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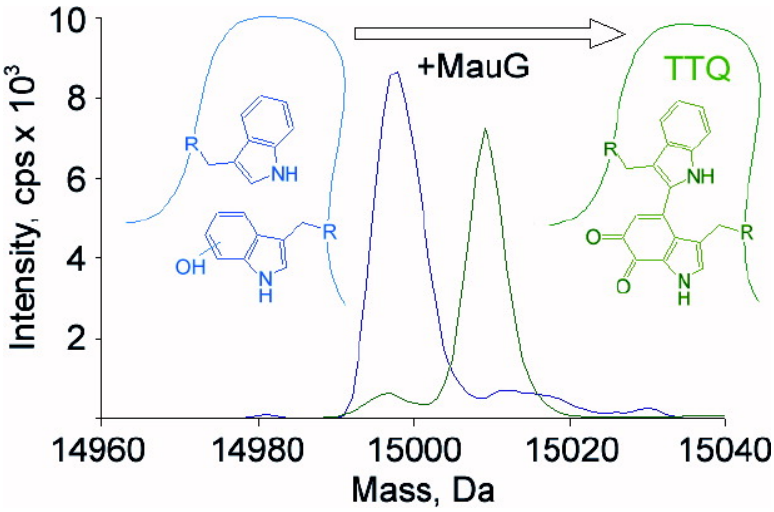

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MauG-Dependent in Vitro Biosynthesis of Tryptophan Tryptophylquinone in Methylamine Dehydrogenase

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Tryptophan tryptophylquinone[1] (TTQ) (Figure 1) is the prosthetic group of methylamine dehydrogenase (MADH) and aromatic amine dehydrogenase.[2] It is synthesized through post-translational modification of two endogenous tryptophan residues. This modification involves two oxygenation reactions and one cross-linking reaction. MADH exhibits an $\alpha_2\beta_2$ heterotetrameric structure.[3] In MADH from *Paracoccus denitrificans*, Trp^{57} and Trp^{108} from the β subunit are the two residues from which TTQ is derived. During the process, two oxygens are added onto Cζ1 and Cη1 of βTrp^{57}, and a cross-link is made between Cϵ3 of βTrp^{57} and Cδ1 of βTrp^{108}.

The genes that encode the MADH subunits, together with nine other genes that relate to MADH expression and function, are clustered in the methylamine utilization (*mau*) locus.[4] Four genes, *mauFEDG*, were shown to be essential for MADH maturation.[4,5] One of these, *mauG*, has been shown to be required for TTQ formation during MADH biosynthesis.[6] The MauG protein has been expressed and characterized.[7] It contains two *c*-type hemes as predicted from the sequence, which contains two CXXCH motifs. In contrast to typical *c*-type cytochromes, the reduced form of MauG binds and reacts with oxygen. The electron paramagnetic resonance spectrum of oxidized MauG exhibits signals corresponding to one high-spin heme and one low-spin heme. The *g* values exhibited by these hemes are atypical of *c*-type cytochromes and much more similar to those of hemes that bind and activate oxygen, including ligand complexes of cytochrome P450CAM and the complex of heme oxygenase with heme.

To test the role MauG plays in TTQ maturation, MauG was inactivated in vivo by site-directed mutagenesis in the recombinant MADH expression system.[8] This resulted in the production of MADH with incompletely formed TTQ.[6] The major species that was isolated was inactive and possessed a biosynthetic intermediate of TTQ with a monohydroxylated βTrp^{57} and no covalent cross-link between βTrp^{57} and βTrp^{108} (Figure 1). Furthermore, while MADH with this biosynthetic intermediate was isolated as a holoenzyme, it exhibited subunit−subunit interactions weaker than that of native MADH, as evidenced by native polyacrylamide gel electrophoresis (PAGE).[6]

In this study, the isolated monohydroxylated biosynthetic intermediate was incubated in vitro with purified MauG to determine whether the latter is competent to catalyze the remaining steps in TTQ biosynthesis. The inactive biosynthetic intermediate of MADH was analyzed before and after incubation with purified MauG by mass spectrometry and electrophoretic and steady-state kinetic analyses. The results indicate that, in the presence of MauG, incorporation of the second oxygen into βTrp^{57} and formation of the cross-link with βTrp^{108} to form TTQ occurs in vitro. The product of this reaction also exhibits normal subunit−subunit interactions.

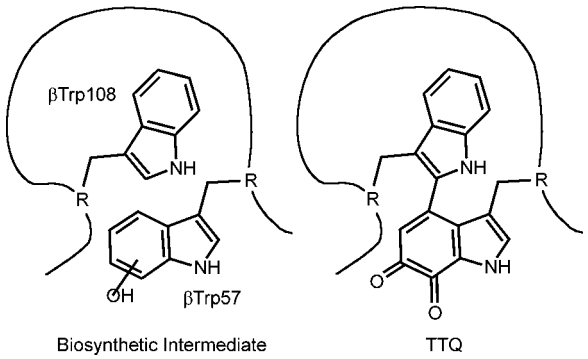

Figure 1. Structure of TTQ and its biosynthetic intermediate. The intermediate that accumulates in vivo in the absence of MauG contains a monohydroxylated βTrp^{57} and no cross-link between βTrp^{57} and βTrp^{108}. The position at which the OH is attached to the ring is not known. In mature TTQ, the quinone oxygens are present on carbons Cζ1 and Cη1 of the indole ring of βTrp^{57}, and it is covalently cross-linked to the indole ring of βTrp^{108}.

ESI mass spectrometry of the substrate was performed before and after incubation with MauG under aerobic conditions to determine whether TTQ had been correctly synthesized in vitro. The β subunit of MADH with incompletely synthesized TTQ showed a major peak corresponding to a β subunit containing the six disulfides and a single extra oxygen[6] (Figure 2). After incubation with MauG, the major peak now corresponds to the mass of a β subunit with fully synthesized TTQ. There was also a minor peak that corresponds to a β subunit containing the six disulfides and single extra oxygen, which corresponds to a portion of unreacted substrate. Similar results were obtained using intermediates that were isolated from expression systems possessing either mutations in or deletion of *mauG*.

Electrophoretic analysis was performed by nondenaturing PAGE and compared with native MADH. Native MADH migrates as a single band, corresponding to the $\alpha_2\beta_2$ heterotetramer. Before incubation with MauG, the intermediate migrates primarily as two bands, which correspond to the positions of migration of the isolated α and β subunits of MADH (Figure 3). This is due to weakened subunit−subunit interactions, relative to native MADH, in the absence of mature correctly synthesized TTQ.[6,9] After incubation with MauG, the major species now migrates as a single band, with a position corresponding to that of the MADH $\alpha_2\beta_2$ heterotetramer. An in-gel stain for MADH activity showed that incubation with MauG leads to formation of active MADH. The appearance of MADH activity was also observed by assaying the product of the reaction in solution using the established steady-state assay for

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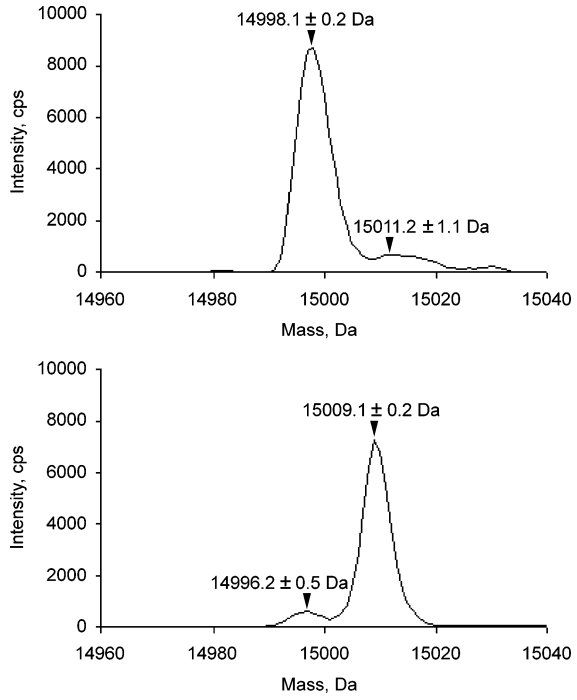

Figure 2. Evidence of in vitro biosynthesis of TTQ and MADH from mass spectrometry. The methods for sample preparation and analysis have been previously described.[6,9] Deconvoluted mass spectra of the β subunit of the biosynthetic intermediate of MADH (top) before and (bottom) after aerobic incubation overnight with MauG. Deconvolution was performed with a mass range to search for deconvoluted peaks of 14 000−45 000 Da, using the Bayesian Protein Reconstruct tool in the ABI BioAnalyst software.

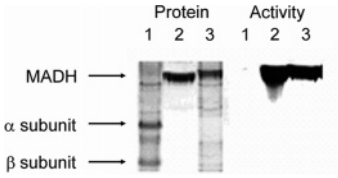

Figure 3. Evidence of in vitro biosynthesis of MADH from electrophoretic analysis. Samples were subjected to nondenaturing PAGE using 4−20% gradient gels. Gels were stained for protein with Coomassie Blue G250 and stained for MADH activity by incubation overnight in a solution of 0.05 M potassium phosphate, pH 7.5, which contained an excess of methylamine and nitroblue tetrazolium. Positions that correspond to those of the purified α and β subunits and the $\alpha_2\beta_2$ holoenzyme are indicated. (1) Biosynthetic intermediate. (2) Native MADH. (3) Biosynthetic intermediate after 30 min aerobic incubation with MauG.

MADH activity. These results correlated with those shown for the in-gel assays. Significantly, the results in Figure 3 not only show the appearance of activity but also clearly demonstrate the correlation of appearance of activity with the strengthening of subunit−subunit interactions, both of which are linked to TTQ formation. The activity correlated with MADH migrates as the $\alpha_2\beta_2$ heterotetramer, and only this band had positive activity.

These results were obtained by aerobic incubation of the biosynthetic intermediate with MauG in the absence of any added factors. The MauG used in these studies is essentially pure, but the biosynthetic intermediate does contain some impurities from the *Rhodobacter sphaeroides* expression system.[8] It is not possible to further purify the intermediate because of its instability. As such, we cannot rule out the possibility that some other factor is present in these preparations that may be required in combination with MauG for efficient completion of TTQ biosynthesis. The rates of TTQ biosynthesis varied with different batches of biosynthetic intermediate that were used as substrate, and the number of turnovers that could be catalyzed by MauG varied from one to several. The observed reactions were oxygen-dependent, but it was difficult to obtain controls with zero activity under anaerobic conditions, suggesting a very high affinity for oxygen in this process. Under no conditions in these experiments was in vitro biosynthesis of TTQ ever observed in the absence of added MauG. Further studies are in progress to determine the factors which are required for consistent multiple turnovers in the in vitro system.

It is now recognized that redox-active prosthetic groups may be derived from post-translational modifications of peptide amino acid residues.[10] For two such tyrosine-derived cofactors, 2,4,5-trihydroxyphenylalanine quinone (topaquinone) of copper amine oxidases[11] and the cross-linked tyrosine−cysteine of galactose oxidase,[12] the cofactor is synthesized by a self-processing mechanism. In contrast, the biogenesis of TTQ is not autocatalytic and requires at least one other accessory gene product, MauG, to complete TTQ biosynthesis after insertion of the first oxygen into βTrp^{57}.

These results are the first description of an enzyme-mediated biosynthesis of a protein-derived cofactor in vitro. It is clearly shown that the incorporation of the second oxygen into βTrp^{57} and the covalent cross-linking of βTrp^{57} to βTrp^{108} are MauG-dependent processes. These reaction steps are severely compromised in vivo when *mauG* is mutated or deleted. These steps may then be catalyzed in vitro upon addition of MauG to the isolated biosynthetic intermediate. These results also show that TTQ formation is linked to proper assembly of subunits during MADH biosynthesis. Last, these results demonstrate a novel function for the *c*-type heme protein, MauG, which is consistent with its atypical physical properties.[7]

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Note Added after ASAP Publication. Due to a production error, a cofactor name was incorrect in the next-to-last paragraph in the version published ASAP on May 18, 2005. The name was corrected to "2,4,5-trihydroxyphenylalanine quinone" in the version published ASAP on May 20, 2005.

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