Understanding Quinone Cofactor Biogenesis in Methylamine Dehydrogenase through Novel Cofactor Generation†

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ABSTRACT: Cofactors made from constitutive amino acids in proteins are now known to be relatively common. A number of these involve the generation of quinone cofactors, such as topaquinone in the copper-containing amine oxidases, and lysine tyrosylquinone in lysyl oxidase. The biogenesis of the quinone cofactor tryptophan tryptophylquinone (TTQ) in methylamine dehydrogenase (MADH) involves the post-translational modification of two constitutive Trp residues ($Trp^{\beta57}$ and $Trp^{\beta108}$ in *Paracoccus denitrificans* MADH). The modifications for generating TTQ are the addition of two oxygens to the indole ring of $Trp^{\beta57}$ and the formation of a covalent cross-link between Cϵ3 of $Trp^{\beta57}$ and Cδ1 of $Trp^{\beta108}$. The order in which these events occur is unknown. To investigate the role $Trp^{\beta108}$ may play in this process, this residue was mutated to both a His (βW108H) and a Cys (βW108C) residue. For each mutant, the majority of the protein that was isolated was inactive and exhibited weaker subunit−subunit interactions than native MADH. Analysis by mass spectrometry suggested that the inactive protein was a biosynthetic intermediate with only one oxygen atom incorporated into $Trp^{\beta57}$ and no cross-link with residue β108. However, in each mutant preparation, a small percentage of the mutant enzyme was active and appears to possess a functional tryptophylquinone cofactor. In the case of βW108C, this cofactor may be identical to cysteine tryptophylquinone, recently described in the bacterial quinohemoprotein amine dehydrogenase. In βW108H, the active cofactor is presumably a histidine tryptophylquinone, which has not been previously described, and represents the synthesis of a novel quinone protein cofactor.

Methylamine dehydrogenase (MADH),[1] from *Paracoccus denitrificans*, is a periplasmic $\alpha_2\beta_2$ heterotetramer that catalyzes the conversion of methylamine to formaldehyde and ammonia (*1*). The genes encoding the α and β subunits of MADH are located in the methylamine utilization (*mau*) cluster (*2*), which is induced in response to growth on methylamine as a sole source of carbon. The gene encoding the natural electron acceptor for MADH, amicyanin, is also present in this gene cluster (*3*). In addition to the amicyanin and α and β subunit structural genes, the *mau* cluster also encodes several other genes (*4*−*6*), the products of which have not yet been isolated (Table 1). From the results of gene deletion studies (*4*, *5*), it is known that four of these genes, *mauDEFG*, are required for the correct biogenesis of MADH. From the deduced sequences of these four genes, it is possible to assign putative functions to some of them. The *mauD* gene encodes a C-X-X-C motif and may be involved in the processing of the six disulfide bonds of the β subunit of MADH; the sequence of the *mauD* gene is similar to that of disulfide isomerases. The *mauE* product appears to be a membrane protein, and it is possible that it plays a role in the translocation of MADH into the periplasm. The *mauF* gene product shows no homology to any class of protein. The *mauG* gene encodes two heme *c*-binding motifs (C-X-

Table 1: *mau* Gene Cluster of *P. denitrificans*

gene	function or features inferred from sequence	required for MADH biosynthesis	ref
mauR	LysR-type transcription activator	no	*6*
mauF	unknown	yes	*4*
mauB	MADH α subunit structural gene	yes	*2*
mauE	putative membrane protein	yes	*5*
mauD	C-X-X-C motif and similarity to disulfide isomerases	yes	*5*
mauA	MADH β subunit structural gene	yes	*2*
mauC	amicyanin structural gene	no	*3*
mauJ	unknown	no	*4*
mauG	two C-X-X-C-H motifs and similarity to diheme cytochrome *c* peroxidases	yes	*4*
mauM	similarity to ferredoxins	no	*4*
mauN	similarity to ferredoxins	no	*4*

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[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; CTQ, cysteine tryptophylquinone; HTQ, histidine tryptophylquinone; QHAmDH, quinohemoprotein amine dehydrogenase.

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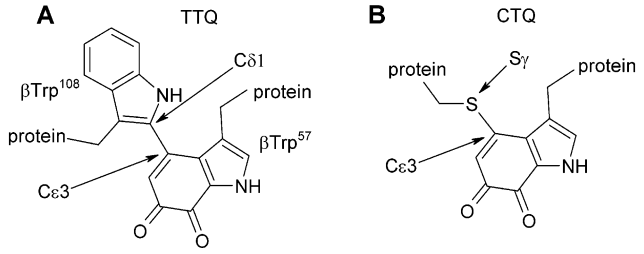

FIGURE 1: Structures of the quinone cofactors: (A) TTQ from methylamine dehydrogenase (residue numbering is that of the *P. denitrificans* enzyme) and (B) CTQ from quinohemoprotein amine dehydrogenase.

X-C-H) and is similar in sequence to diheme cytochrome *c* peroxidase. Its gene product could be involved in the oxygenation and redox reactions that are required for the biogenesis of the cofactor of MADH, tryptophan tryptophylquinone (TTQ) (*7*).

TTQ is a protein-derived cofactor that is formed via the post-translational modification of two tryptophan residues in the β subunit: Trp$^{\beta57}$ and Trp$^{\beta108}$ in the *P. denitrificans* enzyme (*8*). The existence of protein-derived cofactors has been demonstrated relatively recently, so their abundance and diversity are only just coming to light (*9*, *10*). In some cases, biogenesis is self-processing, as is seen with topaquinone (TPQ) in amine oxidases (*11*−*13*). This may represent an evolutionary pathway that allowed proteins to harness organic chemistries that were beyond the capabilities of the 20 amino acids, without the need for an exogenously synthesized cofactor. Alternatively, other complex cofactors have evolved that require other enzymes for biogenesis, as in the case of TTQ. To generate the TTQ cofactor, two oxygen atoms are incorporated into the indole ring of Trp$^{\beta57}$ and a covalent bond is formed between the indole rings of Trp$^{\beta57}$ and Trp$^{\beta108}$ (Figure 1A).

Other than the requirement for the four gene products described above, nothing is known about the mechanism of TTQ biogenesis. Unlike the Trp$^{\beta57}$ portion of TTQ, Trp$^{\beta108}$ appears to play no obvious role in the catalytic mechanism of oxidative deamination by MADH. It does play a role in the reoxidation of MADH by its protein electron acceptor, amicyanin, by connecting the active site chemistry to the subsequent surface-mediated interprotein electron transfer reaction (*1*). It could also be involved in stabilizing the reduced forms of MADH, the semiquinone radical in particular, through partial delocalization of electrons across the two indole rings (*14*). The results presented in this study indicate that Trp$^{\beta108}$ also plays a critical role in the biogenesis of TTQ.

It has been recently reported (*15*, *16*) that another protein, quinohemoprotein amine dehydrogenase (QHAmDH), possesses a cysteine tryptophylquinone cofactor (CTQ), which differs from TTQ in that cysteine occupies the position of Trp$^{\beta108}$ and is cross-linked to a Trp residue via a thioether bond (Figure 1B). To gain insight into and to understand the minimum requirements for TTQ biosynthesis in MADH, Trp$^{\beta108}$ was altered by site-directed mutagenesis. Trp$^{\beta108}$ was converted to a cysteine to determine whether a QHAmDH-like CTQ could be generated in MADH. Trp$^{\beta108}$ was also converted to a histidine as molecular modeling studies indicated that the histidine side chain could occupy a position similar to that of the pentameric ring of Trp$^{\beta108}$, and potentially form a cross-link to Trp$^{\beta57}$ generating a novel cofactor, histidine tryptophylquinone (HTQ).

βW108H and βW108C MADH mutants were heterologously expressed using a construct that also contained the four required genes, *mauDEFG* (*17*). The resultant mutant proteins possessed a small amount of enzymatic activity that correlated with the presence of a functional tryptophylquinone cofactor that was identified in each of the protein samples. The majority of each mutant protein preparation contained a partially biogenesized cofactor. This biosynthetic intermediate was characterized by mass spectrometry, and a relationship between TTQ biogenesis and intersubunit association in MADH was determined. These results provide new insight into the mechanism of biosynthesis of protein-derived cofactors, particularly complex quinone cofactors, such as TTQ.

EXPERIMENTAL PROCEDURES

Native MADH was purified from *P. denitrificans* as described previously (*18*). Recombinant polyhistidine-tagged wild-type MADH and βW108H and βW108C mutants of MADH were heterologously expressed in *Rhodobacter sphaeroides* (*17*) and purified as described previously for other MADH mutants (*19*).

Molecular Biology. Site-directed mutagenesis was performed on double-stranded pMEG976 (*17*) using the QuickChange site-directed mutagenesis kit (Stratagene) and two mutagenic primers following a previously described procedure (*17*). In this construct, the gene that encodes the MADH β subunit possesses a six-histidine tag at its C-terminus that facilitates purification of the expressed protein. The primers used to create the site-directed mutations were 5′-CAACGACATCATCCATTGCTTCGGCGCCGAGG-3′ for βW108H and 5′-CGAATTCGCCAACGATATCATCTGCTGCTTCGGCGC-3′ for βW108C. For each, the complementary sequence was used as the second primer for the mutagenesis. The underlined bases are those that were changed to create the desired mutation, as well as additional changes that were incorporated to generate or remove a restriction site to facilitate screening for the mutation. The mutations were confirmed by sequencing 70 base pairs around the mutated site.

Kinetic Assays. Steady state kinetic assays of MADH were performed with artificial (*18*) and natural (*20*) electron acceptors. Assays were performed in 10 mM potassium phosphate (pH 7.5) at 30 °C. The artificial electron acceptor assay mixture contained 16 nM MADH, varied concentrations of methylamine, 4.8 mM phenazine ethosulfate, and 170 μM 2,6-dichlorophenolindophenol (DCIP). The reaction was monitored at 600 nm to determine the rate of reduction of DCIP. The natural electron acceptor assay mixture contained 16 nM MADH, 0.1 mM methylamine, and varied concentrations of amicyanin. This reaction was monitored by the change in absorbance caused by the reduction of amicyanin at 595 nm. Data were fit to eq 1

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

where v is the measured initial rate, E is the concentration of MADH, [S] is the concentration of the varied substrate, k_{cat} is the turnover number, and K_m is the Michaelis constant.

Electrophoretic Techniques. Samples containing MADH and site-directed mutants of MADH were subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and nondenaturing PAGE using a 12.5% gel. Gels were stained for protein with Coomassie Blue G250. The nondenaturing gels were also stained for MADH activity by incubation overnight in a solution of 0.5 M potassium phosphate (pH 7.5) which contained an excess of methylamine and nitroblue tetrazolium.

Mass Spectrometry. MADH samples for mass spectral analysis were desalted by dialysis against water using 0.025 μm MF-Millipore membrane filters (Millipore, Bedford, MA). Samples were then diluted with an equal volume of 70% methanol and 3% formic acid in water to a final concentration of approximately 1 μg/μL, and then 3 μL was loaded into a coated nanoelectrospray capillary (Protana Engineering). In some preparations, dialysis was insufficient to remove all salts, and samples were further desalted using 20 μm Poros R2 resin [Applied Biosystems, Inc. (ABI), Foster City, CA; polystyrene divinylbenzene] in a glass purification capillary. Briefly, the sample was loaded onto the R2 resin, washed three times with ~7 μL of 5% acetonitrile and 0.5% formic acid, and eluted with ~1.5 μL of 70% acetonitrile and 0.5% formic acid into a coated nanoelectrospray capillary.

ESI mass spectra were acquired using a QSTAR Pulsar *i* quadrupole-TOF (time-of-flight) mass spectrometer equipped with a nano-ESI source (Protana Engineering). The ESI voltage was 1000 V; the TOF region acceleration voltage was 4 kV, and the injection pulse repetition rate was 6.0 kHz. External calibration was performed using human angiotensin II [monoisotopic mass (MH^+) of 1046.5417; Sigma-Aldrich, St. Louis, MO] and adrenocorticotropin hormone (ACTH) fragment 18−39 [monoisotopic mass (MH^+) of 2465.1989; Sigma-Aldrich]. Mass spectra were the average of approximately 300 scans collected in the positive mode over a 5 min acquisition period.

RESULTS

Extracts of cells that expressed the recombinant MADH mutants were prepared by ultrasonic disruption. Each extract was applied to a Ni^{2+}−NTA superflow column (Qiagen) and then eluted with buffer that contained increasing concentrations of imidazole. The 6-His-tagged MADH eluted at approximately 50 mM imidazole. In contrast to wild-type MADH, the fractions containing the βW108H and βW108C MADH mutants exhibited relatively low activity and no clearly discernible visible absorption spectrum. These mutant enzymes were characterized by electrophoresis, steady state kinetics, and mass spectrometry.

Electrophoretic Analysis. The βW108H and βW108C MADH mutants which were expressed in *R. sphaeroides* were analyzed by SDS−PAGE and nondenaturing PAGE and compared with native MADH from *P. denitrificans* (Figure 2). Analysis by SDS−PAGE revealed that the α and β subunits of βW108H and βW108C MADH were each present and migrated to the expected position (Figure 2A). An additional band at approximately 30 kDa was present in all extracts from *R. sphaeroides*. This is apparently an endogenous protein that binds to the affinity column. Its presence has no effect on activity. While this and the other minor contaminants could be removed by further purification, this also resulted in a significant loss of mutant MADHs due to their relative instability compared to native MADH. Thus, for these electrophoretic analyses, samples were compared that were isolated directly from cell extracts after elution from the affinity column. From the SDS−PAGE analysis, it can be concluded that the α and β subunits of βW108H and βW108C MADH are correctly processed with respect to overall polypeptide size.

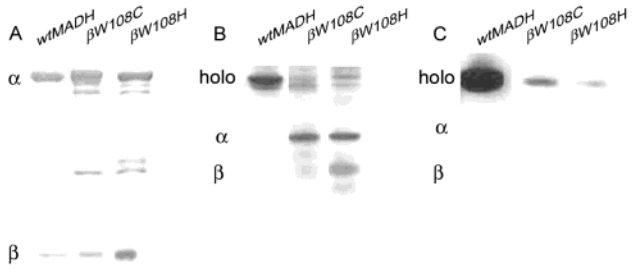

FIGURE 2: Electrophoretic analysis of wild-type and mutant methylamine dehydrogenase. The positions that correspond to those of the purified α and β subunits and the $\alpha_2\beta_2$ holoenzyme are indicated. The wild-type MADH shown on these gels is the native enzyme purified from *P. denitrificans*. (A) SDS−PAGE stained for protein. (B) Native nondenaturing PAGE stained for protein. (C) Native nondenaturing PAGE stained for MADH activity.

In contrast with the results of the SDS−PAGE analysis, the analysis of the proteins by nondenaturing PAGE revealed significant differences between the mutant and native MADHs. The native MADH migrates as a single band, corresponding to the $\alpha_2\beta_2$ heterotetramer. The βW108H MADH mutant migrates primarily as two bands, which correspond to the positions of migration of the isolated α and β subunits of native MADH (Figure 2B). A small amount of the mutant, however, migrates to a position corresponding to that of the native MADH $\alpha_2\beta_2$ heterotetramer. A similar result is seen with βW108C MADH, but the presence of the β subunit is less evident. For the βW108H and βW108C MADH mutants, an activity stain of the gel showed that only the minor band migrating to the position of the $\alpha_2\beta_2$ heterotetramer stained positive for activity (Figure 2C). Thus, the small amount of activity which was observed in the samples corresponds to a subpopulation of each mutant in which the α and β subunits remain associated during nondenaturing PAGE.

As stated above, the relative amount of the β subunit of W108C MADH in the nondenaturing gel is significantly smaller than the amount of W108H MADH. The relative intensity of the β subunit band in the SDS gel in Figure 2A is also smaller in magnitude for W108C than for W108H. This appears to be due to the decreased stability of the W108C β subunit, relative to that of the W108H and native subunits.

Kinetic Analysis. βW108H and βW108C MADH were analyzed by steady state kinetics using both artificial and natural electron acceptors. It was difficult to determine an accurate turnover number for the mutant enzymes since the absence of characteristic spectral features does not allow the concentration to be determined from the extinction coefficient of the cofactor, as is done with the native enzyme. The concentration of mutant MADH was estimated by comparison with samples with known concentrations of MADH after

Table 2: Assignment of Peaks from Mass Spectral Analysis

MADH β subunit	modification	calcd mass (Da)[a]	observed mass (Da)[b]
wild-type	fully biogenesized	15009.5	15009.3 ± 0.1
βW108H	formation of six disulfide bonds, incorporation of one carbonyl oxygen	14947.5	14948.2 ± 0.4
βW108H	fully biogenesized	14960.5	14959.9 ± 0.1
βW108H	formation of six disulfide bonds, no additional oxygen	14932.5	14932.9 ± 0.5
βW108C	formation of six disulfide bonds, incorporation of one carbonyl oxygen	14913.5	14914.3 ± 0.4
βW108C	fully biogenesized	14926.5	14927.0 ± 0.1
βW108C	formation of six disulfide bonds, no additional oxygen	14898.5	14897.7 ± nd

[a] Theoretical masses were calculated using GPMAW 5.02b (Lighthouse Data). Wild-type and mutant subunits each possess a six-His tag at the C-terminus. Fully biogenesized subunits possess six intrasubunit disulfide bonds, two quinone carbonyl oxygens, and a covalent cross-link between tryptophan residues as described in the text. [b] Errors in the observed mass were calculated using the following equation:

$$\sqrt{\frac{\sum (zE_z)^2 I_z}{\sum I_z}} = \frac{1}{2}E_{\text{overall}}$$

where z is the charge species, E_z is the error in the measurement of charge species z, and I_z is the intensity of the charge species. E_z, z, and I_z are output by the deconvolution software for each peak used in protein zero mass determination.

SDS−PAGE and staining for protein. For βW108H MADH, k_{cat} values of 0.2 and 0.5 s^{-1} were obtained in the steady state assays with artificial and natural electron acceptors, respectively. For βW108C, a k_{cat} value of 0.5 s^{-1} was obtained with artificial electron acceptors. These k_{cat} values for the βW108H and βW108C MADH mutants are ~1−3% of those obtained for native MADH and recombinant MADH (16 s^{-1}) (*17*). This result may reflect a small percentage of active enzyme within the total population, a uniform population of enzyme with low activity, or some combination of both. Given the results of the in-gel activity stain (Figure 2C), these results are most likely due to a subpopulation of mutant MADH with some activity. K_m values for methylamine of 19 and 13 μM were obtained for βW108H and βW108C, respectively, values which are similar to that of the native MADH (9 μM). This suggests that each active mutant enzyme binds substrate in a manner similar to that of native MADH. To determine whether the activity of βW108H MADH could be attributed to the reactivity of a carbonyl-containing cofactor, the βW108H MADH was pretreated with phenylhydrazine which forms a covalent adduct with TTQ in native MADH (*21*). This treatment eliminated nearly all of the observed activity, consistent with the presence of at least some functional tryptophylquinone cofactor that is responsible for the observed activity.

Mass Spectrometry. Native and mutant MADH were analyzed by ESI mass spectrometry. The *m*/*z* envelopes of the α and β subunits could be clearly distinguished (Figure 3). The series of multiply charged protein peaks was deconvoluted to provide protein zero-charge mass using the Bayesian Protein Reconstruct tool, which is based on maximum entropy, in the ABI BioAnalyst software package. These results are summarized in Table 2. Theoretical masses were calculated from the amino acid sequence, taking into account formation of the six disulfide bonds, the cross-link between $Trp^{\beta 57}$ and residue β108, and the incorporation of two carbonyl oxygens into $Trp^{\beta 57}$ in the β subunit. Native and recombinant MADH showed a single mass for the β subunit corresponding to a β subunit containing a fully biogenesized TTQ (Figure 4A). Both mutants exhibited a major peak corresponding to a species containing a single extra oxygen, with respect to the mass of a β subunit species containing six disulfides (panels B and C of Figure 4, and

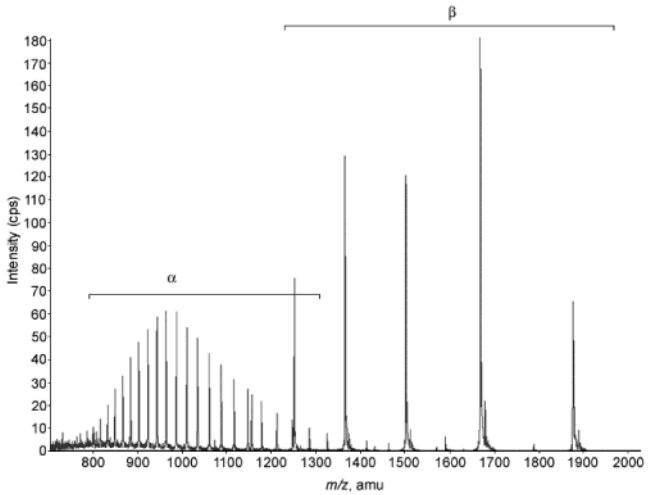

FIGURE 3: Mass spectrum showing the two *m*/*z* envelopes corresponding to the α and β subunits of recombinant wild-type MADH.

Table 2). The masses suggest that this oxygen is in the form of a hydroxyl rather than a carbonyl. In addition to the major peak, both mutant MADHs had two minor peaks. The larger of these was at a mass corresponding to a fully biogenesized HTQ or CTQ. The second minor mass peak corresponded to a β subunit with six disufide bonds but no additional oxygen. In wild-type MADH samples, there was no detectable amount of β subunit containing only the six disulfides. There was also no evidence for nonspecific insertion of oxygen into residues other than $Trp^{\beta 57}$. This suggests that mutation of β108 strongly affects the insertion of the second oxygen into $Trp^{\beta 57}$, and to a much lesser extent also affects the efficiency of the initial monooxygenation.

In an effort to provide additional support for these conclusions, fragmentation analysis of peptides after proteolysis was investigated. Unfortunately, repeated attempts have not yielded definitive results. While it was possible to identify some of the predicted peptides by mass spectrometry, it was not possible to identify the critical peptides of interest. One possible cause may be the unusual nature of the structure of the MADH β-subunit, which is difficult to unfold. It has six disulfide bonds, as well as the covalent TTQ cross-link, when present. To achieve proper results, prior to proteolytic digestion, it is necessary to reduce and modify each of the 12 sulfhydryls and to derivatize the reactive quinone to

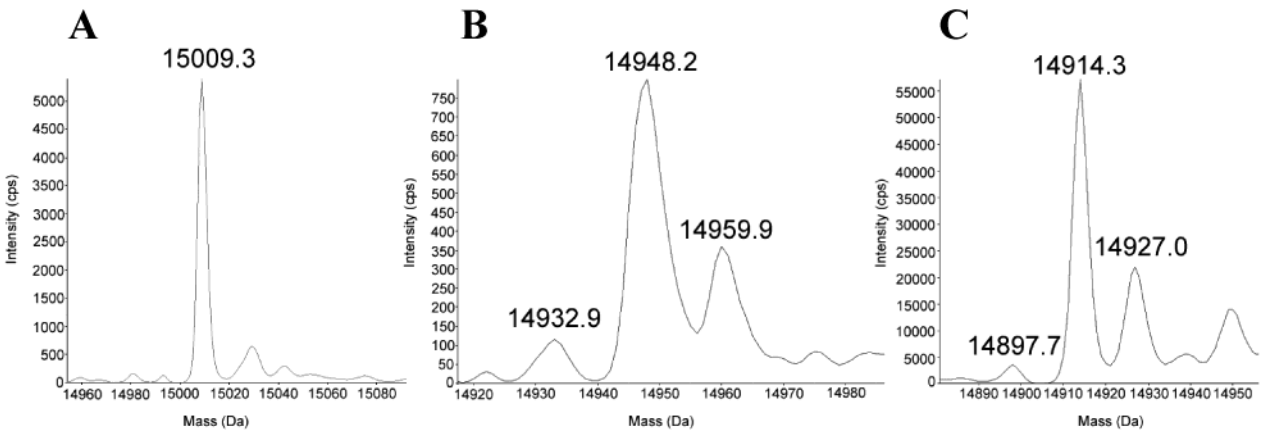

FIGURE 4: Deconvoluted mass spectra of (A) recombinant wild-type MADH, (B) βW108H MADH, and (C) βW108C MADH. Deconvolution was performed with the following parameters. The mass range for searching for deconvoluted peaks was 14000−16000 Da. The *m*/*z* range used in deconvolution was 1200−2000 (which encompasses the β subunit *m*/*z* envelope). Other parameters were left at the software default values (Bayesian Protein Reconstruct tool in the ABI BioAnalyst software package).

prevent reaction with free amino groups. Furthermore, if the putative monooxygenated tryptophan is present, there is the possibility that it may participate in covalent adduct formation with nucleophilic species and complicate the fragmentation pattern. There is also the possibility that the peptides of interest are simply not ionizing (charging) well. Further proteolytic digestion and mass spectrometry studies are being carried out to localize the position of the monooxygenation, and the presence or absence of a cross-link. Determination of the X-ray crystal structures of the β-subunits would be the definitive experiment, and crystallization trials are being actively pursued.

DISCUSSION

To date, other than the requirement for the coexpression of the *mauDEFG* genes for the production of active MADH, nothing was known about the mechanism of TTQ biogenesis. It is clear from the final structure of TTQ that its formation requires a minimum of two discrete reaction steps: insertion of two oxygens into Trp$^{\beta57}$ and covalent cross-linking of the side chains of Trp$^{\beta57}$ and Trp$^{\beta108}$. The oxygenation could conceivably occur in a single dioxygenation or in two monooxygenation steps. In addition, other redox reactions may also be required. However, the order of these events and the role of the processing enzymes were unknown.

The results described in this study provide good evidence for two features of the mechanism of TTQ biosynthesis. (i) Insertion of oxygen occurs in two independent monooxygenation steps. (ii) Insertion of the first oxygen does not require the presence of Trp$^{\beta108}$ and appears to be the initial event in the biosynthesis of TTQ. Furthermore, these results suggest either that insertion of the second oxygen does not occur in the absence of the cross-link or conversely that the formation of the cross-link is dependent on the insertion of the second oxygen. It is also conceivable that these two chemical steps are concomitant.

The data that were obtained by mass spectrometry, while not absolutely definitive, are most consistent with the absence of a cross-link between residue β108 and Trp$^{\beta57}$ for the majority species in expressed βW108H and βW108C MADH. One possible explanation for the inability to form a cross-link between Trp$^{\beta57}$ and either Cys$^{\beta108}$ or His$^{\beta108}$ is that the substituted side chain is in the wrong position or orientation to efficiently form the covalent bond with Trp$^{\beta57}$. Examination of the crystal structure of MADH and modeling of the substituted residues indicate that the mutation of β108 to either cysteine or histidine should place the substituted residue sufficiently close and with the correct attack geometry to Trp$^{\beta57}$ to form a cross-link. However, the loss of the bulky Trp$^{\beta108}$ indole ring will allow the smaller mutant residues to move around, and adopt additional conformations that are not suitable for attack to form the cross-link. This suggests that the cross-linking process might be less efficient when Trp is replaced with a smaller residue. It is also possible that some rearrangement of the peptide backbone has occurred in the mutants, further altering the position of residue β108.

Alternatively, the second oxygen may have to be incorporated into Trp$^{\beta57}$ to activate the ring for cross-linking. In this case, the reduced efficiency of biogenesis would suggest that Trp$^{\beta108}$ has a structural role in orienting the Trp$^{\beta57}$ indole ring for oxygen insertion. The smaller side chains of His and Cys in the mutants would allow more movement and flexibility in the position of the Trp$^{\beta57}$ side chain, allowing it to adopt a nonproductive conformation for oxygen insertion, thus reducing the efficiency of TTQ formation. Since no significant accumulation of a dioxygenated, non-cross-linked species is observed, cross-linking and insertion of the second oxygen are either concomitant or strongly coupled, one proceeding rapidly following the other.

Another unresolved question is whether the cross-linking of Trp$^{\beta108}$ and Trp$^{\beta57}$ is an autocatalytic or enzyme-mediated process. If it is the latter, then another explanation for the much lower efficiency of cross-linking in βW108C and βW108H MADH is that the enzyme that catalyzes the cross-link formation does not efficiently recognize either Cys$^{\beta108}$ or His$^{\beta108}$ as a substrate.

From a mechanistic point of view, a cross-link with Cys$^{\beta108}$ should be possible as another CTQ enzyme, QHAmDH, has been recently characterized (*15*, *16*). Since the cross-link in CTQ in QHAmDH is between the Cys sulfur and Trp and forms a thioether bond, it seems likely that nucleophilic attack of Trp by Cys is a part of the biosynthetic reaction mechanism in that enzyme. Such a mechanism may account for the formation of the cofactor in the active subpopulation of βW108C MADH. By analogy, nucleophilic attack of

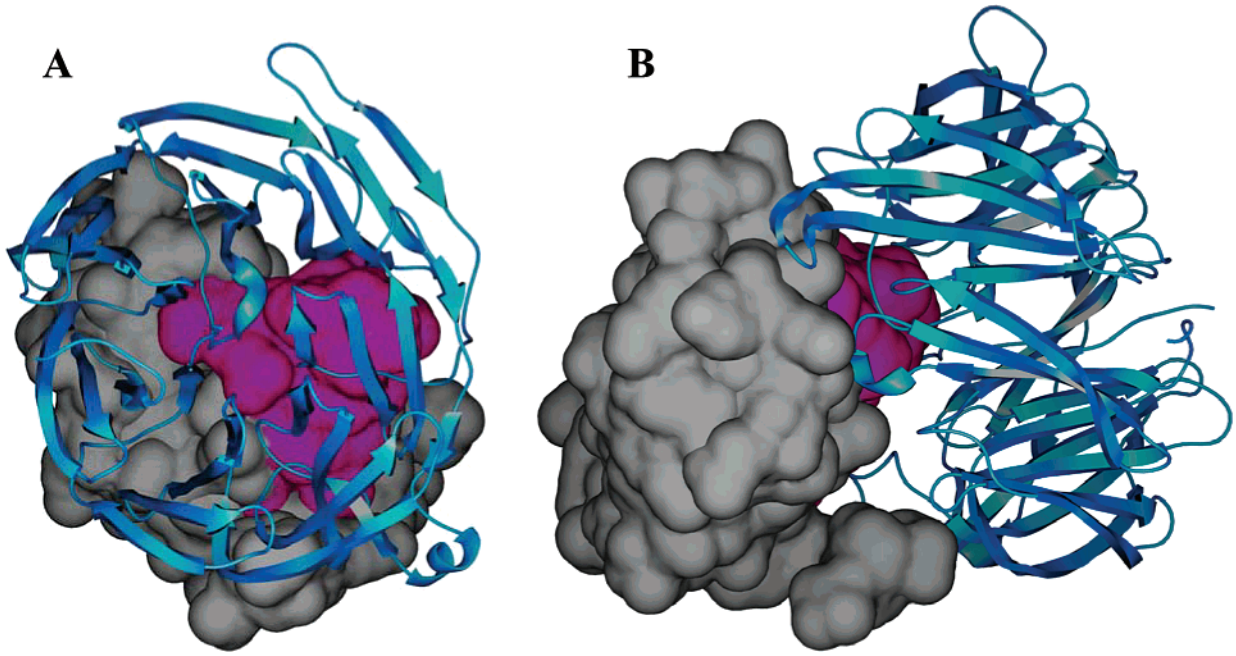

FIGURE 5: Two views (rotated by 90° about a vertical axis through the molecular center of gravity) showing the interaction of the β subunit loop (β90−β108) with the α subunit for one half of the active heterotetramer of MADH ($\alpha_2\beta_2$). The β subunit is represented as a molecular accessible surface, with residues 90−108 colored magenta. The α subunit is represented as a secondary structure ribbon and is colored cyan. The image was generated using SPOCK (*23*).

Trp$^{\beta57}$ by an imidazole nitrogen of His$^{\beta108}$ could account for the formation of the cofactor in the active portion of βW108H MADH. In native MADH, however, the TTQ cross-link is between two carbons. While nucleophilic attack of Trp$^{\beta57}$ by the Cδ1 atom of Trp$^{\beta108}$ is still a possible biosynthetic reaction step, it is not clear what would activate the native system for such a reaction. Indeed, it is also possible in the case of βW108H that the imidazole ring of histidine mimics the pentameric ring of tryptophan, and that the same activation process may occur to form a HTQ cofactor possessing a carbon−carbon cross-link between the His$^{\beta108}$ Cδ atom and Trp$^{\beta57}$.

The mass spectral evidence for a minor species at a mass corresponding to a fully biogenesized HTQ, combined with steady state kinetic results and in-gel activity stains, strongly suggests that we have indeed generated the novel HTQ cofactor. Similar evidence obtained with βW108C MADH suggests that we have also generated a CTQ cofactor. It is important to note that while QHAmDH has been shown to possess a CTQ cofactor, that enzyme is not significantly similar in sequence or structure to MADH other than the presence of a tryptophylquinone cofactor. Thus, the ability to generate a CTQ cofactor in MADH by site-directed mutagenesis of the single residue is a similarly remarkable result. Although the mass demonstrates that the QHAmDH CTQ and βW108C MADH CTQ are identical in chemical composition, it is conceivable that they may be structurally different. However, modeling does suggest that the most likely structure for the βW108C MADH CTQ will be identical to that of the QHAmDH CTQ.

It is significant that for each mutant, some active quinone is formed. This means that the requirement for Trp in position β108 is not absolute and that any structural rearrangement of the active site as a result of the mutation of Trp$^{\beta108}$ is not so large that it completely prevents the formation of a cross-linked tryptophylquinone cofactor. Biosynthesis of CTQ and the novel HTQ does apparently occur in the MADH mutants, albeit less efficiently than TTQ biosynthesis.

There is another structural consequence of the βW108C and βW108H mutations. These mutations affect the strength of the subunit−subunit interactions in MADH. Residues β108 and β57 are situated near the interface between the α and β subunits, and the active site is made up of residues from both subunits. However, neither residue interacts directly with α subunit residues. Significant subpopulations of βW108H and βW108C MADH do migrate as the heterotetramer despite the mutation of Trp$^{\beta108}$. From the activity staining of the PAGE gels, this fraction appears to correspond to the subpopulation that possesses a correctly processed tryptophylquinone cofactor. Thus, the weakening of interactions between the α and β subunits of βW108H and βW108C MADH is not due to the replacement of Trp$^{\beta108}$ with Cys or His, but rather to incomplete formation of a cross-linked tryptophylquinone. The basis for this effect on subunit−subunit interaction may be inferred from examination of the crystal structure of MADH.

The crystal structure of MADH with the fully biogenesized cofactor indicates that the cross-link between residues β108 and β57 serves to anchor the β subunit loop (β90−β108) that is primarily responsible for interactions with the α subunit, comprising 52% of the buried surface area at the α−β subunit interface (surface area calculations were carried out using SPOCK) (Figure 5). Thus, the reduced level of the subunit−subunit interaction may be explained by a disordering of this loop in the absence of a cross-link. In the native structure, this loop is folded over β57, preventing access to it, except via the substrate binding channel (Figure 6). If the incorporation of the second oxygen and formation of the cross-link are indeed catalyzed by a processing enzyme, flexibility of this loop, allowing it to "fold back", would enable the processing enzyme to access Trp$^{\beta57}$.

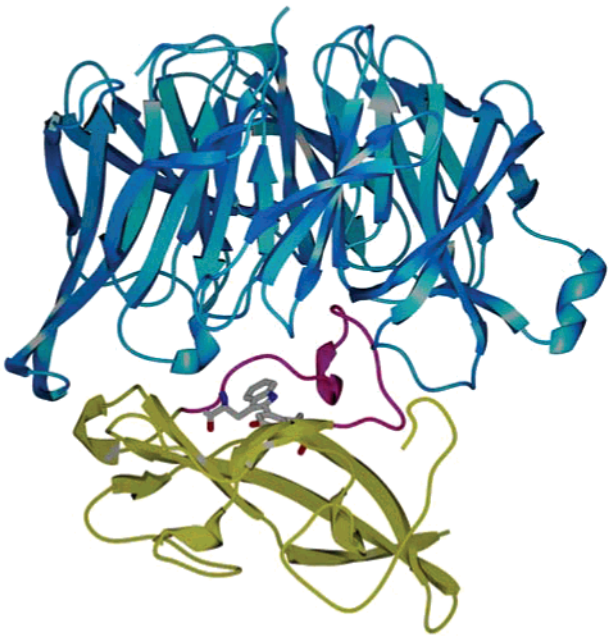

FIGURE 6: View of the $\alpha-\beta$ dimer of the MADH heterotetramer represented as secondary structure ribbons. The α subunit is colored cyan, and the β subunit is colored yellow, except for residues β90−β108 which are colored magenta. TTQ is drawn explicitly. The image was generated using SPOCK (*23*).

The indication that $Trp^{\beta57}$ may be efficiently monooxygenated in the absence of the cross-link is somewhat remarkable. In the absence of the cross-link, there is nothing obviously distinguishing about $Trp^{\beta57}$. It is not known whether this monooxygenation reaction is autocatalytic or enzymatic, but in either case, it must be highly specific. Tryptophan residues of proteins are not routinely oxygenated. An important question which has been raised by these new results, and which is as yet unresolved, is what makes $Trp^{\beta57}$ so special. The masses of the major species in both mutants are most consistent with the addition of oxygen in its reduced hydroxyl form. In this case, further oxidation to carbonyl will be required at a later stage in biogenesis. Recent work with copper-containing amine oxidases suggests that the two oxygens in the TPQ cofactor are initially inserted in their reduced form to yield a quinol, prior to oxidation to the quinone (*22*). This is consistent with our TTQ data as well, and suggests a similar mechanistic order may occur in MADH for generation of the quinone. One of the major questions remaining for the biosynthetic intermediate of the βW108H and βW108C mutants is the carbon position on the indole ring to which the first oxygen has been attached. Efforts to obtain crystals of βW108H and βW108C for X-ray studies are ongoing.

In summary, mutation of $Trp^{\beta108}$ to a cysteine or histidine produces in the main a partially processed tryptophyl cofactor containing a single oxygen. These incompletely biogenesized mutants show a decreased level of $\alpha-\beta$ subunit interaction, which is a consequence of the lack of a fully biogenesized cofactor rather than the loss of βW108. A small percentage of expressed MADH in each mutant preparation appears to contain a fully processed CTQ or HTQ, suggesting that the presence of Trp at position β108 is not absolutely required for cofactor biogenesis but greatly increases the efficiency of the process. CTQ has already been observed as the cofactor in the functionally related but structurally distinct enzyme QHAmDH. HTQ has never been previously detected in a native or engineered protein. Further studies of these mutant MADHs should help us to understand the mechanisms by which the specific oxygenation of aromatic residues and cross-linking of amino acid residues occurs in proteins. Such processes are now known to occur in a range of enzymes with protein-derived amino acid cofactors.

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REFERENCES

1. Davidson, V. L. (2001) *Adv. Protein Chem. 58*, 95−140.
2. Chistoserdov, A. Y., Boyd, J., Mathews, F. S., and Lidstrom, M. E. (1992) *Biochem. Biophys. Res. Commun. 184*, 1181−1189.
3. Van Spanning, R. J., Wansell, C. W., Reijnders, W. N., Oltmann, L. F., and Stouthamer, A. H. (1990) *FEBS Lett. 275*, 217−220.
4. van der Palen, C. J., Slotboom, D. J., Jongejan, L., Reijnders, W. N., Harms, N., Duine, J. A., and van Spanning, R. J. (1995) *Eur. J. Biochem. 230*, 860−871.
5. van der Palen, C. J., Reijnders, W. N., deVries, S., Duine, J. A., and van Spanning, R. J. (1997) *Antonie van Leeuwenhoek 72*, 219−228.
6. Van Spanning, R. J., van der Palen, C. J., Slotboom, D. J., Reijnders, W. N., Stouthamer, A. H., and Duine, J. A. (1994) *Eur. J. Biochem. 226*, 201−210.
7. McIntire, W. S., Wemmer, D. E., Chistoserdov, A., and Lidstrom, M. E. (1991) *Science 252*, 817−824.
8. Chen, L. Y., Doi, N., Durley, R. C. E., Chistoserdov, A. Y., Lidstrom, M. E., Davidson, V. L., and Mathews, F. S. (1998) *J. Mol. Biol. 276*, 131−149.
9. Xie, L., and van der Donk, W. A. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 12863−12865.
10. Okeley, N. M., and van der Donk, W. A. (2000) *Chem. Biol. 7*, R159−R171.
11. Matsuzaki, R., Fukui, T., Sato, H., Ozaki, Y., and Tanizawa, K. (1994) *FEBS Lett. 351*, 360−364.
12. Cai, D., and Klinman, J. P. (1994) *J. Biol. Chem. 269*, 32039−32042.
13. Ruggiero, C. E., Smith, J. A., Tanizawa, K., and Dooley, D. M. (1997) *Biochemistry 36*, 1953−1959.
14. Singh, V., Zhu, Z. Y., Davidson, V. L., and McCracken, J. (2000) *J. Am. Chem. Soc. 122*, 931−938.
15. Datta, S., Mori, Y., Takagi, K., Kawaguchi, K., Chen, Z. W., Okajima, T., Kuroda, S., Ikeda, T., Kano, K., Tanizawa, K., and Mathews, F. S. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 14268−14273.
16. Satoh, A., Kim, J. K., Miyahara, I., Devreese, B., Vandenberghe, I., Hacisalihoglu, A., Okajima, T., Kuroda, S., Adachi, O., Duine, J. A., Van Beeumen, J., Tanizawa, K., and Hirotsu, K. (2002) *J. Biol. Chem. 277*, 2830−2834.
17. Graichen, M. E., Jones, L. H., Sharma, B. V., van Spanning, R. J. M., Hosler, J. P., and Davidson, V. L. (1999) *J. Bacteriol. 181*, 4216−4222.
18. Davidson, V. L. (1990) *Methods Enzymol. 188*, 241−246.
19. Zhu, Z. Y., Jones, L. M. H., Graichen, M. E., and Davidson, V. L. (2000) *Biochemistry 39*, 8830−8836.
20. Brooks, H. B., Jones, L. H., and Davidson, V. L. (1993) *Biochemistry 32*, 2725−2729.
21. Davidson, V. L., and Jones, L. H. (1995) *Biochim. Biophys. Acta 1252*, 146−150.
22. Kim, M., Okajima, T., Kishishita, S., Yoshimura, M., Kawamori, A., Tanizawa, K., and Yamaguchi, H. (2002) *Nat. Struct. Biol. 9*, 591−596.
23. Christopher, J. A. (1997) Ph.D. Thesis, Texas A&M University, College Station, TX.

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