Binding of Monovalent Cations to Methylamine Dehydrogenase in the Semiquinone State and Its Effect on Electron Transfer[†]

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ABSTRACT: The binding of monovalent cations to methylamine dehydrogenase in the semiquinone state ($MADH_{sq}$) at a site close to the tryptophan tryptophylquinone (TTQ) active center is demonstrated in experiments which show that the radical EPR signal of $MADH_{sq}$ is considerably broadened in the presence of Cs^+, NH_4^+, and, to a smaller extent, Na^+. The cations also stabilize the semiquinone state, as is evident from the increase of the EPR intensity they induce. On the basis of the optical absorbance spectra, two slightly different forms of $MADH_{sq}$ can be discerned. One form, with the main band at 425 nm, is observed at low pH and in the presence of NH_4^+, whereas the other, with the main band at 429 nm, is observed at high pH and in the presence of Cs^+ or Na^+. Stopped-flow studies of the oxidation by amicyanin of $MADH_{red}$ via $MADH_{sq}$ to $MADH_{ox}$ show a strong stimulation of the first step by monovalent cations. It is shown that it is primarily the actual electron transfer rate, rather than the affinity of $MADH_{red}$ for amicyanin, that is affected by cations. Values for the dissociation constants of the monovalent cations for $MADH_{red}$, estimated from the kinetic experiments, are higher than those that were previously determined for $MADH_{ox}$, and can be deduced to be higher than those for $MADH_{sq}$ as well. The results are discussed within the context of the electron transfer theory.

Methylamine dehydrogenase [MADH,[1] EC 1.4.99.3; for recent reviews, see Davidson (1993), Duine (1993), and Klinman and Mu (1994)] 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; Kenney & McIntire, 1983; Vellieux et al., 1986; Husain & Davidson, 1987). The enzyme, which is unique in containing tryptophan tryptophylquinone (TTQ) as the active center (McIntire et al., 1991; 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; van Houwelingen et al., 1985; van Spanning et al., 1990).

It has been demonstrated that monovalent cations have profound effects on both the spectral and kinetic properties of MADH. The binding of monovalent cations to $MADH_{ox}$ is manifest from changes in the optical absorbance spectrum (Kuusk & McIntire, 1994; Gorren & Duine, 1994), and monovalent cations competitively inhibit the reduction of $MADH_{ox}$ by methylamine (Gorren & Duine, 1994). By extending these observations from simple monovalent cations to several nonconvertible cationic substrate analogues such as trimethylamine, it was shown that the inhibition of the reduction of $MADH_{ox}$ is due to competition between $CH_3NH_3^+$ and other monovalent cations for a site in close proximity to the TTQ *o*-quinone group, that serves as the methylammonium binding site in catalysis (Kuusk & McIntire, 1994; Gorren et al., unpublished results).

Monovalent cations not only decrease the reduction rate of $MADH_{ox}$ but also increase the oxidation rate of $MADH_{red}$ by amicyanin (Gorren & Duine, 1994). The oxidation necessarily has to occur in two steps, as TTQ_{red} is a two-electron donor, whereas amicyanin is a one-electron acceptor. In the absence of monovalent cations, the intermediate semiquinone form was not accumulated, indicating that $MADH_{sq}$ reacts much faster with amicyanin than does $MADH_{red}$. In the presence of monovalent cations, however, the transient formation of $MADH_{sq}$ could be observed, which shows that the cations mainly or solely increase the rate of the oxidation of $MADH_{red}$ to $MADH_{sq}$ (Gorren & Duine, 1994).

Whereas the inhibitory effect of monovalent cations on the reduction of $MADH_{ox}$ by methylamine can, on the basis of both spectral and kinetic data, be ascribed to the existence of a methylammonium binding site on MADH, the origin of the stimulation of the oxidation of $MADH_{red}$ by amicyanin is unknown. In the present paper, we aim to establish if monovalent cations bind to $MADH_{sq}$, as they do to $MADH_{ox}$, by investigating the effect of monovalent cations on the EPR signal of $MADH_{sq}$. Furthermore, additonal studies of the stimulation by monovalent cations of the oxidation of $MADH_{red}$ by amicyanin were performed to obtain values for

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[1] Abbreviations: $MADH_{ox}$, $MADH_{sq}$, and $MADH_{red}$, oxidized, semireduced, and reduced methylamine dehydrogenase, respectively; amic, amicyanin; MeAm, methylamine; TriMeAm, trimethylamine; TTQ, tryptophan tryptophylquinone; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; n.a., no additions.

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the apparent dissociation constants of cations to $MADH_{red}$, and to determine whether it is the actual electron transfer rate or the affinity of $MADH_{red}$ for amicyanin that is affected.

MATERIALS AND METHODS

Thiobacillus 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 by spectrophotometric methods, adopting an A_{280}/A_{440} ratio of 6.7 (van Wielink et al., 1990) and an 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 from the amount of methylamine needed to reduce the oxidized enzyme, as judged from the changes in the optical absorbance spectrum. Reduced MADH was prepared just before use by addition of a stoichiometric amount of methylamine to the oxidized enzyme. 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). Methylamine•HCl and trimethylamine•HCl were purchased from Aldrich. All reagents were analytical grade and used without further purification. All experiments were performed at 20 °C.

Optical absorbance spectra were measured on a Hewlett-Packard 8452A diode array spectrophotometer. Pre-steady-state kinetics were performed with a Hi-Tech Scientific PQ/SF-53 preparative quench/stopped-flow spectrophotometer. The kinetic traces for the oxidation by amicyanin of $MADH_{red}$ via $MADH_{sq}$ to $MADH_{ox}$ were fitted to the function:

$$\Delta A = [\mathrm{MADH}]*\left[\left(\Delta\epsilon_{\mathrm{ox-red}} + \Delta\epsilon_{\mathrm{sq-red}}\frac{k_1}{k_2 - k_1}\right)*\mathrm{e}^{-k_1 t} - \Delta\epsilon_{\mathrm{sq-red}}*\frac{k_1}{k_2 - k_1}*\mathrm{e}^{-k_2 t}\right]$$

with the underlying assumption that the reaction can be described by two consecutive pseudo-first-order reactions.

EPR spectra were measured with a Varian E9 spectrometer at room temperature. $MADH_{sq}$ samples were prepared by three different methods:

(A) "Photoinduced" Samples. MADH (500 μL of 53.3 μM tetramer in 20 mM HEPES, pH 7.3, and in the presence or absence of cations) was reduced by the addition of 1 mM methylamine and illuminated for 1 h with a 50 W quartz−halogen light source. The excess of methylamine was removed by four cycles of concentration by ultracentrifugation (Centriprep-30; Amicon) and dilution in 15 mL of 20 mM HEPES, pH 7.3. Samples were then transferred to EPR aqueous sample cells and measured.

(B) "Methylamine-Induced" Samples. MADH (500 μL of 53.3 μM tetramer in 20 mM CHES, pH 8.9, and in the presence or absence of cations) was divided into two equal portions, one of which was then stoichiometrically reduced by the addition of 1−5 μL aliquots of a 500 μM solution of methylamine. The reduction was monitored spectrophotometrically. The MADH portions (one oxidized, one reduced) were then recombined, transferred to EPR aqueous sample cells, and measured.

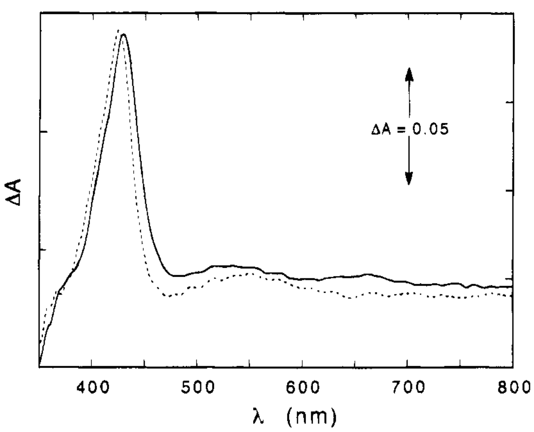

FIGURE 1: Optical absorbance difference spectra obtained by continuous illumination of $MADH_{red}$. Experimental procedure: 13.2 μM $MADH_{ox}$ was reduced by 1 mM methylamine and illuminated for 20 min by a 50 W quartz−halogen light source. Shown are the light-minus-dark absorbance difference spectra observed at pH 7.0 (20 mM HEPES) (dotted line) and at pH 9.1 (20 mM CHES) (continuous line).

(C) "Dithionite-Induced" Samples. MADH (2000 μL of 53.3 μM tetramer in 20 mM CHES, pH 9.0) was made anaerobic by flushing the air-tight samples with argon (Air-Products). The sample was then semireduced by the addition of 5−20 μL aliquots of a freshly prepared anaerobic 500 μM solution of sodium dithionite with a gas-tight Hamilton syringe until the concentration of $MADH_{sq}$, which was monitored spectrophotometrically, had reached its maximum level. From this solution, which was then kept on ice anaerobically, 200 μL portions were taken, under readmittance of air. Subsequently the appropriate cation concentrations were added to these samples, after which they were transferred to EPR aqueous sample cells and measured. In contrast to the samples obtained by the other methods, the EPR intensity of the "dithionite-induced" samples slowly decreased in time, probably due to the presence of air during the experiments (Kenney & McIntire, 1983).

RESULTS

When $MADH_{ox}$ and an excess of methylamine were mixed in a stopped-flow spectrophotometer, a rapid reduction of MADH ensued (Gorren & Duine, 1994). Unexpectedly, however, upon longer incubation this fast $2e^-$-reduction was followed by a very slow reaction, which could be identified as a $1e^-$-oxidation by comparing the absorbance difference spectrum with that obtained for $MADH_{sq}$-minus-$MADH_{red}$ in titrations of MADH with sodium dithionite (Kenney & McIntire, 1983). This reaction turned out to be light-induced and was slowly reversed in the dark. When MADH was reduced with a stoichiometric amount of methylamine instead of a large excess, formation of $MADH_{ox}$ as well as of $MADH_{sq}$ could be observed, and re-reduction no longer occurred. No photooxidation was observed in frozen samples, indicating that a bimolecular event is involved.

The photoinduced $MADH_{sq}/MADH_{red}$ absorbance difference spectra at high and neutral pH exhibited subtle differences (Figure 1). At pH 7.0, the main band, centered at 425 nm, was accompanied by one low band at 550 nm, whereas at pH 9.1 the main band had shifted to 429 nm and two low bands at 520 and 660 nm could be discerned.

Table 1: Optical Absorbance Properties of $\mathrm{MADH_{sq}}$ in the Presence and Absence of Monovalent Cations[a]

	sample	peak position (nm)	type of spectrum[b]
pH 7.0	n.a.	425	I
	0.1 M $\mathrm{Cs^+}$	427	II
	1 M $\mathrm{K^+}$	426	I-II
pH 9.1	n.a.	429	II
	0.1 M $\mathrm{Cs^+}$	429	II
	0.5 M $\mathrm{Na^+}$	429	II
	0.1 M $\mathrm{NH_4^+}$	427	I
	0.5 M $\mathrm{NH_4^+}$	427	I

[a] See the legends to Figure 1 for experimental details. [b] Spectral type I has one low absorbance band at 550 nm (see dotted line from Figure 1); spectral type II has two low bands at 520 and 660 nm (see continuous line from Figure 1).

Table 2: EPR Parameters of $\mathrm{MADH_{sq}}$ in the Absence and Presence of Monovalent Cations[a]

sample type; pH[b]	cation	*g*-value	line width (G)	relative intensity[c]
photoinduced (pH 7.3)	n.a.	2.0036	8.6	
	0.1 M $\mathrm{Cs^+}$	2.0043	10.8	
	0.02 M $\mathrm{TriMeAm^+}$	2.0038	8.8	
methylamine-induced (pH 8.9)	n.a.	2.0041	8.0	≡1
	0.5 M $\mathrm{Na^+}$	2.0041	8.4	1.12
	0.1 M $\mathrm{^{14}NH_4^+}$	2.0041	9.0	1.47
	0.1 M $\mathrm{^{15}NH_4^+}$	2.0042	9.2	1.48
	0.1M $\mathrm{Cs^+}$	2.0045	12.0	1.96
dithionite-induced (pH 9.0)	n.a.	2.0038	6.9	≡1
	0.001 M $\mathrm{MeAm^+}$	2.0038	6.8	1.01
	0.01 M $\mathrm{TriMeAm^+}$	2.0038	6.8	0.86
	0.5 M $\mathrm{Na^+}$	2.0038	7.5	1.52
	0.02 M $\mathrm{^{14}NH_4^+}$	2.0036	9.0	1.48
	0.5 M $\mathrm{^{14}NH_4^+}$	2.0035	9.1	2.15
	0.05 M $\mathrm{Cs^+}$	2.0042	12.2	2.52

[a] See the legends to Figure 2 for spectrometer settings. [b] See Materials and Methods for experimental details. [c] The signal intensities were obtained by double integration of the EPR signal, and are expressed relative to the intensities of the signals in the absence of cations. The absolute intensities of the latter signals were 46.9 and 29.3 μM for the "methylamine-" and "dithionite-induced" samples respectively (TTQ concentration: 106.6 μM).

Monovalent cations also affected the spectrum of the photoinduced $\mathrm{MADH_{sq}}$: in the presence of CsCl, the "high-pH" form was obtained both at pH 7.0 and at 9.1, whereas in the presence of $\mathrm{NH_4Cl}$ the "low-pH form" prevailed at both pH values. The small monovalent cations $\mathrm{Na^+}$ and $\mathrm{K^+}$ apparently both favored formation of the "high-pH" form (Table 1). The photooxidation rate and the maximally obtainable $\mathrm{MADH_{sq}}$ concentration by and large did not depend strongly on pH or the presence of cations, yielding calculated values of 0.010–0.014 $\mathrm{min^{-1}}$ for the rate of photooxidation of $\mathrm{MADH_{red}}$ to $\mathrm{MADH_{sq}}$, of 0.021–0.035 $\mathrm{min^{-1}}$ for the reverse reaction, and of 27–39% for the fraction of $\mathrm{MADH_{sq}}$ maximally formed during illumination (with a 50 W quartz–halogen light source).

Table 2 shows the EPR parameters of the $\mathrm{MADH_{sq}}$ radical signal measured for various $\mathrm{MADH_{sq}}$-containing samples. Small but reproducible differences could be discerned between samples that were obtained by photooxidation at pH 7.3 and those that were obtained by incubation at pH 8.9 of MADH that was semireduced by methylamine, the latter ones displaying slightly higher *g*-values (Figure 2A). Samples that were obtained by anaerobic semireduction of MADH by sodium dithionite at pH 9.0 had *g*-values similar to those of the "photoinduced" samples, but slightly narrower signals than either of the other two sample types (Table 2). Addition of methylamine to this sample type had no effect on the EPR spectrum (Table 2).

Despite their different EPR properties, all three types of sample reacted in the same fashion to the addition of monovalent cations. $\mathrm{Cs^+}$ induced a strong broadening of the signal and a small increase of the *g*-value (Figure 2B). $\mathrm{NH_4^+}$ also broadened the EPR signal, though to a lesser extent; in the "dithionite-induced" samples, a small negative *g*-shift was detected (Figure 2C). With $\mathrm{^{15}NH_4^+}$, a spectrum was obtained with very similar parameters, but a slightly different shape (Figure 2D). With $\mathrm{Na^+}$, the broadening, if any, was small (Table 2). Surprisingly, trimethylamine did not affect the EPR spectrum whatsoever (Table 2). The cations also affected the intensity of the EPR signal. $\mathrm{Cs^+}$, $\mathrm{NH_4^+}$, and, to a lesser extent, $\mathrm{Na^+}$ all increased the intensity of the signal. Trimethylamine, on the other hand, caused a significant decrease. Methylamine had no effect (Table 2).

The oxidation of $\mathrm{MADH_{red}}$ by amicyanin is strongly stimulated by the presence of monovalent cations (Gorren & Duine, 1994). Figure 3 shows how the oxidation rate of $\mathrm{MADH_{red}}$ by amicyanin depended on the concentration of various cations for both consecutive oxidation steps, i.e., for $\mathrm{MADH_{red}}$ to $\mathrm{MADH_{sq}}$ and for $\mathrm{MADH_{sq}}$ to $\mathrm{MADH_{ox}}$ separately. The results confirm the previously reported order of effectiveness for the cations in stimulating the oxidation rate ($\mathrm{Cs^+} > \mathrm{NH_4^+} \geq \mathrm{Rb^+} > \mathrm{K^+}$; the $\mathrm{Rb^+}$ data are for clarity left out of the figure), as well as the fact that the effect is primarily on the first oxidation step (Gorren & Duine, 1994). In contrast to the other cations, trimethylammonium had hardly any effect on the oxidation rate at all. Values for the dissociation constants (K_d) as well as for the rate constants at saturating cation concentrations (k_{ox}') were estimated from Figure 3 for both oxidation steps (Table 3).

By measuring the oxidation rate in the absence and presence of CsCl at varying concentrations of amicyanin we were able to establish that first and foremost the maximal rate of oxidation was affected; however, a smaller effect on the affinity of MADH for amicyanin cannot be excluded (Table 4).

DISCUSSION

The determination of the effects of monovalent cations on the absorbance spectrum of $\mathrm{MADH_{sq}}$ proved to be less straightforward than was the case for $\mathrm{MADH_{ox}}$ and $\mathrm{MADH_{red}}$ (for the latter we observed no spectral effects) for two reasons. The effects were smaller than those found for $\mathrm{MADH_{ox}}$ and the monovalent cations shift the redox equilibrium 2:

$$2\mathrm{MADH_{sq}} \leftrightarrow \mathrm{MADH_{ox}} + \mathrm{MADH_{red}} \qquad (2)$$

Because of this, the usual way to obtain the $\mathrm{MADH_{sq}}$ absorbance spectrum, by anaerobic titration of $\mathrm{MADH_{ox}}$ by dithionite at high pH (Kenney & McIntire, 1983; Husain et al., 1987; Davidson et al., 1990), is not applicable, since the effects of the cations on the $\mathrm{MADH_{sq}}$ absorbance spectrum will be drowned by shifts in the redox equilibrium. Therefore, we chose to generate the $\mathrm{MADH_{sq}}$ state by photooxidation of $\mathrm{MADH_{red}}$ in the presence of an excess of methylamine. To our knowledge, there is no mention of the photooxidation of $\mathrm{MADH_{red}}$ in the literature. Perhaps the photosensitivity of methylamine-reduced MADH from

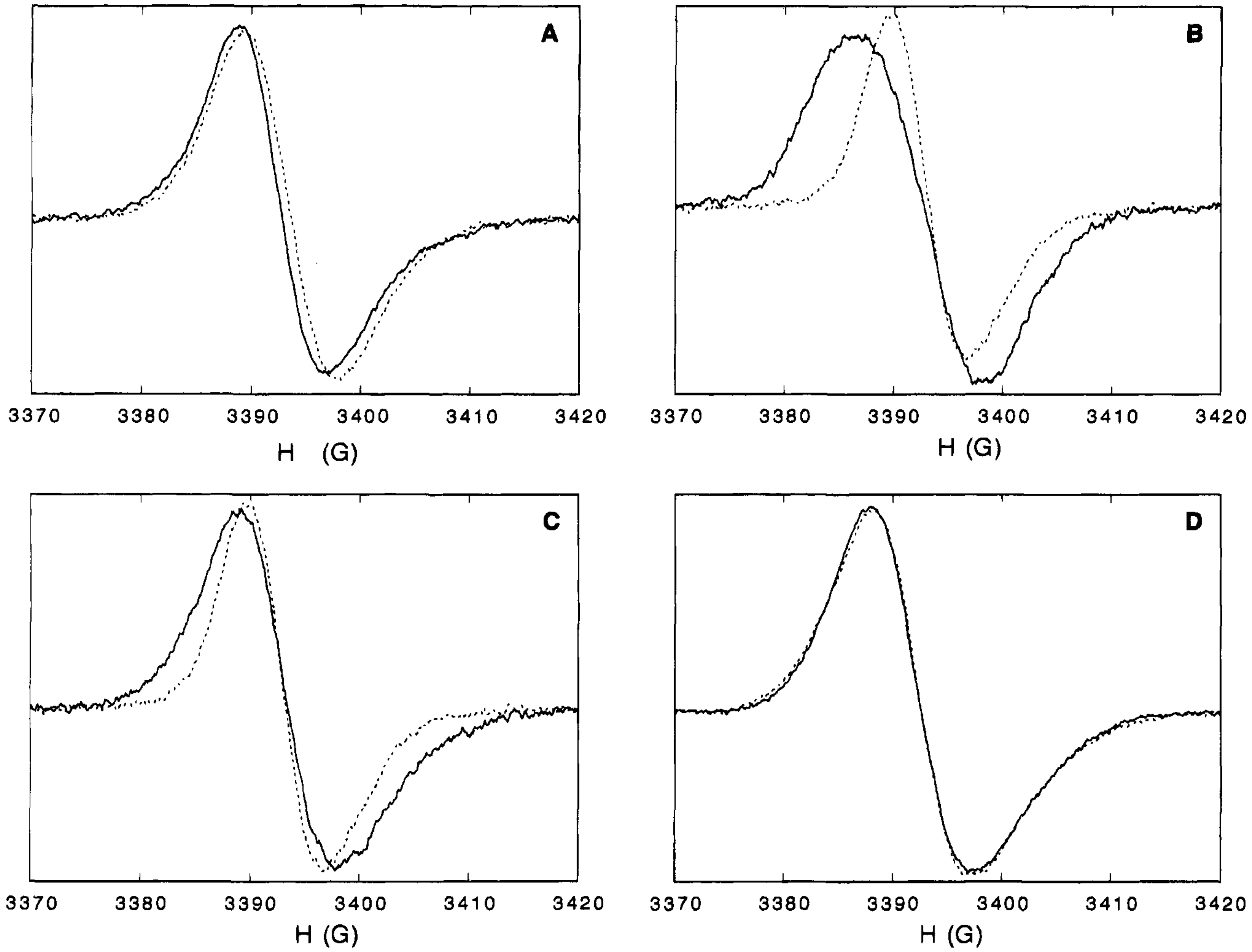

FIGURE 2: Dependence of the EPR spectra of $MADH_{sq}$ on pH and monovalent cations. Panel A: Comparison of the spectra of a "methylamine-induced" sample at pH 8.9 (continuous line) and a "photoinduced" sample at pH 7.3 (dotted line). Panel B: Comparison of the spectra of "dithionite-induced" samples in the presence (continuous line) and absence (dotted line) of 50 mM CsCl (pH 9.0). Panel C: Comparison of the spectra of "dithionite-induced" samples in the presence (continuous line) and absence (dotted line) of 20 mM $^{14}NH_4Cl$ (pH 9.0). Panel D: Comparison of the spectra of "methylamine-induced" samples (pH 8.9) in the presence of 100 mM $^{15}NH_4Cl$ (continuous line) and 100 mM $^{14}NH_4Cl$ (dotted line). See Materials and Methods for details on sample preparation. The amplitudes of the signals were arbitrarily set so as to facilitate comparison of the signal shapes. Spectrometer settings: microwave frequency, 9515 GHz; modulation amplitude, 0.2 mT; microwave power, 1.0 mW; scan rate, 1.25 mT•min^{-1}; temperature, 21 °C.

Methylobacterium extorquens AM1 upon prolongued illumination by a strong light source, as reported by Eady and Large (1971), may be taken as evidence that the photooxidation is not limited to the enzyme from *T. versutus.*

In the absence of excess methylamine, illumination of $MADH_{red}$ eventually led to the formation of $MADH_{ox}$, either by photooxidation of $MADH_{sq}$ or according to eq 2. Excess methylamine, however, will re-reduce all $MADH_{ox}$ formed, thereby allowing the determination of a pure $MADH_{sq}$ − $MADH_{red}$ absorbance difference spectrum, devoid of $MADH_{ox}$. A further advantage of the method is that it is applicable at neutral as well as at high pH. On the other hand, since we have no information on the exact nature of the photoreaction, other than that it must involve an external agent, we cannot be certain that the enzyme form thus created is identical to the $MADH_{sq}$ obtained by dithionite titration, although in this respect the very close resemblance of the aborbance spectrum is reassuring. Also, only the shape and not the absolute amplitude of the absorbance spectrum can be determined, since $MADH_{sq}$ may not be fully formed. As a consequence, a general bleaching, as was observed for some cations in the case of $MADH_{ox}$ (Gorren & Duine, 1994), will go unnoticed.

As was the case for $MADH_{ox}$, two different spectral effects of monovalent cations could be discerned, but the distribution of the cations between the two classes was totally different. Low pH and the presence of NH_4^+ favored a form with the major band centered at 425 nm, whereas high-pH conditions, as well as the other monovalent cations investigated, induced a form with the band at 429 nm. One way of explaining this dichotomy is that, while being bound at the same location in the MADH active site pocket, NH_4^+ and H_3O^+, unlike the other cations, provide a possibility for additional hydrogen bonds. A slight blue-shift of the $MADH_{sq}$ absorbance peak from 429 to 427 nm in the presence of NH_4^+ at pH 9.0 has also been reported for the enzyme from *Methylophilus* W3A1 (Kenney & McIntire, 1983).

EPR spectra of $MADH_{sq}$ were gathered for samples prepared by three quite different methods. Nevertheless, differences between the three sample types were generally small. The *g*-values for all samples, which ranged from 2.0035 to 2.0046, were far more similar to each other and to a previously reported value for the *Thiobacillus* enzyme ($g = 2.0043$; de Beer et al., 1980), than to the value found for MADH from *M.* W3A1 ($g = 2.0086$; Kenney & McIntire, 1983). Because of the uncertainties with regard to the exact

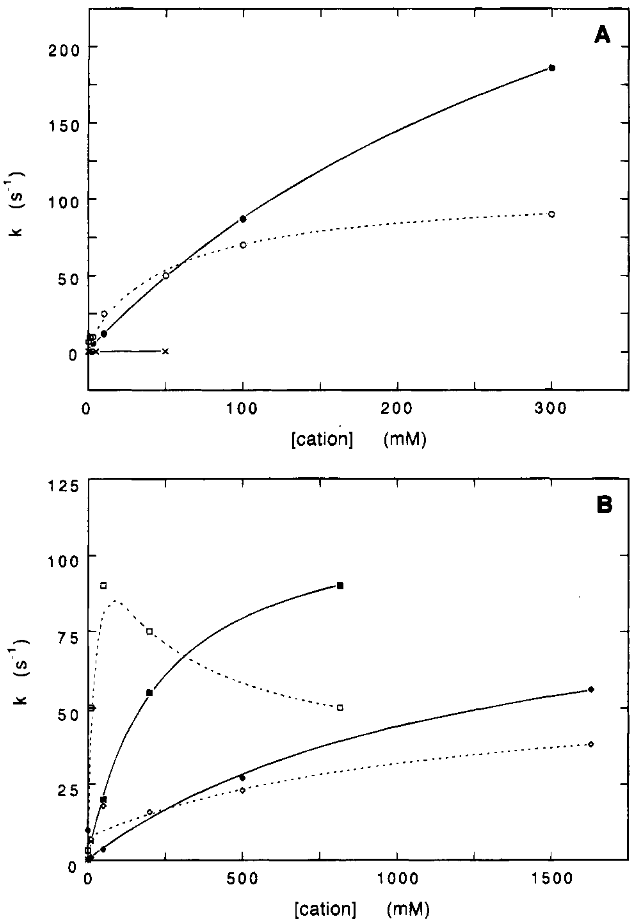

FIGURE 3: Effect of monovalent cations on the rate of oxidation of $MADH_{red}$ and $MADH_{sq}$ by amicyanin. Rate constants were calculated as described under Materials and Methods. Shown are the oxidation rate constants for $MADH_{red}$ (continuous lines; closed symbols) and $MADH_{sq}$ (dotted lines; open symbols) in the presence of CsCl (circles; panel A), NH_4Cl (squares; panel B), KCl (diamonds; panel B), and trimethylamineHCl (crosses; panel A). Experimental conditions: 3.3 μM $MADH_{red}$, 40 μM amicyanin, 20 mM HEPES (pH 7.0). For the lines drawn through the data points (except for the oxidation of $MADH_{sq}$ in the presence of NH_4^+), see Table 3.

Table 3: Estimated Kinetic Constants for Cation Binding to $MADH_{red}$ and Cationic Stimulation of the $MADH_{red}$ Oxidation Rate[a]

	$MADH_{red}$		$MADH_{sq}$	
cation	K_d (mM)	k_{ox}' (s^{-1})	K_d (mM)	k_{ox}' (s^{-1})
Cs^+	400 (50)	430 (50)	50 (20)	110 (20)
NH_4^+	230 (20)	120 (10)		≤50
K^+	1400 (300)	100 (20)	1100 (600)	60 (20)

[a] Values were estimated by fitting plots of the calculated rate constants for the oxidations of $MADH_{red}$ to $MADH_{sq}$ and $MADH_{sq}$ to $MADH_{ox}$ (see Figure 3) to the function $k_{obs} = (k_{ox}K_d + k_{ox}'[\text{cation}])/(K_d + [\text{cation}])$ in which k_{ox} and k_{ox}' are the apparent rate constants for the oxidation of $MADH_{red}$ or $MADH_{sq}$ by amicyanin in the absence and presence of cations, respectively, and K_d represents the dissociation constant for the cation. For k_{ox}, values of 0.31 (1) and 7 (1) s^{-1} were obtained for the oxidation of $MADH_{red}$ and $MADH_{sq}$, respectively. If only the maximal oxidation rates are affected by the cations, the true oxidation rate constants in the absence (k^0) and presence (k^x) of cations are obtained by multiplication of k_{ox} and k_{ox}' with $(K_s + [\text{amic}])/[\text{amic}]$, with K_s representing the dissociation constant for amicyanin. Assuming a value for K_s of 30 μM (see Table 4) and with 40 μM amicyanin, this yields a multiplication factor of 1.75. Estimated accuracies are indicated in parentheses.

Table 4: Kinetic Parameters for the Oxidation of $MADH_{red}$ by Amicyanin in the Absence and Presence of Cations[a]

conditions	k_{ox} (s^{-1})	K_s (μM)
20 mM HEPES (pH 7.0)	0.57 (8)	30 (10)
idem + 50 mM CsCl	103 (7)	10 (5)
50 mM KP_i (pH 7.0)	15 (5)	50 (20)

[a] The kinetic parameters were determined by fitting the plots of the rate of oxidation of $MADH_{red}$ to $MADH_{sq}$ vs the amicyanin concentration to the function $k_{obs} = k_{ox}[\text{amic}]/(K_s + [\text{amic}])$. Estimated accuracies are indicated in parentheses.

nature of the photoinduced form of $MADH_{sq}$, the slightly lower *g*-values observed for these samples, as compared to those obtained at pH 9, cannot be confidently ascribed to the pH difference. An indication, however, that the pH difference is causing the shift is perhaps provided by the observation that Cs^+ increases the *g*-value, whereas NH_4^+, at least for the "dithionite-reduced" samples as well as for the enzyme from *M.* W3A1 ($g = 2.0075$; Kenney & McIntire, 1983), appears to lower it, which exactly mirrors the alignment of low pH and NH_4^+ vs high pH and Cs^+ we established for the optical absorbance changes of $MADH_{sq}$. The width of the signal also differed slightly between the different sample types. To which extent the use of different reducing agents (methylamine or dithionite) or different pHs has contributed to those variations must remain unresolved at present. That it was not the mere presence of methylamine that induced the differences is clear from the observation that methylamine did not affect the EPR properties of the "dithionite-induced" samples.

Interaction of monovalent cations with the TTQ active center in $MADH_{sq}$ is evident from the EPR spectra. The considerable broadening of the EPR signal in the presence of NH_4^+ and, especially, Cs^+ is consistent with a coupling between the electron spin of the TTQ radical and the nuclear spin of the cation ($I = {}^1/_2$, 1, and ${}^7/_2$ for ^{15}N, ^{14}N, and ^{133}Cs, respectively). The broadening with both cations is too large for alternative explanations. For the small effect we observed for Na^+, on the other hand, alternative sources, such as increased *g*-strain due to protein conformational changes, cannot be ruled out. It is also possible that the binding constant for Na^+ to $MADH_{sq}$ at pH 9 is too small to get $MADH_{sq}{\cdot}Na^+$ completely formed in the presence of 500 mM NaCl.

A significant broadening of the $MADH_{sq}$ EPR signal in the presence of NH_4Cl for the enzyme from *M.* W3A1 (from 7.5 to 11 G; Kenney & McIntire, 1983) has previously been taken as evidence for some form of covalent interaction between NH_4^+ and TTQ. However, the present results, particularly those with Cs^+, demonstrate that, although the broadening of the radical signal in the presence of monovalent cations furnishes strong evidence for the binding of the cations close to the TTQ, a covalent bond need not be invoked. This may also be relevant to conclusions drawn from recently reported ESEEM spectra of [^{14}N]- and [^{15}N]-methylamine-derived $MADH_{sq}$ samples (Warncke et al., 1993). On the basis of the significant differences between the spectra observed for both isotopes, it was assumed that the MADH semiquinone, formed during catalysis, has the methylamine nitrogen atom incorporated into the TTQ, the main argument being that the differences are too big to be explained by an interaction that is mediated by a hydrogen bond, and therefore require a covalent bond between the methylamine nitrogen and the TTQ. Our observations, however, demonstrate that a strong coupling is also consistent with an electrostatically bound cation. Perhaps ESEEM spectra of "dithionite-induced" MADH samples in the absence and presence of various cations can shed more light on this matter.

The kinetic experiments generally confirmed prior results we obtained with regard to the stimulatory effect of monovalent cations on the rate of oxidation of $MADH_{red}$ by amicyanin (Gorren & Duine, 1994). At pH 7, the order of effectiveness of different cations at a concentration of 200 mM was $Cs^+ > NH_4^+ \sim Rb^+ > K^+ > Na^+ >$ n.a., and the cations primarily affected the first oxidation step ($MADH_{red}$-to-$MADH_{sq}$). The apparent dissociation constants with regard to $MADH_{red}$ we arrived at for the various cations, however, differ substantially from previous estimates [cf. Table 3 of Gorren and Duine (1994)]. The origin of the difference lies in the fact that, as for the previous paper we were content to determine the order of magnitude of K_d, values were obtained from fits of the observed rate constants at one wavelength as a function of the cation concentrations. This can, depending on the applied wavelengths, lead to substantial underestimation of K_d, when the rates for the two oxidation steps start to diverge and when the absorbance spectra of the MADH redox states change, as they do in the presence of cations. The present values for the apparent rate constants, on the other hand, were extracted by fitting the time-resolved absorbance changes at various wavelengths to a sum of two exponential powers (see Materials and Methods), enabling the determination of apparent rate constants for the first and the second oxidation step separately. It should be noted, however, that the K_d and k_{ox}' values, which were then estimated from fits of the apparent rate constants vs the cation concentration, still represent rather crude approximations, since the cation affinities of $MADH_{red}$ turned out to be so low that saturating cation concentrations could not be attained. For the second oxidation step ($MADH_{sq}$-to-$MADH_{ox}$), an additional source of incertitude in the determined K_d and k_{ox}' values lies in the fact that at low cation concentrations the second oxidation step is faster than the first one, which introduces a large margin of error in the determination of the rate constants. The decrease that appears to occur for the rate of the oxidation of $MADH_{sq}$ to $MADH_{ox}$ at very high NH_4Cl levels may represent a genuine effect, since it could be due to the binding of a second ammonium ion to $MADH_{sq}$, as was previously shown to occur for $MADH_{ox}$ (Gorren & Duine, 1994; Kuusk & McIntire, 1994). However, in view of the uncertainties in the k values, inherent to our analysis, the phenomenon requires further verification.

The experiments in which we added varying concentrations of amicyanin confirm our earlier suspicion (Gorren & Duine, 1994) that cations primarily affect the maximal rate of oxidation, with minor effects at best on the MADH–amicyanin binding constant. A comparable set of parameters for the $MADH_{red}$–amicyanin reaction was reported for the enzyme from *Paracoccus denitrificans*, with values of 4.0 μM and 6–7 s^{-1} [estimated from Figure 3 from Brooks and Davidson (1994)] for K_s and k_{ox}, respectively, as well as a value for K_s of 4.5 μM, derived from direct binding studies (Davidson et al., 1993). These values, which were all obtained in the presence of 10 mM KP_i at pH 7.5 and 20 °C, fall fairly well within the range of our observations, when the different measuring conditions are taken into account. We found no clear evidence for the increase in K_s in the presence of salts, that was observed in those studies. A second set of parameters for the *P. denitrificans* enzyme (with values for K_s and k_{ox} of 1.9 μM and 24 s^{-1}, respectively) cannot easily be compared to our results, as the reaction conditions were not specified (Brooks & Davidson, 1993).

The bulk of the results presented in this paper can, at least qualitatively, be explained by assuming that monovalent cations affect the oxidation rate by preferentially binding to the semireduced (and oxidized) states of MADH or, to put it differently, by increasing the driving force for the oxidation of $MADH_{red}$ to $MADH_{sq}$ (eq 3):

$$\begin{tikzcd} MADH_{red} \arrow[r, shift left] \arrow[d, shift left] & MADH_{sq} \arrow[l, shift left] \arrow[d, shift left] \\ MADH_{red}{*}M^+ \arrow[u, shift left] \arrow[r, shift left] & MADH_{sq}{*}M^+ \arrow[l, shift left] \arrow[u, shift left] \end{tikzcd} \qquad (3)$$

According to the electron transfer theory (Marcus & Sutin, 1985; Sutin & Brunschwig, 1990), an increase in the driving force does not necessarily lead to an increase of the electron transfer rate, but will cause a decrease when the driving force exceeds the reorganization parameter. However, in cases involving biological electron transfer, the correlation between the driving force and the electron transfer rate is usually positive (Cusanovich et al., 1988, Sutin & Brunschwig, 1990).

If the model presented above governs the electron transfer rates, the low rate of the $MADH_{red}$-to-$MADH_{sq}$ reaction, relative to the rate of the $MADH_{sq}$-to-$MADH_{ox}$ transition we observed at low pH and in the absence of cations (Gorren & Duine, 1994), should be ascribed to a relatively low reduction potential of $MADH_{sq}$. In agreement with this, one finds that at low pH and in the absence of cations 50% reduction of MADH yields no $MADH_{sq}$, but a 50/50% mixture of $MADH_{red}$ and $MADH_{ox}$, indicating that the equilibrium of eq 2 is lying far to the right. At high pH, on the other hand, $MADH_{sq}$ will be present under otherwise similar conditions, implying a shift to the left of equilibrium 2. Moreover, double integration of the radical EPR signals demonstrated that the $MADH_{sq}$ concentration was increased by the presence of the monovalent cations, and that the extent, to which the various cations enhanced the $MADH_{sq}$ concentration, afforded the same order of cations ($Cs^+ > NH_4^+ > Na^+ >$ n.a.) as was observed for the enhancement of the oxidation rate. Strong support comes from the remarkable observation that trimethylamine, which previously was shown to bind particularly strongly to $MADH_{ox}$ (Kuusk & McIntire, 1994; Gorren and Duine, unpublished results) and to inhibit strongly the reduction of $MADH_{ox}$ by methylamine (Gorren and Duine, unpublished results), did not affect the width of the EPR signal, did not enhance the $MADH_{red}$ oxidation rate, and lowered the $MADH_{sq}$ concentration. Finally, from the low affinity of Cs^+ and NH_4^+ for $MADH_{red}$ (Table 3) as compared to that for $MADH_{ox}$ (Gorren & Duine, 1994), in combination with the observation that the cations shift equilibrium 2 toward the semiquinone state, it follows that the K_d values for cation binding to $MADH_{red}$ must be higher than those for $MADH_{sq}$.

Interestingly, an increase of the rate of $MADH_{sq}$ formation from $MADH_{ox}$ and $MADH_{red}$ with increasing buffer concentration (KP_i) was previously reported for the enzyme from *P. denitrificans* (Davidson et al., 1990). The phenomenon, which was ascribed to an effect of ionic strength on the bimolecular association, may well have been due to a similar stabilization of the $MADH_{sq}$ state by K^+ ions as observed by us.

Although the *qualitative* correlation between the cation affinities and the oxidation rate is striking, a quantitative assessment shows that the oxidation rate increase is unlikely to be solely due to a greater driving force. The rate constant for electron transfer is given by

$$k = \mathrm{A}^{*}\mathrm{e}^{-(\lambda/4RT)(1+G^{\circ\prime}/\lambda)^2} \tag{4}$$

in which A is a preexponential factor, $\Delta G^{\circ\prime}$ is the standard free energy change, and λ is the reorganization parameter (Marcus & Sutin, 1985). The way in which a change in driving force affects the reaction rate is described by

$$\frac{\mathrm{d}\log k}{\mathrm{d}\log K} = \frac{1}{2}\left(1 - \frac{RT}{\lambda}\log K\right) \tag{5}$$

with K representing the redox equilibrium constant. From this, one can derive the relation between rates and driving forces in the presence and absence of cations:

$$\log\left(\frac{k^{\mathrm{x}}}{k^{0}}\right) < \frac{1}{2}\left(1 - \frac{RT}{\lambda}\log K^{0}\right)^{*}\log\left(\frac{K^{\mathrm{x}}}{K^{0}}\right) \tag{6}$$

with k^0, k^x, K^0, and K^x representing the rate constants and equilibrium constants in the absence and presence of cations, respectively. The redox equilibrium constants can be expressed in the cation dissociation constants according to equilibrium 3 as

$$K_{\mathrm{d}}^{\mathrm{red}}K^{0} = K_{\mathrm{d}}^{\mathrm{sq}}K^{\mathrm{x}} \tag{7}$$

with K_d^{red} and K_d^{sq} representing the cation dissociation constants for $\mathrm{MADH_{red}}$ and $\mathrm{MADH_{sq}}$, respectively. Introduction of the estimated values for k^x/k^0 and $K^x/K^0 = K_d^{red}/K_d^{sq}$ (Table 3) into eq 6 yields $(-RT \log K^0)/\lambda > 6$ in the case of $\mathrm{Cs^+}$. This implies that, in order to ascribe the rate increase completely to the increase in driving force, one must adopt an extremely low value for λ and/or an extremely negative driving force, whereas a high value for λ (225 kJ/mol) has been reported (Brooks & Davidson, 1994). Therefore, it appears likely that a major contribution to the rate increase derives from a change in λ, as might be exerted by the cations directly, by interaction with the TTQ, or indirectly, by way of inducing protein conformational changes. A more definitive analysis, however, must await the determination of more accurate K_d^{sq} values for the monovalent cations. We expect to be able to obtain these values by determining the widths of the radical EPR signal as a function of the cation concentrations.

In summary, we have demonstrated the binding of monovalent cations, but not of the bigger substrate analogue trimethylamine, to $\mathrm{MADH_{sq}}$ at a site close enough to the TTQ to broaden the radical EPR signal, most probably at the C6-carbonyl oxygen of the negatively charged *o*-semiquinone group of the TTQ active center. We also demonstrated a positive correlation between the binding of monovalent cations to $\mathrm{MADH_{sq}}$, on the one hand, and the stimulation by monovalent cations of the rate of oxidation of $\mathrm{MADH_{red}}$ by amicyanin on the other. We have put forward a hypothesis that links the kinetic effects of the monovalent cations partly to a modulation of the $\mathrm{MADH_{sq}}$ reduction potential and partly to a lowering of the reorganizational barrier.

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