Expression and characterization of copper-containing proteins: galactose oxidase and tyrosinase
by Ejan Marie Kamlin

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University

Abstract:
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Tyrosinase, another copper-containing protein, has been expressed and purified from Streptomyces. Due to low protein yields, expression was attempted in Escherichia coli. Although the E. coli expression systems were not successful, other options exist which should be examined in order to obtain adequate amounts of recombinant protein in order for characterization to proceed.

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Ejan Marie Kamlin

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

December 2002

APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the thesis 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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ACKNOWLEDGEMENTS

I would like to thank past and present members of the Dooley group, especially Dr. Melanie Rogers for her expertise and friendship. I would also like to thank Dr. David Dooley for his continued support throughout my stay at Montana State University.

TABLE OF CONTENTS

1. INTRODUCTION....1
Overview of Protein Copper Centers....1
Galactose Oxidase....3
Applications of Galactose Oxidase....4
Structure....5
Tyrosyl Radical....9
Catalytic Mechanism....11
Research Goals....11
Tyrosinase....13
Structure....14
Molecular Mechanism....16
Biological Role of Tyrosinase....18
Research Goals....20

2. EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF GALACTOSE OXIDASE MUTANTS....22

Introduction....22
Materials, Methods, and Results....23
Construction of Expression Plasmids....23
Aspergillus Expression Vectors for W290H & W290G....23
Pichia pastoris Expression Vectors for Wild type and W290F..24
Transformation of *Aspergillus* and *Pichia*....25
Aspergillus Transformation....25
Pichia Transformation....26
Small-scale Expression of W290F....27
Results of Transformation and Small-scale Expression of W290F....28
Expression and Purification of GOase....30
Aspergillus Expression and Purification of W290H and W290G....30
Pichia pastoris Expression and Purification of W290F....31
Results of Protein Preparations....32
Galactose Oxidase Enzyme Assay and Kinetic Analysis....33
Kinetic Analysis Results....34
Atomic Absorption Spectrometry....38
Atomic Absorption Spectrometry Results....38
Proton Uptake....39
Proton Uptake Results....40
Dissociation Constants for Azide Binding....42
Results of K_d Analysis with Azide....42
Azide Complexes of Oxidized W290H, W290G, and Semi-Reduced W290G....43
Results of Optical Spectra of Azide complexes....44
Dependence of Activity on pH....46
Results of pH Dependence of Activity....46

TABLE OF CONTENTS-CONTINUED

Radical Decay....47
Results of Radical Decay Experiments....47
Discussion and Conclusions....50
Crystal Structures....50
Protein Purification....54
Specific Activity....55
Kinetic Analysis....55
Copper Atomic Absorption Spectrometry....56
Proton Uptake....56
Dissociation Constant for Azide....58
Optical Spectra of Oxidized and Semi-Reduced W290 Mutants and their Azide Complexes....58
Dependence of Enzyme Activity on pH....59
Radical Decay....59
Conclusion....61
Future Experiments....61

3. EXPRESSION TRIALS AND PURIFICATION OF TYROSINASE....63

Introduction....63
Experiments and Results....65
Expression Vector pAH1....65
PEG-Assisted Transformation of *Streptomyces lividans* Protoplasts....65
Small-scale Expression of *Streptomyces glaucescens* Tyrosinase in *Streptomyces lividans*....67
Transformation and Small-scale Expression Results....69
Scale-up of Expression and Purification of Tyrosinase....70
Results of Scale-up and Purification....71
Metal-Free Protein Preparation....73
NusA-Tyrosinase Fusion Protein....74
Vector Construction....74
Small-scale Expression Trial of NusA-Tyrosinase....75
NusA-Tyrosinase Fusion Results....76
Codon Bias-Based *E. coli* Expression....76
Expression System....77
Codon Bias Expression Results....79
Discussion....79
Future Experiments....80

REFERENCES CITED....81

LIST OF TABLES

Table Page

1. GOase Activity of Transformants During Expression Trial at Certain Time-points....29

2. Specific Activity of GOase and W290 Mutants Using D-galactose as Substrate....34

3. Summary of Kinetic Parameters Using D-Galactose as Substrate....36

4. Summary of Kinetic Parameters Using 2-methylene-1,3-propanediol....38

5. Copper Content of GOase and the Mutants....39

6. Proton Uptake Values....42

7. Azide Binding Constants for Wild type and the Mutants....43

8. Decay Rates of Wild Type and Mutant GOase....49

9. Radical Decay of W290H in Various Buffers....50

10. Purification Table for Tyrosinase....72

LIST OF FIGURES

Figure Page

1. Copper Centers....2

2. Cross-links of GOase and Tyrosinase....3

3. Overall 3-D Structure of GOase....6

4. Active Site of GOase (Copper Coordination)....7

5. Active Site of Mature GOase....8

6. EPR Spectra of the Radical Site....11

7. Amino Acids that Replaced W290....13

8. Derivatives of the Tyrosinase Active Site....15

9. Similarities in the Spectroscopic Features of Ocyhemocyanin and Tyrosinase....16

10. Generation of *Aspergillus* Expression Plasmids....23

11. Mutagenesis Strategy....24

12. *Pichia* Expression Plasmid for Wild type or W290F....26

13. SDS-PAGE of KM71 Transformants at Different Time-Points....29

14. Elution Profiles....32

15. SDS-PAGE from a W290G Prep....33

16. Kinetics of Wild type and Mutants Using D-Galactose as Substrate....35-36

17. Kinetics of Wild type and Mutants Using 2-methylene-1,3-propanediol as Substrate....37

18. Typical Spectra of a Proton Uptake Experiment....41

19. A_{600} vs volume NaOH....41

20. Typical Graph of K_d Experiment....43

LIST OF FIGURES – CONTINUED

Figure Page

21. Optical Spectra of Oxidized and Semi-reduced Azide Complexes....44-46

22. pH Dependence on Activity....47

23. W290G pH Dependence on Activity....47

24. Typical Radical Decay Spectra....48

25. Radical Decay A_{445} vs Time....48-49

26. Active Site Structures (wild type and mutants)....53

27. Shuttle Vector pAH1....65

28. Activity Time Course of Tyrosinase Expression....69

29. SDS-PAGE of Expression Time-points....70

30. Mono-Q Elution Profile of Tyrosinase....72

31. SDS-PAGE of Mono-Q Elution....72

32. SDS-PAGE of a Metal-Free Prep of the MelC1:Tyrosinase Complex....73

33. Expression Vector pET 43a(+)....73

34. *MelC* Operon....75

ABSTRACT

Copper-containing proteins have been the focus of many biological and structural studies. The structure and function of galactose oxidase has been studied intensely. In this study, three mutants of galactose oxidase, W290H, W290F, and W290G were analyzed and compared to wild type through structural and functional studies in order to probe the role of tryptophan 290 within the protein. In the wild type protein, tryptophan 290 stacks over a tyrosine-cysteine thioether bond. That same tyrosine plays a role in the catalytic action of galactose oxidase as a tyrosyl radical. The stacking tryptophan was found to protect the cross-link thus stabilizing the active, radical form of galactose oxidase.

Tyrosinase, another copper-containing protein, has been expressed and purified from *Streptomyces*. Due to low protein yields, expression was attempted in *Escherichia coli*. Although the *E. coli* expression systems were not successful, other options exist which should be examined in order to obtain adequate amounts of recombinant protein in order for characterization to proceed.

INTRODUCTION

Overview of Protein Copper Centers

Transition metal ions such as copper, iron, molybdenum, and manganese play major roles in biology because they exist in multiple oxidation states *in vivo*. Copper exists biologically in the +1 and +2 states(1). Cu(I) can have coordination numbers of two, three, or four while Cu(II) favors five (most common), although four and six coordinate complexes are also well known(2). Almost all known biological applications of copper proteins involve oxidation-reduction (redox) reactions, either to carry out an electron transfer event, or to effect a redox transformation of a substrate molecule.

The active sites of copper proteins have been categorized into three classes based on their geometric and electronic structures: type 1, or blue copper, type 2, and type 3 (3-5). Recently, this list has expanded to include other copper centers including trinuclear copper clusters composed of a type 2 and type 3 center, the mixed-valent binuclear Cu_A site, the Cu_B-heme center of cytochrome c oxidase, as well as the tetranuclear Cu_Z center of nitrous oxide reductase(3). Type 1 copper centers such as azurin are characterized by a single Cu atom in the active site, an intense blue color, and a narrow hyperfine coupling in the EPR spectrum(4). They are usually coordinated by three strong ligands, a cysteine and two histidines, and may also have one or two weaker ligands such as methionine (4). Type 2 copper centers such as amine oxidase contain a single Cu atom, exhibit weak optical absorption bands, and a normal Cu EPR spectrum with values $g\perp$ 2.06, $g\|$ 2.29, and $A\|$ 16.1

mT(6). Generally, the type 2 sites are coordinated by two or three histidine residues and a water molecule or hydroxide ion(4). The type 3 copper center such as catechol oxidase contains a coupled binuclear copper site, strong absorption in the near-UV with λ max = 330nm, and no EPR signal due to a pair of Cu atoms which are antiferromagnetically coupled(3). Type 3 copper sites are usually coordinated by three histidines and a bridging ligand such as oxygen or hydroxide (Figure 1).

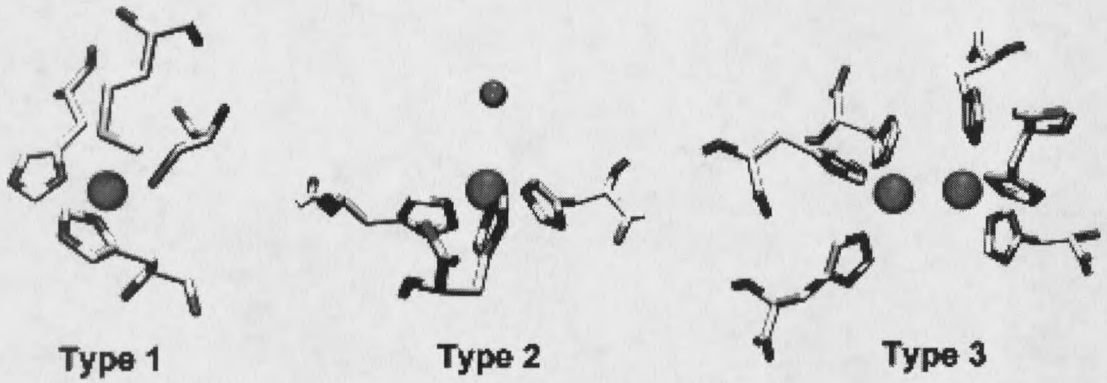

Figure 1. Copper centers. The models shown come from azurin, (type 1), nitrite reductase, (type 2), and catechol oxidase, (type 3) (7).

This study centers on proteins from two of the three different classes of copper centers. First, galactose oxidase (GOase, EC 1.1.39), which has a type 2 copper center and post-translational modification (PTM) of a tyrosine residue. This tyrosine, (Y272) which also participates in the catalytic mechanism as a tyrosyl radical, is covalently linked to the sulfur of C228 in a thioether bond (Figure 2). The cross-link is believed to modulate the reactivity and redox potential of the tyrosyl radical(9;10). Second, tyrosinase (EC 1.14.18.1) which has a type 3 binuclear copper center and, in some proteins, a PTM of a His-Cys cross-link (Figure 2). The cross-

link does not seem to play a role in catalysis; however, in tyrosinase, as well as GOase, the mechanism of formation of the covalent linkage remains unknown(8).

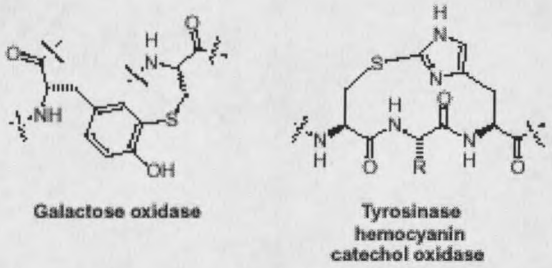

Figure 2. Cross-links of GOase and Tyrosinase (8).

Galactose Oxidase

Galactose oxidase is an extracellular copper containing enzyme that is secreted by *Fusarium sp*(11). The *Fusarium* GOase enzymes are the ones primarily studied, however, recently putative GOase enzymes have been discovered in *Stigmatella aurantiaca*, a gram-negative soil bacteria as well as *Streptomyces coelicolor* A3(2), a gram-positive soil bacteria(12;13). Galactose oxidase catalyzes the oxidation of primary alcohols to their corresponding aldehydes while reducing dioxygen to hydrogen peroxide (H_2O_2) as shown in Equation 1(14).

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \quad \text{(Eq. 1)}$$

Although the enzyme can utilize a wide variety of substrates, it is stereospecific in its substrate requirements(15). For example, D-galactose is a substrate, however, L-galactose and D-glucose are not(16). Although the biological role for GOase is unknown, its broad substrate range may exist in order for the organism to rapidly form H_2O_2 rapidly as a defense against bacteria(17).

Applications of Galactose Oxidase

Galactose oxidase has many potential uses ranging from medical to synthetic applications. Assays have been developed to detect galactose in blood and other biological fluids in order to detect disorders such as galactosemia, or the inability to break down galactose into glucose (18-20). If left undetected, galactose accumulates in the blood and body tissues. This build-up of galactose can lead to jaundice, an enlarged liver, cataracts, mental retardation, and possibly death(21). Treatment is the lifetime elimination of dairy products from the diet. Attractive industrial applications of GOase involve the biotransforamtion of glyco-biopolymers (glycolipids, glycoproteins, polysaccharides) into desirable materials such as sweeteners, flavorants, or paper strength additives (22-27). As GOase liberates one molecule of H_2O_2 per molecule of aldehyde that is formed, it can be used to indirectly quantitate the number of aldehyde residues introduced into a biomolecule or cell surface (23-27). Synthetic chemists are also mimicking GOase activity with small, low molecular weight catalysts for applications as synthetic tools and pharmaceuticals(22). The high stereospecificity, mild reaction conditions, and environmentally-sound GOase based system holds advantages over conventional chemical systems that are often hard to control, non-specific, costly, or hazardous(22). Recently, GOase has been modified by directed evolution to introduce glucose 6-oxidase activity(28). The selective oxidation of the 6-hydroxy group of D-glucose to introduce an aldehyde functionality is not catalyzed by known oxidase enzymes(28). Selective functionalization at the glucose C-6 position in oligo- and polysaccharides is a synthetically useful reaction that would facilitate further modifications for food, pharmaceutical and materials

applications. Recently, GOase has been used in the detection of rectal and lung cancer(29). This technique takes advantage of both GOase and Schiff's reagent. GOase forms aldehydes while Schiff's reagent (basic fuschin) reacts with the aldehydes to produce a magenta color(29). The galactose oxidase Schiff's (GOS) reaction can detect D-galactose-β [1-3]-N-acetyl-D-galactosamine, or Thomsen Friedenreich antigen, which is present in neoplastic tissues and remote non-neoplastic mucosa from individuals with cancer but not in individuals without cancer(29). Applications of GOase will no doubt increase as more is known about its mechanism and large scale production of the enzyme is achieved.

Structure

Dactylium dendroides, recently reclassified as a *Fusarium* species galactose oxidase is a single polypeptide chain of 639 amino acids with a molecular weight of 68 kDa(30). GOase is interesting in that it undergoes several processing events to achieve its mature form. First, the signal sequence for extracellular secretion is cleaved, followed by cleavage of a seventeen amino acid N-terminal prosequence upon exposure to copper and dioxygen(31). The role of this prosequence is unknown, but may involve protein folding or maintaining inactive protein intracellularly. Lastly, a Y272-C228 cross-link is formed upon exposure to copper and dioxygen in a self-processing reaction(31). This cross-link aids in the catalytic cycle of the enzyme(31). GOase is part of an increasing number of enzymes that are posttranslationally modified via self-processing reactions to form the active site.

The crystal structure of GOase has been solved to 1.7 Å resolution(32;33). The crystals were grown in acetate buffer at pH 4.5 where the protein is inactive.

They were subsequently transferred to PIPES buffer to obtain a structure at pH 7 and additionally treated with a copper chelator, diethyldithiocarbamate (DDC) to obtain the apo structure(33).

The structure of GOase is divided into three domains that are mostly β structure (Figure 3). Domain I (residues 1-155) has a β-sandwich structure which is linked to domain II by a well ordered stretch of amino acids. Domain II (residues 156-532) looks like a seven-bladed propeller where each blade is made of a four stranded antiparrallel β-sheet. Domain III (residues 533-639) is located on the top of domain II and two of its seven β-strands reach down through the middle of the propeller of domain II providing a ligand to the copper(34).

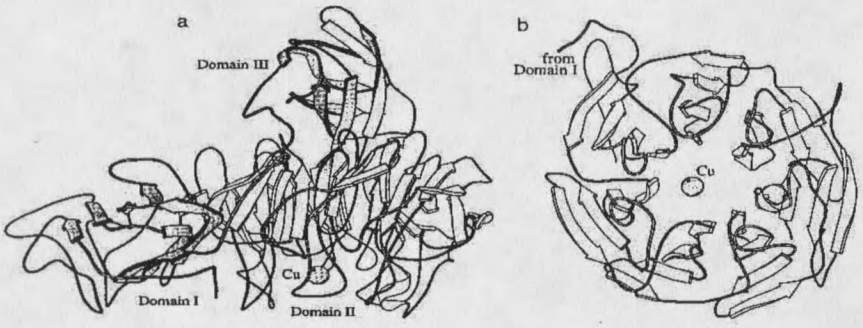

Figure 3. Overall 3-D structure of GOase as ribbon diagrams drawn using the program MOLSCRIPT. (a) Side view of the molecule with domains I and III shaded. (b) View of domain II approximately along the pseudo seven-fold axis (34).

The active site sits at the solvent accessible surface of domain II, close to the seven-fold axis (Figure 4). It has an apparent square pyramidal coordination and is ligated by two equatorial histidines (H496, H581) and two tyrosine residues, one equatorial (Y272) and one axial (Y495). The axial tyrosine is considered a weak ligand because it is 2.69Å from the Cu ion. Tyrosine 495 is the active site base,