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Minireview

Structure and mechanism of tryptophylquinone enzymes

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Abstract

Tryptophylquinone cofactors are formed by posttranslational modifications that result in the incorporation of two oxygens into a tryptophan side chain, and the covalent cross-linking of that side chain to another amino acid residue. Tryptophylquinone enzymes catalyze the oxidative deamination of primary amines, and utilize other redox proteins as electron acceptors. Mechanistic and structural studies of these enzymes are providing insight into how these enzymes utilize these highly reactive protein-derived quinones in a controlled manner to facilitate biologically important catalytic and electron transfer reactions.
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Keywords: Amine dehydrogenase; Quinoprotein; Posttranslational modification; Protein-derived cofactor; Redox protein; Hydrogen tunneling

1. Introduction

Recent advances in enzymology have extended the scope of the field of cofactor-dependent enzyme catalysis. It has been documented that catalytic and redox-active prosthetic groups may be derived from posttranslational modification of peptide

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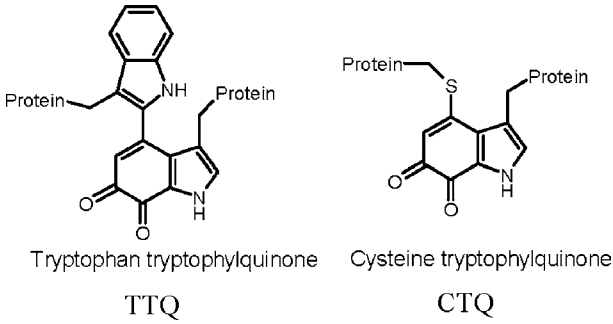

Fig. 1. Structures of tryptophylquinone cofactors of quinoprotein dehydrogenases. Tryptophan tryptophylquinone (TTQ) and cysteine tryptophylquinone (CTQ) are derived from amino acid residues of the polypeptide chain.

amino acid residues [1–3]. Such "new" prosthetic groups include covalently cross-linked amino acid residues, stable amino acid-based free radicals, and quinones derived from tyrosine and tryptophan residues. This review focuses on tryptophylquinone enzymes. Tryptophan tryptophylquinone (TTQ)[1] is the prosthetic group of methylamine dehydrogenase (MADH). This enzyme was first characterized in 1968 by Eady and Large [4], but the exact nature of its prosthetic group remained unknown for several years. The structure of the prosthetic group of MADH was determined in 1991 by McIntire et al. [5] using chemical and NMR spectroscopic methods, to be 2′,4-bitryptophan-6,7-dione. It was given the common name of TTQ. The structure was subsequently confirmed by X-ray crystallographic analyses of MADH [6]. In addition to MADH, TTQ has also been identified as the prosthetic group of aromatic amine dehydrogenase (AADH) [7]. Recently, the enzyme quinohemoprotein amine dehydrogenase (QHNDH) was shown to possess not TTQ, but cysteine tryptophylquinone (CTQ) as a cofactor [8,9]. In each of these enzymes two oxygens have been incorporated into a tryptophan side chain to generate a quinone, and the side chain has also become covalently cross-linked to another amino acid residue (either another tryptophan or a cysteine) to form the protein-derived cofactor (Fig. 1).

2. Physiological roles of tryptophylquinone enzymes

The TTQ and CTQ-dependent enzymes that have been characterized thus far are soluble enzymes localized in the periplasmic space of gram negative bacteria. Each is an inducible enzyme that allows the host bacterium to utilize particular primary amines as a sole source of carbon and energy. Each enzyme catalyzes the oxidative deamination of the primary amine. These dehydrogenases do not utilize NAD^+ or $NADP^+$ as electron acceptors. Instead, during the reductive half-reaction the

[1] *Abbreviations used:* CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase; AADH, aromatic amine dehydrogenase; QHNDH, quinohemoprotein amine dehydrogenase.

Table 1
Tryptophylquinone enzymes and their physiological electron acceptors

Enzyme	Cofactor(s)	Electron acceptor (cofactor)	References
Methylamine dehydrogenase	TTQ	Amicyanin (copper)	[11,59]
Aromatic amine dehydrogenase	TTQ	Azurin (copper)	[7,60]
Quinohemoprotein amine dehydrogenase	CTQ + 2 heme *c*	Cytochrome *c* (heme *c*) or Azurin (copper)[a]	[8,9,61,62]

[a] The electron acceptor depends upon the organism.

quinone cofactor transfers electrons to a specific redox protein [10], either a copper protein or a *c*-type cytochrome, which mediates the transfer of these electrons to the membrane-bound respiratory chain (Table 1).

3. Structures of tryptophylquinone enzymes

The physical properties of the MADHs that have been characterized thus far indicate that they are a relatively well-conserved class of enzymes [11]. Each MADH is a tetramer of two identical larger α subunits of molecular weight of 40,000–50,000, and two identical smaller β subunits of molecular weight of approximately 15,000. The β subunits each possess TTQ and display a high degree of structural similarity, and sequence homology. Crystal structures have been determined for MADH from *Paracoccus denitrificans* [12] (Fig. 2), *Thiobacillus versutus* [13] and *Methylobacterium extorquens* AMI [14]. AADH possesses an $\alpha_2\beta_2$ structure similar to MADH [7], and the β subunit of AADH displays significant sequence identity to the β subunit of MADH [15].

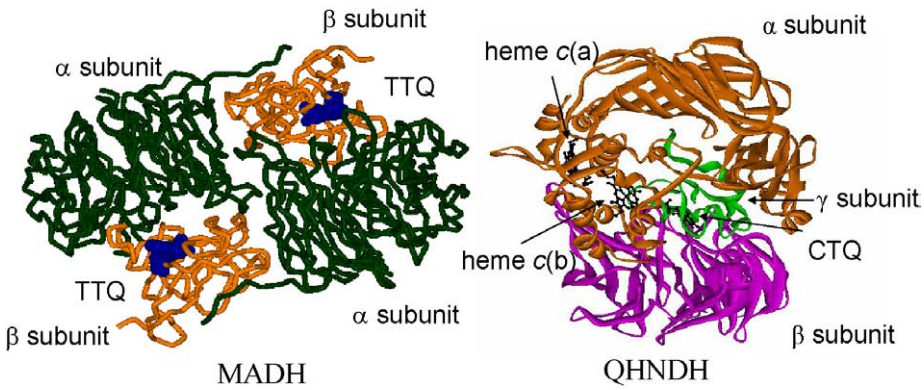

Fig. 2. The structures of methylamine dehydrogenase (MADH) and quinohemoprotein amine dehydrogenase (QHNDH). The MADH structure is that of the enzyme from *P. denitrificans* [12] (Protein Data Bank entry 2BBK). Only the protein backbone is shown. The TTQ prosthetic groups on each β subunit are presented as space fill in blue. The QHNDH structure is that of the enzyme from *P. denitrificans* [9] (Protein Data Bank entry 1JJU). Only the protein backbone is shown. The relative positions of three redox centers are shown in ball-and-stick model and colored black.

In contrast to the TTQ-bearing enzymes, QHNDH is an αβγ heterotrimeric protein. The crystal structures of QHNDH from *P. denitrificans* [9] (Fig. 2) and *Pseudomonas putida* are known [8]. The smallest 82-residue γ subunit contains CTQ. In addition to CTQ, the γ subunit contains three novel thioether cross-links that are formed between cysteine sulfurs and either the β- or γ-methylene carbon of an aspartic or glutamic acid residue. The largest α subunit is a 489-residue, four-domain polypeptide chain, which contains two *c*-type hemes. One heme *c*(a) is solvent-accessible and has His and Met as the axial ligands. The other heme *c*(b) has bis-histidyl axial ligands and is fully buried within the α subunit and located approximately 9 Å from the tryptophylquinone moiety of CTQ. The α and γ subunits sit on the surface of the β subunit that with the γ subunit forms the enzyme active site.

4. Spectroscopic and redox properties of tryptophylquinone enzymes

MADH and AADH exhibit distinct visible absorption spectra for the different redox states of TTQ; fully oxidized quinone, one-electron reduced semiquinone, and two-electron reduced quinol [16] (Fig. 3). In the normal reaction cycle, oxidized TTQ is reduced by two electrons from the amine substrate, and then reoxidized in two one-electron steps by one-electron carriers, amicyanin for MADH and azurin for AADH. The electronic properties of the semiquinone form of TTQ in MADH have been characterized by EPR, electron double nuclear resonance (ENDOR), and electron spin echo envelope modulation (ESEEM) spectroscopies [8,17,18]. The oxidation–reduction midpoint potential (E_m) value for the two-electron oxi-

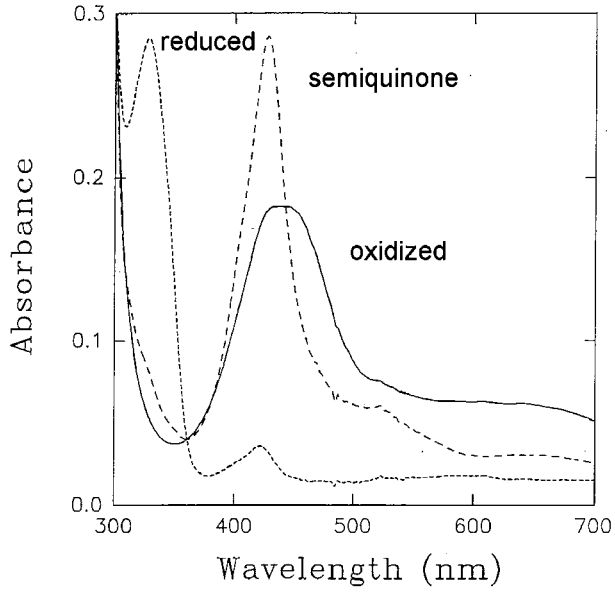

Fig. 3. Visible absorption spectra of different redox forms of methylamine dehydrogenase.

dized/reduced couple of *P. denitrificans* MADH has been determined by spectrochemical titration [19]. At pH 7.5 it is +95 mV (vs. SHE) and over the range of pH from 6.5 to 8.5 it is pH-dependent and exhibits a change of approximately −30 mV per pH unit. This indicates that the two-electron transfer is linked to the transfer of a single proton. In the reduced form, the quinol oxygen at C7 is shielded from solvent and hydrogen-bonded to an amide hydrogen of the polypeptide backbone. Therefore, the quinol is singly protonated at C6 and the semiquinone is unprotonated and anionic. Kinetic studies have determined that the E_m values at pH 7.5 for the one-electron couples are oxidized/semiquinone = +14 mV and semiquinone/reduced = +190 mV [20]. QHNDH is a multisubunit enzyme which also possesses two covalent hemes which obscure the absorption spectrum of CTQ. The spectrum of the isolated CTQ-bearing subunit after denaturation of the enzyme is similar to that of the TTQ enzymes. For QHNDH, the E_m value for the two-electron couple of CTQ in the isolated subunit was determined using mediator-assisted continuous-flow column electrolytic spectroelectrochemistry to be +65 mV [21].

5. Mechanistic studies of tryptophylquinone enzymes

5.1. Reaction mechanism of MADH

On the basis of results of studies of MADH from *P. denitrificans*, a detailed chemical reaction mechanism for the overall oxidation–reduction reaction of MADH with methylamine and amicyanin has been proposed (Fig. 4).

The first step is the formation of a covalent Schiff base, imine, adduct between the amino nitrogen of the methylamine substrate and the C6 carbon of TTQ. It is known that the pK_a value of methylamine is 10.6. Thus, nucleophilic attack of TTQ by the substrate amine requires an active-site residue to deprotonate the methylammonium substrate to generate the reactive uncharged methylamine. The formation of the imine proceeds via a carbinolamine intermediate (II).

The next reaction step is the conversion of the oxidized TTQ-substrate adduct to the reduced TTQ-product adduct (III). This step is initiated by the abstraction of a proton from the methyl carbon of the substrate by an active-site base. At the same time, the TTQ is reduced with a rate constant of approximately 275 s^{-1} [22]. This reaction step exhibits a deuterium kinetic isotope effect (KIE) of 17.2 with CD_3NH_2 as a substrate for MADH [22]. Even correcting for possible contributions to this value from secondary isotope effects from the other two methyl deuteriums, the value of the observed KIE appears to exceed the semiclassical limit for a hydrogen abstraction reaction. This suggests that quantum mechanical proton tunneling may play a role in the proton abstraction step, a possibility supported by subsequent computational studies [23,24]. Similar studies of AADH also yielded an anomalously large deuterium KIEs [25,26]. Thus it appears that quantum mechanical effects may play a common role in the hydrogen abstraction steps of the reactions catalyzed by all TTQ-dependent enzymes and that these quinoproteins are particularly useful systems for the study of enzymatic hydrogen tunneling [27].

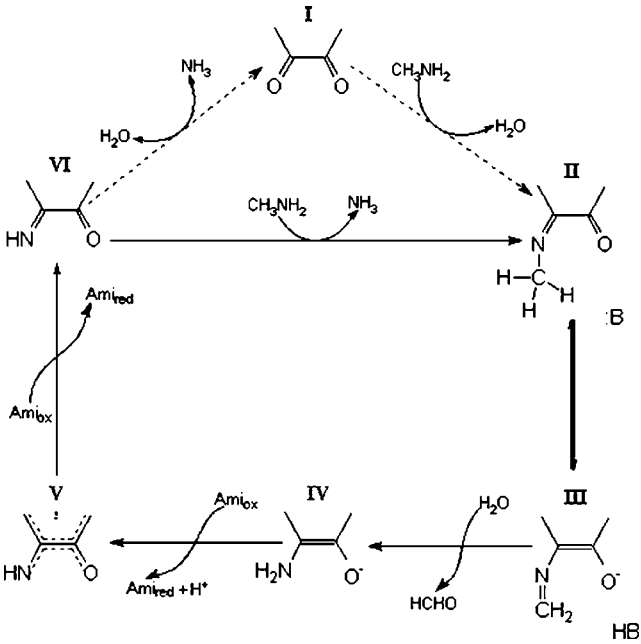

Fig. 4. Proposed chemical reaction mechanism for the conversion of methylamine to formaldehyde plus ammonia by methylamine dehydrogenase. Only the quinone portion of TTQ is shown. B and BH represent active-site residues that may function as general acids or bases in the reaction mechanism. Ami indicates amicyanin. The details of the reaction mechanisms are presented in the text.

On reduction of TTQ, the imine bond between the substrate nitrogen and C6 of TTQ is converted to a single bond and a new imine bond is formed between this nitrogen and the methyl carbon of the substrate. This new imine bond is then hydrolyzed to release the formaldehyde product and yield the reduced *N*-quinol form of TTQ (IV) as a reaction intermediate [28]. The rate constant for the release of the formaldehyde product is 19 s^{-1} [29], and this is the rate-determining reaction step in the overall steady-state reaction with methylamine under physiologic reaction conditions.

After the aldehyde product is released, the *N*-quinol form of the enzyme is stable. The identity of the *N*-quinol was proven by NMR analysis of the reactions of MADH with ^{13}C- and ^{15}N-labeled methylamine [28]. It was demonstrated that the products of the reductive half-reaction are an equivalent of formaldehyde hydrate and the reduced *N*-quinol form of TTQ that possesses covalently bound substrate-derived nitrogen. Similar results were obtained in studies of the reaction of the TTQ-dependent AADH with ^{13}C- and ^{15}N-labeled amine substrates [30].

After reduction by methylamine, *N*-quinol MADH is reoxidized in two one-electron transfers to amicyanin molecules. The substrate-derived amino nitrogen remains bound to TTQ after the first electron transfer step to yield an iminosemiquinone (*N*-semiquinone) form of TTQ (V). This reaction intermediate

has been isolated and characterized in detail by electron paramagnetic resonance techniques [18].

The oxidation of the *N*-semiquinone intermediate yields an oxidized imine form of TTQ with substrate-derived nitrogen still bound to the C6 carbon (VI) [31]. In the absence of another molecule of substrate, this imine intermediate will be hydrolyzed to the quinone (I) in a relatively slow reaction. In the steady-state reaction with excess substrate present, the amino nitrogen of another molecule of substrate, rather than water, reacts directly with this iminoquinone to form the next enzyme–substrate adduct with concomitant release of the ammonia product [31].

5.2. Reaction mechanism of QHNDH

Study of the reaction mechanism of QHNDH is more complicated than that of the TTQ enzymes. QHNDH possesses not only CTQ but also two covalent *c*-type hemes. Thus, the immediate electron acceptors for CTQ are present in the enzyme. This makes it difficult to separate the reductive and oxidative half-reactions of the quinone cofactor. Transient kinetic studies of the CTQ-dependent reduction of heme in QHNDH by amine substrates yielded different rate constants for different substrates (72, 190, and 162 s^{-1} for methylamine, butylamine, and benzylamine, respectively). Deuterium KIE values of 5.3, 3.9, and 8.5 were observed, respectively, for the reactions of methylamine, butylamine, and benzylamine [32]. These results suggest that the abstraction of a proton from the α-methylene group of the substrate, that occurs concomitant with CTQ reduction, is the rate-limiting step in the CTQ-dependent reduction of hemes in QHNDH by these amine substrates. Interestingly, the reaction of 2-phenylethylamine with QHNDH does not exhibit a significant KIE ($^{H}k_3/^{D}k_3 = 1.05$) and exhibits a much smaller rate constant of 16 s^{-1}. It was shown that for 2-phenylethylamine the rate-limiting step in the single-turnover reaction is not proton abstraction but instead hydrolysis of the imine reaction intermediate from CTQ and product release prior to intraprotein electron transfer. Analysis of the products of the reactions of QHNDH with chiral deuterated 2-phenylethylamines demonstrated that the enzyme abstracts the pro-S proton of substrate in a highly stereospecific manner [32]. Analysis of the structure of phenylhydrazine-inhibited QHNDH and molecular modeling provided explanations for these observations [32].

5.3. Role of active-site residues in hydrogen abstraction

It has not yet been proven, for any quinoprotein amine dehydrogenase, which residue is the one that abstracts the proton from the α-carbon of the bound substrate. The active sites in the structures of MADH from *P. denitrificans* and QHNDH from *P. denitrificans* are overlaid in Fig. 5. Each contains two Asp residues in close proximity to the active quinone carbonyl. $Asp33_\gamma$ of QHNDH and $Asp76_\beta$ of MADH each has its side chain oriented towards the C_6 carbonyl. $Asp13_\gamma$ of QHNDH and $Asp32_\beta$ of MADH each has its side chain pointing away from the quinone, but each has its main-chain carbonyl oxygen pointed towards the C_6. These similarities are remarkable given the very different overall structures of MADH and QHNDH.

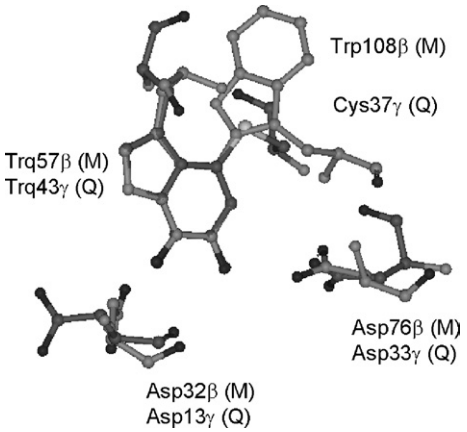

Fig. 5. Overlay of tryptophylquinone cofactors and structurally conserved aspartic acid residues in the structures of MADH and QHNDH.

Unfortunately, it was not possible to determine the role in catalysis of $Asp76_{\beta}$ of MADH because site-directed mutagenesis of that residue resulted in undetectable levels of MADH production [33]. However, inspection of the crystal structure of phenylhydrazine-inhibited QHNDH suggests that $Asp33_{\gamma}$ is the residue that performs the proton abstraction [32]. Another crystal structure of QHNDH from *Ps. putida* bound with *p*-nitrophenylhydrazine [34] also supports the assignment for $Asp33_{\gamma}$ to be the catalytic base.

5.4. Role of active-site residues in determining substrate specificity

MADH has been shown to be a good system with which to investigate the structural determinants of substrate specificity. On inspection of the crystal structure of MADH, a water-filled channel at the interface between the α and β subunits was observed (Fig. 6A). This is most likely the pathway through which the substrate enters the active site and forms a covalent enzyme–substrate adduct with TTQ. One of the amino acid residues in this channel, $Phe55_{\alpha}$, has been shown to regulate the entry of substrate from the channel into the active site. This is because the side-chains of $Phe55_{\alpha}$ and $Tyr119_{\beta}$ form a "gate" where the substrate channel enters the active site. Site-directed mutagenesis studies showed that the phenyl group of $Phe55_{\alpha}$ serves two functions in determining substrate specificity. It interacts with the methyl group of methylamine to help orient the substrate's amino group for nucleophilic attack of TTQ, and it excludes long-chain amines from the active site (Fig. 6B). Site-directed mutagenesis was used to create αF55A MADH which exhibits a dramatically altered substrate specificity in which long-chain amines are preferred to short-chain amines [35] (Table 2).

In addition to directly mutating residue $Phe55_{\alpha}$, site-directed mutagenesis was used to alter the position of this residue. To reposition the phenyl side-chain of

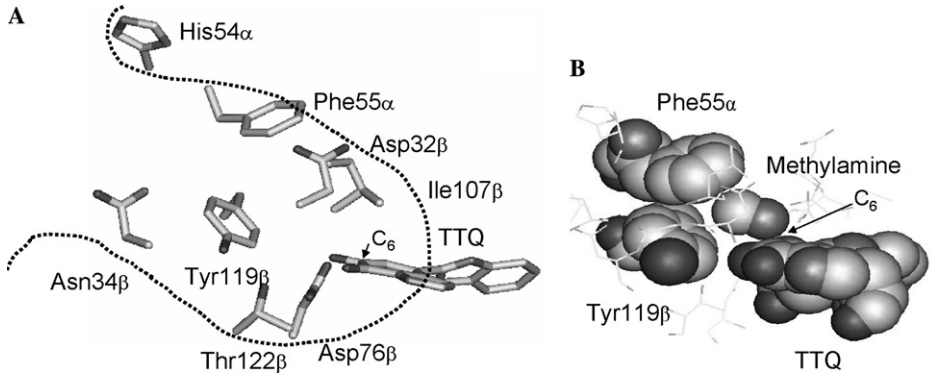

Fig. 6. (A) The substrate channel into the active site of methylamine dehydrogenase. The residues which line the channel that allows substrate access from the protein surface to the enzyme active site are shown. The α and β designations indicate whether the residue is a part of the α or β protein subunit. The C_6 of TTQ, which is the site at which substrate binds to the cofactor, is labeled. (B) Orientation of residues $Phe55_\alpha$ and $Tyr119_\beta$ in methylamine dehydrogenase. The TTQ cofactor and residues $Phe55_\alpha$ and $Tyr119_\beta$ are shown in space fill to indicate their van der Waals' radii. A methylamine molecule has been modeled into the active-site structure in the appropriate position for nucleophilic attack of the C_6 of TTQ.

Table 2
Substrate specificities of native and mutant MADHs

Substrate	k_{cat}/K_m (s^{-1} μM^{-1}) MADH				
	Native	βI107V	βI107N	αF55A	αF55I
Methylamine	**3.3**	0.2	0.1	0.005	0.03
Propylamine	0.8	**0.5**	0.5	0.02	0.03
1-Aminopentane	0.007	0.02	**1.1**	0.4	0.02
1,7-Diaminoheptane	0.07	0.06	0.2	**4.6**	0.06

Data are taken from [35,36].

$Phe55_\alpha$, mutations were made of residue Ile107 of the β subunit [36]. This residue is located adjacent to $Phe55_\alpha$ at the interface of the α and β subunits. From the crystal structure, it appears that the side-chain of $Ile107_\beta$ restricts the movement of the side-chain of $Phe55_\alpha$. Residue 107_β was mutated to valine and asparagine. The βI107V MADH exhibits a strong preference for propylamine, and the βI107N MADH exhibits a preference for 1-aminopentane. Thus, it has been possible to create forms of MADH which exhibit preference for amines with carbon-chain lengths of one, three, five, or seven carbons. Furthermore, conversion of $Phe55_\alpha$ to isoleucine yields an enzyme which exhibits no clear substrate specificity and shows relatively poor activity with amines of any carbon-chain length (Table 2). Changing the substrate specificity of an enzyme by protein engineering typically requires replacement, addition, or deletion of several amino acid residues [37–39]. The studies with MADH represent one of the few examples in which the substrate

specificity of an enzyme has been significantly changed by single site-directed mutations.

6. Electron transfer studies of tryptophylquinone enzymes

The physiological electron acceptors of tryptophylquinone enzymes are not pyridine nucleotides or oxygen, but other soluble redox proteins (Table 1). This makes these enzymes and their electron acceptors excellent systems with which to study mechanisms of long range interprotein electron transfer reactions. The electron acceptors for TTQ in MADH and AADH are type I copper proteins, amicyanin [40] and azurin [41], respectively. The immediate electron acceptor for CTQ in QHNDH is a *c*-type heme which is present on a different protein subunit [9]. The most extensively studied of these systems is the complex formed between MADH, amicyanin and its electron acceptor, cytochrome *c*-551i [42]. The results of those studies have demonstrated the applicability of Marcus Theory to biological electron transfer reactions [20,40,43], provided some insight into how aspects of protein structure may influence electron transfer parameters such as electronic coupling and reorganization energy [44,45], and illustrated how the kinetic mechanisms of such complex systems may determine the rate-determining step for protein electron transfer reactions [46–48]. These studies will not be discussed here but more detailed information may be found in other recent reviews [10,49,50].

7. Mechanism of tryptophylquinone cofactor biogenesis

Relatively little is known about the mechanisms of biogenesis of TTQ and CTQ. In contrast to the well-characterized mechanism of topaquinone biogenesis in amine oxidases [51–53], tryptophylquinone biogenesis is not a self-processing autocatalytic event. The biogenesis of MADH and TTQ requires four accessory gene products [54,55]. One of these genes, *mauG*, encodes a di-*c*-type heme protein [56]. Inactivation of *mauG* in vivo results in the biosynthesis of MADH with an incompletely formed cofactor, in which only a single hydroxyl has been incorporated into residue Trp57 and the cross-link between Trp57 and Trp108 is absent [57]. Similar biosynthetic intermediates were observed when Trp57 was mutated to either histidine or cysteine [58]. These results indicate that for TTQ biosynthesis, incorporation of oxygen into Trp57 occurs in two independent monooxygenation steps, the second of which requires MauG. Furthermore, the cross-linking of Trp57 and Trp108, and the second monooxygenation, appear to be coupled. Remarkably, the gene cluster which encodes QHNDH is completely different from that which encodes MADH and possesses no gene analogous to *mauG* [9]. Present in the QHNDH operon is an open reading frame which encodes a putative radical SAM protein, which is not present in the MADH operon. Several details of the mechanisms of biogenesis of TTQ and CTQ remain to be elucidated. This information will have a significant impact

on our fundamental understanding of the scope and mechanisms of posttranslational modification of proteins.

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