Mechanistic Studies of Aromatic Amine Dehydrogenase, a Tryptophan Tryptophylquinone Enzyme†

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ABSTRACT: Aromatic amine dehydrogenase (AADH) is the second enzyme known to possess the tryptophan tryptophylquinone (TTQ) prosthetic group. Its ability to catalyze the oxidative deamination of a wide range of aromatic and aliphatic amines has been investigated. Steady-state and transient kinetic studies of the reaction of AADH with a series of *p*-substituted phenylethylamines were performed to determine structure−reactivity correlations. The K_m values correlated strongly with hydrophobic effects. The microscopic rate constant associated with TTQ reduction, k_3, correlated with electronic substituent effects, particularly field/inductive effects, in a manner consistent with the formation of a carbanionic reaction intermediate in the reductive half-reaction. Transient kinetic studies were also performed with a series of *p*-substituted benzylamines, which were not substrates in the steady-state assay, but which did stoichiometrically reduce TTQ. The k_3 for the reaction with benzylamines also correlated well with electronic effects. The rate constant associated with the release of the aldehyde product was also determined for the phenylethylamines and appears to be the most rate-limiting step in the overall oxidation−reduction reaction. This rate constant correlated with hydrophobicity. AADH also reacted with primary aliphatic amines, preferring longer chain, more hydrophobic amines. This substrate specificity for aliphatic amines is opposite of that of methylamine dehydrogenase (MADH), the other known TTQ enzyme. On the basis of these studies, a reaction mechanism is proposed for AADH. These data are discussed in relation to the results of structure−reactivity correlation studies of the reactions catalyzed by MADH and two eukaryotic quinoproteins with different quinone prosthetic groups, plasma amine oxidase and lysyl oxidase.

Aromatic amine dehydrogenase (AADH)[1] was first purified and characterized from the soil bacterium, *Alcaligenes faecalis*, by Iwaki et al. (1983). After their preliminary studies of the kinetic, spectral, and structural properties of AADH, a detailed characterization of the enzyme was recently reported by Govindaraj et al. (1994). AADH catalyzes the oxidation of a primary amine to its corresponding aldehyde and ammonia. The two electrons that are derived from the amine substrate are transferred to some electron acceptor by the enzyme (eq 1).

$$RCH_2NH_2 + H_2O \rightarrow RCHO + NH_3 + 2e^- + 2H^+ \quad (1)$$

AADH exhibits an $\alpha_2\beta_2$ structure with subunit molecular weights of 39 000 and 18 000. Each small subunit contains a covalently bound quinone prosthetic group, which is involved both in catalysis and in the subsequent electron transfer to its physiologic electron acceptor. This prosthetic group has been identified as tryptophan tryptophylquinone (TTQ); the absorption spectra for the oxidized, semiquinone, and reduced forms of AADH have been characterized, and accurate extinction coefficients for each redox form have been determined (Govindaraj et al., 1994). These physical, spectral, and structural properties of AADH are very similar to those of methylamine dehydrogenase (MADH) (Davidson, 1993), which is the only other known TTQ-containing enzyme. TTQ in MADH is formed by posttranslational modifications of two gene-encoded tryptophan residues (Chistoserdov et al., 1990), which lead to covalent attachment between the indole rings of the two tryptophan residues and oxidation of one indole ring to an *o*-quinone. The TTQ structure (Figure 1), on the basis of mass spectroscopy and nuclear magnetic resonance studies of derivatized prosthetic group-containing peptides from MADH of bacterium W3A1, was proposed by McIntire et al. (1991b). This structure was verified by X-ray crystallographic studies of MADH from *Thiobacillus versutus* and *Paracoccus denitrificans* (Chen et al., 1991). Resonance Raman spectroscopy was also used to describe the TTQ prosthetic group of MADH from *T. versutus*, *P. denitrificans*, and bacterium W3A1 (Backes et al., 1991; McIntire et al., 1991a) and to identify TTQ as the prosthetic group of AADH (Govindaraj et al., 1994).

It now seems that while most bacterial quinoproteins, such as methanol dehydrogenase and glucose dehydrogenase, have tightly, but noncovalently, associated PQQ, certain eukaryotic amine oxidases possess other novel covalently bound quinone species at their active sites. For bovine serum amine oxidase, it has been shown that the redox center is a modified tyrosine residue, 6-hydroxydopa (topa), which may exist as either an *o*- or a *p*-quinone (Janes et al., 1990). For mammalian lysyl oxidase, the precise nature of the covalently bound organic

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[1] Abbreviations: AADH, aromatic amine dehydrogenase; DCIP, 2,6-dichlorophenolindophenol; MADH, methylamine dehydrogenase; PES, phenazine ethosulfate; PQQ, pyrroloquinoline quinone; topa, 6-hydroxydopa; TTQ, tryptophan tryptophylquinone σ_p, electronic substituent parameter; *R*, resonance substituent parameter; *F*, field/inductive substituent parameter; π, hydrophobicity substituent parameter; E_s, steric substituent parameter.

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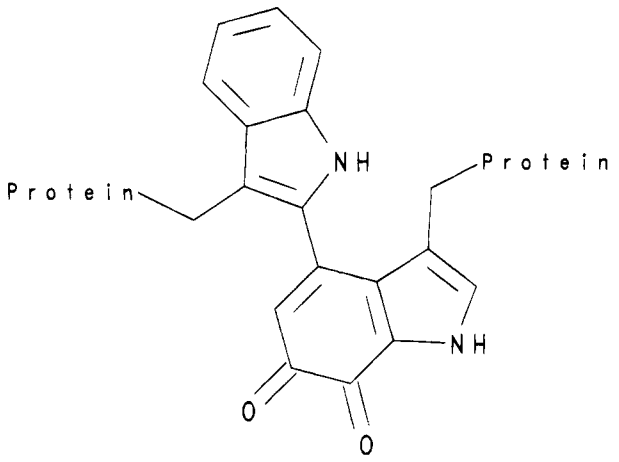

FIGURE 1: Structure of the tryptophan tryptophylquinone (TTQ) prosthetic group of aromatic amine dehydrogenase.

cofactor is uncertain, but data suggest that it is an *o*-quinone, which is structurally similar to topa (Kagan & Trackman, 1993). MADH and AADH are atypical bacterial quinoproteins with novel covalently bound and amino acid-modified prosthetic groups. In this respect they are more similar to the eukaryotic quinoproteins, particularly the amine oxidases, in that they possess a covalently bound, amino acid-derived quinone at the active site.

Substituted benzylamines have been used to demonstrate structure−reactivity correlations in the reactions of bovine serum amine oxidase (Hartmann & Klinman, 1991), lysyl oxidase (Williamson & Kagan, 1987), and MADH (Davidson et al., 1992). These three enzymes have different covalently bound quinone cofactors, different substrate specificities, and different levels of reactivity with benzylamines. However, the reductive half-reactions of these three enzymes were similar in their mechanisms of formation of the enzyme−substrate complex and the involvement of a carbanionic reaction intermediate.

In this paper, we report steady-state and transient kinetic parameters for the reactions of various aliphatic and aromatic amines with AADH. These kinetic data are used to demonstrate structure−reactivity correlations for the various substrates that are oxidized by the TTQ prosthetic group of AADH. From these results, it was possible to propose a mechanism and to determine steady-state and microscopic rate constants for the reductive half-reaction of AADH with amine substrates. The relationships between the reaction mechanisms and prosthetic group structures of the two prokaryotic TTQ-bearing enzymes, MADH and AADH, and the eukaryotic quinoprotein amine oxidases are also discussed.

EXPERIMENTAL PROCEDURES

Purification of AADH from *A. faecalis* (IFO 14479) was as described previously, and protein concentrations were calculated from previously determined extinction coefficients (Govindaraj et al., 1994). The chemicals that were used in this study were obtained from either Aldrich or Sigma.

Steady-state kinetic assays for AADH activity were performed in 0.25 M potassium phosphate buffer (pH 7.5) at 30 °C using a spectrophotometric dye-linked assay similar to that described previously for the assay of MADH (Davidson, 1990). The reaction mixture contained 4.8 mM phenazine ethosulfate (PES), the immediate electron acceptor, and 0.17 mM 2,6-dichlorophenolindophenol (DCIP), a redox-sensitive dye to which the reduction of PES is coupled. AADH (1−2 μg/mL) was preincubated in the reaction mixture, and the reactions were initiated by the addition of the amine substrate. Initial velocities were determined from the rate of reduction of DCIP, which was monitored at 600 nm (ϵ = 21 500 M^{-1} cm^{-1}). To determine steady-state kinetic parameters, initial rates were measured at different concentrations of each substrate, and those data were fit to the Michaelis−Menten equation (eq 2). It should be noted

$$v/[E_o] = k_{cat}[S]/(K_m + [S]) \quad (2)$$

that the reported k_{cat} values are really apparent values that were obtained by varying only the amine substrate in the presence of saturating PES (approximately 20-fold greater concentration than the K_d for PES). For tyramine, the k_{cat} measured under these conditions was within error of the true k_{cat}, which we obtained by varying each substrate (data not shown).

Transient kinetic experiments were performed using an On-Line Instrument Systems (OLIS, Bogart, GA) stopped-flow sample handling unit coupled to Durrum optics. A 486-class computer controlled by OLIS software was used to collect and analyze the data. Experiments were performed at 30 °C in 0.25 M potassium phosphate buffer (pH 7.5). The reaction of oxidized AADH with amine substrate was monitored by the decrease in absorbance at 456 nm ($\Delta\epsilon$ = 25 500 M^{-1} cm^{-1} for the conversion of oxidized AADH to reduced AADH). The reaction of reduced AADH and PES was monitored by the increase in absorbance at 483 nm ($\Delta\epsilon$ = 19 800 M^{-1} cm^{-1} for the conversion of reduced AADH to oxidized AADH). This wavelength is isosbestic for the oxidized and reduced forms of PES.

Reduced AADH was prepared by the addition of 2 molar equiv of tyramine to AADH. Typically three or four data sets, each containing 500−1000 data points, were collected and averaged. Under conditions where the varied reactant was in excess of the concentration of the fixed reactant, the observed rate constant (k_{obs}) could be determined from data fit to the equation for a single-exponential decay:

$$A = Ce^{-kt} + b \quad (3)$$

where C is a constant related to the initial absorbance and b represents an offset value to account for a nonzero baseline. Observed absorbance changes were monophasic, indicating that the reactions of AADH with substrate and with PES were pseudo-first order under these conditions. It was assumed that the observed reactions obeyed the scheme shown in eq 4, where E and E′ represent different redox forms of AADH.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E'P \quad (4)$$

To determine the microscopic rate constant for each reaction step, values of k_{obs} obtained at different substrate concentrations were fit to either eq 5, which was derived by Strickland et al. (1975), or eq 6, which was derived by Hiromi (1979). Equation 5 provides a simple method for

$$k_{obs} = \{k_3[S]/(K_d + [S])\} + k_4 \quad (5)$$

$$k_{obs} = 0.5\{k_1[S] + k_2 + k_3 + k_4 - ((k_1[S] + k_2 + k_3 + K_4)^2 - 4(k_1k_3[S] + k_1K_4[S] + k_2k_4))^{0.5}\} \quad (6)$$

extracting these rate constants, provided that $k_2 \gg k_3$. In cases where this condition is not met, the more rigorous method (eq 6) should be applied. Data for the reactions of oxidized AADH with amine substrates fit well to eq 5. Data for the reaction of reduced AADH with PES were better fit to eq 6. In all experiments the concentration of substrate was sufficiently greater than the enzyme concentration, so that k_{obs} was independent of the enzyme concentration, as predicted by eqs 5 and 6. It should be noted that according to this model (eq 4) the value obtained for k_4 will be valid only if E'P accumulates, but not if the dissociation of P is faster than k_4. Nonlinear curve fitting of data was performed with the Sigma plot 5.0 (Jandel Scientific, San Raphael, CA) computer program.

RESULTS

Steady-State Kinetic Analysis. Steady-state kinetic experiments were performed with various primary aliphatic and aromatic amines. The steady-state kinetic parameters for the oxidation of amines that served as substrates for AADH in this assay are listed in Table 1. AADH exhibited broad substrate specificity for primary aliphatic and aromatic amines. The most efficient substrates for AADH were substituted phenylethylamines. In addition to phenylethylamines, primary aliphatic amines were substrates for AADH under these experimental conditions, but with apparent K_m values that were significantly greater than those of the phenylethylamines. Longer chain primary aliphatic amines were better substrates for AADH than shorter chain amines. This substrate specificity of AADH for aliphatic amines was opposite that of MADH (Davidson, 1989), the other known TTQ-bearing enzyme, which prefers methylamine as a substrate. Benzylamines were also tested as substrates. It is interesting to note that although AADH has broad substrate specificity and phenylethylamines were efficient substrates, benzylamine-dependent activity was not observed under these steady-state experimental conditions. Also, para-substituted benzylamines that possessed the following substituents, nitro, methyl, methoxy, fluoro, chloro, and bromo, were tested and found not to be substrates for AADH in this assay. To determine whether any turnover of AADH occurred in the steady state, the assay was repeated with excess *p*-nitrobenzylamine and a much higher concentration of AADH (68 μg/mL). This higher level of enzyme increased the amount of product formation to detectable levels. The observed k_{cat} was less than 0.01 s^{-1}; however, this confirmed that the benzylamine was a substrate for AADH, albeit one that was turned over very inefficiently.

Benzylamine-Dependent Reduction of AADH. Benzylamines, which were not effective substrates for AADH in the steady-state kinetic assay, were observed to stoichiometrically reduce the protein-bound TTQ prosthetic group. The addition of benzylamines to oxidized AADH caused changes in its visible absorption spectrum that indicated that the protein-bound TTQ was being reduced. Complete reduction of the enzyme was achieved by the addition of a 2:1 molar ratio of benzylamine to enzyme (1:1 per TTQ). These spectral changes (Figure 2) were identical to those observed upon the addition of amines that were substrates for the steady-state assay. The reactions of AADH with this series of para-substituted benzylamines were further examined by stopped-flow spectroscopy (described in the following sections).

Table 1: Steady-State Kinetic Parameters for the Reactions of Aliphatic and Aromatic Amines with AADH

amine	k_{cat} (s^{-1})	K_m (μM)
methylamine	1.8 ± 0.1	(3.8 ± 0.6) × 10^4
ethylamine	4.8 ± 0.2	(6.1 ± 0.8) × 10^4
propylamine	10 ± 1	(3.6 ± 0.3) × 10^4
butylamine	8.9 ± 0.1	(7.0 ± 0.3) × 10^3
ethanolamine	9.1 ± 0.3	(1.8 ± 0.1) × 10^5
phenylethylamine	13 ± 1	2.3 ± 0.2
(*p*-hydroxyphenyl)ethylamine	13 ± 1	6.9 ± 0.6
(*p*-fluorophenyl)ethylamine	8.5 ± 0.3	1.2 ± 0.1
(*p*-aminophenyl)ethylamine	14 ± 1	18 ± 2
(*p*-methylphenyl)ethylamine	2.4 ± 0.2	1.1 ± 0.3
(*p*-chlorophenyl)ethylamine	0.6 ± 0.03	0.9 ± 0.1
dopamine	18 ± 1	147 ± 10
histamine	12 ± 1	(5.6 ± 0.3) × 10^3

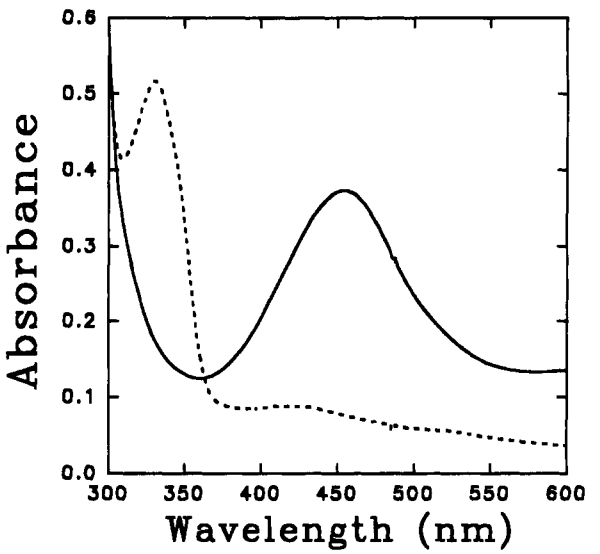

FIGURE 2: Spectral changes caused by the addition of benzylamine to AADH. Oxidized AADH (11.8 μM) was present in 0.25 M potassium phosphate (pH 7.5). Spectra were recorded before (solid line) and after (dashed line) the addition of 2.0 molar equiv of benzylamine.

Stopped-Flow Kinetic Studies of the Reductive Half-Reaction of AADH. The three redox states of AADH exhibit distinct absorption spectra (Govindaraj et al., 1994). The chromophore exhibits a broad peak centered at 456 nm, which increases in absorbance upon conversion from the oxidized to the semiquinone state and is essentially bleached in the reduced state. The absorbance changes at 456 nm with time, which were observed upon mixing oxidized AADH with each amine substrate, could be fit to a single-exponential decay, suggesting a direct two-electron reduction of TTQ by substrate with no involvement of the semiquinone in the reductive half-reaction.

As stated earlier, benzylamines were also able to stoichiometrically reduce AADH. Therefore, the reactions of benzylamines, as well as of phenylethylamines, with AADH were examined by stopped-flow spectroscopy (Figure 3). For each amine, k_{obs} was measured at several different substrate concentrations. For most of the benzylamines, it was possible to observe a reasonable variation in k_{obs} with concentration, as shown in Figure 4. In these cases, direct

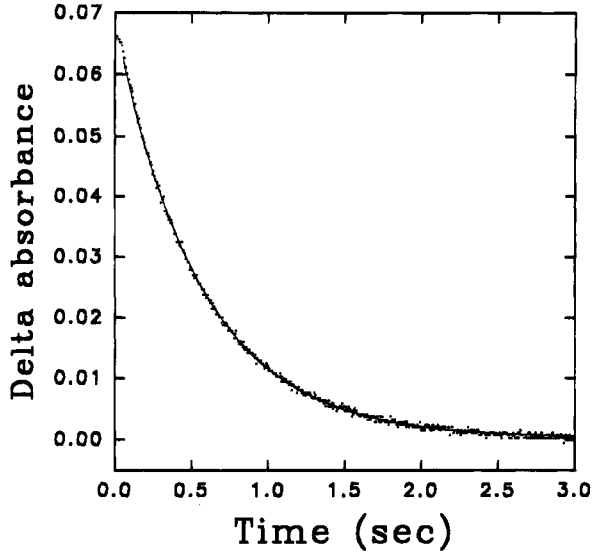

FIGURE 3: Reduction of AADH by benzylamine. AADH (1.5 μM) was mixed with benzylamine (250 μM) in 0.25 M potassium phosphate buffer (pH 7.5) at 30 °C. Absorbance changes were monitored at 456 nm. The solid line represents a fit of these data to eq 3, which yielded a k_{obs} of 1.8 s^{-1}.

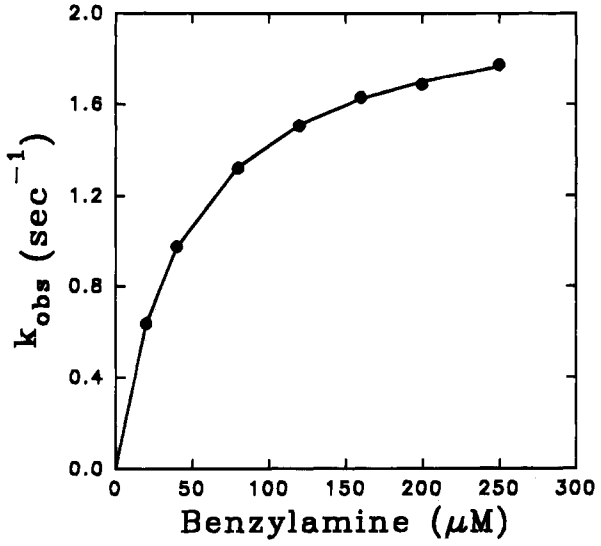

FIGURE 4: Plot of k_{obs} against benzylamine concentration for several reactions, such as that shown in Figure 3. The values of k_{obs} were calculated by fitting the decrease in A_{456} to eq 3. The line drawn through these points is the fit of these data to eq 5.

plots of k_{obs} against substrate concentration for the benzylamines passed through the origin, suggesting that either k_4 was approximately equal to zero (Strickland et al., 1975) or dissociation of the aldehyde product was very rapid relative k_4. Consistent with this interpretation was the observation that complete irreversible reduction of AADH occurred upon the addition of a stoichiometric amount of each amine substrate. For these benzylamines, the data were fit to eq 5 and values for k_3 and K_d were obtained (Table 2).

Unfortunately, it was not possible to obtain fitted values of k_3 and K_d for chloro- and bromobenzylamines and the phenylethylamines from these stopped-flow kinetic experiments. This was because of the requirement that the varied reactant, substrate, be in excess of the fixed reactant, AADH, in order to maintain pseudo-first-order reaction conditions, which are necessary to allow the use of eqs 5 and 6 to fit the data. The minimum concentration of AADH that allowed

Table 2: Stopped-Flow Kinetic Parameters for the Reactions of Para-Substituted Benzylamines with AADH

p substituent	k_3 (s^{-1})	K_d (μM)
H	2.1 ± 0.01	46 ± 1
NO_2	62 ± 1	16 ± 1
CH_3	0.3 ± 0.002	6.2 ± 0.2
OCH_3	9 ± 0.04	110 ± 3
F	7.6 ± 0.1	25 ± 1
Cl	1.5 ± 0.01	<5
Br	1.6 ± 0.05	<5

Table 3: Microscopic Rate Constants for the Reactions of Para-Substituted Phenylethylamines with AADH

p substituent	k_3 (s^{-1})[a]	k_5 (s^{-1})
H	78	18.7
OH	390	15.7
F	170	10.2
NH_2	55	26.6
CH_3	77	2.6
Cl	130	0.6

[a] Standard errors are not included in this table because these are not fitted values. As discussed in the text, k_3 is the limiting value of k_{obs} in the concentration-independent range, and k_5 is calculated from eq 9.

a sufficiently large absorbance change to collect reliable data was 1 μM, and at substrate concentrations less than 5 μM the reaction was no longer pseudo-first order. With most of the phenylethylamines and chloro- and bromobenzylamines, k_{obs} at substrate concentrations in excess of 5 μM was not dependent upon substrate concentration, suggesting that the lowest concentration of substrate used was well in excess of its K_d value. It was assumed, therefore, that for these amines the K_d values were in the low micromolar or submicromolar range. The limiting value of k_{obs}, which was observed at concentrations of substrate much larger than K_d, will be equal to $k_3 + k_4$ (Strickland et al., 1975). As k_4 is effectively equal to zero for the reduction of AADH by these amines (discussed earlier), this limiting value of k_{obs} may be taken to be k_3. These values are listed in Tables 2 and 3. It is evident from Tables 2 and 3 that the efficiency of enzyme reduction (k_3) was influenced by the nature of the para substituent on both benzyl- and phenylethylamines. In general, the benzylamines exhibited a significantly slower k_3 for TTQ reduction and a higher dissociation constant (K_d) than did the phenylethylamines.

Stopped-Flow Kinetic Studies of the Oxidative Half-Reaction of AADH with PES. It has been shown that the steady-state reaction of AADH with PES obeys a ping-pong mechanism (Iwaki et al., 1983), as described by eq 7, in which the second substrate (oxidized PES) reacts with reduced AADH after release of the first product (aldehyde). E_o is oxidized AADH, E_r is reduced AADH, A is the amine substrate, P is the corresponding aldehyde product, B is oxidized PES, and Q is reduced PES.

$$E_o + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_o{-}A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_r{-}P \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_r + P$$

$$E_r + B \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_r{-}B \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_o{-}Q \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_o + Q \qquad (7)$$

The reaction of reduced AADH with oxidized PES was examined by stopped-flow spectroscopy, as described under Experimental Procedures, and k_{obs} was measured at several

different PES concentrations. The absorbance changes at 483 nm with time, which were observed upon mixing AADH with PES, could be fit to a single-exponential decay. A systematic deviation was observed when the data obtained at different PES concentrations were fit to eq 5. However, these data fit quite well to eq 6. This plot of k_{obs} against the concentration of PES (data not shown) yielded the following fitted values: for the forward electron transfer from AADH to PES (k_3 in eq 4, k_9 in eq 7), 73 ± 5 s^{-1}; for the reverse reaction (k_{10}), 9 ± 4 s^{-1}; and for K_d (k_8/k_7), 217 ± 57 μM.

Calculation of the Rate of the Aldehyde Product Release Step from AADH. A rate equation (eq 8) for the ping-pong mechanism for AADH (eq 7) was derived by using the method of King and Altmann (1956). The rate constant for

$$k_{cat} = k_3k_5k_9k_{11}/[k_9k_{11}(k_3 + k_4 + k_5) + k_3k_5(k_9 + k_{10} + k_{11})] \quad (8)$$

the reverse reaction of the catalytic step (k_4) was assumed to be either zero or significantly less than k_5. It was also assumed that the value of the rate of release of reduced PES (k_{11}) was much greater than k_9 and k_{10}. This has been shown to be true for the analogous step in the reaction of PES with MADH.[2] Further justification for this assumption is the observation that when reduced AADH is mixed with oxidized PES and allowed to come to equilibrium, only partial oxidation of AADH occurs (data not shown). This is true even in the presence of a large excess of PES and suggests that the rate constant for the back reaction (k_{10}) is significant and comparable to k_9. Since the fitted value for k_{10} is relatively insignificant, the dissociation step for reduced PES (k_{11}) must be significantly faster than k_{10} or k_9. This simplified eq 8 to eq 9. Each of the variables in eq 9 has

$$k_{cat} = k_3k_5k_9/(k_3k_5 + k_3k_9 + k_5k_9) \quad (9)$$

been determined experimentally except for k_5, which is the rate of the release step for the aldehyde product. Values of k_{cat} were obtained from steady-state kinetic studies, k_3 values were obtained from stopped-flow experiments of the reactions of AADH with amine substrates, and k_9 was determined from stopped-flow experiments of the reaction of AADH with PES. From these data, it was possible to calculate the rates of product release for the aldehyde products, which correspond to each of the para-substituted phenylethylamines listed in Table 3. Comparison of the k_5 values in Table 3 with the steady-state k_{cat} values listed in Table 1 indicates that the aldehyde product release step is likely the most rate-limiting step in the overall oxidation−reduction reactions of AADH with substituted phenylethylamines and PES. The relatively small values for k_5 also support the conclusion that the irreversibility of the reactions of the amines with AADH is due to a relatively insignificant k_4 and not to rapid dissociation of aldehyde.

Structure−Reactivity Correlations. To examine the basis for the influence on the kinetic constants of the para substituents of the benzylamines and phenylethylamines, structure-correlation analyses were performed. Steady-state and transient kinetic parameters were correlated with substituent constants. Hammett plots were constructed in which

[2] V. L. Davidson, M. E. Graichen, and L. H. Jones, unpublished results.

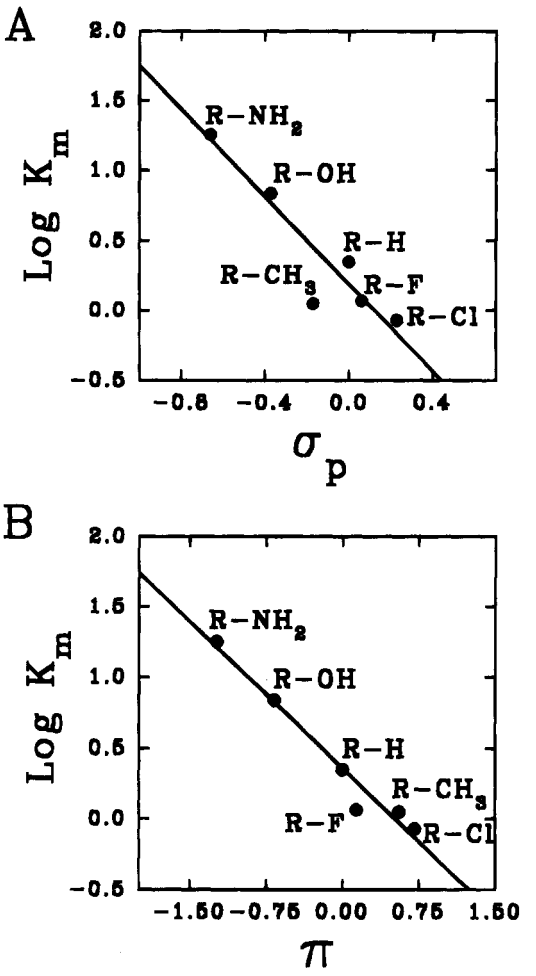

FIGURE 5: Hammett plots of log K_m versus σ_p (A) and π (B) for the reactions of AADH with *p*-substituted phenylethylamines. The correlation coefficients (r^2) and slopes are 0.85 and −1.56, respectively, in panel A and 0.96 and −0.7, respectively, in panel B.

the logarithm of each kinetic constant was plotted against different substituent constants for the series of substituted benzyl- and phenylethylamines. Those substituent effects that were examined are hydrophobic effects (π), steric effects (E_s), and electronic effects (σ_p), which were also factored into their component parts, field/inductive effects (F) and resonance effects (R). These substituent constants were obtained from Hansch and Leo (1979) and Hansch et al. (1991). 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 substituents 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 σ-bonds by polarization of those bonds (inductive). Unlike the resonance effect, the field/inductive effect is attenuated with distance. The F and R parameters that were used here are modified Swain−Lupton constants that have been adjusted to place them on the same scale as the Hammett constants (Hansch et al., 1991).

Hammett plots of log K_m for para-substituted phenylethylamines indicated that K_m was most strongly influenced by electronic and hydrophobic effects (Figure 5). The correlation coefficients (r^2) were 0.85 for the plot of log K_m against σ_p and 0.96 for the plot of log K_m against π. These plots

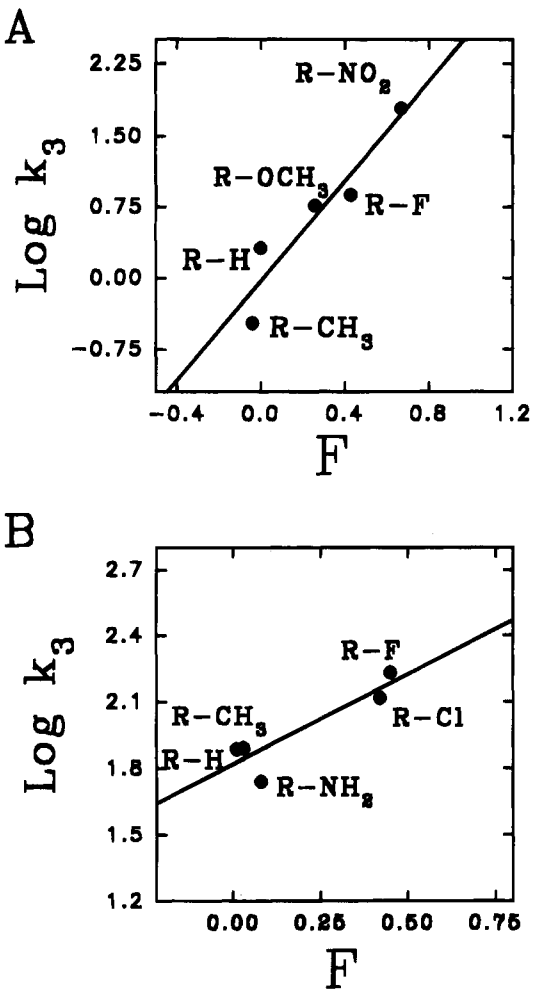

FIGURE 6: Hammett plots of log k_3 versus F for the reactions of AADH with p-substituted benzylamines (A) and phenylethylamines (B). Correlation coefficients (r^2) and slopes are 0.89 and +2.6, respectively, in panel A and 0.81 and +0.8, respectively, in panel B.

exhibited negative slopes of −1.5 for the dependence on σ_p and −0.7 for the dependence on π. This indicated that increased electron-withdrawing character and hydrophobicity of substituent groups of the substituted phenylethylamines decreased K_m. A Hammett plot of log K_m for aliphatic amines against log P, the octanol−water partition coefficient for that amine, which reflects the hydrophobicity of the entire molecule, also showed good correlation. The r^2 value was 0.81 and the slope was −0.51 (data not shown), further suggesting that increased hydrophobicity enhances the interaction of substrate amines with AADH. Values for k_5, the aldehyde product release step, also exhibited some correlation with hydrophobicity ($r^2 = 0.65$, slope = −0.71).

Strong correlations were revealed between field/inductive effects (F) for both para-substituted benzylamines ($r^2 = 0.89$) and phenylethylamines ($r^2 = 0.81$) with log k_3 (Figure 6). Linear regression analysis of plots of log k_3 versus F yielded slopes of +2.6 for benzylamines and +0.8 for phenylethylamines. Therefore, the more electron-withdrawing substituents increased the rate of reduction of TTQ in the enzyme−substrate complex. This suggests the formation of a carbanionic intermediate in this process. Field/inductive effects depend upon proximity and the number of bonds separating the reactive site of the molecule and the substituent group, and they become progressively attenuated with distance. The decrease in the magnitude of the slope of the plot of log k_3 versus F for the phenylethylamines relative to the benzylamines is, therefore, quite reasonable and expected since the substituent is further removed from the active site on the phenylethylamines. Good correlation was also observed between k_3 and hydrophobicity for benzylamines ($r^2 = 0.82$, slope = −2.4), but not for phenylethylamines ($r^2 = 0.38$, slope = 0.2). This means that increasing hydrophobicity decreases the rate of TTQ reduction by benzylamines, but has little effect on phenylethylamines. The possible significance of this will be discussed later.

DISCUSSION

A reaction mechanism for AADH with amine substrates is proposed in Figure 7. It is essentially identical to that proposed previously for MADH (Brooks et al., 1993). The reaction is initiated by nucleophilic attack by the amine nitrogen on one of the quinone carbonyls. This results in the formation of a carbinolamine intermediate, which loses water to form an imine intermediate. The kinetically determined K_d describes the two combined steps, leading to reversible imine formation. Next, an active site nucleophile abstracts a proton from the α-carbon, forming a carbanionic intermediate concomitant with the reduction of TTQ. This step is described by k_3. Release of the aldehyde product then occurs by hydrolysis of the newly formed imine bond between the methyl carbon and the amino group. This is essentially the reverse of the process of imine formation, and these two steps that result in the release of the aldehyde are described by k_5.

The positive slopes of the Hammett plots of log k_3 against field/inductive effects (F) for the reactions of AADH with substituted benzylamines and phenylethylamines support the proposed proton abstraction step in the reaction mechanism. The 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 that leads to carbanion formation. Strong correlations of k_3 with F imply that the ability of a substituent to reduce the negative charge density on the α-carbon is mediated primarily by through-space electrostatic interactions and polarization of the intervening bonds. These effects will be attenuated with distance, and this interpretation is consistent with the larger magnitude of the effect that was observed for benzylamines, relative to phenylethylamines in which one more methylene group separates the reacting group and the substituent. Very little correlation was observed between k_3 and resonance electronic effects (R).

Although K_d values could not be determined from transient kinetic data for the reactions of AADH with phenylethylamines (discussed earlier), it was possible to obtain K_m values from steady-state kinetic experiments. These K_m values were strongly correlated with hydrophobic effects. For this reaction mechanism, K_m is a term that comprises several rate constants that describe the overall oxidation−reduction reaction. It is also defined as the concentration of amine substrate that yields half-maximal activity with a saturating concentration of PES present. If one assumes that these K_m values reflect the affinity of the substrate for the enzyme, then these data would be consistent with the notion that the substrate binding site of AADH is relatively hydrophobic. This is also consistent with the good correlation between

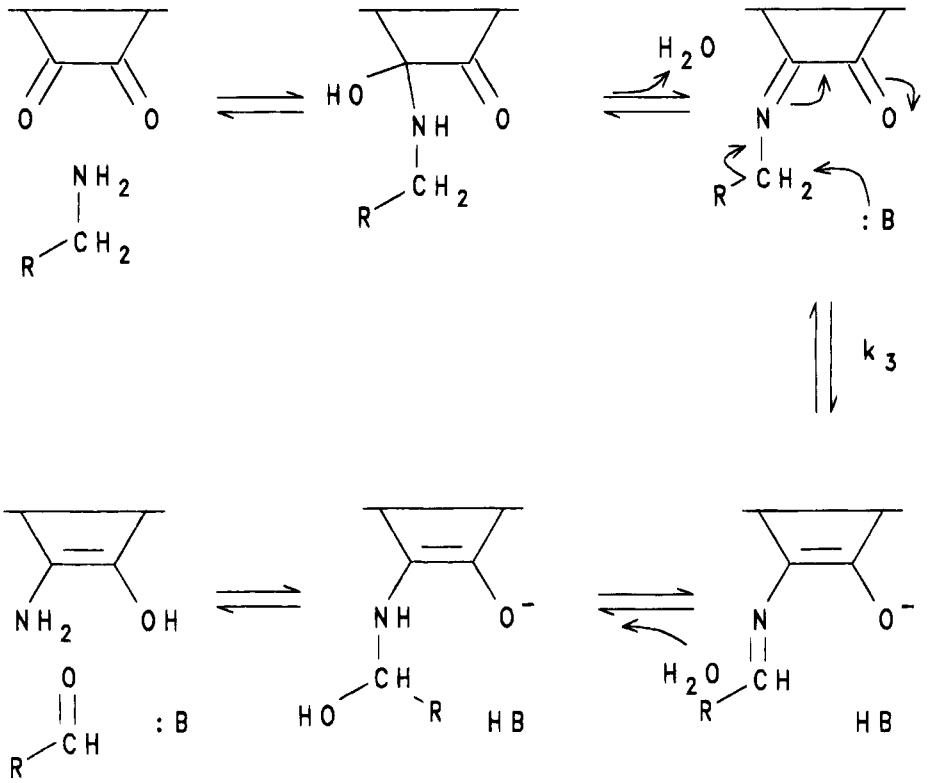

FIGURE 7: Proposed mechanism for the reductive half-reaction of AADH.

log P and K_m for aliphatic amines. Good correlation was also observed between K_m values and σ_p, with the electronic effects primarily arising from resonance effects. This suggests that resonance contributions from the benzene ring can either enhance nucleophilic attack by the substrate amine nitrogen or stabilize the imine intermediate by reducing k_2 relative to k_1. This is surprising because the phenyl ring is separated from the imine by two methylene groups, and therefore, such an effect would require hyperconjugation between the benzyl ring and nitrogen. It should be noted that the K_m may also reflect kinetic parameters that are related to TTQ reduction and subsequent reaction steps, and this may be influencing the observed correlation. The values of k_5, which describe the release of the aldehyde product, correlated most strongly with hydrophobicity. The observation that increasing hydrophobicity decreases the rate of product release is consistent with other data that suggest that the substrate binding site is hydrophobic.

A curious observation was that increasing hydrophobicity decreased k_3 for substituted benzylamines, but not for phenylethylamines. Kinetically determined K_d values for substituted benzylamines did not show strong correlations with hydrophobicity or any other substituent constant. The K_d values for benzylamines were, however, significantly higher than those for phenylethylamines, and the k_3 values were lower. The lack of any observed correlations for the K_d for benzylamines may be due to the fact that they do not fit well into a binding site that has evolved to specifically bind phenylethylamines. It is possible that the benzylamines bind to a hydrophobic cavity in such a way that does not provide an optimal orientation for interaction with the active site cofactor and catalytic base. Some movement within the active site after binding may be required, therefore, for the benzylamines to reduce TTQ. The more hydrophobic substituted benzylamines may be less able to achieve this reorientation. This could explain the correlations for benzylamines, but not for phenylethylamines, which fit perfectly into the active site. This also supports the notion that phenylethylamines, rather than benzylamines, are the physiologically relevant substrates for AADH.

It is of interest to compare the results of these structure–reactivity correlations for the reduction of AADH by benzylamines with analogous studies that have been performed with other quinoprotein amine oxidases and dehydrogenases. 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). Structure–reactivity correlation studies for the reactions of para-substituted benzylamines with plasma amine oxidase (Hartmann & Klinman, 1991) demonstrated good correlation, with a positive slope between k_3 and σ_p. These data supported the involvement of a carbanionic intermediate in the reaction mechanism of that enzyme. Studies of the reduction by benzylamines of MADH, the other known TTQ enzyme (Davidson et al., 1992), revealed strong correlation between k_3 and F with a positive slope, very much like what was observed in these studies with AADH. Thus, each of the enzymes appears to share a common mechanism for the reduction of the quinone cofactor within the enzyme–substrate complex.

A significant difference in the reaction mechanisms of the TTQ-dependent amine dehydrogenases and the quinoprotein amine oxidases appears to involve the release steps for the aldehyde product. With the TTQ enzymes, the presence of the benzyl ring directly attached to the α-carbon greatly impedes turnover of the enzyme in the steady state. It was previously speculated for MADH that this structural feature made the enzyme–product complex more resistant to hydrolysis. Consistent with this notion is the recent observation

that allylamine also efficiently reduces TTQ in MADH, but is not a substrate in the steady-state assay because of very slow product release.[2] The same appears to be true for AADH, which has a binding preference for aromatic amines but does not react well with benzylamines in the steady state. As indicated in this study, aldehyde release apparently is the rate-determining step in the steady-state reaction of AADH. Thus, it is also likely that slow product release from AADH is caused by the benzyl moiety attached to the α-carbon. In contrast to AADH, kinetic analysis of the reaction of plasma amine oxidase with amine substrates indicated that benzylamines and phenylethylamines reacted with comparable rates (Palcic & Klinman, 1983). These results raise the interesting question of why the extended conjugation should affect the release of product from enzyme-bound TTQ in a manner different from that of the enzyme-bound topa in the amine oxidases.

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