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Whence topa? Models for the biogenesis of topa quinone in copper amine oxidases

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Abstract

Copper amine oxidases are dual function enzymes that first make their own cofactor, and then use this cofactor to catalyze amine oxidation. The cofactor, oxidized 2,4,5-trihydroxyphenylalanine (TPQ), is formed by the addition of cupric ion and dioxygen to precursor protein. Drawing on the mechanism of the oxidative half reaction in the copper amine oxidases, together with knowledge from other metal-containing enzyme systems, a new model is put forth for TPQ biogenesis. © 2000 Elsevier Science B.V. All rights reserved.

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1. Dedication

Beginning in the 1980s, work by Hans Duine and his co-workers focused attention on a possible role for covalently bound quinones as cofactors in eukaryotic systems. Although their original suggestion of bound pyrroloquinoline quinone in a range of eukaryotic enzymes was not correct, their proposal of a novel role for quinones as enzymatic cofactors has been substantiated. A new class of cofactors, referred to collectively as quino-cofactors, is now acknowledged. This class includes the originally described pyrroloquinoline quinone (as a freely dissociable cofactor) in alcohol dehydrogenases from gram negative bacteria and the cross-linked tryptophan tryptophanyl quinone in select bacterial amine oxidases. There is also the ubiquitous topa quinone (found in copper amine oxidases from bacteria, plants and mammals), and the quite recently described lysine tyrosyl quinone in mammalian lysyl oxidases. This chapter is dedicated to the role played by Hans Duine and his colleagues in focusing international attention on the existence of a new family of biological catalysts.

2. Introduction to topa quinone

2,4,5-Trihydroxyphenylalanine (topa) quinone (TPQ) is the redox cofactor of the copper-containing amine oxidases [1]. As such, it is very widely distributed in nature, with copper amine oxidases having been identified throughout the bacteria and eukarya [2–4]. These enzymes catalyze the oxidative deamination of primary amines to aldehydes, with the concomitant reduction of molecular oxygen to hydrogen peroxide. They are homodimers with subunit

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molecular masses of 75–85 kDa, each subunit containing one Cu^{II} ion in addition to the TPQ.

Unlike pyrroloquinoline quinone (PQQ), the redox prosthetic group that is associated non-covalently with certain bacterial dehydrogenases, TPQ is linked within the primary structure of the copper amine oxidases through standard peptide bonds [1]. Edman sequencing of polypeptide fragments containing stably modified TPQ has revealed the local amino acid sequence with an absence of a residue at one round of the cycle corresponding to the TPQ derivative. By comparison of this amino acid sequence to that derived from the relevant cDNA it was demonstrated that the precursor to TPQ is a specific, gene-encoded Tyr residue [5]. Two possible mechanisms were put forth for the post-translational modification of the Tyr to TPQ: the first made use of a separate (unknown) class of oxidative enzymes capable of processing precursor amine oxidases to their mature form; the second invoked self-processing of the proteins. In the latter case, it was suggested that proximity of the active site copper to the Tyr precursor might be sufficient for a metal ion catalyzed ring oxidation [5].

Two independent approaches were conceived and conducted to resolve the mechanistic issue of TPQ biogenesis. Cai and Klinman [6,7] expressed an amine oxidase from *Hansenula polymorpha* in *Saccharomyces cerevisiae* (which lacks an endogenous amine oxidase). The wild-type *H. polymorpha* enzyme, as well as a mutant enzyme with an altered amino acid sequence flanking the precursor Tyr, were expressed and found to be active and to contain TPQ. By contrast, a mutant form of enzyme with altered copper binding failed to produce TPQ. These results were interpreted in the context of a copper-dependent, self-processing mechanism. Tanizawa and co-workers pursued a different approach, isolating copper-depleted, TPQ-deficient forms of two different *Arthrobacter globiformis* amine oxidases [8,9]. This was accomplished by protein expression in *Escherichia coli* under conditions of limiting copper. They demonstrated that TPQ production can be initiated in vitro, by the addition of cupric ion to purified protein in the presence of molecular oxygen. Although the reaction is absolutely dependent on dioxygen, no other exogenous factors were required. Subsequently, the *H. polymorpha* amine oxidase was also obtained in a copper free form following expression in *E. coli* and shown to generate TPQ upon addition of cupric ion and molecular oxygen [10].

3. Dilemma

The ability of copper-containing, cofactor-deficient proteins to generate TPQ, with molecular oxygen as the only other requirement, presents a quandary. The initial proposal for a self-processing mechanism focused on a copper-hydroperoxide intermediate as the catalyst in ring hydroxylation of Tyr to yield dopa [5,6]. This mechanism is analogous to one proposed for the tyrosinase reaction in which a 2-electron reduction of dioxygen precedes the ring hydroxylation process (see Ref. [11] and discussion below). However, no likely candidate for a two electron donor has been identified in TPQ biogenesis. Although many of the amine oxidases contain Cys residues as potential electron donors, the *E. coli* amine oxidase does not [12] and there is some evidence that this enzyme undergoes self-processing in a manner analogous to the *A. globiformis* and *H. polymorpha* enzymes [13]. This, then, defines the central problem in understanding the mechanism of TPQ biogenesis: first, molecular oxygen with a triplet electronic state is unreactive with singlet organic substrate to give singlet product(s) in a one step process; second, the redox potential of the protein bound Cu^{II} (ε (vs. NHE) = 0.3–0.4 V, by analogy with other type 2 copper proteins [14]) appears insufficient to oxidize Tyr to Tyr radical in an efficient process (ε (vs. NHE) = 0.76–1 V for the one electron reaction of the redox-active tyrosines in photosystem II [15]).

There are few chemical precedents or model studies that bear on this problem. One earlier study did report that Cu^{II} catalyzes an oxidation of phenol to *p*-benzoquinone in protic solvents and it was noted that the reaction may proceed via the formation of phenoxy radical-Cu^{I} pairs [16]. However, the mechanism of this reaction is ambiguous [16] and the system may not be of direct relevance to TPQ biogenesis.

A working model, nevertheless, has been developed for the mechanism of TPQ formation [17,18]. A Cu^{II}-Tyr enzyme complex, in equilibrium with a Cu^{I}-tyrosyl radical complex, is suggested to form initially; the Cu^{I} species then reacts with molecular oxygen to yield an activated oxygen complex which is capable of insertion into the Tyr ring. This scheme is somewhat analogous to that proposed for the oxidative half reaction in the amine oxidase catalytic cycle. The oxidation of reduced cofactor has been proposed to yield a Cu^{I}-semiquinone radical intermediate prior to the binding and reduction of dioxygen (see Ref. [19] and discussion below). With regard to TPQ biogenesis, neither Ruggiero et al. [18] nor we (Williams and Klinman, unpublished results) have been able to detect tyrosyl radical formation or a diminution in Cu^{II} EPR signal in precursor forms of various amine oxidases. To account for the discrepancy between the model and experimental observations, it is necessary to propose that an initial equilibrium between Cu^{II}-Tyr and Cu^{I}-Tyr radical, if formed, must strongly favor the Cu^{II} state (see discussion below). Curiously, chemical reduction of a precursor enzyme to the Cu^{I} state with dithiothreitol did not prevent TPQ biogenesis [18]; this observation is difficult to reconcile with a model in which Cu^{II} is used to initiate oxidization of the tyrosine ring.

4. Models from other copper-containing systems

The traditional view of metallo-monooxygenases and oxidases is that molecular oxygen is activated at the metal center by one or two electron reduction. Monooxygenase mechanisms are exemplified by dopamine β-monooxygenase (DβM), peptidylglycine α-hydroxylating monooxygenase (PHM) and tyrosinase, whereas the catalytic cycle of amine oxidases offers a model for metallo-oxidases (see Refs. [11,20] for recent reviews). DβM oxidizes dopamine to noradrenaline by the insertion of one atom of dioxygen into the benzylic position of the ethylamine side chain. The other oxygen is reduced to water, requiring 2 electrons to be supplied by an exogenous donor such as ascorbate. The enzyme contains 2 mononuclear copper sites, only one of which appears to interact with substrate. The 2 Cu^{II} sites of the resting enzyme are reduced by ascorbate, and the $2Cu^{I}$ form then interacts with substrate and oxygen (in a random fashion). The initial chemical event within the productive ternary complex is proposed to be a two electron and one proton transfer from the copper sites and an active site acidic group of the protein, respectively, to oxygen to yield a copper-hydroperoxy intermediate. Cleavage of the O–O bond and hydrogen extraction from the substrate forms water as well as copper-oxo and substrate radicals, which subsequently combine to form hydroxylated product. PHM is an homologous enzyme to DβM, both at the structural and mechanistic level. Recently the crystal structure of PHM was solved [21]. The 2 copper atoms are separated by 11 Å and are located on separate domains of the protein, facing an interdomain cleft accessible to solvent.

In contrast to DβM and PHM, tyrosinase contains 2 closely associated metal ions forming a binuclear copper center. Tyrosinase catalyzes the ortho-hydroxylation of monophenols and their subsequent oxidation to orthoquinones. The deoxy form of the enzyme binds oxygen reversibly and contains 2 cuprous atoms. The resulting complex consists of a side-on, μ–η^2:η^2 peroxide and is activated for ring hydroxylation or catechol oxidation depending on the nature of the incoming substrate. As intro-

duced above, the requirement for a 2-electron reduction of dioxygen, prior to involvement of substrate, makes the DβM, PHM and tyrosinase systems poor models for TPQ biogenesis [39].

The oxidative half reaction of the TPQ-containing amine oxidases offers an interesting model for TPQ biogenesis. In the reductive first half reaction of amine oxidases, the ammonia from substrate is transferred to the C5 position of the cofactor, subsequent to hydrolysis and release of product aldehyde from a product Schiff base complex. The resulting aminoquinol must be reoxidized by dioxygen to regenerate TPQ in the resting enzyme [22]. A TPQ semiquinone has been directly observed in EPR studies of enzyme reduced with substrate under anaerobic conditions and is accompanied by a reduction in the Cu^{II} signal [19]. Cyanide ion drives the equilibrium toward the semiquinone-Cu^{I} enzyme form [19], presumably because of its high affinity for the reduced form of copper (Cu^{I}). Further work by Turowski et al. [23] has demonstrated that electron transfer between the copper and the cofactor occurs at a rate that is extremely rapid and kinetically competent. The model for cofactor reoxidation emerging from these data involves two one electron transfers to dioxygen. It is presumed that oxygen binds to the cuprous ion and is reduced thereby in the formation of a $Cu^{II}–O_2^{\cdot}$ complex. The second electron is then transferred to this complex from the semiquinone to give a peroxide anion complex and iminoquinone. As noted above, a one-electron transfer to Cu^{II} prior to dioxygen binding may be the best model we currently have for TPQ biogenesis. However, there is a critical distinction between the oxidative half reaction of the catalytic cycle of the amine oxidases and TPQ formation: the redox potential for (free) TPQ (vs. NHE) is approximately 0.1 V [24], at least 0.7 V more negative than the estimated redox potential for a protein bound tyrosine side chain!

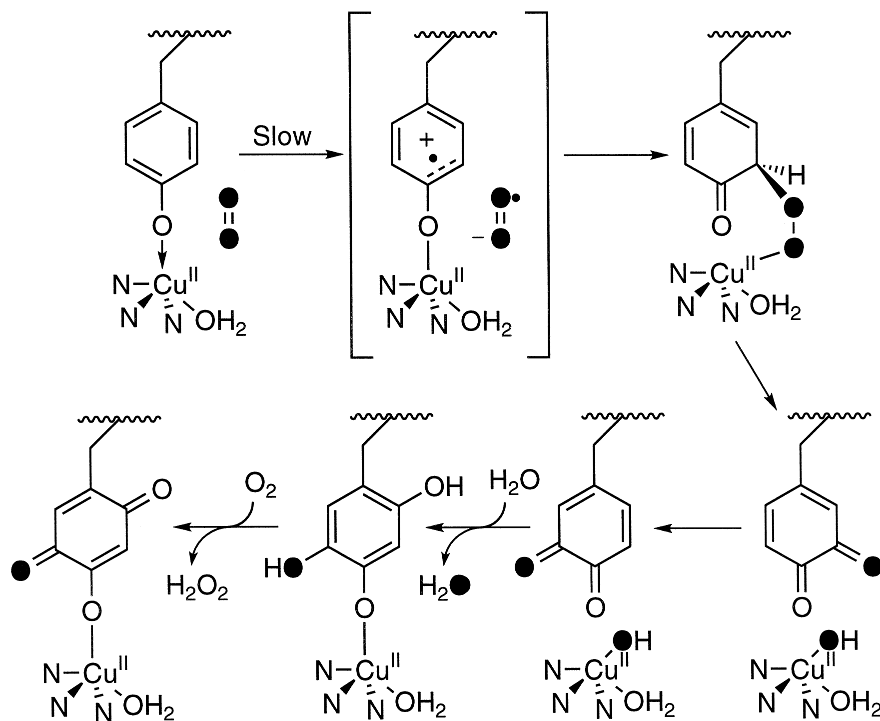
Additionally, recent work by Su and Klinman [25] has suggested another view for reoxidation of the aminoquinol form of cofactor. Through a combination of ^{18}O and solvent isotope effects, stopped-flow kinetics and viscosity studies, a detailed mechanism has been inferred for the oxidative half reaction in bovine serum amine oxidase. The magnitude of the ^{18}O kinetic isotope effect on V_{max}/K_m for O_2 suggests that the rate determining step is the first electron transfer to dioxygen. Additionally, there is no effect of solvent viscosity on V_{max}/K_m, indicating that dioxygen must prebind to enzyme before it is reduced. Recognizing that Cu^{II} does not interact with dioxygen and that Cu^{I} will transfer electron density to O_2 concomitant with its complexation to the reduced metal site, it is inferred that oxygen must pre-bind to the enzyme at a non-metal site. According to such a mechanism, the initial one electron reduction of dioxygen occurs from the reduced cofactor/Cu^{II} complex and not from a preformed Cu^{I} species. We note that preliminary data also suggest that dioxygen prebinds to the Cu^{II} precursor form of protein during biogenesis, (Williams and Klinman, unpublished results).

5. Models from non-heme iron systems

Two alternative mechanisms for oxygen activation have been presented for the non-heme iron proteins, lipoxygenase and protocatechuate 3,4-dioxygenase (3,4-PCD). In both cases, it has been proposed that substrate is activated by the enzyme for direct electrophilic attack by dioxygen. Lipoxygenase catalyzes the oxidation of unsaturated fatty acids to hydroperoxides. Extreme isotopic sensitivity is observed in the initial hydrogen abstraction from substrate and this step in the reaction has received considerable attention from this laboratory. In its active state the enzyme contains a high-spin Fe^{III} center with 5 endogenous ligands and an hydroxide in the sixth position [26]. It is not expected that oxygen would bind to such a center and, indeed, C–H bond cleavage occurs prior to oxygen binding in the steady state [27]. The mechanism, therefore, can be formalized as two half reac-

tions. In the first half reaction, hydrogen abstraction from substrate to Fe^{III}–OH is proposed to lead to an Fe^{II}–OH_2 complex as well as a substrate-derived radical. In view of the model for TPQ biogenesis it is interesting to note the energetics of this reaction pair. The redox potential of the active-site Fe^{III} (vs. NHE) is estimated to be 0.6 V [28] whereas the redox potential for 1,4-cyclohexadiene as a model for substrate is 1.1 V (cf. Ref. [29]). In the second half reaction, consideration of the relative reactivity of the Fe^{II} and substrate radical species to molecular oxygen implies that the site of the subsequent interaction with oxygen is at the substrate radical rather than the ferrous ion [30]. This leads to a hydroperoxyl radical intermediate, which is converted to product hydroperoxide by the transfer of a proton and electron from Fe^{II}–OH_2 to regenerate active enzyme. This suggests that the function of the redox metal center in lipoxygenase is to activate the substrate, rather than to control the reactivity of oxygen. In attempting to relate TPQ biogenesis to the mechanism of the lipoxygenase reaction, we note that the redox potential for the Cu^{II}/Cu^{I} cycle in the copper amine oxidases is not known, although there is no reason to suspect that it will be very different from the value (ca. 0.3 to 0.4 V vs. NHE) found in other type-2 copper proteins [14]. The lowered redox potential for the copper center in the amine oxidases, when compared to the iron potential in lipoxygenase, would explain the inability to observe any Tyr radical in precursor proteins leading to TPQ. We cannot ascertain at this time whether the difference in potential between the copper center and Tyr precursor is too great to allow the generation of a low level of a tyrosyl radical-Cu^{I} pair (below our detection limit) as the reactive intermediate in TPQ production.

An even more unusual mechanism is proposed for 3,4-PCD. Like lipoxygenase 3,4-PCD



Scheme 1. A possible mechanism for TPQ biogenesis (modified from Ref. [18]). Note that the hydroxide derived from splitting of the hydroperoxy intermediate is not the source of the oxygen at C-2 of the mature cofactor (cf. Ref. [34]).

is active in a high-spin Fe^{III} state and a wealth of kinetic and spectroscopic data are available (for reviews see [29,31]) Additionally, crystal structures of the enzyme complexed with substrate and analogous, competitive inhibitors and exogenous ligands have been reported recently [32,33]. 3,4-PCD catalyzes the oxidative intradiol ring opening of catechols concomitant with the cleavage of molecular oxygen. The substrates are bound sequentially, catechol preceding O_2 and various data indicate that iron maintains its ferric state throughout the reaction cycle [31]. Lipscomb and colleagues have proposed that substrate is metal-coordinated and deprotonated first through one hydroxyl then the other. This chelation requires the release and reorientation of an endogenous Tyr ligand and the formation, thereby, of a specific (non-metal) O_2 binding site. The substrate is now dianionic and, it is suggested, activated for electrophilic attack by dioxygen. It is expected that the site specificity of this attack is controlled by the relative orientation of the aromatic ring and bound O_2, as well as the influence of the ligands in *trans* to the substrate oxygens. That some electron density transfer occurs from the substrate to the metal may serve to relieve the spin forbidden nature of reaction while not requiring that the iron be reduced fully [29,31]. Alternatively, a 2-step reaction is feasible producing superoxide and substrate-derived radical as the initial intermediates.

What implications do these non-heme iron systems have for TPQ biogenesis? As noted, the energetics of the initial reactive pair in lipoxygenase are not so dissimilar from that expected in the amine oxidase precursor enzyme as to preclude lipoxygenase as a model for TPQ biogenesis. However, an alternative mechanism of substrate activation is also given precedence by the 3,4-PCD reactions. If there exists in the amine oxidases a discrete (non-metal) O_2 binding site, as seems likely, a rate limiting first electron transfer to dioxygen from activated substrate (Cu^{II}-liganded tyrosinate) would not lead to accumulation of radical (see Scheme 1). *This predicts that the cupric state is maintained throughout the biogenesis reaction and is in keeping with our observations and general outline for the oxidative half reaction in amine oxidase catalytic turnover* [25]. Several features of the mechanism in Scheme 1 have supporting evidence. These include the demonstration that the origin of the C2 oxygen in the mature cofactor is derived from solvent [34] and the finding that formation of hydrogen peroxide occurs during biogenesis [40]. Nevertheless, validation of any mechanism requires the identification of the key intermediates in the initial steps of Tyr oxidation. The recent addition of crystallographic data on the amine oxidases [35–38] to that already produced from kinetic and spectroscopic studies may help provide this information. In particular, structural studies on precursor forms of protein as well as proteins that have been mutagenized to alter or retard the biogenetic process may provide the missing keys to our understanding of this cryptic process.

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