The Effects of pH and Cations on the Spectral and Kinetic Properties of Methylamine Dehydrogenase from *Thiobacillus versutus*†

Antonius C. F. Gorren* and Johannis A. Duine

Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Received February 15, 1994; Revised Manuscript Received July 18, 1994®

ABSTRACT: The catalytic parameters of *Thiobacillus versutus* methylamine dehydrogenase (MADH) with the physiological substrates methylamine and amicyanin show a pH profile that is quite different from the one found in commonly used assays with artificial electron acceptors. The optimum at pH 7.5, observed for k_{cat} in the latter case, is absent with amicyanin as the reoxidizing substrate. With amicyanin k_{cat} scarcely depends on pH; the same is true for the maximal rate of reduction of MADH by methylamine (k_{red}). Conversely, both the specificity constant (k_{cat}/K_m) of MADH for amicyanin and the apparent second-order rate constant for the reduction of MADH by methylamine (k_{assoc}^{app}) increase very sharply with pH. MADH has a high- and a low-affinity binding site for monovalent cations. Cation binding to the high-affinity site, which only binds the larger cations (Cs^+, Rb^+, and NH_4^+), is accompanied by a red shift in the absorbance spectrum, whereas cation binding to the low-affinity site, which, less specifically, favors binding of the smaller cations, leads to a bleaching of the visible spectrum with a concomitant increase in the near-UV. Cation binding to either site strongly affects the reactivity of MADH. The reduction of MADH by methylamine is inhibited by monovalent cations, whereas the oxidation of reduced MADH by amicyanin is strongly stimulated. For the former reaction it was established that cations affect only k_{assoc}^{app}, not k_{red}. Some speculations about the molecular basis for the effects of pH and cations are presented.

Methylamine dehydrogenase (MADH)[1] (EC 1.4.99.3) occurs in a number of methylotrophic bacteria that can grow on methylamine as the sole carbon and energy source (Eady & Large, 1968; Mehta, 1977; Matsumoto, 1978; Haywood et al., 1982; Kenney & McIntire, 1983; Lawton & Anthony, 1985; Vellieux et al., 1986; Husain & Davidson, 1987). The enzyme, which is unique in containing tryptophyltryptophan quinone (TTQ) as the active center (McIntire et al., 1991a; Chen et al., 1991), catalyzes the oxidation of primary amines, as exemplified in eq 1 for the preferred substrate methylamine.

$$CH_3NH_3^+ + H_2O + 2A \rightarrow HCHO + NH_4^+ + 2H^+ + 2A^- \quad (1)$$

Whereas several artificial dyes can serve as the electron acceptor (A) *in vitro*, the blue copper protein amicyanin is the *in vivo* electron acceptor in most organisms (Tobari & Harada, 1981; Lawton & Anthony, 1985; van Houwelingen et al., 1985; Ubbink et al., 1991), as was unequivocally shown to be the case for *Paracoccus denitrificans* (van Spanning et al., 1990).

There have been a number of reports on the enzyme kinetics of MADH, but in most cases artificial electron acceptors were used (Eady & Large, 1968, 1971; Matsumoto, 1978; Chandrasekar & Klapper, 1986; Husain & Davidson, 1987). In an assay with PMS (or PES) and DCPIP the reaction proceeds optimally at pH 7.5 (Eady & Large, 1968; Mehta, 1977; Matsumoto, 1978; Husain & Davidson, 1987). However, relatively few data are available on the reaction of MADH with amicyanin. Sets of K_m and V_{max} values at pH 7.0 were reported for the enzymes from *Pseudomonas* AM1 (Tobari & Harada, 1981) and Organism 4025 (Lawton & Anthony, 1985) and, at pH 7.5, from *P. denitrificans* (Brooks et al., 1993). In addition, studies on the pre-steady-state reaction at pH 7 between MADH and amicyanin from *Thiobacillus versutus* yielded an apparent association rate constant of 2×10^4 $M^{-1} \cdot s^{-1}$ (van Wielink et al., 1989). A much higher value was recently derived from similar experiments in the case of *P. denitrificans* (Brooks & Davidson, 1993). The results reported in this paper represent the first in-depth kinetic study of a methylamine/MADH/amicyanin system.

MATERIALS AND METHODS

T. versutus (ATCC 25364^T) was grown, and MADH and amicyanin were purified as described previously (van Houwelingen et al., 1985; van Wielink et al., 1990). The purity of the proteins was checked both by polyacrylamide gel electrophoresis (Pharmacia PhastSystem, SDS-gradient gel 8–25) and spectrophotometrically, adopting a A_{280}/A_{440} ratio of 6.7 (van Wielink et al., 1990) and a A_{280}/A_{596} ratio of 4.2 (van Houwelingen et al., 1985) for pure MADH and amicyanin, respectively. The concentration of MADH was determined spectrophotometrically using an absorbance coefficient at 444 nm of 21 000 $M^{-1} \cdot cm^{-1}$ (van Wielink et al., 1990). The amount of redox-active MADH was independently checked by measuring the amount of methylamine needed to reduce the oxidized enzyme, as judged from the changes in the optical absorbance spectrum. The concentration of amicyanin was determined spectrophotometrically using an absorbance coefficient at 596 nm of 3900 $M^{-1} \cdot cm^{-1}$ (van Houwelingen et al., 1985).

† This study was partly funded by the Netherlands Organisation for Scientific Research (NWO) under the auspices of the Netherlands Technology Foundation (STW), and partly financed by the EC (Contract No. CHRX-CT93-0189).

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: $MADH_{ox}$, $MADH_{sq}$, and $MADH_{red}$, oxidized, semireduced, and reduced methylamine dehydrogenase, respectively; TTQ, tryptophyltryptophan quinone; SDS, sodium dodecyl sulfate; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; PES, phenazine ethosulfate; KP_i, potassium phosphate buffer; Tris, tris(hydroxymethyl)aminomethane; BisTris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; n.a., no additions.

0006-2960/94/0433-12202$04.50/0

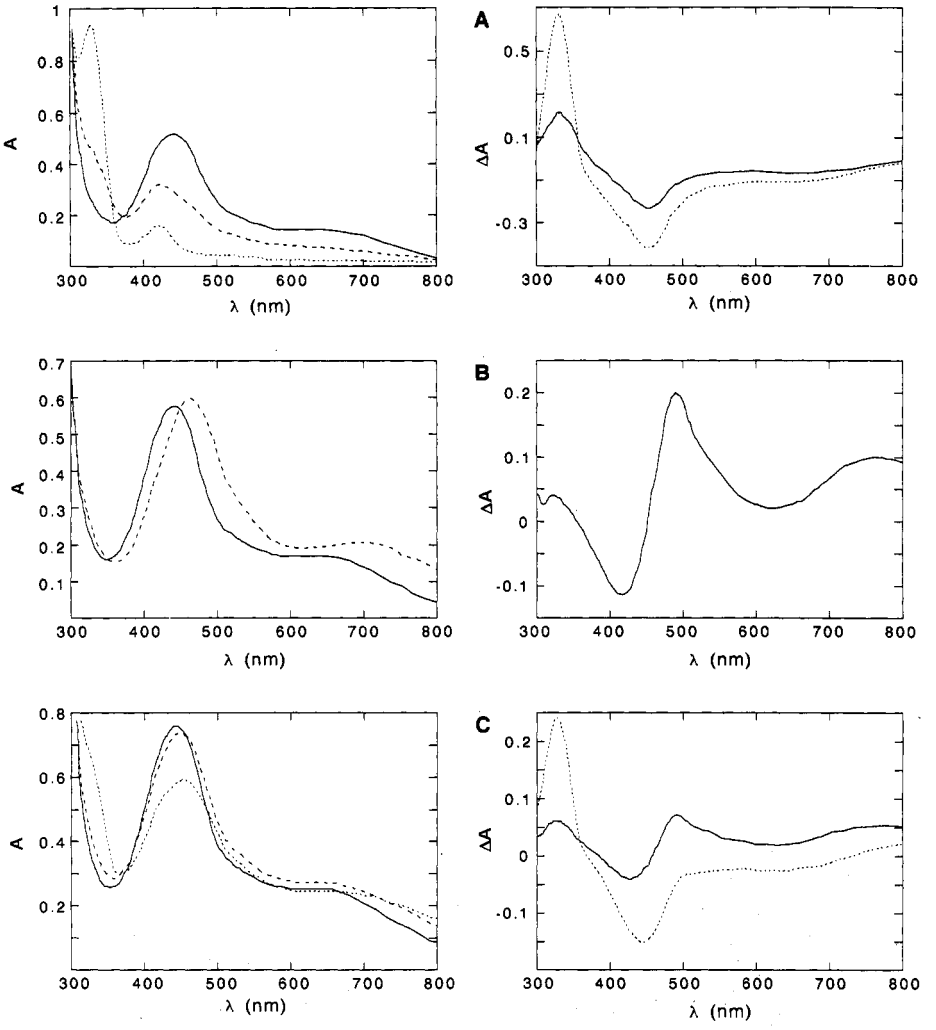

FIGURE 1: Effect of salts on the absorbance spectrum of $MADH_{ox}$. Left-hand panels: Panel A: effect of KCl at pH 9. (—) 28 μM MADH in 20 mM CHES (pH 9.0); (- - -) idem in the presence of 1 M KCl; (···) idem after reduction by 75 μM methylamine. Panel B: effect of CsCl at pH 6; (—) 28 μM MADH in 10 mM HEPES/sodium acetate (pH 6.0); (- - -) in the presence of 250 mM CsCl. Panel C: effect of NH_4Cl at pH 6: (—) 36 μM MADH in 10 mM HEPES/sodium acetate (pH 6.0); (- - -) idem in the presence of 100 mM NH_4Cl; (···) idem in the presence of 500 mM NH_4Cl. The right-hand panels show the corresponding absorbance difference spectra. Panel A: (—) absorbance changes induced by the addition of KCl; (···) red-minus-ox absorbance difference spectrum (reduced spectrum in the presence, oxidized spectrum in the absence of KCl). Panel B: absorbance changes induced by the addition of CsCl. Panel C: (—) absorbance changes induced by the addition of 100 mM NH_4Cl; (···) difference between the absorbance spectra observed in the presence of 500 and 100 mM NH_4Cl.

Optical absorbance spectra and steady-state kinetics were measured on a Hewlett-Packard 8452A diode-array spectrophotometer. Pre-steady-state kinetics as well as some of the steady-state experiments were performed with a Hi-Tech Scientific PQ/SF-53 preparative quench/stopped-flow spectrophotometer.

In the methylamine/MADH/PMS–DCPIP assay the reaction was monitored by measuring the absorbance changes caused by the reduction of DCPIP at 600 nm. The red-minus-ox absorbance–difference coefficient at each pH for DCPIP was determined according to Armstrong (1964). In the methylamine/MADH/amicyanin assay the reaction was followed by measuring the rate of bleaching of amicyanin at 596 nm. Reduced MADH was prepared just before use by addition of a stoichiometric amount of methylamine to the oxidized enzyme. All reagents were analytical grade and were used without further purification. All experiments were performed at 20 °C.

RESULTS

Effects of Cations and pH on the Optical Absorbance Spectrum of MADH. The absorbance spectrum of MADH in its fully oxidized state is characterized by a peak at 445 nm and a broad low band centered at about 650 nm. We investigated the absorbance changes induced by the addition of the chlorides of several monovalent cations (Figure 1). Two different effects could be discerned: KCl (Figure 1) and NaCl (not shown) caused the bleaching of all absorbance bands in the visible spectrum, with a concomitant increase at 328 nm, not unlike the absorbance changes that are observed upon reduction of MADH by methylamine. No reduction had occurred, however, as was checked by the addition of oxidized amicyanin. The second effect was observed with CsCl and consisted of a red shift of the visible absorbance spectrum, with the new peak position at 465 nm. The absorbance difference spectrum featured maxima at 490 and 760 nm and minima at 417 and 625 nm. With NH_4Cl and RbCl both effects were observed, the former effect at high and the latter at low concentrations. $MgCl_2$ (250 mM) had no effect on the absorbance spectrum. Addition of salts (CsCl, KCl, or NH_4Cl) to $MADH_{red}$ did not lead to significant absorbance changes (not shown).

Values for the apparent dissociation constants (K_d^{app}), based on optical titrations, demonstrate that the red shift requires

Table 1: Dependence on pH of K_d^{app} for the Binding of Cations to $MADH_{ox}$

cation	spectral effect[a]	K_d^{app} (mM)[b] pH 7	pH 8	pH 9
Na^+	bleaching	3200	360	45
K^+	bleaching	1600	540	110
NH_4^+	red shift	20[c]	8	1.5
	bleaching	440	280	340
Cs^+	red shift	4	3.4	1.5
	bleaching	3800	1600	1300

[a] The red shift is characterized by absorbance decreases at 417 and 625 nm and increases at 490 and 760 nm. The bleaching is characterized by an absorbance decrease of the visible spectrum and an increase in the near-UV. [b] K_d values were estimated by plotting $\Delta A_{\lambda 1} - \Delta A_{\lambda 2}$ against the salt concentration for several wavelength pairs (λ_1, λ_2) and fitting the resulting curves to the function:

$$\Delta A = \frac{(\Delta\epsilon)[\text{cation}]}{K_d + [\text{cation}]}$$

or, for biphasic curves, to the function:

$$\Delta A = \frac{\Delta\epsilon_1[\text{cation}]}{K_{d1} + [\text{cation}]} + \frac{\Delta\epsilon_2[\text{cation}]}{K_{d2} + [\text{cation}]}$$

Since the cation-binding sites are mutually dependent, this function is only valid if the two K_d values are very different. K_d values far above the highest concentration of added salt ($K_d \geq 1$ M) were estimated using a value for $\Delta\epsilon$ obtained for the same spectral phase under conditions with a lower K_d. [c] At pH 7 the fits for the red shifts observed with NH_4^+ were not quite satisfactory, probably because the K_d values for the red shift and the bleaching were too similar. A good fit could be obtained with the function:

$$\Delta A = \frac{\Delta\epsilon_1[\text{cation}]/K_{d1} + \Delta\epsilon_2[\text{cation}]/K_{d2} + \Delta\epsilon_2[\text{cation}]^2/K_{d1}K_{d2}'}{1 + [\text{cation}]\{1/K_{d1} + 1/K_{d2}\} + [\text{cation}]^2/K_{d1}K_{d2}'}$$

in which K_{d1} and K_{d2} are the dissociation constants for the red shift and the bleaching, respectively, $\Delta\epsilon_1$ and $\Delta\epsilon_2$ are the corresponding absorbance difference coefficients, and K_{d2}' is the apparent dissociation constant for the bleaching with the high-affinity (red shift) site occupied. The underlying assumption is that the absorbance spectrum after cation binding to the low-affinity site is the same, whether or not a cation is bound at the high-affinity site. The values derived from fits to this function were 20 mM, 50 mM, and 350 mM for K_{d1}, K_{d2}, and K_{d2}', respectively.

much lower salt concentrations than the bleaching and that for both effects there is a decrease in K_d^{app} with increasing pH (Table 1). In addition to being pH dependent, the two spectral effects were also mutually dependent: at pH 9 the presence of 500 mM NaCl induced an 11-fold increase of K_d^{app} for the red shift with Cs^+; similarly, a greater than 10-fold increase was found for K_d^{app} for the bleaching with Na^+ in the presence of 100 mM CsCl.

The effect of pH itself on the absorbance spectrum closely resembled the effects observed with KCl or NaCl. Between pH 5 and 9 the pH effects were reversible, whereas outside that range irreversible processes became apparent. Upon longer incubation at high pH, particularly in the presence of CsCl and NH_4Cl, a spectrum resembling that of the semiquinone was generated, with a maximum at about 420 nm, that could not be reduced by methylamine.

pH Dependence of the Reactions of MADH with Methylamine and Amicyanin. Since thus far most kinetic data on MADH were gathered in steady-state experiments with PMS/DCPIP as the electron acceptor couple, we studied the pH dependence in the same assay system and found that the *T. versutus* enzyme behaves similar to MADH's from other organisms, exhibiting an optimum at pH 7.5 (not shown). The pH profile of the steady-state kinetic parameters of

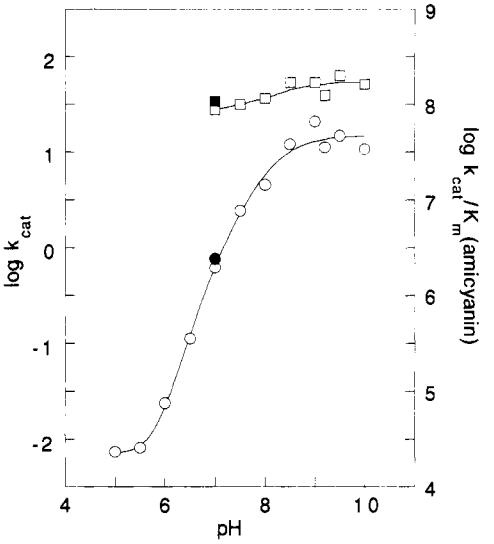

FIGURE 2: Dependence on pH of the activity of MADH with methylamine and amicyanin. Reaction rates were measured at 596 nm in stopped-flow experiments. Experimental conditions: 1 mM methylamine; 8 μM amicyanin; 50 nM MADH (dimer); 50 mM buffer (sodium acetate, BisTris, HEPES, Bicine, or CHES), 100 mM KCl. Values for k_{cat}/K_m(amicyanin) (open circles) and k_{cat} (open squares) were estimated by analysis of the time-resolved traces with the Simfit fitting program developed in our laboratory and were plotted logarithmically, with k_{cat} and k_{cat}/K_m expressed in s^{-1} and $M^{-1}\cdot s^{-1}$, respectively. The closed symbols represent values that were derived from Eadie–Hofstee plots; amicyanin concentrations were varied between 2 and 40 μM. The line drawn through the k_{cat}/K_m data points is a best fit to the function:

$$\log k = \log\left\{k_{low} + \frac{k_{high}}{(1 + 10^{pK_a^1 - pH})(1 + 10^{pK_a^2 - pH})}\right\}$$

The optimal fitting parameters were as follows: $k_{low} = (2.3 \pm 0.5) \times 10^4\ M^{-1}\cdot s^{-1}$; $k_{high} = (4.7 \pm 0.6) \times 10^7\ M^{-1}\cdot s^{-1}$; $pK_a^1 = 6.7 \pm 0.2$; $pK_a^2 = 8.2 \pm 0.2$. For this function to be valid it must be assumed that the specificity constant for the intermediate state with one group protonated is negligible in comparison to that of the fully deprotonated state. This was checked by simulating curves using a more general function that takes into account the reactivity of the intermediate state; acceptable fits were only obtained with the specificity constant for the intermediate state smaller than $5 \times 10^6\ M^{-1}\cdot s^{-1}$; the values for the other parameters were not affected.

MADH, obtained in the presence of saturating concentrations of methylamine, exhibits a steep increase with pH for the specificity constant (k_{cat}/K_m) of MADH for amicyanin, until above pH 8.5 a maximal value of about $6 \times 10^7\ M^{-1}\cdot s^{-1}$ is reached (Figure 2). On the other hand, k_{cat} varied only slightly, increasing from 30 s^{-1} at pH 7 to 55 s^{-1} at pH 10. At pH 7.0 and 7.5, where a comparison can be made, there is good agreement between our values for K_m and k_{cat} and those reported for other organisms (Tobari & Harada, 1981; Lawton & Anthony, 1985; Brooks et al., 1993).

Below pH 7 k_{cat} was not determined because of the large amounts of amicyanin this would require. For the same reason we also did not measure the specificity constant of MADH for methylamine in steady-state experiments with amicyanin, but instead performed stopped-flow measurements of the reduction of $MADH_{ox}$ by methylamine.

The pre-steady-state reduction of MADH by an excess of methylamine was measured in stopped-flow experiments for a wide pH range. The reaction was simultaneously monitored at 424 nm and at 440 nm with identical results. Pseudo-first-order rate constants were determined for varying concentrations of methylamine and the results fitted to the

function:[2]

$$k_{obs} = \frac{k_{red} k_{assoc}^{app}[\text{methylamine}]}{k_{red} + k_{assoc}^{app}[\text{methylamine}]} \quad (2)$$

in which k_{obs}, k_{assoc}^{app}, and k_{red} represent the observed first-order rate constant, the apparent association rate constant and the (maximal) reduction rate constant, respectively. It was found that k_{assoc}^{app} strongly increases when the pH is raised, whereas k_{red} only slightly varies with pH, yielding values of 105–115 s^{-1} between pH 6 and 9 (Figure 3). The values derived from our data for k_{assoc}^{app} and k_{red} at pH 7.0 and pH 7.5 are in good agreement with previous reports for *T. versutus* (van Wielink et al., 1989), bacterium W3A1 (McWhirter & Klapper, 1989), and *P. denitrificans* (Brooks et al., 1993).

Effects of Cations on the Reactivity of MADH. Determination of the ionic strength dependence, both with KCl and with NH_4Cl, of the steady-state activity of MADH in an assay with methylamine and amicyanin demonstrated that the ionic strength hardly affects the overall maximal rate (k_{cat}) (not shown). However, when we studied the reduction of $MADH_{ox}$ by methylamine and its reoxidation by amicyanin separately in stopped-flow experiments, substantial effects of salts on the reaction rates were observed. Moreover, the extent of the effects strongly depended on the identity of the cation. The rate of reduction of $MADH_{ox}$ by methylamine at pH 7 was slowed down considerably in the presence of NH_4Cl and

[2] The kinetic scheme from which we derived rate equation 2 is as follows:

$$MADH_{ox} + MeAm \underset{k_{diss}}{\overset{k_{assoc}}{\rightleftarrows}} MADH_{ox}{*}MeAm \xrightarrow{k_{red}} MADH_{red} + P$$

in which MeAm stands for methylamine and P represents the reaction products ammonium and formaldehyde. For reaction schemes of this type usually a distinction is made between the two extreme cases, with either $k_{diss} \gg k_{red}$ (the rapid equilibrium assumption) or $k_{diss} \ll k_{red}$ (no complex dissociation). In both cases, however, the pseudo-first-order rate constant as a function of the methylamine concentration will yield a similar hyperbolic plot. In the former case the rate constant will be given by

$$k_{obs} = \frac{k_{red}[MA]}{k_{diss}/k_{assoc} + [MA]}$$

whereas in the latter case the rate constant will be

$$k_{obs} = k_{assoc}[MA] \quad \text{for } [MA] \ll k_{red}/k_{assoc}$$

$$k_{obs} = k_{red} \quad \text{for } [MA] \gg k_{red}/k_{assoc}$$

In principal, the two cases can be distinguished by analysis of the progress curves for the intermediate concentration range ($[MA] \cong k_{red}/k_{assoc}$), which will remain first order for a rapid equilibrium, but will become sigmoidal otherwise. In our case all progress curves were comfortably fitted by a single exponential, suggesting that rapid equilibrium conditions apply ($k_{red} \ll k_{diss}$). However, since for the, in this respect, crucial progress curves (with $k_{obs} \sim {}^1/_2 k_{red} \sim 50\ s^{-1}$) between 5% and 15% of the reaction occurs within the dead time of the apparatus, we are not confident that we would be able to discern a small sigmoidal deviation from first-order behavior. Therefore, starting from the more general rate equation:

$$k_{obs} = \frac{k_{red}[MA]}{(k_{red} + k_{diss})/k_{assoc} + [MA]}$$

and with

$$k_{assoc}^{app} = \frac{k_{red}}{k_{red} + k_{diss}}(k_{assoc})$$

we arrive at our eq 2, leaving undecided whether the apparent association rate constant is the true association rate constant, the product of the (maximal) reduction rate constant and the equilibrium association constant, or a hybrid of these extreme cases.

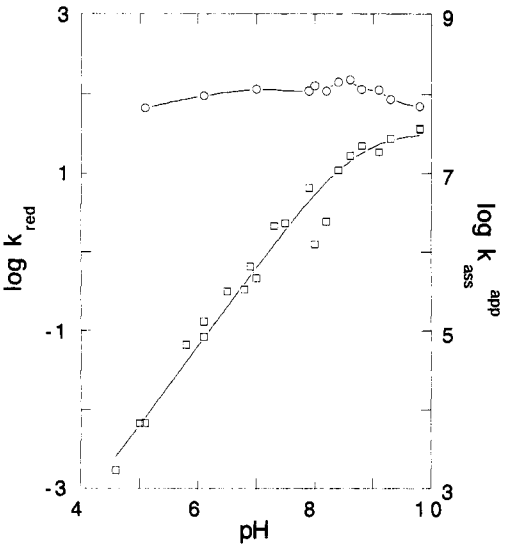

FIGURE 3: The dependence on pH of the rate of reduction of MADH by methylamine. Values for k_{red}(methylamine) (circles) and k_{assoc}^{app}-(methylamine) (squares) were derived as explained in the Results and were plotted logarithmically with k_{red} and k_{assoc}^{app} expressed in s^{-1} and $M^{-1}{\cdot}s^{-1}$, respectively. Experimental conditions: 10 mM buffer (potassium acetate, BisTris, MES, HEPES, Bicine, or CHES); 50 mM KCl; 1–4 μM MADH (dimer); 2.5 μM–100 mM methylamine. The line drawn through the k_{assoc}^{app}(methylamine) data was fitted to the function:

$$\log k = \log k_{max} - \log(1 + 10^{pK_a - pH})$$

The optimal fitting paramers were as follows: $k_{max} = (3 \pm 1) \times 10^7$ $M^{-1}{\cdot}s^{-1}$; $pK_a = 8.7 \pm 0.1$.

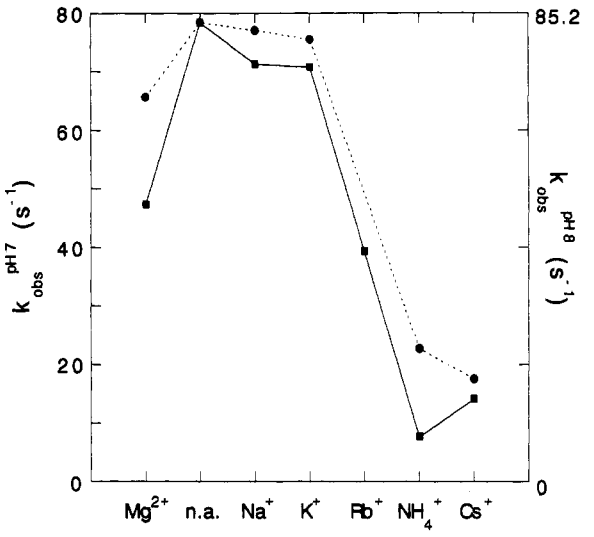

FIGURE 4: Effect of cations on the rate of reduction of $MADH_{ox}$ by methylamine. Plotted are the first-order rate constants observed at 440 nm in the presence and absence of salts. Experimental conditions: 5 μM MADH, 500 μM methylamine, and 10 mM HEPES (pH 7.0) (circles, dotted line); 5 μM MADH, 50 μM methylamine, and 10 mM Bicine (pH 8.0) (squares, continuous line). The salt concentration was 200 mM.

CsCl (Figure 4), whereas very small effects were observed in the presence of KCl and NaCl. At pH 7 the effectivity of the cations (at a concentration of 200 mM) in inhibiting $MADH_{ox}$ reduction increases with the cationic radius. At pH 8 all cations are more effective inhibitors, and a slightly different order of effectivity (at 200 mM) is observed ($NH_4^+ > Cs^+ > Rb^+ > K^+ \sim Na^+ >$ n.a.). The divalent cation Mg^{2+} also inhibits the reaction between MADH and methylamine.

Experiments performed with varying concentrations of methylamine demonstrated that the inhibitory effect of NH_4-

Table 2: Effect of Cations on k_{red} and k_{assoc}^{app} for the Reduction of MADH by Methylamine

measuring conditions[a]	k_{red}[b] (s^{-1})	k_{assoc}^{app}[b] ($M^{-1}\cdot s^{-1}$)	K_d[c] (mM)	k_{assoc}^{app}(sat.)/ k_{assoc}^{app}(n.a.)[c]
pH 7:				
n.a.	109	5.19×10^5		
+0.1 M CsCl	120	0.67×10^5	7.0	0.13
+0.5 M NH_4Cl	107	0.41×10^5	40	≤0.02
pH 8				
n.a.	125	5.43×10^6		
+1 M KCl	121	1.55×10^6	500	0.18

[a] The applied buffers were 10 mM HEPES and 10 mM Bicine at pH 7 and 8, respectively. n.a.: no salt added. [b] Values for k_{red} and k_{assoc}^{app} were calculated using eq 2. [c] The inhibition constants and the maximal inhibitory effects of cations on k_{assoc}^{app} were estimated by fitting plots of the observed reduction rates (measured at a constant methylamine concentration) vs the cation concentration to the function:

$$k_{obs} = \frac{k_{red}[\text{MeAm}](1/K_s + [\text{cation}]/K_dK_s')}{[\text{MeAm}](1/K_s + [\text{cation}]/K_dK_s') + [\text{cation}]/K_d + 1}$$

in which K_d is the dissociation (inhibition) constant for the cation, and K_s and K_s' are the dissociation constants for methylamine in the absence and presence of bound cation. The maximal inhibitory effects are represented in the table as the ratio between the rate constants in the presence [k_{assoc}^{app}(sat.)] and absence [k_{assoc}^{app}(n.a.)] of saturating cation concentrations and are calculated as K_s/K_s'. The equation is valid if cations affect K_s but not k_{red}.

Cl, CsCl, and KCl was on k_{assoc}^{app} only, with no change in k_{red} (Table 2). As was the case for the absorbance spectral effects, the main origin for the different effectivities is the difference in affinity of MADH for the various cations. From measurements of the reduction rate at varying cation concentrations, apparent dissociation constants for some of the cations were calculated, as well as the maximally attainable inhibition (Table 2).

The effects of salt on the oxidation of $MADH_{red}$ by amicyanin are opposite to those observed for the reduction of MADH. All monovalent cations increase the oxidation rate, in essentially the same order of effectivity (at 200 mM) as found for the decrease of the reduction rate: $Cs^+ > Rb^+ \sim NH_4^+ > K^+ > Na^+ >$ n.a. (Figure 5). In this case the monovalent cations as a group behave differently from the divalent cations Mg^{2+} and Ca^{2+}, which inhibit the oxidation rate. The stimulation that can be achieved by the addition of cations (200 mM; pH 7) is quite remarkable: with Cs^+ a 170-fold increase in rate is observed and with NH_4^+ the apparent second-order rate constant (k_{assoc}^{app}) increases from 1.4×10^4 $M^{-1}\cdot s^{-1}$, close to a previously reported value (van Wielink et al., 1989), to 1×10^6 $M^{-1}\cdot s^{-1}$, which is in better agreement with steady-state data on MADH. The oxidation of $MADH_{red}$ by amicyanin necessarily has to occur in two separate reactions, with a semiquinone ($MADH_{sq}$) intermediately formed. One can to some extent discriminate the effects of salt on the two subsequent steps by simultaneously monitoring the absorbance changes at both 420 and 450 nm, since these absorbance changes primarily represent the $MADH_{red}$-to-$MADH_{sq}$ and $MADH_{sq}$-to-$MADH_{ox}$ transitions, respectively. In the absence of salts and at pH 7 no intermediate is observed, with the same apparent rate at both wavelengths, indicating that $MADH_{sq}$ reacts far more rapidly with amicyanin than does $MADH_{red}$. In the presence of salts the apparent rates at 420 and 450 nm start to diverge (Figure 5), which implies that the $MADH_{red}$-to-$MADH_{sq}$ transition is affected more than the subsequent step.

For some ions (Cs^+, NH_4^+, and K^+ at pH 7) $MADH_{red}$ oxidation rates were measured at varying cation concentrations. The interpretation of the results is less straightforward than those obtained for the reduction of $MADH_{ox}$ by methylamine, since data on the amicyanin concentration dependence are lacking. Nevertheless, K_d^{app} values were estimated under the assumption that cations affect the maximal rate of oxidation only and are presented along with the values obtained from the optical titrations and the reductive half-reaction in Table 3.

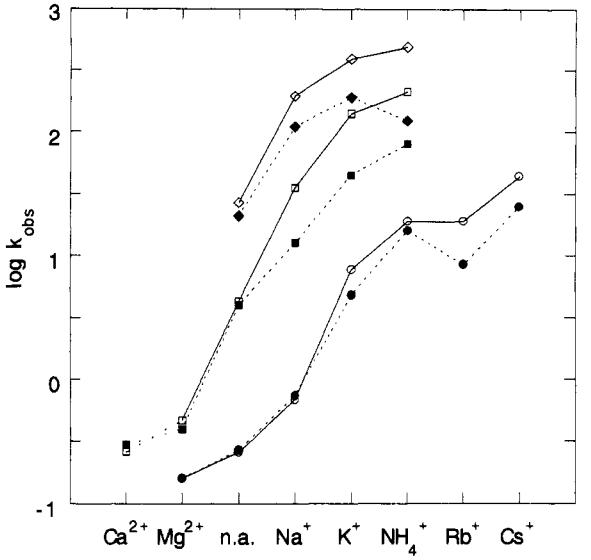

FIGURE 5: Effect of cations on the rate of oxidation of $MADH_{red}$ by amicyanin. The observed apparent rate constants in the presence and in the absence of salts measured at 420 nm (mainly the $MADH_{red}$-to-$MADH_{sq}$ conversion; open symbols, continuous lines) and 450 nm (mainly the $MADH_{sq}$-to-$MADH_{ox}$ conversion; closed symbols, dotted lines) are plotted logarithmically, with k_{obs} expressed in s^{-1}. Experimental conditions: 5 μM $MADH_{red}$, 20 μM amicyanin, and 10 mM HEPES (pH 7.0) (circles); 10 mM Bicine (pH 8.0) (squares); or 10 mM CHES (pH 9.0) (diamonds). The salt concentration was 200 mM, except for $CaCl_2$, in which case it was 50 mM.

DISCUSSION

Effects of Cations and pH on the Optical Absorbance Spectrum of MADH. Since NH_4^+ is one of the reaction products of the catalytic conversion of methylamine, the effect of NH_4^+ on the absorbance spectrum of MADH has been the object of prior studies. With the enzyme from bacterium W3A1, NH_4^+ was found to induce a red shift and intensification of the visible absorbance band (Kenney & McIntire, 1983; McWhirter & Klapper, 1989). Previously such absorbance changes were not observed with MADH from *P. denitrificans* and *T. versutus*, but rather a semiquinone-like absorbance spectrum with, for *Thiobacillus*, a maximum at 420 nm was reported (Backes et al., 1991). The experiments, presented in this paper, demonstrate, however, that the *Thiobacillus* enzyme does exhibit a red shift in the presence of NH_4^+. The conversion to an enzyme form with a semiquinone-type spectrum was also found by us, but this transition required longer incubation times and high pH values and may involve an irreversible intramolecular reaction, since attempts to reduce or oxidize this enzyme form were unsuccessful. In addition to the red shift, high concentrations of NH_4^+ also caused a hitherto not reported bleaching of the visible absorbance spectrum. From the observation that the nature of the effect of NH_4^+ is concentration-dependent, with the red shift occurring at low, and the bleaching at high concentrations, it follows that two different binding sites on MADH are involved: a high-affinity binding site that specifically binds the bigger monovalent cations Cs^+, NH_4^+, and Rb^+, and a less specific low-affinity binding site that

Table 3: Apparent Dissociation Constants of MADH–Cation Complexes for Monovalent Cations at pH 7

	K_d (M)		
cation	optical titration[a]	inhibition of reduction[b]	stimulation of oxidation[c]
Cs^+	0.004 ± 0.001	0.007 ± 0.002	0.02 ± 0.01
NH_4^+	0.02 ± 0.01[d]	0.04 ± 0.01	0.10 ± 0.02
K^+	1.6 ± 0.5	2.7 ± 0.5	1.0 ± 0.1[e]
K^+ (pH 8)	0.5 ± 0.2	0.5 ± 0.2	n.d.

[a] Calculated from the optical titrations of $MADH_{ox}$ as in Table 1. [b] Calculated from the inhibition of the reduction of $MADH_{ox}$ as in Table 2. [c] If cations affect the maximal oxidation rate only, the observed rate can be described by the function:

$$k_{obs} = \frac{(k_{ox}K_d + k_{ox}'[\text{cation}])[\text{Amic}]}{(K_s + [\text{Amic}])(K_d + [\text{cation}])}$$

in which K_d and K_s are the dissociation constants for the cation and amicyanin, respectively, and k_{ox} and k_{ox}' are the maximal rates of oxidation of $MADH_{red}$ by amicyanin in the absence and presence of cations. Since no values are available for K_s, k_{ox}, and k_{ox}', values for K_d were calculated by fitting plots of k_{obs} vs cation concentration to the function:

$$k_{obs} = \frac{C_1K_d + C_2[\text{cation}]}{K_d + [\text{cation}]}$$

in which C_1 and C_2 represent $k_{ox}[\text{Amic}]/(K_s + [\text{Amic}])$ and $k_{ox}'[\text{Amic}]/(K_s + [\text{Amic}])$, respectively. [d] The value found for the red shift. [e] Estimated assuming that the maximal stimulation by K^+ equals that obtained with Cs^+.

preferentially binds smaller cations. When the pH is increased both sites display stronger cation-binding affinities as well as a tendency toward the binding of smaller cations.

A loss of absorbance in the visible spectrum upon increasing pH has been previously reported for the enzyme from *P. denitrificans* (Davidson, 1989). In view of the similarity of the spectral effects exerted by high pH and small cations, and of the strong pH dependence of the apparent K_d values for K^+ and particularly for Na^+, it seems likely that the effect of pH is secondary, originating from stronger binding of residual K^+ or Na^+ at high pH. To ascertain this, additional experiments are needed to determine the true pK_a and K_d values.

pH Dependence of the Reactions of MADH with Methylamine and Amicyanin. In the commonly used assay with PMS and DCPIP as the electron acceptor couple, the enzyme from *T. versutus* displayed a sharp optimum in the activity at pH 7.5, in agreement with previous reports on the pH dependence of the activity of MADH with artificial electron acceptors (Eady & Large, 1968; Mehta, 1977; Matsumoto, 1978; Kenney & McIntire, 1983; Chandrasekar & Klapper, 1986; Husain & Davidson, 1987). In the physiological reaction of MADH with its natural redox partner amicyanin, however, there is no pH optimum for k_{cat}. Since with amicyanin the values for k_{cat} were higher than with PMS, the optimum at pH 7.5 is peculiar to the PMS/DCPIP assay and therefore irrelevant to the *in vivo* situation. The data presented in this paper do not allow the identification of all steps that determine k_{cat}. By comparing k_{red} and k_{cat}, however, we can conclude that between pH 7 and 9 k_{red} contributes significantly to k_{cat}.

Unlike k_{cat} and k_{red} the specificity constants of MADH for amicyanin and methylamine, the latter as estimated from stopped-flow experiments, turned out to be extremely pH dependent, both increasing with pH to values in the order of 10^7 $M^{-1}\cdot s^{-1}$, displaying optima at pH 9.2 and above 10, respectively. The pH profile of k_{assoc}^{app}(methylamine) could be fitted assuming that the activity depends on the deprotonation of a single group with a pK_a^{app} of 8.7. However, the dependence on pH of the apparent K_d values of the smaller cations implies that the apparent pK_a value will be decreased in the presence of K^+ or Na^+. Since a value of 8.7 was obtained in the presence of 50 mM KCl, the true pK_a value must be higher. Concerning the molecular basis of this pH dependence, one might think of the protonation of the as yet unidentified base that is proposed to participate in catalysis (Davidson et al., 1992). This would, however, affect not only the apparent association rate constant (k_{assoc}^{app}) for methylamine but also the maximal MADH-reduction rate constant (k_{red}). Since the latter rate constant varies little over the whole pH range investigated, the explanation for the pH profiles must be sought for elsewhere. We cannot rule out the possibility that only the deprotonated form of methylamine, which has a pK_a of 10.6, reacts with MADH. Such a difference in reactivity might arise from the lack of nucleophilicity of the protonated form. It should be borne in mind, however, that this, too, would affect k_{red}, rather than K_m, contrary to observations. Furthermore, the competitive inhibition by monovalent cations of the reaction between $MADH_{ox}$ and methylamine suggests a common binding site for methylamine and monovalent cations, which implies that methylamine is bound as a cation as well. Supporting evidence for this idea comes from the fact that both a red-shifted absorbance spectrum and competitive inhibition of the MADH–methylamine reaction are also observed with the nonconvertible substrate analogue trimethylamine (unpublished results). Finally, the pH dependent change in MADH, that affects its absorbance spectrum (Davidson, 1989), resonance Raman spectrum (McIntire et al., 1991b; Backes et al., 1991), reactivity with amicyanin, and affinity for monovalent cations, is expected to affect the reduction of MADH by methylamine as well. Therefore, the pH effect is likely to originate from the (de)protonation of a group in the substrate-binding pocket.

The absence of a pH effect on k_{red} can be explained in several ways. It may be that the pK_a of the enzyme group, the protonation of which affects substrate affinity, is lowered by substrate binding from a value of 8.7 or higher to a value below pH 7, which would effectively render k_{red} pH independent in the experimental pH range. However, it is also conceivable that k_{red} simply is not affected by the protonation state of the enzyme group. Our results do not allow us to discriminate between these two options.

For k_{cat}/K_m(amicyanin) a satisfactory fit to the data required two protonatable groups, with apparent pK_a values of 6.7 and 8.2, as well as a nonzero reaction rate at low pH. With regard to the lower of the two pK_a values the obvious candidate is a histidine on amicyanin with a pK_a of 6.9, the protonation of which was reported to result in abolishment of the self-exchange of amicyanin (Lommen et al., 1988; Lommen & Canters, 1990). The higher of the two pK_a values agrees with the value of 8.2 that has been reported for the pH dependent absorbance changes of MADH from *P. denitrificans* (Davidson, 1989). The exactness of the agreement must be coincidental, since both the pH dependent absorbance changes and the rate of reoxidation by amicyanin were determined in the presence of cations (Na^+ and K^+, respectively), which implies that in both cases the true pK_a values are higher. Nevertheless, both the spectral and kinetic effects are likely to originate from the (de)protonation of the same cation binding site on MADH.

Effects of Cations on the Reactivity of MADH. The lack of ionic strength dependence of k_{cat} agrees with observations with the enzymes from bacterium W3A1 and *P. denitrificans* (McIntire, 1987; Davidson, 1989). Those studies had yielded

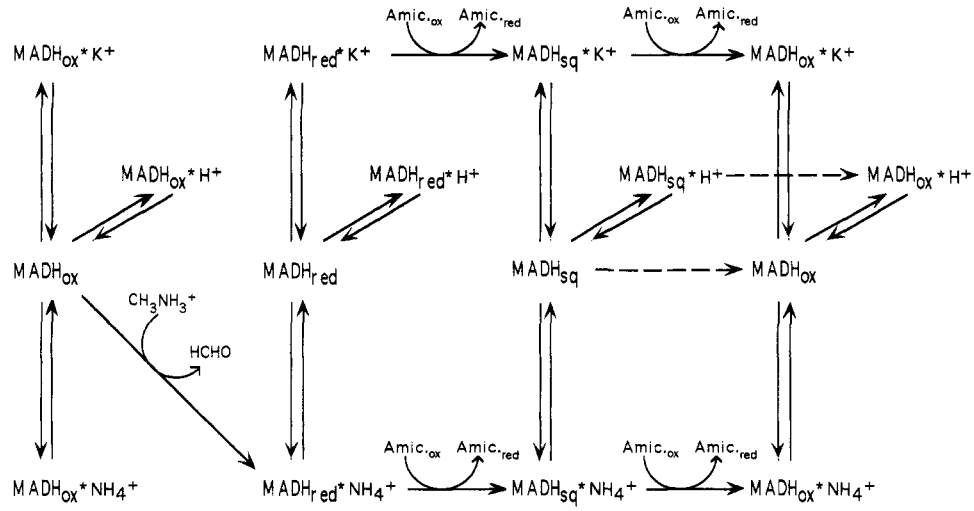

FIGURE 6: Schematic representation of the electron flow in the MADH catalytic cycle. Horizontally, the different redox states of MADH are shown. The enzyme state with a cation bound to the high-affinity binding site is represented as $MADH^*NH_4^+$, since ammonium ions are the only cations that may occupy this site *in vivo*. The enzyme state with a cation bound to the low-affinity binding site is represented as $MADH^*K^+$; however, Na^+ or NH_4^+ can also fulfill this role. The enzyme state found at low pH is represented as $MADH^*H^+$. For the sake of clarity enzyme states with more than one binding site occupied are omitted. The oxidations of the $MADH_{sq}$ and the $MADH_{sq}{}^*H^+$ states are represented by broken arrows, since from the present data it cannot be established if these enzyme states react with amicyanin.

contradictory results with regard to the behavior of the specificity constant for methylamine on varying the ionic strength, since no significant effect was found for *P. denitrificans* MADH, whereas a considerable decrease with increasing ionic strength was reported for MADH from W3A1. Our data suggest that the rate decrease observed in the latter case is due to the specific binding of K^+ to MADH. The use of different salts in the two previous studies (KCl and NaCl for the enzymes from W3A1 and *P. denitrificans*, respectively) may have contributed to the discrepancy. The actual ionic strength dependence of the affinity of MADH for either one of its substrates appears to be rather small. Regarding amicyanin, this agrees well with X-ray crystallographic studies of the MADH–amicyanin complex, which also indicated that the nature of the interaction between both proteins is primarily hydrophobic (Chen et al., 1992).

The differences in the effectivities, with which the various cations enhance the reactivity of $MADH_{red}$ with amicyanin and inhibit the reduction of $MADH_{ox}$ by methylamine, largely derive from the different affinities of the cations for MADH. This can be deduced from the agreement between the apparent K_d values obtained from the optical titrations on the one hand and from the reactivities of MADH with methylamine and amicyanin on the other (Table 3). Whether there are also intrinsic differences in the extent to which cations affect the reactivity of MADH is less clear. However at pH 8, 200 mM NH_4^+ inhibits the reaction of $MADH_{ox}$ with methylamine stronger than 200 mM Cs^+, even though the optical titrations demonstrate that the K_d^{app} for the latter ion is smaller; furthermore, extrapolations of the titrations at pH 7 of the reactivity of MADH with methylamine in the presence of different cations suggested that at saturating concentrations K^+ and Cs^+ only cause partial inhibition, whereas complete inhibition can be attained with NH_4^+ (Table 2). Taken together, these results suggest that both binding sites must be occupied for inhibition to be complete.

The K_d^{app} values calculated from the optical titrations and the reduction of $MADH_{ox}$ by methylamine display a slightly greater similarity to each other than to the ones derived from the reaction between $MADH_{red}$ and amicyanin. This may represent a true difference, since $MADH_{red}$ does not necessarily have the same cation-binding properties as $MADH_{ox}$. However, it should also be noted that, in the case of the reaction with amicyanin, it was assumed that the affinity of amicyanin is not affected by cation binding. Therefore, the deviations could also arise from relatively small effects of cations on the binding between MADH and amicyanin, as were recently reported in the case of Na^+ (Davidson et al., 1993).

Both activation and inhibition of the activity of MADH by NH_4^+ were reported previously for the enzyme from bacterium W3A1 (McIntire, 1987). Until now it was assumed that the enzymes from *T. versutus* and *P. denitrificans* did not exhibit such effects (Backes et al., 1991). Our results demonstrate that at least *Thiobacillus* MADH behaves completely similar to the W3A1 enzyme and that activation and inhibition involve the oxidative and reductive half-reactions, respectively.

Molecular Basis for the Spectral and Kinetic Effects. Although the type of experiments described in this paper does not allow the elucidation of the underlying mechanism, some inferences can be made. At first sight the observation of opposite effects of cations on the reduction and reoxidation of MADH might suggest that, ultimately, cations affect the MADH reactivity by lowering the redox potential of the TTQ. However, at least for the reaction with methylamine this cannot be so, since in that case k_{red} should be affected, contrary to our observations. We therefore believe that the competitive inhibition by cations is caused by binding to—or close to—a site that serves as a methylammonium-binding site in the catalytic reaction. The effect of cations on the reoxidation rate of $MADH_{red}$ must be explained differently, since amicyanin does not bind at the same site as does methylamine (Chen et al., 1992). Although we cannot *a priori* exclude the possibility that the affinity of MADH for amicyanin is affected, it seems more likely that cation binding changes the actual rate of electron transfer between MADH and amicyanin, particularly since for *P. denitrificans* determinations of the apparent dissociation constants of the MADH–amicyanin complex demonstrated that 200 mM NaCl, rather than stimulating the formation of the complex, induced an 8-fold increase of K_d (Davidson et al., 1993).

The apparent pK_a value of 8.9 that we found for the pH dependence of k_{assoc}^{app}(methylamine) provides us with a lower limit for the true pK_a of the protein group involved in the pH effect. The pK_a^{app} of 8.2 for the oxidative half-reaction, which

also represents a lower limit, may well derive from the same group. Within the active site pocket the best candidate for this role seems to be Tyr 119.

On the basis of the crystal structure the best candidate within the active site pocket for the high-affinity, large cation-binding site is Asp 76, which has been proposed to play a crucial role in catalysis (Huizinga et al., 1992). We propose that this residue serves as a substrate-binding site in catalysis. The red shift may be explained by the proximity of the TTQ to the charge of the cation. Previously, the red shift with NH_4^+ has been ascribed to a specific ammonia adduct of TTQ (Kenney & McIntire, 1983; McWhirter & Klapper, 1989). Our observation of similar spectroscopic changes in the presence of Cs^+ and Rb^+ rules out that possibility.

Against the identification of Asp 76 as the high-affinity cation-binding site one might raise the objection that the pronounced pH dependence of cation binding suggests that the group involved has a pK_a far higher than is expected for aspartate. However, whereas for some cations the data for the pH dependence of cation binding yield a straightforward relationship between pH and K_d^{app}, with K_d^{app} increasing 1 order of magnitude per pH unit, for other cations the increase is considerably smaller than that (Table 1). From this it follows that indirect effects are involved in the pH dependence of cation binding. This is not surprising, as within the narrow bounds of the active site pocket the protonation state of any one group is expected to have profound effects on the cation-binding properties of another. Therefore, we postulate that the protonation state of Tyr 119, i.e., the same group that according to us determines the pH dependence of the reactivity of MADH, also affects cation binding to Asp 76, which itself may remain deprotonated in the experimental pH range. Although not readily identifiable, the low-affinity cation-binding site is likely to be found within the active site pocket as well, since the reduction of $MADH_{ox}$ by methylamine is competitively inhibited by cation binding to this site, and since we observed strong anticooperativity between high- and low-affinity cation binding.

The absence of spectral effects with divalent cations, which, in contrast to monovalent cations, inhibit not only the reduction but also the reoxidation of MADH, suggests that these ions do not bind in the active site pocket.

CONCLUSIONS

We have demonstrated the presence of two specific monovalent cation-binding sites in the substrate-binding pocket of MADH. When either of these sites is occupied, the reduction of $MADH_{ox}$ by methylamine is competitively inhibited, whereas the rate of reoxidation of $MADH_{red}$ by amicyanin is greatly enhanced. There is at least one group in the active site, the protonation of which results in the complete inhibition of the reduction of $MADH_{ox}$ by methylamine and the almost complete inhibition of the reoxidation by amicyanin. In Figure 6 the various enzyme states as well as the effects of pH and cations on the catalytic cycle are summarized.

ACKNOWLEDGMENT

The authors thank A. Besse and A. C. Mulder for their contributions to the experimental work. The Simfit program was developed in our laboratory by Dr. J. B. A. van Tol.

REFERENCES

Armstrong, J. McD. (1964) *Biochim. Biophys. Acta 86*, 194–197.
Backes, G., Davidson, V. L., Huitema, F., Duine, J. A., & Sanders-Loehr, J. (1991) *Biochemistry 30*, 9201–9210.
Brooks, H. B., & Davidson, V. L. (1993) *Biochem. J. 294*, 211–213.
Brooks, H. B., Jones, L. H., & Davidson, V. L. (1993) *Biochemistry 32*, 2725–2729.
Chandrasekar, R., & Klapper, M. H. (1986) *J. Biol. Chem. 261*, 3616–3619.
Chen, L., Mathews, F. S., Davidson, V. L., Huizinga, E. G., Vellieux, F. M. D., Duine, J. A., & Hol, W. G. J. (1991) *FEBS Lett. 287*, 163–166.
Chen, L., Durley, R., Poliks, B. J., Hamada, K., Chen, Z., Mathews, F. S., Davidson, V. L., Satow, Y., Huizinga, E., Vellieux, F. M. D., & Hol, W. G. J. (1992) *Biochemistry 31*, 4959–4964.
Davidson, V. L. (1989) *Biochem. J. 261*, 107–111.
Davidson, V. L., Jones, L. H., & Graichen, M. E. (1992) *Biochemistry 31*, 3385–3390.
Davidson, V. L., Graichen, M. E., & Jones, L H. (1993) *Biochim. Biophys. Acta 1144*, 39–45.
Eady, R. R., & Large, P. J. (1968) *Biochem. J. 106*, 245–255.
Eady, R. R., & Large, P. J. (1971) *Biochem. J. 123*, 757–771.
Haywood, G. W., Janschke, N. S., Large, P. J., & Wallis, J. M. (1982) *FEMS Microbiol. Lett. 15*, 79–82.
Huizinga, E. G., van Zanten, B. A. M., Duine, J. A., Jongejan, J. A., Huitema, F., Wilson, K. S., & Hol, W. G. J. (1992) *Biochemistry 31*, 9789–9795.
Husain, M., & Davidson, V. L. (1987) *J. Bacteriol. 169*, 1712–1717.
Kenney, W. C., & McIntire, W. (1983) *Biochemistry 22*, 3858–3868.
Lawton, S. A., & Anthony, C. (1985) *Biochem. J. 228*, 719–726.
Lommen, A., & Canters, G. W. (1990) *J. Biol. Chem. 265*, 2768–2774.
Lommen, A., Canters, G. W., & van Beeumen, J. (1988) *Eur. J. Biochem. 176*, 213–223.
Matsumoto, T. (1978) *Biochim. Biophys. Acta 522*, 291–302.
McIntire, W. S. (1987) *J. Biol. Chem. 262*, 11012–11019.
McIntire, W. S., Wemmer, D. E., Chistoserdov, A. E., & Lidstrom, M. E. (1991a) *Science 252*, 817–824.
McIntire, W. S., Bates, J. L., Brown, D. E., & Dooley, D. M. (1991b) *Biochemistry 30*, 125–133.
McWhirter, R. B., & Klapper, M. H. (1989) in *PQQ & Quinoproteins* (Jongejan, J. A., & Duine, J. A., Eds.) pp 259–268, Kluwer Academic Publishers, Dordrecht.
Mehta, R. J. (1977) *Can. J. Microbiol. 23*, 402–406.
Tobari, J., & Harada, Y. (1981) *Biochem. Biophys. Res. Commun. 101*, 502–508.
Ubbink, M., van Kleef, M. A. G., Kleinjan, D.-J., Hoitink, C. W. G., Huitema, F., Beintema, J. J., Duine, J. A., & Canters, G. W. (1991) *Eur. J. Biochem. 202*, 1003–1012.
van Houwelingen, T., Canters, G. W., Stobbelaar, G., Duine, J. A., Frank, J. Jzn., & Tsugita, A. (1985) *Eur. J. Biochem. 153*, 75–80.
van Spanning, R. J. M., Wansell, C. W., Reijnders, W. N. M., Oltmann, F., & Stouthamer, A. H. (1990) *FEBS Lett. 275*, 217–220.
van Wielink, J. E., Frank, J. Jzn., & Duine, J. A. (1989) in *PQQ & Quinoproteins* (Jongejan, J. A., & Duine, J. A., Eds.) pp. 269–278, Kluwer Academic Publishers, Dordrecht.
van Wielink, J. E., Frank, J., & Duine, J. A. (1990) *Methods Enzymol. 188*, 235–241.
Vellieux, F. M. D., Frank, J. Jzn., Swarte, M. B. A., Groendijk, H., Duine, J. A., Drenth, J., & Hol, W. G. J. (1986) *Eur. J. Biochem. 154*, 383–386.