Kinetic and chemical mechanisms for the effects of univalent cations on the spectral properties of aromatic amine dehydrogenase

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Univalent cations and pH influence the UV–visible absorption spectrum of the tryptophan tryptophylquinone (TTQ) enzyme, aromatic amine dehydrogenase (AADH). Little spectral perturbation was observed when pH was varied in the absence of univalent cations. The addition of alkali metal univalent cations (K^+ , Na^+ , Li^+ , Rb^+ or Cs^+) to oxidized AADH caused significant changes in its absorption spectrum. The apparent K_d for each cation, determined from titrations of the spectral perturbation, decreased with increasing pH. Transient kinetic studies involving rapid mixing of AADH with cations and pH jump revealed that the rate of the cation-induced spectral changes initially decreased with increasing cation concentration to a minimum value, then increased with increasing cation concentration. A kinetic model was developed to fit these data, determine the true pH-independent K_d values for K^+ and Na^+ , and explain the pH-

dependence of the apparent K_d . A chemical reaction mechanism, based on the kinetic data, is presented in which the metallic univalent cation facilitates the chemical modification of the TTQ prosthetic group to form an hydroxide adduct which gives rise to the spectral change. Addition of NH_4^+/NH_3 to AADH caused changes in the absorption spectrum which were very different from those caused by addition of the metallic univalent cations. The kinetics of the reaction induced by addition of NH_4^+/NH_3 were also different, being simple saturation kinetics. Another reaction mechanism is proposed for the NH_4^+/NH_3 -induced spectral change that involves nucleophilic addition of the unprotonated NH_3 to TTQ. The general relevance of these data and models to the physiological reactions of TTQ-dependent enzymes and to the roles of univalent cations in modulating enzyme activity are discussed.

INTRODUCTION

Quinoprotein amine dehydrogenases are produced by some bacteria and allow them to grow on biological amines as a source of carbon and energy. These enzymes possess covalently bound tryptophan tryptophylquinone (TTQ) [1] (Figure 1) as a prosthetic group which participates in catalysis and electron transfer. Methylamine dehydrogenase (MADH) is produced by several methylotrophic and autotrophic bacteria and allows them to grow on methylamine as a sole source of carbon [2]. Aromatic amine dehydrogenase (AADH), which is most specific for phenylethylamines, is produced by *Alcaligenes faecalis* and allows growth on phenylethylamine as a carbon source. This enzyme, which was first identified by Iwaki et al. [3], has an $\alpha_2\beta_2$ structure with each β -subunit possessing one TTQ [4]. It catalyses the reaction shown in eqn. (1), where Φ is a benzene (phenyl) ring. In nature, the oxidant A in eqn. (1) is a blue copper protein, azurin [5,6].

TTQ in MADH is formed by a co- or post-translational modification of two tryptophan residues [7]. It is the chromophore responsible for the visible spectra of MADH and AADH. It has been reported that univalent cations (e.g. Li^+ , Na^+ , K^+ , Rb^+ , Cs^+) and ammonium influence the absorption of MADH [8,9]. The apparent K_d value for each cation was dependent on pH. Kuusk and McIntire [8] have proposed that metallic univalent cations, and ammonium and methylammonium, bind to two different sites in MADH. Resonance Raman spectroscopy studies of MADH by Moenne-Loccoz et al. [10] indicate that ammonium, methylammonium and Cs^+ interact directly with the carbonyl oxygen of TTQ at neutral pH, but that Na^+ does not. The exact mechanisms by which these univalent cations influence the

absorption spectrum of MADH and the mechanisms by which they interact with TTQ are not known.

In this paper we show that the TTQ-dependent AADH also exhibits univalent cation-induced spectral changes similar to, but in some respects different from, those reported for MADH. In addition to titrations of cation- and pH-induced spectral perturbations, transient kinetic studies were performed to investigate the kinetics of the cation- and pH-induced spectral changes. On the basis of these results, we propose a kinetic mechanism that allows the true pH-independent K_d value for cation binding to be determined. We also propose a chemical reaction mechanism that explains the basis for the influence of univalent cations on the absorption spectra of TTQ-dependent enzymes. Differences in the kinetic and chemical reaction mechanisms of NH_4^+ relative to the metallic cations are also characterized.

EXPERIMENTAL

Materials

Purification of AADH from *A. faecalis* (IFO 14479) was as described previously [4]. Fully oxidized AADH was prepared by incubation with $K_3[Fe(CN)_6]$. Protein concentrations were calculated from previously determined absorption coefficients [4]. H_2O (99.9%) was obtained from C/D/N Isotopes. All other reagents were obtained from either Sigma or J. T. Baker.

All experiments were performed in BisTris Propane buffer (BTP). Buffers of different pH were made by mixing solutions of pure BTP base and BTP plus HCl. Stock solutions of all salts, except ammonia, were prepared in 10 mM BTP buffer. Solutions of NH_4^+/NH_3 of different pH were made by mixing solutions of NH_4OH and NH_4Cl plus HCl.

Abbreviations used: TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase; AADH, aromatic amine dehydrogenase; BTP, BisTris propane {1,3-bis[tris(hydroxymethyl)amino]propane}; PQQ, pyrroloquinoline quinone.

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Figure 1 Structure of the TTQ prosthetic group

Spectral titrations

Absorption spectra were recorded using a Milton-Roy 3000 spectrophotometer. AADH was incubated at 25 °C with solutions of different pH containing different concentrations of the following salts: LiCl, NaCl, KCl, RbCl, CsCl and $\text{NH}_4^+/\text{NH}_3$. The A_{455}/A_{280} ratios for oxidized AADH were determined and used to quantify changes in the absorption spectra as a function of salt concentration and pH. Ratios of absorbance at other wavelengths were also determined as a control. For oxidized AADH analysis of changes in A_{334}/A_{280} yielded results statistically identical with those determined from changes in A_{455}/A_{280} . Data on the cation-dependent changes in absorbance were initially fitted to eqn. (2) to determine apparent K_d values

$$\Delta A = \frac{(\Delta A_{\text{max}})[\text{M}^+]}{K_d + [\text{M}^+]} \quad (2)$$

for each cation. $[\text{M}^+]$ is the concentration of cation and ΔA_{max} is the maximum change in absorbance caused by addition of saturating M^+ .

Transient kinetic experiments

An On-Line Instrument Systems (OLIS) RSM1000 rapid-scanning stopped-flow spectrophotometer was used for transient kinetic experiments. Data were either fitted by global kinetic and factor analysis using the OLIS software or by analysis of the change in absorbance with time at 455 nm. All data were best described by a single exponential and fitted to either eqn. (3a) or eqn. (3b)

$$A_{455} = Ce^{-k_{\text{obs}}t} + b \quad (3a)$$

$$A_{455} = C(1 - e^{-k_{\text{obs}}t}) + b \quad (3b)$$

where C is a constant related to the absorbance change and b represents an offset value to account for a non-zero baseline. Non-linear curve fitting of data was performed with OLIS software and Sigma Plot 5.0 (Jandel Scientific, San Raphael, CA, U.S.A.).

Kinetics of cation-induced spectral changes

Rapid mixing of AADH with different salts was carried out to determine the kinetics of the cation-induced spectral change. In

these experiments one syringe contained AADH (10 μM) in 10 mM BTP and the other syringe contained the salt in the same buffer at twice the desired final concentration.

Kinetics of pH-induced spectral changes

The pH of solutions containing AADH was rapidly changed by rapid mixing to determine the kinetics of the pH-induced spectral change. In experiments in which pH was rapidly changed, one syringe contained AADH (10 μM) in 5 mM BTP plus the desired concentration of a specific salt. The other syringe contained the same salt concentration in 100 mM BTP at a different pH. The final pH after mixing was determined by measuring the pH value of the reaction mixture. These pH-jump experiments were performed in both directions. Control experiments were performed to verify that the change in concentration of BTP alone did not cause any significant spectral perturbation.

Kinetic solvent isotope effects

To determine the extent of any deuterium kinetic solvent isotope effect on the pH-induced spectral change some of the reactions described above were repeated in buffered $^2\text{H}_2\text{O}$. All buffered $^2\text{H}_2\text{O}$ solutions were prepared as described by Schowen and Schowen [11]. Buffers of specific pD were prepared using a standard pH-meter soaked in $^2\text{H}_2\text{O}$, and correction was made for the effect of $^2\text{H}_2\text{O}$ on the electrode response. The value of pD was obtained by adding 0.4 to the observed pH in solutions in $^2\text{H}_2\text{O}$ [12]. With solutions that contained protein samples, H_2O was completely exchanged for $^2\text{H}_2\text{O}$ by repeated ultrafiltration using Amicon centripeps. After solvent exchange, these protein solutions were incubated for 8 h at 4 °C to ensure the complete exchange of all solvent-exposed titratable protonium ions for deuterium ions.

RESULTS

Effects of specific cations and pH on the absorption spectrum of AADH

Titration of oxidized AADH were performed in which spectra were recorded after incremental additions of Li^+ , Na^+ , Rb^+ , Cs^+ or $\text{NH}_4^+/\text{NH}_3$ at pH 9.0 (Figure 2). The spectral changes observed after addition of Li^+ , Na^+ , K^+ and Rb^+ are quite similar to each other (Figures 2A–2D). In each case, addition of cation causes a decrease in absorbance of the peak centred at 455 nm, appearance of a shoulder at approx. 340 nm and an isosbestic point at approx. 370 nm. The spectral changes observed after addition of $\text{NH}_4^+/\text{NH}_3$ (Figure 2F) are quite different. Addition of $\text{NH}_4^+/\text{NH}_3$ causes a decrease in A_{455} concomitant with a shift in the maximum of this peak to 410 nm. An isobestic point is maintained at approx. 410 nm. The spectral changes caused by addition of Cs^+ (Figure 2E) are also different, but more similar to those caused by the other metallic cations than to those induced by $\text{NH}_4^+/\text{NH}_3$. From the data shown in Figure 2 and similar titrations it was possible to determine, using eqn. (2), apparent K_d values associated with the spectral perturbations caused by each cation at pH 9.0.

These titrations were also performed at pH 7.0, 8.0 and 10.0 with Na^+ and K^+ . The titrations for Na^+ are shown in Figure 3. For the metallic cations, the apparent K_d varied with pH, decreasing substantially as pH was increased from 7.0 to 10.00 (Table 1). As discussed later, these apparent K_d values do not accurately describe cation binding but are complex terms that include the intrinsic K_d as well as other rate and equilibrium constants that reflect pH-dependent effects on the enzyme and cofactor.

Figure 2 Effects of univalent cations on the absorption spectrum of oxidized AADH

All titrations were performed in 10 mM BTP, pH 9.0, at 25 °C. After recording, each spectrum was normalized so that A_{280} was equal to 1.0. The arrows indicate the directions of the spectral changes. (A) LiCl was present at 0, 50, 100, 190, 370, 690 and 1200 mM; (B) NaCl was present at 0, 50, 100, 190, 370, 690, 1200, 1950 and 2800 mM; (C) KCl was present at 0, 40, 155, 570 and 1700 mM; (D) RbCl was present at 0, 10, 20, 40, 80, 155, 300, 570, 1000 and 1700 mM; (E) CsCl was present at 0, 50, 100, 190, 370, 690 and 1200 mM; (F) $\text{NH}_4^+/\text{NH}_3$ was present at 0, 5, 10, 40, 80, 155 and 570 mM.

The effects described above are specific for univalent cations. Addition of up to 250 mM Ca^{2+} at pH 9.0 had very little effect on the absorption spectrum of AADH (results not shown). Anions also have no significant effect on the absorption spectrum of AADH. The effect of using different potassium salts was investigated. Solutions were prepared that contained the chloride, nitrate, phosphate and acetate salts of potassium in the same K^+ concentration and pH. The spectral perturbations caused by the presence of each of these salts and apparent K_d values were essentially identical. These results demonstrate that the stable perturbation of the absorption spectrum of AADH is caused specifically by univalent cations.

Similar titrations were also performed at pH 7.0, 8.0, 9.0 and 10.0 with $\text{NH}_4^+/\text{NH}_3$ (Table 2). If one examines the apparent K_d values at different pH for $\text{NH}_4^+/\text{NH}_3$ and uses the total concentration of NH_4^+ plus NH_3 in solution, one observes a pattern for the pH-dependence of K_d values which is qualitatively similar to that of the metallic cations. However, taking into account that the $\text{p}K_a$ of ammonia is 9.24, it is possible to determine the exact concentrations of NH_4^+ and NH_3 present in solution. It has been shown [13] that, for a model compound of TTQ, the actual effector in solution is NH_3 . Spectral changes were observed with the model compound that were very similar to those shown in Figure 2(F). Those changes were attributed to formation of an iminoquinone by direct reaction with NH_3 . Our analysis (Table

2) reveals that, if one assumes that NH_3 , and not NH_4^+ is the reactive compound, then the K_d is essentially unchanged over the range of pH 7.0–10.0. These observations are consistent with NH_3 rather than NH_4^+ being responsible for the distinct spectral perturbations.

It appears that there are two different mechanisms by which the spectrum of TTQ is perturbed: one induced by metallic univalent cations (Li^+ , Na^+ , K^+ , Rb^+ , Cs^+) and another induced by $\text{NH}_4^+/\text{NH}_3$. Furthermore, for the latter it appears that the unprotonated NH_3 , not the cation, may be responsible for the spectral perturbations.

Kinetic analysis of metallic cation-induced spectral changes

To obtain information on the mechanism by which univalent cations induce spectral perturbations of AADH, the kinetics of the K^+ - and Na^+ -induced spectral changes were studied by stopped-flow spectroscopy. On mixing oxidized AADH with either cation, a monophasic change in absorbance with time was observed. These data were fitted to eqn. (3) to obtain k_{obs} . The variation in k_{obs} with K^+ concentration at different pH values is shown in Figure 4. The rate of the spectral change decreased with increasing cation concentration, to a minimum value, and then increased with increasing cation concentration. The variation in k_{obs} with Na^+ concentration exhibited a similar pattern (Figure

Figure 3 Effects of pH on the spectral changes caused by addition of NaCl to AADH

All titrations were performed in 10 mM BTP, at the indicated pH, at 25 °C. After the recording, each spectrum was normalized so that A_{280} was equal to 1.0. The arrows indicate the directions of the spectral changes. **(A)** pH 7.0; NaCl was present at 0, 240, 450, 830, 1400 and 2200 mM. **(B)** pH 8.0; NaCl was present at 0, 50, 100, 190, 370, 690, 1200 and 1950 mM. **(C)** pH 9.0; NaCl was present at 0, 50, 100, 190, 370, 690, 1200, 1950 and 2800 mM. **(D)** pH 10.0; NaCl was present at 0, 5, 10, 80, 150, 280, 510 and 1260 mM.

Table 1 Apparent and true dissociation constants for metallic univalent cation binding to AADH

The apparent K_d values were determined using eqn. (2). The pH-independent true dissociation constants described in Scheme 1 was determined using eqn. (5). Nd, not determined. Results are means $\pm$ S.E.M. ($n = 3-10$)

Cation	pH	Apparent K_d (mM)	K_{M^+} (mM)	K_{M^+}' (mM)
K ⁺	7.0	1200 $\pm$ 300	33.5 $\pm$ 4.1	0.286 $\pm$ 0.031
	8.0	1000 $\pm$ 100		
	9.0	140 $\pm$ 15		
	10.0	20 $\pm$ 1		
Na ⁺	7.0	910 $\pm$ 230	80.8 $\pm$ 19.2	3.99 $\pm$ 0.56
	8.0	880 $\pm$ 190		
	9.0	360 $\pm$ 22		
	10.0	52 $\pm$ 6		
Li ⁺	9.0	623 $\pm$ 129	Nd	Nd
Rb ⁺	9.0	222 $\pm$ 10	Nd	Nd
Cs ⁺	9.0	479 $\pm$ 98	Nd	Nd

5). This non-linear dependence of rate on concentration indicates that the spectral changes are not the result of a simple one-step cation-binding process.

Kinetic model for the reactions of AADH with metallic univalent cations

A kinetic model that describes the effects of metallic univalent cations and pH on the spectral properties of AADH is given in

Table 2 Apparent K_d values for NH_4^+/NH_3 binding to AADH

Values were calculated from the data shown in Figures 2 and 4 and similar titrations using eqn. (2) using either the total concentration of $NH_4^+ + NH_3$ or only the concentration of NH_3 which was calculated using a pK_a value of 9.24. Results are means $\pm$ S.E.M. ($n = 3-10$).

pH	K_d (mM)	
	$NH_4^+ + NH_3$	NH_3
7.0	2000 $\pm$ 470	11 $\pm$ 1
8.0	279 $\pm$ 15	15 $\pm$ 2
9.0	34 $\pm$ 2	12 $\pm$ 1
10.0	10 $\pm$ 1	10 $\pm$ 1

Scheme 1. This model accounts for the observed dependencies of the rates and extents of spectral perturbations on specific cations and $[H^+]$. According to this model, the spectral perturbations are caused not simply by cation binding, but by conversion of E into another enzyme form $[E^*]$ with distinct spectral properties. The presence of the metallic cation stabilizes E^* relative to E. E^* could represent a different conformation of the enzyme or, as discussed below, enzyme with a modified cofactor. This model assumes that some active-site group with a dissociable proton, when unprotonated, provides a ligand for the metallic cation (M). M-E represents AADH in the quinone form with a metallic cation bound at the active site. H-E represents AADH with this

Figure 4 Effects of pH and K^+ concentration on the rate of cation-induced spectral changes

All stopped-flow experiments were performed in 10 mM BTP, at the indicated pH, at 25 °C. K^+ concentrations are those after mixing. The solid lines are fits of the data to eqn. (5). ▽, pH 8.0; ■, pH 9.0; ○, pH 10.0.

Figure 5 Comparison of the effects of Na^+ and K^+ on the rate of the cation-induced spectral change

All stopped-flow experiments were performed in 10 mM BTP, pH 10.0, at 25 °C. Cation concentrations are those after mixing. The solid lines are fits of the data to eqn. (5). ○, K^+ ; ●, Na^+ .

Scheme 1

group protonated and therefore unable to serve as a ligand for M. E represents AADH with this group unprotonated but without M bound at the active site. K_a is the dissociation constant for the H–E proton. K_{M^+} is the dissociation constant for the M–E complex. Since this constant may be different for E and

E^* , it is designated K_{M^+}' for the latter. A rate equation for Scheme 1 is given in eqn. (4).

$$k_{\text{obs}} = \frac{k_1 K_{M^+} (1 + [H^+]/K_a) + k_2 [M]}{K_{M^+} (1 + [H^+]/K_a) + [M]} + \frac{k_{-1} K_{M^+}' (1 + [H^+]/K_a) + k_{-2} [M]}{K_{M^+}' (1 + [H^+]/K_a) + [M]} \quad (4)$$

The following assumptions were made in the derivation of eqn. (4). The binding and dissociation of M and H^+ to all enzyme forms were assumed to be in rapid equilibrium. All E forms are spectroscopically indistinguishable from each other and all E^* forms are spectroscopically indistinguishable from each other. According to this model $k_{-1} \gg k_1$ and $k_2 \gg k_{-2}$. This allows eqn. (4) to be simplified to eqn. (5).

$$k_{\text{obs}} = \frac{k_2 [M]}{K_{M^+} (1 + [H^+]/K_a) + [M]} + \frac{k_{-1} K_{M^+}' (1 + [H^+]/K_a)}{K_{M^+}' (1 + [H^+]/K_a) + [M]} \quad (5)$$

As seen in Figures 4 and 5, the experimental data can be fitted quite well to this model. It is also clear that the apparent K_d values determined from fits of the static spectral titrations (Table 1) are not true K_d values, but describe the overall equilibrium shown in Scheme 1. The values of K_{M^+} and K_{M^+}' for K^+ and Na^+ are listed in Table 1. Fits of the data also yielded a value of k_{-1} of $5.5 \pm 1.3 \text{ s}^{-1}$, which was independent of the cation, and values of k_2 of $2.05 \pm 0.31 \text{ s}^{-1}$ for K^+ and $1.84 \pm 0.22 \text{ s}^{-1}$ for Na^+ .

To be able to fit the data to eqn. (5), it was necessary to know the value of K_a . That value was obtained by fitting the titration data at different values of pH for Na^+ and K^+ , which was described in Figures 1 and 2, to eqn. (6).

$$A/A_0 = \frac{1 + C_1 \{[M]/(1 + [H^+]/K_a)\}}{1 + C_2 \{[M]/(1 + [H^+]/K_a)\}} \quad (6a)$$

$$C_1 = \frac{1/K_{M^+} + \alpha K_1/K_{M^+}'}{1 + \alpha K_1} \quad (6b)$$

$$C_2 = \frac{1/K_{M^+} + \alpha K_1/K_{M^+}}{1 + K_1} \quad (6c)$$

This equation was derived for Scheme 1 and describes the system after it has reached equilibrium. C_1 and C_2 are defined as composites of equilibrium constants, the values of which are independent of $[H^+]$ and $[M]$. Combining these constants [eqns. (6b) and (6c)] reduces the number of unknown variables in eqn. (6a) so that a reliable value of K_a may be obtained from the fit of the data. The term α is the ratio of the absorption coefficients for E^*/E at 455 nm and K_a is k_1/k_{-1} . It is assumed that all E forms exhibit the same spectral features and that all E^* forms exhibit the same spectral features. When the titration data at different values of pH for Na^+ and K^+ were fitted to eqn. (6), an identical value of $K_a = 5.0 (\pm 1.0) \times 10^{-11}$ ($pK_a = 10.3$) was obtained for the Na^+ titrations and the K^+ titrations. That the same K_a was obtained from the fits of each data set is consistent with the model in Scheme 1 in which the K_a value is expected to be independent of the cation. A value of $\alpha = 0.24 \pm 0.03$ was obtained for each cation.

Kinetic analysis of pH-induced spectral changes

The pH of solutions containing AADH was a fixed concentration of salt was rapidly changed by rapid mixing as described in the Experimental section. These pH-jump experiments were performed in both directions. Control experiments were performed to verify that the change in concentration of BTP alone did not cause any spectral perturbation. After the pH jump, a monophasic change in absorbance with time was observed. These data were fitted to eqn. (3) to obtain k_{obs} . These experiments were

Figure 6 Effects of K^+ concentration on pH-induced spectral changes of AADH

These pH-jump experiments were performed as described in the Experimental section. (A) In each experiment pH was changed from 9.9 to 8.3. (B) In each experiment pH was changed from 7.9 to 9.8. The solid lines are fits of the data to eqn. (5).

repeated in the presence of different concentrations of K^+ (Figure 6). When pH was decreased during the pH jump, k_{obs} decreased in the presence of increasing concentrations of K^+ (Figure 6A). When pH was increased during the pH jump, k_{obs} initially decreased in the presence of increasing concentrations of K^+ , then increased with increasing concentrations of K^+ (Figure 6B). These data could also be fitted very well to eqn. (5).

Kinetic solvent isotope effect

To investigate this process further, kinetic solvent isotope effect experiments were performed. The cation mixing experiment was repeated in buffered $^2\text{H}_2\text{O}$ and the rates were compared with those obtained in buffered H_2O . In these experiments, AADH in 10 mM BTP buffer was mixed with 200 and 400 mM KCl in the same buffer. At each salt concentration a kinetic solvent isotope effect of 3.0 ± 0.4 was observed. The magnitude of the kinetic solvent isotope effect is consistent with a H^+ -transfer step involving an exchangeable proton being a part of the reaction which causes the cation- and pH-dependent spectral perturbation. The H^+ which competes with M^+ for the proposed cation-binding site in Scheme 1 is in rapid equilibrium, and would not give rise to this effect. These data suggest that the slower conversions of E into E^* (k_1 and k_2 in Scheme 1) require the transfer of an exchangeable H^+ (discussed below).

Kinetic analysis of NH_3 -induced spectral changes

To obtain information on the mechanism by which $\text{NH}_4^+/\text{NH}_3$ induces spectral perturbations of AADH, the kinetics of the $\text{NH}_4^+/\text{NH}_3$ -induced spectral changes were studied by stopped-flow spectroscopy. On mixing oxidized AADH with $\text{NH}_4^+/\text{NH}_3$, a monophasic change in absorbance with time was observed. These data were fitted to eqn. (3) to obtain k_{obs} . In contrast with that observed with the metallic cations, the rate increased with increasing concentration, and saturation behaviour was observed (Figure 7). These data could be well fitted to the simple kinetic model [eqn. (7a)] and equation [eqn. (7b) [14]] given below

$$k_{\text{obs}} = \frac{k_3[\text{NH}_3]}{[\text{NH}_3] + K_d} + k_4 \quad (7b)$$

Figure 7 Effects of NH_3 concentration on the rate of the NH_3 -induced spectral change of AADH

All stopped-flow experiments were performed in 10 mM BTP, pH 9.0, at 25 °C. NH_3 concentrations are the concentration after mixing. The solid line represents a fit of the data to eqn. (7b).

where k_3 describes the rate of a reaction that occurs after binding in which AADH is modified to yield a form that is spectroscopically distinct from the native oxidized AADH. The dependence of k_{obs} on NH_3 concentration is totally different from that observed with K^+ and Na^+ (Figure 5). The fit of the data in Figure 7 to eqn. (7b) yielded values of $K_d = 3.9 \pm 0.3$ mM, $k_3 = 266 \pm 20$ s $^{-1}$ and $k_4 = 3.5 \pm 0.3$ s $^{-1}$.

DISCUSSION

The two known TTQ-dependent enzymes, MADH and AADH, exhibit many similarities with respect to catalytic mechanism and electron-transfer properties [5,15]. The effects of different univalent cations and pH on the spectral properties of MADH are well documented [8–10]. We demonstrate here that these similarities extend to the influence of univalent cations on the spectral properties of AADH. To gain insight into the precise cause of these spectral perturbations and the mechanism by which univalent cations induce these changes, we have performed a detailed kinetic analysis in addition to static spectral titrations.

Reactions of metallic cations with AADH

The features of the spectral perturbations which are caused by the addition of metallic cations to AADH (Figure 2) are very similar to those reported for MADH [8]. Differences are evident, however, in the apparent affinities of the metallic cations for each enzyme. The apparent K_d values determined here for AADH at high pH (Table 1) are in general greater than those previously reported for MADHs, and the specificity exhibited for K^+ at pH 9 is much more pronounced than was seen with MADH [8,9]. As discussed above, the apparent K_d for the metallic cations is actually a complex term which reflects more than cation binding (Scheme 1). These studies demonstrate that, to determine the true K_d for cation binding, transient kinetic analysis at different pH values is required. Analysis only of data from static titrations will give apparent and potentially misleading K_d values.

For metallic cations, the kinetic data indicate that we are not observing changes due to a simple binding phenomenon. The spectral perturbations do not appear to be due simply to electrostatic or proximity effects with TTQ. The kinetic data reveal that the cation-induced spectral changes to AADH are relatively slow (i.e. much less than diffusion controlled) and that the rate-limiting step in the process by which cations induce spectral perturbations involves the transfer of an exchangeable proton. On the basis of the results of spectral titrations, transient kinetic studies and kinetic solvent isotope effect experiments, we propose that the mechanism by which univalent cations induce spectral changes is that they facilitate covalent modification of TTQ to form an hydroxide adduct.

A chemical reaction mechanism for the hydration of enzyme-bound TTQ is shown in Scheme 2. It is proposed that the C-6 position of TTQ is the site of reaction. This has been shown to be the reactive carbonyl of TTQ in MADH [16] and TTQ model compounds [17]. R_1 represents the second indole ring of TTQ and R_2 is the polypeptide chain to which TTQ is linked at that point. B_1 and B_2 in Scheme 2 represent amino acid residues at the active site. One residue serves as a ligand for the univalent cation (M^+) when it is unprotonated. The other serves as a general base which activates water for nucleophilic attack of the C-6 carbonyl carbon of TTQ. The formation of an hydroxide adduct of TTQ in MADH at high pH has previously been demonstrated by resonance Raman spectroscopy [18].

Reactions of NH_3 with AADH

It is clear from the data that the spectral changes caused by addition of NH_4^+/NH_3 reflect a different process from that for the metallic cations. The difference in the features of the spectral perturbations suggest that a different product is formed and the kinetic results indicate a different type of reaction mechanism. The saturation kinetics (Figure 7) suggest a simple two-step process of rapid binding followed by a rate-limiting catalytic step. A possible reaction mechanism is given in Scheme 3. This mechanism requires that unprotonated NH_3 be the nucleophile

that reacts with TTQ. As discussed above in the Results section, if one assumes that NH_3 and not NH_4^+ is the reactive compound, then the K_d is essentially unchanged over the pH range 7.0–10.0.

It must be noted that for MADH from *Thiobacillus versutus*, resonance Raman spectroscopy studies [10] indicated that the influence of NH_4^+ was due to its proximity to TTQ and not covalent modification. Addition of NH_4^+ to *T. versutus* MADH caused a red shift of the absorption maximum of its UV-visible spectrum from 440 nm to 453 nm, which is similar to the change caused by addition of Cs^+ [19]. AADH and MADH are similar in that the spectral changes induced by NH_4^+/NH_3 are different from those induced by metallic cations. However, whereas NH_4^+/NH_3 was shown to cause a red shift in the absorption spectrum of oxidized MADH [8,9,18], it causes a blue shift in the absorption spectrum of oxidized AADH (Figure 2F). Itoh et al. [13] have shown that the absorption spectrum of a TTQ model compound exhibits a similar blue shift when it reacts with NH_3 to form an iminoquinone adduct. Thus NH_4^+/NH_3 may exert different effects on MADH and AADH, but for AADH the reaction mechanism shown in Scheme 3 is most consistent with the experimental data.

Significance

Alkali metal cations, because they are relatively inert with respect to chemical reactions, are often overlooked as potential participants in enzyme-catalysed reactions. Several enzymes are known to be activated or inhibited by univalent metal cations [20]. However, there is relatively little known about the nature of these cation-binding sites and the mechanisms of action of these univalent cations. There are only a few reports of the crystal structures of univalent-cation-dependent enzymes with their cation-binding sites identified. These include dialkylglycine decarboxylase [21], pyruvate kinase [22], fructose 1,6-bisphosphatase [23] and thrombin [24]. In the case of thrombin it is clear that Na^+ binding is related to allosteric properties of the enzyme [24]. For tryptophan synthase it has been shown that univalent cations control both the allosteric linkage between subunits and the equilibrium distribution of reaction intermediates [25,26]. For tryptophanase, it was proposed that univalent cations interacted at or near the active site and either participated directly in catalysis or were needed for proper alignment of functional groups [27]. It is still unclear to what extent univalent alkali metal cations play a direct role in enzyme-mediated catalysis, and by what mechanisms such univalent-cation-dependent catalysis may occur.

It has been shown for MADH that univalent cations affect the stability of the TTQ semiquinone [19]. Recent studies show that univalent cations enhance the rate of the proton-transfer reaction that gates the electron-transfer reaction from the substrate-reduced aminoquinol TTQ of MADH to amicyanin (G. R. Bishop and V. L. Davidson, unpublished work). The crystal

Scheme 2

Scheme 3

structure of AADH has not yet been determined. Crystal structures of MADH are available, but as yet no cation-binding site has been identified. The structure of MADH does, however, include several potentially electrophilic and negatively charged residues at the active site near the reactive C-6 carbonyl which could possibly bind cations near the TTQ cofactor [16]. (the 1.75 Å crystal structure of *Paracoccus denitrificans* MADH is available in the Brookhaven databank, entry 2BBK.) The data presented here for AADH and previously published work on MADH [8–10] strongly suggest a direct rather than an allosteric role for univalent cations at the active sites of TTQ-dependent enzymes.

The previous studies on the cation effects on the spectral properties of MADH [8–10] have raised questions as to the physiological relevance of these phenomena as the reported K_d values for Na^+ and K^+ at neutral pH were so large. An important finding of this study is that these apparent K_d values are misleading. For AADH, the true K_d values are actually much lower than the apparent K_d values (Table 1). These results demonstrate that the lack of a spectral change in AADH does not necessarily mean that cation is not bound at the active site (see Scheme 1). Univalent cation binding at the active site is quite possible at physiological pH in the presence of physiological concentrations of these cations. Furthermore the K_d value for the cation is different for the E and E* forms of the enzyme. This means that different redox forms of AADH and possibly different reaction intermediates may bind cation with different affinities and that the cation may preferentially stabilize different enzyme forms. This is quite consistent with observed effects of univalent cations on the reactivity of MADH ([28]; G. R. Bishop and V. L. Davidson, unpublished work) at concentrations of cation much lower than the previously reported K_d values obtained from spectral titrations.

It is interesting to note that another class of quinoproteins, pyrroloquinoline quinone (PQQ)-dependent dehydrogenases have a requirement for bivalent cations [29]. For methanol dehydrogenase it has been proposed that Ca^{2+} may facilitate formation of and stabilize a hemiketal reaction intermediate between the substrate alcohol and PQQ carbonyl. It has also been demonstrated *in vitro* that Ca^{2+} facilitates formation of an alcohol adduct with a PQQ model compound [30]. Thus the role of bivalent cations in the reaction mechanisms of PQQ-dependent enzymes bears at least some similarity to the mechanism shown in Scheme 2 for the role of univalent cations in facilitating hydration of TTQ in TTQ-dependent enzymes.

Further studies to identify and characterize roles for univalent cations in physiological reactions of AADH and MADH are in progress in this laboratory. The TTQ-dependent enzymes provide an excellent system for examining the mechanisms of enzyme-mediated proton-transfer reactions and the roles of univalent cations in influencing these reactions. The characterization of

these processes in AADH and MADH may reflect and be representative of a more universal role for univalent cations in enzymic processes.

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