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Uncovering novel biochemistry in the mechanism of tryptophan tryptophylquinone cofactor biosynthesis

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Summary

Catalytic quinone cofactors derived from posttranslational modification of amino acid residues within the enzyme polypeptide have roles in a variety of biological processes ranging from metabolism in bacteria to inflammation and connective tissue maturation in humans. In recent years, studies of the biosynthesis of one of these cofactors, tryptophan tryptophylquinone (TTQ), have provided examples of novel chemistry that is required for the generation of these protein-derived cofactors. A novel *c*-type diheme enzyme, MauG, catalyzes a six-electron oxidation that completes TTQ biosynthesis in a 119-kDa protein substrate. The posttranslational modification reactions proceed *via* an unprecedented Fe(V) equivalent catalytic intermediate comprised of two hemes; one an Fe(IV)=O and the other a six-coordinate Fe(IV) with axial ligands provided by amino acid residues. This high valent diheme species is an alternative to Compound I, an Fe(IV)=O heme with a porphyrin or amino acid cation radical, which is typically the reactive intermediate of heme-dependent oxygenases and peroxidases.

Introduction

Several enzymes have recently been characterized with cofactors formed by post-translational modification of amino acid residues within the polypeptide [1,2]. Over 30 such protein-derived cofactors have so far been identified. In contrast to the acquisition of an exogenous cofactor, their evolution provided an endogenous route to increase the diversity of chemistry that could be catalyzed by enzymes synthesized only from natural amino acids.

Protein-derived quinone cofactors are formed from tyrosine or tryptophan residues into which one or two oxygens have been incorporated (Figure 1). Sometimes the quinolated amino acid residue is also covalently cross-linked to another amino acid residue in the polypeptide. Tryptophan-derived quinone cofactors [3] are present in two independently evolved bacterial dehydrogenases possessing either tryptophan tryptophylquinone (TTQ) [4] or cysteine tryptophylquinone (CTQ) [5,6]. These enzymes enable the bacteria to use primary amines as sole sources of carbon, nitrogen and energy. Two types of tyrosine-derived quinone cofactors [7] are present in oxidases from bacterial, mammalian, insect and plant sources. Copper amine oxidases, with functions ranging from metabolism in bacteria to inflammatory response in

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humans [8], possess topaquinone (TPQ) [9]. Lysyl oxidase, a copper-containing enzyme required for maturation of collagen and elastin in mammals [10], contains lysine tyrosylquinone (LTQ) [11]. Enzymes with these four cofactors employ similar mechanisms in their catalytic reductive half-reactions (equation 1) [12]. However, their respective mechanisms of cofactor biosynthesis are very different. Copper is required for TPQ and LTQ biosynthesis, and the process is self-

catalyzed [13-15]. Iron is required for TTQ biosynthesis in the form of a diheme enzyme, MauG [16,17]. The mechanism of CTQ biosynthesis is unknown [18]. This review will focus on the biosynthesis of TTQ in methylamine dehydrogenase (MADH) from *Paracoccus denitrificans*, and the unusual catalytic mechanism of MauG.

Overview of amine dehydrogenase maturation

TTQ has been identified in amine dehydrogenases from a variety of bacterial sources [3]. To form TTQ, two atoms of oxygen are incorporated into the indole ring of residue β Trp57 and a covalent bond is formed between the indole rings of β Trp57 and β Trp108. This process requires the action of other enzymes that are subject to the same genetic regulation as the structural genes of MADH [19,20]. These genes are clustered in the methylamine utilization (*mau*) locus with a gene order of *mauRFBEDACJGMN* [19,20]. The α and β subunits of MADH are encoded by *mauB* and *mauA*, respectively [21]. Four other genes, *mauFEDG*, are essential for MADH biogenesis [19,20] and of these a specific role in TTQ biosynthesis has been demonstrated for *mauG* [17,22].

MauG is a novel c-type diheme protein

MauG was homologously expressed in *P. denitrificans* [16]. It is a 42.3 kDa monomer, and has sequence similarities to bacterial diheme cytochrome *c* peroxidases (CCPs) [23] but exhibits negligible peroxidase activity [16]. The MauG sequence contains two CXXCH motifs for *c*-type hemes. Within the motif the two cysteine residues covalently link the porphyrin to the protein *via* thioether linkages, and the histidine residue is the proximal axial heme ligand [24]. The presence of two *c*-type hemes was confirmed by mass spectrometry [16]. The electron paramagnetic resonance (EPR) spectrum of MauG revealed signals for one high-spin and one low-spin heme, each with *g* values atypical for *c*-type hemes, including diheme CCPs [16]. Diferrous MauG binds carbon monoxide and reoxidizes in air, which is atypical behavior for *c*-type cytochromes. MauG also exhibits negative redox cooperativity [25]. The two hemes have a similar oxidation-reduction midpoint potential (E_m) value, but act as a diheme unit due to facile electron transfer between them, resulting in the addition of a second electron ($E_m = -240\text{mV}$) being more difficult than addition of the first ($E_m = -159\text{mV}$) [25].

MauG completes biosynthesis of TTQ

TTQ biosynthesis was studied using a recombinant system that contained the structural genes for *P. denitrificans* MADH as well as *mauFEDG* expressed in *Rhodobacter sphaeroides* [26]. To define the role of MauG, the *mauG* gene was inactivated and wild-type MADH was expressed in its absence. This allowed isolation of a biosynthetic precursor of MADH (preMADH) [22]. Proteolysis and mass spectrometry demonstrated that preMADH contains an incompletely synthesized TTQ with a single hydroxyl group on β Trp57 and no covalent crosslink to β Trp108 [22].

Incubation of preMADH with MauG resulted in formation of catalytically active MADH with mature TTQ [17]. Mass spectrometry of the reaction product synthesized in the presence of either $^{18}\text{O}_2$ or H_2^{18}O demonstrated that the second oxygen insertion catalyzed by MauG is at C6 of the cofactor [27] (Figure 2). This was concluded using the knowledge that rapid exchange with solvent occurs at the C6 position of TTQ [28]; however this also meant that the source of the oxygen atom could not be ascertained. The mechanism of initial hydroxylation at C7 of $\beta\text{Trp}57$ is unknown. These studies demonstrated that MauG catalyzes the six-electron oxidation of preMADH to yield oxidized MADH with the mature TTQ cofactor, the process requiring oxygen insertion (two-electrons), cross-linking of $\beta\text{Trp}57$ to $\beta\text{Trp}108$ (two-electrons), and oxidation to the quinone (two-electrons). Oxidation equivalents ($[\text{O}]$) could be provided by O_2 plus an electron donor or by H_2O_2 [29]. An electron donor is only necessary when O_2 is the oxidant, and reduces the enzyme to the diferrous state which then reacts with O_2 . Low potential electron donors such as dithiothreitol with E_m values more negative than MauG are the most effective *in vitro* [29]. However, even ascorbate ($E_m = +58\text{mV}$) can drive TTQ biosynthesis when present in large excess, albeit at a much slower rate. If O_2 is the natural substrate, this promiscuity of MauG may enable it to accept electrons from a wide range of possible donors in the oxidizing periplasmic environment where MauG resides. However, the natural source of $[\text{O}]$ is unknown. Whether there is a naturally occurring and reliable source of H_2O_2 in the periplasm is also unknown, but as the periplasm contains cytochrome *c* peroxidase some H_2O_2 must be present, at least under certain conditions.

Characterization of a novel high-valent iron species required for TTQ biosynthesis

As a first step towards identifying intermediates in the biosynthetic reaction, oxidized MauG was mixed with stoichiometric H_2O_2 (i.e., one-third of the requirement for complete TTQ biosynthesis) and monitored spectroscopically. This caused a rapid decrease in intensity of the Soret peak of MauG and a shift in its maximum from 405 to 407 nm. This relatively stable intermediate decayed with at a rate of $2 \times 10^{-4} \text{ s}^{-1}$ [30]. The EPR spectrum of this intermediate demonstrated that both hemes had become EPR-silent [31]. Mössbauer spectroscopy revealed the presence of two distinct Fe(IV) species in the intermediate [31]. One was consistent with an Fe(IV)=O species ($\delta = 0.06 \text{ mm/s}$, $\Delta E_Q = 1.70 \text{ mm/s}$). The other was assigned to a unprecedented Fe(IV) species with two axial ligands from protein ($\delta = 0.17 \text{ mm/s}$, $\Delta E_Q = 2.54 \text{ mm/s}$) [31]. There was no evidence for the presence of Compound I, an Fe(IV)=O heme with a porphyrin cation radical [32], which is utilized in peroxidase and cytochrome P450 catalyzed chemistry.

Kinetics of the first two-electron catalytic cycle of MauG

The formation of the *bis*-Fe(IV) MauG intermediate and its two-electron oxidation of preMADH were studied by stopped-flow spectroscopy [30]. Formation of the intermediate on addition of H_2O_2 was rapid ($k > 300 \text{ s}^{-1}$). The reaction of *bis*-Fe(IV) MauG with preMADH exhibited saturation behavior with a limiting first-order rate constant of 0.8 s^{-1} and a K_d of $\leq 1.5 \text{ }\mu\text{M}$ for the MauG-preMADH complex. The order of addition of H_2O_2 and preMADH to MauG was random, with the presence of preMADH neither stimulating nor impeding the reaction with H_2O_2 [30]. It had also been shown that diferrous MauG reacts with O_2 in the absence of substrate [16]. This phenomenon was further studied with the O_2 analog CO [30]. The reaction of diferrous MauG with CO was biphasic with a major transition with a bimolecular rate constant of $5.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, and a minor transition with a rate of 16 s^{-1} which was independent of $[\text{CO}]$. As observed with H_2O_2 , the reactivity of MauG towards CO was insensitive to the presence or absence of preMADH. These results are in contrast to what is typically seen with cytochrome P450-dependent monooxygenases. These are unreactive

toward molecular oxygen until binding of substrate triggers a conformational change that allows the high-spin heme to bind and activate O₂ [33].

Intermediates in MauG-dependent TTQ biosynthesis

Our current postulated mechanism for MauG-dependent TTQ biosynthesis is shown in Figure 3. This six-electron oxidation requires three moles of H₂O₂, which can be equated to three two-electron cycles; (1) hydroxylation at C6 of β Trp57 to form a quinol, (2) cross-link formation between β Trp57 and β Trp108, and (3) oxidation of the quinol to the quinone. Although the order of these three cycles is unknown (other than (3) must follow (1)), there is evidence that the final cycle is (3); quinol to quinone oxidation (Figure 3). A transient intermediate with a λ_{max} at 330 nm was observed during steady-state MauG-dependent TTQ biosynthesis [29]. This feature is characteristic of the reduced MADH quinol, whereas oxidized quinone has a broad absorbance at 440 nm [34]. To test this hypothesis, the quinol was formed by dithionite reduction of mature MADH. Oxidation of the chemically reduced MADH quinol to TTQ did not occur in the presence of either H₂O₂ or MauG alone. However, in the presence of both components, MauG H₂O₂-dependent oxidation of the quinol to TTQ was observed, consistent with this being the final step in TTQ biosynthesis.

While additional intermediates in the overall mechanism remain to be elucidated, it appears that conversion of preMADH to quinol MADH (requiring two two-electron cycles) proceeds *via* a radical mechanism. EPR spectroscopy of the products of the initial two-electron reaction of *bis*-Fe(IV)MauG with preMADH revealed the formation of a new protein-based free radical species that occurred concomitant with conversion of the hemes to the diferric state [31]. This radical species was very stable and exhibited an EPR signal similar to that of an aromatic protein-based radical. Its precise nature remains to be characterized. Although it is convenient to think in two-electron cycles, there is the possibility that the insertion of the second oxygen atom into β Trp57 and cross-link formation could be a concerted four-electron process. The long-lived preMADH aromatic radical that results from the reduction of *bis*-Fe(IV) MauG would be consistent with this possibility. Although the physiological source of [O] is not known, the availability of H₂O₂ (a possible substrate), or electrons in the oxidizing periplasm (which are required if O₂ is the substrate), could be limiting, leading to a lag between each two-electron cycle. Although the stable aromatic radical only accounts for one electron, long-lived radical intermediates may be beneficial as they would reduce the loss of energy through decay, and the possibility of deleterious non-specific oxidative damage to preMADH and MauG.

It should be noted that consistent with the kinetically determined K_d of ≤ 1.5 μ M, it was demonstrated that MauG and preMADH co-elute during high-resolution size-exclusion chromatography [35]. In contrast MauG and MADH with mature TTQ do not co-elute. This suggests the possibility that the preMADH substrate and subsequent reaction intermediates may remain bound to MauG throughout the three-step six-electron oxidation.

Comparison of MauG and bacterial diheme cytochrome c peroxidases

Although MauG exhibits sequence homology to bacterial diheme CCPs, its redox and catalytic properties are very different. The majority of diheme CCPs studied are isolated in an inactive diferric state, where the heme that reacts with H₂O₂ (P-heme) is six-coordinate with His/His axial ligation. Reduction of the other six-coordinate His/Met ligated electron transfer heme (E-heme) in diheme CCP generates a mixed-valence state (Fe(III)-Fe(II)) that triggers a conformational change in which the distal His of the P-heme is replaced by water [36,37]. The enzyme can then react with H₂O₂ to form Fe(IV)=O at the P-heme. Delivery of two protons and two more electrons releases water and generates a transient diferric species which is further reduced to the mixed-valence state (Figure 4). The E_m values of the hemes of diheme CCP are

separated by more than 600 mV [38] which allows for a stable mixed-valence state. In contrast, the as-isolated diferric MauG enzyme is active, and due to the similar intrinsic E_m values of the hemes and the redox cooperativity between them, it cannot attain a mixed-valence state [25].

Interestingly, the diheme CCP from *Nitrosomonas europaea* (NeCCP) is active in the diferric state [23,39]. The crystal structure of diferric NeCCP confirmed that it adopted the active conformation of the mixed-valence diheme CCPs [40]. Both the mixed-valence and diferric states of NeCCP can react with H_2O_2 [39] and Compound I is detected in the latter. NeCCP forms part of a substantial homology cluster that includes the *E. coli* diheme CCP [23], which lies sequentially between the diferric H_2O_2 -inactive CCPs and the MauGs, forming a mechanistic bridge between the “classic” diheme CCPs and the MauG enzymes.

Conclusions

Heme and non-heme high-valent iron species are powerful oxidizing species in chemistry and biology. The best characterized form of an Fe(V) equivalent in biological systems is Compound I, a non-covalently bound *b*-type heme in the Fe(IV)=O oxidation state and containing a porphyrin cation radical. During TTQ biosynthesis MauG stabilizes a diheme *bis*-Fe(IV) reaction intermediate with the second oxidizing equivalent stored not as a porphyrin radical but as a second Fe(IV) heme. This high-valent iron intermediate is unusually stable, and when added to its protein substrate produces a stable preMADH based radical intermediate. Elucidation of how MauG and preMADH so effectively stabilize highly reactive high-valent metal and radical species will significantly advance our understanding of radical-based chemistry and enzymology. MauG is sequentially related to diheme CCPs, but exhibits different redox and catalytic behavior. Further characterization of MauG should provide insight into how the protein dictates the function of the heme as a peroxidase, oxygenase, oxygen transporter or electron transfer mediator. This should elucidate previously unrecognized strategies for enzyme-catalyzed post-translational modifications and suggest potential applications for biocatalysis.

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TPQ **LTQ** **CTQ** **TTQ**

Figure 1. Protein-derived quinone cofactors. TPQ, 2,4,5-trihydroxyphenylalanine quinone; LTQ, lysine tyrosylquinone; CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone.

Figure 2.
Overview of TTQ biosynthesis. Oxidation equivalents ([O]) may be provided by O_2 plus an electron donor or by H_2O_2

Figure 3.
Postulated mechanism for MauG-dependent completion of TTQ biosynthesis.

Figure 4.
General mechanism for activation and reaction of di-heme CCP.