Mechanisms Whereby Mononuclear Copper Proteins Functionalize Organic Substrates

Judith P. Klinman

Departments of Chemistry and Molecular and Cell Biology, University of California, Berkeley, California 94720

Received April 2, 1996 (Revised Manuscript Received July 29, 1996)

Contents

Judith P. Klinman received her A.B. and Ph.D. degrees in Chemistry from the University of Pennsylvania. She was a postdoctoral fellow at the Weizmann Institute of Science, Rehovot, Israel, where she had access to highly enriched oxygen-18 water for the study of reaction mechanism. She spent 10 years at the Institute for Cancer Research in Philadelphia, first as a postdoctoral fellow with Irwin Rose and later as a Staff Scientist. She has been on the faculty of the University of California, Berkeley, since 1978. Her research is currently focused on three areas: (i) nuclear tunneling in enzyme-catalyzed reactions and the relationship of this phenomenon to the origin of enzymatic rate accelerations; (ii) the mechanism of dioxygen activation by metalloenzymes; and (iii) the biogenesis and catalytic mechanism of quinoproteins. In addition to her lifelong fascination with enzymes, Dr. Klinman enjoys hikes in the mountains or at the beach, "hanging out" with friends and family (especially grandson Alex), and long weekends at a cabin in Sonoma County.

I. Introduction

Although the number of proteins that utilize copper as an essential cofactor is not exceptionally large, the roles played by many of these proteins appear central to the success of the organism. For example, the ubiquitous copper/iron protein cytochrome oxidase functions in oxidative phosphorylation, catalyzing the final reduction of dioxygen to water coupled to the generation of a proton gradient and ATP formation (cf., review by Ferguson-Miller and Babcock, in this issue). A second biologically essential protein interconverting dioxygen and its reduced forms is the copper/zinc superoxide dismutase, which catalyzes a reduction of 1 mol of superoxide to hydrogen peroxide coupled to an oxidation of a second mole of superoxide to molecular oxygen. Copper is found to be especially important in the nervous system of higher eukaryotes, appearing in proteins responsible for the biogenesis of both catecholamine and peptidic hormones/neurotransmitters.

Over the last decade, great advances have been made in our understanding of copper proteins that utilize dioxygen in the course of the functionalization of organic substrates. These types of enzymes can be categorized, depending on whether the copper centers involved in substrate functionalization exist alone (mononuclear) or in a complex with other copper ions (bi- or polynuclear). This review is focused on the former category of proteins, and the reader is referred to the review by Solomon, Sundaram, and Machonkin (in this issue) for a description of the bi- and polynuclear centered proteins. One important feature of the mononuclear copper catalysts is the difficulty inorganic chemists have encountered in devising suitable model systems for the generation of biological activity. For this reason, the biological systems have often "led the way," providing new paradigms for catalysis that can then be tested in appropriately designed model reactions.

S0009-2665(95)00047-1 CCC: $25.00

II. Dopamine β-Monooxygenase and Peptide Amidating Enzyme

A. Dopamine β-Monooxygenase (DβM)

1. General Background

The literature on DβM is voluminous, primarily because of the important role this enzyme plays in controlling the levels of the neurotransmitters/hormones dopamine and norepinephrine (for reviews, see refs 1 and 2). The conversion of dopamine to norepinephrine involves an insertion of an atom of oxygen into the benzylic position of the ethylamine side chain of dopamine and other phenethylamine analogs:

$$RCH_2CH_2NH_2 + O2 \xrightarrow{2e^-,\ 2H^+} RCHOHCH_2NH_2 + H_2O \quad (1)$$

As the reaction in eq 1 implies, the reduction of oxygen to water is a four-electron process, with two electrons originating from substrate and two electrons from an exogenous electron donor. The naturally occurring electron donor is ascorbic acid (vitamin C), which undergoes oxidation in two sequential steps to generate 2 mol of semidehydroascorbate:[3,4]

$$2(\text{ascorbate}) \rightarrow 2(\text{semidehydroascorbate}) \quad (2)$$

DβM is found in both soluble and membranous forms, primarily within neurosecretory vesicles of the adrenal gland and the sympathetic nervous system. This compartmentalization of DβM requires a mechanism for the transfer of reducing equivalents from the exterior to the interior of the vesicle, in order to maintain a constant pool of reduced ascorbic acid for enzyme turnover. It is generally assumed that the intravesicular semidehydroascorbic acid formed in norepinephrine production is reduced back to ascorbate by a transmembrane electron-transfer process.[2] Since dehydroascorbate is not believed to be a substrate for transmembrane electron-transfer processes, this process must be kinetically competitive with the diffusion-controlled, bimolecular disproportionation of semidehydroascorbate to ascorbate and dehydroascorbate:

$$2(\text{semidehydroascorbate}) \rightarrow \text{ascorbate} + \text{dehydroascorbate} \quad (3)$$

Early studies of DβM had shown that this protein contained copper and that the oxygen at the benzylic position of product was derived from molecular oxygen and not water. These properties were sufficient to describe DβM as a copper-dependent monooxygenase, but provided little insight into mechanism. DβM binds copper relatively weakly[5] and when purified to homogeneity contains low levels of copper.[6] Early efforts to determine the relationship between the stoichiometry of copper reconstitution and enzyme activity were hampered by the pervasive contamination of buffers and glassware by trace copper at the nanomolar (nm) level of protein used in steady-state kinetic studies. Not until rapid mixing experiments were performed, using much higher protein concentrations (micromolar, μM), was it possible to demonstrate that optimal activity

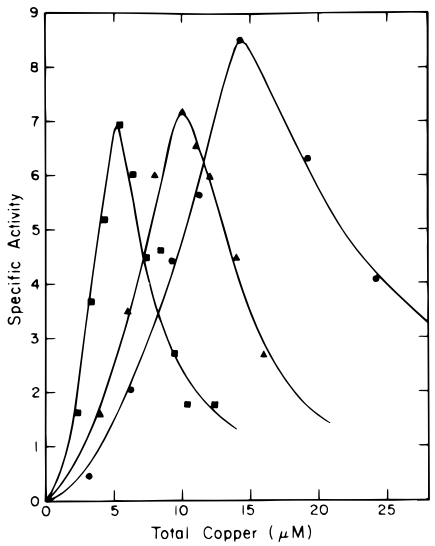


Figure 1. Dependence of DβM activity on copper concentration for three different enzyme concentrations (increasing from left to right: 2.5, 5.0, and 7.1 μM DβM subunits). The solid lines are the result of a computer fit for a model involving two catalytic copper sites with comparable affinity (10 nM) and a weaker inhibitory site. (Reprinted from ref 7. Copyright 1984 Journal of Biological Chemistry.)

correlated with the addition of *two* coppers per protein polypeptide chain of ~73 KDa,[7] Figure 1. A similar requirement for two coppers per subunit was demonstrated using milligram levels of enzyme in the characterization of a mechanism based inactivator.[8] These findings set the stage for all subsequent structure function studies of DβM.

2. Copper Binding Sites

Low temperature EPR spectra of DβM indicate a typical "type 2" cupric ion pattern which is similar to Cu^{II}-EDTA standards.[5] Spin counting of fully reconstituted enzyme shows two cupric ions per subunit. These data immediately ruled out a binuclear complex in which the active site coppers are connected by a bridging ligand, distinguishing DβM both structurally and functionally from "type 3" copper containing enzymes such as tyrosinase[9,10] (cf., review by Solomon, Sundaram, and Machonkin, this issue). Early kinetic studies had indicated that ascorbate and dopamine/dioxygen interact with different forms of DβM, via a ping-pong mechanism.[11] The initial description of such a process indicated reduction of *free* enzyme by ascorbate to convert the resting Cu(II) form of enzyme to Cu(I):

$$E\text{-}Cu^{II}_2 + 2(\text{Asc}) \rightarrow E\text{-}Cu^{I}_2 + 2(\text{semidehydroascorbate}) \quad (4)$$

This was followed by the recycling of $E\text{-}Cu^{I}$ to $E\text{-}Cu^{II}$ with dopamine (DA) and dioxygen, to yield norepinephrine (NE) and water as products:

$$E\text{-}Cu^{I}_2 + DA + O_2 \rightarrow E\text{-}Cu^{II}_2 + NE + H_2O \quad (5)$$

Under conditions of saturating ascorbate, it was possible to isolate kinetically the reaction in eq 5 and to determine the effect of dopamine deuteration on turnover rates. Such studies show that under conditions of low dopamine concentration (which allows

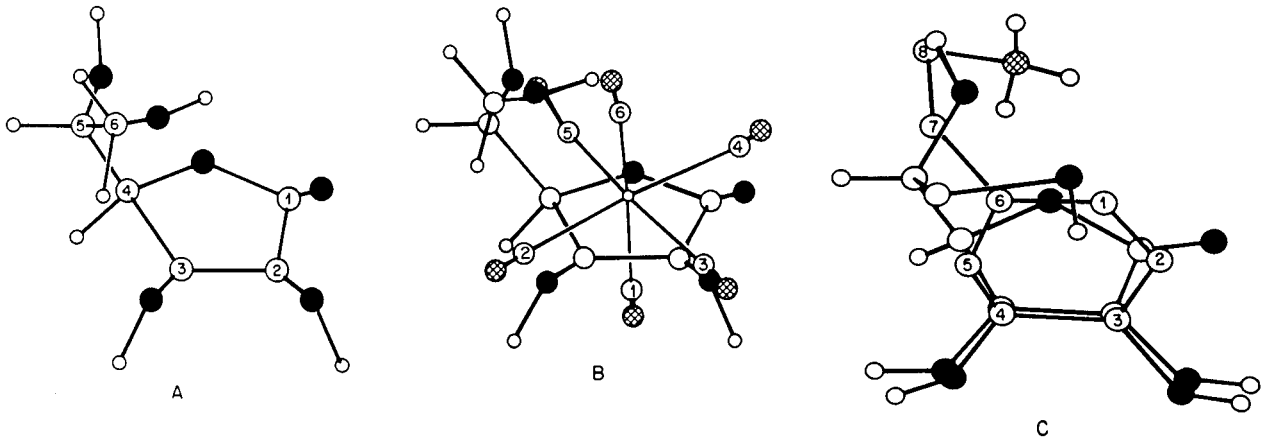


Figure 2. Structural comparison among ascorbate, ferrocyanide, and dopamine as reductants for DβM (open circles are carbon, closed circles are oxygen, hatched circles are nitrogen, and small open circles are hydrogen). Panel A is ascorbate alone; panel B is ferrocyanide superimposed on ascorbate (numbering refers to ferrocyanide); and panel C is a superposition of dopamine on ascorbate (numbering refers to dopamine). (Reprinted from ref 15. Copyright 1987 American Chemical Society.)

measurement of the kinetic parameter $V_{max}/K_{m(DA)}$), insertion of oxygen into the β-carbon bond of DA is partially rate determining, whereas under conditions of saturating dopamine (which leads to measurement of V_{max}), dissociation of products from enzyme becomes the major rate-limiting step:[12]

$$E{\cdot}O_2 + DA \rightleftharpoons E{\cdot}O_2{\cdot}DA \longrightarrow E{\cdot}NE{\cdot}H_2O \longrightarrow E + NE + H_2O$$

$V_{max}/K_{m(DA)}$ (partially limited by C-H cleavage)

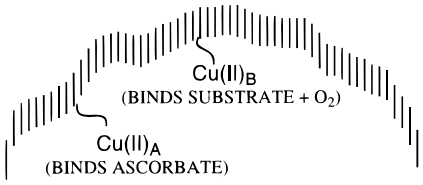
V_{max} (limited mainly by product release) (6)

As predicted from these steady state isotope effects, a close to stoichiometric accumulation of the E·NE·H_2O complex was later confirmed under pre-steady-state conditions using rapid mixing, acid quench procedures.[13] At this juncture, it was expected that the enzyme product complex would be in the oxidized, Cu(II) form. However, when rapid mixing, freeze quench studies were performed in the presence of saturating ascorbate, EPR spectra indicated a very low level of Cu(II) in the E·NE·H_2O complex. In a companion study, using enzyme which had been prereduced by a *stoichiometric* amount of ascorbate, Cu(II) and hydroxylated product were produced in a ratio of 2:1.[14] These rapid mixing experiments led to the conclusion that high levels of ascorbate in reaction mixtures reduce the E·NE·H_2O complex to its Cu(I) form, i.e., that ascorbate is able to interact productively with the enzyme product complex:

$$E\text{-}Cu^{II}_2{\cdot}NE{\cdot}H_2O + 2(Asc) \rightarrow E\text{-}Cu^{I}_2{\cdot}NE{\cdot}H_2O + 2(\text{semidehydroascorbate}) \quad (7)$$

This key observation provided the first evidence that there was a separate binding site on enzyme for reductant and substrate/product. It was proposed that each of the copper centers performs a separate function, with Cu_A catalyzing electron transfer from ascorbate into the active site and Cu_B catalyzing oxygen insertion into substrate,[14] Scheme 1. Thus, while DβM contains two coppers per subunit, it can be categorized as a mononuclear copper protein with regard to the functionalization of substrate by dioxygen.

Scheme 1. Schematic of the DβM Subunit, Showing the Different Roles for Cu_A (in Electron Transfer) and Cu_B (in Substrate Hydroxylation) (Adapted from Ref 14)



What additional evidence has been brought to bear on this property of distinct copper sites with different functions? In the initial studies of copper reconstitution of DβM, it was found that the activity vs copper curves were best fit by a model which assumed equal affinities of Cu_A and Cu_B to protein[7] (Figure 1). Similarly, EPR experiments of protein samples containing either 1 or 2 mol of copper consistently show identical spectra when normalized for differences in concentration of enzyme bound copper.[6] By contrast, detailed kinetic studies using reductants of varied structure have indicated that ascorbate and ferrocyanide are almost equally efficient as electron donors to DβM, whereas catecholamines are much less efficient.[15] Since this difference in behavior does not appear to correlate with a trend in redox potentials, it was attributed to different binding requirements at a reductant and substrate catecholamine binding sites. Molecular modeling of ascorbate and ferrocyanide supports this idea, since it may be possible to accommodate both of these (good) reductants into a single site with relatively little structural perturbation (Figure 2).

Blackburn and co-workers have successfully used copper-binding ligands to deconvolute the properties of the separate copper sites. Carbon monoxide has been shown to bind to reduced DβM in a manner which is competitive with dioxygen and at a stoichiometry of only 1 mol per enzyme subunit; these results have led to a model in which CO mimics dioxygen and binds exclusively at the substrate hydroxylation site.[16] It has been possible to prepare

protein samples which contain only a single copper per subunit and to demonstrate that these samples retain their ability to bind CO; hence, the preferential loss of Cu_A has been inferred.[17] Isocyanides have also proved useful in distinguishing the copper sites in DβM. Using 2,6-dimethylphenyl isocyanide and DβM in its Cu(I) valence state, Reedy *et al.* have observed monoisocyanide complexes with different frequencies (2148 and 2129 cm^{-1}), indicative of inequivalent coordination at each copper site.[18]

EXAFS and edge absorption studies of DβM have been conducted by numerous research groups, often with conflicting results.[19−22] It is now generally agreed that each copper center in the oxidized form of DβM contains 2−3 histidine ligands and 1−2 O/N ligands.[22] Data for the reduced form of enzyme indicate the appearance of a single heavy atom scatterer, ascribed to either a chloride ion or sulfur atom, but generally believed to be the latter.[21−22] Since reduced enzyme samples depleted at the Cu_A site still show a heavy atom scatterer, the copper site containing the sulfur ligand is attributed to the site responsible for substrate hydroxylation (Cu_B).[17] The active-site sulfur atom is likely to be a methionine residue, found to be conserved between peptide amidating enzyme and DβM[23] (see discussion on Peptidylglycine α-Amidating Enzyme below). When a complex of reduced DβM with CO complex was examined by IR, it was found to have a stretching frequency ~20 wave numbers higher than model Cu^I/CO complexes. This has been attributed to less back-bonding from copper to CO, arising from a decrease in the number of ligands or their basicity at the reduced Cu_B center.[16,17,22] In the absence of a three-dimensional structure for DβM, it is difficult to infer the outer-sphere environment of the active-site copper centers. However, in the course of mapping the intrachain disulfide bonds in bovine DβM, Villafranca and co-workers have observed that five of these covalent linkages are clustered in the region of the densest concentration of histidine residues. Also, contained within this linear sequence are side chains known to undergo alkylation by mechanism-based inhibitors. These authors have proposed that the copper binding domains lie in regions defined by an extensive network of disulfide-linked structures.[24]

The mechanistic importance of the significant structural changes that occur at the copper sites upon conversion of the oxidized to reduced form of DβM are not well understood. The structural changes due to enzyme reduction may be the signal for the formation of a kinetically productive complex of substrate and dioxygen. These structural changes may also be the signal that prevents any further interaction of enzyme with reductant until the product E·NE·H_2O complex is formed. Kinetic studies of dopamine hydroxylation using either dopamine or ascorbate as reductant indicate the same rate constant ($V_{max}/K_{m(O_2)}$) for reaction of the enzyme dopamine complex with dioxygen to form product:

$$E\cdot DA + O_2 \rightleftharpoons E\cdot DA\cdot O_2 \rightarrow E\cdot NE\cdot H_2O \qquad (8)$$

Since dopamine is a much poorer electron donor than ascorbate, this finding rules out any donation of electrons to the ternary complex of dopamine and dioxygen prior to the formation of enzyme-bound norepinephrine and water.[15] In the context of understanding the structural features controlling electron flux to Cu_A and between Cu_A and Cu_B, it should be borne in mind that both copper centers in DβM are expected to be reoxidized *prior* to substrate hydroxylation (in the course of dioxygen activation for insertion into substrate, see discussion on Mechanism of Substrate Hydroxylation below). We currently have no information regarding the structure of the copper sites in catalytic intermediates formed following the binding of dopamine and oxygen to reduced enzyme, including that of the product norepinephrine and water complex. For the future, it may be feasible to trap some of these species and characterize their properties by EXAFS and edge absorption studies.

3. Mechanism of Substrate Hydroxylation

As the above discussion indicates, DβM can be placed into the category of mononuclear, metallomonooxygenases. In contrast to the extensive information which has become available for heme-iron systems (cf., review by Sono, Roach, Coulter, and Dawson, this issue) and binuclear copper (and more recently iron) metalloproteins (cf., reviews by Solomon, Sundaram, and Machonkin and by Lipscomb and Sträter, this issue), little has been understood regarding how a single iron or copper center can catalyze C−H bond activation. Many investigators, including those in my own laboratory, have attempted to "peel back the layers" of the DβM enigma. A large number of steady-state kinetic studies have been focused on elucidating the kinetic mechanism of DβM, in particular, the sequence of binding of substrates to enzyme and the nature of rate-limiting steps. It is generally accepted that (i) ascorbate reacts with an enzyme form that is irreversibly connected to the one that interacts with dopamine and oxygen (cf., eqs 4, 5, and 7, above), and (ii) either dopamine or oxygen can bind first to the reduced enzyme.[12] Rapid mixing studies confirm the ability of preformed Cu^I-DβM to react with substrate and oxygen in a kinetically competent manner.[14] The Cu(I) form of DβM is sufficiently stable in the absence of substrate on a second time scale that it can be produced aerobically (cf. ref 14). It is clear that substrate must trigger a conformation change within the E-Cu^I·DA·O_2 complex that allows the activation of dioxygen to proceed on a millisecond time scale.

Given the relative kinetic inertness of molecular oxygen, together with the fact that the catalytic form of DβM is two electron reduced, it is normally assumed that the first species to form in DβM catalysis is a copper−peroxy intermediate:

$$E\text{-}Cu^I{}_2\cdot O_2\cdot DA \rightarrow E\text{-}Cu^{II}{}_2\text{-}OOH\cdot DA \qquad (9)$$

Early studies, showing the absolute requirement for a proton in substrate hydroxylation, support the view that the reduced oxygen is in a hydroperoxy form.[12] Given the prevalence of the belief that Cu^{II}−OOH is on the catalytic path of DβM, what *direct* evidence (if any) exists for this species? In an effort to generate a stable analog of the E-Cu^{II}−OOH·DA complex, the unreactive β,β-difluoro analog of phenethylamine was synthesized:

$$\text{Ph-CF}_2\text{-CH}_2\text{NH}_2$$
1

Rapid mixing of the ascorbate reduced E-Cu^I form of enzyme with **1** failed to give rise to a detectable (>10% of E_{tot}) Cu(II) signal.[25] This unexpected result could have three possible origins. First, fluorine may not be a sufficiently good analog of hydrogen, such that the greater electron density at the β-carbon of **1** impedes its correct binding within the active site (note $K_i \cong 26$ mM). A second possibility is that an E- Cu^{II}-OOH·**1** intermediate actually formed, but that it was spin coupled to the second cupric ion at the Cu_A site. Although there is no evidence for spin coupling within any of the complexes of DβM thus far characterized, an interaction of this type cannot be rigorously eliminated for intermediates along the reaction path. A final possible explanation relates to the nature of the species responsible for substrate hydroxylation. As will be discussed in greater detail below, recent data suggest that a precursor E-Cu-OOH intermediate undergoes further reductive activation to generate a Cu^{II}-O$^\bullet$ species as the active hydroxylating agent.[26] If a reaction of this type were to occur in the presence of **1**, spin-coupling processes could diminish the EPR signals of the active-site cupric ions. One argument against this latter explanation is the stability of DβM when incubated with **1** under turnover conditions.[25] If a Cu^{II}-O$^\bullet$ species were to form in the absence of an activatable substrate, enzyme inactivation might be expected to rapidly ensue.

In relating substrate activation to oxygen chemistry, it is important to know the mode of C−H activation, in particular, whether this occurs via the production of radical intermediates. Studies of the rate of C−H bond cleavage as a function of substrate structure offer the opportunity to assess changes in charge at the reacting carbon in proceeding from the ground state to transition state:

$$\text{X-Ph-CH}_2\text{-CH}_2\text{NH}_2 \rightarrow [\text{TS}] \rightarrow \text{X-Ph-CH}^-\text{-CH}_2\text{-NH}_2\,(\text{carbanion}) \text{ or } \text{X-Ph-CH}^\bullet\text{-CH}_2\text{-NH}_2\,(\text{radical}) \quad (10)$$

To the extent that the TS resembles the intermediate state, it is possible to infer the chemical nature of this intermediate. One recurring difficulty in steady-state kinetic studies is the complexity of the measured parameters, which most often reflect multiple steps. When studying a series of substrates, there is the added complexity of a possible change in rate-determining step with change in substrate structure. Both pre-steady state kinetics and isotopic probes have been used to circumvent these problems. While rapid kinetic studies require high levels of protein, these studies can provide a direct measure of chemical processes. By contrast, isotopic probes are less direct, but far more suited to extensive studies of a protein which is available in only limited amounts. Given the absence, until recently, of a suitable high level expression system for DβM,[27] the majority of kinetic studies on this protein have used steady-state methods.

As already shown in eq 6 above, early measurement of deuterium isotope effects with DA as substrate had indicated either partial (for $V_{max}/K_{m(DA)}$) or almost no (V_{max}) rate limitation by the C−H cleavage step. Full interpretation of steady-state isotope effects requires a frame of reference, which is the intrinsic isotope effect on the isolated C−H bond cleavage step. Northrop has published a method for extracting this intrinsic value from measured values in complex enzyme reactions.[28] Application of his method to the DβM reaction has yielded an intrinsic value of 10.9 ± 1.9 using DA as substrate.[29] The availability of this intrinsic isotope effect for DβM allowed the calculation of the rate constant for the C−H bond cleavage step with a series of eight ring-substituted phenethylamine substrates through the use of eq 11:[30,31]

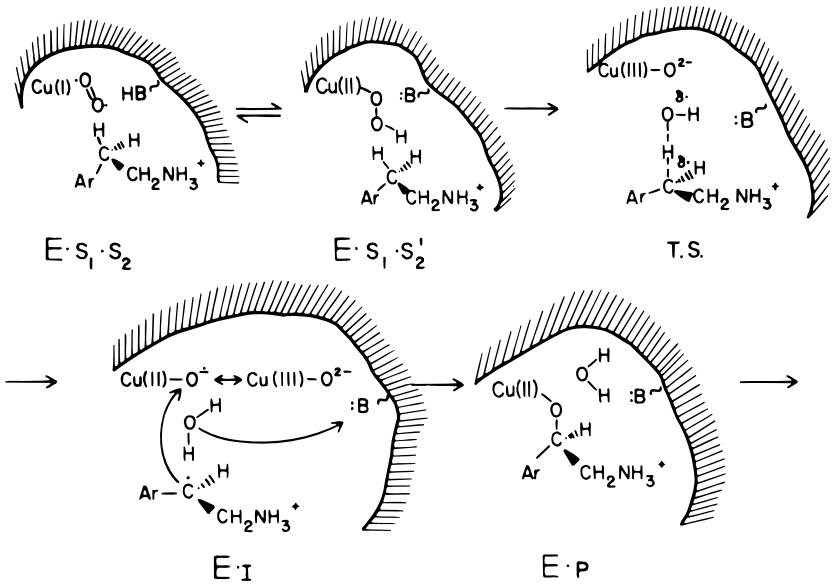
$$k_{C-H} = V_H\,({}^Dk - 1)/{}^DV - 1 \quad (11)$$

where k_{C-H} is the desired rate constant, V_H is the observed rate constant for protonated substrate, ${}^Dk$ is the intrinsic isotope effect (assigned the value measured for dopamine), and ${}^DV$ is the observed isotope effect on V_{max}. As discussed by Miller and Klinman,[30] limitations in the application of eq 11 could, in principle, arise when ${}^DV$ is too close to unity or the value of ${}^Dk$ changes greatly as a function of substrate structure. In the case of DβM, a smooth trend in k_{C-H} was observed as a function of substrate structure, leading to a ρ value of -1.5.[31] ρ values of this sign and magnitude, which indicate significant carbocation character at the reacting carbon center, are often seen in radical-generating systems. Although a negative ρ value would also be consistent with a hydride abstraction process, this was considered a highly unlikely mechanism from the outset. Similar evidence for the generation of radical intermediates in substrate activation has come from structure-reactivity studies using a series of mechanism-based inhibitors.[32]

With the implication of a hydrogen atom abstraction process in the course of substrate hydroxylation and the highly likely (but still unproven) involvement of a copper−hydroperoxy intermediate, the major challenge in DβM chemistry has been a correlation of oxygen and substrate chemistry. Very specifically: what is the nature of the oxygen species responsible for C−H cleavage? It was initially argued that simple binding of substrate in close proximity to a metal−hydroperoxide center should be sufficient to initiate a hydrogen atom abstraction from the benzylic position of substrate. The overall thermodynamic driving force for such a process was proposed to arise from the large and favorable difference in bond dissociation energy between a benzylic C−H bond (~85 kcal/mol) and the O−H bond of product water (~119 kcal.mol),[31] Scheme 2. The type of transition state envisaged in Scheme 2 is consistent with a demonstration of tight binding by a bis-substrate analog designed to bridge the substrate binding site and the active-site copper using a sulfur-containing metal ligand,[33,21] Figure 3.

If a metal-linked hydroperoxy species is sufficiently "hot" to functionalize organic substrates, this property has enormous implications for the understanding of analogous nonheme, metalloenzyme reactions and for the design and investigation of appropriate model systems. Tian *et al.* undertook a series of

Scheme 2. Mechanism for DβM in Which Copper Hydroperoxide Acts as the Hydroxylating Agent (Adapted from Ref 31)



oxygen-18 isotope effect studies, in an effort to provide independent evidence for the postulated transition-state structure.[26] By using dioxygen labeled with oxygen-18 at the level of natural abundance, and an apparatus that allows conversion of unreacted dioxygen to carbon dioxide for analysis by isotope ratio mass spectrometry, it was possible to follow time-dependent changes in the oxygen-18 content of unreacted dioxygen. Initial measurement of the O-18 isotope effect in this way using dopamine as substrate indicated a kinetic oxygen isotope effect of 1.0197 ± 0.0003. This value reflects the portion of the DβM reaction involving all steps from binding of free oxygen to the enzyme−substrate complex up to and including the conversion of substrate to hydroxylated product (eq 8, above). The intrinsic oxygen isotope effect is expected to be larger than the measured one due to some kinetic complexity. Indeed, measurement of the O-18 isotope effect with deuterated dopamine led to an elevated value of $^{18}(V/K_{(O_2)}) = 1.025 \pm 0.0003$, which has been shown to be very close to the intrinsic O-18 isotope effect.

Two important features emerged at this point: first, that the value of the measured O-18 isotope effect in the DβM reaction was significantly larger than that expected for metal−hydroperoxide formation [where $^{18}(V/K_{(O_2)}) \cong 1.010$ (ref 34)], as expected for a decrease in bond order through cleavage of the O−O bond of the intermediate hydroperoxide in the course of C−H bond abstraction; and second, that a single O-18 isotope effect could not distinguish direct hydrogen abstraction by a hydroperoxyl intermediate from a mechanism in which copper hydroperoxide is reductively cleaved prior to substrate activation. In an effort to clarify the latter key issue, it was decided to examine the sensitivity of the experimental O-18 isotope effect to changes in substrate structure. The series of ring-substituted phenethylamines previously characterized with DβM had shown rate constants for C−H bond cleavage that varied almost 3 orders of magnitude, from 680 s^{-1} for dopamine to 2 s^{-1} for *p*-(trifluoromethyl)phenethylamine.[31] It was anticipated that the position of the transition state for hydrogen atom abstraction would vary as well, becoming more product like for the slower substrates. For a simple hydroperoxide mechanism, Scheme 2, the magnitude of the O-18 isotope effect would be expected to increase as the substrate became less reactive, due to the formation of a transition state with more extensive O−O bond cleavage. By contrast, a mechanism in which copper hydroperoxide is reductively cleaved prior to C−H abstraction to give $C^{II}-O^{\bullet}$ as the hydroxylating species (together with water as a second product) predicts an increase in bond order to oxygen as the transition state for C−H cleavage becomes more productlike. In the latter case, the O-18 isotope effect is expected to decrease with decreasing substrate reactivity. Quite unexpectedly, when the data became available, the latter trend in $^{18}(V/K_{(O_2)})$ was observed (Table 1).

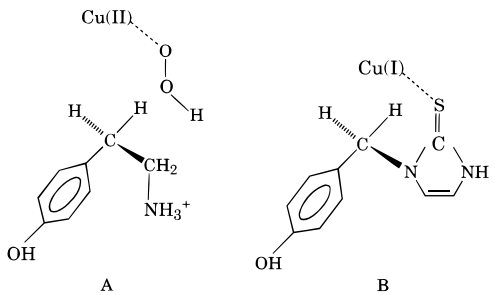
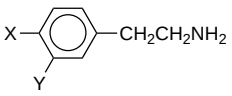


Figure 3. Possible binding mode for a bissubstrate inhibitor of DβM [as designed by Kruse *et al.*, ref 33], structure B, for comparison to the enzymatic transition state structure proposed by Miller and Klinman [ref 31], structure A. A direct interaction of the bissubstrate inhibitor with copper is supported by changes in the Cu(I) XAFS in the presence of inhibitor [ref 21].

As a result of these new O-18 isotope effect data, it has become necessary to reformulate the DβM mechanism. Two independent properties of DβM have been important in this context. First, characterization of a number of mechanism-based inhibitors has established that there are three reactive tyrosines at or near the DβM active site (Tyr 477, 216, and 357 in the bovine enzyme),[35,36,162] and second, DβM has been shown to be capable of catalyzing

Table 1. Relationship between Intrinsic Oxygen-18 Isotope Effects and Substrate Reactivity in the DβM Reaction



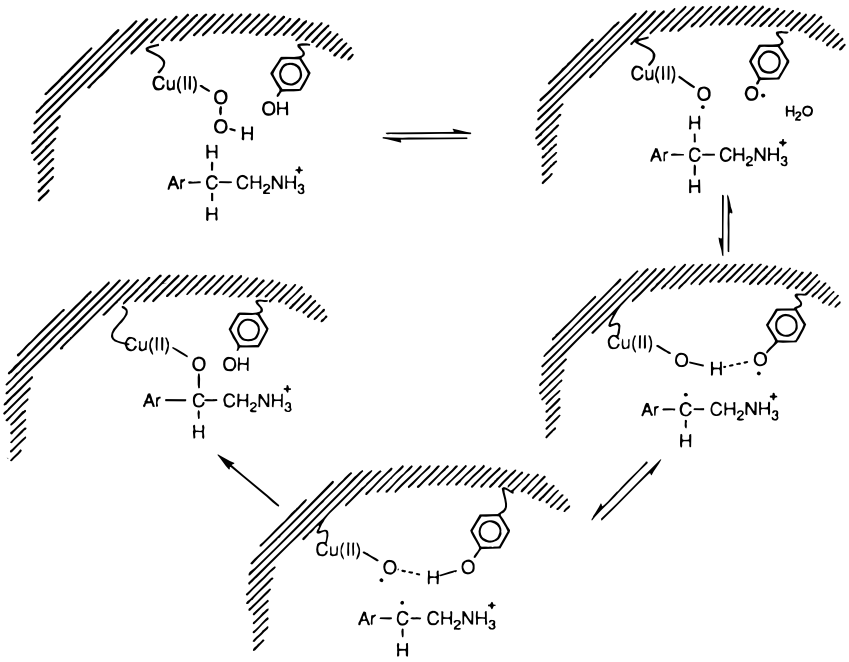
X	Y	$^{18}(V/K_{(O_2)})$	k_{C-H} (s^{-1})
OH	OH	1.0281 ± 0.0012	680
H	H	1.0256 ± 0.0008	500
CH_3	H	1.0236 ± 0.0011	140
Br	H	1.0215 ± 0.0001	22
CF_3	H	1.0216 ± 0.0003	2

hydrogen abstraction from either the phenolic or the methyl group of cresols, with the latter leading to enzyme turnover and the former leading to enzyme inhibition.[37] A new mechanism which takes into account all of the known properties of DβM is shown in Scheme 3. As illustrated, a copper hydroperoxide is formed initially. Reductive activation of this intermediate leads to expulsion of water, the generation of a Cu^{II}-O$^{\bullet}$ as the hydroxylating species and a concomitant, transient accumulation of a tyrosyl radical. Within this intermediate, there are four unpaired spins, with two spins coming from Cu(II) (at the Cu_A and the Cu_B sites), one spin coming from the metal-bound oxygen radical and the last from tyrosyl radical. The position of the tyrosyl radical may lie between the active site Cu_B site and the electron transfer Cu_A site with an appropriate distance for spin coupling. In this way, it is conceivable that this intermediate has the overall properties of a diamagnetic species, consistent with our inability to detect Cu(II) signals in the EPR spectrum of enzyme which has been reacted with **1** (see above). Abstraction of the benzylic hydrogen from substrate by Cu^{II}-O$^{\bullet}$ is proposed to lead to a hydrogen-bonded unit bridging the copper-bound oxygen atom and the ring oxygen of tyrosine. This species provides a pathway for the transfer of the substrate-derived hydrogen atom to the tyrosyl radical, regenerating tyrosine and allowing for the combination of the substrate-derived radical center with the metal-bound oxygen radical to form an inner-sphere alkoxide product. Such an inner-sphere product complex has been inferred from structure function studies of the rate of release of products formed from the hydroxylation of a series of phenethylamine derivatives.[31]

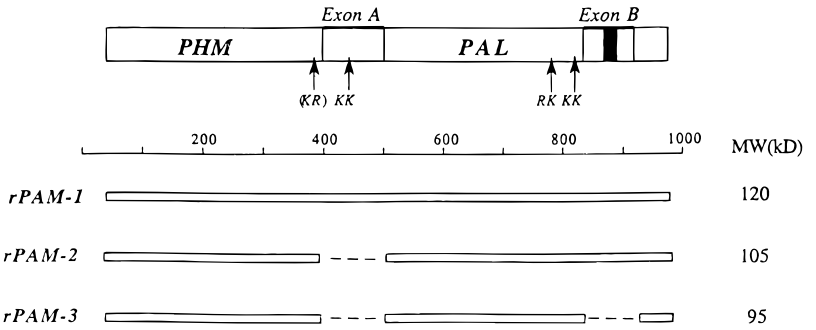
If this *radically* new mechanistic proposal for DβM is correct, it is likely to pertain to peptide-amidating enzyme as well (see below) and may also be relevant to other nonheme metallomonooxygenases. It would be extremely valuable to have a model system available which mimics many of the features of the DβM mechanism (cf., ref 38). To date, the only model system to show a regiospecific hydroxylation of the aliphatic portion of a phenethylamine precursor has come from Itoh *et al.*[39] These investigators have devised a mononuclear copper center liganded to two pyridines and the nitrogen of phenethylamine. Exposure of this ligand system to either Cu(II) and appropriate reductants or Cu(I) has been shown to lead to a highly efficient production of β-hydroxylated phenylethylamine product. One curious feature of this model is that the product complex is binuclear in copper, with the β-hydroxy-groups of product bridging the two copper centers. Because of this feature, it is impossible to know at what stage of the reaction the copper has moved from a mononuclear to a binuclear configuration. The authors propose a Cu^{II}-O$^{\bullet}$ intermediate as the hydroxylating agent but also state that they cannot rule out a μ-η^2:η^2-peroxo dicopper complex.

One question that has arisen in the context of the mechanism of Scheme 3 is why a copper hydroperoxy intermediate would react with an amino acid side chain when it could react with the benzylic side chain of substrate directly (the bond dissociation energy for the benzylic C−H and phenolic O−H are very close). The proposed mechanism for DβM may reflect an

Scheme 3. Mechanism for DβM in Which Copper Hydroperoxide Is Cleaved To Generate Cu^{II}-O$^{\bullet}$ as the Hydroxylating Agent (Reprinted from Ref 26. Copyright 1994 American Chemical Society)



Scheme 4. Gene Structure for PAM[a] (Adapted from Ref 72)



[a] As indicated, all PAM RNA's derive from a single gene (rectangular box at the top) by alternative splicing. The optional exons are A, which encodes a proteolytic processing site and B, which encodes a membrane-anchoring region.

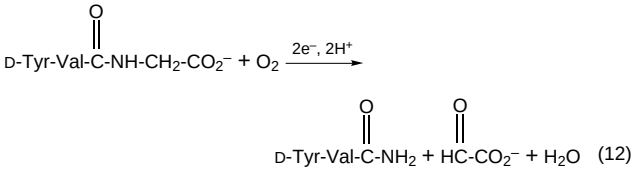
evolutionary strategy for the generation of a self-contained catalytic unit with the capability of performing a wide range of chemical reactions. It is well known that DβM catalyzes many different types of oxidations, which include sulfoxidation, oxygenative ketonization, olefin oxidation, alkyne oxidation, selenooxidation, and oxygenative N-dealkylation.[2] For the future, it will be critical to assess possible contributions of active-site side chains in the catalytic mechanism. In fact, this has already been initiated with the peptide amidating enzyme (see below). One caveat in the origination of this new DβM mechanism has been the assumption of classical behavior, i.e., that the structure of the transition state is predicted to become more like the product as the substrate reactivity decreases. In recent years, my laboratory has demonstrated the occurrence of quantum mechanical tunneling in numerous enzyme-catalyzed hydrogen transfer reactions.[40−43] The degree to which quantum mechanics dominates each reaction has been shown to vary from system to system. At this juncture, it is not possible to rule out such a phenomenon in the DβM reaction, and experiments aimed at addressing this question are in order.

B. Peptidylglycine α-Amidating Enzyme

1. General Background

The characterization of peptidylglycine α-amidating enzyme (PAM) is a relatively recent phenomenon, coinciding with the increasing awareness of the physiologic importance of peptidic hormones which contain an amide linkage at their C-terminus (for recent reviews, see refs 44−46). In fact, at least half of the existing peptide hormones are modified in this way, with well-known examples being substance P, oxytocin, thyrotropin, and calcitonin. It is not currently understood why peptide hormones exist in two distinct classes, although it is clear that amidation at the C-terminus eliminates the negative charge normally found at this position; this property has led to the speculation that the recognition site on the hormone receptor for C-terminally amidated peptides is hydrophobic in character.[45]

The first demonstration of a PAM activity occurred as recently as l982, using the short peptide, D-Tyr-Val-Gly as substrate. This important study demonstrated first, that glyoxylate was a product and second, that the nitrogen from glycine was transferred to the second product.[47] With subsequent demonstrations of the essentiality of dioxygen, and a two-electron reductant (e.g., ascorbate),[48] it was possible to write the general mechanism for PAM:



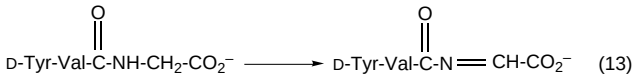
Thus, PAM represents an unique example of an enzyme-catalyzed oxidative cleavage of a peptide bond. Given the overall similarity of the PAM reaction, eq 12 to that of DβM, eq 1, it did not take long for investigators to establish that enzyme activity was eliminated by chelators and that copper was the only ion capable of restoring activity.[49]

Curiously, early purifications of PAM from a variety of sources indicated proteins that varied in size from about 40 kDa to close to 100 kDa. It is now known that multiple forms of PAM are produced *in vivo*, the result of alternative splicing. As shown in Scheme 4, a single gene for PAM encodes two separate enzyme activities (PHM and PAL), as well as two functionally relevant exons (A and B).[67] PHM (peptidylglycine α-hydroxylating monooxygenase) is the enzyme activity analogous to DBM, whereas PAL (peptidyl-α-hydroxyglycine α-amidating lyase) catalyzes the hydrolysis of a catalytic intermediate (see below). Exon A encodes a dibasic site which leads to proteolytic processing of the gene product, and exon B contains sequence information for membrane insertion. A large number of gene products are formed with the dominant forms being PAM- 1, −2, and −3.[67−71] PAM-1 is unique in that it contains exon A and, consequently, is the only gene product capable of post-translational processing to give separate polypeptide chains for PHM and PAL. PAM-1 is also a membranous protein, undergoing insertion near its C-terminus. Analogous to DβM, PAM is localized to secretory vesicles, with the enzyme active site(s) facing the interior of the vesicle. In the case of PAM-1, the C-terminus of the protein is found in the cytosol, whereas the bulk of the protein resides within the vesicle interior [cf., ref 45]. PAM-2 and -3 are similar in that they lack exon A and, therefore, are incapable of post-translational cleavage to give separate PHM and PAL activities. PAM-2 contains exon B, undergoing insertion into the membrane and PAM-3 is soluble. Genetic engineering has led to constructs for either PHM and PAL as separate

activities[50,51] and to a construct encoding a bifunctional, soluble protein of 75 kDa.[52,53] In both cases, fairly high-level expression systems are currently available. Given the vanishingly small amount of PAM available from natural sources, the development of these expression systems was an essential step in facilitating biophysical and mechanistic studies.

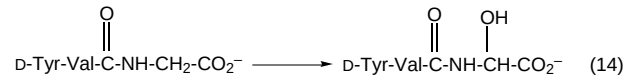
2. Copper-Binding Sites and Mechanism of Substrate Hydroxylation: Analogies to Dopamine β-Monooxygenase

When the PAM activity was first described, a dehydrogenase mechanism to yield a carbinol amide intermediate was postulated as the first step in the oxidative cleavage of substrate,[47] eq 13:



(13)

Subsequent hydrolysis of this carbinol amide product structure would yield D-Tyr-Val amide and glyoxylate. A critical test of such a mechanism is the source of O-18 in the carbonyl of glyoxylate, since the dehydrogenase mechanism of eq 13 predicts that O-18 will be derived from water. One difficulty in an analysis of this type is the extreme lability of the aldehydic functionality of glyoxylate toward exchange with solvent.[46] An alternate mechanism for PAM is the one analogous to Dβ M, in which dioxygen hydroylates the α-carbon of glycine to give an α-hydroxylated intermediate,[54,55] eq 14:



(14)

2

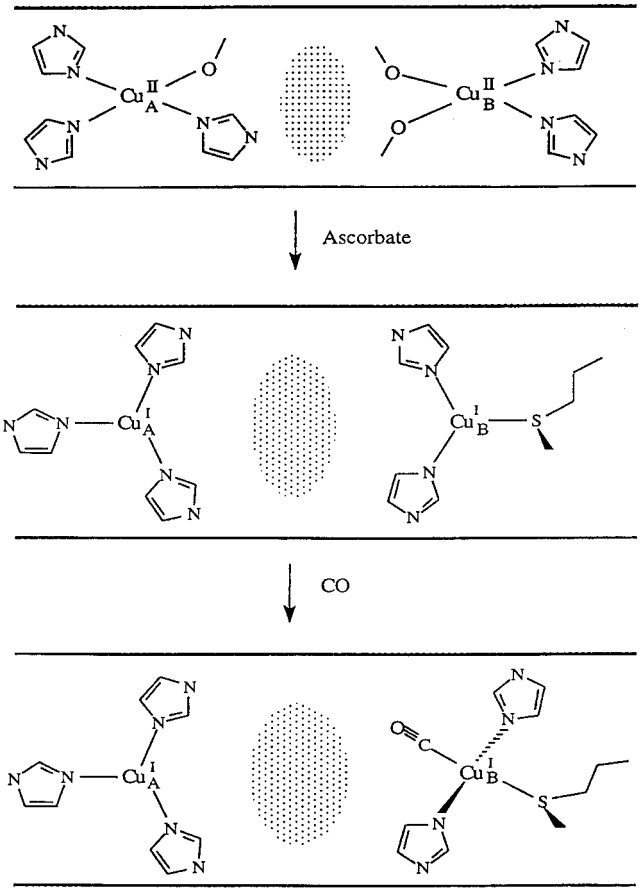
Many lines of evidence now support the validity of eq 14. Early studies of the stereochemistry of glycine oxidation had shown abstraction of its pro-S hydrogen.[56] In l989, Young and Tamburini were able to prepare both diastereomers of the α-hydroxyglycine intermediate **2** and show that only one of the diastereomers was enzymatically converted to product.[57] Subsequent studies by Ping *et al.* demonstrated that the active diastereomer has the (*S*) configuration;[58] this is the product expected from hydroxylation of glycine by retention of configuration (the observed stereochemical path of all monooxygenases, including Dβ M).[57−61] These experiments required the ability to prepare stable samples of **2**. Although **2** might appear to be a highly unstable structure, it is quite long-lived at low pH [half-time of about 7 days at pH 5.5[58]]. Since the intragranular pH of the storage vesicles where PAM is localized is acidic,[45] the requirement for an enzyme activity capable of hydrolysis of **2** is immediately apparent. May and coworkers were the first to isolate and characterize a separate enzyme function catalyzing the hydrolysis of α-hydroxylated glycine derivatives.[63] The elegant and detailed characterizations of the gene structure for PAM (see above) clearly predict that PAL will exist both as a separate activity and within a bifunctional enzyme. In retrospect, the initial ability of investigators to assay samples of monofunctional PHM (in the absence of PAL) was likely due to the high pH of the assay mixtures (conditions where **2** and its analogs hydrolyze rapidly). Additional experimental findings in support of eq 14 are (i) the ability to isolate O-18-labeled α-hydroxyglycine intermediate when the oxidative reaction is run with O-18-labeled dioxygen[64,65] [no label is incorporated from O-18 water[65]] and (ii) the demonstration of O-18 incorporation into pyruvate (converted *in situ* to lactate) when dansyl-Tyr-Val-D-Ala was used as substrate.[66]

In the context of the strong chemical analogy between the PHM portion of PAM and Dβ M, Southan and Kruse investigated sequence similarity between these two proteins.[73] It was found that a region of about 300 amino acids showed strong homology. Although Dβ M is significantly larger than PHM, the conserved region represents the central ∼300 amino acids for both proteins. Within this region, the authors could locate all of the residues which were known to become covalently labeled in the course of mechanism-based inhibition of Dβ M, a His-His and His-Thr-His motif, a conserved His lying between these two motifs, two conserved methionines, and five of the cysteines in Dβ M. Integrating these findings into a structural model for Dβ M subsequently proposed by Robertson *et al.*,[24] it is possible to propose two putative copper-binding domains. The first domain contains the His-His motif at positions 248 and 249 (using the numbering scheme of Dβ M) and a disulfide linkage surrounding these residues (between 218 and 269). The second putative domain contains His-Thr-His at residues 398−400, a disulfide bond between residues 452 and 474 and methionine at 473. Recent mutagenesis studies on PHM (see below) implicate methionine 473 as the sulfur ligand which becomes bound to the Cu_B site in reduced enzyme.

Consistent with the noted sequence homologies, it has been demonstrated that two copper atoms per subunit are required for optimal PHM activity.[75] Both of these copper centers undergo reduction to an EPR silent form in the presence of ascorbate, followed by reoxidation to Cu(II) in the presence of substrate.[76] Using the recombinant bifunctional PAM, Blackburn and his co-workers have begun to explore structural details of the copper binding sites by EPR, EXAFS, and FTIR.[72] Some of their most important findings are: (i) an ability to observe both copper centers by EPR in oxidized enzyme, implicating mononuclear sites; (ii) the presence of 2−3 histidines and 1−2 oxygen or nitrogen ligands at the copper sites in oxidized enzyme; and (iii) a large structural perturbation on enzyme reduction. Of particular note is the appearance of a heavy atom scatterer (Cl^-/S) as a ligand at one of the copper sites in reduced enzyme. Of potential importance, the EXAFS data for the Cu(I) form of PAM appear more resolved than those for Dβ M, suggestive of 3-coordination or possibly lower at the copper center. The model put forth by Boswell *et al.* for PHM is shown in Scheme 5. At this stage of investigations, it can be concluded that PAM shows a strong similarity to Dβ M with regard to the nature of the copper-binding sites.

Two advantages of the PHM system over that Dβ M are first, that PHM can be isolated from a relatively high level expression system and second, that expressed PHM is comparatively small. The second

Scheme 5. Model for the Geometry and Ligands to the Copper Sites in PAM (Adapted from Ref 72)



property should facilitate future crystallographic characterization of PHM, while the first has already allowed site specific mutagenesis experiments to be performed. In the first study of this kind,[74] Eipper *et al.* have mutated His 108 (within the His-His motif) to Ala and His 244 (within the His-Thr-His motif) to Ala; both of these changes completely eliminate PHM activity. Mutation of two methionines was also undertaken, with the finding that methionine 314 (number 473 in DβM), but not 109 is critical for activity. These results provide strong support for the postulated copper-binding sites, as well as for an essential role of a sulfur-containing side chain in the binding of reduced copper to DβM and PAM. With regard to the catalytic mechanism proposed for DβM (see Scheme 3 above), Eipper *et al.* mutated one of the tyrosines known to be alkylated by mechanism-based inhibitors in DβM to a Phe (Tyr 79 in PAM, number 216 in DβM). Analysis of enzyme activity with Y79F indicated at most a sevenfold decrease in k_{cat}/K_m with no effect on k_{cat}. Unfortunately, no detailed kinetic experiments have yet been conducted on PHM, although it is anticipated that this enzyme will be similar to DβM. If this is the case, the measured k_{cat}/K_m for PAM is expected to reflect steps for the hydroxylation of substrate, whereas k_{cat} will reflect product release. In any case, the decrease in k_{cat}/K_m is not dramatic and argues that tyrosine 79 is not essential for activity. If the mechanism in Scheme 3 above is obligatory, a different residue would be required to function in cleavage of the copper hydroperoxide intermediate. This may be Tyr 318, the analog of a second Tyr in DβM (477) demonstrated to be alkylated by mechanism-based inhibitors. It is also possible that an alternate pathway can predominate when the residue interacting with copper hydroperoxide is altered or absent. As indicated in the discussion of DβM mechanism, the energy for abstraction of a benzylic hydrogen is similar to that for abstraction of a hydrogen atom from a phenolic group, such that direct interaction of substrate, rather than an amino acid side chain, with a copper-hydroperoxide intermediate may occur with compromised enzyme. However, caution is needed at this point, lest premature interpretation occurs. Additional in-depth studies of PHM are much needed, in particular, the measurement of deuterium and O-18 isotope effects, such that mechanism and transition-state structures can be directly compared between the two enzymes.

III. Copper Amine Oxidases

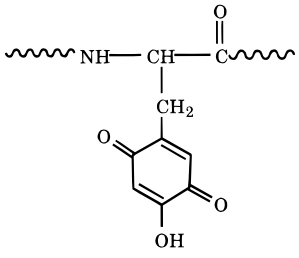
A. General Background

In contrast to DβM and PAM, the copper amine oxidases (CAO's) are ubiquitous, occurring in bacteria, yeast, plants, and mammals (for recent reviews see refs 77−79 and 159). These proteins are almost always observed to be dimers of 75−80 kDa subunits, with each subunit containing a single cupric ion.

The function of the CAO's in prokaryotes appears fairly straightforward, allowing growth of microorganisms on primary amines as a nitrogen source via an oxidative release of ammonium ion:

$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_4^+ \quad (15)$$

While it is clear that the CAO's in higher eukaryotes must regulate biogenic amine (e.g., dopamine and histamine) levels through oxidative metabolism, the precise number, location, and regulation of these amine oxidases remains to be elucidated. A second feature distinguishing the CAO's from DβM and PAM is the presence of an organic cofactor in the CAO's. The nature of this elusive cofactor has only recently been shown to be a peptide-bound quinone (topa quinone or TPQ):[80]



Thus, the copper amine oxidases belong to the new class of proteins designated quinoproteins. This class includes the prokaryotic proteins, which use pyrroloquinoline quinone (PQQ) as a dissociable cofactor,[81] and several bacterial methylamine oxidases which use peptide-bound tryptophan tryptophylquinone (TTQ).[82] Additionally, a new variant of quino-cofactor has recently been discovered in lysyl oxidase, indicating that lysyl oxidase can be formally distinguished from the family of copper amine oxidases.[83]

The presence of an organic cofactor in the CAO's raises many questions with regard to the structure and function of copper. First, where does the copper reside in relation to cofactor? Second, how does the cofactor interact functionally with copper in the course of the catalytic mechanism? Lastly, what is the role of copper in the biogenesis of TPQ? This field has been moving quite rapidly in recent years, and at least partial answers are now available for each of these questions.

B.Structural Characterization of the Copper-Binding Site and Relation to Topa Quinone

Extensive biophysical characterizations of the copper amine oxidases have been conducted over the past 15 years. Through a combination of EXAFS, pulsed EPR, and NMR solvent relaxation studies, the copper site in resting enzyme was concluded to be in the cupric state and to be of square-pyramidal geometry, containing three equatorial histidines, an equatorial water, and an axial water as ligands.[84−86,160] Distance mapping experiments using NMR, EPR, and fluorescent energy transfer provided an estimate of the relationship between copper and the reactive carbonyl of the active site organic cofactor. Although the original data were interpreted in the context of active site PQQ as cofactor, subsequent reanalysis of the data in the context of TPQ implicated a distance of ∼3.0Å between the C-2 oxygen of the quinocofactor and copper.[87,88]

These basic features have been beautifully confirmed by the recent crystal structure of a phenethylamine oxidase from *E. coli*.[89] Structures of two crystal forms have been published: the first structure (at 2 Å resolution) is from inactive crystals grown from ammonium sulfate, whereas a second, lower resolution structure is from crystals prepared in citrate buffer. A schematic of the active site from this work is shown in Figure 4a. The three histidine ligands are shown in blue, and the two water molecules are shown as small yellow spheres. Although the TPQ ring in the inactive crystals is actually coordinated to copper, the cofactor ring has flipped by 180° in the active form and is facing toward Asp 383. A water molecule now bridges the copper ion and the ring of TPQ.

Prior to the availability of a crystal structure for a copper amine oxidase, numerous DNA-derived protein sequences had been aligned and used to predict the histidines ligands for copper. A conserved His-X-His motif was observed in all proteins at ∼50 amino acids toward the C-terminus from the TPQ precursor.[90−92] The crystal structures indicate that this motif does indeed provide two of the three histidine ligands to copper (His 524 and His 526 in *E. coli* enzyme). The third histidine ligand was more controversial and was originally attributed to a conserved histidine at ∼30 amino acids toward the N-terminus from the TPQ precursor.[92] However, this conclusion was based on an incomplete sequence for the lentil seedling amine oxidase. A recent alignment of 10 copper amine oxidases (containing the corrected lentil seedling sequence) showed a fourth conserved histidine near the C-terminus of all copper amine oxidases.[93] The crystal structure confirms that this histidine is the third ligand to copper (His 689 in *E. coli* enzyme), with the other conserved histidine (His 440 in *E. coli* enzyme) playing a role in intersubunit interactions.[89] From Figure 4a, it can be seen that a Tyr 369 and Glu 695 lie near the copper ligands. These residues are conserved in all amine oxidases and are expected to play critical roles in copper binding and/or reaction mechanism. Two of the conserved amino acids surrounding the TPQ, Thr 462 and Asp467, undergo unanticipated electrostatic/H-bonding interactions with the conserved His 440 of the second subunit. From Figure 4b, it can be seen how hairpin loops containing TPQ and His 440 on one subunit interact with Thr 462 and Asp 467 on a second subunit. The functional role of this subunit−subunit interaction is currently unknown.

C. Interaction between the Copper and Cofactor in the Course of the Catalytic Mechanism.

The finding of a new organic cofactor in the copper amine oxidases raises many questions regarding its catalytic mechanism and its interdependence on copper. The reaction of the CAO's can be formally divided into reductive and oxidative half-reactions as shown below:

reductive half-reaction

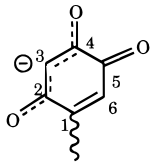
$$E_{ox} + RCH_2NH_2 \rightarrow E_{red} + RCHO \qquad (16)$$

oxidative half-reaction

$$E_{red} + O_2 + H_2O \rightarrow E_{ox} + H_2O_2 + NH_3 \qquad (17)$$

In support of eqs 16 and 17, steady-state kinetics for the CAO's indicate a ping-pong pattern with regard to amine substrate and dioxygen [reviewed in refs 94 and 95]. Under pre-steady-state conditions, it is possible to monitor the reduction of enzyme by substrate in the absence of dioxygen; kinetic constants obtained under these conditions agree well with steady-state parameters for the reductive half-reaction.[96]

A number of experimental approaches have shown that E_{ox}, containing topaquinone, **3** is converted to the aminoquinol **4** in E_{red}:



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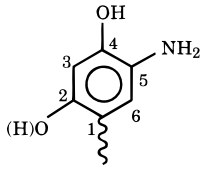


4

This indicates an aminotransferase reaction for the copper amine oxidases, in which substrate nitrogen is transferred to the cofactor ring (cf., ref 78). The involvement of the C-5 carbonyl of TPQ in this process is supported by a large number of studies showing the reactivity of the C-5 position of TPQ analogs toward nucleophilic reagents.[97−100] It also has been possible to synthesize an analog of **4** and show that it has the absorbance properties of reduced enzyme.[97]

As indicated in structure **3**, TPQ will be ionized under physiologic conditions, a consequence of its low pK_a[97] (4.2 in model compounds and ∼3 in bovine

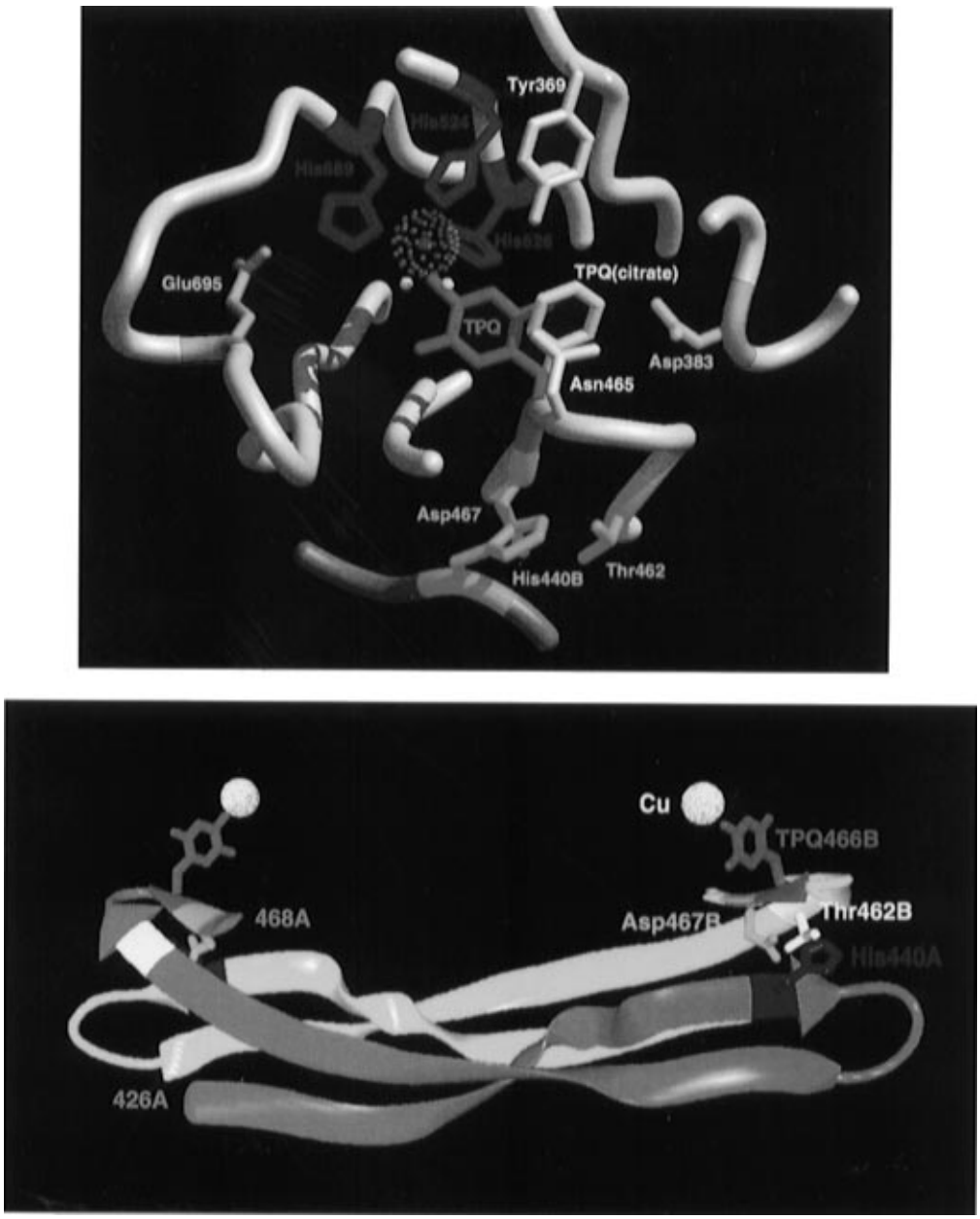
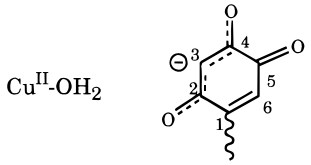


Figure 4. (a, top) Active site of the *E. coli* amine oxidase. The polypeptide chain is white for subunit A and grey for subunit B. The position of TPQ in the inactive crystal is shown complexed to copper (both are green). In the active crystal, the cofactor ring has rotated (shown in yellow). Note the presence of two water molecules (shown as yellow balls). (b, bottom) β-Hairpins linking the active sites of the *E. coli* amine oxidase monomers. Subunit A is in red and subunit B in cyan. The copper atoms are shown as green van der Waals dot surfaces. Note the interaction of His 440 on one subunit with Thr 462 and Asp 467 on the other subunit. (Reprinted from ref 89. Copyright 1995 Current Biology Ltd.)

serum amine oxidase). The acidity of the C-4 hydroxyl group leads to a resonance-stabilized structure, with reduced electrophilicity at the C-2 and C-4 positions. Recent resonance Raman studies by Saunders-Loehr and co-workers on CAO's from a number of sources have indicated a band at about 1680 cm^{-1}, attributed to a localized carbonyl at C-5, and a band with less double-bond character at about 1390 cm^{-1}, attributed to a resonance delocalized structure involving carbons 2 and 4.[101,102] Extensive delocalization of charge between the oxygens on carbons 2 and 4 is also indicated from UV/vis absorbance spectra, which show identical λ_{max} values for free and protein bound TPQ.[80]

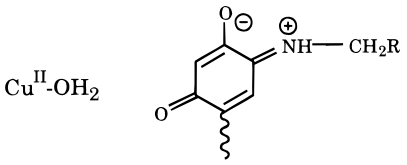
From the available crystal structure of the active form of the *E. coli* amine oxidase, it is not yet possible to delineate the precise position of the TPQ ring. However, the currently available structures clearly indicate the position of Asp 383, a conserved residue likely to function as the catalytic base in substrate oxidation. If we use Asp 383 to orient the topa ring, the following relationship of TPQ to active site copper can be inferred:



This indicates that a copper-bound water is, at least in principle, in a position suitable for hydrogen bonding to the oxyanion at C-2 of the cofactor. However, as indicated above, there is no evidence for

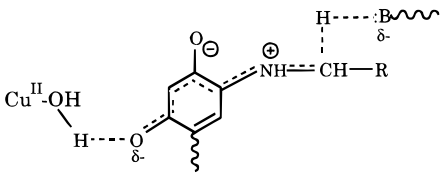
any increased localization of charge at the oxygen at C-2, i.e., comparison of spectroscopic data for CAO's to TPQ model compounds supports full delocalization of charge between C-2 and C-4.

Hartman and Klinman have carried out extensive chemical and kinetic studies to characterize intermediates in the conversion of **3** to **4**. Their studies show the presence of a substrate Schiff base with absorbance at around 350 nm that can be reductively trapped by cyanoborohydride.[103] From spectroscopic characterization of adducts of amines with model TPQ compounds, the structure for the enzyme-bound substrate Schiff base complex has been concluded to have considerable *p*-quinostructure, attributed to increased charge localization on the oxygen at C-4 through an electrostatic interaction with protonated imine at C-5,[99] **5**. Since little charge is anticipated at the C-2 oxygen, interaction with copper-bound water may be minimal for this intermediate.

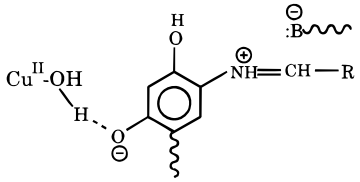


5

The conversion of **5** to product Schiff base, **7**, is generally agreed to occur via a base-catalyzed abstraction of a proton from the α-carbon of substrate. Studies of structure-reactivity correlations with bovine serum amine oxidase, using a series of ring-substituted benzylamines, indicate a transition-state structure for this C−H abstraction process with considerable development of negative charge,[104] **6**:



6



7

For bovine serum amine oxidase, the kinetic pK_a for the interaction of substrate with free enzyme is 8.0, and this pK_a is reduced to 5.6 in the enzyme−substrate complex.[105] Examination of the recent crystal structure of the *E. coli* enzyme indicates that Asp 383 is buried in a hydrophobic pocket, such that a pK_a of 8.0 would not be out of line for the resting form of enzyme. Binding of substrate to cofactor to generate a protonated Schiff base would introduce an electrostatic interaction capable of lowering the pK_a of Asp 383 to 5.6. Thus, the kinetic data obtained with bovine serum amine oxidase, together with the crystal structure for the *E. coli* enzyme, support the idea that a single carboxylate gives rise to the observed pK_a values in catalysis.

One feature that initially appeared to cloud the interpretation of the amine oxidase mechanism was the possibility that the ring hydroxyl of TPQ would function as the catalytic base. However, the finding of the same pK_a of 5.6 for both substrate oxidation (occurring from the oxidized form of TPQ) and tritium exchange from the product Schiff base of phenethylamine (occurring from the reduced form of TPQ)[105]) rules out this mechanism. Model studies clearly show an ∼5 order of magnitude increase in the pK_a values for TPQ in going from its oxidized to reduced forms.[97]

In contrast to structures **3** and **5**, examination of the structure of the product Schiff base, **7**, suggests an important interaction with the copper-bound water. Note that the cofactor is formally reduced within the product Schiff base complex. Since the pK_a values of reduced cofactor are ≥9,[97] the ring oxygens are expected to undergo protonation as cofactor gets reduced. One of the required protons has previously been proposed to be transferred from the transiently protonated active site base to the oxygen at C-4,[105] but what about the oxygen at C-2? Since this is the position proposed to be in closest proximity to the copper-bound water, a reasonable expectation is that the oxyanion at C-2 of reduced cofactor will enter into a hydrogen bond with or accept a proton from the copper-bound water. A similar interaction may also occur in the transition state leading to **7** and in the aminoquinol, **4**, formed following the hydrolysis of aldehyde from the product Schiff base.

The above discussion suggests that one of the water ligands to copper may play an important role in the neutralization of charge in species **4**, **6**, and **7**. Of the two water molecules bound to the active site copper, spectroscopic characterizations lead to the conclusion that it is the equatorial water which undergoes replacement by exogenously added ligands such as azide and cyanide.[106] Regarding the simple exchange of bound waters with bulk solvent, the amine oxidases appear to fit into two separate classes: those that show rapid exchange of both waters (e.g., the pig plasma amine oxidase) and those that undergo rapid exchange solely at the axial water (e.g., bovine serum amine oxidase).[107] From the crystal structure for a 2-hydrozidopyridine derivative of the active form of the *E. coli* amine oxidase, Knowles and co-workers have concluded that it is the axial water which is proximal to the C-2 position of TPQ.[108]

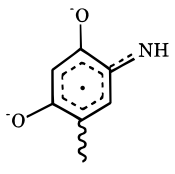
One question that has never been fully resolved is whether the copper-free form of amine oxidase can oxidize substrate under single turnover conditions. In this context, Renaldi *et al.* have reported that the apo form of the lentil seedling amine oxidase catalyzes the release of one mole of aldehyde product per enzyme subunit using *p*-diaminobenzylamine as substrate. This finding needs to be explored further with the lentil seedlings and other amine oxidases. Recent model studies have established that TPQ analogs catalyze the oxidation of amines in the absence of copper.[97−100] In the case of the enzymatic reduction of dioxygen, it is generally agreed that copper is a

key component. For many years, it was thought that the active site copper remained in its +2 valence state throughout the catalytic cycle. However, an important early experiment indicated the appearance of an organic radical upon reduction of enzyme by substrate in the presence of cyanide ion.[106] Although cyanide has been shown to interact with amine oxidases at multiple sites,[110] a reasonable explanation for the observed behavior was the binding of CN^- to a reduced copper site, thereby trapping a semiquinone form of cofactor.[106] More recent experiments by Dooley and co-workers have provided direct evidence for copper reduction following the interaction of amine oxidase with substrate under anaerobic conditions.[111] By varying the temperature at which EPR spectra were collected, these authors have shown a diminution of the enzyme bound Cu(II) signal, concomitant with the appearance of a signal for an organic radical. They deduced that the position of equilibrium for the conversion of oxidized copper and substrate reduced cofactor to reduced copper and cofactor semiquinone, eq 18, is affected by temperature:

$$\text{E-Cu}^{\text{II}}\text{-TPQ}_{\text{RED}} \underset{\text{low temperature}}{\overset{\text{physiologic temperature}}{\rightleftharpoons}} \text{E-Cu}^{\text{I}}\text{-TPQ}_{\text{SQ}} \quad (18)$$

Only when EPR spectra were analyzed at elevated temperature was it possible to detect the intramolecular electron transfer leading to $\text{E-Cu}^{\text{I}}\text{-TPQ}_{\text{SQ}}$. The kinetic competence of the process in eq 18 is supported by temperature jump studies, which indicate that all but 11% of the observed absorbance changes (arising from semiquinone) occur with a rate constant greater than or equal to 4000 s^{-1}.[112] This value is far in excess of earlier measured rate constants for amine oxidase catalysis under either steady-state or pre-steady-state conditions.

Several features of the enzyme-bound TPQ semiquinone are worth noting. First, the amount of semiquinone detected in EPR experiments is always less than expected from the loss of the Cu(II) spectrum, attributed to an intra- or intermolecular disproportionation of semiquinone to form oxidized quinone and reduced aminoquinol.[111] Second, the unpaired electron in semiquinone couples to a nitrogen nucleus which is shown to derive from substrate using isotopic labeling.[113] Third, analysis of the structure of the semiquinone by electron spin echo envelope modulation and continuous wave ENDOR indicates only a single proton at the substrate derived nitrogen,[114] **8**:



8

In this way, the enzyme-bound TPQ_{SQ} appears structurally closer to the oxidized than reduced cofactor.

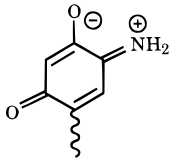
With the implication of a Cu^{I}−semiquinone intermediate in amine oxidase catalysis, it becomes of interest to inquire whether ligation at the Cu(I) site will be altered relative to that seen in the oxidized form of enzyme. Analogous to other copper proteins (e.g., Scheme 5), a ligand(s) at the copper site in CAO is expected to be lost as the metal is reduced. One likely candidate in the amine oxidase is the equatorial water, known to be displaced by exogenous ligands.[106] This would create a site for complexation to oxygen intermediates as dioxygen undergoes reduction to hydrogen peroxide. Presumably, dioxygen is first reduced to superoxide, concomitant with the oxidation of copper and formation of a $\text{Cu}^{\text{II}}\text{-O}_2^{\bullet}$ complex. Subsequent formation of a metal peroxide anion, by transfer of one electron to the metal superoxide intermediate (see eq 19, below), may be quite favorable, especially if the driving force for conversion of the semiquinone of cofactor to oxidized TPQ is high:

$$\text{E-Cu}^{\text{I}} + \text{O}_2 \rightleftharpoons \text{E-Cu}^{\text{II}}\text{-O}_2^{\bullet} \rightleftharpoons \text{E-Cu}^{\text{II}}\text{-O}_2^{-} \rightleftharpoons \text{E-Cu}^{\text{II}}\text{-O}_2\text{H} \rightarrow \text{E-Cu}^{\text{II}} + \text{H}_2\text{O}_2$$

(TPQ_{SQ}, TPQ_{SQ}, TPQ_{ox}, H^+, TPQ_{ox}, H^+, TPQ_{ox}) (19)

Using $HO_2^{\bullet}$ and HO_2^- as models for metal-complexed intermediates, the half-potential for $\text{Cu}^{\text{II}}\text{-O}_2^{\bullet} \rightarrow \text{Cu}^{\text{II}}\text{-O}_2^-$ can be estimated as 0.8 V vs NHE.[158] Although we do not know the potential for $\text{TPQ}_{\text{SQ}} \rightarrow \text{TPQ}_{\text{ox}}$, cyclic voltammetric studies of TPQ model compounds consistently show a two-electron wave [$E_m = -0.15$ V vs SCE for the hydantoin of TPQ[97]], with no evidence for stable radical intermediates.[97] Unfortunately, electrochemical studies of TPQ at the enzyme active site have thus far been precluded by the presence of two redox centers (Cu(II) and TPQ), as well as the absence of any apparent channel for a redox mediator to access the TPQ. The sequence of events shown in eq 19 is expected to be completed by an initial proton-transfer step to generate a metal-bound hydroperoxide and oxidized TPQ and a final proton transfer coupled to release of bound H_2O_2.

At this stage in the reaction cycle, TPQ_{ox} will be in an iminoquinone form, **9**:



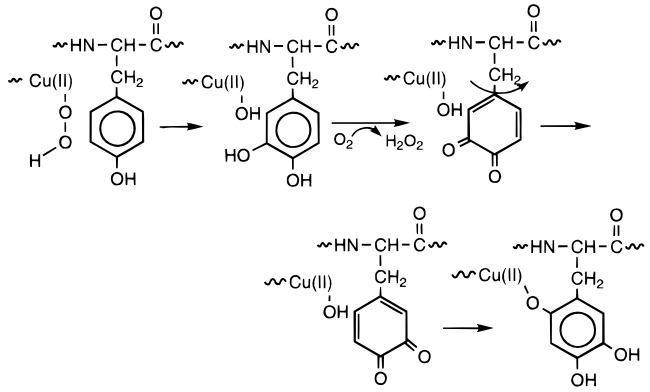
9

Under steady-state conditions, the lifetime of **9** will determine whether this species undergoes hydrolysis by water or a transimination reaction with substrate to generate **5** directly. For the future, much remains to be learned regarding this oxidative half-reaction, in particular, the coordination number and geometry of reduced copper in the $\text{E-Cu}^{\text{I}}\text{-TPQ}_{\text{SQ}}$ complex, the nature of rate-limiting steps in oxygen reduction, and the relative stabilities of the postulated intermediates.

D. Role of Copper in Topa Quinone Biogenesis

One of the unusual features of TPQ is its extreme toxicity as a free amino acid (where it is referred to as 6-hydroxydopa).[115] Neurobiologists have used 6-hydroxydopa as a neuron-specific cytotoxic agent for decades. The mechanism by which 6-hydroxydopa kills cells is not known with certainty, but it is

Scheme 6. Postulated Mechanism for the Biogenesis of TPQ from Tyrosine (Reprinted from Ref 122. Copyright 1994 American Chemical Society)



likely to derive both from the ability of 6-hydroxydopa to bind as an agonist to the glutamate receptor[116]) and the relative ease with which this amino acid is oxidized [E_m = 0.079 V vs NHE at pH 7[117]]. Whatever the pathway for TPQ biogenesis, it is to be expected that this process will be carefully regulated.

Once it had been demonstrated that the amino acid precursor to TPQ in proteins was tyrosine[118] and that this precursor amino acid was contained within a consensus sequence of Asn-Tyr-Asp/Glu[119] two hypotheses were advanced for TPQ formation: the first postulated a separate set of enzyme(s) capable of recognizing the consensus sequence containing the Tyr precursor, while the second proposed a "self processing" mechanism using the protein-bound copper.[118] As illustrated in Scheme 6, Mu *et al.* postulated that the first step in TPQ formation would involve hydroxylation of Tyr to dopa, followed by an oxidation of dopa to dopa quinone. Rotation of the dopa quinone by 180° was predicted to place the C-2 position of this reactive intermediate near a copper-bound hydroxide ion or water. Subsequent nucleophilic attack by water (or hydroxide) on the dopa ring leads directly to the quinol form of TPQ. This intermediate was expected to undergo rapid oxidization in the presence of dioxygen, analogous to the oxidation of the aminoquinol formed during catalytic turnover.

The most straightforward way to distinguish a self-processing mechanism from one that requires additional enzyme(s) is to express an amine oxidase gene under conditions of low copper and to show that the addition of copper to apoprotein leads to the concomitant production of quino-cofactor and enzyme activity. This approach has been successfully applied in the expression of two *Arthrobacter globiformis* amine oxidases in *E. coli.* Although *E. coli* produces its own amine oxidase, this does not appear to be present at a high enough level to compromise the experiments. In the case of both a phenethylamine oxidase[120] and histamine oxidase[121] from *A. globiformis*, it was possible to isolate and purify copper-free enzyme and show that the addition of cupric ion leads to generation of topa quinone, together with the appearance of enzyme activity. In both instances, the conversion of precursor to active protein was absolutely dependent on molecular oxygen.

A different approach was taken with a eukaryotic system in which the gene for a methylamine oxidase from *Hansenula polymorpha* was expressed in *Saccharomyces cerevisiae. S. cerevisiae* appears to be ideal for expression of amine oxidases, since no evidence exists for an endogenously produced amine oxidase in this strain of yeast. When the recombinant *H. polymorpha* enzyme was isolated from *Saccharomyces cerevisiae* cells grown on normal media, it was found to be fully active.[122] Given the presumed lack of processing enzymes for topa quinone production in *S. cerevisiae*, this initial result pointed toward a self-processing mechanism. It was subsequently shown that enzyme produced from media depleted in copper contained approximately 30% copper, together with 30% topa quinone and enzyme activity. However, attempts at further addition of copper failed to give any increase in levels of copper or quinocofactor. It is now known that expression of the eukaryotic enzyme under reduced copper leads to production of a zinc metallo-enzyme.[123]

In light of the inability to carry out simple metal addition experiments to the eukaryotic amine oxidase, a different approach was taken using site-specific mutagenesis.[124] The first mutant to be prepared was within the consensus sequence, involving replacement of the Asp/Glu at the +1 position toward the C-terminus from TPQ with an Asn. It was reasoned that disruption of the putative recognition consensus sequence would lead to failure to produce quinocofactor and enzyme activity. Instead, fully active enzyme was obtained when either ethylamine or benzylamine was used to assay for enzyme activity. Sequencing of the active-site peptide showed that Asn was present, i.e., that it had not been hydrolyzed to Asp. This result ruled out a critical role for the highly conserved sequence in TPQ biogenesis. The second site of mutation involved one of the His ligands to copper. Although *H. polymorpha* amine oxidase contains a His-X-His-Y-His motif, rather than the usual His-X-His motif, the central His of the extended motif was assumed to bind copper. Mutation of this His to Asp eliminated copper binding, TPQ production and enzyme activity. Clearly, disruption of the copper binding site had prevented conversion of the Tyr precursor to TPQ in the yeast amine oxidase. Taken together, the available data implicate a role for the protein-bound copper in the biogenesis of TPQ production in both pro- and eukaryotic systems.

More recent experiments of Hanlon *et al.* have used an *E. coli* K-12 phenethylamine oxidase gene with a mutated leader sequence.[125] This gene leads to accumulation of inactive, cytoplasmic protein. Dilution of crude extracts of protein from cell lysates showed a time-dependent production of both amine oxidase activity and quinone. These authors have postulated that an inhibitor may be present in cell extracts which loses its potency on dilution. Another, more simple explanation for their data is that the ratio of exogenous copper to the apoform of protein increases with increasing dilution, leading to faster rates for copper binding and cofactor biogenesis.

With the evidence in support of a self-processing mechanism for TPQ formation, the challenge is to elucidate the chemical pathway for this process. In a recent, beautiful experiment, Tanizawa, Sanders-Loehr, and co-workers[102] have shown that incubation

of the apoforms of the *Arthrobacter globiformis* phenethylamine oxidase and histamine oxidase with O-18 labeled water and cupric ion leads to incorporation of O-18 into the C-2 position of TPQ in both cases. These results are as predicted by Mu *et al.*,[118] for a mechanism in which an intermediate dopa quinone undergoes nucleophilic addition from a metal bound water/hydroxide (Scheme 6). A useful model for this portion of the reaction comes from a recent study showing the oxidative conversion of 4-methylcatechol to 2-hydroxy-5-methyl-1,4-benzoquinone.[126] The far more difficult and less understood portion of the biogenesis reaction is the oxidative conversion of Tyr to dopa. For example, is there a copper hydroperoxide formed as the hydroxylating agent or is the Tyr precursor directly oxidized by copper? As a corollary to these questions, at what stage of the reaction does dioxygen enter, i.e., is O_2 necessary to generate oxidized Tyr intermediates, or is O_2 the agent that traps a preformed Tyr radical?

The only study to address these questions thus far comes from the laboratory of Tanizawa and coworkers on the apoform of the phenethylamine oxidase from *A. globiformis*.[127] These authors have shown that reconstitution of enzyme with cupric ion in the absence of dioxygen leads to enzyme with surprisingly little signal in the EPR. The subsequent addition of a limiting amount of oxygen leads to a time-dependent increase in the Cu(II) signal, as well as the slower appearance of an organic radical. Since addition of excess oxygen eliminates the signal due to organic radical, this is concluded to be a transient intermediate in cofactor biogenesis. An analysis of the fine structure of the organic radical indicates that it bears a strong resemblance to the semiquinone formed from aminoquinol in enzyme which has been reduced by substrate under anaerobic conditions. Thus, Matsuzaki *et al.*, propose that the ϵ-amino group of a lysine adds to the C-2 position of tyrosine in the course of TPQ formation.[127] This conclusion is puzzling for a number of reasons: first, if an amine were to add to C-2 of a TPQ precursor, it would soon be required to undergo displacement by water, i.e., there does not appear to be any chemical advantage to such a process. Second, there is no evidence for an active-site lysine in the crystal structure of the *E. coli* amine oxidase. It is, of course, possible that the three-dimensional structure of the precursor form of amine oxidase is quite different from that of mature protein. Although Frébort *et al.* have reported that 1,4-diamino-2-butyne labels a lysine (at position 356) in the course of its inhibition of an amine oxidase from *Aspergillus niger*,[128] it is possible that oxidation of this inhibitor leads to an intermediate with sufficient lifetime to diffuse away from the cofactor and label a non-active-site side chain. Clearly, a full understanding of the time course for the changes in EPR signal following addition of copper to apoenzyme, as well as the nature of the intermediates leading from tyrosine to TPQ, must await further investigations.

IV. Galactose Oxidase

A. General Background

Galactose oxidase (GO) is a fungal protein, comprised of a single polypeptide chain (68 kDa) with one copper atom per active center [for reviews, see refs 129−131]. This enzyme catalyzes the two-electron oxidation of primary alcohols to aldehydes, which themselves can serve as substrates to yield carboxylic acids.[134] In the course of a catalytic cycle, dioxygen becomes two-electron-reduced to hydrogen peroxide:

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \qquad (20)$$

Galactose oxidase shows a broad specificity, oxidizing low molecular weight alcohols such as D-galactose and dihydroxyacetone, as well as the non-reducing end of D-galactose bound to a polysaccharide chain.[129,131] Although the physiological function is not known for GO, it has been speculated to center on the production of hydrogen peroxide for use in lignin degradation.[130]

The mechanistic history of this protein is long and quite fascinating. For many years, it was recognized that GO was isolated in a partially inactive, EPR-detectable form. Production of active enzyme could be effected by oxidants such as Mn^{3+}-EDTA, iridium hexachloride salts, porphyrexide, and ferrocyanide, leading to enzyme that no longer displayed an EPR signal. In an effort to explain the diamagnetic properties of active enzyme, two proposals had been put forth. The first invoked production of a Cu(III) species,[133] while the second proposed a Cu(II) protein containing residual paramagnetic oxidant bound close enough to the metal center to permit spin coupling.[134,136]

B. Reconciliation of Cu-EPR Spectra: A Copper Complexed Organic Radical

Our current understanding of GO began in the late 1980's, with the elegant spectroscopic studies of Whittaker and co-workers. Native enzyme, with an absorbance maximum at 445 nm was reduced to its inactive form using ferrocyanide.[137] Characterization of this inactive enzyme by EPR and UV/vis spectroscopy showed that it had an increased EPR signal but had lost its absorbance peak at 445 nm. Enzyme could then be fully reactivated with ferricyanide, leading to a restoration of the absorbance peak and elimination of the EPR signal. Activated enzyme had been gel filtered to remove excess oxidant, and the authors concluded that protein was free of adventitiously bound oxidant. As a result of these studies, the active form of GO was concluded to contain a Cu(II) site in close proximity to an organic radical. Reduction of this organic radical converted the enzyme to its inactive form:

$$\underset{\text{active}}{\text{E-Cu}^{II}\text{-X}^{\bullet}} \rightarrow \underset{\text{inactive}}{\text{E-Cu}^{II}\text{-X}} \qquad (21)$$

Definitive characterization of $X^{\bullet}$ became possible with the preparation of a copper-free form of galactose oxidase.[138] Oxidation of this enzyme yielded an intermediate with UV, near-IR, and EPR spectra of an aromatic radical. It was confirmed that enzyme had been oxidized at a catalytically relevant side chain, since addition of copper led to restoration of the activity and absorbance properties of fully active enzyme. On the working assumption that the organic radical was tyrosine, fungi were grown on

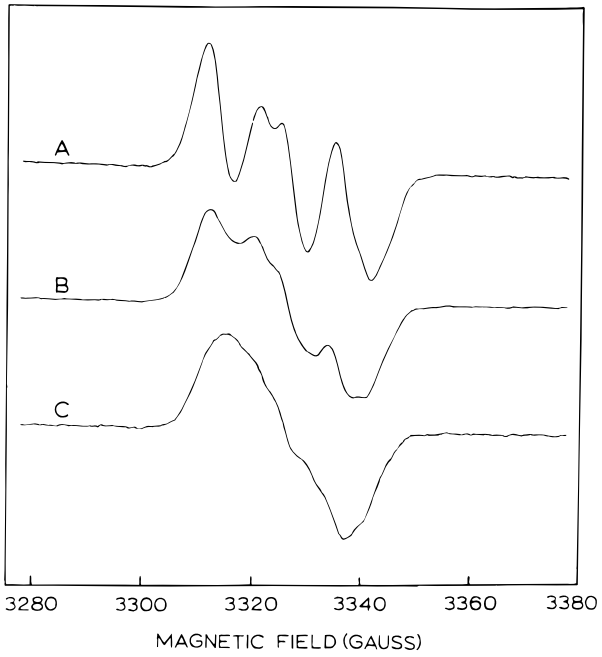
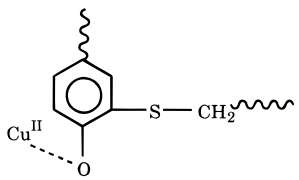


Figure 5. EPR spectra of the radical site in GO. A is the oxidized spectrum of native copper-free enzyme; B is the oxidized apoenzyme prepared from 70% [^{2}H]tyrosine-labeled protein; and C is a difference spectrum, obtained by subtracting 30% of spectrum A from spectrum B. (Reprinted from ref 138. Copyright 1990 Journal of Biological Chemistry.)

media containing [β,β-^{2}H]tyrosine, together with an inhibitor of aromatic amino acid synthesis. When the resulting GO, 70% deuterated at tyrosine, was demetalated and examined by EPR, it showed the expected loss of hyperfine structure arising from spin coupling processes with protonated tyrosine, Figure 5, thereby proving that X$^\bullet$ in galactose oxidase is Tyr$^\bullet$. Quite recent studies indicate a similar structure in an aldehyde oxidase (glyoxal oxidase) from the fungus *Phanerochaete chrysosporium*.[161]

In the initial characterizations of the Tyr radical in apogalactose oxidase, it became evident that this structure possessed unique structure and reactivity. With the availability of a 1.7 Å crystal structure for galactose oxidase,[139] two features became immediately apparent: first, that the sulfur of an active-site cysteine residue was within bonding distance of the C-3 carbon of Tyr 272 and second, that the ring oxygen of Tyr 272 was directly complexed to copper:

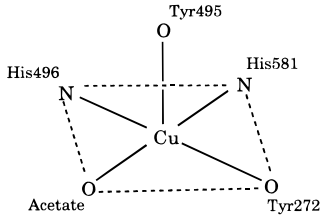


Although biochemical studies, showing a tyrosine cross-linked via a thioether to cysteine, have not yet been performed on GO, numerous experiments provide full support for the structure shown above. There is the finding that a mutant form of GO, in which Cys 228 has been changed to glycine, is 10 000 less active and migrates more slowly than wild type enzyme on gel electrophoresis.[140,141] The EPR and ENDOR spectroscopic properties observed with GO are consistent with the properties of thioether-substituted phenol models.[142] Of particular interest with regard to chemical reactivity, the redox potential for galactose oxidase is estimated as 0.4 to 0.5 V,[138] a much less positive value than seen for free tyrosine (0.93 V)[143] or the redox active tyrosines in photosystem II (0.76−1 V).[144] The lowered GO redox potential, which can be attributed in part to the electron-donating properties of the sulfur atom in the ring, explains the ease with which the active-site Tyr 272 undergoes oxidation. One unanswered question concerns the extent to which the properties of Tyr 272 are altered by π stacking with a second aromatic residue (Trp 290, see below). In a recent mutagenesis experiment, enzyme in which Trp 290 had been converted to His was reported to lack the 800 nm band of expressed wild-type enzyme.[141] While this observation suggested that the long wavelength absorbance in GO was due to a discrete interaction between the modified Tyr 272 and Trp 290, more recent experiments indicate that the 800 nm band is present initially in W290H but decays with time as the protein is desalted.[145] Clearly, the details of the modified tyrosine/tryptophan interaction will require more investigation.

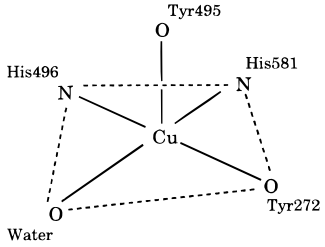
C. Structure of Copper Site and Relation to Substrate Oxidation

The crystal structure for the Cu(II) form of GO has been determined at pH values of 4.5 and 7.0, yielding the structures in Scheme 7.[146] As shown, the copper is square pyramidal, containing His 496, His 581, Tyr 272, and either an acetate or water as fourth ligand. A fifth, more weakly bound Tyr (495) is in the axial position of copper at a distance of 2.69 Å. Although not indicated in Scheme 7, Trp 290 is located above Tyr 272 in a position suitable for π stacking. Clark *et al.* have studied the oxidation states of the various forms of GO by XANES, finding an edge absorption characteristic of Cu(II) for both the fully active and reductively inactivated species[147,148] (cf., eq 21, above). When GO was complexed with substrate under

Scheme 7. Schematic Diagrams for the Copper-Binding Sites in GO (Adapted from Ref 146)

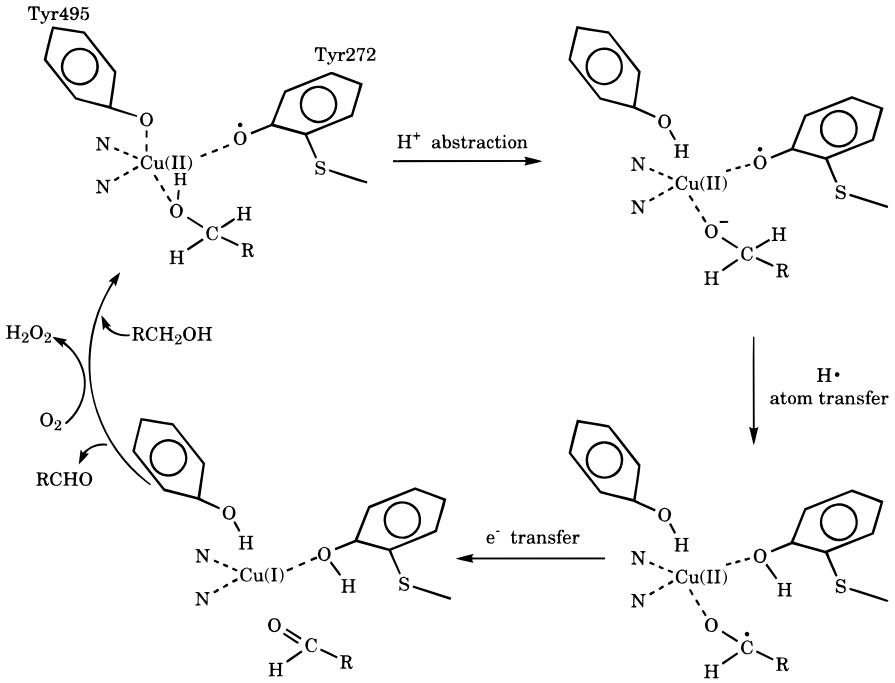


pH 4.5



pH 7.0

Scheme 8. Postulated Reaction Mechanism for GO (Adapted from Ref 149)



anaerobic conditions, the edge moved to a value characteristic of Cu(I), indicating a reduction of the copper site by substrate. Structural characterization of both the Cu(II) and Cu(I) forms of GO by EXAFS indicates that the Cu(I) in reduced enzyme has a changed environment, most likely via a decrease in its coordination number to 3.

Ligand binding to GO has been studied in an effort to explore the reactivity of the copper site. A particularly interesting finding is that binding of azide leads to the uptake of 0.8 protons per active site, attributed to the conversion of tyrosinate 495 to tyrosine by exogenous ligands.[149] Recent mutagenesis experiments in which Tyr 495 was converted to Phe show a reduction in k_{cat}/K_m of 1100-fold and, importantly, a loss of proton uptake on azide binding.[150] Resonance Raman studies of active and azide inhibited GO by McGlashen *et al.* have supported the conclusion that contributions from the bound tyrosinate are lost on azide binding.[151] In recent nuclear magnetic relaxation dispersion (NMRD) measurements, Knowles *et al.*, have also implicated a loss of a rapidly exchanging equatorial water in the presence of azide.[152] Unpublished crystallographic results are noted to support this result. Of mechanistic relevance, NMRD experiments in the presence of the substrate dihydroxyacetone look very similar to those with azide. A model which will accommodate all of these findings involves binding of the alcoholic position of substrate to copper in the position of the original equatorial water[146] (Scheme 8). Bound substrate is proposed to lose its proton which is taken up by tyrosinate at position 495. Subsequent oxidation of copper-bound alcoholate occurs via a hydrogen atom transfer to the modified tyrosine radical followed by electron transfer to copper to form the aldehydic product. The intermediary of a substrate-derived radical is supported by the demonstration of highly efficient inactivation of GO using a radical probe molecule which undergoes ring opening subsequent to reduction of the tyrosyl radical to tyrosine.[153] Wachter and Branchaud have also raised the possibility of a concerted mechanism in which the hydrogen atom and electron from substrate undergo simultaneous transfer to the tyrosyl radical and copper, respectively.[154]

According to the mechanism drawn in Scheme 8, dioxygen interacts with enzyme only after product aldehyde has dissociated from the active site. There is, however, some uncertainty regarding the point in the reaction sequence where dioxygen binds and whether galactose oxidase can be formally described by a ping-pong mechanism.[130,155] Although numerous kinetic studies had been performed on GO prior to the identification of its cofactor, many of these experiments used only partially active enzyme (cf., ref 129). Branchaud has now reported a useful procedure for the generation of stably activated enzyme.[156] One feature that has been seen consistently with GO is a large primary kinetic deuterium isotope effect.[129,155,157] With the extensive crystallographic and spectroscopic data as a back-up, this system is now "ripe" for an in-depth examination of the properties of the C−H bond cleavage step as well as the details of the interaction of dioxygen with enzyme.

V. Unifying Features and Perspectives for the Future

The four copper systems described in this review share the common property of reversible interconversion of protein-bound copper between its cuprous and cupric states. In general, an organic reagent (ascorbate in DβM and PAM, reduced TPQ in the CAO's, and alcoholic substrate in GO) reduces the cupric form of enzyme while dioxygen oxidizes the cuprous form. In all three cases, peroxide is formed (by two-electron reduction of O_2) as either an intermediate (DβM and PAM) or a product (GO and CAO's). A general mechanism appears to involve initial one-electron reduction of dioxygen by the

active site Cu(I) with the second electron coming from a remote Cu(I) site (DβM and PAM) or an organic cofactor (CAO's and GO). There is evidence in three of these enzyme systems (DβM, PAM, and GO) that the number of ligands coordinating to copper is reduced on metal reduction. These changes in ligand number and/or structure are expected to create a site for dioxygen interaction with the Cu(I) forms of enzyme and may also produce protein structural changes that control the course of the reaction. In addition to these similarities at the copper site that interacts with dioxygen, DβM and PAM must also catalyze an intersite electron transfer between their electron-accepting, Cu_A sites, and oxygen-reducing, Cu_B sites. In all cases, the characterized copper centers are found to contain at least two histidines as ligands.

Although the substrate that reduces copper is also the primary substrate of the reaction in GO, this is not so with the CAO's and DβM and PAM. In the case of DβM and PAM, the chemistry between copper and oxygen creates an oxidant capable of hydroxylation at the benzylic position of phenethylamines or the α-carbon of peptide-bound glycine. Analogous to GO, however, substrate activation occurs via hydrogen atom abstraction. As now understood in some detail, the oxidation of amine substrate in the CAO's occurs by proton activation at TPQ, in a mechanism with strong analogies to pyridoxal phosphate chemistry. The role of the new cofactor TPQ appears to be as a "transducer", converting an electrophilic catalyst (which reacts with substrate) into a reductant (capable of one-electron transfer to copper with concomitant formation of an organic semiquinone).

Although quantum mechanical tunneling has been demonstrated to contribute to the hydrogen transfer from substrate to cofactor at room temperature in the CAO's, this phenomenon has not yet been investigated in GO or DβM and PAM. As discussed in the context of the DβM mechanism, existing proposals for the nature of the oxidant in substrate hydroxylation are based on the assumption of largely classical behavior for hydrogen abstraction. It will be extremely important and interesting to ascertain whether quantum effects also play a significant role in DBM, PAM, and GO.

Unlike DβM and PAM, which as hydroxylases form a ternary complex between enzyme, dioxygen, and substrate, the reactions catalyzed by the CAO's and (most likely) GO can be formalized as two half-reactions, involving reduction of enzyme by substrate followed by reoxidation of reduced enzyme by molecular oxygen. In contrast to the better understood reductive half-reactions, relatively little is known regarding the oxidative half-reactions of the CAO's and GO. For example, is superoxide formed as an intermediate, and what are the lifetimes of the plausible intermediates in the conversion of free dioxygen to a protein−copper complexed hydroperoxy species (cf., eq 19, above)? In particular, what is the rate-limiting step(s) that controls the two-electron, two-proton addition to dioxygen in its conversion to hydrogen peroxide?

In addition to catalysis, the active-site structures of GO and the CAO's indicate new organic cofactors which must be generated from tyrosine precursors. In the case of the CAO's, there is convincing evidence that the active site copper is responsible for the biogenesis of TPQ. It is likely that this is also the case for the modified tyrosyl radical in GO, given the production of active recombinant protein expressed in an organism *(Aspergillus nidulans)* lacking GO (cf., ref 141). Although the modified tyrosyl radical in mature GO can be reversibly generated in the presence of mild reductants and oxidants, this process is not necessarily related to the *in vivo* biogenetic pathway. For the *in vivo* process, a precursor protein structure containing an unmodified tyrosine and cysteine must be activated to yield the final thioether product. Since the precursor form of GO is likely to contain copper in its reduced state, the biogenetic process may be initiated by one-electron oxidations of the bound metal and active-site tyrosine, accompanied by a two-electron reduction of dioxygen to copper hydroperoxide. In the case of TPQ biogenesis, the initial hydroxylation of tyrosine to dopa was initially proposed to have formal analogies to the DβM mechanism, in which an active-site copper hydroperoxide catalyzes hydroxylation of an organic substrate. However, *in vitro* biogenetic studies performed thus far fail to implicate an exogenous donor, as would be anticipated for copper hydroperoxide formation. In the absence of such a donor, the biogenetic pathway for TPQ may have a stronger analogy to the pathway for cofactor production in GO than to the catalytic mechanism in DβM. It will be extremely exciting to see how our understanding of these processes evolves over the next few years.

VI. Abbreviations

EPR	electron paramagnetic resonance
NMR	nuclear magnetic resonance
EXAFS	extended X-ray absorption fine structure
XANES	X-ray absorption near-edge absorption
DβM	dopamine β-monooxygenase
PAM	peptidylglycine α-amidating enzyme
PHM	peptidylglycine α-hydroxylating monooxygenase
PAL	peptidyl-α-hydroxyglycine α-amidating lyase
CAO	copper amine oxidase
TPQ	topaquinone
PQQ	pyrroloquinoline quinone
TTQ	tryptophan tryptophylquinone
GO	galactose oxidase
ENDOR	electron nuclear double resonance spectroscopy
NMRD	nuclear magnetic relaxation dispersion
DA	dopamine
NE	norepinephrine

VII. Acknowledgments

This work was supported by grants from the National Institutes of Health (GM25765-18 and GM39296-09). The author thanks Dr. Rob Scarrow for valuable comments and criticism of the manuscript.

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CR950047G