Complex Formation between Methylamine Dehydrogenase and Amicyanin from Paracoccus denitrificans*

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Two proteins isolated from Paracoccus denitrificans, the copper-containing electron carrier amicyanin and the pyrroloquinoline quinone-containing enzyme methylamine dehydrogenase, have been shown to form a complex. Complex formation between methylamine dehydrogenase and either oxidized or reduced amicyanin resulted in alterations in the absorbance spectrum of the pyrroloquinoline quinone prosthetic group of methylamine dehydrogenase. Binding of amicyanin to the enzyme exhibited positive cooperativity. Complex formation with methylamine dehydrogenase shifted the oxidation-reduction midpoint potential of amicyanin by 73 mV, from +294 to +221 mV, making electron transfer from amicyanin to cytochrome c551 (Em = +190 mV) thermodynamically possible.

It has recently been established that a number of oxidoreductases from a variety of sources contain pyrroloquinoline quinone (PQQ) as a prosthetic group (reviewed in Ref. 1). This family of enzymes includes a number of bacterial quinoproteins that contain either covalently or noncovalently bound PQQ and eukaryotic amine oxidases (e.g. plasma amine oxidase, diamine oxidase, and lysyl oxidase) which contain bound PQQ and eukaryotic amine oxidases (e.g. plasma amine oxidase). Previous work in our laboratories has extensively characterized methylamine dehydrogenase and has provided values of +100 mV for the oxidation-reduction midpoint potential (Em) of PQQ in methylamine dehydrogenase (8) and of +294 mV for the Em of the copper center in amicyanin (9). Growth of P. denitrificans on methylamine also induces the synthesis of two soluble periplasmic c-type cytochromes, cytochrome c551 (10). Kinetic studies established that amicyanin served as the immediate acceptor from methylamine dehydrogenase (3, 9) and that amicyanin was required to mediate the transfer of electrons from methylamine dehydrogenase to cytochrome c551. However, the Em value of +190 mV measured for cytochrome c551 suggested that electron flow from reduced amicyanin to cytochrome c551 should have been thermodynamically unfavorable (9). Furthermore, it was shown that in the absence of methylamine dehydrogenase reduced amicyanin was not able to reduce cytochrome c551. Reduction of the cytochrome by amicyanin did not occur in the presence of methylamine dehydrogenase (9). As a possible explanation for these phenomena, we proposed that complex formation between two or more of the three proteins involved in electron transfer from methylamine to cytochrome c551 might shift the Em value of at least one of the proteins sufficiently to produce thermodynamically favorable electron transfer (9). Complex formation between protein oxidation-reduction partners is, in fact, a common phenomenon (9-22). In the case of at least one such complex, that between spinach ferredoxin and spinach ferredoxin:NADP+ oxidoreductase, complex formation alters the Em values of both proteins making electron transfer from reduced ferredoxin to the FAD group of the reductase more favorable (23, 24). However, to date there are no reports of complex formation causing an otherwise thermodynamically unfavorable electron transport to become possible. This report presents evidence for the formation of a complex between the amicyanin and methylamine dehydrogenase that shifts the Em value of amicyanin to a sufficiently more negative value so that electron transfer from amicyanin to cytochrome c551 is facilitated.

MATERIALS AND METHODS

Amicyanin, cytochrome c551, and methylamine dehydrogenase were purified from P. denitrificans (ATCC 13543) as described previously (2, 3, 10). Plastocyanin was a gift from Dr. Richard Malkin (Division of Molecular Plant Biology, University of California, Berkeley). Electrophoretical titrations were performed as described previously (23) using an optically transparent gold electrode in a thin-layer cell. Absorbance spectra for the electrochemical titrations were recorded at 5 °C using an Aminco DW-2a spectrophotometer. Control titrations of equine cytochrome c, spinach ferredoxin, and methyl viologen gave Ec values within 5 mV of literature values. All titrations were fully reversible.

Difference spectra resulting from complex formation were obtained at 5 °C using a 1-cm pathlength quartz split cell, as described previously (25), and a Perkin-Elmer Lambda 5 spectrophotometer. The oxidized forms of amicyanin and methylamine dehydrogenase were generated by addition of excess potassium ferricyanide, followed by passage over Sephadex G-10 to remove any excess ferricyanide. Reduced amicyanin was generated by similar treatment of the protein with sodium ascorbate and passage over Sephadex G-10. Hill coefficients were calculated from plots of ΔA versus amicyanin concentration using the Cricket Graph software of Cricket Software, Inc. on a Macintosh SE computer. Membrane filtration binding assays were conducted with Centricon-30 microconcentrators (Amicon Corp., 30,000 cutoff) which were centrifuged at 4 °C for 1 h at 4,000 × g.
Protein concentrations were calculated from previously determined extinction coefficients (3, 8, 10).

RESULTS

The difference spectrum obtained by mixing oxidized amicyanin with oxidized methylamine dehydrogenase exhibited a peak at 408 nm and a trough centered around 468 nm (Fig. 1A, dotted line). As oxidized amicyanin contains no absorbance maxima in this spectral region, it is likely that interactions between the two proteins are perturbing spectral features of the PQQ group of methylamine dehydrogenase. Addition of 200 mM NaCl largely, but not completely, eliminated the absorbance changes caused by mixing the two proteins (Fig. 1A, solid line), suggesting that the protein-protein interactions responsible for these absorbance changes are predominantly electrostatic in nature. A difference spectrum essentially identical to that shown by the solid line in Fig. 1A was obtained if the proteins were mixed in buffer containing 200 mM NaCl (data not shown). The difference spectrum resulting from the interaction of oxidized methylamine dehydrogenase with reduced amicyanin exhibited a broad trough centered at 464 nm (Fig. 1B, dotted line). As reduced amicyanin exhibits no appreciable absorbance in the visible, the negative feature at 464 nm probably reflects a perturbation of the spectrum of methylamine dehydrogenase. The spectral perturbations were again almost completely eliminated by the addition of 200 mM NaCl (Fig. 1B, solid line). Treatment of the sample used to generate the spectrum of Fig. 1B with excess sodium ascorbate, followed by analysis of the resulting absorbance spectrum, indicated that no electron transfer from reduced amicyanin to the enzyme had occurred on mixing (data not shown). Plots of ΔA against the molar ratio of either oxidized or reduced amicyanin to methylamine dehydrogenase (Fig. 2) yielded sigmoidal binding curves which suggest positive cooperativity in the binding process. Hill plots (26) of these data (not shown) were linear and yielded Hill coefficients of 1.8 ± 0.2 and 2.1 ± 0.2 for the binding to the enzyme of oxidized and reduced amicyanin, respectively.

To demonstrate the specificity of the amicyanin-methylamine dehydrogenase interaction, similar experiments were conducted in which methylamine dehydrogenase was mixed with either spinach plastocyanin or P. denitrificans cytochrome c551. Plastocyanin, which serves as electron donor to plant Photosystem I (27), resembles amicyanin in molecular weight, spectral characteristics, and E<sub>m</sub> value and shows significant amino acid sequence homology to amicyanin (3, 9, 27, 28). However, plastocyanin is a very poor electron acceptor for P. denitrificans methylamine dehydrogenase. No spectral changes were observed when plastocyanin was mixed with methylamine dehydrogenase at low ionic strength, at molar ratios as high as 6:1. No spectral changes were observed when cytochrome c551 was mixed with methylamine dehydrogenase at a molar ratio of 4:1.

Independent evidence for complex formation between amicyanin and methylamine dehydrogenase was obtained from membrane ultrafiltration experiments (Table I). As expected for a 14.5-kDa protein (3), amicyanin alone passed readily through the membrane filter at either low or high ionic strength. In the presence of methylamine dehydrogenase ([amicyanin]:[methylamine dehydrogenase] = 2:1), no amicyanin could be detected passing through the membrane at low ionic strength, consistent with the hypothesis that all of the amicyanin was complexed to the dehydrogenase. At high ionic strength, where an electrostatically stabilized complex

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**Fig. 1.** The effect of the interaction between amicyanin and methylamine dehydrogenase on the visible absorbance spectra of the proteins. A, the sample cuvette contained in a 1.0-ml volume: 9.4 nmol of oxidized methylamine dehydrogenase, 10 μmol of potassium phosphate buffer (pH 7.5), and 54 nmol of oxidized amicyanin. B, the sample cuvette contained in a 1.0-ml volume: 6.45 nmol of oxidized methylamine dehydrogenase, 10 μmol of potassium phosphate buffer (pH 7.5), and 39.6 nmol of reduced amicyanin. The reference cuvettes contained identical concentrations of the components, but the two proteins were present in separate compartments. Dotted lines in both A and B show the spectra before and solid lines immediately after the addition of NaCl (final concentration = 200 mM) to the sample and reference cuvettes.

**Fig. 2.** Binding curves for oxidized (●) and reduced (□) amicyanin with methylamine dehydrogenase. For oxidized amicyanin, ΔA at 408-468 nm was measured and for the reduced amicyanin, ΔA at 420-465 nm was measured. The absorbance changes were normalized so that the maximal change at each wavelength pair was set = 100%. Experimental conditions were as in Fig. 1.

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<sup>2</sup> V. L. Davidson, M. Husin, and R. Malkin, unpublished observations.
TABLE I
Membrane ultrafiltration of the amicyanin-methylamine dehydrogenase complex

| Amicyanin in filtrate | | |
|-----------------------|--|---|
| Amicyanin             | 80 ± 5 |
| Amicyanin + methylamine dehydrogenase | 0 |
| Amicyanin + methylamine dehydrogenase (200 mM NaCl) | 75 ± 5 |

would be expected to dissociate, as much amicyanin was detected in the filtrate in the presence of methylamine dehydrogenase as in the absence of the enzyme (Table I). Similar results were obtained using an Amicon stirred-cell concentrator and an XM-50 membrane (molecular weight cutoff = 50 kDa) instead of the Centricon concentrators. Gel filtration chromatography on Sephadex G-75, another technique that has been used in our laboratory to document complex formation between proteins (15, 22), produced no detectable co-chromatography of amicyanin and methylamine dehydrogenase. The latter result suggests that the two proteins form a relatively weak complex (K_d > 10^{-6} M), consistent with the high concentrations of amicyanin required to saturate binding to the dehydrogenase (Fig. 2).

To determine whether the electrostatic interaction between amicyanin and methylamine dehydrogenase played a role in the amicyanin-mediated transfer of electrons from methylamine dehydrogenase to cytochrome c_551, the dependence of this reaction on ionic strength was examined. As noted previously (3, 9, 10), no cytochrome c_551 reduction by methylamine plus methylamine dehydrogenase occurred in the absence of amicyanin (Fig. 3). The rate of amicyanin-dependent electron flow from methylamine to cytochrome c_551 in the presence of the enzyme decreased substantially when 200 mM NaCl was present. Furthermore, the reduction of the cytochrome was not complete in the presence of 200 mM NaCl. Addition of another 200 pmol of amicyanin caused the complete reduction of the remaining cytochrome c_551 (data not shown). The slower rate of cytochrome c_551 reduction in 200 mM NaCl was less pronounced at higher ratios of amicyanin to methylamine dehydrogenase. This latter observation was expected, as the decreased binding affinity at high ionic strength can be compensated for by increasing the amicyanin concentration which, in turn, results in a higher concentration of the complex. The direct reduction of amicyanin by methylamine plus methylamine dehydrogenase, as monitored by absorbance changes at 595 nm, was also dependent on the ionic strength. The initial rate of amicyanin reduction by methylamine dehydrogenase was 40% slower in 20 mM potassium phosphate buffer (pH 7.2) containing 200 mM NaCl than in 20 mM phosphate buffer alone (data not shown). These inhibitory effects of high ionic strength appear to result from disruption of protein-protein interactions, as the rate of the dehydrogenase-catalyzed reduction of a nonphysiological acceptor, phenazine methosulfate, by methylamine was unaffected by NaCl concentrations as high as 1.5 M.²

Having established conditions under which complex formation between amicyanin and methylamine dehydrogenase occurred, it was then possible to measure the E_m value for amicyanin in the complex in an attempt to resolve the apparent contradiction that rapid electron flow from amicyanin to the cytochrome occurred despite a 104-mV unfavorable difference in E_m values between the two proteins (9). Results of oxidation-reduction titrations of a mixture of amicyanin and methylamine dehydrogenase in the presence or absence of 200 mM NaCl (Fig. 4) provide at least a partial resolution of this

² V. L. Davidson, unpublished observations.
apparent paradox. In the presence of methylamine dehydrogenase, an $E_m$ value of $+221 \pm 7$ mV ($n = 0.9$) was measured for amicyanin at low ionic strength. In the presence of 200 mM NaCl, an $E_m$ value of $+278 \pm 16$ mV ($n = 0.9$) was measured for amicyanin. A titration of amicyanin alone on the presence of 200 mM NaCl yielded an $E_m$ value of $+287 \pm 5$ mV (data not shown), close to the 294 $\pm 6$ mV $E_m$ value previously obtained for amicyanin alone at low ionic strength (9), indicating that the $E_m$ value of amicyanin itself does not vary appreciably with ionic strength over this range. The midpoint potential of amicyanin is thus shifted by complex formation with the enzyme to a value close enough to that of cytochrome $c_{551}$ ($E_m = +190 \pm 5$ mV, Ref. 9) so that, at equilibrium, significant electron transfer from reduced amicyanin to the cytochrome will have occurred. Conversely, the presence of methylamine dehydrogenase produced no shift in the $E_m$ value of cytochrome $c_{551}$ (data not shown).

**DISCUSSION**

The data presented above clearly indicate that the *P. denitrificans* methylamine dehydrogenase and amicyanin form a complex stabilized predominantly by electrostatic interactions. Additional evidence for complex formation has been obtained from preliminary x-ray crystallographic data on methylamine dehydrogenase and its complex with amicyanin (29). The results of the ultrafiltration experiments (Table I) suggest a minimal stoichiometry of 2 amicyanin:1 methylamine dehydrogenase in the complex. The $\alpha\beta_2$ structure of methylamine dehydrogenase, which contains two FQ groups (2), also suggests that there are likely to be at least two amicyanin-binding sites on the enzyme. The significant decrease in the rate of amicyanin-mediated electron transfer observed at high ionic strength (Fig. 3) further suggests that the formation of an electrostatically stabilized amicyanin-enzyme complex is important for efficient electron transport from methylamine dehydrogenase to cytochrome $c_{551}$ via amicyanin. The observation that a small spectral perturbation persists (Fig. 1) in the presence of 200 mM NaCl suggests that hydrophobic interactions may also contribute to complex formation. However, the ultrafiltration data (Table I) suggest that if some amicyanin-enzyme interactions do occur in the presence of 200 mM NaCl, the interactions are considerably weaker than those that occur at low ionic strength. Although a 9-mV shift in the $E_m$ value of amicyanin at high ionic strength was observed in the presence of methylamine dehydrogenase, this small shift is within the experimental uncertainty of the measurements.

The most striking result obtained in this study is the 73-mV decrease in the $E_m$ value of amicyanin that results from complex formation with methylamine dehydrogenase. These thermodynamic considerations argue that such a shift in $E_m$ reflects stronger binding of the oxidized amicyanin to the enzyme than of the reduced amicyanin. This is consistent with the observation that higher concentrations of reduced amicyanin, compared to the oxidized protein, were required to saturate binding to the enzyme (Fig. 2). The shift in the $E_m$ value of amicyanin, caused by complex formation, results in a significant narrowing of the difference between the $E_m$ values of amicyanin and cytochrome $c_{551}$ from 104 (±10) mV for the separate proteins at low ionic strength (9) to 31 (±12) mV for the proteins in in complex. Thus, formation of this complex will significantly facilitate electron transfer from amicyanin to cytochrome $c_{551}$. As the presence of methylamine dehydrogenase caused no shift in the $E_m$ value of cytochrome $c_{551}$, the enhancement of electron transfer from amicyanin to cytochrome $c_{551}$ observed in the complete system must be due to interactions between methylamine dehydrogenase and amicyanin. These results would seem to resolve the apparent paradox concerning electron transfer from amicyanin to cytochrome $c_{551}$ and provide an explanation for our earlier observations that while amicyanin-mediated electron transfer from methylamine dehydrogenase to cytochrome $c_{551}$ occurred, no electron transfer from reduced amicyanin to the cytochrome occurred in the absence of this quinoprotein.

The effect of complex formation with methylamine dehydrogenase on the $E_m$ value for the copper center of amicyanin has broad implications regarding mechanisms of intermolecular electron transfer. These studies emphasize that neither redox potential nor kinetic data alone should be used as the sole basis for assigning sequences of electron transfer proteins. Given the significant difference in the $E_m$ value for free and complexed amicyanin and the fact that crystallographic data will soon be available for amicyanin, methylamine dehydrogenase, and their complex, these *P. denitrificans* electron carriers will provide an excellent model to study the influence of protein environments on the redox properties of a prosthetic group and the effects of protein-protein interactions on the structure and function of redox proteins.

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