Re-Engineering Monovalent Cation Binding Sites of Methylamine Dehydrogenase: Effects on Spectral Properties and Gated Electron Transfer†

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Received June 15, 2001; Revised Manuscript Received August 20, 2001

ABSTRACT: Methylamine dehydrogenase (MADH) is a tryptophan tryptophylquinone (TTQ)-dependent enzyme that catalyzes the oxidative deamination of primary amines. Monovalent cations are known to affect the spectral properties of MADH and to influence the rate of the gated electron transfer (ET) reaction from substrate-reduced MADH to amicyanin. Two putative monovalent cation binding sites in MADH have been identified by X-ray crystallography [Labesse, G., Ferrari, D., Chen, Z.-W., Rossi, G.-L., Kuusk, V., McIntire, W. S., and Mathews, F. S. (1998) *J. Biol. Chem. 273*, 25703−25712]. One requires cation−π interactions involving residue αPhe55. An αF55A mutation differentially affects these two monovalent cation-dependent phenomena. The apparent K_d associated with spectral perturbations increases 10-fold. The apparent K_d associated with enhancement of the gated ET reaction becomes too small to measure, indicating that either it has decreased more than 1000-fold or the mutation has caused a conformational change that eliminates the requirement for the cation for the gated ET. These results show that of the two binding sites revealed in the structure, cation binding to the distal site, which is stabilized by the cation−π interactions, is responsible for the spectral perturbations. Cation binding to the proximal site, which is stabilized by several oxygen ligands, is responsible for the enhancement of the rate of gated ET. Another site-directed mutant, αF55E MADH, exhibited cation binding properties that were the same as those of the native enzyme, indicating that interactions with the carboxylate of Glu can effectively replace the cation−π interactions with Phe in stabilizing monovalent cation binding to the distal site.

Methylamine dehydrogenase (MADH,[1] EC 1.4.99.3) is a periplasmic quinoprotein found in several methylotrophic and autotrophic bacteria (*1*, *2*). This paper describes results obtained with the MADH from *Paracoccus denitrificans*. This enzyme has an $\alpha_2\beta_2$ structure with each β-subunit possessing one covalently bound tryptophan tryptophylquinone (TTQ) prosthetic group (*2*, *3*) (Figure 1). TTQ is formed by posttranslational modification of two gene-encoded tryptophan residues, Trp57 and Trp108 in the *P. denitrificans* enzyme. TTQ participates in catalysis and electron transfer (ET). It catalyzes the oxidative deamination of primary amines to their corresponding aldehydes with release of two electrons and two protons according to eq 1.

$$RCH_2NH_3^+ + H_2O + 2A_{oxi} \rightarrow RCHO + NH_4^+ + 2A_{red} + 2H^+ \quad (1)$$

In most bacteria, including *P. denitrificans*, the electron acceptor for MADH (A in eq 1) is the blue copper protein, amicyanin (*4*). The ET reactions from *P. denitrificans* MADH to amicyanin have been extensively studied (*5*−*9*), as have the catalytic reactions of MADH (*10*, *11*).

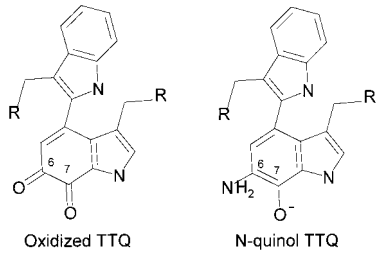

FIGURE 1: Structures of oxidized and substrate-reduced tryptophan tryptophylquinone (TTQ). The C6 and C7 carbonyl carbons are labeled. R denotes connections to the polypeptide chain.

The product of the reductive half-reaction of MADH with the amine substrate is an *N*-quinol form of MADH (*12*), in which the substrate-derived amino group has displaced O6 of TTQ (Figure 1). It was shown that the ET rate from the *N*-quinol MADH to amicyanin was dependent on the presence of monovalent cations such as Na^+ and K^+. At very low cation concentrations, the ET rate is very low, but with increasing monovalent cation concentrations, the rate of the reaction increases by orders of magnitude (*7*). It was demonstrated that this effect was dependent specifically on monovalent cations. It was not a general ionic strength effect, and neither divalent cations nor anions influenced the observed rate. Studies with the *P. denitrificans* enzyme revealed that the observed rate of the redox reaction is not the true ET rate, but that of a rate-limiting proton transfer step (*7*, *8*). A model was presented to explain how monovalent cations bind to the enzyme active site and influence

† This work was supported by National Institutes of Health Grant GM-41574.

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[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; ET, electron transfer; AADH, aromatic amine dehydrogenase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BTP, BisTris propane; KSIE, kinetic solvent isotope effect.

10.1021/bi011246z CCC: $20.00
Published on Web 09/19/2001

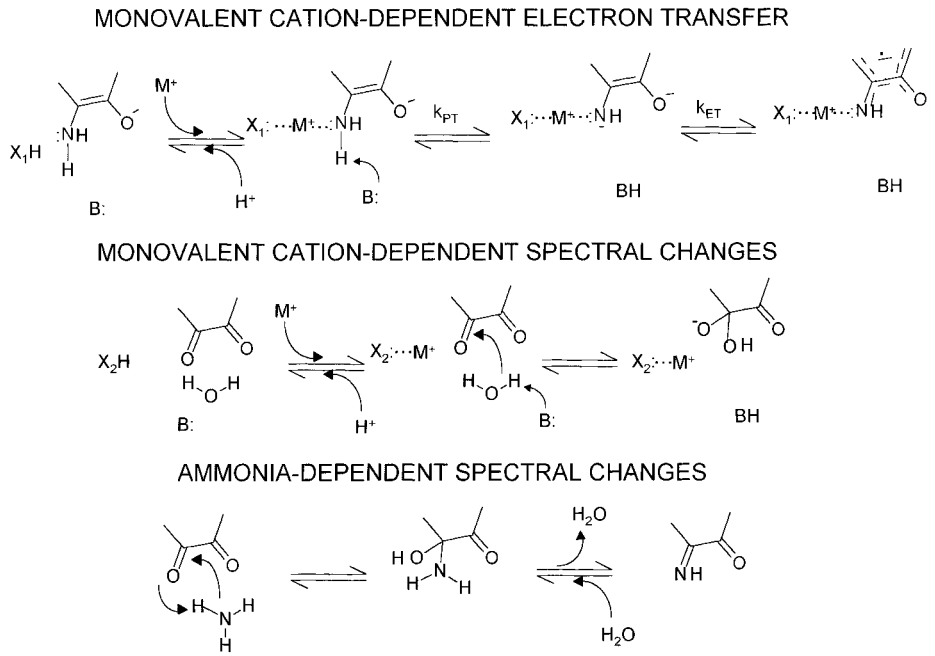

FIGURE 2: Proposed reaction mechanisms for monovalent cation- and ammonia-dependent effects on MADH. X and B represent functional groups in the enzyme active site. M^+ represents a monovalent cation. Only the relevant portions of TTQ are shown. These schemes are adapted from those presented in refs *7* and *16*.

the rate of proton transfer in this reaction (*7*) (Figure 2). This rate enhancement was attributed to the cation facilitating the deprotonation of the TTQ-bound *N*-quinol amino group by stabilizing the anionic product of the deprotonation from which rapid ET occurs.

It has also been reported that monovalent cation binding to MADH changes its absorption spectrum. This phenomenon has been studied with MADHs from *Methylophilus methylotrophus* W3A1 (*13*), *Thiobacillus versutus* (*14*), and *Methylobacterium extorquens* AM1 (*15*). It has also been studied with another TTQ-dependent enzyme, aromatic amine dehydrogenase (AADH) (*16*). This phenomenon is most easily observed at high pH and is not known to be physiologically relevant. However, it has provided a useful handle for investigating monovalent cation binding to the active site of TTQ enzymes. We have previously proposed a model to account for these cation-dependent spectral changes in which the cation stabilizes and facilitates formation of a hydroxide adduct of TTQ at high pH (*16*) (Figure 2).

Ammonium salts also influence the spectral and ET properties of MADH. Stimulation of the rate of the reaction of *N*-quinol MADH with amicyanin is observed (*7*). Spectral perturbations of MADH and AADH are observed as well; however, the nature of the spectral change is different from that which is caused by Na^+ and K^+ (*13*−*16*). We have proposed that these spectral changes are caused by nucleophilic attack of the TTQ carbonyl by the unprotonated NH_3 and are unrelated to the Na^+- and K^+-dependent spectral changes (*16*) (Figure 2).

While these monovalent cation-dependent effects on MADH have been well documented, it is not known whether cations bind to a single site and induce these different effects or whether there are two distinct cation binding sites which are associated with different cation-dependent phenomena. Some insight into this question was provided by X-ray crystallographic studies (*15*). Two potential cation binding sites were identified in the crystal structure of MADH from *M. extorquens* AM1 after crystals were soaked in Cs^+ (discussed later). In one of these putative sites, cation binding is stabilized primarily by cation−π interactions involving the aromatic side chains of Tyr119 of the β subunit and Phe55 of the α subunit.[2] We have prepared an αF55A MADH by site-directed mutagenesis and reported that this mutation dramatically alters the specificity of MADH for its amine substrate (*17*). In this paper, we investigate the effects of the αF55A mutation on these monovalent cation-dependent phenomena. We also prepared another site-directed mutant, αF55E MADH, and examined the effect of this mutation as well. From the results of this study, we were able to determine which of the two putative cation binding sites is associated with each of the different monovalent cation-dependent phenomena. Furthermore, it appears that the binding of the cations to the two sites may be coupled since the αF55A mutation has different and opposite effects on cation-dependent spectral changes and ET rate enhancement. These studies also provide examples of how monovalent cation binding sites may be re-engineered by site-directed mutagenesis to alter enzyme function.

EXPERIMENTAL PROCEDURES

Materials. Native MADH was purified from *P. denitrificans* as described previously (*18*). The αF55A and αF55E site-directed mutants of MADH were expressed in *Rhodobacter sphaeroides* (*19*) and purified as described previously for the αF55A MADH mutant (*17*). Amicyanin was purified from *P. denitrificans* as described previously (*4*). All reagents

[2] The numbering system used here for the MADH α subunit is based on the refined crystal structure of MADH (*3*). The residue number is different from that found in the Protein Data Bank file. To convert to the numbering system which is used in this paper, one must add 13 to the residue number as listed in the PDB files. Thus, residue αPhe55 corresponds to αPhe42 in PDB entry 2BBK. The numbering for the β subunit is unchanged.

were obtained from Sigma or Aldrich and used without further purification. The following buffers were used in these studies: potassium phosphate, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and BisTris propane (BTP).

Site-Directed Mutagenesis. Site-directed mutagenesis was performed on double-stranded pMEG976 (*20*) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and two mutagenic primers following a previously described procedure (*20*). In this construct, the gene which encodes the MADH β subunit possesses a polyhistidine tag at its C-terminus to facilitate purification of the expressed protein. The primers used to create the mutation to convert αPhe55 to Glu were 5′-GTCAACGACCCGGCGCATGACGCCGCG-GTCACCCAGCAATTCG-3′ and its complementary sequence. The underlined bases are those which were changed to create the desired mutation. The mutation was confirmed by sequencing 70 base pairs around the mutated site.

Analysis of Monovalent Cation-Dependent Absorption Changes. All experiments were performed in BTP which was adjusted to pH 9.0 with HCl. Stock solutions of all salts were prepared in 10 mM BTP, except for NH_4Cl which was prepared in 200 mM BTP. The fully oxidized MADH was prepared by incubation with $K_3[Fe(CN)_6]$. Before titration with monovalent cations, the $K_3[Fe(CN)_6]$ was removed by seven cycles of washing with 10 mM BTP buffer using a Centricon-30 centrifuge concentrator (Amicon).

Absorption spectra were recorded using a Shimadzu MultiSpec-1501 spectrophotometer with HYPER−UV 1.51 as the operating software. Native and mutant MADH samples were incubated at 25 °C in 10 mM BTP buffer, pH 9.0. The cation concentration was adjusted by sequential addition from 4 M stock solutions of each salt. For comparison of different MADH forms, spectra were normalized using their absorbance at 280 nm to account for any pH-, salt-, or mutation-dependent effects on the extinction coefficient of the chromophore.

To quantitate the effects of the cation-dependent spectral changes, the change in absorbance with increasing cation concentration was measured at appropriate wavelengths. The apparent K_d value associated with the cation-dependent spectral perturbation was calculated using eq 2, where $[M^+]$ is the concentration of cation and ΔA_{max} is the maximum change in absorbance

$$\Delta A = \Delta A_{max}[M^+]/(K_d + [M^+]) \quad (2)$$

at saturating $[M^+]$.

Electron Transfer Kinetics. All transient kinetic experiments were performed using an On-Line Instrument Systems (OLIS) RSM rapid-scanning stopped-flow spectrophotometer. Reactions were performed in either potassium phosphate or HEPES buffer with salt concentrations and temperature as indicated. MADH which had been reduced by addition of the substrate methylamine was mixed with oxidized amicyanin, and the reactions were monitored between 320 and 560 nm to determine the rate of MADH oxidation by amicyanin. MADH was the limiting reactant with its concentration fixed at 2 μM, and amicyanin concentrations were sufficiently high that pseudo-first-order conditions were maintained and that amicyanin was at saturating concentrations, well above the K_d for complex formation, at each pH and salt concentration. The details of the experimental protocol and methods of data analysis have been previously described (*8*).

To determine the kinetic solvent isotope effect (KSIE) on the apparent ET rate, experiments were performed exactly as described above except that all buffers and salt solutions were made in D_2O, rather than H_2O. Prior to each experiment, MADH and amicyanin were incubated in buffered D_2O overnight and washed with the appropriate buffered D_2O solution to ensure the complete exchange of all solvent exchangeable H^+ for D^+. The value of pD was obtained by adding 0.4 to the observed pH in solutions in D_2O (*21*). The details of our experimental method for such KSIE studies have been previously described (*8*).

RESULTS

Effects of Mutations on Monovalent Cation-Dependent Spectral Perturbations. It has previously been shown that monovalent cations cause pH-dependent perturbations of the absorption spectrum of oxidized MADH (*13*−*15*). Because these changes are more readily observed at high pH, these experiments were performed at pH 9.0. MADH is stable at this pH, and any cation-induced changes in spectral or kinetic properties that are observed are reversible.

In BTP buffer at pH 9.0 in the absence of added cations, subtle differences in the absorption spectra of native, αF55A, and αF55E MADH are evident (Figure 3). The absorption maximum of each mutant MADH is shifted to a higher wavelength than that of the native MADH, with maxima of 430 nm for native, 434 nm for αF55E, and 441 nm for αF55A MADH. For αF55A MADH, the peak centered at 441 nm also appeared to be broader than those of the native and αF55E MADH.

Each MADH form was titrated with increasing concentrations of KCl. The initial and final spectra from each titration are shown in Figure 3. Although the final spectra of the mutants are somewhat different than the final spectrum of the native, this is because their initial spectra were different. The actual change in each spectrum that was caused by the addition of K^+ is essentially the same. This can be seen by comparing the final minus initial difference spectra that are shown in the insets of Figure 3. In each case, addition of K^+ causes a decrease in absorbance of the peak centered at approximately 430−440 nm and the appearance of a shoulder at approximately 340 nm. These titrations were repeated using Na^+ in place of K^+. The spectral changes for each MADH that were observed after addition of Na^+ were essentially the same as that for K^+ addition (data not shown). Thus, it appears that even after the mutations, the αF55A and αF55E MADH still bind Na^+ and K^+ in a position very similar to that in the native MADH.

While the nature of the spectral changes caused by the addition of monovalent cations was the same for native and mutant MADHs, their relative affinities for each cation were different. The dependence of the spectral changes on the concentrations of K^+ and Na^+ is shown in Figure 4. It is evident that the αF55A mutation has decreased the affinity for the monovalent cations, whereas the αF55E mutation had essentially no effect. The apparent K_d values that were calculated from these data are shown in Table 1.

Effects of Mutations on Ammonia-Dependent Spectral Perturbations. Addition of ammonium salts to oxidized

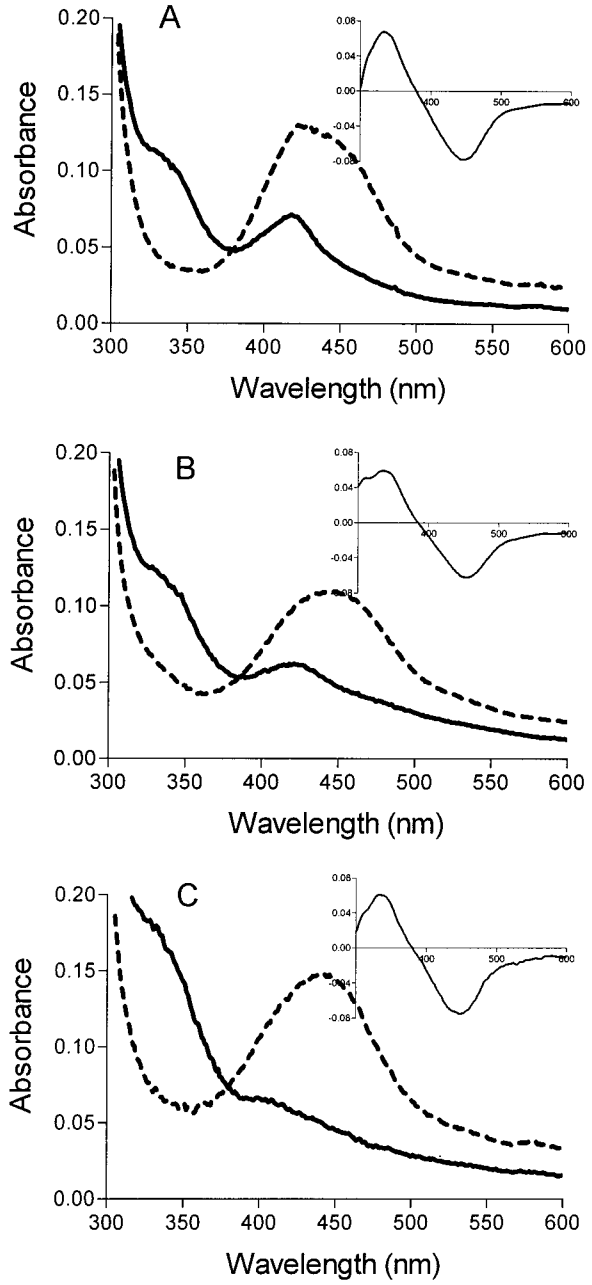

FIGURE 3: Changes in the absorption spectra of native and mutant MADH caused by the addition of KCl. Spectra were recorded in 10 mM BTP buffer (pH 9.0) before (- - -) and after (—) titration with KCl. The final minus initial difference spectrum for each titration is shown in each inset: (A) native MADH, (B) αF55A MADH, and (C) αF55E MADH.

MADH also causes spectral perturbations. As seen in Figure 5, these spectral changes are quite different from those which are caused by the addition of Na^+ and K^+. Each MADH form was titrated with increasing concentrations of NH_4Cl. The initial and final spectra from each titration are shown as well as the final minus initial difference spectra. In each case, addition of NH_4Cl caused a decrease in absorbance of the peak centered at approximately 430−440 nm and an increase in absorbance at approximately 500 nm. In further contrast to the effects of Na^+ and K^+, no absorbance increase around 340 nm was observed. The dependence of the spectral changes on NH_4Cl concentration is shown in Figure 4C. Whereas the αF55A mutation decreased the affinity for K^+ and Na^+, this mutation had essentially no effect on the NH_4-

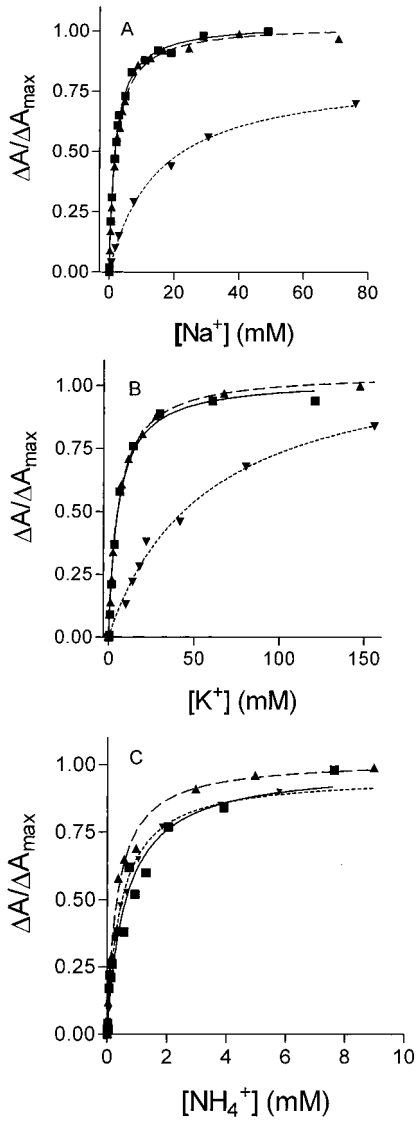

FIGURE 4: Concentration dependence of changes in the absorption spectra of native and mutant MADHs on (A) NaCl, (B) KCl, and (C) NH_4Cl. Spectra were recorded in 10 mM BTP buffer (pH 9.0). The three sets of data in each panel are for native MADH (■), αF55A MADH (▼), and αF55E MADH (▲). The lines are fits of each data set to eq 2.

Table 1: Apparent K_d Values for Cation- and Ammonia-Dependent Spectral Changes

	apparent K_d (mM)		
added salt	native MADH	αF55A MADH	αF55E MADH
KCl	5.5 ± 0.5	45 ± 5	5.7 ± 0.2
NaCl	2.2 ± 0.08	23 ± 6	1.9 ± 0.07
NH_4Cl	0.50 ± 0.03	0.49 ± 0.02	0.35 ± 0.03

Cl-dependent changes. The apparent K_d values that were calculated from these data are shown in Table 1.

Effects of Mutations on the Rate of Monovalent Cation-Dependent Gated Electron Transfer. The electron transfer (ET) reactions from different redox forms of MADH to amicyanin have been extensively studied (*5*−*9*). It was shown that ET from the substrate-reduced *N*-quinol MADH to amicyanin is gated by a proton transfer reaction. In other words, the apparent electron transfer rate (k_{ET}) is really the rate of the slower preceding proton transfer reaction. This proton transfer reaction requires the presence of a monovalent

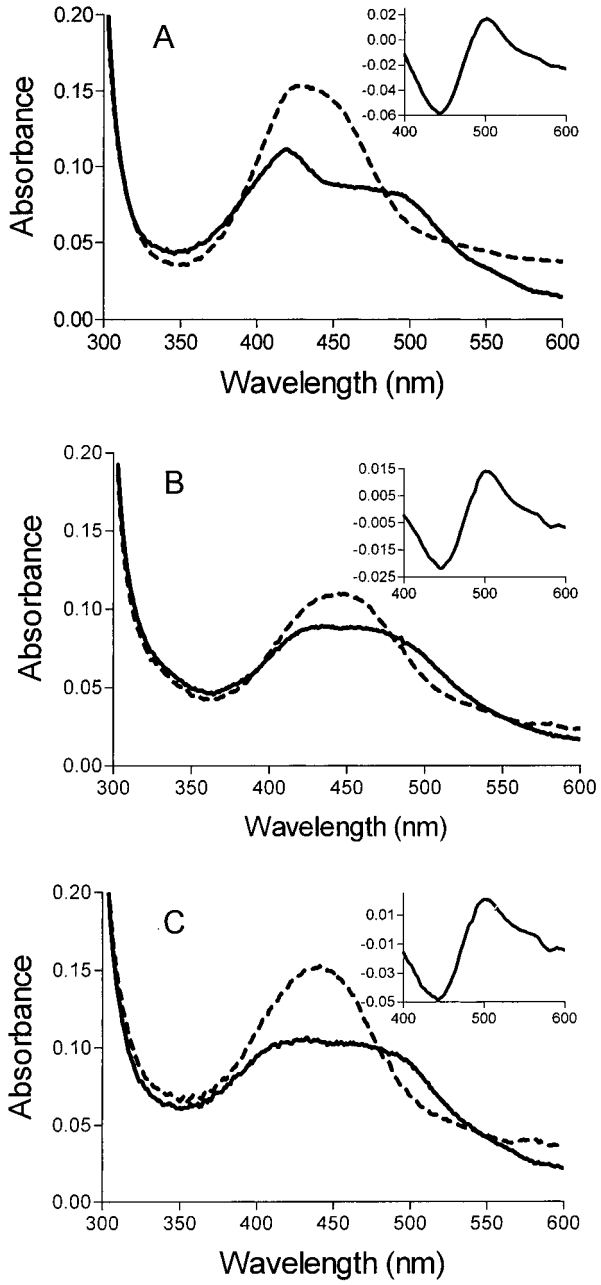

FIGURE 5: Changes in the absorption spectra of native and mutant MADH caused by the addition of NH_4Cl. Spectra were recorded in 10 mM BTP buffer (pH 9.0) before (- - -) and after (—) titration with NH_4Cl. The final minus initial difference spectrum for each titration is shown in each inset: (A) native MADH, (B) αF55A MADH, and (C) αF55E MADH.

cation at the active site (*7*). Therefore, the apparent k_{ET} is dependent on the concentration of monovalent cation present.

Preliminary examination of the ET reaction from αF55A MADH to amicyanin revealed that the concentration of cation required to stimulate k_{ET} was much lower than that needed with native MADH. To minimize background levels of monovalent cations, the ET studies were performed in HEPES buffer with the pH of the buffer adjusted with calcium hydroxide since divalent cations do not have any effect on k_{ET} (*7*). As shown in Figure 6A, the rate of reaction of native MADH in the absence of added cation is very slow (i.e., 0.4 s^{-1}). As the concentration of added K^+ increases from 0 to 200 mM, the rate increases to 80 s^{-1}. This apparent k_{ET} is similar to what was previously reported at this pH

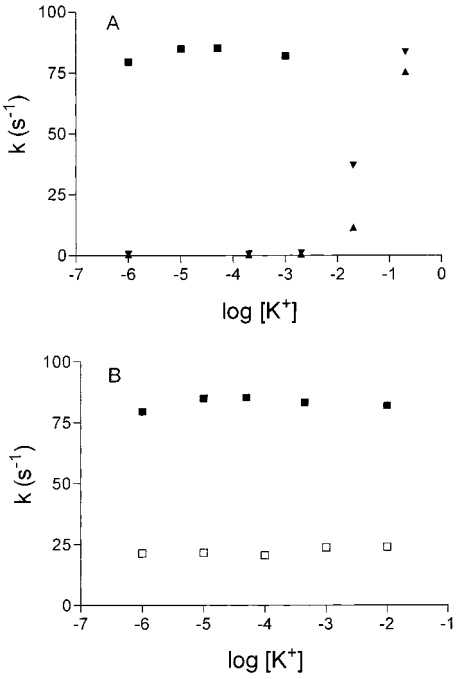

FIGURE 6: (A) Effect of KCl on the reaction of native and mutant *N*-quinol MADHs with amicyanin. Experiments were performed in 10 mM HEPES (pH 7.5) at 18 °C. The rate constant (*k*) is the apparent k_{ET} for the gated ET reaction. The three data sets are for native MADH (▲), αF55A MADH (■), and αF55E MADH (▼). (B) Kinetic isotope effect study of the reaction of *N*-quinol αF55A MADH with amicyanin. The reactions were performed in buffered H_2O (■) and D_2O (□) as described in the text.

and temperature at saturating concentrations of monovalent cation (*7*). The approximate K_d value associated with this phenomenon can be estimated from the data in Figure 6A to be approximately 100 mM. The αF55E MADH exhibited an apparent k_{ET} and a K^+ dependence of that rate which was essentially identical to that of the native MADH. In contrast, with the αF55A MADH, the observed rate was independent of added K^+ and identical to the rate of the native MADH at saturating cation concentrations. The apparent k_{ET} for the αF55A MADH was approximately the same in the absence of added salt as was the k_{ET} for the native MADH at saturating concentrations of K^+.

It is essentially impossible to prepare a buffer that is completely free of monovalent cations. On the basis of information provided by the manufacturers, we estimate that the monovalent cation concentration of buffers used in this study is approximately 1 μM, due to residual monovalent cations in the reagents. The data shown in Figure 6 suggest that for the αF55A MADH, the cation binding site that is associated with stimulation of the apparent k_{ET} is already saturated in the absence of added K^+. This suggests that the K_m for this cation binding is <1 μM, compared to approximately 100 mM for native and αF55E MADH.

We have previously shown that the role of monovalent cations in ET from *N*-quinol MADH to amicyanin is to facilitate a proton transfer that is rate-determining for the subsequent ET step. This was demonstrated by KSIE studies that showed a significant KSIE on the apparent k_{ET}. To confirm that the ET from αF55A MADH is occurring by the same mechanism as in native MADH, KSIE studies were performed with that mutant. As shown in Figure 6B, in both the absence and presence of added cation, the apparent k_{ET}

for the αF55A MADH exhibits a significant KSIE. The KSIE ($^{H_2O}k/^{D_2O}k$) of approximately 4 is similar to what was observed previously for the reaction with native MADH (*7*).

DISCUSSION

Monovalent cations have previously been shown to have two distinct effects on MADH, perturbation of the absorption spectrum and stimulation of the rate of gated ET from the *N*-quinol to amicyanin. Ammonium salts also perturb the absorption spectrum of MADH but in a manner different from that of K^+ and Na^+. On the basis of the results of our studies with MADH from *P. denitrificans* (*7*) and another TTQ enzyme, AADH (*16*), we have previously proposed chemical reaction mechanisms to account for each of these three effects (Figure 2). K^+- and Na^+-dependent spectral changes were attributed to the cation stabilizing and facilitating formation of a hydroxide adduct of TTQ at high pH. K^+-dependent stimulation and Na^+-dependent stimulation of rates of gated ET were attributed to the cation facilitating the deprotonation of the TTQ-bound *N*-quinol amino group by stabilizing the anionic product of the deprotonation from which rapid ET occurs. The pH-dependent spectral changes caused by ammonium salts were proposed to be caused by nucleophilic attack of the TTQ carbonyl by the unprotonated NH_3 which will be present as a significant fraction of the total NH_3 and NH_4^+ ($pK_a = 9.2$) present at pH 9.0. It was not known previously whether the monovalent cation-dependent changes in spectral properties and gated ET were caused by cation binding to the same site or to different sites. The results presented here clearly show that different cation binding sites must be associated with the nonphysiologic spectral perturbations, and the physiologically relevant control of the rate of ET. The αF55A mutation caused opposite effects on the K_d values associated with the two processes. The lack of an effect on the K_d for the NH_4Cl-dependent changes further shows that these effects are not mediated by either cation binding site and supports our proposal that it is the unprotonated NH_3 that is responsible for this effect.

Two potential cation binding sites have been identified in the crystal structure of MADH from *M. extorquens* AM1. When crystals were soaked in Cs^+, the cation was present at two sites in the proximity of the enzyme active site (*15*). The residues that are present in the active site of MADH from *M. extorquens* AM1 are completely conserved in the crystal structure of MADH from *P. denitrificans*. These two Cs^+ binding sites are shown in Figure 7. In the proximal cation binding site nearer O6 of TTQ, several carbonyl groups point toward the cation (O6 from TTQ and amide oxygens from Ile106, Asp105, Asn104, and Asp32). In contrast, the distal cation binding site involves primarily cation−π interactions (*22*) with the aromatic sides chains of Tyr119 and αPhe55. The amide oxygen of Asp32 may also participate in the binding at this site.

Mutation of αPhe55 to Ala should disrupt cation binding to the distal site. Consistent with this, the K_d value associated with the cation-dependent spectral perturbation increased 10-fold with the αF55A mutant. This result also shows that monovalent cation binding to the distal site, not the proximal site, is required for the spectral perturbations. Furthermore, these results show that it is monovalent cation binding to the proximal site that is required to facilitate the proton transfer reaction that gates ET from *N*-quinol MADH.

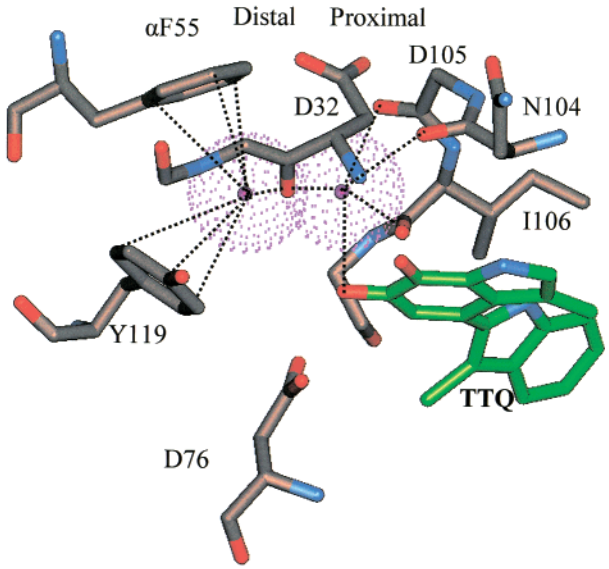

FIGURE 7: Putative monovalent cation binding sites in the crystal structure of MADH. This figure is adapted from that of the crystal structure of the Cs^+-bound MADH from *M. extorquens* AM1 (*15*). The structures of that MADH and *P. denitrificans* MADH are highly conserved, and the residues which comprise the cation binding sites are identical in each enzyme. In this figure, the relevant portion of the crystal structure of the *P. denitrificans* enzyme is shown with two ions present in the positions which correspond to the Cs^+ binding sites in the *M. extorquens* structure. The coordinates for this MADH structure are available as PDB entry 2BBK (*3*).

While it was expected that the αF55A mutation would disrupt the distal binding site, it is interesting that the affinity did not decrease more than 10-fold. The mutation deletes one of the aromatic rings that we assume to be a major force in stabilizing the bound cation by cation−π interactions. The αF55A MADH only has a phenol ring from Tyr119 and a carboxyl oxygen atom from the Asp32 to bind the cation at the distal site. There is, however, precedence for a cation binding site stabilized in large part by a single aromatic residue, such as would be present in the distal site of the αF55A mutant. The crystal structure of rhodanese reveals a Cs^+ binding site in which a single tryptophan side chain and two oxygen atoms are used to stabilize Cs^+ binding (*23*).

Another surprising result of this study is that the αF55A mutation, which involves a residue that participates in the distal binding site, appears to cause the affinity of the proximal site to increase by several orders of magnitude, based on the observation that the rate of gated ET is enhanced in the absence of added cation. One can only speculate about the reasons for this. It should be noted that in the Cs^+-bound crystal structure, each of the two Cs^+ cations was present in approximately 50% occupancy (*15*). Thus, for Cs^+ it appears that binding to the two sites is mutually exclusive. This may be a consequence of the relatively large size of Cs^+ (ionic radius = 1.69 Å). K^+ and Na^+ are smaller with ionic radii of 1.33 and 0.95 Å, respectively, so their binding to the two sites may not be mutually exclusive. However, they will be very close to each other, and as such, steric hindrance and charge repulsion could cause one site to exhibit a much higher affinity if the other site is not occupied. It may also be significant that Tyr119 and αPhe55 are positioned at the end of a substrate channel from the protein surface which opens into the enzyme active site. The position of these two

residues restricts access of large substrates to the active site. This has been proven by the results of mutagenesis studies of αPhe55 which dramatically altered the substrate specificity for MADH (*17*). The K_m values for methylamine were increased by several orders of magnitude, and K_m values for long chain amines were decreased by orders of magnitude. As is true for substrates, monovalent cations must pass through the constriction formed by Tyr119 and αPhe55 to gain access to the proximal cation binding site. The αF55A mutation would provide a clearer path for access to the proximal site relative to the native MADH. Another possible consequence of the increased solvent accessibility of the active site and removal of the phenyl side chain is to affect the ionization of other side chains in the active site. If potential ligands for the proximal site, Asp32 and Asp76, are more exposed to the solvent and more readily deprotonated, then the affinity of the proximal site could be much stronger. The increased binding affinity of the proximal site may be the result of a combination of the factors discussed above.

An alternative explanation for enhancement of the rate of gated ET in the mutant is that the αF55A mutation has caused a conformational change which eliminates the need for cation binding to the proximal site. We cannot rule out this possibility. It should be noted, however, that the enhanced ET rate is still that of a proton transfer that gates the true ET since we measure the same KSIE that is exhibited by the native MADH. The proposed mechanism for this gated ET reaction (*7*) indicated that the cation was necessary to stabilize a TTQ−NH^- intermediate from which rapid ET occurred (see Figure 2). A conformational change that could stabilize this intermediate without a cation at the proximal site is difficult to rationalize from the crystal structure. For this reason, we favor the interpretation that the results shown in Figure 6 reflect a large increase in the affinity of the proximal site for the monovalent cation.

To further probe the role of αPhe55 in monovalent cation binding, that residue was also converted to Glu by site-directed mutagenesis. The αF55E MADH exhibited cation binding properties which were nearly identical to those of native MADH. There is a small shift in the absorbance maximum of the resting absorption spectrum, but the K_d values for the cation-dependent spectral perturbations and cation-dependent gated ET are essentially the same as that of native MADH. It is interesting that the binding affinity is so similar since stabilizing cation−π interactions are being replaced with ionic and electrostatic interactions with Glu. These results indicate that the carboxyl group of Glu can effectively replace the aromatic Phe side chain to provide ligands at the distal cation binding site.

Conclusions. These studies have yielded several significant observations. (i) Monovalent cation-dependent enhancement of the rate of gated ET from *N*-quinol MADH requires cation binding to the proximal site. (ii) Monovalent cation-dependent spectral perturbations of oxidized MADH require cation binding to the distal site. (iii) Ammonia-dependent spectral changes are not associated with either the proximal or distal cation binding site. (iv) Alteration of the distal binding site either significantly increases the affinity of the proximal site for cations or causes a conformational change that eliminates the need for cation binding to the proximal site. (v) Glutamate may effectively replace phenylalanine in stabilizing monovalent cation binding to the distal site.

We were able to use protein engineering to enhance the rate of the physiologically relevant gated ET reaction from the *N*-quinol. This result, however, was unexpected and would have been very difficult to predict from the structure. This highlights the importance of precisely characterizing enzyme and ET reaction mechanisms if one wishes to use site-directed mutagenesis as a tool to rationally redesign enzyme functions. The demonstration that different binding sites are associated with spectral perturbations and enhancement of gated ET rates illustrates the need to use caution when correlating effects that are observed under nonphysiologic conditions with physiologically relevant effects. These results indicate that the spectral perturbations are unrelated to the effects on the gated ET reaction. Proof of the involvement of an aromatic residue, αPhe55, in stabilizing monovalent cation binding provides an example of the relevance of cation−π interactions in biological systems.

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BI011246Z