Commentary

How many ways to craft a cofactor?

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Standard textbooks define enzymatic cofactors as low molecular weight structures that are separate from and can bind reversibly to their cognate protein. As with all established paradigms, change is almost guaranteed, and work over the last decade has forced us to expand our definition of cofactor to include structures that are derived from the protein itself. Early studies of ribonucleotide reductase had indicated the presence of a protein-derived tyrosyl radical as the storage site for the free radical that initiates the conversion of ribonucleotides to deoxyribonucleotides (1). This was followed by the equally unorthodox finding of a protein-bound glycyl radical in select anaerobic proteins (2). More complex posttranslationally derived redox cofactors appeared on the scene in l990, with the discovery of the tyrosine-derived cofactor TPQ in a eukaryotic copper amine oxidase (3) (Fig. 1). The field of quino-cofactors has turned out to be structurally rich, with variants being reported that are formed from tryptophan as well as tyrosine. In a recent issue of PNAS, Datta *et al*. (4) amaze us further with a new quino-cofactor derived from the cross-linking of oxidized tryptophan and cysteine and designated CTQ (Fig. 1).

This exciting discovery follows an earlier report of a cofactor in which a tryptophyl quinone is cross-linked to a second tryptophan to form TTQ (5) (Fig. 1) and is related to the finding that tyrosine-based quinone cofactors also have been observed to be cross-linked to a second amino acid, i.e., the lysine tyrosyl quinone in lysyl oxidase, LTQ (6) (Fig. 1). What is remarkable is the lack of both sequence and structural homology among the tyrosyl-containing and tryptophyl quinone-containing proteins. It appears that nature has found multiple pathways to generate cofactors that are chemically and mechanistically similar.

The extensive mechanistic work on TPQ has served as a guide to understanding the catalytic role of the protein-derived quinones (7). In all cases, the substrates of these diverse enzymes are primary amines that are capable of covalent adduct formation with the quinone functional group of cofactor. The resulting covalent complex contributes an electrophilic sink, analogous to the cofactor pyridoxal phosphate, thereby increasing the acidity of the α-proton of substrate (Fig. 2). In each case where a structure is available for a quino-protein, an aspartic acid side chain has been found to lie in close proximity to the cofactor, thereby implicating this residue as the catalytic base for proton abstraction (8, 9). The x-ray structure for the CTQ containing quinohemoprotein amine dehydrogenase by Datta *et al.* (4) repeats this theme, showing aspartic acid (Asp-33) as the singly charged amino acid side chain in proximity to the quino-cofactor on the γ subunit. Remarkably, the side chain of Asp-33γ is held in place by a covalent, thioether linkage at its β-carbon to the sulfur of a cysteine (Cys-78γ). This theme of thioether formation is repeated at two other positions within the γ subunit.

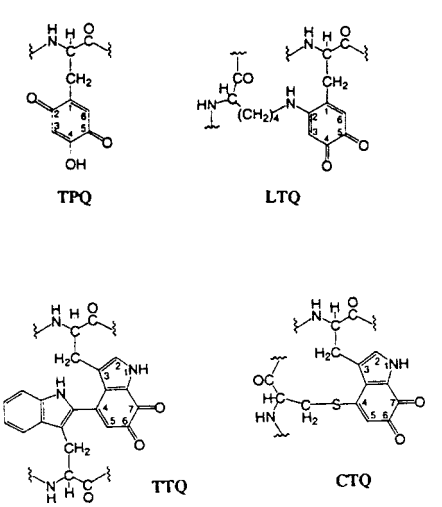

Fig. 1. Quino-cofactors derived from protein-bound tyrosine (TPQ, LTQ) and tryptophan (TTQ, CTQ).

In the mechanism shown in Fig. 2, the protein-bound cofactor has been converted to a reduced, aminoquinol form, which must be recycled to the initial oxidized cofactor to complete the catalytic cycle. A divergence between the tryptophan- and tyrosine-derived cofactors is the pathway for aminoquinol reoxidation. In the case of TPQ and LTQ, molecular oxygen is the acceptor of two protons and two electrons, forming hydrogen peroxide as a final product (10). The tryptophan-derived cofactors, which are found in the periplasmic space of Gram-negative bacteria, catalyze electron transfer, one at a time to an exogenous acceptor (11). The presence of an active site metal ion, Cu(II), in the TPQ- and LTQ-containing proteins had originally led to the proposal of a redox role for the metal ion in cofactor reoxidation. Recent studies implicate, instead, a nonmetal binding site for O_2 with the role of the active site metal being stabilization of reduced oxygen intermediates (12). In contrast, the proteins containing TTQ (13) and CTQ must exclude O_2, directing electrons to their external acceptor. The structure by Datta *et al.* (4) shows how this intramolecular electron transfer may occur in the CTQ-containing quinohemoprotein, identifying an N-terminal heme with a solvent-accessible edge and a buried heme closer to the CTQ cofactor on the γ subunit.

The discovery of each of the structures in Fig. 1 has generated some serious head scratching as to how such structures may arise. Studies of the biogenesis of TPQ are the most developed, indicating that addition of copper ion and O_2 are sufficient to generate TPQ from tyrosine (14, 15). An x-ray structure of an unprocessed protein, in which the active site Cu(II) has been replaced by the nonredox metal Zn(II), shows the hydroxyl group of the precursor tryosine complexed to the metal (16). Kinetic and spectroscopic studies indicate that binding of O_2 is required for the formation of a tyrosine-copper charge transfer complex during productive biogenesis, implicating O_2 as the trigger for the initiation of biogenesis (17, 18). A comparison of the precursor, zinc-containing structure to the mature, copper- and TPQ-containing protein indicates only small and subtle structural changes (16). The TPQ-containing proteins are truly dual-function enzymes, catalyzing both the production of their own cofactor

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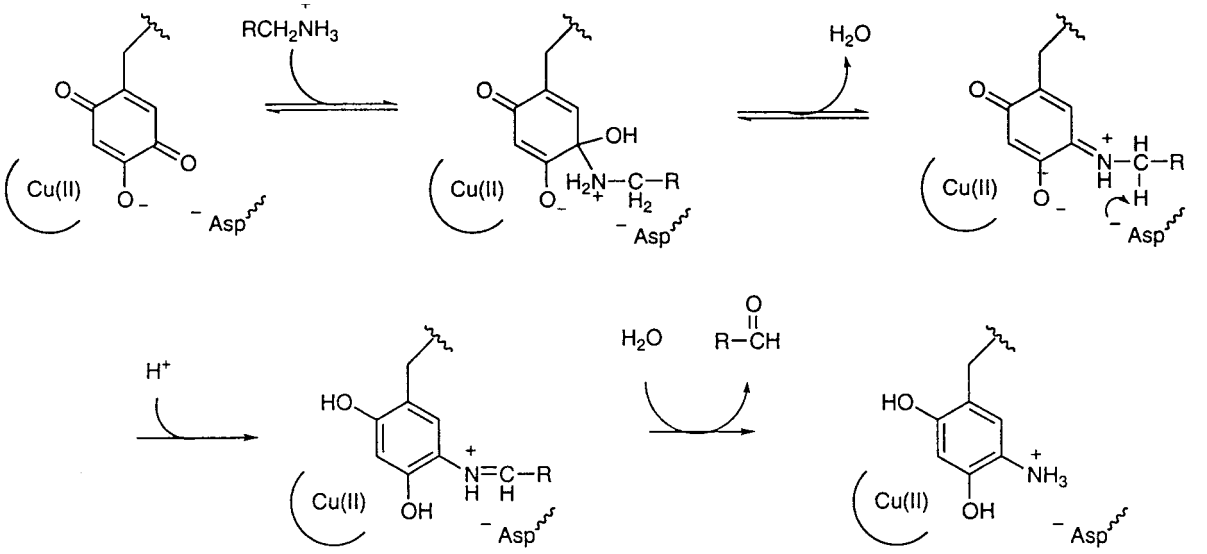

Fig. 2. Mechanism for reductive half-reaction of TPQ enzymes.

and amine oxidation without significant structural rearrangement.

The proteins that contain the tryptophan-derived cofactors are conspicuous by their lack of bound, redox active metal ions as well as an absence of O_2 binding during catalytic turnover. In the case of both TTQ and CTQ, exogenous proteins are almost certainly involved in cofactor formation. The lack, thus far, of x-ray data for the precursor forms of either a TTQ- or CTQ-containing enzyme leaves open the possibility of large structural differences between the precursor and mature forms of protein. A recent x-ray study of unprocessed galactose oxidase (19), a protein that contains a cross-linked cofactor derived from cysteine and tyrosine in its mature form (20), indicates significant structural differences that include the presence of an N-terminal peptide that is cleaved in the process of cofactor production (21).

Datta *et al.* (4) identify four ORFs for quinohemoprotein, three of which encode the subunits of the amine dehydrogenase. A fourth ORF may provide the key to the biogenesis of CTQ, showing weak but significant homology to proteins that belong to a newly identified superfamily called the radical SAM proteins (22). These proteins have been shown to use an iron sulfur cluster to reductively cleave *S*-adenosylmethionine (SAM), producing a deoxyadenosyl radical center capable of initiating free radical conversions (23). These radical reactions include the formation of the glycyl radical found in pyruvate formate lyase (24) and the anaerobic ribonucleotide reductase (25).

It is premature, however, to jump to conclusions regarding the role of this fourth protein in CTQ production. A deoxyadenosyl radical may be critical, for example, in the production of the three thioether linkages found to stabilize the mature quinohemoprotein amine dehydrogenase structure. A separate protein may catalyze the production of an intermediate, tryptophyl quinone, that would then be susceptible to nucleophilic attack by a neighboring Cys (33 γ). In this manner the tryptophan-cysteine cross-link seen in CTQ would arise from heterolytic as opposed to homolytic chemistry (cf. ref. 26). A similar process has been proposed to explain the structures seen in the tyrosine-derived cofactors, TPQ and LTQ (6) (Fig. 3).

A compelling question in this field concerns the mechanistic imperatives for the production of derivatized quinones as cofactors. In the case of the cysteine tyrosyl radical seen in galactose oxidase, the thioether at the ortho position of the tyrosyl radical does not appear to alter the redox potential appreciably (27) and may play primarily a structural role. In the case of the various quinones (Fig. 1), it is conceivable that the requisite chemistry (Fig. 2) could be performed with underivatized ortho-quinones (e.g., **2** in Fig. 3) (28). One difficulty with such a scenario would be the inherent reactivity of ortho-quinone ring structures toward nucleophilic attack by active site residues and/or substrate itself. A major advantage of ortho-quinone modification to yield the structures shown in Fig. 1 is to direct the substrate toward one of the carbonyls of cofactor for Schiff base formation (Fig. 2), as opposed to direct attack on the ring itself.

The above considerations introduce the possibility that the nature of the functional group that modifies the intermediate ortho-quinone is a consequence of the protein active site structure in which the quinone has evolved and is not driven by the requirements for catalytic activity. This raises the possibility of additional, alternate side chains, e.g., a histidine or the carboxylates of aspartate and glutamate, functioning as cross-linking agents. Melville *et al.* (29) addressed this question by preparing a model for the carboxylate analog of TPQ and LTQ, in which an ester linkage replaces the hydroxyl group and lysyl side chain, respectively, at the 6 position of the ring (refer to Fig. 3 for numbering). The first hint that the carboxylate ester derivative may be unsuitable as a cofactor was its hydrolytic instability near neutral pH. More significantly, the redox potential for the carboxylate ester derivative is elevated to +133 mV vs. SCE (29), in contrast to the redox potentials for models of cofactors known to occur in protein active sites [e.g., E_m = −150 mV for TPQ at pH 6.8 (30), E_m = −182 for LTQ at pH 7.0 (6), and E_m = −150 for TTQ at pH 6.8 (31)]. This small range of redox potentials for TPQ, LTQ, and TTQ model compounds suggests that a potential of −150 to −180 mV is linked to cofactor function, implicating the nature of the ring substitution as a critical factor in the production of viable quinocofactor. It will be important and very interesting to learn the redox properties of the newly discovered CTQ structure reported by Datta *et al* (4).

In addition to the above considerations, the evolution of cross-linked cofactors may reflect differences in conformational flexibility required for cofactor during biogenesis vs. catalytic turnover. As discussed in the context of TPQ formation, ring mobility appears critical for the

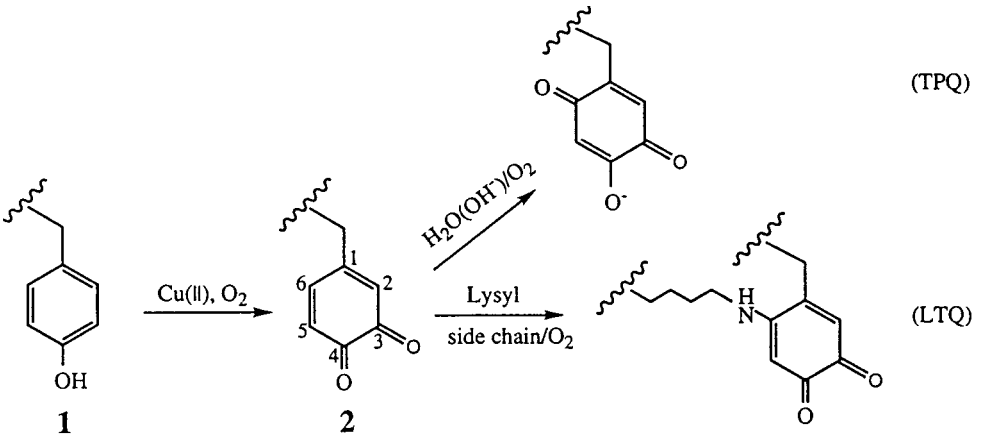

Fig. 3. An ortho-quinone (**2**) proposed as the common intermediate in TPQ and LTQ production.

achievement of a variety of chemical intermediates, each with a different mode of interaction with the active site copper ion (32). Once the mature cofactor has been formed, however, TPQ immobilization appears necessary to allow for precise interactions between active site side chains and TPQ during the catalytic turnover. For TPQ, the one extant example of a noncross-linked quino-cofactor, restriction of movement in the mature cofactor is achieved by a chain of active site residues that create a "wall" behind the back face of the cofactor (33). In the case of LTQ, TTQ, and CTQ, the requirement for this type of protein architecture is obviated by the presence of a covalent linkage to a second amino acid side chain.

Many more aspects of this exciting and evolving field will be discussed at the upcoming Gordon Research Conference on Protein-Derived Cofactors, Radicals, and Quinones in January.

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