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Deuterium Kinetic Isotope Effect and Stopped-Flow Kinetic Studies of the Quinoprotein Methylamine Dehydrogenase†

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ABSTRACT: Stopped-flow kinetic studies of the reductive half-reaction of methylamine dehydrogenase from *Paracoccus denitrificans* yielded kinetic constants for the reversible formation of the imine intermediate formed between the substrate and the tryptophan tryptophylquinone (TTQ) prosthetic group and for the hydrogen abstraction step which occurs concomitantly with TTQ reduction. When CD_3NH_2 was used as a substrate, deuterium kinetic isotope effects of 4.2 and 3.8, respectively, were measured for the rate constants that correspond to the formation and dissociation of the iminoquinone intermediate. A deuterium kinetic isotope effect of 17.2 was measured for the hydrogen abstraction step. The maximum deuterium kinetic isotope effect which was measured in steady-state kinetic experiments was 3.0. These data are discussed in relation to the reaction mechanism of methylamine dehydrogenase and the similar large deuterium kinetic isotope effect for hydrogen abstraction which has been observed for another quinoprotein, plasma amine oxidase.

Methylamine dehydrogenase from *Paracoccus denitrificans* is a soluble enzyme which catalyzes the oxidation of methylamine to formaldehyde and ammonia. It possesses an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500 (Husain & Davidson, 1987). Each small subunit contains a covalently bound quinone prosthetic group, which is involved both in catalysis and in the subsequent electron transfer to a Type I copper protein, amicyanin (Husain & Davidson, 1985), which is its physiological reoxidant. This enzyme is a member of a newly characterized family of bacterial and eukaryotic oxidoreductases which are referred to as quinoproteins [reviewed in Duine and Jongejan (1989) and Davidson (1993)]. Most bacterial quinoproteins, such as methanol and glucose dehydrogenases, possess tightly but noncovalently associated pyrroloquinoline quinone (PQQ)[1] (Salisbury et al., 1979) as a prosthetic group. Certain eukaryotic amine oxidases possess other novel covalently-bound quinone species at their active sites. For bovine plasma amine oxidase it has been shown that the organic redox cofactor is a modified tyrosine residue, 6-hydroxydopaquinone or topaquinone (Janes et al., 1990). For mammalian lysyl oxidase the precise nature of the covalently-bound organic cofactor is uncertain but data suggest that it is an *o*-quinone (Williamson et al, 1986). Methylamine dehydrogenase is atypical of bacterial quinoproteins and similar to eukaryotic quinoproteins, particularly the amine oxidases, in that it possesses a covalently-bound, amino acid-derived *o*-quinone at its active site. Recent biochemical (McIntire et al., 1991), X-ray crystallographic (Chen et al., 1991), and resonance Raman spectroscopic (Backes et al., 1991) studies have identified this prosthetic group as tryptophan tryptophylquinone (TTQ, Figure 1). Like topaquinone, TTQ is derived from a posttranslational modification of gene-encoded amino acid residues, in this case two tryptophan residues of the polypeptide chain (Chistoserdov et al., 1990).

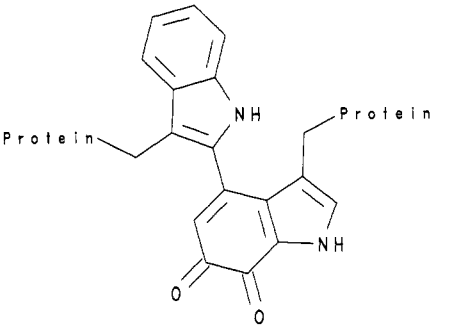

FIGURE 1: Structure of the tryptophan tryptophylquinone (TTQ) prosthetic group of methylamine dehydrogenase.

We have previously characterized the steady-state kinetic parameters for methylamine dehydrogenase with artificial electron acceptors (Davidson, 1989) and performed mechanistic studies of the reductive half-reaction of this enzyme with a variety of alternative substrates and inhibitors (Davidson & Jones, 1991a, 1992; Davidson et al., 1992). Those studies have indicated a number of mechanistic similarities between methylamine dehydrogenase and the eukaryotic quinoproteins. We present here the first detailed report of stopped-flow kinetic studies of a TTQ-bearing enzyme. These data indicate that methylamine dehydrogenase, like the topaquinone-bearing plasma amine oxidase (Palcic & Klinman, 1983), exhibits a very large primary deuterium kinetic isotope effect which appears to exceed predicted semiclassical limits.

EXPERIMENTAL PROCEDURES

The purification of methylamine dehydrogenase (Davidson, 1990), and amicyanin (Husain & Davidson, 1985) from *P. denitrificans* (ATCC 13543) was as described previously. Protein concentrations were calculated from previously determined extinction coefficients (Husain & Davidson, 1985; Husain et al., 1987). CH_3NH_2·HCl and CD_3NH_2·HCl (98% deuterated) were obtained from Sigma Chemical Co. (St. Louis, MO). Steady-state kinetic mixing experiments with 50:50 mixtures of protonated and deuterated methylamine

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[1] Abbreviations: PQQ, pyrroloquinoline quinone; TTQ, tryptophan tryptophylquinone; PES, phenazine ethosulfate; DCIP, 2,6-dichlorophenolindophenol.

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were performed to verify the absence of enzyme inhibitors in the deuterated samples.

Two steady-state kinetic assays of methylamine dehydrogenase activity were used. One employed a spectrophotometric dye-linked assay with phenazine ethosulfate (PES) as an electron acceptor, in which the oxidation of methylamine was coupled to a change in the absorbance of a redox-sensitive dye, 2,6-dichlorophenolindophenol (DCIP) (Davidson, 1990). The second assay employed amicyanin as the electron acceptor. The assay mixture contained 8 nM methylamine dehydrogenase and saturating levels (30 μM) of amicyanin in 0.01 M potassium phosphate buffer, pH 7.5. The reaction was initiated by the addition of substrate, and activity was monitored by the change in absorbance caused by the reduction of amicyanin at 595 nm. Both assays were performed at 30 °C. Values of k_{cat} are per site.

Stopped-flow experiments were performed using an On-Line Instrument Systems (OLIS, Bogart, GA) stopped-flow sample handling unit instrument coupled to Durrum optics. A 486-class computer controlled by OLIS software was used to collect data. All experiments used glass-distilled water. Methylamine solutions were made fresh every day in appropriate buffer. All experiments were performed at 30 °C. Data were collected after the flow period (approximately 0.004 s) using a gain of 10 for between 0.1 and 2 s depending on the reaction rate. The reaction was monitored at 440 nm. Typically three to four data sets, each containing 1000 data points, were averaged, and the data were fit using OLIS software to eq 1 for a single-exponential decay, where C is a

$$A_{440} = Ce^{-kt} + b \quad (1)$$

constant related to the initial absorbance and b represents an offset value to account for a nonzero baseline. Because some of the reactions being monitored are relatively fast (up to 275 s^{-1}), we are not able to observe the entire reaction. However, k_{obs} is independent of enzyme concentration (discussed below). Since the reaction is fit to a single exponential and we are working at concentrations of substrate much greater than enzyme, an accurate value for k_{obs} may be obtained from the portion of the reaction which we observe. This was confirmed with real data and with computer simulations, in which the k_{obs} determined from as little as 20% of the total reaction was equivalent to that determined from the entire reaction.

It was assumed that the observed reactions obeyed the scheme shown in eq 2, where E and E′ represent, respectively, the oxidized and reduced forms of methylamine dehydrogenase. In the mechanism shown later in Figure 5, E corresponds

$$E + S \underset{k2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E'P \quad (2)$$

to I, ES corresponds to III, and E′P corresponds to IV. To determine these rate constants, k_{obs} was fit to eq 3, where [S] is the concentration of methylamine. Methylamine concentration was varied between 0.01 and 10 mM with methylamine dehydrogenase concentrations of 1–10 μM. In all experiments the concentration of substrate was sufficiently greater than the enzyme concentration so that k_{obs} was independent of substrate concentration as predicted by eq 3. Nonlinear curve-fitting of these data was performed with the Enzfitter computer program (Elsevier-Biosoft, Cambridge).

$$k_{obs} = 0.5(k_1[S] + k_2 + k_3 - ((k_1[S] + k_2 + k_3)^2 - 4k_1k_3[S])^{0.5}) \quad (3)$$

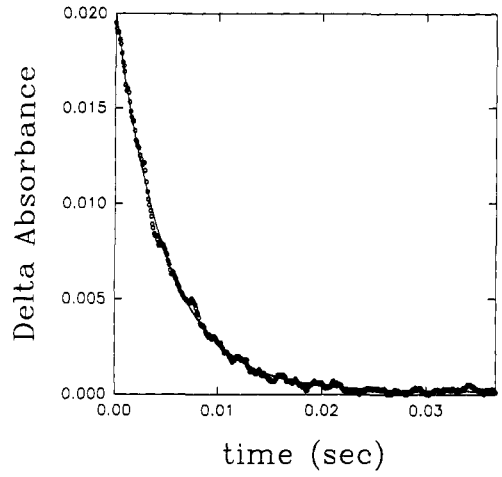

FIGURE 2: Reduction of methylamine dehydrogenase by methylamine. Methylamine (71 μM) was mixed with methylamine dehydrogenase (1.0 μM) in 0.01 M potassium phosphate, pH 7.5, at 30 °C. Absorbance was monitored at 440 nm. The solid line drawn through the points represents the fit of these data to eq 1. That fit gave a value for k_{obs} of 207 ± 1 for this data set.

RESULTS

Stopped-Flow Studies of the Reductive Half-Reaction. Like flavoproteins, quinoproteins are able catalyze one- and two-electron transfer reactions. Methylamine dehydrogenase mediates electron transfer from a two-electron donor, methylamine, to a one-electron acceptor, amicyanin. The three redox states of methylamine dehydrogenase exhibit distinct absorption spectra (Husain et al., 1987; Davidson et al., 1990). The chromophore exhibits a broad peak centered at 440 nm which increases in absorbance on conversion from the oxidized to the semiquinone state and is essentially bleached in the reduced state. The absorbance change which was observed on mixing of methylamine dehydrogenase with methylamine was best fit to a single-exponential decay (Figure 2), suggesting a direct two-electron reduction of TTQ by substrate with no involvement of the semiquinone in the reductive half-reaction. Attempts to fit the data in Figure 2 and similar data to fits with two exponentials failed to significantly alter the value obtained for a single-exponential fit of k_{obs}, and plots of the residuals indicated no improvement on the fit to a single exponential. Biphasic curves were only observed with low concentrations of methylamine which approached that of the enzyme. This can be attributed to the fact that under these conditions the reaction is actually second-order heterogenous and no longer pseudo-first-order as is required by eq 3. All data obtained under conditions where eq 3 is valid were best described by a single exponential.

Previous studies have characterized absorption spectra of the carbinolamine (Backes et al., 1991) and putative iminoquinone (Kenny & McIntire, 1983) forms of methylamine dehydrogenase. The former exhibits an absorption maximum at 425 nm and the latter an absorption maximum at 491 nm. As such, the reaction of methylamine with methylamine dehydrogenase was also monitored at 425 and 491 nm. Even at temperatures as low as 5 °C, no evidence for formation of either of these species was observed. Only changes in absorbance consistent with the reduction of TTQ were seen. It is, therefore, likely that these reactions which occur prior to the reductive step (k_3) are very fast relative to k_3 and are reflected in the binding (k_1) step of the simple kinetic model (eq 2).

Table I: Stopped-Flow Kinetic Constants[a]

kinetic constant	substrate		
	CH_3NH_2	CD_3NH_2	k^H/k^D
k_1 ($s^{-1}M^{-1}$)	(8.96 ± 1.07) × 10^6	(2.15 ± 0.48) × 10^6	4.2 ± 1.1
k_2 (s^{-1})	117 ± 38	30.5 ± 8.3	3.8 ± 1.6
$K_d = k_2/k_1$ (M)	(1.30 ± 0.45) × 10^{-5}	(1.42 ± 0.5) × 10^{-5}	0.9 ± 0.4
k_3 (s^{-1})	275 ± 9	16.0 ± 0.1	17.2 ± 0.6

[a] Experiments were performed in 0.01 M potassium phosphate buffer, pH 7.5, at 30 °C. These kinetic constants were obtained by fitting the data in Figure 3 to eq 3.

To obtain rate constants for this reaction, values of k_{obs} were obtained at several different concentrations of methylamine. These data were analyzed by different methods to determine which gave the best fit for the data. When these data were analyzed by the method of Strickland et al. (1975) (eq 4), reciprocal plots of $1/k_{obs}$ versus 1/[methylamine]

$$k_{obs} = k_1k_3[S]/(k_1[S] + k_2 + k_3) \quad (4)$$

appeared to be concave upward rather than linear and attempts to fit the data to eq 4 directly by nonlinear methods were unsuccessful. The failure of the Strickland model to fit these data suggests that the simplifying assumption that k_2 is much greater than k_3 is not valid (Ramsay et al., 1987). A more rigorous solution for the model in eq 2 has been derived by Hiromi (1979) and is given in eq 5. This equation is identical

$$k_{obs} = 0.5(k_1[S] + k_2 + k_3 + k_4 - ((k_1[S] + k_2 + k_3 + k_4)^2 - 4k_1k_3[S] - 4k_1k_4[S] - 4k_2k_4)^{0.5}) \quad (5)$$

to eq 3 except that it includes the k_4 term, which is neglected in eq 3. Attempts to fit the data to this equation consistently gave relatively small or negative values for k_4 and yielded significantly larger errors in k_1, k_2, and k_3 than were obtained using eq 3. As such, it was concluded that for these reactions k_4 was negligible and that eq 3 was the most appropriate for analyzing the data.

The rate constants for the reaction of methylamine dehydrogenase with methylamine and deuteromethylamine are listed in Table I. These experiments (Figure 3) were performed in 0.01 M potassium phosphate buffer. Methylamine dehydrogenase has traditionally been assayed using artificial electron acceptors such as PES. These assays have been routinely performed in 0.1 M phosphate buffers. Examination of the reaction of methylamine dehydrogenase with its natural electron acceptor, amicyanin, indicated that the reaction was optimal at low ionic strength (Gray et al., 1988; Davidson & Jones, 1991b), and as such the assays which use amicyanin as an electron acceptor are performed in 0.01 M phosphate buffer. To compare data obtained from assays performed with the natural and artificial acceptors, stopped-flow kinetic studies were performed in both 0.1 and 0.01 M buffer to ascertain whether the ionic strength difference had any effect on the reductive half-reaction. In the higher ionic strength buffer an identical value of k_3 was obtained. The value of K_d (k_2/k_1) was, however, approximately 8-fold higher in the higher ionic strength buffer.

Comparison of the kinetic constants obtained with CH_3NH_2 and CD_3NH_2 (Table I) reveals a kinetic isotope effect $^Dk_3$ of 17.2. An essentially identical isotope effect was observed when experiments were performed in 0.1 M buffer. It should be noted that this isotope effect was obtained with methylamine in which the carbon was trideuterated. The value of $^Dk_3$ will reflect, therefore, the product of the primary and secondary isotope effects. If this value is corrected for the contribution of the secondary effects, using an upper limit of 1.36 for the maximum magnitude of a secondary isotope effect (Klinman, 1978), the primary deuterium isotope effect on k_3 can be estimated to be in the range 9.3–17.2.

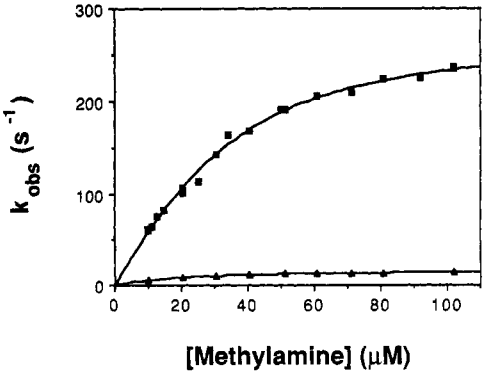

FIGURE 3: Plot of k_{obs} against substrate concentration for the reduction of methylamine dehydrogenase. All experiments were done in 0.01 M potassium phosphate, pH 7.5, at 30 °C. The values of k_{obs} were calculated by fitting the decrease in A_{440} to eq 1. The line drawn through these points is the fit of these data to eq 3. Squares, CH_3NH_2; triangles, CD_3NH_2.

Although K_d was essentially the same for CH_3NH_2 and CD_3NH_2, significant deuterium kinetic isotope effects of 4.2 and 3.8, respectively, were observed on the individual rate constants k_1 and k_2 (Table I) for experiments performed in 0.01 M buffer. When experiments were performed in 0.1 M buffer, deuterium kinetic isotope effects of 1.8 and 2.5 were observed, respectively, for k_1 and k_2.

Steady-State Kinetic Studies. Methylamine dehydrogenase has routinely been assayed using PES as an electron acceptor in 0.1 M potassium phosphate, pH 7.5. We have previously reported a deuterium kinetic isotope effect, $^Dk_{cat}$, of 3.0 for the oxidation of methylamine by methylamine dehydrogenase when assayed under these conditions (Davidson, 1989). We describe here a steady-state kinetic assay which employs amicyanin (Figure 4A), the natural electron acceptor for methylamine dehydrogenase, instead of PES. This assay is optimum at low ionic strength and was performed in 0.01 M buffer, conditions identical to those used to obtain the stopped-flow kinetic data in Table I. To provide an accurate and complete description of the reaction catalyzed by this enzyme, given the historical use of artificial electron acceptors, the steady-state studies with PES were also repeated in 0.01 M buffer (Figure 4B). The kinetic constants which were obtained in steady-state studies performed under these conditions are listed in Table II. In contrast to the value of $^Dk_3$ of 17.2 which was obtained in stopped-flow studies, the largest deuterium kinetic isotope effect which was obtained in any of the steady-state studies was 3.0.

DISCUSSION

A mechanism for the reductive half-reaction of methylamine dehydrogenase with methylamine is proposed in Figure 5. The reaction is initiated by a nucleophilic attack by the amine nitrogen on one of the quinone carbonyls. This most likely results in formation of a carbinolamine intermediate (II) which loses water to form an imine intermediate (III). The initial formation of a carbinolamine on addition of the amine substrate is supported by recent resonance Raman spectroscopic studies of this enzyme (Backes et al., 1991). It is next proposed that an active-site nucleophile abstracts a proton from the α-carbon,

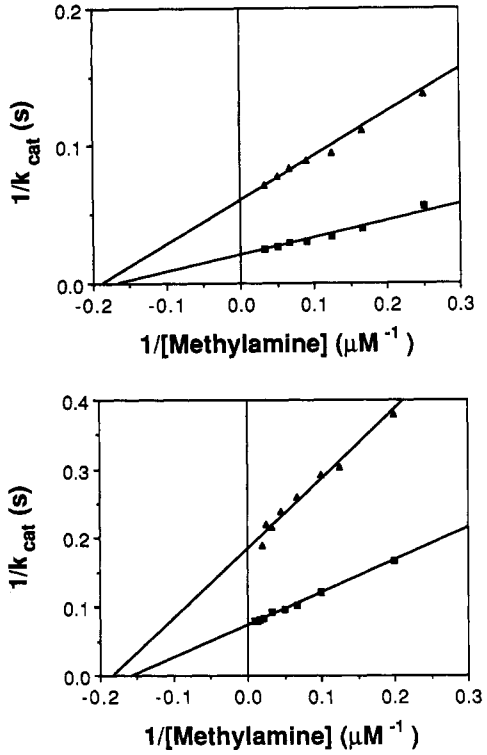

FIGURE 4: Steady-state kinetic studies of methylamine dehydrogenase. (A) Reciprocal plot of methylamine dehydrogenase activity against substrate concentration with amicyanin as an electron acceptor. (B) Reciprocal plot of methylamine dehydrogenase activity against substrate concentration with PES as an electron acceptor. Reaction conditions were as described under Experimental Procedures. Squares, CH_3NH_2; triangles, CD_3NH_2.

Table II: Steady-State Kinetic Constants[a]

kinetic constant	substrate			
	PES/DCIP assay		amicyanin assay	
	CH_3NH_2	CD_3NH_2	CH_3NH_2	CD_3NH_2
k_{cat} (s^{-1})	13.6 ± 0.1	5.4 ± 0.1	48.5 ± 1.4	16.4 ± 0.1
K_m (M)	(6.4 ± 0.2) × 10^{-6}	(5.5 ± 0.7) × 10^{-6}	(5.9 ± 0.5) × 10^{-6}	(5.3 ± 0.9) × 10^{-6}
k_{cat}/K_m (s^{-1} M^{-1})	(2.1 ± 0.1) × 10^6	(1.0 ± 0.1) × 10^6	(8.2 ± 0.7) × 10^6	(3.1 ± 0.5) × 10^6
$^Dk_{cat}$	2.5 ± 0.1		3.0 ± 0.1	
$^D[k_{cat}/K_m]$	2.1 ± 0.2		2.6 ± 0.5	

[a] Experiments were performed in 0.01 M potassium phosphate buffer, pH 7.5, at 30 °C. These kinetic constants were obtained by fitting the data in Figure 4 to the Michaelis–Menten equation. K_m values are apparent K_ms obtained in the presence of saturating levels of the electron acceptor.

thus forming a carbanionic intermediate concomitant with the reduction of the TTQ prosthetic group and formation of IV. Release of the aldehyde product then occurs by hydrolysis of the newly formed imine bond between the methyl carbon and the amino group. In the simplified kinetic mechanism described in eq 2, k_3 represents the proton abstraction step, k_1 represents the combined two steps leading to imine formation, and k_2 represents the reverse of k_1. This kinetic description of k_1 and k_2 is necessary as we are not able to distinguish the carbinolamine and imine intermediates.

Significant deuterium kinetic isotope effects were observed for k_1 and k_2 (Table I). These effects are too large to be attributable to secondary isotope effects and seem to reflect a primary effect in the reversible process of imine formation. We have previously (Davidson et al., 1992) reported that resonance contributions can stabilize imine formation between

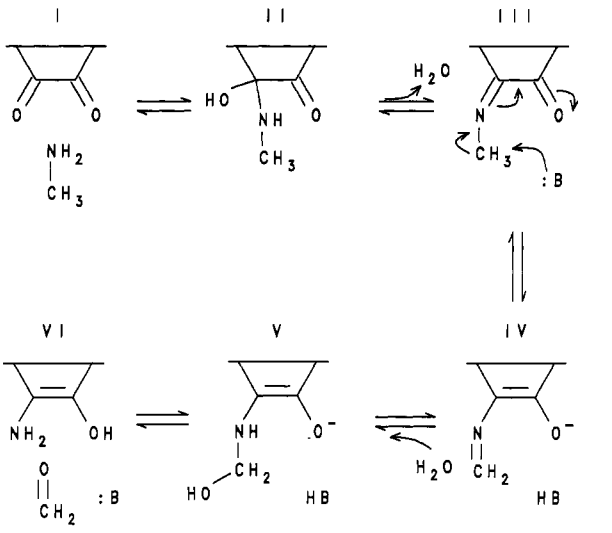

FIGURE 5: Proposed mechanism for the reductive half-reaction of methylamine dehydrogenase.

p-substituted benzylamines and TTQ in methylamine dehydrogenase, despite the fact that the imine bond and benzyl ring are separated by a methylene group. This led us to postulate that hyperconjugation was somehow involved in this process. The magnitudes of $^Dk_1$ and $^Dk_2$ can also be rationalized by a mechanism which involves hyperconjugation of the methyl group of the substrate.

The very large value of $^Dk_3$ indicates that the hydrogen abstraction step is the slowest of the first three steps shown in Figure 5 and that the imine intermediate must accumulate prior to the proton abstraction. Values for $^Dk_{cat}$ and $^D(k_{cat}/K_m)$ of 3.0 or less were observed in the steady-state assays (Table II) compared with the value of 17.2 observed for $^Dk_3$ in stopped-flow studies. This suggests that hydrogen abstraction is not the rate-limiting step in the overall oxidation–reduction reaction. The suppression of the isotope effect is also predicted from isotope effect theory and a knowledge of the steady-state kinetic mechanism of the enzyme. Methylamine dehydrogenase exhibits a ping-pong steady-state mechanism with PES as an electron acceptor (Davidson, 1989). Such a mechanism is described in eq 6, and for this mechanism k_{cat} is defined by eq 7. Because k_4 for this reaction is zero,

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} FP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} F + P$$

$$F + B \underset{k_8}{\overset{k_7}{\rightleftharpoons}} FB \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EQ \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E + Q \qquad (6)$$

$$k_{cat} = (k_3k_5k_9k_{11})/[k_8k_{10}(k_2 + k_3 + k_4) + k_2k_4(k_8 + k_9 + k_{10})] \qquad (7)$$

it is possible to simplify this equation by eliminating many of the terms in the denominator. If we further assume that k_3 and k_2 are the only terms in this expression that are subject to an isotope effect, then one obtains the relationship given in eq 8. Substituting into this equation the values for the

$$^Dk_{cat} = \frac{k_3(^Dk_2 + ^Dk_3)}{^Dk_3(k_2 + k_3)} \qquad (8)$$

kinetic constants from Table I which were obtained from stopped-flow studies, one obtains a predicted value of 2.04 for

$^{D}k_{cat}$ for the steady-state reaction in the PES/DCIP assay. This is quite close to the observed value of 2.5 (Table II). Furthermore, we have assumed in this exercise that the aldehyde release step (k_5) is not subject to an isotope effect. As the aldehyde product is dideuterated, a secondary isotope effect on this step is possible and, if present, would increase the magnitude of the predicted value for $^{D}k_{cat}$ and provide even closer correlation between the experimental and predicted values. The predicted magnitude of $^{D}(k_{cat}/K_m)$ could not be determined because the equation which defines that term includes rate constants for the oxidative half-reaction, the values of which are not known. It is also not possible to make a similar calculation for the steady-state reaction with amicyanin as an electron acceptor because the steady-state mechanism is not known. It is most likely more complicated than the one given above since amicyanin is a one-electron acceptor and methylamine dehydrogenase is a two-electron donor. The analysis presented above would seem to lend strong support both for the validity of the values for the microscopic rate constants and also for the reported isotope effects on each of these constants. Furthermore, it is noteworthy that when deuterated methylamine was used as a substrate for the steady-state assay with amicyanin, the value which was obtained for k_{cat} was essentially identical to that obtained for k_3 in stopped-flow studies with deuterated methylamine. This suggests that with this substrate, in the amicyanin assay, the deuterium abstraction step has become the rate-limiting step in the overall oxidation–reduction reaction.

The magnitude of the primary deuterium kinetic isotope effect on k_3 of 9.3–17.2 would seem to exceed the semiclassical limit for a hydrogen abstraction reaction (Klinman, 1978). A similar large primary deuterium kinetic isotope effect in the range of 9.6–13.5 was measured for the hydrogen abstraction step in the reaction catalyzed by another quinoprotein, bovine plasma amine oxidase (Palcic & Klinman, 1983). For that enzyme it was subsequently shown that the large isotope effect could be explained by a mechanism involving proton tunneling (Grant & Klinman, 1989). The data presented here suggest the possibility that similar quantum mechanical effects may also play a role in the hydrogen abstraction step of the reaction catalyzed by methylamine dehydrogenase. We have previously characterized similarities between methylamine dehydrogenase and the eukaryotic quinoprotein amine oxidases in their reactions with benzylamines (Davidson et al., 1992) and nucleophilic amines such as hydrazines (Davidson & Jones, 1992). These similarities in the reductive half-reactions are intriguing given that methylamine dehydrogenase exhibits a very different substrate specificity than plasma amine oxidase, does not utilize O_2 as a reoxidant, and possesses TTQ rather than topaquinone as a prosthetic group. It will be of great interest to examine other quinoprotein oxidoreductases to learn whether the occurrence of very large primary isotope effects and possibly proton tunneling are properties common to this class of enzymes.

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