Intermolecular Electron Transfer from Substrate-Reduced Methylamine Dehydrogenase to Amicyanin Is Linked to Proton Transfer†

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ABSTRACT: Within the methylamine dehydrogenase−amicyanin complex, intermolecular electron transfer (ET) occurs between tryptophan tryptophylquinone (TTQ) and copper. The ET reactions from two chemically distinct reduced forms of TTQ were studied. The quinol form of TTQ was generated by reduction by dithionite. An aminoquinol form of TTQ, in which an amino group displaces the carbonyl oxygen, was generated by reduction by the substrate methylamine. Thermodynamic analysis of the ET reactions suggested that the ET event was rate-limiting for the redox reaction between quinol TTQ and copper, but not for the ET reaction from aminoquinol TTQ to copper. Solvent kinetic isotope effect studies indicated that proton transfer was involved in the rate-limiting reaction step for the ET from the substrate-reduced enzyme, but not the dithionite-reduced enzyme. Solvent deuterium kinetic isotope effects of 1.5 and 12.2 were obtained, respectively, for the ET reactions from dithionite-reduced and substrate-reduced methylamine dehydrogenase. These results demonstrate that application of ET theory to the analysis of thermodynamic data for the intermolecular protein ET reactions can potentially be used to distinguish between true ET reactions and those which are gated or attenuated by adiabatic events. Kinetic models are presented to explain how the incorporation of the substrate-derived amino group into TTQ may alter the rate-limiting step for ET.

Long range electron transfer (ET)[1] between proteins is a process which is fundamental to respiration, photosynthesis, and redox reactions of intermediary metabolism. The mechanisms by which such reactions occur and the factors which regulate the observed rates of these reactions remain poorly understood. ET rates are predicted to depend upon driving force, temperature, and the distance and/or pathway which separates the redox centers [reviewed in Marcus and Sutin (1987), Winkler and Gray (1992), Moser et al. (1992), and McLendon and Hake (1992)]. Protein conformational changes and reorientation of proteins within an ET complex may also regulate the observed rate of biological ET reactions (Hoffman & Ratner, 1987; Brunschwig & Sutin, 1989). Solvent conditions such as viscosity (Zhou & Kostic, 1993) and ionic strength (Hazzard et al., 1991; Harris et al., 1994) can alter the observed ET rate by affecting these dynamic conformational fluctuations of protein redox partners. The concept of a rate-limiting process which occurs as a prerequisite for ET is known as gating. In the case where ET is regulated by a rate-determining conformational change, the system may be thought of as being conformationally gated. Alternatively, if ET is regulated by a rate-limiting catalytic event, such as the making or breaking of specific bonds at or near the active site, the system may be thought of as being catalytically gated. An apparent example of the latter is described in this paper.

Methylamine dehydrogenase (MADH) (Davidson, 1993) catalyzes the oxidative deamination of methylamine to formaldehyde plus ammonia and possesses the tryptophan tryptophylquinone (TTQ) (McIntire et al., 1991) prosthetic group which participates in catalysis and electron transfer. MADH forms a complex with its physiologic electron acceptor amicyanin (Husain & Davidson, 1985), a type I copper protein. The structure of the protein matrix which separates TTQ and copper is known from crystallographic analyses of a binary complex of MADH and amicyanin (Chen et al., 1992) and a ternary complex of these proteins in association with cytochrome *c*551i (Chen et al., 1994). These and the cytochrome *c*−cytochrome *c* peroxidase complex (Pelletier & Kraut, 1992) are the only physiologic complexes of soluble redox proteins for which detailed crystal structures are known. During the physiologic reaction of MADH, reduction of TTQ by methylamine is associated with release of formaldehyde and incorporation of the substrate-derived amino group to yield an aminoquinol form of MADH (Brooks et al., 1993). Furthermore, it has been shown that the amino group remains incorporated after the one-electron oxidation of the aminoquinol to form an aminosemiquinone (Warncke et al., 1993). Release of the ammonia product from TTQ occurs after or concomitant with the oxidation of the aminosemiquinone to the quinone. MADH may also be reduced chemically to generate the quinol and semiquinone forms of MADH (Husain et al., 1987). Thus, structurally different forms of reduced TTQ can be generated by titrating MADH with reducing equivalents of either dithionite or the substrate methylamine. TTQ is a two-electron carrier, and amicyanin is a one-electron carrier. It is, therefore, possible to study multiple ET

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[1] Abbreviations: ET, electron transfer; MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; H_{AB}, electronic coupling; λ, reorganizational energy; KSIE, kinetic solvent isotope effect.

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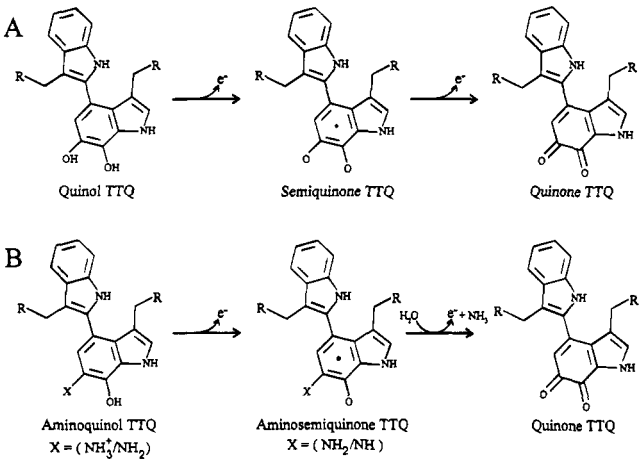

FIGURE 1: Products of successive one-electron oxidations of dithionite-reduced (A) and substrate-reduced (B) TTQ in MADH. The protonation states of the amino group in B are unknown. The actual distribution of spin density of the radical in semiquinone forms is currently under study and should not be inferred from the figure.

reactions between the different redox forms of MADH and amicyanin (Figure 1).

Nonadiabatic ET reactions may be described by eqs 1 and 2 which are derived in part from Marcus theory (Marcus & Sutin, 1985). H_{AB} is the electronic coupling between redox centers and describes the degree of wave function overlap occurring between donor and acceptor sites. λ is the reorganizational energy which describes the energy barrier for ET. h is Planck's constant. R is the gas constant. T is temperature. $\Delta G°$ is the standard free energy difference for the reaction. k_o is the characteristic frequency of the nuclei which is usually assigned a value of 10^{13} s^{-1}. r is the distance between donor and acceptor. r_o is the close contact distance (3 Å). β is the electronic decay factor which is related to the nature of the intervening medium between redox centers. According to eqs 1 and 2, it should be possible to experimentally vary k_{ET} as a function of either temperature or $\Delta G°$. We have previously examined the temperature dependence of the ET reaction from quinol MADH to amicyanin (Brooks & Davidson, 1994a). The $\Delta G°$ dependence of this reaction was also characterized by monitoring the forward and reverse redox reactions between quinol and semiquinone MADH and amicyanin (Brooks & Davidson, 1994b). These temperature and $\Delta G°$ dependence studies yielded comparable values for H_{AB} and λ for the ET reaction from quinol MADH to amicyanin and demonstrated that this redox reaction behaved in a manner consistent with the predictions of ET theory (eqs 1 and 2). Good correlation has also been reported for values of λ which were obtained from temperature and $\Delta G°$ dependence studies of the ET reaction between cytochrome c and cytochrome c peroxidase (Conklin & McLendon, 1988).

This paper addresses the question of how modification of TTQ by substrate affects the ET reactions from reduced MADH to amicyanin. It is shown that ET is rate-determining for the redox reactions of quinol and semiquinone MADH with amicyanin, but not for the ET reaction from aminoquinol MADH. Furthermore, it is demonstrated that the adiabatic process which is rate-limiting for ET from substrate-reduced MADH involves proton transfer.

$$k_{ET} = \frac{4\pi^2 H_{AB}^{\ 2}}{h\sqrt{4\pi\lambda RT}} e^{-(\Delta G°+\lambda)^2/4\lambda RT} \quad (1)$$

$$k_{ET} = [k_o e^{-\beta(r-r_0)}] e^{-(\Delta G°+\lambda)^2/4\lambda RT} \quad (2)$$

EXPERIMENTAL PROCEDURES

Purifications of MADH (Davidson, 1990) and amicyanin (Husian & Davidson, 1985) from *Paracoccus denitrificans* were as previously described. Protein concentrations were calculated from known extinction coefficients (Husain & Davidson, 1985; Husain et al., 1987). Reduced MADH was prepared by titration with either methylamine or sodium hydrosulfite (dithionite). D_2O (99.9%) was obtained from C/D/N Isotopes. All other reagents were obtained from Sigma.

Reactions were performed in either H_2O or D_2O in 10 mM potassium phosphate which included 200 mM KCl. The pH or pD of the buffers was 7.5. All buffered D_2O solutions were prepared according to Schowen and Schowen (1982). Buffers of specific pD were prepared using a standard pH meter soaked in D_2O, and correction was made for the effect of D_2O on the electrode response. The value of pD was obtained by adding 0.40 to the observed pH in solutions in D_2O (Glasoe & Long, 1960). 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 hydrogen ions for deuterium ions.

An On-Line Instrument Systems (OLIS) RSM1000 stopped-flow spectrophotometer was used for stopped-flow kinetic experiments. Reactions were monitored at 443 nm where MADH exhibits a $\Delta\epsilon$ of 26 200 M^{-1} cm^{-1} on conversion from the reduced to the semiquinone form (Husain et al., 1987). This wavelength is isosbestic for the semiquinone and oxidized forms of MADH. The spectra of the dithionite-reduced and substrate-reduced forms of MADH are essentially identical. The observed amplitude for each reaction correlated with the expected amplitude calculated from the known extinction coefficients. The $\Delta\epsilon$s showed no significant temperature dependence over the range studied. All data were best described by a single exponential, and each observed rate constant (k_{obs}) was obtained from a nonlinear fit to the exponential rise. For each set of mixing experiments, the concentration of MADH was fixed at 1.0 μM and the amicyanin concentration was varied. In the buffer used in these studies, the K_d for amicyanin is sufficiently high that it was possible to maintain pseudo-first order conditions at all amicyanin concentrations. In each case, saturation behavior was observed and data were fit according to eq 3 (Strickland et al., 1975). Nonlinear curve fitting of data was

$$k_{obs} = \frac{k_3[\text{amicyanin}]}{[\text{amicyanin}] + K_d} + k_4 \quad (3)$$

performed with OLIS software and the Sigma Plot 5.0 (Jandel Scientific, San Raphael, CA) computer program.

RESULTS AND DISCUSSION

Kinetic Analysis. The oxidative half-reaction of MADH proceeds through two sequential one-electron reductions as

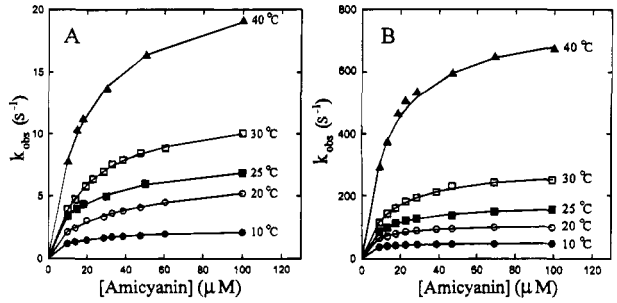

FIGURE 2: Concentration and temperature dependence of k_{obs} for the reactions of (A) dithionite-reduced and (B) substrate-reduced MADH with amicyanin. The temperature at which each set of experiments was performed is indicated. The solid lines represent the fits of these data to eq 3.

2 Step Mechanism $\quad E + A \underset{}{\overset{K_d}{\rightleftharpoons}} EA \underset{-k_3}{\overset{k_3}{\rightleftharpoons}} EP$

3 Step Mechanism $\quad E + A \overset{K_d}{\rightleftharpoons} EA \underset{-k_C}{\overset{k_C}{\rightleftharpoons}} EA^* \underset{-k_{ET}}{\overset{k_{ET}}{\rightleftharpoons}} EP$

1) 'True' ET: (2 step mechanism or $k_{ET} << k_C$)

$k_3 = k_{ET} \qquad \lambda_{obs} = \lambda_{ET}$

2) 'Gated' ET ($k_C << k_{ET}$)

$k_3 = k_C \qquad \lambda_{obs} = f(\Delta G_C^*)$

3) 'Coupled' ET ($k_{ET} << k_C$ but $K_C << 1$)

$k_3 = K_C \cdot k_{ET} \qquad \lambda_{obs} = f(\lambda_{ET} + \lambda_C)$

FIGURE 3: Kinetic models for intermolecular ET reactions. The definition of k_3 for coupled ET was derived in Harris et al. (1994). k_c describes the rate of a conformational reorientation or catalytic event which is required to achieve an activated form of the protein complex in which ET occurs. K_c is the equilibrium constant for that step. K_d is the dissociation constant for the complex.

described by the minimal scheme below (eq 4) in which M_r, M_s, and M_o are reduced, semiquinone, and oxidized MADH,

$$M_r + A_o \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M_rA_o \underset{k_4}{\overset{k_3}{\rightleftharpoons}} M_sA_r \underset{k_6}{\overset{k_5}{\rightleftharpoons}} M_s + A_r$$

$$M_s + A_o \underset{k_8}{\overset{k_7}{\rightleftharpoons}} M_sA_o \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} M_oA_r \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} M_o + A_r \qquad (4)$$

respectively. A_o and A_r are oxidized and reduced amicyanin, respectively. At the wavelength being monitored, all steps beyond k_3/k_4 are spectroscopically invisible. For the reactions of reduced MADH with amicyanin, data for the concentration dependence of k_{obs} were fit to eq 3 (Figure 2), which is derived for a simplified model given in eq 5. In each set of

$$M_r + A_o \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M_rA_o \underset{k_4}{\overset{k_3}{\rightleftharpoons}} M_sA_r \qquad (5)$$

experiments, the fitted curve passed through the origin, indicative of an irreversible reaction. This will be true if either k_4 is equal to 0 or if k_5 in eq 4 is much greater than k_4. In either case, the limiting first order rate constant will be equal to k_3, which strictly describes the redox change of MADH from the reduced to the semiquinone state. This will be referred to as an apparent ET rate constant (k_{ET}). It is apparent because in this kinetic model any spectroscopically invisible reaction steps which may occur subsequent to binding and before ET may be reflected in k_3 (Figure 3). The k_3 will only be a true k_{ET} if no such additional reaction

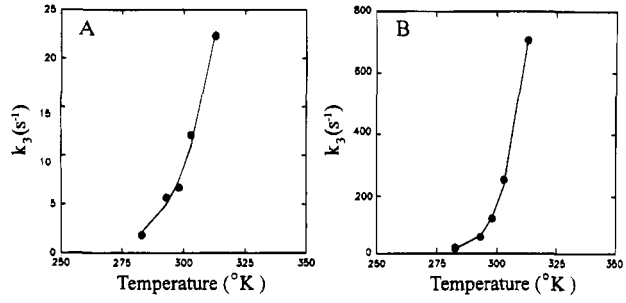

FIGURE 4: Analysis of the temperature dependence of apparent k_{ET} for the reactions of (A) dithionite-reduced MADH and (B) substrate-reduced MADH with amicyanin. Values of k_3 were determined from the data shown in Figure 2, which were fit to eq 3. The solid lines represent the fits of these data to eqs 1 and 2. The fits to the two equations are superimposable.

steps occur or if ET is rate-limiting in a multistep mechanism where all other steps are energetically favorable (Harris et al., 1994).

Thermodynamic Analysis. The temperature dependence of k_3 was determined under identical conditions for the reactions of amicyanin with both dithionite-reduced and substrate-reduced MADH (Figure 2). These data reveal that incorporation of the substrate-derived amino group into reduced TTQ affects both the magnitude and temperature dependence of k_3. The temperature dependence of k_3 values obtained from Figure 2 was analyzed by eqs 1 and 2 (Figure 4). For the reaction between dithionite-reduced MADH and amicyanin, $\Delta G°$ is -3.0 kJ mol^{-1} (Brooks & Davidson, 1994b). We have assumed the same value for the analysis of the reaction with substrate-reduced MADH. Although this may not be correct, it is a reasonable approximation and, as noted previously (Brooks & Davidson, 1994a), the value for λ associated with this reaction is so large (>2 eV) relative to $\Delta G°$ that any error associated with our assumed value for $\Delta G°$ will not be significant.[2] In each case, reasonable fits of the data were obtained (Figure 4). The analysis of the reaction with dithionite-reduced MADH yields values for λ and H_{AB} which are consistent with the apparent k_{ET} describing an ET event (Table 1). The range of fitted values for the distance between redox centers was obtained with β values ranging from 0.7 to 1.4 Å^{-1} (Moser et al., 1992; Onuchic et al., 1992) and agrees well with the distance of 9.4 Å which is seen in the crystal structures of the ET complex (Chen et al., 1992, 1994). In contrast, the fitted Marcus parameters for the reaction of substrate-reduced MADH appear to be physically meaningless and suggest that, for that reaction, k_{ET} is not describing an ET event but some adiabatic process.

[2] For example, if one assumes that $\Delta G°$ is -9.64 kJ mol^{-1} ($\Delta E°$ of $+100$ mV rather than $+31$ mV), fits of the temperature dependence of k_3 to eq 1 yield values for H_{AB} and λ of 23 000 cm^{-1} and 3.66 eV, respectively. Alternatively, if one assumes that $\Delta G°$ is $+9.64$ kJ mol^{-1} ($\Delta E°$ of -100 mV), values for H_{AB} and λ of 22 400 cm^{-1} and 3.26 eV, respectively, are obtained. These fitted values are nearly identical to those given in Table 1, and in each case, a fit statistically similar to the one shown in Figure 4B was obtained. Thus, even given some uncertainty in the value of $\Delta G°$ for the reaction of substrate-reduced MADH, this reaction clearly yields values of $H_{AB} \gg 80$ cm^{-1}, $\lambda \gg 2.0$ eV, and $d < 0$ Å (eq 2) which are diagnostic for a gated reaction. It should be also noted that, while these fitted parameters that are obtained from the temperature dependence are relatively insensitive to $\Delta G°$, changes in $\Delta G°$ can significantly affect k_{ET}. For example, given the values for H_{AB} and λ in Table 1, if $\Delta G°$ were increased from -3.0 to -9.64 kJ mol^{-1} at 30 °C, then, according to eq 1, k_{ET} for the reaction of substrate-reduced MADH would increase from 275 to 1006 s^{-1}.

Table 1: Thermodynamic Parameters for Electron Transfer from Dithionite- and Substrate-Reduced MADH to Amicyanin

parameter[a]	TTQ redox form	
	quinol	aminoquinol
ΔS^* (J/mol K)	−32	+71
ΔH^* (kJ/mol)	+59	+82
λ (eV)	2.41	[3.53][b]
H_{AB} (cm^{-1})	20.2	[22 850]
r (Å)[c]	7.4−12.1	[<0][d]

[a] These parameters were determined from the k_3 values obtained from analysis of the data shown in Figure 2. ΔH^* and ΔS^* were determined from fits of that data to eq 6, λ and H_{AB} were determined from fits to eq 1, and r was determined from fits to eq 2. [b] Brackets indicate that these parameters are not physically meaningful because the formalism used to analyze the data may be inappropriate. [c] The range of values for r is for fits of the data to eq 2 using a range of β values from 0.7 to 1.4 $Å^{-1}$. [d] Fitted values of r equal to −8.9 and −2.9 Å were obtained, respectively, using β values of 0.7 and 1.4 $Å^{-1}$.

Thus, application of eqs 1 and 2 to this reaction is inappropriate for obtaining values of λ and H_{AB} but useful in that it suggests the possibility that the reaction is gated (see Figure 3).

A potential complication which must be considered when examining the temperature dependence of ET rate constants is that $\Delta G°$ may be temperature dependent. This could arise from temperature dependent changes in the conformation of MADH which affect its redox potential. Given the relatively large λ, any changes in $\Delta G°$ with temperature would have to be substantial to compromise our analysis.[2] Fortunately, MADH and amicyanin are quite thermally stable toward denaturation and the K_d for the MADH−amicyanin complex does not vary over this range of temperatures (Brooks & Davidson, 1994a). Thus, the likelihood of significant temperature-dependent changes in $\Delta G°$ is small.

These temperature dependence data were also analyzed by transition state theory, which provides information about the formation of the activated complex which leads to the conversion of reactants into products in an adiabatic reaction. For reactions occurring in solution, a specialized form of transition state theory known as the Eyring equation (eq 6) describes the temperature dependence of a reaction rate. In

$$\ln\left(\frac{k_3 h}{k_B T}\right) = \frac{-\Delta H^*}{RT} + \frac{\Delta S^*}{R} \qquad (6)$$

eq 6, h is Planck's constant, R is the gas constant, T is temperature, k_B is Boltzman's constant, ΔH^* is activation enthalpy, and ΔS^* is activation entropy. Each data set was fit by eq 6, and the fitted parameters are given in Table 1.

An adiabatic process is a reaction which proceeds to completion every time the transition state energy is achieved and is most appropriately described by transition state theory. Conversely, in a nonadiabatic process, achievement of the transition state energy does not always lead to product formation. The probability that the achievement of the activation energy leads to the formation of product is less than unity and approaches 0 as H_{AB} approaches 0. If the actual ET step is truly rate-limiting for the experimentally determined k_{ET}, then H_{AB} should be less than 80 cm^{-1}. This nonadiabatic limit is calculated from the dynamic relaxation rate of water and occurs when the reorganization of water, as the bulk solvent, becomes rate-limiting (Winkler & Gray, 1992). Long range ET reactions are nonadiabatic and have

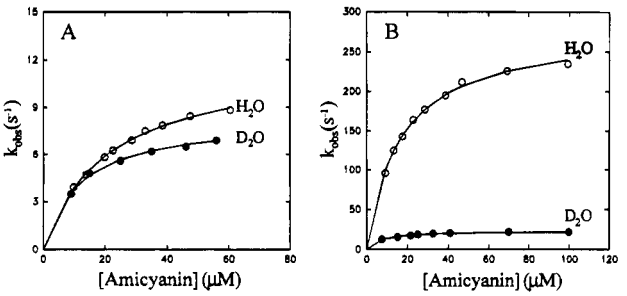

FIGURE 5: Concentration dependence of k_{obs} for the reactions of (A) dithionite-reduced MADH and (B) substrate-reduced MADH with amicyanin in buffered H_2O (○) and D_2O (●). The solid lines represent the fits of these data to eq 3.

questionable reaction coordinates. Because they occur within an electron transfer complex, they are collisionless. As such, the standard activation entropy and enthalpy changes are best described in terms of λ and H_{AB}. This is the case for the reaction of dithionite-reduced MADH. Conversely, the value of H_{AB} of 22 850 for the reaction of the substrate-reduced MADH is diagnostic of a reaction which is rate-limited by an adiabatic process. This is also dramatically indicated by the fitted value for the ET distance which is a negative number. Thus, analysis of these thermodynamic data by ET theory strongly implies that the ET reaction from substrate-reduced MADH to amicyanin is a gated process. As such, the parameters derived from eqs 1 and 2 for this reaction are inappropriate, and instead, the temperature dependence of this reaction is best described by ΔH^* and ΔS^* determined from analysis by eq 6.

Solvent Kinetic Isotope Effects. To gain insight into the nature of the process which gates the ET from substrate-reduced MADH to amicyanin, the reactions of reduced MADH with oxidized amicyanin were examined (Figure 5) in buffered deuterium oxide (D_2O). Solvent kinetic isotope effects, unlike substrate kinetic isotope effects, are global in nature. Incubation of an enzyme in D_2O leads to a multitude of isotopic exchanges within the enzymatic framework. By performing the reactions in H_2O and D_2O, a kinetic solvent isotope effect (KSIE = $^{H_2O}k/^{D_2O}k$) can be determined. A primary KSIE (5−10) is only observed when a hydrogen nucleus is transferred in the rate-limiting step. Interpretation of KSIEs for enzyme-catalyzed reactions may be complicated by changes in the protein caused by exchange of all ionizable protons with deuterons. The observed KSIE may reflect changes in the ionization behavior of the active form of the enzyme or substrate which will yield a KSIE of 3−4. Secondary effects, defined as those involving conformational changes in the enzyme caused by changing the properties of hydrogen bonds, hydrophobic bonds, and other factors will yield a KSIE of less than 2.0 and do not involve hydrogen transfer in the rate-limiting step (Schowen & Schowen, 1982).

For the reaction of dithionite-reduced MADH, a relatively small secondary KSIE on k_3 of 1.5 was observed (Figure 5A). This is consistent with k_3 describing an ET event, which would not be expected to exhibit a primary KSIE. Under the same conditions, the reaction between substrate-reduced MADH and amicyanin exhibited a KSIE on k_3 of 12.2 (Figure 5B). If it is assumed that the same secondary effects which are present in the reaction with dithionite-reduced MADH are also present in the reaction with substrate-reduced MADH, then correction for those secondary effects yields a

corrected value of 8.1, consistent with a KSIE normally observed for primary kinetic deuterium isotope effects. This is consistent with k_3 for the reaction of substrate-reduced MADH describing an adiabatic process which gates ET and indicates that the rate-limiting process involves the transfer of an exchangeable proton.

The term gating has been used to describe an ET reaction which is rate-limited by an adiabatic process such as a protein conformational change or solvent reorganization (Hoffman & Ratner, 1987). It is also possible that the reaction may be kinetically gated by a catalytic step such as product release or proton transfer. For example, in xanthine oxidase, it has been shown that the rate of intramolecular ET between FAD and an iron−sulfur center is linked to protonation/deprotonation (Hille, 1991). Our results suggest that the intermolecular ET reaction from aminoquinol MADH to amicyanin is catalytically gated by protonation/deprotonation.

Conclusion. The difference in the rate-limiting step for the ET reactions from dithionite- and substrate-reduced MADH to amicyanin may be explained by one of two possibilities. The presence of the amino group on TTQ may require an additional step to precede ET which is not needed with the quinol, and which gates the reaction. Alternatively, both the quinol and aminoquinol forms of TTQ may require the same prerequisite step for ET, but the relative rates of k_c and k_{ET} (see Figure 3) may be different for the two TTQ forms.

It is interesting to note that the limiting value of k_{obs} for the reaction of substrate-reduced MADH is actually faster than that for dithionite-reduced MADH, despite the fact that the former is a gated reaction. Thus, it is possible that k_c may be the same for the two TTQ forms, but because k_{ET} is much faster for the aminoquinol, the overall reaction becomes gated by k_c. The faster k_{ET} for the aminoquinol could be due to differences in electronic or structural properties relative to the quinol which are caused by the modification of TTQ by substrate. This could cause a decrease in λ or increase in H_{AB} for the true k_{ET}, which cannot be directly measured because the reaction is gated. It could also alter the redox potential of TTQ sufficiently to increase k_{ET} without significantly altering the Marcus parameters for the reaction.[2] Further experiments are in progress to help to further characterize this phenomenon.

Analysis of the temperature dependence of the ET reactions from dithionite- and substrate-reduced MADH to amicyanin indicates that modification of TTQ by substrate changes the rate-limiting step for ET. The ET reaction from dithionite-reduced MADH to amicyanin appears to be either a true or coupled ET and is best described by ET theory. The ET reaction from substrate-reduced MADH to amicyanin appears to be linked to the protonation state of a critical ionizable species and is best described by transition state theory. These results demonstrate that the application of ET theory to a thermodynamic analysis of an ET reaction not only may provide valid estimates for λ and H_{AB} for ungated ET but also may be used as a diagnostic tool to identify gated ET reactions (see Table 1). These results will help to establish criteria for classifying long range intermolecular ET reactions between proteins and illustrate a methodology for characterizing the nature of the processes which influence the observed rates of ET reactions.

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