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Tyrosine-Derived Quinone Cofactors

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ABSTRACT
Copper amine oxidases (CAOs) and lysyl oxidase (LOX) both contain Cu^{2+} and quinone cofactors that are derived from a tyrosine residue in the active site. In CAOs, the cofactor is 2,4,5-trihydroxyphenylalanine quinone (TPQ), and in LOX it is lysine tyrosyl quinone (LTQ). The mechanism of oxidative deamination by CAOs is well understood, but there is a controversy surrounding the role of Cu^{2+} in cofactor reoxidation. The chemistry of LTQ in LOX, by contrast, has not been as extensively studied. This Account discusses the strategies that CAOs have evolved to control the mobility of TPQ to optimize activity. In addition, some recent studies on CAOs whose active-site Cu^{2+} has been replaced with Co^{2+} or Ni^{2+} are summarized. Finally, there is a discussion on the properties of a model compound of LTQ and their relevance to the chemistry of LOX.

Introduction

Quinoproteins constitute a class of enzymes that contain quinones as their cofactors.[1] The first quinone cofactor to be identified was pyrroloquinoline quinone (PQQ, Figure 1) in bacterial alcohol and glucose dehydrogenases. PQQ is dissociable and is bound to the protein via ionic interactions through its three carboxylate groups. A second class of nondissociable quinone cofactors (Figure 1) was first observed in copper amine oxidases (CAOs). While originally thought to be covalently bound PQQ, the cofactor was later identified as 2,4,5-trihydroxyphenylalanine quinone (TPQ).[2] The discovery of TPQ led to the identification of a series of new quinone cofactors, namely, lysine tyrosylquinone (LTQ) in lysyl oxidase (LOX)[3] and tryptophan tryptophylquinone (TTQ)[4] and cysteine tryptophylquinone (CTQ)[5] in bacterial amine dehydrogenases. Although the concept of PQQ as a cofactor in eukaryotic enzymes quickly diminished, a recent report supports its function as a vitamin in mammals.[6] TPQ and LTQ are derived from a tyrosine residue in the active site via posttranslational modifications.[7] The biogenesis of TPQ requires only the active-site Cu^{2+} and O_2. Cu^{2+} is proposed to catalyze hydroxylation of the tyrosine ring to generate dopa, which undergoes oxidation, hydration, and finally oxidation to yield TPQ. A recent X-ray crystal structure analysis of the biogenesis intermediates supports this proposal.[8] LTQ contains a cross-link between an ϵ-amino side chain of a lysine and a modified tyrosine within the same polypeptide chain.[3] It has not been established whether the biogenesis proceeds via a self-processing mechanism as in TPQ.

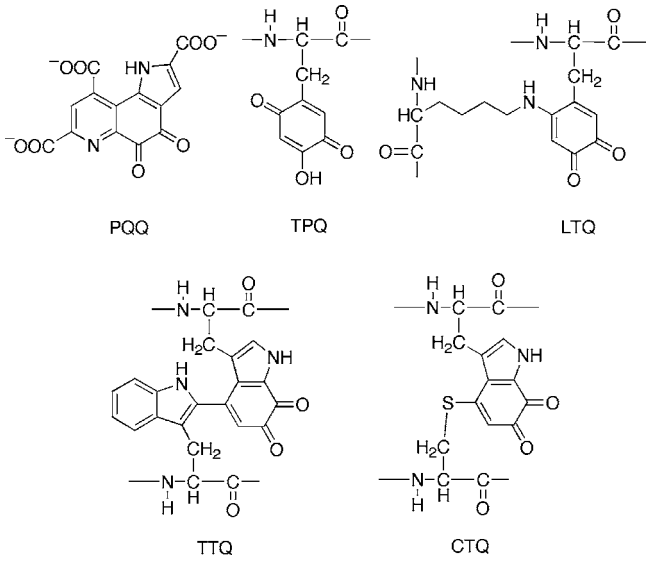

FIGURE 1. Structures of quinonoid cofactors.

Many reviews are available on quinoproteins,[1,7,9] mechanism of CAOs,[10] and properties of LOX and LOX-like proteins.[11] In this Account, recent findings on the mechanism of CAOs and the properties of the LTQ cofactor in LOX will be described. The first part concerns TPQ in CAOs, illustrating how reactivity and conformational flexibility are carefully modulated in the enzyme active site, together with a discussion of the role of Cu^{2+} in cofactor reoxidation, a matter of much contention. The second part will describe a comparison of spectroscopic and chemical properties of LTQ and TPQ.

Copper Amine Oxidases

CAOs are widely dispersed throughout nature and are found in bacteria, yeasts, fungi, plants, and mammals, although their precise physiological roles are still largely undefined. In bacteria, fungi, and yeast, CAOs enable utilization of amines as sources of nitrogen and carbon for growth. In plants, they are involved in wound healing and play a role in growth. In mammals, they seem to function as regulatory enzymes, catabolizing a wide range of biogenic amines. In addition, new roles have been proposed for mammalian cell surface CAOs in regulation of glucose uptake, signaling, and cell adhesion.[12] Of the organisms whose complete genomes are available, it is notable that *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* have no genes encoding CAOs.

The reaction catalyzed by CAOs follows a classical ping-pong mechanism composed of distinct half-reactions. In the reductive half-reaction, a substrate amine is oxidatively deaminated to the product aldehyde, leaving the enzyme reduced by $2e^-$ (eq 1). In the oxidative half-reaction, O_2 oxidizes the reduced enzyme back to the resting state,

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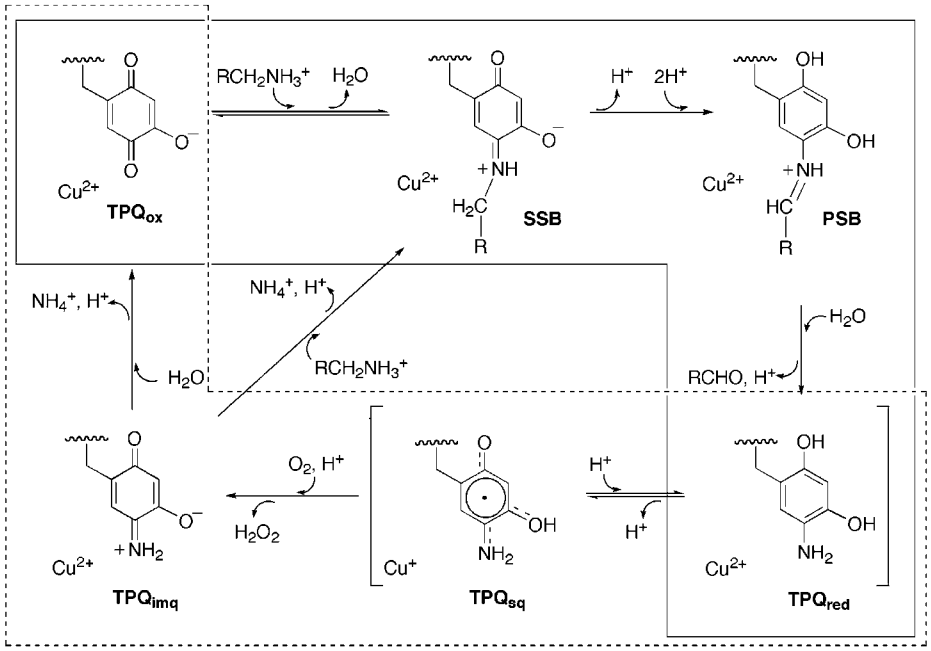
Scheme 1. Reaction Mechanism of CAOs[a]



[a] TPQ_{ox}, oxidized form of TPQ; SSB, substrate Schiff base intermediate; PSB, product Schiff base intermediate; TPQ_{red}, reduced form of TPQ; TPQ_{sq}, semiquinone form of TPQ; TPQ_{imq}, iminoquinone form of TPQ. The reductive and oxidative half-reactions are shown in solid and dotted boxes, respectively.

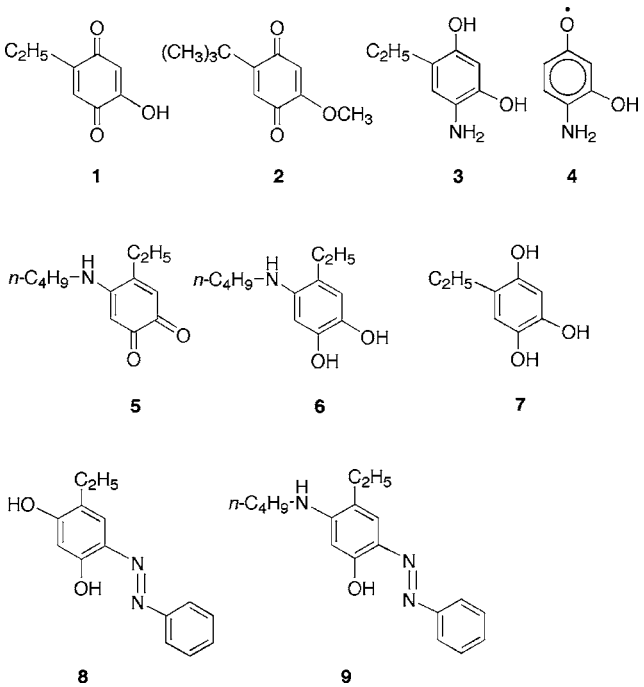


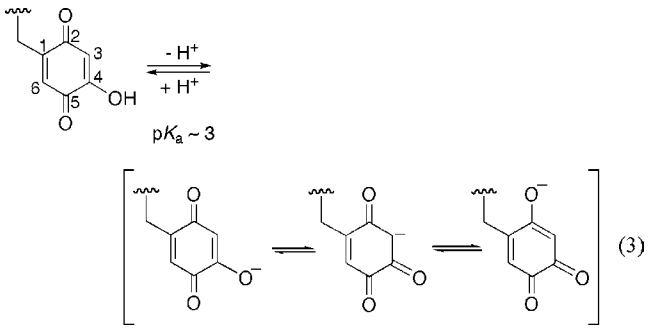
FIGURE 2. Structures of model compounds.

releasing NH_4^+ and H_2O_2 (eq 2). The current understanding of the reaction mechanism is shown in Scheme 1.[10] Nucleophilic addition of an amine at the C5 position of the TPQ cofactor and subsequent dehydration produces the substrate Schiff base intermediate (SSB). C1 proton abstraction from the SSB results in the reduction of the TPQ ring to yield the product Schiff base intermediate (PSB). Hydrolysis of the PSB forms the aminoquinol form of TPQ (TPQ_{red}) and releases a product aldehyde, completing the reductive-half reaction. In the oxidative-half reaction, a $2e^-$-transfer from TPQ_{red} to O_2 yields the iminoquinone form of TPQ (TPQ_{imq}) and H_2O_2. Hydrolysis of TPQ_{imq} releases NH_4^+, regenerating TPQ_{ox}.

$$E\text{-}TPQ_{ox} + RCH_2NH_3^+ \rightleftharpoons E^{\text{-}RCH_2NH_3^+}_{\text{-}TPQ_{ox}} \longrightarrow E\text{-}TPQ_{red} + RCHO + H_2O \quad (1)$$

$$E\text{-}TPQ_{red} + O_2 + H_2O \longrightarrow E\text{-}TPQ_{ox} + NH_4^+ + H_2O_2 \quad (2)$$

At physiological pH, TPQ_{ox} exists as a resonance-stabilized monoanion (eq 3), exhibiting a characteristic broad visible absorption band at around 480 nm. The λ_{max} of TPQ_{ox} reflects both the hydrophobicity of the surrounding environment and the degree of charge delocalization. The pK_a of the 4-hydroxyl group is 4.1 in the model compound (**1**, Figure 2) and ~3.0 in the enzyme.[13] Protonated TPQ_{ox} loses the 480 nm absorbance, displaying a spectrum characteristic of *p*-quinones (i.e., **2**, Figure 2).[14] The electron delocalization through O4 to O2 directs the nucleophilic addition of an amine to the C5 carbonyl carbon, preventing 1,4-addition to the quinone ring.[14]



(3)

Structural Considerations. To achieve optimum activity, the positioning and orientation of the TPQ ring are

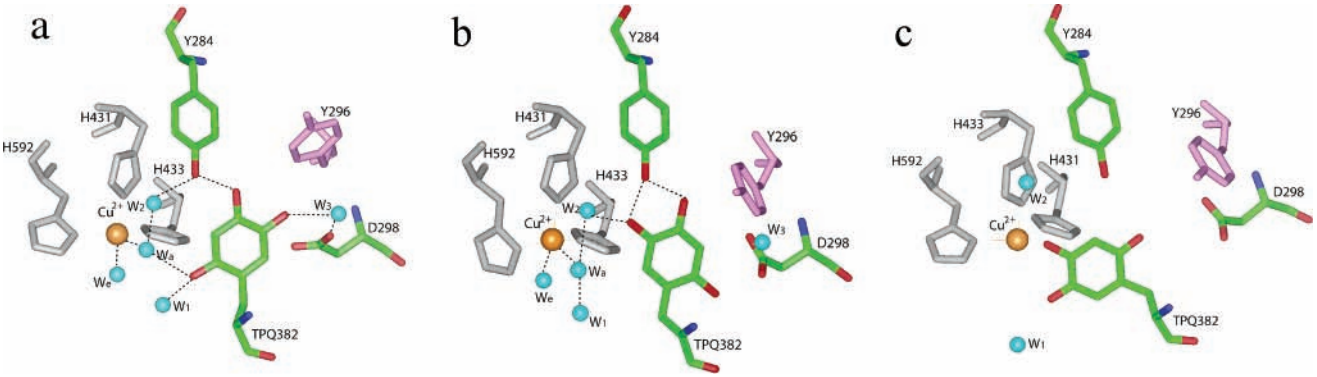


FIGURE 3. Active-site structures of WT-AGAO: (a) active conformation of TPQ_{ox}; (b) flipped conformation of TPQ_{ox}; (c) on-copper form of TPQ_{ox}. Y296 is located at the base of the proposed substrate channel.

Table 1. Residue Numbers in AGAO, EGAO, and HPAO

AGAO	ECAO	HPAO
M280[a]	M365[a]	M301[a]
V282[b]	V367[b]	V303[b]
Y284[a]	Y369[a]	Y305[a]
Y296[a]	Y381[a]	A317[a]
D298[a,c]	D383[a,c]	D319[a,c]
N381[b]	N465[b]	N404[b]
TPQ382	TPQ466	TPQ405
D383	D467	E406
Y384[a,d]	Y468[a,d]	Y407[a,d]
A402[d]	A486[d]	L425[d]
F407[a]	A491[a]	N430[a]
H431[e]	H524[e]	H456[e]
H433[e]	H526[e]	H458[e]
H592[e]	H689[e]	H624[e]
M602[d]	M699[d]	M634[d]

[a] Residues surrounding wedge-shaped pocket. [b] Residues creating wedge-shaped pocket. [c] Active-site base. [d] Nonmetal O_2 binding site. [e] Ligands for Cu^{2+}.

carefully modulated through interactions with the active-site residues and several conserved water molecules. Table 1 lists the active-site residues in *Arthrobacter globiformis* (AGAO), *Hansenula polymorpha* (HPAO), and *Escherichia coli* (ECAO). Unless otherwise stated, the residue numbers used herein correspond to the CAO from AGAO.

Figure 3a shows the active conformation of TPQ that was first observed in HPAO at pH 6.5[15] and later in AGAO at pH 6.8.[16] The C5 carbonyl of TPQ_{ox} faces the active-site base residue (D298) and the proposed substrate channel, and the C2 carbonyl group lies near the Cu^{2+} site. The O4 of TPQ_{ox} forms a short hydrogen bond with the hydroxyl group of a conserved tyrosine residue (Y284). W3 forms a hydrogen-bonding bridge between O5 and D298. Cu^{2+} is generally observed as five-coordinate, with three histidine and two water ligands (axial water denoted as Wa and equatorial water denoted as We) in a distorted square pyramid. There is a conserved hydrogen-bonding network through O4, Y284, W2, and Wa to O2. In ECAO at pH 7.0, TPQ is in the active conformation, but the electron density for TPQ suggests some limited mobility (i.e., pivoting).[17] When crystals are grown in the presence of ammonium or lithium sulfates (>1.0 M) or at an acidic pH (<5.0), TPQ is seen in altered conformations where it has flipped (Figure 3b) or ligated to Cu^{2+} through O4, termed the on-copper form (Figure 3c).[18−20] In the flipped form, the TPQ ring has rotated around C_β–C_γ, retaining

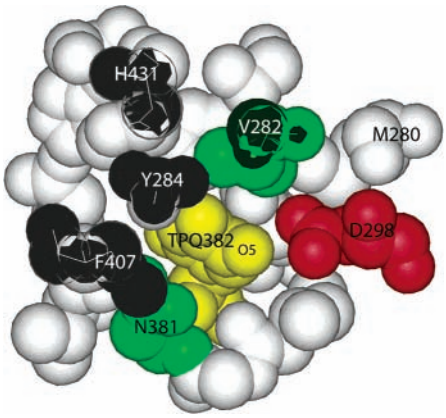


FIGURE 4. Space-filling representation of the region surrounding TPQ_{ox} (yellow) in the active conformation of WT-AGAO (100 K). V282 and N381, forming a wedge-shape pocket, are shown in green. O5 of TPQ is facing D298 (in red). Labeled residues are all conserved except F407 (see Table 1). The figure was generated based on the wedge structure first reported for WT-ECAO.[17]

the hydrogen-bonding interaction between O4 and Y284 (Figure 3b). The crystals where TPQ is bound to copper are colorless, indicating charge localization of the O4 anion through ligation to Cu^{2+}.

A wedge-shaped pocket surrounding TPQ (Figure 4) was first found in ECAO and was subsequently seen in all CAO structures.[17] When this cavity is disrupted by mutation of Y284, D298, or N381, TPQ_{ox} can swing out, resulting in a significant loss of activity. In the Y284F form of ECAO, the majority of the TPQ_{ox} has flipped and swung out from the wedge.[21] The 50-fold reduced activity of this mutant reflects a small population of TPQ_{ox} in the active conformation. In the D298A form of ECAO, TPQ_{ox} is greatly disordered.[17] In the D298E form of ECAO, TPQ_{ox} is locked in a single conformation where the extra methylene group of E298, as compared to D298, pushes the TPQ ring into the side of the wedge.[17] This mutant has greatly reduced activity compared to the WT, implying that some limited mobility of TPQ is critical for optimal activity.[17] The equivalent mutant in HPAO (D319E) has 15-fold decreased activity compared with WT, where resonance Raman (RR) spectroscopy indicates that TPQ has greater mobility in this mutant, contrasting with ECAO.[22]

Observation of alternate conformations for TPQ_{ox} shows that TPQ can have considerable mobility in the active site.

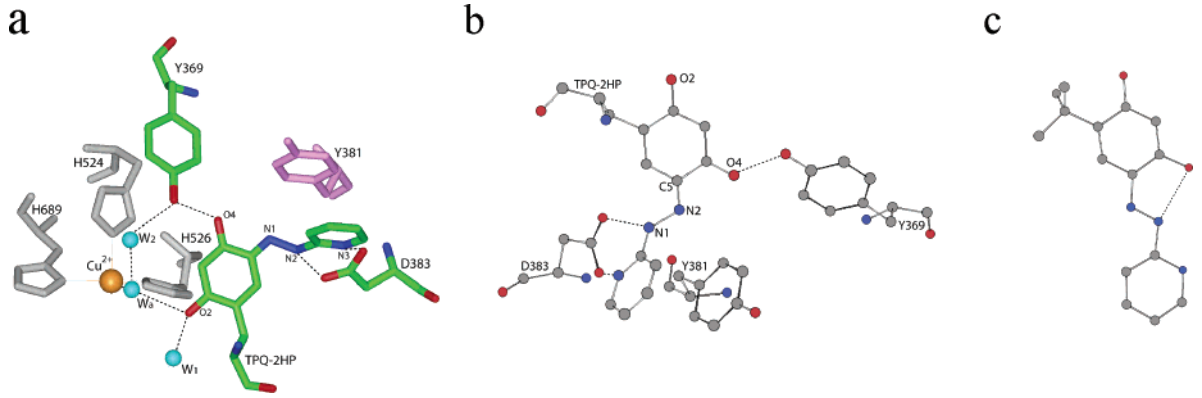


FIGURE 5. (a) Active-site structure of adduct I in WT-ECAO. (b) Alternative view of adduct I in WT-ECAO. (c) Structure of model adduct I. Carbons, nitrogens, and oxygens are shown as gray, blue, and red spheres, respectively.

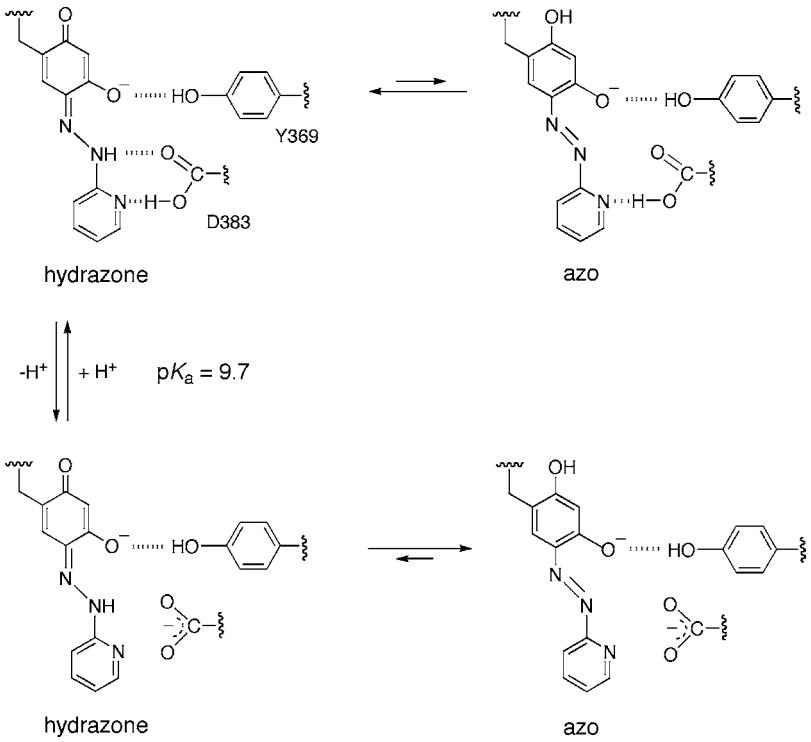


FIGURE 6. Hydrazone−azo tautomerization of adduct I in WT-ECAO. Below the pK_a of adduct I, the equilibrium favors the hydrazone.

However, as turnover is either significantly or completely inhibited under conditions where these conformations are observed, it is most likely that they represent inactivation. During turnover, the mobility of TPQ is tightly controlled, and the alternate conformations of TPQ are not on the reaction pathway.

Factors Controlling Mobility of SSB and PSB. The mechanism of the reductive half-reaction is well established. A recent review summarizes the identification of intermediates and discusses the importance of the protonation state of the SSB, PSB, and TPQ_{red} (neutral, monoprotonated, and neutral, respectively) for full activity.[10] This section contains a discussion of some recent work on ECAO that provides direct evidence that the SSB and PSB intermediates potentially have conformational flexibility.[23] As for TPQ_{ox}, this flexibility is controlled by hydrogen-bonding interactions with the active-site residues.

In HPAO, mutation of Y284 and the residues flanking TPQ (N381 and D383) to alanine causes accumulation of a neutral form of the PSB, which hydrolyzes slowly compared to the monoprotonated PSB (in Scheme 1).[24−26] In these mutants, the PSB is proposed to be in a flipped conformation, where this is thought to result from a rotation of the PSB away from the active-site base.[11] A study of 2-hydrazinopyridine-inhibited ECAO (ECAO-2HP) has clearly shown that D298 and, particularly, Y284 limit the mobility of the SSB and PSB.[23] The structure of ECAO−2HP (adduct I) contained the first direct evidence that D298 (D383 in ECAO) is the active-site base as it forms short hydrogen bonds with N2, which corresponds to the C1 of the SSB and N3 (Figure 5a,b).[27] A model compound (azo form, Figure 5c) revealed that the interaction with D298 keeps the adduct I predominantly as the hydrazone form by preventing the pyridine and TPQ rings from becoming coplanar (Figure 6).[23] Mutation of

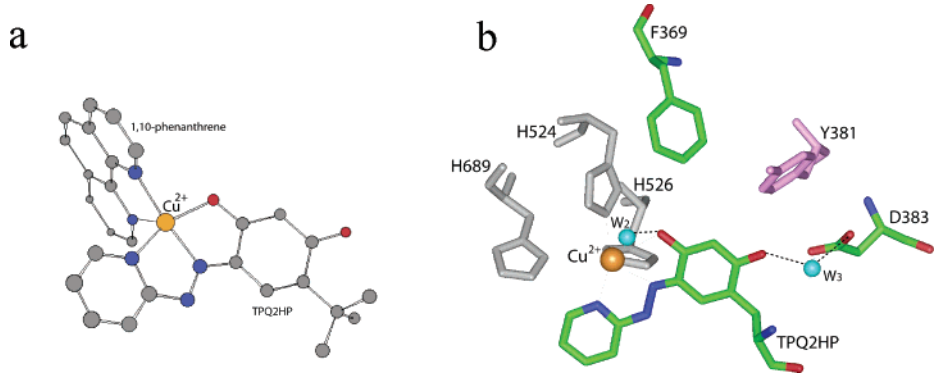
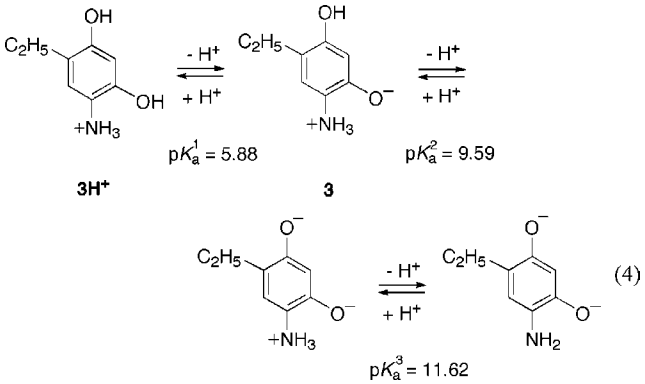


FIGURE 7. (a) Structure of model adduct II. Carbons, nitrogens, and oxygens are shown as gray, blue, and red spheres, respectively. (b) Structure of Y369F−ECAO adduct II.

D298 to A, N, or E or of Y284 to F alters the position of equilibrium between the hydrazone and azo tautomers, where the hydrazone completely dominates in D298E.[23]

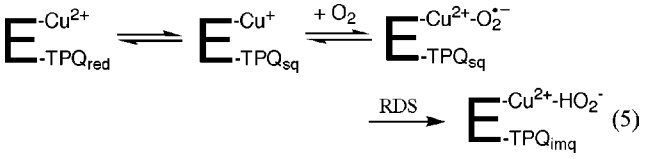
In WT, adduct I can be converted to a distinct purple species, termed adduct II, by heating or by increasing the pH.[27] Addition of Cu^{2+} to the adduct I model produced a species with UV−vis and RR spectra strikingly similar to those of adduct II (Figure 7a), leading to the surprising proposal that adduct II is the on-copper form of TPQ-2HP in ECAO.[23] In the Y284F mutant (Y369F-ECAO), the conversion is facile compared to WT, occurring at room temperature.[23] The structure of Y369F-ECAO confirmed that TPQ-2HP has migrated onto copper, displacing one of the histidine ligands (Figure 7b).[23] This result is significant as it clearly demonstrates that the hydrogen-bonding interaction between O4 and Y284 is key in keeping TPQ in the active conformation throughout the reductive half-reaction. Loss of this interaction gives TPQ significant freedom to move in the active site, showing that both the SSB and PSB have the potential to swing away from D298 in Y284 mutants. This translation away from the negative charge of D298 could alter the pK_a of the imino nitrogen of the PSB, explaining why the neutral from of the PSB persists in some active-site mutants of HPAO. This means that Y284 likely needs to remain protonated throughout the reductive half-reaction.

For optimal activity, the PSB must be monoprotonated.[10] The proton abstraction from the neutral SSB to form the protonated PSB requires that the latter pick up two protons from the surrounding environment. One is donated back from D298, since it was shown to be deprotonated at the PSB state.[28] Wa could be the source of the second proton in BSAO and HPAO where the pK_a of Cu^{2+}-OH_2 was inferred to be ~7.0.[13,29−31] Hydrolysis of the PSB yields protonated TPQ_{red}, but the active species observed in the turnover is neutral.[22,29] $TPQ_{red}H^+$ has a pK_a around 7 in the enzyme[29] (5.9 in solution;[13] see eq 4) and so can readily release a proton at the optimal pH for catalysis. It is possible that Wa could act as a proton shuttle but does not remain deprotonated after the formation of TPQ_{red}. Transient deprotonation of Wa would allow hydrolysis of the PSB, where the proton is donated back from $TPQ_{red}H^+$.

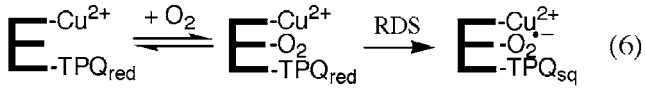


Role of Copper in the Oxidative Half-Reaction. In a recent review, the species involved in the oxidative half-reaction have been discussed, and a summary of the kinetic evidence for the mechanism of BSAO (bovine serum amine oxidase) and HPAO was also presented.[10] While it is generally accepted that Cu^{2+} does not play a redox role in the reductive half-reaction, the precise role of Cu^{2+} in the oxidative half-reaction still remains controversial. The argument centers on whether Cu^{2+} is directly involved in the formation of the semiquinone (TPQ_{sq}), or in other words, whether Cu^{2+} changes its redox state during the oxidative half-reaction. In this section, a recent study on AGAO will be described which shows that bacterial CAOs may react in a different manner from eukaryotic CAOs.

Following anaerobic substrate-reduction of CAO to form TPQ_{red}, a disproportionation reaction occurs with Cu^{2+} to generate TPQ_{sq} and Cu^+. The yield of TPQ_{sq} is greater at room temperature than at 77 K and in the presence of ligands stabilizing Cu^+ (e.g., CN^-),[32] and variable amounts of TPQ_{sq} have been observed in all forms of CAOs. The observation of Cu^+/TPQ_{sq} led to the proposal that Cu^+ reduces O_2, forming Cu^{2+}-superoxide, where a further $1e^-$ reduction produces TPQ_{imq} and Cu^{2+}-hydroperoxide (eq 5).[32] A Cu^{2+}-peroxide/hydroperoxide has been observed both in a crystal structure (ECAO)[33] and spectroscopically (AGAO).[34] The TPQ_{sq} observed in CAOs (pH 7.0) is a neutral species with substantial quinonoid character.[35,36]



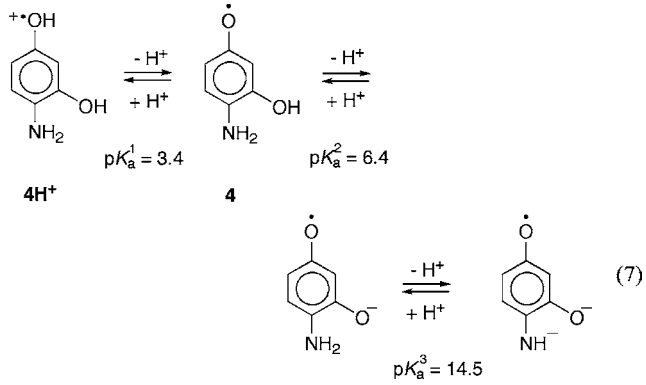
It is the role of Cu^+/TPQ_{sq} in O_2 reduction that has been a highly contentious issue. Studies of HPAO and BSAO were interpreted as showing that the catalytically active species is Cu^{2+}/TPQ_{red}.[29,37] Here, O_2 reacts directly with TPQ_{red} to form TPQ_{sq} and Cu^{2+}-superoxide, where the role of Cu^{2+} is to stabilize the reduced oxygen species (eq 6), and Cu^+ is not on the reaction pathway. O_2 preassociates with the enzyme at a hydrophobic, nonmetal O_2-binding site near Cu^{2+} comprising Y407, L425, and M634 (HPAO numbering), prior to reacting with TPQ_{red}. In support of this idea, mutation of M634 to a smaller amino acid increased the K_m for O_2 without affecting k_{cat}.[31] TPQ_{sq} is still an intermediate in both pathways, but the discussion centers around the mechanism of its formation.



To directly address the controversy surrounding the redox state of Cu, metal-substituted forms of CAOs have been prepared and their reactivity has been characterized in comparison with WT. Previously, there was always significant activity in the Cu-free forms due to incomplete removal of Cu. The residual activities (~13%) of Cu-free HPAO were abolished by incubation with phenylhydrazine, prior to reconstitution of the enzyme with Co^{2+}.[37] Nearly complete removal of Cu^{2+} (>99.5%) was achieved for AGAO.[16]

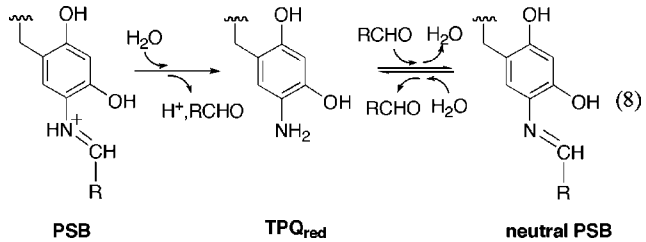
Co-HPAO exhibits 19% activity campared with WT-HPAO. The Co substitution caused ca. 70-fold increase in the apparent K_m for O_2, but k_{cat} was virtually unchanged.[37] TPQ_{sq} was not detected in substrate-reduced Co-HPAO. The reduced activity of Co-HPAO is not the result of a change in the rate-limiting step but rather arises from a reduced efficiency in TPQ_{red} oxidation caused by a change in net charge at the adjacent metal site from +1 (cupric hydroxide) in Cu-HPAO to +2 (cobaltous H_2O) in Co-HPAO.[30] Wa was proposed to be deprotonated in Cu-HPAO, thereby functioning as a proton acceptor from cationic TPQ_{sq}, formed immediately after $1e^-$ transfer from TPQ_{red} to O_2. In Co-HPAO, Wa remains protonated, and the formation of TPQ_{sq} and superoxide is impaired.

A model study determined a pK_a of 3.8 for $TPQ_{sq}H^+$ (**4H**$^+$ in eq 7), indicating that this species might not need assistance from the active site to lose a proton.[36] The model compound for TPQ_{red} (**3**) readily undergoes aerobic oxidation in the absence of added metals, suggesting that a redox partner may not be required for TPQ_{red} to react with O_2.[37] However, the mechanism in solution involves autoxidation leading to a radical chain reaction[38] and so could be intrinsically different from the enzyme, where the cofactor cannot freely diffuse.



Co- and Ni-AGAO exhibited 2.2% and 0.9% activities, respectively, compared with WT-AGAO.[16] In contrast to Co-HPAO, their K_m values for amine and dioxygen were comparable to that of WT, but k_{cat} was reduced 50−100-fold. In Co- and Ni-AGAO, the metal substitution did not alter the hydrogen-bond network around TPQ_{ox} but did modify the coordination environment of the metal center to octahedral, gaining one extra water ligand over Cu-AGAO (Figure 8). There was no effect on the electronic state of TPQ_{ox} or TPQ_{red}, and the rate of the reductive half-reaction was also unaffected. As in the case of Co-HPAO, TPQ_{sq} was not detected in either Co- or Ni-AGAO.

Under anaerobic conditions, the accumulation of the neutral form of the PSB was observed in Co- and Ni-AGAO after the completion of the reductive half-reaction. This was a result of a back-reaction between the product aldehyde and TPQ_{red} (eq 8). The reduction in the rate of cofactor reoxidation in Co- and Ni-AGAO is partly due to this back-reaction. Upon exposure to O_2, the oxidation of TPQ_{red} to TPQ_{imq} is 1000 times slower in metal-substituted AGAO compared to WT. The hydrolysis of TPQ_{imq} to TPQ_{ox} is also 1000 times slower in the former. Although this hydrolysis step is less likely involved in the steady state, where the transimination reaction between TPQ_{imq} and an amine to form the SSB is much preferred,[39] the significant rate reduction in metal-substituted AGAO confirms the change in local environment (i.e., extent of hydration in the active site). Clearly, in AGAO the electron-transfer reaction between O_2 and TPQ_{red} is greatly accelerated by Cu^{2+}. Cu^+/TPQ_{sq} can be involved in the electron transfer reaction, and although it is not strictly required for the reaction with O_2, oxidation is much slower in those lacking a redox metal-centered pathway to TPQ_{sq}.



Lysyl Oxidase

LOX is distinct from CAOs, having a monomeric structure (32 kDa) and LTQ as the cofactor. LOX plays an essential

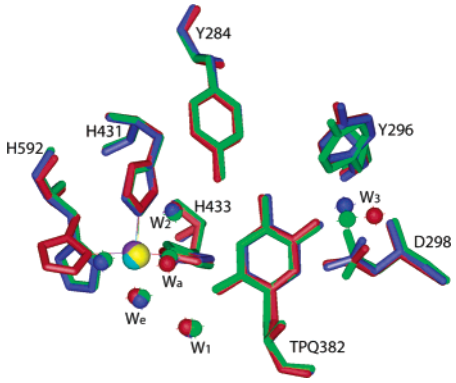


FIGURE 8. Superimposed active sites of Cu-, Co-, and Ni-AGAO, shown in red, blue, and green, respectively. Cu^{2+}, Co^{2+}, and Ni^{2+} are represented by purple, sky blue, and yellow spheres, respectively.

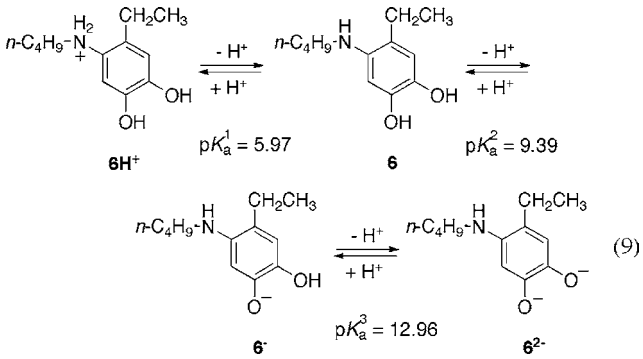
role in the development of the extracellular matrix, catalyzing cross-linking of collagen and elastin in birds and mammals. Recently, *lox*-like genes (*loxl*) containing scavenger receptor cysteine-rich (SRCR) domains at their 5′ end, in addition to the highly conserved LOX catalytic domain, were found.[11] Aberrant LOX and LOXL expression has been linked to a number of diseases such as heritable connective tissue disorders, liver fibrosis, and breast cancer.[40,41] It has been suggested that LOX and LOXL may possess alternative functions in, for instance, developmental regulation, tumor suppression, cell growth control, and cell adhesion.[11] In particular, the sequence of the murine *rrg* gene (anti-oncogene of *ras*) has been shown to be identical to that of rat *lox*, where levels of mRNA expression of *rrg* have been correlated to LOX activity.[42] In contrast to CAOs, the reaction mechanism of LOX is not well characterized. In this section, the current understanding of the chemical properties of LTQ in solution in comparison with TPQ will be discussed.

Catalytic Properties of LOX. LOX can oxidatively deaminate peptidyl lysines, lysine, diamines, and primary amines in vitro. Steady-state kinetics indicate that LOX follows a ping-pong mechanism. Stoichiometric product aldehyde formation was observed under anaerobic conditions. Kinetic studies have shown that α-proton abstraction is partially rate-limiting and indicate the presence of an active-site base residue with a pK_a of 7.0.[43] LOX contains one Cu^{2+} in an octahedral coordination geometry involving at least three nitrogen ligands.[44] Removal of Cu^{2+} from LOX can be achieved by dialyzing against α,α′-bipyridyl in 6 M urea.[44,45] The resulting Cu-free LOX retained 40% of WT activity, where the loss of activity is due to the inactivation of LTQ.[45] It was proposed that Cu^{2+} does not play a redox role in catalysis but is essential in maintaining the structural integrity of LTQ and/or the protein.[45]

How Does LTQ Differ from TPQ in Solution?

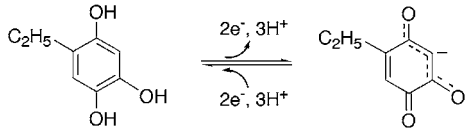
The UV−vis spectra of model compounds of LTQ_{ox} and TPQ_{ox} (**5** and **1**, respectively; Figure 2) and PQQ at physiological pH are shown in Figure 9. **5** and **1** are very similar, particularly in the visible region. PQQ has a much smaller extinction coefficient at ~500 nm but has an extra strong absorbance at 350 nm. Either protonation (pK_a = 1.96) or deprotonation (pK_a = 10.33) of the *n*-butylamino side chain of **5** causes bleaching of the absorbance at 504 nm, and the resulting species are not stable.[46] The broad visible absorbance of **1** is due to the resonance delocalization of the 4-oxoanion where the C-3 proton exchanges with solvent protons (eq 3). In contrast, neither the ring protons of **5** nor the LTQ cofactor in LOX shows any exchange.[46,47] The C1 carbonyl oxygen of **5** is exchangeable with solvent water, indicating that this position undergoes nucleophilic addition of the substrate. Although their overall UV−vis spectral features are similar, TPQ_{ox} exists as a resonance-stabilized monoanion but LTQ_{ox} exists as a neutral *o*-quinone at physiological pH.

The reduced form of LTQ, LTQ_{red} (**6**, Figure 2), has no absorbance in the visible region. The UV−vis spectrum and its pH dependency are very close to those of **3**. Three pK_as were determined for **6** (5.97, 9.39, and 12.96) by spectroscopic and electrochemical pH titrations (eq 9), similar to **3**.[46] On the basis of the similarity in the UV−vis spectroscopic properties of **3** and **6** at pH 7, upon reduction with substrate, LTQ should lose its visible absorbance.

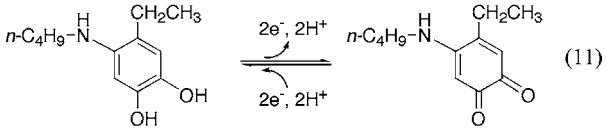


(9)

Recently the redox potential of the TPQ cofactor in AGAO (pH 7) was determined to be very close to that of **1**.[48] **5** has the same redox potential as **1** (0.061 V vs NHE), showing that **5** can act as the redox cofactor analogous to **1**.[3,46] The difference between **1** and **5** is that the former undergoes $3H^+$, $2e^-$ redox chemistry (eq 10)[13] and the latter undergoes $2H^+$, $2e^-$ redox chemistry at physiological pH (eq 11).[46]



(10)



(11)

Identification of LTQ in Enzymes. Hydrazines are potent inhibitors of both LOX and CAOs, forming stable adducts with their quinone cofactors. Model studies

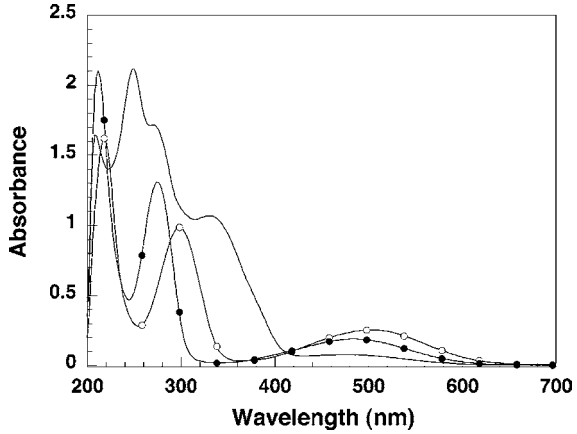


FIGURE 9. UV−vis spectra of PQQ (—), **5** (○), and **1** (●) at pH 7.0.

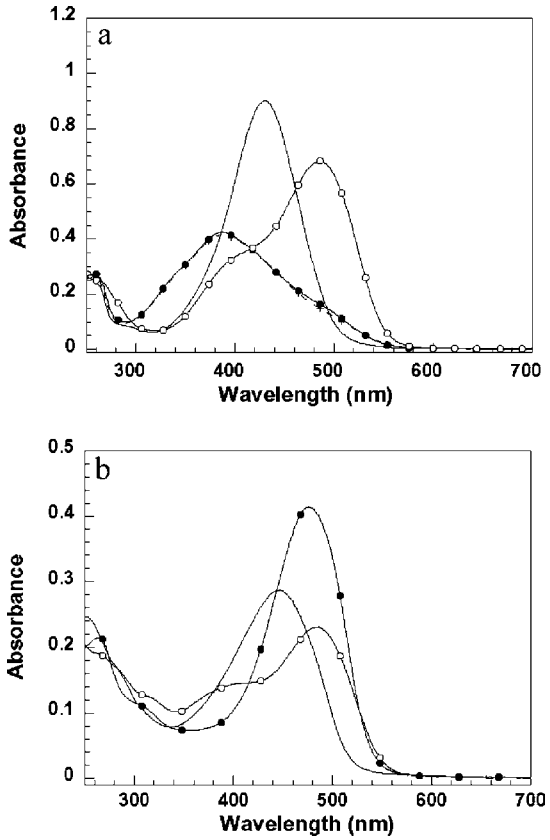


FIGURE 10. UV−vis spectra of (a) **8** at pH 7.1 (—), **8** at pH 13.2 (○), **8** at pH 4.4 (---), and **8** at pH 1.6 (●); (b) **9** at pH 7.0 (—), **9** at pH 13.3 (○), and **9** at pH 1.3 (●).

revealed that the nucleophilic addition of hydrazines occurs at the C1 carbonyl carbon of LTQ, consistent with the ^{18}O exchange results described earlier.[46] As in the case of the TPQ−phenylhydrazine derivative (**8**), the LTQ−phenylhydrazine adduct (**9**) exists predominantly as the azo tautomer. A detailed comparison of their UV−vis spectroscopic characteristics shows that they can potentially be used for the primary assignment of LTQ versus TPQ.[46] Further characterization by a combination of RR spectroscopy, mass spectrometry, and NMR can then be used to provide a definitive characterization.[2−4]

At high alkaline pH (>13), TPQ−PHZ (**8**) exhibits a red shift in λ_{max} from 442 to 482 nm (Figure 10a). This was designated as the purple shift after the distinct color of **8** and was initially thought to be a characteristic of TPQ alone.[49] However, LTQ−PHZ (**9**) also shows the purple shift (Figure 10b).[46] These spectral changes result from acid dissociation of the hydroxyl group at C2 of **8** and **9**, respectively. The purple shift alone cannot distinguish between LTQ and TPQ in enzymes. However, **9** possesses an acidic pK_a (2.12), assigned to the *n*-butyl amino group, that results in a red shift in λ_{max} from 446 to 478 nm (Figure 10b), giving a spectrum distinct from that of **8** at the same pH. A second red shift in λ_{max} below pH 3.0 should provide an initial indication of the presence of LTQ rather than TPQ.

Conclusions

In CAOs, the conformational flexibility of TPQ and TPQ-derived intermediates is restricted by hydrogen-bonding interactions with active-site residues to achieve full activity. Cu^{2+} is not essential but is clearly required for full activity in AGAO and for efficient binding of O_2 in HPAO, where its role in the oxidation of TPQ_{red} to TPQ_{imq} is still unresolved. The mechanism of TPQ_{red} oxidation seems to depend on the source of the enzyme, but the reasons for this are unclear and further investigations are needed.

In LOX, LTQ exists as a neutral *o*-quinone in contrast to TPQ, which is a resonance-stabilized monoanion at physiological pH. The *o*-quinone character of LTQ may mean that its chemistry is distinct from that of TPQ. Despite these structural differences, they have the same redox potential in solution. To distinguish LTQ from TPQ, it is necessary to undertake a UV−vis spectroscopic pH titration of the phenylhydrazine adduct at acidic pHs in addition to the purple shift assay.

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