Cloning, sequence analysis, and characterization of the lysyl oxidase from *Pichia pastoris*
by Jason Andrew Kuchar

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
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Abstract:

Lysyl oxidase from *Pichia pastoris* has been successfully isolated, sequenced, cloned, and over-expressed. EPR and resonance Raman experiments have shown that copper and TPQ are present, respectively. Lysyl oxidase from *P. pastoris* has a similar substrate specificity to the mammalian enzyme (both have been shown to oxidize peptidyl lysine residues) and is 30% identical to the human kidney diamine oxidase, KDAO (the highest of any non-mammalian source). PPLO also has a relatively broad substrate specificity compared to other amine oxidases. It has been demonstrated that it can oxidize recombinant human tropoelastin, the *in vivo* substrate of lysyl oxidase. Molecular modeling data suggest that the substrate channel in lysyl oxidase from *P. pastoris* permits greater active site access than observed in structurally-characterized amine oxidases. This larger channel may account for the diversity of substrates that are turned over by this enzyme.

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APPROVAL

of a dissertation submitted by

Jason Andrew Kuchar

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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ABBREVIATIONS

ABTS - 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid

AGAO - *Arthrobacter globiformis* amine oxidase

BALO - bovine aorta lysyl oxidase

BPAO - bovine plasma amine oxidase

DLLO - *Drosophila melangaster* lysyl oxidase

ECAO - *Escherichia coli* amine oxidase

EPAO - equine plasma amine oxidase

HCTL - homocysteine thiolactone

HSAO - human amine oxidase

KDAO - human kidney diamine oxidase

LTQ - lysine tyrosylquinone

PAAO - *Pichia angusta* (previously *Hansenula polymorpha*) amine oxidase

PSAO - *Pisum sativum* amine oxidase

RKAO - rat amiloride binding protein

TPQ - topa quinone

ABSTRACT

Lysyl oxidase from *Pichia pastoris* has been successfully isolated, sequenced, cloned, and over-expressed. EPR and resonance Raman experiments have shown that copper and TPQ are present, respectively. Lysyl oxidase from *P. pastoris* has a similar substrate specificity to the mammalian enzyme (both have been shown to oxidize peptidyl lysine residues) and is 30% identical to the human kidney diamine oxidase, KDAO (the highest of any non-mammalian source). PPLO also has a relatively broad substrate specificity compared to other amine oxidases. It has been demonstrated that it can oxidize recombinant human tropoelastin, the *in vivo* substrate of lysyl oxidase. Molecular modeling data suggest that the substrate channel in lysyl oxidase from *P. pastoris* permits greater active site access than observed in structurally-characterized amine oxidases. This larger channel may account for the diversity of substrates that are turned over by this enzyme.

INTRODUCTION

Overview of Amine Oxidases

Amine oxidases can be divided into two broad classes: those that are flavin containing enzymes (EC 1.4.3.4) and those that have copper and a covalently attached quinone cofactor, designated topa quinone (TPQ) (EC 1.4.3.6). A review of the flavin enzymes, which have no sequence homology to the copper containing enzymes, can be found elsewhere (1). This dissertation discusses the copper amine oxidases, specifically, the "lysyl oxidase" from *Pichia pastoris*. Thus, future reference to amine oxidases will be assumed to mean the copper-containing amine oxidases.

Amine oxidases catalyze the oxidative deamination of amines to aldehydes and ammonia, concomitant with a two-electron reduction of dioxygen to hydrogen peroxide (Equation 1):

These enzymes are widespread in nature and have been isolated from bacteria, fungi, plants, and animals (2). Four amine oxidases have been structurally characterized by X-ray crystallographic techniques (3-6). All of the crystallographically characterized amine oxidases are homodimers of approximately 150 – 180 kD. As is apparent from Fig. 1, the structures are very similar, except for the presence of a unique N-terminal domain present in the *Escherichia coli* enzyme. This domain is present to varying extents in other amine

Figure 1. Secondary structure rendering of the four available amine oxidase crystal structures - barrels represent α -helical structure, arrows represent β -sheet structure, the light gray loops represent random coil structure and the dark gray sections on the loops represent turns. A) AGAO B) ECAO C) PSAO D) PAAO

oxidases (RKAO, KDAO, BPAO, HSAO and PPLO) and are similar to each other.

However, they have no homology to ECAO and thus, are unlikely to have a similar fold.

In fact, residues 5-27 in HSAO have been proposed to be a transmembrane domain (7).

Figure 2. Active site of PAAO. The substrate channel extends from the upper left corner towards TPQ (5).

Both copper and a quinone, TPQ, are located within the active site and are required for catalytic activity (Fig. 2) (5). Other similarities include the presence of a large solvent filled cavity present at the subunit interface, a second metal site (whose function is currently unknown), and a proposed substrate-binding channel which extends from the surface of the protein to the active site. The electron density from the crystal structure of AGAO allows partial occupancy by a second row transition metal or full occupancy by a first row metal (Mg or Na) in the second metal site (4). ICP analysis of

various amine oxidases suggests that the site is probably occupied by Ca *in vitro* (8).

TPQ is generated by the post-translational modification of a conserved active-site tyrosine residue via a novel self-processing reaction (Fig. 3) (9). This tyrosine is found in the active site consensus sequence TXXNY(D/E). The processing requires only Cu and O₂ to be completed. Tyr is proposed to coordinate to the copper and become oxidized by one electron. This activates the Tyr ring for addition of oxygen. The ring is then thought to rotate allowing addition of another oxygen, resulting in the TPQ structure below.

Amine oxidases typically display broad substrate specificities, catalyzing the turnover of numerous primary amines, and selected di- and polyamines. Their relatively broad specificity has complicated efforts to determine a definitive role for amine oxidases in many organisms, because of the enzyme's possible involvement in numerous metabolic pathways. Proposed cellular processes that may involve amine oxidases include programmed cell death (10), cell division (11), glucose transport in rat small intestine or adipocytes (12-14), and vascular adhesion (7,15,16). They have also been implicated in playing a role in the following diseases: atherosclerosis (17-20); cancer (21); and diabetes (22-24). Amine oxidases may also be involved in modulating the response(s) of higher organisms to amines, or to the H₂O₂ and aldehyde products generated by oxidation, not

TPQ

LTQ

Figure 3. Proposed mechanism of TPQ biogenesis.

only in the tissues, but also in the environment. For example, amines have been implicated in such diverse roles as control of protein and nucleic acid synthesis, cell proliferation, and cell differentiation (25) (26). Hydrogen peroxide is postulated to be an important signaling molecule (27). In plants, for example, it may be necessary for proper cell wall formation (28). The unambiguous identification of definitive roles for amine oxidases has also been impeded by the presence of multiple amine oxidase genes in many species (29,30). A primary example is *Homo sapiens*, where several genes have been identified, i.e....kidney diamine oxidase, retinal amine oxidase, lysyl oxidase, lysyl oxidase-like proteins, and semicarbazide sensitive amine oxidase (7,31-33).

Overview of Lysyl Oxidases

Mammalian lysyl oxidase (EC 1.4.3.13) has a different coding sequence than the structurally-characterized amine oxidases (34). Furthermore, lysyl oxidase does not have the same active site consensus sequence present in other amine oxidases. The catalytic domain sequence is DIDCQWWIDITDVXPGNY for lysyl oxidase (35) versus the active site consensus sequence of TXXNY(D/E) for amine oxidases. Recently, an active site peptide, including the carbonyl cofactor from bovine aorta lysyl oxidase, was isolated and characterized (36). The cofactor was found to be lysine tyrosylquinone (LTQ). LTQ has a covalently attached lysine residue in the position that corresponds to the O₂ position in TPQ. The biogenesis of LTQ is thought to proceed by a similar mechanism as that of amine oxidases (Fig. 4) (36). Nonetheless, there are clear mechanistic parallels between lysyl oxidase and other amine oxidases (37). The proposed mechanisms for enzyme turnover of amine oxidases and lysyl oxidases are shown in Figures 5A and 5B, respectively.

Despite the pronounced specificity toward peptidyl lysine residues, lysyl oxidase also catalyzes the oxidation of a variety of primary amines. However, some primary amines have also been shown to act as competitive inhibitors that irreversibly inactivate the enzyme upon prolonged exposure (~50% after 90 min.) (38). Owing to lysyl oxidase's relative insolubility and tendency for aggregation, structural and spectroscopic studies of the protein are difficult. The protein has yet to be crystallized in a form amenable to X-ray diffraction studies. Thus, relatively little is known about the structure of the enzyme.

Figure 4. Proposed mechanism for the generation of lysine tyrosylquinone (36).

Along with lysyl oxidase, three additional lysyl oxidase-like genes have been found. It has been hypothesized that this is a multigene family present in distinct cellular and tissue locations, each with a related but different function (35). The carboxy-terminal end shows significant sequence homology among all four genes and include the copper-binding site containing four histidines (WEWHSCHQHYH), two metal-binding domains, a cytokine receptor-like motif (C-x₉-C-x-W-x₂₆₋₃₂-C-x₁₀₋₁₃-C), ten cysteines and

Figure 5A. Proposed Mechanism for TPQ Turnover

Figure 5B. Proposed Mechanism for LTQ Turnover

the catalytic domain (35). The cysteines are believed to form five specifically linked disulfide bonds.

The primary function ascribed to lysyl oxidase has been the oxidation of selected lysine residues in collagen and elastin (39). This role is critical for the maturation of connective tissue in vertebrates. Numerous pathologies have been associated with defects in this pathway (40). Tropoelastin is secreted into the extracellular space where it associates and aligns itself with other tropoelastin fibers by a process termed coacervation. Lysyl oxidase has a higher affinity for this insoluble form than for monomers in solution, emphasizing the importance of this process. Lysyl oxidase oxidizes lysine residues in the tropoelastin fibers to α -aminoadipic- δ -semialdehyde (allysine), which is able to condense with lysine or allysine residues on adjacent fibers and form the crosslinks associated with mature elastin or collagen (40). Figure 6 illustrates some of the common cross-links found in tropoelastin and the necessary steps for their formation. As noted above, multiple coding sequences have been elucidated that code for lysyl oxidase. It has been hypothesized that this is a family of proteins with different, but not unrelated functions (35). Lysyl oxidase activity has also been suggested to play a role in wound healing (41), oncogenic activity (42-45), and the regulation of intercellular and intracellular concentrations of polyamines (25).

Yeast Amine Oxidases

When microorganisms are grown on monoamines as their sole nitrogen source, the nutritional function of the induced amine oxidase(s) appears unambiguous (46).

Figure 6. Chemical structures of selected intermediates and lysine-derived cross-links in collagen and elastin

Yeasts and other fungi can have important roles in the environment with regard to the decomposition of biomaterials, and frequently live in environments with substantial amounts of decaying organic matter. In such environments, most of the nitrogen may be present as organic compounds or complex macromolecules, rather than ammonia or nitrogen oxides. It is therefore an advantage for yeasts to catalyze the deamination of as many of these molecules as possible. If oxidative deamination is a significant source of biological nitrogen, amine oxidases may be important in controlling the environmental fates and distributions of amines and/or their deamination products. Furthermore, amine oxidase activity may provide a nitrogen source directly from proteinaceous material in the yeasts' environment. This would be especially true once the nitrogen, in forms available to other organisms, has been depleted.

If yeasts are grown on ammonia or nitrate for their nitrogen source, then amine oxidase activity is generally undetectable. In contrast, when yeasts are grown on either methylamine or n-butylamine, not only is amine oxidase activity present (and possibly translocated to the peroxisome (47)), but a different specific activity profile results for the various substrates. Additionally, multiple amine oxidase active bands can be resolved on a polyacrylamide gel under these conditions. This implies that most yeasts are capable of producing at least two different amine oxidases that differ in substrate specificity and that are differentially expressed depending on the exogenous amine environment. Frequently, these two enzymes have been designated methylamine oxidase and benzylamine oxidase. Whether one or both are expressed depends upon the yeast strain and what amine is used as the nitrogen source (29,48). For example, when *Candida nagoyaensis* was grown on

methyllamine and the cell extract run on a gel, there were three active bands. Only two bands were present when *C. nagoyaensis* was grown on n-butylamine. Furthermore, the specific activity toward methyllamine was reduced by half when grown with n-butylamine, but the specific activity was ten-fold higher toward benzylamine. These observations indicate the presence of multiple amine oxidase coding sequences. Moreover, these data indicate that growth conditions can modulate the expression profile for this family of proteins.

Notably, the benzylamine oxidase isolated from *Pichia pastoris* grown on spermidine was found to have an unusually broad substrate specificity (46). Of particular interest is the report by Tur and Lerch that the *P. pastoris* enzyme, grown on butylamine, has a preference for peptidyl lysine as a substrate, analogous to the substrate specificity of the mammalian lysyl oxidase enzyme (49). The enzyme has been designated the *P. pastoris* lysyl oxidase (PPLO) since that report, and is the only non-animal lysyl oxidase yet described to our knowledge.

Research Goals

Two major factors led to the investigation of PPLO. First, PPLO was reported to oxidase substrates similar to those preferred by the mammalian lysyl oxidases. Specifically, PPLO was able to turn over peptidyl lysyl groups. This suggests PPLO could be the first lysyl oxidase identified from a non-mammalian source. It would be advantageous to work with PPLO because it does not have the problems with aggregation and solubility associated with the mammalian lysyl oxidase. We anticipated that it would

be easier to get large quantities of protein from *P. pastoris* for characterization since over-expression trials of active mammalian lysyl oxidase has thus far proven unsuccessful. PPLO is likely to be more closely related to the other amine oxidases, as opposed to lysyl oxidase, especially considering that PPLO is relatively large with an apparent $M_r = 120$ kD by SDS/PAGE gel electrophoresis compared to an apparent $M_r = 32$ kD for the mammalian lysyl oxidase.

The second motivation for these studies was consideration of PPLO's unique ability to turn over a large variety of amines. Thus, in addition to understanding the relationship between PPLO and other types of amine oxidases, it remains an attractive target for structural and mechanistic studies with the goal of elucidating the molecular basis for the recognition and oxidative deamination of substrates, including peptidyl lysine residues. It will be valuable to generate substrate binding models to help understand what allows this enzyme to work on such a large variety of substrates. Does PPLO specifically recognize certain peptide sequences or protein motifs (as implied by the results of Tur and Lerch (49))? Alternatively, how does PPLO accommodate structurally diverse amines as substrates?

CHAPTER 2

SEQUENCE ANALYSIS AND OVER-EXPRESSION OF PPLO

Introduction

In the past, only about one mg of PPLO was obtained per liter of culture after purification. The construction of an over-expression system is therefore necessary in order to obtain sufficient quantities of protein for enzymatic and structural characterization. Also, experiments with site-directed mutants were envisioned to help elucidate the roles of key residues in the protein. Selected mutants could be expressed with the same over-expression system. Identifying the coding sequence of PPLO was critical before a number of these and other experiments can be performed. The identity, isolation, and cloning of the coding sequence are all essential elements of the process needed to develop an over-expression system.

The basic strategy employed consisted of three stages. First, it was necessary to identify the whole coding sequence, because neither the PPLO protein nor gene had previously been sequenced. Next, the coding sequence would be determined and isolated, thereby permitting the generation of various constructs. This construct would then be integrated into the yeast genome and expression trials performed. Finally, construct stocks would be sequenced to check for errors in positive expression candidates.

A sequence analysis of the PPLO gene was needed in order to compare its' similarity to amine oxidases and the mammalian lysyl oxidase. A line-up of amine

oxidases from various sources was developed and key similarities and differences were observed. This was important in deciding whether PPLO is more similar to the amine oxidase enzyme class, the lysyl oxidase enzyme class, or if it belonged to a unique class of amine oxidases because of its unusual molecular weight (~120 kD/monomer). This information suggested that PPLO was more similar structurally to the family of amine oxidases than the family of lysyl oxidases. This helped us to plan appropriate experiments to test this hypothesis.

Materials and Methods

Gene Sequence

Genomic DNA was isolated from *Pichia pastoris* (ATCC# 28,485) (50). Two sets of primers were designed to amplify part of the lysyl oxidase gene from this genome. The design of the first set of degenerate primers was based on the topa consensus region (TXXNYD/EY - 5'ACIGTIGCIAAYTAYGARTA3') from *Pichia angusta* (previously *Hansenula polymorpha* (51)) and a semi-conserved region about 250 codons downstream of the topa region (EDFPIMP - 5'GGCATIAIIGGIMAITCYTC3'). A second set of primers was also employed. One, (5'RTARTCRTARTTNCCIATNGT3'), was designed from the active site peptide sequence (T-X-X-N-Y-D/E-Y). This primer is the reverse complement of the topa based sequence above. This is necessary since the second primer site is now upstream rather than downstream of this priming site. The second primer, (5'RTNACNSARCCNSARGG3'), was designed from the conserved upstream region

(T/V-T-Q/E-P-E/Q-G). The second set of primers were used to generate a 550 bp PCR product to use as a probe for isolating the PPLO gene. The MegaPrime DNA labeling system from Amersham was used along with cytidine 5'-[α - 32 P] triphosphate for generating the labeled probe.

Two different methods were initially used to obtain the genomic sequence of PPLO. The ~550 bp DNA fragment was used as a probe for both Southern hybridization experiments (50) and against a genomic library (50) (this library was generously supplied by Dr. James Cregg from the Keck Graduate Institute of Applied Life Sciences, Claremont, CA). Isolated fragments were circularized by ligation and inverse PCR amplified. This inverse PCR method is extremely useful for isolating adjacent fragments to known sequences in the genomic DNA. The PCR products were then sequenced by the dideoxy chain-termination method (52) using gene specific primers to walk along the gene. The primers were ($[\gamma^{33}\text{-P}]\text{ATP}$) end labeled with the *fmol* Sequencing kit (Promega, Madison WI) and the sequences determined by gel analysis. Both sequences were independently determined at least four total times and at least once in either direction.

Sequences were compiled and analyzed with the software package GCG (Version 8, Madison WI). The consensus coding sequence was translated and then lined-up against other amine oxidase sequences using the "pileup" protocol in GCG. These were manually manipulated in order to maximize the total number of conserved residues in each sequence.

Primers

All primers were synthesized by Midland Chemical Company (Midland, TX) or Life Technologies (Carlsbad, CA). These primers were desalted prior to shipping with the exception of the primers used for mutagenesis (*vida infra*) which were desalted and HPLC purified.

Design of the Over-expression System

Two separate constructs were engineered for over-expression of PPLO. The first construct was obtained by first amplifying the coding sequence using PCR. Concurrently, the ends were modified to yield the appropriate restriction sites for cleavage by the restriction enzymes, *Bam* HI and *Not* I. Upon digestion the amplified sequence was ligated into the pPIC3 vector (Invitrogen, Carlsbad CA) and electroporated into InV α F' *E. coli* cells (Fig. 7) (50). Colonies were selected by their resistance to ampicillin and screened by PCR. Constructs from positive colonies were then linearized by *Eco*RI and electroporated into *P. pastoris* GS115 cells (Invitrogen, Carlsbad CA) (Fig. 8) (50). Putative integrants were screened by PCR and the sequence checked for errors using the dideoxy chain-termination method (52).

The second construct was also obtained by PCR amplification of the genomic coding sequence. During this amplification the ends were modified by introducing the restriction sites *Mfe* I and *Not* I. After digestion, this product was ligated into the pPIC Z B vector and electroporated into InV α F' *E. coli* cells (Fig. 7) (50). Selection was achieved through resistance to the antibiotic Zeocin and screening by PCR. Positives were linearized with *Pme* I and electroporated into GS 115 cells

Figure 7. Vectors and constructs used for the over-expression systems. A) pPIC3 plasmid B) pPIC3 plasmid with the PPLO coding sequence C) pPICZB plasmid D) pPICZB plasmid with the PPLO coding sequence

(Fig. 8) (50). Positive integrant sequences were checked for errors through sequencing of the stock constructs using the dideoxy chain-termination method (52). Five individual colonies were selected and assayed for over-expression. Stocks were made from the colony with the highest PPLO activity 24 hours after induction.

Figure 8. Cloning strategy for PPLO over-expression. 1) The coding sequence (dark gray) was ligated into the pPIC3 vector adjacent to an alcohol oxidase promoter sequence. 2) The construct was linearized by the restriction enzyme *EcoRI* and electroporated into GS115 cells (light gray represents the chromosomal copy). 3) Putative integrants were screened by PCR.

Results and Discussion

Gene Isolation and Sequencing

The first set of primers amplified part of the methylamine oxidase gene from *P. pastoris*. This was concluded based on the amount of identity (71%) found between the translated methylamine peptide sequence from *Pichia angusta* and the translated PCR product obtained from these primers. Amplification with the second set of primers resulted in a product of ~550 basepairs, the anticipated length of the fragment between these primers for an amine oxidase gene. This sequence was thought to be part of the PPLO coding sequence because it maintained the conserved amine oxidase residues on the one hand, but was not very similar to the methylamine oxidase sequence on the other. The identity was 25% between this translated fragment and the translated methylamine oxidase gene from *P. angusta*. This DNA fragment was used as a probe for both a genomic library screening and a Southern hybridization experiment. The desired product was not found in the library and was determined to either be absent or present in low abundance. In contrast, the Southern hybridization experiment yielded two positives that corresponded to the desired product. Digests with *Bam*HI, *Hind*III, *Kpn*I, *Pst*I and *Eco*RI were conducted. *Eco*RI yielded the best restriction digest pattern and resulted in two fragments of ~2,000 basepairs each. These fragments contained the entire PPLO coding sequence.

Sequence Homologies

Surprisingly, comparison of the translated *P. pastoris* sequence to proteins in the GenBank database revealed the highest homology (50% similar and 30% identical) to human kidney diamine oxidase (Table 1). A lineup of nine different amine oxidase sequences (Fig. 9) revealed only 29 residues that were absolutely conserved, including those in the TPQ consensus sequence (T-X-X-N-Y-D/E-Y), and the three histidine ligands for copper. These histidines have been unambiguously established as copper ligands in the structures of amine oxidases (3-6).

Table 1. Percent Identity Among Amine Oxidases

	RKAO	KDAO	BPAO	HSAO	PPLO	AGAO	PAAO	PSAO	ECAO
RKAO		38.7	71.9	83.2	24.0	21.2	18.3	16.5	20.3
KDAO			41.9	41.4	29.8	23.6	22.0	24.1	24.3
BPAO				80.8	27.5	23.4	20.4	22.3	22.6
HSAO					27.3	25.3	22.1	22.8	23.5
PPLO						23.3	20.9	24.5	23.0
AGAO							32.8	24.3	28.7
PAAO								25.7	28.1
PSAO									30.3

Of special interest, the alignment in Fig. 9 reveals a number of regions that show substantial homology between PPLO and various mammalian amine oxidases. Several of the homologous regions among PPLO and the mammalian enzymes are not present in ECAO, PSAO, PAAO, or AGAO. Specifically, there appear to be three regions that show the greatest amount of homology between PPLO and the mammalian amine oxidases (Fig 10). The region between PPLO residues 57-148 has 22 absolutely conserved residues, including the last five in this region, only one of which is conserved among all the amine oxidases. This is the longest string (five) of absolutely

271

360

RKAO	LHPTGLEILL	DHGSTDVQDW	RVEQLWY...	...NGKFYNN	PEELARKYAV	GEVDT..VVL	EDPLPNG...	..TEKPPLFS	SYKPRGEFHT
KDAO	LHPTGLELLV	DHGSTDAGHW	AVEQVWY...	...NGKFYGS	PEELARKYAD	GEVDV..VVL	EDPLPGGKGH	DSTEEPPLFS	SHKPRGDFPS
BPAO	LHPVGLELLV	DHKALDPADW	TVQKVFF...	...QGRYYEN	LAQLEEQFEA	GQVNV..VVI	PDDGTGG...	FW	SLKS....QV
HSOA	LHHVGLELLV	NHKALDPARW	TIQKVFF...	...QGRYYDS	LAQLEAQFEA	GLVNV..VLI	PDNGTGG...	SW	SLKS....PV
PPLO	LVPTGFFFKF	DMTGRDVSQW	KMLEYIY...	...NNKVYTS	AEELYEAMQK	DDFVT..LPK	IDVDNLDWTV	IQRNDSAPIR	HLDDR.....
AGAO	VAPLSAGVFE	YAEER..GRR	ILRGLAFVQD	FPEDSAWAHP	VDGLVAYVDV	VSKEVTRVID	TGV.....FP	VPAEHGNYTD	PEL...TGPL
PAAO	CDPWTIG...	YDERWGTGKR	LQQALVYYRS	DEDDSQYSHP	LD....FCPI	VDTEEKKVIF	IDIPNRRRKV	SKHKHANFYP	KHMIEKVGAM
PSAO	CSSFTMGWFG	EEKNV....R	TVRLDCFMKE	.STVNIYVRP	ITGITI...V	ADLDLMKIVE	YHDRDIEAVP	TAENTY...	.QVSKQSPPF
ECOA	TTPLTVVIFD	GKDGLKQDAR	LLKVIISYLD	.VGDGNYWHI	IENLVA...V	VDLEQKKIVK	IEEGPVVPVP	MTARPF...	.GRDRVAPAV
CONS	L---G-----	---:---D---W	---:--- ---	----- ---Y--	-- L-----	-----:---	-D-----	-----	-----

361

450

RKAO	PVNVAGPHVV	QPSGPRYKLE	GNTVLVYGGWS	FSY..RLRSS	SGLQIFNVLF	GG.....ER	VAYEVSVQEA	VALYGGHTPA	GMQTKYIDVG
KDAO	PIHVSQPRLV	QPHGPRFRLE	GNAVLYGGWS	FAF..RLRSS	SGLQVLNVHF	GG.....ER	IAYEVSVQEA	VALYGGHTPA	GMQTKYLDVG
BPAO	PPGPTPPLQF	HPQGPRFSVQ	GNRVASSLWT	FSF..GLGAF	SGPRVFDVRF	QG.....ER	LAYEISLQEA	GAVYGGNTPA	AMLTRYMDSG
HSOA	PPGPAPPLQF	YPQGPRFSVQ	GSRVASSLWT	FSF..GLGAF	SGPRIFDVRF	QG.....ER	LVYEISLQEA	LAIYGGNSPA	AMTRYVDGG
PPLO	...KSPRLV	EPEGRWAYD	GEEEFYSWMD	WGFYTSWSRD	TGISFYDITF	KG.....ER	IVYELSLQEL	IAEYGSDDPF	NQHTFYSDIS
AGAO	RTTQKPISIT	QPEGPSFTVT	GGNHI.EWEK	WSLDVGFVDV	EGVVLHNI AF	RD..GDRLRP	IINRASIAEM	VVPYGDPSPI	RSWQNYFDTG
PAAO	RPEAPPINVT	QPEGVSFKMT	G.NVM.EWSN	FKFHIGFNVR	EGIVLSDVSY	ND..HGNVRP	IFHRISLSEM	IVPYGSPEFP	HQRKHALDIG
PSAO	GPKQHSLTSH	QPQGPQFQIN	G.HSV.SWAN	WKFHIGFDVR	AGIVISLASI	YDLEKHKSR	VLYKGYISEL	FVPYQDPTEE	FYFKTFFDSG
ECOA	KPMQ....II	EPEGKNYTTIT	G.DMI.HWRN	WDFHLSMNSR	VGPMISTVTY	ND..NGTKRK	VMYEGSLGGM	IVPYGDPDIG	WYFKAYLDSG
CONS	-----P---	-P-G-R ---	G-----:---	: -----	G--- -F	-G-----ER	--YE:S:QE-	-A-YG:--P-	---T-Y-D--

451

540

RKAO	.WGLGSVTHE	LAPGIDCPET	ATFLDAFHYY	DSDGPVHYPH	ALCLFEMPTG	VPLRRHFNSN	FKGGFNFYAG	LKGYVLVLR	TSTVYNYDYI
KDAO	.WGLGSVTHE	LAPGIDCPET	ATFLDTFHYY	DADDPVHYPR	ALCLFEMPTG	VPLRRHFNSN	FKGGFNFYAG	LKGQVLVLR	TSTVYNYDYI
BPAO	.FGMGYFATP	LIRGVDCPYL	ATYMDWHFVV	ESQTPKTLHD	AFCVFEQNKG	LPLRRHHSDF	LS...HYFGG	VAQTVLVFRS	VSTMLNYDYV
HSOA	.FGMGKYTTP	LTRGVDCPYL	ATYVDWHFLL	ESQAPKTIRD	AFCVFEQNQG	LPLRRHHSDF	YS...HYFGG	LAETVLVFRS	MSTLLNYDYV
PPLO	.YGVGN.RFS	LVPGYDCPAT	AGYFTT.DTF	EYDEFYNRTL	SYCVFENQED	YSLLRHTGAS	YS.....AI	TQNPTLNVR	ISTIGNYDYN
AGAO	EYLVGQYANS	LELGDCDCLD	ITYLSPVISD	AFGNPREIRN	GICMHEEDWG	ILAK.H.SDL	.WSGI..NYT	RRNRMRVISF	FTTIGNYDYG
PAAO	EYGAGYMTNP	LSLGCDCCKV	IHYLDAHFSD	RAGDPITVKN	AVCIHEEDDG	LLFK.H.SDF	.RDNFATSLV	TRATKLVSQ	IFTAANYEYC
PSAO	EFGFGLSTVS	LIPNRDCPPH	AQFIDTYVHS	ANGTPILLKN	AICVFEQYGN	IMWR.H.TEN	GIPNESIEES	RTEVNLIVRT	IVTVGNYDYN
ECOA	DYGMGTLTSP	IARGKDAPSN	AVLLNETIAD	YTGVPMEIPR	PIAVFERYAG	PEYK.H.QEM	GQPNVSTERR	...ELVVRW	ISTVGNYDYI
CONS	- G:G-----	L--G-DCP--	A: -----	-----	: -C:FE----	--:L-RH----	----- -	-----L--R-	-ST:-NYDY-

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541

630

RKAO WDFIFYSNGV MEAKMHATGY VHATFY.... TPEG.L RHGTRLQTHL LGNIHTHLVH YRVDMDVAGT KNSFQTLTMK LENLTPWSP
 KDAO WDFIFYPNGV MEAKMHATGY VHATFY.... TPEG.L RHGTRLHTHL LGNIHTHLVH YRVDLDVAGT KNSFQTLQMK LENITNPWSP
 BPAO WDMVFYPNGA IEVKLHATGY ISSAFL.... FGAA.R RYGNQVGEHT LGPVHTSAH YKVDLDVGGL ENWVWAEDMA FVPTAIPWSP
 HSAO WDTVHPSGA IEIRFYATGY ISSAFL.... FGAT.G KYGNQVSEHT LGTVHTSAH FKVDLDVAGL ENWVWAEDMV FVPMVWPWSP
 PPLO FLYKFFLDGT LEVSVRAAGY IQAGYW.... NPETSA PYGLKIHDLV SGSFHDHVLN YKVDLDVGGT KNRASKYVMK DVDVEYPWAP
 AGAO FYWYLYLDGT IEFEEKATGV VFTSAF.... PEGGSDN I..SQLAPGL GAPFHQHIFS ARLDMAIDGF TNRVEEEDVV RQTM...GPG
 PAAO LYWVFMQDGA IRLDIRLTGI LNTYIL.... GDDEEAG PWGTRVYPNV NAHNHQHLFS LRIDPRIDGD GNSAAACDAK SSPYPLGSPE
 PSAO IDWEFKASGS IKPSIALSGI LEIKGTNIK. HKDEIKEDL H.GKLVSANS IGIYHDHFI YLDFDIDGT HNSFEKTSLK ..TVRIKDG
 ECAO FDWIFHENGIT IGIDAGATGI EAVKGVKAKT MHDETAKDDT RYGTLDHNI VGTTHQHIYN FRLDLVDGE NNSLVAMD.P ..VVKPNTAG
 CONS |---F:--G- :E----A:GY |---:|----- :G-:----- -G--H-H--- ||VD|DV|G- :N-----M- -----PW:P

631

720

RKAO SHSLVQPTLE QTQYSQEHQA AFRFGQTLPK YLLFSSPQK. NCW.GHRRSY RLQIHSMAEQ VLPPGWQEER AV.....TW ARYPLAVTKY
 KDAO RHRVVQPTLE QTQYSWERQA AFRFKRKLPK YLLFTSPQE. NPW.GHKRSY RLQIHSMAEQ VLPPGWQEEQ AI.....TW ARYPLAVTKY
 BPAO EHQIQRLQVT RKQLETEEQA AFPLGGASPR YLYLASKQS. NKW.GHPRGY RIQTVSFAGG PMPQNSPMER AF.....SW GRYQLAITQR
 HSAO EHQLQRLQVT RKLLEEMEEQA AFLVGSATPR YLYLASNHS. NKW.GHPRGY RIQMLSFAGE PLPQNSSMAR GF.....SW ERYQLAVTQR
 PPLO GTVYNTKQIA REVLEKEDFN GINWPENGQG ILLIESAET NSF.GNPRAY NIMPGGGGVH RIVKNSRSGP ET.....QNW ARSNLFLTKH
 AGAO NERGNAFSRK RTVLTRSEA VREADARTGR TWIISNPESK NRLNE.PVGY KLHAHNQPTL LADPGSSIARRAAF ATKDLWVTRY
 PAAO NMYGNAFYSE KTTFKTVKDS LTNYESATGR SWDIFNPNKV NPYSGKPPSY KLVSTQCPL LAKEGSLVAKRAPW ASHSVNVVPY
 PSAO SKRKSYYTTE TQTAKTESDA KITIGLAPAE L.VVVPNPNK TAV.GNEVG YRLIP.AIPAH PL.....LTE DDYPQIRGAF TNYNVWVTAY
 ECAO GPRTS..TMQ VNQYNIGNEQ DAAQKFDPGT IRLLSNPNKE NRM.GNPVSY QIIPYAGGTH PVAKGAQFAP DEWIYDRLSF MDKQLWVTRY
 CONS -----: :-----E--- |----- -L---S---- N-|-G--R:Y -:-:-:-| -:-:-:-:-W -R--L--T:-

721

810

RKAO RESERYSSSL YNQNDPWDP VVFEEFL.RN NENIEDDLV AWWTVGFLHI PHSEDVPNTA TPGNSVGFL RPFNFFPEDP SLASRDTVIV
 KDAO RESELCSSSI YHQNDPWHP VVFEQFL.HN NENIENEDLV AWWTVGFLHI PHSEDIPNTA TPGNSVGFL RPFNFFPEDP SLASRDTVIV
 BPAO KETEPSSSSV FNQNDPWPT VDFSDFI..N NETIAGKDLV AWWTAGFLHI PHAEDIPNTV TVGNGVGFFL RPYNFFDQEP SMDSADSIYF
 HSAO KEEEPSSSSV FNQNDPWAPT VDFSDFI..N NETIAGKDLV AWWTAGFLHI PHAEDIPNTV TVGNGVGFFL RPYNFFDQEP SFYSADSIYF
 PPLO KDEELRSSTA LNTNALYDPP VNFNAFL..D DESLDGEDIV AWWNLGLHLH PNSNDLPNTI FSTAHASFML TPFNYFDSN SRDTTQOVFY
 AGAO ADDERYPTGD FVNQHSFGA. GLPSYIAQD RD.IDGQDIV VWHTFGLTHF PRVEDWP..I MPVDTVGFKL RPEGFFDRSP VLDVPANPSQ
 PAAO KDNRLYPSGD HVPQWSGDGV RGMREWIGDG SENIDNTDIL FFHTFGITHF PAGEDFP..I MPAEPITLML RPRHFFTENP GLDIQPSYAM
 PSAO NRTEKWAGGL YVDHSRGDDT LAVWT...KQ NREIVNKDIV MWHVVGIIHV PAQEDFP..I MPLLSTSFEL RPTNFFERNP VLKTLSPRDV
 ECAO HPGERFPEGK YPNRSTHDTG LGQYS...KD NESLDNTDAV VMMTTGTTHV ARAEWP..I MPTIEWHTLL KPWNFFDET TLGALKKDK*
 CONS ||-E--SS|- ---N--|-P: V-F--F:--| |E-:--:D:V AWW--G--H: P-:~D:PNT- -:-:-:-F-L -P|N|F--|- S--|---|--

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	811				865
RKAO	W.PQDKGLNR	VQRW..IPED	RRCLVSPPPFS	YNGTYKPV..	
KDAO	W.PRDNGPNY	VQRW..IPED	RDSCMPPPPFS	YNGTYRPV*.	
BPAO	REGQDAGSCE	INPLACLPQA	ATCAPDLPVF	SHGGYPEY..	
HSAO	RGDQDAGACE	VNPLACLPQA	AACAPDLPAF	SHGGFSHN..	
PPLO	TYDDETEESN	WEFYG..NDW	SSCGLEVPEP	NFEDYTYGRG	TRINKKMTNS DEVY*
AGAO	SGSHCHG...				
PAAO	TTSEAKRAVH	KETKDKTSRL	AFEGSCCGK.		
PSAO	AWPGCSN*..				
ECAO					
CONS	---- -----	-----	--C---P--	---- -----	-----

Figure 9. Alignment of structurally characterized amine oxidases by X-ray crystallography and selected mammalian amine oxidases with PPLO. Bold letters for amino acids indicate conservation between the sequences. All the amino acids in a column are shown in bold to designate absolute conservation. If only the mammalian sequences and PPLO are shown in bold, this indicates either a conservative or absolute conservation among them. Homology was determined by using an amino acid hierarchy alphabet, class I (53). Absolutely conserved amino acids received a value of five and are designated by the amino acid letter on the consensus line; conservatively substituted amino acids scored a value of 3 and are designated by the (|) symbol on the consensus line; semi-conserved amino acids scored a value of 2 and are designated by the (:) symbol on the consensus line. ECAO - *Escherichia coli* amine oxidase (I40923), PAAO - *Pichia angusta* (previously *Hansenula polymorpha*) (S04963), AGAO - *Arthrobacter globiformis* (JC2139), PSAO - *Pisum sativum* (C44239), BPAO - Bovine plasma amine oxidase (A54411), KDAO - human kidney diamine oxidase (A54053), RKAO - rat amiloride binding protein (S34656), HSAO - human amine oxidase (JC5234).

Figure 10 PPLO model with the homologous domains shared with the mammalian amine oxidases highlighted. Region 1 in light gray (residues 83-148), region 2 in black (residues 363-386), and region 3 also in light gray (residues 637-704).

conserved residues in the lineup. In addition, there are also 21 conserved or semi-conserved amino acid residues. In the known structures this region lies on the surface of the protein. The secondary structure starts as α -helix, continues as a connecting region with a small bend, and ends with β -sheet. The next homologous region, between residues 363-386, has 12 identical and 6 conserved or semi-conserved residues. This region consists of the second half of a β -sheet, which lies on the surface, and is followed by another β -sheet which extends into the protein and passes near the active site. The residues in this region are much more conserved between the mammalian and PPLO sequences when compared to the sequences of the structurally-characterized

amine oxidases. This might imply the presence of an important structural feature maintained in these enzymes that is not present in PSAO, ECAO, AGAO, or PAAO. Lastly, a long region toward PPLO's C-terminal end has numerous conserved and semi-conserved residues which includes His 664, one of the Cu ligands, and two ligands for the putative second metal ion site, Asp 653 and the backbone carbonyl from Ile 654. The latter part of this region interacts with the other sub-unit near the region forming the inter-subunit cavity present between the two subunits. This region may be involved in defining the size and shape of the inter-subunit cavity. The secondary structure in this region consists mostly of β -sheet.

The two sequences that have the highest homology to PPLO (Fig. 9) are human kidney diamine oxidase (KDAO) and rat amiloride-binding protein (RKAO). Both of these proteins have been found to bind amiloride (54). Since amiloride resembles some of the amine substrates that are oxidized by these enzymes, it likely binds to or near the active site. Amiloride is one among a family of guanidine containing compounds that have been found to inhibit KDAO (aminoguanidine is a very potent inhibitor) (55). KDAO and RKAO also have a heparin-binding motif present, RFKRKLPK, which is not found in the other amine oxidases or PPLO (Fig. 9).

Amiloride

Aminoguanidine

Most of the residues proposed in AGAO to line or be near the substrate channel are not conserved in PPLO or other amine oxidases. The exceptions are listed below (all the residue numbers below refer to AGAO unless stated otherwise) (4). The side chain proposed to be the gate for substrate access to the active site corresponds to Tyr 296 in PPLO. It is a Tyr in all sequences except PSAO and PAAO where it is a Phe and Ala, respectively. Asp 298 is absolutely conserved and has been identified as the active site base (56). Thr 378 and Asn 381 are part of the active site consensus sequence and are absolutely conserved. Tyr 302 is conserved as an aromatic residue in the lineup. Additionally, three other residues that are highly conserved among amine oxidases, other than PPLO, are Trp 168, Gly 300, and Phe 297. In other amine oxidases, Trp 168 is conserved as an aromatic residue, but in PPLO it is a Val. Gly 300 lies between substrate channel residues in the primary sequence. However, in PPLO this residue corresponds to Ser. Phe 297 is conserved as a hydrophobic residue and lies between substrate channel residues, but it is a Ser in PPLO.

The Cys residues that form a disulfide bond in the structurally characterized amine oxidases, with the exception of ECAO, are conserved in the sequence of PPLO as Cys 415 and Cys 440. Next, the second metal site ligands are proposed to originate from the conserved residues Asp 537 and 682 in PPLO. Asp 539 also in PPLO is only semi- conserved being replaced by Ala in AGAO and Arg in PAAO. Two other second metal site ligands in PPLO are proposed to originate from carbonyl groups on the polypeptide backbone, Leu 538 and Ile 683. Based on the lineup, PPLO's second metal site would resemble the ECAO and PSAO sites most closely (PPLO has all three Asp

groups similar to ECAO and PSAO). PPLO also has five potential N-linked glycosylation sites (Asn-X-Ser/Thr, X≠Pro) where a polysaccharide may be attached: Asn 81, Asn 104, Asn 191, Asn 309, and Asn 434.

Over-expression of PPLO

Over-expression trials for the mammalian enzymes have to date been largely unsuccessful. Active lysyl oxidase has been modestly over-expressed in mammalian cells (34) and *E. coli* cells (57). However, the bacterial results do not seem to be reproducible. In contrast, the PPLO coding sequence, the sequence with the highest similarity to the mammalian coding sequences, was successfully over-expressed with a ten-fold increase in PPLO expression (ten mgs / liter of culture) compared to wild-type through homologous recombination (Fig. 8).

Conclusions

It was found that *P. pastoris* has at least two amine oxidase genes. The methylamine oxidase gene was only partially sequenced, but PPLO was completely determined. This was not surprising considering many yeasts express multiple amine oxidase genes. It is conceivable that *P. pastoris* could have other amine oxidase genes apart from the two sequenced in this work. The identification and isolation of these would be necessary for helping to understand the role of this family of enzymes in *P. pastoris*.

Based on the amine oxidase line-up, PPLO is more closely related to the family of amine oxidases rather than the mammalian lysyl oxidases (Fig. 9). For example,

PPLO has the topa consensus sequence (TXXNY(D/E)) not LTQ's (DIDCQWWIDITDVXPGNY). Although it is interesting to point out PPLO, among the amine oxidases, is more similar to mammalian amine oxidases, particularly KDAO, than either bacteria, plants, or even other yeasts, this raises some intriguing questions. More sequencing of other amine oxidase sequences needs to be done to see if any other non-mammalian proteins will also be similar to PPLO or if PPLO is something of an anomaly. Another representation demonstrating the sequence homology was generated (Fig. 11) and illustrates again that PPLO is more similar to the mammalian family of amine oxidases than any of the other determined sequences (58).

Figure 11. Phylogenetic tree of amine oxidases from 21 species. Created with 96 amino acid sequences from four structurally conserved regions, using software PROTDIST (Dayhoff PAM matrix) and NEIGHBOR (58).

Since an over-expression system has been developed, numerous spectroscopic, mechanistic, and structural experiments can readily be performed that would otherwise

be very difficult. Many such experiments use large amounts of protein and so must be carefully planned to optimize the amount of enzyme used. In contrast, it is difficult to purify even modest amounts of BALO (bovine aorta lysyl oxidase). Thus, PPLO is an excellent candidate for structural and mechanistic studies with the goal of defining the recognition and oxidation of peptidyl lysines.

CHAPTER 3

STRUCTURAL AND MECHANISTIC STUDIES OF PPLO

Introduction

It is essential to isolate substantial amounts of homogeneous protein in order to carry out detailed structural and mechanistic studies. The growth conditions and purification procedures needed to achieve this goal for PPLO were developed and employed as part of this dissertation research. Previously published methods of purification provided low expression levels and used only gel analysis under denaturing or non-denaturing conditions to analyze protein purity (29). Often, low-abundant contaminants with a chromophore can be detected by UV-VIS spectroscopy, but not gel electrophoresis. Based on this early work, PPLO was already recognized to have some unusual properties, such as a relatively large $M_r = 120$ kD and a broad substrate specificity. It was unclear if PPLO belonged to the TPQ, LTQ, or a novel group of amine oxidases.

Among the first experiments were a molecular weight analysis and a spectroscopic survey which included resonance Raman, EPR, UV-VIS, and CD. Many of these techniques had already been performed for various amine oxidases and were compared to the data collected for PPLO. X-ray crystallization trials were initiated with Dr. Hans Freeman (University of Sydney, Department of Biochemistry, Sydney, Australia) so that a detailed structural comparison could be made between PPLO and the other known amine oxidase structures. A homology model of PPLO was also developed

to help direct future experiments in advance of a crystallographic structure and allow some structural comparisons. It would also allow exploration of the novel substrate specificity of PPLO.

Although some kinetic parameters had been previously determined for various PPLO substrates (29), additional kinetic experiments were performed on the homogeneous enzyme available from this thesis work. The reasons for this were threefold: discrepancies in the literature for some kinetic parameters needed to be addressed; parameters of additional substrates were sought; and comparisons of wild-type parameters to mutant PPLO parameters were anticipated.

Three PPLO enzymes with alternate coding sequences were designed to probe the role of specific protein residues. The first was Tyr384 → Phe. This tyrosine is absolutely conserved among amine oxidases and is hydrogen bonded to TPQ in the known X-ray structures. The effect on substrate turnover was investigated and compared to wild-type values. The second alternate sequence designed was His453 → Ala. This histidine is one of four conserved in amine oxidases (the other three are copper ligands). This residue resides on the "arm" that reaches across the protein surface to the second subunit and partially obstructs the substrate channel. The last alternate sequence designed was Thr474 → Leu. This threonine is part of the active site consensus sequence (T-X-X-N-Y-D/E). The effect of this mutation on formation of TPQ and substrate turnover were investigated.

Materials and Methods

Growth Conditions

The following protocol was used to generate supplies of protein for sufficient characterization of the native enzyme. *Pichia pastoris* cells from stab cultures (ATCC# 28,485) were plated on YPD plates (YPD: 1% yeast extract, 2% peptone, and 2% dextrose). Minimal media cultures of two mL were inoculated with single colonies from these plates. The cultures were incubated at 30° C and shaken at 300 rpm. The media consisted of 0.68% YNB (yeast nutrient broth) without amino acids or ammonia sulfate, 0.68% dextrose, and 10 mM n-butyl amine. These two mL cultures were used to inoculate one Liter cultures which were grown at 30° C and shaken at 125 rpm. Cultures were harvested 72 hours later and stored at -20° C.

The following growth conditions were used to express the recombinant enzyme. A histidine minus strain of *P. pastoris* (ATCC# GS115 *his4*) was used along with either a His⁺/Ampicillin or Zeocin marker for selection of recombinant cells. Both were designed and yielded similar over-expression characteristics. Ampicillin is only effective against *E. coli*. The His⁻ cells were necessary in conjunction with Ampicillin so that only transformed yeast cells with the His⁺ vector grew on the histidine deficient plates. Zeocin is preferred because it is an effective antibiotic toward both *E. coli* and yeast and, thus, simplifies the selection of over-expression candidates (it eliminates the need for the His⁺ selection step). However, this plasmid was not available when initial construct designs began.

Cells with the appropriate construct were also grown on YPD plates. These were started in minimal media cultures of two mL grown at 30° C and 300 rpm. However, instead of 10 mM n-butyl amine, 1% ammonium sulfate was used as the nitrogen source and 2% dextrose rather than 0.68% was employed. This media mix was continued into the one Liter cultures which was incubated at 30° C and 125 rpm. After 48 hours the cultures were spun down and induced with a one Liter broth consisting of 1% yeast extract, 2% peptone, 2% methanol, and 100 µM copper sulfate media mix. Cells were harvested after 24 hours and stored at -20° C.

Generation of Alternate PPLO Sequences

A flow chart of the protocol used is provided (Fig. 12). Three different oligonucleotides with the desired alternate sequence were designed (the changed nucleotides are underlined). The first was Tyr384 → Phe. The primer used to generate this was (5'TAATTGCCGAGTTCGGTTCAGATG3'). The second alternate sequence designed was His453 → Ala. The primer used to generate this was (5'ACTGCTACGTGCCACTGGTGCTTC3'). The last mutant designed was Thr474 → Leu. The primer used to generate this was (5'TTTATTTCTCTTATTGGAAACTAC3').

The MORPHTM site-specific plasmid DNA mutagenesis kit from 5 Prime → 3 Prime, Inc. was used to generate all three mutants. The appropriate alternate sequence primer and target plasmid (pPIC Z B with the PPLO coding sequence construct) were denatured together and allowed to anneal. The replacement strand was synthesized from the alternate sequence oligonucleotide using T4 DNA Polymerase and T4 DNA ligase. The mixture was digested with *DPN* I. This step fragmented the non-mutagenized target

Figure 8. An outline of the MORPH™ site-specific plasmid DNA mutagenesis kit protocol.

plasmids. The alternate sequence constructs were then transformed into an *E. coli* mutS strain which theoretically results in half of the colonies with wild-type and half with alternate coding sequences. Positive alternate sequence constructs were confirmed by dideoxy sequencing. These were then linearized and integrated into the genome of *P. pastoris*.

Purification

Effectiveness of possible cell lysis protocols were evaluated using a microscope and analyzing the change in cell morphology. By this criterion, the use of the French Press method was deemed inefficient. Thus, a second method was tried, proved effective, and subsequently used. This method requires the addition of each of the following in equal volumes: glass beads; buffer (0.1 M KPO₄, pH 7.0); and cell paste (generally 60 mL of each in a 450 mL centrifuge bottle). The solution was vigorously shaken by hand for 10 minutes and centrifuged at 7,000 rpm for 10 minutes (a 40% head space helps to ensure adequate shaking). The protein was located in the supernatant. It was saved and set aside. Additional buffer was added to the cells and the cells were shaken again. This process was repeated until cell lysis was 90% or higher (usually 2-3 times).

The supernatants were pooled, concentrated, and spun again (18,000 rpm for 10 minutes) to help remove cellular debris. The protein solution was filtered through Whatman #4 filter paper and loaded onto a Sigma DEAE fast flow column (anion exchange) previously equilibrated in 0.1 M KPO₄, pH 7.0. The fraction containing PPLO was eluted with 1.0 M KPO₄, pH 7.0 which was subsequently concentrated and

the conductivity reduced to < 10 mS. The second column employed was a Poros PI column from Pharmacia, also an anion exchange step. Although this column also was equilibrated with 0.1 M KPO_4 , pH 7.0 and eluted with 1.0 M KPO_4 , pH 7.0, the salt gradient was ramped rather than stepped using a Perceptive Biosystems BioCad. Active fractions were pooled and concentrated. Further purification was carried out for experiments that required protein to be $>99\%$ homogeneous. Generally, the major contaminant following the anion exchange column was a protein that had a sharp absorbance peak around 400 nm which is thought to be catalase. Since catalase is a tetramer with a molecular weight of 59 kD and PPLO is a dimer with an apparent molecular weight of 119 kD, gel filtration was not very effective at separating the two proteins. However, two additional columns have been successfully employed. The first, a concanavalin A column, binds proteins based on their carbohydrate content. A gradient of 0.1 M to 1.0 M methyl α -D-glucopyranoside was used. The second, an anion exchange step again, was run at a reduced pH (pH 5.6 rather than 7.0 with the Poros PI column used earlier in the purification). The gradient was 0.1 M to 1.0 M MOPSO + NaCl. The NaCl was added until the conductivity was > 90 mS.

During purification the enzyme activity was monitored by a literature method (59) using 1 mM benzylamine as the substrate. Enzyme purity was determined via SDS polyacrylamide gel electrophoresis and UV-VIS spectroscopy. Only protein of greater than 99% purity was used for the experiments herein. The best protein samples (greatest specific activity) were submitted for crystallization trials.

Molecular Weight Analysis

Three different approaches have been used to determine a molecular weight for PPLO. The first gave a predicted pI/MW based on the amino acid sequence alone. The sequence was submitted via the web to the ExPASy Molecular Biology Server on the world wide web (<http://www.expasy.ch/>). The result was a predicted MW of 90 kD and a predicted isoelectric point of 4.5. The second method tried was MALDI-TOF. Unfortunately, these experiments were not successful. Sinnapinic acid was to provide the matrix used in either a 50:50 or a 70:30 ratio to a 10 mg/ml protein sample. The control, bovine serum albumin, flew successfully under the same conditions. Lastly, PPLO was deglycosylated by PNGase F at 37° C for 24 hrs, which removes N-linked carbohydrate moieties. The deglycosylated protein was compared with freshly isolated protein by their apparent molecular weights determined by SDS/PAGE (10 - 20% gel).

Spectroscopy

PPLO samples for resonance Raman consisted of either purified PPLO or purified PPLO followed by derivatization, using a 20-fold excess of phenylhydrazine or *p*-nitrophenylhydrazine. Subsequently, samples were extensively microdialyzed (microdialysis system from Bethesda Research laboratories, Inc.) against 500 mL of 0.1 M KPO₄, pH 7.0 to remove any unreacted derivative. Raman spectra were obtained using a Spex Triplemate (model 1877) spectrograph (0.60 m, 1800-groove grating), a Spex Spectrum One liquid N₂-cooled CCD detector, and a Coherent Innova Ar⁺ laser as the excitation source. Samples were placed in glass capillaries and spectra were measured at room temperature. The excitation wavelength used was 457.9 nm with a 40

mW power setting unless stated otherwise. X-band EPR data was obtained using a Bruker 220D SRC instrument interfaced to a PS/2 computer and controlled by SSI software. CD spectra were measured using a Jasco J-710. The step resolution was 1 nm, the scan speed 100 nm/min, slit width of 500 μ m and a sensitivity of 10 mdeg.

Kinetics

All turnover experiments were conducted at 30° C. A coupled peroxidase assay with ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) was used to monitor the turnover of amine substrates (60). The concentration of ABTS was kept constant at 2 mM. Horseradish peroxidase, 11.6 purpurogallin units (61), and four μ g of protein were used per assay. Substrate concentrations were varied from 3 to 3000 μ M. Data were collected at thirteen substrate concentrations (except for ornithine, where nine concentrations were used). Triplicate runs were carried out at each concentration. Reaction mixtures had a final volume of two mL. Kinetic parameters were obtained from analysis of a Hanes plot ($[S]/v$ versus $[S]$) (62). All of the correlation coefficients exceeded 0.99.

Alternate sequence Tyr384 $\rightarrow$ Phe was more difficult to analyze due to its smaller k_{cat} values. Accordingly, the standard deviation was greater than that of the recombinant wild-type enzyme. Unfortunately, kinetic data could not be collected for the other two alternate sequences.

Homology Modeling of PPLO

The program Insight II (Molecular Simulations) was used on an Octane Silicon Graphics computer to overlay the four crystallographically determined structures of amine oxidases (*Escherichia coli* amine oxidase (ECAO), pea seedling amine oxidase (PSAO), *Pichia angusta* (previously *Hansenula polymorpha*) amine oxidase (PAAO), and *Arthrobacter globiformis* amine oxidase (AGAO)). The amine oxidase sequences were aligned based on sequence identity along with PPLO in the subprogram Homology. Homologous regions were superimposed on the crystal structures and connected by random loops. Coordinates were then assigned to PPLO. There were no coordinates to residues for portions of the N and C termini (1-82 and 745-779), so they were not included. Copper was omitted from the structure and Tyr was used instead of TPQ in the active site. The subprogram Discover was then used to perform an energy minimization on the PPLO enzyme which was not solvated. The first routine used the method of steepest descent. The default CVFF forcefield parameters were used without cross terms or Morse bond potentials (63). The new molecule was then minimized using cell multiple cutoff values for the conjugate gradient method (64). The resulting molecule in Fig. 13 is a surface rendering and in Fig. 14 an α -carbon backbone trace. This method has been successfully used by researchers modeling other systems (65,66).

Figure 13. Comparison of the PPLO model to the X-Ray crystallographic structure of AGAO. Gray and dark gray each represent one of the subunits, while black represents TPQ. A) AGAO - This view is looking down the substrate-binding channel at TPQ, which is barely visible. B) PPLO - This view is also looking down the substrate-binding channel at TPQ which is clearly visible. Also visible is a large V-shaped depression, not present in AGAO, with TPQ sitting in the cleft of the V.

Figure 14. Overlaid backbone structures of AGAO in black and PPLO in light gray.

Results and Discussion

Spectroscopic Properties

The absorbance spectrum of PPLO is very similar to previously reported amine oxidase spectra (Fig. 15). Protein concentrations were determined from the absorbance at the λ_{max} of 280 nm ($\epsilon_{280} = 140,150 \text{ M}^{-1} \text{ cm}^{-1}$ per monomer). This was calculated by counting the number of tyrosines and tryptophans found in the coding sequence, and multiplying by their corresponding conversion factors ($\epsilon = 1210 \text{ M}^{-1} \text{ cm}^{-1}$ and $5500 \text{ M}^{-1} \text{ cm}^{-1}$, respectively). A smaller absorption band at 480 nm ($\epsilon_{480} = 2100 \text{ M}^{-1} \text{ cm}^{-1}$ per monomer) was calculated by comparing the ratio of the absorbance at 280 nm vs. 480 nm and multiplying by ϵ_{280} . This second band arises from electronic transitions of TPQ,

Figure 15. The absorbance spectrum of PPLO in 100 mM KPO₄, pH 7.0. The protein concentration was 98 μM and measured in a 1.0 cm pathlength cell.

and gives amine oxidases their distinctive pink color. The CD data shows a band at 480 nm corresponding to TPQ and a band at 800 nm due to Cu (II) d-d transitions (Fig. 16). The PPLO spectrum is most similar to the CD spectrum of BPAO reported by Suzuki *et al* (67). However, a positive feature at 600 nm and a minor peak near 500 nm that are present in many other amine oxidase CD spectra are absent in that of PPLO.

Figure 16. CD spectrum of PPLO. The CD data was collected at room temperature with a constant slit width of 500 μm . The enzyme concentration was 120 μM in 100 mM KPO_4 , pH 7.0.

The EPR spectrum is also typical of amine oxidases which is consistent with a Cu(II) in a $d_{x^2-y^2}$ ground state (Fig. 17). The experimental spectrum may be satisfactorily simulated with values of $g_{\parallel} = 2.273$, $g_{\perp} = 2.056$, and $A_{\parallel} = 184$ G. Double integration of the signal indicated 0.87 Cu(II) ions were present per monomer. The $g_{\perp}$ and $g_{\parallel}$ values for amine oxidases range from 2.04 - 2.078 and 2.229 - 2.31,

respectively; $A_{||}$ is typically between 149 - 175 Gauss. These values are consistent with a five-coordinate square pyramidal geometry (4) with oxygen and/or nitrogen ligands. A five-coordinate Cu(II) with three histidine imidazole and two water ligands is observed in the crystal structures of ECAO and AGAO at acidic pH values. In contrast, the equatorial water ligand is less well-defined in the PAAO crystal structure (3). If a tetrahedral geometry for the Cu(II) was present, then a much smaller $A_{||}$ value would be expected. The spectrum is not consistent with either a tetrahedral or square planar geometry.

Figure 17. X-band EPR data of PPLO at 76 K. The power was 6.32 mW, the frequency at 9.4 GHz, and the modulation amplitude at 12.5. The enzyme concentration was 120 μ M in 100 mM KPO_4 , pH 7.0.

An active site peptide from PPLO has been isolated and sequenced (68). Resonance Raman spectroscopy (69) of the phenylhydrazine derivatives of this peptide, the whole enzyme, and the model compound TPQ-hydantoin identified TPQ as the active site cofactor in PPLO. Mass spectroscopy analysis indicated that the labeled cofactor was coded by tyrosine 382 (68). The *p*-nitrophenylhydrazine derivative of the intact enzyme has also been prepared and examined in order to confirm this conclusion. The resonance Raman spectra of both the phenylhydrazine and *p*-nitrophenylhydrazine derivatives are similar to the Raman spectra of the corresponding derivatives of the model compound, TPQ hydantoin (Fig. 18). The spectra are distinctly different from the spectra of LTQ in bovine aorta lysyl oxidase (36). Therefore, these data confirm that the

Figure 18. Resonance Raman spectra of derivatized PPLO and the model compound TPQ-hydantoin. All samples were run in 0.1 M KPO₄ buffer, pH 7.0. The two top panels were derivatized with phenylhydrazine and the lower two with nitrophenylhydrazine.

active site carbonyl cofactor in PPLO is TPQ and not LTQ (70). Both the absorbance spectrum and resonance Raman data establish the presence of TPQ. Therefore, the relatively high activity of PPLO (compared to other TPQ-containing amine oxidases) in peptidyl lysyl oxidation is not due to the structure of the cofactor *per se* (70).

Specificity

Steady-state kinetics data establish that 1,6-hexanediamine was the best substrate and ornithine the poorest among those examined (Table 2). When compared with the results of Haywood and Large (Table 3) (29), the K_M values are within a factor of two with the exception of 1,4-butanediamine. However, when compared with the work done by Tur and Lerch (49), significant differences are apparent with some K_M values differing by more than a factor of two. Furthermore, we observe different effects for N- or C-terminal modifications on lysine oxidation. Tur and Lerch's data indicate that acetylating the α amino group significantly decreases the turnover number. In contrast, methylation of the carboxyl group results in an increase in the turnover number. Finally, the combination of these two modifications produces an even greater increase in

Table 2. Kinetic Parameters of Various Substrates for PPLO

	K_M (μM)	V_{max}	k_{cat} (min^{-1})	k_{cat}/K_M
n-butyl amine	81 ± 7	.396	241 ± 7	2.99
benzylamine	58 ± 7	.368	224 ± 9	3.88
β -phenethylamine	11 ± 4	.430	262 ± 8	23.50
1,4-butanediamine	34 ± 5	.531	324 ± 11	9.61
1,6-hexanediamine	3 ± 7	.418	255 ± 10	92.3
ornithine	549 ± 19	.290	177 ± 8	0.32
spermidine	31 ± 2	.581	353 ± 11	11.39
lysine	47 ± 4	.255	155 ± 6	3.31
lysine methylester	39 ± 3	.267	163 ± 6	4.19
N- α -acetyl lysine methylester	70 ± 3	.419	255 ± 10	3.67

Table 3. Comparison of K_M Values Obtained by Different Researchers.

Substrate	Apparent K_M Values (mM)		
	(Tur and Lerch)	(Large and Haywood)	(Kuchar and Dooley)
Butylamine	0.235	0.083	0.0806
Ornithine	0.80		0.549
Lysine	0.091	0.063	0.0468
Lysine methylester	0.0047		0.0388
N-a-acetyllysine	2.97		
N-a-acetyllysine methylester	0.013		0.0697
b-phenethylamine		0.023	0.0112
Benzylamine		0.035	0.0577
1,4-butanediamine		0.0005	0.0337
1,6-hexanediamine		a	0.0028
Spermidine		a	0.0310
Spermine		a	
Histamine		a	

^a Active but K_M value not determined

turnover number, compared with methylation alone. Our data displays the same trend for the methylation of the carboxyl group. However, we find that the combined modifications had a cancellation effect, resulting in a similar turnover number to unmodified lysine (Table 2).

A family of lactone compounds has recently been identified as lysyl oxidase inhibitors (71). This class included the compound HCTL, homocysteine thiolactone. It was found that PPLO did not have the same specificity towards this inhibitor. HCTL was not an effective inhibitor of PPLO (no effect at 50 μ M), whereas, it was a fairly potent inactivator of the bovine aorta lysyl oxidase enzyme ($K_I = 21 \pm 3 \mu$ M) (71). This may suggest a difference in reactivity of TPQ (PPLO) and LTQ (mammalian lysyl oxidase).

HCTL

Some of the differences between our results and previous measurements (29) (49) of kinetic parameters could arise from different states of glycosylation. We have observed at least three states of the enzyme by SDS/Page (Fig. 19). After initial purification a 120 kD form is found and was the form characterized spectrally and kinetically. However, if the sample is stored for more than 2 months at 4 °C at > 10 mg/ml (less time at greater concentrations) a white precipitate is formed; after centrifugation the protein migrates to about 112 kD. This 112 kD form is also

Figure 19. SDS/Page of different glycosylation states of PPLO. Lanes 1,2, and 3 are PPLO stored at 4° C for > two months. Lane 1 is a PPLO control taken directly from 4° C. Lane 2 was run under deglycosylation conditions minus PNGase F. Lane 3 was run under deglycosylation conditions with PNGase F. Lanes 4, 5, and 6 are freshly isolated PPLO. Lane 4 is a PPLO control directly taken from 4° C. Lane 5 was run under deglycosylation conditions minus PNGase F. Lane 6 was run under deglycosylation conditions with PNGase F.

associated with a higher specific activity toward benzylamine. Both of these forms when subjected to deglycosylation conditions (PNGase-F at 37 °C for 24 hrs) migrate to 107 kD. The value reported by Tur and Lerch was 106 kD (49). Differences in the molecular masses of the enzyme, such as that reported here and by Tur and Lerch, could also be due to different glycosylation patterns among different cell strains of *P. pastoris*; Tur and Lerch do not state which strain they used to isolate the enzyme.

Alternate Sequences

Sufficient amounts of protein for the alternate sequence Tyr384 → Phe was obtained for both spectroscopic and kinetic analysis (~5 mg/L were expressed). The resonance Raman and CD spectra of the alternate sequence were similar to the recombinant wild-type protein (Figs. 20 and 21, respectively). However, the turnover

Figure 20 Resonance Raman of wild-type PPLO and Y384F. Spectra were obtained under identical conditions with the exception of the excitation laser line used. The line at 514 nm was used for the wild-type and the line at 496 nm was used for Y384F.

kinetics were very different (Table 4). The k_{cat} values were similarly depressed for all three substrates tested. Although, the K_M values increased for all three substrates, the effects were varied. Butylamine changed only slightly, benzylamine increased two-fold, and 1,6-hexanediamine increased ten-fold.

Figure 21 CD spectra of wild-type and Y384F PPLO, 120 μM .

Table 4. Mutant Y384F and Wild-type Kinetic Parameters.

Substrate	K_M (μM) WT	K_M (μM) Y384F	V_{max} Y384F	k_{cat} (min^{-1}) WT	k_{cat} (min^{-1}) Y384F	k_{cat}/K_M (mM) Y384F
Butylamine	80.6	93.8	0.180	241	55	0.586
Benzylamine	57.7	115.7	0.213	224	65	0.562
1,6-hexanediamine	2.76	29.0	0.206	255	62	2.14

Other researchers, working with different organisms, have changed this Tyr which is hydrogen bonded to TPQ in the known crystal structures (Tyr305 in Fig. 2). Klinman and coworkers changed this residue to an Ala, Cys, or Phe for PAAO (72).

The Ala and Cys alternate sequences behaved similarly towards substrates with a decrease in k_{cat} of 4-7 fold. However, the Phe alternate sequence k_{cat} decreased > 100-fold and $k_{\text{cat}}/K_{\text{M}}$ decreased > 500-fold. Klinman proposes that the Phe alternate sequence disrupts an extensive hydrogen bonding network in the active site, inhibiting proton transfer to oxygen during turnover. In contrast Ala or Cys can maintain this network and has only a small decrease in catalytic efficiency.

The Leeds group changed this residue to a Phe in ECAO (73). The $k_{\text{cat}}/K_{\text{M}}$ for the substrate β -phenethylamine was reduced by 50-fold at pH 7.0. This is very similar to the result (*vide infra*) for PPLO Y384F using 1,6-hexanediamine as the substrate. However, they report that this is mostly due to a decrease in k_{cat} because the K_{M} values were 1.2 μM for the wild-type and 1.5 μM for the alternate sequence enzymes. In contrast, PPLO Y384F had a large increase in K_{M} and only a modest decrease in k_{cat} . They propose that TPQ can rotate into a "non-productive" orientation more readily for this alternate sequence than in the wild-type, thus reducing k_{cat} by a factor of 40.

The alternate sequence, Thr474 $\rightarrow$ Leu, yielded very little protein (~ 0.5 mg/L). Of the protein present very little TPQ was reactive with nitrophenylhydrazine (18-fold less than wild-type). Furthermore, the activity toward 1,6-hexanediamine had decreased by 950-fold. It could be that low expression is seen because the enzyme is improperly folded and readily degraded. A suitable over-expression candidate has not been found for the last alternate sequence, His453 $\rightarrow$ Ala.

Modeling of PPLO

Among the four crystallographically determined structures ECAO, AGAO, PSAO, and PAAO, the substrate channel is quite narrow and access to TPQ from the solvent appears to be sterically limited. In contrast, the PPLO model shows TPQ at the base of a V-shaped depression in the surface of the protein. This depression appears to be much larger than the channel present in the structurally characterized enzymes (Fig. 13).

Of the structurally characterized amine oxidases, PPLO is most homologous to AGAO. The PPLO model was overlayed with the AGAO crystal structure. It was observed that the interior of the protein was very similar, whereas the solvent exposed regions did not overlay nearly as well. This included the region around the substrate channel. Furthermore, there are no absolutely conserved residues in the channel leading to the active site based on the lineup or the crystal structures.

The modeling data suggests PPLO has a substrate channel that can accommodate large molecules more readily than other TPQ-containing amine oxidases (Fig. 13). It appears that the lysyl oxidase activity of PPLO may be a consequence of a protein fold that results in an especially accommodating active-site channel. Variations among the substrate-channel X-ray structures and their corresponding amino acid sequences suggest that this feature may be important in determining substrate specificity. Alternatively, variations in the dynamics or energetics of conformational changes in the active-site region (including the channel) may also influence substrate specificity.

Crystallography

Very pure protein samples have been submitted for crystallization trials. This work has been done in collaboration with Dr. Hans Freeman (University of Sydney, Department of Biochemistry, Sydney, Australia). Crystals have been grown and have diffracted to 2.65 Å (Table 5). Currently, heavy-atom derivative soaks are underway and collection of X-ray data on suitable derivatives are planned.

Table 5. Current Status of *Pichia pastoris* Lysyl Oxidase Crystals.

Crystal forms 1 & 2	Orthorhombic	Orthorhombic
Space group	P2 ₁ 2 ₁ 2 ₁	I2 ₁ 2 ₁ 2 ₁
Unit cell dimensions	a = 84.6, b = 163.7, c = 315.3 Å	a = 115.2, b = 144.8, c = 192.2 Å
Asymmetric unit	2 – 4 molecules (depending on solvent content)	1 molecule
Data recorded	3.5 Å data set (rotating-anode X-ray generator) 2.65 Å data sets (2) at synchrotron	2.7 Å data set (rotating-anode X-ray generator)

Conclusions

Spectroscopically, PPLO is very similar to other amine oxidases. UV-VIS, CD, EPR, and resonance Raman all indicate that PPLO belongs to the amine oxidase family of proteins rather than to the lysyl oxidase family. Based on this data, it is expected that the active site structure will closely resemble other currently available amine oxidase structures. In contrast, it is interesting to note that the kinetic parameters of PPLO are quite dissimilar to other amine oxidases. For example, PPLO turns over the substrates putrescine, ornithine, lysine, spermine, and spermidine, whereas, neither the methylamine oxidase nor the benzylamine oxidase from *Candida boidinii* is active

towards these amines. Furthermore, PPLO has a substrate specificity similar to the mammalian lysyl oxidase.

The kinetic parameters of Y384F indicate an important, but not essential role for this residue. In PPLO the catalytic activity for the three substrates investigated had uniformly decreased, but the affinity for these substrates had been altered incongruously. Other groups found varying effects when this residue was mutated in other amine oxidases (72,73). One similarity found through structural analysis for the ECAO and PAAO enzymes found this Tyr important for maintaining TPQ in a conformation likely to promote catalysis. The increase in TPQ flexibility (TPQ is able to rotate into a non-productive conformation) determined from the crystal structures may explain the decreased catalytic efficiency of these mutants. The extent of this effect and its variability especially when using different substrates indicates that either this residue plays at least a slightly different role in each enzyme or the environment of each active site is being effected uniquely. In fact, it has been proposed that this reflects the abilities of the active site waters or residues to compensate for the altered sequence during turnover (72,73). Lastly, the Thr474 → Leu alternate sequence results indicate an essential role for this residue in efficient reactive TPQ formation.

Clearly, it is not the cofactor structure in and of itself that determines the substrate specificity. Lysyl oxidase and PPLO are from different kingdoms, have different translocation profiles (one is expressed intracellularly and the other is secreted), and have completely different coding sequences. Yet, PPLO and lysyl oxidase appear to have similar substrate specificities towards peptidyl lysines (49) (*vida*

infra). PPLO may still be a useful model for determining the recognition signal of lysyl oxidases for lysine and peptidyl lysine residues. In lieu of a crystal structure, a homology model was generated in order to investigate possible structural differences among the amine oxidase structures and the PPLO model that could help explain PPLO's substrate preferences. It appears PPLO has a much larger substrate channel and it is this feature that is responsible for PPLO's ability to turn over such a large variety of substrates. It is critical to complete the crystal structure and confirm, revise, or replace this hypothesis. Additionally, the crystal structure comparison to the PPLO model would speak directly to the validity of the methods employed to generate the model. If validated, this could be an attractive alternative for proteins in this family that are resistant to crystallization. This lab has also submitted the EPAO, equine plasma amine oxidase, for crystallization trials, but these have resulted in only poor crystals and when analyzed contain a very large space group. This protein is heavily glycosylated which is notorious for disrupting the formation of high quality crystals for collecting X-ray data.

Obtaining the lysyl oxidase structure and comparing it to PPLO and other amine oxidases should remain a high priority. This would address whether it has an open substrate channel similar to PPLO. If lysyl oxidase does have an open channel, this would indicate how PPLO and the family of lysyl oxidases are able to turnover such large substrates such as tropoelastin. Otherwise, this may indicate a specific recognition of tropoelastin by lysyl oxidase and an adventitious interaction by PPLO.

CHAPTER 4

EXPRESSION OF BOVINE AORTA LYSYL OXIDASE (BALO) AND ANALYSIS OF ITS ACTIVITY WITH TROPOELASTIN IN COMPARISON TO PPLO

Introduction

As pointed out previously, PPLO is a member of the amine oxidase family. However, prior results have indicated that PPLO and the lysyl oxidase family have a similar substrate specificity (49). Rather than use lysyl derivatives or small peptides, we sought a direct comparison of PPLO and bovine aorta lysyl oxidase (BALO) to determine their specificity towards the *in vivo* substrate, tropoelastin. The form of tropoelastin used in this work is the best substrate model currently available for the true substrate(s). In order to compare these enzymes it was necessary to express and isolate not only BALO but also the radiolabelled substrate, tropoelastin. Various enzymes were incubated with tropoelastin and the amount of disintegrations per minute from radiolabelled water generated from the condensation of lysyl groups or lysyl derivatives counted. This is an indirect measurement of the amount of substrate turned over.

Materials and MethodsIsolation and Radiolabelling of Tropoelastin

A slightly modified method of that developed by Dr. Herbert Kagan (Department of Biochemistry, Boston University School of Medicine, Boston MA) for isolating and radiolabelling tropoelastin was employed. A recombinant *E. coli* strain containing recombinant human tropoelastin (generously provided by Dr. Kagan) was inoculated

onto a LB plate and incubated at 37° C overnight. One colony was transferred to a 50 mL LB culture with 50 µg/mL ampicillin. This was also incubated overnight (16-18 hrs.) at 37° C. The total volume was then increased to 1 L and incubated for an additional 2 hours.

Next, the cells were sterilely centrifuged at 5,000 rpm for 10 min. These were washed three times with lysine-deficient RPMI-1640 medium (Invitrogen, Carlsbad CA). The cell pellet was suspended in 500 mL of the RPMI medium and shaken for 10 min at 37° C. Protein expression was stimulated at this point by addition of 30 mg of nalidixic acid and allowed to incubate for 2 hours. Radiolabelled 4,5-[3H]-lysine (1 mCi in 1 mL of water) was then added and incubated for 3 more hours at 37° C.

Cells were spun down at 5,000 rpm for 10 min and washed twice with PBS (Phosphate Buffer Saline - 0.2 g KCl, 0.2 g KH₂PO₄, 8.0 g NaCl, 2.89 g Na₂HPO₄ + 12 H₂O, diluted to 1 L with sterile deionized H₂O). The cell pellet was resuspended in buffer A (50 mM Tris - pH 8.0, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 5% glycerol). Next, lysozyme (2 mg/10 mL of sample) was added and incubated at 0° C for 30 min. The sample was then spun down at 10,000 rpm for 20 min. The pellets were resuspended in buffer A and 0.05% deoxycholic acid. After mixing the cells were homogenized using a Dounce-pestle B (15 strokes). The sample was then centrifuged at 10,000 rpm for 30 min.

The pellet was then treated with 4 mL of 70% formic acid and 630 mg of CNBr. The reaction was kept in the hood with stirring overnight at room temperature. Water was added (1/3 of the volume) and left uncovered on ice for 4 hours. The sample was

then spun down again at 10,000 rpm for 10 min. The supernate was dialyzed against three exchanges of 1 L of 0.1 M acetic acid. The samples were aliquoted at 0.5 mL each and stored at -80°C .

Purification of Bovine Aorta Lysyl Oxidase

The protocol followed is basically that of Kagan and Cai (74). Bovine aortas from 1-3 week old calves were obtained from A Arena and Sons Inc. (Hopkinton, MA). The purification was started with ~600 g of aortas. First, The aortas were frozen in liquid nitrogen and a Waring blender was used to grind them into small pieces. The tissue was extracted twice with 1500 ml of Buffer I (0.4 M NaCl, 16 mM KPO_4 , pH 7.8) at 4°C . The pellet was then extracted with 1500 ml of Buffer II (16 mM KPO_4 , pH 7.8) at 4°C . Finally, the pellet was extracted three more times with 1500 ml of Buffer III (4.0 M urea, 16 mM KPO_4 , pH 7.8) at 4°C . The Buffer III extracts were combined (4.5 L total) and mixed with 500 g of Bio-Gel hydroxyapatite already equilibrated in Buffer III. The suspension was stirred for 10 min at 4°C and allowed to settle for 30 min. The supernate was centrifuged at 10,000 rpm for 10 min and concentrated to 750 mL using two 250 mL Amicon ultrafiltration membranes (YM10). All the extracts and hydroxyapatite protocol were completed the first day. However, the two 250 mL Amicons were very inefficient at concentrating the protein solution and took three full days to complete.

This was then dialyzed against two exchanges of 25 L of Buffer II overnight. The sample was precipitated by addition of an equal volume of 1.0 M KPO_4 , pH 7.8. The precipitate was dissolved in Buffer IV (6.0 M urea, 16 mM KPO_4 , pH 7.8) and run

through a Sephacryl S-200 column (100 x 5 cm) using Buffer IV which took ~1.5 days for the active fractions to elute. Fractions were analyzed by SDS/PAGE and the ABTS assay previously described (60). Active fractions were pooled and concentrated to 25 mL using the 60 mL Amicon (YM10). The sample was further purified by running through a Sephacryl S-100 (100 x 2.0 cm) column with Buffer IV. The active fractions eluted after ~12 hours, were pooled, and stored at 4° C.

Tropoelastin Assay

Reactions consisted of the substrate (tropoelastin, 50,000 dpm (disintegrations per minute)), 100 picomoles of the oxidase monomeric subunit being studied, and buffer (either 1.2 M or 140 mM urea in 0.1 M KPO₄, pH 7.6). These were incubated for 2 hours at 37° C. Two different literature methods were used to analyze the reaction products. The first method developed by Shackleton and Hulmes (75) determined the enzyme activity by scintillation counting of the ultrafiltrate from an Amicon C-10 microconcentrator and subtracting the number of counts in the presence of BAPN, a specific inhibitor of BALO. The method was slightly modified for this work since BAPN is not specific for all amine oxidases and lysyl oxidases. Instead, a control reaction was run along with the enzymatic reactions. The control which omitted the addition of enzyme, was counted directly while the enzymatic reactions were evaporated and the precipitate resuspended in water. The difference in the number of counts between the control and the enzymatic reactions represent the amount of labelled water that had condensed, evaporated, and thus, represent the relative activity of each enzyme.

The second method developed by Bedell-Hogan *et al* (76) employs an analysis of the radiolabelled water. The reactions are evaporated and collected in a cold trap. The distillate is scintillation counted which also indirectly measures the amount of enzymatic activity present.

Results and Discussion

Tropoelastin and BALO Purification

Typical yields of radiolabelled tropoelastin resulted in a volume of 8 mL with counts of ~7500 dpm/ μ L and ranged in size from < 10 kD to > 100 kD. BALO was ~90% homogeneous judged by SDS/PAGE after purification. The quantity (3 mg) and purity were sufficient for carrying out the desired kinetic experiments. The enzyme was stored at 4° C as opposed to the -80° C described. Thus, the protein slowly loss activity over time. Initially, the specific activity was 1.18×10^6 dpm mg^{-1} assaying at 37° C for 2 hours against 50×10^5 dpm of human recombinant tropoelastin substrate which is similar to the values obtained by other researchers (74).

Assays Versus Tropoelastin

The first method for analyzing the reactions which used the microconcentrators, had a large number of counts in every reaction which suggested that every amine oxidase employed had activity toward tropoelastin. This had not been reported previously. Additionally, the control reaction had a large number of counts and the standard deviation for each data set were high. Therefore, it was concluded that this assay was inherently unreliable. So, the more rigorous method described by Kagan *et*

al (76) was then implemented. This data contradicted the results found from the first method, but were consistent with previous findings (49). Additionally, the control reactions had relatively few counts and a much smaller standard deviation. The reactions were performed with various amine oxidases and compared to two different lysyl oxidase enzymes (Table 6). BALO, DLLO (*Drosophila melangaster* lysyl oxidase), and PPLO all had similar activities which were ~5 times the background rate. In contrast, AGAO, PSAO, KDAO, and EPAO had activities at background levels.

Table 6. Activity of Various Oxidases Versus Tropoelastin.

Enzyme	DPM
Control	142±13
BALO	833±52
DLLO	813±60
PPLO	767±25
AGAO	140±22
PSAO	142±10
KDAO	124±6
EPAO	134±10

These reactions were run in triplicate in the presence of 140 mM urea and 0.1 M KPO₄, pH 7.6.

Conclusions

PPLO was the only amine oxidase examined that was able to oxidize tropoelastin. It was anticipated that AGAO and PSAO would not be active toward tropoelastin since these enzymes previously showed no activity toward certain bulky amines (i.e... spermine, histamine, and dopamine). However, the results for KDAO, EPAO, and PPLO were not straight forward. KDAO had been shown to be active versus some of these compounds (77,78). Although neither the sequence nor even

basic characteristics of EPAO are known, it is assumed to be very homologous to KDAO. Thus, these two enzymes are expected to behave similarly and do in this work. Not only had PPLO also been shown to be active versus these compounds but, it was demonstrated that it could turnover certain lysyl peptides (49). Based on those studies, it was not surprising that PPLO had activity toward tropoelastin. Remarkably, PPLO turned over tropoelastin at a similar rate to BALO and DLLO. This is quite surprising considering tropoelastin is a natural substrate for BALO, but this protein is not even found in yeast. It was thought that KDAO and perhaps EPAO could also be active toward tropoelastin considering KDAO is the most homologous protein to PPLO known. This was obviously not the case. Even though they share the unique ability to turnover certain bulky amine substrates, this does not extend to recombinant human tropoelastin.

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APPENDIX A

BASIC MOLECULAR BIOLOGY METHODS

Southern Hybridization

The method employed for the Southern hybridization experiment was that found in "Current Protocols in Molecular Biology" (49), Section 2.9.1 and 2.10.1 with the deviations listed below. The first deviation was the omission of the depurination step, since the fragments of interest were smaller than 4 kb. The transfer method used Whatman 3MM paper as a wick. The DNA was immobilized to the membrane using a UV transilluminator. The dried membrane was stored at -20° C.

The hybridization analysis used a probe generated by random oligonucleotide priming. The denaturation step of this probe was omitted. The last deviation was that the probe was allowed to hybridize for 48 hours at 65° C.

Library Search for PPLO

The method employed for the library screening experiment was that found in "Current Protocols in Molecular Biology" (49), Section 6.2.1 and 6.4.1 with the deviations listed below. The probe was allowed to anneal for 20 hours at room temperature. Then the membranes were washed four times for 5 min. at room temperature with 2 X SSC, 0.1% SDS. The final two washes were for 1 hour at 60° C with 1 X SSC, 0.1% SDS.

Electroporation of E. coli and yeast

The method employed for the electroporation of E. coli was that found in "Current Protocols in Molecular Biology" (49), Section 1.8.4 without deviation and for

the electroporation of yeast section 13.7.5 was used with omission of the lithium acetate and DTT treatment.

Isolation of yeast genomic DNA

The method employed for the isolation of yeast genomic DNA was that found in "Current Protocols in Molecular Biology" (49), Section 13.11.1 without deviation.

Ligation or Digestion of constructs

The protocols followed were those outlined by the manufacturer without deviation (New England Biolabs, Beverly MA or Promega, Madison WI).

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