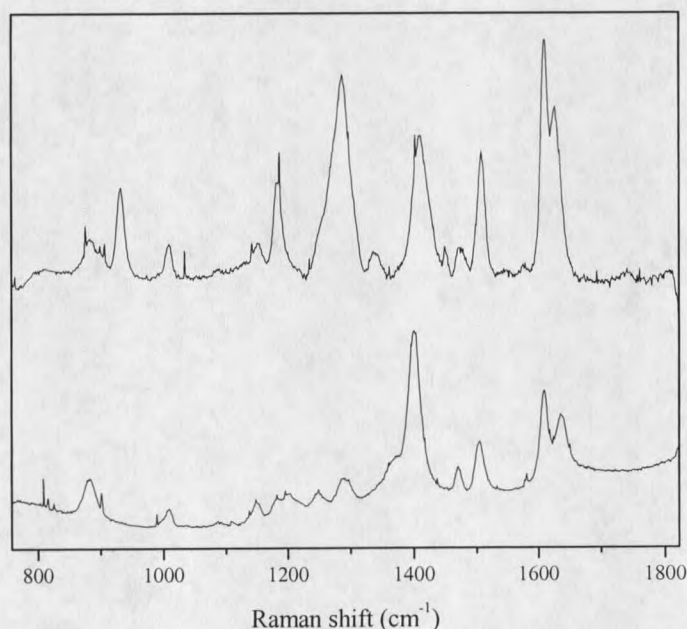
**Figure 78.** Circular dichroism spectrum of equine plasma amine oxidase.



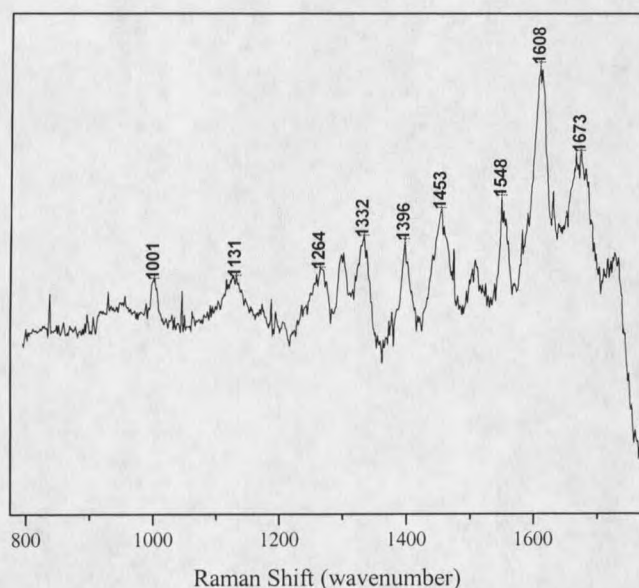
**Figure 79.** Phenylhydrazine titration of EPAO. This shows a cofactor to protein stoichiometry of 0.85 TPQ per monomer or 1.7 per dimer.

### Resonance Raman Spectroscopy.

Resonance Raman spectra of the phenylhydrazine derivatives of amine oxidases are definitive for identification of the TPQ cofactor in copper amine oxidases. The resonance Raman spectrum of the phenylhydrazine adduct of EPAO is given in Figure 80 and is essentially identical to the spectrum of Carter et al. [235]. The visible and resonance Raman spectra of the phenylhydrazine adduct indicate that this carbonyl reagent reacts only with TPQ and not with the source of the other visible spectral features of the native enzyme. Figure 81 shows the resonance Raman spectrum of highly concentrated native EPAO.



**Figure 80.** Resonance Raman spectrum of the phenylhydrazine derivative of EPAO (upper spectrum). The lower spectrum is of the model compound TPQ-hydantoin reacted with phenylhydrazine. Experimental conditions: excitation 457.9 nm, power 40 mW, integration 1 minute, 10 accumulations.



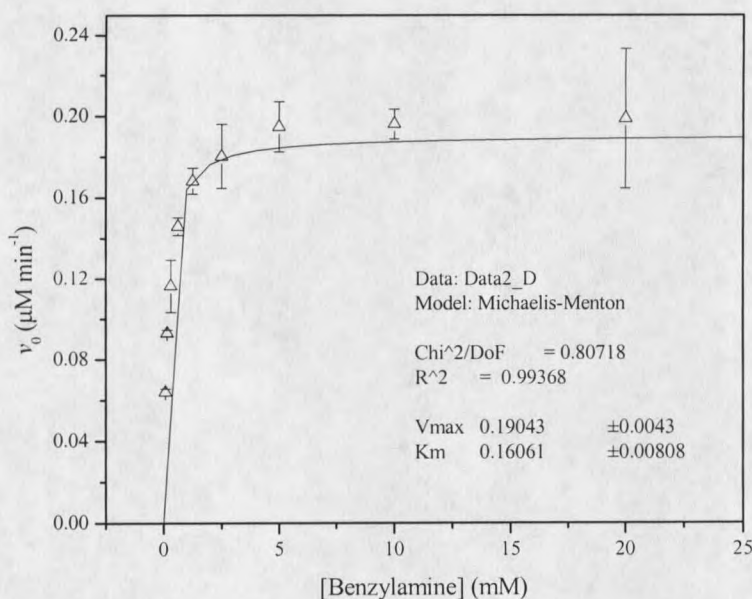
**Figure 81.** Resonance Raman spectra of native EPAO. Experimental conditions: excitation 476.5 nm, power 40 mW, integration 1 minute, 15 accumulations.

#### Steady State Kinetics for Benzylamine Oxidation.

The steady state parameters for benzylamine oxidation were determined with pH, ionic strength and temperature approximating physiological conditions. The fit to the Michaelis-Menten equation indicates a  $K_M$  of  $160 \pm 10 \mu\text{M}$  and a  $V_{\text{max}}$  of  $1714 \pm 39$  International Units per liter (Figure 82). Division of  $V_{\text{max}}$  by the enzyme concentration gives  $k_{\text{cat}} = 3.95 \pm 0.2 \text{ min}^{-1}$ . Carter et al. [235] reported a  $K_M$  of  $130 \mu\text{M}$  for benzylamine oxidation, in close agreement to the value reported here, Note that Carter et al. used a different buffer, 100 mM  $\text{KP}_i$ , pH 7.2, which could account for the difference in  $K_M$  values. The Michaelis constant reported in this work is quite similar to that given for the porcine plasma amine oxidase[239], but is about an order of magnitude less than that reported for the bovine plasma amine oxidase, 1.4 mM [240]. The steady state



parameters for benzylamine oxidation by BPAO have been derived and indicate a  $K_M$  of  $2.7 \pm 0.3$  mM (data not shown). Interestingly, BPAO steady state kinetics for benzylamine oxidation, when analyzed under the same conditions given for EPAO, demonstrate substrate inhibition at benzylamine concentrations above 5 mM.



**Figure 82.** Steady state kinetics of benzylamine oxidation by EPAO.

### Crystallization and Diffraction.

EPAO from two preparations has yielded crystals and preliminary diffraction data in a collaborative effort with Professor Hans Freeman (University of Sydney, Australia). The space group is orthorhombic, C-centered with  $001 = 2n$ , i.e. C2221. The unit cell is enormous,  $110.9 \times 676.3 \times 379.1 \text{ \AA}^3$ , with an asymmetric unit estimated as 18 molecules. The volume of the unit cell is  $\sim 20$  larger than the units cells for amine oxidases from

*Arthrobacter globiformis* and *Pisum sativum*, with the resulting diffraction proportionally weaker. On a rotating-anode X-ray generator a resolution of about 4 Å is obtained.

### Discussion

#### EPAO Purification.

Polyethylene glycol precipitation was selected as the initial step of the developed purification protocol because it is a commonly used and well-studied procedure for the fractionation of blood plasma proteins [241-243]. Dilution of plasma with an equal part of buffer results in better separation of plasma components, increased yield of EPAO activity, and has the practical benefit of producing a less viscous solution for the following chromatographic step. 12% PEG (w/v) precipitation conditions were determined empirically to give a high recovery of total EPAO activity and to reduce deleterious protein aggregation and precipitation.

The remaining anion exchange and size exclusion chromatography steps give a high yield of very pure EPAO. The total enzyme in the fraction with the highest specific activity is similar to the yields in the published protocol of Carter et al. [235]. However, the purification protocol given here also gives substantial quantities of EPAO with lower, but still acceptable, specific activities.

The highest specific activities recovered by this method approach 1000 spectroscopic units per mg, markedly lower than the 1400 spectroscopic units per mg reported by Carter et al. The reasons for this discrepancy are not yet understood. Although the specific activities between the two protocols are comparable throughout

much of the preparations, the published protocol reports a much greater increase in specific activity following gel filtration than is observed in the purifications described here. This remains puzzling, especially considering both protocols use the same column, conditions, and gel filtration medium. It is also possible that genetic variation is a factor.

One drawback of the protocol developed here is the considerable time investment, an average of about four week. In comparison, Carter's method can be completed in two week. One cause of the time difference is due to the high viscosity of the polyethylene glycol-plasma solution, which increases the time required for the following anion exchange step. After development of this protocol it was determined that the deleterious effects attributed to ammonium sulfate fractionation in the purification of amine oxidases were in fact an artifact from the use of low quality ammonium sulfate (D.M. Dooley, pers. comm.). Therefore it may prove beneficially to incorporate ammonium sulfate precipitation into subsequent purifications..

In my hands it was found to be necessary to divide the protein solutions into aliquots for multiple chromatography runs over the same column and media in order to give acceptable purification factors. This was not required by Carter et al. Furthermore, Carter reported a significant increase in specific activity by the addition of an anion exchange chromatography step on a Mono-Q column (Amersham-Pharmacia) following gel filtration. When tried here, EPAO eluted in a broad, diffuse peak with no increase in specific activity.

The visible absorption spectra of EPAO is atypical for copper amine oxidase in that addition peaks and shoulders are present. This suggests the presence of contaminants

in the purified enzyme. However, no contaminants are discernible by Coomassie Blue staining of SDS/PAGE, native AGE, and isoelectric focusing gels. Therefore any contaminants would be present at very low levels. No correlation between specific activity and the ratio of absorbance at 590 nm and 466 nm was observed (data not shown).

#### Copper, calcium and TPO.

Analysis of metal ion content by ICP-AES, ICP-AAS, and quantitative EPR is consistent with two Cu(II) ions per EPAO dimer, as expected for a copper amine oxidase. Although the molar extinction coefficient reported by Carter et al. is reasonable and has been used throughout this work, it was determined by correlating the absorbance at 280 nm with protein concentration estimated from the Bradford assay. It is possible for a more accurate value to be calculated through magnetic circular dichroism [33], which may result in more accurate cofactor quantification.

Calcium was determined to be present at an stoichiometry of 3 atoms per EPAO dimer. The inability to remove calcium by dialysis against 2 mM EDTA leads to the conclusion that the calcium ions are very tightly bound at a high affinity site. The equine enzyme provided the first indication that calcium likely occupies the second metal binding site of mammalian copper amine oxidases, and this result was later confirmed by analysis of rhKDAO. Calcium binding in proteins and possible functions are discussed in detail in the chapter devoted to expression and characterization of rhKDAO.

TPQ quantification by phenylhydrazine titration is consistent with two organic cofactors per EPAO dimer. Unlike rhKDAO, specific activity does not correlate well with either copper or TPQ quantification.

#### Visible absorption, circular dichroism, EPR and resonance Raman

Visible absorption and circular dichroism spectroscopy of purified EPAO are consistent with copper amine oxidases although, as mentioned above, the features at 592, 550 and 380 nm remain to be assigned. Most likely they indicate the presence of minor contaminants that are quite difficult to remove during purification. In developing this purification protocol several different chromatographic techniques were applied under several conditions, including alternative anion exchange, cation exchange, hydrophobic interaction, and concanavalin A chromatographies. None of these decreased the intensities of these anomalous spectral features nor significantly increased the specific activity.

EPR of the native enzyme and resonance Raman of the phenylhydrazine adduct for the enzyme purified by this protocol are entirely consistent with the characterized amine oxidases and virtually identical to Carter et al.

Resonance Raman of the native enzyme gives a relatively clean spectrum, unlike native rhKDAO that was highly fluorescent. Thus native EPAO is an attractive target for the characterization of trapped intermediates or covalently bound inhibitor compounds in a mammalian CAO. A recent article provides an example of the use of resonance Raman on native amine oxidases, in which rates of solvent exchange with the C5 carbonyl and

the C3 hydrogen of TPQ were measured . The data was then used to infer the orientation and mobility of the cofactor [244]

### Conclusions

Although the primary goal of this particular project, that is to obtain equine plasma crystal suitable for the collection of x-ray diffraction data and eventually a solved molecular structure for a mammalian amine oxidase, has yet to be achieved, considerable progress has been made. Two enzyme purifications have provided crystals, albeit unsuitable for structure determination. The fact that EPAO does crystallize encouraging one, and the crystallographers are optimistic that minor tweaking of the crystallization conditions may result in suitable crystals. Crystallization trials are an ongoing effort.

A purification protocol has been developed that gives large quantities, up to 100 mg, of highly purified enzyme. Such quantities are very conducive to detailed spectroscopic and biochemical investigations and provide an additional source of amine oxidase, a *mammalian* source, for future study and comparative analysis

An unexplored and attractive alternative source of EPAO would be to attempt development of a recombinant enzyme using the *Drosophila* S2 cell. Several equine cDNA libraries have been acquired in an attempt to deduce the amino acid sequence of this enzyme. Unfortunately, the preliminary selection attempts by PCR using degenerate primers designed from the mammalian amino acid consensus sequences have failed. A clone containing the mature coding sequence would be a major advancement and would allow efforts to produce a recombinant enzyme.

The purified enzyme has been used in studies which have so far resulted in two publications in collaboration with Professor Lawrence Sayre (Case Western Reserve University, OH) [219,220]. This work into irreversible amine oxidase inhibition and the logical design of specific inhibitors continues in the Dooley research group under the capable hands of Eric Shepard, Ph.D. candidate.

APPENDICES



APPENDIX A  
AMINO ACID SEQUENCE ALIGNMENT

A search of databases was conducted using the program TBLASTN v2.2.2 [245]. The databases searched include the National Center for Biotechnology Information nucleotide database (NCBI, <http://ncbi.nlm.nih.gov>), the NCBI microbial genomes databases, The Institute for Genomic Research Microbial Genomes databases (TIGR, <http://tigr.org>), the TIGR Unfinished Microbial databases, the TIGR Rice Genome Project database, the TIGR Unique Gene Indexes database, The Genome Database for Cyanobacteria (Cyanobase, <http://www.kazusa.org.jp/cyanobase>), The Sanger Center databases (<http://www.sanger.ac.uk>), The Genome Database (<http://gdb.org>), The Fugu Genomics Project (<http://hgmp.mrc.ac.uk>), and multiple databases available through the National Science Foundation Plant Genome Research Program (<http://www.nsf.gov/biol/dbi/pgrsites.html>). Input files for database searching were constructed from amine oxidases amino acid sequence data from both a microbial (*E. coli*) and a mammalian source (*H. sapiens* KDAO). The search of databases has been an ongoing exercise, and the data herein represent searches up until July, 2002.

The goal of the selection criteria for sequences to include in the lineup was to represent as many different sequences as available, to avoid duplicates, and to contain the catalytic Domain IV. Three arbitrary criteria were used: 1) the sequences must contain the TPQ consensus sequence, 2) the sequence must be at least fifty residues in length, and 3) sequences must differ from another sequence from the same organism in at least three amino acid positions. For example, two different sequences for the common pea, *Pisum sativum*, are included in this alignment although they are nearly identical, however, the

sequences are from two different cultivars or strains of pea and differ sufficiently so that both sequences were included in this lineup.

No attempt has been made to verify the accuracy of the sequences used herein, and there are certainly sequencing and analysis errors present. To illustrate, it is improbable that the carboxyl end of *Medicago truncatula*, common name barrel medic, is extended for some 300 residues beyond most copper amine oxidase sequences. Nor is it likely that *Schizosaccharomyces pombe* amine oxidase contains an eight residue histidine "tag" near its C-terminus. Furthermore, *Hordeum vulgare* 1, *Aspergillus niger* 1, and *Sorghum bicolor* sequences actually lack one or more of histidines that ligate copper.

One sequence, from the megaplasmid MP1 of the bacterium *Dienococcus radiodurans*, has a confirmed frameshift mutation that results in a prematurely terminated sequence that would certainly result in a nonfunctional enzyme if the polypeptide was expressed.

Presented below is a list of the organisms for which a copper amine oxidase sequence has been found, the common name of the organism where available, and its taxonomic lineage. Following this is the complete amino acid sequence alignment. The sequences were aligned using the CLUSTALW program included in the Vector NTI software package (Informax, Inc.) [246], and were ordered according to taxonomy. Multiple amine oxidase sequences from a single organism are distinguished by numbers (e.g. *Arabidopsis thaliana* 1, *Arabidopsis thaliana* 2, etc.). Each sequence in the alignment also contains the NCBI Accession Number, TIGR Unique Gene Identifier (UGI), or the database from which it originated.

List of Organisms1) *Homo sapiens*

Preferred common name: human

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo2) *Mus musculus*

Preferred common name: house mouse

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus3) *Rattus norvegicus*

Preferred common name: Norway rat

Rattus sp. strain Wistar [equivalent name]

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Rattus4) *Bos taurus*

Preferred common name: cow

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Mammalia; Eutheria; Cetartiodactyla; Ruminantia; Pecora; Bovoidea; Bovidae;  
Bovinae; Bos5) *Takifugu rubripes*Other names: *Fugu rubripes*

Preferred common name: torafugu

Other common name: pufferfish

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Actinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei;  
Acanthomorpha; Acanthopterygii; Percomorpha; Tetraodontiformes;  
Tetraodontidae; Takifugu6) *Lycopersicon esculentum*Other names: *Solanum lycopersicum*[synonym]

Preferred common name: tomato

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;  
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Asteridae;  
euasterids I; Solanales; Solanaceae; Solanum; Lycopersicon

7) *Solanum tuberosum*

Preferred common name: potato

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Asteridae; euasterids I; Solanales; Solanaceae; Solanum

8) *Arabidopsis thaliana*

Preferred common name: thale cress

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids II; Brassicales; Brassicaceae; Arabidopsis

9) *Cicer arietinum*

Preferred common name: chickpea

Other common name: garbanzo

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Cicereae; Cicer

10) *Canavalia lineata*

Preferred common name: none (same genus as jack bean)

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Canavalia

11) *Glycine max*

Preferred common name: soybean

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Phaseoleae; Glycine

12) *Medicago truncatula*

Preferred common name: barrel medic (*Medicago sativum* common name: alfalfa)

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Trifolieae; Medicago

13) *Pisum sativum*

Other names: *Pisum abyssinicum*[synonym]

Preferred common name: pea

Other common name: garden pea

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Viciae; Pisum

14) *Lens culinaris*

Preferred common name: lentil

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots; Rosidae; eurosids I; Fabales; Fabaceae; Papilionoideae; Viciae; Lens

15) *Oryza sativa*

Preferred common name: rice

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; Ehrhartoideae; Oryzeae; Oryza

14) *Triticum aestivum*

Other name: *Triticum vulgare*

Preferred common name: bread wheat

Other common names: common wheat, Canadian hard winter wheat, wheat

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; Pooideae; Triticeae; Triticum

15) *Hordeum vulgare* subspecies vulgare

Preferred common name: barley

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; Pooideae; Triticeae; Hordeum; Hordeum vulgare

16) *Zea mays* subspecies mays

Preferred common name: maize

Other common names: corn, Indian corn

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACC clade; Panicoideae; Andropogoneae; Zea; Zea mays

17) *Sorghum bicolor*Other name: *Sorghum vulgare*

Preferred common name: sorghum

Other common names: broomcorn, milo

Lineage: Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACC clade; Panicoideae; Andropogoneae; Sorghum

18) *Pichia angusta*Other name: *Hansenula polymorpha*

Lineage: Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Pichia

19) *Pichia pastoris*

Lineage: Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Pichia

20) *Candida albicans*Other names: *Candida stellatoidea*

Lineage: Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida

21) *Schizosaccharomyces pombe*Other name: *Schizosaccharomyces malidevorans*

Preferred common name: fission yeast

Lineage: Eukaryota; Fungi; Ascomycota; Schizosaccharomycetes; Schizosaccharomycetales; Schizosaccharomycetaceae; Schizosaccharomyces

22) *Aspergillus niger*

Lineage: Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Aspergillus

23) *Aspergillus fumigatus*

Lineage: Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Aspergillus

24) *Escherichia coli* K12

Lineage: Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae; Escherichia; Escherichia coli

25) *Klebsiella pneumoniae*

Other names: *Klebsiella pneumoniae*, *Bacterium pneumoniae crouposae*, *Bacterium pneumoniae crouposae*, *Hyalococcus pneumoniae*, *Hyalococcus pneumoniae*, *Bacillus pneumoniae*

Lineage: Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae; Klebsiella

26) *Legionella pneumophila*

Lineage: Bacteria; Proteobacteria; gamma subdivision; Legionellaceae group; Legionellaceae; Legionella

27) *Mesorhizobium loti*

Other names: *Rhizobium loti*

Lineage: Bacteria; Proteobacteria; alpha subdivision; Rhizobiaceae group; Phyllobacteriaceae; Mesorhizobium

28) *Arthrobacter globiformis*

Other names: *Achromobacter globiforme*, *Bacterium globiforme*, *Mycobacterium globiforme*, *Corynebacterium globiforme*, *Bacterium globiforme*, *Achromobacter globiformis*, *Mycobacterium globiforme*, *Corynebacterium globiforme*

Lineage: Bacteria; Firmicutes; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter

29) *Arthrobacter* sp. P1

Other names: *Arthrobacter* sp. (strain P1), *Arthrobacter* P1

Lineage: Bacteria; Firmicutes; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter

30) *Deinococcus radiodurans*

Other names: *Micrococcus radiodurans*, *Deinococcus radiidurans*

Lineage: Bacteria; Thermus/Deinococcus group; Deinococci; Deinococcales; Deinococcaceae; Deinococcus

31) *Mycobacterium smegmatis*

Other names: *Bacillus smegmatis*, *Bacterium smegmatis*, *Mycobacterium paratuberculosis smegmatis*,

Lineage: Bacteria; Firmicutes; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium

32) *Anabaena* sp.

Lineage: Bacteria; Cyanobacteria; Nostocales; Nostocaceae



33) *Nostoc punctiforme*

Lineage: Bacteria; Cyanobacteria; Nostocales; Nostocaceae; Nostoc

34) *Sulfolobus solfataricus*

Lineage: Archaea; Crenarchaeota; Thermoprotei; Sulfolobales; Sulfolobaceae;  
Sulfolobus

### Amino Acid Sequence Alignment

Key:

- Y Completely conserved consensus residue
- Y Consensus residue from similar residues at a given position
- Y Consensus residue from the occurrence of greater than 50% of a single residue at a given position
- Y Residue weakly similar to consensus residue
- H Tyrosine that forms a hydrogen bond to the C4 carbonyl of TPQ
- ◇ “Gate” residue, proposed to limit access to active site
- ⊕ Catalytic base
- ⊗ Tyrosinyl residue that is post-translationally modified to TPQ
- Δ Copper-coordinating histidinyl residue
- Second metal binding site, carboxylate ligand donor
- Second metal binding site, backbone carbonyl ligand donor
- The three residues suggested to define a hydrophobic, “pre-binding” site for dioxygen [20].

2° structure    Secondary structure of pea seedling amine oxidase: alpha helices represented by  $\alpha$ ,  $\beta$ -sheet by  $\beta$ , Domain II in light blue, Domain III in blue, Domain IV in purple. The numbering of secondary structure elements are also given [12].

|                                   |                     | 1                                                 | 20                                  | 40               | 50       |
|-----------------------------------|---------------------|---------------------------------------------------|-------------------------------------|------------------|----------|
| <i>Homo sapiens</i> DAO           | (NP_001082)         | -----                                             |                                     |                  |          |
| <i>Homo sapiens</i> SSAO          | (NP_003725)         | -----                                             |                                     |                  |          |
| <i>Homo sapiens</i> retinal SSAOb | (NP_033720)         | -----                                             |                                     |                  |          |
| <i>Homo sapiens</i> retinal SSAOa | (NP_001149)         | -----                                             |                                     |                  |          |
| <i>Mus musculus</i> DAO           | (AAH21880)          | -----                                             |                                     |                  |          |
| <i>Mus musculus</i> SSAO          | (AAD09199)          | -----                                             |                                     |                  |          |
| <i>Rattus norvegicus</i> DAO      | (NP_075224)         | -----                                             |                                     |                  |          |
| <i>Bos taurus</i> DAO             | (TIGR UGI TC108675) | -----                                             |                                     |                  |          |
| <i>Bos taurus</i> aorta SSAO      | (BAA88896)          | -----                                             |                                     |                  |          |
| <i>Bos taurus</i> liver BPAO      | (Q29437)            | -----                                             |                                     |                  |          |
| <i>Bos taurus</i> lung SSAO       | (O46406)            | -----                                             |                                     |                  |          |
| <i>Takifugu rubripes</i> 1        | (Cyanobase)         | -----                                             |                                     |                  |          |
| <i>Takifugu rubripes</i> 2        | (Cyanobase)         | -----                                             |                                     |                  |          |
| <i>Lycopersicon esculentum</i>    | (TIGR UGI TC90443)  | -----                                             | VRPKVTLVVRMAASVANYDYIVDWEFQNDGLIRPK |                  |          |
| <i>Solanum tuberosum</i>          | (TIGR UGI TC28345)  | -----                                             |                                     |                  |          |
| <i>Arabidopsis thaliana</i> 1     | (NP_174448)         | MVNRDNSIVALSFMLFLLVLHLHFETTTAARKPVRVFGPPSSIEWSPPS |                                     |                  |          |
| <i>Arabidopsis thaliana</i> 2     | (AAB87690)          | -----                                             |                                     |                  |          |
| <i>Arabidopsis thaliana</i> 3     | (NP_176469)         | -----                                             |                                     | MAEPSFARLFLLF    |          |
| <i>Arabidopsis thaliana</i> 4     | (NP_181777)         | -----                                             |                                     | MASASKKTSACPH    |          |
| <i>Arabidopsis thaliana</i> 5     | (NP_189953)         | -----                                             |                                     |                  |          |
| <i>Arabidopsis thaliana</i> 6     | (AAF19542)          | -----                                             |                                     |                  |          |
| <i>Cicer arietinum</i>            | (CAA08855)          | -----                                             |                                     |                  |          |
| <i>Canavalia lineata</i>          | (AAD49420)          | -----                                             |                                     | MESRNLLRFLVLSVCV |          |
| <i>Glycine max</i>                | (AAD40979)          | -----                                             |                                     |                  |          |
| <i>Medicago truncatula</i> 1      | (TIGR UGI TC38359)  | -----                                             |                                     |                  |          |
| <i>Medicago truncatula</i> 2      | (TIGR UGI TC37663)  | -----                                             |                                     |                  | RCXSSAYC |
| <i>Pisum sativum</i> 1            | (Q43077)            | -----                                             |                                     |                  |          |
| <i>Pisum sativum</i> 2            | (BAA77206)          | -----                                             |                                     |                  |          |
| <i>Lens culinaris</i> 1           | (P49252)            | -----                                             |                                     |                  |          |
| <i>Lens culinaris</i> 2           | (AAB34918)          | -----                                             |                                     |                  |          |
| <i>Oryza sativa</i>               | (TIGR UGI TC62332)  | -----                                             |                                     |                  |          |
| <i>Triticum aestivum</i>          | (TIGR UGI TC18533)  | -----                                             |                                     |                  |          |
| <i>Hordeum vulgare</i> 1          | (TIGR UGI TC12664)  | -----                                             |                                     |                  |          |
| <i>Hordeum vulgare</i> 2          | (TIGR UGI TC8886)   | -----                                             |                                     |                  |          |

|                                    |                    | 1     | 20 | 40 | 50                        |
|------------------------------------|--------------------|-------|----|----|---------------------------|
| <i>Zea mays</i>                    | (TIGR UGI TC85570) | ----- |    |    |                           |
| <i>Sorghum bicolor</i>             | (TIGR UGI TC24328) | ----- |    |    |                           |
| <i>Pichia angusta</i>              | (P12807)           | ----- |    |    |                           |
| <i>Pichia pastoris</i>             | (AF358434)         | ----- |    |    |                           |
| <i>Candida albicans</i> 1(6;2370)  | (NCBI)             | ----- |    |    |                           |
| <i>Candida albicans</i> 2(6;1984)  | (NCBI)             | ----- |    |    |                           |
| <i>Schizosaccharomyces pombe</i> 1 | (CAB83008)         | ----- |    |    |                           |
| <i>Schizosaccharomyces pombe</i> 2 | (T39171)           | ----- |    |    |                           |
| <i>Aspergillus niger</i> 1         | (S71320)           | ----- |    |    |                           |
| <i>Aspergillus niger</i> 2         | (AF362473_1)       | ----- |    |    |                           |
| <i>Aspergillus fumigatus</i> 1     | (NCBI)             | ----- |    |    |                           |
| <i>Aspergillus fumigatus</i> 2     | (NCBI)             | ----- |    |    |                           |
| <i>Escherichia coli</i>            | (P46883)           | ----- |    |    | MGSPSLYSARKTTTLALAVALSFAW |
| <i>Klebsiella aerogenes</i>        | (P49250)           | ----- |    |    | MANGLKFSPRKTALALAVAVVCAW  |
| <i>Klebsiella pneumoniae</i>       | (NCBI)             | ----- |    |    |                           |
| <i>Legionella pneumophila</i>      | (NCBI)             | ----- |    |    |                           |
| <i>Mesorhizobium loti</i>          | (NP_106786)        | ----- |    |    |                           |
| <i>Arthrobacter globiformis</i> 1  | (Q59118)           | ----- |    |    |                           |
| <i>Arthrobacter globiformis</i> 2  | (P46881)           | ----- |    |    |                           |
| <i>Arthrobacter</i> strain P1      | (Q07123)           | ----- |    |    |                           |
| <i>Deinococcus radiodurans</i>     | (NCBI)             | ----- |    |    |                           |
| <i>Mycobacterium smegmatis</i> 1   | (NCBI)             | ----- |    |    |                           |
| <i>Mycobacterium smegmatis</i> 2   | (NCBI)             | ----- |    |    |                           |
| <i>Anabaena</i> sp PCC7120         | (Cyanobase)        | ----- |    |    |                           |
| <i>Nostoc punctiforme</i>          | (NCBI)             | ----- |    |    |                           |
| <i>Sulfolobus solfataricus</i>     | (NP_343112)        | ----- |    |    |                           |

Consensus

|                         | 51                                                                               | 60                                | 80                           | 100                               | 120          | 129         |
|-------------------------|----------------------------------------------------------------------------------|-----------------------------------|------------------------------|-----------------------------------|--------------|-------------|
| <i>H. sapiens</i> DAO   | -----                                                                            | MPALGWAVAAAILMLQTAMAEP            | -----                        | S--PG--TLPRKAGVFSD                | LSNQELKAVHS  |             |
| <i>H. sapiens</i> SSAO  | ----                                                                             | MNQKTILVLLILAVITIFALVCVLLVGRG     | --                           | GDGGEPSQLPHCPSPSAQPWTHPGQSQLFAD   | LSREELTAVMR  |             |
| <i>H. sapiens</i> SSAOb | ----                                                                             | MHLKIVLAFLALSLITIFALAYVLLTSPG     | -----                        | GSSQPPHCPSVSHRAQPWPHPGQSQLFAD     | LSREELTAVMR  |             |
| <i>H. sapiens</i> SSAOa | ----                                                                             | MHLKIVLAFLALSLITIFALAYVLLTSPG     | -----                        | GSSQPPHCPSVSHRAQPWPHPGQSQLFAD     | LSREELTAVMR  |             |
| <i>M. musculus</i> DAO  | -----                                                                            | MSLAFGWAAVILLQTADTPS              | -----                        | A--VT--TPHDKARIFAD                | LSPQEIKAVHS  |             |
| <i>M. musculus</i> SSAO | ----                                                                             | MTQKTTLVLLALAVITIFALVCVLLAGRS     | --                           | GDGGGLSQPLHCPSVLPSVQPRTHPSQSQPFAD | LSPEELTAVMS  |             |
| <i>R. norvegicus</i>    | -----                                                                            | MCLAFGWAAVILVLQTVDTAS             | -----                        | A--VR--TPYDKARVFAD                | LSPQEIKAVHS  |             |
| <i>B. taurus</i> DAO    | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>B. taurus</i> aorta  | ----                                                                             | MNQKTTLVLLALAVITIFALVCVLIAGRG     | --                           | GDGGEASQPHYCPSGTPSVQPWTHPGQNQLFAD | LSREELTAVMS  |             |
| <i>B. taurus</i> BPAO   | -----                                                                            | MFIFIFLSLWTLVLMGREGGVGSEEGVGKQCH  | PSLPPRCPSRSPSDQPWTHPDQSQLFAD | LSREELTTVMS                       |              |             |
| <i>B. taurus</i> lung   | -----                                                                            | MFIFIFLSLWTLVLMGREGGVGSEEGVGKQCH  | PSLPPRCPSRSPSDQPWTHPDQSQLFAD | LSREELTAVMS                       |              |             |
| <i>T. rubripes</i> 1    | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>T. rubripes</i> 2    | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>L. esculentum</i>    | VGLSGILMVKGSPYVNMNEVNQNEYLYGTLLSENIVGVIHDHYVTFHLDMDIDGPSNNSFLKVNLLKE             |                                   |                              | MTSFGESPRRS                       |              |             |
| <i>S. tuberosum</i>     | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>A. thaliana</i> 1    | PPKDDFEWFEINIYKNIEQTAFRPTGQGPSQGIGHKD                                            | -----                             | PPVFSSVFVIGSLSFIPPPHPFDP     | LTEIELNLVRN                       |              |             |
| <i>A. thaliana</i> 2    | -----                                                                            | MHINVNVLLSSTSITTPSMNTS            | -----                        | ILAILFLIQCVFTLGLHFHPLDP           | LTPQEINKTSF  |             |
| <i>A. thaliana</i> 3    | FSFLLI FATYSWVFGPD SGFLFGTRVRKTLGS                                               | -----                             | NRQVHVDHSLEKPHHPLDP          | LTVREINRVRT                       |              |             |
| <i>A. thaliana</i> 4    | HGGS LPPP KLVSAAPDTVAVWSDADDQRASKVSLESVIRPVDSFPDNTAKKPANKGISVMPRTETKHP           |                                   | LDPLSAAEISV                  |                                   |              |             |
| <i>A. thaliana</i> 5    | ----                                                                             | MVELSFSQLLVLLLSLLFLFTTLASS        | -----                        | SKTPRFKYSLEKPHHPLDP               | LTTPPEIKRVQT |             |
| <i>A. thaliana</i> 6    | --                                                                               | FLLI FATYSWVFGPD SGFLFGTRVRKTL GS | -----                        | NRQVHVDHSLEKPHHPLDP               | LTVREINRVRT  |             |
| <i>C. arietinum</i>     | -----                                                                            | MASTTIKLSFFF                      | -----                        | AITLI FLQAVT ---                  | PLNLQHPLDP   | LTKEEFSIVQT |
| <i>C. lineata</i>       | ALVLLLTLFHSPTSLSNKGVLDCNLYSGWCSSKNRFSSTKPKVIKKQPSFTYQNNHESEVPQHPLDP              |                                   | LTIQEFNKVRN                  |                                   |              |             |
| <i>G. max</i>           | -----                                                                            |                                   | -----                        | MVRAQTSHPLDPLTAAEISV              |              |             |
| <i>M. truncatula</i> 1  | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>M. truncatula</i> 2  | XEILITGFQNRVHHL DQNSIVLLVINLKVLA FRSMVTSVSWANWKFHIGFDVRAGIVISLASIYDLEKHKSRRLVLYK | GY                                |                              |                                   |              |             |
| <i>P. sativum</i> 1     | -----                                                                            | MASTTTMRLALFS                     | -----                        | VLTLLSFHAVVSVTPLHVQHPLDP          | LTKEEFLAVQT  |             |
| <i>P. sativum</i> 2     | -----                                                                            | MASTTTMKLALFS                     | -----                        | VLTLLSFHAVVSVTPLHVQHPLDP          | LTKEEFLAVQT  |             |
| <i>L. culinaris</i> 1   | -----                                                                            | KFALFS                            | -----                        | VLTLLSFHAVFSFTPLHTQHPLDP          | ITKEEFLAVQT  |             |
| <i>L. culinaris</i> 2   | -----                                                                            | KFALFS                            | -----                        | VLTLLSFHAVFSFTPLHTQHPLDP          | ITKEEFLAVQT  |             |
| <i>O. sativa</i>        | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>T. aestivum</i>      | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>H. vulgare</i> 1     | -----                                                                            |                                   | -----                        |                                   |              |             |
| <i>H. vulgare</i> 2     | -----                                                                            |                                   | -----                        |                                   |              |             |

|                  | 51                                                                               | 60 | 80 | 100 | 120 | 129 |
|------------------|----------------------------------------------------------------------------------|----|----|-----|-----|-----|
| Z. mays          | -----                                                                            |    |    |     |     |     |
| S. bicolor       | -----                                                                            |    |    |     |     |     |
| P. angusta       | ----- MERLRQIASQATAASAAPARPAHP LDPLSTAEIKA                                       |    |    |     |     |     |
| P. pastoris      | ----MRLTNLLSLTTLVALAVAVPDFYQKREAVSSKEAALLRRDASAECVSNENVEIEAPKTNIWTS LAKEEVQEVLD  |    |    |     |     |     |
| C. albicans 1    | ----- HPLDPLSPAIEKS                                                              |    |    |     |     |     |
| C. albicans 2    | ----- HPFHPVKDYEIRLA                                                             |    |    |     |     |     |
| S. pombe 1       | ----- MAYYPHLPYHHHHHTSVPGERLSDP LDPLSADELKL                                      |    |    |     |     |     |
| S. pombe 2       | ----- MAEESKAAEYFDP LDPLSFNELRY                                                  |    |    |     |     |     |
| A. niger 1       | ----- MLPHPLAILSEEETNI                                                           |    |    |     |     |     |
| A. niger 2       | -----                                                                            |    |    |     |     |     |
| A. fumigatus 1   | -----                                                                            |    |    |     |     |     |
| A. fumigatus 2   | -----                                                                            |    |    |     |     |     |
| E. coli          | QAPVFAHGGEAHMVPMDKTLKEFGADVQWDDYAQLFTLIKDGAYVKVKPGAQTAIVNGQPLALQVPVV MKDNKAWVSDT |    |    |     |     |     |
| K. aerogenes     | QSPVFAHGSEAHMVPLDKTLQEFGADVQWDDYAQMFTLIKDGAYVKVKPGAQTAIVNGKSLDLPVPVV MKEGKAWVSDT |    |    |     |     |     |
| K. pneumoniae    | -----                                                                            |    |    |     |     |     |
| L. pneumophila   | -----                                                                            |    |    |     |     |     |
| M. loti          | ----- MNTKMTVPTKAVCGCSKPADATA                                                    |    |    |     |     |     |
| A. globiformis 1 | ----- MTLQTPSTPLVQDPPVPATLVHAA                                                   |    |    |     |     |     |
| A. globiformis 2 | ----- MTPSTIQ                                                                    |    |    |     |     |     |
| A. strain P1     | ----- MTLNAESEALVG                                                               |    |    |     |     |     |
| D. radiodurans   | -----                                                                            |    |    |     |     |     |
| M. smegmatis 1   | -----                                                                            |    |    |     |     |     |
| M. smegmatis 2   | -----                                                                            |    |    |     |     |     |
| Anabaena PCC7120 | -----                                                                            |    |    |     |     |     |
| N. punctiforme   | -----                                                                            |    |    |     |     |     |
| S. solfataricus  | ----- MVEEEQVAISKSPDLP LNEEE-IRKSV                                               |    |    |     |     |     |

Consensus

L

2° structure

αααααααααα

h1

|                         | 130                                                        | 140   | 160               | 180     | 200             | 208 |
|-------------------------|------------------------------------------------------------|-------|-------------------|---------|-----------------|-----|
| <i>H. sapiens</i> DAO   | FLWSKKELRLQPSSTTTMAKNTVFLIEMLLPKKYHVLRF                    | LDKG  | -----             | ER----- | HPVREARAVIFFG   |     |
| <i>H. sapiens</i> SSAO  | FLTQRLGPGLVDAAQARPSDNCVFSVELQLPPKAAALAH                    | LDRG  | -----             | SP----- | PPAREALAIVFFG   |     |
| <i>H. sapiens</i> SSAOb | FLTQRLGPGLVDAAQAQPSDNCIFSVELQLPPKAAALAH                    | LDRG  | -----             | SP----- | PPAREALAIVLFG   |     |
| <i>H. sapiens</i> SSAOa | FLTQRLGPGLVDAAQAQPSDNCIFSVELQLPPKAAALAH                    | LDRG  | -----             | SP----- | PPAREALAIVLFG   |     |
| <i>M. musculus</i> DAO  | FLMSRKELGLESSKNLTAKNSVFLIEMLLPKKKNVLKFL                    | DEG   | -----             | RK----- | SPVREARAIFFG    |     |
| <i>M. musculus</i> SSAO | FLTKHLGPGLVDAAQARPSDNCVFSVELQLPAKAAALAH                    | LDRG  | -----             | GP----- | PPVREALAIFFG    |     |
| <i>R. norvegicus</i>    | FLMNREELGLQPSKEPTLAKNSVFLIEMLLPKKKHVLKFL                   | DEG   | -----             | RK----- | GNPREARAVIFFG   |     |
| <i>B. taurus</i> DAO    | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>B. taurus</i> aorta  | FLTQKLGPDLVDAAQARPSDNCIFSVELQLPPKAAALAH                    | LDRR  | -----             | SP----- | PPAREALAIVFFG   |     |
| <i>B. taurus</i> BPAO   | FLTQQLGPDLVDAAQARPSDNCVFSVELQLPPKAAALAH                    | LDRG  | -----             | SP----- | PPAREALAIVFFG   |     |
| <i>B. taurus</i> lung   | FLTQKLGPDLVDAAQARPSDNCIFSVELQLPPKAAALAH                    | LDRR  | -----             | SP----- | PPAREALAIVFFG   |     |
| <i>T. rubripes</i> 1    | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>T. rubripes</i> 2    | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>L. esculentum</i>    | YLKAVRNVAKTEKDAQIKLKLYDPSEFHVINSNKK                        | S -R- | -----             | -----   | VGNPVGVTKLFLVEL |     |
| <i>S. tuberosum</i>     | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>A. thaliana</i> 1    | IINERYPIGLEHRTFTQYVGLNEPDKSLVLSWVSSQYH                     | ----- | -----             | -----   | NVKSPPRQ-AFV    |     |
| <i>A. thaliana</i> 2    | IVKKSH-LGNLKDITFHYLDLEPNKSHVLQWLSPNPS                      | ----- | -----             | -----   | KKPPPPRRRSFV    |     |
| <i>A. thaliana</i> 3    | ILSNHDPGFGSGSATIHSMALDEPEKSRVVQWKKGN                       | ----- | -----             | -----   | KLLSRR-AAV      |     |
| <i>A. thaliana</i> 4    | AVATVRAAGANPEVRDGMRFIEVASVEPDKQVVALADAYFFPPFQPSLLPRTKSGPVI | PMKLP | PPRRARLVVYNQKSNET |         |                 |     |
| <i>A. thaliana</i> 5    | ILSGHDPGFGSGSTIIHAMALDEPDKQRVIRWKKGD                       | ----- | -----             | -----   | RLPPRR-AEI      |     |
| <i>A. thaliana</i> 6    | ILSNHDPGFGSGSATIHSMALDEPEKSRVVQWKKGN                       | ----- | -----             | -----   | KLLSRR-AAV      |     |
| <i>C. arietinum</i>     | VVLHKYPTSKNS-VAFHYIGLDDPDKDDVLKYETQP                       | ----- | -----             | -----   | TLITIPRK-SFV    |     |
| <i>C. lineata</i>       | ILSTHPLFKSPSSYILHSIVLDEPDKKLVLKWKKG                        | D     | -----             | -----   | PLLPRK-ASV      |     |
| <i>G. max</i>           | AVATVRAAGATPEVRDGMRFIEVDLVEPEKQVVALADAYFFPPFQPSLLPRTKGGPVI | PTKLP | PPRKARLVVYNKKSNET |         |                 |     |
| <i>M. truncatula</i> 1  | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>M. truncatula</i> 2  | ISELFVPYQDPTEEFYFKTFFDSGEFGFGLSTVSLIPN                     | ----- | -----             | -----   | RDCPPHAQFIDTY   |     |
| <i>P. sativum</i> 1     | IVQNKYPISNNR-LAFHYIGLDDPEKDHVLRVYETHP                      | ----- | -----             | -----   | TLVSIIPRK-IFV   |     |
| <i>P. sativum</i> 2     | IVQNKYPISKNN-LAFHYIGLDDPEKDHVLRVYETHP                      | ----- | -----             | -----   | TLVSIIPRK-SFV   |     |
| <i>L. culinaris</i> 1   | IVQNKYPISNNK-LAFHYIGVDDPEKDLVLKYETSP                       | ----- | -----             | -----   | TLISIPRK-IFV    |     |
| <i>L. culinaris</i> 2   | IVQNKYPISNNK-LAFHYIGVDDPEKDLVLKYETSP                       | ----- | -----             | -----   | TLISIPRK-IFV    |     |
| <i>O. sativa</i>        | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>T. aestivum</i>      | -----                                                      | ----- | -----             | -----   | -----           |     |
| <i>H. vulgare</i> 1     | -----                                                      | ----- | -----             | -----   | LIDDDKL         |     |
| <i>H. vulgare</i> 2     | -----                                                      | ----- | -----             | -----   | -----           |     |



|                  | 130                                                                             | 140 | 160 | 180 | 200 | 208 |
|------------------|---------------------------------------------------------------------------------|-----|-----|-----|-----|-----|
| Z. mays          | -----                                                                           |     |     |     |     |     |
| S. bicolor       | -----                                                                           |     |     |     |     |     |
| P. angusta       | ATNTVKSYFAGKKISFNTVTTLREPARKAYIQWKE ----- QGGPLPPRLAYY                          |     |     |     |     |     |
| P. pastoris      | LLHSTYNITEVTKADFFSNYVLWIETLKPNKTEALTYLDEDGDLF ----- PRNARTVVYFG                 |     |     |     |     |     |
| C. albicans 1    | VSNIVKSKFPNKSISFNTVTTLKEPIKRAYYEWKE ----- KKGPLPSRLAYY                          |     |     |     |     |     |
| C. albicans 2    | SKLVKDAYPASANVHFVQIDRVDPKKDMKQYIVAEREGLP ----- LPKI ----- PR ----- ILY          |     |     |     |     |     |
| S. pombe 1       | AVEIIRHEYPSKHFAFNVTLEPPKAKYLHWKYSK ----- EDAHKPERIALA                           |     |     |     |     |     |
| S. pombe 2       | VVNLVRKSYPEKQISFDVVTLSEPHKEEYVHWRYSS ----- AHEGIPDRRAYV                         |     |     |     |     |     |
| A. niger 1       | ARNVILAQHPNTVIDFREIYLSEPPKAQLLEFLALEHSGRL ----- SPTS ----- PRPPRLALCQYD         |     |     |     |     |     |
| A. niger 2       | -RNVILAQHPNTVIDFREIYLSEPPKAQLLEFLALEHSGRL ----- SPTS ----- PRPPRLALCQYD         |     |     |     |     |     |
| A. fumigatus 1   | -----                                                                           |     |     |     |     |     |
| A. fumigatus 2   | -----                                                                           |     |     |     |     |     |
| E. coli          | FINDVFQSGLDQTFQVEKRPHPLNALTAEI KQAVEIVKASADFKPNTRFTEISLLPPDKEAVWAFALENKVPDQPRKA |     |     |     |     |     |
| K. aerogenes     | FINDVFQSGLDQTFQVEKRPHPLNSLSAAEISKAVTIVKAAPEFQPNTRFTEISLHEPDKAAVWAFALQGTVPDAPRTA |     |     |     |     |     |
| K. pneumoniae    | -----                                                                           |     |     |     |     |     |
| L. pneumophila   | -----                                                                           |     |     |     |     |     |
| M. loti          | SAHPLDPLSKAELLSVFEIVRDDAAGKDFYFETVELLEPAKG ----- SVRSFRRGNPISRKARV              |     |     |     |     |     |
| A. globiformis 1 | AQHPLEQLSAEEIHEARRILAEAGLVGESTRFAYLGLIEPPKT ----- TRQGDVTGAARLV RAML            |     |     |     |     |     |
| A. globiformis 2 | TASPFRLASAGEISEVQGILRTAGLLGPEKRIAYLGVLDP ----- ARGAGSEAEDRRFRVFI                |     |     |     |     |     |
| A. strain P1     | VSHPLDPLSRVEIARAVAILKEGPAAAESFRFISVELREPSK ----- DDLRAGVAVAREADA                |     |     |     |     |     |
| D. radiodurans   | -----                                                                           |     |     |     |     |     |
| M. smegmatis 1   | -----                                                                           |     |     |     |     |     |
| M. smegmatis 2   | -----                                                                           |     |     |     |     |     |
| Anabaena PCC7120 | -----                                                                           |     |     |     |     |     |
| N. punctiforme   | -----                                                                           |     |     |     |     |     |
| S. solfataricus  | EVLKRQLNLDAKVVKFFSVELKEPKKQEYLEWRNNK ----- NIKIERESL                            |     |     |     |     |     |

Consensus L

|              |         |         |            |    |         |
|--------------|---------|---------|------------|----|---------|
| 2° structure | ααααααα | βββββββ | αααααααα α | αα | β-βββββ |
|              | h1      | βA1     | h2         | h3 | βA2     |



|                         | 209            | 220                    | 240           | 260          | 280               | 287                    |
|-------------------------|----------------|------------------------|---------------|--------------|-------------------|------------------------|
| <i>H. sapiens</i> DAO   | DQEHPNVTEFAVGP | LPGPCYMRALSPR          | -PGY-QSSWASRP | ISTAELYHTLQ  | EATKPLHQFFLNTTGFS | FQDCHDR                |
| <i>H. sapiens</i> SSAO  | RQPQPNVSELVVGP | LPHPSYMRDVTVERHGG      | -PLPYHRRPVLFQ | EYLDIDQMI    | FNRELPOAS         | GLLHHCCFYKH --RGRN     |
| <i>H. sapiens</i> SSAOb | GQPQPNVSELVVGP | LPHPSYMRDVTVERHGG      | -PLPYHRRPVLR  | AEFTQMWRHLK  | DVELPKAPIFLS      | SSTFNYN ----GST        |
| <i>H. sapiens</i> SSAOa | GQPQPNVSELVVGP | LPHPSYMRDVTVERHGG      | -PLPYHRRPVLR  | AEFTQMWRHLK  | DVELPKAPIFLS      | SSTFNYN ----GST        |
| <i>M. musculus</i> DAO  | AQDHPNVTEFAVGP | LPRPCYVQALSPR          | -PGH-HLSWSSRP | ISTAEDLLYHM  | LNRAITPLHQFFL     | DTTGFS FLGCDDRF        |
| <i>M. musculus</i> SSAO | GQPKPNVSELVVGP | LPHPSYMRDVTVERHGG      | -PLPYHRRPVLD  | REYQDIEEMI   | FHRELPOAS         | GLLHHCCFYKH --QGQN     |
| <i>R. norvegicus</i>    | AQDYPNVTEFAVGP | LPRPYIRALSPR           | -PGH-HLSWSSRP | ISTAEDLLYHTL | KRATMPLHQFFL      | DTTGFS FLGCDDRC        |
| <i>B. taurus</i> DAO    | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>B. taurus</i> aorta  | GQPQPNVTELVVGP | LPQPSYMRDVTVERHGG      | -PLPYHRRPVLL  | REYLDIDQMI   | FNRELPOAA         | GVLHHCCSYKQ --GGGN     |
| <i>B. taurus</i> BPAO   | GQPQPNVTELVVGP | LPQPSYMRDVTVERHGG      | -PLPYHRRPVLL  | REYLDIDQMI   | FNRELPOAA         | GVLHHCCSYKQ --GGQK     |
| <i>B. taurus</i> lung   | GQPQPNVTELVVGP | LPQPSYMRDVTVERHGG      | -PLPYHRTVLL   | REYLDIDQMI   | FNRELPOAA         | GVLHHCCSYKQ --GGGN     |
| <i>T. rubripes</i> 1    | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>T. rubripes</i> 2    | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>L. esculentum</i>    | QLVYLTIMILLRKE | LLLLITKFGSHLIMNLS      | -NGLLACLF     | IKVKVMILLF   | GLDRDRAIENK       | DIVLWYTLGFHHIPQCE      |
| <i>S. tuberosum</i>     | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>A. thaliana</i> 1    | IARDHGKTREIVVD | FASQAIVS --EKIHVGN     | -GYPMLTIDE    | QQATSELVLK   | FKPFRDSIRRR       | G---LNVSEVVVTSTM       |
| <i>A. thaliana</i> 2    | VVRAGGQTYELIID | LITSKIAS --SRIYTG      | H-GFPSFTF     | IELFKASKLPL  | TYPPFKKSILDR      | S---LNISEVSCIPFTV      |
| <i>A. thaliana</i> 3    | VAYWGGQTHEITVD | LDSEGRVVS --DVINRTS    | -GYPILTLND    | VFAASQVPLKS  | LEFNRSIEAR        | G---VKFSDLACITPFA      |
| <i>A. thaliana</i> 4    | SVWIVALS       | EVHAVTRGGHHRGRVSSQVIPD | -VQPPMDAAEY   | AECEAIVKDF   | PPFIEAMKRR        | G-IEDMDLVMVDPWCVG      |
| <i>A. thaliana</i> 5    | LAMNGESHVLTVD  | LKSEGRVVS --DLVNPTF    | -GYPILTMKD    | IIAVSQVPYKS  | VEFNRSIEAR        | G---IPFSGLICITPFA      |
| <i>A. thaliana</i> 6    | VAYWGGQTHEITVD | LDSEGRVVS --DVINRTS    | -GYPILTLND    | VFAASQVPLKS  | LEFNRSIEAR        | G---VKFSDLACITPFA      |
| <i>C. arietinum</i>     | VAIINSQNHEIVID | LRLKTIVS --DNIHKG      | Y-GFPILSA     | DEQGVAIKLPL  | KYPPFIASVNKR      | G---LNLSEIVCSTFSM      |
| <i>C. lineata</i>       | IARVNRVAYVLTVD | LSTGEVTGGERDLGSVS      | -GYPTMTLE     | EMVGVLEVPLKS | PEFNKTISK         | R-G---VNLADLTICIPVAS   |
| <i>G. max</i>           | STWIVELREVHAT  | TRGGHHRGKVISSTVVPD     | -VQPPMDAVEY   | AECEAVVKDF   | PPPFREAN          | EGRGGIEEMDLVMVDPWCAG   |
| <i>M. truncatula</i> 1  | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>M. truncatula</i> 2  | IHSADGTPSLLKNA | ICVFEQYGNIMWRHTET      | -GIPNEFVEE    | SRTEVNLI     | VRTVTVGN          | YDNLVDWEFKASGSIKPAIAL  |
| <i>P. sativum</i> 1     | VAIINSQTHEILIN | LRIRSIVS --DNIHNGY     | -GFPIILSV     | DEQSLAIKLPL  | KYPPFIDS          | VKKR G---LNLSEIVCSSFTM |
| <i>P. sativum</i> 2     | VAIINSQTHEILID | LRIRSIVS --DNIHNGY     | -GFPIILSV     | DEQSLAIELPL  | KYPPFIDS          | VKKR G---LNLSEIVCSSFTM |
| <i>L. culinaris</i> 1   | VAIINSQTHEILID | LTIKSIVS --DNIHNGY     | -GFVLSAAEQ    | FLAIDLPLK    | YPPFIASVNKR       | G---LNISEIVCSSFTM      |
| <i>L. culinaris</i> 2   | VAIINSQTHEILID | LTIKSIVS --DNIHNGY     | -GFVLSAAEQ    | FLAIDLPLK    | YPPFIASVNKR       | G---LNISEIVCSSFTM      |
| <i>O. sativa</i>        | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>T. aestivum</i>      | -----          | -----                  | -----         | -----        | -----             | -----                  |
| <i>H. vulgare</i> 1     | VLXKRGXVIDTX   | APRVDAFEKSGPPXAAGFG    | -TRPAMDAMEY   | AECEATVKNY   | PAFVEAMKKR        | G-VDDMELVMVDAWCAG      |
| <i>H. vulgare</i> 2     | -----          | -----                  | -----         | -----        | -----             | -----                  |







|                         | 288                                          | 300                               | 320                 | 340                | 360 | 366 |
|-------------------------|----------------------------------------------|-----------------------------------|---------------------|--------------------|-----|-----|
| <i>H. sapiens</i> DAO   | LAFTDVAPRGVASGQRRSWLI IQ                     | --RYVEG-----                      | YFLHPTGLELLVDHG--   | STDAGHWAVEQVWYNGKF |     |     |
| <i>H. sapiens</i> SSAO  | LVTMTTAPRGLQSGDRATWFGLY                      | --YNISGA-----                     | GFFLHHVBLELLVNHK--  | ALDPARWTIQKVFYQGRY |     |     |
| <i>H. sapiens</i> SSAOb | LAHVHATPRGLRSRERTTWALY                       | --HNISGV-----                     | GLFLHPVBLELLLDHR--  | ALDPAHWTVQQVFYLGHY |     |     |
| <i>H. sapiens</i> SSAOa | LAHVHATPRGLRSRERTTWIGLY                      | --HNISGV-----                     | GLFLHPVBLELLLDHR--  | ALDPAHWTVQQVFYLGHY |     |     |
| <i>M. musculus</i> DAO  | LTFTDVAPRGVESGQRRSWLIVQ                      | --RYVEG-----                      | YFLHPTGLEILVDHS--   | STDVQDWRVEQLWYNGKF |     |     |
| <i>M. musculus</i> SSAO | LLTMTTAPRGLQSGDRATWFGLY                      | --YNLSGA-----                     | GFYHPHPIGLELLIDHK-- | ALDPALWTIQKVFYQGRY |     |     |
| <i>R. norvegicus</i>    | LTFTDVAPRGVASGQRRSWFIVQ                      | --RYVEG-----                      | YFLHPTGLEILLDHG--   | STDVQDWRVEQLWYNGKF |     |     |
| <i>B. taurus</i> DAO    | -----                                        | -----                             | -----               | -----              |     |     |
| <i>B. taurus</i> aorta  | LVTMTTAPRGLQSGDRATWFGLY                      | --YNISGA-----                     | GYLHPVBLELLVDHK--   | ALDPAQWTIQKVFFQGRY |     |     |
| <i>B. taurus</i> BPAO   | LLTMNSAPRGVQSGDRSTWFGIY                      | --YNITKG-----                     | GPYLHPVBLELLVDHK--  | ALDPADWTVQKVFFQGRY |     |     |
| <i>B. taurus</i> lung   | LVTMTTAPPGLQSGDRATWFGLY                      | --YNISKA-----                     | GYLHPVBLELLVDHK--   | ALDPAQWTIQKVFFQGRY |     |     |
| <i>T. rubripes</i> 1    | -----                                        | -----                             | -----               | -----              |     |     |
| <i>T. rubripes</i> 2    | -----                                        | -----                             | -----               | -----              |     |     |
| <i>L. esculentum</i>    | DFPIMPTVSSSFEIKPVNFFESNPILNIPPNSPKDLPQFAKLQL | LLDHILIVLYVYDLIXTLNKLLEIFDEGE---- | S                   |                    |     |     |
| <i>S. tuberosum</i>     | -----                                        | -----                             | -----               | -----              |     |     |
| <i>A. thaliana</i> 1    | GWFGGEAKPER----                              | LIKRPFFY--LNGSVN-----             | TYLRPIEGMTIIVNLD--  | QMKVTKFRDRF-----   | T   |     |
| <i>A. thaliana</i> 2    | GWYGETTTRR----                               | ELKASCFY--RDGSVN-----             | VFTRPIEGITITIDVD--  | SMQVVKYSDRF-----   | R   |     |
| <i>A. thaliana</i> 3    | GWFGSEEEGR----                               | RVIRVQCFT--LQGTTN-----            | YFMRPLEGLYVTVDDL--  | KLEVIKIIDKG-----   | P   |     |
| <i>A. thaliana</i> 4    | YHSEADAPSRRLAKPLIYCRTDS                      | --DSPMEN-----                     | GYARPVEGIYVLVDMQ--  | NMVVIEFEDR-----    |     |     |
| <i>A. thaliana</i> 5    | GWYGPDEEGR----                               | RVIKIQCF--KQDTVN-----             | FYMRPIEGLYLTVDMD--  | KLEI IKIVDNG-----  | P   |     |
| <i>A. thaliana</i> 6    | GWFGSEEEGR----                               | RVIRVQCFT--LQGTTN-----            | YFMRPLEGLYVTVDDL--  | KLEVIKIIDKG-----   | P   |     |
| <i>C. arietinum</i>     | GWFGEEKNSR----                               | TVRVDCFM--KENTVN-----             | IYVRPISGITIVVDLQ--  | LMKIVEYNDRG-----   | I   |     |
| <i>C. lineata</i>       | GWYGTQVEEEN--                                | GRLIKVQCYS--HKGTVN-----           | FYMKPIEGVNALVDMD--  | RKKVLAILDNGQ-----  | N   |     |
| <i>G. max</i>           | YHSEADAPSRRLAKPLIFCRTES                      | --DCPMEN-----                     | GYARPVEGIHVLVDMQ--  | NMVVLEFEDRKLVP---- |     |     |
| <i>M. truncatula</i> 1  | -----                                        | -----                             | -----               | -----              |     |     |
| <i>M. truncatula</i> 2  | SGILEIKGTNIKHKDEIKEDLHG                      | --KLVSANSIGIYHDFYMY               | LDFDIDGVQNSFEKT--   | SLKTVKITDGSSKRKSYW |     |     |
| <i>P. sativum</i> 1     | GWFGEEKNVR----                               | TVRLDCFM--KESTVN-----             | IYVRPITGITIVADLD--  | LMKIVEYHHRD-----   | I   |     |
| <i>P. sativum</i> 2     | GWFGEEKNVR----                               | TVRLDCFM--KESTVN-----             | IYVRPITGITIVADLD--  | LMKIVEYHHRD-----   | I   |     |
| <i>L. culinaris</i> 1   | GWFGEEKNSR----                               | TVRVDCFM--KESTVN-----             | IYVRPITGITIVADLD--  | LMKIVEYHHRD-----   | T   |     |
| <i>L. culinaris</i> 2   | GWFGEEKNSR----                               | TVRVDCFM--KESTVN-----             | IYVRPITGITIVADLD--  | LMKIVEYHHRD-----   | T   |     |
| <i>O. sativa</i>        | -----                                        | -----                             | GPVEGIHVIVDMQ--     | NNVVIEFEDRKLVP---- |     |     |
| <i>T. aestivum</i>      | -----                                        | -----                             | -----               | -----              |     |     |
| <i>H. vulgare</i> 1     | YYSDADAPSRRLARPLIFCRTES                      | --DSPMEN-----                     | GYARPVEGIHVVDVDMQ-- | NNVVIEFEDRKLVP---- |     |     |
| <i>H. vulgare</i> 2     | -----                                        | -----                             | -----               | -----              |     |     |



|                  | 288                     | 300                  | 320                    | 340                  | 360   | 366   |
|------------------|-------------------------|----------------------|------------------------|----------------------|-------|-------|
| Z. mays          | -----                   | -----                | -----                  | -----                | ----- | ----- |
| S. bicolor       | -----                   | -----                | -----                  | -----                | ----- | ----- |
| P. angusta       | GYDERWGTGKRLQQALVYYS    | -----DEDD-----       | SQYSHPLDFCPIVDTE       | --EKKVIFIDIPNRR      | ----  | R     |
| P. pastoris      | MGCHSAAPQLHDPATGATVDYGT | -CNINTEND-----       | AENLVPTGFFFKFDMTG      | --RDVSQWKMLEYIYNNKV  |       |       |
| C. albicans 1    | GYDERWGASRRLLQQALMYWRS  | -----DEDD-----       | SQYSHPLDFCPIVDMN       | --AKKVVFIDIPQRR      | ----  | R     |
| C. albicans 2    | ATDDANETRPLIQFYMYVLG    | ---NGHSES-----       | NHYSPLPKFSPVFECF       | --TKKFIRIDYLPG       | ----  | G     |
| S. pombe 1       | GYDERYGNTRRMQQAMMYYS    | -----NEDD-----       | SQYSHPLDFCPIIDTE       | --DQKVVAIDIPPVR      | ----  | R     |
| S. pombe 2       | GYDERYGSRRLLQQALMYYP    | -----GDSG-----       | HLRSIPLDFCPIIDVD       | --QKKVIAIDIPKVR      | ----  | R     |
| A. niger 1       | GGLDYVEEKRRYFQGLCFATDKR | --KNNPDA-----        | NFYSYPLPLIPVMDGA       | --YPGDHSGRPSRHRCKGEG |       |       |
| A. niger 2       | GGLDYVEEKRRYFQGLCFATDKR | --KNNPDA-----        | NFYSYPLPLIPVMDAL       | --TQEIIIRVDRPATGGKGE |       |       |
| A. fumigatus 1   | GIDERWGFERRLLQQALPYYS   | -----SQHD-----       | NQYAHPLDFTVVADTE       | --TQEILSVDVRRVNG     | --ER  |       |
| A. fumigatus 2   | -----                   | -----                | -----                  | -----                | ----- | ----- |
| E. coli          | GYFDGKDGLQDARLLKVISY    | -L--DVGDGN-----      | YWAHP IENLVAVVDLE      | --QKKIVKIEEGPVVPVPM  |       |       |
| K. aerogenes     | GFFDGKDGLQDARLLKVVS     | -L--DTGDGN-----      | YWAHP IENLVAVVDLE      | --AKKIIKIEEGPVIPVPM  |       |       |
| K. pneumoniae    | GFFDGKDGLQDARLLKVVS     | -L--DTRRRQ-----      | LLAHP IENLVAVVDLE      | --AKKIIKIEEGPVIPVPM  |       |       |
| L. pneumophila   | GYFDEEEEREQ             | --RIVKGISFYR--DNLKDN | -----GYARPIEGVVAIVDLG  | --NEKVLRVIDDGRNV     | ----  |       |
| M. loti          | GNFGVPDEEGR             | --YIAHVFAWLR--LRENEN | -----FYAHP IGLNAIVDIR  | --TNRVLRVDDHEIIP     | ----  |       |
| A. globiformis 1 | GVFEYGNEEGK             | --RLLRGLGFRQ--DHPADH | -----PWAHP IDGLVAFVDVE | --NRRVNHLLIDGVPV     | ----  |       |
| A. globiformis 2 | GVFEYAEERGR             | --RILRGLAFVQ--DFPEDS | -----AWAHPVDGLVAYVDV   | --SKEVTRVIDTGVFP     | ----  |       |
| A. strain P1     | GYFGEDNEGRR             | ---LMRALVFVR--DEADDS | -----PYAHP IENFIVFYDLN | --AGKVVRLEDDQAIP     | ----  |       |
| D. radiodurans   | -----                   | -----                | -----                  | -----                | ----- | ----- |
| M. smegmatis 1   | YYYGAQDHYDNSPLVCRPLTFVR | --AAPSEN-----        | GYARPVEGLIVTFDLD       | --KMEVLDIEDHGVVP     | ----  |       |
| M. smegmatis 2   | -----                   | YK--STAGAN-----      | PYAHPVSGLHCVIDLN       | --AMEVLRVEDDGTNE     | ----  |       |
| Anabaena PCC7120 | LSQEEAATGGRLCRTLLFYRG   | -----ERWN-----       | YYGSPIEGVIATVDLN       | --KGTVTNFIDQGIVPIS   | ----  |       |
| N. punctiforme   | -----                   | RLCRSLSYQQG-----QNNW | -----YYGSPIEGVATVNLN   | --TGKIVSFIDKGNVPFS   | ----  |       |
| S. solfataricus  | GHVDEELRGRR             | ----VAIGYMWVKKDIEDNG | -----YGRPVGHLMPWVDLD   | --KMEVIRIDDHG        | ----- |       |

Consensus

Y I GL VVDL VI D

2° structure

ββββββββ

βB2

ααα

ββββββ

h7

βB3

ββββββββ

βB4



|                         | 367         | 380                          | 400           | 420            | 440       | 445                          |
|-------------------------|-------------|------------------------------|---------------|----------------|-----------|------------------------------|
| <i>H. sapiens</i> DAO   | YGSPEELARKY | YADGEVDVVVLEDPLPGGKGHDSTEEPP | PLFSSHK       | PRGDFPSP       | PIHVS     | GPR LVQPHGPRFRLEG--NAVLY     |
| <i>H. sapiens</i> SSAO  | YDSLAEQLEAQ | FEAGLVNVVLIP                 | -----         | DNGTGGSWSLKSPV | PPG-----  | PAPPLQFY PQGPRFSVQG--SRVAS   |
| <i>H. sapiens</i> SSAOb | YADLGQLERE  | FKSGRLEVVRVP                 | -----         | LPPPNGASSLRSR  | SPG-----  | PLPPLQFS PQGSQYSVQG--NLVVS   |
| <i>H. sapiens</i> SSAOa | YADLGQLERE  | FKSGRLEVVRVP                 | -----         | LPPPNGASSLRSR  | SPG-----  | PLPPLQFS PQGSQYSVQG--NLVVS   |
| <i>M. musculus</i> DAO  | YNSPEELAQK  | YAVGEVEAVVLEE                | VVLEDPLPGATEQ | PPLFSSYK       | PRGEFHTP  | PTVAGPH VVQPSGPRYKLEG--NVVLY |
| <i>M. musculus</i> SSAO | YESLTQLEDQ  | FEAGLVNVVLVP                 | -----         | NNGTGGSWSLKSSV | PPG-----  | PAPPLQFHPQGPRFSVQG--SQVSS    |
| <i>R. norvegicus</i>    | YNNPEELARKY | YAVGEVD                      | ----          | VVLEDPLPNGTEK  | PPLFSSYK  | PRGEFHTP                     |
| <i>B. taurus</i> DAO    | -----       | -----                        | -----         | -----          | -----     | -----                        |
| <i>B. taurus</i> aorta  | YESLAQLEEQ  | FEAGRVNVVVIP                 | -----         | NNGTGGSWSLKSQV | PPG-----  | PTPPLQFHPQGTRFSVQG--SRVTS    |
| <i>B. taurus</i> BPAO   | YENLAQLEEQ  | FEAGQVNVVVIP                 | -----         | DDGTGGFWSLKSQV | PPG-----  | PTPPLQFHPQGPRFSVQG--NRVAS    |
| <i>B. taurus</i> lung   | YESLVQLEEQ  | FEAGRVNVVVIP                 | -----         | NNGTGGSWSLKSQV | PPG-----  | PTPPLQFHPQGTRFSVQG--SRVTS    |
| <i>T. rubripes</i> 1    | -----       | -----                        | -----         | -----          | -----     | QLFHPEGKRYISD--NHVLY         |
| <i>T. rubripes</i> 2    | -----       | -----                        | -----         | -----          | -----     | KLVEPQGPRYRTDQ--NFVEY        |
| <i>L. esculentum</i>    | IPIPKAANTD  | YRYSRIKK                     | -----         | NKQKINLLK      | PIS-----  | IEQPNGPSFTIENN--HLVKW        |
| <i>S. tuberosum</i>     | -----       | -----                        | -----         | -----          | -----     | -----                        |
| <i>A. thaliana</i> 1    | SPLPNAKGTE  | FRISKLK                      | -----         | PPFGPSLQNAV    | -----     | LFQSEGPFGKIDG--HTNRW         |
| <i>A. thaliana</i> 2    | KPLPEKEGND  | FRKKHKP                      | -----         | FPFSCNVS       | -----     | DT-----GFKILG--NRVKW         |
| <i>A. thaliana</i> 3    | IPIPKASGTE  | YRFGVQNK                     | -----         | PVHMDRIN       | PIS-----  | MEQPDGPSFRVEDG--HLVKW        |
| <i>A. thaliana</i> 4    | -----       | E-----                       | -----         | SRGGVDRSDVK    | PLQ-----  | IIQPEGPSFRVRGY--FVEW         |
| <i>A. thaliana</i> 5    | VPVPKSTGTE  | YRYGFLNE                     | -----         | TVYMDRVN       | PMS-----  | MEQPDGPSFQVEDG--YLVKW        |
| <i>A. thaliana</i> 6    | IPIPKASGTE  | YRFGVQNK                     | -----         | PVHMDRIN       | PIS-----  | MEQPDGPSFRVEDG--HLVKW        |
| <i>C. arietinum</i>     | EAVPTAEKTD  | YRFSHQN                      | -----         | PPFGPKLNSLV    | -----     | SHQPQGS                      |
| <i>C. lineata</i>       | IPVSSGINTD  | YRYSIQKL                     | -----         | NGELSLFN       | PIS-----  | LEQPKGPSFTVDG--HLVKW         |
| <i>G. max</i>           | -----       | LPPADPLRNYTSGE               | -----         | TQGGVDRSDVK    | PLQ-----  | IIQPEGPSFRVNGH--FIEW         |
| <i>M. truncatula</i> 1  | -----       | -----                        | -----         | -----          | -----     | -----                        |
| <i>M. truncatula</i> 2  | TTETQIAKTES | DRCXSSAYCX                   | -----         | EILITGFQNRVHH  | LDQN----- | SIVLLVINLKVLAFRSMV--TSVSW    |
| <i>P. sativum</i> 1     | EAVPTAENTE  | YQVSKQS                      | -----         | PPFGPKQHS      | SLT-----  | SHQPQGPFGQING--HSVSW         |
| <i>P. sativum</i> 2     | EAVPTAENTE  | YQVSKQS                      | -----         | PPFGPKQHS      | SLT-----  | SHQPQGPFGQIEG--HSVSW         |
| <i>L. culinaris</i> 1   | EAVPTAENTE  | YQVSKQS                      | -----         | PPFGPKQHS      | SLT-----  | SHQPQGPFGQING--TSVSW         |
| <i>L. culinaris</i> 2   | EAVPTAENTE  | YQVSKQS                      | -----         | PPFGPKQHS      | SLT-----  | SHQPQGPFGQING--TSVSW         |
| <i>O. sativa</i>        | -----       | LPPPDHSRNYTPGE               | -----         | TRGGVDRSDLK    | PLI-----  | INQPEGPSFRINGY--FVEW         |
| <i>T. aestivum</i>      | -----       | -----                        | -----         | -----          | -----     | -----                        |
| <i>H. vulgare</i> 1     | -----       | LPTPDHLRNYTPGE               | -----         | TRGGVDRSDVK    | PLI-----  | INQPEGPSFRINGY--FVEW         |
| <i>H. vulgare</i> 2     | -----       | -----                        | -----         | -----          | -----     | -----                        |



|                  | 367          | 380                | 400        | 420          | 440         | 445   |
|------------------|--------------|--------------------|------------|--------------|-------------|-------|
| Z. mays          | -----        | -----              | -----      | -----        | -----       | ----- |
| S. bicolor       | -----        | -----              | -----      | -----        | -----       | ----- |
| P. angusta       | KVSKHKHANFY  | PKHMIKVG           | -----      | AMRPEAP      | PIN         | ----- |
| P. pastoris      | YTSAEELYEAM  | QKDDFVTL           | PKIDVDNLDW | TVIQRNDS     | APIRHLDDRK  | ----- |
| C. albicans 1    | KVSKHKHSNF   | HPKHVADKY          | GSK        | -----        | ENPSGYRQEDF | PIN   |
| C. albicans 2    | FDHKTVP      | TGPWQEV            | PCVQYHPD   | -----        | LNGETQMRDVK | PLL   |
| S. pombe 1       | PLSKHKHSNF   | NKKDIEAELG         | -----      | KMREVK       | PIS         | ----- |
| S. pombe 2       | PIPQDVNSD    | NNLKKLEQEME        | -----      | AMKMLK       | PLR         | ----- |
| A. niger 1       | LTEQTFKRDI   | IGHCKDSYVPE        | -----      | LLPGGTRKDLK  | PLN         | ----- |
| A. niger 2       | LTEQTFKRDI   | IGHCKDSYVPE        | -----      | LLPGGTRKDLK  | PLN         | ----- |
| A. fumigatus 1   | TPVPLDEHNY   | LPQFIKDQYR         | -----      | PERLKA       | IE          | ----- |
| A. fumigatus 2   | -----        | -----              | -----      | -----        | -----       | ----- |
| E. coli          | ARPF         | DGRDRVAP           | -----      | AVK          | PMQ         | ----- |
| K. aerogenes     | PRPYDGRDRNAP | -----              | -----      | AVK          | PLE         | ----- |
| K. pneumoniae    | PRPYDGRDRNAP | -----              | -----      | RNRWRP       | -----       | ----- |
| L. pneumophila   | PIPK         | ---EKINYDSDSYP     | -----      | EKRKDLK      | PLD         | ----- |
| M. loti          | IPMK         | ---EANYEAEFIKE     | -----      | PRQPYK       | PLN         | ----- |
| A. globiformis 1 | VPEV         | ---NGNYTDPAIRG     | -----      | ELRTDLL      | PIE         | ----- |
| A. globiformis 2 | VPAE         | ---HGNYTDPELTG     | -----      | PLRTTQK      | PIS         | ----- |
| A. strain P1     | VPSA         | ---RGNYLPKYVG      | -----      | EARTDLK      | PLN         | ----- |
| D. radiodurans   | -----        | -----              | -----      | -----        | -----       | ----- |
| M. smegmatis 1   | LPPK         | ---AGNYTEQFMFDADNR | -----      | PAFTEFRQDVKK | IE          | ----- |
| M. smegmatis 2   | RPDV         | ---MGEYVPRH        | IPERIRA    | -----        | ASTREPLK    | PLE   |
| Anabaena PCC7120 | K-E          | ---NWNYDLQSLGKLLS  | -----      | PPKLLQ       | -----       | ----- |
| N. punctiforme   | K-E          | ---NWNYDLKSLGKLLS  | -----      | APKALK       | -----       | ----- |
| S. solfataricus  | TSPLPLQDAN   | YTPEKLG            | -----      | KIFGDDLK     | PIE         | ----- |

Consensus

Y

PL

I Q P G F I G V W

2° structure

ββ  
βx

βββ

ββββ  
βC1

βC2



|                         | 446                                                                               | 460                                                               | 480                                             | 500 | 520 524 |
|-------------------------|-----------------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------|-----|---------|
| <i>H. sapiens</i> DAO   | GGWSFAFRLRSSS-GLQVLNVHFGGER-----                                                  |                                                                   | IAYEVSQVEAVALYGGHTPAGMQTKYLDVGWGLGS-VTHELAPG--  |     |         |
| <i>H. sapiens</i> SSAO  | SLWTFSGGLGAFS-GPRIFDVRFQGER-----                                                  |                                                                   | LVYEISLQELALAIYGGNSPAAMTTRYVDGGFGMGK-YTTPLTRG-- |     |         |
| <i>H. sapiens</i> SSAOb | SLWSFTFGHGVFS-GLRIFDVRFQGER-----                                                  |                                                                   | IAYEVSQVECVSIYGADSPKTMLTRYLDSSFGLGR-NSRGLVRG--  |     |         |
| <i>H. sapiens</i> SSAOa | SLWSFTFGHGVFS-GLRIFDVRFQGER-----                                                  |                                                                   | IAYEVSQVECVSIYGADSPKTMLTRYLDSSFGLGR-NSRGLVRG--  |     |         |
| <i>M. musculus</i> DAO  | GDWSFSYRLRSSS-GLQIFNVLFQGER-----                                                  |                                                                   | VAYEVSQVEAVALYGGHTPAGMQTKYIDVGWGLGS-VTHELAPG--  |     |         |
| <i>M. musculus</i> SSAO | SLWAFSGGLGAFS-GPRIFDIRFQGER-----                                                  |                                                                   | VAYEISVQEAIALYGGNSPASMSTCYVDGSFGIGK-YSTPLIRG--  |     |         |
| <i>R. norvegicus</i>    | GGWSFSYRLRSSS-GLQIFNVLFQGER-----                                                  |                                                                   | VAYEVSQVEAVALYGGHTPAGMQTKYIDVGWGLGS-VTHELAPG--  |     |         |
| <i>B. taurus</i> DAO    | -----                                                                             |                                                                   | -----PAGMQTKYMDVGWGLGS-VTYELAPG--               |     |         |
| <i>B. taurus</i> aorta  | SLWTFSGGLGAFS-GPRIFDIRFQGER-----                                                  |                                                                   | LAYEISLQEAVAIYGGNTPAAMLTRYMDGCFGMGK-FATPLTRG--  |     |         |
| <i>B. taurus</i> BPAO   | SLWTFSGGLGAFS-GPRVFDVRFQGER-----                                                  |                                                                   | LAYEISLQEAGAVYGGNTPAAMLTRYMDSGFGMGY-FATPLIRG--  |     |         |
| <i>B. taurus</i> lung   | SLWTFSGGLGAFS-GPRIFDIRFQGER-----                                                  |                                                                   | LAYEISLQEAVAIYGGNTPAAMLTRYMDACFGMGK-FATPLTRG--  |     |         |
| <i>T. rubripes</i> 1    | MDWSFAFGLSSLT-GMRVFDVRFKDER-----                                                  |                                                                   | ILYELSVQEAMSVYGSVTPGMGLTKFFDTSIGIGR-FAHQLVRG--  |     |         |
| <i>T. rubripes</i> 2    | AGWSFAYRVRSLs-GIQVFDLRYNGER-----                                                  |                                                                   | IAYEISLQEAIAIYAGDTPAAMQTKYIDSGWAMGV-STHELAPG--  |     |         |
| <i>L. esculentum</i>    | ANWEFHLKPDPRAG-VIISRVMFEDPDTGKMRNVMYKGFTSELFPYMDPSDAWYFKTYMDAGEYGFGLQAMPLDPLN-    |                                                                   |                                                 |     |         |
| <i>S. tuberosum</i>     | -----                                                                             | AYIDGSR-GRRPVAHRLSFIEMVVPYGDPNEPHYRKNAFDAGEDGLGKNAHSLKKG--        |                                                 |     |         |
| <i>A. thaliana</i> 1    | ANWEFHMSFDVRA-GLVISLASIFDMVDNKYRQVLYKGHLSEIFVPYMDPSDWYFRTTFDCGEFGCGQYAVSLEPYT-    |                                                                   |                                                 |     |         |
| <i>A. thaliana</i> 2    | ANWKFHVGFTARA-GVTISTASVLDPRTKRFRRVMYRGHVSETFVPYMDPTYEWYYRTFMDIGFGRSAVNQLPLI-      |                                                                   |                                                 |     |         |
| <i>A. thaliana</i> 3    | ANWVFHVKADQRA-GMISQATVRDSETGEPRSVMYKGFPSSELPYMDPEEGWYKGYMDAGELGLGPTAMPLVPLN-      |                                                                   |                                                 |     |         |
| <i>A. thaliana</i> 4    | QKWNFRIGFTPRE-GLVIHSVAYVDGSR-GRRPVAHRLSFVEMVVPYGDPNEPHYRKNAFDAGEDGLGKNAHSLKKG--   |                                                                   |                                                 |     |         |
| <i>A. thaliana</i> 5    | ANWKFHVKPDQRA-GMISQATVRDSETGEPRSVMYKGFPSSELPYMDPEEGWYKGYMDAGELGLGPTAMPLVPLN-      |                                                                   |                                                 |     |         |
| <i>A. thaliana</i> 6    | ANWVFHVKADQRA-GMISQATVRDSETGEPRSVMYKGFPSSELPYMDPEEGWYKGYMDAGELGLGPTAMPLVPLN-      |                                                                   |                                                 |     |         |
| <i>C. arietinum</i>     | ANWRFHIGFDARA-GIVISLASIYDLEKHKSRRVLYKGYISELPYQDPTEEFYFKTFDSDGEFGFGLSTVSLIPNR-     |                                                                   |                                                 |     |         |
| <i>C. lineata</i>       | ANWEFHVKPDPRAG-GTIIISQVKVRDPDTSKLRNVMYKGFTSELFPYMDPTTEGWYFKTYMDAGEYGFGLQAMPLDPLN- |                                                                   |                                                 |     |         |
| <i>G. max</i>           | QKWNFRIGFTPRE-GLVIHSVAYIDGSR-GRRPVAHRLSFVEMVVPYGDPNDPHYRKNAFDAGEDGLGKNHSLKKG--    |                                                                   |                                                 |     |         |
| <i>M. truncatula</i> 1  | -----                                                                             | FIIYSVAYVDGSR-GRRPVAHRLSFVEMVVPYGDPTTEPHYRKNAFDAGEDGLGKNAHSLKKG-- |                                                 |     |         |
| <i>M. truncatula</i> 2  | ANWKFHIGFDVRA-GIVISLASIYDLEKHKSRRVLYKGYISELPYQDPTEEFYFKTFDSDGEFGFGLSTVSLIPN--     |                                                                   |                                                 |     |         |
| <i>P. sativum</i> 1     | ANWKFHIGFDVRA-GIVISLASIYDLEKHKSRRVLYKGYISELPYQDPTEEFYFKTFDSDGEFGFGLSTVSLIPNR-     |                                                                   |                                                 |     |         |
| <i>P. sativum</i> 2     | ANWKFHIGFDVRA-GIVISLASIYDLEKHKSRRVLYKGYISELPYQDPTEEFYFKTFDSDGEFGFGLSTVSLIPNR-     |                                                                   |                                                 |     |         |
| <i>L. culinaris</i> 1   | ANWKFHIGFDVRA-GIVISLASIYDLEKHKSRRVLYKGYISELPYQDPTEEFYFKTFDSDGEFGFGLSTVSLIPNR-     |                                                                   |                                                 |     |         |
| <i>L. culinaris</i> 2   | ANWKFHIGFDVRA-GIVISLASIYDLEKHKSRRVLYKGYISELPYQDPTEEFYFKTFDSDGEFGFGLSTVSLIPNR-     |                                                                   |                                                 |     |         |
| <i>O. sativa</i>        | QKWNFRIGFTPKE-GLVIYSVAYADGSR-GRRPIAHRLSFVEMVVPYGDPNEPHYRKNAFDAGEDGLGKNAHSLKRG--   |                                                                   |                                                 |     |         |
| <i>T. aestivum</i>      | -----                                                                             |                                                                   |                                                 |     |         |
| <i>H. vulgare</i> 1     | QKWNFRIGSTPKE-GLVIYSVAYVDGSR-GRRPIAHRLTFVEMVVPYGDPSSEPHYRKNAFDAGEDGLGKNAHSLKKG--  |                                                                   |                                                 |     |         |
| <i>H. vulgare</i> 2     | -----                                                                             |                                                                   |                                                 |     | G--     |







|                         | 525   | 540                | 560                   | 580                       | 603               |
|-------------------------|-------|--------------------|-----------------------|---------------------------|-------------------|
| <i>H. sapiens</i> DAO   | ----- | IDCPETATFLDTFHYYDA | -DDPVHYPRALCLFEMPTGVP | LRRHFNSNFKGGFNFYAGLKGQV   | LV                |
| <i>H. sapiens</i> SSAO  | ----- | VDCPYLATYVDWHFLLS  | -QAPKTIRDAFCVFEQNQGLP | LRRHHSGLYS                | ---HYFGGLAETV LV  |
| <i>H. sapiens</i> SSAOb | ----- | VDCPYQATMVDIHLVGK  | -GAVQLLPGAVCVFEEAQGLP | LRRHHNYLQN                | ---HFYGGGLASSA LV |
| <i>H. sapiens</i> SSAOa | ----- | VDCPYQATMVDIHLVGK  | -GAVQLLPGAVCVFEEAQGLP | LRRHHNYLQN                | ---HFYGGGLASSA LV |
| <i>M. musculus</i> DAO  | ----- | IDCPETATFLDAFHYYDS | -DGPVLYPRALCLFEMPTGVP | LRRHFDSNFKGGFNFYAGLKGIV   | LV                |
| <i>M. musculus</i> SSAO | ----- | VDCPYLATYVDWHFLLS  | -QAPKTLRDAFCVFEQNQGLP | LRRHHSDFYS                | ---HYFGGVVGTV LV  |
| <i>R. norvegicus</i>    | ----- | IDCPETATFLDAFHYYDS | -DGPVHYPRALCLFEMPTGVP | LRRHFNSNFKGGFNFYAGLKGIV   | LV                |
| <i>B. taurus</i> DAO    | ----- | IDCPEMATFVDALHYDD  | -DGPVLYPRALCLFEMPTGVP | IRRHFNSNFGGFFNFYAGLKGQALV |                   |
| <i>B. taurus</i> aorta  | ----- | VDCPYLATYVDWHFLLS  | -QAPKTLHDAFCVFEQNKGLP | LRRHHSDFIS                | ---QYFGGVVETV LV  |
| <i>B. taurus</i> BPAO   | ----- | VDCPYLATYMDWHFVVS  | -QTPKTLHDAFCVFEQNKGLP | LRRHHSDFLS                | ---HYFGGVAQTV LV  |
| <i>B. taurus</i> lung   | ----- | VDCPYLATYVDWHFLLS  | -QAPKTLHDAFCVFEQNKGLP | LRRHHSDFIS                | ---QYFGGVVETV LV  |
| <i>T. rubripes</i> 1    | ----- | VDCPYEAAIITTYRYIDA | -PNPIKFEDSICIFEHNLGQP | LRRHFSDFFH                | ---NSYGGMVNSALV   |
| <i>T. rubripes</i> 2    | ----- | IDCPEIATFIDLHYHYDT | -DKPKRYRNALCIFEMTTAIP | LRRHFNSNFKGGYNFYGGDLNTV   | LV                |
| <i>L. esculentum</i>    | ----- | DCPRNAYYMDGVFVAAD  | -GTPYVRSNMICVFERYAGD  | IGWRHAECPTG               | -LPIREVRPKVT LV   |
| <i>S. tuberosum</i>     | ----- | CDCLGYIKYFDAHFTNFT | -GGVETIENCVCLHEEDH    | -GILWKHQDWRTGLA           | -EVRRS ---RRLT    |
| <i>A. thaliana</i> 1    | ----- | DCPGNAAFMDGVFASQD  | -GTPIKITNVMCIFEKYAGD  | IMWRHTEIEIPGL             | -KVRP ---DVS LV   |
| <i>A. thaliana</i> 2    | ----- | DCPQNAAFEDGHVAGPD  | -GTAQKMTNVMCVFEKNGYG  | ASFRHTEINVPGQ             | -VITSGAAEIS LV    |
| <i>A. thaliana</i> 3    | ----- | DCPRNSYYIDGVFASPD  | -GKPIVQPNMICLFERYAGD  | ISWRHSEILFANA             | -DIRESRPKVT LV    |
| <i>A. thaliana</i> 4    | ----- | CDCLGSIKYFDAHFTNFT | -GGVETIENCVCLHEEDH    | -GILWKHQDWRTGLA           | -EVRRS ---RRLT    |
| <i>A. thaliana</i> 5    | ----- | DCPRNAYYIDGFFASPE  | -GIPILQPNMICLFERYAGD  | TSWRHSEILLPGV             | -DIRESRAKVT LV    |
| <i>A. thaliana</i> 6    | ----- | DCPRNSYYIDGVFASPD  | -GKPIVQPNMICLFERYAGD  | ISWRHSEILFANA             | -DIRESRPKVT LV    |
| <i>C. arietinum</i>     | ----- | DCPSHAQFIDTYIHSAD  | -GSPILLKNAICVFEQYG    | -NIMWRHTETGIPNE           | -FPEESRTEVN LI    |
| <i>C. lineata</i>       | ----- | DCPRNAYYMDGVFASAD  | -GTPYLQPNMICVFESYAGD  | VAWRHAECPTNM              | -KVTEARPKVT LV    |
| <i>G. max</i>           | ----- | CDCLGYIKYFDAHFTNFT | -GGVETIENCVCLHEEDH    | -GILWKHQDWRTGLA           | -EVRRS ---RRLT    |
| <i>M. truncatula</i> 1  | ----- | CDCLGYIKYFDAHFTNFT | -GGVLKLSKIVYVCMKR     | ---IMGFSGSIKTGEL          | -AXQKSGGPED FQ    |
| <i>M. truncatula</i> 2  | ----- | RDCPPHAQFIDTYIHSAD | -GTPSLKNAICVFEQYG     | -NIMWRHTETGIPN            | -EFVEESRTEVN LI   |
| <i>P. sativum</i> 1     | ----- | DCPPHAQFIDTYVHSAN  | -GTPILLKNAICVFEQYG    | -NIMWRHTENGIPNE           | -SIEESRTEVN LI    |
| <i>P. sativum</i> 2     | ----- | DCPPHAQFIDTYIHSAN  | -GTPILLKNAICVFEQYG    | -NIMWRHTENGIPNE           | -SIEESRTEVN LI    |
| <i>L. culinaris</i> 1   | ----- | DCPPHAQFIDTYIHSAD  | -GTPIFLENAICVFEQYG    | -NIMWRHTETGIPNE           | -SIEESRTEVD LA    |
| <i>L. culinaris</i> 2   | ----- | DCPPHAQFIDTYIHSAD  | -GTPIFLENAICVFEQYG    | -NIMWRHTETGIPNE           | -SIEESRTEVD LA    |
| <i>O. sativa</i>        | ----- | CDCLGFIKYFDAHFTNFT | -GGVETIENCVCLHEEDH    | -GILWKHQDWRTGLA           | -EVRRS ---RRLT    |
| <i>T. aestivum</i>      | ----- |                    |                       | TGLA                      | -EVRRS ---RRLT    |
| <i>H. vulgare</i> 1     | ----- | CDCLGYIKYFDAHFTNFT | -GHVETIENCVCLHEEDH    | -GILWKHQDWRTGLA           | -EVRRS ---RRLT    |
| <i>H. vulgare</i> 2     | ----- | CDCLGYIKYFDAHFTNFT | -GHVETIENCVCLHEEDH    | -GILWKHQDWRTGLA           | -EVRRS ---RRLT    |







|                         | 604        | 620                               | 640                                   | 660            | 682            |
|-------------------------|------------|-----------------------------------|---------------------------------------|----------------|----------------|
| <i>H. sapiens</i> DAO   | LRTTSTVY   | NYDYIWDFIFYPNGVMEAKMHATGYVHATFYTP | -----                                 | EGLRHGTRLHTHLI | GNIHTHLVHYRV   |
| <i>H. sapiens</i> SSAO  | VRSMSTLL   | NYDYVWDTV FHP                     | SGAIEIRFYATGYISSAFLFG                 | -----          | ATGKYGNQVSEHTL |
| <i>H. sapiens</i> SSAOb | VRSVSSVG   | NYDYIWDFVLYP                      | NGALEGRVHATGYINTAFLKGGE               | -----          | EGLLFGNRVGERVL |
| <i>H. sapiens</i> SSAOa | VRSVSSVG   | NYDYIWDFVLYP                      | NGALEGRVHATGYINTAFLKGGE               | -----          | EGLLFGNRVGERVL |
| <i>M. musculus</i> DAO  | LRTTSTVY   | NYDYIWDFIFYPNGVMETKMHATGYVHATFYTP | -----                                 | EGLRHGTRLQTHLL | GNIHTHLVHYRV   |
| <i>M. musculus</i> SSAO | VRSVSTLL   | NYDYIWD                           | MVFHPNGAIEVKFHATGYISSAFFFG            | -----          | AGEKFGNRVGAHTL |
| <i>R. norvegicus</i>    | LRTTSTVY   | NYDYIWDFIFYSNGVMEAKMHATGYVHATFYTP | -----                                 | EGLRHGTRLQTHLL | GNIHTHLVHYRV   |
| <i>B. taurus</i> DAO    | LRTTSTVY   | NYDYIWDFIFYPNGVMEAKMHATGYIHATFYTP | -----                                 | EGLRHGTRLHTHLI | GNMHTHLVHYRV   |
| <i>B. taurus</i> aorta  | FRSVSTLL   | NYDYVWDMV FHP                     | NGAIEVKFHATGYISSAFFFG                 | -----          | TAQKYGNQVRENTL |
| <i>B. taurus</i> BPAO   | FRSVSTML   | NYDYVWDMV FYP                     | NGAIEVKFHATGYISSAFLFG                 | -----          | AARRYGNQVGEHTL |
| <i>B. taurus</i> lung   | FRSVSTLL   | NYDYVWDMV FHP                     | NGAIEVKFHVTGYISSAFFFG                 | -----          | TAQKYGNQVRENTL |
| <i>T. rubripes</i> 1    | MRTITAIGNY | DYMWDFIF                          | YQSGSVEAKVHATGYISSYMMEG               | -----          | SLNYGHQVAEKVL  |
| <i>T. rubripes</i> 2    | LRTTSTVY   | NYDYIWDFL                         | FYQNGVMEVKMSATGYIHSTFFTS              | -----          | NGLQYGTKVYENGL |
| <i>L. esculentum</i>    | VRMAASV    | ANYDYIVD                          | WEFQNDGLIRPKVGLSGILMVKGSPYVNMNEVNQ    | ----           | NEYLYGTLLSENIV |
| <i>S. tuberosum</i>     | VSFICTV    | ANYEYGFY                          | WHFYQDGKIEAXQADWNSQLR CSSTGE          | -----          | VRKYGTTIAPGLY  |
| <i>A. thaliana</i> 1    | VRMVTTV    | GNNDYIVD                          | YEFKPSGSIKIGVGLTGVLEV                 | ----           | KPVKYVNTSEIKED |
| <i>A. thaliana</i> 2    | VRMVATL    | GNNDYIVD                          | WEFKKSGAIRVGDLTGVLEV                  | ----           | KATSYTSNEQITEN |
| <i>A. thaliana</i> 3    | ARMATSV    | GNNDYIFD                          | WEFQTDGLIRVTVAASGMLMVKGTPYDNVDDLGDRED | ----           | DAGPLISENVI    |
| <i>A. thaliana</i> 4    | VSFLCTV    | ANYEYGFY                          | WHFYQDGKIEAEVKLTGILSLGALQPGE          | -----          | TRKYGTTIAPGLY  |
| <i>A. thaliana</i> 5    | ARMACSV    | GNNDYIFD                          | WEFQMDGVIRVTVAASGMLMVKGTYENVEDLGEKED  | ----           | DSGPLISENVI    |
| <i>A. thaliana</i> 6    | ARMATSV    | GNNDYIFD                          | WEFQTDGLIRVTVAASGMLMVKGTPYDNVDDLGDRED | ----           | DAGPLISENVI    |
| <i>C. arietinum</i>     | VRTVTV     | GNNDVLD                           | WEFKTSGSIKPAIALSGILEIKGANIKHKDEIKED   | ----           | QYGTLSANSI     |
| <i>C. lineata</i>       | VRMAAAV    | ANYDYIVD                          | WEFQTDGLIRSKVGLSGILMVKGSSYENMNQVPEQED | ----           | LYGTLLSENSI    |
| <i>G. max</i>           | VSFICTV    | ANYEYGFY                          | WHFYQDGKIEAEVKLTGILSLGALQPGE          | -----          | TRKYGTTIAPGLY  |
| <i>M. truncatula</i> 1  | FLLYCTV    | ANYEYAFF                          | WHFYQDGKIEAEVKLTGILSLGALMPGE          | -----          | VRKYGTMIAPGLY  |
| <i>M. truncatula</i> 2  | VRTVTV     | GNNDVLD                           | WEFKASGSIKPAIALSGILEIKGTNIKHKDEIKED   | ----           | EDLHGKLVANSI   |
| <i>P. sativum</i> 1     | VRTIVTV    | GNNDVID                           | WEFKASGSIKPAIALSGILEIKGTNIKHKDEIKED   | ----           | LHGKLVANSI     |
| <i>P. sativum</i> 2     | VRTIVTV    | GNNDVID                           | WEFKASGSIKPAIALSGILEIKGTNIKHKDEIKED   | ----           | LHGKLVANSI     |
| <i>L. culinaris</i> 1   | IRTVTV     | GNNDVLD                           | WEFKTSGWMKPSIALSGILEIKGTNIKHKDEIKEE   | ----           | IHGKLVANSI     |
| <i>L. culinaris</i> 2   | IRTVTV     | GNNDVLD                           | WEFKTSGWMKPSIALSGILEIKGTNIKHKDEIKEE   | ----           | IHGKLVANSI     |
| <i>O. sativa</i>        | VSFICTV    | ANYEYGFY                          | WHFYQDGKIEAX                          | -----          |                |
| <i>T. aestivum</i>      | VSFICTV    | ANYEYGFY                          | WHFYQDGKIEAEVKLTGILSLGALMPGE          | -----          | SRKYGTTIAPSLY  |
| <i>H. vulgare</i> 1     | VSFICTV    | ANYEYGFY                          | WHFYQDGQIEAEVKLTGILSLGALMPEE          | -----          | STKYGTTIAPRLY  |
| <i>H. vulgare</i> 2     | VSFICTV    | ANYEYGFY                          | WHFYQDGKIEAEVKLTGILSLGALMPGE          | -----          | SRKYGTTIAPSLY  |



|                  | 604                                        | 620                                | 640           | 660                            | 682                           |
|------------------|--------------------------------------------|------------------------------------|---------------|--------------------------------|-------------------------------|
| Z. mays          | VSFICTVANYEYGFYWHFYQ                       | -----                              | -----         | -----                          | -----                         |
| S. bicolor       | VSFICTVANYEYGFYWHFYQ                       | DGKIEAEVKLTGILSLGALMPGE            | -----         | SRKYGTTIAPGLYAPVHQHFFVARMDMA   |                               |
| P. angusta       | VSQIFTAANYEYCLYWVFMQDGAIRLDIRL             | TGILNTYILGDDEE                     | -----         | AGPWGTRVYPNVNAHNHQHFLSLRIDPR   |                               |
| P. pastoris      | VRFISTIGNYDYNFLYKFFLDGTL                   | EVSVRAAGYIQAGYWNPET                | -----         | SAPYGLKIHVDVLSGSFHDHVLNLYKVDLD |                               |
| C. albicans 1    | ISQIFTAANYEYCVYWILRQDGTIKLEIRL             | TGILNTYICADGED                     | -----         | IGPWGTVVYPNVNAHNHQHFLSLRIHPR   |                               |
| C. albicans 2    | VQTIATVANYEYIINLK                          | FVTDGSDIEVRATGILSTMPIDENV          | -----         | KVPWGTIVGPNVMAAYHQHILGFRIIDPA  |                               |
| S. pombe 1       | ISQIFTAANYEYMVYWIFHMDGVIECELKL             | TGILNTYAMNEGED                     | -----         | LKGWGTQVYPQVNAHNHQHFLCLRLNPM   |                               |
| S. pombe 2       | VSQVFTAANYEYLVYWIFRMDGVIECEIRL             | TGILNTNAINED                       | -----         | LKGHGTQVYPKISAENHEHFLCLRLNPM   |                               |
| A. niger 1       | VQFIITLANYEYIFAYKFDQSGGITGRVACHGYLERGQHRWQ | -----                              | GQVRVQRRQWRRV | GPPEPAHFLCAHRPG                |                               |
| A. niger 2       | VQFIITLANYEYIFAYKFDQSGGITGRVACHGYLERGQHRWQ | -----                              | VSEYGNVVS     | GGVLAQNQHIFCVRIDPA             |                               |
| A. fumigatus 1   | ISQIITAANYEYAFYHTFTLDGTYKLEVKL             | TGMLNTYCLHPSEQ                     | -----         | AAPFGTEIARGLDAQNHQHIFSLRVDPE   |                               |
| A. fumigatus 2   | LQTIITVGNIEYIFAFQFTQDASVTYEV               | RATGILSTAPINLGQ                    | -----         | TVPYGTIVAPGVMAPYHQHFLSLRIDPA   |                               |
| E. coli          | VRWISTVGNIDYIFDWIFHENG                     | TIGIDAGATGIEAVKGVKAKTMHDETAKDDTR   | YGT           | LIDHNIVGTTHQHIYNFRDLDD         |                               |
| K. aerogenes     | VRWISTVGNIDYIFDWVFDHNGT                    | TIGIDAGATGIEAVKGVLA                | KTMHDPSAKEDTR | YGT                            | LIDHNIVGTTHQHIYNFRDLDD        |
| K. pneumoniae    | VRWISTVGNIDYIFDWFHN                        | -----                              | -----         | -----                          |                               |
| L. pneumophila   | VSFFTTIGNYDYGFWYRFGQDGS                    | LKLEIKLTGIVQTAAIFP                 | EA            | -----                          | QYEWGGKLTPELAAPTHQHFFNARLHMM  |
| M. loti          | VSCICTVANYEYGFIFWYLHTDGAIEFELKATGI         | INTTACQPGKP                        | -----         | GR                             | YGKEVSPGVLGHIHQHIFCARLDMS     |
| A. globiformis 1 | VSFFTTVGNIDYGFYWYLYLDGTIEFEAKATGI          | VFTAALP                            | --DK          | -----                          | DYAYASEIAPGLGAPYHQHFLSARLDMM  |
| A. globiformis 2 | ISFFTTIGNYDYGFWYWYLYLDGTIEFEAKATG          | VVFTSAFP                           | --EG          | -----                          | GSDNISQLAPGLGAPFHQHIF SARLDMA |
| A. strain P1     | ISFIATVANYEYAFYWHFLFLDGSIEFLVKATGIL        | STAGQLPGEK                         | -----         | NPYQGS                         | LNNDGLYAPIHQHMFNVRMDFE        |
| D. radiodurans   | -----                                      | LGNYDYGFWYLYQDGTIQLEAKLTGIIQTAAIEP | GQ            | -----                          | PYPYGGMVDEGLGGPTHQHFFNARLDMM  |
| M. smegmatis 1   | ISMICTVGNIEYGFYLYNDAGIEMEVLKLTGVL          | TTGSIPEGE                          | -----         | TPRWGKMVAPGMYGPNHQHFFNFRMDMS   |                               |
| M. smegmatis 2   | LSFHVTVANYEYLVYWRLYQDGNIECEVRATGIM         | VTTPLPAGAD                         | -----         | AHPNGALVDERTYAPYHQHFLIARLDLD   |                               |
| Anabaena PCC7120 | ITITAAVDNYDGINWIFHQDGTLEVNDLTGIVLV         | QGTEAETQP                          | -----         | EDNFYGRLLAKNIFGVNHQHFFNYRLDMD  |                               |
| N. punctiforme   | MSMTTAVDNYDYSLNWIFHQDGTLEVQNELTGI          | VLAQGTVAQKQS                       | -----         | EDDSYGRLLAKNIFGVNHQHFFNYRLDFD  |                               |
| S. solfataricus  | VSWATLANIDYGFYFYQDGSIEFLVKLTGII            | INDDSISEKDP                        | -----         | TPKYGTRVTPEVYAPIHIHFFNIRLNIN   |                               |

|              |        |   |        |         |        |    |     |    |     |      |       |  |      |              |     |   |   |   |        |
|--------------|--------|---|--------|---------|--------|----|-----|----|-----|------|-------|--|------|--------------|-----|---|---|---|--------|
| Consensus    | VR     | I | TV     | ANYDYIF | W      | FY | DG  | IE | V   | TGIL | G     |  | YGT  | VA           | LIG | H | H | F | YRLDMD |
|              |        |   |        | ♠       | ■      |    |     |    |     | ■    |       |  |      |              |     |   | ▲ | ▲ | ●●●    |
| 2° structure | ββββββ |   | ββββββ |         | ββββββ |    | βββ |    | ααα |      | βββββ |  | ββββ | ββββββββββββ |     |   |   |   |        |
|              | βD3    |   | βD4    |         | βD5    |    | βE1 |    | h9  |      | βE2   |  | βE3  | βC6          |     |   |   |   |        |



|                         | 683                              | 700               | 720              | 740                 | 761          |
|-------------------------|----------------------------------|-------------------|------------------|---------------------|--------------|
| <i>H. sapiens</i> DAO   | VAGTKNSFQTLQMKLENITNPWSPRHRVQ    | --PTLEQTQYSW      | ERQAAFRFKRKL     | P KYLLFTSPQENP      | --WGHKRSYRLQ |
| <i>H. sapiens</i> SSAO  | VAGLENWVVAEDMVFVPMAPWSPPEHQLQR   | --LQVTRKL         | LEMEEQAAFLVGSATP | RYLYLASNHSNK        | --WGHPRGYRIQ |
| <i>H. sapiens</i> SSAOb | VAGLKNWVVAEDVVF KPVAAPWNPEHWLQR  | --PQLTRQVLGK      | EDLTAFSLGSP      | PLP RYLYLASNQTN     | --WGHQRGYRIQ |
| <i>H. sapiens</i> SSAOa | VAGLKNWVVAEDVVF KPVAAPWNPEHWLQR  | --PQLTRQVLGK      | EDLTAFSLGSP      | PLP RYLYLASNQTN     | --WGHQRG---- |
| <i>M. musculus</i> DAO  | VAGTKNSFRTLKTKLENITNPWSPSHSLVQ   | --PTLEQTQYSH      | EHQAAFRFGQTL     | P KYLLFSSPQKNR      | --WGHRRSYRLQ |
| <i>M. musculus</i> SSAO | VAGLKNWAWAEDMAFVPTIVPWQ PEYQMQR  | --LQVTRKL         | LETEEEAAFP       | LGATP RYLYLASNHSNK  | --WGHRRGYRIQ |
| <i>R. norvegicus</i>    | VAGTKNSFQTLTMKLENLTNPWSPSHSLVQ   | --PTLEQTQYS       | QEHQAAFRFGQTL    | P KYLLFSSPQKNC      | --WGHRRSYRLQ |
| <i>B. taurus</i> DAO    | VAGTKNSFQTLQMKLENITNPWSPGHRLVH   | --PALQQMRYSR      | ERQAAFRFGQTL     | P KYLLFTSPEQNP      | --WGHQRSYRVQ |
| <i>B. taurus</i> aorta  | VGLENWVVAEDMAFVPTTVPWSPPEHQIQR   | --LQVTRKQ         | LETEEQAAFP       | LGASP RYLYLASKQSNK  | --WGHPRGYRIQ |
| <i>B. taurus</i> BPAO   | VGLENWVVAEDMAFVPTAIPWSPPEHQIQR   | --LQVTRKQ         | LETEEQAAFP       | LGASP RYLYLASKQSNK  | --WGHPRGYRIQ |
| <i>B. taurus</i> lung   | VGLENWVVAEDMAFVPTTVPWSPPEHQIQR   | --LQVIRKQ         | LETEEQAAFP       | LGGSPP RYLYLASKQSNK | --WGHPRGYRIQ |
| <i>T. rubripes</i> 1    | VAGK-----                        |                   |                  |                     |              |
| <i>T. rubripes</i> 2    | IAG-----                         |                   |                  |                     |              |
| <i>L. esculentum</i>    | IDGPSNNSFLKVNLLKEMTSFGESPR       | ---RSYLKAVRNVAKTE | KDAQIKLKLYDPSEFH | VINSNKKS            | -RVGNPVGVTKL |
| <i>S. tuberosum</i>     | VDCKSGESHNQVVEVNARVEPPGENNV      | -HNNAFYAEKFL      | ETELEAMRDCNPLTA  | RHWIIRNTRTVN        | -RTGQLTGYKLV |
| <i>A. thaliana</i> 1    | IDGTDN-SFVRSELVTKRTPKSVNTP       | -RKSYWTTKRLKAE    | -----            | ELLVVNPSRKT         | -KHGNEVGYRL  |
| <i>A. thaliana</i> 2    | VDGNGN-SLVKAKLKTVRVTDVNKTSSRRKSY | WTVVKETA          | TEADGRVRLGS      | -EPVELLIVNPNKKT     | -KIGNTVGYRLI |
| <i>A. thaliana</i> 3    | IDGPMNNSLVKVHLEKQRVPTGKSPR       | ---KSYLKVKKYIAKTE | KDAQIKLSLYDPYEFH | IVNPNRKS            | -RVGNPAGYRIV |
| <i>A. thaliana</i> 4    | VDCKPAEAFNQVVEVNVVRVDERGENNV     | -HNNAFYAEKLLKSE   | AVAMRDCDPLSA     | RHWIVRNTTRTVN       | -RTGQLTGYKLV |
| <i>A. thaliana</i> 5    | IDGSANNSFVKVHLEKQRLPPGESRR       | ---KSYLKVKKYVAKTE | KDAQIKMSLYDPYEFH | LVNPNRLS            | -RLGNPAGYKLV |
| <i>A. thaliana</i> 6    | IDGPMNNSLVKVHLEKQRVPTGKSPR       | ---KSYLKVKKYIAKTE | KDAQIKLSLYDPYEFH | IVNPNRKS            | -RVGNPAGYRIV |
| <i>C. arietinum</i>     | VDGVNN-SFEKTSCLKTVRITDGSSKR      | ---KSYWTTETQTAKTE | SDAKITIGL        | -SPAELVVVNPNKKT     | -AVGNDVGYRLI |
| <i>C. lineata</i>       | IDGSQN-SFVKVNLKKQETSPGESPR       | ---KSYLKAVRKVAKTE | KDAQIRLKLYDPCEFH | LVNPLKKT            | -KVGNPVGYKIV |
| <i>G. max</i>           | VDCKPGEAFNQVVEVNVKVEKPGDNNV      | -HNNAFYAEKLLKSE   | MEAMRDCDPLSA     | RPWIVRNTTRTVN       | -RTGHLTGYKLV |
| <i>M. truncatula</i> 1  | VDSRPGALNQVVEVNMKVEEPGEKNI       | -HNNAFYAEETLLRSE  | LEAMRDCNPLTA     | RHWIVRNTRTGN        | -RTGQLTGYKLV |
| <i>M. truncatula</i> 2  | IDGVQN-SFEKTSCLKTVKITDGSSKR      | ---KSYWTTETQIAKTE | SD-----          |                     |              |
| <i>P. sativum</i> 1     | IDGTHN-SFEKTSCLKTVRIKDGSSKR      | ---KSYWTTETQTAKTE | SDAKITIGL        | -APAELVVVNPNIKT     | -AVGNEVGYRLI |
| <i>P. sativum</i> 2     | IDGTHN-SFEKTSCLKTVRIKDGSSKR      | ---KSYWTTETQTAKTE | SDAKITIGL        | -APAELVVVNPNIKT     | -AVGNEVGYRLI |
| <i>L. culinaris</i> 1   | IDGTQN-SFEKTSCLKTVRIVDEVQE       | ---KSYWTTETQTAKTE | SDAKITIGL        | -APAELVVVNPNIKT     | -AVGNEVGYRLI |
| <i>L. culinaris</i> 2   | IDGTQN-SFEKTSCLKTVRIVDGGSKR      | ---KSYWTTETQTAKTE | SDAKITIGL        | -APAELVVVNPNIKT     | -AVGNEVGYRLI |
| <i>O. sativa</i>        | -----                            |                   |                  |                     |              |
| <i>T. aestivum</i>      | VDCKPNEAHNQVVEVNVKVESAGTNNV      | -HNNAFYAEKILKSE   | ELQAMEIVTLHLR    | DIGLLGTQXAVN        | -RTGQPTGFRLV |
| <i>H. vulgare</i> 1     | GDCNLTKLINQVGEKVNVESAGSNNV       | -HYNAFIVNEKNRNF   | DFVIERLLPLFL     | DIGLVNPKG           | -----        |
| <i>H. vulgare</i> 2     | VDCKPNEAHNQVVEVNVNVESAGTNNV      | -HNNAFYAEKILKSE   | ELQAMEIVTLHLR    | DIGLLGTXRAVN        | -RTGQPTGFRLV |



|                  | 683                         | 700                   | 720                 | 740                | 761                |
|------------------|-----------------------------|-----------------------|---------------------|--------------------|--------------------|
| Z. mays          | -----VVEVNVKVESAGTHNV       | -HNNAFYAEKLLKSE       | ELQAMRCDPSSA        | RHWIVRNTRTVN       | -RTGQPTGYGSY       |
| S. bicolor       | VDCKPNEAHNHVVEVNVKVESAGTHNV | -HNNAFYAEKLLKSE       | ELQAMRCDPSSA        | RHWIVRNTRTVN       | -RTGQPTGYRLV       |
| P. angusta       | IDG-DGNSAAACDAKSSPYPLGSPENM | -YGNAFYSEKTTFKTVKDSL  | TNYESATGRSWDIF      | NPKNVPYSGKPPSYKLV  |                    |
| P. pastoris      | VGGTKNRASKYVMKDVDVEYPWAPG   | --TVYNTKQIAREVLE      | KEDFNGINWPENGQ      | GILLIESAEETN-SFGN  | PRAYNIM            |
| C. albicans 1    | IDG-DNNSAATSDAKPSPFPTGSTQNM | -YGNAFYCEKTTFKTVKESIT | NFESATAR            | RTWDMYNPSSIHKYS    | GKPATYKLV          |
| C. albicans 2    | VDG-HMNSVVYDDAIKLPR---      | DEFNP-YGIGFVTD        | RHFVEKAGYIEQ--      | RPFDNRTYKII        | NENVINPISKT        |
| S. pombe 1       | LDG-YSNSVAVVDGVSGPGEPSKENY  | -YGNAFYTTERTVPKTVKE   | AICDYNSDTS          | RTWDICNP           | NKLHPYS            |
| S. pombe 2       | LDG-LRNSVATVDALRDKN---      | G-----TLVSKYI         | IPEITVTEAISNYDS     | STGR               | RTWDICNLNKLHPYS    |
| A. niger 1       | YYG-PNNSVQVEESHVPVPMN--     | AVTNP-NGNFYKVN        | TETMERAGFFDA--      | APELNRTVKM         | VNPHKKNPISQK       |
| A. niger 2       | IDG-PNNSVQVEESHVPVPMN--     | AVTNP-NGNFYKVN        | TETMERAGFFDA--      | APELNRTVKM         | VNPHKKNPISQK       |
| A. fumigatus 1   | IDG-PGNTVVQSDAVPMADPVGSPANP | -YGNGFYAKKTPLRTALQ    | GASDYCHETS          | RGWDIINPSRL        | NPSTRKPIAYKIL      |
| A. fumigatus 2   | IDG-HMNSLMVEESHAMPLNDPSVHNP | -FGVGYTTVNHI          | VESETPLDL--         | DVTGKRVFKI         | INENVKNPMS         |
| E. coli          | VDG----ENNSLVAMD            | PVVKPNTAGGP           | -RTSTMQVNQYNIG      | NEQDAAQKFD         | PGTIR--LLSNPNKEN   |
| K. aerogenes     | VDG----ENNTLVAMD            | PEVKPNTAGGP           | -RTSTMQVNQYTID      | SEQKAAQKFD         | PGTIR--LLSNTSKEN   |
| K. pneumoniae    | -----                       |                       |                     |                    |                    |
| L. pneumophila   | VDG----EKNSFSEHEFYPIAMGGKNP | -YGNAFGSSQKT          | FEHEGEAARSACAQTQ    | RTWKIFNPNSVN       | -GIGTAPAYKL-       |
| M. loti          | VDG----DRNSVVELNTYAAPEGPDNP | -HGNAFYEEETVLPSE      | ELQACRRANLETH       | RAWKIASADRKN       | -HVGEPTAYKLE       |
| A. globiformis 1 | IDG----DANRVEELDLVRLPKGPGNP | -HGNAFTQKRTLLARE      | ESEAVRDADGAKGRVWHIS | NPDSLNLHGH         | PVGYTLY            |
| A. globiformis 2 | IDG----FTNRVEEEDVVRQTMGPGNE | -RGNAFSRKRTVLTRE      | ESEAVREADARTGRTWI   | ISNPESKN           | -RLNEPVGYKLH       |
| A. strain P1     | LDG----VKNAVYEVD---         | MEYPEHNP-TGTA         | FMAVDRLLLETEQK      | AIRKTNEAKH         | RFWKIANHESKN       |
| D. radiodurans   | VDG----VQNSVTEHEFQAPAWSDTNP | -YGNVFHTTSTRTLTRE     | LDACREADGKTGRYWKII  | NPNVKN-SVGN        | PTGYKLI            |
| M. smegmatis 1   | IDG----PGNSVYEVD            | SIPEPDPENLNP-HHNA     | WITKDTLVASE         | SEGARDWEFSTGRYWKVV | NPSKLN-EFGAPVGYKIM |
| M. smegmatis 2   | IDG----PDNTVYMTESYAAPIGPDNP | -YGLSVVVRNEALRTE      | ADGIQDVNFATQ        | RAWKVVNTNVN        | -GL-----           |
| Anabaena PCC7120 | VDG----QANNV                | MEMNVASLPIGKNNP       | -IGNAIVVKDTP        | LKTEKAAVRDL        | DIKHSREWMIANADKKN  |
| N. punctiforme   | VDG----QANSV                | MEMNVKALPMDKKNP       | -LGNAIAVAETPLTKETAA | IRDLD              | MKSSREWMIVSADKKN   |
| S. solfataricus  | VDG----LRNRI                | YEVNLRGEPITEKNP       | -VGNAFFAEENLLENE    | ADARRHVN           | PQTGRYWKIVNIQKKN   |

|           |     |   |   |  |   |  |   |   |   |   |  |   |  |   |   |  |   |   |       |
|-----------|-----|---|---|--|---|--|---|---|---|---|--|---|--|---|---|--|---|---|-------|
| Consensus | VDG | N | S |  | F |  | L | T | E | A |  | R |  | I | N |  | G | P | GYRLV |
|-----------|-----|---|---|--|---|--|---|---|---|---|--|---|--|---|---|--|---|---|-------|

|              |              |     |            |  |      |  |                |  |        |  |  |  |  |  |  |  |     |  |  |
|--------------|--------------|-----|------------|--|------|--|----------------|--|--------|--|--|--|--|--|--|--|-----|--|--|
| 2° structure | BBBBBBBBBBBB |     | BBBBBBBBBB |  | αααα |  | BBBBBBBBBBBBBB |  | BBBBBB |  |  |  |  |  |  |  |     |  |  |
|              |              | βF1 |            |  | βF2  |  | h10            |  | βF3    |  |  |  |  |  |  |  | βF4 |  |  |



|                         | 762           | 780              | 800                   | 820                    | 840               |
|-------------------------|---------------|------------------|-----------------------|------------------------|-------------------|
| <i>H. sapiens</i> DAO   | IHSM-----     | ADQVLPPGWQEEQAIT | WARYPLAVTKYRES----    | ELCSSSIYHQNDPWHF-----  | PVVFEQFLHNN       |
| <i>H. sapiens</i> SSAO  | MLSF-----     | AGEPLPQNSSMARGFS | WERYQLAVTQRKEE----    | EPSSSSVFNQNDPWAP-----  | TVDFSDFINN-       |
| <i>H. sapiens</i> SSAOb | IHSP-----     | LGIQIPLESDMERALS | WGRYQLVVTQRKEE----    | ESQSSSIYHQNDIWTP-----  | TVTFADFINN-       |
| <i>H. sapiens</i> SSAOa | -----         | YQLVVTQRKEE----  | ESQSSSIYHQNDIWTP----- | TVTFADFINN-            |                   |
| <i>M. musculus</i> DAO  | IHSM-----     | AEQVLPPGWQEERA   | VTWARYPLAVTKYRES----  | ERYSSSLYNQNDPWPDP----- | PVVFEEFLRNN       |
| <i>M. musculus</i> SSAO | ILSF-----     | AGKPLPQESPIEKAF  | TWGRYHLAVTQRKEE----   | EPSSSSIYNQNDPWTP-----  | TVDFTDIFSN-       |
| <i>R. norvegicus</i>    | IHSM-----     | AEQVLPPGWQEERA   | VTWARYPLAVTKYRES----  | ERYSSSLYNQNDPWPDP----- | PVVFEEFLRNN       |
| <i>B. taurus</i> DAO    | IHSM-----     | ADQVLPPGWQEERA   | VTWARYPLAVTKYRES----  | ERYSSSIYNQNDPWPDP----- | PVVFEEFLRNN       |
| <i>B. taurus</i> aorta  | TVSF-----     | AGRPLPQNSSTERAIS | WGRYQLAVTQRKET----    | EPSSSSVFNQNDPWTP-----  | TVDFADFINN-       |
| <i>B. taurus</i> BPAO   | TVSF-----     | AGGPMPQNSPMEAFS  | WGRYQLAITQRKET----    | EPSSSSVFNQNDPWTP-----  | TVDFSDFINN-       |
| <i>B. taurus</i> lung   | TVSF-----     | AGRPLPQNSSTERAIS | WGRYQLAVTQRKET----    | EPSSSSVFNQNDPWTP-----  | TVDFADFINN-       |
| <i>T. rubripes</i> 1    | -----         | -----            | -----                 | -----                  | -----             |
| <i>T. rubripes</i> 2    | -----         | -----            | -----                 | -----                  | -----             |
| <i>L. esculentum</i>    | FLVELQLVYLTIM | ILLRKELLLLI      | TKFGSHLIMNLSNGLLACLF  | KVKVMILLFGLDRDR-----   | AIEN----          |
| <i>S. tuberosum</i>     | PGSN-----     | CLPLAGSEAKFLR    | RAAFLKHNLWVTPYSRE---- | EIFPGGEFPNQNPRVG-----  | EGLATWVQQR--      |
| <i>A. thaliana</i> 1    | HGPAS-----    | EGPLLAQDDYPQI    | RAAFTNYNVWITPYNNT---- | EVWASGLYADRSQG----     | D---DTLAVWSQRNR-- |
| <i>A. thaliana</i> 2    | PEHLP-----    | ATSLTDDDDYPEI    | RAGYTKYPVWVTAYDRS---- | ERWAGGFYSDRSRG----     | D---DGLAVWSSNRN-- |
| <i>A. thaliana</i> 3    | PGGN-----     | AASLLDHDDPPQI    | RGAFNNQIWVTPYNRS----  | EQYAGGVLIYQSOG----     | D---DTLQVWSDRDR-- |
| <i>A. thaliana</i> 4    | PGSN-----     | CLPLARPEAKFLR    | RAAFLKHNLWVTRYAPD---- | EKFPGGEFPNQNPRAG-----  | EGLATWVKQNR--     |
| <i>A. thaliana</i> 5    | PGGN-----     | AASLLDHDDPPQM    | RGAFNNQIWVTRYNRS----  | EQWAGGLLMYQSRG----     | E---DTLQVWSDRDR-- |
| <i>A. thaliana</i> 6    | PGGN-----     | AASLLDHDDPPQI    | RGAFNNQIWVTPYNRS----  | EQYAGGVLIYQSOG----     | D---DTLQVWSDRDR-- |
| <i>C. arietinum</i>     | PAIP-----     | AHPLLTEDDYPQI    | RGAFNNYNVWVTPYNRT---- | EKWAGGLYVDHSRG----     | D---DTLAVWTQKNR-- |
| <i>C. lineata</i>       | PGGT-----     | AASLLDAEDPPQK    | RAAFTNNQIWVTPYNKT---- | EQWAGGLFVYQSKG----     | D---DTLQVWSNRNR-- |
| <i>G. max</i>           | PGSN-----     | CLPLAGSEAKFLR    | RAAFLKHNLWVTPYARD---- | EMHPGGEFPNQNPRVG-----  | EGLATWVKQNR--     |
| <i>M. truncatula</i> 1  | PGSN-----     | CLPLAGSEAKFLR    | RAAFLKHNVWVTAYSRD---- | EMFPGGEFPNQNPRVG-----  | EGLATWIKQNR--     |
| <i>M. truncatula</i> 2  | -----         | -----            | -----                 | -----                  | -----             |
| <i>P. sativum</i> 1     | PAIP-----     | AHPLLTEDDYPQI    | RGAFNNYNVWVTAYNRT---- | EKWAGGLYVDHSRG----     | D---DTLAVWTKQNR-- |
| <i>P. sativum</i> 2     | PAIP-----     | AHPLLTEDDYPQI    | RGAFNNYNVWVTAYNRT---- | EKWAGGLYVDHSRG----     | D---DTLAVWTKQNR-- |
| <i>L. culinaris</i> 1   | PAIP-----     | AHPLLTEDDYPQI    | RGAFNNYNVWVTXNNRT---- | EKWAGGLYVDXSRG----     | D---DTLAVWTKKNR-- |
| <i>L. culinaris</i> 2   | PAIP-----     | AHPLLTEDDYPQI    | RGAFNNYNVWVTPYNRT---- | EKWAGGLYVDHSRG----     | D---DTLAVWTKKNR-- |
| <i>O. sativa</i>        | -----         | -----            | -----                 | -----                  | -----             |
| <i>T. aestivum</i>      | PGSN-----     | CLPFALPEAKFLR    | RAGFLKHNLWVTPYKSD---- | EKFPGGEFPNQNPRH-----   | EGLATWVKKDR--     |
| <i>H. vulgare</i> 1     | -----         | -----            | -----                 | -----                  | -----             |
| <i>H. vulgare</i> 2     | PGSN-----     | CLPFALPEAKFLR    | RAGFLKHNLWVTPYKSD---- | EKFPGGEFPNQNPRH-----   | EGLATWVKQDR--     |



|                  | 762            | 780              | 800                    | 820                  | 840                                |
|------------------|----------------|------------------|------------------------|----------------------|------------------------------------|
| Z. mays          | LVXN-----      | CLPLALPEAKFLR    | RAGFLKHNLWVTQYKRG      | -----                | EMFPGGEFPNQNPRIH-----EGLPTWVKNDR-- |
| S. bicolor       | PGSN-----      | CLPLALPEAKFLR    | RAGFLKHNLWVTQYKRD      | -----                | EMFPGGEFPNQNPRIH-----EGLPTWVKKDR-- |
| P. angusta       | STQC-----      | PPLLAKEGSLVAK    | RAPWASHSVNVVPYKDN      | -----                | RLYPSGDHVPQWSGDG----VRGMREWIGDG--S |
| P. pastoris      | PGGGG-----     | VHRIVKNSRSGPETQN | WARSNLFLTKHKDE         | -----                | ELRSSTALNTNALYDP-----PVNFNAFLDD    |
| C. albicans 1    | STFC-----      | SPLLAQEGSLVKK    | RAPWAASHTQVVPYVDDKLGYG | RLYPSGDHVPQWSGDG---- | LRGMREWIGDG--T                     |
| C. albicans 2    | MPAR-----      | QMLLADPDSFNAK    | RAKFATQQVWVTKYQDH      | -----                | QLFAAGEFTNQSKIDT-----GITEWANG---I  |
| S. pombe 1       | SRET-----      | PRLMARPGSLVSN    | RAGFARHHIHVTPYKDG      | -----                | QIYPAGDYVPQTSSEP----TKGLPEWIAEEP-D |
| S. pombe 2       | SRDT-----      | SPVLSQPGTTNSDC   | SGFAENNIYVTPYMDD       | -----                | QIFPTGDYAPQASDDT----PKGLSKWISDDP-N |
| A. niger 1       | PLAT-----      | QRLPADPNSIQAR    | RAQFAQHVVWVTKYRDG      | -----                | ELYAGGRYTLQSQ--E-----EIEGVSDAVKRG  |
| A. niger 2       | PLAT-----      | QRLPADPNSIQAR    | RAQFAQHVVWVTKYRDG      | -----                | ELYAGGRYTLQSQ--E-----EIEGVSDAVKRG  |
| A. fumigatus 1   | NNNC-----      | PRLLAKEGSLVYK    | RAGFARHALWVLPYRDH      | -----                | EIFPAGQYVCQSTGEENHPHNPTIVDWAARN--- |
| A. fumigatus 2   | PHYS-----      | QMVLAHPSSYHFK    | RSEFAEHAVWVTRYSD       | -----                | ERYPAGEHTMQSLGGE-----GIYSWIRSRPAP  |
| E. coli          | PYAGGTHPVAKGAQ | FAPDEWIYH        | RLSFMDKQLWVTRYHPG      | -----                | ERFPEGKYPNRSTHDTG-----LGQYSKDN     |
| K. aerogenes     | PYAGGTHPAATGAK | FAPDEWIYH        | RLSFMDKQLWVTRYHPT      | -----                | ERYPEGKYPNRSADHTG-----LGQYAKDD     |
| K. pneumoniae    | -----          | -----            | -----                  | -----                | -----                              |
| L. pneumophila   | -----          | -----            | -----                  | -----                | -----                              |
| M. loti          | ATHP-----      | VTPYVAAHSPSGR    | RSTFTSNHLWVTAFDPE      | -----                | ERYPAGEYMNHSDGSGG-----VADFVKNDR--  |
| A. globiformis 1 | PEGN-----      | PTLAMADDSSIAS    | RAAFARHHLWVTRHAEE      | -----                | ELYAAGDFVNQHPGGAV-----LPAYVAQDR--  |
| A. globiformis 2 | AHNQ-----      | PTLLADPGSSIA     | RAAFATKDLWVTRYADD      | -----                | ERYPTGDFVNQHS GGAG-----LPSYIAQDR-- |
| A. strain P1     | PTNG-----      | IQLAARDDAYVSK    | RAQFARNNLWVTAYDRT      | -----                | ERFAAGEYPNQATGADDG-----LHIWTQKDR-- |
| D. radiodurans   | -----          | -----            | -----                  | -----                | -----                              |
| M. smegmatis 1   | P-----         | -----            | -----                  | -----                | -----                              |
| M. smegmatis 2   | -----          | -----            | -----                  | -----                | -----                              |
| Anabaena PCC7120 | PGGN-----      | TVFFPVEGAKIRQ    | KAEFATHHVVWVTRYKPH     | -----                | ELYAGGDYPNQAPP GK-----GLPEYIAD---  |
| N. punctiforme   | P-----         | -----            | -----                  | -----                | -----                              |
| S. solfataricus  | PGHN-----      | VLPLPLDDSYVRR    | RGAYINYHLWVTPYNEE      | -----                | ERYASGDYPYLRANDG-----LPKYILKKR--   |

Consensus

L

RA F YNLWVT Y

E YA G Y Q

L W R

2° structure

ααααα ααα ββββββ

h11 h12 βC7

αααααααα

h13



|                         | 841                  | 860            | 880                 | 900                                           | 919           |
|-------------------------|----------------------|----------------|---------------------|-----------------------------------------------|---------------|
| <i>H. sapiens</i> DAO   | ENIENEDLVAWVTVGFLHI  | PHSEDI         | PNTATPGNSVG         | FLLRPFNFFPEDPSLASRDTVIVWPRDNG ---             | PNYVQRWIPED   |
| <i>H. sapiens</i> SSAO  | ETIAGKDLVAWVTAGFLHI  | PHAEDI         | PNTVTVGNGVG         | FLLRPFYNFFDEDPSFYADSIIYFRGDQDAGACEVNPLACLPA   |               |
| <i>H. sapiens</i> SSAOb | ETLLGEDLVAWVTASFLHI  | PHAEDI         | PNTVTTLGNRVG        | FLLRPFYNFFDEDPSIFSPGSVYFEKGQDAGLCSINPVACLPLDL |               |
| <i>H. sapiens</i> SSAOa | ETLLGEDLVAWVTASFLHI  | PHAEDI         | PNTVTTLGNRVG        | FLLRPFYNFFDEDPSIFSPGSVYFEKGQDAGLCSINPVACLPLDL |               |
| <i>M. musculus</i> DAO  | ENIENEDLVAWVTVGFLHI  | PHSEDP         | PNTATPGNCVG         | FLIRPFNFFFEEDPSLASRDTVIVWPQDNG ---            | LNHVQRWIPEN   |
| <i>M. musculus</i> SSAO | ETIAGEDLVAWVTAGFLHI  | PHAEDI         | PNTVTAGNSVG         | FLLRPFYNFFDEDPSFHSADSIYFREGQDATAACEVNPLACLSQT |               |
| <i>R. norvegicus</i>    | ENIEDEDLVAWVTVGFLHI  | PHSEDP         | PNTATPGNSVG         | FLLRPFNFFPEDPSLASRDTVIVWPQDKG ---             | LNRVQRWIPED   |
| <i>B. taurus</i> DAO    | ENIEDEDLVAWVTVGFLHI  | PHAEDI         | PNTTTPGNSVG         | FLLRPFNFFPEDPSLA-----                         |               |
| <i>B. taurus</i> aorta  | ETIAGKDLVAWVTAGFLHI  | PHAEDI         | PNTVTVGNGVG         | FLLRPFYNFFDEDPSINSADSIYFQKHQDAGSCEVNSLACLPA   |               |
| <i>B. taurus</i> BPAO   | ETIAGKDLVAWVTAGFLHI  | PHAEDI         | PNTVTVGNGVG         | FLLRPFYNFFDQEPSMDSADSIYFREGQDAGSCEINPLACLPA   |               |
| <i>B. taurus</i> lung   | ETIAGKDLVAWVTAGFLHI  | PHAEDI         | PNTVTVGNGVG         | FLLRPFYNFFDQEPSMDSADSIYFREGQDAGSCEINPLACLPA   |               |
| <i>T. rubripes</i> 1    | -----                |                |                     |                                               |               |
| <i>T. rubripes</i> 2    | -----                |                |                     |                                               |               |
| <i>L. esculentum</i>    | -----                | KDIVLWYTLGFHHI | PCQEDFPIMPTVS--     | SSFEIKPVNFFESNPILNIPPNSPKDLPQFAKLQL           | LLDHILIVLY    |
| <i>S. tuberosum</i>     | -SLEETQIVLWYIFGLIHV  | PRLEDWPVMPVE-- | HIGFMLQPHGFFNCSPA   | VDVPP-SMSDSDIKEN                              | -----         |
| <i>A. thaliana</i> 1    | -KIEKTDIVMWTYVGFHHV  | PCQEDFPIMPTL-- | FGGFELRPTNFFEQNPDL  | KTPIKLNTTPTCTARND                             | -----         |
| <i>A. thaliana</i> 2    | -EIEKNDIVMWTYVGFHHI  | PCQEDFPIMPTL-- | HGGFTLRPSNFFDNDPLIG | -----                                         |               |
| <i>A. thaliana</i> 3    | -SIENKDIVLWYTLGFHHV  | PCQEDYPVMPTV-- | AASFELKPANFFESNPIL  | GSAPFFEKDLPVCRPFASS                           | -----         |
| <i>A. thaliana</i> 4    | -SLEESDVVLWYVFGITHV  | PRLEDWPVMPVE-- | HIGFTLMPHGFFNCSPA   | VDVPP-NPCELETKESEVKEVVAPKALQT                 |               |
| <i>A. thaliana</i> 5    | -SIENKDIVLWYTLGFHHV  | PCQEDFPIMPTI-- | ASSFELKPVNFFESNPVL  | GISPFFEKDLPVC                                 | -----         |
| <i>A. thaliana</i> 6    | -SIENKDIVLWYTLGFHHV  | PCQEDYPVMPTV-- | AASFELKPANFFESNPIL  | GSAPFFEKDLPSQHTGCCFVEFASLNEA                  |               |
| <i>C. arietinum</i>     | -DIENKDIVLWHVVGIIHHV | PAQEDFPIMPLL-- | STSFELRPTNFFERNPVL  | NLTLSPRDVNWPGCSN                              | -----         |
| <i>C. lineata</i>       | -PIENKDIVLWYTLGFHHI  | PCQEDYPIMPTV-- | SSSFDLKPVNFFERNPIL  | RVPPNFEDDLPVCKAHGSA                           | -----         |
| <i>G. max</i>           | -SLEEADIVLWYVFGVTHI  | PRLEDWPVMPVE-- | RIGFMLMPHGFFNCSPA   | VDVPP-NQSDLDDKEN                              | ----GLPAKPIQN |
| <i>M. truncatula</i> 1  | -SLEETNIVLWYVFGITHF  | IIYSVAYVDGSR-- | GRRPVAHRLSFVEMVVPY  | GDPTEPHYRKNAFDAG                              | EDGLGKNAHSLK  |
| <i>M. truncatula</i> 2  | -----                |                |                     |                                               |               |
| <i>P. sativum</i> 1     | -EIVNKDIVMWHVVGIIHHV | PAQEDFPIMPLL-- | STSFELRPTNFFERNPVL  | KTLSPRDVAWPAGCSN                              | -----         |
| <i>P. sativum</i> 2     | -EIVNKDIVMWHVVGIIHHV | PAQEDFPIMPLL-- | STSFELRPTNFFERNPVL  | KTLSPRDVAWPAGCSN                              | -----         |
| <i>L. culinaris</i> 1   | -EIVNKDIVMWHVVGIIHHV | PAQEDFPIMPLL-- | STSFELRPTNFFERNPAL  | KTLPPIDFTWPGCSN                               | -----         |
| <i>L. culinaris</i> 2   | -EIVNKDIVMWHVVGIIHHV | PAQEDFPIMPLL-- | STSFELRPTNFFERNPVL  | KTLPPRDFTWPGCSN                               | -----         |
| <i>O. sativa</i>        | -----                |                |                     |                                               |               |
| <i>T. aestivum</i>      | -PLEETDIVLWYVFGLTTHI | PRLEDWPVMPVE-- | HIGFMLMPHGFFNCSPA   | VDVPPGTSDAADVKEAESPKAIQXRQPCF                 |               |
| <i>H. vulgare</i> 1     | -----                |                |                     |                                               |               |
| <i>H. vulgare</i> 2     | -PLEETDIVLWYVFGLTTHI | PRLEDWPVMPVE-- | RIGFMLMPHGFFNCSPA   | VDVPPGTSDAADVKEAESPKAIRTVALSP                 |               |



|                  | 841      | 860       | 880       | 900          | 919               |                                 |
|------------------|----------|-----------|-----------|--------------|-------------------|---------------------------------|
| Z. mays          | -PLEETD  | IVLWYVFG  | LTHIPRLED | WPVMPVE--    | HIGFMLMPHGFFNCSPA | VDVPP-SSSDADVKEAESPKAIQNSLX-F   |
| S. bicolor       | -PLEETD  | IVXLVRVR  | SHYPQAGRL | ASHAG---     | GAYRLHAHA         | TWILQLLTCGRRASQLVRS             |
| P. angusta       | ENIDNTD  | ILFFHTFG  | ITHFPAPED | FPLMPAE--    | PITLMLRPRHFFTEN   | PGLDIQPSYAMTTSEAKRAVHKETKDKTSRL |
| P. pastoris      | ESLDGED  | IVAVVNLGL | HHLNPSNDL | NPNTIFSTAHAS | FMLT              | PFNYFDSSENSRD                   |
| C. albicans 1    | DKVDNTD  | ILLFHTFG  | ITHFPAPED | FVMPTE--     | IFDLQLRPRNIHTEN   | PVL-----                        |
| C. albicans 2    | DPVRNED  | IVVWATMG  | FTHIPRVED | FVMPVE--     | THNIQLAPANFFDRN   | PAL-----                        |
| S. pombe 1       | ASVDNTD  | IVVWHTFG  | ITHFPAPED | FPLMPAE--    | PIRLLLRPRNFFLRN   | PALDVPPSKNVT                    |
| S. pombe 2       | AQIKNTD  | IVVWHTFG  | MIHFPAPED | FPIMPAE--    | SIHLFLQPRNFFKHN   | PALDTSSSVNSTSEATSPNTHHENLRDTSQK |
| A. niger 1       | DSVVDTD  | IVVWSTFG  | ITHNPRVED | WPVMPVE--    | IFQLMIRPADFFTAN   | PSLDVPSDKNISSRVVGNRCCVNAHI----  |
| A. niger 2       | DSVVDTD  | IVVWSTFG  | ITHNPRVED | WPVMPVE--    | IFQLMIRPADFFTAN   | PSLDVPSDKN-----                 |
| A. fumigatus 1   | ESIENTD  | IVCYIQFG  | LTHFPRTED | FPIMPAE--    | PVSVMLRASNFFQKN   | PAL-----                        |
| A. fumigatus 2   | VSVRNED  | IVIWHTFG  | TTHNPRVED | WPVMPAE--    | KMSVT             | LKPVNFFWRN                      |
| E. coli          | ESLDNTD  | IVVWMTTG  | TTHVARAE  | EWPIMPTE--   | WVHTLLK           | PWNFFDETPTLGALKKDK-----         |
| K. aerogenes     | ESLTNHDD | IVVWITTG  | TTHVARAE  | EWPIMPTE--   | WALALLK           | PWNFFDETPTLGEKKK-----           |
| K. pneumoniae    | -----    | -----     | -----     | -----        | -----             | -----                           |
| L. pneumophila   | -----    | -----     | -----     | -----        | -----             | -----                           |
| M. loti          | -PLVDED  | IVLWHTFG  | VHHQVRP   | EDFPVQPCI--  | FTGFKLMPSGFFDQNP  | GIDLVGETNAASCHASADR-----        |
| A. globiformis 1 | -DIDGQD  | IVVWHSFGL | THFPRPED  | WPIMPVD--    | TTGFTLKPHGFFDEN   | PTLNVPSSAAGHCGTGSEREHAAPGGTAVGH |
| A. globiformis 2 | -DIDGQD  | IVVWHTFG  | LTHFPRVED | WPIMPVD--    | TVGFKLRPEGFFDRS   | PVLDVPANPS--Q-SGS-----          |
| A. strain P1     | -NIVDTD  | IVVWYTFGM | HVVRL     | EDWPMPRQ--   | NIGFMLEPHGFFNQNP  | PTLNLPSTSTTTQTGEADTCCHTDK-----  |
| D. radiodurans   | -----    | -----     | -----     | -----        | -----             | -----                           |
| M. smegmatis 1   | -----    | -----     | -----     | -----        | -----             | -----                           |
| M. smegmatis 2   | -----    | -----     | -----     | -----        | -----             | -----                           |
| Anabaena PCC7120 | -----    | -----     | -----     | -----        | -----             | -----                           |
| N. punctiforme   | -----    | -----     | -----     | -----        | -----             | -----                           |
| S. solfataricus  | -SIVDED  | IVIWYTLG  | VEHVVRIED | WPVMPVE--    | MAGFRLIPDGFFDKN   | PTIYLPRQLRINGQPLHREHKSH-----    |

Consensus            I    DIVLW T G    HIP    EDFPVMP            F LRP NFF    P L

2° structure            ββββββββββββ    ααα            β-ββββ    ββββ  
                                  βc8            h14            βD6    βF5

|                         | 920                                                                             | 940   | 960   | 980   | 998   |
|-------------------------|---------------------------------------------------------------------------------|-------|-------|-------|-------|
| <i>H. sapiens</i> DAO   | RDCSMPPPFSYNGTYRPV                                                              | ----- | ----- | ----- | ----- |
| <i>H. sapiens</i> SSAO  | AACAPDLPAFSGHGGFSHN                                                             | ----- | ----- | ----- | ----- |
| <i>H. sapiens</i> SSAOb | AACVPDLPPFSYHGF                                                                 | ----- | ----- | ----- | ----- |
| <i>H. sapiens</i> SSAOa | AACVPDLPPFSYHGF                                                                 | ----- | ----- | ----- | ----- |
| <i>M. musculus</i> DAO  | RDCLVSPPPFSYNGTYKPV                                                             | ----- | ----- | ----- | ----- |
| <i>M. musculus</i> SSAO | ATCAPEIPAFSGHGGFAYRDN                                                           | ----- | ----- | ----- | ----- |
| <i>R. norvegicus</i>    | RRCLVSPPPFSYNGTYKPV                                                             | ----- | ----- | ----- | ----- |
| <i>B. taurus</i> DAO    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>B. taurus</i> aorta  | PACAPDLPAFSGHGGFFTN                                                             | ----- | ----- | ----- | ----- |
| <i>B. taurus</i> BPAO   | ATCAPDLPVFSHGGEYPEY                                                             | ----- | ----- | ----- | ----- |
| <i>B. taurus</i> lung   | ATCAPDLPVFSHGGEYPEY                                                             | ----- | ----- | ----- | ----- |
| <i>T. rubripes</i> 1    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>T. rubripes</i> 2    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>L. esculentum</i>    | VYD                                                                             | ----- | ----- | ----- | ----- |
| <i>S. tuberosum</i>     | GTVAK                                                                           | ----- | ----- | ----- | ----- |
| <i>A. thaliana</i> 1    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>A. thaliana</i> 2    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>A. thaliana</i> 3    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>A. thaliana</i> 4    | GLLSKL                                                                          | ----- | ----- | ----- | ----- |
| <i>A. thaliana</i> 5    | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>A. thaliana</i> 6    | EKALETKKGETFYSRKIILDVAEMAPDPFRLKYNLVKKLCYQDFQQRPERQQIKSLFCGKKTIISSDD            | ----- | ----- | ----- | ----- |
| <i>C. arietinum</i>     | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>C. lineata</i>       | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>G. max</i>           | GLIAKL                                                                          | ----- | ----- | ----- | ----- |
| <i>M. truncatula</i> 1  | KGCDCLGYIKYFDAHFTNFTGGVLKLSKIVYVCMKRIMGFSGSIKTGELAXQKSGGPEDFQFLLYCTVANYEYAFFWHF | ----- | ----- | ----- | ----- |
| <i>M. truncatula</i> 2  | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>P. sativum</i> 1     | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>P. sativum</i> 2     | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>L. culinaris</i> 1   | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>L. culinaris</i> 2   | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>O. sativa</i>        | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>T. aestivum</i>      | QALKLRGSSKEQTGNKSYLLVV                                                          | ----- | ----- | ----- | ----- |
| <i>H. vulgare</i> 1     | -----                                                                           | ----- | ----- | ----- | ----- |
| <i>H. vulgare</i> 2     | SSEASRLSSKEQTGNKSYLLVV                                                          | ----- | ----- | ----- | ----- |



|                  | 920                                                                             | 940   | 960   | 980   | 998   |
|------------------|---------------------------------------------------------------------------------|-------|-------|-------|-------|
| Z. mays          | EAVSFAVLPRKKDQRAAA                                                              | ----- | ----- | ----- | ----- |
| S. bicolor       | CELSKGEISARLL                                                                   | ----- | ----- | ----- | ----- |
| P. angusta       | AFEGSCCGK                                                                       | ----- | ----- | ----- | ----- |
| P. pastoris      | CGLEVPEPNFEDYTYGRGTRINKKMTNSDEVY                                                | ----- | ----- | ----- | ----- |
| C. albicans 1    | -----                                                                           | ----- | ----- | ----- | ----- |
| C. albicans 2    | -----                                                                           | ----- | ----- | ----- | ----- |
| S. pombe 1       | KS--LTDATSEFAFGEKFCEKHKGDHFF                                                    | ----- | ----- | ----- | ----- |
| S. pombe 2       | RESHSTPHDYEPHVSDKNDKSVEDKLHFVQKDESRPKPEVVDAAQKHEGRSETLAQPGQONANQSEEKQGGQNGSNGGH | ----- | ----- | ----- | ----- |
| A. niger 1       | -----                                                                           | ----- | ----- | ----- | ----- |
| A. niger 2       | -----                                                                           | ----- | ----- | ----- | ----- |
| A. fumigatus 1   | -----                                                                           | ----- | ----- | ----- | ----- |
| A. fumigatus 2   | -----                                                                           | ----- | ----- | ----- | ----- |
| E. coli          | -----                                                                           | ----- | ----- | ----- | ----- |
| K. aerogenes     | -----                                                                           | ----- | ----- | ----- | ----- |
| K. pneumoniae    | -----                                                                           | ----- | ----- | ----- | ----- |
| L. pneumophila   | -----                                                                           | ----- | ----- | ----- | ----- |
| M. loti          | -----                                                                           | ----- | ----- | ----- | ----- |
| A. globiformis 1 | SGPDTGGQGHCCH                                                                   | ----- | ----- | ----- | ----- |
| A. globiformis 2 | -----HCHG-----                                                                  | ----- | ----- | ----- | ----- |
| A. strain P1     | -----                                                                           | ----- | ----- | ----- | ----- |
| D. radiodurans   | -----                                                                           | ----- | ----- | ----- | ----- |
| M. smegmatis 1   | -----                                                                           | ----- | ----- | ----- | ----- |
| M. smegmatis 2   | -----                                                                           | ----- | ----- | ----- | ----- |
| Anabaena PCC7120 | -----                                                                           | ----- | ----- | ----- | ----- |
| N. punctiforme   | -----                                                                           | ----- | ----- | ----- | ----- |
| S. solfataricus  | -----                                                                           | ----- | ----- | ----- | ----- |

Consensus

|                 |                                                                                |                       |
|-----------------|--------------------------------------------------------------------------------|-----------------------|
| S. pombe 2      | HHHHHHHYITGHVYGGYHKHSGSGGHLVDMMKNISDVTHDFAMGNFRYHKYD                           | -----                 |
| M. truncatula 1 | YQDGKIEAEVKLTGILSLGALMPGEVRKYGTMIAPGLYAPVHQHFFVARMDMSVDSRP                     | GEALNQVVEVNMKVEEPGEKN |
| M. truncatula 1 | IHNNAFYAEETLLRSELEAMRDCNPLTARHWIVRNTRTGNRTGQLTGYKLVPGSNCLPLAGSEAKFLRRAAFLKHNWV |                       |
| M. truncatula 1 | TAYSRDEMFPGGEFPNQNPVGEGLATWIKQNRSLREETNIVLWYVFGITH                             |                       |

Amino Acid Sequence Alignment of Mammalian CAO

Key:

- Y Completely conserved consensus residue
- Y Consensus residue from similar residues at a given position
- Y Consensus residue from the occurrence of greater than 50% of a single residue at a given position
- Y Residue weakly similar to consensus residue



|                                   |                 | 1   | 10               | 20                 | 30               | 40                  |
|-----------------------------------|-----------------|-----|------------------|--------------------|------------------|---------------------|
| 50                                |                 |     |                  |                    |                  |                     |
| <i>Homo sapiens</i> DAO           | (NP_001082)     | (1) | -----MPALGWAVAAI | MLQTAMAEP-----     | S--P             |                     |
| <i>Rattus norvegicus</i> DAO      | (NP_075224)     | (1) | -----MCLAFGWA    | AVILVLQTVDTAS----- | A--V             |                     |
| <i>Mus musculus</i> DAO           | (AAH21880)      | (1) | -----MSLAFGWA    | AVILLQLTADTPS----- | A--V             |                     |
| <i>Bos taurus</i> DAO             | (TIGR TC108675) | (1) | -----            | -----              | -----            |                     |
| <i>Homo sapiens</i> SSAO          | (NP_003725)     | (1) | MNQKTI           | LVLLILAVITIFALVC   | VLLVGRG--GDGGEP  | SQLPHCPSVSPSA       |
| <i>Homo sapiens</i> retinal SSAOb | (NP_033720)     | (1) | MHLKIV           | LAFLLALSLITIFALAY  | VLLTSPG-----GSSQ | PPHCPSVSHRA         |
| <i>Homo sapiens</i> retinal SSAOa | (NP_001149)     | (1) | MHLKIV           | LAFLLALSLITIFALAY  | VLLTSPG-----GSSQ | PPHCPSVSHRA         |
| <i>Mus musculus</i> SSAO          | (AAD09199)      | (1) | MTQKTT           | LVLLALAVITIFALVC   | VLLAGRS--GDGGGL  | SQLPHCPSVLPSV       |
| <i>Bos taurus</i> aorta SSAO      | (BAA88896)      | (1) | MNQKTT           | LVLLALAVITIFALVC   | VLIAGRG--GDGGEA  | SQPHYCPSGTPSV       |
| <i>Bos taurus</i> lung SSAO       | (O46406)        | (1) | ---MFIFIF        | LSLWTL             | LLVMGREGGVGSEEGV | GKQCHPSLPPRCPSRSPSD |
| <i>Bos taurus</i> liver BPAO      | (Q29437)        | (1) | ---MFIFIF        | LSLWTL             | LLVMGREGGVGSEEGV | GKQCHPSLPPRCPSRSPSD |
| Consensus                         |                 | (1) | L                | LALAVI             | I AL VLL S       | S P CPS S           |

|                             |      | 51      | 60       | 70     | 80       | 90     | 100      | 110                  |                       |
|-----------------------------|------|---------|----------|--------|----------|--------|----------|----------------------|-----------------------|
| 120                         |      |         |          |        |          |        |          |                      |                       |
| <i>H. sapiens</i> DAO       | (24) | G--     | TLPRKAGV | FSDL   | SNQELK   | AVHSFL | WSKKEL   | RQLQPSSTTTMAKNTV     | FLIEMLLPKKYHVL        |
| <i>R. norvegicus</i> DAO    | (24) | R--     | TPYDKARV | FADL   | SPQEIK   | AVHSFL | MNREEL   | GLQPSKEPTLAKNSV      | FLIEMLLPKKKHVL        |
| <i>M. musculus</i> DAO      | (24) | T--     | TPHDKAR  | I      | FADL     | SPQEIK | AVHSFL   | MSRKELGLESSKNL       | TLAKNSVFLIEMLLPKKKNVL |
| <i>B. taurus</i> DAO        | (1)  | -----   | -----    | -----  | -----    | -----  | -----    | -----                | -----                 |
| <i>H. sapiens</i> SSAO      | (49) | QPWTHPG | QSQLFADL | SREELT | AVMRFLTQ | RLGPG  | LVDAQAQ  | PSDNCVFSVELQLPPKAAAL | AHLDRGSP              |
| <i>H. sapiens</i> rSSAOB    | (45) | QPWPHPG | QSQLFADL | SREELT | AVMRFLTQ | RLGPG  | LVDAQAQ  | PSDNCIFSVELQLPPKAAAL | AHLDRGSP              |
| <i>H. sapiens</i> rSSAOa    | (45) | QPWPHPG | QSQLFADL | SREELT | AVMRFLTQ | RLGPG  | LVDAQAQ  | PSDNCIFSVELQLPPKAAAL | AHLDRGSP              |
| <i>M. musculus</i> SSAO     | (49) | QPRTHPS | QSQPFADL | SPEELT | AVMSFLTQ | HLGPG  | LVDAQAQ  | PSDNCVFSVELQLPAKAAAL | AHLDRGGP              |
| <i>B. taurus</i> aorta SSAO | (49) | QPWTHPG | QSQLFADL | SREELT | AVMSFLTQ | KLGP   | LDVDAQAQ | PSDNCIFSVELQLPPKAAAL | AHLDRRSP              |
| <i>B. taurus</i> lung SSAO  | (48) | QPWTHPD | QSQLFADL | SREELT | AVMSFLTQ | KLGP   | LDVDAQAQ | PSDNCIFSVELQLPPKAAAL | AHLDRRSP              |
| <i>B. taurus</i> liver BPAO | (48) | QPWTHPD | QSQLFADL | SREELT | TVMSFLTQ | QLGP   | LDVDAQAQ | PSDNCVFSVELQLPPKAAAL | AHLDRGSP              |
| Consensus                   | (51) | QPWTHP  | QSQLFADL | SREELT | AVMSFLTQ | RLGPG  | LVDAQAQ  | PSDNCVFSVELQLPPKAAAL | AHLDRGSP              |



|                             |       | 121                                                              | 130                     | 140          | 150           | 160           | 170  | 180 |
|-----------------------------|-------|------------------------------------------------------------------|-------------------------|--------------|---------------|---------------|------|-----|
| 190                         |       |                                                                  |                         |              |               |               |      |     |
| <i>H. sapiens</i> DAO       | (92)  | HPVREARAVIFFGDQEH                                                | PNVTEFAVGPLPGPCYMRALSPR | -PGYQSSWASRP | ISTA          | EYALLYHTLQ    | EATK |     |
| <i>R. norvegicus</i> DAO    | (92)  | GNPREARAVIFFGAQDY                                                | PNVTEFAVGPLPRPYIIRALSPR | -PGHHSWSSRP  | ISTA          | EYDLYHTLKRATM |      |     |
| <i>M. musculus</i> DAO      | (92)  | SPVREARAIFFGAQDHPNVTEFAVGPLPRPCYVQALSPR                          | -PGHHSWSSRP             | ISTA         | EYDLYHMLNRAIT |               |      |     |
| <i>B. taurus</i> DAO        | (1)   | -----                                                            |                         |              |               |               |      |     |
| <i>H. sapiens</i> SSAO      | (119) | PPAREALAIVFFGRQPQPNVSELVVGPLPHPSYMRDVTVERHGGPLPYHRRPVL           | FQ                      | EYLDIDQMI    | FNREL         |               |      |     |
| <i>H. sapiens</i> rSSAOb    | (115) | PPAREALAIVLFGGQPQPNVSELVVGPLPHPSYMRDVTVERHGGPLPYHRRPVL           | RAEFTQMWRHLKDVEL        |              |               |               |      |     |
| <i>H. sapiens</i> rSSAOa    | (115) | PPAREALAIVLFGGQPQPNVSELVVGPLPHPSYMRDVTVERHGGPLPYHRRPVL           | RAEFTQMWRHLKDVEL        |              |               |               |      |     |
| <i>M. musculus</i> SSAO     | (119) | PPVREALAIIFFGGQPKPNVSELVVGPLPHPSYMRDVTVERHGGPLPYRRPVLDREYQDIEEMI | FHREL                   |              |               |               |      |     |
| <i>B. taurus</i> aorta SSAO | (119) | PPAREALAIVFFGGQPQPNVTELVVGPLPQPSYMRDVTVERHGGPLPYRRPVLLREYLDIDQMI | FNREL                   |              |               |               |      |     |
| <i>B. taurus</i> lung SSAO  | (118) | PPAREALAIVFFGGQPQPNVTELVVGPLPQPSYMRDVTVERHGGPLPYRHTVLLREYLDIDQMI | FNREL                   |              |               |               |      |     |
| <i>B. taurus</i> liver BPAO | (118) | PPAREALAIVFFGGQPQPNVTELVVGPLPQPSYMRDVTVERHGGPLPYRRPVLLREYLDIDQMI | FNREL                   |              |               |               |      |     |
| Consensus                   | (121) | PPAREALAIVFFGGQPQPNVTELVVGPLP                                    | PSYMRDVTVERHGGPLPYRRPV  | EY           | I             | MI            | EL   |     |

|                             |       | 191                                                       | 200               | 210         | 220             | 230             | 240   | 250 |
|-----------------------------|-------|-----------------------------------------------------------|-------------------|-------------|-----------------|-----------------|-------|-----|
| 260                         |       |                                                           |                   |             |                 |                 |       |     |
| <i>H. sapiens</i> DAO       | (161) | PLHQFFLNTTGFSFQDCHDRCLAFTD                                | VAPRGV            | ASGQRRSWLI  | IQRYVEG--       | YFLHPTGLELLVDHG | STDA  |     |
| <i>R. norvegicus</i> DAO    | (161) | PLHQFFLDTTGFSFLGCDDRCLTFTD                                | VAPRGV            | ASGQRRSWF   | IVQRYVEG--      | YFLHPTGLEILLDHG | STDV  |     |
| <i>M. musculus</i> DAO      | (161) | PLHQFFLDTTGFSFLGCDDRCLTFTD                                | VAPRGVESGQRRSWL   | IVQRYVEG--  | YFLHPTGLEILVDHS | STDV            |       |     |
| <i>B. taurus</i> DAO        | (1)   | -----                                                     |                   |             |                 |                 |       |     |
| <i>H. sapiens</i> SSAO      | (189) | PQASGLLHHCCFYKH--RGRNLVTMTTAPRGLQSGDRATWFGLYYNISGAGFFLHHV | GLELLVNHKALDP     |             |                 |                 |       |     |
| <i>H. sapiens</i> rSSAOb    | (185) | PKAPIFLSSTFNYN---GSTLAAVHATPRGLRSRERTTW                   | MALYHNISGVGLFLHPV | GLELLLDHRA  | LDP             |                 |       |     |
| <i>H. sapiens</i> rSSAOa    | (185) | PKAPIFLSSTFNYN---GSTLAAVHATPRGLRSRERTTW                   | IGLYHNISGVGLFLHPV | GLELLLDHRA  | LDP             |                 |       |     |
| <i>M. musculus</i> SSAO     | (189) | PQASGLLHHCCFYKH--QGQNLVTMTTAPRGLQSGDRATWFGLYYNLSGAGFY     | PHPIGLELLIDH      | KALDP       |                 |                 |       |     |
| <i>B. taurus</i> aorta SSAO | (189) | PQAAGVLHHCCSYKQ--GGGNLVTMTTAPRGLQSGDRATWFGLYYNISGAGYYLHPV | GLELLVDH          | KALDP       |                 |                 |       |     |
| <i>B. taurus</i> lung SSAO  | (188) | PQAAGVLHHCCSYKQ--GGGNLVTMTTAPPGLQSGDRATWFGLYYNISKAGYYLHPV | GLELLVDH          | KALDP       |                 |                 |       |     |
| <i>B. taurus</i> liver BPAO | (188) | PQAAGVLHHCCSYKQ--GGQKLLTMNSAPRGVQSGDRSTWFGIYYNITKGGPYLHPV | GLELLVDH          | KALDP       |                 |                 |       |     |
| Consensus                   | (191) | P A L Y G L M                                             | APRGL SGDR        | TWFGLYYNISG | GYFLHPV         | GLELLVDH        | KALDP |     |



|                                      |       | 261                                   | 270                               | 280 | 290 | 300 | 310    | 320 |
|--------------------------------------|-------|---------------------------------------|-----------------------------------|-----|-----|-----|--------|-----|
| 330                                  |       |                                       |                                   |     |     |     |        |     |
| <i>H. sapiens</i> DAO                | (229) | GHWAVEQVWYNGKFYGSPEELARKYADGEVDV----  | VVLEDPLPGGKGHDSTEEPPLFSSHKPRGDFPS |     |     |     |        |     |
| <i>R. norvegicus</i> DAO             | (229) | QDWRVEQLWYNGKFYNNPEELARKYAVGEVDT----  | VVLEDPLPN-----GTEKPPLFSSYKPRGEFHT |     |     |     |        |     |
| <i>M. musculus</i> DAO               | (229) | QDWRVEQLWYNGKFYNSPEELAQKYAVGEVEAVVLEE | VVLEDPLPG-----ATEQPPLFSSYKPRGEFHT |     |     |     |        |     |
| <i>B. taurus</i> DAO                 | (1)   | -----                                 | -----                             |     |     |     |        |     |
| <i>H. sapiens</i> SSAO               | (257) | ARWTIQKVIFYQGRYYDSLAQLEAQFEAGLVNV---- | VLI PDNGTGG-----SWSLKSP----V      |     |     |     |        |     |
| <i>H. sapiens</i> rSSAO <sub>b</sub> | (251) | AHWTVQQVFYLGHHYADLGQLEREFKSGRLEV----  | VRVPLPPPNG-----ASSLRSR----N       |     |     |     |        |     |
| <i>H. sapiens</i> rSSAO <sub>a</sub> | (251) | AHWTVQQVFYLGHHYADLGQLEREFKSGRLEV----  | VRVPLPPPNG-----ASSLRSR----N       |     |     |     |        |     |
| <i>M. musculus</i> SSAO              | (257) | ALWTIQKVIFYQGRYYESLTQLEDQFEAGLVNV---- | VLVPNNGTGG-----SWSLKSS----V       |     |     |     |        |     |
| <i>B. taurus</i> aorta SSAO          | (257) | AQWTIQKVFFQGRYYESLAQLEEQFEAGRVNV----  | VVIPNNGTGG-----SWSLKSQ----V       |     |     |     |        |     |
| <i>B. taurus</i> lung SSAO           | (256) | AQWTIQKVFFQGRYYESLVQLEEQFEAGRVNV----  | VVIPNNGTGG-----SWSLKSQ----V       |     |     |     |        |     |
| <i>B. taurus</i> liver BPAO          | (256) | ADWTVQKVFFQGRYYENLAQLEEQFEAGQVNV----  | VVIPDDGTGG-----FWSLKSQ----V       |     |     |     |        |     |
| Consensus                            | (261) | A WTVQ VFYQGRYY SL QLE F AG V V       | VVIP GG                           |     |     |     | S SLKS |     |

|                                      |       | 331                                                  | 340                  | 350           | 360        | 370 | 380 | 390 |
|--------------------------------------|-------|------------------------------------------------------|----------------------|---------------|------------|-----|-----|-----|
| 400                                  |       |                                                      |                      |               |            |     |     |     |
| <i>H. sapiens</i> DAO                | (294) | PIHVS GPRLVQPHGPRFRLEGNVLYGGWSFAFRLRSS               | SGLQVLNVHFGGERIAYEVS | SVQEAV        | ALYGGHT    |     |     |     |
| <i>R. norvegicus</i> DAO             | (289) | PVN VAGPHVVQPSGPRYKLEGN TVLYGGWSFSYRLRSS             | SGLQIFNVLFGGERVAYEVS | SVQEAV        | ALYGGHT    |     |     |     |
| <i>M. musculus</i> DAO               | (294) | PVT VAGPHVVQPSGPRYKLEGNV VLYGDWSFSYRLRSS             | SGLQIFNVLFGGERVAYEVS | SVQEAV        | ALYGGHT    |     |     |     |
| <i>B. taurus</i> DAO                 | (1)   | -----                                                | -----                |               |            |     |     |     |
| <i>H. sapiens</i> SSAO               | (307) | PPGPAPPLQFY PQGPRFSVQGS RVASSLWTF SFG LGAFSGPRIFD    | VRFQGERLVYEISLQ      | EALAIYGGNS    |            |     |     |     |
| <i>H. sapiens</i> rSSAO <sub>b</sub> | (301) | SPGPLPPLQFSPQGSQYSVQGNLVSSSLWSFTFGHGVFSGLRIFD        | VRFQGERIAYEVS        | SVQECVSIYGADS |            |     |     |     |
| <i>H. sapiens</i> rSSAO <sub>a</sub> | (301) | SPGPLPPLQFSPQGSQYSVQGNLVSSSLWSFTFGHGVFSGLRIFD        | VRFQGERIAYEVS        | SVQECVSIYGADS |            |     |     |     |
| <i>M. musculus</i> SSAO              | (307) | PPGPAPPLQFHPQGPRFSVQGSQVSSSLWAFSFG LGAFSGPRIFD       | IRFQGERVAYEIS        | SVQEAI        | ALYGGNS    |     |     |     |
| <i>B. taurus</i> aorta SSAO          | (307) | PPGP TPPLQFHPQGT RFSVQGS RV TSSSLWTF SFG LGAFSGPRIFD | IRFQGERL             | AYEISLQ       | EAVAIYGGNT |     |     |     |
| <i>B. taurus</i> lung SSAO           | (306) | PPGP TPPLQFHPQGT RFSVQGS RV TSSSLWTF SFG LGAFSGPRIFD | IRFQGERL             | AYEISLQ       | EAVAIYGGNT |     |     |     |
| <i>B. taurus</i> liver BPAO          | (306) | PPGP TPPLQFHPQGPRFSVQGNRVASSLWTF SFG LGAFSGPRV       | FDVRFQGERL           | AYEISLQ       | EAGAVYGGNT |     |     |     |
| Consensus                            | (331) | PPGP PPLQF PQGPRFSVQGN V SSLWSFSFG LGAFSG RIFD       | VRFQGERL             | AYEISVQ       | EAVAIYGG T |     |     |     |



|                             |       | 401         | 410         | 420          | 430         | 440        | 450        | 460         |
|-----------------------------|-------|-------------|-------------|--------------|-------------|------------|------------|-------------|
| 470                         |       |             |             |              |             |            |            |             |
| <i>H. sapiens</i> DAO       | (364) | PAGMQTKYLDV | GWGLGSVTHE  | LAPGIDCPET   | ATFLDTFHYYD | DADDPVHYPR | ALCLFEMPTG | VPLRRHFNS   |
| <i>R. norvegicus</i> DAO    | (359) | PAGMQTKYIDV | GWGLGSVTHE  | LAPGIDCPET   | ATFLDAFHYYD | SDGPVHYPH  | ALCLFEMPTG | VPLRRHFNS   |
| <i>M. musculus</i> DAO      | (364) | PAGMQTKYIDV | GWGLGSVTHE  | LAPGIDCPET   | ATFLDAFHYYD | SDGPVLYPR  | ALCLFEMPTG | VPLRRHFDS   |
| <i>B. taurus</i> DAO        | (1)   | PAGMQTKYMDV | GWGLGSVTYEL | LAPGIDCPEMAT | FVDALHYYDD  | DDGPVLYPR  | ALCLFEMPTG | VPIRRHFNS   |
| <i>H. sapiens</i> SSAO      | (377) | PAAMTTRYVDG | GFGMGKYTTP  | LTRGVDCPYL   | ATYVDWHFL   | LESQAPKTI  | RDACVFEQN  | QGLPLRRHSD  |
| <i>H. sapiens</i> rSSAOb    | (371) | PKTMLTRYLD  | SSFGLGRNSR  | GLVRGVDCPY   | QATMVDIHIL  | VKGAVQLLP  | GAVCVFEEA  | QGLPLRRHHNY |
| <i>H. sapiens</i> rSSAOa    | (371) | PKTMLTRYLD  | SSFGLGRNSR  | GLVRGVDCPY   | QATMVDIHIL  | VKGAVQLLP  | GAVCVFEEA  | QGLPLRRHHNY |
| <i>M. musculus</i> SSAO     | (377) | PASMSTCYVDG | SFGIGKYSTP  | LIRGVDCPYL   | ATYVDWHFL   | LESQAPKTL  | RDACVFEQN  | QGLPLRRHSD  |
| <i>B. taurus</i> aorta SSAO | (377) | PAAMLTRYMDG | CFGMGKFATP  | LTRGVDCPYL   | ATYVDWHFL   | LESQAPRTL  | HDACVFEQN  | KGLPLRRHSD  |
| <i>B. taurus</i> lung SSAO  | (376) | PAAMLTRYMDA | CFGMGKFATP  | LTRGVDCPYL   | ATYVDWHFL   | LESQAPKTL  | HDACVFEQN  | KGLPLRRHSD  |
| <i>B. taurus</i> liver BPAO | (376) | PAAMLTRYMDS | GFGMGYFATP  | LIRGVDCPYL   | ATYMDWHFV   | VVESQTPKTL | HDACVFEQN  | KGLPLRRHSD  |
| Consensus                   | (401) | PAAM TRYMD  | GFGLGK T    | L RGVDCPYL   | ATYVD H     | LLES AP    | LP ALCVFE  | GLPLRRHH    |

|                             |       | 471         | 480        | 490         | 500            | 510        | 520           | 530              |
|-----------------------------|-------|-------------|------------|-------------|----------------|------------|---------------|------------------|
| 540                         |       |             |            |             |                |            |               |                  |
| <i>H. sapiens</i> DAO       | (434) | NFKGGFNFYA  | GLKGQVLVLR | TTSTVYNYDYI | WDFIFYPNGV     | MEAKMHATGY | VHATFYTP--    | EGLRHGTRL        |
| <i>R. norvegicus</i> DAO    | (429) | NFKGGFNFYA  | GLKGYVLVLR | TTSTVYNYDYI | WDFIFYSNGV     | MEAKMHATGY | VHATFYTP--    | EGLRHGTRL        |
| <i>M. musculus</i> DAO      | (434) | NFKGGFNFYA  | GLKGYVLVLR | TTSTVYNYDYI | WDFIFYPNGV     | METKMHATGY | VHATFYTP--    | EGLRHGTRL        |
| <i>B. taurus</i> DAO        | (71)  | NFNGGFNFYA  | GLKGQALVLR | TTSTVYNYDYI | WDFIFYPNGV     | MEAKMHATGY | IHATFYTP--    | EGLRHGTRL        |
| <i>H. sapiens</i> SSAO      | (447) | LYS---HYFG  | GLAETVLVVR | SMSTLLNYDY  | VWDTVFHPSG     | AEIRFYATGY | ISSAFLFG--    | ATGKYGNQV        |
| <i>H. sapiens</i> rSSAOb    | (441) | LQN---HFYGG | LASSALVVR  | SVSSVGN     | YDYIWDFVLY     | PNGALEGRV  | HATGYINTA     | FLKGGEGLLF       |
| <i>H. sapiens</i> rSSAOa    | (441) | LQN---HFYGG | LASSALVVR  | SVSSVGN     | YDYIWDFVLY     | PNGALEGRV  | HATGYINTA     | FLKGGEGLLF       |
| <i>M. musculus</i> SSAO     | (447) | FYS---HYFG  | GVVGTVLVVR | SVSTLLNYDY  | IWDMVFHPN      | GAIEVKFHAT | GYISSAFFFG--  | AGEKFGNRV        |
| <i>B. taurus</i> aorta SSAO | (447) | FIS---QYFG  | GVVETVLVFR | SVSTLLNYDY  | VWDMVFHPN      | GAIEVKFHAT | GYISSAFFFG--  | TAQKYGNQV        |
| <i>B. taurus</i> lung SSAO  | (446) | FIS---QYFG  | GVVETVLVFR | SVSTLLNYDY  | VWDMVFHPN      | GAIEVKFHV  | TGYISSAFFFG-- | TAQKYGNQV        |
| <i>B. taurus</i> liver BPAO | (446) | FLS---HYFG  | GVAQTVLVFR | SVSTMLNYDY  | VWDMVFYPN      | GAIEVKLHAT | GYISSAFLFG--  | AARRYGNQV        |
| Consensus                   | (471) | F           | NFYGGL     | TVLVLR      | SVSTV NYDYIWDF | VFYPNGAIE  | KMHATGYI      | SAFY G EGLRHGNRV |



|                                      |       | 541                                          | 550                       | 560  | 570  | 580  | 590    | 600              |       |
|--------------------------------------|-------|----------------------------------------------|---------------------------|------|------|------|--------|------------------|-------|
| 610                                  |       |                                              |                           |      |      |      |        |                  |       |
| <i>H. sapiens</i> DAO                | (502) | HTHLIGNIHTHLVHYRVDLDVAGTKNSFQTLQMKLENITNPWS  | PRHRVQPTLEQTQYSWE         | ERQA | AF   | RFKR |        |                  |       |
| <i>R. norvegicus</i> DAO             | (497) | QTHLLGNIHTHLVHYRVDMDVAGTKNSFQTLTMKLENLTNPWS  | SPSHSLVQPTLEQTQYSQ        | EHQA | AF   | RFQG |        |                  |       |
| <i>M. musculus</i> DAO               | (502) | QTHLLGNIHTHLVHYRVDLDVAGTKNSFRTLTQTKLENITNPWS | SPSHSLVQPTLEQTQYSH        | EHQA | AF   | RFQG |        |                  |       |
| <i>B. taurus</i> DAO                 | (139) | HTHLIGNMHTHLVHYRVDLDVAGTKNSFQTLQMKLENITNPWS  | PGHRLVHPALQQMRYSR         | ERQA | AF   | RFQG |        |                  |       |
| <i>H. sapiens</i> SSAO               | (512) | SEHTLGTVHTHSAHFVLDLDVAGLENWVVAEDMVFPMAVPWS   | PEHQLQRLQVTRKLE           | EEQA | AF   | LVGS |        |                  |       |
| <i>H. sapiens</i> rSSAO <sub>b</sub> | (508) | GERVLGTVHTHAFHFKLDDVAGLKNWVVAEDVVFQPVAA      | PWNPEHWLQRPQLTRQVLGK      | EDLT | AF   | SLGS |        |                  |       |
| <i>H. sapiens</i> rSSAO <sub>a</sub> | (508) | GERVLGTVHTHAFHFKLDDVAGLKNWVVAEDVVFQPVAA      | PWNPEHWLQRPQLTRQVLGK      | EDLT | AF   | SLGS |        |                  |       |
| <i>M. musculus</i> SSAO              | (512) | GAHTLGTVHTHSAHFVLDLDVAGLKNWAWAEDMAFVPTIV     | PWQPEYQMQRQLQVTRKLE       | TE   | EEQA | AF   | PLGG   |                  |       |
| <i>B. taurus</i> aorta SSAO          | (512) | RENTLGTVHTHSAHYKVLDLDVGGLKNWVVAEDMAFVPTTV    | PWSPEHQIQRLQVTRKQLE       | TE   | EEQA | AF   | PLGG   |                  |       |
| <i>B. taurus</i> lung SSAO           | (511) | RENTLGTVHTHSAHYKVLDLDVGGLKNWVVAEDMAFVPTTV    | PWSPEHQIQRLQVIRKQLE       | TE   | EEQA | AF   | PLGG   |                  |       |
| <i>B. taurus</i> liver BPAO          | (511) | GEHTLGPVHTHSAHYKVLDLDVGGLKNWVVAEDMAFVPTAI    | PWSPEHQIQRLQVTRKQLE       | TE   | EEQA | AF   | PLGG   |                  |       |
| Consensus                            | (541) | EHL                                          | LGTVHTHSAHYKVLDLDVAGLKNWV | AEDM | F    | PIT  | PWSPEH | LQRPQLTR QL EEQA | AF LG |

|                                      |       | 611 | 620                                                       | 630                  | 640                    | 650      | 660      | 670 |
|--------------------------------------|-------|-----|-----------------------------------------------------------|----------------------|------------------------|----------|----------|-----|
| 680                                  |       |     |                                                           |                      |                        |          |          |     |
| <i>H. sapiens</i> DAO                | (572) | KL  | PKYLLFTSPQENPWGHKRSYRLQIHSMADQVLPPGWQEE                   | QA                   | ITWARYPLAVTKYRESE      | LC       | SSSIYHQN |     |
| <i>R. norvegicus</i> DAO             | (567) | TL  | PKYLLFSSPQKNCWGHRRSYRLQIHSMADQVLPPGWQEE                   | RA                   | VTWARYPLAVTKYRESE      | RY       | SSSLYNQN |     |
| <i>M. musculus</i> DAO               | (572) | TL  | PKYLLFSSPQKNRWGHRRSYRLQIHSMADQVLPPGWQEE                   | RA                   | VTWARYPLAVTKYRESE      | RY       | SSSLYNQN |     |
| <i>B. taurus</i> DAO                 | (209) | TL  | PKYLLFTSPEQNPWGHQRSYRVQIHSMADQVLPPGWQEE                   | RA                   | VTWARYPLAVTKYRESE      | RY       | SSSIYNQN |     |
| <i>H. sapiens</i> SSAO               | (582) | AT  | PRYLYLASNHSNKWGHPRGYRIQMLSFAGEPLPQNSSMARGFSWERYQLAVTQRKEE | EP                   | SSSVFNQN               |          |          |     |
| <i>H. sapiens</i> rSSAO <sub>b</sub> | (578) | PL  | PRYLYLASNQTNAWGHQRGYRIQIHSPGLIQIPLES                      | DMERALS              | WGRYQLVVTQRKEE         | ESQ      | SSSIYHQN |     |
| <i>H. sapiens</i> rSSAO <sub>a</sub> | (578) | PL  | PRYLYLASNQTNAWGHQ                                         | RG                   | -----YQLVVTQRKEE       | ESQ      | SSSIYHQN |     |
| <i>M. musculus</i> SSAO              | (582) | AT  | PRYLYLASNHSNKWGHRRGYRIQILSFAGKPLPQESPIE                   | KAFTWGRYHLAVTQRKEE   | EP                     | SSSIFNQN |          |     |
| <i>B. taurus</i> aorta SSAO          | (582) | AS  | PRYLYLASKQSNKWGHPRGYRIQTVSFAGRPLPQNSS                     | TERAISWGRYQLAVTQRKET | EP                     | SSSVFNQN |          |     |
| <i>B. taurus</i> lung SSAO           | (581) | GS  | PRYLYLASKQSNKWGHPRGYRIQTVSFAGRPLPQNSS                     | TERAISWGRYQLAVTQRKET | EP                     | SSSVFNQN |          |     |
| <i>B. taurus</i> liver BPAO          | (581) | AS  | PRYLYLASKQSNKWGHPRGYRIQTVSFAGGPM                          | PQN                  | SPMERAFSWGRYQLAITQRKET | EP       | SSSVFNQN |     |
| Consensus                            | (611) | L   | PRYLYLAS QSNKWGH RGYRIQI S AG LP S                        | ERAISWGRYQLAVTQRKESE | SSSIYNQN               |          |          |     |



|                      |            |       | 681       | 690         | 700        | 710        | 720          | 730         | 740                    |
|----------------------|------------|-------|-----------|-------------|------------|------------|--------------|-------------|------------------------|
| 750                  |            |       |           |             |            |            |              |             |                        |
| <i>H. sapiens</i>    | DAO        | (642) | DPWHPPVVF | EQFLHNNENI  | ENEDLVAWVT | VGFLHIPHSE | DI PNTATPGN  | SVGFLLRPF   | NFFPEDPSLASR           |
| <i>R. norvegicus</i> | DAO        | (637) | DPWDPPVVF | EFLRNNENI   | EDLVAWVT   | VGFLHIPHSE | DVPNTATPGN   | SVGFLLRPF   | NFFPEDPSLASR           |
| <i>M. musculus</i>   | DAO        | (642) | DPWDPPVVF | EFLRNNENI   | ENEDLVAWVT | VGFLHIPHSE | DVPNTATPGN   | CVGFLLRPF   | NFFFEEDPSLASR          |
| <i>B. taurus</i>     | DAO        | (279) | DPWDPPVVF | EFLRNNENI   | EDLVAWVT   | VGFLHIPHA  | EDI PNTTTPGN | SVGFLLRPF   | NFFPEDPSLA--           |
| <i>H. sapiens</i>    | SSAO       | (652) | DPWAPTVD  | FSDFINN-ETI | AGKDLVAWVT | AGFLHIPHA  | EDI PNTVTVG  | NGVGFFLRP   | YNFFDEDPSFYSA          |
| <i>H. sapiens</i>    | rSSAOb     | (648) | DIWTPTVT  | FADFINN-ETL | LGEDLVAWVT | ASFLHIPHA  | EDI PNTVTLGN | RVGFLLRP    | YNFFDEDPSIFSP          |
| <i>H. sapiens</i>    | rSSAOa     | (621) | DIWTPTVT  | FADFINN-ETL | LGEDLVAWVT | ASFLHIPHA  | EDI PNTVTLGN | RVGFLLRP    | YNFFDEDPSIFSP          |
| <i>M. musculus</i>   | SSAO       | (652) | DPWTPTVD  | FTDFISN-ETI | AGEDLVAWVT | AGFLHIPHA  | EDI PNTVTAGN | SVGFLLRP    | YNFFDEDPSFHSA          |
| <i>B. taurus</i>     | aorta SSAO | (652) | DPWTPTVD  | FADFINN-ETI | AGKDLVAWVT | AGFLHIPHA  | EDI PNTVTVG  | NGVGFFLRP   | YNFFDEDPSINSA          |
| <i>B. taurus</i>     | lung SSAO  | (651) | DPWTPTVD  | FADFINN-ETI | AGKDLVAWVT | AGFLHIPHA  | EDI PNTVTVG  | NGVGFFLRP   | YNFFDQEPSMDSA          |
| <i>B. taurus</i>     | liver BPAO | (651) | DPWTPTVD  | FSDFINN-ETI | AGKDLVAWVT | AGFLHIPHA  | EDI PNTVTVG  | NGVGFFLRP   | YNFFDQEPSMDSA          |
| Consensus            |            | (681) | DPWTPTV   | FADFINN     | ETI        | GEDLVAWVT  | AGFLHIPHA    | EDI PNTVTVG | NGVGFFLLRPYNFFDEDPSL S |

|                      |            |       | 751       | 760          | 770       | 780      | 790           |
|----------------------|------------|-------|-----------|--------------|-----------|----------|---------------|
| <i>H. sapiens</i>    | DAO        | (712) | DTVIVWPRD | NG---PNYVQRW | IPEDRDC   | SMPPPF   | SYNGTYRPV--   |
| <i>R. norvegicus</i> | DAO        | (707) | DTVIVWPQD | KG---LNRVQRW | IPEDRRCL  | VSPPF    | SYNGTYKPV--   |
| <i>M. musculus</i>   | DAO        | (712) | DTVIVWPQD | NG---LNHVQRW | IPENRDCL  | VSPPF    | SYNGTYKPV--   |
| <i>B. taurus</i>     | DAO        | (347) | -----     | -----        | -----     | -----    | -----         |
| <i>H. sapiens</i>    | SSAO       | (721) | DSIYFRGD  | QDAGACEVN    | NPLACLPQA | AACAPDL  | PAFSHGGS      |
| <i>H. sapiens</i>    | rSSAOb     | (717) | GSVYFEKG  | QDAGLCSIN    | PVACLPDLA | ACVPDL   | PPFSYHGF----  |
| <i>H. sapiens</i>    | rSSAOa     | (690) | GSVYFEKG  | QDAGLCSIN    | PVACLPDLA | ACVPDL   | PPFSYHGF----  |
| <i>M. musculus</i>   | SSAO       | (721) | DSIYFREG  | QDATAACEVN   | NPLACLSQT | ATCAPEI  | PAFSHGGS      |
| <i>B. taurus</i>     | aorta SSAO | (721) | DSIYFQKH  | QDAGSCEVN    | SLACLPKDP | ACAPDL   | PAFSHGGS      |
| <i>B. taurus</i>     | lung SSAO  | (720) | DSIYFREG  | QDAGSCEIN    | PVACLPQA  | AATCAPDL | PVFSHGGY      |
| <i>B. taurus</i>     | liver BPAO | (720) | DSIYFREG  | QDAGSCEIN    | PVACLPQA  | AATCAPDL | PVFSHGGY      |
| Consensus            |            | (751) | DSIYF     | QDAG C       | INPLACLP  | A        | CAPDLP FSHGGF |

APPENDIX B  
GENERATION OF PHYLOGENETIC TREES

Phylogenetic trees, commonly called evolutionary trees, were calculated in order to generate a reasonable model of the evolutionary relationships among the copper amine oxidases. These trees graphically show the range of amine oxidase distribution in taxonomical groups, the relative difference in sequences in various taxa, and may be used to infer physiochemical properties of some proteins (i.e. two protein sequences that are quite similar and therefore show little distance on the phylogenetic tree may be predicted to share certain physical and chemical characteristics). Nei and Kumar have an excellent and recent introduction to phylogenetics [247].

PHYLP v3.6a (Phylogeny Inference Package), the software package used in this work, is one of the most used phylogenetic software packages, and is available free of charge on the Internet[248]. This package includes various statistic methods, including parsimony, distance matrix, and likelihood methods, and accepts various data sets for input, such as nucleic acid sequences, amino acid sequences, gene frequencies, distance matrices, and 0/1 characters.

It is unreasonable to use the complete amino acid sequences from the lineup in Appendix A for data input for the following reasons: 1) the sequences are of widely varying lengths and are often incomplete, 2) the use of very long input sequences would greatly increase the required computation time, and 3) the phylogenetic software does not handle insertions or deletions very well and treats characters that are not recognized as representing any amino acid. Therefore, an input file has been created from a subset of the complete amino acid sequences, which is assumed to reasonably represent the complete sequences and allow for the inference of apparent phylogenetic relationships.



The input file was constructed using the following criteria: 1) the residues are all from the catalytic domain, Domain IV, 2) the input file contains the TPQ consensus sequence, the three histidine residues that coordinate copper, and the residues that correspond to the second metal binding site ligands, and 3) empty positions, either from insertions or deletions, are not included. Thus the number of sequences suitable for inclusion is 45 out of the 60 available sequences. Using the numbering scheme in the amino acid sequence lineup shown in Appendix A, the following sets of residues were selected for the input file: residues 609-642, 658-685, 775-801, 807-818, 847-871, and 875-891. The length of the input file is 138 amino acids, of which 62 % are in the structurally conserved regions identified by Kumar et al [12]. The first line in the input file gives the total number of sequences and the length of each sequence.

Distance matrix and maximum likelihood methods were chosen. Parsimony programs were not used, although these statistical algorithms are the simplest and require the least computational time, because the assumptions of parsimony programs were deemed to be overly simplistic for this data set. Parameters for the PHYLIP programs used are the default settings except that input sequences were randomized and global rearrangements were allowed. Randomization and global rearrangement greatly increase the computational time but result in more refined trees. No assumption of a molecular clock was used.

The PHYLIP program PROTDIST was used to construct a distance matrix from the input sequences. The distance value for each pair of sequences provides an approximation of the total branch length between the two species. To construct the

distance matrix, I chose to use the Jones-Taylor-Thornton model of amino acid substitution, a matrix based upon the empirical probability of a substitution of a specific amino acid to any other amino acid [249]. The Jones-Taylor-Thornton matrix of probability is quite similar to the earlier Dayhoff PAM matrix [250] but is an updated matrix consisting of a much greater sample size of protein sequences. The output of PROTDIST, a distance matrix, was then used as input into the program FITCH, which uses the least-squares method of Fitch and Margoliash for fitting trees to distance matrices and give a possible phylogenetic tree as the output [251].

ProML, the Protein Maximum Likelihood program, also utilized the Jones-Taylor-Thornton probability matrix. However, in maximum likelihood methods the probability of a given set of sequence for a specific substitution model (Jones-Taylor-Thornton) is maximized for each tree topology, that is, for each tree branching pattern. The topology that gives the maximum probability is then chosen as the final tree.

The output phylogenetic trees from PHYLIP programs were then imported into TREEVIEW software [252], which increases the graphic quality and simplifies the manipulation and editing of the trees, although no editing was used for the trees shown herein. The trees were then saved as enhanced metafiles (\*.emf) and imported into Microsoft PowerPoint, and then the ten letter headers for each row of sequences were replaced with the correct binomial nomenclature and, if required, the amine oxidase gene number used in Appendix A.

Phylogenetic trees shown here are unrooted trees, and the number,  $t$ , of possible branching patterns (topologies) for an unrooted tree of  $m$  taxa (in this case the taxa are amino acid sequences) are given by the equation:

$$t = [(2m - 4)!] / [2^{m-3}(m - 3)!]$$

Thus, the number of possible topologies for the 45 sequences used in this input file is approximately  $4.56 \times 10^{64}$ . Thus the computation time required to derive the optimal tree can be considerable.

The generated evolutionary trees are statistical models limited by the input data set and the necessary assumptions used to derive the models, and these models are only approximations of the true and unknown evolutionary relationships. It is important to note that the trees shown herein are not species tree (i.e. do not reflect the evolutionary relationships or "family tree" among species) but are only "gene trees" that indicate the relationships within the family of copper amine oxidase genes. Remember that these trees are were constructed from a subset of the primary sequences from the sequenced copper amine oxidase gene products.

Amino Acid Sequence Input File for PHYLIP Programs

45 138

Homo KDAO

TVYNYDYIWDFIFYPNGVMEAKMHATGYVHATFHGTRLHTHLIGNIHThLVHYRVDLDVAGVLP PGWQEEQAITWARYPLAVTKYELCSSSIYHQND  
LVAVVTVGFLHIPHSEDIPNTATPVGFLLRPFNFFPEDPSL

Homo SSAO

TLLNYDYVWDTVFHPSGAIEIRFYATGYISSAFYGNQVSEHTLGTVHTSAHFVKVDLDVAGPLPQNSSMARGFSWERYQLAVTQREPSSSSSVFNQND  
LVAVVTAGFLHIPHAEDIPNTVTVGFFLRPYNFFDEDPSF

Homo SSAOb

SVGNyDYIWDFVLYPNGALEGRVHATGYINTAFFGNRVGERVLGTVHTAHFHKLDLDVAGQIPLESdMERALSWGRYQLVVTQRESQSSSIYHQND  
LVAVVTASFLHIPHAEDIPNTVTLVGFLLRPYNFFDEDPSI

Mus DAO

TVYNYDYIWDFIFYPNGVMETKMhATGYVHATFHGTRLQTHLLGNIHThLVHYRVDLDVAGVLP PGWQEERAVTWARYPLAVTKYERYSSSLYNQND  
LVAVVTVGFLHIPHSEDVPNTATPVGFLIRPFNFFEDPSL

Mus SSAO

TLLNYDYIWDmVfHPNGAIEVKFhATGYISSAFFGNRVGAHTLGTVHTSAHFVKVDLDVAGPLPQESPIEKaFTWGRYHLAVTQREPSSSSIFNQND  
LVAVVTAGFLHIPHAEDIPNTVTAVGFFLRPYNFFDEDPSF

Rattus DAO

TVYNYDYIWDFIFYSNGVMEAKMHATGYVHATFHGTRLQTHLLGNIHThLVHYRVDMDVAGVLP PGWQEERAVTWARYPLAVTKYERYSSSLYNQND  
LVAVVTVGFLHIPHSEDVPNTATPVGFLLRPFNFFPEDPSL

Bos DAO

TVYNYDYIWDFIFYPNGVMEAKMHATGYIHATFHGTRLHThLIGNMHThLVHYRVDLDVAGVLP PGWQEERAVTWARYPLAVTKYERYSSSIYNQND  
LVAVVTVGFLHIPHAEDIPNTTTPVGFLLRPFNFFPEDPSL

Bos aorta

TLLNYDYVWDMVfHPNGAIEVKFhATGYISSAFYGNQVRENTLGTVHTSAHYKVDLDVGGPLPQNSSTERaISWGRYQLAVTQREPSSSSSVFNQND  
LVAVVTAGFLHIPHAEDIPNTVTVGFFLRPYNFFDEDPSI

Bos BPAO

TMLNYDYVWDMVfYPNGAIEVKLhATGYISSAFYGNQVGEHTLGPVHTSAHYKVDLDVGGPMPQNSPMERaFSWGRYQLAITQREPSSSSSVFNQND  
LVAVVTAGFLHIPHAEDIPNTVTVGFFLRPYNFFDQEPSM

Bos lung

TLLNYDYVWDMVfHPNGAIEVKFhVTGYISSAFYGNQVRENTLGTVHTSAHYKVDLDVGGPLPQNSSTERaISWGRYQLAVTQREPSSSSSVFNQND  
LVAVVTAGFLHIPHAEDIPNTVTVGFFLRPYNFFDQEPSM



tomato

SVANYDYIVDWEFQNDGLIRPKVGLSGILMVKGYGTTLLSENIVGVIHDHYVTFHLDMDIDGILLRKELLLLITKFGSHLIMNLSNKVKVMILLFGL  
IVLWYTLGFHHIPQCQEDFPIMPTVSSFEIKPVNFFESNPIL

Solanum

TVANYEYGFYWHFYQDGKIEAXGQADWNSQLRCYGTTIAPGLYAPVHQHFFVARMDMAVDCLAGSEAKFLRRAAF LKHN LWVTPYEIFPGGEFPNQ  
IVLWYIFGLIHVPRLEDWPVMPVEIGFMLQPHGFFNCSPAV

Athaliana1

TVGNYDYIVDYEFKPSGSIKIGVGLTGVLVLEVKPHGTIVADNTIGVNHDHFVTYRLDLDIDGLLAQDDYPQIRAAFTNYNVWITPYEVWASGLYADRS  
IVMWYTVGFHHVPCQEDFPTMPTLGGFELRPTNFFEQNPDL

Athaliana2

TLGNYDYIVDWEFKKSGAIRVGVDLTGVLEVKAYGTQVAKNTIAVNHDHYLTYYLDLDVDGLLTDDDYPEIRAGYTKYPVWVTAYERWAGGFYSDRS  
IVMWYNVGFHHIPYQEDFPVMP TLGGFTLRPSNFFDNDPLI

Athaliana3

SVGNYDYIFDWEFQTDGLIRVTVAASGMLMVKGAGPLISENVIGVVHDHFI TFHLDMDIDGLLDHDDPPQIRGAFTNNQIWVTPYEQYAGGVLIYQS  
IVLWYTLGFHHVPCQEDYPVMP TVASFELKPANFFESNPIL

Athaliana4

TVANYEYGFYWHFYQDGKIEAEVKLTGILSLGAYGTTIAPGLYAPVHQHFFIARMDMSVDCLARPEAKFLRRAAF LKHN LWVTRYEKFPGGEFPNQ  
VVLWYVFGITHVPRLEDWPVMPVEIGFTLMPHGFFNCSPAV

Athaliana5

SVGNYDYIFDWEFQMDGVIRVTVAASGMLMVKGSGPLISENVIGVVHDHFI SFHLDMDIDGLLDHDDPPQMRGAFTNNQIWVTRYEQWAGGLLMYQS  
IVLWYTLGFHHVPCQEDFPVMP TISSFELKPVNFFESNPVL

Athaliana6

SVGNYDYIFDWEFQTDGLIRVTVAASGMLMVKGAGPLISENVIGVVHDHFI TFHLDMDIDGLLDHDDPPQIRGAFTNNQIWVTPYEQYAGGVLIYQS  
IVLWYTLGFHHVPCQEDYPVMP TVASFELKPANFFESNPIL

Chickpea

TVGNYDNVLDWEFKTSGSIKPAIALSGILEIKGYGTLVSANSIGIYHDHFYMYLDFDVGLLTEDDYPQIRGAFTNYNVWVTPYEKWAGGLYVDHS  
IVLWHVVGIIHVP AQEDFPIMPLLT SFELRPTNFFERNPVL

C. lineata

AVANYDYIVDWEFQTDGLIRSKVGLSGILMVKGYGTTLLSENSIGVIHDHFLTYLDIDIDGLLDAEDPPQKRAAFTNNQIWVTPYEQWAGGLFVYQS  
IVLWYTLGFHHIPQCQEDYPIMPTVSSFDLKPVNFFERNPIL

G. max

TVANYEYGFYWHFYQDGKIEAEVKLTGILSLGAYGTTIAPGLYAPVHQHFFVARMDMAVDCLAGSEAKFLRRAAF LKHN LWVTPYEMHPGGEFPNQ  
IVLWYVFGVTHI PRLEDWPVMPVEIGFMLMPHGFFNCSPAV

Medicago 1

TVANYEYAFFWHFYQDGKIEAEVKLTGILSLGAYGTMIAPGLYAPVHQHFFVARMDMSVDSL AGSEAKFLRRAAF LKHN VWVTAYEMFPGGEFPNQ  
IVLWYVFGITHFI IYSVAYVDGSRRRPVAHRLSFVEMVVPY

*P. sativum*1

TVGNYDNVIDWEFKASGSIKPSIALSGILEIKGHGKLVANSIGIYHDHFIYYLDFDIDGLLTEDDYPQIRGAFTNYNVWVTAYEKWAGGLYVDHS  
IVMWHVVGIHHVPAQEDFPIMPLLTSELRPTNFFERNPVL

*P. sativum*2

TVGNYDNVIDWEFKASGSIKPAIALSGILEIKGHGKLVANSIGIYHDHFIYYLDFDIDGLLTEDDYPQIRGAFTNYNVWVTAYEKWAGGLYVDHS  
IVMWHVVGIHHVPAQEDFPIMPLLTSELRPTNFFERNPVL

*Lens* 1

TVGNYDNVLDWEFKTSGWMKPSIALSGILEIKGHGKLVANSIGIYHDHFIYYLDFDIDGLLTEDDYPQIRGAFTNYNVWVTXNEKWAGGLYVDXS  
IVMWHVVGIHHVPAQEDFPIMPLLTSELRPTNFFERNPAL

*Lens* 2

TVGNYDNVLDWEFKTSGWMKPSIALSGILEIKGHGKLVANSIGIYHDHFIYYLDFDIDGLLTEDDYPQIRGAFTNYNVWVTPYEKWAGGLYVDHS  
IVMWHVVGIHHVPAQEDFPIMPLLTSELRPTNFFERNPVL

*T. aestivum*

TVANYEYGFYWHFYQDGKIEAEVKLTGILSLGAYGTTIAPSLYAPVHQHFFVARMDMAVDCFALPEAKFLRRAGFLKHNLWVTPYEKFPGGGEFPNQ  
IVLWYVFGLTTHIPRLDWPVMPVEIGFMLMPHGFFNCSPAV

*H. vulgare*2

TVANYEYGFYWHFYQDGKIEAEVKLTGILSLGAYGTTIAPSLYAPVHQHFFVARMDMAVDCFALPEAKFLRRAGFLKHNLWVTPYEKFPGGGEFPNQ  
IVLWYVFGLTTHIPRLDWPVMPVEIGFMLMPHGFFNCSPAV

*P. angusta*

TAANYEYCLYWVFMQDGAIRLDIRLTGILNTYIWGTRVYPNVNAHNHQHLFSLRIDPRIDGLAKEGSLVAKRAPWASHSVNVVFPYRLYPSGDHVPQW  
ILFFHTFGITHFPAPEDFPLMPAEITLMLRPRHFFTENPGL

*P. pastoris*

TIGNYDYNFLYKFFLDGTLEVSVRAAGYIQAGYYGLKIHDLVLSGSFHDHVLNYKVDLDVGGIVKNSRSGPETQNWARSNLFLTKHELRSSTALNTNA  
IVAWVNLGLHLPNSNDLPNTIFSASFMLTPFNYFDSNSR

*Candida* 1

TAANYEYCVYWILRQDGTIKLEIRLTGILNTYIWGTVVYPNVNAHNHQHLFSLRIHPRIDGLAQEGSLVKKRAPWAASHTQVVPYRLYPSGDHVPQW  
ILLFHTFGITHFPAPEDFPVMPTEFDLQLRPRNIHTENPVL

*Candida* 2

TVANYEYIINLKFTDGSIDIEVRATGILSTMPWGTIVGPNVMAAYHQHILGFRIDPAVDGLADPDSFNAKRAKFATQQVWVTQYQLFAAGEFTNQS  
IVVWATMGFTHIPRVEDFPVMPVEHNIQLAPANFFDRNPAL

*S. pombe* 1

TAANYEYMVYWIFHMDGVIECELKLTGILNTYAWGTQVYPQVNAHNHQHLFCLRLNPMLDGMARPGSLVSNRAGFARHHIHVTPYQIYPAGDYVPQT  
IVVWHTFGITHFPAPEDFPLMPAEIRLLLRPRNFFLRNPAL

*S. pombe* 2

TAANYEYLVYWIFRMDGVIECEIRLTGILNTNAHGTQVYPKISAENHEHLFCLRLNPMLDGLSQPGTTNSDCSGFAENNIYVTPYQIFPTGDYAPQA  
IVVWHTFGMIHFPAPEDFPIMP AEIHLFLQPRNFFKHNPAL

*A. niger* 1

TLANYEYIFAYKFDQSGGITGRVACHGYLERGQVRQRRQWRVVGPEPPAHFLCAHRPGYYGLADPNSIQARRAQFAQHVVWVTKYELYAGGRYTLQS  
VVVWSTFGITHNPRVEDWPVMPVEFQLMIRPADFFTANPSL

*A. niger* 2

TLANYEYIFAYKFDQSGGITVESRATGILNVVNYGNVSSGGVLAQNHQHIFCVRIDPAIDGLADPNSIQARRAQFAQHVVWVTKYELYAGGRYTLQS  
VVVWSTFGITHNPRVEDWPVMPVEFQLMIRPADFFTANPSL

*Afumigatus*

TAANYEYAFYHTFTLDGTYKLEVKLTGMLNTYCFGTEIARGLDAQNHQHIFSLRVDPEIDGLAKPGSTVYKRAGFARHALWVLPYEIFPAGQYVCQS  
IVCYIQFGLTHFPRTEDFPIMPAEVSVMRLASNFFQKNPAL

*fumigatus*2

TVGNYEYIFAFQFTQDASVTYEV RATGILSTAPYGTIVAPGVMAPYHQHLFSLRIDPAIDGLAHPSSYHFKRSEFAEHAVWVTRYERY SAGEHTMQS  
IVIWHTFGTTHNPRVEDWPVMPAEMSVTLKPVNFFWRNPAL

*E. coli*

TVGNYDYIFDWIFHENG TIGIDAGATGIEAVKGYGT LIDHNIVGTTHQHIYNFRLLDLDVDGQFAPDEWIYHRLSFMDKQLWVTRYERFPEGKYPNRS  
AVVWMTTGTHVARAEW PIMPTEVHTLLKPNWFFDETPTL

*Kaerogenes*

TVGNYDYIFDWVFDNGTIGIDAGATGIEAVKGYGT LIDHNIVGTTHQHIYNFRLLDLDVDGKFAPDEWIYHRLSFMDKQLWVTRYERYPEGKYPNRS  
DVVWITTGTHVARAEW PIMPTEALALLKPNWFFDETPTL

*M. loti*

TVANYEYGIFWYLHTDGAIEFELKATGIINTTAYGKEVSPGVLGHIHQHIFCARLDMSVDGYVAAHSPSGRRSTFTSNHLWVTA FERYPAGEYMNHS  
LVLWHTFGVHHQVRPEDFPVQPCITGFKLMPSGFFDQNP GI

*AGAO* 1

TVGNYDYGFYWYLYLDGTIEFEAKATGIVFTAAYASEIAPGLGAPYHQHLFSARLDMMIDGAMADDSSIASRAAFARHHLWVTRHEL YAAGDFVNQH  
LVVWHSFGLTHFPRPEDWPIMPVDTGFTLKP HGFFDENPTL

*AGAO* 2

TIGNYDYGFYWYLYLDGTIEFEAKATGVVFTSANISQLAPGLGAPFHQHIF SARLDMAIDGLADPGSSIARRAAFATKDLWVTRYERYPTGDFVNQH  
IVVWHTFGLTHFPRVEDWPIMPVDVGFKLRPEGFFDRSPVL

*A. strainP1*

TVANYEYAFYWHLFLDGSIEFLVKATGILSTAGGQSLNNDGLYAPIHQHMFNVRMDFELDGAARDDAYVSKRAQFARNNLWVTAYERFAAGEYPNQA  
LVVWYTFGMHHVVRLEDWPVMPRQIGFMLEPHGFFNQNP TL

*Solfolobus*

TLANYDYGFFWYFYQDGSIEFLVKLTGIINDDSYGTRVTPEVYAPIHIHFFNIRLNINVDGPLPDDSYVRRRGAYINYHLWVTPYERYASGDYPYLR  
LVIWYTLGVEHVVRIEDWPVMPVEAGFRLIPDGFFDKNPTI

Parameters Used for the Program PROTDIST

Input data is the amino acid sequence file given above.

Protein distance algorithm, version 3.6a3

Settings for this run:

P Use JTT, PAM, Kimura or categories model? Jones-Taylor-Thornton

matrix

G Gamma distribution of rates among positions? No

C One category of substitution rates? Yes

W Use weights for positions? No

M Analyze multiple data sets? No

I Input sequences interleaved? Yes

0 Terminal type (IBM PC, ANSI)? (none)

1 Print out the data at start of run No

2 Print indications of progress of run Yes

Are these settings correct? (type Y or the letter for one to change)



Output from PROTDIST; A Distance Matrix with Zero Elements Along the Diagonal

|            |        |        |        |        |        |        |        |        |        |        |        |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Homo KDAO  | 0.0000 | 0.6377 | 0.5565 | 0.0953 | 0.5032 | 0.0782 | 0.0698 | 0.5498 | 0.5963 | 0.5833 |        |
| 1.8683     | 1.8013 | 1.3573 | 1.5054 | 1.7138 | 1.8546 | 1.6621 | 1.7138 | 1.8179 | 1.5843 | 1.8367 | 2.5329 |
| 1.8440     | 1.8400 | 1.8043 | 1.8051 | 1.8391 | 1.8391 | 1.6637 | 1.1721 | 1.8603 | 1.6074 | 1.5048 | 1.6024 |
| 2.1282     | 1.7258 | 1.7653 | 1.7848 | 1.4186 | 1.4593 | 1.7370 | 1.6015 | 1.7604 | 1.7763 | 1.7539 |        |
| Homo SSAO  | 0.6377 | 0.0000 | 0.4061 | 0.6110 | 0.1698 | 0.6265 | 0.5936 | 0.1236 | 0.1689 | 0.1499 |        |
| 2.0293     | 2.0543 | 1.4700 | 1.5318 | 1.6986 | 2.0321 | 1.7363 | 1.6986 | 1.9612 | 1.7742 | 2.0147 | 2.8450 |
| 2.0129     | 2.0160 | 2.0223 | 2.0474 | 2.0049 | 2.0049 | 1.9827 | 1.3892 | 2.2346 | 1.6743 | 1.9233 | 2.0288 |
| 2.2163     | 1.6836 | 2.1040 | 2.0298 | 1.4611 | 1.4956 | 1.7037 | 1.6332 | 1.8982 | 1.9573 | 1.6978 |        |
| Homo SSAOb | 0.5565 | 0.4061 | 0.0000 | 0.5770 | 0.3324 | 0.5799 | 0.5362 | 0.3654 | 0.3828 | 0.4077 |        |
| 2.0371     | 1.9513 | 1.5619 | 1.5823 | 1.7640 | 1.9740 | 1.7655 | 1.7640 | 2.0383 | 1.8815 | 1.9173 | 2.8067 |
| 2.0888     | 2.0919 | 2.0680 | 2.0930 | 1.9592 | 1.9592 | 1.9465 | 1.3472 | 2.1180 | 1.7141 | 1.8441 | 2.0242 |
| 2.2317     | 1.8151 | 1.9378 | 1.8591 | 1.3760 | 1.4093 | 1.6001 | 1.6238 | 1.8314 | 1.7909 | 1.5858 |        |
| Mus DAO    | 0.0953 | 0.6110 | 0.5770 | 0.0000 | 0.5107 | 0.0310 | 0.0867 | 0.5468 | 0.5818 | 0.5801 |        |
| 1.8255     | 1.8072 | 1.3221 | 1.4789 | 1.6840 | 1.8085 | 1.6323 | 1.6840 | 1.7497 | 1.5443 | 1.8363 | 2.4173 |
| 1.7756     | 1.7717 | 1.7365 | 1.7384 | 1.7932 | 1.7932 | 1.7194 | 1.2077 | 1.9286 | 1.5834 | 1.5541 | 1.6246 |
| 2.0524     | 1.6310 | 1.7490 | 1.6970 | 1.3895 | 1.4153 | 1.5972 | 1.5898 | 1.6283 | 1.7402 | 1.6457 |        |
| Mus SSAO   | 0.5032 | 0.1698 | 0.3324 | 0.5107 | 0.0000 | 0.5145 | 0.4729 | 0.1377 | 0.1818 | 0.1639 |        |
| 2.0515     | 1.9499 | 1.5254 | 1.6005 | 1.7525 | 1.9279 | 1.7700 | 1.7525 | 2.0322 | 1.7732 | 1.9109 | 2.6863 |
| 2.0913     | 2.0869 | 2.1010 | 2.1265 | 1.9065 | 1.9065 | 1.9171 | 1.3323 | 2.0825 | 1.5636 | 1.8133 | 1.9499 |
| 2.3546     | 1.8348 | 2.0095 | 1.9414 | 1.4547 | 1.4890 | 1.7438 | 1.5939 | 1.8904 | 1.9231 | 1.6336 |        |
| Rattus DAO | 0.0782 | 0.6265 | 0.5799 | 0.0310 | 0.5145 | 0.0000 | 0.0699 | 0.5508 | 0.5861 | 0.5844 |        |
| 1.8644     | 1.7330 | 1.3665 | 1.4963 | 1.6629 | 1.7354 | 1.6116 | 1.6629 | 1.7833 | 1.5545 | 1.7611 | 2.4269 |
| 1.8093     | 1.8053 | 1.7700 | 1.7713 | 1.7197 | 1.7197 | 1.6997 | 1.2194 | 1.9048 | 1.6168 | 1.5276 | 1.6294 |
| 2.0753     | 1.6571 | 1.7459 | 1.6935 | 1.4235 | 1.4497 | 1.5712 | 1.5643 | 1.6019 | 1.7380 | 1.6484 |        |
| Bos DAO    | 0.0698 | 0.5936 | 0.5362 | 0.0867 | 0.4729 | 0.0699 | 0.0000 | 0.5181 | 0.5536 | 0.5514 |        |
| 1.9549     | 1.8090 | 1.3599 | 1.4754 | 1.6765 | 1.8040 | 1.6666 | 1.6765 | 1.7831 | 1.5885 | 1.8320 | 2.5326 |
| 1.8092     | 1.8052 | 1.7697 | 1.7710 | 1.7885 | 1.7885 | 1.6802 | 1.1922 | 1.8811 | 1.5817 | 1.5099 | 1.6349 |
| 2.0480     | 1.6601 | 1.7349 | 1.6677 | 1.3757 | 1.4015 | 1.6701 | 1.6491 | 1.6391 | 1.7428 | 1.6506 |        |
| Bos aorta  | 0.5498 | 0.1236 | 0.3654 | 0.5468 | 0.1377 | 0.5508 | 0.5181 | 0.0000 | 0.1025 | 0.0304 |        |
| 1.9361     | 1.9455 | 1.4619 | 1.4364 | 1.6388 | 1.9235 | 1.6764 | 1.6388 | 1.9439 | 1.6485 | 1.9065 | 2.7833 |
| 2.0025     | 1.9983 | 2.0118 | 2.0369 | 1.9208 | 1.9208 | 1.9166 | 1.2780 | 2.0903 | 1.5941 | 1.8875 | 1.9269 |
| 2.3220     | 1.7490 | 2.0542 | 1.9780 | 1.4184 | 1.4512 | 1.6229 | 1.6024 | 1.8571 | 1.9090 | 1.6328 |        |

|            |        |        |        |        |        |        |        |        |        |        |        |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Bos BPAO   | 0.5963 | 0.1689 | 0.3828 | 0.5818 | 0.1818 | 0.5861 | 0.5536 | 0.1025 | 0.0000 | 0.0863 |        |
| 2.0201     | 1.8989 | 1.5034 | 1.5888 | 1.7493 | 1.8694 | 1.7862 | 1.7493 | 2.0713 | 1.7672 | 1.8529 | 2.6578 |
| 2.1312     | 2.1269 | 2.1407 | 2.1659 | 1.8437 | 1.8437 | 1.9986 | 1.2257 | 2.2004 | 1.6232 | 1.8856 | 1.9986 |
| 2.3551     | 1.8102 | 2.0537 | 1.9329 | 1.5250 | 1.5605 | 1.6707 | 1.5682 | 1.7764 | 1.8455 | 1.6691 |        |
| Bos lung   | 0.5833 | 0.1499 | 0.4077 | 0.5801 | 0.1639 | 0.5844 | 0.5514 | 0.0304 | 0.0863 | 0.0000 |        |
| 1.9147     | 1.9778 | 1.4387 | 1.4654 | 1.6601 | 1.9127 | 1.6973 | 1.6601 | 1.9224 | 1.6331 | 1.8958 | 2.7445 |
| 1.9801     | 1.9759 | 1.9892 | 2.0139 | 1.9100 | 1.9100 | 1.9409 | 1.2669 | 2.1160 | 1.6156 | 1.8680 | 1.8976 |
| 2.3490     | 1.7967 | 2.0454 | 2.0008 | 1.4600 | 1.4939 | 1.6598 | 1.6620 | 1.8560 | 1.9168 | 1.6561 |        |
| tomato     | 1.8683 | 2.0293 | 2.0371 | 1.8255 | 2.0515 | 1.8644 | 1.9549 | 1.9361 | 2.0201 | 1.9147 |        |
| 0.0000     | 1.9802 | 0.9035 | 1.1121 | 0.6030 | 1.8962 | 0.6253 | 0.6030 | 1.0304 | 0.4804 | 1.8042 | 2.4774 |
| 1.0859     | 1.0831 | 1.0710 | 1.0905 | 1.7923 | 1.7923 | 1.7758 | 2.1377 | 1.8188 | 1.5261 | 1.6987 | 1.7032 |
| 2.4393     | 1.8171 | 1.8570 | 1.7482 | 1.5404 | 1.4934 | 1.8233 | 1.9115 | 2.0820 | 1.9993 | 1.9249 |        |
| Solanum    | 1.8013 | 2.0543 | 1.9513 | 1.8072 | 1.9499 | 1.7330 | 1.8090 | 1.9455 | 1.8989 | 1.9778 |        |
| 1.9802     | 0.0000 | 1.4856 | 1.5303 | 1.4030 | 0.1866 | 1.4779 | 1.4030 | 1.5090 | 1.4058 | 0.1576 | 0.5955 |
| 1.6162     | 1.6162 | 1.6414 | 1.6068 | 0.1718 | 0.1718 | 1.3992 | 1.9590 | 1.5269 | 1.1083 | 1.1532 | 1.2017 |
| 1.5746     | 1.1212 | 1.0809 | 1.0661 | 1.2976 | 1.3686 | 1.1189 | 0.8585 | 0.7884 | 0.8075 | 1.0234 |        |
| Athaliana1 | 1.3573 | 1.4700 | 1.5619 | 1.3221 | 1.5254 | 1.3665 | 1.3599 | 1.4619 | 1.5034 | 1.4387 |        |
| 0.9035     | 1.4856 | 0.0000 | 0.3984 | 0.6117 | 1.4363 | 0.6322 | 0.6117 | 0.4462 | 0.5683 | 1.3984 | 1.8999 |
| 0.4359     | 0.4312 | 0.4659 | 0.4580 | 1.4524 | 1.4524 | 1.4729 | 1.8539 | 1.4288 | 1.0242 | 1.3710 | 1.2638 |
| 1.7410     | 1.2516 | 1.3591 | 1.2094 | 1.0847 | 1.0883 | 1.3980 | 1.4143 | 1.5137 | 1.3699 | 1.2076 |        |
| Athaliana2 | 1.5054 | 1.5318 | 1.5823 | 1.4789 | 1.6005 | 1.4963 | 1.4754 | 1.4364 | 1.5888 | 1.4654 |        |
| 1.1121     | 1.5303 | 0.3984 | 0.0000 | 0.7643 | 1.3577 | 0.7798 | 0.7643 | 0.5948 | 0.6792 | 1.3835 | 1.8778 |
| 0.6076     | 0.6015 | 0.6495 | 0.6446 | 1.3461 | 1.3461 | 1.5469 | 1.9939 | 1.6667 | 1.1913 | 1.4316 | 1.4243 |
| 1.7707     | 1.3171 | 1.4549 | 1.3841 | 1.1879 | 1.1991 | 1.3736 | 1.4379 | 1.5978 | 1.4680 | 1.2112 |        |
| Athaliana3 | 1.7138 | 1.6986 | 1.7640 | 1.6840 | 1.7525 | 1.6629 | 1.6765 | 1.6388 | 1.7493 | 1.6601 |        |
| 0.6030     | 1.4030 | 0.6117 | 0.7643 | 0.0000 | 1.4222 | 0.1132 | 0.0000 | 0.5975 | 0.2837 | 1.3962 | 1.9472 |
| 0.6329     | 0.6285 | 0.6436 | 0.6201 | 1.4387 | 1.4387 | 1.6436 | 1.7906 | 1.6456 | 1.0845 | 1.4890 | 1.4803 |
| 1.6883     | 1.3011 | 1.5225 | 1.2726 | 1.0869 | 1.0547 | 1.2869 | 1.3002 | 1.3157 | 1.3322 | 1.3073 |        |
| Athaliana4 | 1.8546 | 2.0321 | 1.9740 | 1.8085 | 1.9279 | 1.7354 | 1.8040 | 1.9235 | 1.8694 | 1.9127 |        |
| 1.8962     | 0.1866 | 1.4363 | 1.3577 | 1.4222 | 0.0000 | 1.4397 | 1.4222 | 1.3587 | 1.3219 | 0.0897 | 0.4307 |
| 1.4375     | 1.4256 | 1.4480 | 1.4527 | 0.0909 | 0.0909 | 1.2599 | 1.9755 | 1.3294 | 1.0171 | 1.0079 | 1.1311 |
| 1.5128     | 1.0515 | 1.0387 | 0.8799 | 1.1856 | 1.2562 | 0.9521 | 0.7365 | 0.7269 | 0.7200 | 0.8651 |        |
| Athaliana5 | 1.6621 | 1.7363 | 1.7655 | 1.6323 | 1.7700 | 1.6116 | 1.6666 | 1.6764 | 1.7862 | 1.6973 |        |
| 0.6253     | 1.4779 | 0.6322 | 0.7798 | 0.1132 | 1.4397 | 0.0000 | 0.1132 | 0.6185 | 0.3176 | 1.4811 | 1.9798 |
| 0.6308     | 0.6263 | 0.6460 | 0.6335 | 1.5130 | 1.5130 | 1.6746 | 1.7622 | 1.6695 | 1.1032 | 1.5182 | 1.4660 |
| 1.7182     | 1.3239 | 1.5128 | 1.2426 | 1.0945 | 1.0861 | 1.2993 | 1.2917 | 1.2996 | 1.3993 | 1.4443 |        |

|             |        |        |        |        |        |        |        |        |        |        |        |
|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Athaliana6  | 1.7138 | 1.6986 | 1.7640 | 1.6840 | 1.7525 | 1.6629 | 1.6765 | 1.6388 | 1.7493 | 1.6601 |        |
| 0.6030      | 1.4030 | 0.6117 | 0.7643 | 0.0000 | 1.4222 | 0.1132 | 0.0000 | 0.5975 | 0.2837 | 1.3962 | 1.9472 |
| 0.6329      | 0.6285 | 0.6436 | 0.6201 | 1.4387 | 1.4387 | 1.6436 | 1.7906 | 1.6456 | 1.0845 | 1.4890 | 1.4803 |
| 1.6883      | 1.3011 | 1.5225 | 1.2726 | 1.0869 | 1.0547 | 1.2869 | 1.3002 | 1.3157 | 1.3322 | 1.3073 |        |
| Chickpea    | 1.8179 | 1.9612 | 2.0383 | 1.7497 | 2.0322 | 1.7833 | 1.7831 | 1.9439 | 2.0713 | 1.9224 |        |
| 1.0304      | 1.5090 | 0.4462 | 0.5948 | 0.5975 | 1.3587 | 0.6185 | 0.5975 | 0.0000 | 0.5071 | 1.3913 | 1.8749 |
| 0.0716      | 0.0632 | 0.0823 | 0.0644 | 1.4079 | 1.4079 | 1.4948 | 2.0847 | 1.5364 | 1.2343 | 1.3714 | 1.3615 |
| 1.7740      | 1.4253 | 1.5161 | 1.3104 | 1.2447 | 1.2455 | 1.3259 | 1.4510 | 1.5744 | 1.4430 | 1.3130 |        |
| C. lineata  | 1.5843 | 1.7742 | 1.8815 | 1.5443 | 1.7732 | 1.5545 | 1.5885 | 1.6485 | 1.7672 | 1.6331 |        |
| 0.4804      | 1.4058 | 0.5683 | 0.6792 | 0.2837 | 1.3219 | 0.3176 | 0.2837 | 0.5071 | 0.0000 | 1.2827 | 1.7681 |
| 0.5536      | 0.5518 | 0.5680 | 0.5473 | 1.3134 | 1.3134 | 1.5391 | 1.9852 | 1.5066 | 1.0382 | 1.3469 | 1.3278 |
| 1.7424      | 1.3580 | 1.3980 | 1.2677 | 1.1611 | 1.1515 | 1.2846 | 1.3884 | 1.3601 | 1.3995 | 1.3013 |        |
| G. max      | 1.8367 | 2.0147 | 1.9173 | 1.8363 | 1.9109 | 1.7611 | 1.8320 | 1.9065 | 1.8529 | 1.8958 |        |
| 1.8042      | 0.1576 | 1.3984 | 1.3835 | 1.3962 | 0.0897 | 1.4811 | 1.3962 | 1.3913 | 1.2827 | 0.0000 | 0.4050 |
| 1.5037      | 1.4916 | 1.5252 | 1.4965 | 0.0668 | 0.0668 | 1.2214 | 1.8873 | 1.3113 | 0.9839 | 0.9856 | 1.1206 |
| 1.4696      | 1.0112 | 1.0106 | 0.9008 | 1.2705 | 1.3062 | 0.9326 | 0.7643 | 0.7229 | 0.7669 | 0.8436 |        |
| Medicago 1  | 2.5329 | 2.8450 | 2.8067 | 2.4173 | 2.6863 | 2.4269 | 2.5326 | 2.7833 | 2.6578 | 2.7445 |        |
| 2.4774      | 0.5955 | 1.8999 | 1.8778 | 1.9472 | 0.4307 | 1.9798 | 1.9472 | 1.8749 | 1.7681 | 0.4050 | 0.0000 |
| 1.9558      | 1.9396 | 2.0381 | 2.0065 | 0.4804 | 0.4804 | 1.7175 | 2.4294 | 1.8333 | 1.4834 | 1.3898 | 1.5442 |
| 2.2157      | 1.4669 | 1.4356 | 1.3452 | 1.7331 | 1.8050 | 1.3697 | 1.2899 | 1.2732 | 1.2102 | 1.4265 |        |
| P. sativum1 | 1.8440 | 2.0129 | 2.0888 | 1.7756 | 2.0913 | 1.8093 | 1.8092 | 2.0025 | 2.1312 | 1.9801 |        |
| 1.0859      | 1.6162 | 0.4359 | 0.6076 | 0.6329 | 1.4375 | 0.6308 | 0.6329 | 0.0716 | 0.5536 | 1.5037 | 1.9558 |
| 0.0000      | 0.0077 | 0.0486 | 0.0397 | 1.5204 | 1.5204 | 1.6055 | 2.0887 | 1.6137 | 1.2871 | 1.4472 | 1.3995 |
| 1.7579      | 1.4427 | 1.5939 | 1.3579 | 1.3075 | 1.3131 | 1.3301 | 1.4827 | 1.5787 | 1.4106 | 1.3853 |        |
| P. sativum2 | 1.8400 | 2.0160 | 2.0919 | 1.7717 | 2.0869 | 1.8053 | 1.8052 | 1.9983 | 2.1269 | 1.9759 |        |
| 1.0831      | 1.6162 | 0.4312 | 0.6015 | 0.6285 | 1.4256 | 0.6263 | 0.6285 | 0.0632 | 0.5518 | 1.4916 | 1.9396 |
| 0.0077      | 0.0000 | 0.0568 | 0.0477 | 1.5082 | 1.5082 | 1.5910 | 2.1240 | 1.6003 | 1.2766 | 1.4351 | 1.3879 |
| 1.7605      | 1.4308 | 1.5808 | 1.3468 | 1.2962 | 1.3017 | 1.3192 | 1.4706 | 1.5659 | 1.4194 | 1.3939 |        |
| Lens 1      | 1.8043 | 2.0223 | 2.0680 | 1.7365 | 2.1010 | 1.7700 | 1.7697 | 2.0118 | 2.1407 | 1.9892 |        |
| 1.0710      | 1.6414 | 0.4659 | 0.6495 | 0.6436 | 1.4480 | 0.6460 | 0.6436 | 0.0823 | 0.5680 | 1.5252 | 2.0381 |
| 0.0486      | 0.0568 | 0.0000 | 0.0159 | 1.5422 | 1.5422 | 1.6530 | 2.0662 | 1.7349 | 1.3391 | 1.4733 | 1.4229 |
| 1.7694      | 1.4489 | 1.6161 | 1.3952 | 1.3423 | 1.3440 | 1.4294 | 1.4907 | 1.6554 | 1.5126 | 1.4404 |        |
| Lens 2      | 1.8051 | 2.0474 | 2.0930 | 1.7384 | 2.1265 | 1.7713 | 1.7710 | 2.0369 | 2.1659 | 2.0139 |        |
| 1.0905      | 1.6068 | 0.4580 | 0.6446 | 0.6201 | 1.4527 | 0.6335 | 0.6201 | 0.0644 | 0.5473 | 1.4965 | 2.0065 |
| 0.0397      | 0.0477 | 0.0159 | 0.0000 | 1.5129 | 1.5129 | 1.6008 | 2.1002 | 1.6454 | 1.3531 | 1.4458 | 1.3980 |
| 1.7680      | 1.4537 | 1.5817 | 1.4009 | 1.3412 | 1.3428 | 1.3973 | 1.5114 | 1.6086 | 1.4961 | 1.4194 |        |

|            |        |        |        |        |        |        |        |        |        |        |               |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------------|
| T.aestivum | 1.8391 | 2.0049 | 1.9592 | 1.7932 | 1.9065 | 1.7197 | 1.7885 | 1.9208 | 1.8437 | 1.9100 |               |
|            | 1.7923 | 0.1718 | 1.4524 | 1.3461 | 1.4387 | 0.0909 | 1.5130 | 1.4387 | 1.4079 | 1.3134 | 0.0668 0.4804 |
|            | 1.5204 | 1.5082 | 1.5422 | 1.5129 | 0.0000 | 0.0000 | 1.2708 | 1.9642 | 1.3548 | 0.9948 | 0.9802 1.0843 |
|            | 1.5657 | 1.0945 | 0.9928 | 0.9365 | 1.2690 | 1.3308 | 0.9974 | 0.7941 | 0.7513 | 0.7576 | 0.8969        |
| H.vulgare2 | 1.8391 | 2.0049 | 1.9592 | 1.7932 | 1.9065 | 1.7197 | 1.7885 | 1.9208 | 1.8437 | 1.9100 |               |
|            | 1.7923 | 0.1718 | 1.4524 | 1.3461 | 1.4387 | 0.0909 | 1.5130 | 1.4387 | 1.4079 | 1.3134 | 0.0668 0.4804 |
|            | 1.5204 | 1.5082 | 1.5422 | 1.5129 | 0.0000 | 0.0000 | 1.2708 | 1.9642 | 1.3548 | 0.9948 | 0.9802 1.0843 |
|            | 1.5657 | 1.0945 | 0.9928 | 0.9365 | 1.2690 | 1.3308 | 0.9974 | 0.7941 | 0.7513 | 0.7576 | 0.8969        |
| P. angusta | 1.6637 | 1.9827 | 1.9465 | 1.7194 | 1.9171 | 1.6997 | 1.6802 | 1.9166 | 1.9986 | 1.9409 |               |
|            | 1.7758 | 1.3992 | 1.4729 | 1.5469 | 1.6436 | 1.2599 | 1.6746 | 1.6436 | 1.4948 | 1.5391 | 1.2214 1.7175 |
|            | 1.6055 | 1.5910 | 1.6530 | 1.6008 | 1.2708 | 1.2708 | 0.0000 | 2.1655 | 0.2322 | 0.9960 | 0.4599 0.7163 |
|            | 1.5575 | 0.9567 | 0.7311 | 0.9280 | 1.5292 | 1.5591 | 1.2523 | 1.0834 | 1.1630 | 1.3024 | 1.3186        |
| P.pastoris | 1.1721 | 1.3892 | 1.3472 | 1.2077 | 1.3323 | 1.2194 | 1.1922 | 1.2780 | 1.2257 | 1.2669 |               |
|            | 2.1377 | 1.9590 | 1.8539 | 1.9939 | 1.7906 | 1.9755 | 1.7622 | 1.7906 | 2.0847 | 1.9852 | 1.8873 2.4294 |
|            | 2.0887 | 2.1240 | 2.0662 | 2.1002 | 1.9642 | 1.9642 | 2.1655 | 0.0000 | 2.4343 | 1.7814 | 2.0631 1.9185 |
|            | 2.4528 | 1.9447 | 1.8222 | 1.8910 | 2.0913 | 2.0593 | 1.8829 | 1.6193 | 1.8019 | 1.6469 | 2.0572        |
| Candida 1  | 1.8603 | 2.2346 | 2.1180 | 1.9286 | 2.0825 | 1.9048 | 1.8811 | 2.0903 | 2.2004 | 2.1160 |               |
|            | 1.8188 | 1.5269 | 1.4288 | 1.6667 | 1.6456 | 1.3294 | 1.6695 | 1.6456 | 1.5364 | 1.5066 | 1.3113 1.8333 |
|            | 1.6137 | 1.6003 | 1.7349 | 1.6454 | 1.3548 | 1.3548 | 0.2322 | 2.4343 | 0.0000 | 1.0095 | 0.5000 0.7123 |
|            | 1.7401 | 1.0374 | 0.8371 | 1.0291 | 1.5575 | 1.6195 | 1.2753 | 1.1188 | 1.1673 | 1.4007 | 1.3320        |
| Candida 2  | 1.6074 | 1.6743 | 1.7141 | 1.5834 | 1.5636 | 1.6168 | 1.5817 | 1.5941 | 1.6232 | 1.6156 |               |
|            | 1.5261 | 1.1083 | 1.0242 | 1.1913 | 1.0845 | 1.0171 | 1.1032 | 1.0845 | 1.2343 | 1.0382 | 0.9839 1.4834 |
|            | 1.2871 | 1.2766 | 1.3391 | 1.3531 | 0.9948 | 0.9948 | 0.9960 | 1.7814 | 1.0095 | 0.0000 | 0.8808 0.9578 |
|            | 1.1569 | 0.6610 | 0.9624 | 0.5566 | 0.9861 | 1.0383 | 1.0759 | 1.0081 | 0.9058 | 1.0128 | 1.2077        |
| S. pombe 1 | 1.5048 | 1.9233 | 1.8441 | 1.5541 | 1.8133 | 1.5276 | 1.5099 | 1.8875 | 1.8856 | 1.8680 |               |
|            | 1.6987 | 1.1532 | 1.3710 | 1.4316 | 1.4890 | 1.0079 | 1.5182 | 1.4890 | 1.3714 | 1.3469 | 0.9856 1.3898 |
|            | 1.4472 | 1.4351 | 1.4733 | 1.4458 | 0.9802 | 0.9802 | 0.4599 | 2.0631 | 0.5000 | 0.8808 | 0.0000 0.3442 |
|            | 1.4429 | 0.9130 | 0.6677 | 0.8814 | 1.3080 | 1.3417 | 0.9943 | 0.8888 | 0.9501 | 1.0459 | 1.1625        |
| S. pombe 2 | 1.6024 | 2.0288 | 2.0242 | 1.6246 | 1.9499 | 1.6294 | 1.6349 | 1.9269 | 1.9986 | 1.8976 |               |
|            | 1.7032 | 1.2017 | 1.2638 | 1.4243 | 1.4803 | 1.1311 | 1.4660 | 1.4803 | 1.3615 | 1.3278 | 1.1206 1.5442 |
|            | 1.3995 | 1.3879 | 1.4229 | 1.3980 | 1.0843 | 1.0843 | 0.7163 | 1.9185 | 0.7123 | 0.9578 | 0.3442 0.0000 |
|            | 1.6931 | 1.0688 | 0.8463 | 0.9309 | 1.3005 | 1.4116 | 1.1944 | 1.1407 | 1.1251 | 1.1417 | 1.3625        |
| A. niger 1 | 2.1282 | 2.2163 | 2.2317 | 2.0524 | 2.3546 | 2.0753 | 2.0480 | 2.3220 | 2.3551 | 2.3490 |               |
|            | 2.4393 | 1.5746 | 1.7410 | 1.7707 | 1.6883 | 1.5128 | 1.7182 | 1.6883 | 1.7740 | 1.7424 | 1.4696 2.2157 |
|            | 1.7579 | 1.7605 | 1.7694 | 1.7680 | 1.5657 | 1.5657 | 1.5575 | 2.4528 | 1.7401 | 1.1569 | 1.4429 1.6931 |
|            | 0.0000 | 0.3856 | 1.5172 | 1.0652 | 1.7801 | 1.8432 | 1.9491 | 1.6216 | 1.5359 | 1.5945 | 1.8155        |



|            |        |        |        |        |        |        |        |        |        |        |        |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| A. niger 2 | 1.7258 | 1.6836 | 1.8151 | 1.6310 | 1.8348 | 1.6571 | 1.6601 | 1.7490 | 1.8102 | 1.7967 |        |
|            | 1.8171 | 1.1212 | 1.2516 | 1.3171 | 1.3011 | 1.0515 | 1.3239 | 1.3011 | 1.4253 | 1.3580 | 1.4669 |
|            | 1.4427 | 1.4308 | 1.4489 | 1.4537 | 1.0945 | 1.0945 | 0.9567 | 1.9447 | 1.0374 | 0.6610 | 1.0688 |
|            | 0.3856 | 0.0000 | 0.9336 | 0.6341 | 1.1785 | 1.2134 | 1.0768 | 1.0661 | 1.0379 | 1.1586 | 1.2840 |
| Afumigatus | 1.7653 | 2.1040 | 1.9378 | 1.7490 | 2.0095 | 1.7459 | 1.7349 | 2.0542 | 2.0537 | 2.0454 |        |
|            | 1.8570 | 1.0809 | 1.3591 | 1.4549 | 1.5225 | 1.0387 | 1.5128 | 1.5225 | 1.5161 | 1.3980 | 1.4356 |
|            | 1.5939 | 1.5808 | 1.6161 | 1.5817 | 0.9928 | 0.9928 | 0.7311 | 1.8222 | 0.8371 | 0.9624 | 0.8463 |
|            | 1.5172 | 0.9336 | 0.0000 | 0.7801 | 1.2897 | 1.3273 | 1.2868 | 0.9310 | 0.9084 | 1.0730 | 1.3910 |
| fumigatus2 | 1.7848 | 2.0298 | 1.8591 | 1.6970 | 1.9414 | 1.6935 | 1.6677 | 1.9780 | 1.9329 | 2.0008 |        |
|            | 1.7482 | 1.0661 | 1.2094 | 1.3841 | 1.2726 | 0.8799 | 1.2426 | 1.2726 | 1.3104 | 1.2677 | 1.3452 |
|            | 1.3579 | 1.3468 | 1.3952 | 1.4009 | 0.9365 | 0.9365 | 0.9280 | 1.8910 | 1.0291 | 0.5566 | 0.9309 |
|            | 1.0652 | 0.6341 | 0.7801 | 0.0000 | 1.0852 | 1.0956 | 1.0961 | 0.9176 | 0.8623 | 0.9979 | 1.1693 |
| E. coli    | 1.4186 | 1.4611 | 1.3760 | 1.3895 | 1.4547 | 1.4235 | 1.3757 | 1.4184 | 1.5250 | 1.4600 |        |
|            | 1.5404 | 1.2976 | 1.0847 | 1.1879 | 1.0869 | 1.1856 | 1.0945 | 1.0869 | 1.2447 | 1.1611 | 1.7331 |
|            | 1.3075 | 1.2962 | 1.3423 | 1.3412 | 1.2690 | 1.2690 | 1.5292 | 2.0913 | 1.5575 | 0.9861 | 1.3005 |
|            | 1.7801 | 1.1785 | 1.2897 | 1.0852 | 0.0000 | 0.0694 | 1.3078 | 1.1088 | 1.1197 | 1.1552 | 1.2317 |
| Kaerogenes | 1.4593 | 1.4956 | 1.4093 | 1.4153 | 1.4890 | 1.4497 | 1.4015 | 1.4512 | 1.5605 | 1.4939 |        |
|            | 1.4934 | 1.3686 | 1.0883 | 1.1991 | 1.0547 | 1.2562 | 1.0861 | 1.0547 | 1.2455 | 1.1515 | 1.8050 |
|            | 1.3131 | 1.3017 | 1.3440 | 1.3428 | 1.3308 | 1.3308 | 1.5591 | 2.0593 | 1.6195 | 1.0383 | 1.4116 |
|            | 1.8432 | 1.2134 | 1.3273 | 1.0956 | 0.0694 | 0.0000 | 1.3054 | 1.1063 | 1.1493 | 1.2049 | 1.2297 |
| M. loti    | 1.7370 | 1.7037 | 1.6001 | 1.5972 | 1.7438 | 1.5712 | 1.6701 | 1.6229 | 1.6707 | 1.6598 |        |
|            | 1.8233 | 1.1189 | 1.3980 | 1.3736 | 1.2869 | 0.9521 | 1.2993 | 1.2869 | 1.3259 | 1.2846 | 1.3697 |
|            | 1.3301 | 1.3192 | 1.4294 | 1.3973 | 0.9974 | 0.9974 | 1.2523 | 1.8829 | 1.2753 | 1.0759 | 1.1944 |
|            | 1.9491 | 1.0768 | 1.2868 | 1.0961 | 1.3078 | 1.3054 | 0.0000 | 0.7392 | 0.8380 | 0.9002 | 0.8800 |
| AGAO 1     | 1.6015 | 1.6332 | 1.6238 | 1.5898 | 1.5939 | 1.5643 | 1.6491 | 1.6024 | 1.5682 | 1.6620 |        |
|            | 1.9115 | 0.8585 | 1.4143 | 1.4379 | 1.3002 | 0.7365 | 1.2917 | 1.3002 | 1.4510 | 1.3884 | 1.2899 |
|            | 1.4827 | 1.4706 | 1.4907 | 1.5114 | 0.7941 | 0.7941 | 1.0834 | 1.6193 | 1.1188 | 1.0081 | 1.1407 |
|            | 1.6216 | 1.0661 | 0.9310 | 0.9176 | 1.1088 | 1.1063 | 0.7392 | 0.0000 | 0.3370 | 0.7055 | 0.8720 |
| AGAO 2     | 1.7604 | 1.8982 | 1.8314 | 1.6283 | 1.8904 | 1.6019 | 1.6391 | 1.8571 | 1.7764 | 1.8560 |        |
|            | 2.0820 | 0.7884 | 1.5137 | 1.5978 | 1.3157 | 0.7269 | 1.2996 | 1.3157 | 1.5744 | 1.3601 | 1.2732 |
|            | 1.5787 | 1.5659 | 1.6554 | 1.6086 | 0.7513 | 0.7513 | 1.1630 | 1.8019 | 1.1673 | 0.9058 | 1.1251 |
|            | 1.5359 | 1.0379 | 0.9084 | 0.8623 | 1.1197 | 1.1493 | 0.8380 | 0.3370 | 0.0000 | 0.8747 | 0.9979 |
| A.strainP1 | 1.7763 | 1.9573 | 1.7909 | 1.7402 | 1.9231 | 1.7380 | 1.7428 | 1.9090 | 1.8455 | 1.9168 |        |
|            | 1.9993 | 0.8075 | 1.3699 | 1.4680 | 1.3322 | 0.7200 | 1.3993 | 1.3322 | 1.4430 | 1.3995 | 1.2102 |
|            | 1.4106 | 1.4194 | 1.5126 | 1.4961 | 0.7576 | 0.7576 | 1.3024 | 1.6469 | 1.4007 | 1.0128 | 1.1417 |
|            | 1.5945 | 1.1586 | 1.0730 | 0.9979 | 1.1552 | 1.2049 | 0.9002 | 0.7055 | 0.8747 | 0.0000 | 0.8127 |

|            |        |        |        |        |        |        |        |        |        |        |        |        |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Solfolobus | 1.7539 | 1.6978 | 1.5858 | 1.6457 | 1.6336 | 1.6484 | 1.6506 | 1.6328 | 1.6691 | 1.6561 |        |        |
|            | 1.9249 | 1.0234 | 1.2076 | 1.2112 | 1.3073 | 0.8651 | 1.4443 | 1.3073 | 1.3130 | 1.3013 | 0.8436 | 1.4265 |
|            | 1.3853 | 1.3939 | 1.4404 | 1.4194 | 0.8969 | 0.8969 | 1.3186 | 2.0572 | 1.3320 | 1.2077 | 1.1625 | 1.3625 |
|            | 1.8155 | 1.2840 | 1.3910 | 1.1693 | 1.2317 | 1.2297 | 0.8800 | 0.8720 | 0.9979 | 0.8127 | 0.0000 |        |

Parameters Used for the Program FITCH

Input data is the distance matrix output from PROTDIST.

Fitch-Margoliash method version 3.6a3

Settings for this run:

D Method (F-M, Minimum Evolution)? Fitch-Margoliash  
 U Search for best tree? Yes  
 P Power? 2.00000  
 - Negative branch lengths allowed? No  
 O Outgroup root? No, use as outgroup species 1  
 L Lower-triangular data matrix? No  
 R Upper-triangular data matrix? No  
 S Subreplicates? No  
 G Global rearrangements? Yes  
 J Randomize input order of species? YES  
 M Analyze multiple data sets? No  
 0 Terminal type (IBM PC, ANSI, none)? (none)  
 1 Print out the data at start of run No  
 2 Print indications of progress of run Yes  
 3 Print out tree Yes  
 4 Write out trees onto tree file? Yes

Y to accept these or type the letter for one to change

Parameters Used for the Program ProML

Input data is the amino acid sequence file above.

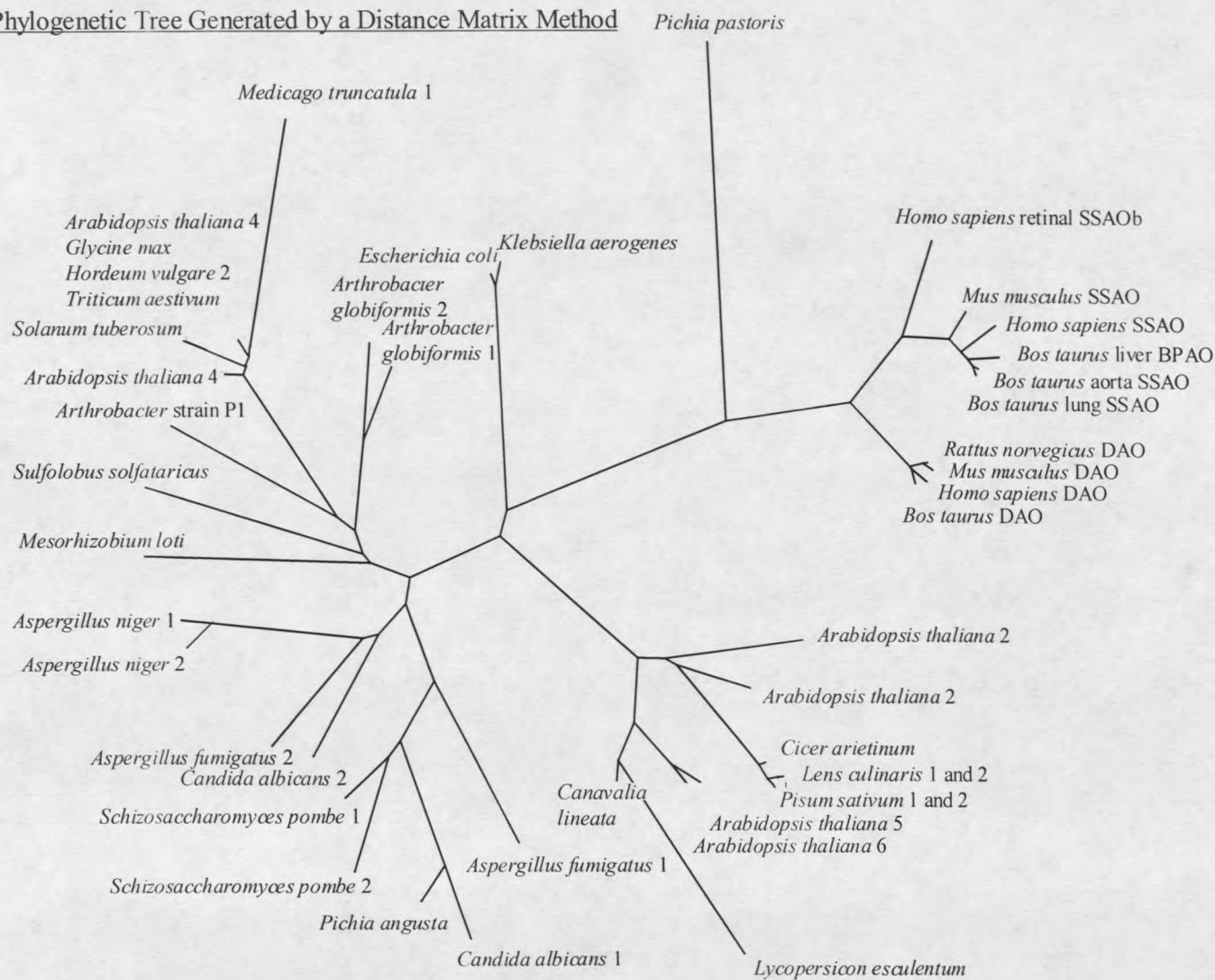
Amino acid sequence Maximum Likelihood method, version 3.6a3

Settings for this run:

- U           Search for best tree? Yes
- P   JTT or PAM amino acid change model? Jones-Taylor-Thornton model
- C           One category of sites? Yes
- R           Rate variation among sites? constant rate of change
- W           Sites weighted? No
- S   Speedier but rougher analysis? No
- G           Global rearrangements? Yes
- J   Randomize input order of sequences? Yes
- O           Outgroup root? No, use as outgroup species 1
- M   Analyze multiple data sets? No
- I   Input sequences interleaved? Yes
- 0   Terminal type (IBM PC, ANSI, none)? (none)
- 1   Print out the data at start of run No
- 2   Print indications of progress of run Yes
- 3           Print out tree Yes
- 4   Write out trees onto tree file? Yes
- 5   Reconstruct hypothetical sequences? No

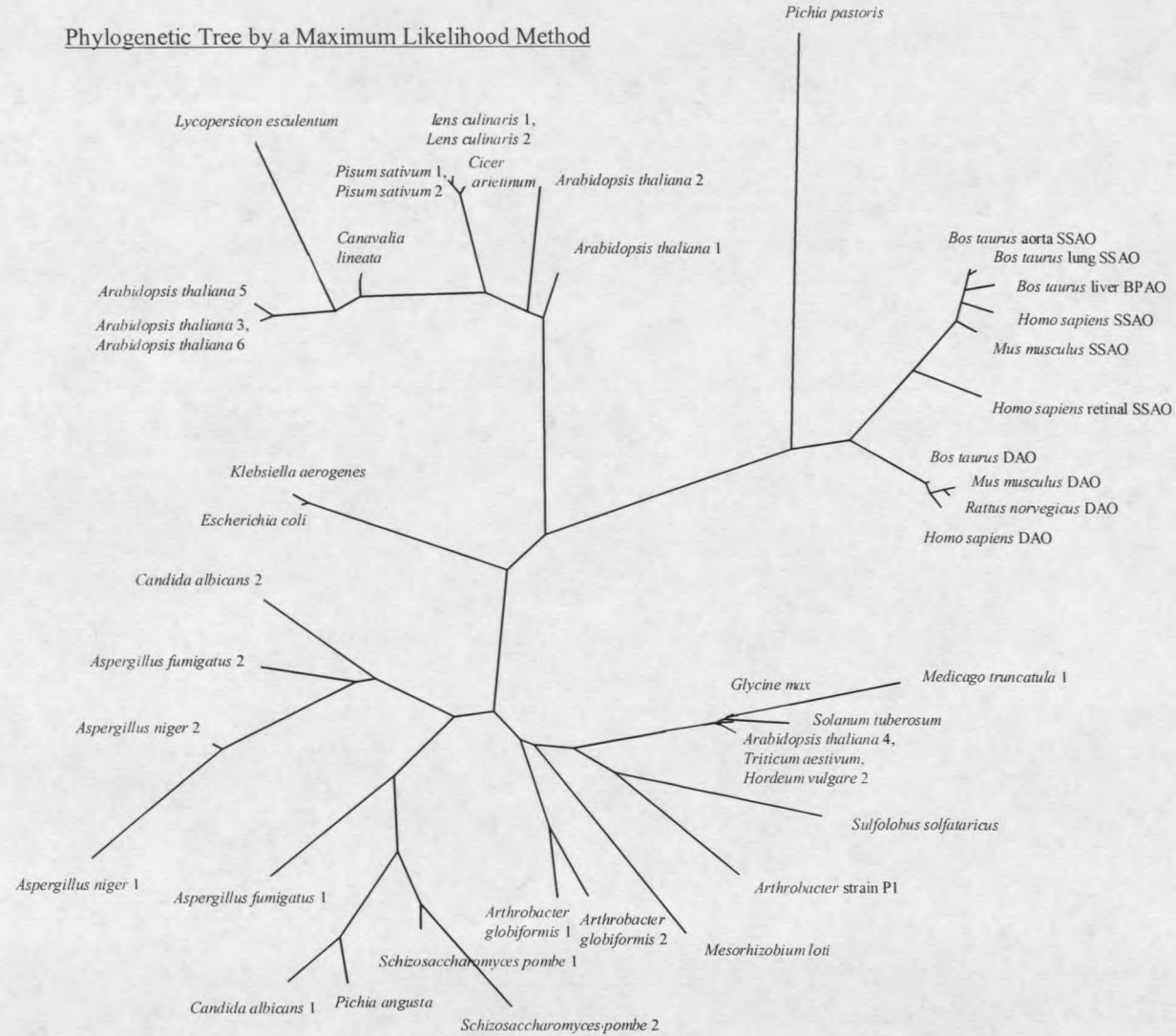
Y to accept these or type the letter for one to change

# Phylogenetic Tree Generated by a Distance Matrix Method





# Phylogenetic Tree by a Maximum Likelihood Method



## APPENDIX C

## USE OF GRÖBNER BASES TO DERIVE STEADY STATE RATE EQUATIONS

Several methods to derive steady state rate equations for enzyme-catalyzed reactions are available, for example, the determinant method, the schematic method of King and Altman and its several modifications, the use of graph theory by Volkenstein and Goldstein, etc. [253] However, these methods often require the manipulation of very large algebraic equations, which invites the introduction of significant human error and considerable consternation. I have utilized the Gröbner Bases package included in the symbolic algebra software package Maple 8 (Waterloo Maple, Inc). Note that “Gröbner” is often alternatively spelled as “Groebner” and is spelled as “grobner” in Maple. Introductory information for the use of Gröbner Bases can be found at <http://anstel.wins.uva.nl/~heck/AIHENP96/AIHENP96.html> and in reference [254], and examples of use for biochemical kinetics can be found at <http://www.mapleapps.com/categories/science/biochemistry/html/Grobner.html>.

Gröbner Bases is a technique for solving systems of multivariable polynomials and is an algorithm based upon Polynomial Ideal Theory and was originally developed by Buchberger in the mid 1960's. [255]. It is used to solve systems of equations of the type:

$$\begin{aligned} f_1(x_1, x_2, \dots, x_n) &= 0 \\ f_2(x_1, x_2, \dots, x_n) &= 0 \\ &\vdots \\ f_m(x_1, x_2, \dots, x_n) &= 0 \end{aligned}$$

where  $f_1, f_2, \dots, f_m$  are polynomials in  $n$  unknowns.

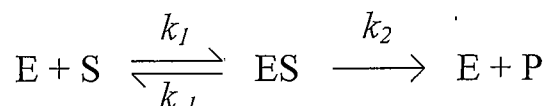
This appendix will illustrate the use of Gröbner Bases for solving steady state enzymatic rate equations and demonstrate the specific applications to this thesis. In Maple syntax, the command prompt is ">", user inputs and commands are given in red font, and Maple outputs are in blue font.

Rate equations derived are:

- 1) Michaelis-Menten equation
- 2) Substrate inhibition
- 3) Bi Ter Ping Pong
- 4) Reversible substrate inhibition at two enzyme state in the mechanism
- 5) Reversible substrate inhibition at two enzyme form and an additional pathway for product formation

### Use of Gröbner Bases to Derive the Michaelis-Menton Equation

Using the following enzyme reaction mechanism where **E** is Enzyme; **S** is Substrate; **ES** is Enzyme-substrate complex and **P** is Reaction product



```
> restart:with(grobner):
```

Rate equation for the formation (v0 of the product P

```
f1:=v=(diff(P(t),t)=k[2]*ES):f1;
```

```
>
```

$$v = \left( \frac{d}{dt} P(t) = k_2 ES \right)$$

Rate equation for the change of the enzyme-substrate complex, ES

```
> f2:=diff(ES(t), (t))=k[1]*E*S-(k[-1]+k[2])*ES:f2;
```

$$\frac{d}{dt} ES(t) = k_1 E S - (k_{-1} + k_2) ES$$

Equation for the total concentration of enzyme, Et

```
> f3:=Et=E+ES:f3;
```

$$Et = E + ES$$

Rearrange equations to the form of polynomials equal to zero. Under the assumptions of steady state, the concentration of the enzyme-substrate complex does not change.

```
> eq1:=rhs(rhs(f1))-v:
```

```
eq2:=rhs(f2):
```

```
eq3:=rhs(f3)-Et:eq1;eq2;eq3;
```

$$k_2 ES - v$$

$$k_1 ES - (k_{-1} + k_2) ES$$

$$E + ES - Et$$

Determine Groebner Bases

```
> Bases:=gbasis([eq1,eq2,eq3],[E,ES,v],plex);
```

$$Bases := [E k_{-1} + k_2 E + k_1 ES - k_{-1} Et - k_2 Et, ES k_{-1} + k_2 ES + ES k_1 S - k_1 S Et,$$

$$-k_2 k_1 S Et + k_{-1} v + k_2 v + k_1 S v]$$



Solve for v

> eq4:=solve(Bases[3],v):eq4;

$$\frac{k_2 k_1 S E t}{k_{-1} + k_2 + k_1 S}$$

Define the maximal rate of reaction as  $V_{max} = k_2 * E t$

> eq5:=algsubs(k[2]\*Et=V[max],eq4):eq5;

$$\frac{k_1 S V_{max}}{k_{-1} + k_2 + k_1 S}$$

Simplify by division of numerator and denominator by  $k_1$

> eq6:=numer(eq5)/k[1]:eq6;

$$S V_{max}$$

> eq7:=denom(eq5)/k[1]:eq7;

$$\frac{k_1 S + k_{-1} + k_2}{k_1}$$

Combine rate constants into a single constant -- the Michaelis constant,  $K_M$

> eq8:=expand(eq7):eq8;

$$S + \frac{k_{-1}}{k_1} + \frac{k_2}{k_1}$$

> eq9:=algsubs((k[-1]+k[2])/k[1]=K[M],eq8):eq9;

$$S + K_M$$

### Solve rate equation

Combine numerator and denominator terms to give the complete rate equation, which gives a common form of the Michaelis-Menten equation.

> eq10:=v=eq6/eq9:eq10;

$$v = \frac{S V_{max}}{S + K_M}$$

Where:

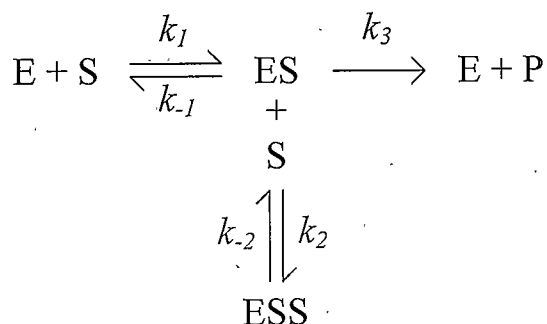
> 'K[m]'=(k[-1]+k[2])/k[1];

>

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

### Rate Equation for a Simple Model of Substrate Inhibition

A classical explanation of substrate inhibition is that two molecules of substrate have parts of themselves stuck in the active site in such a way that prevents the reaction from proceeding. The derivation below gives an equation identical in form to that given by Cleland [85]. However, Cleland's example is for the case of total substrate inhibition, that is the **ESS** complex is a dead-end, irreversible state, and my rate equation is for the partial substrate inhibition case, where the **ESS** complex formation is reversible; therefore  $K_i$  given herein is not equivalent to Cleland's  $K_i$ .



where **E** is Enzyme; **S** is Substrate; **ES** is Enzyme-Substrate complex, **ESS** is Enzyme-Substrate-Substrate complex, and **P** is reaction Product

```
> restart:with(grobner):
```

**Define the set of polynomials equal to zero.**

Rate of formation for the product P

```
> eq1:=v-k[3]*ES:eq1;
```

$$v = k_3 ES$$

Rate of formation of ES

```
> eq2:=k[1]*E*S+k[-2]*ESS-(k[-1]+k[3]+k[2]*S)*ES: eq2;
```

$$k_1 E S + k_2 ESS - (k_{-1} + k_3 + k_2 S) ES$$

Rate of formation of ESS

$$> \text{eq3} := k[2] * ES * S - k[-2] * ESS : \text{eq3};$$

$$k_2 ES S - k_{-2} ESS$$

Equation for conservation of total enzyme

$$> \text{eq4} := Et - E - ES - ESS : \text{eq4};$$

$$Et - E - ES - ESS$$

### Determine Groebner Bases

$$> \text{Bases} := \text{gbasis}([\text{eq1}, \text{eq2}, \text{eq3}, \text{eq4}], [E, ES, ESS, v], \text{plex});$$

$$\text{Bases} := [k_2 S^2 E k_1 + k_{-2} E k_{-1} + k_3 k_{-2} E + k_{-2} S E k_1 - k_{-1} k_{-2} Et - k_3 k_{-2} Et,$$

$$k_2 S^2 ES k_1 + ES k_{-1} k_{-2} + k_3 ES k_{-2} + S ES k_1 k_{-2} - k_{-2} S k_1 Et,$$

$$k_2 S^2 ESS k_1 - k_2 S^2 k_1 Et + k_{-1} k_{-2} ESS + k_3 k_{-2} ESS + k_1 S k_{-2} ESS,$$

$$-k_3 k_{-2} S k_1 Et + k_2 S^2 k_1 v + k_{-1} k_{-2} v + k_3 k_{-2} v + k_1 S k_{-2} v]$$

Solve for v

$$> \text{eq5} := \text{solve}(\text{Bases}[4], v) : \text{eq5};$$

$$\frac{k_3 k_{-2} S k_1 Et}{k_2 S^2 k_1 + k_{-1} k_{-2} + k_3 k_{-2} + k_1 S k_{-2}}$$

### Rearrangement and Simplification

Define Vmax as k3 multiplied by total enzyme

$$> \text{eq6} := \text{algsubs}(k[3] * Et = V[\text{max}], \text{eq5}) : \text{eq6};$$

$$\frac{k_1 S k_{-2} V_{\text{max}}}{k_2 S^2 k_1 + k_{-1} k_{-2} + k_3 k_{-2} + k_1 S k_{-2}}$$

Simplify by A) division of numerator by k1 \* k-2

$$> \text{eq7} := \text{numer}(\text{eq6}) / (k[1] * k[-2]) : \text{eq7};$$

$$S V_{\text{max}}$$

And B) division of denominator by k1 \* k-2

$$> \text{eq8} := \text{denom}(\text{eq6}) / (k[1] * k[-2]) : \text{eq8};$$

$$\frac{k_2 S^2 k_1 + k_{-1} k_{-2} + k_3 k_{-2} + k_1 S k_{-2}}{k_1 k_{-2}}$$

Expand the denominator polynomial

$$> \text{eq9} := \text{expand}(\text{eq8}) : \text{eq9};$$

$$\frac{k_2 S^2}{k_{-2}} + \frac{k_{-1}}{k_1} + \frac{k_3}{k_1} + S$$

Substitution:  $K_m = (k_{-1} + k_3)/k_1$ ;  $K_i = k_{-2}/k_2$

> eq10:=expand(algsubs(k[2]/k[-2]=1/K[i],eq9)):eq10;

$$\frac{S^2}{K_i} + \frac{k_{-1}}{k_1} + \frac{k_3}{k_1} + S$$

> eq11:=expand(algsubs((k[-1]+k[3])/k[1]=K[m],eq10)):eq11;

$$\frac{S^2}{K_i} + K_m + S$$

### Solve rate equation

Combine numerator and denominator

> eq12:=v=eq7/eq11:eq12;

$$v = \frac{S V_{max}}{\frac{S^2}{K_i} + K_m + S}$$

Where:

> 'K[M]'=(k[-1]+k[3])/k[1];

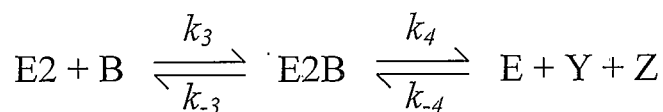
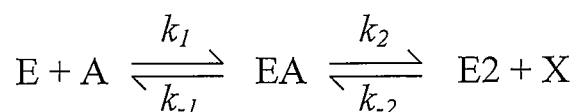
$$K_M = \frac{k_{-1} + k_3}{k_1}$$

> 'K[i]'=k[-2]/k[2];

$$K_i = \frac{k_{-2}}{k_2}$$

### Rate Equation for a Bi Ter Ping Pong Mechanism

This section demonstrates the use of Gröbner Bases to solve rate equations for more complex reaction mechanisms. Copper-containing amine oxidases have a Bi Ter Ping Pong type of mechanism, where the first substrate is a primary amine, the first product is an aldehyde, the second substrate is dioxygen, and the products are ammonia and hydrogen peroxide. The rate equation given here is identical to that given by Cornish-Bowden [205].



Where **E** is the resting form of the Enzyme; **A** is the first substrate; **EA** is Enzyme-first substrate complex,; **E2** is the modified enzyme form; **B** is the second substrate; **E2B** is the modified enzyme-second substrate complex; and **X**, **Y**, and **Z** are the reaction products.

#### **Define set of polynomials to represent reaction scheme**

```
> restart:with(grobner):
```

Rate (v) of overall reaction

```
> eq1:=v-k[4]*ES2+k[-4]*E*Y*Z:eq1;
```

Formation of ES

```
> eq2:=k[1]*E*A+k[-2]*E2*X-(k[2]+k[-1])*ES:eq2;
```

Formation of E2

```
> eq3:=k[2]*ES+k[-3]*ES2-(k[3]*B+k[-2]*X)*E2:eq3;
```

Formation of ES2

```
> eq4:=k[3]*E2*B+k[-4]*E*Y*Z-(k[-3]+k[4])*ES2:eq4;
```



Conservation of enzyme

> eq5:=Et-E-ES-E2-ES2: eq5;

$$v - k_4 ES2 + k_4 E YZ$$

$$k_1 E A + k_2 E2 X - (k_2 + k_{-1}) ES$$

$$k_2 ES + k_3 ES2 - (k_3 B + k_{-2} X) E2$$

$$k_3 E2 B + k_4 E YZ - (k_3 + k_4) ES2$$

$$Et - E - ES - E2 - ES2$$

**Determine Groebner Bases and solve for v**

> Bases:=gbasis([eq1,eq2,eq3,eq4,eq5],[E,ES,E2,ES2,v],plex);

$$Bases := [XYZ k_4 k_2 E k_{-1} + XYZ k_4 k_2 k_3 E - X k_2 k_{-1} k_3 Et + X k_2 k_{-1} k_3 E$$

$$+ X k_2 k_{-1} k_4 E - X k_2 k_{-1} k_4 Et + X k_2 k_1 E A k_3 + X k_2 A k_1 k_4 E + YZ k_4 k_2 k_3 E$$

$$+ YZ k_4 k_2 E k_3 B + YZ k_4 k_{-1} k_3 E + YZ k_4 k_{-1} E k_3 B + k_2 k_1 E A k_3 + k_2 E k_1 A k_3 B$$

$$+ k_2 A k_1 k_4 E + k_2 k_4 E k_3 B - k_2 k_4 k_3 B Et - k_{-1} k_4 k_3 B Et + k_{-1} k_4 E k_3 B$$

$$+ A k_1 k_4 E k_3 B, XYZ k_4 k_2 ES k_{-1} + XYZ k_4 k_2 k_3 ES - XYZ k_4 k_2 k_3 Et$$

$$+ X k_2 k_{-1} k_3 ES + X k_2 k_{-1} k_4 ES - X k_2 k_3 k_1 A Et + X k_2 k_3 A k_1 ES$$

$$+ X k_2 A k_1 k_4 ES - X k_2 k_1 A k_4 Et + YZ k_4 k_2 k_3 ES + YZ k_4 k_2 ES k_3 B$$

$$+ YZ k_4 k_{-1} k_3 ES + YZ k_4 k_{-1} ES k_3 B + k_2 k_3 A k_1 ES + k_2 A k_1 k_4 ES + k_2 k_4 ES k_3 B$$

$$+ k_2 ES k_1 A k_3 B + k_4 ES k_3 B k_{-1} - k_3 B k_1 A k_4 Et + A k_1 k_4 ES k_3 B, k_4 YZ k_{-1} k_2 E2 X$$

$$+ XYZ k_4 k_2 k_3 E2 + X k_2 k_{-1} k_3 E2 + X k_2 k_{-1} k_4 E2 + X k_2 A k_1 E2 k_3$$

$$+ X k_2 A k_1 k_4 E2 + YZ k_4 k_{-1} k_3 E2 - YZ k_4 k_{-1} k_3 Et + k_4 YZ k_{-1} k_3 E2 B$$

$$- YZ k_4 k_3 k_2 Et + YZ k_4 k_3 k_2 E2 + k_4 YZ k_2 k_3 E2 B + k_4 k_3 E2 B k_{-1} + k_3 k_2 k_1 A E2$$

$$- k_3 k_2 k_1 A Et + A k_1 k_4 k_3 E2 B + k_2 k_1 A k_3 E2 B - k_2 k_1 A k_4 Et + k_4 k_2 k_3 E2 B$$

$$+ k_2 k_1 A k_4 E2, -XYZ k_4 k_2 k_{-1} Et + XYZ k_4 k_2 k_{-1} ES2 + XYZ k_4 k_2 k_3 ES2$$

$$+ X k_2 k_{-1} k_3 ES2 + X k_2 k_{-1} k_4 ES2 + X k_2 A k_1 ES2 k_3 + X k_2 A k_1 k_4 ES2$$

$$+ k_4 YZ k_{-1} k_3 ES2 - YZ k_4 k_{-1} k_3 B Et + YZ k_4 k_{-1} k_3 B ES2 + k_4 YZ k_2 k_3 ES2$$

$$- YZ k_4 k_2 k_3 B Et + YZ k_4 k_2 k_3 B ES2 + B k_3 k_4 ES2 k_{-1} + k_2 k_1 A ES2 k_3$$

$$+ k_3 B k_1 A k_4 ES2 + k_2 k_1 A ES2 k_4 + B k_2 k_3 k_4 ES2 + k_2 k_1 A k_3 B ES2 - k_2 k_1 A k_3 B Et$$

$$, XYZ k_4 k_2 k_{-1} k_3 Et + k_4 YZ k_{-1} k_2 X v + XYZ k_4 k_2 k_3 v + X k_2 k_3 k_{-1} v$$

$$+ X k_2 k_{-1} k_4 v + X k_2 k_1 A k_3 v + X k_2 k_1 A k_4 v + k_4 YZ k_2 k_3 B v + YZ k_4 k_2 k_3 v$$

$$+ k_4 YZ k_{-1} k_3 B v + YZ k_4 k_3 k_{-1} v + k_2 k_3 B k_4 v + k_2 k_1 A k_3 B v + k_2 k_1 A k_3 v$$

$$+ k_2 k_1 A k_4 v - k_2 k_3 B k_1 A k_4 Et + k_3 B k_4 k_{-1} v + k_3 B k_1 A k_4 v]$$

> eq6:=solve(Bases[5],v):eq6;

> eq7:=expand(eq6):eq7;

$$\begin{aligned}
 & -Et(k_{-4}YZk_{-1}k_{-2}Xk_{-3} - k_3Bk_2k_1Ak_4) / (k_{-4}YZk_{-1}k_{-2}X + XYZk_{-4}k_{-2}k_{-3} + Xk_{-1}k_{-2}k_{-3} \\
 & + Xk_{-2}k_{-1}k_4 + XAk_{-2}k_1k_4 + XAk_1k_{-2}k_{-3} + k_{-4}YZk_{-1}k_3B + k_{-4}YZk_{-1}k_{-3} \\
 & + k_{-4}YZk_2k_{-3} + k_{-4}YZk_2k_3B + Bk_3k_4k_{-1} + k_2k_1Ak_3B + k_3Bk_1Ak_4 + Bk_2k_3k_4 \\
 & + k_2k_1Ak_{-3} + k_2k_1Ak_4) \\
 & -Et k_{-4}YZk_{-1}k_{-2}Xk_{-3} / (k_{-4}YZk_{-1}k_{-2}X + XYZk_{-4}k_{-2}k_{-3} + Xk_{-1}k_{-2}k_{-3} + Xk_{-2}k_{-1}k_4 \\
 & + XAk_{-2}k_1k_4 + XAk_1k_{-2}k_{-3} + k_{-4}YZk_{-1}k_3B + k_{-4}YZk_{-1}k_{-3} + k_{-4}YZk_2k_{-3} \\
 & + k_{-4}YZk_2k_3B + Bk_3k_4k_{-1} + k_2k_1Ak_3B + k_3Bk_1Ak_4 + Bk_2k_3k_4 + k_2k_1Ak_{-3} \\
 & + k_2k_1Ak_4) + Et k_3Bk_2k_1Ak_4 / (k_{-4}YZk_{-1}k_{-2}X + XYZk_{-4}k_{-2}k_{-3} + Xk_{-1}k_{-2}k_{-3} \\
 & + Xk_{-2}k_{-1}k_4 + XAk_{-2}k_1k_4 + XAk_1k_{-2}k_{-3} + k_{-4}YZk_{-1}k_3B + k_{-4}YZk_{-1}k_{-3} \\
 & + k_{-4}YZk_2k_{-3} + k_{-4}YZk_2k_3B + Bk_3k_4k_{-1} + k_2k_1Ak_3B + k_3Bk_1Ak_4 + Bk_2k_3k_4 \\
 & + k_2k_1Ak_{-3} + k_2k_1Ak_4)
 \end{aligned}$$

### Simplification by reasonable assumption

Assume formation of E2 + product aldehyde(X) and E + products peroxide and ammonia (Y, Z) are irreversible

> k[-2]:=0;k[-4]:=0;eq7;

$$k_{-2} := 0$$

$$k_{-4} := 0$$

$$\frac{Et k_3 B k_2 k_1 A k_4}{B k_3 k_4 k_{-1} + k_2 k_1 A k_{-3} + k_2 k_1 A k_3 B + B k_2 k_3 k_4 + k_2 k_1 A k_4 + k_3 B k_1 A k_4}$$

### Simplification by rearrangement and substitution

Define Vmax as k4 \* Et

> eq8:=algsubs(k[4]\*Et=V[max],eq7):eq8;

$$\frac{k_2 k_1 A k_3 B V_{max}}{k_2 k_1 A k_{-3} + k_2 k_1 A k_3 B + (k_{-1} k_3 B + k_2 k_3 B + A k_2 k_1 + k_3 B k_1 A) k_4}$$

Simplify by division of numerator and denominator by k1 \* k2 \* k3

> eq9:=numer(eq8)/(k[1]\*k[2]\*k[3]):eq9;

$$A B V_{max}$$

> eq10:=denom(eq8)/(k[1]\*k[2]\*k[4]):eq10;

$$\frac{B k_3 k_4 k_{-1} + k_2 k_1 A k_{-3} + k_2 k_1 A k_3 B + B k_2 k_3 k_4 + k_2 k_1 A k_4 + k_3 B k_1 A k_4}{k_2 k_1 k_4}$$

> eq11:=collect(eq10,A):eq11;

$$\frac{(k_1 k_2 k_3 + B k_1 k_3 k_2 + k_2 k_1 k_4 + B k_4 k_1 k_3) A}{k_2 k_1 k_4} + \frac{B k_3 k_4 k_{-1} + B k_2 k_3 k_4}{k_2 k_1 k_4}$$

> eq12:=collect(eq11,B):eq12;

$$\left( \frac{(k_4 k_1 k_3 + k_1 k_3 k_2) A}{k_2 k_1 k_4} + \frac{k_4 k_3 k_2 + k_4 k_3 k_{-1}}{k_2 k_1 k_4} \right) B + \frac{(k_1 k_2 k_3 + k_2 k_1 k_4) A}{k_2 k_1 k_4}$$

> eq13:=subs((k[2]\*k[1]\*k[-

3]+k[1]\*k[2]\*k[4])/k[1]/k[2]/k[4]\*A=K[1]\*A,eq12):eq13;

$$\left( \frac{(k_4 k_1 k_3 + k_1 k_3 k_2) A}{k_2 k_1 k_4} + \frac{k_4 k_3 k_2 + k_4 k_3 k_{-1}}{k_2 k_1 k_4} \right) B + A K_1$$

> eq14:=subs((k[4]\*k[3]\*k[2]+k[4]\*k[3]\*k[-

1])/k[4]/k[1]/k[2]=K[2],eq13):eq14;

$$\left( \frac{(k_4 k_1 k_3 + k_1 k_3 k_2) A}{k_2 k_1 k_4} + K_2 \right) B + A K_1$$

>

eq15:=subs((k[3]\*k[2]\*k[1]+k[3]\*k[4]\*k[1])/k[4]/k[2]/k[1]\*A

=K[3]\*A,eq14):eq15;

$$(K_3 A + K_2) B + A K_1$$

> eq16:=sort(expand(eq15),[K[1],K[2],K[3]]):eq16;

$$A K_1 + B K_2 + B A K_3$$

**Combine the numerator and denominator for complete rate equation:**

> eq17:=v=eq9/eq16:eq17;

$$v = \frac{A B V_{max}}{A K_1 + B K_2 + B A K_3}$$

Where:> K[1]=(k[-3]\*k[2]\*k[1]+k[4]\*k[2]\*k[1])/k[4]/k[2]/k[1];

> K[2]=(k[4]\*k[3]\*k[2]+k[4]\*k[3]\*k[-1])/k[4]/k[1]/k[2];

> K[3]=(k[3]\*k[2]\*k[1]+k[3]\*k[4]\*k[1])/k[4]/k[2]/k[1];

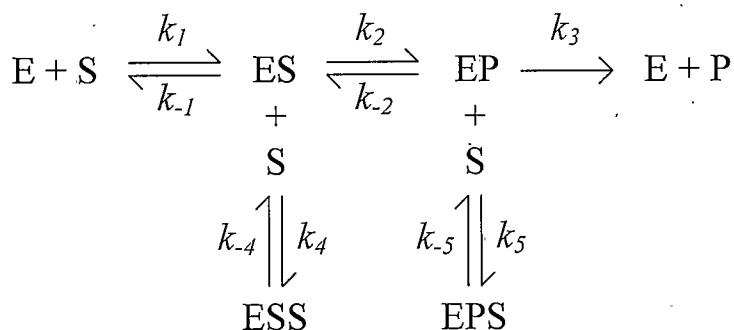
$$K_1 = \frac{k_1 k_2 k_3 + k_2 k_1 k_4}{k_2 k_1 k_4}$$

$$K_2 = \frac{k_4 k_3 k_2 + k_4 k_3 k_{-1}}{k_2 k_1 k_4}$$

$$K_3 = \frac{k_4 k_1 k_3 + k_1 k_3 k_2}{k_2 k_1 k_4}$$

### Reversible Substrate Inhibition at Two Steps in Mechanism

The simple model of substrate inhibition above is a greatly simplified scheme for the catalytic cycle of copper amine oxidases, nor does it fit the substrate inhibition data for rhKDAO with histamine or agmatine. One can speculate that an excess of substrate might result in substrate binding during additional steps during the catalytic cycle and thus might inhibit the overall reaction at multiple steps. To investigate this I constructed a model in which substrate inhibits two step in the mechanism. The rhKDAO steady state data was then fit to the derived rate equation.



Where **E** is the resting form of the Enzyme; **S** is the Substrate; **ES** is the Enzyme-Substrate complex; **ESS** is the Enzyme-Substrate-Substrate complex; **EP** is a modified form of the Enzyme; **EPS** is a modified form of the Enzyme with bound Substrate; and **P** is the Product.

```
> restart:with (grobner):
```

```
Define set of polynomials that represent reaction scheme
rate of reaction
```

```
> eq1:=v-k[3]*EP:eq1;
```

ES at steady state

> eq2 := k[1] \* E \* S + k[-2] \* EP + k[-4] \* ESS - (k[1] + k[2] + k[4] \* S) \* ES : eq2;

EP at steady state

> eq3 := k[2] \* ES + k[-5] \* EPS - (k[-2] + k[3] + k[5] \* S) \* EP : eq3;

ESS substrate inhibited complex at steady state

> eq4 := k[4] \* ES \* S - k[-4] \* ESS : eq4;

EPS substrate inhibited complex at steady state

> eq5 := k[5] \* EP \* S - k[-5] \* EPS : eq5;

Conservation of enzyme

> eq6 := Et - E - ES - ESS - EP - EPS : eq6;  

$$v - k_3 EP$$

$$k_1 ES + k_2 EP + k_4 ESS - (k_1 + k_2 + k_4 S) ES$$

$$k_2 ES + k_5 EPS - (k_2 + k_3 + k_5 S) EP$$

$$k_4 ESS - k_4 ES S$$

$$k_5 EPS - k_5 EP S$$

$$Et - E - ES - ESS - EP - EPS$$

### Determine Groebner Bases

> Bases := gbasis([eq1, eq2, eq3, eq4, eq5, eq6], [E, ES, ESS, EP, EPS, v], plex);

Bases :=  $[k_2 k_1 E S^2 k_{-4} k_5 + k_1 E S^2 k_{-5} k_4 k_{-2} + k_3 k_1 E S^2 k_{-5} k_4 + k_3 E k_{-4} k_{-1} k_{-5}$   
 $+ E k_{-5} k_{-4} k_{-1} k_{-2} + k_3 k_2 E k_{-4} k_{-5} + k_2 k_1 E S k_{-5} k_{-4} + k_1 E S k_{-5} k_{-4} k_{-2} + k_3 k_1 E S k_{-4} k_{-5}$   
 $- k_{-1} k_{-2} k_{-5} k_{-4} Et - k_3 k_{-1} k_{-5} k_{-4} Et - k_3 k_2 k_{-5} k_{-4} Et, k_2 ES k_{-4} k_{-1} S^2 k_5 + ES k_{-5} k_{-1} S^2 k_4 k_{-2}$   
 $+ k_3 ES k_{-5} k_{-1} S^2 k_4 + k_3 ES k_{-4} k_{-1} k_{-5} + ES k_{-5} k_{-4} k_{-1} k_{-2} + k_3 k_2 ES k_{-4} k_{-5}$   
 $+ k_2 ES k_{-5} k_{-4} k_{-1} S + ES k_{-5} k_{-4} k_{-1} S k_{-2} + k_3 ES k_{-4} k_{-1} S k_{-5} - k_{-5} k_3 k_{-4} k_{-1} S Et$   
 $- k_2 k_{-5} k_{-4} k_{-1} S Et, k_2 k_{-4} ESS k_{-1} S^2 k_5 + ESS k_{-5} k_{-1} S^2 k_4 k_{-2} + k_3 ESS k_{-5} k_{-1} S^2 k_4$   
 $+ k_3 k_{-4} ESS k_{-1} k_{-5} + k_{-4} ESS k_{-5} k_{-1} k_{-2} + k_3 k_2 k_{-4} ESS k_{-5} + k_2 k_{-4} ESS k_{-5} k_{-1} S$   
 $+ k_{-4} ESS k_{-5} k_{-1} S k_{-2} + k_3 k_{-4} ESS k_{-1} S k_{-5} - k_4 S^2 k_3 k_{-5} k_{-1} Et - k_4 S^2 k_{-2} k_{-5} k_{-1} Et,$   
 $EP k_{-4} k_2 k_{-1} S^2 k_5 + EP k_{-5} k_{-1} S^2 k_4 k_{-2} + k_3 EP k_{-5} k_{-1} S^2 k_4 + k_3 EP k_{-4} k_{-1} k_{-5}$   
 $+ EP k_{-5} k_{-4} k_{-1} k_{-2} + k_3 EP k_{-4} k_2 k_{-5} + EP k_{-5} k_{-4} k_2 k_{-1} S + EP k_{-5} k_{-4} k_{-1} S k_{-2}$



$$\begin{aligned}
& + k_3 EP k_4 k_1 S k_5 - k_5 k_4 k_2 k_1 S Et, EPS k_4 k_2 k_1 S^2 k_5 + k_5 EPS k_1 S^2 k_4 k_2 \\
& + k_3 k_5 EPS k_1 S^2 k_4 + k_3 k_5 EPS k_4 k_1 + k_5 EPS k_4 k_1 k_2 + k_3 k_5 EPS k_4 k_2 \\
& + k_5 EPS k_4 k_2 k_1 S + k_5 EPS k_4 k_1 S k_2 + k_3 k_5 EPS k_4 k_1 S - k_5 S^2 k_4 k_2 k_1 Et, \\
& k_4 k_2 k_1 S^2 k_5 v + k_5 k_1 S^2 k_4 k_2 v + k_5 k_1 S^2 k_4 k_3 v + k_5 k_4 k_3 k_1 v + k_5 k_4 k_1 k_2 v \\
& + k_5 k_3 k_2 k_4 v - k_5 k_3 k_4 k_2 k_1 S Et + k_5 k_4 k_2 k_1 S v + k_5 k_4 k_1 S k_2 v + k_5 k_4 k_3 k_1 S v]
\end{aligned}$$

**Solve Groebner Bases for reaction rate, v**

> eq6:=solve(Bases[6],v):eq6;

$$\begin{aligned}
& k_5 k_3 k_4 k_2 k_1 S Et / (k_4 k_2 k_1 S^2 k_5 + k_5 k_1 S^2 k_4 k_2 + k_5 k_1 S^2 k_4 k_3 + k_3 k_4 k_1 k_5 \\
& + k_5 k_4 k_1 k_2 + k_3 k_4 k_2 k_5 + k_5 k_4 k_2 k_1 S + k_5 k_4 k_1 S k_2 + k_3 k_4 k_1 S k_5)
\end{aligned}$$

**Simplification**

Define Vmax as k3 \* Et

> eq7:=algsubs(k[3]\*Et=v[max],eq6):eq7;

$$\begin{aligned}
& k_5 k_4 k_2 k_1 S V_{max} / (k_4 k_2 k_1 S^2 k_5 + k_5 k_1 S^2 k_4 k_2 + k_5 k_4 k_2 k_1 S + k_5 k_4 k_1 S k_2 \\
& + k_5 k_4 k_1 k_2 + (k_4 k_2 k_5 + k_5 k_1 S^2 k_4 + k_4 k_1 k_5 + k_4 k_1 S k_5) k_3)
\end{aligned}$$

Simplify by dividing numerator and denominator by k-5 \* k-4 \* k2 \* k1

> eq8:=numer(eq7)/(k[-5]\*k[-4]\*k[2]\*k[1]):eq8;

$$S V_{max}$$

> eq9:=denom(eq7)/(k[-5]\*k[-4]\*k[2]\*k[1]):eq9;

$$\begin{aligned}
& (k_4 k_2 k_1 S^2 k_5 + k_5 k_1 S^2 k_4 k_2 + k_5 k_1 S^2 k_4 k_3 + k_3 k_4 k_1 k_5 + k_5 k_4 k_1 k_2 + k_3 k_4 k_2 k_5 \\
& + k_5 k_4 k_2 k_1 S + k_5 k_4 k_1 S k_2 + k_3 k_4 k_1 S k_5) / (k_2 k_4 k_1 k_5)
\end{aligned}$$

Simplify by rearrangement and substitution

> eq10:=collect(expand(eq9),S):eq10;

$$\left( \frac{k_4 k_2}{k_2 k_4} + \frac{k_4 k_3}{k_2 k_4} + \frac{k_5}{k_5} \right) S^2 + \left( 1 + \frac{k_3}{k_2} + \frac{k_2}{k_2} \right) S + \frac{k_1 k_2}{k_2 k_1} + \frac{k_3}{k_1} + \frac{k_3 k_1}{k_2 k_1}$$

> eq11:=algsubs(1/k[1]\*k[3]+1/k[2]/k[1]\*k[-1]\*k[-2]+1/k[2]/k[1]\*k[3]\*k[-1]=K[1],eq10):eq11;

$$\left( \frac{k_4 k_2}{k_2 k_4} + \frac{k_4 k_3}{k_2 k_4} + \frac{k_5}{k_5} \right) S^2 + \left( 1 + \frac{k_3}{k_2} + \frac{k_2}{k_2} \right) S + K_1$$

> eq12:=algsubs(1/k[2]\*k[3]+1/k[2]\*k[-2]=K[2],eq11):eq12;

$$\frac{S^2 (k_4 K_2 k_5 + k_5 k_4)}{k_4 k_5} + S(1 + K_2) + K_1$$

> eq13:=expand(eq12):eq13;

$$\frac{S^2 k_4 K_2}{k_{-4}} + \frac{S^2 k_5}{k_{-5}} + S + S K_2 + K_1$$

> eq14:=collect(algsubs(k[4]/k[-4]=1/K[iA],eq13),S):eq14;

$$\frac{(K_2 k_{-5} + k_5 K_{iA}) S^2}{K_{iA} k_{-5}} + \frac{(K_{iA} k_{-5} + K_2 K_{iA} k_{-5}) S}{K_{iA} k_{-5}} + K_1$$

> eq15:=expand(collect(eq14,S)):eq15;

$$\frac{S^2 K_2}{K_{iA}} + \frac{S^2 k_5}{k_{-5}} + S + S K_2 + K_1$$

> eq16:=expand(algsubs(k[5]/k[-5]=1/K[iB],eq15)):eq16;

$$\frac{S^2 K_2}{K_{iA}} + \frac{S^2}{K_{iB}} + S + S K_2 + K_1$$

**Combine numerator and denominator for complete rate equation**

> eq15:=v=eq8/eq16:eq15;

$$v = \frac{S V_{max}}{\frac{S^2 K_2}{K_{iA}} + \frac{S^2}{K_{iB}} + S + S K_2 + K_1}$$

> eq17:=K[iA]=k[-4]/k[4]:eq17;

> eq18:=K[iB]=k[-5]/k[5]:eq18;

> eq19:=K[1]=1/k[1]\*k[3]+1/k[2]/k[1]\*k[-1]\*k[-2]+1/k[2]/k[1]\*k[3]\*k[-1]:eq19;

> eq20:=K[2]=1+1/k[2]\*k[3]+1/k[2]\*k[-2]:eq20;

Where:

$$K_{iA} = \frac{k_{-4}}{k_4}$$

$$K_{iB} = \frac{k_{-5}}{k_5}$$

$$K_1 = \frac{k_{-1} k_{-2}}{k_2 k_1} + \frac{k_3}{k_1} + \frac{k_3 k_{-1}}{k_2 k_1}$$

$$K_2 = 1 + \frac{k_3}{k_2} + \frac{k_{-2}}{k_2}$$

Further simplification in which terms  $K_1A$  and  $K_1B$  are combined into a single constant,  $K_3$

> eq21:=eq10:eq21;

$$\left( \frac{k_4 k_3}{k_{-4} k_2} + \frac{k_5}{k_{-5}} + \frac{k_4 k_{-2}}{k_{-4} k_2} \right) S^2 + \left( 1 + \frac{k_3}{k_2} + \frac{k_{-2}}{k_2} \right) S + \frac{k_3 k_{-1}}{k_2 k_1} + \frac{k_3}{k_1} + \frac{k_{-1} k_{-2}}{k_2 k_1}$$

> eq22:=collect(algsbss(1/k[-4]/k[2]\*k[4]\*k[3]+k[5]/k[-5]+1/k[-4]/k[2]\*k[4]\*k[-2]=K[3],eq21),S):eq22;

$$S^2 K_3 + \left( 1 + \frac{k_3}{k_2} + \frac{k_{-2}}{k_2} \right) S + \frac{k_3 k_{-1}}{k_2 k_1} + \frac{k_3}{k_1} + \frac{k_{-1} k_{-2}}{k_2 k_1}$$

> eq23:=collect(algsbss(1/k[2]/k[1]\*k[3]\*k[-1]+1/k[1]\*k[3]+1/k[2]/k[1]\*k[-1]\*k[-2]=K[1],eq22),S):eq23;

$$\left( 1 + \frac{k_3}{k_2} + \frac{k_{-2}}{k_2} \right) S + K_1 + S^2 K_3$$

> eq24:=sort(expand(algsbss(1/k[2]\*k[3]+1/k[2]\*k[-2]=K[2],expand(eq23))),S):eq24;

$$K_3 S^2 + S + K_2 S + K_1$$

Combine numerator and denominator to solve complete rate equation

> eq25:=eq8/eq24:eq25;

$$\frac{S V_{max}}{K_3 S^2 + S + K_2 S + K_1}$$

> eq26:=K[1]=1/k[2]/k[1]\*k[3]\*k[-1]+1/k[1]\*k[3]+1/k[2]/k[1]\*k[-1]\*k[-2]:eq26;

> eq27:=K[2]=1/k[2]\*k[3]+1/k[2]\*k[-2]:eq27;

> eq28:=K[3]=1/k[-4]/k[2]\*k[4]\*k[3]+k[5]/k[-5]+1/k[-4]/k[2]\*k[4]\*k[-2]:eq28;

Where:

$$K_1 = \frac{k_3 k_{-1}}{k_2 k_1} + \frac{k_3}{k_1} + \frac{k_{-1} k_{-2}}{k_2 k_1}$$

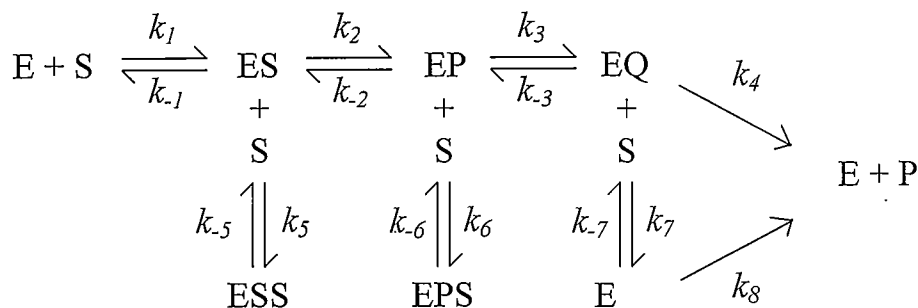
$$K_2 = \frac{k_{-2}}{k_2} + \frac{k_3}{k_2}$$

$$K_3 = \frac{k_4 k_3}{k_{-4} k_2} + \frac{k_5}{k_{-5}} + \frac{k_4 k_{-2}}{k_{-4} k_2}$$

### Substrate Inhibition at Two Enzyme Forms and an Alternative Path to Product

This mechanistic scheme was concocted in an effort fit the data of rhKDAO oxidation of histamine and agmatine. In this case, substrate acts as a reversible inhibitor at two forms of the enzyme and also provides an alternative means of product formation.

Unfortunately the derived rate equation proved to be too complex to fit to the data. However, this exercise demonstrates the use of Gröbner Bases and Maple software to derive rate equations for fairly complex mechanistic schemes. Maple commands are given, but the majority of Maple output is not shown.



Where **E** is the resting form of the Enzyme; **S** is the Substrate; **ES** is Enzyme-Substrate complex; **ESS** is the Enzyme-Substrate-Substrate complex; **EP** is a modified form of the Enzyme; **EPS** is a modified form of the Enzyme with bound Substrate; **EQ** is another modified form of the enzyme, **EQS** is a modified form of the Enzyme with bound Substrate, and **P** is the Product.

**> restart:with(grobner):**

**Write polynomial equations to represent reaction scheme**

Rate of formation of product P

> eq1:=-v+k[4]\*EQ+k[8]\*EQS:eq1;  

$$-v + k_4 EQ + k_8 EQS$$

ES at steady state

> eq2:=k[1]\*E\*S+k[-2]\*EP+k[-5]\*ESS-(k[-1]+k[2]+k[5]\*S)\*ES:eq2;  

$$k_1 ES + k_2 EP + k_5 ESS - (k_1 + k_2 + k_5 S) ES$$

ESS at steady state

> eq3:=k[5]\*ES\*S-k[-5]\*ESS:eq3;  

$$k_5 ESS - k_5 ESS$$

EP at steady state

> eq4:=k[2]\*ES+k[-3]\*EQ+k[-6]\*EPS-(k[-2]+k[3]+k[6]\*S)\*EP:eq4;  

$$k_2 ES + k_3 EQ + k_6 EPS - (k_2 + k_3 + k_6 S) EP$$

EPS at steady state

> eq5:=k[6]\*EP\*S-k[-6]\*EPS:eq5;  

$$k_6 EPS - k_6 EPS$$

EQ at steady state

> eq6:=k[3]\*EP+k[-7]\*EQS-(k[-3]+k[4]+k[7]\*S)\*EQ:eq6;  

$$k_3 EP + k_7 EQS - (k_3 + k_4 + k_7 S) EQ$$

EQS at steady state

> eq7:=k[7]\*EQ\*S-(k[-7]+k[8])\*EQS:eq7;  

$$k_7 EQS - (k_7 + k_8) EQS$$

Conservation of enzyme

> eq8:=Et-ES-ESS-EP-EPS-EQ-EQS:eq8;  

$$Et - ES - ESS - EP - EPS - EQ - EQS$$

**Determine Groebner Bases**

> eq9:Bases:=gbasis([eq1,eq2,eq3,eq4,eq5,eq6,eq7,eq8],  
 [E,ES,ESS,EP,EPS,EQ,EQS,v],plex);



**Solve for v**

```
> eq10:=solve(Bases[8],v):eq10;
> eq11:=expand(eq10):eq11;
```

**Simplification**

Separate numerator and define  $V_{\max 1} = k_4 * EQ$ ,  $V_{\max 2} = k_8 * EQS$  and  $V_{\max} = V_{\max 1} + V_{\max 2}$

```
> eq12:=expand(numer(algsubs(k[4]*Et=V[max1],eq11))):eq12;
> eq13:=algsubs(k[8]*Et=V[max2],eq12):eq13;
> eq14:=algsubs(V[max1]+V[max2]=V[max],eq13):eq14;
> eq15:=collect(eq14,V[max1]):eq15;
```

Combine numerator and denominator. Define  $K_1$  and  $K_2$  as composite rate constants

```
> eq16:=denom(eq11):eq16;
> eq17:=eq15/eq16:eq17;
> eq18:=algsubs(k[-6]*k[3]*k[-7]*k[-5]*k[2]+k[-6]*k[8]*k[3]*k[-5]*k[2]=K[1],eq17):eq18;
> eq19:=algsubs(k[-6]*k[3]*k[7]*S*k[-5]*k[2]=K[2],eq18):eq19;
```

Now simplification of denominator

```
> eq20:=collect(expand(denom(eq19)),S):eq20;
> eq21:=collect(algsubs(k[-6]*k[7]*k[8]*k[3]*k[5]+k[-6]*k[7]*k[8]*k[5]*k[-2]+k[-5]*k[2]*k[6]*k[7]*k[8]=K[3],eq20),S):eq21;
> eq22:=expand(eq21):eq22;
> eq23:=collect(eq22,S):eq23;
> eq24:=algsubs(k[3]*k[-6]*k[7]*k[8]*k[-5]+k[-6]*k[4]*k[8]*k[3]*k[5]+k[-5]*k[2]*k[6]*k[-3]*k[8]+k[-6]*k[7]*k[8]*k[-5]*k[-2]+k[-6]*k[4]*k[-7]*k[3]*k[5]+k[-6]*k[4]*k[8]*k[5]*k[-2]+k[-6]*k[8]*k[5]*k[-2]*k[-3]+k[-5]*k[2]*k[6]*k[4]*k[8]+k[-6]*k[7]*k[8]*k[-5]*k[2]+k[-5]*k[2]*k[6]*k[4]*k[-7]+k[-6]*k[4]*k[-7]*k[5]*k[-2]+k[-6]*k[-7]*k[5]*k[-2]*k[-3]+k[-5]*k[2]*k[6]*k[-3]*k[-7]=K[4],eq23):eq24;
> eq25:=collect(algsubs(k[-6]*k[4]*k[8]*k[-5]*k[-2]+k[3]*k[-6]*k[4]*k[8]*k[-5]+k[-6]*k[4]*k[-7]*k[-5]*k[2]+k[-6]*k[4]*k[-7]*k[-5]*k[-2]+k[3]*k[-6]*k[4]*k[-7]*k[-5]+k[-6]*k[-7]*k[-5]*k[-2]*k[-3]+k[-6]*k[-7]*k[-5]*k[2]*k[-3]+k[-6]*k[8]*k[-5]*k[-2]*k[-3]+k[-6]*k[4]*k[8]*k[-5]*k[2]+k[-6]*k[8]*k[-5]*k[2]*k[-3]=K[5],eq24),S):eq25;
```

Recombine numerator and denominator for complete rate equation

> eq26:=v=collect(numer(eq19)/eq25,S):eq26;

$$v = \frac{V_{max1} K_1 - V_{max1} K_2 + V_{max} K_2}{S K_4 + K_5 + S^2 K_3 + K_1 + K_2}$$

Where: > eq27:=V[max2]=k[8]\*Et:eq27;

> eq28:=V[max]=k[4]\*Et+k[8]\*Et:eq28;

> eq28:=K[1]=k[-6]\*k[3]\*k[-7]\*k[-5]\*k[2]

+k[-6]\*k[8]\*k[3]\*k[-5]\*k[2]:eq28;

> eq29:=K[2]=k[-6]\*k[3]\*k[7]\*S\*k[-5]\*k[2]:eq29;

> eq30:=K[3]=k[-6]\*k[7]\*k[8]\*k[3]\*k[5]+k[-6]\*

k[7]\*k[8]\*k[5]\*k[-2]+k[-5]\*k[2]\*k[6]\*k[7]\*k[8]:eq30;

> eq31:=K[4]=(k[3]\*k[-6]\*k[7]\*k[8]\*k[-5]+k[-6]\*k[4]\*k[8]

\*k[3]\*k[5]+k[-5]\*k[2]\*k[6]\*k[-3]\*k[8]+k[-6]\*k[7]\*k[8]

\*k[-5]\*k[-2]+k[-6]\*k[4]\*k[-7]\*k[3]\*k[5]+k[-6]\*k[4]\*k[8]

\*k[5]\*k[-2]+k[-6]\*k[8]\*k[5]\*k[-2]\*k[-3]+k[-5]\*k[2]\*k[6]

\*k[4]\*k[8]+k[-6]\*k[7]\*k[8]\*k[-5]\*k[2]+k[-5]\*k[2]\*k[6]

\*k[4]\*k[-7]+k[-6]\*k[4]\*k[-7]\*k[5]\*k[-2]+k[-6]\*k[-7]

\*k[5]\*k[-2]\*k[-3]+k[-5]\*k[2]\*k[6]\*k[-3]\*k[-7]):eq31;

> eq32:=K[5]=k[-6]\*k[4]\*k[8]\*k[-5]\*k[-2]+k[3]\*k[-6]\*k[4]

\*k[8]\*k[-5]+k[-6]\*k[4]\*k[-7]\*k[-5]\*k[2]+k[-6]\*k[4]\*k[-7]

\*k[-5]\*k[-2]+k[3]\*k[-6]\*k[4]\*k[-7]\*k[-5]+k[-6]\*k[-7]

\*k[-5]\*k[-2]\*k[-3]+k[-6]\*k[-7]\*k[-5]\*k[2]\*k[-3]+k[-6]

\*k[8]\*k[-5]\*k[-2]\*k[-3]+k[-6]\*k[4]\*k[8]\*k[-5]\*k[2]

+k[-6]\*k[8]\*k[-5]\*k[2]\*k[-3]:eq32;

$$V_{max2} = k_8 Et$$

$$V_{max} = k_4 Et + k_8 Et$$

$$K_1 = k_{-6} k_8 k_3 k_{-5} k_2 + k_{-6} k_3 k_{-7} k_{-5} k_2$$

$$K_2 = k_{-6} k_3 k_7 S k_{-5} k_2$$

$$K_3 = k_{-6} k_7 k_8 k_3 k_5 + k_{-6} k_7 k_8 k_5 k_{-2} + k_{-5} k_2 k_6 k_7 k_8$$

$$K_4 = k_{-6} k_7 k_8 k_{-5} k_{-2} + k_{-6} k_4 k_{-7} k_5 k_{-2} + k_{-5} k_2 k_6 k_{-3} k_{-7} + k_{-6} k_4 k_{-7} k_3 k_5 + k_{-6} k_4 k_8 k_5 k_{-2} \\ + k_{-6} k_4 k_8 k_3 k_5 + k_3 k_{-6} k_7 k_8 k_{-5} + k_{-6} k_{-7} k_5 k_{-2} k_{-3} + k_{-6} k_7 k_8 k_{-5} k_2 + k_{-6} k_8 k_5 k_{-2} k_{-3} \\ + k_{-5} k_2 k_6 k_{-3} k_8 + k_{-5} k_2 k_6 k_4 k_{-7} + k_{-5} k_2 k_6 k_4 k_8$$

$$K_5 = k_{-6} k_4 k_8 k_{-5} k_{-2} + k_3 k_{-6} k_4 k_8 k_{-5} + k_{-6} k_{-7} k_{-5} k_2 k_{-3} + k_{-6} k_{-7} k_{-5} k_{-2} k_{-3} + k_3 k_{-6} k_4 k_{-7} k_{-5} \\ + k_{-6} k_4 k_{-7} k_{-5} k_2 + k_{-6} k_8 k_{-5} k_2 k_{-3} + k_{-6} k_4 k_8 k_{-5} k_2 + k_{-6} k_8 k_{-5} k_{-2} k_{-3} + k_{-6} k_4 k_{-7} k_{-5} k_{-2}$$

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