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Reactions of Benzylamines with Methylamine Dehydrogenase. Evidence for a Carbanionic Reaction Intermediate and Reaction Mechanism Similar to Eukaryotic Quinoproteins†

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ABSTRACT: It had been previously reported that aromatic amines were not substrates for the bacterial quinoprotein methylamine dehydrogenase. In this study, benzylamine-dependent activity was also not observed in the steady-state assay of this enzyme with the artificial electron acceptor phenazine ethosulfate (PES). Benzylamines did, however, stoichiometrically reduce the protein-bound tryptophan tryptophylquinone (TTQ) prosthetic group and acted as reversible competitive inhibitors of methylamine oxidation when the enzyme was assayed with PES. When methylamine dehydrogenase activity was monitored using a steady-state assay which employed its physiological electron acceptor amicyanin instead of PES, very low but detectable benzylamine-dependent activity was observed. The reactions of a series of para-substituted benzylamines with methylamine dehydrogenase were examined. A Hammett plot of the log of K_i values for the competitive inhibition by these amines against σ_p exhibited a negative slope. Rapid kinetic measurements allowed the determination of values of k_3 and K_s for the reduction of TTQ by each of these amines. A Hammett plot of log k_3 versus σ_p exhibited a positive slope, which suggests that the oxidation of these amines by methylamine dehydrogenase proceeds through a carbanionic reaction intermediate. A negative slope was observed for the correlation between log K_s and σ_p. Plots of log k_3 and log K_s against substituent constants which reflected either resonance or field/inductive parameters for each para substituent indicated that the magnitude of k_3 was primarily influenced by field/inductive effects while K_s was primarily influenced by resonance effects. No correlation was observed between either k_3 or K_s and the relative hydrophobicity of the para-substituted benzylamines or steric parameters. The K_i values which were obtained from steady-state kinetic experiments correlated strongly with the K_s values which were obtained from rapid kinetic experiments. On the basis of these results, a mechanism is proposed for the reactions of benzylamines with this enzyme. These data are also discussed in light of results of similar studies of the reactions of para-substituted benzylamines with two eukaryotic quinoproteins, lysyl oxidase and plasma amine oxidase.

Methylamine dehydrogenase from *Paracoccus denitrificans* is a soluble enzyme which catalyzes the oxidation of methylamine to formaldehyde plus 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 by Duine and Jongejan (1989)]. These enzymes had all been thought to possess pyrroloquinolinequinone (PQQ)[1] (Salisbury et al., 1979) as a prosthetic group. It now seems that while most bacterial quinoproteins, such as methanol and glucose dehydrogenases, do possess tightly, but noncovalently-associated, PQQ, certain eukaryotic amine oxidases possess other novel covalently-bound quinone species at their active sites. For bovine plasma amine

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[1] Abbreviations: PQQ, pyrroloquinolinequinone; TTQ, tryptophan tryptophylquinone; PES, phenazine ethosulfate; DCIP, 2,6-dichlorophenolindophenol; R, resonance parameter; F, field/inductive parameter; P, octanol-water partition coefficient; E_s, steric parameter.

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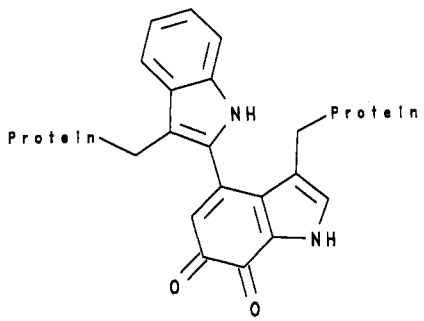

FIGURE 1: Structure of the TTQ prosthetic group of methylamine dehydrogenase.

oxidase it has been shown that the redox center is a modified tyrosine residue, 6-hydroxydopa or TOPA, which may exist either as an *o*- or *p*-quinone (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 which is structurally similar to PQQ (van der Meer & Duine, 1986; 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 *o*-quinone at its active site. This cofactor was previously identified as PQQ (van der Meer et al., 1987). However, recent biochemical (McIntire et al., 1991), X-ray crystallographic (Chen et al., 1991), and resonance Raman spectroscopic (Backes et al., 1991) studies indicate that the cofactor is not PQQ but another unique *o*-quinone species, tryptophan tryptophylquinone (TTQ) (Figure 1), which is derived from two gene-encoded tryptophan residues of the polypeptide chain (Chistoserdov et al., 1990).

It has been reported that substituted benzylamines may serve as either substrates for, or potent reversible ground-state inhibitors of, lysyl oxidase (Williamson & Kagan, 1987). Substituted benzylamines have also been used to demonstrate structure–reactivity correlations in the reaction of bovine plasma amine oxidase (Hartmann & Klinman, 1991). Unlike these two eukaryotic quinoproteins, the substrate specificity of methylamine dehydrogenases is for short-chain primary aliphatic amines (Husain & Davidson, 1987; Davidson, 1989; Eady & Large, 1971; McIntire, 1987; Matsumoto, 1978), and benzylamine was reported not to be a substrate for this enzyme. In light of reports on the reactions of lysyl oxidase and plasma amine oxidase with substituted benzylamines, we have examined more closely the interactions of benzylamines with methylamine dehydrogenase. We report here that while these compounds are not effective substrates in the steady-state assay of activity, benzylamines do reduce the enzyme-bound TTQ and act as competitive inhibitors of methylamine oxidation. The efficiencies of a series of para-substituted benzylamines to act as reductants and inhibitors of methylamine dehydrogenase have been examined. These data provide strong support for an intermediate carbanionic species in the mechanism of the reductive half-reaction of methylamine dehydrogenase. The relationship between the reaction mechanisms and prosthetic group structures of methylamine dehydrogenase, lysyl oxidase, and plasma amine oxidase is also discussed.

Experimental Procedures

The purification of methylamine dehydrogenase (Davidson, 1990), amicyanin (Husain & Davidson, 1985), and cytochrome *c*-551i (Husain & Davidson, 1986) from *P. denitrificans* (ATCC 13543) was as described previously. Protein concentrations were calculated from previously determined extinction coefficients (Husain & Davidson, 1985, 1986; Husain et al., 1987). The chemicals which were used in this study were obtained from either Aldrich or Sigma.

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 and cytochrome *c*-551i as electron acceptors, and activity was monitored by the change in absorbance caused by the amine-dependent reduction of the cytochrome (Davidson & Jones, 1991a). Both assays were performed at 30 °C. When necessary, excess reagents and noncovalently-bound species were separated from the enzyme by passage over a small Ultrogel AcA 202 (IBF Biotechnics) desalting column.

Direct measurements of the rate of reduction of methylamine dehydrogenase were made with a Milton Roy Spectronic 3000 Array spectrophotometer. As the different redox states of this enzyme are quite insensitive to oxygen (Husain et al., 1987), anaerobic conditions were not necessary. The reduction of the enzyme was monitored by following either the increase in absorbance at 330 nm or the decrease in absorbance at 440 nm, both of which occur on reduction (Husain et al., 1987). These experiments were also performed at 30 °C. The observed rate constant was determined by fitting data to either eq 1 for a single exponential rise or eq 2 for a single exponential

$$A_{330} = C(1 - e^{-kt}) + b \quad (1)$$

$$A_{440} = C(e^{-kt}) + b \quad (2)$$

decay. In each equation C is a constant related to the initial absorbance and b represents an offset value to account for a nonzero baseline. It was assumed that the observed reactions obeyed the scheme shown in eq 3, where E and E′ represent

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E'P \quad (3)$$

respectively the oxidized and reduced forms of methylamine dehydrogenase. The values of k_4 were estimated from direct plots of k_{obs} against substrate concentration (Strickland et al., 1975). For each of the samples examined in this study, small but finite values of k_4 were observed. In such a situation eq 4 is applicable, and values for k_3 and K_s were determined from

$$\frac{1}{k_{obs} - k_4} = \frac{K_s}{k_3}\frac{1}{[S]} + \frac{1}{k_3} \quad (4)$$

analysis of double-reciprocal plots of $k_{obs} - k_4$ versus substrate concentration (Strickland et al., 1975). According to this derivation, K_s will be equal to either k_2/k_1 or $(k_2 + k_3)/k_1$ depending upon whether equilibrium or steady-state conditions apply. Data from kinetic experiments were analyzed with the Enzfitter computer program (Elsevier-BIOSOFT, Cambridge).

Results

Inhibition of Methylamine Dehydrogenase by Benzylamines. Although *P. denitrificans* methylamine dehydrogenase is able to use a variety of substituted primary alkylamines as substrates (Davidson, 1989), benzylamine was not a substrate for this enzyme in the steady-state kinetic assay using PES as an electron acceptor. Neither were substituted benzylamines which possessed the following para substituents: methyl, methoxy, bromo, and nitro. Addition of up to 5 mM each of these caused no detectable enzyme-mediated reduction of PES and DCIP. Each substituted benzylamine did, however, inhibit the reaction of methylamine dehydrogenase with

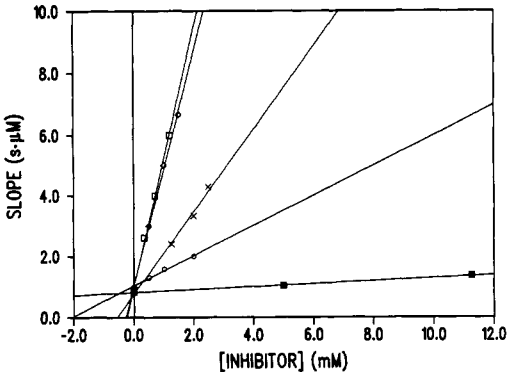

FIGURE 2: Secondary plots of the inhibition of methylamine dehydrogenase by substituted benzylamines. Initial rates of methylamine oxidation were measured while the methylamine concentration was varied at different fixed concentrations of each of the para-substituted benzylamines. For each benzylamine, reciprocal plots of $1/k_{cat}$ against $1/[S]$ produced a family of lines which intersected on the $1/k_{cat}$ axis. Secondary plots of these data are shown here. Each line represents the study with the indicated amine as the competitive inhibitor: (□) *p*-nitrobenzylamine, (◇) *p*-bromobenzylamine, (×) *p*-methylbenzylamine, (○) benzylamine, and (■) *p*-methoxybenzylamine.

Table I: Kinetic Parameters for the Interactions of Para-Substituted Benzylamines with Methylamine Dehydrogenase

para substituent	K_i (mM)	K_s (mM)	k_3 (s^{-1})	k_4 (s^{-1})[a]
$-OCH_3$	17 ± 1	57 ± 40	1.0 ± 0.7	0.001
$-CH_3$	0.57 ± 0.10	11 ± 2	0.71 ± 0.11	0.003
-H	2.1 ± 0.2	2.3 ± 0.2	0.39 ± 0.03	0.004
-Br	0.28 ± 0.03	5.7 ± 2.2	1.4 ± 0.5	0.010
$-NO_2$	0.24 ± 0.02	0.7 ± 0.1	2.0 ± 0.1	0.005

[a] No standard errors are given for the values of k_4. This is because these values were estimated from the ordinate intercept of plots of k_{obs} versus substrate and not fitted to a curve as were the other parameters.

methylamine. To determine the mechanism of inhibition of each of these benzylamines and the K_i value of each, steady-state kinetic experiments were performed. Initial rates of methylamine oxidation were measured while methylamine concentration was varied at different fixed concentrations of each of the para-substituted benzylamines. For each benzylamine, reciprocal plots of $1/k_{cat}$ against $1/[S]$ produced a family of lines which intersected on the $1/k_{cat}$ axis. This indicated that, in each case, inhibition was competitive with respect to methylamine. Secondary plots of slopes against inhibitor concentration for each of these benzylamines were linear (Figure 2). Different K_i values were obtained for each of the benzylamines (Table I). To be absolutely certain that the inhibition of the enzyme was reversible, samples of methylamine dehydrogenase which had been incubated with a large excess of each of the benzylamines were passed over a small desalting column to remove any excess reagents and noncovalently-bound species. After gel filtration, each of these enzyme samples exhibited normal enzymatic activity and exhibited a visible absorption spectrum which was characteristic of the reduced form of the enzyme.

Reduction of Methylamine Dehydrogenase by Benzylamines. The reaction of methylamine dehydrogenase with benzylamines was further examined spectroscopically. Addition of *p*-nitrobenzylamine to oxidized methylamine dehydrogenase caused changes in its visible absorption spectrum (Figure 3) which indicated that the protein-bound TTQ was being reduced (Husain et al., 1987). Complete reduction of the enzyme was achieved by addition of a 2:1 molar ratio of *p*-nitrobenzylamine to enzyme (1:1 per TTQ). The reaction was complete in approximately 20 min. Addition to oxidized

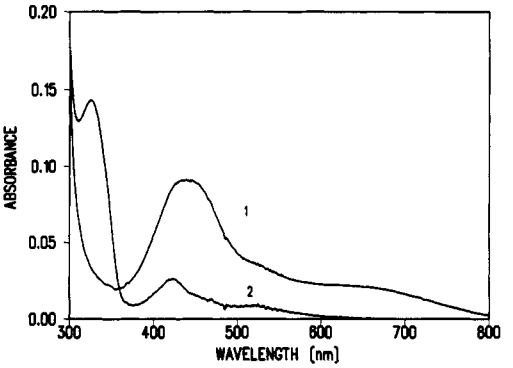

FIGURE 3: Spectral changes caused by addition of benzylamine to methylamine dehydrogenase. Oxidized methylamine dehydrogenase (2.8 μM) was present in 50 mM potassium phosphate, pH 7.5. Spectra were recorded before (1) and after (2) addition of 2.0 molar equiv of benzylamine.

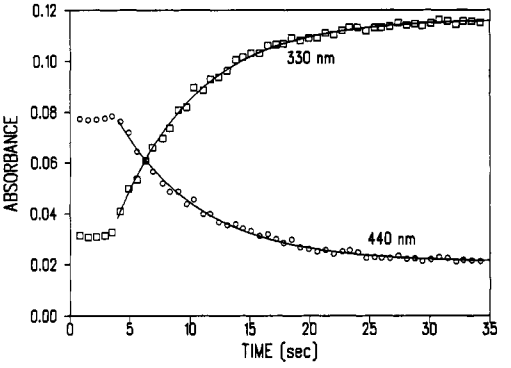

FIGURE 4: Reduction of methylamine dehydrogenase by benzylamine. The reaction was initiated by addition of 1.2 mM benzylamine to 2.9 μM methylamine dehydrogenase in 50 mM potassium phosphate, pH 7.5, with rapid mixing. Absorbance was measured at 330 (□) and 440 (○) nm at 0.7-s intervals. Data were analyzed as described under Experimental Procedures.

methylamine dehydrogenase of benzylamine caused identical spectral changes. Again complete reduction of the enzyme was achieved by addition of a 2:1 molar ratio of amine to enzyme (1:1 per TTQ). The reaction with benzylamine, however, was very slow and required approximately 3 h for completion. Thus, despite not functioning as substrates in the steady-state assay of enzyme activity, these benzylamines were able to reduce the TTQ prosthetic group of methylamine dehydrogenase. Furthermore, the efficiency of enzyme reduction appeared to depend upon the nature of the para substituent. A detailed examination of this phenomenon is described below.

The reduction of oxidized methylamine dehydrogenase was measured by monitoring either the increase in absorbance at 330 nm or the decrease in absorbance at 440 nm, or both simultaneously, on addition of varying concentrations of each of the benzylamines to the enzyme. In all cases, the absorbance changes with time could be fit to the equation for a single exponential (Figure 4). Direct plots of k_{obs} against concentration for each of these benzylamines passed through the ordinate at a positive value and allowed the estimation of k_4 (Table I). For each benzylamine, a reciprocal plot of $k_{obs} - k_4$ against substrate concentration was constructed (Figure 5). From these data it was possible to obtain values of K_s and k_3 for the reactions of each of these benzylamines with methylamine dehydrogenase (Table I). Semilogarithmic plots of k_{obs} against time for each reaction were linear and showed no detectable lag, suggesting that for these reactions $k_2 \gg k_3$ and that K_s was, therefore, equivalent to K_d. This does not, however, constitute absolute proof that K_s is identical to K_d,

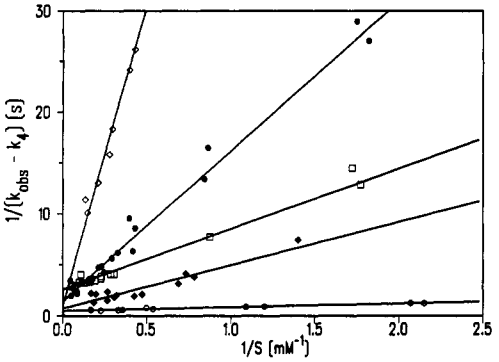

FIGURE 5: Reciprocal plots of the observed rate constants for the reduction of methylamine dehydrogenase by substituted benzylamines. Each line represents the study with the indicated amine as the substrate: (◇) *p*-methoxybenzylamine, (●) *p*-methylbenzylamine, (□) benzylamine, (◆) *p*-bromobenzylamine, and (○) *p*-nitrobenzylamine.

Table II: Correlations between the Kinetic Parameters for the Interactions of Para-Substituted Benzylamines with Methylamine Dehydrogenase and Hammett Substituent Constants and Field/Inductive and Resonance Parameters

kinetic parameter	r^2				
	σ_p	F	R	log P^a	$E_s{}^b$
K_i	0.509	0.004	0.523	0.519	0.352
K_s	0.725	0.114	0.956	0.031	0.055
k_3	0.468	0.867	0.006	0.032	0.430

[a] The correlations with log P did not include *p*-bromobenzylamine, for which a value of log P could not be found. [b] The correlations with E_s did not include *p*-nitrobenzylamine, for reasons discussed in the text.

and one cannot rule out the possibility that other kinetic parameters are contributing to K_s. It should also be noted that the relatively high standard errors for the parameters for the reaction with *p*-methoxybenzylamine were due to solubility problems. It was not possible to dissolve this poor substrate at sufficiently high concentrations to obtain as complete a data set as was obtained for the other compounds.

Relationships between Kinetic Parameters and Substituent Constants. To examine the influence of the electronegativity of the different para substituents on the ability of these substituted benzylamines to reduce and to inhibit methylamine dehydrogenase, a series of Hammett plots were constructed from the kinetic data and values of σ_p (Hansch et al., 1991). Plots of K_i and K_s exhibited negative slopes, whereas k_3 exhibited a positive slope. The plots of k_3 and K_s exhibited ρ values of 0.46 ± 0.26 and −1.5 ± 0.5, respectively. In each case, the correlation was significant but not very strong (Table II). To gain further insight into the nature of the substituent effects which influence the reactivities of these para-substituted benzylamines with methylamine dehydrogenase, the logs of K_i, k_3, and K_s were each plotted against substituent constants reflective of field/inductive (F) and resonance (R) parameters. The electronic effects described by σ can be factored into these two component parts. The constant R describes the ability of a substituent to withdraw electrons from or release electrons into the conjugated system of π-electrons. This will be observed for appropriate substituents in the para position with which strong resonance interaction can occur. The constant F describes the ability of a substituent to influence a process by electrostatic interactions through space (field) and through intervening bonds by polarization of those bonds (inductive). Unlike the resonance effect the field/inductive effect is attenuated with distance. The F and R parameters which were used here are modified Swain–Lupton constants which have been adjusted to place them on the same scale as the Hammett constants (Hansch et al., 1991).[2] Very strong correlations were observed between log k_3 and F and between log K_s and R. The correlation coefficients for all possible plots are given in Table II. The constants of proportionality (equivalent of ρ values) for these graphs are 0.93 ± 0.20 for log k_3 versus F and −2.7 ± 0.3 for log K_s versus R. In assessing possible electronic effects on reactivity, it is appropriate to consider also the possible roles of hydrophobicity and steric effects. Possible correlations between substituent hydrophobicity and these kinetic parameters were examined (Table II) by plotting the logs of each of these kinetic parameters against log P, the octanol–water partition coefficient, of the substituted benzylamines (Hansch et al., 1968). Some correlation was observed with K_i. The slope of that graph was negative. No correlations were observed with K_s and k_3. It is difficult to assess steric substituent effects on enzyme-catalyzed reactions. Several different steric parameters have been described in the literature. The choice of an appropriate steric parameter is complicated because it will depend upon the nature of the substrate binding site of the enzyme and solvent conditions at that site, neither of which are known. The steric parameters (E_s) used here were calculated from average van der Waals radii (Kutter & Hansch, 1969). The data which were obtained for *p*-nitrobenzylamine were not included in this analysis as the E_s value for the nitro substituent will be dramatically different depending upon whether it is coplanar or perpendicular to the reaction center. Using these values, it can be seen (Table II) that no significant correlations were observed between the kinetic parameters and E_s. From the sum of the data listed in Table II it appears that K_s is influenced primarily by resonance effects, whereas the rate of TTQ reduction (k_3) is influenced primarily by field/inductive effects.

Table III: Correlations of Collinearity of the Kinetic Parameters for the Interactions of Para-Substituted Benzylamines with Methylamine Dehydrogenase

kinetic parameter	r^2		
	k_3	K_s	K_i
k_3	1.000		
K_s	0.030	1.000	
K_i	0.037	0.956	1.000

Methylamine dehydrogenase obeys a ping-pong kinetic mechanism (Davidson, 1989) in which release of the aldehyde product precedes reoxidation by PES. Thus, given the competitive pattern of reversible inhibition which was observed with the benzylamines, one would expect that K_i would be related to the dissociation constant of the enzyme–benzylamine complex. Examination of the collinearity of the kinetic parameters which were obtained in this study does reveal a strong correlation between K_i and K_s (Table III), consistent with the previously established kinetic mechanism for this enzyme.

Steady-State Kinetic Assay of Methylamine Dehydrogenase with Physiological Electron Acceptors. To further explore the question of why benzylamines are not substrates for methylamine dehydrogenase in the steady-state assay with PES, these amines were tested as substrates in a recently developed assay which employs the physiological electron acceptors for the enzyme (Davidson & Jones, 1991a). In contrast to the results of the assay with PES, low but detectable rates of reaction were observed with the physiological electron acceptors. The K_{cat} values which were obtained with the benzylamines ranged from 10 to 50 min^{-1}, compared to 1100 min^{-1} for methylamine. The enzyme could be saturated with mi-

[2] The reader is referred to Hansch et al. (1991) for tables of these values and a review of the contribution of field/inductive and resonance parameters to overall values for substituent constants.

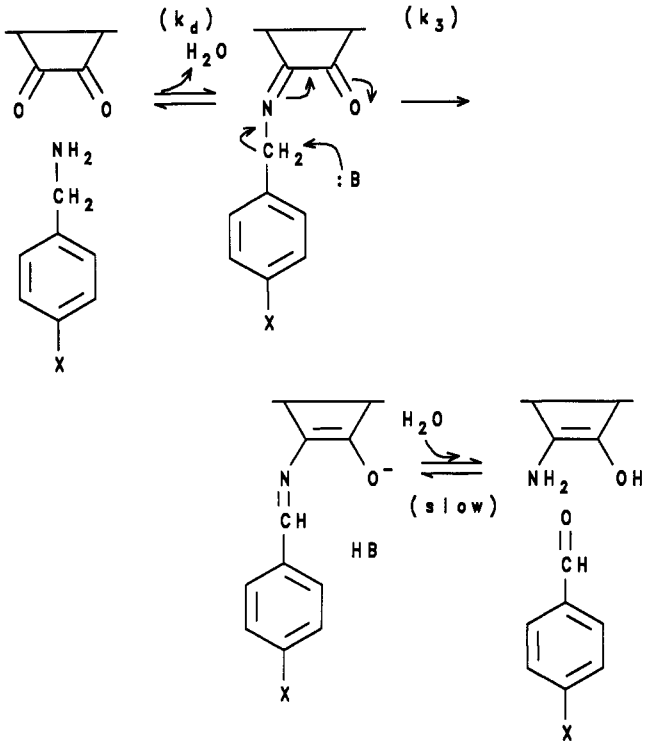

FIGURE 6: Proposed mechanism for the reaction of benzylamines with methylamine dehydrogenase.

cromolar concentrations of the benzylamines. Because of the nature of this assay, with such low rates it was not possible to obtain data of sufficient quality to examine structure–reactivity correlations, as was done above with the other kinetic data. It is noteworthy, however, that depending upon the nature of the electron acceptor used in the steady-state assay, benzylamines behave either strictly as competitive inhibitors or as substrates, albeit poor ones. A possible explanation for this is discussed later.

Discussion

A mechanism for the reaction of methylamine dehydrogenase with benzylamines is proposed in Figure 6. 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 which loses water to form the imine intermediate shown. 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). Our data indicate that the magnitude of K_s is influenced primarily by resonance effects. Furthermore, analysis of the kinetic data according to Strickland (1975) suggests that K_s is equivalent to k_2/k_1 (K_d). This suggests that resonance contributions from the benzene ring can stabilize the initial imine intermediate by reducing k_2 relative to k_1, despite the fact that the ring substituent is separated from the imine by a methylene group. If true, this would require hyperconjugation between the benzyl ring and iminoquinone. Alternatively, the kinetically determined K_s may not be truly equivalent to K_d and may also reflect kinetic parameters which are related to TTQ reduction and subsequent reaction steps. The positive slope of the Hammett plot of log k_3 versus σ_p is diagnostic of a carbanionic intermediate in that reaction. An increase in rate which correlates with an increase in the ability of a substituent to withdraw electrons from the reaction site is consistent with a proton abstraction mechanism which leads to carbanion formation. It is next, therefore, proposed that an active site nucleophile abstracts a proton from the α-carbon, thus forming a carbanionic intermediate concomitant with the reduction of the prosthetic group. Previous steady-state kinetic studies have characterized a deuterium kinetic isotope effect on k_{cat} of 3.0 for the oxidation of methylamine by methylamine dehydrogenase (Davidson, 1989), consistent with proton abstraction as the rate-limiting step for this reaction. Our data indicate that k_3 is influenced primarily by field/inductive effects (described earlier). That is to say that the ability of a substituent to reduce the negative charge density on the α-carbon by through-space electrostatic interactions and polarization of the intervening bonds will stabilize the transition state of this intermediate and enhance the rate of reaction. Resonance effects do not appear to correlate with k_3 (Table II). As σ_p is composed of both resonance and field/inductive parameters, it can be understood why the correlation between log k_3 and σ_p ($r^2 = 0.468$) is much less than that between log k_3 and F ($r^2 = 0.867$) and why the magnitude of the constant of proportionality for the graph of log k_3 versus σ_p (0.46 ± 0.26) is less than that for log k_3 versus F (0.93 ± 0.2). This step, k_3, is essentially irreversible. Kinetic analysis indicated that k_4 was very small compared to k_3 (Table I). Furthermore, after removal of excess benzylamine at this point, the fully reduced form of the enzyme was obtained. These data suggest that product formation must occur. The reduced enzyme–product complex shown in Figure 6 will be more stabilized by the benzyl moiety attached to the α-carbon and more resistant to hydrolysis than the complex formed by reaction with the normal substrate, methylamine. Electrons may be delocalized in a manner which significantly lowers the relative concentration of the tautomeric form of the intermediate which is susceptible to hydrolysis. Hydrolysis and the resulting release of the aldehyde product occurs, therefore, very slowly relative to the reaction with aliphatic substrates. The release is apparently so slow that one is unable to detect significant turnover of the enzyme in the steady-state assay with PES.

It should be noted that the K_s values which were obtained for the benzylamines are significantly larger than the K_m value for methylamine which is 10 μM (Davidson, 1989). This is quite likely due to steric factors due to the presence of the relatively bulky benzyl ring. In fact, it is surprising that an active site designed to accommodate methylamine would even be accessible to something as large as benzylamines. The data here, however, are consistent with previous observations that compounds much larger than methylamine, such as cyclopropylamine (Davidson & Jones, 1991b) and phenylhydrazine (Davidson & Jones, 1992), are cofactor-directed inhibitors of *P. denitrificans* methylamine dehydrogenase. X-ray crystallographic studies (Vellieux et al., 1989; Chen et al., 1992) have indicated that the TTQ prosthetic group of this enzyme is located in a narrow, relatively hydrophobic, channel at the interface between the two dissimilar subunits. The fact that the active site can accommodate benzylamines may suggest some flexibility in that channel and is also in keeping with the hydrophobic nature of the channel.

Quinoprotein dehydrogenases are routinely assayed with artificial electron acceptors such as PES. In vivo, however, these enzymes do not normally donate electrons to small soluble species but to other proteins or membrane components (Anthony, 1988). For methylamine dehydrogenase, electron transfer to amicyanin is a through-protein process which does not involve direct contact between redox centers.[3] Amicyanin

[3] L. Chen, R. Durley, B. J. Poliks, K. Hamada, Z. Chen, F. S. Mathews, V. L. Davidson, Y. Satow, E. Huizinga, F. M. D. Vellieux, & W. G. J. Hol, manuscript submitted for publication.

binds to a surface domain of the dehydrogenase which is distinct from the active site. The observed very low turnover number for the reaction of benzylamines in the assay with amicyanin as an electron acceptor is consistent with the proposal of a very slow product release relative to aliphatic substrates. The absence of activity when assayed with PES may be because the longer lifetime of the enzyme–product complex allows it to block access of PES to the reduced prosthetic group, or it may be that once released from the cofactor, the benzaldehyde products may not readily diffuse out of the active site and block entry of PES to the active site. This not a factor when amicyanin is used in the assay because, as stated above, it does not require access to the active site to reoxidize the enzyme. It should be noted that while no correlations between K_s or k_3 and hydrophobicity were observed, some correlation was observed between K_i and the hydrophobicity of the amine substrate, with K_i decreasing with increasing hydrophobicity. Again, this may relate to the position of the active site which in this enzyme resides at the end of a relatively hydrophobic channel. These data further suggest that caution should be used in assigning substrate specificities to quinoprotein dehydrogenases on the basis of steady-state kinetic studies with artificial electron acceptors.

The reduction and inhibition by benzylamines of methylamine dehydrogenase is particularly relevant in light of recent reports on the reactions of such amines with eukaryotic quinoprotein amine oxidases. Benzylamine derivatives with strongly electronegative para substituents were shown to be effective ground-state inhibitors of lysyl oxidase by virtue of their ability to stabilize an enzyme-bound carbanion intermediate (Williamson & Kagan, 1987). The Hammett plot of K_i versus σ_p which was obtained in that study is very similar to that which was obtained here. Furthermore, as in the present study, investigations of the reactions of para-substituted benzylamines with plasma amine oxidase (Hartmann & Klinman, 1991) indicated a positive slope for the correlation between k_3 and σ_p. Those data supported the involvement of a carbanionic intermediate in the reaction mechanism of that enzyme. Thus, while methylamine dehydrogenase exhibits a very different substrate specificity than lysyl and plasma amine oxidases, while it does not utilize O_2 as a reoxidant, and while it now appears that each of these enzymes possesses a different quinone prosthetic group, these data suggest that the mechanisms by which the reductive half-reaction of amine oxidation is catalyzed by methylamine dehydrogenase and the eukaryotic quinoprotein amine oxidases are very similar. Whether benzylamine is an effective substrate or inhibitor of the overall reaction of each of these quinoproteins is likely correlated with the efficiency of hydrolysis and concomitant release of product. This step in the reaction mechanism is probably influenced by the active site environment of the quinone prosthetic group in its respective enzyme, which will dictate the nature of the reaction of the quinone with the amine.

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