

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF ENZYMES OF A NOVEL
GROUP OF TRYPTOPHYLQUINONE COFACTOR CONTAINING OXIDASES

by

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A dissertation submitted in partial fulfillment for the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term
2016

Major Professor: Victor L. Davidson

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ABSTRACT

Protein-derived cofactors are redox and catalytic centers that are generally formed by the posttranslational modifications of one or more amino acids. An important class of these cofactors are the quinones derived from tyrosine and tryptophan. Amongst redox proteins, it has been known till now that oxidases either contain a flavin or a tyrosylquinone cofactor, whereas tryptophylquinone ones are present within the dehydrogenases. In recent times, oxidases from a marine bacterium, *Marinomonas mediterranea*, have been shown to possess the latter. This study involves the characterization of two such proteins, lysine- ϵ -oxidase (LodA) and glycine oxidase (GoxA). They have been reported to contain the same cysteine tryptophylquinone (CTQ) cofactor. Both require the co-expression of a second protein, LodB and GoxB respectively to generate matured CTQ containing active protein. Kinetic analysis of the reaction catalyzed by LodA showed that it followed the usual Michaelis-menten mode of interaction with its substrates. GoxA on the other hand exhibited allosteric cooperativity for its substrate glycine. This was attributed to the dimeric conformation of the wildtype GoxA based on size exclusion chromatographic studies. Mutagenesis study of amino acid residues based on the crystal structure of LodA and a homology model of GoxA, have given a detailed idea about their structure-function relationship. Kinetic studies on mutants of Tyr211 of LodA along with Lys530 present at the substrate channel, showed effects on both K_m for the substrates and k_{cat} for the reaction. As a result these residues have shown their involvement in forming a gate-like structure to control the to and fro movement of the substrate and products. Corresponding to this residue, the Phe237 of

GoxA has proved to be important in maintaining the allostericity, by mediating the stable dimer formation. From the kinetic parameters, Cys448 of LodA was found to be responsible for substrate specificity and affinity. Whereas, mutants of His466 of GoxA that correspond to the Cys448 residue, were unable to yield CTQ containing active GoxA. On the other hand, Asp512 of LodA and Asp547 of GoxA that correspond to each other, have been implicated for their involvement in CTQ biogenesis. This study therefore highlights how even though this new pool of enzymes have great degrees of similarity in terms of the cofactors and conserved active site residues, there are major differences in the mechanism of the reaction that they catalyze which on a broader sense could influence the overall physiological importance of the enzyme in the biological system.

This work is dedicated to my parents, Mr. Prasanta Sehanobish and Mrs. Mridula Sehanobish.

ACKNOWLEDGMENTS

I would like to start by thanking the University of Central Florida for giving me the opportunity to pursue my doctoral studies. Being an international student the transition to a new country was difficult. But it was made easy by a lot of people to whom I am immensely grateful. First and foremost my PhD. advisor, Dr. Victor L. Davidson for offering me the position of graduate research assistant in his extremely established lab. Most of my research experience and knowledge has been gathered while being a part of his lab. I would like to thank my committee members, Dr. Annette Khaled, Dr. William Self and Dr. Suren Tatulian for their useful feedback and ideas. I would like to thank Dr. Antonio Sanchez-Amat, our collaborator, for introducing us to the whole new type of oxidases which formed the basis of my dissertation and also for his input in various publications. I would then like to thank my lab members Dr. Moonsung Choi, Dr. Sooim Shin, Yu tang, Zhongxin Ma and a special mention to Dr. Heather Williamson for the extensive and very helpful discussions and Dr. Brain Dow for being a great support during the last 5 years. At this point I would also like to thank 3 very important friends who have been a constant source of encouragement and support throughout the journey, Sambuddha, Debarati and Satadru. Lastly I would like to thank the people without whom achieving this degree would not have been possible. It is the belief that my husband, Dr. Saptarshi Debroy had in me, helped me overcome the difficult times. His words and actions of support have been the pillar of my success. It is worth mentioning the importance of the presence of my in laws, Mr. Samarjit Debroy and Mrs. Mahua Debroy who have shown tremendous belief in my ability to succeed.

Last but not the least and most importantly I would like to thank my parents, Mr. Prasanta Sehanobish and Mrs. Mridula Sehanobish for all their hard work, for all the values that they have instilled in me and for their unconditional love and support in every step of my life. Without them this dream would not have been a reality.

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LIST OF ABBREVIATIONS

AADH	Aromatic amine dehydrogenase
CAO	Copper amine oxidase
CTQ	Cysteine tryptophylquinone
GoxA	Glycine oxidase
k_{cat}	Turnover no.
K_m	Michaelis constant
LodA	Lysine- ϵ -oxidase
LTQ	Lysine Tyrosylquinone
MADH	Methylamine dehydrogenase
NAD	Nicotinamide adenine dinucleotide
PES/DCIP	Phenazine Ethosulphate/ Dichlorophenolindophenol
preMADH	precursor protein of MADH with incompletely synthesized TTQ
QHNDH	Quinohemoprotein amine dehydrogenase
TTQ	Tryptophan tryptophylquinone
V_{max}	Maximal velocity

WT..... wild-type

CHAPTER 1: GENERAL INTRODUCTION

Oxidases and dehydrogenases belong to the oxidoreductase group of redox enzymes. They are involved in catalyzing the transfer of electrons between different substances in these redox reactions¹. The basic difference between an oxidase and a dehydrogenase lies in the type of the final electron acceptor they use in the reactions catalyzed by them. Oxidases as per the name, use O₂ whereas dehydrogenases use molecules such as NAD⁺ ² or other redox proteins to accept their electrons. Since redox enzymes are of great importance in various biological pathways such as respiration and metabolism³, studying different proteins of this category is of utmost importance.

Most of these redox proteins and enzymes have cofactors that act as the redox active centers. Protein cofactors can be of different types. There are the inorganic ions such as Zn, Mg, Cu, Fe which are readily found in metalloproteins. The organic cofactors include the NAD, FAD (Flavin Adenine dinucleotide) and FMN (Flavin mononucleotide). Most of these cofactors can be easily detected by Uv/Vis spectroscopy. The latter two are generally found in flavoproteins. For e.g. changes in the absorbance spectrum of FAD around 450 nm indicates the oxidation state of the Flavin containing proteins⁴. The other class of cofactors include the ones that have been generated by posttranslational modification of already existing amino acids. In recent years a lot of interesting research has been done on this and continues to be a growing area for more investigations. Certain amino acids especially the aromatic ones undergo post translational modification and further crosslink to adjacent amino acids to generate redox active centers. The earliest

known form of such cofactors was that of pyrroloquinoline quinone (PQQ) and proteins containing these cofactors were specifically called quinoproteins⁵. From the data available it has been observed that the tryptophan derived cofactors are generally present in dehydrogenases for e.g. MADH containing TTQ. Oxidases that are quinoproteins generally contain tyrosine derived cofactors, for e.g. amine oxidase containing TPQ⁶.

It is known that L-amino acid oxidases are generally flavoproteins containing Flavin-derived cofactors⁷. For the first time an oxidase was discovered from a marine melanogenic bacteria, *Marinomonas mediterranea*, which showed lysine- ϵ -oxidase activity⁸. The unique property of this enzyme is that it contained a posttranslationally modified tryptophylquinone cofactor, CTQ, just like a dehydrogenase, but enzymatically behaved as an oxidase. This protein was named LodA and was encoded by the *lodA* gene. The only other reported example of a CTQ containing enzyme was a dehydrogenase called quinohemoprotein amine dehydrogenase (QHNDH)⁹. Due to complexity in its structure QHNDH wasn't studied completely. LodA was found to catalyze the oxidative deamination of L-lysine generating hydrogen peroxide that made it antimicrobial in nature¹⁰. Hence this was an interesting protein to study to understand how even though it contained an intrinsic property of a dehydrogenase (i.e. CTQ), it still managed to behave as an oxidase.

In this study the mechanism of action has been studied. To understand the significance and importance of the enzyme, the elucidation of the reaction mechanism was necessary, as it is the presence of the CTQ that makes the enzyme active. Precursor

forms of the enzyme without the CTQ were found to be inactive¹¹. From the data obtained it has been shown that LodA carries out the oxidative deamination by following a ping-pong mechanism¹² and occurs via the formation of a Schiff base intermediate. Its reductive half reaction resembles those of all other tryptophylquinone containing dehydrogenases and tyrosyl quinone containing oxidase. Yet it is different from both of these categories of redox proteins. To further characterize this and understand the structure-function relationship, site-directed mutagenesis was done. Cys516 and Trp581 were involved in the CTQ formation. These were mutated to Alanine. None of the variants produced any active protein and no CTQ was detected in any of those mutants. Various possible significant residues were mutated like the Cys448, Tyr211 and Lys530 to see their effects on both structure and function of LodA¹³. Cys448 was found to be involved in the determining the substrate specificity and binding especially for lysine. An aromatic amino acid was required at the 211 position (Tyr211) which along with the Lys530 formed a hydrophobic pocket as they Hydrogen (H) bonded with each other. This affected the entry and exit of products and intermediates hence modulating the reaction rate and affinities for both O₂ and L-lysine.

On further genome analysis it was seen that 2 other genes from the same bacteria were found to encode LodA-like proteins¹⁴. This led one to believe that there is a whole reservoir of such oxidases that contain tryptophylquinone cofactors. Amongst the two, one of them showed glycine oxidase activity¹⁴ and was named as GoxA. This was first discovered in *Marinomonas mediterranea* and was then recombinantly expressed in *E.coli*. It was found to be inactive in the precursor form that was devoid of a mature CTQ

¹¹. In this study we further characterize GoxA and try to elucidate the structure-function relationship. Since this was similar to LodA, it was thought that probably the mode of reaction mechanism was the same with the major difference being in the substrate which is Glycine for Gox. Hence kinetic analysis of the enzyme for the oxidative deamination of glycine was studied. This enzyme showed allosteric cooperativity for glycine which was absolutely unique and different from LodA. The dimeric form of the enzyme was thought to support this mode of reaction. Presence of glycine facilitates the binding of substrate at the other subunits thus increasing the overall reaction rate since the enzyme shows positive cooperativity. The fact that it showed an allosteric mechanism could mean that this has more metabolic function than antimicrobial as in the case of LodA. The crystal structure of GoxA has not yet been determined. This poses a problem in general to study any type of enzymes in terms of their structure-function. So its homology model was created with LodA as the reference and site directed mutagenesis was done. Residue His466 was analogous to Cys448 and Phe237 was analogous to Tyr211. H466 was found to be directly or indirectly involved in the CTQ biosynthesis because mutating this residue generated inactive precursor protein. On the other hand mutation of F237 resulted in the loss of cooperativity for glycine by forming weaker dimer with the subunits. This study therefore shows how apparently similar enzymes have different mechanism of catalyzing reactions and how just from this mechanism one can predict the overall physiological function of the enzyme.

CHAPTER 2: LITERATURE REVIEW

Cofactors of a protein

Cofactors are known to be molecules associated with proteins that make a protein active. In the absence of these cofactors proteins are still in their precursor form and are inactive in nature. A lot of the functional groups present in a protein may account for some of the catalysis, nucleophilic and electrophilic reactions but cannot account for the catalysis of many other enzymatic reactions. It is here that the presence of cofactors becomes very important. An inactive enzyme without the cofactor has been termed as the apoenzyme and the entire active protein has been named as the holoenzyme ¹⁵. The cofactors are not only involved in accelerating the reaction rate of the catalyzed reaction but later studies also showed the involvement of cofactors in protein folding and protein stability ¹⁶. According to these studies, the cofactor was found to be involved in stabilizing the native protein. This was possible by the interaction of the cofactor with unfolded polypeptide. Often this interaction prior to the protein folding step could speed up the overall folding process. Thus along with enhancing the reaction rates these cofactors adds diversity in the functions of the enzymes at times making them redox active centers or allowing the coordination with different substrates¹⁵.

There are mainly 4 different types of cofactors. There are the metalloproteins which contain metal ions as their primary cofactors. A large number of these metal ions have been found associated with protein activity. Some of them include Copper (Cu), Iron (Fe), Zinc (Zn), Magnesium (Mg), Cobalt (Co), Molybdenum (Mo)¹⁷. The second category of

cofactors are the organic ones which since they are directly involved in enzymatic reactions hence could also be termed as coenzymes. The most common example for coenzymes are the FAD, NAD⁺ ². Another category of cofactors are the organometallic ones. These have metal ions tightly associated to the organic moieties in the protein and cannot be dissociated from it easily. The most common example for this is heme that contains Fe associated to a porphyrin ring. The final category of cofactors are the ones that have been generated by the posttranslational modification of amino acid residues in proteins. Some of these are self-generated and some require the presence of other proteins to catalyze the biosynthesis of the cofactors⁶. Examples of some of these cofactors include tryptophylquinone cofactors that have been formed from modifications in tryptophan residues¹⁸. In general many of these cofactors have spectroscopic UV/Vis and fluorescence properties and protein cofactor interactions may influence these properties. If these cofactors are present at the active site of the enzyme then its interaction with the protein is thought to influence the enzyme kinetics and thus the steady state analysis is a good tool to study any such interactions. If the protein is a redox protein then the presence of these cofactors often influence the redox potentials and thus doing redox titrations can give one a good understanding of its affects¹⁹. Midpoint redox potential values maybe affected in the presences and absence of these cofactors^{20, 21}. Metal ions have also been implicated in many infectious diseases²². Hence studying protein-cofactor interactions is a good technique to figure out their importance.

Inorganic metal ions

Metalloproteins as the name suggests are proteins which have inorganic ions as cofactors. These metal ions are important in catalytically and redox active proteins since they often provide different oxidation states that help in redox reactions and electron transfer reactions. Often these metals are coordinated to different amino acid side chains and these are pretty strong interactions such that they don't generally leach out upon purification of these proteins. A large number of metal ions have been found to be present in the protein. These include Fe, Cu, Zn, Mg, Ni, Mn and Zn. Surrounding amino acid residues provide ligands for these metals which in turn tend to influence the stability and the reactivity of the metals. Examples of 2 intriguing redox active metals iron and copper, containing proteins are stated below.

Iron (Fe) containing proteins

Iron is the more abundantly available transition metal ion which when present as a cofactor has physiological importance. Its ability to readily undergo a transition between Fe (II) and Fe (III) redox state makes it indispensable¹⁵. More than one oxidation state of iron allows it to be readily involved in the reactions involving the transfer of electrons. Iron cofactors can be directly bound to the amino acid side chains or in cases such as the heme it might be coordinated to porphyrin rings and thus will be part of organometallic complexes. A common example of Fe being bound to amino acids is in the metalloprotein intradiol catechol dioxygenase²³. This belongs to oxidoreductases and they incorporate dioxygen into the substrates thus carrying out the oxidative cleavage of

catechols. Iron also has been found to be present as a cofactor along with Copper in an oxidase, cytochrome c oxidase which plays a major role in oxidative phosphorylation. Another category of Iron containing proteins are Iron-Sulphur proteins. They contain Fe-S clusters with more than one Fe and sulphide linkages²⁴. These clusters have been found in various different metalloproteins such as the Nitrogenase, Ferredoxins, NADH dehydrogenase and are more commonly known for their involvement in electron transfer in redox reaction with some being integral part of the mitochondrial respiratory chain.

Copper (Cu) containing proteins

A reason for the abundant presence of copper in proteins is its ability to exist both in the oxidized and reduced state. Based on its oxidation state it can either have affinity for thiols or thioether groups or in some cases have can coordinate with O₂ or imidazole nitrogen groups²⁵. Based on the surrounding ligands and the coordination number the copper containing proteins are able to exhibit different chemical and spectroscopic properties²⁶. Type I copper containing proteins have been termed as cupredoxins in which the copper is coordinated by four residues. This is the most studied type of copper containing proteins. Examples of this are plastocyanin and azurin. Plastocyanins are involved in electron transfer during photosynthesis. Azurin too is involved in electron transfer in bacteria. These proteins are blue in appearance and have a strong absorption around 600nm thus depicting the color²⁶. Galactose oxidase which is an oxidoreductase involved in the reduction of galactose contains a Type II copper center²⁷.

Organometallic cofactors

Sometimes the metal may be tightly associated or bound to organic moieties and do not dissociate readily. They have been known to be involved in redox and catalytic reactions. Such cofactors would be referred to as the organometallic ones¹⁵. Common examples are heme with iron attached to the porphyrin ring and cobalamin which has cobalt attached to the corrin ring. Heme containing proteins are discussed below.

Heme

Heme is one of the more important and abundantly available organometallic cofactors, observed in biological systems. It consists of an iron atom present in the center of the porphyrin ring. Based on the substitutions and coordination, the heme group can be classified into different types. The most common one is the heme b. it is coordinated to different ligand amino acids based on the type of protein in which it is present as a prosthetic group. For example it is coordinated to Histidine in hemoglobin²⁸ and myoglobin²⁹. Hemoglobin as is known is the iron containing oxygen transporter present in the red blood cells of vertebrates whereas myoglobin does the same function but is present in the muscle tissues of vertebrates and most mammals. Hence the heme present in these two proteins aid in their overall function as the oxygen-binding proteins. Two other heme b containing proteins are the nitric oxide synthase³⁰ and the cytochrome P450³¹. These have cysteine as their ligand. The other forms are the heme a and heme c. Cytochrome c oxidase contains heme a whereas the other c-type cytochromes such as bacterial diheme cytochrome c peroxidase contains heme c.

Organic cofactors

Coenzymes are organic molecules that act as cofactors and are required for the activity of the protein. Many of these coenzymes have been derived from vitamins B1, B2 and B6. These organic molecules can either be covalently bound to the protein or be loosely bound to it thus being dissociable. Nicotinamide adenine dinucleotide (NAD⁺) is a dissociable cofactor whereas cofactors like Flavin adenine mononucleotide/dinucleotide (FMN, FAD) and pyridoxal phosphate (PLP) are strongly associated and cannot be separated until the protein gets denatured. These cofactors especially the NAD⁺ and the FAD and FMN are involved in electron transfer hence are often found in oxidases and dehydrogenases. These cofactors generally have characteristic binding domains in proteins.

Nicotinamide adenine dinucleotide (NAD)

It is an important coenzyme used in biological systems. It has been implicated in processes such as glycolysis and citric acid cycle. Due to its ability to be involved in redox reactions, it can exist as an oxidized form (NAD⁺) or as a reduced form (NADH). They have characteristic absorption maxima at 250 nm and 340 nm respectively. Many known dehydrogenases use NAD as their cofactor. Glutamate dehydrogenase for example uses NAD⁺ as its cofactor to catalyze the production of ammonia and α -ketoglutarate, based on the biological system and the binding affinity for the substrates. For example it has been found to be central in amino acid catabolism in plants³² and in mammals³³ it has been found to be important in ammonia metabolism all of which requires the ability of the

protein to use NAD as its cofactor rather coenzyme. Another common example of an NAD⁺ using enzyme is the alcohol dehydrogenase which has a definite binding site for the cofactor and catalyzes the oxidation of ethanol.

Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN)

These cofactors have been derived from riboflavin. FADs, like the NAD cofactors are also involved in redox reactions and hence can exist in the oxidized and the reduced form. The oxidized form of FAD shows an absorption maxima at around 450nm. Free Flavin also exhibits fluorescence which gets quenched as and when it binds to the proteins⁴. For most of these proteins denaturation needs to be done in order to release the tightly bound cofactors. Various proteins have been known to contain these cofactors. Nitric oxide synthase contains both the FAD and FMN in a 2:1 ratio³⁴. The only other mammalian protein known to contain the same is the NADPH-cytochrome P-450 oxidoreductase in which the 2 are present in equimolar amounts³⁵. Another protein called thioredoxin reductase, thought to be a key component of the thioredoxin system supplying hydrogen for reduction of ribonucleotides also contained 2 molecules of FAD per molecule of the protein³⁶.

Cofactors generated by posttranslational modification

Posttranslational modification is a process which involves changes in proteins after the process of translation has been complete. It can involve addition or removal of functional groups. Some of them include phosphorylation involving the addition of a phosphate group, glycosylation involving the addition of a carbohydrate molecules and methylation involving the addition of methyl groups. Posttranslational modification occur on amino acid residues present at the surface or at the C or N terminal of the protein. Sometimes the posttranslational modifications involve cleaving off peptides to generate active protein. Since the modifications occur at the surface, the amino acids susceptible to it are easily accessible. These posttranslational modifications tend to affect different important biological processes such as cell signaling.

Within a protein or a peptide, the amino acids that are available tend to have limited chemical functionality in proteins. In most cases they either use already present cofactors or exogenous ones to help in catalysis. But some of these redox proteins in recent times have been discovered to have alternative methods of generating cofactors. They have been able to generate active and catalytic sites by carrying out posttranslational modifications to amino acids present within either a peptide or within a folded protein. Amongst some of the early reported modifications in proteins was the histidine decarboxylase. It was known that amino acid decarboxylases always contained pyridoxal phosphate (PLP) as their cofactor. But there was an exception to this which was found in the histidine decarboxylase. It showed the presence of a serine-derived amino-terminal pyruvoyl group³⁷. There have also been examples of covalent linkages

generated by posttranslational modifications in proteins like cytochrome c oxidases that have been found to crosslink tyrosine and histidine^{38, 39}. Initially quinones, were known to be involved in the transfer of electrons but was not implicated in redox proteins. Recent crystallographic and biochemical studies have shown that some oxidoreductases have been able to undergo posttranslational modification to generate quinones as cofactors. These protein-derived cofactors are synthesized biologically by crosslink formation, cyclization and oxygenation of aromatic amino acids.

Peptide-derived quinone cofactors

Pyrroloquinoline quinone (PQQ)

PQQ was first discovered in bacterial glucose dehydrogenase⁵. It was generated by the posttranslational modification in glutamate and tyrosine that were present in a precursor peptide with incorporation of the Oxygen at the Tyrosine site. Figure 1. shows the chemical structure of the cofactor PQQ. Subsequently PQQ was identified in different bacterial dehydrogenases such as methanol and glucose dehydrogenase^{40, 41} and very recently in alcohol dehydrogenase in *Pseudogluconobacter saccharoetogenes*. PQQ-dependent enzymes oxidizes primary alcohols sugars and aldehydes⁴². Their presence has been widely studied in prokaryotes. Most of these PQQ containing redox enzymes have been found in the periplasmic space of gram negative bacteria.

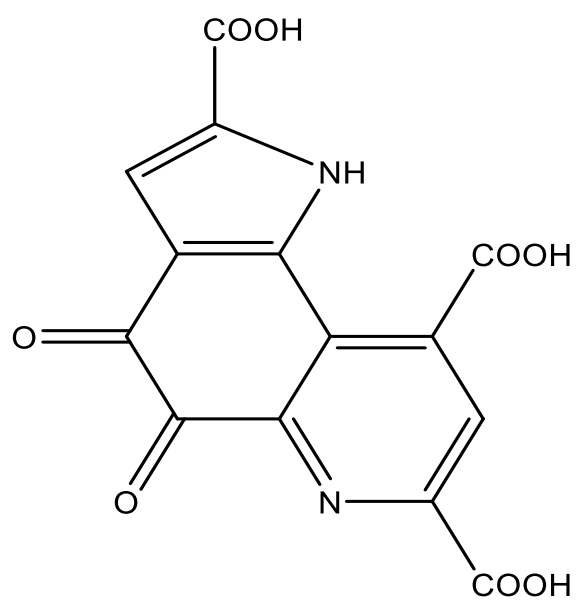


Figure 1 The PQQ cofactor

Gene products of the pqq operon has been found to be responsible for the biosynthesis of the cofactor and these genes expressed PQQ in *E.coli* which doesn't normally produce it⁴³. PQQ can oxidize its corresponding substrates and itself get reduced to a PQQH₂ form by NADPH, glutathione. It can undergo oxidation again to revert to its quinone state via the formation of superoxide radicals. PQQ has also been found in membrane-associated dehydrogenases hence can also transfer electrons to ubiquinones attached to the plasma membrane.

PQQ as an organic molecule has been well studied in prokaryotes but its biosynthesis in eukaryotes and mammalian systems has not been shown. Its importance in human system has been controversial but evidence has shown it to influence physiological and biochemical processes. PQQ isn't synthesized in the body but humans are constantly being exposed to dietary sources of PQQ such as milk⁴⁴, tea, green peppers ⁴⁵. The PQQ molecule has thus been found to have different physiological importance⁴⁶. PQQ has been shown to promote cell proliferation by acting as growth factor when added to human and mouse cell cultures⁴⁷. More recently PQQ dietary supplements have been shown to increase mitochondrial functions and biogenesis and an improved metabolic homeostasis⁴⁸. PQQ has been shown to reduce neuronal cell death in experimental models of stroke and spinal cord injury^{49, 50}. Various recent work also show multiple other functions such as redox activity, radical scavenging along with alterations and changes in the cell signaling processes⁵¹.

Protein-derived quinone cofactors

The discovery of PQQ led to the finding of other quinone cofactors that were derived from posttranslationally modified amino acid residues. These cofactors were protein-derived i.e. amino acids within the protein were modified to generate redox active centers. The biogenesis of some of them were autocatalytic whereas some needed the presence of another catalyzing enzyme to do so. The quinone cofactors that have been obtained by modification of tyrosines and tryptophans are the most studied ones. The most studied tyrosine-derived cofactors are the 2,4,5-trihydroxyphenylalanine quinone or TPQ (Topaquinone), found mainly in copper amine oxidases and Lysine tyrosylquinone (LTQ) found mainly in mammalian lysyl oxidases. There is also a tyrosine-cysteine cross-linked cofactor found in oxidative enzymes in mammals, yeasts and fungi⁵². Galactose oxidase is known to catalyze a variety of alcohols, specifically galactose, to yield aldehydes and H₂O₂. It was shown to possess this posttranslationally generated crosslink between Cys and Tyr⁵².

Modifications involving Tryptophan residues resulted in the generation of tryptophylquinone cofactors. Tryptophan tryptophylquinone (TTQ) was found in amine dehydrogenases such as methylamine dehydrogenase (MADH) and aromatic amine dehydrogenase (AADH), whereas the Cysteine tryptophylquinone (CTQ) was found in quinoxaline amine dehydrogenase (QHNDH) and of late in the oxidases, Lysine- ϵ -oxidase (LodA) and Glycine oxidase (GoxA). Figure 2 shows the chemical structures of the different above mentioned cofactors.

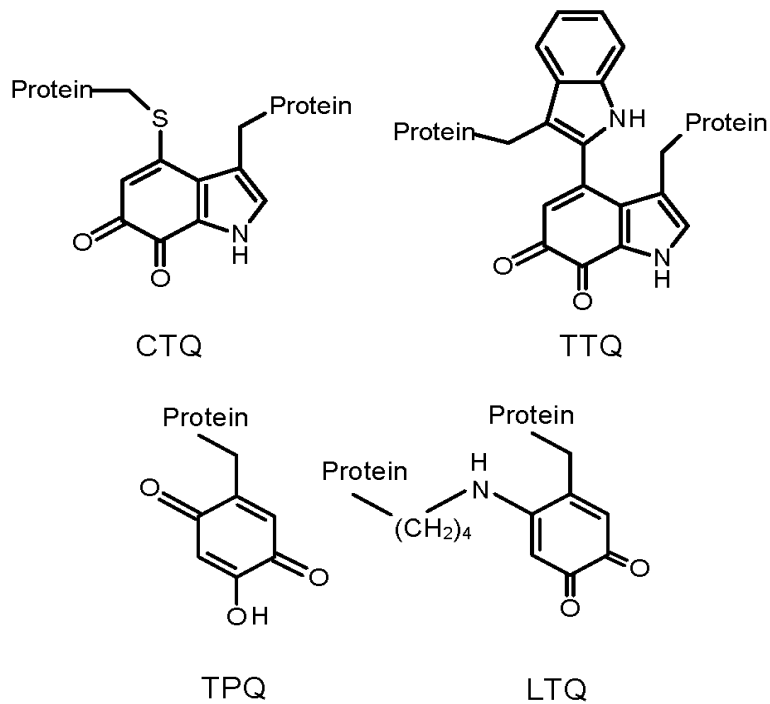


Figure 2. Quinone cofactors that are formed by posttranslational modifications.

CTQ is cysteine tryptophylquinone. TTQ is tryptophan tryptophylquinone. TPQ is 2,4,5-trihydroxyphenylalanine quinone or topaquinone. LTQ is lysine tryptophylquinone.

Tyrosylquinone cofactors

Topaquinone (TPQ): Most copper-containing amine oxidases were initially were thought to contain pyridoxal phosphate PLP or PQQ. The discovery of TPQ and its extensive characterization indicates the importance of such redox cofactors in the proteins. TPQ is a ubiquitous cofactor that has spans over yeast, bacteria and mammals. The TPQ containing enzymes have a wide range of functions, in bacteria it could be involved in metabolism and in humans it could be involved in inflammatory responses. Based on the different organs from which it has been isolated, TPQ containing enzymes have been given various names. They include plasma amine oxidase, histamine oxidase, retinal amine oxidase. The most studied TPQ containing enzyme has been the Copper amine oxidase which was first discovered from bovine serum⁵³.

TPQ is formed by posttranslational modifications in a tyrosine that was found in a consensus site sequence of Asn-Tyr (TPQ)-Asp/Glu-Tyr⁵⁴. It has been confirmed that the synthesis of TPQ is autocatalytic in nature and is not catalyzed by another protein. Instead an active site Copper ion present in close proximity to the Tyrosine in the TPQ, was found to play an important part⁵⁵. The holoenzyme with a completely synthesized TPQ shows an absorption maxima at 480 nm. When the amine oxidases were synthesized in a metal-free media there was no such peak maxima, instead it just had a Tyr at the TPQ position⁵⁵.

⁵⁶.

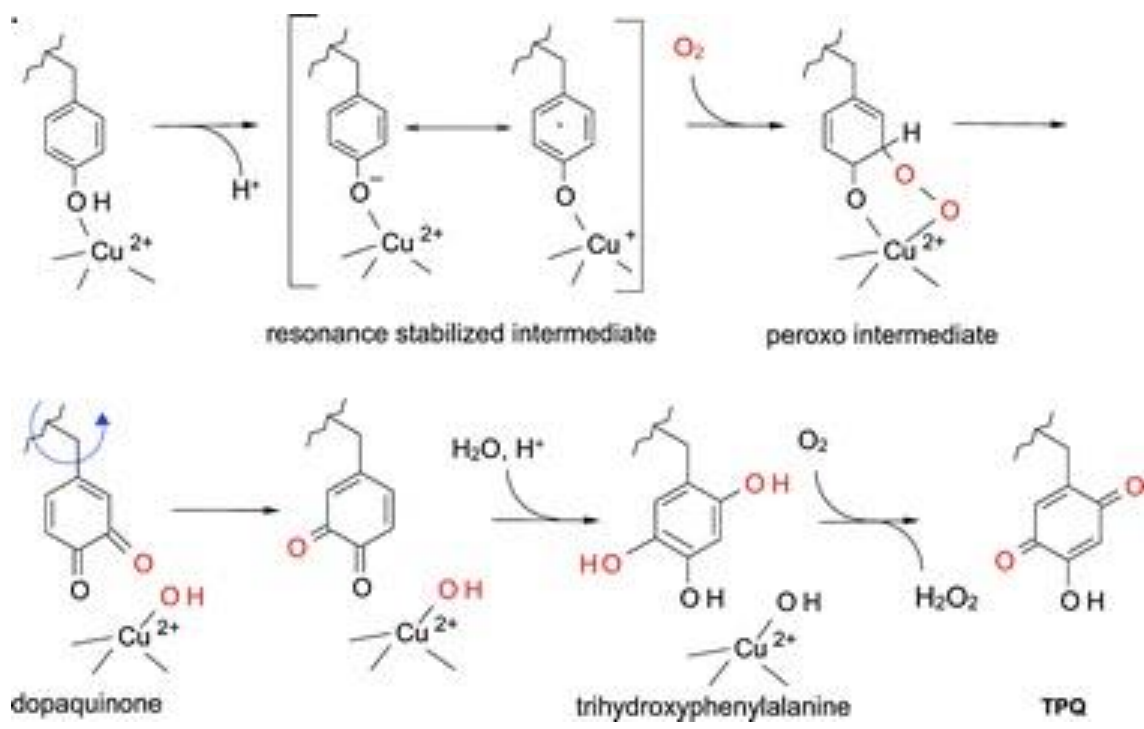


Figure 3 Proposed mechanism for TPQ biosynthesis⁵⁷

These TPQ containing oxidases have dual functions catalyzing both their own cofactor synthesis and also substrate amine oxidation.

Figure 3 shows the mechanism of TPQ biogenesis in copper amine oxidase (CAO). The copper amine oxidase active site is buried deep and contains a type 2 Cu(II) ion that is bound in position by the imidazole groups of 3 His residues at a distance of around 2Å⁵⁸. In case of this oxidase the synthesis takes place in presence of Cu bound to conserved His and other surrounding amino acids^{59, 60}. O₂ reacts with the modified Tyr which gets deprotonated by nearby amino acids and results in the formation of dopaquinone⁶¹. There is a charge transfer complex formation between Cu²⁺ and tyrosinate and generation of a Cu⁺-tyrosyl radical species. O₂ reacts with the intermediate to yield superoxide and reacts with tyrosine to yield the dopaquinone. Following this there is a conformational change which allows hydroxide to be added to it. Then the second O₂ molecule oxidizes it to form the mature TPQ⁶². The copper remains bound at the site and helps in the catalysis.

Once the TPQ has been synthesized, the TPQ containing enzymes carry out the oxidation of amines via the formation of a Schiff base. This is then followed by hydrolysis and release of the aldehyde to produce aminoquinol^{63, 64}. The reaction mechanism is a ping-pong one. The oxidative half reaction is mediated by Cu and O₂ by which the aminoquinol reoxidizes to TPQ, releasing NH₃ and H₂O₂. In humans CAOs can be found indifferent organs and tissues such as kidney, retina and blood and is known to be involved in cell signaling process by peroxide production⁶⁵. Aberrant expression of CAO may lead to congestive heart failure and diabetic complications by oxidative stress on

microvascular system⁶⁶. Hence the extensive characterization of the protein and its cofactor was essential.

Lysine tyrosylquinone (LTQ): The lysine tyrosylquinone (LTQ) is the other tyrosine-derived cofactor that has been studied. LTQ has been found as being an intrinsic part of a mammalian enzyme, lysyl oxidase, being monomeric and extracellular in nature. The enzyme catalyzes the posttranslational cross linking of elastin and collagen to form connective tissues. It uses peptidyl lysine (i.e. lysyl residues) as its substrate and oxidizes the residues to peptidyl- α -amino adipic- δ -semialdehyde residues⁶⁷. Lysyl oxidase is synthesized as an initial 50kDa precursor protein which then undergoes proteolytic cleavage to yield the catalytically active 32kDa⁶⁸. This oxidase was found to be implicated in cell proliferation and tumor suppression⁶⁹ and the propeptide region before the cleavage was thought to be responsible for this⁷⁰.

The limitation in studying the LTQ was the low level of expression of the protein and the unavailability of crystal structures. Hence using techniques like mutagenesis and Resonance Raman spectroscopy, Tyr 349 and Lysine 314 was found to be involved in LTQ formation⁷¹. Just like TPQ, the process of LTQ formation is also autocatalytic and doesn't require another protein to catalyze it⁷². The UV/Vis spectroscopy showed that LTQ had an absorption maximum at 510 nm and this was 20-30 nm red shifted to TPQ containing CAOs⁷¹. A synthetic model for LTQ, NHR-LTQ displayed a maximum at 504 nm which was in excellent accordance with the native enzyme. Since copper was present

in the active site of this LTQ enzyme, its biogenesis was thought to be similar to that of TPQ. The biogenesis occurred via the formation of dopaquinone followed by a nucleophilic attack by the ϵ -amino group of the lysine. The fact that Cu (II) and molecular O₂ were essential for the biosynthesis of LTQ was shown by the isolation of copper depleted form of lysyl oxidase⁷². An exception to this is the discovery of a lysyl oxidase from *Pichia pastoris*⁷³. Instead of having an LTQ this had a topaquinone. It was shown that this lysyl oxidase had similar substrate specificity like the mammalian enzyme thus concluding that the quinone cofactors were not responsible for substrate specificity. Hence even though the mechanism of its biogenesis has been studied a detailed investigation into the same remains to be done based on better expression systems for the proteins.

Tryptophylquinone cofactors

The tryptophylquinone cofactor is formed by modifications at the Trp residue by addition of Oxygen forming quinones and crosslinking with other amino acids. The two known examples are the Tryptophan tryptophylquinone (TTQ) cofactor where it crosslinks to another Tryptophan residue and the Cysteine tryptophylquinone (CTQ) where it crosslinks to another Cysteine residue. These tryptophylquinone cofactor containing enzymes were in the past known to be present only in soluble enzymes that were localized in the periplasmic space of gram negative bacteria. Unlike most of the dehydrogenases these do not use NAD⁺ or NADP⁺ as electron acceptors. Instead they use specific redox proteins as electron acceptors⁷⁴. Methylamine dehydrogenase

(MADH) and aromatic amine dehydrogenase (AADH) contain TTQ and quinohemoprotein amine dehydrogenase (QHNDH) contains CTQ. In recent years 2 other quinoprotein oxidases from *Marinomonas mediterranea* were found to contain CTQ, Lysine- ϵ -oxidase (LodA) and Glycine oxidase (GoxA).

Tryptophan tryptophylquinone (TTQ) cofactor:

Methylamine dehydrogenase (MADH): MADH was the first known enzyme that was found to contain TTQ^{75, 76}. TTQ is a posttranslationally modified protein-derived cofactor that is present as a prosthetic group⁷⁷. MADH is a periplasmic protein which is known to catalyze the oxidative deamination of methylamine to yield formaldehyde and ammonia with transfer of electrons to another redox protein called amicyanin⁷⁸. The only difference with the aromatic amine dehydrogenase (AADH)⁷⁹ is that instead of amicyanin, it is another type 1 copper protein, azurin, that acts as the electron acceptor⁸⁰. These enzymes exist in several bacteria and allow them to use amines as a source of carbon, nitrogen and energy. MADH uses methylamine as its substrate and the reductive reaction begins by an adduct formation between the TTQ and amine substrate. The aminoquinol is then oxidized by electron transfer to amicyanin, via the production of aminosemiquinone then to the oxidized TTQ by releasing ammonia^{81, 82}. It has been possible to detect the different oxidation states of TTQ. The fully oxidized TTQ shows an absorption maxima at 440 nm, the one electron reduced semiquinone has an absorption maxima at 428 nm and the fully reduced quinol is at 330 nm⁸³.

MADH from *P.denitrificans* was present as a quaternary structure with 2 α subunits of 41.5 kDa each and 2 β subunits of 14.4 kDa each, assembled together⁸⁴. The β -subunits have the matured TTQ. The *mau* operon containing *mau* genes, *mauB* and *mauA* encode the α and the β subunit respectively⁸⁵ and *mau C* encodes for amicyanin. It has been found that the biosynthesis of TTQ is based on the expression of MauG protein encoded by the *mauG* gene⁸⁶. In the absence of MauG a precursor form MADH was expressed which was called preMADH with just a single –OH group on the β Trp57 without any crosslink to β Trp108⁸⁷. Crystal structure of the complex of purified MauG with preMADH has been established in vitro depicting the formation of mature TTQ⁸⁸. Figure 4A shows the conversion of the corresponding Trp to the matured TTQ form catalyzed by MauG and 4B shows the crystal structure of the complex. This is interesting as crystal structure of the complex could only be obtained when the 2 purified proteins were incubated together and not from the expression system itself.

The TTQ biogenesis catalyzed by MauG has been completely and very well characterized. MauG is a c-type diheme enzyme that catalyzes the 6-electron oxidation in three, 2 electron steps⁸⁹. The steps involved are the crosslink formation between the 2 Trp residues, the hydroxylation of the β Trp57 to form a quinol and then the oxidation of the quinol to quinone⁹⁰. It is mediated by the hemes one of which is high spin (5-coordinate) and low spin (6-coordinate) thus forming a bis-Fe-(IV) state by either O₂ or addition of H₂O₂^{91, 92}. By site directed mutagenesis studies it was also found that conserved Trp93 between the 2 hemes allowed facile transfer of electrons between them⁹¹.

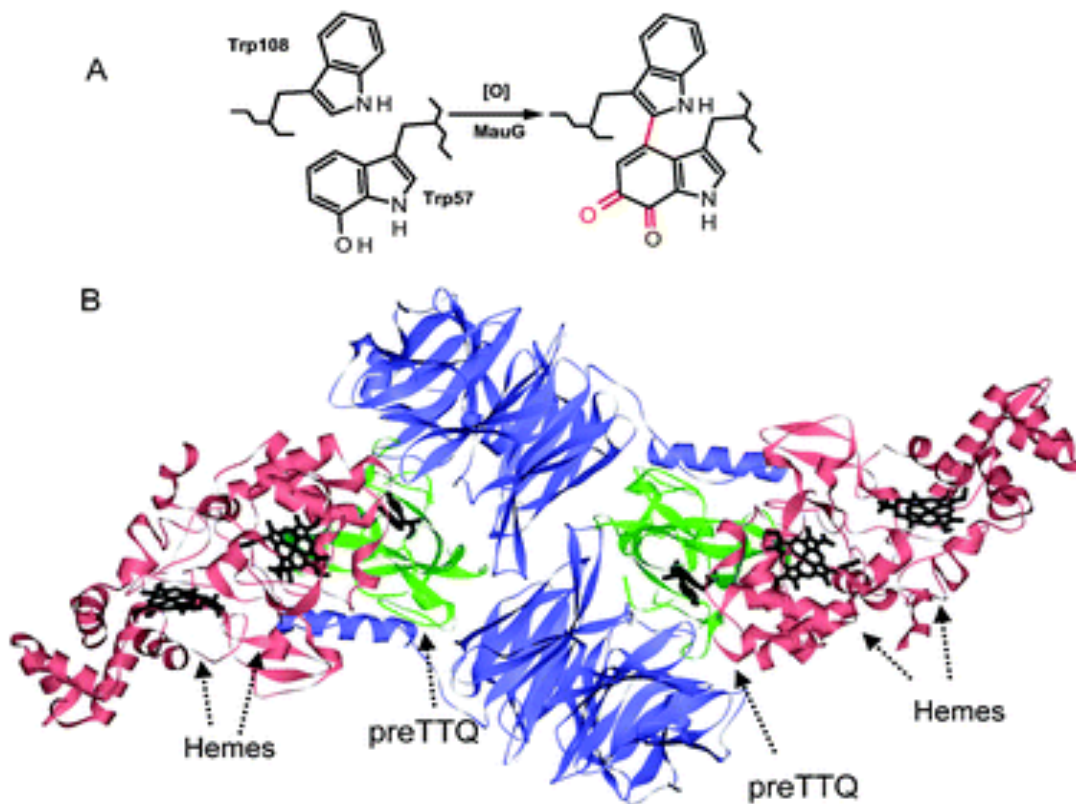


Figure 4 MauG catalyzed TTQ biosynthesis.

A. shows the chemical representation of MauG catalyzing the addition of Oxygen and the crosslinking with Trp in presence of [O] or oxidizing equivalents. B. The crystal structure of the complex of MauG in pink with β -subunits of preMADH, the α -subunits are shown in blue. The β Trp57, β -Trp108 and the hemes are shown in black⁵⁷. The PDB ID for this structure is 3L4M.

Since the dihememes are at a distance of 40 Å from the TTQ generation site, the mechanism took place by a hopping mechanism of electron transfer instead of electron tunneling as normally observed for short distances. This transfer of electrons between the heme and the Trp of the preMADH occurred via the Trp199 residue^{93, 94}. Asp32 and Asp76 have been implicated in their involvement in the biogenesis of TTQ by preventing the proper alignment of the active site residues and preventing the initial hydroxylation⁹⁵.

The other TTQ containing protein was the aromatic amine dehydrogenase which was first discovered in *Alcaligenes faecalis*, a gram negative bacteria⁷⁹. Just like MADH, it too had 2 subunits with the TTQ being present in the smaller one. The different redox states of TTQ were similar to that in MADH showing similar absorption spectra for the 3 states. Even though MADH catalyzes the oxidative deamination of methylamine and the AADH does the same for aromatic amines, their overall mechanism for the reductive half reaction is similar. AADH was also found to react with primary aliphatic amines especially the long and hydrophobic ones⁹⁶.

Cysteine tryptophylquinone (CTQ):

Quinohemoprotein Amine Dehydrogenase (QHNDH): CTQ was first discovered in quinohemoprotein amine dehydrogenase (QHNDH). It is a periplasmic protein and has been isolated from *P. putida*⁹⁷ and *P. denitrificans*⁹⁸, the latter being also common for MADH expression. Expression of the protein takes place in the presence of long carbon chain substrates such as butylamine and also benzylamine which is used up as a source of energy⁹⁹. Based on their source, QHNDH uses either azurin or cytochrome c as its

electron acceptor the latter transferring electrons to cytochrome c oxidase present as a part of the respiratory chain¹⁰⁰. Even though MADH and QHNDH were isolated from the same bacterium, they were found to have structural differences. MADH was a homodimer whereas the crystal structure of QHNDH established that it was a heterotrimeric protein with $\alpha\beta\gamma$ subunits^{9, 101}.

The largest α subunit has 2 heme c groups and the smallest γ subunit has the CTQ cofactor along with additional crosslinks between three other Cys residues to the carboxylic acid of either a glutamate or aspartate¹⁰¹. The cofactor formation involves the Trp 43 and Cys 31 in the γ subunit¹⁰².

Unlike the biogenesis of TTQ, the biogenesis of CTQ has not been well-understood due to structural complexities. An idea was that an AsIB-like protein encoded by ORF2 was responsible for oxidizing the Trp 43 residue¹⁰¹. AsIB is a Fe-S protein that is required for the oxidation of serine and cysteine residues in sulfatases¹⁰³. On the other hand the presence of 2 heme c groups in the α -subunit resembling the MauG protein could also be involved. In recent years, QhpG, a Flavin dependent monooxygenase, encoded by the *qhpG* gene, has been shown to possess sequence similarity with LodB and could be involved in catalyzing the initial hydroxylation at the Trp residue¹⁰⁴. Due to the presence of 3 redox centers and the very high extinction coefficient of the heme groups, it was very difficult to measure the E_m values of them. It was only achieved after dissociating the γ subunit from the rest of the protein¹⁰⁵. In order to aid in the spectroscopic characterization and the kinetic studies, a model compound of CTQ having the same indole-6,7-quinone skeleton with a thioether group at C-4 position was

synthesized¹⁰⁶. QHNDH has been used for the bioelectrocatalytic detection of histamine using its native electron acceptor cytochrome c-550¹⁰⁷. Even though the overall structure of MADH and QHNDH are different, there is some similarity in the Asp residues at the active site that are conserved. Asp 33 of the γ -subunit of QHNDH corresponds to the Asp 76 of the MADH β -subunit. The Asp 13 of the γ subunit of QHNDH corresponds to the Asp 32 of the MADH.

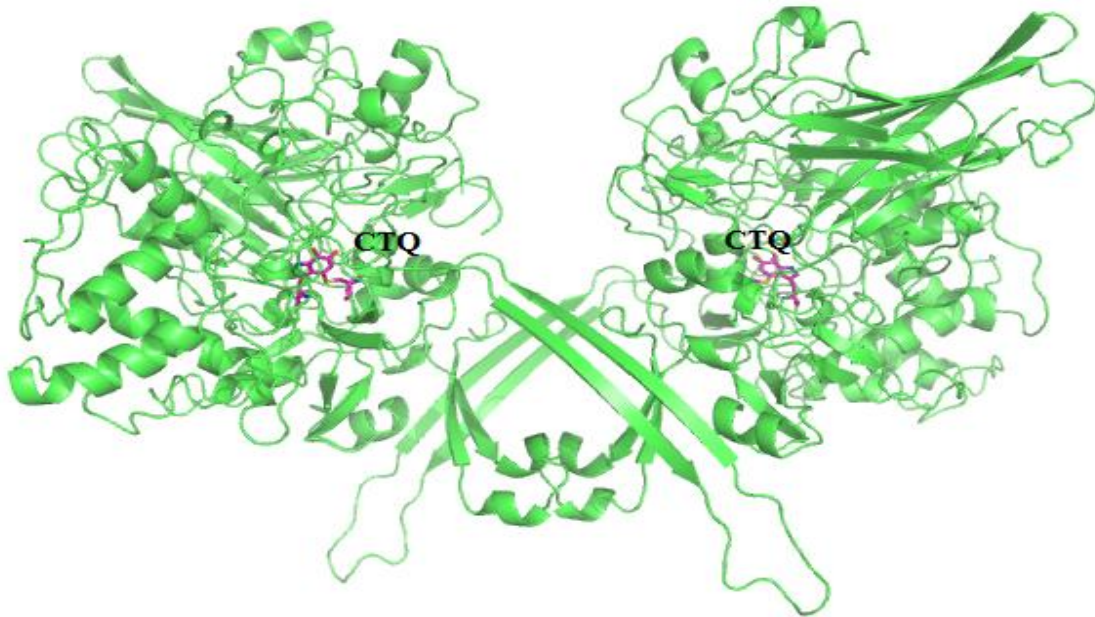
Hence amongst the known tryptophylquinone cofactors, CTQ hasn't been characterized completely. Its mechanism of biogenesis and catalysis hasn't been elucidated completely. In this study the characterization of structural and functional relationship in 2 unique tryptophylquinone cofactor containing oxidases have been elucidated.

Lysinse- ϵ -oxidase (LodA): All previous amino acid oxidase have been known to be flavoproteins¹⁰⁸ and catalyze the oxidative deamination of amino acids to generate α -keto acids, ammonia and hydrogen peroxide. The amino acid oxidation takes place at the α -position of the amino acid chain. The most characterized one are the ones from the snake venom¹⁰⁹. The release of H₂O₂ determines the ability of the protein to execute antimicrobial functions¹¹⁰. Often peptides and proteins have been shown to have antimicrobial properties. In various higher organisms they act as a defense mechanism against pathogens¹¹¹. Many of these LAOs have been found in microorganisms and their antimicrobial activity gives them an added advantage over competing strains¹¹². One of the most studied LAO, is the L-lysine- α -oxidase¹¹³. Since L-lysine is an essential amino

acid that cannot be synthesized in the body, enzymes that are capable of cleaving these have been investigated in context of their possible application in oncology¹¹⁴. Comparative studies of LodA with the L-lysine- α -oxidase and lysl oxidase have also been done. L-lysine- α -oxidase is a flavoprotein. Even though it resulted in a much higher hydrogen peroxide generation, the K_m for L-lysine was 40 μ M¹¹⁵. The kinetic parameter thus indicated that L-lysine- α -oxidase was not specific for L-lysine as its substrate and could use other amino acids as well.

For the first time in a melanogenic marine bacterium, *Marinomonas mediterranea*¹¹⁶, an L-amino acid oxidase was found that contained a quinonic cofactor and not a Flavin¹¹⁷. It was called the Lysine- ϵ -oxidase, LodA. Unlike the other flavoproteins this did not have any FAD binding site¹¹⁸. It also differed from the others as the amino acid oxidation that it catalyzed was at the ϵ -position instead of the very common α -position. The oxidative deamination leads to the generation of a semialdehyde, ammonia and hydrogen peroxide. Due to the release of the latter, it has been found to be antimicrobial against both the gram positive and the gram negative bacteria¹¹⁹. LodA is secreted, thus helping in the differentiation and dispersal of biofilms by forming microcolonies due to its antimicrobial nature¹²⁰. It showed much higher affinity and specificity for L-lysine than that shown by the L-lysine- α -oxidase. The reason for this is the that the available concentration of free amino acids in marine water is very low, therefore the K_m for L-lysine has been adapted accordingly

A.



B.

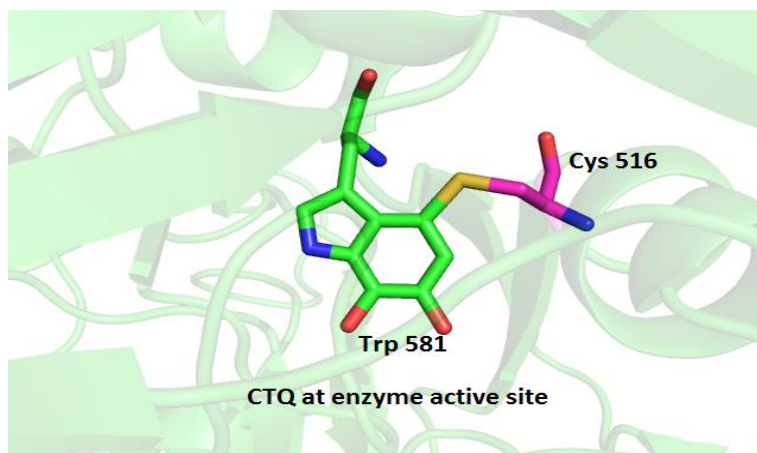


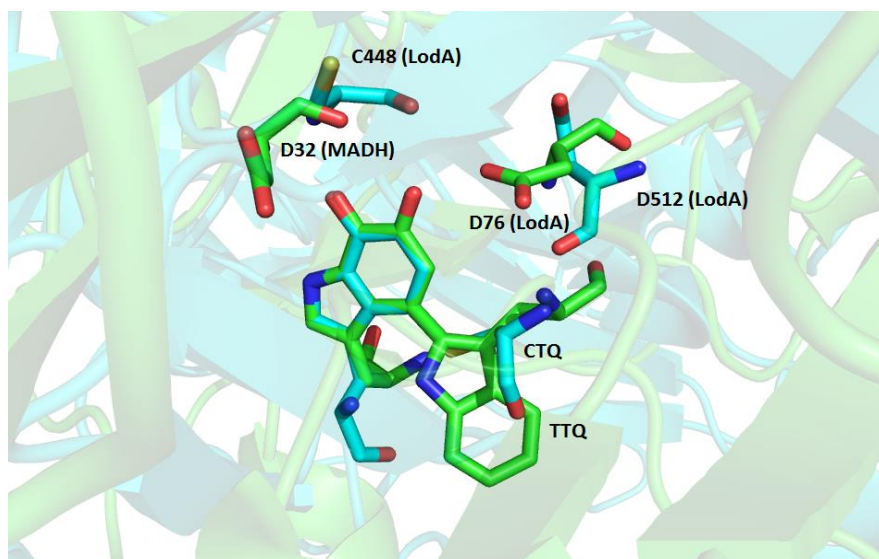
Figure 5 Shows the crystal structure of LodA. (PDB ID 2ymw).

A. show the whole protein as crystallized with the CTQ cofactors. B. Shows the enzyme active site with the Cys 516 and the Trp 581 residues.

Sequence analysis shows the presence of 2 ORFs in an operon-like fashion¹²¹. The first one which was the larger one encoded a 726 amino acid protein of a mass of around 76kDa. This was the LodA. It was seen that Cysteine 516 and Trp 581 did undergo posttranslational modifications to form the CTQ cofactor. Three different crystal structures have been reported, PDB ID – 2YMW, 3WEU¹²², 3WEV. Figure 5A. shows the reported crystal structure for LodA and B. shows the active site with the modified CTQ cofactor. The second ORF which was 2bp from the stop codon of *lodA*, encoded a protein of 369 amino acid and with an approximate molecular mass of 41.4kDa. This was LodB, which had a putative FAD binding domain¹²³. It has been shown that LodB is absolutely required for the expression of the mature CTQ containing LodA¹²⁴, hence indicating that the posttranslational modification of the Cys and the Trp residue could be an FAD-mediated process.

Until the discovery of LodA, only amine dehydrogenases were known to contain tryptophylquinone cofactors. This was the first example of an oxidase showing an unusual cofactor. Hence crystal structure overlays were done with both MADH (TTQ containing) and QHNDH (CTQ containing) to see how the structures correlated. Various residues were found to be analogous to the active site Aspartates of MADH and QHNDH some of which have been shown to be involved in cofactor biogenesis and substrate specific. The presence of Asp 512 in LodA corresponding to the Asp 76 (MADH) and Asp 32 (QHNDH) shows possible conserved function in functionally different proteins. Figure 6A shows the overlay of MADH with LodA and the corresponding cofactor and active site residues, B. shows the overlay of QHNDH with LodA.

A.



B.

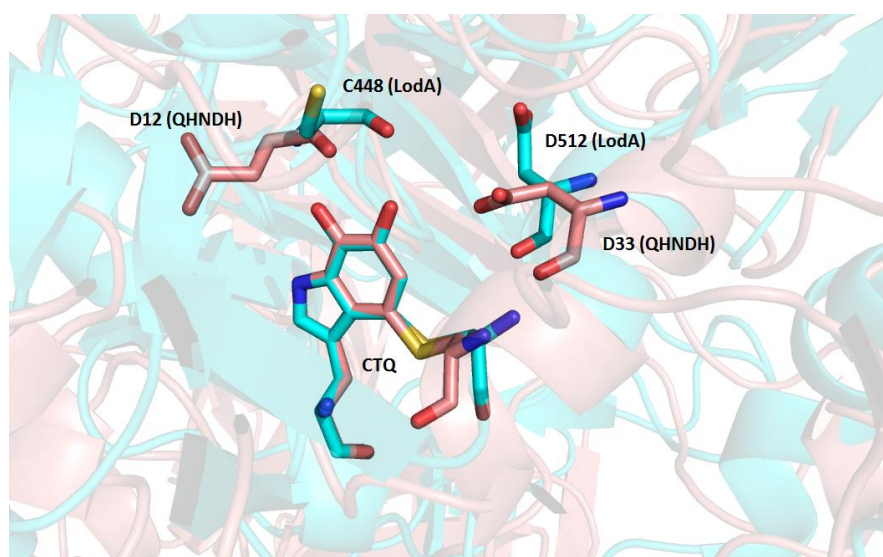


Figure 6 Overlay of reported crystal structures of LodA, MADH, QHNDH.

A. overlay of MADH and LodA with the cofactor forming and active site residues. B. overlay of LodA and QHNDH highlighting the same.

Recent genome sequencing studies on *Marinomonas mediterranea* showed the presence of 2 such similar genes which are both followed by *lodB*-like genes. A wider genome sequencing was done to get an idea about the distribution of these LodA-like proteins. It was found that 168 such similar genes were present in 144 different microbial genomes including some fungal ones. LodA-like proteins were categorized under group IA and a detailed look into their sequence shows the conserved Cys and Trp generating CTQ. All of them are oxidases and are expressed in organisms known for antimicrobial properties¹²⁵.

Glycine oxidase (GoxA): Based on the genome analysis of *Marinomonas mediterranea*, 2 other genes were identified that showed similarity to *lodA*¹²⁶. One of them was thought to show glycine oxidase activity and was called GoxA¹⁴. It too had another gene downstream and overlapping the stop codon for *goxA* which encodes a second LodB-like GoxB protein which too is a flavoprotein. This glycine oxidase contains a quinone cofactor formed from Cys 551 and Trp 566 determined from sequence alignments. This is different from all other known glycine oxidases (GO). The GO from *Bacillus subtilis* is a 45kDa flavoenzyme containing non-covalently bound FAD¹²⁷. This GO was found to carry out the oxidative deamination of a wide range of substrates in order to form the α -keto acids. The GoxA discovered in the marine bacterium did not contain a Flavin cofactor and was specific for Glycine as its substrate.

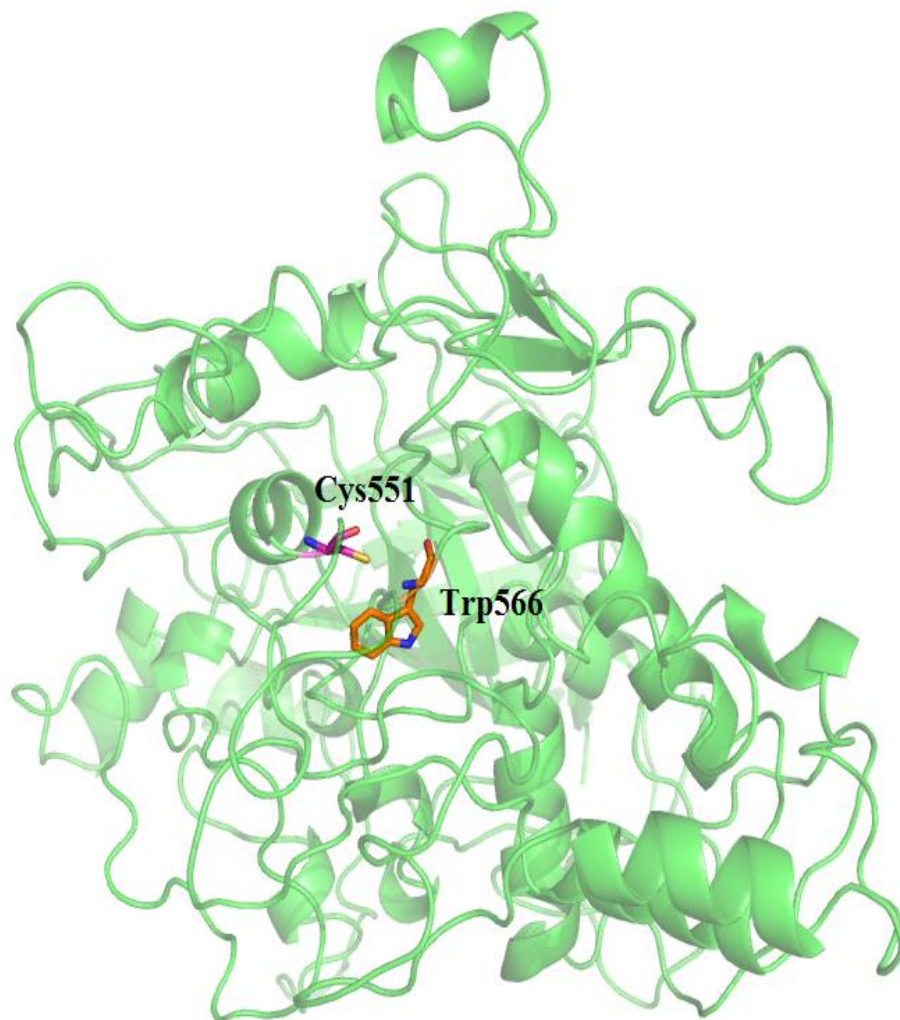
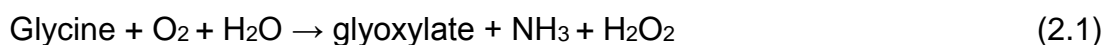


Figure 7 Homology model of GoxA monomer with the highlighted Cys 551 and Trp 566 involved in CTQ biosynthesis.

The crystal structure of GoxA has not been reported. But homology models have been constructed to give an idea about the possible protein structure. Figure 7 shows the homology model of GoxA with the Cys and Trp that form the cofactor.

GoxA catalyzes the following reaction:



It generates peroxide but it isn't known if that is the only physiological function that it possess. Phylogenetic and sequence analysis done on microbial genomes have put GoxA in group II for the Lod-like proteins¹²⁵. LodA and GoxA have been shown to possess 22.8% identity and 34.6% identity.

It has been shown that in the absence of the GoxB and the LodB proteins, an incompletely modified CTQ is synthesized in GoxA and LodA respectively and the roles of the B protein are not interchangeable. The precursor protein that is generated in the absence of GoxB and LodB contains a single hydroxylation at the Trp residue¹¹. and the mass spec of the precursor LodA shows an increase of 16 instead of 28 for a fully formed CTQ¹²⁸.

CHAPTER 3: STEADY-STATE KINETIC MECHANISM OF LODA, A NOVEL CYSTEINE TRYPTOPHYLQUINONE- DEPENDANT OXIDASE

FEBS Lett. 2014 Mar 3;588(5):752-6

Introduction

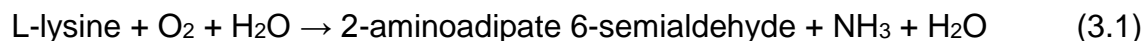
LodA is a novel lysine- ϵ -oxidase which was recently found in the melanogenic marine bacterium, *Marinomonas mediterranea*¹²⁹⁻¹³¹. This enzyme was originally called marinocine but is now referred to as LodA, the gene product of *lodA*. LodA exhibits antimicrobial properties in vivo that result from its production of H₂O₂. It is secreted to the external medium in the biofilms in which the bacterium resides. This causes death of a subpopulation of cells within the *M. mediterranea* biofilm that is accompanied with differentiation, dispersal and phenotypic variation among dispersal cells¹⁰.

Typical amino acid oxidases utilize a flavin cofactor for catalysis and act upon the α -amino group. LodA removes the ϵ -amino group of lysine and the recent crystal structure of LodA reveals that it contains a cysteine tryptophylquinone (CTQ) cofactor¹³². CTQ is a protein-derived cofactor⁵⁷, which is generated by the posttranslational modification of cysteine 516 and tryptophan 581 of LodA. Analysis of LodA by mass spectrometry yields a molecular mass that is also consistent with the presence of CTQ¹²⁸. Another protein LodB is required for the posttranslational formation of CTQ^{124, 128}. All other tryptophylquinone cofactor-containing enzymes are dehydrogenases that use other redox proteins as their electron acceptors¹³³; this is the first time that one has been shown to function as an oxidase. The best known tryptophylquinone enzymes possess tryptophan

tryptophylquinone (TTQ, Fig. 1) where the modified Trp is cross-linked to another Trp rather than a Cys⁷⁷. The known TTQ enzymes are primary amine dehydrogenases^{57, 134}. The one other CTQ-dependent enzyme that has been characterized is a quinoxinohemoprotein amine dehydrogenase (QHNDH) which possesses two covalently attached hemes in addition to CTQ^{9, 101}.

Another class of protein-derived cofactors that contain quinones derived from Tyr residues has been identified¹³⁵. These possess the topaquinone (TPQ, Fig. 1) cofactor and are found only in primary amine oxidases. In each case, these enzymes also possess a tightly bound copper in the active site which is required for biogenesis of the cofactor and subsequently participates in catalysis. A related cofactor is lysine tyrosylquinone (LTQ) which is the catalytic center of mammalian lysyl oxidase⁷¹. This enzyme is also a lysine ϵ -oxidase but it acts only on lysyl residues of a protein substrate rather than free lysine⁶⁷. Like the TPQ-dependent enzymes, lysyl oxidase also possesses a tightly-bound copper in the active site⁷². LodA is the first example of an amino acid or primary amine oxidase that contains neither a flavin nor a metal at its active site.

This paper presents a steady-state kinetic analysis of the reaction that is catalyzed by LodA (eq 1) to determine its steady-state reaction mechanism and kinetic parameters. The results are used to propose a reaction mechanism which is consistent with the crystal structures of a lysine-bound adduct of LodA¹³².



LodA is a novel lysine- ϵ -oxidase which possesses a cysteine tryptophylquinone cofactor. It is the first tryptophylquinone enzyme known to function as an oxidase. A steady-state kinetic analysis shows that LodA obeys a ping-pong kinetic mechanism with values of k_{cat} of $0.22 \pm 0.04 \text{ s}^{-1}$, K_{lysine} of $3.2 \pm 0.5 \text{ }\mu\text{M}$ and K_{O_2} of $37.2 \pm 6.1 \text{ }\mu\text{M}$. The k_{cat} exhibited a pH optimum at 7.5 while k_{cat}/K_{lysine} peaked at 7.0 and remained constant to pH 8.5. Alternative electron acceptors could not effectively substitute for O_2 in the reaction. A mechanism for the reductive half reaction of LodA is proposed that is consistent with the ping-pong kinetics.

Materials and Methods

Recombinant LodA was expressed in *E.coli* Rosetta cells which had been transformed with pETLODAB¹²⁸ which contains *lodA* with an attached 6xHis tag and *lodb* which is required for the posttranslational modification of LodA that forms CTQ. The cells were cultured in LB media with ampicillin and chloramphenicol. Expression levels of active LodA are very sensitive to induction conditions and previously determined optimal conditions¹²⁸ were followed. When the absorbance of the culture reached a value of 0.6, the temperature was decreased to 15 °C and cells were induced by addition of 1 mM IPTG. Cells were harvested after 2 hr and then broken by sonication in 50 mM potassium phosphate buffer (KPi), pH 7.5. The soluble extract applied a Nickel-NTA affinity column and the His-tagged LodA was eluted using 100-120 mM imidazole in 50 mM KPi, pH 7.5. LodA was then further purified by ion exchange chromatography with DEAE cellulose in 50 mM KPi, pH 7.5. The His-tagged LodA bound to the resin and was eluted using 180-

270 mM NaCl in the same buffer. The protein was judged pure by SDS-PAGE and its UV-visible absorption spectrum exhibited a broad peak centered at 400 nm with an absorbance at 400 nm which was about 13-fold less intense than the absorbance at 280 nm. Methylamine dehydrogenase (MADH)¹³⁶, amicyanin⁷⁸ and cytochrome c-550¹³⁷ were purified from *Paracoccus denitrificans* as described previously.

LodA activity had previously been assayed using a standard coupled assay for oxidases in which horseradish peroxidase (HRP) is also present and uses the H₂O₂ product of the oxidase reaction to oxidize Amplex Red to resorufin which leads to a change in fluorescence⁸. This assay was modified to instead follow the change in absorbance associated with production of resorufin which has an extinction coefficient at of 54000 M⁻¹ cm⁻¹ at its absorbance maximum of 570 nm. The assay mixture contained 0.05 mM Amplex Red, 0.1U/ml of HRP and 0.4 μM LodA in 50 mM KPi, pH 7.5 at 25 °C. The concentration of LodA was determined using an $\epsilon_{280} = 125,180 \text{ M}^{-1}\text{cm}^{-1}$ which was calculated from its amino acid sequence¹³⁸. Experiments were performed in which [lysine] was varied in the presence of fixed concentrations [O₂] and vice versa. To vary the concentration of O₂, an appropriate amount of a stock solution of air-saturated buffer ([O₂]=252 μM) was mixed with the reaction mixture which had been made anaerobic by repeated cycles of vacuum and purging with argon. The reactions were initiated by the addition of lysine. In order to account for a small background reaction in which Amplex Red is spontaneously converted to resorufin, control experiments were performed in the absence of LodA to determine the background rate which was subtracted from the experimentally-determined LodA-dependent reaction rates. The reaction was also

assayed by monitoring the release of the ammonia product using a coupled assay. The assay mixture contained 20 U/ml glutamate dehydrogenase, 5 mM α -ketoglutarate, 0.4 μ M LodA and 93.7 μ M NADH in 50 mM KPi, pH 7.5 at 25 °C and the reactions were initiated by the addition of lysine. In assays to test the reactivity of artificial electron acceptors the assay mixture contained 0.4 μ M LodA in 50 mM KPi, pH 7.5 at 25 °C under anaerobic conditions. The reaction was initiated by addition of lysine and monitored by the spectral change associated each electron acceptor, phenazine ethosulfate (PES) plus 2,6-dichloroinophenol (DCIP) ($\epsilon_{600}=21,500 \text{ M}^{-1}\text{cm}^{-1}$), ferricyanide ($\epsilon_{420}=1,040 \text{ M}^{-1}\text{cm}^{-1}$), or NAD⁺ ($\epsilon_{340}=6,220 \text{ M}^{-1}\text{cm}^{-1}$), amicyanin ($\epsilon_{595}=21,500 \text{ M}^{-1}\text{cm}^{-1}$), cytochrome c-550 ($\epsilon_{550}=30,200 \text{ M}^{-1}\text{cm}^{-1}$).

Results and Discussion

Steady-state reaction mechanism and parameter

Steady-state assays of lysine oxidation by LodA were performed in order to determine the kinetic mechanism of this reaction and the kinetic parameters that describe this reaction. The initial rates of reaction were determined at different concentrations of lysine in the presence of a series of fixed concentrations of O₂ (Fig. 8A). Double reciprocal plots of these data exhibited a series of lines which are clearly more parallel than intersecting, consistent with a ping-pong mechanism rather than an ordered or random sequential mechanism (Fig. 8B). Thus, the reaction is described by eq 2. Secondary plots of the intercept ($1/k_{\text{cat}}$ apparent) versus $1/[\text{O}_2]$ (Fig. 8C) and intercept/slope ($1/K_{\text{lysine}}$ apparent) versus $1/[\text{O}_2]$ (Fig. 8D) were each linear. These plots are described by eqs 3

and 4(), respectively. Similarly, when the initial rates of reaction were determined at different concentrations of O₂ in the presence of a series of fixed concentrations of lysine a series of parallel lines was obtained in the reciprocal plots and secondary plots were linear (Figures 8E and 8F) and analysis yielded similar values of $K_{cat} = 0.22 \pm 0.04 \text{ s}^{-1}$, K_m for lysine is $3.2 \pm 0.5 \text{ } \mu\text{M}$, K_m for O₂ is $37.2 \pm 6.1 \text{ } \mu\text{M}$.

$$v/E = \frac{k_{cat}[\text{lysine}][\text{O}_2]}{K_{\text{lysine}}[\text{O}_2] + K_{\text{O}_2}[\text{lysine}] + [\text{lysine}][\text{O}_2]} \quad (3.2)$$

$$\text{intercept} = (K_{\text{O}_2}/k_{cat}) (1/[\text{O}_2]) + (1/k_{cat}) \quad (3.3)$$

$$\text{intercept/slope} = (K_{\text{O}_2}/K_{\text{lysine}}) (1/[\text{O}_2]) + (1/K_{\text{lysine}}) \quad (3.4)$$

This reaction was also studied using another coupled enzyme assay in which the rate of formation of the ammonia product was coupled to NADH oxidation by glutamate dehydrogenase in the presence of α -ketoglutarate. When the assay was performed using saturating concentrations of $100 \text{ } \mu\text{M}$ lysine and $252 \text{ } \mu\text{M}$ O₂ the rate of reaction was $0.25 \pm 0.07 \text{ s}^{-1}$ which is within experimental error of the k_{cat} value obtained in the assay which monitored H₂O₂ production.

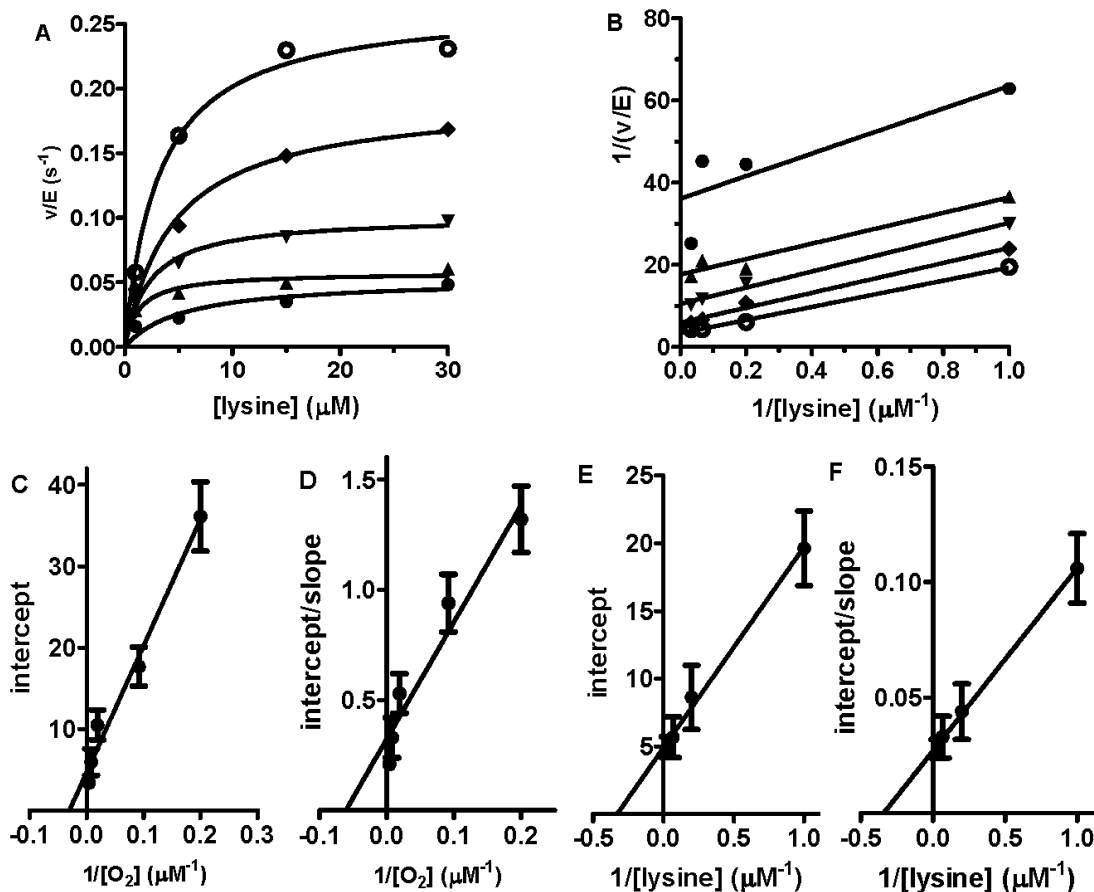


Figure 8 Steady-state kinetic analysis of LodA.

A. Initial rates were determined at different concentrations of lysine in the presence of a fixed concentration of 252 (○), 129 (◊), 54 (▼), 11 (▲) or 5 (●) μM O₂. B. Double reciprocal plot of the data shown in panel A. C. Secondary plots of the y-intercept (1/apparent k_{cat}) from panel B versus $1/[O_2]$. Data are fit by eq 3. D. Secondary plots of the y-intercept/slope (1/apparent K_{lysine}) from panel B versus $1/[O_2]$. Data are fit by eq 4. E. Secondary plots of the y-intercept (1/apparent k_{cat}) from plots of v/E versus $[O_2]$ against $1/[lysine]$. Data are fit by eq 3. F. Secondary plots of the y-intercept/slope (1/apparent K_{O_2}) from plots of v/E versus $[O_2]$ against $1/[lysine]$. Data are fit by eq 4. Error bars are indicated in the secondary plots (C-F).

Dependence of LodA activity on pH

The pH dependence of k_{cat} and k_{cat}/K_m for lysine were determined at 252 $\mu\text{M O}_2$ (Fig. 9). The k_{cat} value exhibited a bell-shaped pH profile with a maximum at pH 7.5. This suggests that there are at least two residues that are required and that one must be protonated and the other must be unprotonated for catalysis. In contrast the k_{cat}/K_m values reached a maximum at pH 7.0 and stayed relatively constant up to pH 8.5. Thus, the catalytic efficiency of LodA is retained at the higher pH as a consequence of a decrease in K_{lysine} .

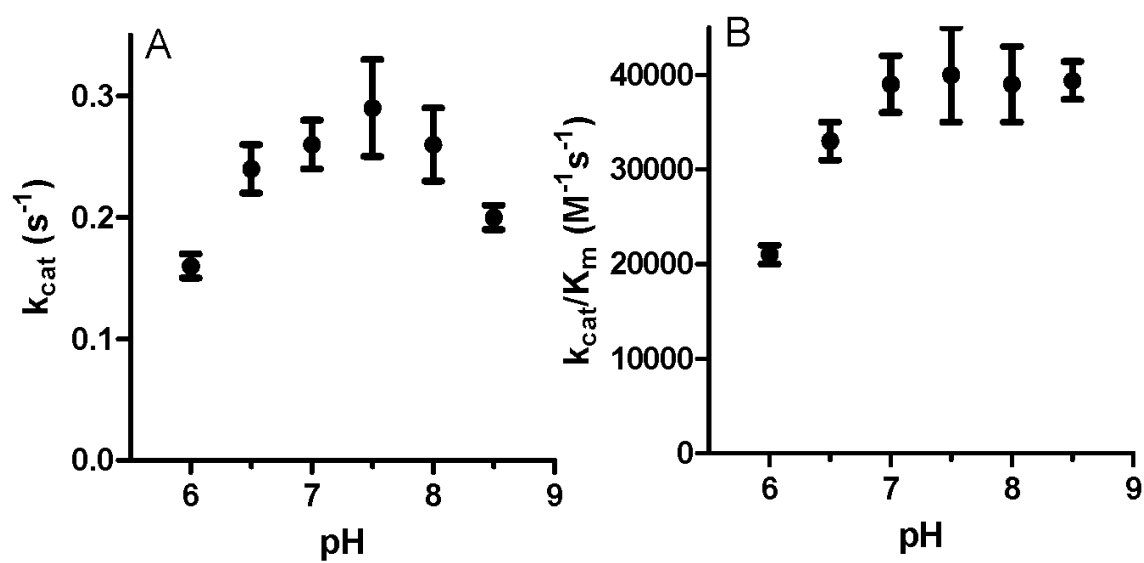


Figure 9 Dependence on pH of k_{cat} and k_{cat}/K_m for lysine at saturating concentration of O₂.

Error bars are indicated.

Reactivity towards alternative electron acceptors.

The distinguishing property between an oxidase and a dehydrogenase is that, the oxidases use oxygen as their final electron acceptor whereas the latter use other redox proteins or non-protein electron acceptors other than oxygen. Since all other known tryptophylquinone enzymes are dehydrogenases the ability of LodA to function as a dehydrogenase was tested using alternative electron acceptors. The most widely used assay for quinoprotein dehydrogenases uses PES coupled to DCIP. For comparison the assay was also performed on MADH which possesses the TTQ cofactor. When assayed in the presence of saturating methylamine with 5 mM PES and 100 μM DCIP MADH exhibited a rate of 36 s^{-1} while LodA when assayed in the presence of saturating lysine under these conditions exhibited a rate of 0.06 s^{-1} . Another commonly used artificial electron acceptor is ferricyanide. When similarly assayed in the presence of 1 mM potassium ferricyanide with saturating lysine LodA exhibited a rate of 0.10 s^{-1} . For a more direct comparison of the efficiency of the artificial electron acceptors, the assays were repeated using 252 μM PES or ferricyanide, a concentration equivalent to that used for O_2 ($K_{\text{O}_2} = 37.2 \pm 6.1 \text{ }\mu\text{M}$). At this concentration the rates with PES/DCIP and ferricyanide were each 0.01 s^{-1} which is barely above background, compared $0.22 \pm 0.04 \text{ s}^{-1}$ for O_2 . NAD^+ (1 mM) was also tested in the presence of 100 μM lysine and no detectable reaction was observed. Two protein electron acceptors were also tested. The copper protein amicyanin is the natural electron acceptor for MADH. In the steady state reaction of methylamine dependent amicyanin reduction by MADH, amicyanin exhibits a k_{cat} of 48 s^{-1} and K_{m} of $6 \text{ }\mu\text{M}$ ¹³⁹. When LodA was assayed with 100 μM amicyanin and saturating

lysine the rate was only 0.008 s⁻¹. Cytochrome *c*-550 from *P. denitrificans* is a relatively promiscuous electron acceptor that shares structural features with many bacterial and mammalian cytochromes *c*¹⁴⁰. When LodA was assayed with 100 μM cytochrome and saturating lysine the rate was 0.002 s⁻¹.

One cannot rule out the possibility that *in vivo* there is an unidentified redox-active molecule or protein that could serve as an electron acceptor for LodA instead of O₂. However, no significant dehydrogenase activity of LodA could be detected in this study. These results support the proposal that the generation of H₂O₂ and consequent antimicrobial activity¹²⁹ is the main physiological function of LodA, which is secreted from cell to perform this function.

Proposed mechanism

The ping-pong kinetic mechanism requires that an intermediate form of the enzyme exist after binding of substrate and release of the first product prior to binding of the second substrate. This finding is consistent with the reported crystal structure of LodA with a covalent lysine adduct with CTQ¹³² and suggests the following reaction mechanism (Fig. 10). The ε-amino group of lysine must first be deprotonated, likely by an active site residue, so that the resulting nucleophilic NH₂ group can attack the electrophilic carbonyl carbon to displace the bound oxygen and yield a Schiff base intermediate, as seen in the crystal structure. An active site residue then abstracts a proton from the ε-carbon reducing the cofactor and resulting in the Schiff base now involving the ε-carbon. Inspection of the crystal structure of the LodA-lysine adduct¹³² reveals that the S of Cys448 is

approximately 5.5 Å from this carbon and could possibly be involved in this step. This covalent adduct is then hydrolyzed to release the aldehyde product prior to O₂ binding. This yields an aminoquinol intermediate form of the enzyme consistent with a ping-pong mechanism. This proposed mechanism for this reductive half-reaction is essentially identical to those of QHNDH¹⁰² and MADH¹³⁹, which possess CTQ and TTQ, respectively. It is also the same mechanism proposed in the reductive half-reactions of the TPQ-dependent amine oxidases¹⁴¹. With the TTQ-dependent MADH it was shown that the ammonia product was not released until after the quinol was oxidized to the quinone by its electron acceptor, and that in the steady-state this occurred by displacement of the amino group from the cofactor by the next amine substrate molecule¹⁰². This may be true for LodA as well. Alternatively, it could be hydrolyzed by water. A key question which remains unanswered is how the quinol or aminoquinol in LodA is reoxidized by O₂. In the TPQ-dependent oxidases Cu²⁺ is also present at the active site. It has been proposed that this interacts with the quinol intermediate to yield a Cu⁺-semiquinone intermediate which would allow reaction of the Cu⁺ with oxygen to generate a superoxide species in the first step of the reoxidation^{142, 143}. LodA, however, does not have a bound metal. Future characterization of the mechanism of the oxidative half-reaction of LodA should be of great interest.

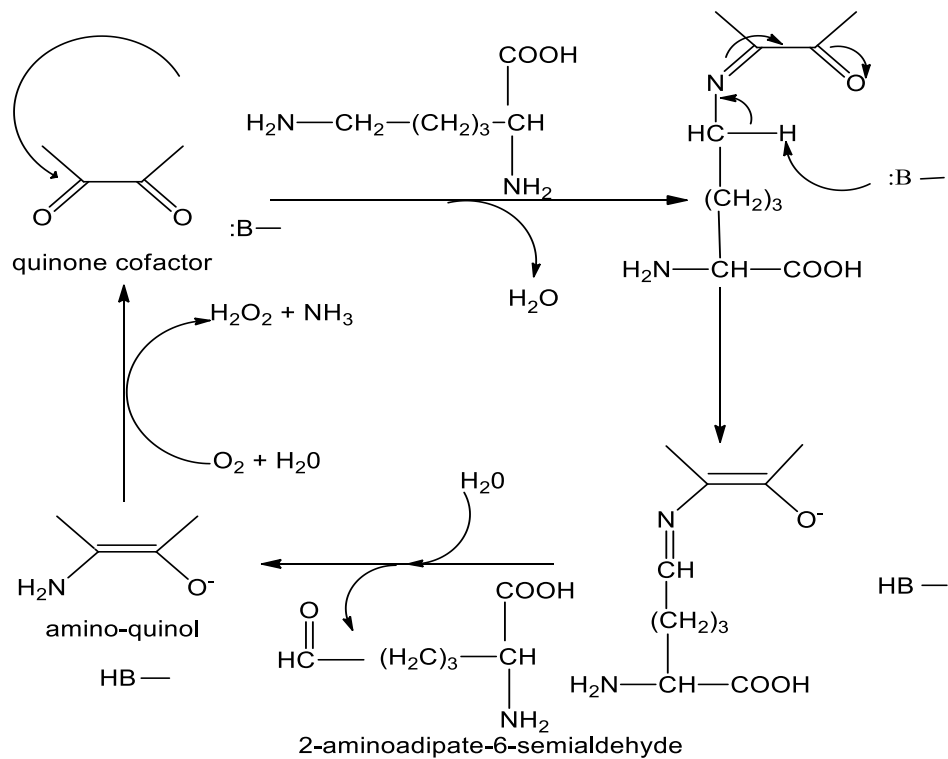


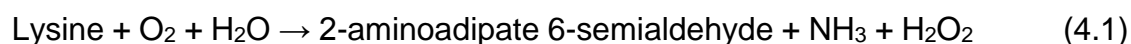
Figure 10. Proposed mechanism for LodA-catalyzed oxidative deamination of lysine.

CHAPTER 4: ROLES OF ACTIVE SITE RESIDUES IN LODA, A CYSTEINE TRYPTOPHYLQUINONE DEPENDENT LYSINE-E-OXIDASE

Arch Biochem Biophys. 2015 Aug 1;579:26-32

Introduction

LodA (previously called marinocine) is an ϵ -lysine oxidase that was first discovered in *Marinomonas mediterranea*, a melanogenic marine bacterium^{8, 129, 130}. It catalyzes the oxidative deamination of lysine by removing the ϵ -amino group and releasing the aldehyde product plus hydrogen peroxide (eq 1). When secreted out into the biofilm in which the bacterium resides, LodA exerts antimicrobial activity as a consequence of the production of H₂O₂. This is beneficial because causing death of a subpopulation of cells facilitates biofilm differentiation and dispersal of surviving cells with phenotypic variation among dispersed cells¹⁰. Wild type (WT) LodA has been recombinantly expressed in *E. coli*¹²⁸ and isolated. LodA contains a protein-derived cofactor⁶ which is generated by posttranslational modifications. The cofactor is cysteine tryptophylquinone (CTQ) which is formed by the di-oxygenation of Trp581 and the crosslinking of this residue with Cys516. Expression of active LodA with correctly synthesized CTQ requires the co-expression of the *lodB* gene¹²⁸, which encodes a flavoprotein that is required for the posttranslational modifications that generate CTQ.



LodA is the first enzyme that was shown to use a tryptophylquinone cofactor that functions as an oxidase. All previously described enzymes that use CTQ or tryptophan tryptophylquinone (TTQ) cofactors are dehydrogenases that use other redox proteins or small molecules other than NAD(P)⁺ as electron acceptors¹³³. LodA is distinct from the other known tryptophylquinone enzymes in using an amino acid as a substrate. LodA is also unusual among amino acid oxidases since the other enzymes previously described in this group utilize a flavin cofactor. Three crystal structures of LodA have been determined, LodA isolated from *M. mediterranea* alone (PDB 3WEU) and with the lysine substrate covalently-bound to CTQ (PDB 3WEV)¹³², and recombinant LodA isolated from *E. coli* (PDB 2YMW). Inspection of these structures identified the residues which are modified to form CTQ, Trp581 and Cys516. It was subsequently shown that mutation of either of these two residues to Ala resulted in no CTQ biosynthesis¹¹. It was also shown that conversion of Asp512, which is in close proximity to CTQ, to Ala led to expression of a precursor of LodA without CTQ¹¹. A similar effect was reported when the structurally analogous Asp was mutated in methylamine dehydrogenase (MADH). That led to expression of a precursor of MADH without TTQ⁹⁵.

A steady-state kinetic study showed that LodA follows a ping-pong mechanism in which it first interacts with lysine forming a Schiff base intermediate with CTQ, then releases the aldehyde product, and then binds O₂ to finally release NH₃ and H₂O₂¹². (Figure 12). From the structure of LodA it was also possible to identify other residues of interest in the active site of LodA which could participate in binding of one or both substrates, or catalysis, as well as CTQ biosynthesis (Figure 11). These residues are the

topic of this study. Cys 448 is located in close proximity to the quinone oxygens of CTQ. Two other quinoproteins, the CTQ-containing quinoxinohemoprotein amine dehydrogenase (QHNDH)¹⁰¹ and the TTQ-containing MADH¹⁴⁴, have a structurally conserved Asp residue in the position corresponding to Cys448 in the LodA structure in the active site. Mutation of the corresponding Asp in MADH affected the efficiency of MauG-dependent TTQ biosynthesis but had no effect on the catalytic activity of the population of the isolated MADH with fully formed TTQ⁹⁵. As such, Cys448 in LodA was converted to Asp, as well as Ala, to ascertain its role in CTQ biosynthesis or catalysis or both. Tyr211 resides in a loop comprised of residues 206 to 215, and it is flanked by one and three Gly residues on either side. This loop which is located close to the entrance of the active site was not visible in the crystals structures of the free and substrate bound native enzymes. In the structure of recombinant LodA, this loop was defined in the structure of one of the two molecules in the asymmetric unit but not in the other. These observations are consistent with mobility of this loop and a possible role in controlling substrate binding or product release, or both, from the active site. To probe the role of Tyr211 it was converted to Phe, Ala and Glu. Lys530 is of interest because it appears to form a hydrogen bond (2.7 Å) between its ϵ -amino N and the phenolic O of Tyr211. It was converted to Ala and Arg. The results of this study demonstrate that these residues in the active site of LodA play multiple roles in binding of lysine and O₂, catalysis and CTQ biosynthesis. Analysis of the Integrated Microbial Genomes database of genome sequences identified 168 genes from 144 different bacterial sequences that encoded LodA-like proteins¹²⁵. LodA from *M. mediterranea* was classified as a member of Group IA LodA-like proteins. The residues

which were mutated in this study were highly conserved in these sequences. These results are discussed in the context of the different evolutionary factors that must be considered for enzymes which possess protein-derived cofactors, in which the catalytic cofactor must be generated by posttranslational modifications.

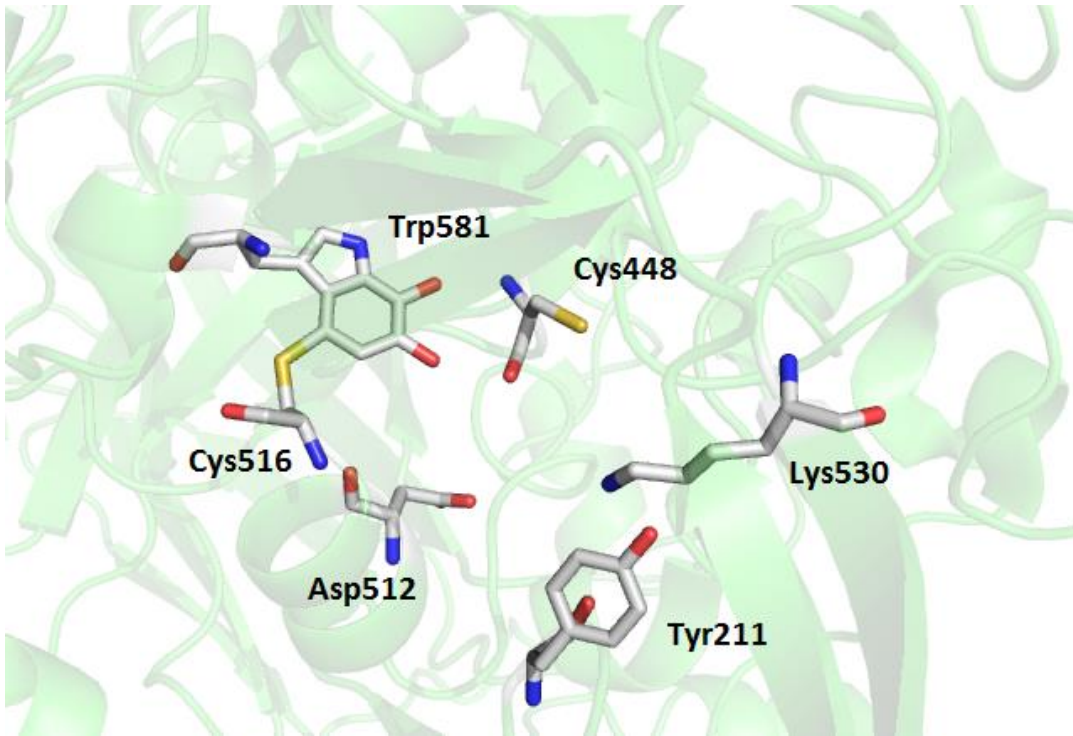


Figure 11 Residues of interest in the active site of LodA.

A portion of the structure of LodA (PDB entry 2YMW) is shown with the residues that form CTQ and other residues of interest in the active site shown as sticks

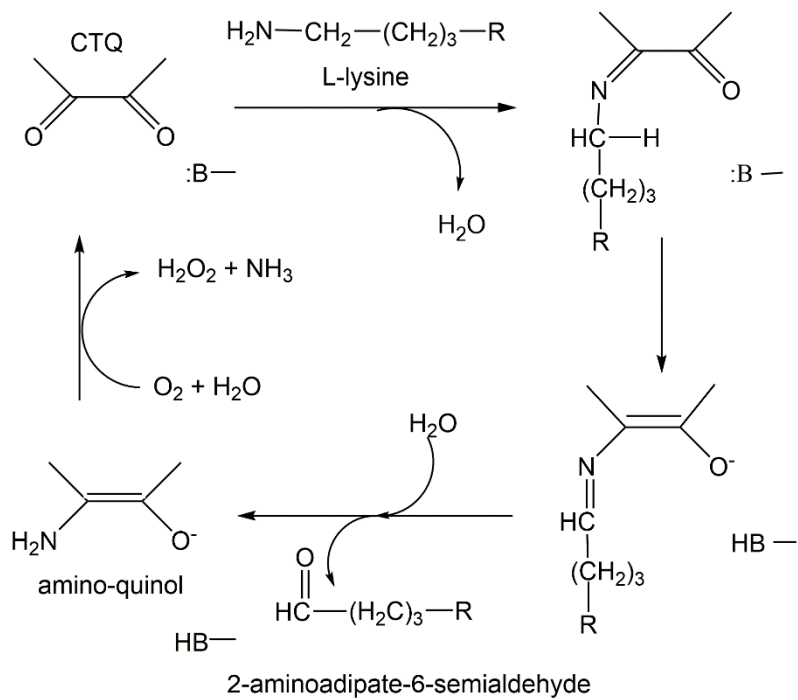


Figure 12 Reaction mechanism for the LodA-catalyzed oxidative deamination of lysine.

Represents an active-site residue which participates in acid–base chemistry

Materials and Methods

Construction, expression and purification of recombinant proteins.

Wild-type (WT) LodA and LodA variants that were generated by site-directed mutagenesis were expressed in *E. coli* Rosetta cells as described previously with the *lodB* gene co-expressed^{12, 128}. The recombinant LodA possessed a 6XHis tag to facilitate purification as previously described¹². The following LodA variants were generated by site directed mutagenesis: C448A, C448D, Y211A, Y211E, Y211F, K530A and K530R. The forward and reverse primers that were used are shown in Table 1. Most of the mutations were made using the QuikChange Lightning kit. The Y211A and C448A mutations were constructed using the overlapping extension method. In the latter case, pETLODAB15 was used as a template for the mutagenesis PCR¹²⁸ using *Pfu* DNA polymerase (Promega). The outer primers used to perform the last PCR step containing the mutations were MARDIRsac1 (5'-CTCTGGTGAGCTCCTACAG-3') and MAREVeco2 (5'-GTGCTTGGGAGAAATTCGCCTC-3'). After PCR amplification, fragments were cloned using the *SacI*-*SacII* sites in pETLODAB15, substituting the wild type copy of *lodA*. All mutations were confirmed by automated DNA sequencing. All cloning steps were performed using *E. coli* DH5 α and the plasmids obtained were electroporated or transformed into *E. coli* Rosetta just before induction.

The concentration of purified LodA was determined using an $\epsilon_{280} = 125,180 \text{ M}^{-1}\text{cm}^{-1}$ which was calculated from its amino acid sequence¹³⁸.

Steady-state kinetic analysis.

The steady-state activity of ϵ -lysine oxidase was monitored using a coupled assay that included horseradish peroxidase (HRP), which uses the H_2O_2 product of the reaction to oxidize Amplex Red to resorufin, which has an extinction coefficient of $54000 \text{ M}^{-1}\text{cm}^{-1}$ at 570 nm ¹². The assay mixture contained 0.05 mM Amplex Red, 0.1 U/ml of HRP and $0.42 \text{ }\mu\text{M}$ LodA (WT or variants) in 50 mM potassium phosphate buffer, $\text{pH } 7.5$, at $25 \text{ }^\circ\text{C}$ with the indicated amounts of lysine and O_2 included. To vary $[\text{O}_2]$ the buffer was made anaerobic by repeated cycles of vacuum and purging argon and mixed with an appropriate amount of air saturated ($[\text{O}_2]=252 \text{ }\mu\text{M}$) or 100% O_2 -saturated ($[\text{O}_2]=1150 \text{ }\mu\text{M}$) buffer¹⁴⁵. In order to investigate the possibility that any of the mutations had converted LodA from an oxidase to a dehydrogenase, reactions were also performed under anaerobic conditions using artificial electron acceptors as described previously¹². In these cases the reaction was initiated by addition of lysine and monitored by the spectral change associated each potential electron acceptor, phenazine ethosulfate (PES) plus 2,6-dichloroinophenol (DCIP) ($\epsilon_{600}=21,500 \text{ M}^{-1}\text{cm}^{-1}$), ferricyanide ($\epsilon_{420}=1,040 \text{ M}^{-1}\text{cm}^{-1}$), or NAD^+ ($\epsilon_{340}=6,220 \text{ M}^{-1}\text{cm}^{-1}$). The data were fit by eq 2.

$$v/E = k_{\text{cat}}[\text{S}]/([\text{S}] + K_{\text{m}}) \quad (4.2)$$

Estimation of the extent of CTQ biosynthesis.

The absorption spectrum of the purified oxidized form of active LodA exhibits characteristic features in the 350-450 nm range (Figure 13A). Inspection of the absorption spectra of the purified variant enzymes revealed the ratio of absorbance at 400 nm to absorbance at 280 nm (A_{400}/A_{280}) was affected by some of the mutations, despite the fact that proteins were pure as judged by SDS-PAGE. The features of the absorption spectra of the variants are essentially identical to that of WT LodA except for the intensity (Figure 13B). The most likely explanation for this is that some mutations affect the extent to which CTQ biosynthesis has occurred, yielding a mixed population of active LodA with mature CTQ and inactive preLodA with incompletely synthesized CTQ. Unfortunately, it was not possible to verify this by determining a molar extinction coefficient for CTQ in LodA by performing a spectrochemical quantitative redox titration because of poor reactivity of the protein-bound CTQ towards dithionite and other reductants. For ease of comparison, the ratio of A_{400}/A_{280} for each variant is presented, normalized with the value for WT LodA set as 1.0 (Table 2). To be sure that the A_{400} was not varying due to changes in the active-site environment that were caused by mutations, the ratio of A_{400}/A_{280} was also determined for proteins after incubation in 6 M guanidine HCl. The values for these unfolded proteins were the same as for the native proteins indicating that the ratio of A_{400}/A_{280} was a valid indicator of the extent of CTQ biosynthesis. For determination of k_{cat} values, the concentration of enzyme was determined from A_{280} . As such one may wish to adjust the k_{cat} values for variants with normalized A_{400}/A_{280} values greater or less than

1.0. The K_m values will not be affected by the variation in A_{400}/A_{280} since the experimentally determined K_m is not dependent on the concentration of the enzyme.

Sequence alignments of LodA-like proteins.

LodA-like proteins belonging to group IA, as defined previously¹³⁸ includes LodA and the most similar proteins. The proteins in this group were aligned using the program clustal omega [17] available at <http://www.ebi.ac.uk/Tools/msa/clustalo/>.

Table 1: Primers used to generate site-directed mutations

Mutation	Primers
C488A	forward 5'-GTTGGATTGCCTATGGCTCCAGGAATAGAAATG-3' Reverse 5'-CATTTCTATTCTGGAGCCATAGGCAATCCACC-3'
C448D	forward: 5' GATTGCCTATGGATCCAGGAAT 3' reverse: 5' ATTCCTGGATCCATAGGCAATC 3'
Y211A	forward 5'-CAGATCTCAGCGGTGCTGGTGGTGGAGATGA-3' Reverse 5'-TCATCTCCACCACCAGCACCGCTGAGATCTG-3'
Y211E	forward: 5' GATCTCAGCGGTGAAGGTGGT 3' reverse: 5' ACCACCTTCACCGCTGAGATC 3'
Y211F	forward: 5' GATCTCAGCGGTTTTGGTGGT 3' reverse: 5' ACCACCAAACCGCTGAGATC 3'
K530A	forward: 5' AGCGTCAATGCAGCAAGTCAG 3' reverse: 5' CTGACTTGCTGCATTGACGCT 3'
K530R	forward: 5' AGCGTCAATCGAGCAAGTCAG 3' reverse: 5' CTGACTTGCTCGATTGACGCT 3'

Table 2 Steady-state kinetic parameters for LodA variants

	Normalized value of A_{400}/A_{280}^a	k_{cat} (s^{-1}) ^b	K_m lysine (μM)	K_m O ₂ (μM)
WT LodA	1.0	0.34 ± 0.01	3.9 ± 0.2	199 ± 20
C448A LodA	1.0	0.39 ± 0.02	48 ± 4	543 ± 94
C448D LodA	1.5	0.29 ± 0.03 (0.19)	142 ± 33	386 ± 70
Y211F LodA	0.8	0.19 ± 0.02 (0.23)	129 ± 22	522 ± 162
Y211A LodA	1.5	No reaction	No reaction	No reaction
Y211E LodA	1.5	No reaction	No reaction	No reaction
K530A LodA	0.7	1.2 ± 0.1 (1.7)	52 ± 5	1043 ± 102
K530R LodA	0.3	0.68 ± 0.04 (2.3)	113 ± 13	706 ± 95

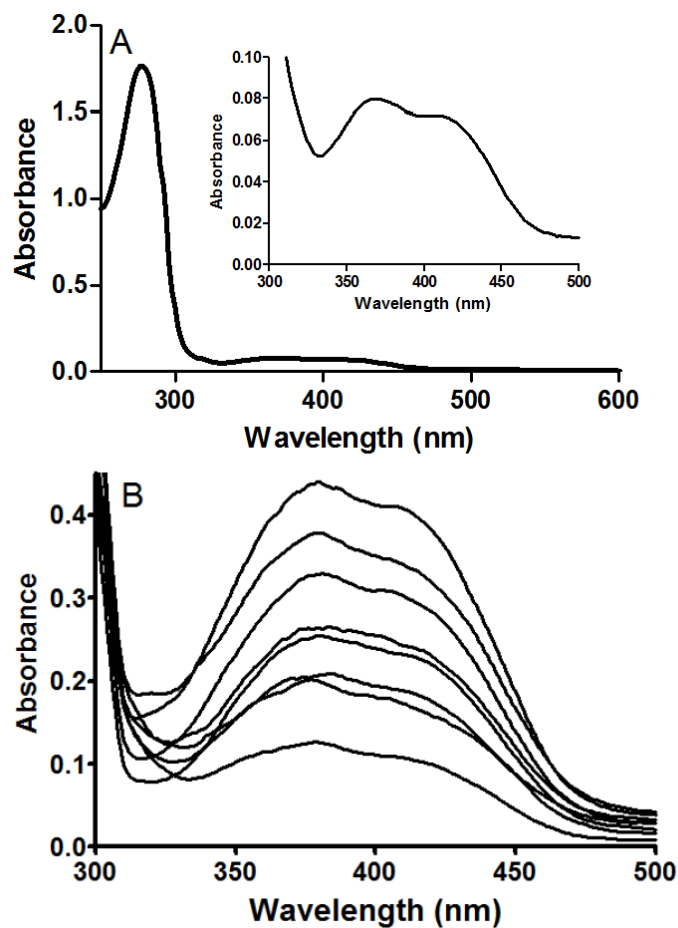


Figure 13 The absorbance spectra of WT LodA and LodA variants.

A. The absorbance spectrum of WT LodA with the visible absorbance features of CTQ magnified in the inset. B. The visible absorbance spectra of LodA variants in this study. The 280 nm absorbance in each of these spectra is identical so the observed variations in the intensity of the absorbance reflect the variation in A_{400}/A_{280} nm of these variant proteins. The spectra from top to bottom are C448D, Y211A, Y211E, WT, C448A, Y211F, K530A and K530R LodA.

Results

Modification of the steady-state assay conditions to allow comparison of LodA variants.

In the previous steady-state kinetic study of LodA, the maximum concentrations of lysine and O₂ that were used were 30 μM and 252 μM, respectively¹². Those values were well above the K_m values that were determined for each substrate. Preliminary examination of the LodA variants which are the topic of the current study revealed that some of them exhibited significantly greater K_m values for these substrates. In order to obtain reasonable estimates for the K_m and k_{cat} values for these variants, it was necessary to extend the range of substrate concentrations used to 1 mM for lysine and 1150 μM for O₂ (100% O₂ saturation). In the presence of 1150 μM for O₂, the reaction with WT LodA exhibited values of k_{cat} of $0.34 \pm 0.01 \text{ s}^{-1}$, K_m for lysine of $3.9 \pm 0.2 \text{ μM}$. These values of k_{cat} and K_m for lysine are similar to the previous reported values of $0.22 \pm 0.04 \text{ s}^{-1}$ and $3.2 \pm 0.2 \text{ μM}$, respectively¹². However in the presence of 1 mM for lysine, a K_m for O₂ of $199 \pm 20 \text{ μM}$ was obtained which is larger than the previously reported value of $37 \pm 6 \text{ μM}$. The reason for this discrepancy is unclear. It may be an artifact caused by the very high lysine concentration which is required to study the variants. It is possible that lysine is binding non-specifically to the enzyme, or perhaps competing with the other substrate (O₂) in the ping-pong mechanism. Despite this peculiarity, in this study all LodA variants and WT LodA were analyzed at the higher concentration ranges so that the values of the kinetic parameters for each enzyme could be compared under the same conditions. Furthermore, as a consequence of some of the mutations, the apparent K_m for O₂

approached the $[O_2]$ in 100% O_2 -saturated buffer. As, such it was not possible to perform experiments in which $[O_2]$ could be varied over an appropriate range so as to obtain true kinetic parameters. As a consequence of this, the K_m values that are reported here should be considered apparent K_m values. However, as can be seen in the results which follow (Table 2), the effects of these mutations on kinetic parameters are quite evident and so the conclusions that can be drawn from these results are not compromised by these limitations imposed by the experimental system.

Effects of Cys448 mutations

Cys448 is present at the active site of LodA in close proximity (5.3 Å) to the CTQ cofactor. This residue was of particular interest because in the TTQ-dependent MADH, mutation of an Asp residue which occupies the analogous position near TTQ in the active site, to Asn caused decreased efficiency of TTQ biosynthesis⁹⁵. C448A and C448D LodA mutations were made and each of these variant proteins was expressed and isolated at yields comparable to that of the WT LodA. The C448D LodA mutation actually increased the A_{400}/A_{280} ratio of the purified protein. This suggests that WT LodA contains a subpopulation with incompletely biosynthesized CTQ and that introducing Asp at this position in LodA has enhanced the efficiency of CTQ biosynthesis relative to WT LodA.

The C448A and C448D LodA variants each exhibited lysine oxidase activity (Figure 14, Table 2). The k_{cat} values of $0.39 \pm 0.02 \text{ s}^{-1}$ for C448A LodA and $0.29 \pm 0.03 \text{ s}^{-1}$ for C448D LodA are similar to that of WT LodA of $0.34 \pm 0.01 \text{ s}^{-1}$. Previous studies of LodA have indicated that an active site base is needed to deprotonate the lysine-CTQ

adduct during catalysis^{12, 132}. As the Cys448 mutations do not significantly decrease k_{cat} , this residue does not appear to function as an active-site base or play any direct role in the catalytic reaction steps. However, the K_m values for both substrates were increased. The K_m for O_2 of $544 \pm 94 \mu\text{M}$ for C448A LodA is about 3-fold greater than that for WT LodA. For C448D LodA the K_m of $386 \pm 70 \mu\text{M}$ is about 2-fold greater. The K_m for lysine of $48 \pm 4 \mu\text{M}$ for C448A LodA is 12-fold greater than that for WT LodA and for C448D LodA the K_m of $142 \pm 33 \text{ s}^{-1}$ is 36-fold greater. These data suggest an important role for Cys448 in lysine binding, or more specifically, facilitating the formation of the lysine-CTQ adduct.

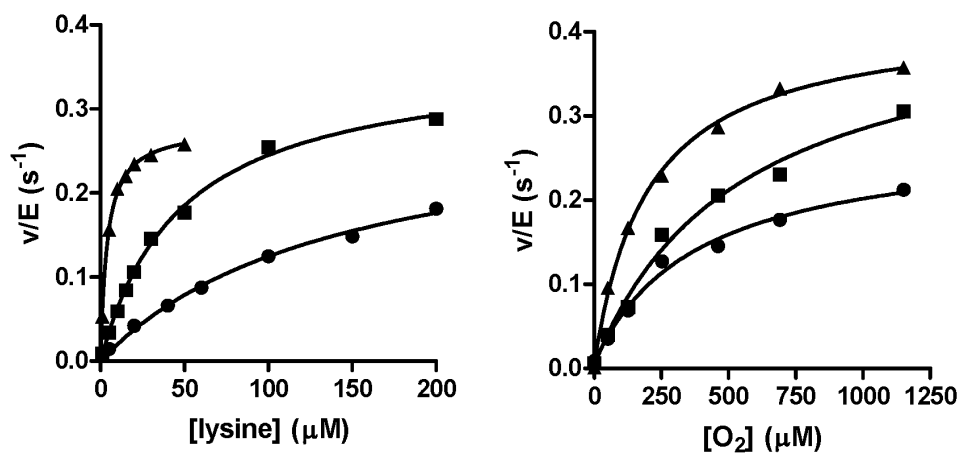


Figure 14 Effects of the C448A and C448D mutations on the steady-state activity of LodA.

A. Assays were performed in the presence of a fixed concentration of 1150 O_2 . B. Assays were performed in the presence of a fixed concentration of 1 mM lysine. The symbols represent WT LodA (▲), C448A LodA (■) and C448D LodA (●). The lines are fits of each data set to eq 1.

Effects of Tyr211 mutations

Tyrosine 211 is situated on a mobile loop in proximity to the active site of LodA (discussed earlier). Tyr211 was mutated to Ala, Glu and Phe. In each case, the amount of protein expressed and isolated was similar to that of WT LodA. However, only the Y211F LodA exhibited activity. Interestingly the A_{400}/A_{280} for the purified Y211A LodA and Y211E LodA were each greater than that of WT LodA and equal to that of C448D LodA which is the highest value observed in this study. This indicates that the lack of enzymatic activity of Y211A and Y211E LodA is not due to lack of CTQ biosynthesis which was not compromised, but apparently optimized by these mutations. In contrast to those mutations, Y211F LodA exhibited an A_{400}/A_{280} less than that of WT LodA suggesting that the biosynthesis of CTQ is a bit less efficient in this variant. However, Y211F LodA was active (Figure 15, Table 2). The k_{cat} value of $0.19 \pm 0.02 \text{ s}^{-1}$ for Y211F is similar to that of WT LodA of $0.34 \pm 0.01 \text{ s}^{-1}$, particularly when one takes into consideration the lower A_{400}/A_{280} for Y211F LodA. The K_m for lysine is $129 \pm 22 \mu\text{M}$ which is 30-fold greater than for WT LodA. The K_m of O_2 is $522 \pm 162 \mu\text{M}$ which is 2.7-fold greater than for WT LodA. These results highlight the importance of an aromatic residue at position 211 for substrate binding. While Y211F LodA has a near normal k_{cat} , the affinity for each substrate is significantly compromised. Y211A and Y211E LodA are completely inactive, despite the presence of CTQ, suggesting that binding of substrates is further compromised in these variants to the extent that activity is undetectable.

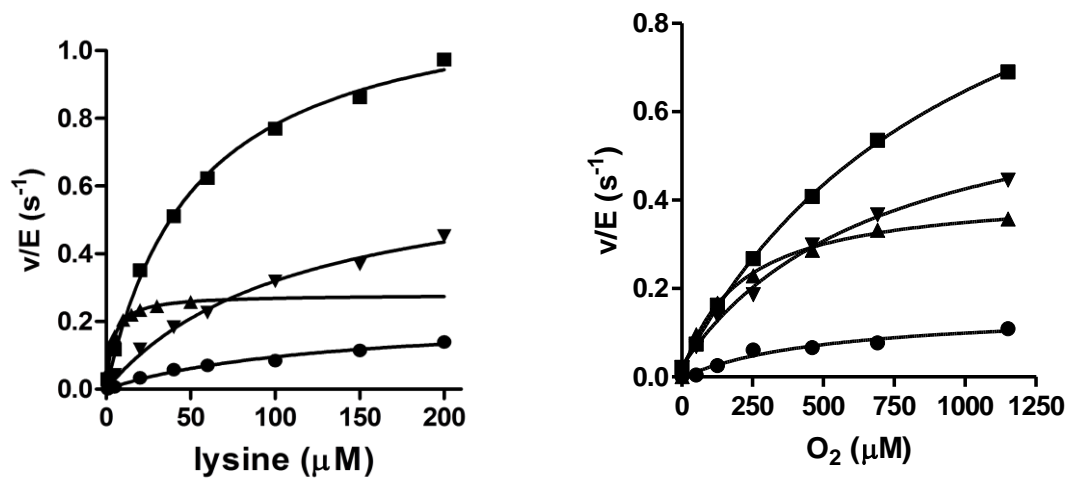


Figure 15 Effects of the Y211A, K530R and K530A mutations on the steady-state activity of LodA.

A. Assays were performed in the presence of a fixed concentration of 1150 O₂. B. Assays were performed in the presence of a fixed concentration of 1 mM lysine. The symbols represent WT LodA (▲), K530R (▼), K530A LodA (■) and Y211F LodA (●). The lines are fits of each data set to eq 1.

Effects of Lys530 mutations

Inspection of the one crystal structure in which Tyr211 is visible (PDB 3WEV) reveals that it forms a hydrogen bond with Lys530 (Figure 11). As such, this residue was mutated to Ala and Arg. The protein was expressed and isolated at levels comparable to WT LodA. However, the A_{400}/A_{280} was significantly decreased indicating that the presence or absence of Lys530 does affect the extent of CTQ biosynthesis. While this residue is not that close to CTQ, it is situated on the outer side of the active site and hence could be involved in CTQ biosynthesis by a direct or indirect involvement with the modifying enzyme LodB.

Interestingly, the mutations of Lys530 increased k_{cat} (Table 2). For the reaction catalyzed by K530A LodA the k_{cat} is $1.2 \pm 0.1 \text{ s}^{-1}$ which is around 3.5-fold greater than for WT LodA and that for K530R is $0.68 \pm 0.04 \text{ s}^{-1}$ which is double that of WT LodA. Considering the lower A_{400}/A_{280} for K530A and K530R LodA, the true k_{cat} may be even greater if one considers the lower level of CTQ that is present. While the k_{cat} has been significantly enhanced by these mutations, the affinity for each substrate was greatly decreased. For K530A, the K_m value for lysine is $52 \pm 5 \text{ }\mu\text{M}$ and that for O_2 is $1043 \pm 102 \text{ }\mu\text{M}$. For K530R, the K_m value for lysine is $112.5 \pm 12.9 \text{ }\mu\text{M}$ and that for O_2 is $706.1 \pm 94.8 \text{ }\mu\text{M}$. While the affinity for O_2 is significantly decreased, because of the increase in k_{cat} , the $k_{cat}/K_m (\text{O}_2)$ values for K530 and K530R LodA of $1150 \text{ s}^{-1}\text{M}^{-1}$ and $960 \text{ s}^{-1}\text{M}^{-1}$ and not that much lower than the value for WT LodA of $1710 \text{ s}^{-1}\text{M}^{-1}$.

Conservation of Cys448, Tyr211 and Lys530 in sequences of proteins encoded by LodA-like genes

Analysis of the Integrated Microbial Genomes database of genome sequences identified 168 genes from 144 different bacterial genomes that encoded LodA-like proteins¹²⁵. LodA from *M. mediterranea* was classified as a member of Group IA LodA-like proteins. Alignment of the sequences of the 20 members of the Group IA LodA-like proteins reveals that the residues which were mutated in this study are highly conserved in these sequences. Alignments of the regions of the sequence in which each of these three residues reside revealed that Cys448 (Table 3) and Lys530 (Table 4) are each conserved in each sequence. Tyr211 is conserved in 15 sequences and the other five have a Phe in this position (Table 5).

Table 3 Alignment of proteins in group IA.

Around Cys448 in LodA, Blue, consensus 100%; Red, >90%; H, hydrophobic residue; 1, N or S

*, IMG gene identification

Locus tag	Organism	Sequence of residues 430-463 in LodA
		<i>xhxxxDxxxhGNcVgXPxCPGIEVTWSxx1xxhY</i>
<i>Marme_2662 LodA</i>	<i>LodA Marinomonas mediterranea MMB-1</i>	PVSDMDTASIGNCVGLPMCPGIEMTWSQLQNPVIY
<i>Mmwy1_3721</i>	<i>Marinomonas sp. MWYL1</i>	PVNEMDAASIGNCVGLPMCPGIEVTWSQLQNSKLY
<i>M320_pool_00037300</i>	<i>Marinomonas sp GOBB3-320</i>	YVNEMDAASIGNCVGLPMCPGIEVTWSQLQNSKLY
<i>PTD2_20217</i>	<i>Pseudoalteromonas tunicata D2</i>	AVHPLDKASIGNCVGLPMCPGIEVTWSQLQNPPLY
<i>G509DRAFT_1169</i>	<i>Flavobacterium subsaxonicum DSM 21790</i>	PVSLDDTASVGNcVGLPMCPGIEVTWSMQNPAVY
<i>PCIT_22609</i>	<i>Pseudoalteromonas citrea NCIMB 1889</i>	PVDPLDQGSVGNcVGLPMCPGIEVTWSQLQNPVKY
<i>Rhein_1334</i>	<i>Rheinheimera sp. A13L</i>	PVNPLDQGSVGNcVGLPMCPGIEVTWSMQNPAVY
<i>H527DRAFT_03370</i>	<i>Aquimarina muelleri DSM 19832</i>	AISEIDMASVGNcVGLPMCPGIEVTWSQLQENIY
<i>PL2TA16_00557</i>	<i>Pseudoalteromonas luteoviolacea 2ta16</i>	GVFYADQGSVGNcVGLPMCPGIEVTWSMQNPAVY
<i>PflaDRAFT_00041100</i>	<i>Pseudoalteromonas flavipulchra 2ta6</i>	GVFYADQGSVGNcVGLPMCPGIEVTWSQLQNPVY
<i>UY7DRAFT_03653</i>	<i>Pseudoalteromonas flavipulchra JG1</i>	GVFYADQGSVGNcVGLPMCPGIEVTWSQLQNPVY
<i>PPIS_19511</i>	<i>Pseudoalteromonas piscicida JCM 20779</i>	GVFYADQGSVGNcVGLPMCPGIEVTWSQLQNPVY
<i>G366DRAFT_02721</i>	<i>Pseudoalteromonas piscicida ATCC 15057</i>	GVFYADQGSVGNcVGLPMCPGIEVTWSQLQNPVY
<i>Gei7105DRAFT_0285</i>	<i>Geitlerinema sp. PCC 7105</i>	GVDDRDRASVGNVGLPQCPGIEVTWTTQNPIVY
<i>NhaI_1391</i>	<i>Nitrosococcus halophilus Nc4</i>	PLCSATSAAMGNcVGLPMCPGIEVTWSMQNPAIY
<i>F460DRAFT_04769</i>	<i>Duganella zoogloeoides ATCC 25935</i>	GIAALDRAAIGNcVGEPMCPGIEVTWSVRNPVIY
<i>2520384729*</i>	<i>Nitrolancetus hollandicus Lb</i>	GIHPLDRVDIGNcVGDPMCPCGIEVTWSVRNPAIY
<i>G559DRAFT_00896</i>	<i>Pseudomonas resinovorans DSM 21078</i>	GVTALDRASVGNcVGGPLCPGIEVTWSQLYSELY
<i>CC1582_00022990</i>	<i>Pseudomonas viridaflava CC1582</i>	NVTDLDRASVGNcVGGPFCPCGIEVTWSQLYKALY
<i>TA043_00024900</i>	<i>Pseudomonas viridaflava TA043</i>	NVTDLDRASVGNcVGGPFCPCGIEVTWSQLYKALY

Table 4 Alignment of proteins in group IA.

Around Lys530 in LodA, Blue, consensus 100%; Red, >90%; h: hydrophobic residue; 1, E or Q; 2, I or V; 3, S or T; 4, I or L
 *, IMG gene identification.

Locus tag	Organism	Sequence of residues 494-590 in LodA
		xGC1PGDLTKRMAxPWQADFFxC32Qx2NFxxPxhN K PxxPxYYxYWWPPQSPWDV4
Marme_2662	LodA <i>Marinomonas mediterranea</i> MMB-1	TGCEPGDLTKRMACPWQADFFNCTIQTVNFSEPSV NK (x)40 PLPPAYYSYWWPPQSPWDVL
Mmwy1_372 1	<i>Marinomonas</i> sp. MWYL1	VGCEPGDLTKRMACPWQADFFNCTVQVNFSEPKV NK (x)40 PLPPAYYSYWWPPQSPWDVL
M320_pool_ 00037300	<i>Marinomonas</i> sp GOBB3-320	GGCEPGDLTKRMACPWQADFFNCTVQVNFSEPK VNK (x)40 PLPPAYYSYWWPPQSPWDVL
PTD2_20217	<i>Pseudoalteromonas tunicata</i> D2	GGCQPGDLTKRMACPWQADFFQCTVQPVNFTEPLI NK (x)74 PITPTYYSYWWPPQSPWDVL
G509DRAFT 1169	<i>Flavobacterium subsaxonicum</i> DSM 21790	GGCEPGDLTKRMACPWQADFFECTIQTFINFS DPAVN K (x)08 PLPPTYYSYWWPPQSPWDVI
PCIT_22609	<i>Pseudoalteromonas citrea</i> NCIMB 1889	GGCEPGDLTKRMACPWQADFFQCTIQNINF T NPAK NK (x)04 PLPPTYYSYWWPPQSPWDVL
Rhein_1334	<i>Rheinheimera</i> sp. A13L	GGCQPGDLTKRMACPWQADFFQCTVQPVNF T VPE VNK (x)04 PLPPTYAYYWWPPQSPWDVL
H527DRAFT _03370	<i>Aquimarina muelleri</i> DSM 19832	GGCEPGDLTKRMACPWQADFFQCTIQYINFSDPEIN K (x)34 PLPPSYTYWWPPQSPWDVL
PL2TA16_00 557	<i>Pseudoalteromonas luteoviolacea</i> 2ta16	GGCQPGDLTKRMACPWQADFFNCTIQNVNF T DPTV NK (x)12 PLAPTYYSYWWPPQSPWDVL
PflaDRAFT_ 00041100	<i>Pseudoalteromonas flavipulchra</i> 2ta6	GGCQPGDLTKRMACPWQADFFNCTIQNVNFTEPTV NK (x)12 PAAPTYYSYWWPPQSPWDVL
UY7DRAFT_ 03653	<i>Pseudoalteromonas flavipulchra</i> JG1	GGCQPGDLTKRMACPWQADFFNCTIQNVNFTEPTV NK (x)12 PAAPTYYSYWWPPQSPWDVL
PPIS_19511	<i>Pseudoalteromonas piscicida</i> JCM 20779	GGCQPGDLTKRMACPWQADFFNCTIQNVNFTEPTV NK (x)12 PAAPTYYSYWWPPQSPWDVL
G366DRAFT _02721	<i>Pseudoalteromonas piscicida</i> ATCC 15057	GGCQPGDLTKRMACPWQADFFNCTIQNVNFTEPTV NK (x)12 PAAPTYYSYWWPPQSPWDVL
Gei7105DRA FT_0285	<i>Geitlerinema</i> sp. PCC 7105	GGCEPGDLTKRMAIPWQADFFNCSIQFVNF T DPNV NK (x)09 PKPPTYYSYWWPPQAPWNVL
Nhal_1391	<i>Nitrosococcus halophilus</i> Nc4	GGCEPGDLTKRMAIPWQADFFNCTIQYVNF T DPDIN K (x)04 PLPPSYAYYWWPPQAPWDVL
F460DRAFT _04769	<i>Duganella zoogloeoidea</i> ATCC 25935	SGCEPGDLTKRMAIPWQADFFYDCSVQMINF DNPDL VK (x)06 PVPPTYAYYWWPPQSPWNVI
2520384729*	<i>Nitrolancetus hollandicus</i> Lb	GGCEPGDLTKRMAIPWQADFFACSVQYINF T DPNV NK (x)04 PKPPTFFAYWWPPQSPMHVI
G559DRAFT _00896	<i>Pseudomonas resinovorans</i> DSM 21078	QGCEPGDLTKRMAIPWQADFFQCTVQYVNF T DPEV NK (x)04 PKAPSYAYYWWPPQSPWNVL
CC1582_000 22990	<i>Pseudomonas viridaflava</i> CC1582	QGCEPGDLTKRMATPWQSDFFQCTVQYINFSDSDA NK (x)04 PKPPTYAYYWWPPQSPWNVI
TA043_0002 4900	<i>Pseudomonas viridaflava</i> TA043	QGCEPGDLTKRMATPWQSDFFQCTVQYINFSDNDA NK (x)04 PKPPTYAYYWWPPQSPWNVI

Table 5 Alignment of proteins in group IA.

Around Tyr211 in LodA, Blue, consensus 100%; Red, >90%; h: hydrophobic residue; 1, G or S; 2, Y or F; 3, G or A; 4, S or A
*, IMG gene identification.

Locus tag	Organism	Sequence of residues 182-230 in LodA
		LGxhxTDxxGRhVhGxGxAGGxxxhx123GxxxWxDDx4DGxVxCxh
Marme_2662	LodA <i>Marinomonas mediterranea</i> MMB-1	LGTLRTDSQGRLIVLGGYGFAGGNTDLGSYGGGDDWYDDISDGSVTCVV
Mmwyl1_3721	<i>Marinomonas</i> sp. MWYL1	LGTLTDDKGRLLVGGYGRAGGNTDLEGYGGGDEWFDDISDGPVTCVV
M320_pool_00037300	<i>Marinomonas</i> sp. GOBB3-320	LGTLRTDNKGRLLVGGYGRAGGNTDLEGYGGGDEWFDDISDGPVTCVV
PTD2_20217	<i>Pseudoalteromonas tunicata</i> D2	LGTLKTDNKGRLLVGGYGHAGGDEALTSYGGSDTWHDDTADGPVYCEV
G509DRAFT_1169	<i>Flavobacterium subsaxonicum</i> DSM 21790	LGNLLTDASGRLLVGGYGNAGGNEPLTSYGGSSTWHDDISDGPVYCTV
PCIT_22609	<i>Pseudoalteromonas citrea</i> NCIMB 1889	LGDLKTDNEGRLIVLGGYGHAGGDKPLVSYGGSDTWHDDIADGPVYCTV
Rhein_1334	<i>Rheinheimera</i> sp. A13L	LGDLLTDDLGRLLVGGYGNAGGDEPLTSYGGSNWTHDDISDGPVYCTV
H527DRAFT_03370	<i>Aquimarina muelleri</i> DSM 19832	LGDLLTDEGRLLVGGYGHAGGNEPLTSYGGSNWTHDDISDGPVYCTI
PL2TA16_00557	<i>Pseudoalteromonas luteoviolacea</i> 2ta16	LGNILTDNEGRLVIGGFGNAGGNEPLESYGGADTWHDDIADGTVYCTV
PflaDRAFT_00041100	<i>Pseudoalteromonas flavipulchra</i> 2ta6	LGDILTDNEGRLVVLGGKGNAGGNLPLESYGGADTWHDDIADGTVYCTV
UY7DRAFT_03653	<i>Pseudoalteromonas flavipulchra</i> JG1	LGDILTDNEGRLVVLGGKGNAGGNQPLESYGGADTWHDDIADGTVYCTV
PPIS_19511	<i>Pseudoalteromonas piscicida</i> JCM 20779	LGDILTDNEGRLVVLGGKGNAGGNQPLESYGGADTWHDDIADGTVYCTV
G366DRAFT_02721	<i>Pseudoalteromonas piscicida</i> ATCC 15057	LGDILTDNEGRLVVLGGKGNAGGNQPLESYGGADTWHDDIADGTVYCTV
Gei7105DRAFT_0285	<i>Geitlerinema</i> sp. PCC 7105	LGTLKTDSSGRLVVLGGFGKSGGDLPLSSYGGADTWYDDISDGSVTCTI
Nhal_1391	<i>Nitrosococcus halophilus</i> Nc4	LGTIKTDRGRLVVLGAYGRAGGDEPITSYGGADTWYDDIADGPVECTV
F460DRAFT_04769	<i>Duganella zoogloeoides</i> ATCC 25935	LGEMRTDSAGNLLVIGAYGRAGGIDPITGFGGGDTWNDDIADGPVTATV
2520384729*	<i>Nitrolancetus hollandicus</i> Lb	LGDLLTDADGRLLVVLGGFGKAGGSEPITSFAGADTWHDDVSDGPVSCKL
G559DRAFT_00896	<i>Pseudomonas resinovorans</i> DSM 21078	LGEMRTDQQGRLLVGGYGHAGGNQSITSFAGADGWHDDISDGPVTCTL
CC1582_00022990	<i>Pseudomonas viridaflava</i> CC1582	LGGVLTDNKGRLLVGGYGHAGGDQSITSFAGADTWHDDTSDGPVTCTL
TA043_00024900	<i>Pseudomonas viridaflava</i> TA043	LGGVLTDKKGRLLVGGYGHAGGDQSITSFAGADTWHDDTSDGPVTCTL

Discussion

Site-directed mutagenesis of residues in the substrate channel of LodA have demonstrated that Cys448, Tyr211 and Lys530 play multiple roles in regulating K_m values of substrates, k_{cat} and the extent of biosynthesis of the protein-derived CTQ cofactor. It may seem intuitively odd that the WT enzyme would have less CTQ formed than some LodA variants. However, the fact that certain mutations either decrease or increase the efficiency of LodB-dependent CTQ biosynthesis is significant. This means that, in addition to furthering our understanding of the functional roles of these residues in the activity of LodA, these results highlight the point that different evolutionary factors must be considered for enzymes which possess protein-derived cofactors. Residues present in the active site that may be optimal for catalytic efficiency may not be the best to participate in CTQ biosynthesis, and *vice versa*. While CTQ biosynthesis is catalyzed by another enzyme, the residues surrounding the nascent CTQ may play important roles in properly positioning the Cys and Trp residues for modification or substrate oxygen for insertion, or both. In this regard, it has been shown that the first modification in the biosynthesis of CTQ takes place auto-catalytically in the absence of LodB, and that some LodA residues are involved in that process¹²⁸.

The residue which corresponds in structure to Cys448 in two other quinoproteins, the CTQ-containing QHNDH¹⁰¹ and the TTQ-containing MADH¹⁴⁴, is a structurally conserved Asp residue. Mutation of this corresponding Asp in MADH affected the efficiency of MauG-dependent TTQ biosynthesis but had no effect on the catalytic

activity of the population of the isolated MADH with fully formed TTQ⁹⁵. Consistent with the results of the MADH study, it can be concluded that the residue at this position, Cys448 in LodA, is not the active-site base that is involved in deprotonation of the lysine-CTQ adduct during catalysis as the C448A mutation has no significant effect on k_{cat} . However, in contrast to the results of the MADH, mutation of Cys448 in LodA to Ala did not result in decreased levels of CTQ biosynthesis. Furthermore, the C448D LodA mutation appears to have enhanced the efficiency of CTQ biosynthesis. This suggests that the native LodA is a mixed population in which some of the protein has incompletely synthesized CTQ and is inactive. Together with the previous study that showed a role for the corresponding Asp in TTQ biosynthesis in MADH, it appears that an Asp residue in this position is preferred for tryptophylquinone cofactor biosynthesis. However, for LodA the presence of Asp at this position is not optimal for catalysis. In particular, this mutation increases the K_m for lysine 35-fold. Thus, the sub-optimal role in CTQ biosynthesis of Cys448 is more than compensated for by the greater catalytic efficiency of the enzyme.

Mutation of Tyr211 and its H-bonding partner Lys530 had significant effects on both k_{cat} and K_m values. As discussed earlier, Tyr211 is present on a mobile loop and it is possible that the H-bond to Lys530 could anchor it in place at certain times during the catalytic cycle. The aromatic character of residue 211 seems critical for activity since the Y211F LodA retains function, whereas Y211A LodA and Y211E LodA are inactive. The K530A and K530R mutations significantly increased the K_m values for the substrates, particularly O₂. For lysine is increased 13- and 29-fold, respectively while for O₂ it increased 5- and 3.5-fold respectively. Since the loop containing Tyr211 was not

observed in the structure of the lysine-CTQ adduct of LodA [8] it may not be important in stabilizing that intermediate. It is possible that Tyr211 in concert with Lys530 could aid in O₂ binding, or retention of O₂ in the active site to poise it for action. This would be consistent with the hydrophobic nature of residue 211 being important. Interestingly, the K530A mutation significantly increased k_{cat} . Since the rate-limiting step for the overall reaction catalyzed by LodA is not known, it is difficult to interpret this effect. Given the position of Lys530 in the active site, and the fact that mutations to Ala and Arg each enhance rather than eliminate activity, these results suggest that Lys530 is not playing a direct role in acid-base chemistry or nucleophilic attack during catalysis. However, it is possible that that it could control the rate of binding of one or both of the substrates, or release of one or more of the products. If the hydrogen-bond between Lys530 and Tyr211 stabilizes the positions of these residues such that they impede the entry into or exit from the active site, then the removal of this blockage of the active site channel caused by the K530A mutation could possibly accelerate a rate-limiting product release step which could account for the increase in k_{cat} . This would also be consistent with the large increases in the K_m values that are caused by mutation of Lys530. In this sense the mobile loop which can be anchored by a hydrogen-bond between Tyr211 and Lys530 could act as a switch that regulates entry of substrates and exit of products to and from the active site.

A couple of points require consideration regarding the kinetic analysis of the LodA variants. As stated earlier, it was not possible to perform a detailed steady-state analysis in which both substrates were varied over an appropriate range of concentrations of each substrate. As such, it is an assumption that each variant obeys a ping-pong mechanism

as does WT LodA. For example, with some flavoprotein oxidases it may be possible for reoxidation of the cofactor to precede or follow the release of the oxidized product. However, in the case of LodA, the substrate forms a covalent adduct with CTQ (Figure 2) and it is difficult to imagine how these mutations could alter the kinetic mechanism to allow O₂ to reoxidize the cofactor prior to release of the aldehyde product. It should also be noted that the mutations in this study involve residues with ionizable groups. It was previously shown¹² that k_{cat} for LodA exhibits a bell-shaped pH profile with a maximum at pH 7.5, implicating the involvement of at least two ionizable groups, and that the K_{m} for lysine also varied in this range. As such, it was not possible to compare kinetic parameters in a pH-independent region.

These results reflect the different evolutionary factors that must be considered for enzymes which possess protein-derived cofactors. Since the catalytic cofactor must be generated by posttranslational modifications, this suggests that the protein must have originally evolved for some other purpose. The modifying enzyme likely also originally evolved for a different purpose. The residues in the active site could not have evolved to optimize function as a lysine oxidase until after the evolution of the LodA that led to its specific interaction with the modifying enzyme LodB and the ability to catalyze CTQ biosynthesis. This is well-described by the observation that some Y211A and Y211E mutations maximize levels of CTQ biosynthesis but are inactive in catalysis. Also consistent with this argument is the observation that Lys530 mutations lead to decreased CTQ biosynthesis but increased k_{cat} . The fact that each of the mutations in this study increased K_{m} values for lysine and O₂ further indicate that the combination of residues

throughout the active site channel together participate in the specificity and catalytic efficiency of the enzyme.

CHAPTER 5: GLYCINE OXIDASE (GOXA)

Introduction

LodA is a unique protein in itself as it is the first among the group of L-amino acid oxidases to be a quinoprotein instead of a flavoprotein. Upon further investigation it was discovered though that there was a complete new reservoir of LodA-like proteins containing CTQ cofactors. The only other CTQ containing protein that has been studied so far has been the QHNDH. But due to the complexity in its structure complete characterization was not possible. Of late a new protein from the same *Marinomonas mediterranea* was found to show glycine oxidase activity and has been referred to as GoxA. It is similar to LodA in a lot of aspects. Both have been found to contain tryptophylquinone (CTQ) cofactor instead of being flavoproteins like the other L-aminoacid oxidases. The Cys and the Trp that are supposedly involved in the CTQ formation have been found to be conserved. Both the *lodA* and the *goxA* genes have a second gene *lodB* and *goxB* respectively present downstream which encodes proteins that are necessary for the mature CTQ generation. LodA and GoxA have been found to show 22.8% identity and 34.6% similarity (distribution in microbial genomes). A crystal structure of LodA has been reported but one for GoxA hasn't been reported yet. Figure 16 shows an overlay of the homology model of GoxA with the crystal structure model of LodA. The major differences lie in the type of amino acid that they use as substrates. LodA carries out the oxidative deamination of L-lysine whereas the GoxA catalyzes the

reaction for glycine. Upon phylogenetic and sequence analysis LodA and GoxA were put in 2 different groups. Group IA included the LodA and similar proteins and group II had the GoxA and similar proteins. Most of the proteins in Group II contain a twin arginine sequence. This is absent in the Group I LodA-like proteins indicating that there could be difference in the final translocation of the proteins which would thus have an effect on the overall function of each of these enzymes in the marine bacterium. A third protein has also been found from the same bacterium and has been thought to be LodA-like, but its function has not yet been discovered. This brings about an interesting observation. *Marinomonas mediterranea* encodes both LodA and GoxA. But based on the sequence and phylogenetic analysis they have been placed in different groups. This could be based on different enzymatic actions. Hence in order to study the diversity in the group of these unusual tryptophylquinone cofactor containing oxidases, GoxA was chosen as the second protein to study. By characterization of GoxA a better understanding could be obtained about the similarities and differences in the structure, function, mechanism of the reaction catalyzed.

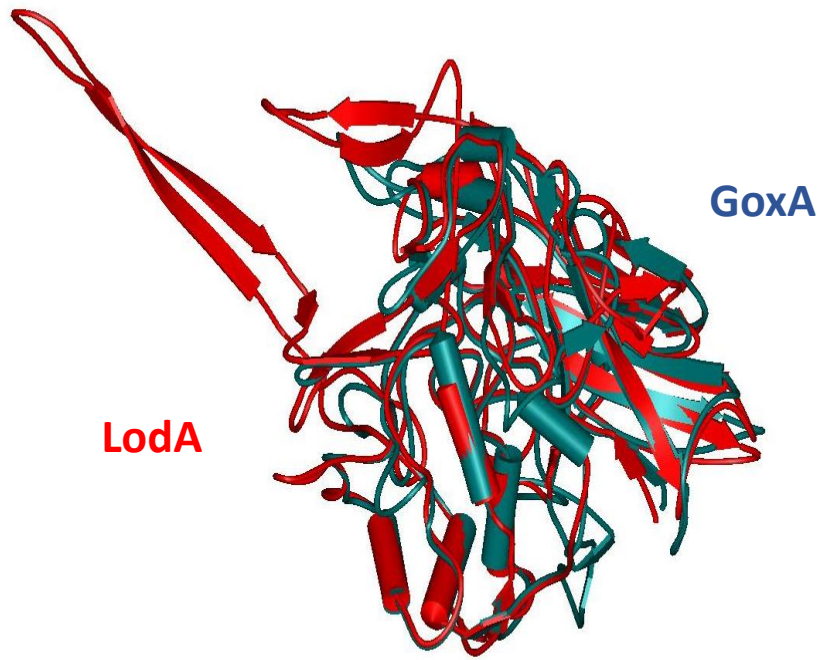


Figure 16 Overlay of the homology model of GoxA with the model for the established crystal structure of LodA.

Red – LodA, Blue – GoxA.

CHAPTER 6: INTERACTION OF GOXA WITH ITS MODIFYING ENZYMES AND ITS SUBUNIT ASSEMBLY ARE DEPENDENT ON THE EXTENT OF CYSTEINE TRYPTOPHYLQUINONE BIOSYNTHESIS

Biochemistry, 2016, 55 (16), pp 2305–2308

Introduction

Quinoproteins may contain exogenous quinone cofactors such as pyrroloquinoline quinone¹³⁴ or protein-derived quinone¹³⁴ cofactors that are formed by irreversible posttranslational modification of amino acid residues, most notably Tyr and Trp (Figure 17)⁶. Tyrosine-derived cofactors include trihydroxyphenylalanine (Topa) quinone (TPQ)⁵⁷ which is found in several copper-containing amine oxidases⁵³ and lysyl tyrosylquinone (LTQ) which is found in lysyl oxidase¹³⁵. Tryptophan-derived cofactors include tryptophan tryptophylquinone (TTQ) which is found in amine dehydrogenases and cysteine tryptophylquinone (CTQ) which was been found in quinohemoprotein amine dehydrogenase (QHNDH)⁷¹, in an ϵ -lysine-epsilon oxidase (LodA)¹⁰¹ and in a glycine oxidase (GoxA)⁸.

The mechanisms of the biosynthesis of the protein-derived quinone cofactors differ from cofactor to cofactor¹⁴. For the Tyr-derived cofactors it is a self-processing event which is dependent on the presence of copper and O₂. The copper facilitates oxygen insertion in the Tyr side chain and subsequently remains in the active site and assists in catalysis^{146, 147}. The biosynthesis of TTQ in methylamine dehydrogenase (MADH) includes posttranslational modifications that are catalyzed by a diheme enzyme called

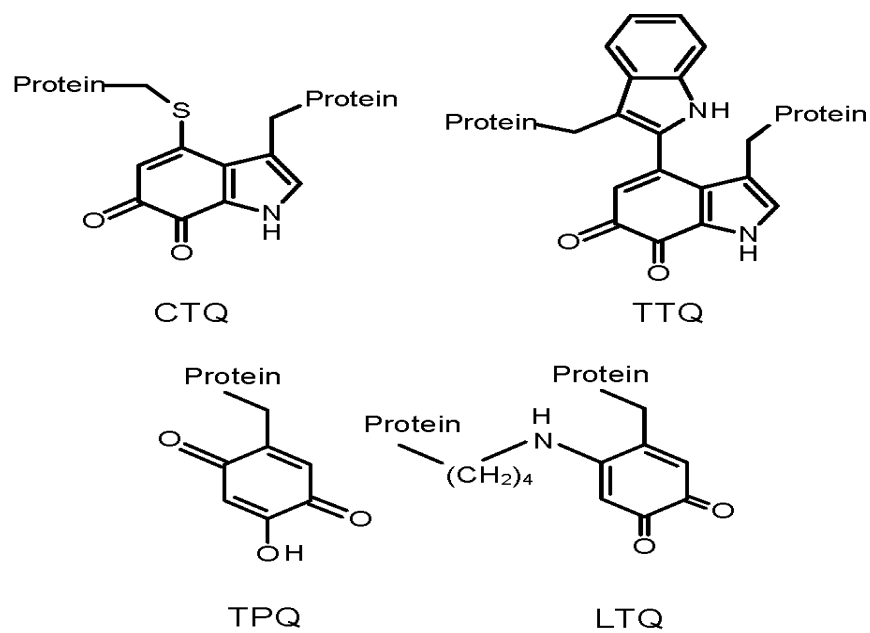


Figure 17 Protein-derived quinone cofactors formed by posttranslational modification.

CTQ is cysteine tryptophylquinone cofactor. TTQ is tryptophan tryptophylquinone cofactor. TPQ is 2,4,5-trihydroxyphenylalanine quinone or Topaquinone. LTQ is lysine tyrosylquinone.

MauG⁷². The mechanism of the biosynthesis of CTQ in QHNDH has yet to be fully characterized, but it does require one or more other gene products⁸. In the case of LodA and GoxA it has been shown that flavoproteins are required for the biosynthesis of CTQ, although no mechanistic studies have been thus far reported. The posttranslational modifications required for CTQ biosynthesis are the incorporation of two oxygen atoms into the side-chain of a specific Trp residue and covalent cross-link formation between that residue and a Cys residue (Figure 17). LodA and GoxA were isolated from the marine bacteria, *Marinomonas mediterranea*^{71, 101}. The gene clusters which encode LodA and GoxA each contain only one other gene, *lodB* and *goxB*⁸⁹. Each of these genes is predicted to encode a putative flavoprotein. Expression of either active LodA or GoxA with the mature CTQ cofactor requires that *lodA* and *goxA* each be co-expressed with *lodB* and *goxB*, respectively¹²⁴. Expression of either active *lodA* or *goxA* in the absence of *lodB* or *goxB*, respectively, leads to the production of inactive forms of the proteins that lack CTQ. These precursor proteins have been termed preLodA and preGoxA¹¹.

This study reports the isolation and characterization of a complex of precursor GoxA and GoxB. It is shown that a flavin is indeed present in this complex with the putative flavoprotein, GoxB, and that mature CTQ is not present in the precursor form of GoxA. An active GoxA with mature, CTQ which is not in complex with GoxB, is also isolated from the same prep. Evidence is presented that the affinity between GoxA and GoxB is dependent upon the extent of CTQ biosynthesis. Furthermore, it is shown that the oligomerization state of GoxA is dependent on the extent of CTQ biosynthesis. These

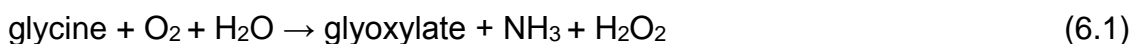
results are compared and contrasted with the features of the biosynthesis of TTQ and TPQ in their respective enzymes.

Materials and methods

GoxA and GoxB were expressed in *E. coli* Rosetta cells. The cells were transformed with the pET15GoxAB plasmid which contained *goxA*, to which a hexahistidine tag had been added and *goxB* downstream of *goxA*. The cells were grown in LB media which also contained ampicillin and chloramphenicol. Cells were grown at 30°C to an OD of 0.6. At that point 1 mM IPTG was added and after four hours cells were harvested. The harvested cells were sonicated in 50 mM potassium phosphate, pH7.5, and centrifuged. The supernatant was passed through a Ni-NTA affinity column. The column was washed using a gradient of imidazole in the same buffer. Protein eluted over a range of 30-150 mM imidazole. The eluted protein was dialyzed and again applied to the affinity column and the process was repeated using a narrower imidazole gradient to improve purity and to better separate the two fractions. As discussed in the results, two fractions were identified, one containing both GoxA and GoxB which eluted at lower imidazole concentration and one which contained GoxA alone that eluted at higher imidazole concentration.

Glycine oxidase activity was determined using a coupled enzyme assay which had been previously described¹⁰¹. The assay was performed under aerobic conditions at 30°C. In addition to the glycine substrate the assay mixture contained 20 U/ml glutamate dehydrogenase 5 mM 2-oxoglutarate and 0.25 mM NADH. The reaction was initiated by addition of 2 mM glycine. The ammonia generated by the oxidative deamination of glycine

(eq 1) was used as a co-substrate by glutamate dehydrogenase (eq 2) leading to the oxidation of NADH which accompanied by a decrease in absorbance at 340 nm (ϵ_{340} of NADH is $6220 \text{ M}^{-1}\text{cm}^{-1}$).



SDS-PAGE was performed by standard methods 7.5% gels. Gels were stained for protein with EZ protein gel staining solution. In order to perform the quinoprotein stain for detection of a covalent quinone proteins, without staining for protein, electrophoretic transfer of the proteins to a nitrocellulose membrane (0.2 μm pure nitrocellulose membrane, BioRad) was performed. This was done at 120 V for 1h at low temperature to prevent overheating. To stain for the presence of quinoproteins¹¹, the nitrocellulose membrane was incubated for 45 min in the dark in a solution of 2 M potassium glycinate, pH 10, containing 0.24 mM Nitro Blue Tetrazolium⁷¹. The quinoproteins will stain. The nitrocellulose membrane was then washed and stored in 0.1 M sodium borate, pH 10.0 until dried.

Size exclusion chromatography (SEC) was performed using a HiLoad 16/600 Superdex 200 column (GE Healthcare). The buffer used for chromatography was 50 mM potassium phosphate buffer, pH 7.5, containing 150 mM NaCl. The flow rate was 0.6 mL/min. Glutamate dehydrogenase (332 kDa), MADH (124 kDa), MauG (42 kDa) and amicyanin (11.5 kDa) were used as standards. Blue dextran was used to determine the void volume. Gaussian fits of the chromatogram (Prism Graph Pad) were used to resolve overlapping peaks in the chromatogram and integrate the areas under the peaks.

Results

During purification of the His-tagged recombinant GoxA, two protein fractions eluted from the Ni-NTA resin affinity column, one at approximately 60 mM imidazole and the other at approximately 100 mM imidazole. When assayed for glycine oxidase activity the first fraction exhibited no activity, whereas the second fraction was active and exhibited a k_{cat} of $14.90 \pm 0.02 \text{ s}^{-1}$ under aerobic conditions ($252 \mu\text{M O}_2$) with 2 mM glycine concentration and 30°C . Each of the fractions exhibited a different visible absorption spectrum. That fraction that was active exhibited spectral features in the 350–420 nm range that are consistent with the presence of CTQ with peaks (Figure 18A). Similar visible absorbance spectra have been reported for LodA¹⁴⁸ and the ϵ subunit of QHNDH¹³ and a synthetic CTQ model compound¹⁰⁵. In contrast, the inactive fraction exhibited an absorbance spectrum with features characteristic of a flavin. (Figure 18B). This suggests that the fraction contained GoxB, although that protein did not have a His-tag. From this spectrum it could not be concluded whether or not CTQ was also present as the absorbance of the flavin would mask the CTQ spectrum.

The two fractions were analyzed by SDS-PAGE (Figure 19A). The inactive fraction contained two species. The larger band migrated as expected for GoxA which has a predicted mass from the gene (Marme_1655) of 76284.8 Da. The other migrated as would be expected for GoxB which has a predicted mass from the gene (Marme_1654) of 41856.3 Da. That active fraction contained only one major species which migrated as expected for GoxA. Analysis by mass spectrometry confirmed that the second species was exactly the molecular weight of the untagged GoxB. Unfortunately the intact GoxA

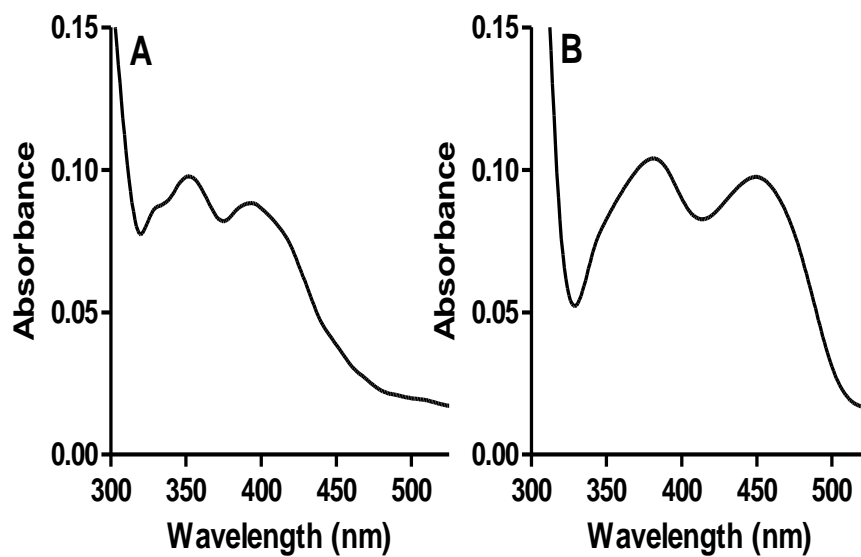


Figure 18 Visible absorption spectra of the two protein fractions that eluted from the Ni-NTA affinity column.

A. Absorption spectrum of the active fraction which exhibits a spectrum characteristic of CTQ. B. Absorption spectrum of the inactive fraction exhibits a spectrum characteristic of flavin.

species did not fly in the mass spectrometer and so it was not possible to determine from the mass whether or not CTQ was present in the GoxA in the inactive fraction which co-eluted with GoxB. It was not possible to separate the two proteins in the inactive fraction by other chromatographic techniques. The sample was subjected to anion exchange (DEAE) chromatography, hydrophobic interaction chromatography using butyl sepharose and size exclusion chromatography. In each case the proteins co-eluted indicating that the two proteins interact with each other to form a tight complex.

Two possible reasons for the lack of activity in the fraction containing the GoxA-GoxB complex are that either the presence of the second protein interferes with the reaction or the GoxA is actually a precursor lacking the mature CTQ cofactor. As the presence or absence of CTQ could not be confirmed by the absorbance spectrum or mass spectrometry analysis, an alternative was used to determine whether CTQ was present. The proteins were subjected to SDS-PAGE followed by electroblot to a nitrocellulose membrane which was subjected to a staining protocol that is specific for covalent quinoproteins¹⁴⁸ (Figure 19B). The active GoxA that eluted by itself stained positive. The GoxA which co-eluted with GoxB did not stain indicating that it does not have the mature CTQ cofactor. Thus, it is actually the precursor GoxA. Additional proteins were included on the gel as positive and negative controls. Two proteins were included in which the presence of the quinone cofactor has been verified in crystal structures. MADH contains two subunits with TTQ present in the smaller subunit¹⁴⁴. The smaller subunit stained positive and the larger one did not. LodA, which contains CTQ¹³² also stained positive.

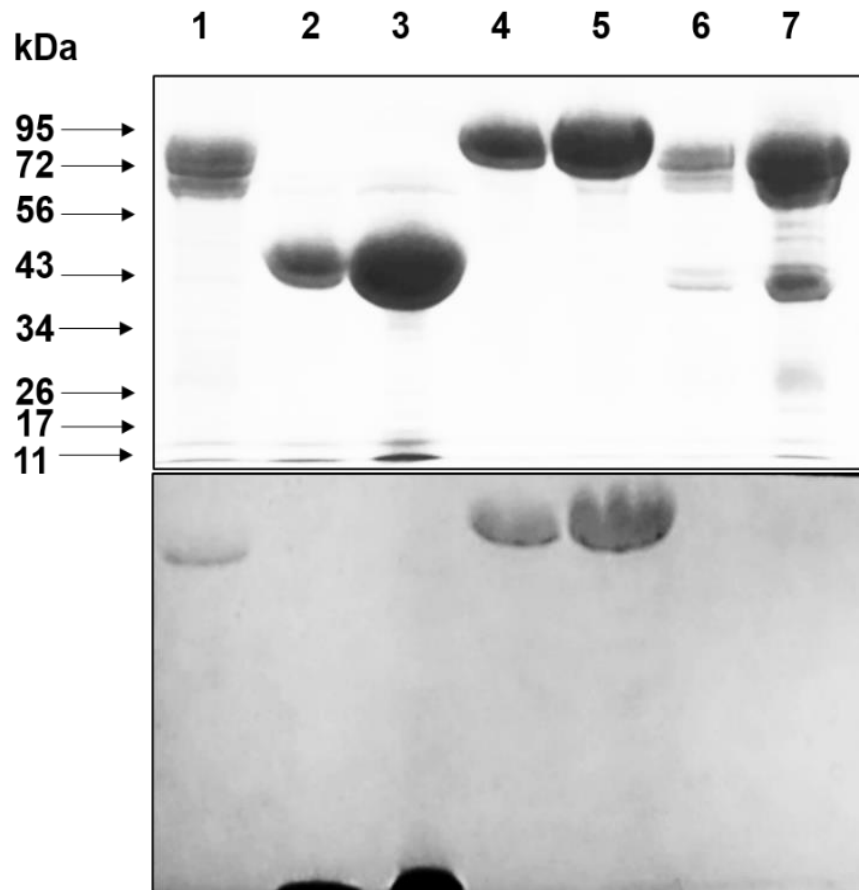


Figure 19 SDS-PAGE and quinoprotein staining of protein fractions.

Top. SDS gel stained for protein. Bottom. Western blot of a gel identical to that in A which was stained for quinoproteins. The lane labeled GoxA is the active fraction gel stained for protein. The lane labeled GoxAB is the inactive fraction. The TTQ-bearing MADH and CTQ-bearing LodA were used as controls.

The GoxB present in complex with the precursor form of GoxA did not stain. Comparable amounts of GoxA, MADH, LodA, and the precursor GoxA-GoxB complex were loaded on the gel. GoxA and LodA did not transfer to the nitrocellulose as efficiently as the other proteins. For that reason the gel in Figure 19A, which is identical to the one which was used in the electroblot transfer to a nitrocellulose membrane was intentionally overloaded to ensure that a negative result was not because of insufficient transfer of proteins. For further confirmation a dot blot was performed in which the protein sample was spotted directly on the nitrocellulose membrane, dried and then subjected to the staining procedure. Again the active protein stained positive and the inactive did not, indicating that whereas the active GoxA has mature CTQ the GoxA in complex with GoxB does not (Figure 20)

The inactive and active fractions were also characterized by size exclusion chromatography. The active GoxA with CTQ eluted from the column primarily as a dimer (Figure 21A). Two peaks were observed. The relative mass of the major peak (84%) was approximately 140 kDa and that of the minor peak (16%) was approximately 76 kDa which is the predicted molecular weight of the monomer. The inactive fraction containing precursor GoxA and GoxB eluted 100% as a single peak with a relative mass of approximately 120 kDa which is approximately the sum of the predicted molecular weights of GoxA and GoxB (Figure 21B).

The implications of these results are profound.

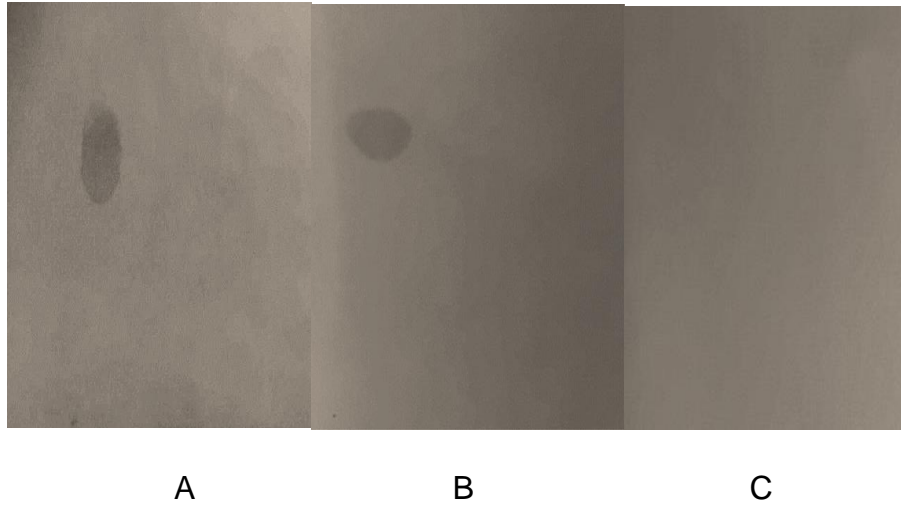


Figure 20 Dot Blot on nitrocellulose membrane.

A. 8µg of GoxA stained positive for quinoprotein stain. B. 8µg of LodA stained positive for quinoprotein stain. C. 8µg of precursor GoxA and GoxB complex which did not stain for the quinoprotein indicating the absence of a mature CTQ.

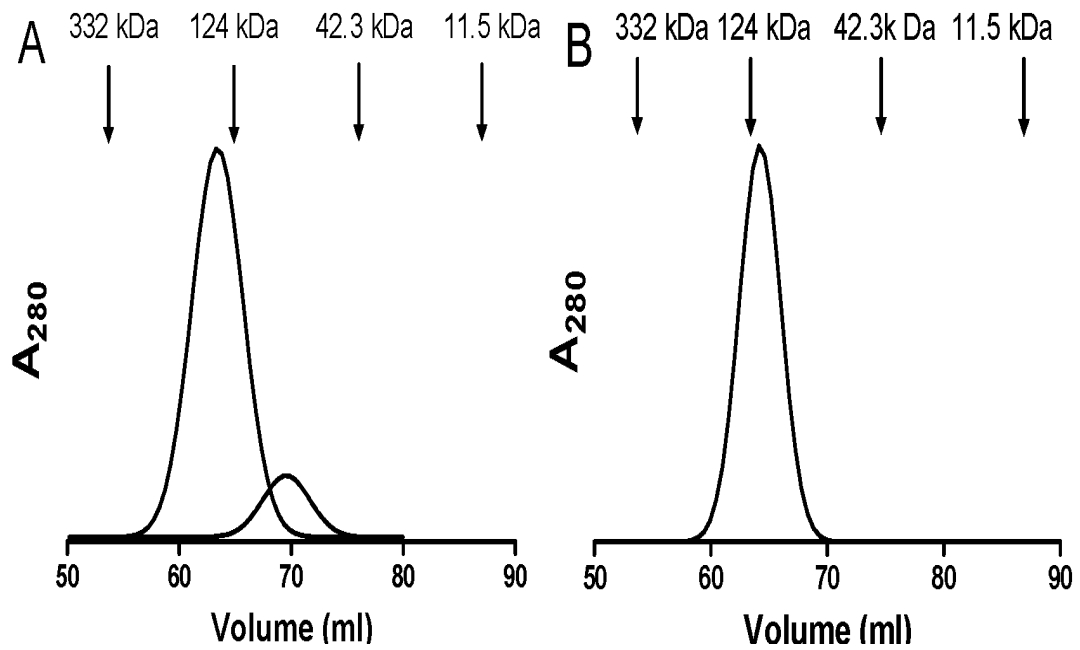


Figure 21 Size exclusion chromatography

The active fraction containing GoxA (A) and the inactive fraction containing precursor GoxA and GoxB (B). The positions of elution and molecular weights of standards used to calibrate the column are indicated.

Together the results indicate that the GoxA precursor forms a tight complex with GoxB, whereas GoxA with mature CTQ does not. Thus, complex formation between the two is strongly dependent upon the extent of CTQ biosynthesis. Furthermore, the native dimeric structure of active GoxA is not achieved until after CTQ formation and release of GoxB. Thus, the oligomerization state of GoxA is also dependent upon the extent of CTQ biosynthesis. The latter point is in stark contrast to what was observed for TTQ biosynthesis in MADH by the diheme enzyme MauG. In that system it was shown that preMADH existed as a heterotetramer and formed a complex with two molecules of MauG, one associated with each halve of the heterotetramer⁸⁸. Thus, in contrast to the results obtained for GoxA, the assembly of MADH showed no dependence on the extent of TTQ biosynthesis.

Discussion

The mechanisms of the biosynthesis of protein-derived cofactors are complex and diverse. The most common protein posttranslational modifications, such as phosphorylation and glycosylation, occur at the surface of the proteins enabling the substrate residues to directly contact the modifying enzyme. In contrast, the amino acid residues that undergo posttranslational modification reside within the protein. Some of these modifications are self-processing events. As discussed earlier, an example of this is the TPQ cofactor of copper-containing amine oxidases. In this case the host protein binds copper which is used to activate oxygen for insertion into the side-chain of a specific Tyr residue¹⁴⁹ and then participates in catalysis^{142, 143}.

The best characterized process of enzyme-catalyzed posttranslational modifications to form a quinone cofactor is the biosynthesis of the TTQ cofactor of MADH by the diheme enzyme MauG. It was shown that the oxidation of the residues of the precursor protein of MADH (preMADH) by a unique bis-FeIV high-valent state of the heme did not occur by direct reaction. Instead it required long range electron transfer from the substrate to the hemes through the protein by a hole-hopping mechanism of electron transfer. It was possible to crystallize a catalytically competent complex of preMADH and MauG. However the proteins did not form a stable complex in solution, as do precursor GoxA and GoxB. Kinetic studies demonstrated that the affinity of MauG for preMADH was approximately 10-fold greater than for MADH with mature TTQ, but the interactions were transient. In the present study difference in affinity of GoxB for the GoxA precursor protein is far greater than for the modified GoxA. In one case a complex forms which cannot be separated and in the other complex formation is undetectable.

LodA which is also isolated from the same bacterium as GoxA also contains a CTQ cofactor. The biosynthesis of the cofactor is dependent upon the flavoprotein LodB. In contrast to what was observed with GoxA, no complex of preLodA or LodA with LodB was ever observed during purification. In fact, the preparation of pure LodA was found to contain preLodA as well which was clearly not in complex with LodB. Thus, while preLodA and LodB must interact to achieve CTQ biosynthesis, the complex formation between the two must be transient as was the case with preMADH and MauG, and different from what was observed for precursor GoxA-GoxB complex. The observation is also consistent with the previous observation that LodB and GoxB cannot substitute for each other and are

specific for their respective LodA and GoxA precursor proteins. It seems that it may not just be specificity but the mechanisms of action of LodB-dependent and GoxB-dependent CTQ biosynthesis may have significant differences.

These results also demonstrate that CTQ formation in GoxA occurs prior to its forming its native dimeric state. This is in stark contrast to what was observed in the MADH-MauG system. This initial characterization of the tight complex formed by GoxA precursor and GoxB is an important step towards for future studies to characterize the details of the mechanism of CTQ formation by GoxB. As GoxB is a flavoprotein, this mechanism will likely describe novel reactions the catalysis of which are unprecedented for a flavin cofactor.

CHAPTER 7: GLYCINE OXIDASE (GOXA) PREPS FOR OPTIMIZING YIELDS OF THE PROTEIN

Introduction

GoxA was being expressed by transforming into the Rosetta cells, the pET15 plasmid vector containing the *goxA* and the *goxB* gene present on the same operon. These preps resulted in the formation of a small amount of the 2 different fractions of protein, the precursor GoxA-GoxB and the matured GoxA. In order to completely characterize an enzyme, its kinetic properties needed to be studied. Since the yield from these cultures were not satisfactory to carry out the studies, other constructs were made to see if the expression could be optimized. Table 6 shows the different constructs that were used to express more active GoxA. Different factors were considered for this.

1. Co-expression of the proteins from genes on different plasmids could improve the yield.
2. The GoxA genes all had N-terminal His tags with a few extra amino acid coding codons. His tags were engineered into the C-terminal end to ensure that the His tag on the N-terminal side wasn't affecting the overall expression of the protein.

Materials and methods

When 2 different plasmids were used with the *goxA* and the *goxB* gene separately, co-transformation was done in order to express the proteins. The pColADuetGoxA The Rosetta cells were grown and induced at 30°C for 4hours. For the pColADuetGoxA + pET11GoxB His tagged, BL21 cells were used and grown passed through Ni-NTA columns for better separation.

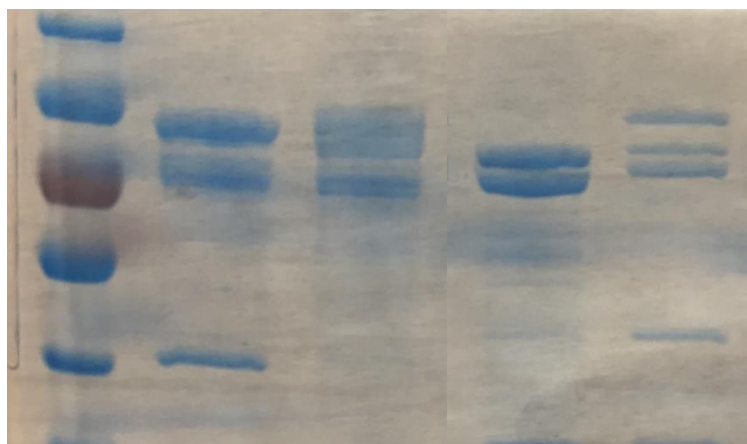
Table 6 Plasmid vectors that were used for co-expression.

1 was co-expressed with 2a and 2b separately.

Vector type	Constituting genes	His-tag
1. pColDuetGoxA	GoxA gene including the leader sequence	C-terminal
2a. pETGoxB11	GoxB gene	C terminal
2b. pETGoxB11	GoxB gene	No His tag

Results and discussion

The C-terminal *goxA* gene engineered into the pColADuet vector was co-expressed with the C-terminal *goxB* gene containing pET11 vector and also with the untagged *goxB* in the pET11 vector. In both cases GoxA protein was expressed. But unlike the pET15 vector containing both *goxA* and *goxB* gene on the same operon, the overall yield of the protein was very low. The fact that the genes were not present on the same operon probably lead to an inefficient complex formation between the GoxA and the GoxB. Due to this essentially GoxA was generated with no or trace amounts of GoxB. Figure 22 shows an SDS gel picture with the corresponding combinations. Samples from the preps that used pET15 vector containing N-terminal His tagged GoxA was also run on the gel for comparison. Upon carrying out the activity assay for these fractions it was observed that they exhibited baseline levels of activity. This probably was due the weak or no heterodimer formation between the 2 proteins as seen in the last chapter. This also indicates the importance of the physical formation of the complex to facilitate the mature CTQ formation in GoxA. The yield too did not improve upon changing the tags from the N-terminal to the C-terminal. This indicated that the expression of matured CTQ containing GoxA was absolutely dependent on its formation of complex with GoxB. Hence having the 2 genes on 2 different operons did not optimize the expression condition. Once the CTQ has been generated the GoxB comes off and is replaced by a monomer of GoxA.



Lane: 1 2 3 4 5

Figure 22 SDS gel picture of the different GoxA expressions.

Lane 1 – protein molecular weight markers, lane 2 – fraction containing precursor GoxA and GoxB complex expressed from the pET15 vector containing *goxA* and *goxB* gene on the same operon, lane 3 – fraction containing matured GoxA from the same prep.

CHAPTER 8: ROLES OF CONSERVED RESIDUES OF GOXA IN CONTROLLING GLYCINE OXIDASE ACTIVITY, COOPERATIVITY, SUBUNIT COMPOSITION AND CYSTEINE TRYPTOPHYLQUINONE BIOSYNTHESIS

Introduction

The glycine oxidase (GoxA)¹⁴ is the second enzyme to be isolated from the melanogenic marine bacterium, *Marinomonas mediterranea* which bears the protein-derived cysteine tryptophylquinone (CTQ) cofactor^{6, 150}. The first such enzyme was the lysine- ϵ -oxidase (LodA)⁸. The biosynthesis of the CTQ cofactor of each enzyme requires posttranslational modifications that are catalyzed by a specific flavoenzyme, GoxB or LodB, respectively^{124, 151}. In each case, the gene clusters contain only two genes, *goxA* and *goxB*, and *lodA* and *lodB*. In addition to the interesting features of the biosynthesis of CTQ in these enzymes, they exhibit atypical activities. All other known tryptophylquinone enzymes that contain either CTQ or tryptophan tryptophylquinone (TTQ) are dehydrogenases^{6, 150}, whereas GoxA and LodA are oxidases. The current study characterizes the steady-state kinetic properties of GoxA and variants generated by site-directed mutagenesis. Despite the similarities of GoxA and LodA, the results reveal that the kinetic mechanisms of the two enzymes and roles of conserved active-site residues are quite different.

LodA catalyzes the oxidative deamination of side-chain of L-lysine to generate a semialdehyde, ammonia and H₂O₂ as products⁸. The enzyme is secreted, and as a consequence of the H₂O₂ generation exhibits antimicrobial properties that lead to dispersal and differentiation of the biofilm in which the host bacterium resides¹⁰. Steady-

state kinetic analysis using its substrates, lysine and O₂, showed that LodA follows a ping-pong kinetic mechanism involving a Schiff base intermediate formed between the ε-amino group and CTQ¹⁵². This was verified by crystal structure of LodA with the lysine-CTQ adduct formed¹³². Roles of residues in and around the active-site of the LodA in catalysis and CTQ biogenesis were identified by site directed mutagenesis¹³.

GoxA catalyzes the oxidative deamination of glycine to generate a glyoxylate, ammonia and H₂O₂ as products¹⁴. Whereas LodA exhibited typical Michaelis-Menten behavior, the current steady-state kinetic study of GoxA reveals that it exhibits allosteric cooperativity towards the glycine substrate. It was previously shown that purified active GoxA eluted primarily as a dimer during size-exclusion chromatography¹⁵³. As the crystal structure of GoxA has yet to be determined, a homology model is constructed from the GoxA sequence and the LodA structure. Given the observed cooperativity and dimeric structure, a docking model is also constructed in which the GoxA monomer is docked with itself to form a putative dimer. The docking model predicts that the entrances to the active-site of each of the two monomers are in the proximity of the protein-protein interface.

Phylogenetic analysis of the Integrated Microbial Genomes database of genome sequences¹²⁵ revealed that LodA-like proteins can be clustered in five different groups that were detected in several classes of bacteria and fungi. LodA is among proteins classified as Group I LodA-like proteins and GoxA is present in Group II. Comparison of the sequences of the Group I and Group II proteins with the structure of LodA and the homology model of GoxA revealed candidates to target for site-directed mutagenesis (Figure 1). Of particular interest was Phe237. Whereas all Group II proteins have a Phe

in this position of the sequences, most Group I proteins including LodA have a Tyr in this position. It is demonstrated herein that mutation of Phe237 to Tyr or Ala eliminates the cooperativity exhibited by GoxA, and weakens dimer formation. His466 was of interest because all Group II proteins each have His in this position, which is in proximity of CTQ in the active-site, while the Group I proteins all have a Cys at the corresponding position. Asp547 which also resides near CTQ was also of interest as this residue is conserved in all Group I and Group II proteins as well as all other structurally characterized TTQ-dependent and CTQ-dependent enzymes ¹⁵⁴⁻¹⁵⁶. These results presented herein provide insight into the roles of specific active-site residues in catalysis and CTQ biogenesis, as well as describing an interesting mechanism by which a single residue can dictate whether or not an enzyme exhibits cooperative allosteric behavior towards a substrate.

Materials and Methods

Construction, expression and purification of GoxA and its variants

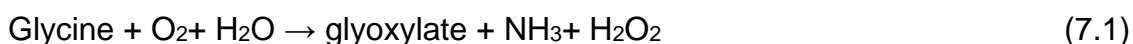
Wild-type (WT) GoxA and GoxA variants that were generated by site-directed mutagenesis were expressed by co-expression of the *goxA* gene with *goxB* in *E. coli* Rosetta cells as described previously ¹⁵³. The GoxA proteins possessed a 6XHis tag which was engineered into the gene. Preparation of cells extracts and purification using a Ni-NTA affinity column was performed as previously described ¹⁵³. The following mutations were made: D547A, H466A, H466D, H466C, F237A and F237Y. The forward and reverse primers that were used are listed in Table 7. Using these primers the mutations were incorporated by PCR using the QuikChange Lightning kit (Agilent).

Table 7 Primers used for site-directed mutagenesis

Mutations	Primers
H466A	Forward - 5'GCATTGCCCCCTGGCGTCG 3' Reverse - 5'CGACGCCAGGGGCGAATGC 3'
H466D	Forward - 5' GGCGCATTTCGATCCTGGCGT 3' Reverse - 5' ACGCCAGGATCGAATGCGCC 3'
H466C	Forward - 5' GGCGCATTCTGTCCTGGCGTC 3' Reverse - 5'GACGCCAGGACAGAATGCGCC 3'
F237A	Forward - 5' GCTATTACGAGTGCCGCCGATAACGAT 3' Reverse - 5' ATCGTTATCGGCGGCACTCGTAATAGC 3'
F237Y	Forward - 5' GCTATTACGAGTTACGCCGATAACGAT 3' Reverse – 5' ATCGTTATCGGCGTAACTCGTAATAGC 3'
D547A	Forward - 5' TGGCAGTGTGCGGCCTTCAG 3' Reverse - 5' CTGAAGGCCCGCACACTGCCA 3'

Steady-state kinetic analysis

GoxA was assayed using a coupled enzyme assay in which the formation of the NH₃ product (eq 1) is monitored by coupling its production to the reaction of glutamate dehydrogenase (eq 2). The standard assay mixture contained GoxA, 5 mM 2-oxoglutarate, 0.25 mM NADH, and 20 U/ml glutamate dehydrogenase at 30°C. The reaction was initiated by the addition of glycine. The initial velocity was determined by monitoring the rate of disappearance of NADH at 340 nm using the ϵ_{340} of NADH of 6220 M⁻¹cm⁻¹. Initial rates were determined in the presence of varying amounts of glycine and O₂. To vary [O₂], a stock solution of the buffer was made anaerobic by repeated cycles of vacuum and purging with argon and then mixed anaerobically with an appropriate amount of either air saturated buffer ([O₂]= 252 μ M) or 100% O₂-saturated buffer ([O₂]= 1150 μ M) buffer ¹⁴⁵.



Two alternative equations were used to fit the data from the kinetic experiments. In the Michaelis-Menten equation (eq 3) k_{cat} is turnover number for the protein, K_m is the Michaelis-Menten constant, $[S]$ is the substrate concentration, $[E]$ is the enzyme concentration and v is the initial reaction rate. In the Hill equation (eq 4) $K_{0.5}$ is the concentration of the substrate that produces half the maximal velocity, and h is the Hill coefficient.

$$v/[E] = k_{\text{cat}}[S]/(K_m+[S]) \quad (7.3)$$

$$v/[E] = k_{\text{cat}}[S]^h / (K_{0.5}^h + [S]^h) \quad (7.4)$$

Size exclusion chromatography

Size exclusion chromatography was performed with an AKTA Prime FPLC system using a HiLoad 16/600 Superdex 200 (GE Healthcare). Chromatography was performed in 50 mM potassium phosphate buffer plus 150 mM NaCl at pH 7.5. The flow rate was 0.6 mL/min. The void volume was calculated using blue dextran. Proteins used as molecular weight markers were a mixture containing glutamate dehydrogenase (332 kDa), methylamine dehydrogenase (124 kDa), MauG (42 kDa) and amicyanin (15 kDa). A plot of the elution volume/void volume versus log molecular weight was used to estimate the masses of the WT and variant GoxA proteins. Gaussian fits of the chromatogram (using Origin and graphed in Prism Graph Pad) were used to resolve overlapping peaks in the chromatogram and integrate the areas under the peaks.

Construction of homology and docking models

A homology model of GoxA was constructed with the sequence of *goxA* gene (Marme_2396) and the structure of LodA (PDB 2YMW) using the SWISS-MODEL tool at www.swissmodel.expasy.org ¹⁵⁷. A docking model of the putative GoxA dimer was constructed by using the ZDOCK utility at <http://zdock.umassmed.edu> ¹⁵⁸. A PDB file generated from the homology model was docked against itself.

Results

Steady-state kinetic analysis of WT GoxA

Initially the concentration of the glycine was varied in the presence of 100% O₂-saturated (1150 μM) buffer. Initial rates were measured and the data were fit to the Michaelis-Menten equation (eq 3) (Figure 23A). While the fit was reasonably good ($R^2=0.97$), the error ranges were high ($k_{cat} = 56 \pm 5 \text{ s}^{-1}$ and $K_m = 999 \pm 172 \text{ μM}$) and a systematic deviation to the fit was evident (i.e., data points clustered above and below the fitted curve). The data were then fit to the Hill equation (eq 4) (Figure 23B) and a much improved fit was obtained ($R^2=0.99$). All data points now fall on the fitted curve. The analysis yield values of $k_{cat} = 39 \pm 2 \text{ s}^{-1}$ and $K_{0.5} = 514 \pm 42 \text{ μM}$. The fit also yielded a Hill coefficient of 1.7 ± 0.2 , indicative of positive cooperativity. Given the previous finding that GoxA is a homodimer¹⁵³, the h value of 1.7 is consistent with a kinetic mechanism in which the binding of the first glycine molecule to one subunit enhances the affinity of the second subunit for glycine. Next the concentration of O₂ was varied in the presence of a saturating concentration of glycine (5 mM). In this case the data were fit well by the Michaelis-Menten equation (Figure 23C) and no improvement of the fit was obtained by using the Hill equation (Figure 23D). Thus, GoxA does not exhibit cooperative behavior towards O₂, but only towards glycine. The analysis of the data using eq 3 yielded values of $k_{cat} = 93 \pm 18 \text{ s}^{-1}$ and $K_m = 2590 \pm 690 \text{ μM}$.

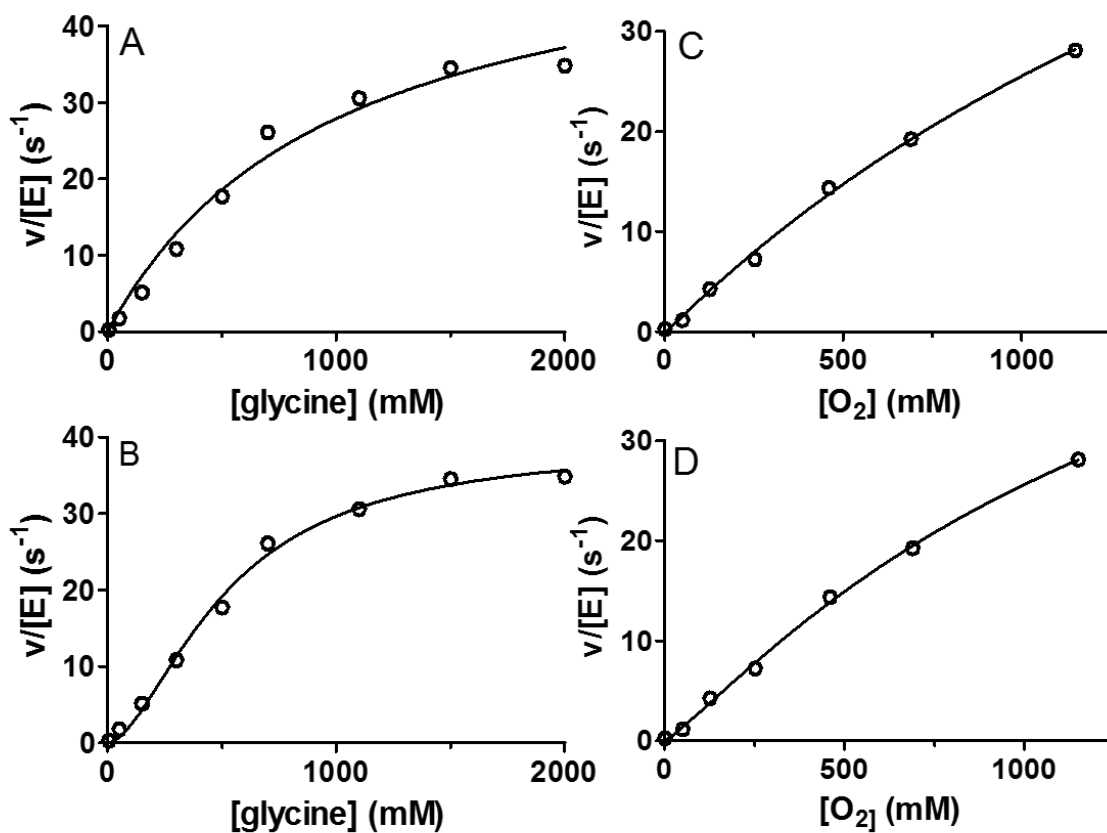


Figure 23 Steady-state kinetic analysis of GoxA.

Assays of glycine oxidase activity were performed with varied concentrations of glycine in the presence of a fixed concentration of 1150 μ M O₂ (A and B) or with varied concentrations of O₂ in the presence of a fixed concentration of 5 mM glycine (C and D). The lines are fits of each data set by either eq 3 (A and C) or eq 4 (B and D).

A couple of points are noteworthy. These results indicate that the K_m for O_2 is actually greater than the $[O_2]$ in a 100% O_2 saturated solution, and much greater than in air-saturated buffer. For this reason the assays were repeated anaerobically in the presence of alternative small molecule electron acceptors to see whether GoxA could function as a dehydrogenase. However, no activity was observed when replacing O_2 with NAD^+ , ferricyanide, phenazine ethosulfate or dichlorophenolindophenol. Also, the k_{cat} obtained when varying O_2 was 93 s^{-1} compared to 39 s^{-1} when varying glycine. The reason for this is that the fixed concentration of O_2 that was used, which is the highest that can be achieved, was not a saturating concentration (i.e. was not much greater than the K_m for O_2). Thus, the lower value is an apparent k_{cat} determined at sub-saturating concentration of the fixed substrate, and the value of 93 s^{-1} determined by varying O_2 at a true saturating concentration of glycine is a more valid estimation of the true k_{cat} . Because of the cooperative behavior towards glycine and the inability to achieve saturating concentrations of O_2 , it was not possible to perform the experiments in which both substrates could be varied over the appropriate range of concentrations necessary to determine whether or not GoxA obeys a ping-pong mechanism, as was done with LodA

152.

Structural comparisons of GoxA with LodA

The crystal structure of GoxA has not yet been determined but it was possible to construct a homology model of the structure based on its sequence and the crystal structure of LodA (Figure 24A). Alignment of the homology model of the structure of GoxA

with LodA reveals strong structural conservation of the active sites (Figure 24B). It shows that the CTQ cofactor in GoxA is formed from Cys551 and Trp566. These residues were previously shown by proteolysis and mass spectrometry of GoxA to form CTQ ¹¹, thus supporting the validity of the model. For reasons discussed earlier, Asp547, His466 and Phe237 of GoxA were chosen as candidates for site-directed mutagenesis to elucidate structure-function relationships. Phe237 is of interest because it corresponds in sequence to Tyr211 of LodA, which is conserved in most Group I proteins and which strongly influenced the K_m values of both lysine and O₂ for LodA ¹³. In the homology model the position of Phe237/Tyr211 is not structurally conserved as are the positions of CTQ and the other two residues. Furthermore, in LodA Lys530 forms a hydrogen bond with Tyr211, but no Lys is present in this position in GoxA.

Effects of site-directed mutagenesis on active-site residues of GoxA

Asp547. Mutation of Asp512 in LodA affected CTQ biosynthesis and so its effect on the activity of the mature enzyme could not be assayed ¹¹. The analogous D547A mutation was made in GoxA. This resulted in protein with no activity. Furthermore, the inactive protein was isolated primarily in complex with the modifying enzyme GoxB. It was previously shown that a precursor of WT GoxA which lacks CTQ forms a tight complex with GoxB ¹⁵³. Thus, Asp547 in GoxA must also be play a role in CTQ biosynthesis on GoxA.

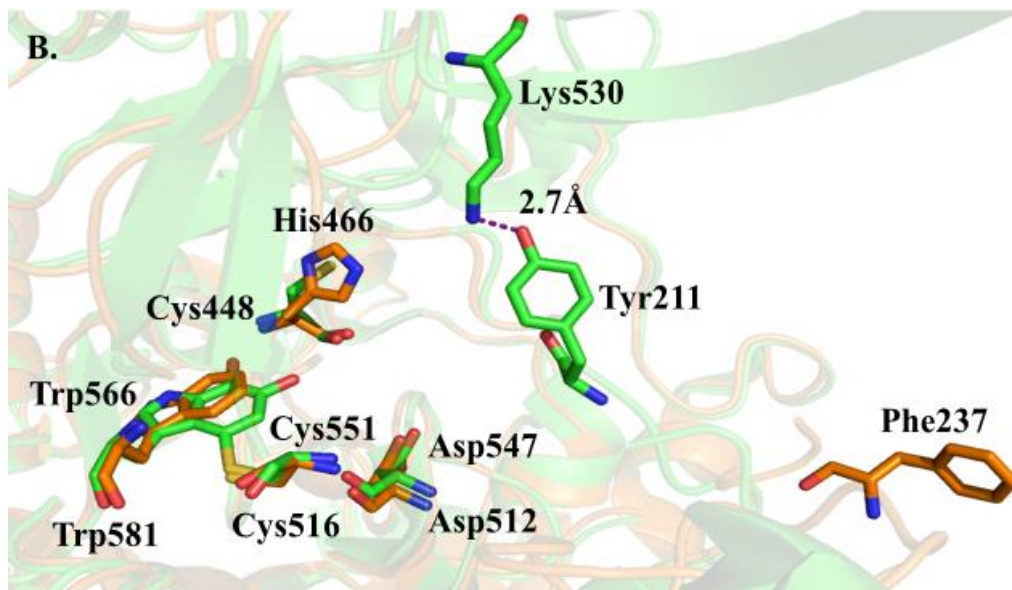
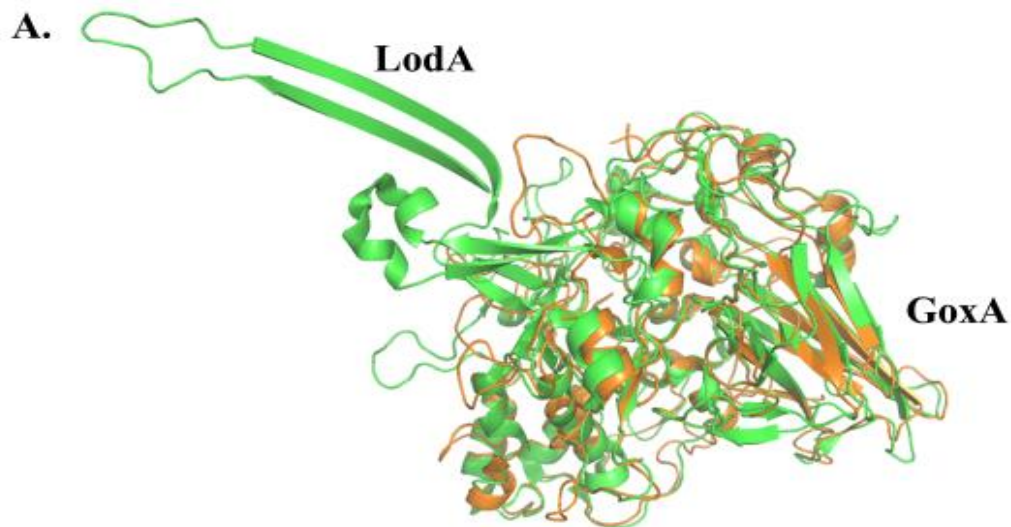


Figure 24 Overlays of the homology model of GoxA with the crystal structure of LodA (PDB ID 2YMW).

(A) The overall structures of monomers of LodA (green) and GoxA (orange) are superimposed. (B) The active sites of GoxA and LodA are superimposed. The Cys and Trp residues which form CTQ and selected other residues are shown as stick. Carbons on residues of LodA and GoxA are colored green and orange, respectively. Oxygen is colored red, nitrogen blue and sulfur yellow.

His466. His466 corresponds to the Cys448 of LodA. That residue was mutated to Ala, Asp and His ¹³. No stable C448H LodA protein was isolated, suggesting that the mutation affected the biosynthesis or stability of the protein, or both. C448A LodA and C448D LodA could be isolated. Those mutations had little effect on k_{cat} but each increased the K_m values for both lysine and O₂ ¹³. The corresponding H466C, H466A and H466D mutations were made in GoxA. As described above for D547A GoxA, each of these three variant proteins could only be isolated in a complex with GoxB and exhibited no activity. Thus, it appears that His466 is also important for GoxB-dependent CTQ biosynthesis in GoxA. As such, the roles His466 and Asp547 in catalysis by GoxA could not be studied, as the mutations compromised CTQ biosynthesis.

Phe237. Phe237 corresponds to the Tyr211 of LodA. That residue was mutated to Phe and Ala. Y211A LodA was inactive and Y211F LodA exhibited a decreased k_{cat} and significantly increased K_m for both lysine and O₂ ¹³. Phe237 in GoxA was converted to Tyr and Ala. The F237Y GoxA variant protein was isolated and assayed. As with WT GoxA, activity was measured at varied concentrations of the glycine in the presence of O₂-saturated (1150 μ M) buffer. In contrast to what was observed for WT GoxA, the data for F237Y GoxA did fit well to the Michaelis-Menten model (Figure 25A). No improvement of the fit was obtained using the eq 4 and the fit to that equation yielded a Hill coefficient value of approximately 1. This indicates that the cooperativity towards glycine exhibited by WT GoxA is lost as a consequence of the F237Y mutation.

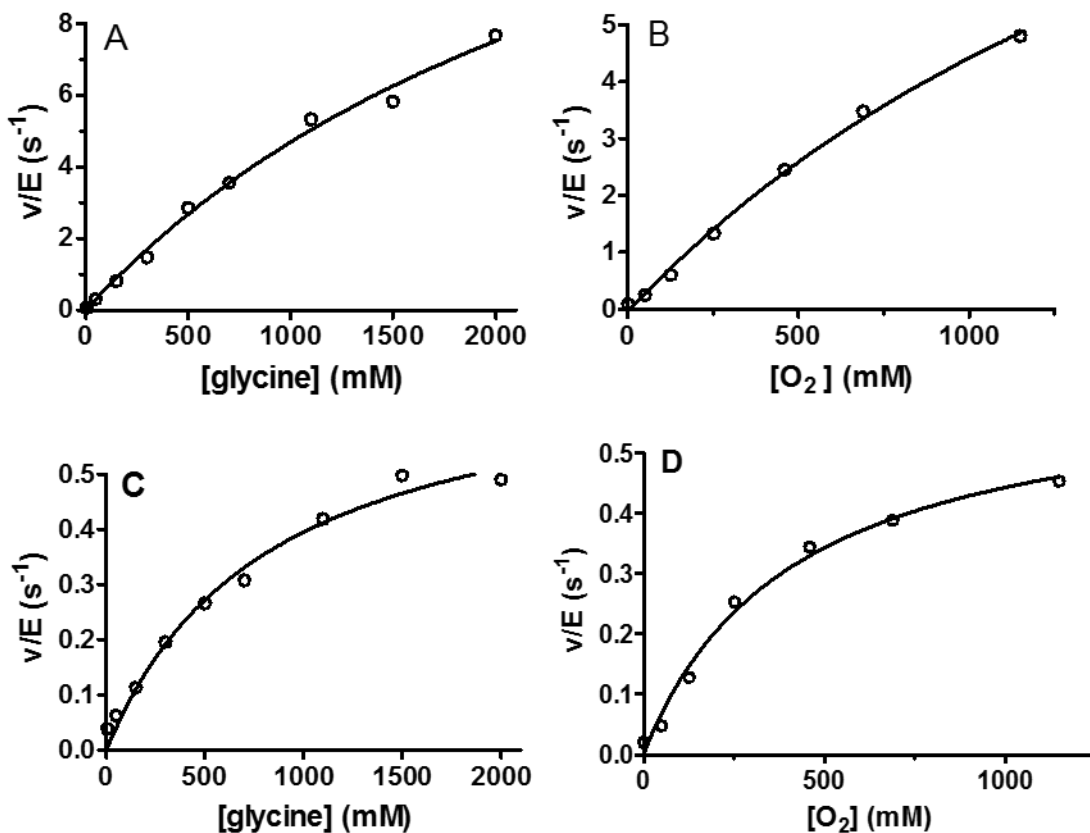


Figure 25 Steady-state kinetic analysis of GoxA variants.

Assays of glycine oxidase activity of F237Y GoxA were performed with varied concentrations of glycine in the presence of a fixed concentration of 1150 mM O₂ (A) or with varied concentrations of O₂ in the presence of a fixed concentration of 5 mM glycine (B). Assays of glycine oxidase activity of F237A GoxA were performed with varied concentrations of glycine in the presence of a fixed concentration of 1150 mM O₂ (C) or with varied concentrations of O₂ in the presence of a fixed concentration of 5 mM glycine (D). The lines are fits of each data set by either eq 3.

Table 8 Steady-state kinetic parameters for GoxA and GoxA variant proteins

	WT GoxA	F237Y GoxA	F237AGoxA	LodA ^a
k_{cat} (s ⁻¹) ^b	93 ± 18	15 ± 5	0.6 ± 0.1	0.22 ± 0.04
$K_{0.5}$ glycine (μM)	514 ± 42 ^c	-	-	-
K_m glycine (μM)	-	3030 ± 840	826 ± 123	-
K_m lysine (μM)	-	-	-	3.2 ± 0.5
K_m O ₂ (μM)	2600 ± 693	2270 ± 1000	416 ± 157	37.2 ± 6.1
k_{cat}/K_{mO_2} (M ⁻¹ s ⁻¹)	36000	6600	1000	5900

^aTaken from reference 12.

^b k_{cat} values are from the experiments in which O₂ was varied rather than from studies where glycine was varied since it was not possible to achieve saturating concentration of O₂

^cWT GoxA fit best to eq 4 and so the $K_{0.5}$ value is used rather than a K_m value.

Activity was also assayed by varying the concentration of O₂ in the presence of a saturating concentration of glycine (Figure 25B). Comparison of the fitted values of the kinetic parameters (Table 8) indicates that the F237Y mutation decreased k_{cat} and increased K_m for glycine with little effect on the K_m for O₂. The F237A GoxA variant protein was also isolated and analyzed (Figure 25C and D). This variant also obeyed Michaelis-Menten kinetics without cooperativity towards glycine. The fitted values of the kinetic parameters (Table 8) indicate that this mutation substantially reduced k_{cat} , had little effect on the K_m for glycine, and substantially decreased the K_m for O₂. Thus, Phe237 not only dictates cooperative behavior towards glycine but also influences the k_{cat} and K_m values for both glycine and O₂.

Effects of Phe237 mutations on the oligomerization state of GoxA

In order to determine whether mutations of Phe237 which abolished cooperativity also affected the stabilization of the native homo-dimeric state of GoxA, the WT and variant proteins were analyzed by size exclusion chromatography. WT GoxA (Figure 26A) elutes from the column primarily as a dimer. One observes a minor peak at about 76 kDa (16%) which corresponds to the predicted mass of a monomer and a major peak at approximately 140 kDa (84%). In contrast, the majority of F237Y GoxA (Figure 26B) elutes as a monomer with a major peak at 76 kDa (62%) and a minor peak at 140 kDa (38%). The majority of F237A GoxA (Figure 26C) also elutes as a monomer with a major peak at 76 kDa (68%) and a minor peak at 140 kDa (32%).

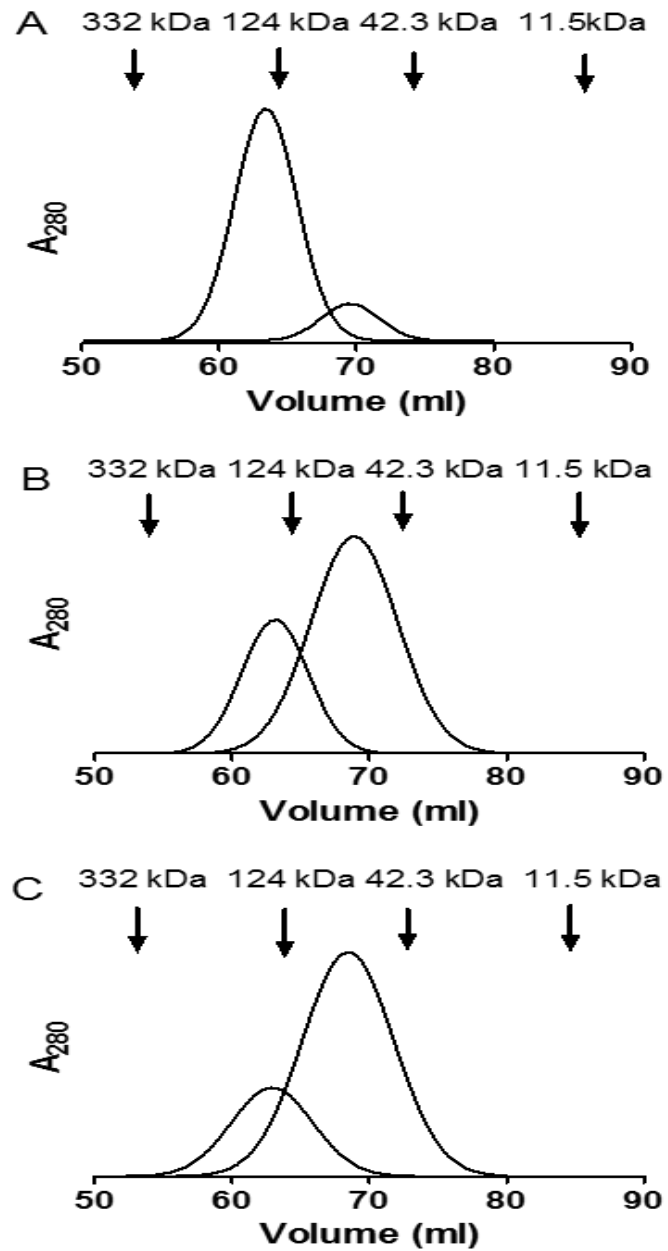


Figure 26 Size exclusion chromatography

WT GoxA (A), F237Y GoxA (B) and F237A GoxA (C). The positions of elution of molecular weight marker proteins are indicated.

A docking model of the GoxA dimer

In order to gain insight into how Phe237 might be involved in the cooperativity and stabilization of dimer formation, a protein docking model was constructed using the ZDOCK program (Figure 27). Interestingly, it shows the Phe237 residues at the protein-protein interface and that the Phe237 residues of each subunit are in close proximity to each other when present as a dimer. Phe237 is also in a position in which it may interact with other aromatic amino acids residues that are present at the interface, Tyr618 and Trp619. In fact, the Tyr618 residues of the two monomers are at a distance of 1.7 Å from each other. The inter-subunit distance between the Trp691 residues of each monomer is about 4 Å. The aromatic residues being in such close proximity suggests the possibility of π -stacking between the side-chains. Since the model used to estimate the distances between these residues is a homology model, the values thus obtained are approximate. However, π -stacking interactions between residues would likely induce the other amino acids to rearrange themselves. Thus, these residues may be important in the stabilization of the dimeric structure and the communication between the two active sites that results in the observed cooperativity of WT GoxA.

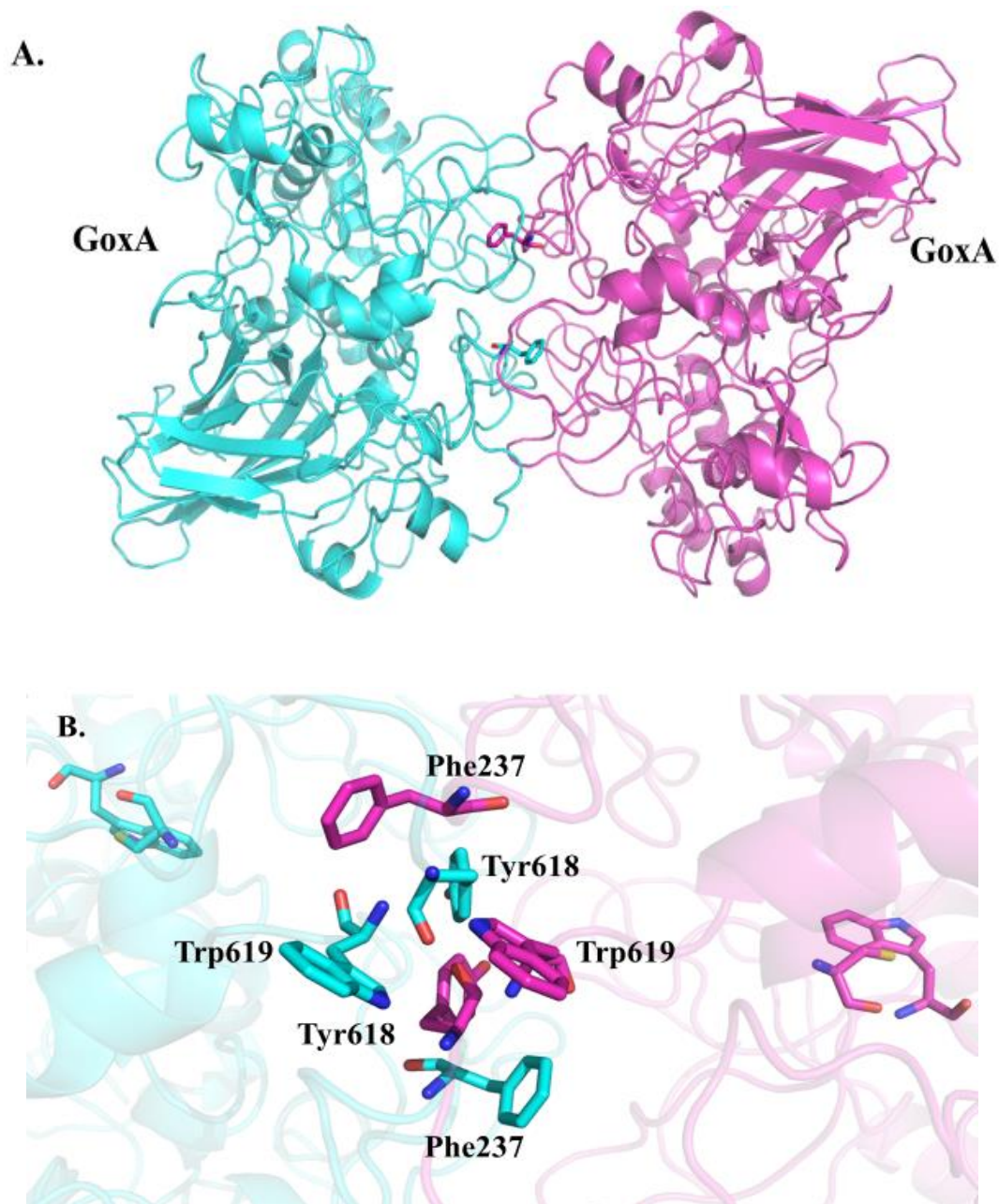


Figure 27 Docking model of the GoxA monomers to form a dimer structure.

The two monomers are colored blue and pink respectively. (A) The overall structure of the dimer with residue Phe237 shown in stick. (B) An expanded view of the protein-protein interface of the two monomers. The Cys and Trp residues which form CTQ and selected residues at the interface are shown as stick. Carbons on residues of LodA and GoxA are colored blue and pink, respectively. Oxygen is colored red, nitrogen blue and sulfur yellow

Discussion

GoxA and LodA are CTQ-containing oxidases discovered in the same bacteria, *M. mediterranea*. Each of them belongs to a new class of unusual tryptophylquinone cofactor-containing oxidases. Analysis of the Integrated Microbial Genomes database of genome sequences identified 168 genes from 144 different species that encode LodA-like proteins¹²⁵. Prior to the characterization of LodA and Gox, all known quinoprotein oxidases contained a tyrosylquinone cofactor¹⁵⁹, and in general all amino acid oxidases contained a flavin cofactor¹⁶⁰. Despite the similarities in sequence and structure exhibited by GoxA and LodA, there are interesting differences in their kinetic mechanisms and steady-state kinetic parameters. This could be a consequence of different physiological roles of the proteins. LodA is a secreted protein which exhibits antimicrobial activity that is important for the health of the biofilm in which the host bacterium resides¹⁰. GoxA has also been reported to exhibit antimicrobial activity, however the finding of cooperativity towards glycine and production of glyoxylate, which is an important metabolite¹⁶¹, suggests a possible metabolic and regulatory functions.

GoxA exhibits cooperative behavior towards its glycine substrate whereas LodA exhibits no cooperativity. The K_m values for glycine and O_2 for GoxA are each much greater than for LodA, however the k_{cat} value of GoxA is also much greater than that for LodA. Thus, despite the very large K_m value which GoxA exhibits for O_2 , the catalytic efficiency k_{cat}/K_{mO_2} for GoxA is actually six-fold greater than that of LodA (Table 2). Thus,

LodA seems to have evolved its catalytic efficiency by increasing affinity for substrates, which GoxA has improved its catalytic efficiency by increasing k_{cat} .

Asp547 and His466 of GoxA appear to be critical for CTQ biosynthesis. The corresponding Asp512 in LodA was also shown to be critical for CTQ biosynthesis, while certain substitutions of Cys448 of LodA, which corresponds to His466, were tolerated ¹³. The tolerated mutations did, however, significantly increase the K_m value for lysine and altered the efficiency of CTQ biosynthesis. These results for the CTQ-dependent enzymes are reminiscent of those from studies of TTQ biosynthesis in MADH ¹⁶². The active site of that enzyme has Asp in each of these two corresponding positions, Asp76 and Asp32. Mutation of the Asp76 that corresponds in position to Asp547 in GoxA resulted in production of only trace amounts of protein which lacked TTQ. Mutation of the Asp32 which corresponds in position to His466 in GoxA resulted in isolation of a mixed population in which some protein was active, albeit with significantly decreased affinity for substrate, and some lacked TTQ ¹⁶². This highlights the fact that these active site residues which likely play roles in catalysis or substrate binding, or both, must have previously evolved to facilitate the process of CTQ biosynthesis.

The protein interface of the GoxA dimer predicted from the modeling studies is completely different from what was observed in the crystal structure of LodA ¹³². LodA crystallized as a tetramer. Each LodA monomer has an unusual structure with two arms of antiparallel β -strands extended from the main body of the subunit. These extensions participate in subunit interactions. The primary sequence of this structural feature of LodA

is not present in GoxA, consistent with it exhibiting a different subunit composition and different subunit-subunit interactions.

The importance of Phe237 of GoxA in stabilizing subunit-subunit interactions and dictating cooperative behavior towards glycine is remarkable. The F237Y and F237A mutations each eliminated cooperativity towards glycine. However, the F237Y mutation decreases k_{cat} about six-fold and decreases K_{mO_2} slightly, while the F237A mutation decreases k_{cat} 155-fold and decreases K_{mO_2} , six-fold (Table 4.4.2). Despite the differences in the effects on the kinetic parameters of substitution with Ala versus Tyr, the destabilization of the dimer by the F237A mutation is only slightly greater than for the F237Y mutation (Figure 4). This indicates that activity is not strictly dependent upon dimer formation. This also means that F237 of GoxA exerts a significant influence on the k_{cat} and affinity for substrates as does Tyr211, the corresponding residue in LodA. In LodA the Y211A mutation eliminated activity while the Y211F mutation caused a small decrease in k_{cat} but a large increase in K_{m} for lysine ¹³.

At this point one can only speculate on the mechanism by which Phe237 stabilizes dimer formation and is involved in the communication between subunit active sites that results in cooperativity. As discussed earlier, the docking model suggests the possibility that strong π -interactions and π -stacking involving Phe237 and surrounding aromatic residues could be involved in these processes. A different structural role for Phe237 in GoxA than for Tyr211 in LodA is also consistent with the absence of a residue that corresponds to Lys530 in LodA. The crystal structure of LodA suggested that Tyr211,

which resides on a flexible loop near the entry to the substrate channel, formed a hydrogen bond with Lys530 creating a gate-like structure to control entry and exit of substrate and the products from the active site. Because of the absence of hydrogen bond to the corresponding Phe237, the corresponding loop in GoxA and consequently Phe237 adopts a different position (Figure 5) which now involves protein-protein interactions.

CHAPTER 9: CONCLUSION

This study involves the structural and functional characterization of two oxidases, LodA and GoxA originally found in the same bacterium, *Marinomonas mediterranea*. They are unique as they are the first reported examples of tryptophylquinone cofactor containing oxidases. Both of them contain CTQ and carry out oxidative deamination of amino acids, L-lysine and glycine respectively. Steady state kinetic analysis of LodA and GoxA showed difference in their mode of reaction catalysis. GoxA displayed allosteric cooperativity for glycine whereas LodA did not for its substrate, L-lysine. The cooperativity of GoxA was supported by the presence of the enzyme as a dimer. Regulation of substrate interaction was also different in the two proteins. In LodA it occurred by the gate-like formation generated by the H-bonded Lys530 and Y211. In GoxA it occurred by the dimer formation mediated by Phe237. Thus the kinetic and site-directed mutagenesis studies have revealed the possibility of two different physiological functions of LodA and GoxA in the microorganism, one being antimicrobial and the other being metabolic, respectively.

Various key points have been identified in this study. Site-directed mutagenesis has shown the importance of a single amino acid residue in stabilizing the structure and conformation of an enzyme. It has also highlighted another very significant aspect regarding the evolution of these enzymes. It is evident that they have been optimized for their functions but not essentially for selectivity, activity and cofactor generations as some mutants did show better CTQ formation than the wildtype, but ended up having compromised or no activity. The kinetic studies have also shown how enzymes

with similar cofactors but different physiological functions could maximize their catalytic efficiencies by merely adjusting kinetic parameters like k_{cat} and K_m . Most importantly since this has been the first detailed structural and functional analysis of CTQ containing oxidases, it will help in broadening the horizon of understanding posttranslational modifications of amino acids in quinoproteins.

In future this study could help in characterizing similar CTQ containing proteins which have been discovered recently as constituting a whole new pool of tryptophylquinone cofactor containing oxidases. The biosynthesis of CTQ cofactor has not yet been well characterized. The mutagenesis studies have already implicated the importance of certain amino acid residues in the CTQ biosynthesis process. Furthermore, the isolation of the complex of the two proteins one of which catalyzes the cofactor formation and the other being in a precursor form will help in determining the mechanism of cofactor biogenesis. Another interesting aspect would be to study the flavoproteins, LodB and GoxB. No flavoprotein has yet been reported to catalyze a quinone cofactor formation. Characterizing these will help in identifying the similarities and differences with the already reported flavoproteins that function as oxidases and catalyze redox reactions.

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