Catalytic Role of Monovalent Cations in the Mechanism of Proton Transfer Which Gates an Interprotein Electron Transfer Reaction†

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ABSTRACT: Within the methylamine dehydrogenase (MADH)−amicyanin protein complex, long range intermolecular electron transfer (ET) occurs between tryptophan tryptophylquinone (TTQ) of MADH and the type I copper of amicyanin. The reoxidations of two chemically distinct reduced forms of TTQ were studied, a quinol (O-quinol) generated by reduction by dithionite and the physiologically relevant aminoquinol (N-quinol) generated by reduction by methylamine. The latter contains a substrate-derived amino group which displaces the C6 carbonyl oxygen on TTQ. ET from N-quinol MADH to amicyanin is gated by the transfer of a solvent exchangeable proton [Bishop, G. R., & Davidson, V. L. (1995) *Biochemistry 34*, 12082−12086]. The factors which influence this proton transfer (PT) reaction have been examined. The rate of PT increases with increasing pH and with increasing salt concentration. The salt effect is due to specific monovalent cations and is not a general ionic strength effect. The rate enhancements by pH and cations do not reflect an elimination of the PT step that gates ET. Over the range of pH from 5.5 to 9.0 and with cation concentrations from 0 to 200 mM, the observed rate of the redox reaction is still that of PT. This is proven by kinetic solvent isotope effect studies which show that a primary isotope effect persists even at the highest values of pH and cation concentration. A model is presented to explain how specific cations contribute to catalysis and influence the rate of PT in this reaction. The pH dependence is attributed to an ionizable group that is involved in cation binding. The effect of the cation is stabilization of a negatively charged reaction intermediate that is formed during the deprotonation of the N-quinol, and from which rapid ET to the copper of amicyanin occurs. The relevance of these findings to other enzymes which exhibit reaction rates that are influenced by monovalent cations is also discussed.

Many redox reactions of intermediary metabolism depend upon long range electron transfer (ET)[1] reactions between donor and acceptor sites. These ET reactions are often linked to the movement of a counterionic proton for the purpose of maintaining charge neutrality (eq 1).

$$AH_{red} \rightleftharpoons A_{ox} + e^- + H^+ \quad (1)$$

Coupled ET and proton transfer (PT) reactions are critical for establishment of an electrochemical gradient which drives the synthesis of ATP during the reactions of oxidative phosphorylation. The molecular mechanisms which link redox and acid−base chemistries in enzymatic systems remain mysterious. This paper describes a process whereby deprotonation of a donor site gates the interprotein transfer of an electron to its physiologic acceptor.

During the reductive half-reaction of its catalytic cycle, methylamine dehydrogenase (MADH) (Davidson, 1993) oxidizes methylamine to formaldehyde and enzyme-bound ammonia (Figure 1A). This mechanism involves covalent amino adduct formation at C6 of the tryptophan tryptophylquinone (TTQ) (McIntire *et al*., 1991) cofactor by an aminotransferase-type mechanism (Davidson *et al*., 1995; Brooks *et al*., 1993; Bishop *et al*., 1996a). Thus, the physiologic reduction of MADH in the absence of oxidant yields a two-electron-reduced N-quinol form of TTQ (Figure

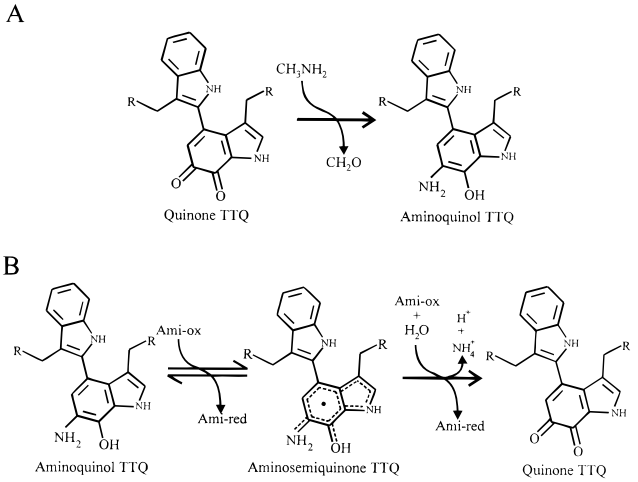

FIGURE 1: Reductive and oxidative half-reactions of MADH. (A) Reduction of the quinone by methylamine results in covalent modification of C6 of TTQ by substrate N which forms the aminoquinol. (B) Reoxidation of the aminoquinol occurs by sequential one-electron transfers to amicyanin (Ami-ox) and proceeds through an aminosemiquinone intermediate.

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[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; ET, electron transfer; PT, proton transfer; O-quinol, fully reduced TTQ with oxygen at C6; O-semiquinone, semiquinone TTQ with oxygen at C6; N-quinol, fully reduced TTQ with nitrogen bonded to C6; N-semiquinone, semiquinone TTQ with nitrogen bonded to C6; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; KSIE, kinetic solvent isotope effect.

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1A). Alternatively, TTQ can be chemically reduced in a nonphysiologic reaction by dithionite to yield an O-quinol form in which oxygen rather than nitrogen is bonded to the C6 carbon (Husain *et al*., 1987). Each reduced form of TTQ may be reoxidized by the type I copper protein amicyanin, the physiologic electron acceptor of MADH (Figure 1B). In vivo, amicyanin transfers the methylamine-derived electrons to a *c*-type cytochrome (Husain & Davidson, 1985). The crystal structure of a complex of MADH, amicyanin, and a physiologic electron acceptor cytochrome c_{551i} has been described (Chen *et al*., 1994).

Since TTQ is a two-electron donor and amicyanin is a one-electron acceptor, the complete oxidations of the N- and O-quinol to the quinone each require two one-electron transfers to 2 equiv of amicyanin (Figure 1B). It was possible to characterize the one-electron-reduced N-semiquinone (Warncke *et al*., 1993; Bishop *et al*., 1996b) and O-semiquinone intermediates (Warncke *et al*., 1995) which are formed following the first oxidation step by amicyanin of the N- and O-quinol TTQ, respectively. Thermodynamic and kinetic solvent isotope effect (KSIE) experiments showed that the rate of the first ET from the N-quinol to amicyanin is catalytically gated by a rate-limiting adiabatic PT reaction (Bishop & Davidson, 1995). This is in contrast to the oxidation of the O-quinol by amicyanin where the ET step was shown to be rate-limiting for the overall redox reaction (Brooks & Davidson, 1994a,b; Bishop & Davidson, 1995).

This paper presents results from experiments that investigate the nature of the rate-limiting PT step which gates ET from N-quinol TTQ of MADH to amicyanin. We show that the rate of PT is attenuated by both pH and monovalent cations and that the effects of pH and cations are distinct. KSIE studies demonstrate that PT is rate-limiting for the ET reaction over a range of pH from 5.5 to 9.0 and a wide range of cation concentrations. We show that the pH dependence of the observed reaction reflects the pK_a of an active site residue whose protonation state influences the binding of monovalent cations. The role of the monovalent cation is proposed to involve the stabilization of a negatively charged intermediate which is formed during deprotonation of the N-quinol amino group, whose pK_a is out of the range of pH studied. Monovalent cations are known to influence the catalytic reaction rates of a large number of enzymes (Suelter, 1970). These results demonstrate a novel role of monovalent cations in the activation of a long range interprotein ET reaction and provide insight into the general mechanisms which couple proton and electron transfer events.

EXPERIMENTAL PROCEDURES

Purifications of MADH (Davidson, 1990) and amicyanin (Husain & Davidson, 1985) from *Paracoccus denitrificans* (ATCC 13543) were as previously described. Protein concentrations were calculated from known extinction coefficients (Husain & Davidson, 1985; Husain *et al*., 1987). Fully reduced MADH was prepared anaerobically by spectrophotometric titration with either methylamine hydrochloride or sodium dithionite. Both reduced forms were stable at room temperature in the presence of atmospheric oxygen. Experiments were performed at 21 °C in 10 mM potassium phosphate (KP_i) at pH 7.5 in the presence or absence of added salts. When Ca^{2+} was present, 10 mM Hepes at pH 7.5 was used to avoid precipitation with phosphate. Buffered D_2O (99.9%, CDN Isotopes) solutions were prepared according to Schowen and Schowen (1982). To examine the pL (L = H or D) dependence of the KSIE, buffers with specific pD were prepared by mixing stock solutions of deuterated mono- and dibasic 10 mM KP_i in the presence of 0, 100, and 200 mM added KCl. Titrations of pD were monitored using a standard pH electrode presoaked in D_2O, and correction was made for the effect of D_2O on the electrode response. For solutions which contained protein, H_2O was completely exchanged for D_2O by repeated ultrafiltration using Amicon centripreps. After solvent exchange, protein solutions were incubated overnight in buffered D_2O at 15 °C to ensure the complete exchange of all solvent-exposed titratable H^+ for D^+.

Kinetic measurements were made using an On-Line Instrument Systems (OLIS) RSM1000 stopped-flow spectrophotometer. MADH was the limiting reactant with its concentration fixed between 1 and 4 mM. Pseudo-first-order conditions were maintained so that the concentration of amicyanin was much greater than that of MADH and well above the K_d for complex formation at each pH and salt concentration. Reactions were monitored between 330 and 490 nm where each of the redox forms of TTQ exhibits a different visible absorbance maximum. The absorbance maximum of amicyanin is at 595 nm, and because of its low extinction coefficient relative to that of MADH, redox-linked changes in amicyanin absorbance do not contribute significantly to the absorbance changes of oxidized, semiquinone, and reduced MADH (Davidson *et al*., 1995). Spectra of O- and N-quinol forms of MADH are essentially identical. The spectra of O-semiquinone and N-semiquinone appear to be similar and each exhibits a maximum absorbance centered at 420 nm (Davidson *et al*., 1995; Bishop *et al*., 1996b).

Kinetic data collected in the rapid-scanning mode were reduced by factor analysis using the singular value decomposition algorithm and then globally fit by the Levenberg and Marquardt nonlinear method of least squares using the fitting routines of the OLIS Global Fit software. Details of the method of data analysis have been described previously (Bishop *et al*., 1996b). Analysis of the sum of the data collected in this study revealed that, for the reaction of N-quinol with oxidized amicyanin, the rate of conversion of N-quinol to N-semiquinone was best analyzed globally between 360 and 443 nm. In contrast, the spectral changes which are observed in the wavelength ranges between 330 and 360 nm, and between 450 and 490 nm, also contain contributions from the subsequent oxidation step in which N-semiquinone is converted to quinone [see Figure 3 in Bishop *et al*. (1996b)]. On the basis of previous studies of the dependence on salt and pH of the absorption spectrum of oxidized MADH (Kuusk & McIntire, 1994; Gorren & Duine, 1994), one would expect the kinetics of the absorbance changes to be biphasic at all wavelengths at high pH in the presence of high salt concentrations. This may be true; however, at pH 9.0 in the presence of 0.2 M KCl, the rate of reaction becomes too fast to measure. The same was true for reactions in the presence of NH_4Cl (Table 1). Under reaction conditions which approach the point where salt-dependent changes in the oxidized spectrum of MADH become significant (e.g., pH 8.5 + 0.2 M KCl and pH 9.0 + 0.1 M KCl), the combination of relatively fast reaction rates and small amplitudes makes it difficult to distinguish biphasic reactions from monophasic reaction. Therefore, under all reaction conditions, the spectral changes over the

Table 1: Effect of Cations on the Reactions of O-Quinol and N-Quinol MADH with Amicyanin

buffer	k_{obs} (s^{-1}) O-quinol	k_{obs} (s^{-1}) N-quinol
10 mM KP_i at pH 7.5	14.0 ± 0.1[a]	42 ± 6
+ 0.2 M KCl	12.1 ± 0.3	144 ± 18
+ 0.4 M KCl	11.6 ± 0.5	480 ± 130
+ 0.2 M NaCl	12.3 ± 0.4	246 ± 74
+ 0.2 M CsCl	16.7 ± 0.6	165 ± 31
+ 0.1 M NH_4Cl	7.0 ± 1.6	>1000
+ 0.2 M NH_4Cl	9.6 ± 0.3	>1000
+ 0.4 M NH_4Cl	11.4 ± 0.5	>1000
+ 0.2 M $(CH_3CH_2CH_2CH_2)_4NCl$	15.9 ± 0.4	52 ± 17
10 mM HEPES at pH 7.5	9.0 ± 0.3	0.60 ± 0.01
+ 0.2 M $CaCl_2$	nd[b]	1.2 ± 0.1
+ 0.4 M $CaCl_2$	nd	0.8 ± 0.1
10 mM KP_i		
at pH 5.0 + 0.2 M KCl	10.6 ± 1.7	5 ± 0.3
at pH 7.5 + 0.2 M KCl	12.0 ± 2.6	144 ± 18
at pH 9.0 + 0.2 M KCl	11.1 ± 2.3	>1000

[a] Observed rates are means of global fits from multiple experiments and are presented ± one standard deviation. [b] nd, not determined.

range of 360−443 nm were globally fit best by the equation for a single-exponential decay with a non-zero background.

Addition of KCl over the range of concentrations used has minimal effects on the spectra of oxidized, semiquinone, and reduced MADH at pH values of <9. At pH 9.0, significant spectral perturbations have been reported at the higher salt concentrations for oxidized MADH (Kuusk & McIntire, 1994; Gorren & Duine, 1994; Davidson *et al*., 1995). Mixing experiments were performed with equal salt in each syringe. This ensured that the spectral changes which were monitored were those that described the redox reaction in the presence of the salt, and that no salt-induced changes in the starting aminoquinol spectrum occurred during the course of the experiment. Cation binding would be expected to be in rapid equilibrium and, therefore, would not effect the observed rates of reaction but may influence the amplitude of the spectral change. This is consistent with our observation of monophasic kinetics for the conversion of aminoquinol to aminosemiquinone.

RESULTS

Analysis of Transient Kinetic Data. The oxidative half-reaction of the catalytic cycle of MADH proceeds by two sequential one-electron transfers to two redox equivalents of amicyanin (Figure 1B). The first ET step from N-quinol TTQ to the Cu^{2+} of amicyanin is irreversible and rate-limited by adiabatic PT (Bishop & Davidson, 1995) (eq 2).

$$MH_{red} + A_{ox} \overset{K_d}{\rightleftharpoons} MH_{red}A_{ox} \underset{k_{-PT}}{\overset{k_{PT}}{\rightleftharpoons}} M_{red}{*}A_{ox} \underset{k_{-ET}}{\overset{k_{ET}}{\rightleftharpoons}} M_{semi}A_{red} \quad (2)$$

In this model, MH_{red}, M_{red}*, and M_{semi} are protonated N-quinol MADH, unprotonated N-quinol MADH (activated for ET), and N-semiquinone MADH, respectively. A_{ox} and A_{red} are oxidized and reduced amicyanin, respectively. Since PT rate limits ET (i.e., $k_{ET} \gg k_{PT}$) (Bishop & Davidson, 1995) and all reaction steps beyond k_{ET}/k_{-ET} are invisible at the wavelengths being monitored, k_{obs} will not be influenced by any reaction steps after k_{PT}/k_{-PT}. Thus, data for the dependence of k_{obs} on amicyanin concentration may be fit by eq 3 (Strickland *et al*., 1975).

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

In each case, random residuals of the fits and low standard deviations of the fitted parameters were obtained. In each case, the fitted curve for plots of k_{obs} versus [amicyanin] passed through the origin, which is indicative of irreversible PT. This is true when $k_{ET} \gg k_{-PT}$. Once K_d was determined for a given set of reaction conditions, it was possible to collect kinetic data at a single saturating amicyanin concentration. Since PT is irreversible, when [amicyanin] $\gg K_d$, eq 3 can be reduced to $k_{obs} = k_{PT}$. In these cases, values of k_{PT} are taken directly from global fits of the reduced raw spectral and kinetic data at saturating amicyanin concentrations.

Dependence of the Rates of O- and N-Quinol Oxidation on Monovalent Cations. Added salts significantly enhance the rate of reaction of N-quinol MADH with amicyanin but have essentially no effect on the reaction of O-quinol MADH (Table 1). This is consistent with the fact that k_{obs} for the former describes a PT reaction whereas for the latter it describes an ET reaction (Bishop & Davidson, 1995). It is also of interest given reports that monovalent cations bind at the active site of oxidized MADH (Kuusk & McIntire, 1994; Gorren & Duine, 1994; Moenne-Loccoz *et al*., 1996). These results suggest that the binding of monovalent cations plays a critical role in the mechanism of PT which regulates the rate of ET from N-quinol TTQ to amicyanin. To determine if this effect is cation-dependent, or a general ionic strength effect, we examined the transient rates of the one-electron oxidations of O- and N-quinol MADH by amicyanin in the absence and presence of varying concentrations of several different salts at pH 7.5 (Table 1). The fact that addition of $CaCl_2$ and addition of $(CH_3CH_2CH_2CH_2)_4NCl$ give essentially no rate enhancement indicates that this is not a general ionic strength effect. Replacement of chloride salts with phosphate salts also had no significant effect (data not shown), indicating that the effects on rate are cation-specific. The lower rates in HEPES buffer compared to those in KP_i buffer may be attributed to the K^+ present in the latter. The rate enhancement by NH_4^+ is much greater than that by alkali metal cations. Possible reasons for this are addressed in the Discussion.

Dependence of the Rates of O- and N-Quinol Oxidation on pH. Given that specific monovalent cations influence the rate of N-quinol TTQ oxidation but not that of O-quinol, the nature of this rate enhancement was further investigated. The reaction of N-quinol MADH with amicyanin was examined at different concentrations of KCl at pH values from 5.5 to 9.0 (Figure 2). It is evident that increasing pH and salt increase the rate of N-quinol oxidation. In contrast to these results, the reaction of O-quinol MADH with amicyanin does not exhibit any significant pH or salt dependence (Table 1). The absence of an effect on the reaction with the O-quinol provides assurance that the effects observed with the N-quinol are not the result of pH-dependent changes in the copper center of amicyanin, or general nonspecific effects on either protein. The pH-dependent effects relate specifically to the substrate-modified TTQ cofactor and its active site environment. When the salt

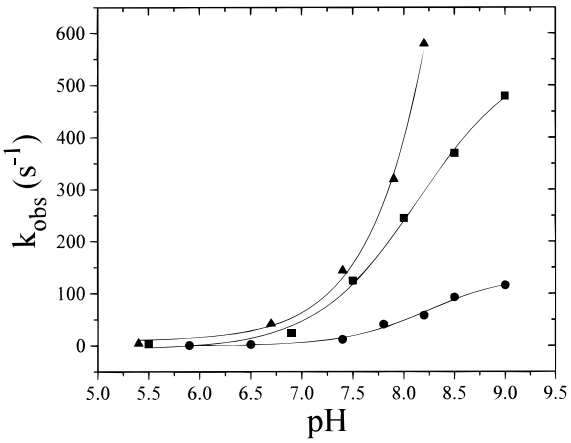

FIGURE 2: Effects of increasing [KCl] on the pH−rate profile for the reaction of aminoquinol MADH with amicyanin. Experiments were performed in 10 mM KP_i in the presence of 0 mM KCl (●), 100 mM KCl (■), and 200 mM KCl (▲). Fits of these data by the Boltzmann equation[2] are shown as solid lines.

dependence of the pH data is fit by a sigmoidal function,[2] a good fit is observed whose midpoint is taken as a crude approximation of the pK_a of the functional group responsible for the observed pH dependence. In 10 mM KP_i buffer, a minimum estimated value[2] of pK_a is 8.1 ± 0.3. It is difficult to compare the apparent pK_a values at different concentrations of added salt because the rates become too fast to accurately measure as the pH optimum is approached.

An important point not resolved by these data is whether this apparent pK_a corresponds to the actual acid−base couple which undergoes PT or if it is due instead to a group which plays an indirect role in PT. If the pH−rate profile is representative of the actual groups which protonate−deprotonate in the reaction coordinate, then PT would not be expected to be rate-limiting for the redox reaction at high pH (i.e., at pH > pK_a, ET would no longer be gated by the deprotonation event). However, if PT is rate-limiting at high pH, then it can be concluded that the pK_a of the acid−base couple which undergoes rate-limiting PT is greater than pH 9.0 and that the pH dependence is related to a different phenomenon, such as cation binding.

Kinetic Solvent Isotope Effect Studies. To gain insight into the precise mechanism of the cation and pH dependence of the PT which gates ET during the reaction from N-quinol MADH to amicyanin, KSIE studies were performed over a range of pL and K^+ concentrations (Figure 3). When rates obtained for the reactions in H_2O and D_2O are plotted against pL, a sigmoidal dependence is observed around an apparent pK_a and fits of the pL-dependent region yield approximate pK_a values[2] of 8.1 ± 0.3 and 8.3 ± 0.2 in H_2O and D_2O, respectively. Thus, replacement of H_2O with D_2O shifts the pK_a of the ionizable group by approximately 0.2 pL unit.

[2] All pH−rate profiles were fit by the Boltzmann equation (presented below) which describes a general sigmoidal curve and is, therefore, model-independent: $y = (y_{initial} - y_{final})/[1 + \exp[(x - x_{0.5})/x_{width}]] + y_{final}$. When fitting pH−rate data with this equation, the y axis is the observed rate constant (k_{obs}) and the x axis is pH. The center of the curve ($x_{0.5}$) was taken to approximate the apparent pK_a; however, it should be recognized that it was not possible to go to high enough pH to completely reach the plateau of the pH−rate profile in any of the reactions measured. This is due to enzyme instability at pH >9.0 and the fact that some reaction rates at high pH and salt concentrations are too fast to detect by stopped-flow (i.e., >500 s^{-1}). Given these limitations, the pK_a values derived from these fits should be considered minimum pK_a values. For the same reasons, the KSIE observed at the highest pL should also be considered a minimum value.

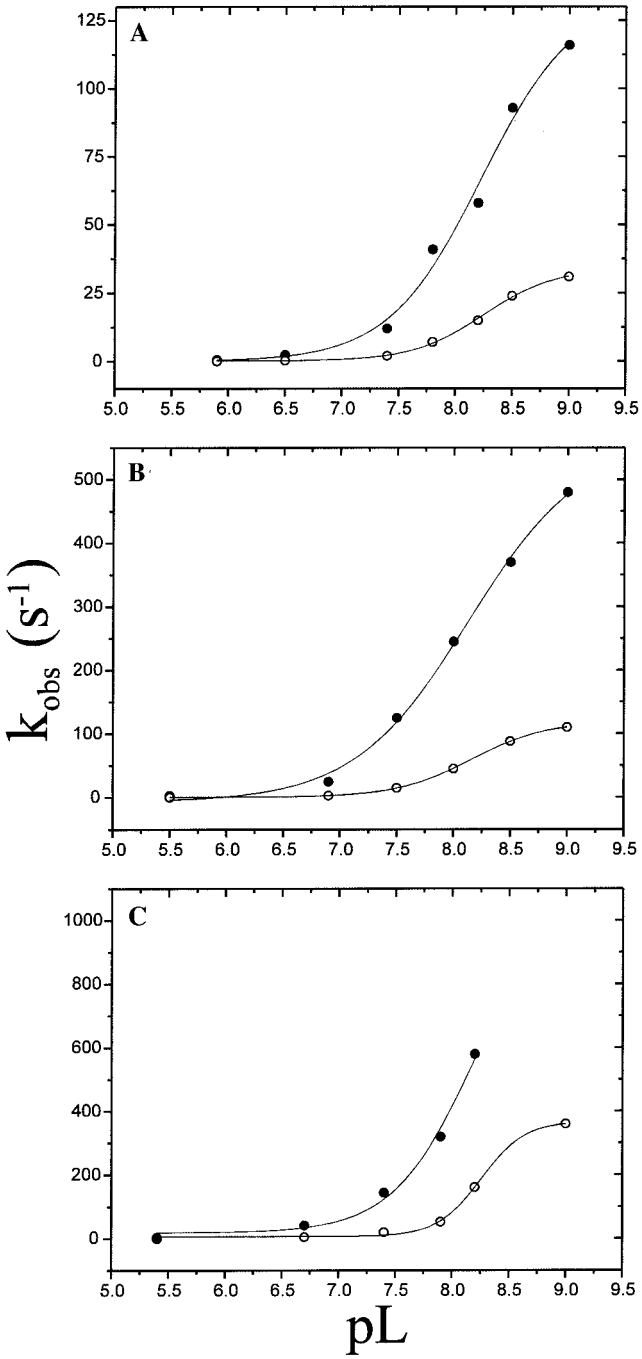

FIGURE 3: Effect of [KCl] and pL on the kinetic solvent isotope effect for the reaction of aminoquinol MADH with amicyanin. Experiments were performed in 10 mM KP_i in the presence of 0 mM KCl (A), 100 mM KCl (B), and 200 mM KCl (C) in buffered H_2O (●) and D_2O (○). Sigmoidal fits[2] are shown as solid lines.

Similar KSIEs are observed at each concentration of K^+ at the corresponding pL (Figure 3). Over the entire range of pHs, primary KSIEs are observed (Figure 4). In the pH-independent region below pL 7.0, the KSIE is approximately 8.0. At the highest pL values, a minimum KSIE of approximately 4.0 persists.[2] In the presence of high salt concentrations at high pH, it becomes difficult to determine this value precisely since the rates in H_2O exceed 500 s^{-1} and become too fast to detect. This means that the KSIE values at the highest pH and salt concentrations may be inaccurate but, if so, will be underestimates. These results confirm that the KSIE measured at an equivalent pL for each of these reactions approximates an actual KSIE and is not an artifact due to comparison of nonequivalent pL values

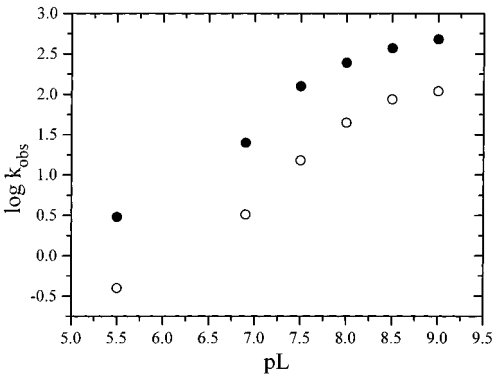

FIGURE 4: Log plot of the effect of [KCl] and pL on the kinetic solvent isotope effect for the reaction of aminoquinol MADH with amicyanin. Experiments were performed in 10 mM KP_i in the presence of 100 mM KCl. These are the same data presented in Figure 3B. By plotting log *k*, the persistance of a primary isotope effect at all pL values is evident.

(Schowen & Schowen, 1982). These data demonstrate that the movement of a solvent exchangeable proton in the reaction coordinate is rate-limiting over the entire range of pL and cation concentrations, and that increasing cation concentration increases the rate of this PT step proportionally in H_2O and D_2O. An alternative mechanism that involves rapid equilibrium protonation may be ruled out because such reactions would give rise to an inverse solvent isotope effect (Schowen & Schowen, 1982). That PT is still rate-limiting in regions of the pL−rate profile, where the rates in H_2O and D_2O are insensitive to solution pL, confirms that the observed pK_a does not reflect the acid−base couple which gates ET, but another group whose protonation state influences the reaction and whose deprotonation is not rate-limiting at high pL. KSIEs obtained at high pL may be representative of the actual isotope effect associated with the group undergoing rate-limiting PT, whereas the larger values obtained at lower pL may include contributions from other isotopically sensitive steps.

DISCUSSION

These results demonstrate that monovalent cations and pH enhance the rate of PT which gates ET from N-quinol MADH to amicyanin. Results from KSIE experiments demonstrate that PT is rate-limiting for N-quinol oxidation and, therefore, gates ET under each of the solution conditions monitored. The effects of pH and cations are distinct, and neither enhances the rate of the redox reaction by removing the gate to ET. KSIE experiments demonstrate that the groups which protonate−deprotonate in the reaction coordinate have a pK_a of >9.0. The dependence of the rate of N-quinol oxidation on monovalent cations and pH is in striking contrast to that of the oxidation of O-quinol MADH by amicyanin. The rate of O-quinol oxidation is independent of salt and pH and agrees with our previous conclusion that this reaction is not gated, but rate-limited by ET.

The active sites of MADH from *Thiobacillus versutus* and bacterium W3A1 were shown to bind ammonium and metallic monovalent cations with varying affinities (Kuusk & McIntire, 1994; Gorren & Duine, 1994; Gorren *et al*., 1995). Kuusk and McIntire (1994) proposed two cation binding sites in close proximity to TTQ in MADH from W3A1: a type I or substrate-binding site which binds $(CH_3)_3NH^+$ and $(CH_3)_4N^+$ and a type II or cation-binding site which binds K^+ and Na^+. Binding of NH_4^+ to the type I site was proposed to be a prelude to binding to the cofactor which gives rise to spectral changes. Subsequently, Moenne-Loccoz *et al*. (1996) concluded on the basis of resonance Raman data that NH_4^+ effects were due not to covalent modification of TTQ but to binding near TTQ. Gorren *et al*. (1995) have also shown that NH_4^+ effects the properties of the MADH semiquinone and concluded that it was due to binding near TTQ. The more pronounced effects of ammonium relative to alkali metal cations presented in Table 1 may reflect such a differential specificity for ammonium and metallic cations at the active site.

Gorren *et al*. (1995) reported that monovalent cations influenced the rate of oxidation of methylamine-reduced MADH from *T. versutus* by amicyanin. Those data agree with our results which show that monovalent cations and pH affect the observed rate of oxidation of N-quinol MADH from *P. denitrificans*. However, that report attributed the influence of monovalent cations on the rate of oxidation of methylamine-reduced MADH to a change in the reorganizational energy associated with the ET reaction. We showed here and previously (Bishop & Davidson, 1995; Bishop *et al*., 1996b) that the rate-limiting step for the oxidation of N-quinol MADH of *P. denitrificans* by amicyanin is not ET but an adiabatic PT reaction. If MADH from *T. versutus* behaves similarly, then using Marcus theory (Marcus & Sutin, 1985) to interpret the results for this reaction is inappropriate since the observed rate does not describe a nonadiabatic reaction.

The data presented herein suggest that the rate enhancement caused by alkali metal cations requires a pH-dependent cation binding step to facilitate rate-limiting PT. To explain these results in the context of a mechanism of PT which gates ET to amicyanin, we propose that the effect of increasing pH is to allow the binding of an alkali monovalent cation at the active site. The active site of MADH from *P. denitrificans* is potentially very electronegative with side chain oxygens of Asp^{32}, Asp^{76}, Tyr^{119}, and Thr^{122} within 3−6 Å of the C6 carbonyl oxygen in the crystal structure of oxidized MADH (Figure 5; Chen *et al*., 1992).[3] The extended electronegative environment within the active site could feasibly exclude anions and preferentially bind and stabilize desolvated cations. Also at the active site are the peptide oxygens of residues 104, 106 (not shown in Figure 5), and 32, and the oxygen bonded to the C7 of TTQ. Tyr^{119} could also potentially participate in cation−π interactions (Dougherty, 1996). Solvent molecules are also present in the crystal structure at the active site. The C7 carbonyl appears to be H-bonded with an amide NH of the polypeptide chain and does not appear to be accessible to solvent.

We believe that the group which undergoes catalytic deprotonation is the substrate-derived amino group present at the C6 position of N-quinol MADH. The reactions of N-quinol MADH undergo PT-gated ET, whereas those of O-quinol MADH do not. The only chemical difference in these two forms of MADH is the presence of the amino group at C6 in the N-quinol that is absent in the O-quinol. The presence of the substrate-derived nitrogen most likely accounts for the change in the rate-limiting step relative to the ET reaction from the O-quinol.

A proposed mechanism for the cation- and pH-dependent deprotonation of N-quinol TTQ that gates ET from MADH

[3] The 1.75 Å crystal structure of *P. denitrificans* MADH is available in the Brookhaven Protein Data Bank (entry 2BBK).

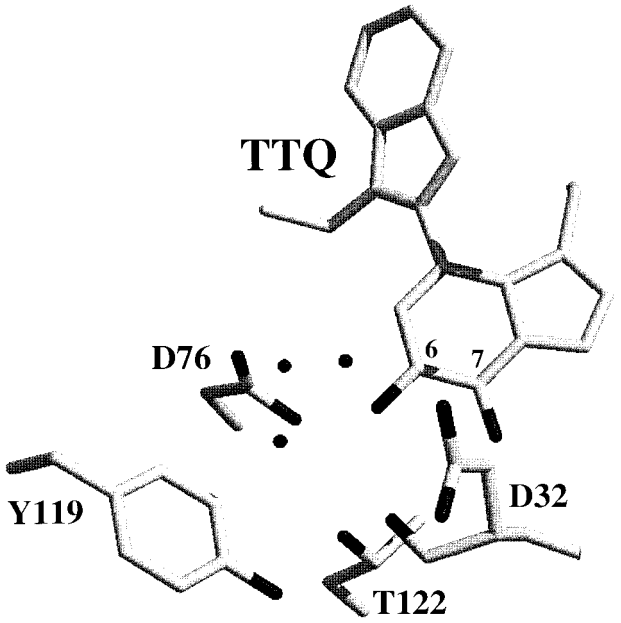

FIGURE 5: Active site structure of oxidized MADH. Carbons 6 and 7 of TTQ are labeled. Oxygen atoms are colored black. Three solvent molecules are presented as black spheres. Coordinates are available in the Brookhaven Protein Data Bank (entry 2BBK).

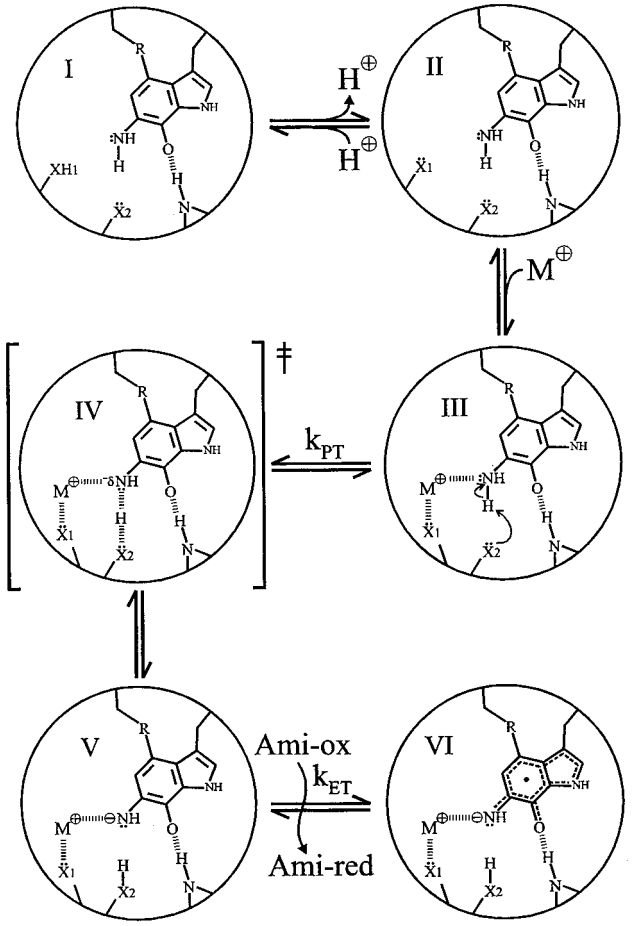

FIGURE 6: Mechanism of monovalent cation (M^+)-dependent proton transfer which gates electron transfer from aminoquinol MADH to amicyanin. Dashed lines indicate electrostatic and H bonding interactions. X_1 and X_2 are ionizable active site residues. Oxidized and reduced amicyanin are represented here as Ami-ox and Ami-red, respectively. Details of this mechanism are presented in the text.

to amicyanin is shown in Figure 6. In this model, binding of a monovalent cation (M^+) plays an essential role in deprotonating N-quinol TTQ and activating it for rapid ET to amicyanin. For M^+ to bind at the active site, an active site base (X_1) that provides a ligand close to the N-quinol nitrogen is required. The pK_a of this residue which is reflected in the pH−rate profiles (Figures 2 and 3) has a minimum value[2] of 8.1. The pH dependence of the PT rate does not describe the ionizable group from which rate-limiting PT occurs but reflects the extent of pH-dependent cation binding which subsequently facilitates the rate-limiting PT step. This explains why the KSIE persists at pL 9.0 and the ET reaction remains gated at this pH. After binding of M^+, TTQ-bound NH_2 is deprotonated by another active site residue (X_2). M^+ may interact with the NH_2 group via the lone pair of electrons on N and weaken the N−H bond to activate it for proton abstraction (III in Figure 6). The formation of a transition-state intermediate for the PT reaction with partial negative charge (IV in Figure 6) is also stabilized by M^+, as is the anionic product following PT (V in Figure 6). Following this rate-limiting deprotonation, rapid ET occurs. ET from this activated intermediate is expected to be highly favored relative to that of the neutral aminoquinol because of the relatively electron-rich character of the intermediate (V). In the absence of M^+, deprotonation of this group is much less favorable. This explains the large rate enhancement by M^+. The immediate product of the ET reaction (VI), an anionic radical may either be reprotonated by X_2H to yield an aminosemiquinone or may rearrange to an iminosemiquinone.

If the apparent pK_a values observed in Figure 3 describe an ionizable group which binds a monovalent cation, then one might expect the apparent pK_a to shift with cation concentration according to eq 4, where K_d describes the monovalent cation binding.

$$pK_a^{app} = pK_a - \log(1 + [M^+]/K_d) \qquad (4)$$

No significant shift is evident in Figure 3. Our model in Figure 6 indicates that the C6 substituent will form part of the cation binding site along with an ionizable residue. The lowest K_d value for K^+ reported in Gorren and Duine (1994) is 110 mM for cation binding at pH 9.0 to the oxidized quinone form of *T. versutus* MADH. *P. denitrificans* MADH is closely related to *T. versutus* MADH, and the active site shown in Figure 5 is very similar in *T. versutus* (Chen *et al.*, 1992). In the substrate-reduced MADH, this will be an NH_2 group rather than the quinone oxygen. One would expect the oxygen to be a much more effective ligand for the cation than the amino nitrogen and, therefore, expect the K_d value for binding of K^+ to the aminoquinol (the relevant species in this study) to be significantly higher than 110 mM at pH 9, and much higher still at lower pH where most of our data is collected. Simple inspection of the variation in rate with K^+ concentration at high pH (see Figure 2) confirms that the K_d for K^+ is relatively high. The rate increases from 125 to 500 to >1000 s^{-1} as $[K^+]$ increases from 10 to 100 to 200 mM. This being the case, the $[M^+]/K_d$ term in eq 4 is negligible under most if not all of our experimental conditions.

Certain features of our proposed mechanism (Figure 6) must be resolved with kinetic data and assumptions made earlier in this paper and in previous studies. For k_{ET} in this study to be much greater than k_{PT}, the true k_{ET} must be much faster than the k_{obs} for the ET reaction from dithionite-reduced quinol MADH to amicyanin (Table 1), which is not a gated

reaction (Brooks & Davidson, 1994a,b; Bishop & Davidson, 1995). If one uses our published values for electronic coupling (H_{AB}) which were obtained for the reaction of O-quinol with amicyanin, and calculates the maximum possible k_{ET} assuming activationless ET ($\Delta G^\circ = -\lambda$), one obtains a value of approximately 10^{10} s^{-1}. Thus, if within the activated intermediate shown in Figure 6 either ΔG° becomes significantly more negative and/or the reorganizational energy (λ) is significantly decreased, then it is possible that k_{ET} may be extremely fast relative to the reaction of the O-quinol. Our kinetic models also require that k_{ET} be greater than k_{-PT} (eqs 2 and 3). This would be difficult to accept if the pK_a of the neutral aminoquinol was comparable to those of free arylamines which are typically >20 (March, 1975). Unless the pK_a for that group is substantially lowered, the ΔpK_a for the proton donor and acceptor would be so great that the reverse reaction would be orders of magnitude greater than k_{PT}. This emphasizes the importance of the monovalent cation which may play multiple roles in facilitating this PT reaction. Stabilization of the transition state for the PT reaction (IV in Figure 6) will lower the activation energy for the reaction. Activation of the N−H bond for proton abstraction (III) and stabilization of the anionic product (V) will also cause the pK_a for this group to substantially decrease. The pK_a must still be >9 since the KSIE persists, but it need not be that much greater. This situation may be analgous to the rate-limiting deprotonation of a carbon acid, which is a common feature of many enzyme-catalyzed reactions, including that of MADH (Brooks *et al*., 1993).

Several enzymes are activated or inhibited by monovalent alkali metal cations (Suelter, 1970). However, there is relatively little known about the nature of these cation binding sites and the mechanisms of action of these monovalent cations. There are only a few reports of the crystal structures of monovalent cation-dependent enzymes with their cation binding sites identified. These include dialkylglycine decarboxylase (Toney *et al*., 1993), pyruvate kinase (Larsen *et al*., 1994), fructose-1,6-bisphosphatase (Villert *et al*., 1995), and thrombin (Di Cera *et al*., 1995). In some cases, this binding is very specific, as is seen for binding of Na^+ and K^+ to dialkylglycine decarboxylase (Toney *et al*., 1993). In the case of thrombin, it is clear that Na^+ binding is related to allosteric properties of the enzyme (Di Cera *et al*., 1995). For tryptophan synthase, Woehl and Dunn (1995) showed that monovalent cations control both the allosteric linkage between subunits and the equilibrium distribution of reaction intermediates. For tryptophanse, Suelter and Snell (1976) proposed that monovalent cations interacted at or near the active site and either participated directly in catalysis or were needed for proper alignment of functional groups. It is still unclear to what extent monovalent alkali metal cations play a direct role in enzyme-mediated catalysis, and to what extent cation binding affects catalysis indirectly by means of allosteric regulation. For MADH, our data are most consistent with a mechanism by which monovalent cations directly participate in the catalytic process.

This paper presents, for the first time, a detailed chemical reaction mechanism for an adiabatic process which gates the transfer of an electron between physiologic protein redox partners. A mechanism by which PT may be directly coupled to ET is described. A new role for monovalent cations in a biological process has also been identified and characterized, that of regulation of the rate of an interprotein ET reaction. The model proposed for that reaction (Figure 6) may also be applied to other enzyme-catalyzed reactions in which monvalent cations can be shown to facilitate otherwise unfavorable PT reactions.

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