New Quinocofactors in Eukaryotes*

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The field of quinoproteins began in the 1980s with the discovery and characterization of a low molecular weight, dissociable cofactor from Gram-negative bacteria, designated pyrroloquinoline quinone (PQQ) (1). PQQ was found to be biologically active both in vitro, via the reconstitution of activity to periplasmal alcohol dehydrogenases, and in vivo, as a stimulant to cell growth. The latter behavior is a consequence of the elevated production of apo-alcohol dehydrogenases relative to PQQ, which could thus be described as a bacterial "vitamin" (cf. Ref. 1 and references within).

By the mid-1980s, interest in PQQ had increased considerably, due to the publication of reports ascribing the active site cofactor in a eukaryotic protein (a copper amine oxidase from bovine serum) to covalently bound PQQ. This was followed by claims for covalently bound PQQ in a range of eukaryotic proteins such as dopamine β-monooxygenase, galactose oxidase, and lipoxynogenase (2). During this period, nutritional studies provided support for an essential role for PQQ in germ-free animals fed chemically defined diets, and the idea began to emerge of PQQ as a "missing vitamin" in mammals (3).

With the wisdom of hindsight, it is now clear that eukaryotic proteins do not contain covalently bound PQQ (cf. Ref. 4 for a full review of the evidence against PQQ in eukaryotic proteins). In a remarkable turn of events, however, the original attribution of a quinone to the active site cofactor in the copper amine oxidases is correct! As illustrated in Fig. 1B, the covalently bound cofactor in the copper amine oxidases resides within the polypeptide chain as a 2,4,5-trihydroxyphenylalanine, designated topa quinone (or TPQ) in its oxidized form. The demonstration of the structure in Fig. 1B followed from the isolation of a cofactor-containing, active site pentapeptide in high yield (from bovine serum amine oxidase) and the determination of the empirical formula for the unknown amino acid via a combination of Edman sequencing and mass spectrometry. Chemical synthesis of a model compound and comparison of its properties to the peptide by UV-visible, resonance Raman, and NMR spectroscopy provided the final proof of structure (5).

Origin of TPQ

At the time of the discovery of TPQ in bovine serum amine oxidase, no DNA sequences were available for mammalian copper amine oxidases. In light of the availability of a DNA sequence encoding a yeast (Hansenula polymorpha) copper amine oxidase, Mu et al. (6) isolated the copper amine oxidase from H. polymorpha and sequenced its active site, cofactor-containing peptide. The subsequent alignment of protein and DNA-derived sequences, although not perfect, indicated tyrosine as the precursor to TPQ. In retrospect, the lack of exact alignment between protein and DNA-derived sequences arose because Mu et al. (6) had isolated a benzylamine oxidase, whereas the cloned gene corresponded to a methylamine oxidase. When Cai and Klinman (7) expressed the H. polymorpha gene in Saccharomyces cerevisiae and characterized the recombinant protein, they were able to demonstrate an exact alignment between protein and DNA-derived active site sequences. The availability of a full-length sequence for the protein in which TPQ was originally characterized (bovine serum amine oxidase) had been stalled by the large size of the protein (82 kDa per subunit, making protein sequencing tedious) and a lack of knowledge regarding the tissue origin for this enzyme (making screening for a cDNA problematic). An important breakthrough occurred in the course of a homology search between the limited peptide sequences available for bovine serum amine oxidase and the Protein Identification Resource data base. Quite unexpectedly, an almost exact match was found between bovine serum amine oxidase peptides and an amiloride binding protein (ABP), earlier reported to correspond to an amiloride-sensitive sodium channel (8). An unusual feature of ABP was the lack of hydrophobic stretch anticipated for a membrane-spanning channel. On the assumption that ABP had been misidentified and was, in fact, a copper amine oxidase, Mu et al. (9) demonstrated that mammalian copper amine oxidases bind amiloride tightly. Subsequent screening of a bovine liver cDNA library, using probes designed from the ABP sequence, led to the isolation of the full-length gene for bovine serum amine oxidase. In confirmation of a precursor-product relationship between tyrosine and TPQ, the following alignment was found between the bovine serum amine oxidase active site peptide and its cDNA-derived sequence (9).

Protein: Leu-Asn-TPQ-Asp-Tyr
cDNA: Leu-Asn-Tyr-Asp-Tyr

SEQUENCE 1

Biogenesis of TPQ

As seen from the above peptide, TPQ is flanked by the amino acid Asn and a hydrophobic amino acid toward its N terminus and Asp and Tyr toward the C terminus. This consensus sequence (10), which is conserved among the sequenced pro- and eukaryotic copper amine oxidases (with the exceptions of the conservative replacement of Asp by Gln in the H. polymorpha and Arthrobacter P1 enzymes and the replacement of Tyr by Asn in the pea seedling and lentil seedling enzymes (11)), can be considered a "signature" for TPQ-containing enzymes. Initially, it appeared reasonable that the recognition signal for the post-translational modification of Tyr to TPQ would lie within this consensus sequence. However, computer searches of the existing protein and DNA data banks revealed numerous, non-quinoproteins that contained this consensus sequence, as well as the appearance of the consensus sequence more than once within the polypeptide chain of the copper amine oxidase from H. polymorpha (12). Clearly, the information contained within the linear consensus sequence is insufficient to encode a highly specific post-translational modification of a single Tyr per 75–85-kDa subunit.

It is well known that copper is an efficient catalyst for the side chain and ring hydroxylation reactions of phenethylamine derivatives (13). Thus, Mu et al. (6) had postulated that the placement of a copper ion proximal to the TPQ precursor would be sufficient for efficient post-translational modification of Tyr. As illustrated in Scheme 1, copper is proposed to catalyze a dioxygen-dependent insertion of an oxygen atom into the tyrosine ring to generate dopa, which upon oxidation gives dopa quinone. The latter compound is a reactive electrophile such that nucleophilic addition of either water or hydroxide ion complexed to the active site copper would be expected to complete the process of TPQ formation.

What evidence has been brought to bear on the mechanism of Scheme 1? Working in a prokaryotic system (expression of a gene for Arthrobacter globiformis amine oxidases in Escherichia coli), Tanizawa and co-workers (14, 15) were able to isolate a precursor, copper-free form of enzyme. Subsequent addition of copper led to full production of enzyme activity and TPQ. Cai and Klinman (7) chose to work with a eukaryotic system (expressing the gene for the yeast H. polymorpha amine oxidase in S. cerevisiae). Although this

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† The abbreviations used are: PQQ, pyrroloquinoline quinone; TPQ, topa (trihydroxyphenylalanine) quinone; ABP, amiloride binding protein; LTQ, l-tyrosine tyrosylquinone.
system did not yield TPQ production upon addition of exogenous copper to copper-depleted enzyme, several lines of evidence were found to support the self-processing mechanism of Scheme 1. First, growth of the transformed *S. cerevisiae* in the presence of copper-containing growth medium produced fully active *H. polymorpha* enzyme, despite the fact that there is no evidence for production of endogenous quinoproteins in *S. cerevisiae* (7). Second, site-specific mutagenesis experiments indicated that disruption of the consensus sequence surrounding the Tyr precursor still produced active amine oxidase, whereas disruption of the copper-binding site eliminated TPQ production (16). The weight of the available data for both prokaryotic and eukaryotic proteins supports a post-translational mechanism involving the active site copper as a catalyst for TPQ formation. Recent experiments indicate that the failure to induce TPQ formation in the copper-depleted recombinant *H. polymorpha* gene product resulted from insertion of zinc into the metal binding site under conditions of expression in *S. cerevisiae*. Expression of the *H. polymorpha* yeast amine oxidase gene in *E. coli* has now been found to lead to precursor protein lacking both copper and zinc; addition of copper in an amount stoichiometric with enzyme subunits leads to fully active protein.2

From a mechanistic perspective, the oxidative portion of the reaction shown in Scheme 1 is particularly puzzling. A reasonable mechanism would involve binding of dioxygen to Cu(II), followed by the generation of Cu(II)-OOH as the reactive oxygen intermediate. However, there is no apparent requirement for exogenous electron donors in the *in vitro* biogenetic process, raising the question of the source of the two electrons that would be required for a reductive activation of dioxygen to hydrogen peroxide. Although it is conceivable that the electron source lies within the protein itself (i.e. in a conversion of two cysteine residues to cystine), two copper amine oxidases have been characterized that are devoid of cysteine residues (the *E. coli* and *Klebsiella* enzymes (11)). As an alternative to dioxygen activation, it is conceivable that the initiation of cofactor biogenesis involves tyrosine oxidation by the active site copper. However, the redox partners are not well matched for this purpose with anticipated reduction potentials of about 0.5–0.4 V for the copper ion and 0.8–1.0 V for the tyrosyl radical (cf. Ref. 13). In principle, an unstable, transient cuprous-tyrosyl radical intermediate could be trapped by molecular oxygen to form a tyrosyl-hydroperoxy species. From the EPR experiments of Tanizawa and co-workers (15), it appears that anaerobic incubation of the precursor *Arthrobacter* protein with cupric ion leads to a loss of signal due to the paramagnetic cupric center. However, no accompanying signal for an organic radical was seen until oxygen was added to the system. Further, the properties of the EPR spectrum for the organic radical formed in the presence of O2 implicate a structure that resembles the semiquinone of the aminoquinol formed along the catalytic path (see below). None of these observations add up to a workable mechanism for the oxidation of tyrosine to dopa, and it is clear that more experiments are needed before the oxidative process will be adequately described. By contrast, O-18 labeling studies recently completed by Sanders-Loehr and co-workers (17) show that the oxygen at position 2 of the mature cofactor derives from solvent water, fully supporting the attack of water or hydroxide ion on dopa quinone to generate TPQ.

**Mechanism and Structure of TPQ Enzymes**

Copper amine oxidases had been the subject of fairly intense mechanistic investigations, both prior to and concomitant with the finding of the TPQ cofactor. An important early breakthrough occurred when kinetic studies of Rius et al. on a porcine plasma amine oxidase implicated the transfer of the substrate amine nitrogen to enzyme during the catalytic cycle (18). In a subsequent study, Hartmann and Klinman (19) were able to trap chemically an adduct between substrate and enzyme and to show that this adduct had the properties expected for a Schiff base complex between the substrate amine and a quino-structure (originally thought to be PQQ). In this way, a transamination mechanism was established, involving a covalent adduct between substrate and a reactive functional group on the enzyme. Utilizing the now known structure for TPQ, it is possible to write a complete reaction mechanism for substrate oxidation (Scheme 2) (20).

As illustrated in Scheme 2, a Schiff base complex between substrate and TPQ occurs at the C-5 position of cofactor. Evidence for the enhanced electrophilicity of this C-5 position comes from solution studies, which show first, preferential attack of TPQ model compounds by nucleophiles at C-5 (21) and second, identical resonance Raman spectra for an active site sample, cofactor-containing peptide derived from enzyme inhibited by phenylhydrazine and a model TPQ compound labeled with phenylhydrazine at the C-5 carbonyl (5). The charge distribution of the substrate Schiff base complex has been the subject of discussion and speculation. From the blue shift in the λ_{max} for this complex (about 340 nm) relative to the uncomplexed TPQ (about 480 nm) (20), it appears that the resonance-stabilized negative charge on the ionized TPQ (pK_{a} ~ 3 for bovine serum amine oxidase (21)) has become more localized upon complexation with substrate. This may occur via an electrostatic interaction between a positively charged, protonated Schiff base and a localized anionic charge on the TPQ derivative at C-4. This view is supported by solution studies showing a shift in the λ_{max} for complexes of model TPQ compounds with amines to shorter wavelengths as the solvent is changed from polar to non-polar (ascribed to enhanced ion pair resulting and resulting localization of charge on the cofactor in the non-polar solvent) (22). The oxidation of amines within the Schiff base complex occurs via proton activation at C-1 of substrate. Structure reactivity correlations using a series of ring-substituted benzylamines indicate an increase in rate with electron- withdrawing substituents (ρ = 1.5) (20). In light of the electron-withdrawing nature of the oxidized TPQ ring, together with its predilection to form Schiff bases, a proton activation mechanism is expected. The similarity of this portion of the reaction to that of another carboxyl cofactor, pyridoxal phosphate, is striking.

As the negative charge formed initially on substrate becomes delocalized into the TPQ ring, the cofactor has been converted from its oxidized to reduced form. Given the large increase in pK_{a} (about 5 pH units) of the TPQ ring upon reduction (21), it is anticipated that the reduced cofactor will be protonated at C-4. This may explain the high reactivity of the product Schiff base toward hydrolysis. Using the reductive trap method of Hartmann and Klinman (19), it has never been possible to trap any product Schiff base under conditions leading to stoichiometric trapping of the substrate Schiff base. While this differential behavior of substrate and product Schiff bases could be due to an altered accessibility of the substrate Schiff base once the cofactor has become reduced. Using a mutant form of the methylamine oxidase from *H. polymorpha*, Cai and

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2 D. Cai, N. Williams, and J. P. Klinman, manuscript in preparation.
Klinman\(^\text{3}\) have recently shown that substrate methylamine gives rise to mechanism-based inhibition in which an intermediate accumulates that has all of the characteristics of a product Schiff base complex. Since the mutation is at the position of the Asp adjacent to TPQ, this implicates a role for the consensus sequence in the maintenance of a catalytically productive geometry for the cofactor-product Schiff base complex. The crystal structure of the dimeric \textit{E. coli} amine oxidase indicates an interaction between the consensus sequence Asp on subunit 1 and a conserved His on subunit 2 (23).

An aminquinol of the cofactor is formed once product aldehyde has been hydrolyzed from the product Schiff base. Janes and Klinman (24) were able to demonstrate that the substrate-derived ammonia remains bound to protein under strict anaerobic conditions. Oxidation of this aminquinol is believed to occur via two one-electron steps with the intermediacy of a semiquinone species (Scheme 1) (25). McCracken and co-workers (26), using N-15-labeled amine to label the cofactor, obtained an EPR spectrum for semiquinone, which is indicative of a covalent attachment of the substrate-derived amino group to the TPQ ring. Thus, the transamination mechanism shown in Scheme 2 appears fully confirmed.

The recent availability of a crystal structure for a copper amine oxidase from \textit{E. coli} provides a beautiful structural context for the mechanism in Scheme 2 (23). First, the single copper ion per subunit is found to be close to the mature TPQ, separated by an axial water molecule. The proximity of copper to TPQ is relevant to the stabilization of reduced cofactor (Scheme 2). The copper-complexed water has been proposed to serve as the nucleophile in the conversion of dopaquinone to topaquinone (Scheme 1) and may serve as a proton source in the oxidative degradation of substrate-derived amine to label the cofactor, obtained an EPR spectrum for semiquinone (Scheme 3) (25). McCracken and co-workers (26), using N-15-labeled amine to label the cofactor, obtained an EPR spectrum for semiquinone, which is indicative of a covalent attachment of the substrate-derived amino group to the TPQ ring. Thus, the transamination mechanism shown in Scheme 2 appears fully confirmed.

**A Second Quinocofactor in Eukaryotes**

Among the copper amine oxidases, the physiologically important lysyl oxidase (28) has been a puzzle. Although formally a member of the copper amine oxidase family, lysyl oxidase shows a number of essential differences. These include the smaller size of lysyl oxidase (monomer of 32 kDa) in relation to other copper amine oxidases (dimers of 75–85-kDa subunits) and the absence of the conserved consensus sequence seen in all known TPQ-containing enzymes (29). Based on the capacity of lysyl oxidase to stain positively in a redox cycling assay developed for the detection of quinone-containing proteins (30) and features of a resonance Raman spectrum for a cofactor-containing peptide (31), lysyl oxidase has alternatively been proposed to contain either PQQ or TPQ.

In contrast to bovine serum amine oxidase (the protein used for the initial characterization of TPQ), lysyl oxidase is available in only limited quantities (typical yield of a lysyl oxidase preparation from bovine calf aorta is optimally 5 mg from 500 g of bovine aorta). Additionally, a high level expression system for lysyl oxidase has not yet been developed. It seems unlikely that the lysyl oxidase cofactor would have yielded its structure in a timely fashion without the experience gained in the course of the discovery and characterization of TPQ.

Using a peptide resulting from a thermolytic digestion of lysyl oxidase derivatized with \(^{14}\text{C}\)phenylhydrazine, Wang \textit{et al.} (32) made the unexpected observation of two amino acids (in comparable yield) at each round of Edman sequencing. Segregation of these amino acids into peptides 1 and 2 was possible by alignment of the detected amino acids with the cDNA-derived protein sequence of porcine lysyl oxidase (28). A blank was found in peptide 1 at the position of tyrosine and in peptide 2 at the position of lysine.

Subdigestion with Asp-N, a protease that cleaves toward the N-terminus of aspartate, yielded two hexapeptides, from which the following structure was inferred.

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\text{Asp-Thr-(Tyr-derivative)-Asn-Ala-Asp} \\
\text{Val-Ala-Glu-Gly-His-(Lys derivative)}
\]

**Structure 1**

Mass spectrometry was used to confirm that the peptide was, indeed, cross-linked and to obtain an accurate mass for the cofactor to four decimal points. The latter yielded a mass of 288.1377 for the (Tyr-derivative)/(Lys-derivative) from which an empirical formula of \text{C}_{15}H_{16}N_{6}O_{8} was computed (32).

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\(^{3}\) D. Cai and J. P. Klinman, manuscript in preparation.
Given a role for a water or hydroxide ion attack on a dopa quinone intermediate in TPQ formation (Scheme 1), it appeared likely that the lysyl oxidase structure would be formed by a similar chemical reaction. This led to the proposal of an attack by the ε-amino group of a lysine side chain on the reactive (C-2) position of dopa quinone to yield the structure (C15H24N4O4) shown in Fig. 1C. Synthesis of an appropriate model compound, Fig. 1D, followed. UV-visible characterization of this model compound indicated a λmax at 504 nm (red shifted about 24 nm relative to TPQ), identical to that observed for native lysyl oxidase. Derivatization of the compound in Fig. 1D with phenylhydrazine and collection of resonance Raman spectra demonstrated a structure quite different from the phenylhydrazine of TPQ (39). Importantly, the phenylhydrazone of the compound in Fig. 1D showed a resonance Raman spectrum identical to that of a phenylhydrazine-labeled, active site-derived peptide from lysyl oxidase (32).

In this manner, a new cross-linked structure, designated lysine tyrosylquinone (or LTQ), was described (32). Site-specific mutagenesis provides further confirmation of the LTQ structure, since conversion of the cross-linking amino acid side chains, Lys-314 and Tyr-349, to Ala and Phe, respectively, yields inactive enzyme. As a control, Wang et al. (32) addressed the possibility that the mature cofactor in lysyl oxidase was the postulated biogenetic intermediate, dopa quinone, as the sequence of the active site peptide. A model for dopa quinone, 4-ethyl-1,2-benzoquinone, reacted with phenylhydrazine to give a derivative with λmax = 330 nm, in contrast to the phenylhydrazone of lysyl oxidase (λmax = 454 nm). Further, the derivatized α-quinone was found to be completely unreactive to incubation overnight with a large excess of an N-alkylamine, thereby ruling out a spurious side reaction as the source of the LTQ structure (32).

The finding of LTQ in lysyl oxidase raises the question of whether this cofactor will be found in other eukaryotic proteins. Recently, Dove et al. (4) examined the cofactor structure for a copper amine oxidase from Pichia pastoris that had been reported to oxidize the ε-amino group of protein or peptide-bound lysyl side chains (38). Despite the similarity in substrate specificity to lysyl oxidase, this yeast copper amine oxidase contains TPQ. This result tells us that the evolution of cofactor structure and substrate specificity is likely to have occurred separately. As described by Palcic and co-workers (35, 36), the family of copper amine oxidases is unique in that stereochemistry has not been conserved. Enzymes have been classified based on the relationship of the stereochemistry for amine oxidation at C-1 to that for exchange at the C-2 position of phenethylamine substrates. The only protein found to be similar to lysyl oxidase is the membrane-associated, semicarbazide-sensitive amine oxidases (37). This latter class of proteins has not yet been cloned and sequenced, and the structure of the active site cofactor remains unknown. It will be of great interest to determine whether the semicarbazide-sensitive amine oxidase contains LTQ, TPQ, or possibly a new variant of quinocofactor.

Given the proposed commonality of dopa quinone as an intermediate in both TPQ and LTQ biogenesis, it is reasonable to question whether additional quinocofactors will be found that differ with regard to the substituent at the C-2 position of the mature cofactor.

In principle, any amino acid side chain with nucleophilic properties (e.g. Ser, His, Asp, or Glu) could couple to a dopa quinone intermediate. However, the specific properties of the final quinone are likely to determine its biological viability. When the redox half-potential for LTQ was determined, it was found to be very similar to TPQ, as well as to the dissociable quinocofactor PQQ (Table I) (32). This suggests that the interaction of reduced cofactor with molecular oxygen, a feature common to the reactions of all known quinocofactors (illustrated for TPQ in Scheme 3), may occur optimally for cofactors with half-potentials in the observed range of 150 to −180 mV (versus standard calomel electrode). At the present time, we have no information concerning the catalytic ability of LTQ model compounds in the oxidation of amine substrates. Comparison of these properties with those for TPQ model compounds (21, 22, 34) may provide critical insight into the selective advantage of one eukaryotic quinocofactor over another.

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