

Mechanistic Possibilities in MauG-Dependent Tryptophan Tryptophylquinone Biosynthesis[†]

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ABSTRACT: Tryptophan tryptophylquinone (TTQ), the prosthetic group of methylamine dehydrogenase, is formed by post-translational modifications of two tryptophan residues that result in the incorporation of two oxygens into one tryptophan side chain and the covalent cross-linking of that side chain to a second tryptophan residue. MauG is a novel 42 kDa di-heme protein, which is required for the biosynthesis of TTQ. An experimental system has been developed that allows the direct continuous monitoring of MauG-dependent TTQ biosynthesis *in vitro*. Four diverse electron donors, ascorbate, dithiothreitol, reduced glutathione, and NADH, were each able to provide reducing equivalents for MauG-dependent TTQ biosynthesis under aerobic conditions. The reaction with NADH was mediated by an NADH-dependent oxidoreductase. Under anaerobic conditions in the absence of an electron donor, H₂O₂ could serve as a substrate for MauG-dependent TTQ biosynthesis. During the reaction with H₂O₂, a discrete reaction intermediate was observed, which is likely the reduced quinol form of TTQ that then is oxidized to the quinone. These results suggest that not only the incorporation of oxygen into the monohydroxylated biosynthetic intermediate but also the subsequent oxidation of quinol MADH during TTQ biosynthesis is a MauG-dependent process. The implications of these results in elucidating the mechanism of MauG-dependent TTQ biosynthesis and identifying potential physiologic electron and oxygen donors for TTQ biosynthesis *in vivo* are discussed.

Tryptophan tryptophylquinone (1) (TTQ¹) is the prosthetic group of methylamine dehydrogenase (MADH) (2). It is synthesized through the post-translational modification of two endogenous tryptophan residues. This modification involves two oxygenation reactions and one covalent cross-linking reaction. In *Paracoccus denitrificans*, 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 with a gene order of *mauRFBEDACJGMN* (3, 4). The first gene, *mauR*, is a Lys R-type transcriptional activator (5). The structural genes for the α and β subunits of MADH are *mauB* and *mauA*, respectively, and *mauC* encodes amicyanin (6), which is the obligate electron acceptor of MADH (7). On the basis of sequence comparison, the last two genes, *mauM* and *mauN*, appear to encode ferredoxin-like proteins with unknown function (4). Four other genes, *mauFEDG*, were shown to be essential for MADH biosynthesis (3, 4). The MauG protein is the only one of these required gene products that

has been expressed and characterized (8). MauG contains two *c*-type hemes, one low-spin and one high-spin (8), which exhibit cooperative redox behavior (9). In contrast to typical *c*-type cytochromes, the reduced form of MauG binds CO and is oxidized by O₂. Furthermore, the EPR parameters for MauG are atypical of *c*-type cytochromes and much more similar to those of hemes that bind and activate oxygen, such as ligand complexes of cytochrome P450CAM and the complex of heme oxygenase with heme (8).

It has been shown that in MADH from *P. denitrificans*, the incorporation of the second oxygen into β Trp⁵⁷ and the covalent cross-linking of β Trp⁵⁷ to β Trp¹⁰⁸ are MauG-dependent processes (Figure 1). These reaction steps are severely compromised *in vivo* when *mauG* is mutated or deleted. Mutation or deletion of *mauG* *in vivo* leads to accumulation of a biosynthetic intermediate of TTQ in which β Trp⁵⁷ is monohydroxylated at the C7 position, and the covalent cross-link between β Trp⁵⁷ and β Trp¹⁰⁸ is absent (10, 11). Incubation of this biosynthetic intermediate *in vitro* with purified MauG results in the incorporation of the second oxygen into β Trp⁵⁷ and the formation of the cross-link with β Trp¹⁰⁸ to form TTQ and yield active MADH (12). This was confirmed by kinetic, electrophoretic and mass spectrometry analysis of the product of the reaction (12). In this earlier study, we noted that the rates of TTQ biosynthesis *in vitro* varied with different batches of biosynthetic intermediate that were used as substrate, and the number of turnovers that could be catalyzed by MauG was limited and variable. This raised the question of whether MauG could function as a

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¹ Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; DTT, dithiothreitol; GSH, reduced glutathione; *E*_m, oxidation-reduction midpoint potential.

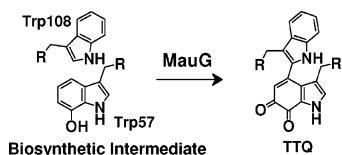


FIGURE 1: MauG-dependent tryptophan tryptophylquinone (TTQ) biosynthesis *in vitro*.

true enzyme capable of reproducibly catalyzing multiple turnovers. Although MauG-dependent TTQ biosynthesis was shown to be O_2 dependent, it was difficult to obtain controls with zero activity under anaerobic conditions, suggesting a very high affinity for oxygen in this process. In this article, we describe a new experimental system that allows direct continuous monitoring of MauG-dependent TTQ biosynthesis *in vitro* and detailed study of factors that are required for MauG-dependent TTQ biosynthesis. The ability of four diverse electron donors to drive TTQ biosynthesis *in vitro* is documented. The ability of H_2O_2 to serve as a substrate for TTQ biosynthesis in the absence of O_2 and an electron donor is also described. The implications of these results in elucidating the mechanism of MauG-dependent TTQ biosynthesis and identifying potential physiologic electron and oxygen donors for TTQ biosynthesis *in vivo* are discussed.

EXPERIMENTAL PROCEDURES

Protein purification. The methods for homologous expression of MauG in *P. denitrificans* and its purification were as described previously (8). The TTQ biosynthetic intermediate of MADH, which contains monohydroxylated Trp⁵⁷ and no cross-link to Trp¹⁰⁸ (10), was heterologously expressed in *Rhodobacter sphaeroides* and purified as described previously (13).

Electrophoretic Analysis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed using 4–20% gradient gels and stained for protein with Coomassie Blue G250. Proteins capable of catalyzing NADH-dependent substrate reduction were stained by incubation for 30 min in a solution of 0.05 M potassium phosphate buffer at pH 7.5, which contained an excess of NADH and nitroblue tetrazolium.

Spectrophotometric Assay of TTQ Biosynthesis *in vitro*. The standard assay for *in vitro* MauG-dependent TTQ biosynthesis contained 0.5 μM MauG and 10 μM biosynthetic intermediate of MADH in 0.01 M potassium phosphate buffer at pH 7.5. The reaction was initiated by the addition of the electron donor to the mixture. Spectral changes were recorded with a Shimadzu MultiSpec-1501 spectrophotometer. TTQ biosynthesis was monitored at 440 nm. The extinction coefficient for oxidized MADH, $\epsilon_{440} = 26\,200\text{ M}^{-1}\text{ cm}^{-1}$ (14), was used to determine the concentration of MADH with mature TTQ, which was produced in the reaction from the ΔA_{440} .

O_2 and H_2O_2 Dependence of TTQ Biosynthesis. In order to determine the O_2 dependence of MauG-dependent TTQ biosynthesis, it was necessary to establish strict anaerobic conditions. Solutions of the biosynthetic intermediate of MADH, MauG, and the electron donor were each placed in sealed cuvettes, which were connected by a tube. The entire system was then made anaerobic by repeated cycles of evacuation and argon replacement. Transfer of reactants was made through the connecting tube. Any spectral changes

associated with the mixing of reactants anaerobically could then be monitored and recorded prior to the addition of O_2 . Air was then inflated into the mixture, and the O_2 -dependent reaction was monitored. Alternatively, after anaerobic incubation of MauG with the biosynthetic intermediate of MADH, H_2O_2 was transferred to the mixture so that the H_2O_2 -dependent reaction could be monitored.

Mass Spectrometry. The methods for sample preparation and analysis were as previously described (10). 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 package.

RESULTS

Effect of Different Electron Donors on Aerobic MauG-Dependent TTQ Biosynthesis. It was previously demonstrated that MauG catalyzes the incorporation of the second oxygen into βTrp^{57} and the covalent cross-linking of βTrp^{57} to βTrp^{108} (10, 12). If molecular oxygen is used as the substrate for the oxygenation reaction, then MauG requires a source of electrons to activate the oxygen. Four potential electron donors were examined: NADH ($E_m = -320\text{ mV}$ (15)), dithiothreitol (DTT, $E_m = -330\text{ mV}$ (16)), reduced glutathione (GSH, $E_m = -240\text{ mV}$ (17)), and ascorbic acid ($E_m = +58\text{ mV}$ (15)). NADH, DTT, and GSH were chosen as potential electron donors because they are relatively low potential donors that exhibit E_m values more negative than that of MauG. We have previously shown that the E_m values of the two one-electron oxidation–reduction reactions of this di-heme protein are -159 and -240 mV (9). With each of these electron donors, an increase in absorbance in the region from 400 to 500 nm was observed (Figure 2), consistent with the formation of oxidized TTQ, which exhibits a broad absorbance in this region centered at 440 nm in mature MADH (14). Even ascorbate, which has a much more positive E_m value, was also able to function as an electron donor when present in large excess compared to the amount of MauG, albeit at a much slower rate than those of the other three. The extent of the absorbance increase indicated that each electron donor was capable of supporting multiple turnovers of MauG-dependent TTQ biosynthesis in the presence of O_2 (Figure 2). The rate of TTQ biosynthesis varied greatly depending upon the electron donor that was used. The k_{obs} values for MauG-dependent TTQ biosynthesis ranged from 0.1 min^{-1} with ascorbate to 25 min^{-1} with NADH. It was not possible to obtain absolute k_{cat} values because some of the reductants at higher concentrations caused damage to MauG. Thus, the reactions had to be performed at subsaturating concentrations of the electron donors. The spectrum of the product of each reaction could be observed from the difference spectrum obtained by subtracting the initial spectrum from the final spectrum (see insets in Figure 2). The difference spectra for the products each exhibit a broad peak centered on 450 nm, which is characteristic of oxidized MADH, although slightly red-shifted compared to that of native MADH. This apparent shift is due to the contribution to the spectral changes of the Soret peaks of the hemes of MauG from their oxidized to reduced states (9), which occurs during the reaction with excess reductant. The combined Soret peaks of the two hemes of MauG shift from an absorbance maximum at 406 nm for

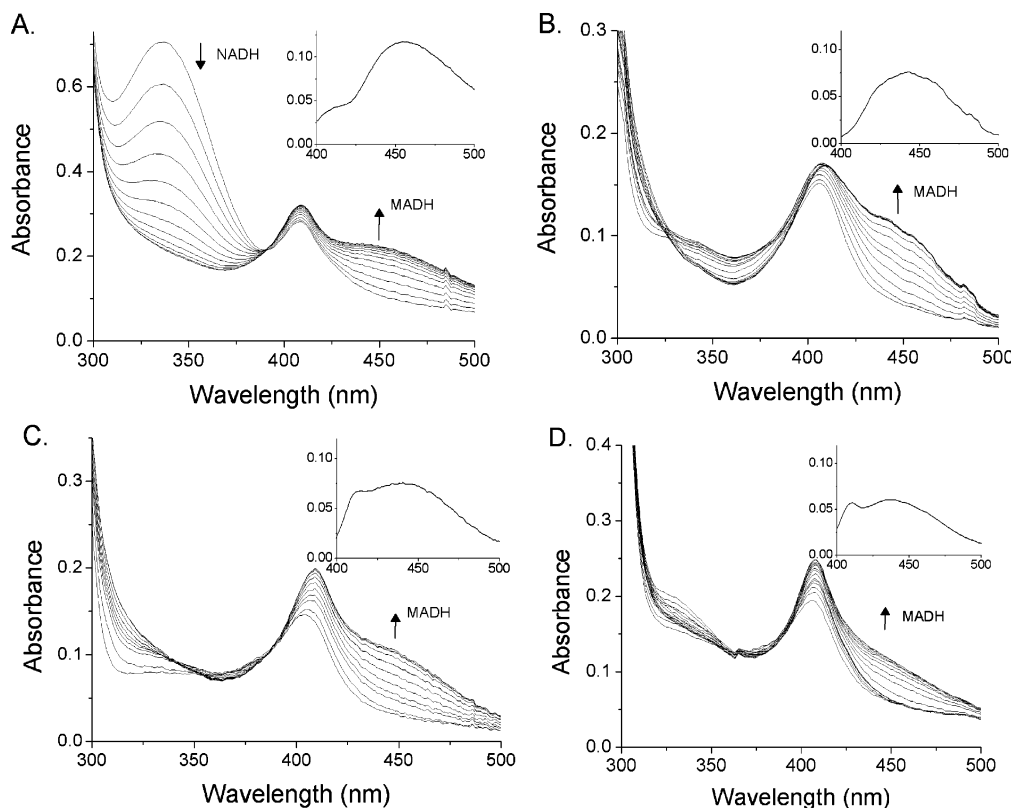


FIGURE 2: Spectrophotometric assay of TTQ biosynthesis. Each reaction mixture contained $0.5 \mu\text{M}$ MauG and $10 \mu\text{M}$ MADH biosynthetic intermediate in 10 mM potassium phosphate at pH 7.5. Reactions were performed under aerobic conditions and initiated by the addition of $100 \mu\text{M}$ NADH (A), $250 \mu\text{M}$ DTT (B), $250 \mu\text{M}$ GSH (C), or $500 \mu\text{M}$ ascorbic acid (D). The arrows indicate the direction of the spectral changes. The spectra were recorded every 2 min for 22 min in A and B, every 10 min for 110 min in C, and every 10 min for 150 min in D. The difference spectrum of the final minus the initial spectrum in the region of interest is shown in each inset. In each spectrum, the absorption peak centered on $406\text{--}416 \text{ nm}$ is due to the MauG present in each sample.

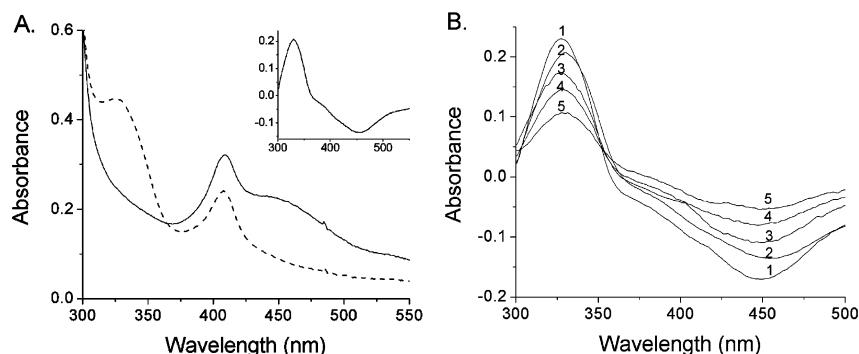


FIGURE 3: Methylamine-dependent reduction of the product of TTQ biosynthesis *in vitro*. (A) The solid line is the spectrum recorded after incubation of MauG and the MADH biosynthetic intermediate with NADH and air as shown in Figure 2A. The dashed line was recorded after the addition of 0.17 mM methylamine. The difference spectrum of the spectra recorded after the addition of methylamine minus that before the addition of methylamine is shown in insert. (B) The difference spectra of the spectra recorded after the addition of 0.17 mM methylamine to wild-type MADH (line 1) and the products of the TTQ biosynthesis reactions in the presence of NADH (line 2), DTT (line 3), GSH (line 4), or ascorbic acid (line 5) that were shown in Figure 2.

the oxidized protein to 416 nm for the fully reduced protein. This is observed as a more distinct shoulder in the difference spectra for the reactions with GSH (Figure 2C) and particularly for the reaction with ascorbate (Figure 2D). This is seen most obviously in the latter reaction because it is so slow. Thus, when the final spectrum was recorded for the ascorbate-mediated reaction, the amount of product, oxidized TTQ, was much smaller relative to the concentration of MauG compared to those from the more efficient reactions with the other electron donors. The large decrease in absorbance in Figure 2A reflects the oxidation of NADH to NAD^+ .

To confirm that the changes in absorbance were indeed due to the synthesis of TTQ and active MADH, methylamine was added to the cuvette after completion of the reaction. This resulted in a bleaching of the absorbance at 450 nm and a corresponding increase in absorbance centered around 330 nm (Figure 3A). These changes are expected for the reduction of TTQ by the amine substrate (18). The methylamine-reduced minus oxidized difference spectra for the reaction products of each of the four electron donors are overlaid with that of the difference spectra for native MADH in Figure 3B. It can be seen that for each of the products of MauG-dependent biosynthesis *in vitro*, the spectral features

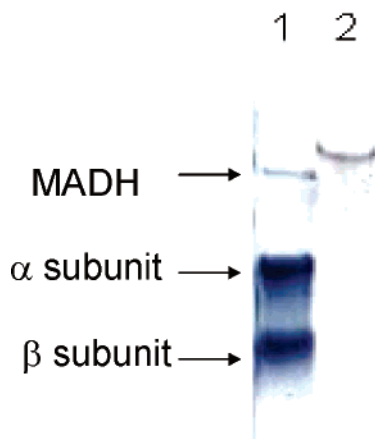


FIGURE 4: Presence of an NADH-dependent oxidoreductase in the preparation of the biosynthetic intermediate of MADH with incompletely synthesized TTQ. Samples were subjected to non-reducing PAGE using 4–20% gradient gel and stained for either protein (lane 1) or NADH-dependent oxidoreductase activity (lane 2). The positions corresponding to α and β subunits of MADH and holoenzyme MADH are indicated.

of the oxidized TTQ (negative peak in the difference spectra) and the reduced TTQ (positive peak in the difference spectra) are very similar to those of TTQ in native MADH. This confirms that the product of each reaction is active MADH with correctly synthesized TTQ.

The observation that NADH could serve as an electron donor for MauG was surprising because NADH oxidation operates via a hydride-transfer mechanism rather than a simple electron-transfer mechanism. NADH-dependent electron-transfer processes are usually mediated by an NADH-dependent oxidoreductase. Because one was not specifically added to this assay, it was suspected that one might be present as a contaminant of one of the protein components of the assay. The recombinant His-tagged biosynthetic intermediate of MADH with monohydroxylated Trp⁵⁷ which is used as a substrate for MauG is isolated from the recombinant *R. sphaeroides* MADH expression system by chromatography over a Ni-NTA column. It is not absolutely pure at this stage, but further purification is difficult because of the instability of the biosynthetic intermediate. Electrophoretic analysis revealed the presence of a contaminant, which stained positive for NADH-dependent oxidoreductase activity (Figure 4). The contaminant did not exhibit a corresponding protein stain on the gel, indicating that it was relatively minor but was clearly catalytically active. It was previously shown that the biosynthetic intermediate of MADH with incompletely synthesized TTQ exhibits weakened subunit–subunit interactions and migrates on non-reducing PAGE primarily as individual subunits but with a small fraction migrating as the holoenzyme (10). The weak protein-staining band above the α subunit is the $\alpha_2\beta_2$ MADH holoenzyme. As a control, extracts of *R. sphaeroides*, which did not harbor the MADH expression plasmid and were incapable of synthesizing either MauG or MADH, were also examined. The same contaminant could be identified from these extracts after chromatography over the Ni-NTA column (data not shown). This indicates that the contaminant is not necessarily associated with the biosynthetic intermediate but simply a protein which binds to the Ni-NTA resin and elutes under the same conditions as those of the biosynthetic intermediate. Although this contaminant is clearly not a

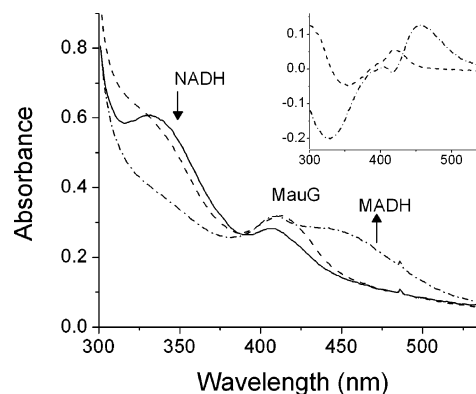


FIGURE 5: Oxygen dependence of MauG-dependent TTQ biosynthesis. The sample contained 0.5 μ M MauG, 10 μ M biosynthetic intermediate of MADH, and 90 μ M NADH intermediate in 10 mM potassium phosphate at pH 7.5. Absorption spectra were recorded immediately after mixing the reactants under anaerobic conditions (—), after 30 min incubation under anaerobic conditions (---), and 30 min after inflating air into the mixture (— · —). The difference spectra after the 30 min incubation minus those before the 30 min incubation under anaerobic conditions (---) and after air inflation minus those before air inflation (— · —) are shown in the insert.

physiological component of the TTQ biosynthesis system, the observation of NADH-dependent TTQ biosynthesis does suggest a possible mechanism for TTQ biosynthesis *in vivo* (discussed later). The MauG preparation used in this study was essentially pure, and no NADH-dependent oxidoreductase activity was exhibited by staining for such activity after electrophoretic analysis.

O₂ Requirement for TTQ Biosynthesis by MauG. In previous studies, it was difficult to absolutely exclude O₂ to establish a true anaerobic baseline for the reaction because the assay required multiple transfers of reagents and product for subsequent analysis (12). In this new continuous spectrophotometric assay, it is possible to completely evacuate each reagent and then mix them and monitor product formation without breaking the system. Under anaerobic conditions, absolutely no MauG-dependent TTQ formation occurs in the presence of electron donors that drive aerobic MauG-dependent TTQ biosynthesis. On mixing the reactants in the absence of O₂, a spectral change is observed in the region of the Soret band of MauG (Figure 5), which is characteristic of the reduction of MauG by the electron donor (8). The NADH-dependent reaction is shown because it exhibits the fastest rate of reaction. No increase in the region of the spectrum corresponding to TTQ absorption is seen at this point. Only after inflating O₂ into the reaction mixture does one observe TTQ biosynthesis (Figure 5).

H₂O₂ Is an Alternative Oxygen Donor for TTQ Biosynthesis by MauG. It has previously been shown that despite approximately 30% sequence identity to di-heme cytochrome *c* peroxidase, MauG does not exhibit any significant peroxidase activity (8). Given the knowledge that H₂O₂ may serve as an alternative source of oxygen for heme-dependent oxygenases (19), the ability of H₂O₂ to serve as a substrate for MauG-dependent TTQ biosynthesis in the absence of electron donors was examined. It can be seen in Figure 6A that MauG-dependent TTQ biosynthesis occurs after the addition of H₂O₂ to the mixture of biosynthetic intermediate and MauG under anaerobic conditions. To confirm that the spectral changes did reflect TTQ biosynthesis, methylamine was added to the mixture, and the spectrum was recorded.

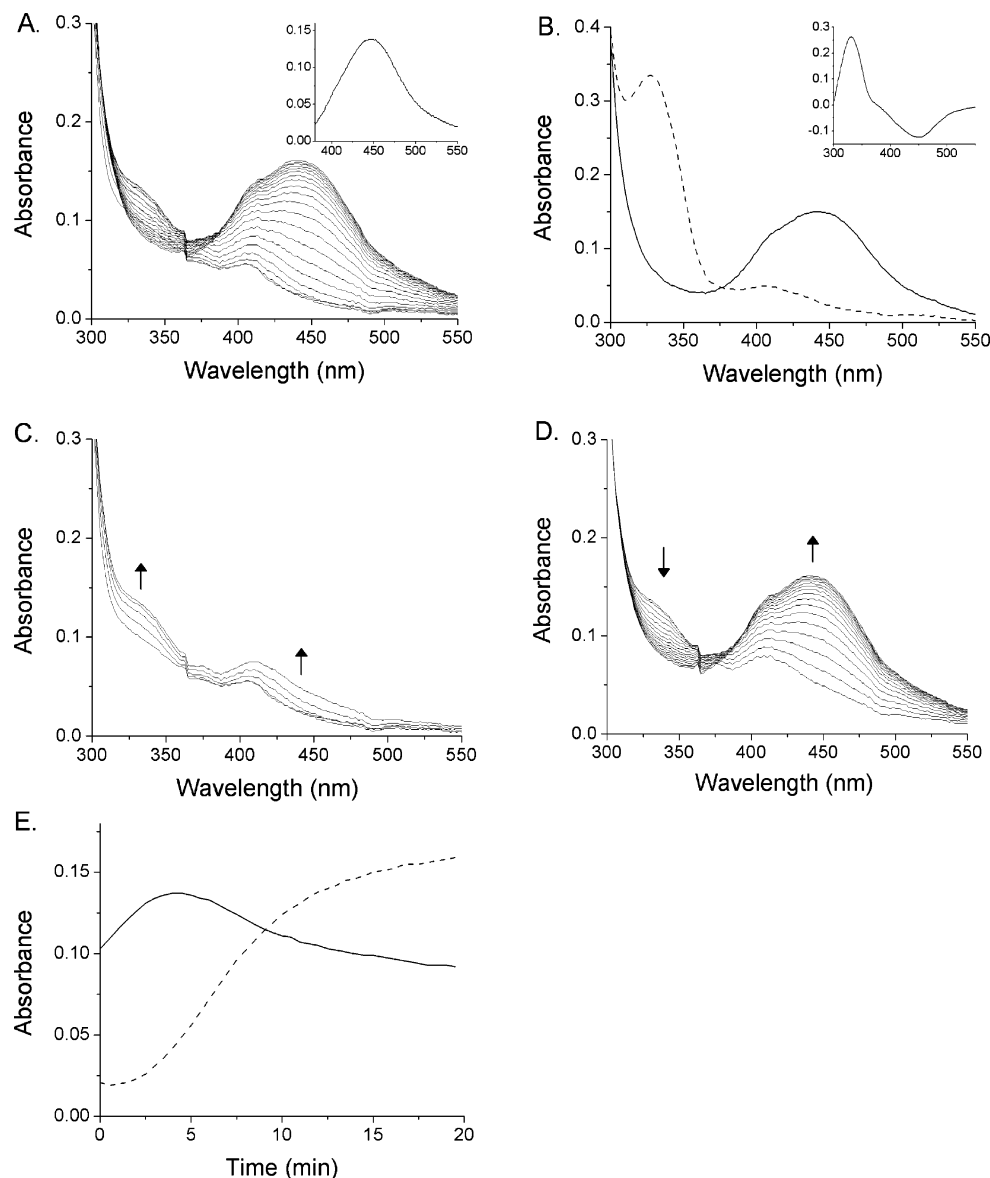


FIGURE 6: Hydrogen peroxide dependence of MauG-dependent TTQ biosynthesis under anaerobic conditions. The sample contained 0.16 μ M MauG and 10 μ M biosynthetic intermediate of MADH intermediate in 10 mM potassium phosphate at pH 7.5. (A) The reaction was initiated by the addition of 0.2 mM H_2O_2 , and absorption spectra were recorded every 1 min. The difference spectrum 20 min after the addition of H_2O_2 minus that before the addition of H_2O_2 is shown in the insert. (B) After completion of the reaction shown in A, 0.17 mM methylamine was added to the mixture. The difference spectrum of the spectra recorded after the addition of methylamine (---) minus that before the addition of methylamine (—) is shown in the insert. (C) The absorption spectra of the first phase of TTQ biosynthesis. (D) The absorption spectra of the second phase of TTQ biosynthesis. In C and D, the arrows indicate the direction of the spectral changes. (E) Time course of absorbance changes at 330 nm (—) and 450 nm (---) after the addition of H_2O_2 .

As expected for mature active MADH, spectral changes characteristic of substrate-dependent TTQ reduction were observed (Figure 6B). The product of the H_2O_2 driven reaction was also confirmed to exhibit steady-state MADH activity, and nondenaturing PAGE indicated that the product MADH exhibited proper subunit–subunit association as seen with native MADH (data not shown). The substrate and product of the reaction were also analyzed by mass spectrometry to confirm that the product of the biosynthetic reaction with H_2O_2 exhibited a mass expected for the formation of mature TTQ (Figure 7) as was previously demonstrated for the O_2 -dependent TTQ biosynthesis reaction (12). The k_{obs} value for MauG-dependent TTQ biosynthesis with H_2O_2 was approximately 10 min^{-1} . Again, it was not possible to obtain an absolute k_{cat} value because the use of higher H_2O_2 concentrations caused damage to MauG. Thus,

the reaction had to be performed at a subsaturating concentration.

The kinetics of the H_2O_2 -dependent MauG-catalyzed reaction was clearly biphasic (Figure 6C–E). During the first phase an increase in absorbance of a peak centered at 330 nm occurred at approximately the same time as the increase in absorbance centered at 450 nm (Figure 6C). In the second phase, the absorbance at 330 nm decreased, whereas the absorbance around 450 nm continued to increase (Figure 6D). The correlation between the decrease in absorbance at 330 nm and the increase at 450 nm during the second phase is clearly seen in Figure 6E. The absorbance at 330 nm exhibits a relatively rapid rise followed by a slow fall back to the initial absorbance. The absorbance at 450 nm exhibits a brief lag followed by an increase on roughly the same time scale as that of the decrease in absorbance at 330 nm. These data

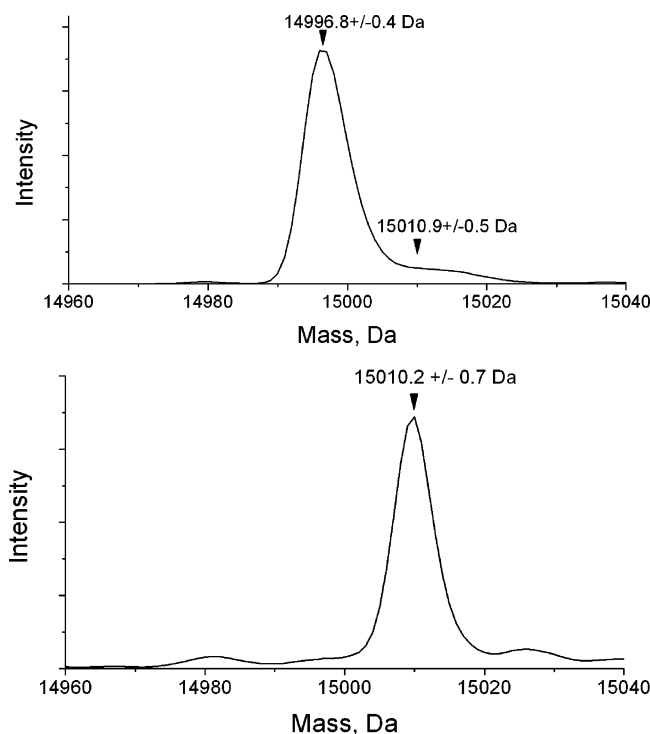


FIGURE 7: Mass spectrometry of the product of H_2O_2 -dependent TTQ biosynthesis by MauG. Deconvoluted mass spectra of the β -subunit of the biosynthetic intermediate of MADH (top) before and (bottom) after incubation with MauG and H_2O_2 for 30 min under anaerobic conditions.

suggest that the peak around 330 nm corresponds to an intermediate in the MauG-dependent formation of mature oxidized TTQ from the biosynthetic intermediate substrate and H_2O_2 .

Biphasic kinetics was not as evident for the O_2 -dependent reactions described earlier. Although a similar increase and decrease at 330 nm was observed for the ascorbate-dependent reaction, no initial increase followed by a decrease in absorbance at 330 nm was observed when using GSH as an electron donor. An initial increase followed by a decrease in absorbance at 330 nm was observed during the O_2 -dependent reaction using DTT as an electron donor, but the initial increase was much smaller than that seen during H_2O_2 -dependent TTQ biosynthesis, indicating barely detectable levels of accumulation of any intermediate. It was not possible to determine whether any intermediate accumulated during the O_2 -dependent reaction with NADH as an electron donor because of the large changes in absorbance in this region of the spectrum associated with the oxidation of NADH.

The identity of the intermediate observed during the H_2O_2 driven reaction may be inferred from a knowledge of the visible absorption spectra of different redox states of MADH, fully oxidized quinone with peak at 440 nm, one-electron reduced semiquinone with the peak at 428 nm, and two-electron reduced quinol with the peak at 330 nm (20). In mature MADH, the extinction coefficient for the absorbance at 330 nm of the quinol ($\epsilon = 56\,400\text{ M}^{-1}\text{ cm}^{-1}$) is approximately twice the extinction coefficient for the absorbance of the quinone at 440 nm ($\epsilon = 26\,200\text{ M}^{-1}\text{ cm}^{-1}$) (14). The maximum increase in absorbance observed at 330 nm is only 26% of that of the change in absorbance at 440 nm, which means that only partial accumulation, about 13%

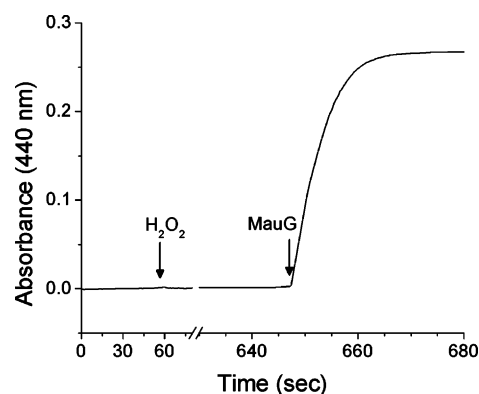


FIGURE 8: Time course of MauG-dependent oxidation of reduced MADH by H_2O_2 . The sample contained $10\text{ }\mu\text{M}$ dithionite-reduced MADH in 10 mM potassium phosphate at pH 7.5. The arrows indicate the addition of 1 mM H_2O_2 at 60 s and $0.6\text{ }\mu\text{M}$ MauG at 648 s.

of the intermediate, occurs during the overall reaction in the steady state. This is because the rate of its formation is only slightly greater than the rate of its decay.

The logical inference is that this transient intermediate is a reduced quinol formed from the insertion of oxygen into the monohydroxylated biosynthetic intermediate, which is the substrate for MauG in this reaction. It could be either the quinol form of the fully synthesized mature TTQ, which is then oxidized, or the quinol of the dihydroxylated Trp⁵⁷ residue prior to cross-linking with Trp¹⁰⁸ to complete TTQ biosynthesis. If it is the former possibility, then the product of the MauG-dependent biosynthesis reaction with H_2O_2 is mature MADH with reduced TTQ, which is then oxidized by the excess H_2O_2 . To test this hypothesis, native MADH was reduced to the quinol state and then mixed with H_2O_2 .

To determine whether H_2O_2 or MauG, or both, played a role in the oxidation of the quinol form of TTQ, fully reduced MADH was generated *in vitro* by reduction with dithionite. After the removal of excess dithionite, H_2O_2 was added to the solution of reduced MADH, and the absorption spectrum was monitored. No spectral changes indicating the oxidation of the reduced MADH were observed. On subsequent addition of MauG to the mixture, spectral changes were observed, consistent with the oxidation of TTQ in the MADH. The increase in A_{440} caused by MauG in the presence of H_2O_2 is shown in Figure 8. Similar results were obtained when reversing the order of addition. On the addition of MauG to reduced MADH, some oxidation of reduced MADH by O_2 was detectable, but it was very slow. Subsequent addition of H_2O_2 then caused rapid oxidation of MADH (data not shown). These results indicate that not only the incorporation of oxygen into the monohydroxylated biosynthetic intermediate is a MauG-dependent process but also the subsequent oxidation of quinol MADH.

The observation that H_2O_2 could serve as an oxygen donor for MauG-dependent TTQ biosynthesis raised the question of whether or not H_2O_2 could also be the actual oxygen donor during NADH-dependent TTQ biosynthesis, which is mediated by an NADH-dependent oxidoreductase (described earlier). NADH-dependent oxidases can form H_2O_2 during turnover. To test this, the NADH-dependent reaction as well as the H_2O_2 -dependent reaction was performed in the presence of catalase. As expected, in the presence of catalase, no MauG-dependent TTQ biosynthesis was observed when

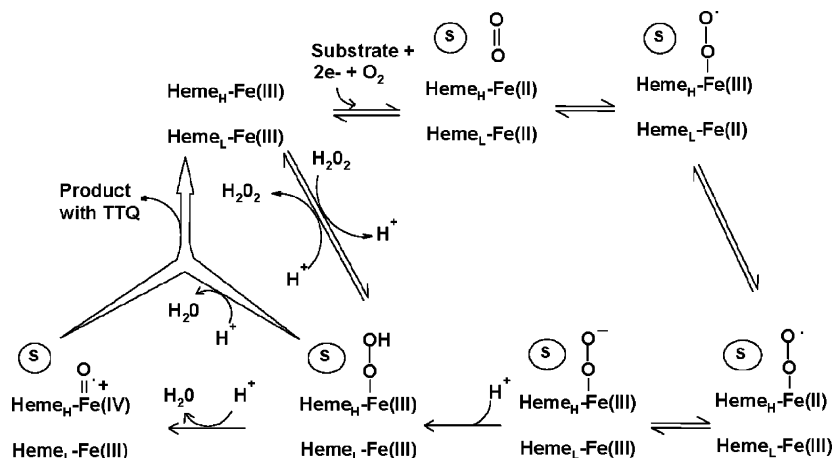


FIGURE 9: Possible intermediates during the reactions of MauG with O_2 or H_2O_2 and substrate. Heme_H and Heme_L designate the high-spin and low-spin hemes, respectively.

H_2O_2 was used as an oxygen donor. In contrast, the presence of catalase had no inhibitory effect on the aerobic NADH-dependent MauG-dependent TTQ biosynthesis (data not shown).

DISCUSSION

One of the unusual features of TTQ biogenesis is that the biosynthetic reactions occur in the periplasmic space. Biosynthetic reactions and most post-translational modifications of bacterial proteins occur in the cytoplasm. Two notable exceptions, which do occur in the periplasm, are covalent attachment of heme to *c*-type cytochromes (21) and disulfide bond formation (22). The latter is a consequence of the relatively oxidizing environment of the periplasm compared to that of the cytoplasm, which results in a dramatically increased tendency of protein thiols to form disulfide bonds. The previous demonstration that the two hemes of MauG exhibit relatively negative E_m values raises the question of what the physiological electron donor in the periplasm could be.

The fact that the rates of turnover with the different electron donors used in this study were dependent on the identity of the electron donor suggest that the electron donation to MauG is the rate-limiting step in the observed reaction *in vitro*. This may be because none of these compounds is the physiological electron donor for MauG. However, if molecular oxygen is the substrate for MauG, then the results of this study do suggest several possible physiologic electron donors for MauG-dependent TTQ biosynthesis *in vivo*. The periplasmic space contains relatively few low molecular weight redox-active compounds. The other redox proteins that are present in the periplasm, which are primarily *c*-type cytochromes and cupredoxins, have E_m values that are significantly more positive than that of MauG. During growth on methylamine, the periplasm of *P. denitrificans* contains significant amounts of three *c*-type cytochromes with E_m values ranging from +148 to +253 mV and cupredoxin amicyanin, which exhibits an E_m value of +294 mV (23). The observation that excess ascorbate is capable of supporting MauG-dependent TTQ biosynthesis *in vitro*, despite its positive E_m value, does suggest the possibility that periplasmic redox proteins may be able to provide electrons for TTQ biosynthesis if their concentrations are sufficiently high relative to that of MauG. This does seem

to be the case because these three cytochromes and amicyanin may be isolated in relatively large amounts from the periplasm of methylamine-grown cells (7, 24). In contrast, MauG has never been detected in these extracts in the native system, suggesting that it is present in only very small amounts.

Glutathione was also an effective electron donor for MauG. While glutathione is usually presumed to function in the cytoplasm, periplasmic glutathione-dependent processes have been described (25). It may be relevant that the biogenesis of MADH also requires the formation of six intrasubunit disulfide bonds in the MADH β -subunit, which harbors TTQ. This process is believed to be facilitated by another inducible periplasmic protein, MauD, which has never been isolated but bears sequence similarity to protein disulfide isomerases (3). The observation that DTT and glutathione can provide electrons for MauG suggests at least the possibility that the multiple disulfide bond formation that is also a part of MADH biogenesis could indirectly be a source of electrons for TTQ biosynthesis.

The fact that this diverse group of electron donors is capable of supporting aerobic MauG-dependent TTQ biosynthesis may also indicate that MauG is relatively nonspecific with respect to electron donor by design. This would allow MauG to utilize a variety of potential electron donors and increase the likelihood of an available source of reducing equivalents being present when MauG and MADH biosyntheses are induced. The observation that NADH can provide electrons via an NADH-dependent oxidoreductase may also be of physiologic relevance. NADH is not present in the periplasm, but it is possible that cytoplasmic reducing equivalents in the form of NADH could be transferred to MauG via a membrane-bound NADH-dependent oxidoreductase or components of the membrane-bound respiratory chain, thus expanding the range of potential sources of reducing equivalents for MauG *in vivo*.

The observation that H_2O_2 is an effective substrate for TTQ biosynthesis has multiple mechanistic implications. It provides strong evidence for the existence of the previously proposed Fe(III) peroxo intermediate in the MauG-dependent oxygenation of the biosynthetic intermediate of MADH to form TTQ (Figure 9). It also provides another example of the mechanistic similarities of MauG and cytochrome P450 enzymes, which are also able to bypass the need for O_2 and

an electron donor via the peroxide shunt on addition of H_2O_2 (19). Furthermore, these results suggest the possibility that H_2O_2 could be a physiologic substrate for MauG. MauG exhibits about 30% sequence similarity to the diheme cytochrome *c* peroxidase of *P. denitrificans*. This and other periplasmic peroxidases normally function to detoxify H_2O_2 to protect the cell (26). The sequence similarity of MauG and the peroxidase suggest the possibility that these proteins evolved from a common ancestor that in one case diverged to form a peroxidase and in the other an H_2O_2 -dependent oxygenase.

MauG is a very unusual oxygenase in several respects. It appears to be the only oxygenase known to use *c*-type hemes for prosthetic groups, and it contains two of them. It functions in the periplasmic space of the host bacterium rather than in the cytoplasm. Its substrate specificity is also somewhat novel in that it inserts oxygen into a single, specific tryptophan residue of a specific protein substrate. The new features of the reactivity of MauG that are described here provide further insight into its reaction mechanism and possible sources of oxygen and reducing equivalents *in vivo*.

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