Copper amine oxidase: a novel use for a tyrosine

The first three-dimensional structure of copper amine oxidase demonstrates that one tyrosine residue is converted into 2,4,5-trihydroxyphenylalanine quinone (TPQ). TPQ binds to copper in the inactive form of the enzyme but not in the active form.

Structure 15 November 1995, **3:**1127–1129

To achieve catalysis, enzymes often bind metals and various cofactors. Additional diversity can be attained by the use of post-translationally modified amino acids. Enzymes performing oxidoreduction reactions may also involve protein residues, preferentially tryptophan and tyrosine, in unusual redox states. Oxidized radical forms of both tyrosine and tryptophan have been shown to be essential for several enzyme reactions. A classical example is the reaction catalyzed by iron-containing class I ribonucleotide reductases, for which it has been shown that a tyrosyl radical is necessary to initiate the reaction [1,2]. Other examples of enzymes containing tyrosyl radicals include the D1 and D2 subunits of photosystem II [3], galactose oxidase [4,5] and prostaglandin H synthase [6,7]. The best characterized tryptophan radical is Trp191 in cytochrome *c* peroxidase, which is involved in electron transfer [8]. Glycine radicals have been shown to be involved in the reactions catalyzed by pyruvate formate lyase [9] and the anaerobic ribonucleotide reductase of *Escherichia coli* [10], and a transient cysteinyl radical is probably involved in the substrate activation step of ribonucleotide reduction [11].

A few enzymes that were originally suggested to have 2,7,9-tricarboxypyrroloquinoline quinone (PQQ) as a cofactor were later shown to contain modified tryptophan and tyrosine side chains [12]. Methylamine dehydrogenase was shown to contain a covalently modified tryptophan residue (TTQ) [13] and in galactose oxidase a tyrosine was found to be covalently bound to a neighboring cysteine residue [5]. Now, the three-dimensional structure of copper amine oxidase from *E. coli* (ECAO) has been determined (Parsons *et al.* [14], this issue of *Structure*). This enzyme contains a novel redox-active modification of a tyrosine residue to 2,4,5-trihydroxyphenylalanine quinone (TPQ) [15]. These properties suggest that tyrosine is the most versatile of all amino acid residues.

Amine oxidases are important regulatory enzymes that catalyze the oxidation of a wide range of biogenic amines including many neurotransmitters, histamines and xenobiotic amines, but little is known about the precise biological function of the copper-containing amine oxidases. In prokaryotes and lower eukaryotic organisms, they appear to be involved in the utilization of amines as a source of nitrogen and carbon. In higher eukaryotes they seem to be involved in detoxification, in wound and resistance responses and secondary metabolism.

The structure of ECAO

ECAO is a homodimeric protein and each subunit consists of four domains. The largest domain, of about 440 residues, folds into an extensive β sandwich which contains the active site and mediates intersubunit interactions. The amine oxidase dimer is formed by very tightly associated β sandwich domains. It is intriguing that two pairs of β hairpins or 'arms' of about 20 residues extend out from the β sandwich to embrace the other subunit. One of these arms reaches from the active site of one subunit to the active site of the other, about 35 Å away. Two fully conserved residues, His440 and Thr462, lie at opposite ends of each arm, such that His440 is close to one active site and Thr462 is close to the other. By this arrangement, a triad is formed with His440 from subunit A hydrogen bonded to the conserved Asp467 of subunit B which also forms a hydrogen bond to the side chain of Thr462 in the B subunit.

During the course of their investigations, Parsons *et al.* [14] noticed that ECAO is irreversibly inactivated by ammonium ions. By removing the ammonium sulfate precipitation step from the enzyme preparation procedure, 10–20 times higher activity was obtained. As preparation of this enzyme usually involves an ammonium sulfate precipitation step, many biochemical investigations may have been performed on partially inactivated enzyme. Because the crystal structure was first determined from crystals precipitated by ammonium sulfate, the crystal structure was also determined in catalytically competent crystals grown from sodium citrate solutions.

Numerous spectroscopic studies have led to a model for the Cu(II) site in amine oxidases in which the Cu(II) is bound to three histidines and two water molecules, with a coordination geometry best described as distorted square pyramidal (reviewed in [16,17]). Two of the histidines were suggested to be His524 and His526 (numbered according to the ECAO sequence) from the conserved H-X-H motif, in agreement with site-directed mutagenesis studies [18]. The third histidine was proposed to be either His440 or His689, both of which are conserved residues. The copper ion is now shown to be coordinated by His524, His526 and His689. Moreover, the crystal structure shows, in agreement with NMR and electron paramagnetic resonance studies [16,17], that the copper–TPQ distance is short, but that TPQ is not directly coordinated to Cu(II). The crystal structure of

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the active form of the enzyme does not allow an accurate evaluation of the copper–TPQ distance but shows that it is significantly longer than 3 Å, the distance estimated from spectroscopic studies. In the active form, the orientation of TPQ brings it close to the side chains of Asp383 and Tyr369, both of which are fully conserved in all known copper amine oxidase sequences. In contrast, in the inactive ammonium sulfate crystals, the TPQ side chain is now coordinated to copper via the oxygen at the 4-position and is directed away from Asp383.

Formation of the quinone residue

The present evidence that the copper ion is close to the TPQ side chain fits well with the finding that the post-translational oxidation of Tyr466 into TPQ is a self-processing reaction that does not require any specific enzyme system such as tyrosine hydroxylases or tyrosinases. The protein-bound copper center was proposed to play a key role in the tyrosine to TPQ conversion reaction [18–21]. Recent studies of site-directed mutants of yeast copper amine oxidase support this theory [18]. Mutation of the glutamic acid residue adjacent to the tyrosine precursor in the consensus Asn-Tyr-Asp/Glu sequence did not affect the capacity for TPQ biogenesis *in vivo*, supporting the prediction that the specificity for TPQ formation is not determined by the consensus sequence. Moreover, the ability to form TPQ was totally abolished by mutation of a histidine residue from the H-X-H motif, and this was associated with very poor copper-binding capacity of the mutant enzyme [18]. Finally, a copper-free and quinone-less inactive form of phenethylamine oxidase could be transformed into an active form, containing TPQ, just by addition of Cu(II) in the presence of air [21].

A likely pathway for the generation of TPQ from Tyr466 is depicted in Figure 1. It was recently observed that, under anaerobic conditions, binding of copper to the quinone-less apo-form of a bacterial oxidase resulted in reduction of Cu(II) to Cu(I) but that reduction did not occur in a mutant where the tyrosine that was the precursor to TPQ had been changed into phenylalanine [21]. It is thus conceivable that Cu(II) is reduced by the tyrosine residue (step 1, Fig. 1), even though there is presently no evidence that a transient tyrosyl radical is formed. Now copper can bind molecular oxygen and generate a superoxide radical-like species (step 2, Fig. 1). A coupling reaction with the tyrosyl radical generates a copper-bound peroxo intermediate (step 3, Fig. 1). Coupling between tyrosyl and superoxide radicals has been reported previously [22]. In the last steps, homolytic cleavage of the O–O bond followed by oxygen transfer to position 2 of the phenyl ring and auto-oxidation generates TPQ (steps 4–6, Fig. 1). The major drawback of such a mechanism is its requirement for a rotation of the side chain, even though this seems feasible. Other mechanisms, albeit less detailed and less rigorous in terms of the electron balance, have been proposed previously with dopa and dopaquinone as intermediates. TPQ could be formed during a nucleophilic attack of dopaquinone by a

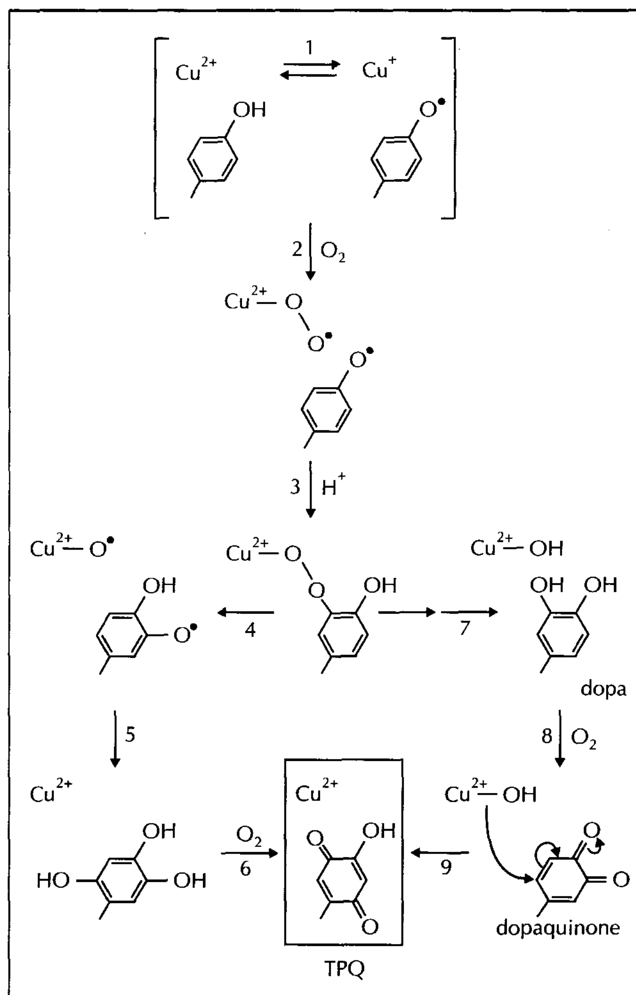

Fig. 1. Proposed mechanism for the generation of TPQ from Tyr466 in ECAO.

copper–hydroxide intermediate (step 9, Fig. 1) [18]. Alternatively, the observation of an intermediate semiquinolamine-like radical during the last steps of the reaction suggested the involvement of an amino group of the protein (a lysine residue) rather than the direct hydration [21]. This suggestion finds very little support from the present structure, because there is no lysine in the active site. Unfortunately, our understanding of the mechanism of copper-catalyzed conversion of tyrosine to TPQ is not improved substantially by the new structural information, and the definitive answers will have to await the outcome of future studies.

Mechanism of amine oxidation

The proposed enzyme mechanism, as presented by Parsons *et al.* [14] and elsewhere [16,17], consists of two half-reactions. The first involves binding of the substrate to TPQ, followed by proton abstraction with concomitant enzyme reduction and hydrolysis of the product imine intermediate to liberate the aldehyde product. The electron-density map of the active enzyme is not sufficiently well defined to reveal the orientation of the TPQ ring. However, the relative location of the side chain of Asp383 makes it a good candidate for the active-site base

responsible for proton abstraction. In the inactive crystal form, Asp383 no longer faces the 5-position of the TPQ ring, where the substrate binds. This may prevent substrate from binding or proton abstraction by Asp383 from occurring and explain the inactivity of the enzyme. The question of whether Asp383 plays a key role during catalysis should be resolved by site-directed mutagenesis. Whether Tyr369 is also important is difficult to tell from the present structural information. However, Tyr369 is suitably located for hydrogen bonding to TPQ and thus controlling its orientation. As shown from the structure of the inactive form, it is crucial to prevent TPQ from binding to the copper ion. One can also suggest that Tyr369 participates with Asp383 in proton transfers. A major issue raised by the crystal structure is the absence of an obvious route for the substrate to reach from the surface to the active site, which is revealed to be deeply buried in the interior of the protein. It is probably advantageous for the enzyme to have a buried active site because of the highly reactive intermediates.

In the second step of the reaction, the reduced organic cofactor transfers two electrons to molecular oxygen, thus producing hydrogen peroxide and ammonia, and regenerating TPQ.

Conclusions

The structure of copper amine oxidase has clarified a number of points of significance for this group of enzymes. The presence of a 2,4,5-trihydroxyphenylalanine is unambiguously demonstrated, and the geometry of the active site suggests how Tyr466 can be converted into a topa-quinone TPQ by a copper-bound oxygen molecule. An important aspect that has emerged from this work is the demonstration that ammonium ions irreversibly inhibit the enzyme. The cause of this effect is still not known, but the differences between the active and inactive enzyme are clearly demonstrated. In the inactive enzyme, TPQ is a ligand to copper but it does not ligate the ion in the active form. The finding that the active site is buried in the protein interior is similar to the situation observed in other radical-based enzymes.

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