Factors Which Stabilize the Methylamine Dehydrogenase−Amicyanin Electron Transfer Protein Complex Revealed by Site-Directed Mutagenesis†

Victor L. Davidson,*,‡ Limei H. Jones,‡ M. Elizabeth Graichen,‡ F. Scott Mathews,§ and Jonathan P. Hosler‡

Department of Biochemistry, The University of Mississippi Medical Center, Jackson, Mississippi 39216-4505, and Department of Biochemistry & Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Received June 6, 1997; Revised Manuscript Received July 25, 1997⊗

ABSTRACT: Methylamine dehydrogenase (MADH) and amicyanin form a physiologic complex within which electrons are transferred from the tryptophan tryptophylquinone (TTQ) cofactor of MADH to the type 1 copper of amicyanin. Interactions responsible for complex formation may be inferred from the crystal structures of complexes of these proteins. Site-directed mutagenesis has been performed to probe the roles of specific amino acid residues of amicyanin in stabilizing the MADH−amicyanin complex and determining the observed ionic strength dependence of complex formation. Conversion of Phe^{97} to Glu severely disrupted binding, establishing the importance of hydrophobic interactions involving this residue. Conversion of Arg^{99} to either Asp or to Leu increased the K_d for complex formation by 2 orders of magnitude at low ionic strength, establishing the importance of ionic interactions which were inferred from the crystal structure involving Arg^{99}. Conversion of Lys^{68} to Ala did not disrupt binding at low ionic strength, but it did greatly diminish the observed ionic strength dependence of complex formation that is seen with wild-type amicyanin. These results demonstrate that the physiologic interaction between MADH and amicyanin is stabilized by a combination of ionic and van der Waals interactions and that individual amino acid residues on the protein surface are able to dictate specific interactions between these soluble redox proteins. These results also indicate that the orientation of MADH and amicyanin when they react with each other in solution is the same as the orientation of the proteins which is seen in the structure of the crystallized protein complex.

The quinoprotein methylamine dehydrogenase (MADH)[1] and a type I copper protein, amicyanin, form a physiologically relevant complex in which intermolecular electron transfer occurs. MADH is a periplasmic enzyme which has been purified from several Gram-negative bacteria (Davidson, 1993) and which catalyzes the oxidation of methylamine to formaldehyde and ammonia. As a prosthetic group, it possesses tryptophan tryptophylquinone (TTQ) (McIntire et al., 1991). In *Paracoccus denitrificans*, amicyanin is an obligatory mediator of electron transfer from MADH to soluble *c*-type cytochromes (Husain & Davidson, 1986). Each protein is induced in this bacterium during growth on methylamine as a carbon source (Husain & Davidson, 1985, 1987). The amicyanin gene is located immediately downstream of that for MADH, and inactivation of the former by gene replacement resulted in loss of the ability to grow on methylamine (van Spanning et al., 1990). *In vivo*, it appears that alternative cytochromes *c* may accept electrons from amicyanin (de Gier et al., 1995). *In vitro*, cytochrome *c*-551i is the most efficient acceptor for the amicyanin-mediated transfer of electrons from MADH. MADH, amicyanin, and cytochrome *c*-551i are isolated as individual soluble proteins, but it was demonstrated in this laboratory that they must form a ternary complex to catalyze methylamine-dependent cytochrome *c*-551i reduction (Husain & Davidson, 1986; Gray et al., 1986, 1988; Davidson & Jones, 1991, 1995). Although it is a thermodynamically favorable reaction, MADH does not reduce cytochrome *c*-551i in the absence of amicyanin (Husain & Davidson, 1986), probably because the proteins are unable to interact in a productive manner. Reduced amicyanin will not significantly reduce oxidized cytochrome in the absence of MADH (Davidson & Jones, 1995). This is because the redox potential of free amicyanin is much more positive than that of the cytochrome. The potential of amicyanin shifts on complex formation with MADH to facilitate the reaction (Gray et al., 1986). The requirement for amicyanin is quite specific. Other structurally similar type I copper proteins, plastocyanin and azurin, do not effectively substitute for amicyanin (Gray et al., 1988; Hyun & Davidson, 1995).

The specific interaction between MADH and amicyanin has been characterized by absorption spectroscopy (Gray et al., 1988), potentiometric studies (Gray et al., 1988), steady-state kinetics (Davidson & Jones, 1991; Brooks et al., 1993), transient kinetics (Brooks & Davidson, 1994a,b; Bishop & Davidson, 1996; Bishop et al., 1996), direct binding assays (Davidson et al., 1993), chemical cross-linking (Kumar & Davidson, 1990), resonance Raman spectroscopy (Backes et al., 1991), and X-ray crystallography (Chen et al., 1992, 1994). The results of these studies raise important questions concerning the relative roles of electrostatic and hydrophobic interactions in stabilizing the functional association between these two proteins. Kinetic, binding, and spectroscopic studies indicated that complex formation was favored at low

† This work was supported by NIH Grant GM-41574 (V.L.D.) and NSF Grant MCB-9419899 (F.S.M.).

* Corresponding author. Telephone: 601-984-1515. Fax: 601-984-1501. E-mail: davidson@fiona.umsmed.edu.

‡ The University of Mississippi Medical Center.

§ Washington University School of Medicine.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; *mau,* methylamine utilization.

S0006-2960(97)01353-6 CCC: $14.00

ionic strength (10 mM buffer) over high ionic strength (10 mM buffer plus 0.2 M NaCl). However, chemical cross-linking studies suggested that both hydrophobic and electrostatic forces were involved in complex formation. A binary complex of MADH and amicyanin (Chen et al., 1992) and a ternary protein complex of these proteins plus cytochrome *c*-551i (Chen et al., 1994) have been crystallized and their structures determined. The ability of MADH to oxidize methylamine and then transfer electrons to cytochrome *c*-551i via amicyanin in these crystals has been demonstrated by single crystal polarized absorption microspectrophotometry (Merli et al., 1996).

The structures of the crystallized complexes of these proteins indicate that the interface between the two proteins is stabilized largely by hydrophobic interactions, despite the presence of some charged and neutral hydrophilic residues in addition to hydrophobic residues (Chen et al., 1992, 1994). One and possibly a second intermolecular salt bridge at the periphery of this hydrophobic interface may also be inferred from the structural data. The actual contributions of these hydrophobic and electrostatic interactions can only be ascertained by probing the structure by site-directed mutagenesis. To better understand the precise mechanism by which this specific protein−protein association is stabilized, we have used site-directed mutagenesis to alter specific amino acid residues of amicyanin which appear from the crystal structure to be important for functional association with MADH. Mutagenesis of certain specific individual amino acid residues caused dramatic decreases in binding affinity and in some cases altered the ionic strength dependence of complex formation which is observed with wild-type amicyanin. These results demonstrate that a combination of hydrophobic and ionic interactions is required to stabilize complex formation and that individual amino acid residues on the protein surface are able to dictate very specific interactions between soluble redox proteins.

EXPERIMENTAL PROCEDURES

Protein Purification. MADH was purified from *P. denitrificans* as previously described (Davidson, 1990). Recombinant amicyanin was purified from *Escherichia coli* using the same methods as described previously for purification of the native protein from *P. denitrificans* (Husain & Davidson, 1985). Protein concentrations were calculated from the known extinction coefficients for MADH (Husain et al., 1987) and amicyanin (Husain & Davidson, 1985). Reduced MADH was prepared by titration with sodium hydrosulfite (dithionite). All reagents were obtained from commercial sources.

Expression of the Amicyanin Gene Product in E. coli. A 555 bp fragment from the methylamine utilization (*mau*) gene cluster of *P. denitrificans* (Chistoserdov et al., 1992) was obtained from Dr. A. Y. Chistoserdov (SUNY, Stony Brook, NY). This fragment was present in pUC18 (pAYC170a). A *Hin*dIII/*Eco*RI fragment from pAYC170a was cloned into pUC19 in order to allow transcription of *mau*C from the pUC *lac* promoter. This fragment includes the region which encodes the signal sequence for export of amicyanin to the periplasm. The resulting plasmid, pMEG201, was transformed into *E. coli* BL21 (DE3) (Novagen) for expression of amicyanin.

Cells were grown in Luria broth containing 100 μg/mL ampicillin and 100 μM Cu^{2+} at 30 °C. Growth of cells at 30 °C produced higher yields of amicyanin than growth at 37 °C. Synthesis of amicyanin via the *lac* promoter site on pMEG201 was induced by the addition of 300 μM isopropyl β-D-thiogalactopyranoside. Cells were harvested 4 h after induction. Fractionation of cells after induction and analysis of cell fractions revealed that the expressed amicyanin was exported to the periplasm and that the signal sequence was cleaved to produce a protein of the correct molecular weight. The amount of amicyanin that was isolated depended upon the copper concentration in the growth medium; 100 μM Cu^{2+} was optimal.

Amicyanin was purified from the periplasmic fraction of *E. coli* by methods essentially identical to those used to fractionate *P. denitrificans* and isolate amicyanin from the periplasmic fraction (Husain & Davidson, 1985; Davidson, 1990). Fractionation of the cells was accomplished by treatment with lysozyme followed by a mild osmotic shock. Amicyanin was purified from this fraction by ion-exchange chromatography over DE-52 (Whatman). While some mutations affected the conditions under which amicyanin eluted from the column, in all cases it was possible to obtain pure protein using this procedure. Yields of wild-type and mutant amicyanins ranged from 10 to 15 mg/L of culture. The kinetic properties of wild-type recombinant amicyanin were verified in steady-state and transient kinetic assays with MADH and cytochrome *c*-551i. The binding and catalytic rate constants observed for recombinant amicyanin are the same as those of the native protein.

Site-Directed Mutagenesis of the Amicyanin Gene. Site-directed mutagenesis was performed on double-stranded pMEG201 using two mutagenic primers with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotide sequences used to construct the site-directed mutants are as follows: R99D, 5′-CATCCCTTCATGGACGGCAAGGTC-3′; R99L, 5′-CATCCCTTCATGCTCGGCAAGGTCGTC-3′; K68A, 5′-GAGGCGGCGTTGGCGGGCCCGATGATG-3′; M71R, 5′-GAAAGGCCCGAGGATGAAGAAGGAG-3′; F97E, 5′-CACTGCACCCCGCATCCCGAGATGCGCGGCAAG-3′. The underlined bases are those which were changed to create the desired change in the amino acid sequence. The sequence of the *mau*C gene from *P. denitrificans* was reported by Van Spanning et al. (1990). In each case, the change in the nucleotide sequence also generated a new restriction site which was used to facilitate screening for the mutation. Mutations were confirmed by dideoxy DNA sequencing of the double-stranded plasmids using the Sequenase Quick-Denature Plasmid Sequencing Kit (Amersham). For each mutation, the entire 555 bp *mau*C-containing fragment was sequenced to ensure that no second site mutations were present, and none were found.

Kinetic Studies. Steady-state kinetic experiments with MADH and amicyanin as an electron acceptor were performed as described previously (Brooks et al., 1993). The assay mixture contained 16 nM MADH and varied concentrations of amicyanin in 10 mM potassium phosphate, pH 7.5, with or without 200 mM KCl. The reaction was initiated by the addition of substrate, and activity was monitored by the change in absorbance caused by the reduction of amicyanin at 595 nm. Assays were performed at 30 °C. Data were fit to eq 1.

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

An On-Line Instrument Systems (OLIS) RSM1000 stopped-flow spectrophotometer was used for transient kinetic experiments. Reactions were monitored at 443 nm, which is an isosbestic point for the semiquinone and oxidized forms of MADH and is where MADH exhibits a $\Delta\epsilon$ of 26 200 M^{-1} cm^{-1} on conversion from reduced to semiquinone (Husain et al., 1987). For each set of mixing experiments, the concentration of MADH was fixed between 0.5 and 2 μM, and the amicyanin concentration was varied. The amicyanin concentration was always in at least 10-fold excess of MADH to maintain pseudo-first-order reaction conditions. Values of k_{obs} were determined from the fits of the raw data to equations describing a single exponential rise (eq 2).

$$A_{443} = C(1 - e^{-kt}) + b \quad (2)$$

$$A_{443} = C_1(1 - e^{-k_1 t}) + C_2(1 - e^{-k_2 t}) + b \quad (3)$$

In the case of the R99D mutant, biphasic kinetics were observed and those data were best fit to eq 3. For reactions of this mutant protein, typically greater than 90% of the total amplitude change associated with the reaction could be attributed to one of the rate constants. Subsequent analysis of k_{obs} for reactions of R99D used the rate constant that was associated with the much larger amplitude. The reason for the biphasic kinetics, which are exhibited only by this mutant, is under further study.

In all reactions, saturation behavior was observed, and data were fit according to the model (eq 4) and the equation (eq

$$\text{MADH}_{red} + \text{amicyanin}_{ox} \overset{K_d}{\rightleftharpoons} \text{MADH}_{red}/\text{amicyanin}_{ox} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} \text{MADH}_{semi}/\text{amicyanin}_{red} \quad (4)$$

$$k_{obs} = \frac{k_3[\text{amicyanin}_{ox}]}{[\text{amicyanin}_{ox}] + K_d} + k_4 \quad (5)$$

5; Strickland et al., 1975) given below. The three kinetic parameters are directly determined from the nonlinear fit of the hyperbolic curve which describes the dependence of k_{obs} on amicyanin concentration. The maximum, concentration-independent, value of k_{obs} is equal to $k_3 + k_4$, and k_4 will equal the *y*-intercept of the curve. K_d is the amicyanin concentration which corresponds to the midpoint of the hyperbola. Nonlinear curve fitting of data was performed with OLIS software and the Enzfitter computer program.

RESULTS

Rationale for Site-Directed Mutagenesis Experiments. The crystal structure of the MADH−amicyanin complex (Chen et al., 1992) reveals that the site on amicyanin that interacts with MADH is the region which surrounds His^{95}, the surface-exposed histidine ligand for the type 1 copper. His^{95} is at the center of a hydrophobic patch of amino acid residues that includes Met^{71}, Met^{51}, Met^{28}, Pro^{52}, Pro^{94}, Pro^{96}, and Phe^{97} (Figure 1). The protein−protein interface with MADH appears to primarily involve hydrophobic (van der Waals) interactions mediated by some of these residues (Figure 2). For example, the side chains of Met^{71} and Phe^{97} are seen in the crystal structure of the complex to be close enough ($\leq$3.5 Å) to make van der Waals contact with residues of MADH. Met^{71} interacts with Thr^{54} of the MADH small subunit. Phe^{97} interacts with Arg^{184} of the MADH small subunit and Pro^{100} of the MADH large subunit. In addition, two possible electrostatic interactions are indicated at the periphery of the hydrophobic interface (Figure 2). The side chain N of Arg^{99}

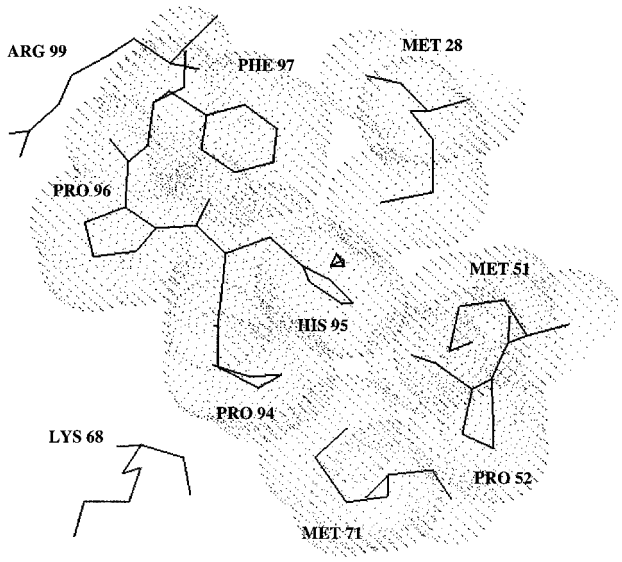

FIGURE 1: Hydrophobic surface domain surrounding the copper site of amicyanin. The surface-exposed His^{95} of amicyanin which serves as a copper ligand is surrounded by seven hydrophobic residues, Met^{71}, Met^{51}, Met^{28}, Pro^{52}, Pro^{94}, Pro^{96}, and Phe^{97}. Van der Waals radii of these residues are shown as dots. Arg^{99} and Lys^{68} are located on the periphery of this hydrophobic patch. Coordinates are available in the Brookhaven Protein Data Bank, entry 2MTA.

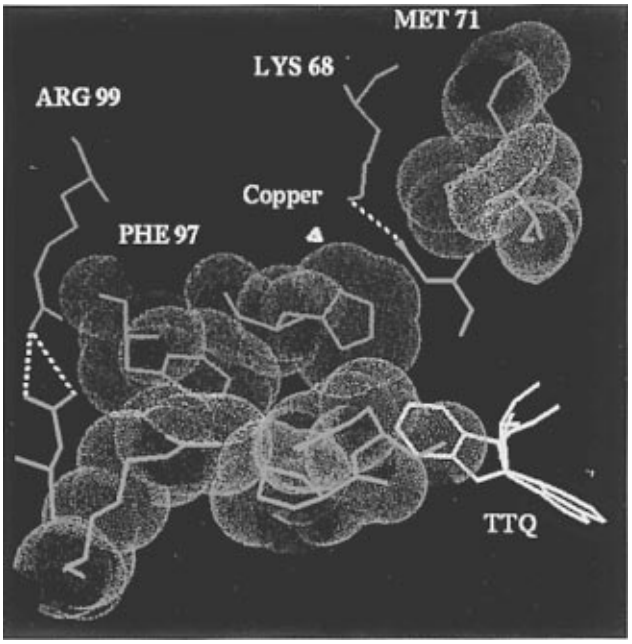

FIGURE 2: Amicyanin−MADH interface. Van der Waals radii of His^{95}, Met^{71}, and Phe^{97} of amicyanin and MADH residues with which they make close contact are shown as dots. Possible ionic interactions between Arg^{99} and Lys^{68} of amicyanin and carboxylic residues of MADH are shown as yellow dashed lines. Amicyanin residues are colored blue and MADH residues are colored red. Met^{71} interacts with Thr^{54} of the MADH small subunit. Phe^{97} interacts with Arg^{184} of the MADH small subunit and Pro^{100} of the MADH large subunit. The side chain N of Arg^{99} of amicyanin and side chain O of Asp^{167} of the MADH large subunit are separated by 2.7 Å. The side chain N of Lys^{68} of amicyanin and side chain O of Asp^{115} of the MADH small subunit are separated by 4.4 Å. The copper of amicyanin and TTQ of MADH are in yellow. Coordinates are available in the Brookhaven Protein Data Bank, entry 2MTA.

Table 1: Effects of Mutagenesis of Specific Amino Acid Residues on Complex Formation between Amicyanin and Methylamine Dehydrogenase

	K_m (μM)[a]		K_d (μM)[b]	
amicyanin	no added KCl[c]	+0.2 M KCl	no added KCl	+0.2 M KCl
wild type[d]	1	8	<5	15
R99D	76	64	90	179
R99L	31	59	59	158
K68A	3	7	<5	5
R99L/K68A	98	60	85	107
M71R	2	23	<5	22
F97E	151	ND[e]	228	ND

[a] Determined using eq 1 from steady-state assays with amicyanin as the terminal electron acceptor. [b] Determined from the analysis of the variation in k_{obs} with amicyanin concentration in pre-steady-state kinetic studies, using eq 5. [c] Values for recombinant wild-type amicyanin from *E. coli* and native amicyanin from *P. denitrificans* are essentially identical. [d] It was not possible to determine K_d values <5 μM because the rate was already maximal at 5 μM, which is the lowest concentration of amicyanin that could be used and still maintain psuedo-first-order reaction conditions under which eq 5 is valid (i.e., [amicyanin] >> [MADH]). [e] ND, not determined.

of amicyanin and side chain O of Asp[167] of the MADH large subunit are separated by only about 2.7 Å. This strongly suggests that these two residues participate in an intermolecular salt bridge. The side chain N of Lys[68] of amicyanin and side chain O of Asp[115] of the MADH small subunit are separated by about 4.4 Å. This suggests the possibility of electrostatic interactions between these two residues. To examine the importance of individual amino acid residues in stabilizing complex formation between amicyanin and MADH, site-directed mutants were prepared in which Arg[99], Lys[68], Met[71], and Phe[97] were changed to other amino acids.

Kinetic Analyses. The relative affinities of MADH for wild-type and mutant amicyanins were determined in kinetic experiments. K_m values were determined using a steady-state enzyme assay in which amicyanin was the terminal electron acceptor in the assay of methylamine-dependent reduction of amicyanin by MADH. K_d values were determined from pre-steady-state stopped-flow studies in which reduced MADH was mixed with oxidized amicyanin. K_m is a term which is comprised of several rate constants that describe the overall oxidation−reduction reaction. It is operationally defined as the concentration of amicyanin which yields half-maximal activity with a saturating concentration of methylamine and a catalytic concentration of MADH. Since K_m is a composite rate constant, it is not surprising that the K_m values do not exactly match the K_d values (Table 1). However, the trends shown in Table 1 are similar for variation in K_m and K_d for the mutant amicyanins. This indicates that the changes in K_m values, like the K_d values, reflect variations in the affinity of mutant amicyanins for MADH. It should also be noted that it was not possible to determine K_d values less than 5 μM because 5 μM is the lowest concentration of amicyanin that could be used and still maintain the psuedo-first-order reaction conditions under which eq 5 is valid (i.e., [amicyanin] >> [MADH]). For some of the mutant amicyanins, the first-order rate constant was maximal and concentration-independent at [amicyanin] $\geq$ 5 μM, so a K_d lower than this value could not be determined.

Analysis of Site-Directed Mutants of Arg[99] and Lys[68]. Arg[99] was changed to Asp and to Leu. Each of these mutations significantly affected complex formation with MADH. The steady-state K_m values and kinetically determined K_d values for each mutant were much greater than for wild type (Table 1). This was true under both the low salt and high salt reaction conditions. The R99L and R99D mutants each exhibited a small salt dependence that is qualitatively similar to what is observed with wild-type amicyanin, an increase in K_m and K_d at the higher ionic strength. However, the ionic strength dependence is less pronounced than that observed with the wild type.

Lys[68] was changed to Ala. The K_m values for the K68A mutant were comparable to those of wild-type amicyanin. The K_d value for the K68A mutant was comparable to that of wild-type amicyanin at the lower ionic strength (i.e., both K_d values are less than 5 μM), but in contrast to what is seen with the wild type, the K_d of K68A did not increase appreciably at the higher ionic strength. Thus, Lys[68] does not appear to be required for binding, but it may play a role in determining the ionic strength dependence of complex formation.

A double mutant in which Arg[99] was changed to Leu and Lys[68] was changed to Ala was also constructed and analyzed. The R99L/K68A mutant exhibited K_m and K_d values which were much greater than wild type and similar to those for the R99L and R99D single mutants. The ionic strength dependence that is apparent in the wild type was not observed in the R99L/K68A mutant. The approximate 3-fold increases in K_m at low ionic strength for the double mutant relative to the single R99L mutant, and K68A relative to wild type, suggest that the K68A mutation may have a small effect on binding at low ionic strength. This effect cannot be discerned from the K_d values for wild type and K68A, which were each below our limit for reliable detection of 5 μM. The small increase in K_d for the double mutant relative to the single R99L mutant at low ionic strength is also consistent with this idea. This indicates that the ionic interaction involving Lys[68] may contribute to stabilization of the complex at low ionic strength but to a much smaller degree than the ionic interaction involving Arg[99]. This conclusion is consistent with the structural data which suggest a strong ionic interaction involving Arg[99] (r = 2.7 Å) but a much weaker ionic interaction involving Lys[68] (r = 4.4 Å).

Analysis of Site-Directed Mutants of Phe[97] and Met[71]. Phe[97] was changed to Glu. This mutation significantly affected complex formation with MADH. The steady-state K_m values and kinetically determined K_d values for the F97E mutant were much greater than those for wild type and the largest of any of the mutants analyzed in this study (Table 1). In fact, the K_d value of 228 μM should be considered a minimum value because it was not possible to achieve high enough concentrations of this mutant amicyanin to saturate MADH in pre-steady-state studies. These results confirm the importance of this hydrophobic residue in stabilizing complex formation between amicyanin and MADH.

Met[71] was changed to Arg. This mutation appeared to have little effect on complex formation with MADH despite the inferred role of hydrophobic interactions involving Met[71] (Figure 2). The steady-state K_m values and kinetically determined K_d values for the M71R mutant were only slightly greater than for wild type (Table 1). A likely explanation for this is that the long aliphatic side chain of Arg is able to replace the side chain of Met[71] at the hydrophobic interface and still position the guanidinium group on the periphery of

Table 2: Comparison of Amino Acid Sequences of Amicyanins and Plastocyanin[a]

T. versutus amicyanin[b]	68 F**R**GE**M**MTKD 76	95 PHP− −**F**M**R**GKV 103
P. denitrificans amicyanin[c]	67 L**K**GP**M**MKKE 75	94 PHP− −**F**M**R**GKV 102
plastocyanin[d]	59 E**E**DL**L**NAPG 67	86 PHQGA**G**M**V**GKV 96

[a] Only relevant portions of the amino acid sequences are displayed. Alignments are based on the complete sequences of each protein as reported in the cited references. [b] Taken from van Beeuman et al. (1991). [c] Taken from van Spanning et al. (1990). [d] This is a concensus sequence of plastocyanin based on the sequences of 25 proteins (van Beeuman et al., 1991).

the hydrophobic interface. Inspection of the structure of the wild-type complex reveals that this is possible.

DISCUSSION

While much has been learned about the mechanisms which stabilize complex formation between tightly associated proteins (e.g., antibody−antigen and ligand−receptor interactions), relatively little is known about the factors which govern specific recognition and stabilization of relatively weakly associated protein complexes. In the case of MADH and amicyanin, specific recognition and efficient complex formation are critical to their physiologic function. The electrons derived from methylamine oxidation by MADH must be transferred to the respiratory chain via amicyanin in order for the cell to be able to grow on this substrate. It is critical that MADH does not accidentally donate electrons to the wrong redox protein. This study provides a picture of how this specific complex formation between MADH and amicyanin occurs.

Interactions between redox proteins are usually thought of as being either hydrophobic or electrostatic in nature. The MADH−amicyanin interaction indicates that the factors which stabilize protein−protein interactions are not necessarily that simple. Both hydrophobic and electrostatic interactions are important. These mutagenesis studies indicate that alteration of Arg^{99} results in a significant weakening of binding, particularly at low ionic strength. Thus, our results show that the ionic interprotein interactions involving this residue, which may be inferred from the crystal structure (Figure 2), are important for stabilizing the complex. The results of mutagenesis of Phe^{97} demonstrate that the hydrophobic interactions involving this residue are also very important.

We propose the following model for complex formation between MADH and amicyanin. The initial recognition and interaction between MADH and amicyanin is strongly influenced by Arg^{99} of amicyanin and an Asp residue of MADH. This initial electrostatic interaction could then allow the hydrophobic surface areas which surround the copper site on amicyanin and the exposed edge of TTQ on MADH to orient opposite each other. Once this occurs, the hydrophobic surfaces are able to interact to form the stable complex. A novel feature of this model is that ionic and electrostatic interactions serve to facilitate nearby hydrophobic protein−protein interactions. Intuitively, this may seem counterproductive. However, it is likely that this feature provides a large degree of the specificity of the interaction.

In considering what dictates the specificity of MADH for amicyanin, it is noteworthy that azurin and plastocyanin have been previously reported to be poor substitutes for amicyanin (Gray et al., 1988; Hyun & Davidson, 1995). Each of these other type 1 copper proteins has an analogous hydrophobic surface domain surrounding the copper site. Comparison of the amino acid sequences of *P. denitrificans* amicyanin with those of another amicyanin from *Thiobacillus versutus* and a plastocyanin consensus sequence (Table 2) (van Beeuman et al., 1991; Durley et al., 1993) reveals that Arg^{99} and Phe^{97} are conserved in the amicyanin sequences but have no counterparts in plastocyanin. These residues reside in a relatively conserved portion of the sequence, but one sees Gly rather than Phe^{97} and Val rather than Arg^{99} in the plastocyanin sequence. The results of this mutagenesis study indicate that Lys^{68} may influence the ionic strength dependence of complex association. It is interesting to note that the corresponding residue in the other amicyanin is Arg, while in plastocyanin it is a negatively charged residue, Glu. Comparison with azurin sequences is more difficult because the similarity with amicyanin is much less and there are considerably more insertions and deletions in the sequences, as well as more deviations in the structures (Durley et al., 1993).

These results are important because they provide strong evidence that the orientation of MADH and amicyanin which is seen in the structure of the crystallized complex is the same as the orientation of the proteins when they react with each other in solution. Only two other physiologic complexes of soluble redox proteins have been crystallized, that of cytochrome *c* with cytochrome *c* peroxidase (Pelletier & Kraut, 1992) and the Fe−protein and MoFe−protein components of nitrogenase (Schindelin et al., 1997). With so few examples it is impossible to generalize about factors which govern the interactions between such protein partners. The type of interaction described here between amicyanin and MADH, with both ionic and hydrophobic components, may be a reasonable model for weakly associated protein complexes. It is important that the hydrophobic region which surrounds the electron transfer site on amicyanin not participate in nonspecific interactions with other proteins that have hydrophobic surface domains. This would lead to competitive inhibition for the electron transfer reaction or, even worse, electron transfer to the wrong protein. This would decrease the efficiency of energy metabolism and be counterproductive to the organism. By requiring one or more specific electrostatic interactions for proper recognition and alignment of hydrophobic surfaces, the chance of nonspecific interactions with other proteins is minimized. It should be noted that while this model may be reasonable, it will certainly not be universal. For example, the crystal structure of the ternary MADH−amicyanin−cytochrome *c*-551i complex (Chen et al., 1994) reveals that the other protein−protein interface between amicyanin and the cytochrome contains relatively polar residues and is stabilized primarily by hydrogen bonds. Clearly, additional studies such as those described here are required to gain a complete understanding of the factors which dictate the specificity of interactions between soluble redox proteins.

ACKNOWLEDGMENT

We thank Dr. Andrei Y. Chistoserdov for providing us with the *mau*C gene from *P. denitrificans*, Rex Williams for technical assistance, and G. Reid Bishop for helpful discussions.

REFERENCES

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

Bishop, G. R., & Davidson, V. L. (1995) *Biochemistry 34*, 12082−12086.

Bishop, G. R., Brooks, H. B., & Davidson, V. L. (1996) *Biochemistry 35*, 8948−8954.

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

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

Chistoserdov, A. Y., Boyd, G., Mathews, F. S., & Lidstrom, M. E. (1992) *Biochem. Biophys. Res. Commun. 184*, 1181−1189.

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. (1995) *J. Biol. Chem. 270*, 23941−23943.

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

de Gier, J.-W., van der Oost, J., Harms, N., Stouthamer, A. H., & van Spanning, R. J. M. (1995) *Eur. J. Biochem. 229*, 148−154.

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

Gray, K. A., Knaff, D. B., Husain, M., & Davidson, V. L. (1986) *FEBS Lett. 207*, 239−242.

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

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. (1987) *J. Bacteriol. 169*, 1712−1717.

Hyun, Y.-L., & Davidson, V. L. (1995) *Biochemistry 34*, 12249−12254.

Kumar, M. A., & Davidson, V. L. (1990) *Biochemistry 29*, 5299−5304.

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

Merli, A., Brodersen, D. E., Morini, B., Chen, Z., Durley, R. C. E., Mathews, F. S., Davidson, V. L., & Rossi, G. L. (1996) *J. Biol. Chem. 271*, 9177−9180.

Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748−1755.

Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., & Rees, D. C. (1997) *Nature 387*, 370−376.

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

van Beeumen, J., van Bun, S., Canters, G. W., Lommen, A., & Chothia, C. (1991) *J. Biol. Chem. 266*, 4869−4877.

van Spanning, R. J. M., Wansell, C. W., Reijnders, W. N. M., Oltmann, L. F., & Stouthamer, A. H. (1990) *FEBS Lett. 275*, 217−220.

BI971353M