Electron Transfer Reactions between Aromatic Amine Dehydrogenase and Azurin†

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ABSTRACT: Binding and electron transfer reactions between the tryptophan tryptophylquinone (TTQ) enzyme, aromatic amine dehydrogenase (AADH), and the type I copper protein azurin have been characterized. In steady-state kinetic assays using azurin as an electron acceptor, it was observed that the apparent K_m for azurin decreased with increasing ionic strength. These results are the opposite of what was observed for the reaction between the TTQ enzyme methylamine dehydrogenase (MADH) and amicyanin, despite the fact that in both cases the pairs of redox proteins are each acidic proteins. It was further demonstrated that azurin does not function as an effective electron acceptor for MADH, and that amicyanin does not function as an effective electron acceptor for AADH. Thus, while the two TTQ enzymes each use type I copper proteins as physiologic electron acceptors, there is a strong specificity for which copper protein serves as a redox partner. The kinetic parameters for the electron transfer reactions from reduced AADH to oxidized azurin were determined by stopped-flow spectroscopy. Different results were obtained depending upon whether AADH was reduced chemically with dithionite or with the substrate tyramine. The values for the limiting first-order apparent electron transfer rate constant (k_{ET}) at 30 °C were 4 and 102 s^{-1}, respectively. Kinetically determined values of K_d also differed by a factor of 2.4. These data suggest that the incorporation of the substrate-derived amino group into the reduced TTQ of AADH significantly increases the apparent k_{ET}. The interaction between AADH and azurin was also quantitated using an ultrafiltration binding assay. This yielded a K_d of 300 μM for the AADH−azurin complex. This K_d correlated well with the kinetically determined K_d values obtained from the stopped-flow kinetic studies. Similarities and differences in the reactivities of the AADH−azurin and MADH−amicyanin redox pairs are discussed.

Aromatic amine dehydrogenase (AADH)[1] from *Alcaligenes faecalis* catalyzes the oxidation of a wide range of primary amines to their corresponding aldehyde plus ammonia. The two electrons that are derived from the amine substrate are transferred to an electron acceptor, believed to be azurin (Edwards et al., 1995). 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, tryptophan tryptophylquinone (TTQ) (McIntire et al., 1991), which is involved both in catalysis and in subsequent electron transfer to its physiologic electron acceptor (Govindaraj et al., 1994). The 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.

Several parallels exist between AADH and MADH. Similarities in the catalytic mechanisms of oxidative deamination of their respective amine substrates have been deduced from steady-state and transient kinetic studies for the reductive half-reactions of AADH (Hyun & Davidson, 1995a) and MADH (Davidson et al., 1992; Brooks et al., 1993). These studies revealed a common mechanism for the reductive half-reaction of each in which reduction of TTQ in the enzyme−substrate complex is coupled to proton abstraction and formation of a carbanionic reaction intermediate. Furthermore, transient kinetic studies indicated that the proton abstraction steps for AADH (Hyun & Davidson, 1995b) and MADH (Brooks et al., 1993) each exhibit anomalously large deuterium kinetic isotope effects of similar magnitude. No structural information is yet available for AADH. However, the absorption spectra of the different redox states of AADH and the resonance Raman spectrum of AADH (Govindaraj et al., 1994) are nearly identical to those of MADH (Husain et al., 1987; Backes et al., 1991), suggesting that the TTQ cofactors occupy similar environments within AADH and MADH. A detailed comparison of circular dichroic spectra of AADH and MADH supported this conclusion and also indicated that both proteins share a very similar secondary structure (Edwards et al., 1995).

The similarities between AADH and MADH appear to extend to the mechanisms by which each is reoxidized *in vivo*. It is well established that in autotrophic and many methylotrophic bacteria (exceptions include *Methylophilus methylotrophus*) the physiologic electron acceptor for MADH is a type I blue copper protein, amicyanin (Husain & Davidson, 1985; Husain et al., 1986), which mediates electron transfer from TTQ to soluble *c*-type cytochromes (Husain & Davidson, 1986). Crystal structures of a binary complex of MADH and amicyanin (Chen et al., 1992) and a ternary complex of MADH, amicyanin, and cytochrome *c*-551i (Chen et al., 1994) from *Paracoccus denitrificans*

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[1] Abbreviations: AADH, aromatic amine dehydrogenase; k_{ET}, apparent electron transfer rate constant; MADH, methylamine dehydrogenase; TFA, trifluoroacetic acid; TTQ, tryptophan tryptophylquinone.

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have been reported. Detailed kinetic and thermodynamic analyses of the physiologic intermolecular electron transfer reaction between MADH and amicyanin have also been reported (Brooks & Davidson, 1994a,b). We have previously reported that a large amount of another type 1 blue copper protein, azurin, is produced by *A. faecalis* when grown under conditions which induce the production of AADH (Edwards et al., 1995). The amino acid sequence (Ambler, 1971) and crystal structure (Baker, 1988) of this azurin have been reported. Preliminary studies suggested that this azurin was a competent electron acceptor for AADH and that, like amicyanin, azurin was able to effectively mediate electron transfer from the dehydrogenase to several physiologic and nonphysiologic *c*-type cytochromes (Edwards et al., 1995).

In this paper, we present steady-state and stopped-flow kinetic analyses of the reactions of AADH with azurin. The association of AADH and azurin is also characterized by direct binding studies. These data are discussed in the context of the known structural and kinetic information on the MADH−amicyanin electron transfer complex, and the known structure of azurin.

EXPERIMENTAL PROCEDURES

Purifications of AADH (Govindaraj et al., 1994) and azurin (Ambler, 1971; Edwards et al., 1995) from *A. faecalis* (IFO 14479) were as described previously, and protein concentrations were calculated from previously determined extinction coefficients (Govindaraj et al., 1994; Rosen et al., 1981). Purifications of MADH (Davidson, 1990) and amicyanin (Husain & Davidson, 1985) from *P. denitrificans* (ATCC 13543) were as described previously, and protein concentrations were calculated from previously determined extinction coefficients (Husain & Davidson, 1985; Husain et al., 1987). The chemicals that were used in this study were obtained from either Aldrich or Sigma.

Steady-state kinetic assays for the reactions between AADH and azurin were performed at 30 °C in either 0.25 M or 0.01 M potassium phosphate, pH 7.5. The reaction mixture contained a fixed concentration of AADH of either 3 nM when using 0.25 M buffer or 7.3 nM when using 0.01 M buffer, and the concentration of azurin was varied. The reactions were initiated by the addition of a saturating concentration (100 μM) of tyramine. Initial velocities of the rate of reduction of azurin were determined from the rate of decrease in absorbance at 625 nm ($\Delta\epsilon = 4000\ M^{-1}\ cm^{-1}$). To determine steady-state kinetic parameters, initial rates were measured at different concentrations of azurin, and those data were fit to the Michaelis−Menten equation (eq 1).

$$v/[E_0] = k_{cat}[S]/(K_m + [S]) \quad (1)$$

Stopped-flow kinetic experiments were performed using an On-Line Instrument Systems (OLIS, Bogart GA) RSM1000 stopped-flow spectrophotometer. A 486-class computer controlled by OLIS software was used to collect and analyze the data. Experiments were carried out by mixing reduced AADH (2.5 μM) with an equal volume of varied concentrations of oxidized azurin at 30 °C in 0.25 M potassium phosphate, pH 7.5. Reduced AADH was prepared by the addition of either 2 molar equiv of tyramine or a few grains of sodium dithionite. The reactions of reduced AADH with oxidized azurin were monitored by the decrease in absorbance at 330 nm ($\Delta\epsilon = 37\,900\ M^{-1}\ cm^{-1}$), which corresponds to the conversion of AADH from the reduced to semiquinone form (Govindaraj et al., 1994). Under conditions where the varied reactant (azurin) was in large excess of the concentration of fixed reactant (AADH), the observed rate constant (k_{obs}) could be determined from data fit to the equation (eq 2) for a single-exponential decay, where C is a

$$A_{330} = Ce^{-kt} + b \quad (2)$$

constant related to the initial absorbance and b represents an offset value to account for a nonzero base line. At concentrations of azurin of 50 μM and greater, observed absorbance changes were monophasic, indicating that the reactions of reduced AADH with oxidized azurin were pseudo-first-order under these reaction conditions. At azurin concentrations less than 50 μM, the observed rates were biphasic, and these data were not used in subsequent analyses. The k_{obs} for each reaction was determined from the average of at least three measurements.

Direct binding studies of the interaction between AADH and azurin by ultrafiltration were performed according to Davidson et al. (1993) with modifications. Solutions containing the proteins were placed in the top compartment of a Centricon-100 (Amicon Inc., Beverly, MA) concentrator, which possessed a 100 000 molecular weight cutoff membrane, and were briefly centrifuged so that only a small amount of solution passed through the membrane. The concentration of each protein on either side of the ultrafiltration membrane was determined by HPLC analysis of the filtrate and retentate using a Vydac C_4 reverse-phase column. The conditions used to separate AADH from azurin were as follows. Solvent A contained 0.06% trifluoroacetic acid (TFA) in water, and solvent B contained 0.052% TFA in 80% acetonitrile in water. Proteins were eluted using a gradient of 75% A/25% B to 35% A/65% B over 26 min at a flow rate of 0.6 mL/min. The small subunit and large subunit of AADH eluted at approximately 16 and 23 min, respectively. The concentration of AADH was monitored by the absorbance of the large subunit at 278 nm, a maximum in this solvent system. Azurin eluted at approximately 20 min and was monitored at 278 nm, also a maximum in this solvent system. Proteins were quantitated by comparison of peak areas to standard curves generated by injection of known concentrations of pure proteins. Peak areas were linearly related to AADH and azurin concentrations covering the range of that used in the binding assay. Control experiments were performed to verify that the free azurin concentrations were identical on either side of the membrane in the absence of AADH. A plot of the concentration of azurin that was retained in the top compartment versus that in the filtrate was linear with a slope of 1.02 ± 0.02 (data not shown). Retention of AADH in the top compartment was greater than 85%.

Nonlinear curve fitting of data was performed with either OLIS software or the Sigma Plot 5.0 (Jandel Scientific, San Raphael, CA) computer program.

RESULTS

Steady-State Kinetic Studies. Steady-state kinetic assays were performed for the reactions of AADH with azurin at 30 °C in either 0.25 M or 0.01 M potassium phosphate,

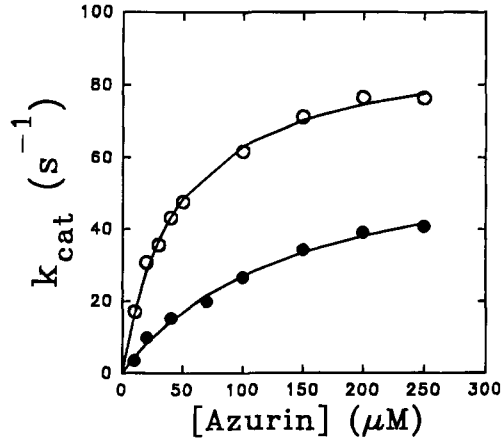

FIGURE 1: Initial rates of the tyramine-dependent reduction of azurin by AADH as a function of azurin concentration and ionic strength. Steady-state kinetic assays were performed as described under Experimental Procedures in 0.25 M potassium phosphate (○) and 0.01 M potassium phosphate (●). The solid lines represent the data fit to eq 1.

Table 1: Steady-State Kinetic Parameters for the Reaction of AADH with Azurin

	buffer concentration (M)	
kinetic parameter	0.01	0.25
k_{cat} (s^{-1})	64 ± 4	91 ± 2
K_m (μM)	137 ± 18	44 ± 3

pH 7.5. The initial rates of tyramine-dependent reduction of azurin by AADH were obtained at different concentrations of azurin in the range of 10−250 μM in the presence of saturating tyramine. Those data were fit to eq 1, and reasonably good fits were obtained (Figure 1). Steady-state kinetic constants that were obtained from these fits are listed in Table 1. The reaction was significantly more favorable at the higher ionic strength. This is due primarily to an approximate 3-fold decrease in the apparent K_m for azurin at the higher ionic strength. This suggests that electrostatic interactions destabilize the protein−protein association, and is consistent with the fact that AADH and azurin are each acidic proteins with pK_a values of 5.2 (Govindaraj et al., 1994) and <6 (Wherland & Pecht, 1978), respectively. These results are, however, in contrast to what was observed for the reaction between MADH and amicyanin from *P. denitrificans*, which is more favorable at lower ionic strength (Gray et al., 1988; Davidson & Jones, 1991), despite the fact that each of those proteins is also acidic.

Cross-Reactivity of Azurin and Amicyanin with AADH and MADH. Experiments were performed to determine the relative abilities of amicyanin to substitute for azurin as an electron acceptor for AADH, and of azurin to substitute for amicyanin as an electron acceptor for MADH. Oxidized azurin was incubated with the appropriate amine substrate in 0.1 M potassium phosphate, pH 7.5, and the reaction was initiated by the addition of a catalytic amount of either AADH or MADH. Whereas azurin was immediately reduced on addition of AADH, complete reduction of azurin by MADH required hours. Similarly, oxidized amicyanin was incubated with the appropriate amine substrate, under identical conditions, and the reaction was initiated by the addition of a catalytic amount of either AADH or MADH. Whereas amicyanin was immediately reduced on addition of MADH, complete reduction of amicyanin by AADH required hours (data not shown).

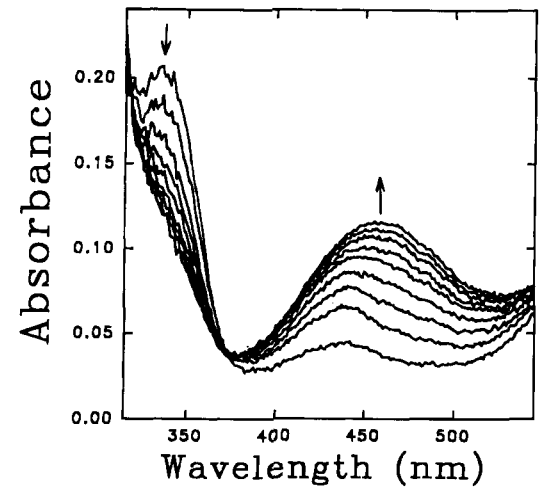

FIGURE 2: Rapid-scanning stopped-flow spectroscopy of the oxidation of AADH by azurin. The reaction was initiated by mixing tyramine-reduced AADH (2.5 μM) with oxidized azurin (26 μM) at 30 °C in 0.25 M potassium phosphate, pH 7.5. Spectra were recorded at 15 ms intervals. The arrows indicate the directions of the spectral changes with time.

Stopped-Flow Kinetic Studies. Because TTQ is a two-electron carrier and azurin is a one-electron carrier, it is assumed that the oxidative half-reaction of AADH proceeds through two sequential one-electron reductions as described by the simple scheme (eq 3), in which A_r, A_s, and A_o are,

$$A_r + Z_o \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A_r{-}Z_o \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A_s{-}Z_r \underset{k_6}{\overset{k_5}{\rightleftharpoons}} A_s + Z_r$$
$$A_s + Z_o \underset{k_8}{\overset{k_7}{\rightleftharpoons}} A_s{-}Z_o \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} A_o{-}Z_r \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} A_o + Z_r \qquad (3)$$

respectively, reduced, semiquinone, and oxidized AADH. Z_o and Z_r are, respectively, oxidized and reduced azurin. When oxidized azurin was mixed with either tyramine-reduced or dithionite-reduced AADH at 30 °C in 0.25 M potassium phosphate, pH 7.5, analysis of the spectral changes which occurred with time (Figure 2) revealed a monophasic conversion from reduced AADH to oxidized AADH with no accumulation of the semiquinone intermediate (A_s). This suggests that all reaction steps after k_3, including the reaction of semiquinone AADH with oxidized azurin (k_9), are much faster than k_3. This observation is consistent with the report that the oxidation of the dithionite-generated semiquinone of MADH by amicyanin was much faster than the oxidation of dithionite-reduced MADH by amicyanin (Brooks & Davidson, 1994b). In this scheme (eq 3), k_3 may be considered an apparent electron transfer rate constant k_{ET}, and the K_d for the protein−protein complex is equal to k_2/k_1.

The reactions of both tyramine-reduced and dithionite-reduced AADH with azurin were examined. The concentration of AADH was fixed at 2.5 μM, and concentrations of oxidized azurin were varied from 50 to 350 μM. It was not possible to obtain data at more saturating azurin because of the required high protein concentrations (>700 μM before mixing). Values of k_{obs} for each reaction were determined from the exponential rate of decrease in absorbance at 330 nm. Because k_3 was rate-limiting in this stopped-flow experiment, it was possible to analyze the concentration

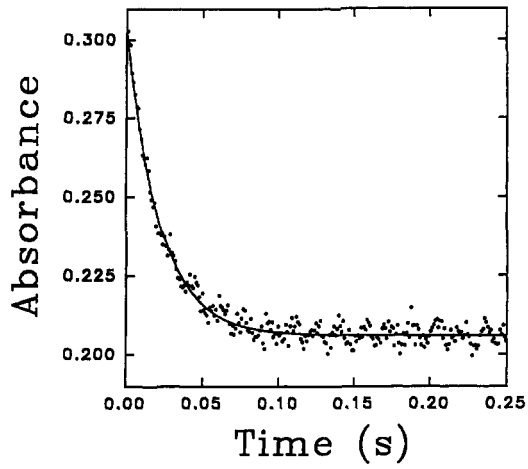

FIGURE 3: Kinetic analysis of the oxidation of AADH by azurin monitored at a single wavelength. The reaction was initiated by mixing tyramine-reduced AADH (2.5 μM) with oxidized azurin (200 μM) at 30 °C in 0.25 M potassium phosphate, pH 7.5. The change in absorbance was monitored at 330 nm. The solid line represents a fit of the data to a single-exponential decay (eq 2), which yielded a k_{obs} of 46 s^{-1} for this reaction.

dependence of k_{obs} according to eq 4 (Strickland et al., 1975).

$$k_{obs} = \frac{k_3[\text{azurin}]}{[\text{azurin}] + K_d} + k_4 \quad (4)$$

For all reactions, the absorbance changes at 330 nm with time could be fit to a single-exponential decay as shown in Figure 3. The direct plot of k_{obs} against azurin concentration (Figure 4) exhibited a hyperbolic concentration dependence, indicating a two-step process in which an initial binding step equilibrates much faster than the following electron transfer step. This will be true for many cases where complex formation of the reacting proteins occurs in fast diffusion-controlled process followed by a slow rate-determining electron transfer step. For the reactions of both substrate-reduced and dithionite-reduced AADH, the plots of k_{obs} versus the concentration of azurin passed through the origin. This indicates that k_4 is either zero or much less than k_5 in eq 3. The values for the apparent k_{ET} (k_3) and K_d values for the each reaction were obtained from fits of these data to eq 4 and are listed in Table 2. Interestingly, the kinetic parameters for the reactions of substrate-reduced and dithionite-reduced AADH with azurin are quite different. The significance of this observation is discussed later.

Direct Binding Assays. An ultrafiltration binding assay was used as a direct method for determining the equilibrium binding constant between AADH and azurin. Variable concentrations of azurin were incubated with AADH and separated by ultrafiltration. When analyzing protein binding data, it was assumed that the concentration of azurin passing through the ultrafiltration membrane represented the free protein, and that the concentration retained by the membrane represented free azurin plus azurin in complex with AADH. Data were fit to eq 5 (Figure 5), which describes ligand

$$[\text{bound azurin}] = \frac{C[\text{free azurin}]}{[\text{free azurin}] + K_d} \quad (5)$$

binding to a single class of sites. No improvement in the fit of the data was obtained by assuming more complicated kinetic models which included two nonidentical binding sites or cooperativity. A specific capacity (moles of azurin bound per moles of TTQ present) of 1.00 ± 0.22 was calculated by dividing the measured capacity (C) for azurin by the AADH concentration. Analysis of these data yielded a K_d of 300 ± 50 μM. It should be noted that this K_d describes the binding of oxidized azurin to oxidized AADH, whereas the kinetically determined K_d describes the binding of oxidized azurin to reduced AADH. The K_d value which was obtained in the direct binding assay correlates reasonably well with the K_d values obtained from the stopped-flow kinetic studies. This is important because it supports the validity of the kinetic models used to analyze the transient kinetic data and the kinetically determined K_d values. The K_d for the interaction of AADH and azurin is significantly higher than that reported for MADH and amicyanin of 4.5 μM (Davidson et al., 1993) but is not necessarily unreasonably high for a physiologic reaction between redox proteins. For example, binding studies of the interaction of methanol dehydrogenase and its physiologic electron acceptor, cytochrome *c*-551i, from *P. denitrificans* yielded a K_d of 380 μM (Harris & Davidson, 1993).

DISCUSSION

Differences in Reactivity of Dithionite-Reduced and Substrate-Reduced AADH. Significant differences in the apparent k_{ET} and K_d were observed for the reactions of azurin with dithionite-reduced and substrate-reduced AADH under identical reaction conditions. Reduction of AADH chemically with dithionite will generate the quinol form of TTQ. Apparently, reduction by tyramine must yield a chemically distinct species. With the other known TTQ enzyme, MADH, it has been shown that on reduction of TTQ by methylamine, formaldehyde is released but the amino group remains bound to the reduced cofactor (Eady & Large, 1971). Thus, for MADH, reduction by substrate yields an aminoquinol form of TTQ in which one of the carbonyl oxygens is replaced by the substrate-derived amino group (Figure 6). It has also been shown for MADH that the amino group remains bound after the one-electron oxidation to yield an aminosemiquinone (Warncke et al., 1993) and that loss of the amino group occurs during the second one-electron oxidation to regenerate the quinone. Previous studies have indicated that the reductive half-reaction of AADH proceeds essentially as does that of MADH (Hyun & Davidson, 1995a). The dramatic differences in the observed kinetic parameters for the reactions of dithionite-reduced and substrate-reduced AADH with azurin support the conclusion that two structurally different forms of reduced TTQ are generated by reduction by dithionite and tyramine, respectively. It is reasonable to assume that, as with MADH, the tyramine-reduced form of AADH contains an aminoquinol form of TTQ. These data indicate that the incorporation of the substrate-derived amino group into reduced TTQ significantly increases the apparent k_{ET} and also affects the K_d. Significant differences have also recently been observed in the apparent k_{ET}s for the reactions of dithionite-reduced and substrate-reduced MADH with amicyanin.[2] In the MADH–amicyanin complex, amicyanin binds to MADH at a site at which the edge of the phenyl ring of the unmodified tryptophan moiety of TTQ is exposed at the protein surface

[2] Bishop & Davidson (1995).

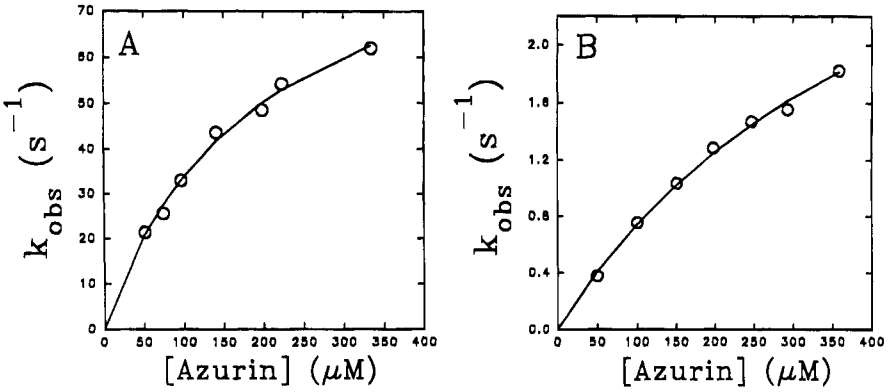

FIGURE 4: Plots of k_{obs} against azurin concentration for the reactions of (A) tyramine-reduced AADH and (B) dithionite-reduced AADH with oxidized azurin. All experiments were performed at 30 °C in 0.25 M potassium phosphate, pH 7.5. The solid lines represent fits of each set of data to eq 4.

Table 2: Stopped-Flow Kinetic Constants for the Reactions of Azurin with AADH

kinetic parameter	tyramine-reduced AADH	dithionite-reduced AADH
k_3 (s^{-1})	102 ± 9	4.3 ± 0.5
K_d (μM)	203 ± 31	490 ± 80

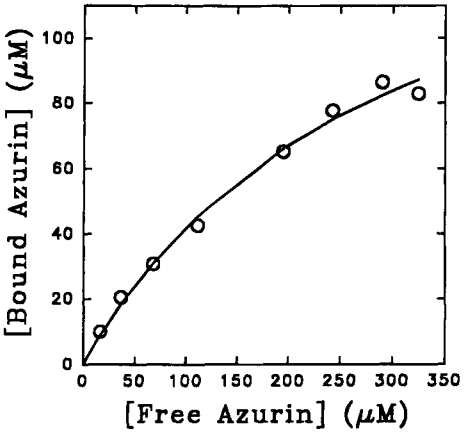

FIGURE 5: Binding of azurin to AADH. Binding was assayed in 0.25 M potassium phosphate, pH 7.5, at room temperature as described under Experimental Procedures. In these experiments, the oxidized forms of each protein were used. The solid line represents the fit of the data to eq 5.

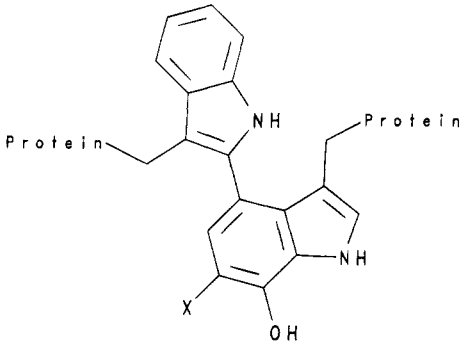

FIGURE 6: Structure of reduced TTQ. The group (X) which is bound to the reactive C-6 carbon will be either a hydroxyl group when TTQ is reduced by dithionite or an amino group when TTQ is reduced by the amine substrate.

(Chen et al., 1992, 1994). This portion of the TTQ is far removed from the carbonyl function at the active site (see Figure 6). Electron transfer from TTQ in MADH to amicyanin is believed to occur from this unmodified tryptophan moiety (Brooks & Davidson, 1994a). Assuming a similar situation exists for the AADH−azurin complex, these results raise an interesting question of how the modification of TTQ at the active site by substrate influences the binding and electron transfer reactions of AADH with azurin.

Specificity of TTQ Enzymes for Their Redox Partners. Given the large body of available structural and kinetic information for MADH and its physiologic electron acceptor, amicyanin, it is worthwhile to determine to what extent the reactions between AADH and azurin are similar and different. In the present paper, we have characterized some interesting differences in the reactivities of azurin and amicyanin with their respective quinoprotein dehydrogenases. Steady-state kinetic analyses for the reactions of AADH and azurin were performed at different ionic strengths. The results indicated that the apparent K_m for azurin decreased with increasing ionic strength. For this reaction mechanism, K_m is a term which is comprised of several rate constants that describe the overall oxidation−reduction reaction. It is also defined as the concentration of azurin which yields half-maximal activity with saturating concentration of tyramine. If one assumes that these K_m values reflect the affinity of azurin for AADH, then these data would be consistent with the knowledge that azurin and AADH are proteins of like charge. These results are, however, in striking contrast to what was observed for the reaction between MADH and amicyanin. The reaction between those proteins was optimal at low ionic strength and decreased with increasing ionic strength (Gray et al., 1988; Davidson & Jones, 1991). These data suggest that the combinations of factors which influence the functional associations of azurin and AADH, and amicyanin and MADH, are different and specific for each pair of redox partners. This is confirmed by the observations that azurin does not function as an effective electron acceptor for MADH, and that amicyanin does not function as an effective electron acceptor for AADH. The findings are also consistent with the previous observation that plastocyanin could not replace amicyanin as an effective electron acceptor for MADH (Gray et al., 1988).

The different ionic strength dependence for the reactions of AADH−azurin and MADH−amicyanin requires some speculation as to what structural features of the reacting proteins may account for these differences. Crystal structures have been reported for the complex of MADH and amicyanin from *P. denitrificans* (Chen et al., 1992, 1994), for free amicyanin (Durley et al., 1993), and for azurin from

Alcaligenes denitrificans (Baker, 1988), which has recently been reclassified as *A. faecalis*. A crystal structure of AADH is not yet available. However, spectroscopic analyses suggest that AADH and MADH have similar secondary structures and that the environments of the TTQ cofactors are similar (Govindaraj et al., 1994; Edwards et al., 1995). Azurin and amicyanin are representative of a class of small type I "blue" copper proteins, also referred to as cupredoxins (Adman, 1991). These proteins contain a single copper which is coordinated to two histidines, a cysteine, and a methionine. These are soluble proteins which mediate electron transfer between other soluble redox proteins. The overall protein topologies of amicyanin and azurin are very similar (Durley et al., 1993). One of the most important common features of azurin and amicyanin, as well as other type I copper proteins, is the hydrophobic patch surrounding the exposed histidine ligand on the northern surface of the molecule which is considered of great importance as a potential electron transfer site. The crystal structure of the MADH−amicyanin complex indicates amicyanin binds to MADH at this site and that hydrophobic interactions predominate at the interface of these two proteins (Chen et al., 1992, 1994). One and possibly a second potential salt bridge, however, are also observed at the interface between the proteins on the periphery of the hydrophobic interface. Formation of these salt bridges may account for the observed ionic strength dependence of the reaction between MADH and amicyanin which suggests an importance of electrostatic interactions. The ionic strength dependence of the reaction of AADH with azurin suggests that hydrophobic interactions are primarily stabilizing the protein−protein association. It is possible that the interface between these two proteins may also be comprised of primarily hydrophobic residues, but lack the potential to form salt bridges as seen in the MADH−amicyanin structure. Further experiments and additional structural information will, of course, be necessary to fully understand the factors which dictate the specificity of the interactions of AADH and MADH with their respective copper proteins.

Conclusions. It is particularly noteworthy that the two known TTQ enzymes, AADH and MADH, appear to preferentially utilize type I copper proteins as physiologic electron acceptors. While these cupredoxins are widely distributed in bacteria, their specific physiologic roles have not been well documented (Adman, 1991). The interaction with AADH represents one of the few well-characterized physiologic functions for an azurin. Despite the significant conservation of structure between azurin and amicyanin, there is strong specificity for which copper protein serves as a redox partner for each TTQ enzyme. Furthermore, chemical changes at the active site of AADH exert a marked effect on the electron transfer reaction with azurin. Further characterization of the AADH−azurin system and comparison with results obtained for the MADH−amicyanin system should provide important information on the factors which govern the specificity of interactions between soluble redox proteins and regulate the observed rates of long-range intermolecular electron transfer reactions.

REFERENCES

Adman, E. T. (1991) *Adv. Protein Chem. 42*, 145−197.

Ambler, R. P. (1971) in *Recent Developments in the Chemical Study of Protein Structures* (Previero, A., Pechere, J.-F., & Colletti-Previero, M. A., Eds.) pp 289−303, INSERM, Paris.

Backes, G., Davidson, V. L., Huitema, F., Duine, J. A., & Sanders-Loehr, J. (1991) *Biochemistry 30*, 9201−9210.

Baker, E. N. (1988) *J. Mol. Biol. 203*, 1071−1095.

Bishop, G. R., & Davidson, V. L. (1995) *Biochemistry* (in press).

Brooks, H. B., & Davidson, V. L. (1994a) *Biochemistry 33*, 5696−5701.

Brooks, H. B., & Davidson, V. L. (1994b) *J. Am. Chem. Soc. 116*, 11201−11202.

Brooks, H. B., Jones, L. H., & Davidson, V. L. (1993) *Biochemistry 32*, 2725−2729.

Chen, L., Durley, R., Poliks, B. J., Hamada, K., Chen, Z., Mathews, F. S., Davidson, V. L., Satow, Y., Huizinga, E., Vellieux, F. M. D., & Hol, W. G. (1992) *Biochemistry 31*, 4959−4964.

Chen, L., Durley, R., Mathews, F. S., & Davidson, V. L. (1994) *Science 264*, 86−90.

Davidson, V. L. (1990) *Methods Enzymol. 188*, 241−246.

Davidson, V. L. (1993) in *Principles and Applications of Quinoproteins* (Davidson, V. L., Ed.) pp 73−95, Marcel Dekker, New York.

Davidson, V. L., & Jones, L. H. (1991) *Anal. Chim. Acta 249*, 235−240.

Davidson, V. L., Jones, L. H., & Graichen, M. E. (1992) *Biochemistry 31*, 3385−3390.

Davidson, V. L., Graichen, M. E., & Jones, L. H. (1993) *Biochim. Biophys. Acta 1144*, 39−45.

Durley, R., Chen, L., Lim, L. W., Mathews, F. S., & Davidson, V. L. (1993) *Protein Sci. 2*, 739−752.

Eady, R. J., & Large, P. J. (1971) *Biochem. J. 123*, 757−771.

Edwards, S. L., Davidson, V. L., Hyun, Y.-L., & Wingfield, P. T. (1995) *J. Biol. Chem. 270*, 4293−4298.

Govindaraj, S., Eisenstein, E., Jones, L. H., Sanders-Loehr, J., Chistoserdov, A. Y., Davidson, V. L., & Edwards, S. L. (1994) *J. Bacteriol. 176*, 2922−2929.

Gray, K. A., Davidson, V. L., & Knaff, D. B. (1988) *J. Biol. Chem. 263*, 13987−13990.

Harris, T. K., & Davidson, V. L. (1993) *Biochemistry 32*, 14145−14150.

Husain, M., & Davidson, V. L. (1985) *J. Biol. Chem. 260*, 14626−14629.

Husain, M., & Davidson, V. L. (1986) *J. Biol. Chem. 261*, 8577−8580.

Husain, M., Davidson, V. L., & Smith, A. J. (1986) *Biochemistry 25*, 2431−2436.

Husain, M., Davidson, V. L., Gray, K. A., & Knaff, D. B. (1987) *Biochemistry 26*, 4139−4143.

Hyun, Y.-L., & Davidson, V. L. (1995a) *Biochemistry 34*, 816−823.

Hyun, Y.-L., & Davidson, V. L. (1995b) *Biochim. Biophys. Acta 1251*, 198−200.

McIntire, W. S., Wemmer, D. E., Christoserdov, A. Y., & Lidstrom, M. E. (1991) *Science 252*, 817−824.

Rosen, P., Segal, M., & Pecht, I. (1981) *Eur. J. Biochem. 120*, 339−344.

Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048−4052.

Warncke, K., Brooks, H. B., Babcock, G. T., Davidson, V. L., & McCracken, J. L. (1993) *J. Am. Chem. Soc. 115*, 6864−6865.

Wherland, S., & Pecht, I. (1978) *Biochemistry 17*, 2585−2591.

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