Articles

Evidence for a Methylammonium-Binding Site on Methylamine Dehydrogenase of *Thiobacillus versutus*†

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ABSTRACT: The nonconvertible substrate analogues di-, tri-, and tetramethylammonium are bound with fairly high affinity to oxidized methylamine dehydrogenase ($MADH_{ox}$) from *Thiobacillus versutus* and induce the same red-shift in the optical absorbance spectrum of $MADH_{ox}$ as do the monovalent cations Cs^+, Rb^+, and NH_4^+. Like the monovalent cations, trimethylamine also competitively inhibits the reduction of $MADH_{ox}$ by methylamine. Rapid-scan experiments show that within the first few milliseconds of the reaction between $MADH_{ox}$ and methylamine a red-shifted intermediate is formed as well. Taken together these experiments demonstrate the existence of a common binding site on $MADH_{ox}$ for the substrate $CH_3NH_3^+$, the substrate analogues $(CH_3)_2NH_2^+$, $(CH_3)_3NH^+$, and $(CH_3)_4N^+$, and the monovalent cations Cs^+, Rb^+, and NH_4^+. Therefore we conclude that, prior to conversion, methylamine is noncovalently bound to $MADH_{ox}$ as a cation. The resonance Raman spectra of $MADH_{ox}$ in the absence and presence of Cs^+, NH_4^+, and $(CH_3)_3NH^+$ are very similar, except for the C=O stretching frequencies of the *o*-quinone carbonyls of the tryptophyltryptophanquinone (TTQ) active center, which show 5−30 cm^{-1} downshifts. From these Raman results and the X-ray crystal structure, we conclude that the $CH_3NH_3^+$ binding site is in close proximity to the O6 carbonyl oxygen of the TTQ.

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 tryptophyltryptophanquinone (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. In most organisms the electron acceptor (A) is the blue copper protein amicyanin (Tobari & Harada, 1981; van Houwelingen et al., 1985; van Spanning et al., 1990).

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

Ammonium ions are known to affect both the optical absorbance of MADH (from *Methylophilus* sp. W3A1) and its reactivity. In the presence of NH_4^+ the absorbance spectrum undergoes a pronounced red-shift, which was tentatively ascribed to a specific adduct of ammonia to TTQ (Kenney & McIntire, 1983; McWhirter & Klapper, 1989). The activity of MADH was reported to be stimulated and inhibited in the presence of low and high concentrations of NH_4^+, respectively (McIntire, 1987).

Recently we showed that, contrary to prior assumptions, these optical and kinetic effects of NH_4^+ are not exclusively observed with the enzyme from *M.* W3A1, but with MADH from *Thiobacillus versutus* as well (Gorren & Duine, 1994). Furthermore it was demonstrated, for the enzymes from both *M.* W3A1 (Kuusk & McIntire, 1994) and *T. versutus* (Gorren & Duine, 1994), that the absorbance red-shift is induced not just by NH_4^+ but also by the large alkali metal cations Cs^+ and Rb^+, which rules out the possibility that the red-shift involves a covalent attachment. Moreover, we established that monovalent cations other than NH_4^+ cause similar enhancement of the oxidative half-reaction and similar inhibition of the reductive half-reaction (Gorren &

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[1] Abbreviations: $MADH_{ox}$, $MADH_{sq}$, and $MADH_{red}$, oxidized, semireduced and reduced methylamine dehydrogenase, respectively; TTQ, tryptophyltryptophanquinone; SDS, sodium dodecyl sulfate; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; 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; EPR, electron spin resonance; ESEEM, electron spin echo envelope modulation; TPQ, topa quinone; AO, amine oxidase.

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Duine, 1994). Since the inhibition of the reaction between $MADH_{ox}$ and methylamine was found by us to be competitive, we proposed that methylamine and the monovalent cations share the same binding site on MADH and consequently that methylamine is bound to MADH as a $CH_3NH_3^+$ cation. The same conclusion was drawn for the enzyme from *M.* W3A1 (Kuusk & McIntire, 1994). In the present paper we intend to put that hypothesis on firmer ground.

The hypothesis that the red-shift-inducing monovalent cations are bound at the substrate-binding site was tested by studying the absorbance changes effected by the addition to $MADH_{ox}$ of di- and trimethylamine as well as tetramethylammoniumchloride. These compounds resemble methylamine but cannot be converted by MADH (Eady & Large, 1968; Mehta, 1977; Matsumoto, 1978; Chandrasekar & Klapper, 1986; Husain & Davidson, 1987). For the enzyme from *M.* W3A1 McIntire and co-workers recently reported that addition of $(CH_3)_3NH^+$ or $(CH_3)_4N^+$ induces a similar red-shift of the absorbance spectrum of $MADH_{ox}$ as observed with Cs^+, Rb^+ or NH_4^+ (Kuusk & McIntire, 1994). In addition to these optical absorbance studies, we investigated the effect of trimethylamine on the reduction of $MADH_{ox}$ by methylamine. Furthermore, rapid-scan studies of the reaction between $MADH_{ox}$ and methylamine are presented that reveal a red-shifted intermediate similar to the species observed with monovalent cations. Finally, evidence for the interaction between TTQ and the bound cations was obtained by resonance Raman spectroscopy.

MATERIALS AND METHODS

The alkylamines were purchased from Aldrich or Janssen Chimica and either used without further purification or, when necessary (*vide infra*), recrystallized from ethanol. Deuterated methylamine was obtained from Janssen Chimica.

T. versutus (ATCC 25364^T) was grown, and MADH was purified as described previously (van Wielink et al., 1990). The purity 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). The concentration was determined spectrophotometrically using an absorbance coefficient at 444 nm of 21 000 M^{-1} s^{-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 by monitoring the changes in the optical absorbance spectrum.

Optical absorbance spectra and steady-state kinetics 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.

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 of DCPIP at each pH was determined according to Armstrong (1964). All experiments were performed at 20 °C.

Resonance Raman spectra were recorded on samples in capillaries at 278 K using a 90° scattering geometry. Spectra were obtained on a McPerson 2061 spectrograph (0.6 m) with a 1800-groove grating and a Kaiser Optical holographic super-notch filter using 488.0 nm (10 mW) excitation, 5 cm^{-1} spectral resolution, 30 s exposure time, and an accumulation of 10 scans. Peak frequencies were calibrated relative to an indene standard and are accurate to ±1 cm^{-1}. The same sample capillaries were used to obtain optical absorbance spectra on a Perkin-Elmer Lambda 9 spectrophotometer before and after resonance Raman experiments. None of the samples were affected by laser irradiation.

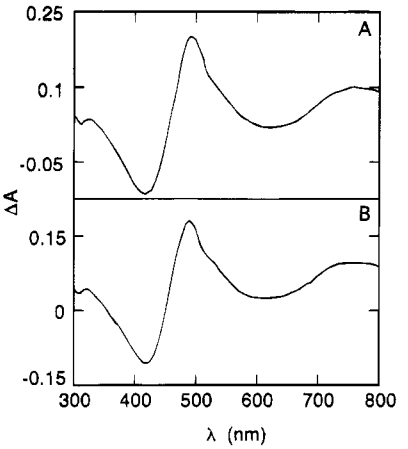

FIGURE 1: Comparison of the effects of Cs^+ and $(CH_3)_3NH^+$ on the absorbance spectra of $MADH_{ox}$. (Spectrum A) Effect of Cs^+. Absorbance difference spectrum induced by the addition of 80 mM CsCl to 22 μM $MADH_{ox}$ in 20 mM HEPES (pH 7.3). (Spectrum B) Effect of $(CH_3)_3NH^+$. Absorbance difference spectrum induced by the addition of 3.5 mM trimethylamine·HCl to 13 μM $MADH_{ox}$ in 20 mM HEPES (pH 7.3).

RESULTS

Effects of Methylamine Analogues on Optical Absorbance and Kinetics of $MADH_{ox}$. Addition of trimethylamine to $MADH_{ox}$ from *T. versutus* induced a red-shift in the absorbance spectrum, with the high absorbance band at 442 nm and the broad low band at 650 nm shifting to 475 and 720 nm, respectively. The shape of the resulting absorbance difference spectrum (Figure 1) closely resembles the one observed upon the addition of Cs^+, Rb^+, or NH_4^+ (Gorren & Duine, 1994). Similar red-shifts were also observed with dimethylamine and tetramethylammonium chloride (not shown).[2] Almost identical results with tri- and tetramethylammonium were recently reported for the enzyme from *M.* W3A1 as well (Kuusk & McIntire, 1994). By monitoring the absorbance changes as a function of the added concentration of $(CH_3)_2NH_2^+$, $(CH_3)_3NH^+$, and $(CH_3)_4N^+$, values for

[2] In the case of dimethylamine and tetramethylammonium chloride, at high concentrations, a second change in the absorbance spectrum occurred, consisting of an increase at 328 nm and a concomitant decrease of all bands in the visible region. The latter effect, however, did not show the hyperbolic concentration dependence expected for reversible binding but increased linearly with the concentration until a sharp cut-off point was reached. The resulting absorbance spectrum in both cases was identical to that of $MADH_{red}$. Addition of oxidized amicyanin to this mixture reversed the absorbance changes and caused the disappearance of the 596 nm absorbance band of $amicyanin_{ox}$, clearly demonstrating that for both compounds the effects are caused by the reduction of MADH, and are unrelated to the similar, but not identical, absorbance changes that are induced by the small monovalent cations (Na^+, K^+, NH_4^+, and Rb^+) at high concentrations (Kuusk & McIntire, 1994; Gorren & Duine, 1994). After recrystallization of either compound, reduction no longer occurred, indicating that it was due to contamination [amounting to 0.020% and 0.008% for $(CH_3)_2NH$·HCl and $(CH_3)_4NCl$, respectively] with methylamine (or some other primary amine).

Table 1: Apparent Dissociation Constants for Alkylammonium Ions to $MADH_{ox}$

cation	K_d^{app} (mM)[a] pH 7	pH 9
NH_4^+ [b]	20	1.5
$CH_3NH_3^+$ [c]	≤0.18	≤0.0049
$(CH_3)_2NH_2^+$	2.3	0.5
$(CH_3)_3NH^+$	0.32	0.12
$(CH_3)_4N^+$	14	7

[a] Series of absorbance spectra were measured in the presence of varying concentrations of the cations in 10 mM HEPES (pH 7.0) or 10 mM CHES (pH 9.0). K_d values were estimated by plotting $\Delta A\lambda_1 - \Delta A\lambda_2$ against the salt concentration for several wavelength pairs $\{\lambda_1;\lambda_2\}$ and fitting the resulting curves to the function

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

[see Gorren and Duine (1994)]. [b] Results taken from Table 1 of Gorren and Duine (1994). [c] Values were obtained by calculating k_{red}/k_{ass}^{app} from the results presented in Figure 3 of Gorren and Duine (1994). They assume the validity of eq 2 and represent upper limits.

the apparent dissociation constants were determined. The $(CH_3)_3NH^+$ ion was found to have the highest affinity, closest to that of the $CH_3NH_3^+$ substrate (Table 1).

Trimethylamine inhibited the reduction of $MADH_{ox}$ by methylamine. In stopped-flow experiments, pseudo-first-order rate constants were determined for varying concentrations of methylamine, both in the absence and presence of 5 mM trimethylamine (in 10 mM HEPES, pH 7.0), and the results fitted to the function (Gorren & Duine, 1994):

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

in which k_{obs}, k_{ass}^{app}, and k_{red} represent the observed first-order rate constant, the apparent association rate constant and the (maximal) reduction rate constant, respectively. A comparison of the kinetic parameters shows that only the apparent dissociation constant for methylamine, not the maximal reduction rate, was affected: whereas k_{red} remained constant at 100−110 s^{-1}, k_{ass}^{app} decreased from 5.3×10^5 M^{-1} s^{-1} to 3.9×10^4 M^{-1} s^{-1}, indicating that trimethylamine is a competitive inhibitor. Assuming that at saturating concentrations of trimethylamine the inhibition is 100%, we could calculate an apparent dissociation constant for trimethylamine of 0.39 mM at pH 7, in good agreement with the value of 0.32 mM, obtained for the red-shift (Table 1). The inhibition by trimethylamine was confirmed in steady-state experiments in an assay with methylamine and PMS/DCPIP (not shown) and is in accordance with results obtained with the enzymes from *Paracoccus denitrificans* and *M.* W3A1 (Davidson & Kumar, 1990).

Rapid-Scan Spectra for the Reduction of $MADH_{ox}$ by Methylamine. To search for intermediates, rapid-scan absorbance spectroscopy was applied to the reaction between $MADH_{ox}$ and methylamine. The bleaching of the visible absorbance spectrum of $MADH_{ox}$, indicative of the formation of $MADH_{red}$, was monophasic for all concentrations of methylamine (between 0.5 and 10 mM), in line with previous papers reporting the absence of intermediates (Brooks et al., 1993; Gorren & Duine, 1994). However, when $MADH_{ox}$ was mixed with 0.5 mM methylamine at pH 6.0, the $MADH_{ox}$ absorbance band was centered at 446 nm, whereas

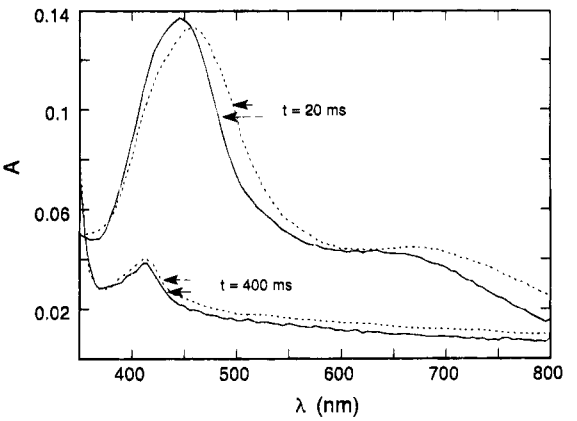

FIGURE 2: Rapid-scan spectra for the reduction of $MADH_{ox}$ by methylamine. Shown are the spectra obtained after 20 and 400 ms. Experimental conditions: 8 μM MADH; 20 mM MES (pH 6.0), and 0.5 mM (continuous lines) or 10 mM (dotted lines) methylamine.

the maximum was red-shifted to 463 nm when a high concentration (10 mM) of methylamine was applied (Figure 2). At pH 8.3, where the reaction between $MADH_{ox}$ and methylamine proceeds much faster (Gorren & Duine, 1994), the peak was centered at 463 nm even for low methylamine concentrations (not shown).

In the hope of observing the intermediate actually being formed, the same experiments were performed with deuterated methylamine (CD_3NH_2), since large kinetic deuterium effects have been reported for the MADH-methylamine reaction (van Wielink et al., 1989; McWhirter & Klapper, 1989; Brooks et al., 1993). Indeed, in this case a distinct red-shift was found to occur prior to the bleaching (Figure 3A). The red-shift is particularly apparent in the 40 ms difference spectrum (dotted line in Figure 3B). By determining the rate of reduction of $MADH_{ox}$ at varying concentrations of CD_3NH_2 (or CH_3NH_2), it was established that deuteration affects the maximal reduction rate rather than the affinity of MADH for methylamine, in line with prior reports (McWhirter & Klapper, 1989; van Wielink et al., 1989; Brooks et al., 1993). At pH 6 a value of 19 for k_{red}^H/k_{red}^D was calculated, lower than the previously estimated value of 30 for the enzyme from *T. versutus* (van Wielink et al., 1989) but in close agreement with the values of 20 and 17 reported for *M.* W3A1 (McWhirter & Klapper, 1989) and *P. denitrificans* (Brooks et al., 1993), respectively.

Effects of Monovalent Cations on the Resonance Raman Spectrum of $MADH_{ox}$. The effect of Cs^+ on the resonance Raman spectrum of $MADH_{ox}$ is illustrated by Figure 4B. The only vibrational modes that were strongly affected are the ones at 1565 and 1618 cm^{-1}. These peaks were previously assigned to carbonyl stretching vibrations of the TTQ-*o*-quinone on the basis of their ^{18}O-isotope dependence (McIntire et al., 1991b; Backes et al., 1991). The addition of Cs^+ caused these peaks to downshift by 11 and 22 cm^{-1}, respectively. An even more marked, but otherwise similar, effect was obtained with $(CH_3)_3NH^+$, with downshifts of 11 and 30 cm^{-1}, respectively (Figure 4C). The likeness of the effects that Cs^+ and $(CH_3)_3NH^+$ binding have on the TTQ is further indicated by other subtle changes in the resonance Raman spectra. Both show a 3 cm^{-1} upshift to 1309 cm^{-1}, intensity increases at 1368 and 1440 cm^{-1}, and an intensity decrease at 1500 cm^{-1} relative to MADH with no added cations (Figure 4).

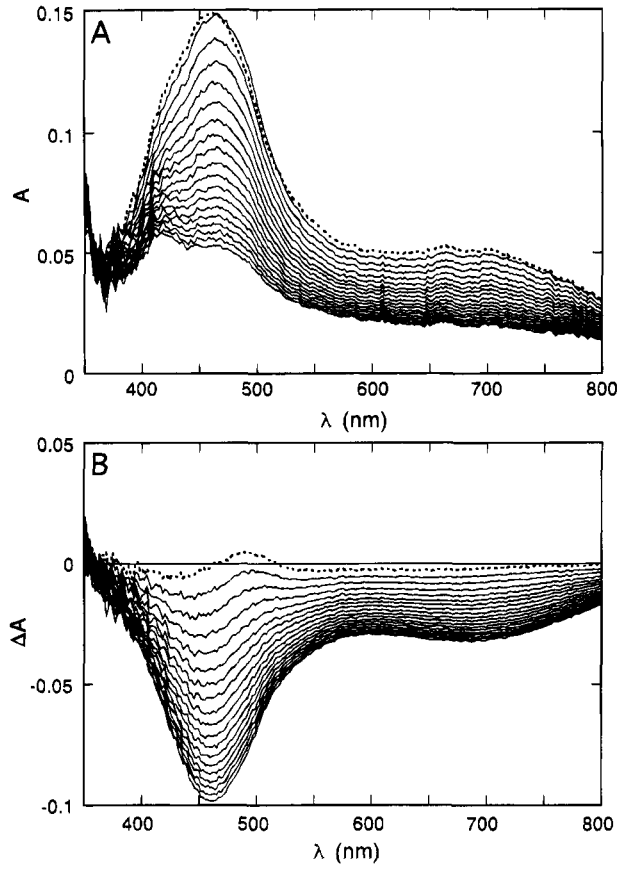

FIGURE 3: Rapid-scan spectra for the reduction of $MADH_{ox}$ by deuterated methylamine (CD_3NH_2). Spectra were measured with 20 ms intervals. Panel A shows the absorbance spectra. For the sake of clarity the first spectrum (20 ms after mixing) is represented by a dotted line. Panel B shows the absorbance difference spectra, in which the first spectrum (20 ms after mixing) has been subtracted from all other spectra. The first absorbance difference spectrum (40 ms minus 20 ms after mixing) is represented by a dotted line. Experimental conditions: 8 μM MADH, 20 mM MES (pH 6.0), and 10 mM CD_3NH_2.

Similar, but smaller, changes were also observed with NH_4^+, with the 1565 and 1618 cm^{-1} peaks downshifting by 5 cm^{-1} (Table 2). The addition of 1 M KCl did not significantly alter the resonance Raman spectrum (Table 2).

DISCUSSION

Interpretation of the Resonance Raman Spectra; The Cation-Binding Site. The resonance Raman spectrum of $MADH_{ox}$ from *T. versutus* (Figure 4A) is better resolved than, but otherwise similar to, the previously published spectrum (Backes et al., 1991). The majority of the spectral features are due to C, N, and H vibrational modes of the tryptophanquinone ring. The fact that these frequencies are essentially unaffected by addition of cations can be taken as evidence that the presence of the cations does not cause major structural changes in the TTQ. The significant shift of the two carbonyl stretching frequencies, however, does imply a direct interaction between the added cation (Cs^+, NH_4^+, or $(CH_3)_3NH^+$) and one or both of the carbonyl oxygens.

The presence of a positive charge close to the carbonyl oxygen would be expected to cause a weakening of the C=O bond, which explains the observed downshifts of the carbonyl stretching frequencies. There have been prior reports on the resonance Raman spectrum of MADH in the presence of

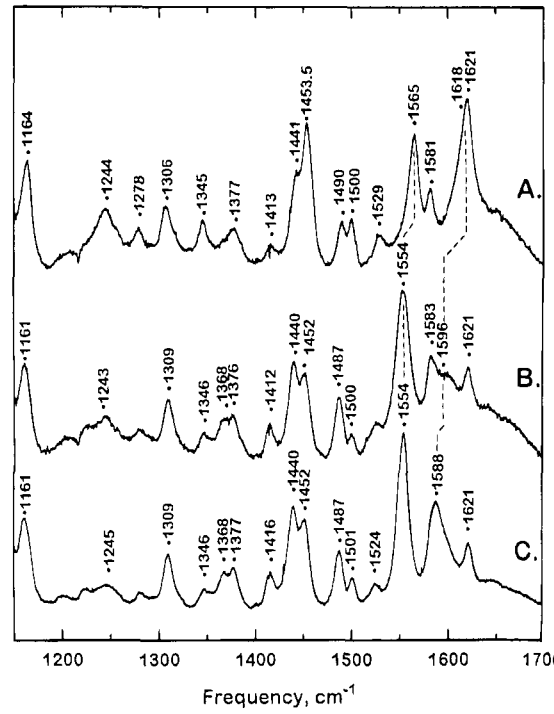

FIGURE 4: Effect of Cs^+ and $(CH_3)_3NH^+$ on the resonance Raman spectra of $MADH_{ox}$. (Spectrum A) 260 μM $MADH_{ox}$ in 20 mM HEPES (pH 7.0). (Spectrum B) As in spectrum A, but with 500 mM CsCl. (Spectrum C) As in spectrum A, but with 10 mM trimethylamine·HCl.

Table 2: TTQ-o-Quinone Raman Vibrational Modes of $MADH_{ox}$ in the Absence and Presence of Monovalent Cations

cation	Raman frequencies (cm^{-1})[a]			
no additions	1565	1581	1618	1621
1 M K^+	1563	1582	1618	1621
1 M NH_4^+	1560	1582	1613[b]	1621
1 M Cs^+	1554	1583	1596[b]	1621
1 M $(CH_3)_3NH^+$	1554	1583	1588	1621

[a] Resonance Raman spectra obtained as in Figure 4, except the NH_4Cl sample, which was buffered with 100 mM acetate (pH 5.0). [b] Band with strongly diminished intensity.

NH_4^+. With the enzyme from *M.* W3A1, the photolability of the complex prevented the successful collection of data (McIntire et al., 1991b). With the enzyme from *P. denitrificans*, however, a spectrum was obtained that was totally different from the one presented here (Backes et al., 1991). In that study the enzymes from *P. denitrificans* and *T. versutus* displayed absorbance maxima near 425 nm, suggesting that significant amounts of the TTQ had been converted to the semiquinone form under the conditions used. This has now been verified by the observation that addition of 1 M NH_4Cl to $MADH_{sq}$ generates the same resonance Raman spectrum (unpublished results) as reported previously for the ammonia adduct of $MADH_{ox}$. Thus, the previous suggestion of NH_4^+ being covalently bound to C6 of TTQ (Backes et al., 1991) was incorrect. The observed differences in the semiquinone Raman spectra with and without ammonia were due to the noncovalent interaction of NH_4^+ with the TTQ semiquinone (Gorren et al., 1995). As with $MADH_{ox}$, the resonance Raman spectrum of the semiquinone (produced by anaerobic titration of $MADH_{ox}$ with sodium dithionite) undergoes a perturbation of its C-O stretching modes in the presence of NH_4^+.

Earlier we demonstrated that at high concentrations the smaller monovalent cations (Na^+, K^+, NH_4^+, and Rb^+) induced a bleaching of the visible absorbance spectrum with a concomitant increase at 328 nm (Gorren & Duine, 1994), as was also the case for the enzyme from *M.* W3A1 (Kuusk & McIntire, 1994). This effect was not reflected in the resonance Raman spectrum in the presence of 1 M K^+ or NH_4^+, because only the residual *chromophoric* species were detected. The lack of any Raman spectral shifts with 1 M K^+ does, however, prove that the shifts observed with Cs^+ and NH_4^+ are specific for the ions in question, rather than representing a general salt effect.

The resonance Raman results are in good agreement with the X-ray crystal structure which shows electron density, tentatively ascribed to NH_4^+, in close proximity to the C6-carbonyl oxygen of the TTQ in *T. versutus* MADH (Huizinga et al., 1992).[3] This cation binding site appears to consist of the C6-carbonyl oxygen, three additional carbonyl residues (peptide oxygens of Asp 32, Asn 104, and Ile 106), and the side chains of Asp 76 and/or Asp 32 (Huizinga et al., 1992). A similar binding site for monovalent cations (Cs^+, NH_4^+, and Na^+) has been identified in the crystal structure of bovine liver rhodanese, where the cation is coordinated to four peptide carbonyl oxygens and an aspartate side chain carboxylate (Kooystra et al., 1988). It should be kept in mind, however, that the MADH X-ray structure was obtained at pH 5.0 and that at low pH the substrate- and cation-affinity of MADH is very low (Kuusk & McIntire, 1994; Gorren & Duine, 1994). Therefore the structure of the cation binding site may be somewhat different at neutral and high pH values. The carboxylates of Asp 76 and/or Asp 32, for instance, which appear to be protonated at pH 5.0 (Huizinga et al., 1992), may be deprotonated at pH 7, which would be expected to allow a stronger participation of the carboxylate group(s) in binding the cation and thus to increase the cation affinity of MADH.

Methylammonium Binding to $MADH_{ox}$: The Red-Shifted Intermediate. The close similarity of the absorbance and Raman spectral changes, induced by Cs^+ and the other large monovalent cations on the one hand and the nonconvertible substrate analogues $(CH_3)_2NH_2^+$, $(CH_3)_3NH^+$, and $(CH_3)_4N^+$ on the other, lends support to the notion that the cations are bound at the substrate-binding site and that the substrate binds to $MADH_{ox}$ as a cation. As suggested before, the optical changes are probably due to the proximity of the positive charge to the TTQ ring system (Gorren & Duine, 1994). $(CH_3)_2NH_2^+$ and $(CH_3)_3NH^+$ bind considerably more strongly to MADH than do the alkali metal cations and NH_4^+ (Table 1), which suggests that the methyl group of CH_3-NH_3^+ provides additional binding energy. The higher K_d^{app} value for $(CH_3)_4N^+$ may be due to steric hindrance by the bulky tetramethyl group. Steric hindrance may also explain why the K_d^{app} values for $(CH_3)_2NH_2^+$ and $(CH_3)_3NH^+$ exceed the derived K_d^{app} value for $CH_3NH_3^+$. However, at present it remains unclear why $(CH_3)_3NH^+$ is a slightly better ligand for $MADH_{ox}$ than $(CH_3)_2NH_2^+$.

In support of our proposal that substrate and substrate analogues are bound at the same (cation-binding) site, we found trimethylamine to be a competitive inhibitor of the reaction between $MADH_{ox}$ and methylamine. Further corroboration comes from the identification of a red-shifted intermediate in the reduction of MADH by methylamine. A red-shifted intermediate has been observed in stopped-flow studies of the reaction of MADH (from *M.* W3A1) with propylamine, which is considerably slower than the reaction with methylamine (McWhirter & Klapper, 1989). In stopped-flow studies of the reaction between $MADH_{ox}$ (from *P. denitrificans* and *T. versutus*) and methylamine, no evidence for an intermediate was found (Brooks et al., 1993; Gorren & Duine, 1994). The present results demonstrate, as was already suggested by Brooks et al. (1993), that the intermediate escaped detection, since at high methylamine concentrations its formation is complete within the mixing time of the stopped-flow apparatus, while, at low methylamine concentrations, the intermediate does not accumulate on account of the fast subsequent reaction [$\sim 100\ s^{-1}$ (Gorren & Duine, 1994)]. The red-shifted intermediate in the reaction with propylamine and the red-shifted complex of $MADH_{ox}$ with NH_4^+ have been ascribed to a quinoneamine (McWhirter & Klapper, 1989) and an *iminoquinone* (Kenney & McIntire, 1983), respectively. However, from the fact that identical absorbance changes were observed with the simple monovalent cations Cs^+ and Rb^+ (Gorren & Duine, 1994), it follows that the red-shifted intermediate does not involve a covalent bond between the TTQ and the alkylamine but an electrostatic interaction between $MADH_{ox}$ and the alkylammonium ion.

Mechanistic Implications. In analogy to the reaction mechanism deduced for the topaquinone-containing amine oxidases (TPQ-AO's), it is generally assumed that the mechanism of MADH-catalyzed amine oxidation involves amino transfer rather than imine elimination. However, whereas there is strong evidence for amino transfer in TPQ-AO [recently reviewed by Klinman and Mu (1994)], the evidence for a similar mechanism for MADH is far more tenuous. Experiments in which substrate-derived radiolabel was incorporated into the active site, which demonstrated the existence of a covalent enzyme−substrate complex and implied the formation of a Schiff base for TPQ-AO, have not been performed with MADH. The observation with TPQ-AO that ammonia release occurs only during the oxidative half-reaction, which is indicative of amino transfer, was announced for MADH (McWhirter & Klapper, 1989) but to the best of our knowledge never published. For TPQ-AO evidence for a carbanion intermediate was obtained from structure reactivity correlations with a number of *para*-substituted benzylamines. Similar results were reported for MADH (Davidson et al., 1992) but with considerably weaker correlation; moreover, the study involved only five derivatives, one of which did not yield sufficiently accurate rate constants, and the maximal variation in observed rates was only 5-fold, as opposed to a 250-fold variation with nine useful derivatives in the TPQ-AO study (Hartmann & Klinman, 1991). Therefore, we regard the MADH structure reactivity correlations, although supportive of a carbanion intermediate, not as compelling evidence by itself.

The red-shift in the absorbance spectrum of $MADH_{ox}$ after addition of NH_4^+ (Kenney & McIntire, 1983), the red-shifted intermediate in the reduction of $MADH_{ox}$ by deuterated propylamine (McWhirter & Klapper, 1989), the broadening of the $MADH_{sq}$ radical EPR-signal in the presence of NH_4^+

[3] The X-ray crystal structure of MADH from *M.* W3A1 shows similar electron density adjacent to the C6-carbonyl oxygen of the TTQ, and it is also ascribed to a nonprotein component (F. S. Mathews, personal communication).

(Kenney & McIntire, 1983), and the optical and resonance Raman studies in the presence of NH_4^+ (Backes et al., 1991) have all been suggested to afford evidence for the existence of a covalent adduct of ammonia to TTQ. As discussed above, the results presented in this paper and in three earlier studies (Kuusk & McIntire, 1994; Gorren & Duine, 1994; Gorren et al., 1995) clearly show that this is not the case. The strongest evidence at present for the formation of a covalent TTQ−ammonia adduct during the reaction of methylamine with $MADH_{ox}$ is provided by the experiments of Warncke et al. (1993), who observed pronounced differences between the ESEEM signals of $MADH_{sq}$ obtained with [^{14}N]- and [^{15}N]methylamine. However, in view of the large effects that are exerted by alkali metal ions on the radical EPR signal of $MADH_{sq}$ (Gorren et al., 1995), the contention by Warncke et al. that the interpretation of the ESEEM data necessarily involves a covalent complex requires further verification. Therefore, we conclude that, although some of the results mentioned above support an amino-transferase mechanism for MADH, definitive proof is lacking at present.

In summary, our results demonstrate that the first step observed in the reduction of $MADH_{ox}$ by alkylamine is the electrostatic binding of the alkylammonium ion in close proximity to the TTQ-*o*-quinone group.

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