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Modeling Novel Quinocofactors: An Overview

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In recent years, three novel quinonoid cofactors, topaquinone (TPQ), tryptophan tryptophylquinone (TTQ), and lysine tyrosylquinone (LTQ) were identified in copper amine oxidase, methylamine dehydrogenase, and lysyl oxidase, respectively. The novel quinocofactors all derive through posttranslational modification of amino acid residues in the backbone of the enzymes, whereas the previously known quinonoid coenzyme, pyrroloquinoline quinone (PQQ), is noncovalently bound to several prokaryotic dehydrogenases. The identification of these new redox cofactors stimulated numerous studies aimed at characterizing their properties and their role in substrate oxidation. Many efforts have been made to shed light on specific points, including (i) the mechanism of posttranslational modification leading to these covalently bound quinonoid coenzymes, (ii) the structural characterization of the copper-binding site and the relation to quinonoid cofactor (TPQ or LTQ), (iii) catalytic mechanism, (iv) modulation of quinocofactor reactivity by the enzyme matrix. In all these cases, recent model studies, disclosing the basic chemical and physicochemical properties of compounds closely resembling the novel quinocofactors, have greatly contributed to answering specific questions, and have offered a frame of reference for interpretation of studies of TPQ, TTQ, and LTQ in enzymatic systems. This minireview is an updated, comprehensive account of these contributions.

Key Words: quinoproteins; model studies; topaquinone; tryptophan tryptophylquinone; lysine tyrosylquinone.

INTRODUCTION

The description, in 1979, of pyrroloquinoline quinone (PQQ; Fig. 1) as a redox cofactor of methanol dehydrogenase (EC 1.1.99.8) from *Pseudomonas* TP1 (*1*), constitutes the starting point of the emergence of an entire new field of research, referred to as quinoenzymes or quinoproteins. Today, this term defines three distinct groups

* This paper is dedicated to the memory of Professor Giacomo Randazzo, clever scientist and good friend, prematurely deceased last year.

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[2] Abbreviations used: AADH, aromatic amine dehydrogenase; AO, copper amine oxidase; BSAO, bovine serum amine oxidase; bpy, 2,2′-bipyridine; ECAO, *Escherichia coli* amine oxidase; LO, lysyl oxidase; LTQ, lysine tyrosylquinone; MADH, methylamine dehydrogenase; PQQ, pyrroloquinoline quinone; RR, resonance Raman; TPQ, topaquinone; TTQ, tryptophan tryptophylquinone.

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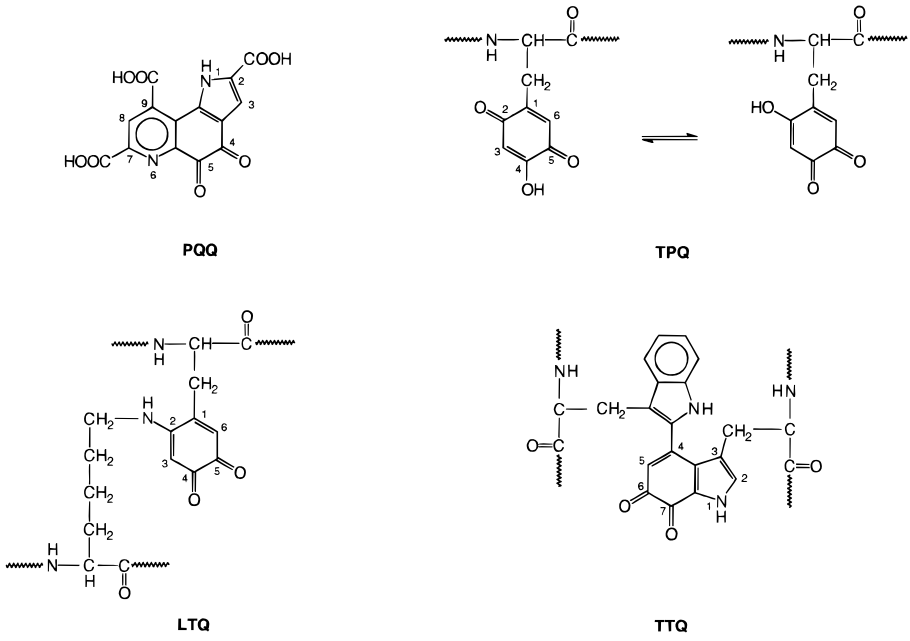


FIG. 1. Structure of PQQ and novel quinocofactors: TPQ, TTQ, and LTQ. TPQ has the potential to exist as either the *o*-quinoid or the *p*-quinonoid form.

of enzymes. The first includes a number of dehydrogenases from gram-negative bacteria that contain PQQ as a dissociable redox cofactor (*2,3*). The amine oxidizer lupanine 17-hydroxylase, isolated from *Pseudomonas lupanini*, also belongs to this class (*4*). Another bacterial dehydrogenase, methylamine dehydrogenase (MADH, EC 1.4.99.3), has been showed as containing a covalently bound cofactor, identified as tryptophan tryptophylquinone (TTQ; Fig. 1), derived through posttranslational modification of two tryptophan residues (*5*). The last group comprises the ubiquitous copper amine oxidases (AOs, EC 1.4.3.6) for which the exact nature of the organic cofactor was clearly elucidated, after much controversy, in 1990 as the quinone of 2,4,5-trihydroxyphenylalanine (topaquinone, TPQ; Fig. 1) (*6*), which has the potential to exist in either the *o*-quinoid or the *p*-quinonoid form. As discussed in detail below, the cofactor of AOs derives from a tyrosine inserted in the polypeptide chain. Moreover, among the AOs, the physiologically important lysyl oxidase (LO, EC 1.4.3.13) was recently proved to contain a modified form of TPQ, designated lysine tyrosylquinone (LTQ; Fig. 1) (*7*).

Various aspects of the biochemistry of quinoproteins are regularly reviewed, and recent comprehensive accounts are available (*4,8–14*). However, the contribution given to these studies by the use of synthetic analogs of quinocofactors has not received wide attention. The purpose of this minireview is to summarize the results obtained from model studies in understanding the structure, chemical properties, and biogenesis of redox quinocofactors and of their function in the corresponding protein matrices. In particular, we will focus on the characterization of the three most recently

discovered quinonoid coenzymes: TPQ, TTQ, and LTQ. The chemistry of PQQ and its derivatives has been recently reviewed by two authors who have greatly contributed to these investigations, Itoh and Ohshiro (*15,16*), and model studies on this topic are still being conducted (*17,18*).

TOPAQUINONE (TPQ)

TPQ Enzymes

Copper containing amine oxidases is a widely distributed class of "Type-2" copper enzymes that catalyze the two-electron oxidative deamination of primary amines to the corresponding aldehydes, ammonia, and hydrogen peroxide:

$$\text{R-CH}_2\text{NH}_3^+ + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{R-CHO} + \text{NH}_4^+ + \text{H}_2\text{O}_2.$$

AOs are α_2 enzymes, with each subunit containing one Cu(II) atom and one TPQ moiety. The M_r value for the dimer usually ranges from 140 to 200 kDa. Typically, these enzymes give pink solutions, with a characteristic broad absorption peak centered around 480–500 nm. Amine oxidation to the corresponding aldehyde is catalyzed by AOs via a transamination mechanism, with the concomitant reduction of TPQ to the aminoquinol form. This is then oxidized by molecular oxygen with the release of ammonia. Figure 2 illustrates the mechanism of the reductive half-reaction. Nucleophilic attack by the amine substrate to the TPQ cofactor (presumably at the C5 carbonyl group) to form a carbinolamine intermediate (I) initiates the reaction. Removal of

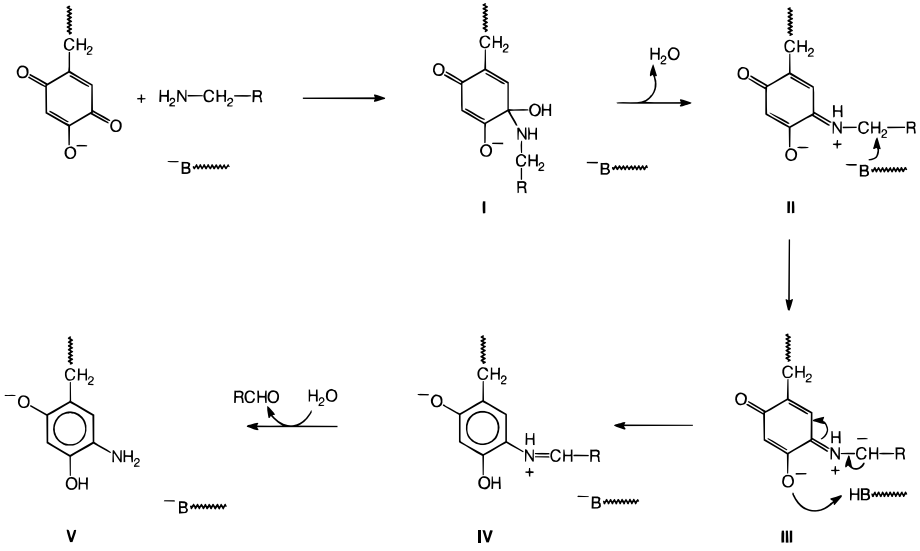


FIG. 2. Reductive half-reaction of the catalytic cycle of copper amine oxidases. **B** indicates the active-site base involved in proton abstraction from the α-carbon of the amine substrate. For more details on the reaction mechanism see main text.

water from the carbinolamine results in a substrate Schiff base complex (II). Base-catalyzed proton abstraction from the substrate Schiff base yields a product Schiff base (IV) via a carbanion intermediate (III). Hydrolysis of the product Schiff base releases the product aldehyde and the aminoquinol form of the reduced cofactor (V). In the oxidative half-reaction of the catalytic cycle, the aminoquinol form of TPQ is then converted back to the oxidized cofactor with the concomitant two-electron reduction of oxygen to hydrogen peroxide and the release of ammonia.

The reader is directed to a series of recent reviews for detailed information regarding various aspects of AOs biochemistry and historical background (*4*,*9*,*10*,*12*,*14*,*19*).

Chemical Properties of TPQ Models

TPQ was first identified as the covalently bound active-site cofactor of bovine serum amine oxidase (BSAO) in 1990 (*6*). The identification of the cofactor has been achieved by the derivatization of the enzyme-bound cofactor with phenylhydrazine or 4-nitrophenylhydrazine to form a stable hydrazine adduct, followed by detailed characterization of active-site cofactor-containing peptides through UV−vis spectroscopy, mass spectroscopy, and NMR (*6*). However, the final elucidation of the cofactor structure required the synthesis of authentic analogs, followed by a comparison of physical properties for the synthetic and protein-derived compounds. To this end, a model compound was prepared in which the peptide moiety was tied back into a cyclic, hydantoin structure (**1a**, **1b**; Fig. 3) (*6*).

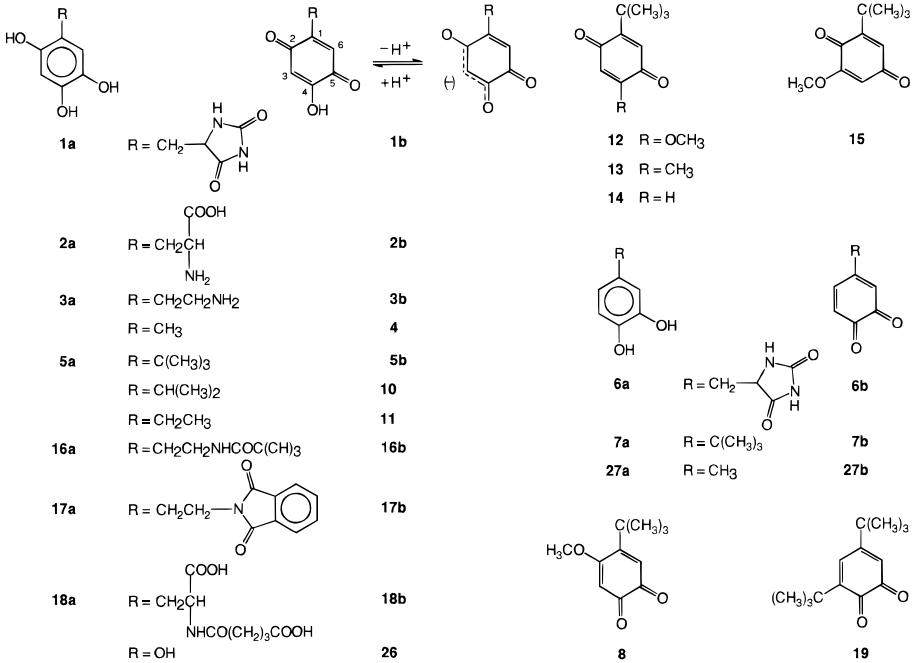


FIG. 3. Structure of *p*- and *o*-quinonoid model compounds for TPQ. For some models, both the reduced and the oxidized forms have been synthesized.

Following the path indicated by Klinman and fellow researchers, several model studies were conducted in order to investigate the mechanistic role of TPQ in enzyme-catalyzed oxidation of substrate and reduction of dioxygen (*20–27*), the interaction of copper with the cofactor (*28,29*), and the biogenetic process of TPQ at the active site of AOs (*30–32*).

The choice of a suitable TPQ model should take into consideration the fact that, although the simplest model, 2-hydroxy-1,4-benzoquinone, is itself commercially available in the reduced form (1,2,4-benzenetriol), the absence of a ring alkyl substituent is known to cause an oxidative intermolecular C–C coupling reaction, with the ultimate formation of 2,2′-bi-*p*-quinones (*33*). Other researchers used the commercially available amino acid topa (**2a**, **2b**; Fig. 3) to evaluate O_2- and Cu(II)-dependent reaction with amines (*34,35*), or to investigate the electrochemical behavior of TPQ cofactor (*35,36*). However, neither this compound nor its decarboxylated derivative topamine (**3a**, **3b**; Fig. 3) (*34,37*) are completely suitable for a rigorous chemical study because of the easy inter- and intramolecular condensation reactions occurring at the quinone stage due to the free amino group (*38,39*). To overcome this problem, a number of model compounds for TPQ cofactor have been synthesized, differing greatly in the nature and bulkiness of the substituent at the C1 position (Fig. 3), which are not subject to cyclocondensation complications.

A note on ring numbering is necessary at this stage of the discussion, since models are usually regarded as alkyl-substituted hydroxybenzoquinones, whereas the rings of topa and TPQ are regarded as 2- (or β-) substituents of the amino acid alanine. For the sake of clarity, the same numbering system adopted for topa (i.e., 2,4,5-trihydroxyphenylalanine) will be used throughout this review. So, 1,2,5-trihydroxy-4-*tert*-butylbenzene will be numbered as 2,4,5-trihydroxy-1-*tert*-butylbenzene in order to maintain the usual numbering system. The same rule will apply to the model compounds for TTQ and LTQ; also in this case reference to ring positions will follow the numbering scheme depicted in Fig. 1.

The UV–vis properties of TPQ analogs **1b**, **4**, and **5b** were investigated in detail (*20,24,25*). These model compounds show a relatively sharp acidic character, being vinylogous carboxylic acids, which can thus exist in two forms, depending on the pH of the solution (Fig. 3). The anionic form of these hydroxyquinones shows a characteristic absorption band in the visible region, centered around 485–490 nm, with reported $\varepsilon \approx 1850–2110\ M^{-1}\ cm^{-1}$ (native enzymes having λ_{max} at around 480–500 nm and $\varepsilon = 2–3 \times 10^3\ M^{-1}\ cm^{-1}$ (*40*)). This is due to the dissociation of the 4-hydroxyl group. The resonance effect involving delocalization of electrons is expected to stabilize the anionic form of these *p*-quinones. The typical absorption band disappears upon protonation, being substituted by a new absorption maximum around 385–390 nm, and the color of the solution changes from pink to pale yellow. The p*K*a value of the hydroxyl group of **1b** and **4** was determined to be 4.13 (*20*) and 4.5 (*24*), respectively, by spectrophotometric titration. These values are very close to that of TPQ when generated by air oxidation of free topa (*35*). Mure and Klinman determined the p*K*a of the oxidized form of BSAO and found a value of 3.0 ± 0.2 (*20*), which is a ca. 1.0 pH unit lower than that of the model compounds. Therefore, the anionic form of the cofactor is more stable in the enzyme active site than a nonbound form in an aqueous solution. This stabilization of the protein-bound ionized

cofactor could be due to the proximity of active-site copper ion or a positively charged amino acid side chain. The evidence reported above indicate that neither the acid dissociation of the 4-hydroxyl group nor the absorption maxima and ε values of the protonated and deprotonated quinones are affected by the nature of the C1-alkyl substituent.

Although energy minimization calculation for the deprotonated TPQ anion in solution indicated an intermediate electronic structure between the *p*-quinone and the *o*-quinone types with three almost equivalent carbonyl groups (*35*), comparison of the catalytic properties of the AOs with the results obtained by reaction of various *p*- and *o*-quinones with amines (see below) indicates that the functional form for TPQ in the enzyme active site is best represented as a *p*-quinonoid structure (*25*,*26*).

Following the discovery of TPQ at the active site of BSAO, resonance Raman (RR) spectroscopy has proved to be an excellent technique for probing the structure and chemistry of the novel quinocofactor (*41–46*), and the results obtained by the study of model compounds of TPQ constituted an essential framework for interpreting RR spectra of AOs. Soon after the first report of TPQ structure, this technique was used to verify the status of the organic cofactor in AOs isolated from a variety of sources, including pig kidney, porcine serum, and pea seedlings (*41*,*42*). In particular, these studies showed that the RR spectra of the *p*-nitrophenylhydrazine derivatives of active-site peptides isolated from these enzymes are identical to the spectra of the *p*-nitrophenylhydrazone of **1b** and of a labeled active-site peptide from BSAO, thus providing support for the identification of TPQ as the organic functional group in BSAO and in AOs in general (*41*,*42*). On the other hand, early attempts to obtain RR spectra of underivatized TPQ in AOs proved unsuccessful owing to the relatively low extinction and high sample fluorescence (*47*). Subsequently, it has been possible to obtain RR spectra of the underivatized model **1b** (*48*). Using highly purified amine oxidase from *Escherichia coli*, Sanders-Loehr and colleagues have recently obtained the RR spectrum of the native TPQ cofactor and found it closely matching that of 1-*tert*-butyl-4-hydroxy-2,5-benzoquinone (**5b**), particularly in the deprotonated state in aqueous solution (*44*). Moreover, the RR spectra of the TPQ cofactor in the native enzyme and model compound **5b** showed a striking similarity of the ^{18}O and D shifts, with oxygen exchange occurring at the carbonyl adjacent to the hydroxyl group (*44*).

Extensive electrochemical studies were conducted on free TPQ (*35*,*36*), TPQ models **1b** and **5b** (*20*,*25*) and dopaquinone analogs **6b**, **7b**, and **8** (Fig. 3) (*20*,*25*). These studies have shown that the pH dependencies of the redox potentials of free TPQ and TPQ model **1b** are almost identical, and that a three-proton transfer is coupled with the two-electron conversion from TPQ to topa (*20*,*35*), in accordance with the fact that TPQ is acid-dissociated at neutral pH. Interestingly, at pH 7.2, the two-electron redox potential of the TPQ model **1b** was shown to be 300 mV less positive than that of dopaquinone analog **6b** but to be similar to that of PQQ (*20*). Therefore, the extra hydroxyl group has a marked effect, increasing the reducing capacity of the reduced form of the quinone by 300 mV. It is well known that simple quinones (especially the *ortho* isomers) are prone to undergo the nucleophilic attack on ring positions other than the carbonyl groups, leading to ring-substituted quinols or catechols. The presence of a ring substituent such as a hydroxyl group just on the ring

position most reactive with nucleophiles drives the reactions with amines and hydrazine derivatives toward the proper carbonyl reactions, resulting in the formation of quinone-imine (iminoquinone) or -hydrazone, respectively.

The electrochemical properties of 5-amino-1-ethylresorcinol (**9**; Fig. 4), synthesized as a model compound of the aminoquinol which has been proposed as a reaction intermediate in amine oxidation by BSAO (*49,50*), were also investigated (*20*). This compound showed practically the same midpoint potentials as the hydantoin model **1** at pH 6.78 and 9.56. As described above for **1**, the redox potential of the aminoquinol model at neutral pH is very close to values previously reported for PQQ (*51,52*), and also for topamine (*53*).

Reactivity of TPQ Models toward Amines

Subsequent to the synthesis and characterization of the TPQ models, several researches focused on the next obvious step, i.e., the examination of the ability of model compounds to act as amine oxidants. The first instance of amine oxidation by a model compound for the active site of AOs was reported by Suzuki and coworkers (*28*). These authors prepared a topa-containing copper (II) complex ([Cu(DL-topa)-(bpy)(H_2O)] $BF_4 \cdot 3H_2O$) and found that this complex led to the oxidation of benzylamine in an aqueous solution (pH 7.0) at room temperature under aerobic conditions and that the rate of this reaction resulted to be about 14-fold greater in comparison with the control reaction with free topa. Under the same conditions, the $[Cu(bpy)(H_2O)_2]^{2+}$ complex showed no oxidation activity toward benzylamine (*28*).

Later, 1-methyl-4-hydroxy-2,5-benzoquinone (**4**), obtained starting from 4-methylcatechol, was showed to oxidize a wide range of primary amines, both benzylic and nonbenzylic, in aqueous solutions and in the presence of Cu(II) ions (*24*). High pH values were found to accelerate sharply the oxidation of the substrates, the optimum being centered around pH 10. In this case, the influence of the alkaline medium on the oxidation rate is not surprising. In fact, the isomerization of the iminoquinone (substrate Schiff base) to the corresponding quinoaldimine (product Schiff base) takes place via an iminoquinone carbanion, which is formed by means of a deprotonation step (III; Fig. 2). This deprotonation is catalyzed by a specific basic residue in native AOs, identified as a conserved aspartate residue (*54–56*), and is conceivably due to the alkaline pH in the case of **4**. The presence of suitable amounts of Cu(II) was shown to be an important requirement for this model system, and an optimal ratio of 4 mol of cupric ions per mole of model compound was found. The broad substrate specificity showed by **4** is probably due to the lack of any sterical hindrance of the quinone when it is free in solution compared to its counterpart inserted in a protein matrix, while the sharp preference for benzylamines is likely to depend on the high stability of the quinoaldimine intermediates, which are favored by the conjugation of an azastilbene system (*24*).

In the same year, Mure and Klinman have carefully investigated structure–reactivity correlation in the catalytic oxidation of benzylamine by a series of TPQ model compounds, which included **1b** as well as several 1-alkyl-4-hydroxy-2,5-benzoquinones which differed in their bulk of alkyl substituent (**4**, **5b**, **10**, **11**; Fig. 3) (*25*). Moreover, since TPQ has the potentiality of existing as a *p*-quinoid or a *o*-quinonoid

structure, these authors prepared also several *p*-quinones (**12**–**15**; Fig. 3) and *o*-quinones (**7b**, **8**; Fig. 3) and compared their ability to oxidize benzylamine with that shown by the TPQ model compounds (*25*). It is important to note that all these studies have been carried out in anhydrous acetonitrile at room temperature, whereas the use of water led to a marked decrease in catalytic efficiency. On the other hand, changing the solvent from acetonitrile to methanol or dichlorometane did not have a significant effect on the reaction (*25*). In this model study, benzylamine was successfully oxidized by TPQ analogs (**1b**, **4**, **5b**, **10**, **11**), and the reactivity of these quinones was found to be dependent on the steric bulk of the alkyl substituent at the C1 position, with hydantoin (**1b**), *tert*-butyl (**5b**), and isopropyl (**10**) being more favorable than small primary alkyl substituents such as ethyl (**11**) and methyl (**4**). On the other hand, the *p*-quinones (**12**–**15**) were catalytically inactive and in some cases (**13**, **14**) underwent ring amination, indicating that the 4-hydroxyl group is essential for optimal catalytic activity (*25*). In the case of **4** and **11**, dimerization of the substrate Schiff base intermediate occurred, while in the cases of TPQ analogs **1b**, **5b**, and **10**, such dimerization was prevented by the presence of a bulky substituent, resulting in higher catalytic turnovers. The *o*-quinones (**7b**, **8**) possessed some activity. In particular, **8** was found to oxidize benzylamine at a rate approaching those of TPQ analogs bearing small alkyl substituents (*25*). The order of the catalytic activity of *o*-quinones **7b**, **8** and TPQ model compound **5b** was found to correlate with their redox potentials, indicating that the reoxidation of aminoquinol intermediate may be a rate-limiting step for *o*-quinone turnover (*25*).

Wang *et al.* synthesized pivalamide quinone (**16a**, **16b**) and phthalimidoethylquinone (**17a**, **17b**) in both reduced (benzenetriol) and oxidized (hydroxyquinone) forms (Fig. 3), as models closely reproducing the context of TPQ as it exists in the protein (*21*). In an attempt to elucidate potential reaction pathways which might occur during aerobic autorecycling deamination, Wang and colleagues reacted the reduced triol form of **16** with alkyl amines to give (alkylamino)resorcinols, via a redox-cycling mechanism that involves the condensation of amines with a trace of hydroxyquinone present in the triol preparation. According to the scheme proposed by these authors, the resulting hydroxyquinoneimine is subsequently reduced by triol to give (alkylamino)resorcinol and to regenerate the trace of hydroxyquinone catalyst (**16b**) (*21*). The regiochemistry of this substitution, demonstrated through a combination of NOE difference and long-range $^{13}C-^{1}H$ coupling data, confirmed that the position of nucleophilic addition of amines to TPQ models is at the electrophilic C5 carbonyl, as indicated also by the structural characterization of arylhydrazine derivatives (*6*,*20*,*21*).

A closer inspection of the mesomeric structures of the anionic form of TPQ models (Fig. 3), reveals that the carbonyl group corresponding to C5 should not share the "enolic" character of the other two oxygen atoms. Therefore, this "true" carbonyl group can be foreseen as being the one involved in reactions with amines, hydrazine derivatives and so on, owing to the relative electron deficiency at the corresponding carbonyl carbon atom.

In another study, the quinone form of the pivalamidoethyl-based model for TPQ (**16b**) was shown to be an effective catalyst for aerobic deamination of benzylamine, didemzylamine, and cinnamylamine in buffered aqueous acetonitrile, especially at pH 10 (*22*). In this model system, the overall yield was limited by the conversion of

16b to the corresponding benzoxazoles, implicating condensation of amines at the more electrophilic C5 carbonyl. On the other hand, "unactivated" primary amines (neopentylamine, α,α-dideuteriobenzylamine, methylamine, and *n*-propylamine) exhibited low deaminative turnover in the model system. Curiously, when (bpy)Cu(II) was added to the reaction's mixture, an inhibitory effect on the catalytic efficiency of **16b** was noted, with the inhibition being greater at higher pH (*22*).

The latter finding raises a number of questions about the effect exerted by Cu(II) on the turnover yield in model systems. In enzymes, the bound copper has been proposed to play an essential role in the reoxidation of the substrate-reduced cofactor (*57,58*), and a similar function is, in principle, conceivable also for model systems. As already been noted, the presence of (bpy)Cu(II) has been reported to be an essential ingredient for the aerobic deamination of benzylamine by topa (*28*). Studies conducted on other model systems have shown that Cu(II) greatly increases the efficiency of different TPQ models to oxidize a vast range of substrate amines in aqueous solutions (*24,34,59,60*). In this context, free topa and topamine were shown scarcely to oxidize peptidyl lysine in elastin in sodium borate buffer, pH 8, but the reaction was markedly stimulated by the addition of Cu(II) (*34*). Moreover, the redox profile of these models was altered by the presence of the metal ion, suggesting that copper–quinone complexes are functional in peptidyl lysine oxidation (*34*). Topa itself has been reported to be an ineffective catalyst for the aerobic deamination of benzylamine (*35*). The oxidized quinonic form of a covalently modified topa-derived compound, *N*-glutaryl-2,4,5-trihydroxyphenylalanine (Q-topa, **18**; Fig. 3), was found to be ineffective in catalyzing the oxidative deamination of putrescine at pH 7, while the addition of Cu(II) at an alkaline pH caused the formation of 1-pyrroline (*59,60*). The amount of 1-pyrroline formed was found to greatly exceed that of oxidized Q-topa, and a marked increase in 1-pyrroline production was observed in the presence of increasing amounts of Cu(II) (*59,60*). The effect exerted by Cu(II) ions on the oxidation of various amines catalyzed by 1-methyl-4-hydroxy-2,5-benzoquinone (**4**) (*24*), has been reported above.

On the other hand, a number of TPQ model compounds have been demonstrated to be active in deaminating benzylamine on their own, both in anhydrous or in buffered aqueous acetonitrile, without the need for added Cu(II) (*21–23,25,26*). Interpreting the observed inhibitory effect of (bpy)Cu(II) in their catalytic system containing **16b** as a TPQ model (*22*), Lee and Sayre proposed that the difference compared to the free topa model must arise from differences in the nature of metal coordination at the amine processing stage (*22,61*). In the case of topa, Cu(II) is coordinated to the free amino carboxylate end of the molecule, so that the coordination of Cu(II) to the free amino group would prevent its cyclization into the quinone ring, thus avoiding what otherwise would be a loss of deamination capability. In the case of **16b**, which lacks the free amino group, the expected coordination of Cu(II) is in a five-membered chelate ring with the oxo-enolate portion of the anionic **16b** nucleus, which would inhibit initial condensation of **16b** with the amine (*22,61*). The fall in efficiency is also explained by the accelerated formation of benzoxazoles in the presence of Cu(II), with the resulting accelerated destruction of the catalyst (*22*). These authors concluded that the aid by Cu(II) in autoxidative recycling in this model system is, if at all, of little significance.

Although the above explanation is consistent with experimental evidences, it is

not generally applicable to other model systems, such as 1-methyl-4-hydroxy-2,5-benzoquinone (**4**) and Q-topaquinone (**18b**), which lack the free amino group and for which a positive effect of Cu(II) on the relative catalytic efficiency has been claimed (*24,59,60*).

In conclusion, although this may not be applicable to all model systems, the TPQ cofactor has per se the capacity for redox cycling in the absence of involvement of a redox-active metal center. This crucial finding may prove to be an important key for the understanding of the true role played by the copper ion in the reoxidation of reduced cofactor in native enzymes, a matter still debated in the literature.

As mentioned above, it has been proposed that copper is required for the oxidative half-reaction. This stems from a number of observations: (i) inhibition of enzyme activity by exogenous copper ligands (*62*); (ii) metal-depleted amine oxidases are catalytically inactive because the reduced, aminoquinol form of the cofactor fails to undergo reoxidation in air (*57*); (iii) detection, upon reduction of various AOs with amine substrate, of a Cu(I)-semiquinone, which has been proposed to be the catalytic intermediate that reacts directly with oxygen during the copper-dependent sequential one-electron reoxidation of the substrate-reduced cofactor (*58*). Recently, Dooley and fellow researches have provided new evidence for the integral role played by copper in enzyme reoxidation and for the existence of a Cu(I)-TPQ$^{\bullet}$ intermediate in the reaction mechanism of AOs (*63,64*).

On the other hand, several reports argue against this scenario. Reconstitution of copper-depleted BSAO with Co(II) was found to restore some amine oxidase activity (*57,65,66*). Since the Co(II)-derivative cannot form a semiquinone radical intermediate, the finding that Co(II)-BSAO is catalytically competent suggests that the Cu(I)-semiquinone is not an obligatory intermediate of the catalytic pathway. On the basis of these findings, the metal ion in BSAO was suggested to have a structural function, i.e., to maintain the active site in a proper conformation, rather than being directly involved in amine oxidation (*57,65*). More recently, Sue and Klinman conducted a very detailed study on the oxidative-half reaction of BSAO and proposed a passive role of copper in the reoxidation of reduced cofactor by molecular oxygen (*67*). Clearly, the mechanism of the oxidative half-reaction of native AOs and the role played by copper in this process need to be studied further. Readers are directed to the quoted references for a more detailed discussion on this portion of the catalytic mechanism of AOs.

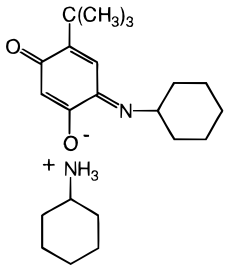
In the panorama of works that made use of model compounds of TPQ as amine oxidants, some studies conducted by Koomen and coworkers should be mentioned. These aimed to demonstrate the biosynthetic routes leading to several plant alkaloids, namely lupin and indole alkaloids (*68,69*). These alkaloids are thought to derive from cadaverine through a multistep biogenetic process, with the presumable involvement of TPQ-containing AOs, the deaminating enzymes for cadaverine in plants. The authors made use of a commercially available *o*-quinone as a model of TPQ, 3,5-di-*tert*-butyl-1,2-benzoquinone (**19**; Fig. 3), the well-known "Corey's reagent" (*68,69*). Model compound **19** was shown as effective in catalyzing the oxidative deamination of tetrahydroanabasine and nazlinin, biogenetic intermediates to lupin and indole alkaloids, respectively, under experimental conditions (*68,69*). The results obtained by Koomen and colleagues support, therefore, the hypothesis that a TPQ-bearing

enzyme might be involved in the biosynthetic pathway of the aforementioned plant alkaloids, probably by catalyzing the oxidative deamination of both cadaverine and cadaverine-derived biogenetic intermediates. Moreover, these studies showed that the oxidative deamination of tetrahydroanabasine and nazlinin with *o*-quinones might serve as a convenient synthetic route for the laboratory synthesis of a number of plant alkaloids. It should be noted that, although **19** is known to be an unsuitable catalyst for the oxidative deamination of primary amines containing an α-unbranched substituent (like benzylamine), since benzoxazoles are formed instead of aldehydes, in the case of tetrahydroanabasine and nazlinin the formation of the corresponding benzoxazoles is prevented by the presence of a second amino group (for more details on these reaction schemes, see Refs *68* and *69*).

Mechanism of Model Reactions and Characterization of Reaction Intermediates

One point of interest is the reaction mechanism for the oxidative deamination of amines catalyzed by model compounds. As explained above, a transamination mechanism appears to be in force for the enzyme, whereas an addition-elimination mechanism was found to be a competing pathway for benzylamine deamination using PQQ (*70,71*). The two mechanisms both involve the formation of an addition compound between the proper carbonyl group of the quinonoid cofactor and the amine substrate. At this point, the cofactor could act like pyridoxal phosphate, with the well known sequence of water elimination, formation of the first Schiff base, isomerization to the second Schiff base, and hydrolysis to the final products. This is conventionally referred to as a transamination mechanism. Alternatively, the adduct formed between the amine and the quinonoid compound could directly undergo a base-catalyzed cleavage, leading to aldimine and reduced quinonoid compound. This is an addition/elimination mechanism, being that the aldimine derived from the amine substrate the eliminated species. A significant difference between the two routes is indicated by the fact that an aminoresorcinol intermediate is formed during the transamination process, from which ammonia is released upon oxidation, whereas ammonia is released from the hydrolytic cleavage of the aldimine provided that the addition/elimination mechanism operates.

Providing support for a transamination reaction mechanism, Mure and Klinman examined the mechanism of interaction of 1-*tert*-butyl-4-hydroxy-2,5-benzoquinone (**5b**) with amines in acetonitrile and succeeded in the synthesis and characterization of model components for each of the intermediate species postulated for the enzymatic reaction (*26*). These include both substrate (**20**–**23**) and product Schiff base (**24**) analogs (Fig. 4), as well as aminoresorcinol analogs (**9**, **25**; Fig. 4) (*20,26*). Monitoring the reaction of **5b** with benzylamine in the absence of oxygen by ^{1}H NMR and UV–vis spectroscopy indicated the formation of intermediates similar to those characterized (*26*). TPQ model compound **5b** formed Shiff base complexes with various amines. When using cyclohexylamine and *n*-propylamine as substrates, the corresponding Schiff bases (**20** and **21**, respectively) were isolated and fully characterized. As revealed by ^{1}H NMR and UV–vis spectroscopy, α-methylbenzylamine also formed the substrate Schiff base **22**, but this was not isolated because of its high solubility. The expected substrate Schiff base **23** which formed during the oxidation of benzylamine by **5b** was not isolated nor detected by spectroscopic methods, presumably due to its



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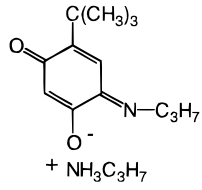

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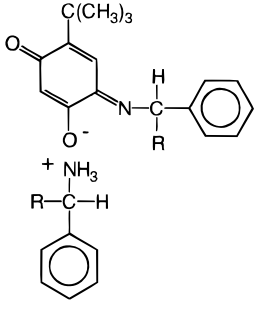
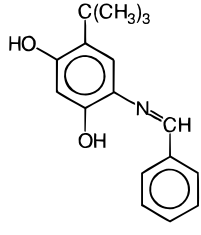


22 R = CH_3

23 R = H



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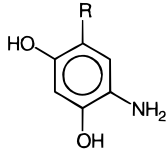

9 R = C_2H_5

25 R = $C(CH_3)_3$

FIG. 4. Model compounds for the intermediates of the TPQ-mediated amine oxidation reaction: substrate Schiff base analogs (**20**–**23**), product Schiff base analog (**24**), aminoresorcinol analogs (**9**, **25**).

unstability and rapid conversion to the product Schiff base **24**. Detailed structural analysis of the substrate Schiff base was performed on **20** using 1D and 2D NMR techniques. The results obtained showed that the position of the nucleophilic addition was at the C5, the carbonyl carbon next to the 4-hydroxyl group. The characterization of substrate Schiff base **20** was further pursued by means of UV–vis spectroscopy in different solvents. In an aprotic solvent (CH_2Cl_2), **20** exists as a contact ion pair with λ_{max} 352 nm. Changing the solvent to a polar protic one (MeOH) causes the disappearance of the 352 nm band and the appearance of a new maximum at 454

nm. In the case of a polar protic solvent, **20** is expected to exist as a fully ionized ion pair, and the observed 100-nm red shift of the λ_{max} is ascribable to the resonance delocalization of the anionic species between oxygens at C2 and C4. These observations support the proposed structure of the substrate Schiff base complex of enzyme (II; Fig. 2), which has been assigned a λ_{max} at 340 nm. This, together with the fact that there is no resonance delocalization in the substrate Schiff base complex of the enzyme, indicates the existence, in the enzymatic system, of a strong electrostatic interaction of the oxyanion at C4 of the cofactor with the neighboring protonated Schiff base (*26*). Alternatively, an interaction with an active-site residue may be in force.

A recent determination of the crystal structure of the complex between the copper amine oxidase from *E. coli* (ECAO) and a covalently bound inhibitor, 2-hydrazinopyridine, permitted to obtain a more detailed picture of the structure and interactions of the substrate Schiff base at the enzyme active site (*72*). In fact, the inhibitor complex is analogous to the substrate Schiff base formed during the reaction with natural substrates. In this case, in addition to the role of electrostatics in localizing the oxyanion in the substrate Schiff base, the oxygen at C4 on the quinone ring was found to be involved in a short hydrogen bond with the hydroxyl of a conserved tyrosine residue (Tyr369 in ECAO) (*72*). This interaction is thus thought to play an important role in stabilizing the position of the substrate Shiff base and is consistent with the oxygen at C4 being ionized (*72*).

The product Schiff base **24** was prepared from the reaction of the aminoresorcinol **25** and benzaldehyde in anhydrous CH_3CN. The UV−vis spectrum of this model compound revealed a λ_{max} at 368 nm and an ε value 10 times larger than that of the substrate Schiff base (**20**, **21**, or **22**), reflecting a double bond in conjugation with the benzene rings. The product Schiff base **24** was highly unstable, especially in an aqueous solution, and rapidly hydrolyzed to the aminoresorcinol and benzaldehyde (*26*).

Aminoresorcinols **9** and **25** were prepared from the corresponding 4-alkylresorcinol, via nitrosation and reduction in two steps, as models of the substrate-reduced form of TPQ in AOs (*20*,*26*). Solutions of aminoresorcinols **9** and **25** are colorless, showing no absorption at around 488 nm, comparable to the reduced form in enzymes. The p*K*a values of the amino and two hydroxyl groups of **9** were found to be 5.88, 9.59, and 11.62 respectively, by spectrophotometric titration (*20*). Therefore, the reduction of the cofactor leads to an increase in p*K*a of the 4-hydroxyl group (2-hydroxyl group in model compounds) of ca. 5 pH units.

In order to determine which structure of the resonance hybrid species (*p*- or *o*-quinonoid) of TPQ takes part in the reaction with amines, Mure and Klinman compared the results obtained using the model compound **5b** with those obtained with the *o*-quinone **8**. At variance from **5b**, which has a *p*-quinonoid structure, and from TPQ-containing enzymes, **8** proved to oxidize *sec*-alkyl primary amines, such as α-methylbenzylamine and cyclohexylamine, efficiently. However, no substrate Schiff bases were detected in the reaction of **8** with both α-methylbenzylamine and *n*-propylamine, while the corresponding product Schiff bases were observed spectroscopically (*26*). Such behavior resembles that described for Corey's reagent **19**, for which Klein and coworkers studied the mechanism of the oxidative deamination of *sec*-alkyl primary

amines (*73*). In this case, a transamination mechanism involving a non-base-catalyzed spontaneous rearrangement of the substrate Schiff base to the product Schiff base was proposed (*73*). It should recalled that the reaction of **5b** with cyclohexylamine and α-methylbenzylamine gave only the corresponding substrate Schiff bases **20** and **22**, respectively, and no further rearrangement to the product Schiff bases was observed (*26*). It has already been described that base-catalyzed proton abstraction is a known requirement for the conversion of the substrate Schiff base to the product Schiff base in TPQ-containing enzymes (Fig. 2). In conclusion, the findings of Mure and Klinman led to the proposal for an enzymatic substrate Schiff base intermediate in a localized *p*-quinone form and strengthened the theory of the existence of a transamination mechanism for amine oxidation (*25,26*). Although these studies support the representation of the functional form for TPQ in the active site of AOs as a 2-hydroxy-1,4-benzoquinone, however, they did not rule out the participation of an *o*-quinone-like species in the initial nucleophilic attack by amine to form the Schiff base complex.

As regards the significance of *ortho*-quinone structure for amine oxidation catalysts, it should be noted that the usually preferred *para*-quinonoid structure of TPQ seems to be in marked contrast to PQQ, TTQ, and LTQ, which all bear an *ortho*-quinone structure (Fig. 1). With this in mind, Kano and coworkers have recently performed a kinetic study on the oxidative deamination of benzylamine catalyzed by PQQ, a model compound for TTQ (see structure **28**; Fig. 7), and TPQ, obtained by air oxidation of commercial free topa (*74*). These authors found a low catalytic activity of TPQ compared with PQQ and TTQ model, and ascribed this to the contribution of the *p*-quinonoid structure of TPQ (*74*). In fact, by examining the catalytic activity of a number of *o*- and *p*-quinones by cyclic voltammetry, Kano *et al.* proposed that an asymmetric *o*-quinonoid structure, with a nonequivalence of the electronic characteristics of the two carbonyl groups, would be essential for catalytic amine oxidation activity (*74*). A role was proposed for Cu(II) at the active site of AOs as a weak ligand of the TPQ cofactor, a coordination that would stabilize the deprotonated form of the cofactor and would make TPQ bear an *o*-quinone-type structure, thus favoring amine oxidizing activity (*74*). Although such TPQ–copper interaction is feasible in principle, there is general agreement, based on the solved crystal structures of various AOs, that TPQ is not directly coordinated to copper (*54–56,75*). In contrast, TPQ and the metal ion are connected through a water molecule, which is coordinated to the copper ion in an axial position and hydrogen bonded to the C2 oxygen of TPQ (*56*). Moreover, the C5 and C4 oxygens of TPQ are connected to the copper site via hydrogen-bonding networks that involve a number of active-site residues and water molecules (*56*).

In a successive attempt to distinguish between transamination and addition–elimination mechanisms, Lee and Sayre used NMR to identify the initial product of model cofactor reduction under anaerobic single-turnover conditions using their pivalamidoethyl model **16b** (*23*). Unfortunately, the occurrence of redox interchange reactions that scrambled the initial cofactor reduction product made any assignment of mechanism ambiguous (*23*). To circumvent these problems, these authors utilized the *tert*-butyl model **5b** for the deamination of benzylamine under reaction conditions that permitted the unambiguous demonstration of a transamination mechanism both in CH_3CN and DMSO (*23*). In order to confirm their findings, Lee and Sayre reacted

5b with the mechanistic probe 5-amino-1,3-cyclohexadienecarboxylic acid (gabaculine). In this case, in fact, the initial single-turnover products tautomerize to aromatic moieties incapable of redox interchange (see above), thus permitting unambiguous mechanistic conclusions. The reaction with 5-amino-1,3-cyclohexadienecarboxylic acid mainly followed transamination, though addition–elimination was found to be a minor competing pathway for this branched primary amine (*23*). It would be of some interest to investigate whether the minor extent of addition-elimination is a general occurrence for branched primary amines in model systems, and if this finding is enzymologically relevant, since TPQ-dependent AOs are very ineffective at deamination of branched primary amines.

The distinction between transamination and addition–elimination mechanisms has been further explored by Sayre and coworkers by reacting model compounds **16b** and **5b** with a secondary amine, pyrrolidine, and with the analog 3-pyrroline, expecting in this case that an addition–elimination mechanism would be observed in their model system (*27*). However, determination of reaction products revealed that in both substrates transamination was the predominating reaction pathway. In particular, reaction with 3-pyrroline led to a pyrrolylated form of the model cofactor, suggesting that 3-pyrroline might act as a cofactor-directed irreversible inactivator of TPQ-containing AOs (*27*). In fact, BSAO was found by the same authors to be inactivated by 3-pyrroline in a concentration- and time-dependent manner, indicating that 3-pyrroline was processed by BSAO through the usual transamination mechanism, leading to the pyrrolylation of TPQ and thus to enzyme inactivation (*27*). Despite the fact that the actual pyrrolylation of TPQ in the enzyme system needs to be confirmed, this finding is interesting if one considers that secondary amines are not substrates of AOs. Sayre and colleagues proposed that the observed inability of AOs to utilize secondary amines as substrates is due to active-site steric exclusion of the amines, rather than their inability to undergo transamination (*27*).

In conclusion, the work conducted by the groups of Klinman and Sayre provided an unambiguous demonstration that the oxidation of amine substrates by TPQ analogs generally follows a transamination mechanism, similar to that of native AOs. This makes model systems particularly suitable for studies aimed at elucidating the mechanistic details of the reaction catalyzed by these enzymes, supporting and integrating the data obtained with native AOs.

Other evidence, based on model studies, of the structure of AOs reaction intermediates and of the role played by the TPQ cofactor has been supplied by Pedersen and coworkers (*37*). Using topamine (**3**; Fig. 3) as a cofactor analog, these authors were able to detect by EPR and optical spectroscopy the formation of an amine-topamine semiquinone radical, when the model compound was incubated with several amines at physiological or alkaline pH values (*37*). The similarity of both EPR and optical spectra recorded in the case of topamine with those obtained by reduction of lentil seedling amine oxidase in anaerobiosis with substrates, led to the conclusion that the enzyme radical is an amine-topa semiquinone (*37*). As mentioned above, the formation of a free radical when AOs are reduced by a substrate was first recognized some time ago, and the generation of a Cu(I)-semiquinone state was demonstrated for a range of substrate-reduced AOs from different sources (58 and references therein).

TPQ–Copper and TPQ–Protein Interactions

Although recent X-ray studies have shown that TPQ is not directly coordinated to the metal atom (see above), earlier works have indicated a considerable interaction between the two redox components (76 and references therein). With the intention of clarifying the structural and the functional relationships between nonblue copper and the organic cofactor in AOs, some coordination studies that involved the synthesis of a complex between copper (or another divalent cation) and a TPQ model were therefore conducted (*28*,*77*). Suzuki and coworkers have constructed a topa-containing ternary Cu(II) complex ([Cu(DL-topa)(bpy)(H_2O)] $BF_4 \cdot 3H_2O$) (*28*). Previous extensive biophysical characterization of the copper site in native AOs through a combination of EXAFS, EPR, ESEEM, ENDOR, and NMR solvent relaxation studies indicated a square pyramidal geometry with three equatorial histidine ligands, together with an equatorial and axial water molecule (*76*). X-ray studies of the topa-containing ternary Cu(II) complex indicated that the metal ion was complexed by the amino and carboxyl groups of topa (*28*). In protein-bound topa, both these functional groups are incorporated into the polypeptide chain. However, it is interesting to note that the copper–topa complex promoted the oxidation of benzylamine in an aqueous solution (*28*). We have already discussed the proposal of Kano *et al.* of a weak coordination of Cu(II) to TPQ (*74*).

Given the proposed existence in the reaction mechanism of AOs of a semiquinonoid radical intermediate (*58*), Kaim and colleagues prepared and characterized by EPR a paramagnetic ruthenium(II) complex of 1-methyl-4-hydroxy-2,5-benzoquinone (**4**) as a model compound of TPQ, (L)Ru$(bpy)_2$, where L is the dianionic semiquinone form of **4** (*77*). The properties shown by this Ru(II) complex of a model compound of TPQ were compared with those of a similar Ru(II)–PQQ complex (*77*). The Ru(II)–TPQ semiquinonoid complex was shown to exist in the neutral deprotonated form and attempts to protonate the phenolate function in the neutral radical complex led to the disappearance of the EPR signal. By comparing the similar though not identical redox and coordination properties of the model system of TPQ relative to PQQ, it emerged that the most important difference is the replacement of basic (and normally protonated) pyrrolic nitrogen by less basic phenolate oxygen in the *para*-position on going from PQQ to TPQ. As a consequence, the persistent semiquinone complex of the deprotonated TPQ model shows less metal contribution to the radical ground state, resulting in a better EPR resolution and a smaller metal coupling constant (*77*).

In another set of model studies, Bossa and fellow researchers confirmed that copper is not within bonding distance of the oxidized cofactor (*29*). These authors performed a detailed analysis of spectroscopic data obtained for low-molecular-weight models of the TPQ cofactor, with the aid of semiempirical molecular orbital calculation methods, in an attempt to gain a deeper insight into the cofactor electronic and basic structural features (*29*,*78–80*). In particular, the investigation of the electronic properties of some adducts of 1,4-dihydroxy-2,5-benzoquinone (**26**; Fig. 3) with bases and hydrazines revealed that all the electronic transitions examined, although highly sensitive to cofactor ionization and hydrogen bonding, could be accounted for without

introducing perturbations due to copper, thus disfavoring the possibility of a direct copper–TPQ interaction (*29*). These studies and, more recently, the AM1 and PM3 semiempirical calculations performed on the adducts between **26** or **4** and 4-(*N*,*N*-dimethyl)aminopyridine (*81*), also provided clues about the TPQ–protein interactions through hydrogen bonding. The ability of TPQ model compounds, namely **26**, in hydrogen bonding to a large variety of proton acceptors like substituted pyridines and imidazole, suggested that the hydroxyl group at C4 of the cofactor exists in the deprotonate, anionic form and is engaged in a proton-transfer bond to an acceptor, either a water molecule or a basic protein residue (*29*). The N-base 4-(*N*,*N*-dimethyl)-aminopyridine was selected by Bossa and coworkers as a suitable proton acceptor that could mimic the proton acceptor present at the enzyme active site and needed for the stabilization of the TPQ anion. Since the adduct between **26** and 4-(*N*,*N*-dimethyl)aminopyridine can exist in two tautomeric forms, namely a hydrogen-bonded ($OH^{\cdots}N$) and a proton transfer one ($O^{-\cdots}HN^{+}$), these authors tested the hypothesis that the system of hydrogen bonds at O2 and O5 of TPQ might play a role in supplying a mechanism for fine tuning the proton position at O4 during the enzyme-catalyzed reaction (*81*). This was done by introducing at specific sites of the adduct between **26** and 4-(*N*,*N*-dimethyl) aminopyridine a number of interacting molecules capable of hydrogen bonding (besides water, different alcohols, chloroform, and methylene chloride were considered) and by evaluating coordination effects on the energetics and geometries of the adduct (*81*). The stability of the proton-transfer tautomer, which is the unfavoured species in the gas phase, was found to increase with the number of proton donors that are coordinated to the system, approaching that of the hydrogen-bonded form when the coordination shell is completely saturated. Moreover, coordination effects were shown appreciably to decrease the activation energy barrier of the proton-transfer reaction (*81*). The authors pointed out that the replacement of water molecules by protein residues as proton donors is not expected to affect substantially the properties of the system. This means that the true identity of the coordinated proton donor species (water or protein residues) which are present at the enzyme active site cannot be ascertained by means of semiempirical calculations.

The recent X-ray study by Li *et al.* of the AO from the yeast *Hansenula polymorpha* gives the first picture of the active site of the enzyme with the TPQ ring in its productive conformation (*56*). This permitted the localization of a network of hydrogen-bonded water molecules in and around the active site of the enzyme. A water molecule was found to be hydrogen bonded to C5 and C4 oxygens of TPQ, which in turn is also hydrogen bonded to Tyr305 (equivalent to Tyr369 in ECAO, see above) (*56*). This water molecule may provide a pathway for transfer of the proton, removed by the active-site basic residue from the amine substrate during its oxidation, to the O4 of the reduced TPQ ring (*56*).

In conclusion, evidence from model studies and from the available X-ray structures of AOs, which confirmed the existence of a network of hydrogen bonds between TPQ and both water molecules and protein residues, indicate that the presence of such coordinated molecules exerts a great influence on the structure and reactivity of the AOs active site.

Biogenesis of TPQ

To date, only a few researchers have tried to address the issue of TPQ biogenesis by means of model studies. Following the finding that a tyrosine codon corresponds to TPQ at the active site of the *H. polymorpha* amine oxidase (*82*), the involvement of tyrosine as the precursor of TPQ was confirmed by comparative studies of DNA and protein sequences from lentil seedlings, bovine serum, and human and porcine kidneys (*83,84*). It was also found that the precursor amino acid is contained within a consensus sequence of Asn–Tyr(TPQ)–Asp/Glu (*42*). Subsequently, studies were conducted by the groups of Klinman and Tanizawa. By using different approaches, they demonstrated the generation of TPQ through the self-processing of the protein with the participation of the copper ion in the active site, thereby ruling out the possibility that a separate set of enzymes, capable of recognizing the tyrosine precursor in the consensus sequence, could be responsible for the generation of TPQ (*85–87*). Moreover, site-directed mutagenesis studies have shown that the copper-ligating histidine residues are required for TPQ formation, confirming that coordinated copper is involved in the biogenesis reaction (*88*). Tanizawa prepared a comprehensive review on early work on TPQ biogenesis (*11*).

As illustrated in Fig. 5, Mu and colleagues (*82*) postulated a pathway for the generation of TPQ that involves initial hydroxylation of Tyr to 3,4-dihydroxyphenylalanine (dopa) (step 1), followed by oxidation of dopa to dopaquinone (step 2). Nucleophilic attack by water (or hydroxide ion) on the dopaquinone ring leads to topa (step 3). Further oxidation of topa would finally give TPQ (step 4). The same authors delineated a detailed chemical mechanism for the entire process. A hydroperoxide ion bound to copper would be used in the initial ring hydroxylation. Subsequent oxidation of dopa to dopaquinone would be followed by bond rotation about the β-carbon, placing the C2 ring carbon in close proximity to a copper-bound hydroxide ion. Nucleophilick attack by the copper hydroxide on the dopaquinone would yield the quinol form of TPQ.

In this mechanism, the rotation of the dopaquinone intermediate was suggested in order to enable the copper ion to catalyze both the initial oxidation of tyrosine and the subsequent attack of water on dopaquinone. The role of copper in the latter step would be reducing the p*K*a for water, thus making it a much more efficient nucleophile by conferring a hydroxide character. In fact, it is conceivable in principle to think that the concentration of hydroxide ion itself at the enzyme active site would be much too low to serve as an effective nucleophile.

The solvent origin of C2 oxygen, deduced form ^{18}O-labeling studies, supports the

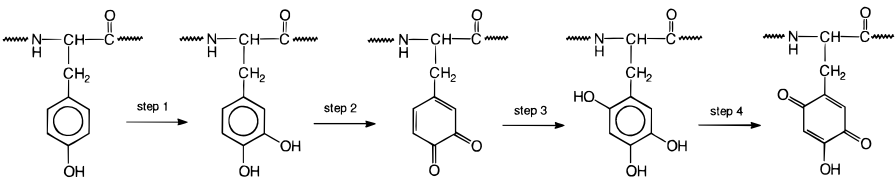

FIG. 5. Postulated pathway for the biogenesis of TPQ cofactor from a tyrosine residue in copper amine oxidases.