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 Philosophy in Biochemistry
Montana State University
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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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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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TABLE OF CONTENTS

Page

1. INTRODUCTION 1

Overview of Amine Oxidases 1
Overview of Lysyl Oxidases 6
Yeast Amine Oxidases 10
Research Goals 13

2. SEQUENCE ANALYSIS AND OVER-EXPRESSION OF PPLO 15

Introduction 15
Materials and Methods 16
    Gene Sequence 16
    Primers 18
    Design of the Over-expression System 18
Results and Discussion 21
    Gene Isolation and Sequencing 21
    Sequence Homologies 22
    Over-expression of PPLO 30
Conclusions 30

3. STRUCTURAL AND MECHANISTIC STUDIES OF PPLO 33

Introduction 33
Materials and Methods 35
    Growth Conditions 35
    Generation of Mutants 36
    Purification 38
    Molecular Weight Analysis 40
    Spectroscopy 40
    Kinetics 41
    Homology Modeling of PPLO 42
Results and Discussion 44
    Spectroscopic Properties 44
    Specificity 48
    Alternate Sequences 51
    Modeling of PPLO 54
    Crystallography 55
Conclusions 55

4. EXPRESSION OF BOVINE AORTA LYSYL OXIDASE (BALO) AND ANALYSIS OF ITS ACTIVITY WITH TROPOELASTIN IN COMPARISON TO PPLO 58

Introduction 58
Materials and Methods 58
Isolation and Radiolabelling of Tropoelastin 58
Purification of Bovine Aorta Lysyl Oxidase 60
Tropoelastin Assay 61
Results and Discussion 62
Tropoelastin and BALO Purification 62
Assays Versus Tropoelastin 62
Conclusions 63

REFERENCES CITED 65

APPENDIX A: BASIC MOLECULAR BIOLOGY METHODS 71

LIST OF TABLES

Table Page

1. Percent Identity Among Amine Oxidases.. 22
2. Kinetic Parameters of Various Substrates for PPLO................................. 48
3. Comparison of K_M Values Obtained by Different Researchers................. 49
4. Mutant Y384F and Wild-type Kinetic Parameters.................................... 52
5. Current Status of *Pichia pastoris* Lysyl Oxidase Crystals......................... 55
6. Activity of Various Oxidases Versus Tropoelastin................................... 63

LIST OF FIGURES

Figure Page

1. Secondary Structure Rendering of the Four Available Amine Oxidase Crystal Structures.......... 2

2. Active Site of PAAO.......... 3

3. Proposed Mechanism of TPQ Biogenesis.......... 5

4. Proposed Mechanism for the Generation of Lysine Tyrosylquinone.......... 7

5A. Proposed Mechanism for TPQ Turnover.......... 8

5B. Proposed Mechanism for LTQ Turnover.......... 9

6. Chemical Structures of Selected Intermediates and Lysine-derived Cross-links in Collagen and Elastin.......... 11

7. Vectors and Constructs Used for the Over-Expression Systems.......... 19

8. Cloning Strategy for PPLO Over-expression.......... 20

9. Alignment of Structurally Characterized Amine Oxidases by X-ray Crystallography and Selected Mammalian Amine Oxidases with PPLO.......... 23

10. PPLO Model.......... 27

11. Phylogenetic Tree of Amine Oxidases.......... 31

12. An Outline of the MORPH™ Site-specific Plasmid DNA Mutagenesis Protocol.......... 37

13. Comparison of the PPLO Model to the X-Ray crystallographic Structure of AGAO.......... 43

14. Overlayed Backbone Structures.......... 43

15. The Absorbance Spectrum of PPLO.......... 44

16. CD Spectrum of PPLO.......... 45

17. X-band EPR Data of PPLO.. 46

18. Resonance Raman Spectra of Derivatized PPLO and the Model
Compound.. 47

19. SDS/PAGE of Different Glycosylation States of PPLO..................... 50

20. Resonance Raman of Wild-type PPLO and Y384F............................ 51

21. CD Spectra of Wild-type and Y384F PPLO....................................... 52

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 amilioride 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):

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2 \qquad (1)$$

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

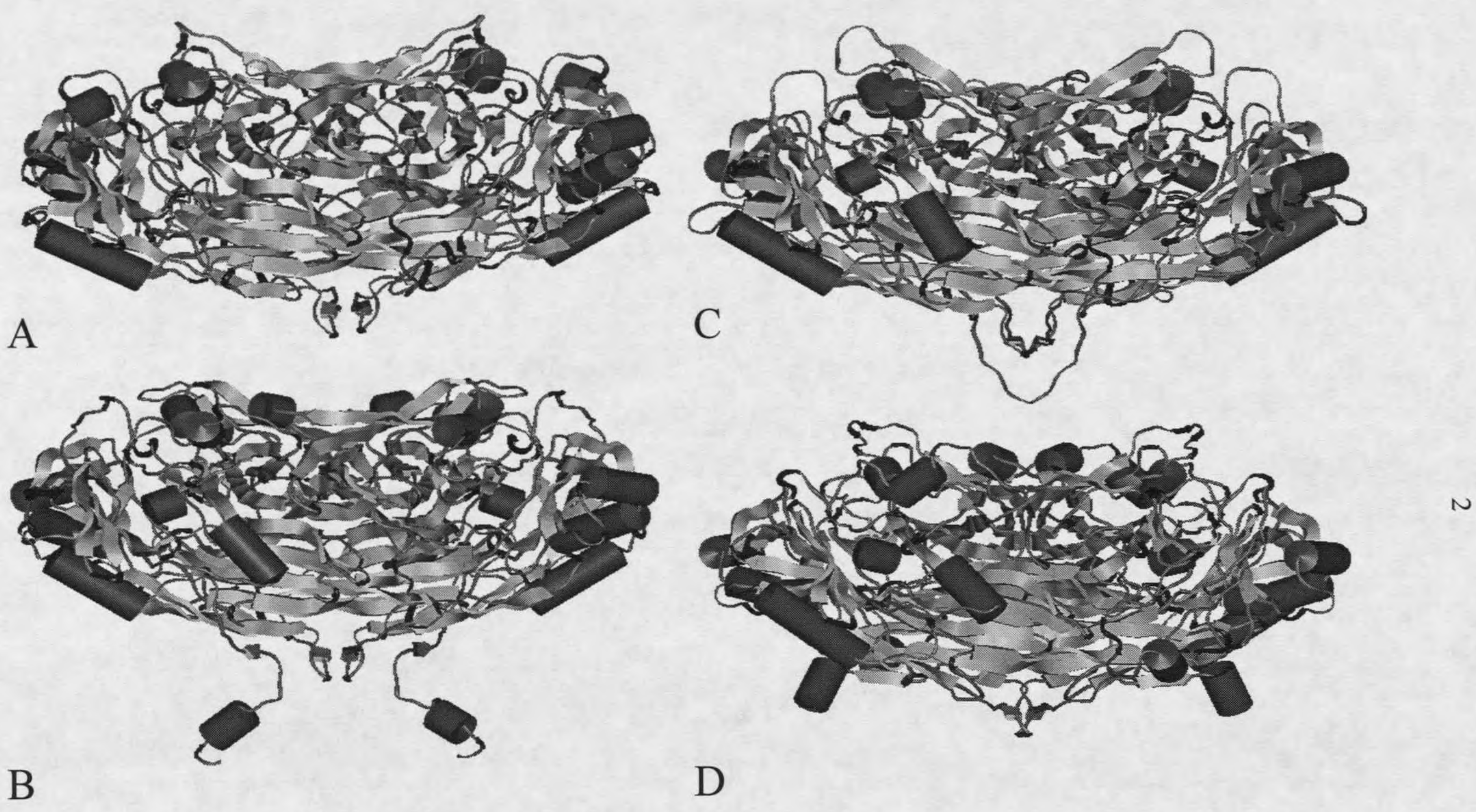

Figure 1. Secondary structure rendering of the four available amine oxidase crystal structures - barrels represent α-helical structure, arrows represent β-sheet structure, the light gray loops represent random coil structure and the dark gray sections on the loops represent turns. A) AGAO B) ECAO C)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

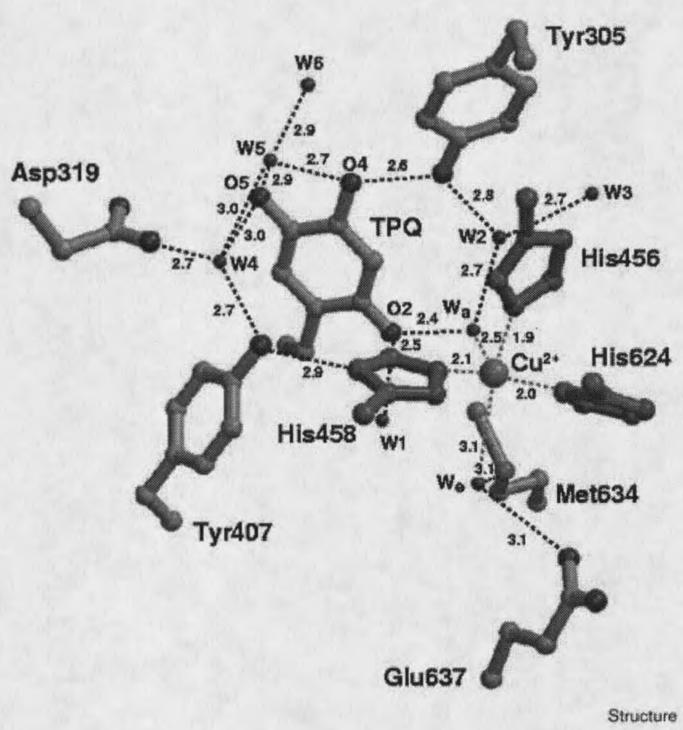

Figure 2. Active site of PAAO. The substrate channel extends from the upper left corner towards TPQ (*5*).

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 O_2 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 H_2O_2 and aldehyde products generated by oxidation, not

O O OH H N O O

TPQ LTQ