Homemade cofactors: Self-processing in galactose oxidase

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Numerous enzymes use a variety of cofactors for achieving their impressive catalytic prowess. Generally, these cofactors are generated via complex multistep biosynthetic pathways involving many proteins. A less commonly encountered means of cofactor biosynthesis, but one that is found with increasing frequency, involves the posttranslational modification of endogenous amino acids in the enzyme. These modifications can occur via autocatalytic processes or may be catalyzed by other auxiliary proteins. In this issue, Firbank *et al.* describe the crystal structure of the precursor of galactose oxidase (GAO), a self-processing enzyme that generates a crosslink between a cysteine and a tyrosine (1). The one-electron oxidized form of this crosslink (a crosslinked tyrosyl radical) functions as the cofactor in the oxidation of primary alcohols. The current work provides an important foundation to study the mechanism of autocatalytic cofactor generation in GAO and may also provide insights into the biogenesis of crosslinked cofactors found in other proteins (2).

A growing number of enzymes have been reported that undergo posttranslational modifications of amino acids within their active sites to create a wide variety of structurally and functionally diverse cofactors. These modifications can be divided into two general classes. One involves proteins that undergo one-electron oxidations of amino acids to provide amino acid radicals on tyrosine, glycine, tryptophan, and cysteine residues (3). The second class undergoes more extensive posttranslational modifications that involve new bond-forming reactions (2). Tyrosines are the most frequently modified residues in this group and are transformed into a wide variety of novel structures (Fig. 1). Amine oxidases and lysyl oxidase contain the quinone cofactors 2,4,5-trihydroxyphenylalanine quinone (TPQ) and lysyl tyrosylquinone (LTQ), respectively (4, 5). The terminal electron transport protein cytochrome *c* oxidase (CcO) is posttranslationally modified through a crosslink between histidine and tyrosine in both bacteria (6) and mammals (7). His

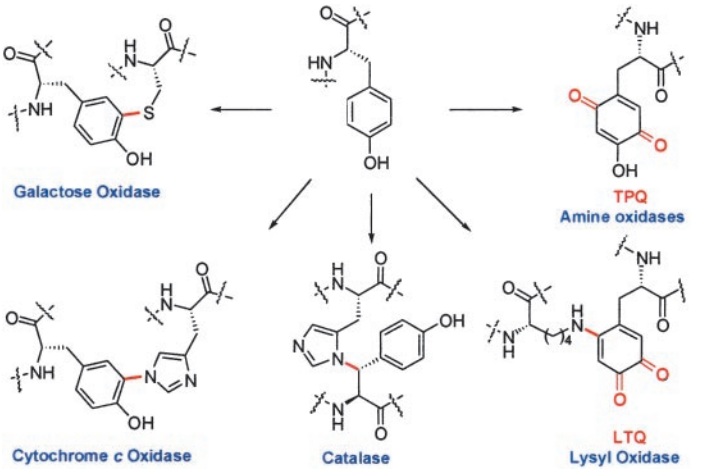

Fig. 1. Structures of posttranslationally modified tyrosines. Newly formed bonds are shown in red.

and Tyr residues are also crosslinked in catalase HPII from *Escherichia coli*, but the linkage in this protein involves a bond between the Cβ of tyrosine and Nδ of histidine (8). In galactose oxidase, as well as glyoxal oxidase, a tyrosine residue is crosslinked by a thioether bond between Cε of the aromatic ring and the sulfur atom of a cysteine (9). This crosslinked tyrosine serves as a ligand to a catalytically essential copper and is oxidized to the tyrosyl radical form in the active state of the protein (10, 11).

The Cu(II)/Cys-Tyr· cofactor in GAO carries out a two-electron oxidation of primary alcohols to the corresponding aldehydes via a radical mechanism (Fig. 2). It is generally agreed upon that the Cys-Tyr· cofactor abstracts a hydrogen atom from the substrate bound to copper. Less clear is the actual role of the crosslink. Initial studies on model compounds noticed the lowering of the one-electron oxidation potential of phenols substituted with a thioether in the *ortho* position (12). This lowered potential nicely correlated with the enormous decrease in the oxidation potential of the Tyr-Cys crosslink from $\approx$1 V for regular tyrosines to 0.4 V in GAO (13). Whereas these reports suggested an electronic role for the crosslink, several other studies have presented support against this hypothesis with DFT calculations predicting only a 1.7 kcal/mol stabilization of the radical because of the crosslink (14). These results have been interpreted to indicate a structural role of the thioether bridge.

The discovery of posttranslationally modified endogenous cofactors has led to great interest into the mechanisms of their formation. Some of these structures, such as tryptophan tryptophyl quinone (TTQ) in methylamine dehydrogenase (15) and formylglycine in sulfatases (16) are generated by accessory proteins (Fig. 3). Others, on the other hand, including TPQ (Fig. 1; refs. 17–19), the MIO structure in phenylalanine ammonia lyase (20), and the chromophore in green fluorescent protein (21) are produced by autocatalytic processes (Fig. 3). Unique among this latter group is galactose oxidase because its self-catalytic maturation involves two very different reactions, the cleavage of a 17-amino acid N-terminal pro-sequence as well as the three-electron oxidation of a Tyr and Cys to the Cys-Tyr· cofactor. Dooley and coworkers previously showed

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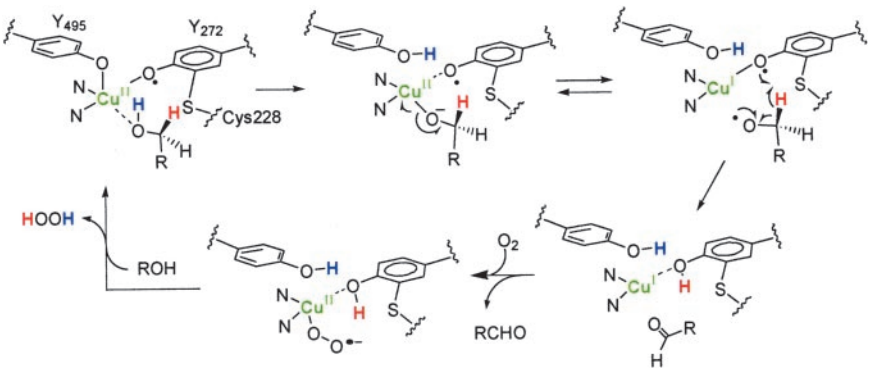

Fig. 2. Proposed mechanism for catalysis by GAO (31). Binding of the substrate to the copper triggers a proton transfer from substrate to the axial tyrosine ligand. Electron transfer from the alkoxide to Cu(II) generates an alkoxy radical from which a hydrogen atom is abstracted by the Cys-Tyr· cofactor. This transformation generates the aldehyde product and a two-electron reduced state of the enzyme, which is oxidized back to the active form of the protein by molecular oxygen. Alternative mechanisms have also been proposed in which the Cys-Tyr· abstracts a hydrogen atom from the alkoxide to generate a ketyl radical anion that then transfers an electron to Cu(II) (i.e., reversal of steps 2 and 3; ref. 14). Both steps may actually occur in an asynchronous concerted step depending on the substrate (32, 33).

that both reactions do not proceed when the protein is heterologously expressed and purified under strictly metal-free conditions (22). On aerobic incubation of this apo-pro-enzyme with copper, the mature active form of GAO was formed. In this issue, Firbank *et al.* follow up on this interesting finding with the determination of the three-dimensional structure of the apo-pro-enzyme (1). The presence of the N-terminal pro-sequence leads to changes in five regions compared with the mature protein. The pro-peptide does not make direct contact with the active site, but prevents several strands and loops to reside at the positions they occupy in the processed protein. As a result, the two residues to be crosslinked, Tyr-272 and Cys-228, as well as Trp-290 that π-stacks with the crosslink once formed (Fig. 4*A*), are in very different positions in the pro-enzyme. The remaining copper ligands, however, are in similar orientations and positions as in the mature protein, suggesting that the pro-enzyme may bind copper at the site to initiate the posttranslational modification events. At least one other protein generates its cofactor by posttranslational modification involving an autocatalytic cleavage of a peptide bond. Histidine decarboxylase is composed of two subunits that originate from the self-processing of an inactive pro-enzyme. During the autocatalytic cleavage, an essential pyruvoyl group is formed at the amino terminus of the α-subunit that derives from Ser-82 of the pro-enzyme (23). In GAO, however, cleavage of the pro-sequence and formation of the cofactor must be separate processes because an intermediate form lacking the N-terminal pro-peptide but without the crosslink has been identified (22). This observation suggests that the mechanisms of both modifications may be elucidated in future investigations.

Interestingly, the authors show that part of Cys-228 may be present in the form of a sulfenate, and they suggest an interesting new proposal for crosslink formation that involves attack by the tyrosine onto an electrophilic sulfur. In this scenario, copper might play a role in both the oxidation of the cysteine and cleavage of the S–O bond of the sulfenate. Sulfenates and sulfinates are present in a number of other proteins, including nitrile hydratase (24), NADH peroxidase, and peroxiredoxins (25). In nitrile hydratase, their formation from cysteine is also likely metal promoted given they are ligands to the catalytically active iron (Fig. 4*D*). Metals are in fact located close to all posttranslationally modified tyrosines in Fig. 1 (see Fig. 4). In

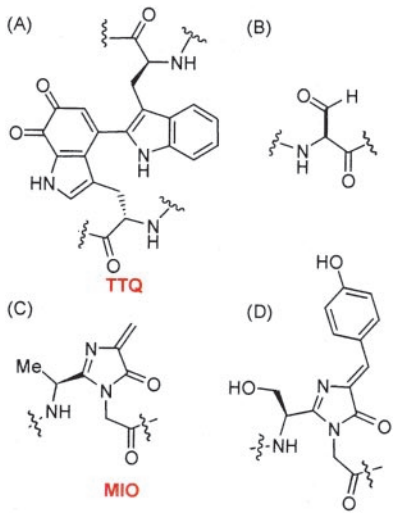

Fig. 3. Structures of cofactors generated through posttranslational modification by accessory enzymes in methylamine dehydrogenase (*A*) and sulfatases (*B*), and structures of cofactors that are generated by autocatalytic processes in phenylalanine ammonia lyase (*C*) and green fluorescent protein (*D*).

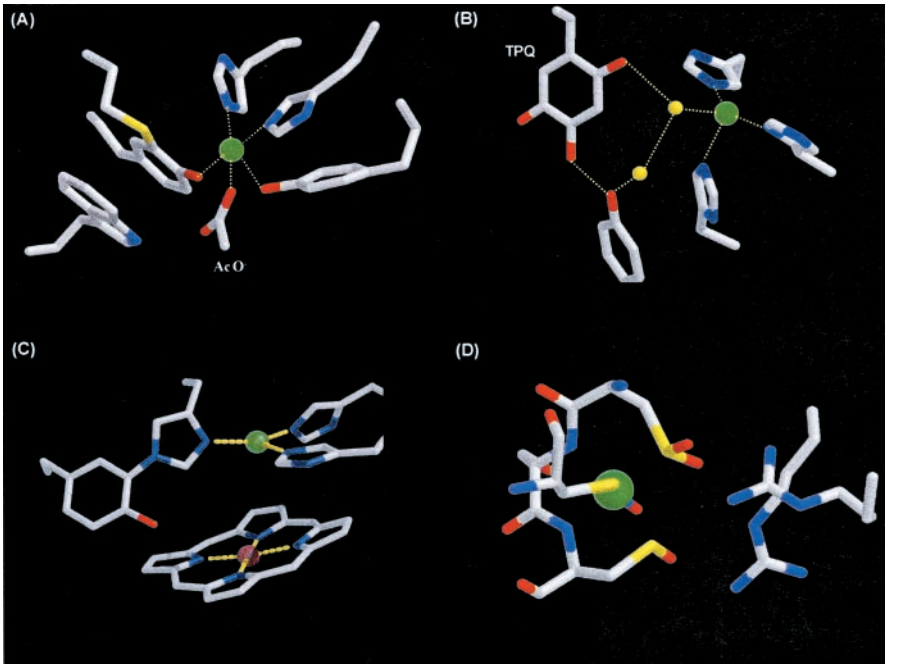

Fig. 4. Active sites of metalloproteins with covalently modified cofactors. (*A*) GAO (ref. 9; PDB 1GOF) and (*B*) *E. coli* amino oxidase (PDB 1SPU). Note that a covalently bound inhibitor present in this structure has been omitted (34). (*C*) CcO from bovine heart (7) (PDB 1OCR), and (*D*) Active site structure of NO inactivated nitrile hydratase (PDB 2AHJ) (24). All figures made with RASMOL.

amine oxidases, oxygen and copper are essential and sufficient for TPQ formation from apo-enzyme (17–19). The copper has been shown to activate the tyrosine for reaction with oxygen (19, 26, 27), and a similar role of the copper can be proposed for the initial oxidation of the tyrosine in GAO (1). The mechanism of formation of the His–Tyr crosslink in cytochrome *c* oxidase is not known, but it is likely that the metals in the bimetallic center will be involved (Fig. 4*C*).

The original reports on the crosslinked tyrosyl radical liganded to Cu(II) led to an explosion in efforts toward development of biomimetic models of the GAO active site and its catalytic properties. These studies have been exceptionally successful, leading to reproduction of the structure, spectroscopy, and catalytic activity of the copper site with low molecular weight copper complexes (28–30). The determination of the structure of unprocessed galactose oxidase reported in this issue may spur similar efforts in trying to understand the mechanism of the formation of the Cu(II)-Cys-Tyr· cofactor, both in GAO itself and in model systems and designed proteins.

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