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Binding constants for a physiologic electron-transfer protein complex between methylamine dehydrogenase and amicyanin. Effects of ionic strength and bound copper on binding

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Two soluble proteins, methylamine dehydrogenase and amicyanin, form a physiologically relevant complex in which intermolecular electron transfer occurs. To characterize and quantitate the binding of these two weakly-associating proteins, an ultrafiltration binding assay has been developed which involves brief centrifugation of mixtures of proteins in centrifuge concentrators followed by quantitation of proteins on each side of the filtration membrane by HPLC. Under low ionic strength conditions which are optimal for the redox reaction between these proteins, a K_d of 4.5 μM was measured for the methylamine dehydrogenase-amicyanin complex. The K_d increased by 8-fold in the presence of added salt. Apoamicyanin, which is known from crystallographic analysis to be structurally very similar to amicyanin, exhibited a much higher K_d and much less specific binding than did the holoprotein. Apoamicyanin also exhibited apparent self-association at low ionic strength which was not observed with amicyanin. These observations are correlated with the known crystal structures of these proteins, free and in complex, and with the available biochemical information on the interactions of these two proteins.

Introduction

A quinoprotein, methylamine dehydrogenase, and a type I copper protein, amicyanin, form a physiologically relevant complex in which intermolecular electron transfer occurs. Methylamine dehydrogenase is a periplasmic enzyme which has been purified from several gram negative bacteria [1]. It catalyzes the oxidation of methylamine to formaldehyde and ammonia. Its prosthetic group is tryptophan tryptophylquinone (TTQ) [2,3], a covalently-bound *o*-quinone which is derived from a posttranslational modification of two gene-encoded tryptophan residues [4]. The primary electron acceptor for methylamine dehydrogenase is amicyanin [5]. Most of the physical, redox and spectroscopic properties of amicyanin are very similar to those of other type I copper proteins, such as plastocyanin and azurin, but available amino acid sequence data indicate that amicyanins are a separate, unique class of copper proteins [6,7]. This claim is supported by resonance Raman spectroscopic [8] and X-ray crystallographic studies [9,10]. The specificity of the interaction between amicyanin and methylamine dehydrogenase is best demonstrated by studies of the isolated proteins from *Paracoccus denitrificans*, in which each of these proteins is synthesized only during growth on methylamine as a carbon source [5,11]. The amicyanin gene has been located immediately downstream of those for methylamine dehydrogenase and inactivation of the former by gene replacement results in complete loss of the ability to grow on methylamine [6]. In *P. denitrificans*, amicyanin mediates the transfer of electrons from methylamine dehydrogenase to soluble periplasmic *c*-type cytochromes, of which cytochrome *c*-551i is the most efficient electron acceptor in vitro [12]. Solution studies indicate that amicyanin may only transfer electrons to this cytochrome when it is in complex with methylamine dehydrogenase [12,13], requiring a ternary protein complex for the methylamine-dependent reduction of cytochrome *c*-551i.

The interaction between methylamine dehydrogenase and amicyanin has been characterized by absorption spectroscopy [13], potentiometry [13], steady-state kinetics [14,15], chemical cross-linking [16], resonance Raman spectroscopy [17] and X-ray crystallography [9]. The results of these studies raised questions as to the

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Abbreviations: TTQ, tryptophan tryptophylquinone; MWCO, molecular weight cutoff.

relative roles of electrostatic and hydrophobic interactions in stabilizing the functional association between these two proteins. On complex formation with methylamine dehydrogenase, the redox potential of amicyanin is lowered by 73 mV from +294 mV to +221 mV, and the absorption spectrum of TTQ is perturbed [13]. This is important physiologically as it facilitates the otherwise unfavorable electron transfer to cytochrome *c*-551i, which has a potential of +190 mV. These complex-dependent changes were observed at low ionic strength (0.01 M buffer) but not observed in the presence of 0.2 M NaCl. In steady-state kinetic studies, the apparent K_m for amicyanin increased with increasing ionic strength [14]. Comparison of resonance Raman spectra of methylamine dehydrogenase and amicyanin, free and in complex, revealed no significant changes, suggesting that neither the TTQ nor copper redox center was structurally altered during complex formation and that the observed complex-dependent changes in redox potential and absorption spectra were due to electrostatic interactions which accompanied complex formation [17]. These data are consistent with the suggestion that electrostatic interactions between amicyanin and methylamine dehydrogenase are involved in stabilizing the proper orientation of the proteins to facilitate intermolecular electron transfer. Chemical cross-linking studies [16] suggested that both electrostatic and hydrophobic forces were involved in complex formation. Crystallization of the complex [18] was accomplished at high ionic strength, and structural analysis indicated that the interface between the proteins was comprised primarily of hydrophobic amino acid residues [9]. Thus, it appears that productive complex formation involves a combination of electrostatic and hydrophobic interactions.

To better understand the precise mechanism by which this specific protein-protein association occurs and is stabilized, we have devised a novel assay with which to directly measure binding constants for the interaction of methylamine dehydrogenase and amicyanin. With this assay it was shown that, despite the apparent predominance of hydrophobic interactions in the crystal structure of the complex, binding is actually favored at low ionic strength. Furthermore, despite the fact that apoamicyanin forms a complex with methylamine dehydrogenase which is structurally very similar to that of amicyanin [9,18], apoamicyanin exhibits a greatly diminished affinity for methylamine dehydrogenase.

Experimental procedures

The purifications of methylamine dehydrogenase [19] and amicyanin [5] from *P. denitrificans* (ATCC 13543), and the preparation of apoamicyanin and method for its reconstitution with copper [20], were as described previously. Protein concentrations were calculated from previously published extinction coefficients [5,20]. The chemicals used in this study were all obtained from commercial sources.

Binding was measured directly by mixing a fixed amount of methylamine dehydrogenase with varying concentrations of amicyanin or apoamicyanin. The proteins, in a total volume of 1.1 ml, were placed in the top compartment of a Centricon-100 (Amicon, Beverly, MA) concentrator, which possesses a 100000 molecular weight cutoff (MWCO) membrane. The molecular weights of methylamine dehydrogenase and amicyanin are 124000 and 12500, respectively. The Centricons were centrifuged for 25 s in a Clay-Adams Safeguard benchtop centrifuge set to low speed. Since the rotor was accelerating during the entire 25 s, a meaningful *g*-force was impossible to estimate. Under these conditions, 75–100 μl passed through the membrane. The entire volume of the filtrate in the bottom compartment was then added back to the top compartment and remixed. The purpose of this first centrifugation was to saturate any protein binding to the membrane or to the plastic of the bottom compartment. Remixed samples were then centrifuged a second time and 70 μl aliquots were removed from both top and bottom compartments for HPLC analysis. All operations were performed at room temperature. As a control, blank assay mixtures were run which contained only amicyanin or apoamicyanin, but no methylamine dehydrogenase.

Samples were analyzed on a Waters 840 Data and Chromatography Control Station, equipped with a model 712 autoinjector, model 490 multi-wavelength detector and Waters Expert software v.6.2 (Milford, MA). Samples were analyzed with either a SynChropak Propyl hydrophobic interaction column (10 cm × 4.6 mm, 300 Å pore size; SynChrom, Lafayette, IN) or a Protein C_4 column (25 cm × 4.6 mm, 300 Å pore size; Vydac, Hesperia, CA). Columns were maintained at 30°C and 50 μl samples were injected. Proteins were eluted from the Propyl column with a linear gradient of 0.025 to 1.2 M ammonium acetate in water (pH 6.8) over 10 min, at a flow rate of 0.6 ml/min. Amicyanin and methylamine dehydrogenase eluted as single peaks, at 3.3 and 7.4 min, respectively *, and were monitored at 280 nm. Proteins were eluted from the C_4 column with a linear gradient of 30:70 to 70:30 solvent A:B over 30 min, at a flow rate of 0.6 ml/min, where A = 0.06% trifluoroacetic acid in water, and B =

* It should be noted that this solvent system is not usual for this type of column, and that the basis of separation is probably weak ion-exchange rather than hydrophobic interaction. Attempts to run these proteins on hydrophobic interaction columns using the standard protocol of decreasing ammonium sulfate concentration were unsuccessful.

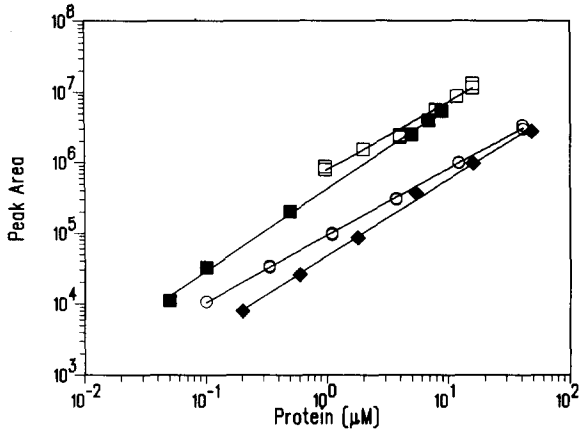

Fig. 1. HPLC peak area as a function of protein concentration. Fifty μl of each standard was injected. Amicyanin (○) and methylamine dehydrogenase (□) in 0.01 M potassium phosphate (pH 7.5) were injected onto the Propyl column and eluted as described under Experimental procedures with the eluant monitored at 280 nm. Under these conditions methylamine dehydrogenase eluted as a single peak. Apoamicyanin (◆) and methylamine dehydrogenase (■) in 0.01 M potassium phosphate (pH 7.5) containing 0.2 M NaCl, were injected onto the C_4 column and eluted as described under Experimental procedures with the eluant monitored at 277 nm. Under these conditions methylamine dehydrogenase split into α and β subunits and was quantitated from the peak which corresponded to the β subunit.

0.052% trifluoroacetic acid in 80% acetonitrile. Amicyanin eluted as a single peak at 20.4 min, while methylamine dehydrogenase split into α and β subunits [11], eluting at 15.4 and 24.7 min, respectively. The β subunit exhibited a sharper peak shape and was used for quantitation. Proteins were monitored at 277 nm, a maximum wavelength in this solvent system. For samples which contained NaCl, the reversed-phase method was used exclusively because the behavior of amicyanin on the Propyl column became erratic when the samples contained as little as 0.5 μmol NaCl. Up to 10 μmol NaCl was without effect on the C_4 column. For both protocols, 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 methylamine dehydrogenase concentration over the range of 0.05 to 10 μM, and to amicyanin or apoamicyanin over the range of 0.2 to 50 μM (Fig. 1). Comparison of standards containing a single protein to those containing methylamine dehydrogenase plus amicyanin or apoamicyanin demonstrated that no interaction between proteins occurred during HPLC with either protocol.

When analyzing protein binding data, it was assumed that the concentration of amicyanin or apoamicyanin passing through the ultrafiltration membrane represented the free protein, and that the concentration retained by the membrane represented bound plus free protein. Data were fit to various ligand binding models (discussed below) by non-linear regression using the Enzfitter computer program (Elsevier-BIOSOFT, Cambridge, UK).

The steady-state kinetics of the methylamine-dependent reduction of amicyanin by methylamine dehydrogenase were assayed as described previously [15]. The assay mixture contained 4.5 nM methylamine dehydrogenase and 2.5 μM amicyanin in 0.01 M potassium phosphate (pH 7.5), at 30°C. The reaction was initiated by the addition of 0.1 mM methylamine and activity was monitored by the change in absorbance caused by the reduction of amicyanin at 595 nm.

Results

We have reported previously [13] that combination of oxidized amicyanin with oxidized methylamine dehydrogenase elicited changes in the visible absorption spectra of the individual proteins. While it has been possible to titrate these changes as a function of added amicyanin it was not possible to obtain a true binding constant from such data. In studies such as this it is often assumed that the total concentration of the added ligand may be taken to be the free concentration because the amount of bound ligand is negligible compared to the total. When this is true, the change in absorbance may be plotted against total ligand to obtain a value for the apparent dissociation constant (K_d). Unfortunately, this assumption is not valid in this experimental system. Because the absorbance changes are very small [13], at least micromolar concentrations of protein are required to yield a detectable signal. The K_d for amicyanin binding is well below the concentration of amicyanin required to obtain the maximum change in absorbance. Therefore, the concentration of free amicyanin cannot be approximated to be the total amicyanin and it is not possible to obtain a K_d for the amicyanin-methylamine dehydrogenase interaction by this technique.

Binding assay

An ultrafiltration method was devised to directly determine the true K_d for complex formation between methylamine dehydrogenase and amicyanin. Ultrafiltration may be used instead of the more traditional equilibrium dialysis methods for measurement of bound and free ligand concentrations [21–23]. The disadvantages of dialysis techniques include the long time necessary to achieve equilibrium conditions and practical difficulties in performing experiments with the large numbers of samples required to study the concentration dependence of binding. Furthermore, equilibrium dialysis has not routinely been used to measure protein-protein interactions because high MWCO dialysis membranes have not been available until very recently. With commercially available ultrafiltration cells with high MWCO membranes it was possible to rapidly

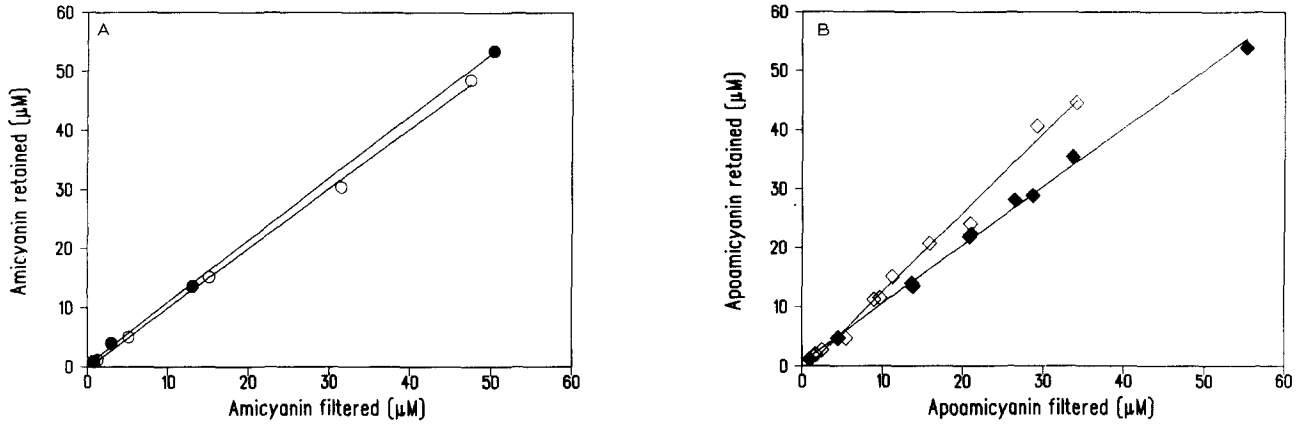

Fig. 2. Ligand concentrations in binding assay blanks. Amicyanin or apoamicyanin were centrifuged in Centricon-100s in the absence of methylamine dehydrogenase. Protein concentrations were then measured on each side of the ultrafiltration membrane. (A) Amicyanin (○) in 0.01 M potassium phosphate (pH 7.5), slope of linear regression line = 1.01; amicyanin (●) in the presence of 0.2 M NaCl, slope = 1.05. (B) Apoamicyanin (◇) in 0.01 M potassium phosphate (pH 7.5), slope = 1.35; apoamicyanin (◆) in the presence of 0.2 M NaCl, slope = 0.99.

measure binding for large numbers of samples (described under Experimental procedures). The volume of sample passing through the membrane was kept to less than 10% of the initial sample volume so as not to appreciably alter the concentrations of ligand or acceptor proteins during the course of the experiment to avoid possible disturbance of the equilibrium. In each experiment, amicyanin blanks which did not contain methylamine dehydrogenase were run to verify that the free amicyanin concentrations were identical on either side of the membrane (Fig. 2A). Preliminary studies demonstrated that, although the molecular weight of amicyanin is 12500, membranes with a MWCO of 30000 slightly restricted its passage, and as such all binding assays were performed with 100000 MWCO membranes. Retention of methylamine dehydrogenase, which has a molecular weight of 124000, by the 100000 MWCO membranes was greater than 99% in the low ionic strength buffer, and 95% in the buffer containing 0.2 M NaCl.

TABLE I

Comparative analysis of alternative models for fitting data on the binding of amicyanin to methylamine dehydrogenase

In this experiment 25 amicyanin concentrations from 1 to 30 μM were mixed with 7 μM methylamine dehydrogenase in 0.01 M potassium phosphate buffer. These data are shown in Fig. 3. Free and bound amicyanin were measured and data were fit to Eqns. 1–3. The specific capacity was calculated by dividing the measured capacity by the final methylamine dehydrogenase concentration. The standard error for each fitted parameter is given.

Model	Dissociation constants	Specific capacity (sites/mol)	Correlation coefficient (r^2)
One site (Eqn. 1)	$K_d = 4.6 \pm 0.5$ μM	1.6 ± 0.1	0.94
Two non-interacting sites (Eqn. 2)	$K_d^1 = 4.5 \pm 1.4$ μM $K_d^2 = 4.1 \pm 3.0$ μM	1.5 ± 0.2 0.1 ± 0.2	0.94
Hill equation (Eqn. 3)	$K = 4.7 \pm 0.4$ μM $n = 1.2 \pm 0.1$	1.4 ± 0.1	0.94

Amicyanin binding to methylamine dehydrogenase

In this first experiment, concentrations of oxidized amicyanin from 1.1 to 30 μM were mixed with 7 μM oxidized methylamine dehydrogenase in 0.01 M potassium phosphate buffer (pH 7.5). Concentrations of free and bound amicyanin were determined after ultrafiltration by HPLC analysis as described under Experimental procedures. These data were fit to three alternative models which are routinely used to describe ligand binding: ligand binding to a single site (Eqn. 1); ligand binding to two distinct non-interacting sites (Eqn. 2); and the Hill equation for cooperative interactions (Eqn. 3). The parameters that were obtained from non-linear least-squares fit of the data to each model are given in Table I. Although the fit to Eqn. 3 indicated the presence of slight positive cooperativity, examination of the standard errors, residuals, and correlation coefficients for each fit yielded no clear justification for assuming any model more complex than the simple single ligand binding model given in Eqn. 1. The fit of the data to Eqn. 1 is shown in Fig. 3. For each subsequent experiment, the three models were also examined, but in no case did Eqns. 2 or 3 yield a significantly better fit to the data than the simple 1 site

$$\text{Bound} = \frac{\text{Capacity} \times \text{Free}}{K_d + \text{Free}} \quad [1]$$

$$\text{Bound} = \frac{\text{Capacity(site 1)} \times \text{Free}}{K_d(\text{site 2}) + \text{Free}} + \frac{\text{Capacity(site 2)} \times \text{Free}}{K_d(\text{site 2}) + \text{Free}} \quad [2]$$

$$\text{Bound} = \frac{\text{Capacity} \times \text{Free}^n}{K + \text{Free}^n} \quad [3]$$

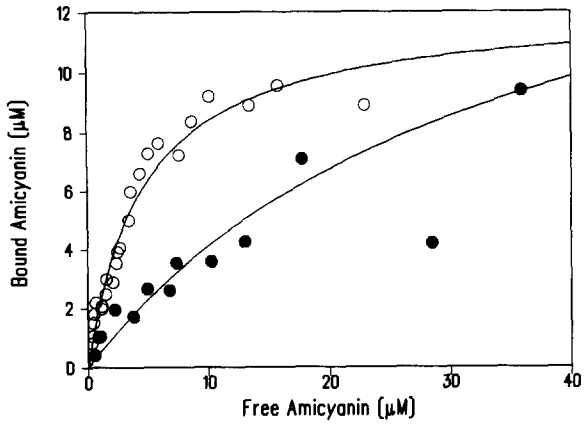

Fig. 3. Binding of amicyanin to methylamine dehydrogenase in the presence and absence of added salt. Binding was assayed in 0.01 M potassium phosphate (pH 7.5) in the absence (○) and presence (●) of 0.2 M NaCl. Solid lines represent the fit of the data to Eqn. 1.

model described by Eqn. 1. The fitted constants to Eqn. 1 for all subsequent experiments are summarized in Table II.

Under the low ionic strength conditions which are optimal for the reaction between methylamine dehydrogenase and amicyanin in vitro, a K_d of 4.5 ± 0.5 μM, and specific capacity of 1.6 ± 0.1 sites occupied per methylamine dehydrogenase molecule were determined. The value of 1.6 binding sites per methylamine dehydrogenase molecule is consistent with X-ray crystallographic studies which indicate that two amicyanins interact specifically with methylamine dehydrogenase [9], which possesses an $\alpha_2\beta_2$ structure. The experimentally determined K_d should be independent of the fixed concentration of methylamine dehydrogenase which is present. To confirm this, the binding of amicyanin was also measured with a fixed concentration of 1 μM methylamine dehydrogenase. Under these conditions a statistically identical K_d of 4.6 ± 1.1 μM was obtained.

As the complex-dependent changes in the absorption spectrum of TTQ and redox potential of amicyanin were not observed in the presence of 0.2 M NaCl [13], the binding of amicyanin to methylamine dehydrogenase was assayed in the presence of 0.2 M NaCl (Fig. 3). In the presence of 0.2 M NaCl, a K_d of 35 ± 10 μM was determined, approx. 8-fold greater than was observed at low ionic strength (Table II). A binding capacity of 2.7 ± 0.5 sites was determined which also suggests that some of the observed binding under these conditions may be non-specific.

TABLE II

Fitted parameters for the binding of amicyanin and apoamicyanin to methylamine dehydrogenase

Assays were conducted in 0.01 M potassium phosphate buffer (pH 7.5), with additions as indicated and data were fit to Eqn. 1. The standard error for each fitted parameter is given.

Amicyanin form present	Added NaCl	K_d (μM)	Specific capacity (sites/mol)
Amicyanin	none	4.5± 0.5	1.6±0.1
Amicyanin	0.2 M	35 ± 10	2.7±0.5
Apoamicyanin	0.2 M	280 ±160	11 ±5
Reconstituted amicyanin	none	4.4± 2.2	1.5±0.3
Reconstituted amicyanin	0.2 M	65 ± 27	3.3±0.9

Apoamicyanin binding

X-ray crystallographic studies indicated that the structure of apoamicyanin is very similar to that of amicyanin [10], and that the structure of an apoamicyanin-methylamine dehydrogenase complex is very similar to the amicyanin-methylamine dehydrogenase complex [9]. As such it was of interest to characterize the parameters for the binding of apoamicyanin to methylamine dehydrogenase.

Unexpectedly, control experiments with apoamicyanin indicated that, unlike amicyanin, the former was significantly retained by 100000 MWCO membranes when suspended in 0.01 M potassium phosphate buffer (Fig. 2B). Because of this apparent aggregation, it was not possible to perform binding studies with apoamicyanin and methylamine dehydrogenase at low ionic strength. In the presence of 0.2 M NaCl, however, apoamicyanin was not restricted by the membranes (Fig. 2B), and binding assays could be performed to compare with those done with amicyanin in the presence of 0.2 M NaCl. The K_d for apoamicyanin was 280 ± 160 μM, and capacity was 11 ± 5 sites (Fig. 4, Table II). Large errors in these fitted parameters are due to the fact that substantial binding of apoamicyanin to methylamine dehydrogenase could not be achieved without using prohibitively large concentrations of apoamicyanin. However, it can be concluded that the binding of apoamicyanin to methylamine dehy-

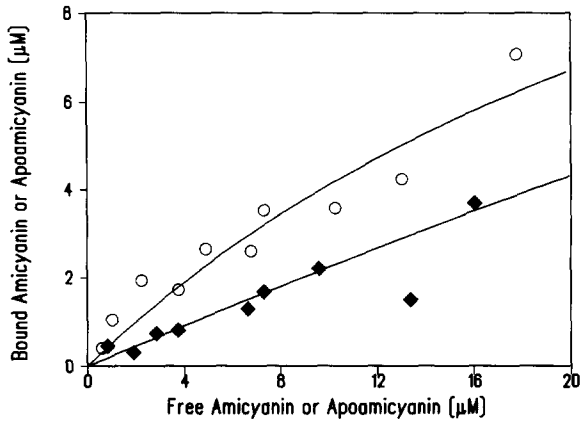

Fig. 4. Binding of amicyanin and apoamicyanin to methylamine dehydrogenase. Binding of amicyanin (○) and apoamicyanin (◆) was assayed in 0.1 M potassium phosphate buffer (pH 7.5) with 0.2 M added NaCl. Solid lines represent the fit of the data to Eqn. 1.

TABLE III

The effect of apoamicyanin on the steady-state kinetic assay of the methylamine-dependent reduction of amicyanin by methylamine dehydrogenase

The assay mixture contained 4.5 nM methylamine dehydrogenase with amicyanin or apoamicyanin or both as indicated in 0.01 M potassium phosphate (pH 7.5) at 30°C in the presence or absence of 0.2 M NaCl. The reaction was initiated by the addition of 0.1 mM methylamine and the rate of reaction was monitored by the decrease in absorbance at 595 nm. Activity (k_{cat}) is reported as mol amicyanin reduced per mol methylamine dehydrogenase per s.

Forms of amicyanin present in assay	k_{cat} (s^{-1})	
	No added NaCl	+0.2 M NaCl
Amicyanin (2.5 μM)	42	11
Apoamicyanin (2.5 μM)	0	0
Amicyanin (2.5 μM) + apoamicyanin (25 μM)	41	12
Amicyanin (2.5 μM) + apoamicyanin (50 μM)	43	13
Reconstituted amicyanin (2.5 μM)	41	13

drogenase was significantly weaker than binding of amicyanin. Furthermore, given the high capacity it may be that much of this binding was non-specific.

To ensure the validity of the results which were obtained with apoamicyanin, binding assays were also performed with apoamicyanin which had been reconstituted to amicyanin by the addition of one molar equivalent of $CuSO_4$ [20]. These studies yielded values for K_d and specific capacity at both high and low ionic strength which were statistically identical to those obtained with native amicyanin (Table II). This confirms that the much different values observed for apoamicyanin binding were not artifacts due to irreversible damage done to the protein during the preparation of the apoprotein.

Kinetic studies

The relative affinities of apoamicyanin and amicyanin for methylamine dehydrogenase were assayed kinetically. The ability of apoamicyanin to act as a competitive inhibitor was measured in a steady-state assay of methylamine-dependent amicyanin reduction by methylamine dehydrogenase (Table III). Such inhibition would occur if apoamicyanin bound specifically to the dehydrogenase with an affinity comparable to that of amicyanin. Under buffer conditions (including 0.2 M NaCl) in which the K_d of apoamicyanin was approx. 8-fold higher than that of amicyanin, no inhibition of amicyanin reduction was observed in the presence of up to a 20-fold excess of apoamicyanin. Also no inhibition was observed in 0.01 M buffer without added salt. Again, as a control, it was demonstrated that amicyanin which was reconstituted from apoamicyanin exhibited normal activity (Table III). These data are consistent with the interpretation that specific apoamicyanin binding to methylamine dehydrogenase is far less efficient than that observed for amicyanin. It should also be noted that the rate of reaction with amicyanin in the presence of 0.2 M NaCl is about 4-fold slower than that observed in the absence of added salt. This is consistent with previous observations [13,14] and with the weaker binding observed for amicyanin in the presence of 0.2 M NaCl (Table II).

Discussion

The data presented here clearly indicate that electrostatic interactions contribute to the actual binding process and stabilize the specific association between these proteins. This is consistent with the results of most solution studies of these proteins, but in apparent contradiction to the crystal structure of the complex of these proteins which indicated that the interface between the proteins was composed of primarily hydrophobic amino acid residues. However, the complex was crystallized in high salt which would favor hydrophobic interactions and as such, oppositely charged groups which may normally interact in solution may point away from each other in the crystalline complex. The validity of this crystal structure has been supported by the recent preliminary structural analysis of crystals of a ternary protein complex between methylamine dehydrogenase, amicyanin and cytochrome *c*-551i [24]. At the present stage of analysis the orientation of amicyanin with respect to methylamine dehydrogenase appears to be quite similar to that in the two-protein complex, suggesting that the interaction between them is specific. The binding data obtained in this study, therefore, will be important in attempting to resolve results from solution studies on the interactions between these proteins with the structural information.

The greatly decreased affinity of methylamine dehydrogenase for apoamicyanin, and the apparent aggregation of apoamicyanin at low ionic strength, were unexpected given the similarities of their crystal structures [9,10]. Those structural data are quite consistent with the prevailing view that the protein, not the copper, determines the active site structure of type I copper proteins. If the only significant difference between the apo- and holoprotein is copper, then removal of this cation must be affecting surface properties of the protein in a way that reduces its affinity for methylamine dehydrogenase, and also causes it to self-associate at low ionic strength. As amicyanin mediates electron transfer between other redox proteins, and copper is not accessible to the surface, electron transfer must occur between copper and one or more amino acid residues on the surface of the protein. This im-

plies that there is always some electronic coupling between copper and surface residues. Removal of copper may, therefore, modify the charge distribution on the surface of the protein. This in turn may affect its ability to bind to other proteins as well as to self-associate.

It should be noted that the binding assay which was developed in this study has several advantages over alternative methods. The technique requires no expensive or unusual equipment. Unlike dialysis techniques, the actual assay takes only a few minutes, thus minimizing degradation of proteins during the assay. It is also a nondestructive technique, and except for the small amount of protein which is needed for HPLC quantitation, the rest of the proteins in the sample may be recycled for further use. As such, this assay may potentially be used as a general technique for characterizing the interactions of weakly associating proteins, provided that they are of sufficiently different molecular weights to be separated by available ultrafiltration membranes.

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