Redox Properties of Tryptophan Tryptophylquinone Enzymes

CORRELATION WITH STRUCTURE AND REACTIVITY*

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The pH dependence of the redox potentials for the oxidized/reduced couples of methylamine dehydrogenase (MADH) and aromatic amine dehydrogenase (AADH) were determined. For each enzyme, a change of ~30 mV/pH unit was observed, indicating that the two-electron transfer is linked to the transfer of a single proton. This result differs from what was obtained from redox studies of a tryptophan tryptophylquinone (TTQ) model compound for which the two-electron couple is linked to the transfer of two protons. This result also distinguishes the redox properties of the enzyme-bound TTQ from those of the membrane-bound quinone components of respiratory and photosynthetic electron transfer chains that transfer two protons per two electrons. This difference is attributed to the accessibility of TTQ to solvent in the enzymes. One of the quinol hydroxyls is shielded from solvent and thus is not protonated. The unusual property of TTQ enzymes of stabilizing the anionic form of the reduced quinol is important for the reaction mechanism of MADH because it allows stabilization of physiologically important reaction intermediates. Examination of the extent to which disproportionation of the MADH and AADH semiquinones occurred as a function of pH revealed that the equilibrium concentration of semiquinone increased with pH. This indicates that the proton transfer is linked to the semiquinone/quinol couple. Therefore, the quinol is singly protonated, and the semiquinone is unprotonated and anionic. It was also shown that the oxidation-reduction midpoint potential for AADH is 20 mV less positive than that of MADH over the range of pH values that was studied and that the TTQ semiquinone of AADH was less stable than that of MADH. This may be explained by differences in the active site environments of the two enzymes, which modulate their respective redox properties.

Redox reactions involving proteins are ubiquitous processes that are fundamental to respiration, photosynthesis, and reactions of intermediary metabolism. Enzyme-bound quinones and flavins are important cofactors of oxidoreductases that function in biological catalysis and electron transfer. These prosthetic groups, as well as membrane-bound components of respiratory electron transfer chains, are particularly important because they are able to couple the two-electron oxidation of substrates to single electron carriers during metabolism and respiration.

Methylamine dehydrogenase (MADH; reviewed in Ref. 1) catalyzes the oxidation of methylamine in the periplasm of many methylotrophic and autotrophic bacteria to form ammonia and formaldehyde concomitant with the two-electron reduction of its redox cofactor (Reaction 1). MADH is

\[
\text{CH}_3\text{NH}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{O}^- + \text{NH}_3 + 2e^- + 2\text{H}^+
\]

**REACTION 1**

an H$_2$L$_2$ heterotetramer with subunit molecular masses of 47 and 15 kDa. The tryptophan tryptophylquinone (TTQ; Fig. 1) (2) prosthetic group, which is located on each small subunit, is derived from two tryptophan side chains that are covalently cross-linked and contain an orthoquinone function through a post-translational modification. In autotrophic bacteria, such as *Paracoccus denitrificans*, the substrate-derived electrons are subsequently passed to a type I copper protein, amicyanin (3), then to one or more c-type cytochromes (4), and finally to a membrane-bound cytochrome oxidase. The crystal structure of a ternary complex of MADH, amicyanin, and cytochrome c-551i from *P. denitrificans* has been determined (5). The second TTQ-dependent enzyme to be characterized thus far is aromatic amine dehydrogenase (AADH) from *Alcaligenes faecalis* (6, 7). It catalyzes the same reaction shown as Reaction 1, except that its preferred substrate is not methylamine but rather phenylethylamines. Like MADH, AADH uses a type I copper protein, in this case azurin, as an electron acceptor (8, 9). These enzymes are members of a newly characterized class of primarily soluble enzymes, referred to as quinoproteins or quinoenzymes (1, 10, 11), which utilize covalently or tightly bound quinones as prosthetic groups.

That TTQ-dependent enzymes donate electrons to another protein that is a one-electron carrier is of interest for two reasons. (i) This requires that the oxidative half-reaction proceeds via a semiquinone intermediate. This means that electron transfer from TTQ to copper occurs from two different redox states of TTQ during the catalytic cycle. (ii) The electron acceptors for most oxidoreductases use small molecules such as NAD$^+$ or O$_2$. When these electron acceptors are present at the active site of the enzyme, it is possible to directly transfer protons, as well as electrons, from substrate or cofactor to acceptor. This cannot occur in these quinoproteins. Their electron acceptors are other proteins that cannot diffuse into the enzyme active site. The electron acceptors for MADH and AADH are reduced by long range electron transfer. Thus, pro-

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‡ The abbreviations used are: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; AADH, aromatic amine dehydrogenase; PMS, phenazine methosulfate; BTP, BisTris propane (1,3-bis[(tris(hydroxymethylamino)propyl]amine); Q-quinol, reduced TTQ with oxygen bonded to C-6; N-quinol, reduced TTQ with an amino group bonded to C-6.

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FIG. 1. The structure of oxidized tryptophan tryptophylquinone (TTQ). The C-6 and C-7 positions are labeled.

Electrons and electrons cannot be transferred together. This raises questions as to the protonation states of the reduced and semiquinone forms of the TTQ cofactor during the catalytic cycle and the fate of the substrate-derived protons. It also suggests functional similarities with membrane-bound quinones in the respiratory chain, such as ubiquinone, which must also separate electron and proton transfer as a part of their physiologic redox reaction (12, 13).

Although much is known about the structure and reactivity of MADH, relatively little is known about its redox properties. The oxidation-reduction midpoint potential (E_m) value for the two-electron oxidized/reduced redox couple of P. denitrificans MADH was determined to be +100 mV at pH 7.5 (14). A similar value was reported for MADH from bacterium W3A1 (15). Recently, the redox properties of a TTQ model compound were reported (16). It exhibited an E_m value similar to that of MADH at pH 7.5. The E_m value of the model compound was pH-dependent, and it was shown that two protons were transferred per two electrons during the conversion from fully oxidized to fully reduced.

In this paper, we report for the first time the pH dependence of the redox potential for a quinoprotein. The E_m values for the oxidized/reduced couples of MADH and AADH were each determined over a range of pH values, and the E_m value of each enzyme exhibited a pH dependence different than that of the TTQ model compound and different from that which is typically observed for biologically active free and membrane-associated quinones. The relative dependence on pH of the one-electron oxidized/semiquinone and semiquinone/reduced couples of MADH and AADH was determined by examining the extent to which disproportionation of each semiquinone occurred as a function of pH. This allowed determination of the protonation states of the TTQ quinol and semiquinone in MADH and AADH. Differences in the redox properties of the protein-bound TTQ and the TTQ model compound and other biogenic quinones are explained by correlation with the crystal structure of MADH (17, 18). The relevance of these findings to the reaction mechanisms of MADH and AADH is discussed. Comparison of the redox properties of MADH and AADH has also allowed us to infer that differences in the active site structures of the two enzymes further modulate their respective redox properties.
The equilibration time for AADH during the oxidative titration performed exactly as described above for MADH. The results of which reductant is used.

These data sets were fit to Equation 1, the reductive and oxidative titrations (Fig. 3). The oxidative titration was performed as described under “Experimental Procedures.” The arrows indicate the directions of the spectral changes.

Redox Titrations of AADH—Redox titrations of AADH were performed exactly as described above for MADH. The results of these studies were very similar with two notable differences. The equilibration time for AADH during the oxidative titration was approximately half of that for MADH. Also, the $E_m$ value of AADH at any given pH was less positive than that of MADH (see below).

**pH Dependence of the $E_m$ Values of MADH and AADH**—The $E_m$ values of MADH were determined over a range of pH. The plot of $E_m$ versus pH (Fig. 4) is linear over the range of pH examined and exhibits a slope $-30.1 \pm 1.5$ mV per pH unit. This indicates that the two-electron redox reaction is linked to the transfer of one proton (24, 28). Since the oxidized quinone is not protonated, this means that only one of the hydroxyl groups on the fully reduced quinol TTQ in MADH is protonated.

Since the plot in Fig. 4 is essentially linear, with no inflection points on either end, it is not possible from these data to determine a pK_a value for either the quinone or quinol. As with a ketone, the pK_a for the quinone oxygens is expected to be very low, and it is not surprising that the acidic end of the curve remains linear. The oxidized cofactor is, therefore, unprotonated TTQ. For the reduced form, one may conclude from these data that the pK_a value for the hydroxyl group that is protonated is greater than 8.5. The reduced cofactor is TTQH^-.

Redox Properties of TTQ

**Redox titration of MADH.** Titrations were performed in 10 mM potassium phosphate, pH 6.8, at 25 °C. In this titration, methylamine is used as a reductant, and O_2 is the oxidant. Data points include those obtained during both the reductive titration (○) and the oxidative titrations (●).
Redox Properties of TTQ

\[ TTQH^+ \rightarrow TTQ + e^- \]
\[ TTQ \rightarrow TTQH^+ + e^- \]
\[ 2TTQH^+ \rightarrow TTQ + TTQH^+ + H^+ \]

**REACTIONS 5–7**

If this were true, then for the disproportionation of the semiquinone (Reaction 7) the equilibrium concentration of TTQH would decrease with pH. The disproportionation reaction will proceed to a lesser extent at low pH than at high pH.

Because the semiquinone forms are kinetically stabilized, it is possible to generate a relatively stable semiquinone by titration with dithionite. With time, however, the semiquinone will disproportionate to the reduced and oxidized form until an equilibrium is established (Fig. 5A). As discussed earlier, the semiquinone is not stable during redox titrations, because the presence of mediators significantly enhances the rate of disproportionation. The semiquinones of MADH and AADH were generated, and the composition of the final equilibrium mixture was monitored after incubation in buffers at pH 5, 7, 9, and 10 (Fig. 5B). Essentially identical results were obtained for MADH and AADH. The only difference was that the rate of the disproportionation reaction of AADH was faster than that of MADH. The extinction coefficients for the different redox states of MADH and AADH vary with pH in the presence of monovalent cations (20, 21). For this reason, BTP buffer was used. For each enzyme, the most semiquinone is observed in the equilibrium mixture at pH 10.0, and somewhat less is observed at pH 9.0. At pH 7.0 and 5.0 the semiquinone has completely disproportionated into a mixture of reduced and oxidized enzyme. Given the known spectrum of each redox form, it was possible to deconvolute the spectra in Fig. 5B and then determine the concentrations of each redox form present in the mixture after equilibrium had been established. The pH dependence of the proportion of MADH semiquinone present at equilibrium is seen in Fig. 6A. The equilibrium concentration of semiquinone increases at high pH. The solid line in Fig. 6A is derived from Reactions 2–4 and describes the predicted fraction of semiquinone if proton transfer is linked to interconversion of the quinol and semiquinone. The dashed line is derived from Reactions 5–7 and describes the predicted fraction of semiquinone if proton transfer is linked to interconversion of the semiquinone and quinone. From these data, it is evident that proton transfer is linked to the interconversion of the fully reduced quinol and semiquinone forms (Reactions 2–4). The semiquinone form must, therefore, be the unprotonated anionic semiquinone.

The redox potentials for the two one-electron couples of MADH were previously determined (29) by kinetic techniques at pH 7.5 at 10 °C to be +190 mV (semiquinone/reduced) and +14 mV (oxidized/semiquinone), which corresponds to an \( E_m \) value of +102 mV for the two-electron couple. After correction for temperature differences, this value is within experimental error of the value determined at pH 7.5 in the present study. The results presented above indicate that for MADH the \( E_m \) value of the oxidized/semiquinone couple will not vary with pH, and that of the semiquinone/reduced couple will vary by approximately 60 mV/pH unit (i.e., one proton transfer is linked to one electron transfer). Thus, while it was not possible to directly measure the \( E_m \) value for each one-electron couple, the

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nonphysiologic pH values outside the range used in this study cause denaturation or non-specific structural perturbations of the enzyme.

The \( E_m \) values of AADH were determined at different values of pH (Fig. 4). The plot of \( E_m \) versus pH for AADH is also linear and exhibits a slope that is essentially the same as that for MADH. Thus, for AADH as well, only one of the quinol hydroxy groups is protonated in the fully reduced enzyme. The \( E_m \) values for AADH were less positive than those for MADH by approximately 20 mV at each pH value.

**pH Dependence of the Disproportionation Reactions of the MADH and AADH Semiquinones**—Although no semiquinone was observed during the redox titrations, semiquinone forms of these enzymes can be generated in vitro and are of physiologic importance in vivo. Since the two-electron interconversion of quinone and quinol involves the transfer of only one proton, this raises the question of whether it is the quinone/semiquinone or semiquinone/quinol couple that is linked to the proton transfer. It was not possible to measure the \( E_m \) values for the individual one-electron couples in this study. At physiologic pH, the semiquinone is kinetically, not thermodynamically, stabilized. In the presence of mediators it is unstable, because they facilitate the transfer of electrons between cofactors and enhance the rate of disproportionation. It was possible, however, to determine which of the one-electron couples is linked to proton transfer by examining the pH dependence of the extent of the disproportionation reaction of the semiquinone.

If proton transfer is linked to the interconversion of quinol and semiquinone (Reactions 2–4), then the \( E_m \) value for the quinone/semiquinone couple will be independent of pH, and the \( E_m \) value for the semiquinone/quinol will vary with pH by approximately -60 mV/pH unit.

\[ \text{TTQH}^+ \rightarrow \text{TTQ} + \text{H}^+ + e^- \]
\[ \text{TTQ} \rightarrow \text{TTQH}^+ + e^- \]
\[ 2\text{TTQH}^+ \rightarrow \text{TTQ} + \text{TTQH}^+ + \text{H}^+ \]

**REACTIONS 2–4**

If this were true, then for the disproportionation of the semiquinone (Reaction 4) the equilibrium concentration of TTQ would increase with pH. The disproportionation reaction will proceed to a lesser extent at high pH than at low pH. If proton transfer is linked to the interconversion of semiquinone and quinone (Reactions 5 and 6), then the \( E_m \) value for the quinone/semiquinone couple will vary with pH by approximately -60 mV/pH unit, and the \( E_m \) value for the semiquinone/quinol will be independent of pH.

\[ \text{TTQH}^+ \rightarrow \text{TTQ} + \text{H}^+ + e^- \]
\[ \text{TTQ} \rightarrow \text{TTQH}^+ + e^- \]
\[ 2\text{TTQH}^+ \rightarrow \text{TTQ} + \text{TTQH}^+ + \text{H}^+ \]

**REACTIONS 5–7**

If this were true, then for the disproportionation of the semiquinone (Reaction 7) the equilibrium concentration of TTQH would decrease with pH. The disproportionation reaction will proceed to a lesser extent at low pH than at high pH.

Because the semiquinone forms are kinetically stabilized, it is possible to generate a relatively stable semiquinone by titration with dithionite. With time, however, the semiquinone will disproportionate to the reduced and oxidized form until an equilibrium is established (Fig. 5A). As discussed earlier, the semiquinone is not stable during redox titrations, because the presence of mediators significantly enhances the rate of disproportionation. The semiquinones of MADH and AADH were generated, and the composition of the final equilibrium mixture was monitored after incubation in buffers at pH 5, 7, 9, and 10 (Fig. 5B). Essentially identical results were obtained for MADH and AADH. The only difference was that the rate of the disproportionation reaction of AADH was faster than that of MADH. The extinction coefficients for the different redox states of MADH and AADH vary with pH in the presence of monovalent cations (20, 21). For this reason, BTP buffer was used. For each enzyme, the most semiquinone is observed in the equilibrium mixture at pH 10.0, and somewhat less is observed at pH 9.0. At pH 7.0 and 5.0 the semiquinone has completely disproportionated into a mixture of reduced and oxidized enzyme. Given the known spectrum of each redox form, it was possible to deconvolute the spectra in Fig. 5B and then determine the concentrations of each redox form present in the mixture after equilibrium had been established. The pH dependence of the proportion of MADH semiquinone present at equilibrium is seen in Fig. 6A. The equilibrium concentration of semiquinone increases at high pH. The solid line in Fig. 6A is derived from Reactions 2–4 and describes the predicted fraction of semiquinone if proton transfer is linked to interconversion of the quinol and semiquinone. The dashed line is derived from Reactions 5–7 and describes the predicted fraction of semiquinone if proton transfer is linked to interconversion of the semiquinone and quinone. From these data, it is evident that proton transfer is linked to the interconversion of the fully reduced quinol and semiquinone forms (Reactions 2–4). The semiquinone form must, therefore, be the unprotonated anionic semiquinone.

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**Fig. 4. Dependence on pH of the \( E_m \) values of MADH and AADH.** For titrations of MADH, either methylamine (○) or ascorbate (■) was used as a reductant. For titrations of AADH (▼) tyramine was used as a reductant.
results obtained here do allow one to estimate these redox potentials over the range of pH studied (Fig. 6B).

**DISCUSSION**

These results reveal the protonation states of the fully reduced and semiquinone forms of TTQ in MADH and AADH. The quinol is singly protonated, and the anionic semiquinone is unprotonated. It was also shown that the \( E_m \) value for AADH is 20 mV less positive than that of MADH. An important aim of this study is to determine how the protein influences the redox properties of the TTQ prosthetic group and whether this influences the catalytic properties of the protein-bound quinone in the quinoprotein. Itoh et al. (16) performed a detailed analysis of the redox properties of a model compound of TTQ in which methyl groups are present at the positions where the rings are covalently attached to the protein. Differences in the redox properties of the protein-bound TTQ in MADH and the model compound may be interpreted in the context of the known crystal structure of MADH (17, 18). These results raise important questions. (i) How do the protein environments of MADH and AADH effect the redox potential of TTQ? (ii) Why is the fully reduced quinol in the enzyme only protonated on one hydroxyl? (iii) What is the significance of the anionic quinol to the reaction mechanisms of these enzymes?

The fact that the redox potential of AADH is more negative than that of MADH indicates that there must be some differences in the way the respective proteins interact with TTQ. It was reported (16) that the dihedral angle of the two indole rings of TTQ could influence its redox properties. It is possible that the polypeptide could influence this angle in the enzymes. The more negative redox potential of AADH may also suggest that the cofactor resides in a less polar environment than it does in MADH. A polar environment would favor the more highly charged redox state (i.e. reduced). It is likely that the active site of AADH will be at least somewhat more hydrophobic than that of MADH based on their substrate specificities. MADH prefers methylamine (30), and AADH prefers phenylethylamines (9). A less polar environment in the active site of AADH may also explain why the anionic semiquinone is less stable (i.e. disproportionates more rapidly) in AADH than in MADH. The conclusion that the semiquinone in MADH and AADH is unprotonated is consistent with results obtained with the TTQ model compound (16). This conclusion is also reasonable because semiquinones typically exhibit \( pK_a \) values <6. These results also support the results of electron nuclear double resonance and electron spin echo envelope modulation studies of the dithionite-generated MADH semiquinone (31). From those studies, it was concluded that both oxygens of the radical were unprotonated and that the radical was negatively charged.

The finding of the pH dependence of the disproportionation reaction of MADH and the conclusion that the semiquinone/reduced couple is linked to a proton transfer help to explain previously reported observations of the reactivity of MADH.
with amicyanin in the crystalline state. Single crystal polarized absorption spectroscopy of a binary protein complex of MADH and amicyanin revealed that the extent to which reduced MADH transferred an electron to amicyanin was dependent on pH (32). Since the proteins are in complex in the crystal, after reduction of MADH one electron is transferred to amicyanin to yield an MADH semiquinone-reduced amicyanin complex, and the proteins can react no further. It was observed that the amount of MADH that was present as semiquinone rather than reduced and the amount of amicyanin that was present as reduced rather than oxidized increased with the pH in which the crystals were incubated. These results suggested that the difference in redox potentials for the TTQ semiquinone/reduced and amicyanin reduced/oxidized couples was pH-dependent.

Our results here are consistent with and provide an explanation for those observations.

Redox studies of the TTQ model compound indicate that the two-electron reduction of the quinone to the quinol is linked to the transfer of two protons. The reduced TTQH₂ model compound exhibited a pKₐ value of 10.1. This value is in the range of what one would expect for a phenolic hydroxyl group. This indicates that the protein environment in MADH and AADH is somewhat important for preventing the protonation of one of the quinol hydroxyls of TTQ in the enzyme. An explanation for this may be inferred from inspection of the crystal structure of MADH (Fig. 7). The structure, which is of the oxidized form of MADH, indicates that only the C-6 carbonyl is accessible to solvent in the active site. The C-7 carbonyl is not, and it is hydrogen-bonded to an amide hydrogen on the peptide backbone. These results indicate that the crystal structure presents an accurate view of the solvent accessibility of TTQ in MADH.

Since the crystal structure is of the oxidized form of MADH, these results also suggest that the accessibility of the C-7 of TTQ to solvent does not change when the enzyme is reduced. The crystal structure of AADH has not yet been determined. These results indicate that the solvent accessibility of only C-6 and not C-7 must be true for AADH as well. This feature of orienting TTQ such that only one of the quinone oxygens is accessible in the active site is apparently a common and probably important feature that is conserved in these TTQ enzymes.

The finding that the reduced form of the enzyme-bound TTQ is anionic is unusual and interesting. Studies of model compounds of the quinoprotein cofactors, TTQ (16), pyrroloquinoline quinone (33), and topaquein (34), each reveal that the interconversion of the fully oxidized and fully reduced forms is a two proton per two electron process. The same is true for ubiquinone (12). The ability of TTQ enzymes to stabilize the anionic singly protonated quinol may be relevant to the reaction mechanisms of MADH and AADH.

It was shown for MADH (27) that during the catalytic reaction cycle, a substrate-derived amino group displaces the accessible quinone oxygen and is covalently attached at the C-6 position of the reduced TTQ cofactor. The physiologically relevant reaction intermediate is an aminquinol. Reoxidation of the aminquinol is a two-step process, since the electron acceptor for MADH is a copper protein that is a one-electron carrier. Spectroscopic (35) and kinetic (36) studies further demonstrate that the substrate-derived amino group remains bound to the semiquinone form of TTQ, which is generated by the first one-electron oxidation of the aminquinol. The presence of the amino group significantly influences the electron transfer properties of the aminquinol MADH relative to quinol MADH (37, 38) and the aminooquinol MADH relative to semiquinone MADH (36). The ability of the TTQ enzymes to maintain a negative charge on the C-7 oxygen in reduced MADH is significant because it will stabilize the amino forms of the reduced cofactor against hydrolysis. The C-6 carbon in the anionic aminquinol will be a much less electrophilic site than it would be in a neutral aminquinol. In fact, in studies of the TTQ model compound it was possible to obtain information on amino forms of the cofactor only by incubating it in the presence of large concentrations of ammonia to compensate for the instability of this intermediate in the absence of the protein. Thus, the structural properties of TTQ enzymes that allow stabilization of the anionic fully reduced state of the cofactor appear to be a critical feature that allows TTQ to function as a cofactor in both catalysis and electron transfer.

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Fig. 7. The active site of MADH. The TTQ cofactor is black, and residues that surround the cofactor are indicated. The dashed lines indicate hydrogen bonding interactions between indole nitrogens on TTQ with main chain oxygen atoms of Ala₁₀³ and Ser₃⁰ and between the O-7 of TTQ and the amide nitrogen of Asp₇⁶. The O-6 is present at the end of a solvent-accessible channel and within hydrogen bonding distance of side chain oxygens of Asp₇⁶ and Thr₁₂².
Redox Properties of TTQ
