Electron Transfer from the Aminosemiquinone Reaction Intermediate of Methylamine Dehydrogenase to Amicyanin†

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ABSTRACT: The tryptophan tryptophylquinone (TTQ) cofactor of methylamine dehydrogenase (MADH) is covalently modified by substrate-derived nitrogen during its two-electron reduction by methylamine to form an aminoquinol (*N*-quinol). An *N*-semiquinone, which retains the substrate-derived N, is the intermediate during the two sequential one-electron oxidations of *N*-quinol MADH by its physiologic electron acceptor, amicyanin. Electron transfer (ET) from *N*-quinol MADH to amicyanin is gated by the deprotonation of the substrate-derived amino group on TTQ in the enzyme active site, whereas ET reactions from dithionite-reduced quinol and semiquinone forms of MADH are rate-limited by the ET event. The ET reaction from the *N*-semiquinone intermediate is shown not to be gated, but rate-limited by the ET step. Marcus analysis of the reaction reveals that the ET reaction from the *N*-semiquinone MADH to amicyanin exhibits the same reorganizational energy and electronic coupling as do the ET reactions of the dithionite-reduced *O*-quinol and *O*-semiquinone forms. The rates of the ET reactions of these three different redox forms of MADH exhibit a $\Delta G°$ dependence which is predicted by Marcus theory. The ET reaction of the *N*-semiquinone is relatively insensitive to pH and salt, and does not exhibit a primary kinetic solvent isotope effect over the range of pH and cation concentrations studied. These properties are similar to those of the ET reaction of quinol MADH and different from those of the gated reaction of *N*-quinol MADH, whose rate varies considerably with pH and concentrations of specific monovalent cations. Thus, the covalent incorporation of substrate-derived N into TTQ is not alone sufficient to cause gating of ET. It affects the rate and $\Delta G°$ for the ET reaction from the TTQ semiquinone by altering its redox potential, but it does not alter the reorganizational energy and electronic coupling associated with ET from TTQ to amicyanin.

The methylamine dehydrogenase (MADH)[1]−amicyanin complex from *Paracoccus denitrificans* is unique in several respects which make it a powerful tool for the study of interprotein electron transfer (ET) reactions. The physiologic ET reactions of the native unmodified proteins are slow enough to be monitored by stopped-flow kinetic techniques under most reaction conditions. Because the tryptophan tryptophylquinone (TTQ) cofactor of MADH is a two-electron carrier, and amicyanin is a one-electron carrier, it is possible to study ET reactions between different redox forms of the proteins. Furthermore, two sets of distinct reduced and semiquinone forms of MADH may be generated by reduction with dithionite and substrate, respectively. Thus, several different ET reactions between different redox states of the proteins (Figure 1), with different driving forces, occur within the same ET protein complex over the same ET distance or pathway. We have previously shown that the redox reactions between amicyanin and the dithionite-generated *O*-quinol and *O*-semiquinone forms of MADH are rate-limited by the ET event, and exhibit a predictable dependence of rate on driving force (*1*). In contrast, the redox reaction between amicyanin and the substrate-generated aminoquinol (*N*-quinol) is gated by a proton-transfer event (*2*). In this paper, we investigate the ET reaction from the substrate-derived *N*-semiquinone MADH to amicyanin to determine whether the covalent incorporation of substrate-derived N into TTQ has also caused ET from the semiquinone redox form to become gated.

MADH catalyzes the oxidative deamination of primary amines to their corresponding free aldehydes plus ammonia (reviewed in *3*). It uses the covalently bound TTQ prosthetic group (*4*) to oxidize substrate and then donate the substrate-derived electrons by two sequential one-electron transfers (Figure 1) to a type I blue copper protein, amicyanin (*5*), which mediates the transfer of electrons to a soluble *c*-type cytochrome (*6*). Amicyanin is an obligatory mediator in this physiologic ET process (*7*), and the crystal structure of the ternary complex of MADH, amicyanin, and cytochrome *c*-551i has been determined (*8*).

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[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; ET, electron transfer; *O*-quinol, fully reduced TTQ with oxygen at the C6 carbon; *O*-semiquinone, semiquinone TTQ with oxygen at the C6 carbon; *N*-quinol, fully reduced TTQ with nitrogen bonded to the C6 carbon; *N*-semiquinone, semiquinone TTQ with nitrogen bonded to the C6 carbon; KSIE, kinetic solvent isotope effect; SVD, singular value decomposition; H_{AB}, electronic coupling; λ, reorganizational energy; E_m, oxidation−reduction midpoint potential.

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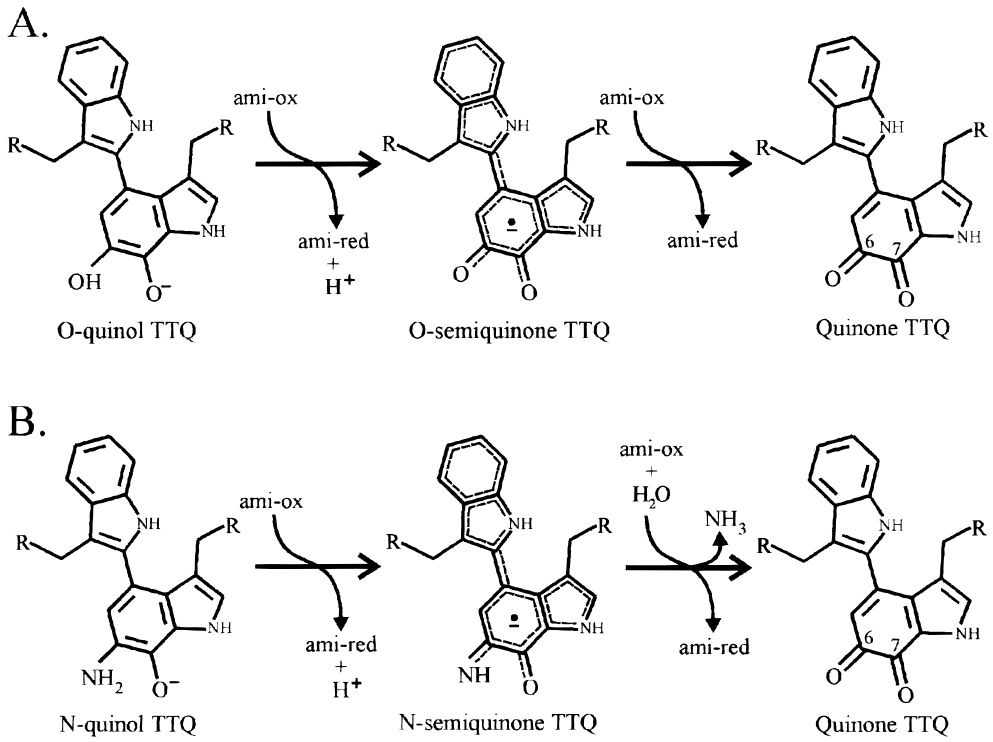

FIGURE 1: One-electron oxidations of dithionite-reduced (A) and substrate-reduced (B) TTQ in MADH. The protonation state of the oxygen on C7 in the O-forms of TTQ has been demonstrated by redox studies (*13*). It has been shown that the distribution of spin density in the *O*-semiquinone extends to the second indole ring but is asymmetric (*32*). A homogeneous distribution should not be inferred from this figure. The C6 and C7 carbonyl carbons are labeled on the quinone TTQ. ami-ox and ami-red signify the oxidized and reduced forms of amicyanin, respectively.

The reductive half-reaction of MADH proceeds via an aminotransferase-type mechanism in which the substrate-derived nitrogen of methylamine is incorporated into the C6 position of the reduced TTQ cofactor (see Figure 1). The existence of a stable *N*-quinol TTQ reaction intermediate was demonstrated by ^{15}N NMR spectroscopy (*9*). The product of the first one-electron transfer from substrate-reduced MADH to amicyanin is a substrate-modified semiquinone TTQ which retains the covalently bound substrate-derived nitrogen (*N*-semiquinone). This was demonstrated by transient kinetic studies (*10*). The rate of the second one-electron transfer from this *N*-semiquinone MADH intermediate to amicyanin is comparable to the rate of reaction of an *N*-semiquinone form of MADH which was generated in vitro (*11*) by comproportionation of substrate-reduced and oxidized MADH. The presence of substrate-derived nitrogen on the in vitro-generated *N*-semiquinone was verified by electron spin echo envelope modulation spectroscopy (*12*). Redox studies (*13*) of MADH indicate that the quinol forms of TTQ are anionic, probably because the oxygen on C7 is shielded from solvent by the protein. The rates of reaction of the *N*-quinol and *N*-semiquinone forms of MADH with amicyanin are much different than the rates of reaction of the corresponding *O*-quinol and *O*-semiquinone forms of the enzyme that are generated by reductive titration with dithionite (*10*).

Analysis by ET theory (*14*) of the temperature dependence (*15*) and driving force dependence (*1*) of the ET reactions from the dithionite-generated *O*-quinol and *O*-semiquinone forms of MADH to amicyanin indicated that the observed redox reactions were rate-limited by ET and exhibited a predictable dependence on $\Delta G°$. In contrast, for the reaction of *N*-quinol MADH with amicyanin, temperature dependence and kinetic solvent isotope effect (KSIE) studies (*2*) demonstrated that the redox reaction was gated (i.e., rate-limited) by an adiabatic proton-transfer step. Furthermore, the rate of the gated ET reaction from *N*-quinol MADH to amicyanin exhibits a strong dependence on pH and monovalent cations, whereas the rate of the ET reaction from the *O*-quinol MADH to amicyanin does not (*16*). The rate of reaction of the *N*-semiquinone MADH with amicyanin is at least an order of magnitude less than that of the reaction of the *O*-semiquinone at pH 7.5 (*10*). This raises the question of whether the modification of TTQ by substrate has also caused the ET reaction from the *N*-semiquinone MADH to amicyanin to become gated.

In this study, we perform a detailed analysis of the ET reaction from the *N*-semiquinone MADH reaction intermediate to amicyanin. The dependence of the reaction rate on temperature, pH, and cation concentration, and KSIEs are investigated. We show that the incorporation of the substrate-derived N into TTQ affects the rate of the ET reaction from the *N*-semiquinone relative to the *O*-semiquinone by altering the driving force for the ET reaction. However, the reaction of the *N*-semiquinone is not gated as is the *N*-quinol reaction. The redox reaction between *N*-semiquinone MADH and amicyanin is rate-limited by the ET event and exhibits Marcus parameters which are identical to those that describe the ET reactions to amicyanin from the *O*-quinol and *O*-semiquinone redox forms of MADH.

EXPERIMENTAL PROCEDURES

Materials. Purifications of MADH (*17*) and amicyanin (*5*) from *P. denitrificans* (ATCC 13543) were as previously described. MADH and amicyanin concentrations were

calculated from known extinction coefficients (*5, 18*) in 10 mM phosphate buffer, pH 7.5. Extinction coefficients of the different redox forms of MADH vary with pH (*11*) and ionic composition of the buffer (*19*). *N*-Quinol MADH was prepared anaerobically by reaction with the substrate, methylamine. Any excess methylamine was removed either by gel filtration using Sephadex G-50 or by dialysis. The *N*-semiquinone MADH was generated by comproportionation of *N*-quinol MADH and quinone MADH as described previously (*11*).

Rapid Kinetic Measurements. Rapid kinetic measurements were made using an On-Line Instrument Systems (OLIS) RSM1000 stopped-flow spectrophotometer. All reactions were monitored over a range of wavelengths between 330 and 490 nm where each of the three redox forms of TTQ exhibits a visible absorbance maximum. The visible absorbance maximum of amicyanin is at 595 nm, and its extinction coefficient is much smaller than that of MADH. Therefore, redox-linked changes in amicyanin do not interfere with the analysis of the absorbance changes of oxidized, semiquinone, or reduced MADH. In mixing experiments, MADH was the limiting reactant with a concentration of 1−4 μM. All experiments were performed under saturating pseudo-first-order conditions with the amicyanin concentration in at least 20-fold excess of that of MADH. For analysis of the rate constants that describe the reaction of *N*-semiquinone MADH with oxidized amicyanin, the model and equation given in eq 1 were used:

$$\mathrm{MADH_{semi}} + \mathrm{amicyanin_{ox}} \overset{K_d}{\rightleftharpoons} \mathrm{MADH_{semi}/amicyanin_{ox}} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} \mathrm{MADH_{ox}/amicyanin_{red}} \quad (1a)$$

$$k_{obs} = \frac{k_3[\mathrm{amicyanin}]}{[\mathrm{amicyanin}] + K_d} + k_4 \quad (1b)$$

The observed reactions were essentially irreversible under all reaction conditions, indicating either that k_4 was zero or that it was much slower than dissociation of the $MADH_{ox}$/$amicyanin_{red}$ complex. Therefore, at saturating concentrations of amicyanin (i.e., [amicyanin] $\gg K_d$), $k_{obs} = k_3$.

Kinetic measurements were often made only at saturating amicyanin concentrations, rather than over a wide range of concentrations. This was done not only to conserve protein but also because under certain reaction conditions it was not possible to accurately determine k_{obs} at subsaturating amicyanin concentrations. This was due in part to the fact that the K_d for amicyanin is near the concentration limit for maintaining pseudo-first-order reaction conditions. Under conditions where data could be obtained over a complete concentration range, a hyperbolic dependence of k_{obs} on amicyanin concentration was observed (Figure 2) with no finite *y*-intercept (i.e., $k_4 = 0$). Analysis of these data by a more rigorous equation which does not incorporate the simplifying assumptions inherent in eq 1b (*20, 21*) did not yield an improved fit to the data. Thus, the assumption that at saturating concentrations of amicyanin $k_{obs} = k_3$ is justified.

Analysis of Transient Kinetic Data. Kinetic data collected in the rapid scanning mode were reduced by factor analysis using Global Fit, the Singular Value Decomposition (SVD) algorithm (*22*) provided by OLIS. SVD reduced data were then globally fit by a robust version of the Levenberg and Marquardt nonlinear method of least squares using the fitting routines of the Global Fit software (*23, 24*).

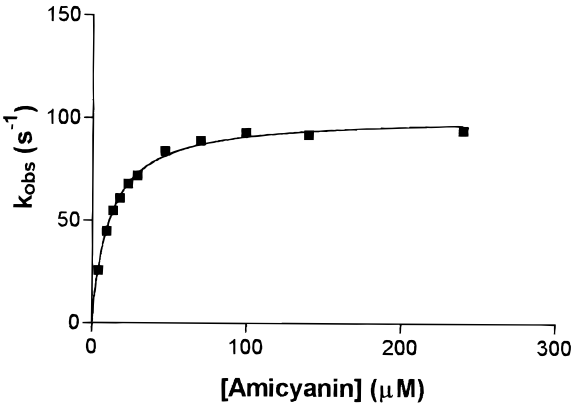

FIGURE 2: Reaction of *N*-semiquinone MADH with oxidized amicyanin. The reactions were performed in 10 mM potassium phosphate plus 0.2 M KCl, at pH 7.5, at 30 °C. Rates were determined as described under Experimental Procedures. The solid line represents a fit of the data to eq 1b. This fit yields values of $k_3 = 101 \pm 4$ and $K_d = 11.3 \pm 1.2$, with $k_4 = 0$.

The oxidative half-reaction of the catalytic cycle of MADH proceeds by two sequential one-electron transfers to 2 redox-equiv of amicyanin (Figure 2). Assignment of individual rate constants to the first and second oxidation steps requires an understanding of the spectral changes that are associated with each reaction step. These experimental details and methods of spectral analysis have been previously described (*10, 16*). As discussed later, the relative rates of the two one-electron oxidations varied with reaction conditions. Under certain conditions, the rate of the first oxidation to form the *N*-semiquinone was so rapid that it occurred in the dead time for mixing and only the rate of the second oxidation could be measured. Under certain other reaction conditions, the rate of the second oxidation was sufficiently faster than that of the first oxidation that no appreciable *N*-semiquinone intermediate accumulated. In these cases, it was only possible to obtain the rate of the slower initial oxidation of the *N*-quinol.

Kinetic Solvent Isotope Effects. In KSIE studies, reactions were performed in buffered solutions of D_2O which were prepared according to Schowen and Schowen (*25*). The value of pD was obtained by adding 0.40 to the observed pH in buffered D_2O (*26*). With solutions which contained protein samples, H_2O was completely exchanged for D_2O by repeated ultrafiltration using Amicon centripreps. After solvent exchange, these protein solutions were incubated overnight in the buffered D_2O at 15 °C to ensure the complete exchange of all solvent-exposed titratable protium for deuterium.

RESULTS

Differential Effects of pH on the ET Reactions from N-Quinol and N-Semiquinone MADH to Amicyanin. The limiting pseudo-first-order rate constants for the sequential oxidations by amicyanin of the *N*-quinol and *N*-semiquinone were determined over a range of pH in 10 mM potassium phosphate buffer plus 0.2 M KCl at 21 °C. The rates of the ET reactions from *N*-quinol MADH to amicyanin and from

Table 1: Differential Effects of pH and KCl on the Rate Constants for *N*-Quinol and *N*-Semiquinone MADH Oxidation by Amicyanin

buffer conditions	*N*-quinol (s^{-1})	*N*-semiquinone (s^{-1})
10 mM potassium phosphate + 200 mM KCl		
pH 5.4	3.2 ± 0.4	nd[a]
pH 6.7	42 ± 3	21 ± 2
pH 7.4	130 ± 22	51 ± 13
pH 7.9	320 ± 49	70 ± 5
pH 9.0	>500	110 ± 16
10 mM potassium phosphate, pH 7.4		
+0 mM KCl	12 ± 3	28 ± 9
+200 mM KCl	130 ± 22	51 ± 13
+400 mM KCl	>500	84 ± 17

[a] Not possible to determine because the *N*-semiquinone does not accumulate under these conditions.

N-semiquinone MADH to amicyanin each exhibit a different dependence on pH. The rate of the gated ET reaction from the *N*-quinol increases dramatically with increasing pH whereas the rate of the ET reaction from the *N*-semiquinone is much less affected by pH (Table 1). It is not possible to obtain the rate of *N*-semiquinone decay under all solution conditions since the extent of accumulation of this intermediate varies with reaction conditions. At pH 5.4, the formation of *N*-semiquinone is so slow relative to its oxidation that there is essentially no accumulation of intermediate and the only rate constant which can be determined is that for the first ET from *N*-quinol. At pH 9.0, the rate of semiquinone formation is too fast to measure by stopped-flow and occurs in the dead time for mixing so that only the rate of *N*-semiquinone oxidation is observed. At intermediate values of pH, the accumulation of the *N*-semiquinone intermediate is sufficient to allow determination of the rate constants for each reaction.

Differential Effects of KCl on the ET Reactions from N-Quinol and N-Semiquinone MADH to Amicyanin. The limiting pseudo-first-order rate constants for the sequential oxidations by amicyanin of the *N*-quinol and *N*-semiquinone were determined over a range of concentrations of added KCl in 10 mM potassium phosphate, pH 7.4, at 21 °C (Table 1). We have previously shown that the salt effect on the rate of oxidation of *N*-quinol MADH by amicyanin is not a general ionic strength effect, but due specifically to the concentration of monovalent cation present (*16*). The rate of the gated ET reaction from the *N*-quinol increases dramatically with increasing [KCl] whereas the rate of the ET reaction from the *N*-semiquinone is much less affected (Table 1).

Marcus Analysis of the Electron-Transfer Reaction from N-Semiquinone MADH to Amicyanin. Analysis of the temperature dependence of a protein ET reaction may be useful for determining whether the redox reaction is gated by an adiabatic, non-ET, reaction step or whether nonadiabatic ET is rate-limiting for the observed reaction (*27*). In the latter case, these data may be used to determine the reorganizational energy (λ) and electronic coupling (H_{AB}) associated with the ET reaction.

The limiting pseudo-first-order rate constants for the sequential oxidations by amicyanin of *N*-quinol and *N*-semiquinone MADH were determined over a range of temperature in pH 7.4 buffer in the presence of 0.2 M KCl. We previously established (*10*) that the *N*-semiquinone which accumulates during the two-step oxidation of *N*-quinol MADH by amicyanin is kinetically identical to the *N*-semiquinone which is generated in vitro by comproportionation of *N*-quinol MADH with quinone MADH at alkaline pH (*11*). The temperature dependence of the reaction of this preformed *N*-semiquinone MADH with oxidized amicyanin was also examined under the same reaction conditions. Although the sample prepared by comproportionation contains a mixture of *N*-semiquinone and *O*-semiquinone, only the reaction of the *N*-semiquinone is observed. That is because under these conditions the reaction of the *O*-semiquinone is very rapid (>500 s^{-1}) and over in the dead time of the mixing experiment (*10*). Essentially identical rates and temperature dependence of the ET rates were observed. These data confirm the validity of the rates that were obtained for the ET reaction from *N*-semiquinone MADH to amicyanin from global kinetic analysis of the overall two-step oxidation of *N*-quinol MADH by amicyanin.

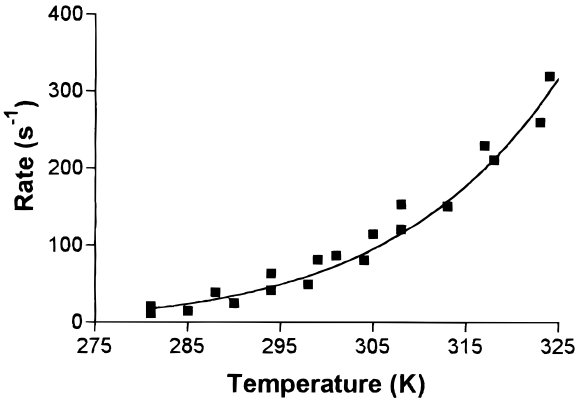

FIGURE 3: Dependence of the rate constant for *N*-semiquinone MADH oxidation by amicyanin on temperature. The solid line represents a fit of the data to eq 2a with $\Delta G° = -15.9$ kJ/mol, $\lambda = 232$ kJ/mol, and $H_{AB} = 15.9$ cm^{-1}.

The temperature dependence of the rate constant which corresponds to the oxidation of *N*-semiquinone MADH by amicyanin was analyzed by eq 2 (Figure 3)

$$k_{ET} = \frac{4\pi^2 H_{AB}{}^2}{h\sqrt{4\pi\lambda RT}} \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \quad (2a)$$

$$k_{ET} = k_o \exp[-\beta(r - r_o)] \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \quad (2b)$$

which describes the dependence of a nonadiabatic reaction on temperature (*14*), where h is Planck's constant and R is the gas constant. In eq 2b, H_{AB} is factored into terms which define the ET distance between redox centers (r is the center to center distance and r_o is the close contact distance which is taken to be 3 Å), and describe the nature of the medium which separates the redox centers (β). The other parameter (k_o) is the characteristic frequency of the nuclei which is set at 10^{-13} s^{-1}. The rate constants obtained from global kinetic fits of the two-step oxidation of the *N*-quinol and those obtained from the oxidation of the preformed *N*-semiquinone are analyzed together. As can be seen in Figure 3, these data are well described by eq 2. The parameters which are obtained from the fits of the data are listed in Table 2 and compared with previously obtained values for the oxidations by amicyanin of *O*-quinol MADH, which is rate-limited by the nonadiabatic ET reaction step (*1, 2, 15*), and *N*-quinol

Table 2: Electron-Transfer Parameters for the Reactions of Different Redox Forms of MADH with Amicyanin

MADH form	λ (eV)	H_{AB} (cm^{-1})	r (Å)[a]
N-semiquinone[b]	2.4 ± 0.1	13 ± 4	9.4 ± 0.7
O-quinol[c]	2.3 ± 0.1	12 ± 7	9.6 ± 1.2
N-quinol[b]	3.4 ± 0.1	20000 ± 2000	<0

[a] Calculated using eq 2b and a β value of 1.0. [b] Parameters were calculated using eq 2. [c] Values taken from ref *15*.

Table 3: Kinetic Solvent Isotope Effects on the Rate Constants for *N*-Quinol and *N*-Semiquinone MADH Oxidation by Amicyanin

buffer conditions	KSIE ($^{H2O}k/^{D2O}k$) for reoxidation of *N*-quinol	KSIE ($^{H2O}k/^{D2O}k$) for reoxidation of *N*-semiquinone
10 mM potassium phosphate + 200 mM KCl		
pL 7.4[a]	6.5 ± 1.2	1.8 ± 0.5
pL 7.9	6.0 ± 1.2	2.4 ± 0.3
pL 8.2	>3.6[b]	2.0 ± 0.4
pL 9.0	>3.1[b]	1.7 ± 0.3
10 mM potassium phosphate, pH 7.4		
+0 mM KCl	6.0 ± 1.9	nd[c]
+200 mM KCl	6.5 ± 1.2	1.8 ± 0.5
+400 mM KCl	>3.8[b]	1.3 ± 0.3

[a] pL refers to pH in buffered H_2O and pD in buffered D_2O. [b] It was not possible to determine the exact KSIE under these reaction conditions because the rate of the reaction in H_2O was too fast to measure. [c] Not possible to determine.

MADH which is gated by an adiabatic proton-transfer reaction (*2, 16*).

The fitted parameters for the reaction of the *N*-semiquinone are essentially identical to the values for the reaction of the *O*-quinol. These data indicate that, in contrast to the reaction of the *N*-quinol, the ET reaction from the *N*-semiquinone is not gated but rate-limited by the ET event. Furthermore, the similarity of the Marcus parameters to those previously obtained for the ET reactions of the *O*-quinol and *O*-semiquinone (*1, 15*) indicates that the presence of the substrate-derived amino group in the *N*-semiquinone does not affect the λ, H_{AB}, or predicted ET distance associated with the true ET event. The observed difference in the rate of the reaction of the *N*-semiquinone, relative to the reactions of the *O*-quinol and *O*-semiquinone, is likely due to differences in $\Delta G°$ for the ET reaction due to differences in the oxidation−reduction midpoint potential (E_m) values of the different redox forms of MADH.

It has not been possible to directly measure the E_m value for the *N*-semiquinone/quinone couple. Electrochemical titrations are equilibrium measurements. When the *N*-quinol or *N*-semiquinone is oxidized to the quinone, the amino group is lost (*9*). Consequently, the oxidation−reduction is not a reversible reaction, and electrochemical titrations of MADH can only yield E_m values for the *O*-quinol/quinone couple (*13*). Information on the effect on the redox potential of modification of TTQ by substrate in MADH may be inferred from related studies on organic model compounds of quinone cofactors. Electrochemical studies of a TTQ model compound (*28*) indicated that the presence of a covalently bound amino group at the C6 position increased the E_m value for the two-electron oxidized/reduced couple by +41 mV. A similar positive shift in the E_m value of +23−25 mV upon iminoquinone formation has been reported for a topaquinone model compound (*29*). When fitting our data to eq 2, a value of +55 mV was used for the *N*-semiquinone/oxidized redox couple. This value is 41 mV more positive than that previously reported (*1*) for the MADH *O*-semiquinone/oxidized redox couple and is a reasonable approximation for this analysis (discussed later). As discussed previously, for ET reactions which exhibit a large λ relative to $-\Delta G°$, some uncertainty in the $\Delta G°$ value used in these calculations will have essentially no effect on the fitted values of H_{AB} and r, and cause an error in the fitted value of λ proportional to the discrepancy in $\Delta G°$ (*27*).[2]

Table 4: Rates and Rate-Limiting Steps for the Oxidation of Different Redox Forms of MADH by Amicyanin

MADH redox form	rate-limiting reaction step	rate constant at 25 °C (s^{-1})[a]	reference
O-quinol MADH	electron transfer	12	*16*
O-semiquinone MADH	electron transfer	>500	*10*
N-quinol MADH	proton transfer	144	*16*
N-semiquinone MADH	electron transfer	51	this work

[a] Buffer conditions of 10 mM potassium phosphate plus 0.2 M KCl at pH 7.5.

Kinetic Solvent Isotope Effect Studies. Given the knowledge that the ET reaction from *N*-quinol MADH to amicyanin is gated by a proton-transfer reaction (*2, 16*), KSIE studies were performed on the reaction of the *N*-semiquinone MADH with amicyanin. The limiting pseudo-first-order rate constants for the sequential oxidations by amicyanin of the *N*-quinol and *N*-semiquinone were determined over a range of pHs and concentrations of KCl at 21 °C in buffered H_2O and D_2O. Whereas the reaction of the *N*-quinol MADH, which is gated by proton transfer, exhibits a significant primary KSIE under each set of reaction conditions, no significant primary KSIE is observed for the reaction of *N*-semiquinone over the range of reaction conditions that were examined (Table 3). The magnitude of the KSIE observed with the *N*-semiquinone is comparable to what was previously reported for the ET reaction of the *O*-quinol (*2*), and consistent with the oxidation of *N*-semiquinone MADH being rate-limited by ET rather than proton transfer.

DISCUSSION

We have previously shown that the redox reactions between amicyanin and the dithionite-generated *O*-quinol and *O*-semiquinone of MADH are rate-limited by the ET event and exhibit a predictable dependence of rate on driving force (*1*). In contrast, the redox reaction between amicyanin and the substrate-generated *N*-quinol is gated by a proton-transfer event (*2*, Table 4). A model for that reaction has been presented in which deprotonation of the substrate-derived amino group on TTQ is required to activate the *N*-quinol for ET (*16*). Given those results, it was important to determine the manner by which ET from the *N*-semiquinone MADH is regulated. We show here that in contrast to the reaction of the *N*-quinol, the redox reaction between amicyanin and *N*-semiquinone MADH is not gated but rate-

[2] For example, if one assumes that the E_m value for the MADH *N*-semiquinone/quinone couple is +155 mV rather than +55 mV, fits of the temperature dependence of the rate to eq 2 yield identical values of H_{AB} and r and a value of λ of 2.2 eV. Alternatively, if one assumes that this E_m value is −45 mV rather than +55 mV, fits of the temperature dependence of the rate to eq 2 again yield identical values of H_{AB} and r and a value or λ of 2.6 eV.

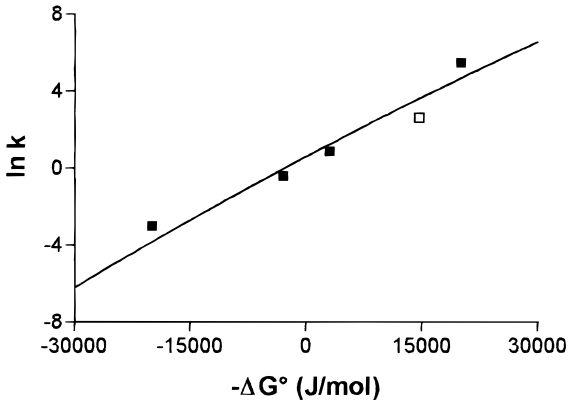

FIGURE 4: Dependence of the rate constants for oxidation of different MADH redox forms by amicyanin on driving force. The solid line represents a fit of the data to eq 2a. The filled squares represent previously determined data (*15*) for the forward and reverse reactions of *O*-quinol and *O*-semiquinone MADH with amicyanin. The open square is the data point obtained in this study for the reaction of *N*-semiquinone MADH with amicyanin. All rate constants are for the reaction at 10 °C.

limited by the ET event. The reaction rate for the ET reaction from the *N*-semiquinone is much less sensitive to pH and salt. It does not exhibit a primary KSIE, and it exhibits Marcus parameters which are identical to those that describe ET from the *O*-quinol and *O*-semiquinone forms of MADH.

We have previously shown that the ET rates of the forward and reverse reactions of *O*-quinol MADH with amicyanin and *O*-semiquinone MADH with amicyanin exhibited a predictable dependence on $\Delta G°$ (*1*). Those four data points could be well described by eq 2 using the Marcus parameters given in Table 2, which were determined from temperature dependence studies of the oxidation of *O*-quinol MADH by amicyanin (*15*). When the data for the reaction of the *N*-semiquinone MADH are included with the four data points for the forward and reverse ET reactions of the O-forms of MADH, and analyzed by eq 2, a good fit for the $\Delta G°$ dependence of the ET rate is obtained (Figure 4). The fitted values for the Marcus parameters are $H_{AB} = 11 \pm 17$ cm^{-1} and $\lambda = 219 \pm 30$ kJ mol^{-1} (2.3 ± 0.3 eV). The values obtained from the fit of the data in Figure 4 have larger errors than those obtained from temperature dependence studies because of the relatively small number of data points and the fact that these data also include some uncertainty in the value of $\Delta G°$. However, these values are essentially identical to the values which were obtained from analysis of the temperature dependencies of the reactions of the *O*-quinol and *N*-semiquinone forms of MADH (Table 2). This analysis confirms that the ET reaction from *N*-semiquinone MADH to amicyanin is not gated, but rate-limited by the ET event. It also supports the assumption that the change in redox potential caused by the incorporation of the substrate-derived N into TTQ in MADH is similar to that reported for the TTQ model compound. Thus, the presence of the substrate-derived N on TTQ is not alone sufficient to cause gated ET. It only does so for the first ET from the fully reduced *N*-quinol. This means that for the reaction of the *N*-semiquinone, either proton transfer (i.e., deprotonation of the substrate-derived N on TTQ) is not required to activate the system for ET, or it does occur and is rapid relative to the ET event.

These results raise the question of why the ET reaction from *N*-quinol MADH to amicyanin requires activation for ET. As discussed earlier, the E_m value for the *N*-quinol/*N*-semiquinone couple cannot be directly determined. If the redox behavior of this redox couple is analogous to that of the TTQ model compound and the *N*-semiquinone/quinone couple, then one may assume that incorporation of N into the C6 position of TTQ raises its E_m value by approximately 41 mV relative to the quinol/semiquinone couple. This would mean that the ΔE_m value for the ET reaction from *N*-quinol MADH to oxidized amicyanin would be approximately -10 mV ($\Delta G° = +960$ J/mol). Not only would this reaction be thermodynamically unfavorable, but according to the relationship shown in Figure 4 this $\Delta G°$ value would correspond to an ET rate constant of only 0.2 s^{-1}. Without some means of activation, this physiologically necessary reaction would not likely occur. It is noteworthy that when the oxidation of the *N*-quinol MADH by amicyanin was studied in buffer in the absence of monovalent cations (i.e., 10 mM HEPES), which are required for activation, a rate constant of 0.6 s^{-1} was reported (*16*). This suggests that when monovalent cations are not available, deprotonation and thus activation of the *N*-quinol are not possible, and the slow ET from the *N*-quinol is the rate-limiting reaction. Deprotonation of the substrate-derived amino group on TTQ in the *N*-quinol will yield a highly reactive reaction intermediate from which the ET to amicyanin is much more favorable (*16*). For the *N*-quinol, this activation step is necessary to compensate for the decrease in the ΔE_m for the ET reaction with amicyanin caused by incorporation of the substrate-derived N. For the *N*-semiquinone, however, despite the decrease in ΔE_m caused by the covalently incorporated N, the ET to amicyanin is still thermodynamically favorable and able to proceed at an adequate rate (see Figure 4). This illustrates an important point. While the experimentally derived values for H_{AB} and r are relatively insensitive[2] to variation in $\Delta G°$, variation in $\Delta G°$ *does* have a substantial effect on the ET rate.

The demonstration that the incorporation of substrate-derived N into TTQ does not alter the λ or H_{AB} associated with ET reactions between TTQ and the copper of amicyanin has important implications. The dithionite-generated *O*-quinol and *O*-semiquinone forms of MADH are much more amenable to study than the N-forms. They are easily preformed and quite stable, and their ET reactions with amicyanin are rate-limited by the ET event. In contrast, the ET reaction of the *N*-quinol is gated, and the *N*-semiquinone is very difficult to preform and relatively unstable. A prior concern with use of the O-forms of MADH was that they are not true physiologic reaction intermediates. Knowledge that the presence of the substrate-derived amino group in the physiologic intermediates does not affect the ET parameters, λ and H_{AB}, demonstrates that the results and conclusions obtained with the O-forms are relevant and descriptive of the physiologic ET process.

An important point which merits discussion is the observation that the three ungated ET reactions to amicyanin from the *O*-quinol, *O*-semiquinone, and *N*-semiquinone exhibit essentially the same relatively large λ value of 2.3−2.4 eV. This value has been obtained from the $\Delta G°$ dependence of ET reactions of the O-forms of MADH (*1*), and from temperature dependence studies of the *O*-quinol (*15*) and

N-semiquinone (Figure 3). The differences in ET rates for these different MADH forms may be attributed to a $\Delta G°$ dependence of the reaction rate which is as predicted by Marcus theory (Figure 4). Since this large λ appears to be a legitimate value and not an experimental artifact, it raises the question of what factors contribute to this relatively large value. One possibility is that this is a reflection of the kinetic complexity of this reaction (*27*). The ET reactions from MADH to amicyanin may be representative of coupled ET in which a rate-limiting ET event is preceded by a relatively rapid but unfavorable non-ET reaction step (*27*, *30*). For such a reaction, the experimentally determined λ value will include contributions from both the λ associated with the ET as well as the energetics associated with the unfavorable prerequisite adiabatic reaction step. One can envision at least two possible non-ET reaction steps that may be required to activate the system for ET. A conformational rearrangement of the proteins within the ET complex may be needed to reorient the proteins from a geometry which is optimal for binding to one which is optimal for ET. Recent results of site-directed mutagenesis experiments (*31*) argue against a contribution to λ from reorientation of the proteins with respect to each other at the interface. Alternatively, some conformational reorientation of the two indole rings (e.g., change in dihedral angle between rings) which comprise the TTQ cofactor may be needed to optimize the system for ET. The need for such a perturbation of the angle between the TTQ rings for ET reactions has also been suggested on the basis of studies with TTQ model compounds (*28*). Such a reaction could conceivably be unfavorable enough ($K_{eq} \ll 1$) to contribute significantly to the experimentally determined λ value. Another possibility which cannot be ruled out is that this is a true ET reaction with a large λ value. It is unknown what intrinsic λ value may be associated with ET from the TTQ cofactor. While much is known about the λ values associated with redox changes of metal redox centers, such as type 1 copper and heme, these systems are very different from TTQ which contains no metal and is comprised of two unfused indole ring systems joined by a single bond. Elucidating the basis for this large λ value associated with ET from TTQ to copper in the MADH−amicyanin complex will be an important goal of future studies on this system.

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