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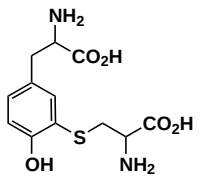
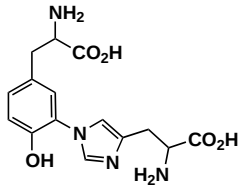
Chemically Modified Amino Acids in Copper Proteins That Bind or Activate Dioxygen**

Malcolm A. Halcrow*

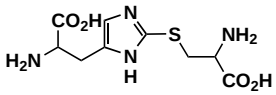
It is an interesting observation that four of the six classes of copper oxidase or monooxygenase enzymes whose structures are known[1–3] contain a chemically modified amino acid either ligated to, or in close proximity to, the active-site copper center. Some of these unusual residues, namely the TyrCys, HisCys, and TyrHis moieties, are derived from a cross-linking reaction between two amino acid side chains. Alternatively, the 2,4,5-trihydroxyphenylalaninequinone (topaquinone, TPQ) and lysyl tyrosyl quinone (LTQ) cofactors are generated by monooxygenation of a tyrosine side chain, followed by nucleophilic attack at the resultant *ortho*-quinone. The HisCys,[4] TyrCys,[5] and TPQ[6] residues were first detected in their respective proteins between nine and eighteen years ago. However, our understanding of the functional roles of these and the other, more recently discovered,[7, 8] residues is often still rather limited. Attention has also now begun to focus on the biosynthesis of these cofactors, which in many cases must involve unusual organic transformations that have not been previously characterized in copper biochemistry.



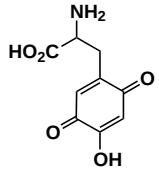
TyrCys



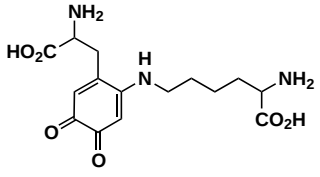
TyrHis



HisCys



TPQ



LTQ

The cross-linked amino acid whose function is best understood is the TyrCys residue found in radical copper oxidases, of which galactose oxidase (GO) is the prototype.[1] This side chain acts as a ligand to a monocopper center in the enzymatic active site (Scheme 1 A),[5] and is oxidized to a [TyrCys]$^{\cdot}$ radical in the active resting enzyme; this phenoxyl radical abstracts a hydrogen atom from the alcohol substrate during turnover.[1] The oxidation potential of the TyrCys moiety is 0.5 V less positive than would be expected for an unmodified tyrosine residue.[9] Consistent with this, several model studies have shown that *ortho*-sulfide substitution of a free or coordinated phenoxide will lower its oxidation potential by 250 – 500 mV.[10] However, recent calculations have shown that the S atom of an *ortho* sulfide substituted phenoxyl group only accepts up to 15 % of the radical unpaired spin, and does not significantly perturb the spin distribution over the rest of the molecule.[11] In addition, it has also been calculated that the O–H homolytic bond dissociation energy (BDE) of an *ortho* sulfide substituted phenol is identical, to within 2 kcal mol^{-1}, to that of an otherwise identical phenol lacking this substituent.[12] Hence, the electronic structure of the [TyrCys]$^{\cdot}$ radical of GO and its reactivity towards H-atom donors are not significantly perturbed by the sulfide substituent, and it appears that the function of this cross-link is purely to lower the oxidation potential of the oxidizable tyrosine ring.

Less is known about the other cross-linked amino acids. The TyrHis cross-link in cytochrome *c* oxidase (CcO) forms part of the heme/copper site of this enzyme (Scheme 1 B),[7] which catalyzes the reduction of dioxygen to water.[3] The currently favored mechanism for this reaction implies that 4-electron reduction of O_2 by CcO occurs in one mechanistic step,[13] and it is thought that the TyrHis residue acts as both as a Brønsted

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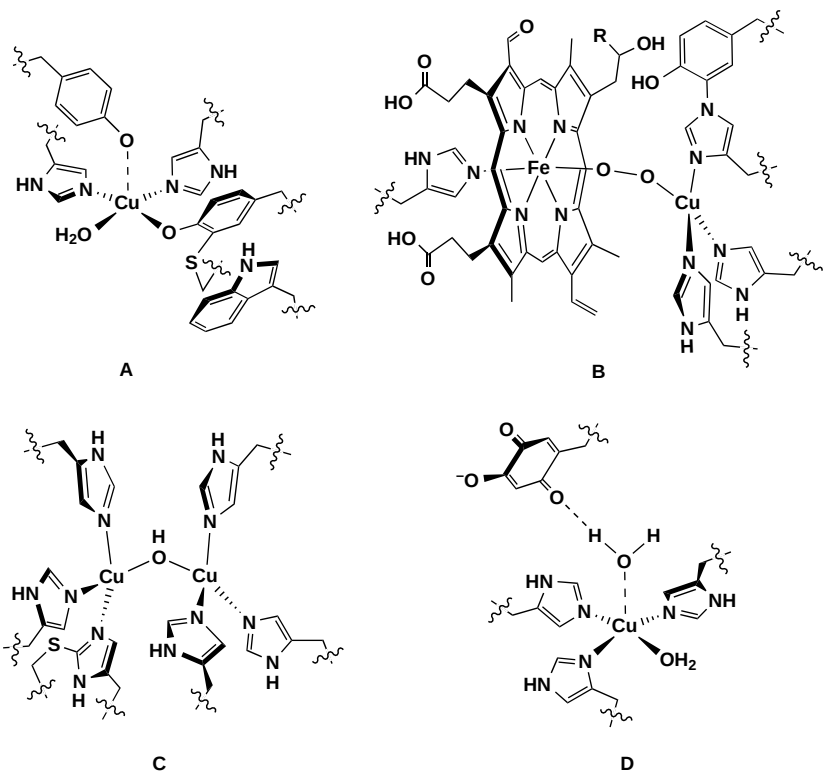


Scheme 1. Molecular structures of the active sites of copper oxidases containing modified amino acid residues: A) galactose oxidase;[5] B) catechol oxidase;[16] C) the O_2 reduction site of cytochrome *c* oxidase;[7] D) copper-containing amine oxidase.[19–21]

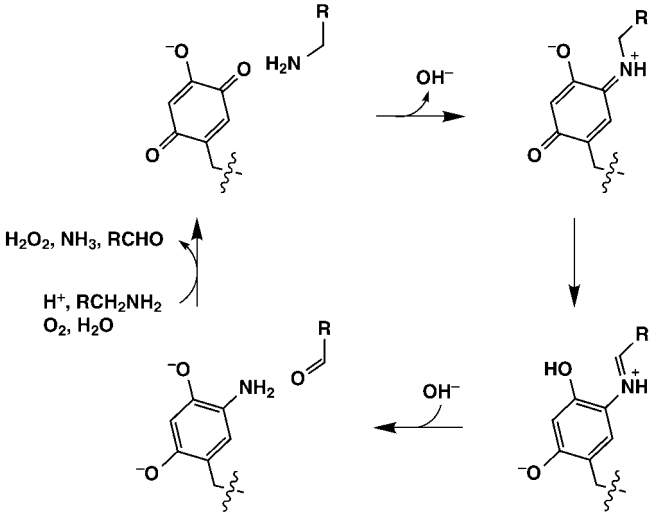
base, and as an H-atom donor to the substrate dioxygen molecule, during this process.[14] However, a model study has shown that the imidazol-1-yl substituent should lower the oxidation potential of the TyrHis side chain by <100 mV, while barely perturbing its O–H homolytic BDE.[15] Hence, it is unclear how the TyrHis cross-link should benefit the radical chemistry that this residue is believed to undergo.

The HisCys side chain is a ligand to many type 3 copper biosites,[2] and has been crystallographically characterized in a catechol oxidase and a molluskan hemocyanin (Scheme 1C).[16] There are no model studies relevant to the properties of the HisCys residue, which is *not* oxidized during substrate binding or catalytic turnover by these proteins.[2] However, since other hemocyanins are known that do not contain a cross-linked histidine side chain,[17] it has been suggested that the HisCys cross-link serves merely to increase the conformational rigidity of the coordinated Cu ion in these proteins.[16]

As yet, the biosynthesis of the cross-linked amino acids has been little studied. It has recently been reported that freshly prepared GO is secreted into the medium as a copper-free proprotein, which lacks the TyrCys cross-link and bears a 17-mer N-terminal extension, which is cleaved off during maturation of the polypeptide.[18] Formation of the TyrCys residue is a self-processing event, which is dependent upon both copper and O_2.[18] Addition of $CuSO_4$ to pro-GO initially affords a species showing a Tyr→Cu ligand to metal charge transfer (LMCT) band at 410 nm and a d–d absorption at 750 nm,[18] which can be attributed to a tetragonal Cu^{II}/tyrosinate species, in which presumably the TyrCys cross-link has not yet formed. Over a period of one hour, this initial species is aerobically converted into active oxidized GO with no observable intermediate at 298 K. Further details of how this is achieved are unknown, however.

Unlike the TyrCys, HisCys, or TyrHis residues, the TPQ cofactor in active copper-containing amine oxidase (CAO) is not directly coordinated to the copper ion in the active enzyme, but lies approximately 5 Å from a $[Cu(His)_3(H_2O)_x]$ ($x=1, 2$) center (Scheme 1D).[19–21] The TPQ ring is the site of substrate oxidation, which occurs by a well-understood transamination mechanism involving nucleophilic attack of the substrate amine at the TPQ C-5 carbonyl group, followed by isomerisation and hydrolysis of the resultant Schiff base (Scheme 2).[1] The reduced amino quinol cofactor is then reoxidized by molecular oxygen, possibly with the involvement of electron transfer to the copper ion although this has not been conclusively demonstrated.

As in GO, the synthesis of the TPQ cofactor in CAO[22] is a self-processing



Scheme 2. Mechanism of amine oxidation by the TPQ cofactor in copper-containing amine oxidase.[1]

event and occurs according to the stoichiometry shown in Equation (1).[23] Aerobic incubation of pro-CAO with Cu^{II}

$$\mathrm{Tyr} + 2\,O_2 + 2\,H^+ \rightarrow \mathrm{TPQ} + H_2O_2 \qquad (1)$$

initially yields two species, which show Tyr→Cu LMCT absorptions at 350 and 380 nm and decay at similar rates.[24] There are several lines of evidence to suggest that the 380 nm species contains Cu^{II} [25] complexed to amino acids outside the active site, and is irrelevant to cofactor synthesis.[24] However,

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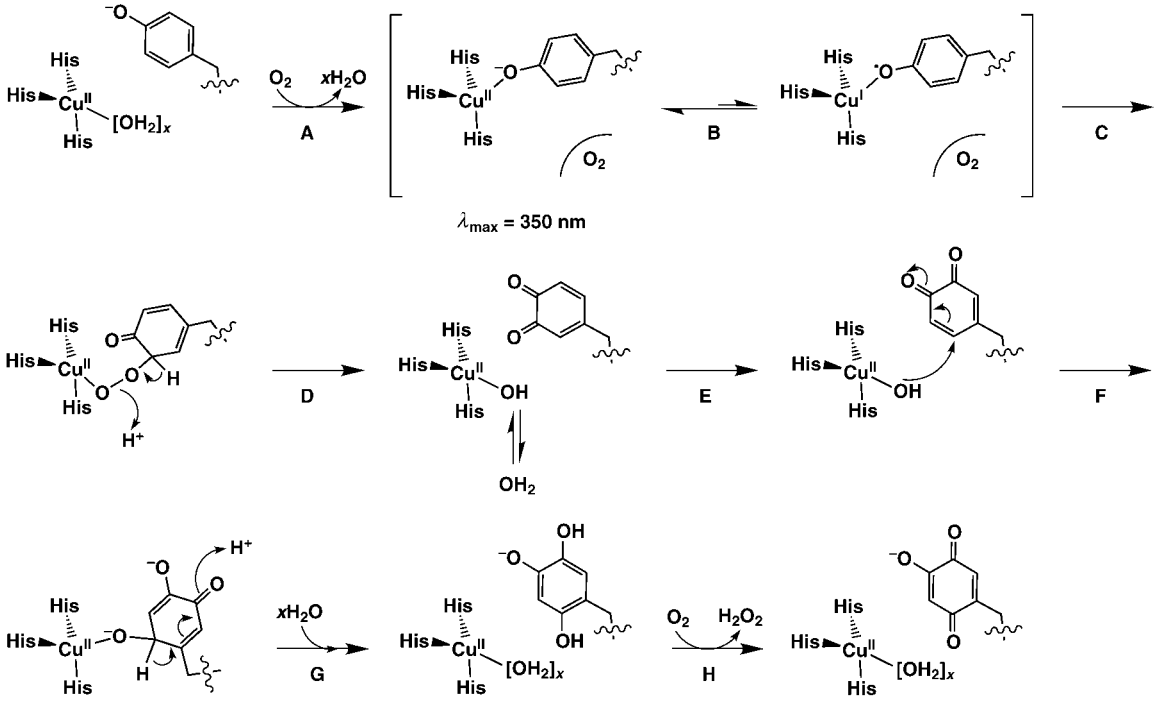
formation of TPQ from the 350 nm intermediate occurs with first-order kinetics,[24] with concomitant uptake of an equimolar amount of O_2.[26] The initial reaction of the 350 nm intermediate with O_2 is the rate-limiting step in TPQ synthesis, and does not involve proton transfer.[26]

The 350 nm intermediate in TPQ biogenesis is thought to contain a tetrahedral $[Cu(His)_3(Tyr)]$ active-site complex.[24] Consistent with this, a single crystal structure of an unprocessed bacterial CAO containing zinc instead of copper, shows an isoelectronic tetrahedral $[Zn(His)_3(Tyr)]^+$ center, with an unmodified tyrosine cofactor residue ligated to the Zn ion.[27] Interestingly, the 350 nm species is not obtained if pro-CAO is incubated with Cu anaerobically, although O_2 is not consumed during its aerobic formation.[24] Since there is no evidence for a $Cu{-}O_2$ bond in this species, it was suggested that there must be a separate dioxygen binding site remote from the Cu ion, and that occupation of this site induces a conformational change to force the Tyr residue to coordinate.

On the basis of these measurements, the mechanism shown in Scheme 3 was proposed for TPQ biogenesis.[26] This mechanism can be considered in two stages: monooxygenation of tyrosine to form 3,4-dihydroxyphenylalanine quinone (DPQ; steps A – D); and hydroxylation and oxidation of the resultant quinone (steps F – H). Chemical precedent has very recently been published for step B of the monooxygenation pathway, namely intramolecular electron transfer with a Cu^{II}/phenoxide complex to yield a Cu^{I}/phenoxyl species.[28] The tyrosyl radical thus formed must then be displaced from the Cu ion to allow O_2 to bind to the Cu center (step C). The resultant arylperoxo complex intermediate has no precedent in copper/oxygen chemistry, but is reminiscent of the initial hydroxylation step effected by monoiron centers in catechol dioxygenases.[29] This species is also structurally similar to the crystallographically characterized substrate – reduced-CAO/O_2 turnover complex, which shows a monodentate dioxygen species bridging between the Cu ion and cofactor ring.[21] Hence, the putative arylperoxo complex in Scheme 3 should be sterically feasible within the CAO active site.

A resonance Raman study has shown that the O atom at the C-2 position in TPQ is derived from H_2O rather than O_2.[30] Since model experiments have shown that aqueous 1,4-hydrolysis of DPQ does not take place at neutral pH values,[31, 32] a water molecule inside the CAO active site would be insufficiently basic to attack the DPQ ring. However, coordination to a Lewis acidic Cu^{II} ion may well make the water nucleophile acidic enough to be deprotonated and react as shown in step F of Scheme 3. It is noteworthy, however, that rotation of the DPQ ring by approximately 180° about its methylene substituent (step E of Scheme 3) would be necessary to allow a Cu-bound nucleophile to attack both the C-2 and C-5 positions of the precursor tyrosine side chain, as is proposed at different stages of this mechanism. Moreover, steps E and F together must be slow compared to exchange of the Cu-bound hydroxide ion with exogenous water, to permit incorporation of a water-derived O atom into the final product.

There are no known examples of phenol monooxygenation by synthetic mononuclear copper complexes. However, steps F – H of Scheme 3 have been modeled by the aqueous autooxidation of 4-alkylcatechols to 2-alkyl-5-hydroxybenzoquinones (Scheme 4). This occurs slowly under ambient conditions, but is catalyzed by Cu^{II} ions.[32, 33] The catalyst substantially increases the rate of oxidation of the catechol precursor (step A in Scheme 4),[32, 33] but does not appear to influence other steps in the reaction.[32] The rate of conversion of the *ortho*-quinones into the hydroxybenzoquinones (steps B and C in Scheme 4) is increased at higher pH values but is not accelerated in the presence of Cu^{II} salts. By analogy



Scheme 3. The mechanism for TPQ biogenesis proposed by Klinman et al.[26]

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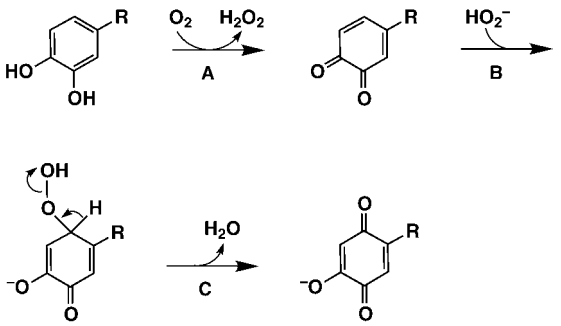

Scheme 4. Mechanism of aerobic catechol autooxidation in vitro.[34]

with the mechanism in Scheme 3, this was taken to show that the active nucleophile in this reaction is a free, rather than Cu-bound, hydroxide ion.[32] However, it has recently been demonstrated that, under these conditions, the O atom at the C-2 position is, in fact, derived by conjugate addition of H_2O_2, which is produced as a by-product of catechol oxidation (Scheme 4).[34] The preference for nucleophilic attack of HO_2^- rather than OH^- in vitro can be attributed to the lower pK_a of H_2O_2 compared to H_2O.

These results mean that the local environment of the CAO active site has changed the chemistry of *ortho*-quinone hydration from that observed in vitro. This precedent makes it difficult to predict what the detailed synthetic mechanisms for the biosynthesis of the TyrCys, HisCys, or TyrHis moieties might be. Close analogues of all three of these cross-linking reactions have been achieved synthetically, albeit by methods that are unlikely to be biochemically relevant.[15, 35] Hence, it will be intriguing to see how nature has evolved pathways to couple nucleophilic phenol or imidazole rings, with thiol or imidazole centers that are not electrophiles, under aerobic ambient conditions.

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