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Kinetic and Physical Evidence That the Diheme Enzyme MauG Tightly Binds to a Biosynthetic Precursor of Methylamine Dehydrogenase with Incompletely Formed Tryptophan Tryptophylquinone[†]

Xianghui Li,[‡] Rong Fu, Aimin Liu,* and Victor L. Davidson*

Department of Biochemistry, The University of Mississippi Medical Center, Jackson, Mississippi 39216

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ABSTRACT: Methylamine dehydrogenase (MADH) contains the protein-derived cofactor tryptophan tryptophylquinone (TTQ) which is generated by the posttranslational modification of two endogenous tryptophan residues. The modifications are incorporation of two oxygens into one tryptophan side chain and the covalent cross-linking of that side chain to a second tryptophan residue. This process requires at least one accessory gene, *mauG*. Inactivation of *mauG* in vivo results in production of an inactive 119 kDa tetrameric $\alpha_2\beta_2$ protein precursor of MADH with incompletely synthesized TTQ. This precursor can be converted to active MADH with mature TTQ in vitro by reaction with MauG, a 42 kDa diheme enzyme. Steady-state kinetic analysis of the MauG-dependent conversion of the precursor to mature MADH with completely synthesized TTQ yielded values of k_{cat} of 0.20 ± 0.01 s^{-1} and K_m of 6.6 ± 0.6 μM for the biosynthetic precursor protein in an in vitro assay. In the absence of an electron donor to initiate the reaction it was possible to isolate the MauG−biosynthetic precursor (enzyme–substrate) complex in solution using high-resolution size-exclusion chromatography. This stable complex is noncovalent and could be separated into its component proteins by anion-exchange chromatography. In contrast to the enzyme–substrate complex, a mixture of MauG and its reaction product, mature MADH, did not elute as a complex during size-exclusion chromatography. The differential binding of MauG to its protein substrate and protein product of the reaction indicates that significant conformational changes in one or both of the proteins occur during catalysis which significantly affects the protein–protein interactions.

Methylamine dehydrogenase (MADH)[1] (*1*) from *Paracoccus denitrificans* catalyzes the oxidative deamination of methylamine to formaldehyde plus ammonia and then transfers substrate-derived electrons to amicyanin, a type 1 copper protein. MADH possesses a heterotetrameric $\alpha_2\beta_2$ structure. Each smaller β subunit possesses a tryptophan tryptophylquinone (TTQ) (*2*) prosthetic group, which mediates both catalysis and electron transfer. TTQ is a protein-derived cofactor (*3*) synthesized through posttranslational modification of two endogenous tryptophan residues. This modification involves the incorporation of two oxygens into βTrp57 and cross-linking of βTrp57 and βTrp108 (*4*). The methylamine utilization (*mau*) gene cluster that encodes the MADH subunits also contains nine other genes that relate to MADH biosynthesis and function (*5*). One of these genes, *mauG*, has been shown to be absolutely required for TTQ biosynthesis (*5–7*). The gene product, MauG, is a 42.3 kDa protein which contains two covalently bound *c*-type hemes, one low spin and one high spin (*8*), which exhibit cooperative redox behavior (*9*). In contrast to typical *c*-type cytochromes, reduced MauG is oxidized by O_2, and 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*).

A heterologous expression system for MADH was developed which included the structural genes for MADH as well as the other genes required for MADH biosynthesis (*10*). The recombinant MADH exhibits spectral and kinetic properties which are indistinguishable from native MADH. Deletion or mutation of *mauG* in this recombinant expression system results in production of a biosynthetic precursor of MADH with incompletely synthesized TTQ, in which βTrp57 is monohydroxylated at C7 and the cross-link with βTrp108 is absent (*6*). This tetrameric protein precursor is catalytically inactive with respect to MADH activity. It also exhibits weakened subunit−subunit interactions relative to mature MADH, as determined by nondenaturing polyacrylamide gel electrophoresis (*6*). Incubation of this precursor with MauG in vitro in the presence of either O_2 plus an electron donor or H_2O_2 results in formation of active MADH with fully synthesized TTQ with the second oxygen incorporated at C6 and the cross-link formed (Figure 1) and normal strengthened subunit−subunit interactions (*7, 11, 12*).

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* Address correspondence to these authors. V.L.D.: tel, 601-984-1516; fax, 601-984-1501; e-mail, vdavidson@biochem.umsmed.edu. A.L.: tel, 601-984-1872; fax, 601-984-1501; e-mail, aliu@biochem.umsmed.edu.

[‡] Present address: Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI.

[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone.

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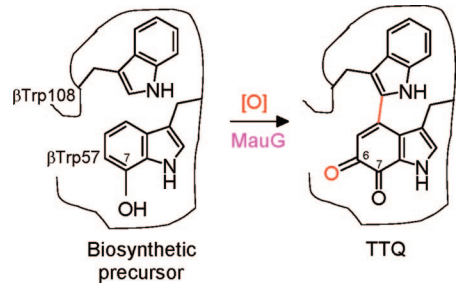

FIGURE 1: MauG-dependent TTQ biosynthesis.

The process of MauG-dependent TTQ biosynthesis is of interest for several reasons. It is the first description of an enzyme-mediated posttranslational modification to generate a protein-derived cofactor (*3*). It is also an atypical enzyme reaction as the substrate is a 119 kDa precursor protein which is much larger than the 42.3 kDa enzyme, MauG. Furthermore, the specific amino acid residue which is modified by MauG is located in the interior of the protein substrate, not on its surface. Thus, the nature of the interactions between enzyme and substrate and the structure of the enzyme–substrate complex are not intuitively obvious. In this study the interaction between MauG and its biosynthetic precursor protein substrate is characterized by steady-state kinetics using the conventional Michaelis–Menten model and analysis by high-resolution liquid chromatography, including size-exclusion and anion-exchange separations. The results provide kinetic parameters for this unusual reaction and demonstrate that MauG is able to strongly discriminate between its substrate, the biosynthetic precursor protein, and its product, mature MADH with oxidized TTQ.

EXPERIMENTAL PROCEDURES

MauG was homologously expressed in *P. denitrificans* and purified as described previously (*8*). The concentration of MauG was determined using its extinction coefficient of 208000 M^{-1} cm^{-1} at 405 nm. Native MADH was purified from *P. denitrificans* as described previously (*13*), and its concentration was determined using its extinction coefficient of 26200 M^{-1} cm^{-1} at 440 nm in the oxidized form (*14*). The biosynthetic precursor of MADH which contains monohydroxylated βTrp57 and no cross-link to βTrp108 (*6*) was heterologously expressed in *Rhodobacter sphaeroides* and purified as described previously (*10*). The yield of this precursor protein, which is very low (approximately 1 mg from a 20 L cell culture), was quantitated using an extinction coefficient of 157000 M^{-1} cm^{-1} at 280 nm. Since the isolated precursor protein was not completely pure, it was routinely run side by side on polyacrylamide gel electrophoresis with known concentrations of pure native MADH to verify concentration. In steady-state kinetic studies the amount of product (mature MADH) after completion of the reaction was determined from its extinction coefficient as described above and compared with the estimated concentration of protein precursor substrate to further verify the substrate concentration in these experiments.

Steady-state kinetic studies of MauG-dependent in vitro TTQ biosynthesis from the biosynthetic precursor protein were performed using a previously described spectrophotometic assay (*11*). Reactions were performed aerobically using ambient dioxygen as the oxygen donor and NADH as an electron donor. Electron donation to MauG was mediated by an NADH-dependent oxidoreductase which is present in the preparation of the biosynthetic precursor (*11*). The reaction was monitored by the rate of appearance of the TTQ chromophore at 450 nm. This wavelength was used rather than the absorption maximum at 440 nm to minimize interference from the strong Soret peak of MauG. Data were fit to eq 1, where [S] is the concentration of the MADH biosynthetic precursor protein, E is the concentration of MauG, v is the initial reaction velocity, k_{cat} is the turnover number (V_{max}/E), and K_m is the Michaelis constant.

$$v/E = k_{cat}[S]/([S] + K_m) \quad (1)$$

High-resolution size-exclusion chromatography of protein mixtures was performed using a HiLoad 16/60 Superdex 200 column on an ÅKTA FPLC system (GE Healthcare Life Science). The column was equilibrated and eluted at 1 mL/min with 50 mM potassium phosphate, pH 7.5, containing 25 mM NaCl. The column was calibrated at the beginning, end, and between runs using the following molecular mass markers (Bio-Rad): thyroglobin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1350 Da). Anion-exchange separation of fractions obtained during size-exclusion chromatography of the mixture of MauG and the MADH biosynthetic precursor was performed using a MonoQ 4.6/100 PE column with a flow rate of 1 mL/min using a linear NaCl gradient in 50 mM potassium phosphate, pH 7.5.

RESULTS AND DISCUSSION

It was previously shown (*11*) that 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. Under anaerobic conditions in the absence of an electron donor, H_2O_2 could serve as a substrate for MauG-dependent TTQ biosynthesis. NADH was used as the electron donor in the current study for the following reasons. The rates of reaction with the other three electron donors were significantly slower than with NADH. For study of the steady-state reaction parameters with respect to the biosynthetic precursor protein substrate it was necessary to choose the fastest electron donor to ensure that reduction of MauG was not the rate-determining step in the reaction. While $H_2O_2$was an efficient alternative substrate, when present in large excess as needed in this experiment it caused damage to MauG. The reaction with NADH is mediated by a nonphysiological NADH-dependent oxidoreductase which purifies under the same conditions used to purify the biosynthetic precursor protein (*11*). The current study exploits this opportunity to initiate the reaction with a safe and efficient source of electrons. As seen below, in this assay the rate and extent of the reaction is clearly dependent on the concentration of the biosynthetic precursor protein–substrate and allows determination of the kinetic parameters of interest.

The biosynthetic precursor protein of MADH with incompletely formed TTQ does not exhibit any visible absorbance. Mature MADH with oxidized TTQ exhibits a broad absorp-

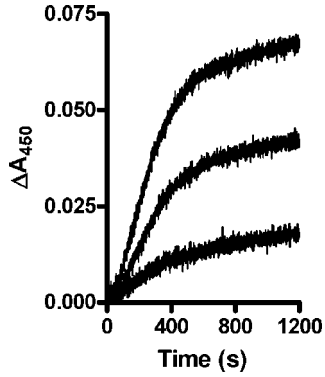

FIGURE 2: MauG-dependent TTQ biosynthesis. Time courses are shown of the absorbance change at 450 nm in the presence of varying concentrations (0.76, 1.95, and 2.63 μM) of the MADH biosynthetic precursor protein. Each reaction mixture also contained 0.23 μM MauG and 300 μM NADH in 10 mM potassium phosphate buffer, pH 7.5.

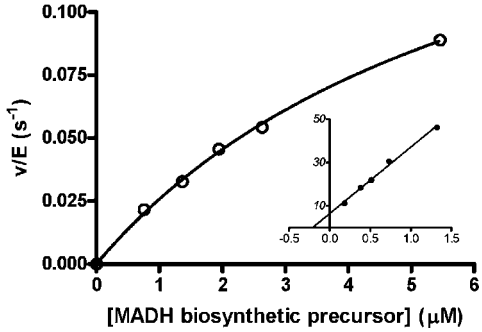

FIGURE 3: Steady-state kinetic analysis of MauG-dependent TTQ biosynthesis. Initial rates were determined from the time courses shown in Figure 2 as well as from additional experiments. The dependence of the initial rates of reaction on the concentration of the MADH biosynthetic precursor is plotted, and the data were fit to eq 1. A double reciprocal plot of these data is shown in the inset.

tion peak centered at 440 nm. In these steady-state kinetic experiments the rate of product formation (i.e., appearance of MADH with completely synthesized TTQ) was monitored by following the increase in absorbance of this peak. The time courses of MauG-dependent TTQ biosynthesis at varying concentrations of the MADH biosynthesis precursor are shown in Figure 2. It should be noted that the substrate precursor protein is purified at very low levels of approximately 1 mg from a 20 L cell culture and is relatively unstable. This prohibited using much higher concentrations of substrate in these steady-state experiments. The initial rate of MauG-dependent TTQ synthesis exhibited a hyperbolic dependence on the concentration of the biosynthetic precursor protein (Figure 3). A fit of these data to eq 1 yielded values of K_m of 6.6 $\pm$ 0.6 μM and k_{cat} of 0.20 $\pm$ 0.01 s^{-1}. MauG-dependent TTQ biosynthesis is an unusual enzymatic reaction in that the substrate, a 119 kDa tetrameric precursor protein, is much larger than the enzyme, a 42 kDa diheme protein. The K_m of 6.6 μM suggests that the enzyme and protein–substrate exhibit a relatively strong affinity toward each other.

It is usually not possible to isolate a stable enzyme–substrate complex since once formed the conversion of substrate to product will occur. In this reaction under aerobic conditions a source of electrons is required for catalysis. Thus, by omitting a source of electrons from the reaction mixture

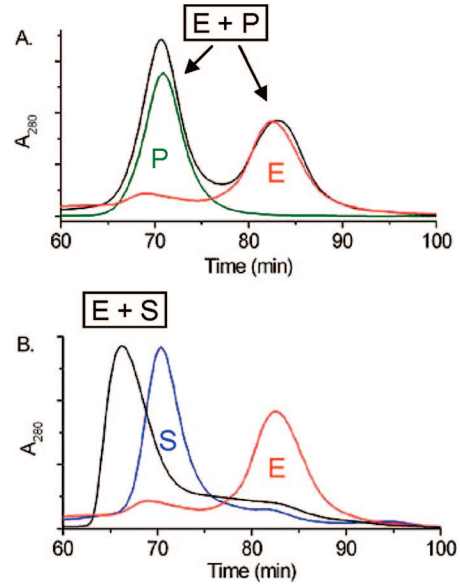

FIGURE 4: Gel filtration analysis of the enzyme MauG (E), the product of its reaction MADH (P), its substrate the MADH biosynthetic precursor (S), and mixtures of E + P and E + S. (A) MauG (E, red), MADH (P, green), and a mixture of 4:1 MauG to MADH (black). (B) MauG (E, red), the MADH biosynthetic precursor (S, blue), and a mixture of 2:1 MauG to the MADH biosynthetic precursor (black). Chromatograms of the mixtures were scaled to size with respect to absorbance to allow easier comparison of peak positions.

it was possible to examine the interactions between the enzyme and substrate without the possibility of conversion of substrate to product. The interactions between MauG, the MADH biosynthetic precursor protein, and mature MADH were studied using high-resolution size-exclusion chromatography. Since no electron donor is present, even under aerobic conditions, the biosynthetic reaction will not occur to a significant extent during the relatively rapid FPLC chromatography. The results clearly indicate that MauG forms a stable protein complex with the MADH biosynthetic precursor in solution but not with mature MADH (Figure 4). MauG alone eluted primarily as a major single peak corresponding to a mass of approximately 43 kDa. The molecular mass calculated from the sequence of MauG including the hemes is 42319.7 Da. MADH alone eluted as a single peak of an apparent mass of 127 kDa. The molecular mass calculated from the sequence of MADH including posttranslational modifications is 119269.7 Da. A mixture of a 4:1 molar ratio of MauG and mature MADH resulted in two well-separated protein fractions corresponding to apparent masses of 43 and 127 kDa, respectively, with no evidence for stable complex formation between the proteins (Figure 4A). The MADH biosynthetic precursor protein alone eluted as a major peak at 131 kDa. The slightly larger apparent mass relative to mature MADH is consistent with previous suggestions that the MADH biosynthetic precursor has a less compact tetrameric complex structure than mature MADH (*6*). A mixture of a 2:1 molar ratio of MauG and the biosynthetic precursor protein eluted primarily as a peak corresponding to an apparent mass of 189 kDa (Figure 4B), indicating significant formation of a stable complex between these two proteins.

To confirm the presence of both MauG and the biosynthetic precursor of MADH in the 189 kDa peak which eluted

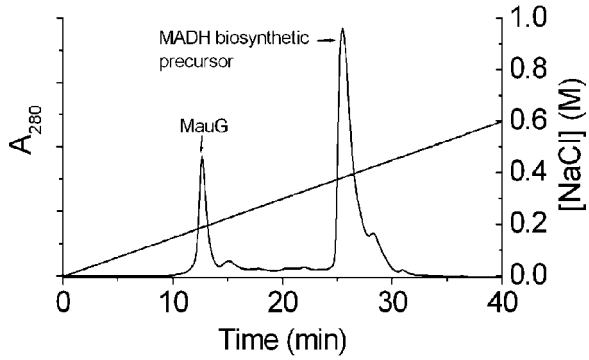

FIGURE 5: Anion-exchange separation of components of the 189 kDa fraction obtained during size-exclusion chromatography of the 2:1 mixture of MauG and the MADH biosynthetic precursor.

when the mixture of the two was applied to the Superdex column, that fraction was collected and immediately subjected to strong anion-exchange chromatography over a MonoQ 4.6/100 PE column (Figure 5). Two fractions were obtained. Visible absorbance spectra and SDS−PAGE showed that the fraction eluting at 210 mM NaCl was MauG and the fraction eluting at 400 mM NaCl was the MADH biosynthetic precursor protein. Thus, the mixture of MauG and the biosynthetic precursor protein forms a stable enzyme–substrate complex which can endure size-exclusion chromatography while the MauG−MADH enzyme–product complex cannot. These data also show that there is no covalent bond between the biosynthetic precursor protein and MauG and that the tight binding is solely due to the protein–protein interactions.

We have previously noted that the reactivity and EPR properties of MauG are similar to those of cytochrome P450s, despite the fact that MauG contains *c*-type hemes and different axial ligation (*8, 11*). The cytochrome P450 catalytic cycle is initiated by substrate binding to the oxidized enzyme with heme in the ferric state (*15*). The current study shows that MauG in the oxidized state is also capable of tightly binding its substrate. The observation that MauG binds tightly to the tetrameric MADH biosynthetic precursor, but not to mature MADH, suggests that there is a significant difference in the conformations of the precursor protein and mature MADH or that the MADH biosynthetic precursor is a more dynamic structure than MADH, or both. It was previously reported (*16*) that a loop comprised of residues β90−β108 on the β subunit of MADH forms about 52% of the interface between α and β subunits of MADH. This is significant as the residue at the end of loop, βTrp108, is cross-linked to form TTQ in the mature enzyme. In mature MADH, this loop is folded over TTQ and is anchored in place by the cross-link between βTrp57 and βTrp108. In the biosynthetic protein precursor substrate which lacks this cross-link, we propose that this loop would be much more flexible and disordered, thereby destabilizing the subunit−subunit interactions and allowing access to the monohydroxylated residue βTrp57 by a processing enzyme, such as MauG. The different structural and dynamic properties of the biosynthetic precursor protein relative to mature MADH allow MauG to bind its substrate precursor protein with its active site, presumably the high-spin heme, accessible to βTrp57 for incorporation of oxygen. Once the MauG-dependent catalysis of TTQ formation is complete, this loop is no longer flexible but is now anchored in place. Subunit−subunit interactions are strengthened, and the binding site for MauG is no longer accessible in the mature MADH.

This work describes the kinetic parameters for MauG-dependent TTQ biosynthesis in vitro. This is the first such description of an enzyme-mediated posttranslational modification to generate a protein-derived cofactor. The relatively small K_m for the biosynthetic precursor protein substrate suggests a strong affinity with MauG despite the fact that the substrate is nearly 3-fold larger than the enzyme, MauG. These kinetic results are supported by the demonstration that MauG and its natural precursor protein–substrate form a complex which is sufficiently stable to survive size-exclusion chromatography. While it is not unique that the substrate of an enzyme is a larger protein molecule, one rarely observes such a stable enzyme–substrate protein complex. In this study we were able to do so by exploiting the fact that the enzyme–substrate complex will not turn over in the absence of a reductant. We have previously shown that MauG and reduced MADH may undergo an interprotein redox reaction (*11*). These data do not exclude the possibility that MauG also interacts with mature MADH in solution, but it clearly does not form such a stable protein–protein complex. This suggests that significant conformational changes in one or both of the proteins occur during catalysis which significantly affects the protein–protein interactions.

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