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 m Biochemistry
Montana State University

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.

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APPROVAL

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Bradley Owen Elmore

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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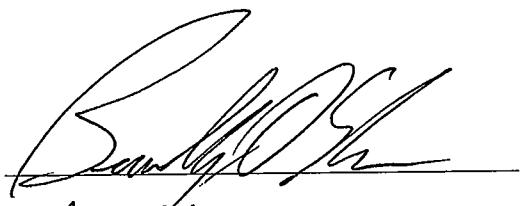
Signature 

Date October 21, 2002

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_B. Ribulose-1,5-bisphosphate carboxylase (rubisco), responsible for the first step of photosynthetic CO_2 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 tyrptophylquinone (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.

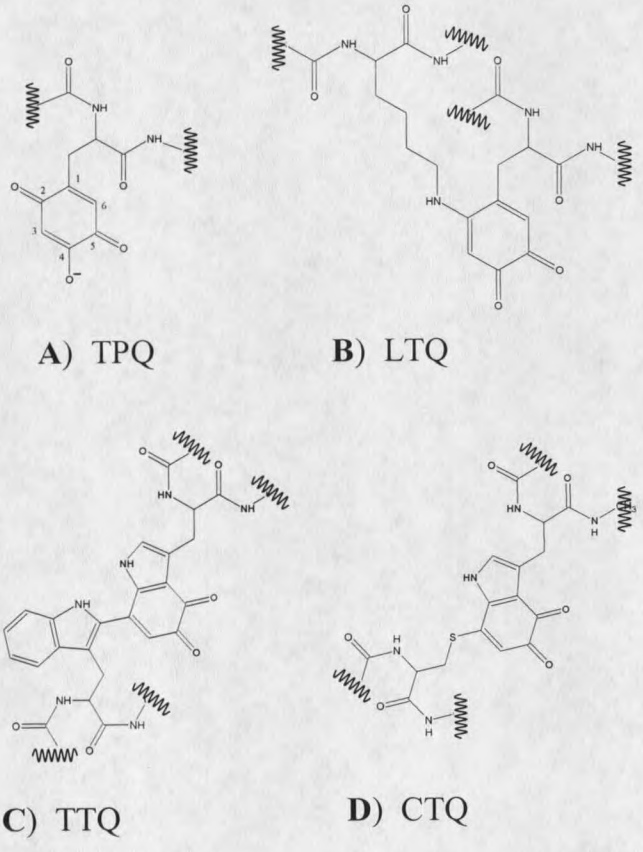

Figure 1. Post-translationally modified quinone cofactors; **A**) 2,4-trihydroxyphenylalanine quinone, TPQ; **B**) lysyl tyrosylquinone, LTQ; **C**) tryptophan tyrphophylquinone, TTQ; **D**) cysteine tryptophyl quinone, CTQ

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 λ_{max} values among CAOs from different sources are thought to reflect the degree of charge localization [10].

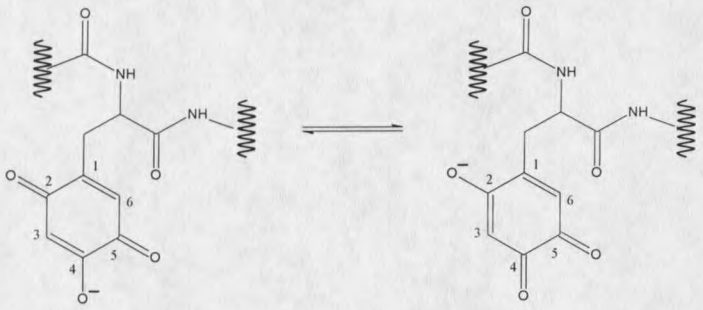

Figure 2. TPQ resonance structures

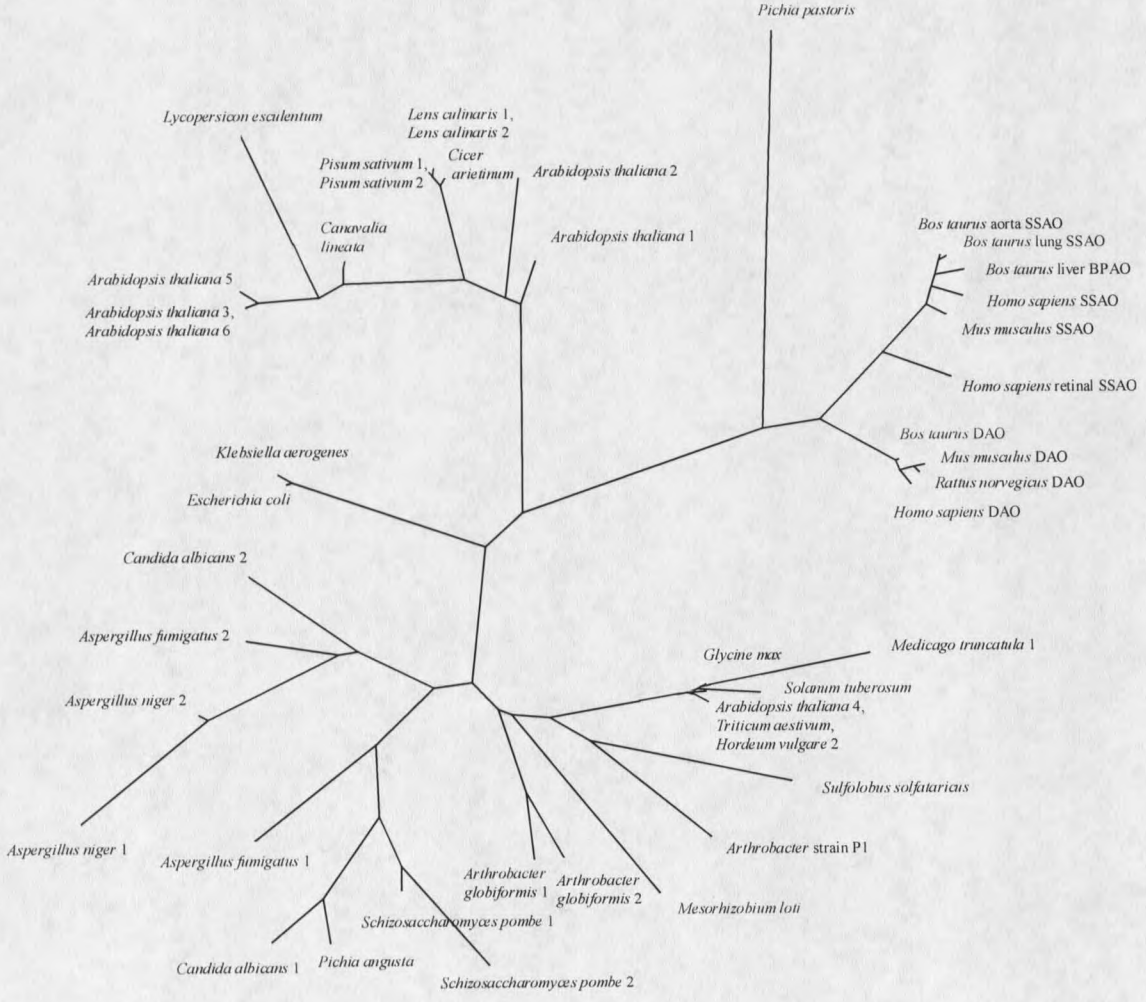

Figure 3. Phylogenetic tree for the copper amine oxidases

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 *Eschericia 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) β-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 α-helix and a four-stranded antiparallel β-sheet. A channel for substrate access and product exit lies between domains D3 and D4.