pH-Dependent Semiquinone Formation by Methylamine Dehydrogenase from *Paracoccus denitrificans*. Evidence for Intermolecular Electron Transfer between Quinone Cofactors†

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ABSTRACT: The quinonoid confactors of *Paracoccus denitrificans* methylamine dehydrogenase exhibited a pH-dependent redistribution of electrons from the 50% reduced plus 50% oxidized to the 100% semiquinone redox form. This phenomenon was only observed at pH values greater than 7.5. The semiquinone was not readily reduced by addition of methylamine, consistent with the view that this substrate donates two electrons at a time to each cofactor during catalysis. Once formed at pH 9.0, no change in redox state from 100% semiquinone was observed when the pH was shifted to 7.5, suggesting that the requirement of high pH was for formation and not stability of the semiquinone. The rate of semiquinone formation exhibited a first-order dependence on the concentration of methylamine dehydrogenase, indicating that this phenomenon was a bimolecular process involving intermolecular electron transfer between reduced and oxidized cofactors. The rate of semiquinone formation decreased with decreasing ionic strength, suggesting a role for hydrophobic interactions in facilitating electron transfer between methylamine dehydrogenase molecules. Methylamine dehydrogenase was covalently modified with norleucine methyl ester in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). This modification did not affect the catalytic activity of the enzyme but greatly inhibited the intermolecular redistribution of electrons at high pH. This modification also prevented subsequent cross-linking by EDC of the large subunit of methylamine dehydrogenase to amicyanin, the natural electron acceptor for this enzyme. This suggests the possibility that a common domain of methylamine dehydrogenase may participate in stabilizing protein–protein interactions which facilitate both intermolecular electron transfer between quinone cofactors and transfer to the copper center of amicyanin.

The oxidation of most biologically relevant organic compounds results in the abstraction of two electrons from the substrate molecule. A key feature of biological energy metabolism is the ability to mediate electron transfer between the two-electron redox centers which participate in such reactions and one-electron carrier redox proteins which participate in the respiratory process. It is well established that flavoproteins may accomplish this task by assuming a radical, semiquinone redox state which is capable of undergoing one-electron oxidation or reduction reactions. The newly discovered redox cofactor pyrroloquinolinequinone (PQQ)[1] (Figure 1) also catalyzes two-electron oxidation–reduction reactions and is able to assume oxidized, reduced, and semiquinone redox states (Husain et al., 1987). PQQ is now known to be present in several diverse oxidoreductases which are referred to as quinoproteins [reviewed by Duine et al. (1987, 1989)]. Nearly all of the quinoproteins characterized thus far are symmetrical dimeric or oligomeric proteins with two PQQ prosthetic groups per holoenzyme. Quinoproteins do not require additional soluble cofactors and, depending upon the particular enzyme, donate electrons in vivo to either cytochromes *c*, copper proteins, or ubiquinone [reviewed by Anthony (1988)].

Methylamine dehydrogenase from *Paracoccus denitrificans* is a soluble periplasmic enzyme which catalyzes the oxidation of methylamine to formaldehyde plus ammonia. It possesses an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500 (Husain & Davidson, 1987). Each small subunit contains a covalently bound quinonoid cofactor which has been reported to be PQQ (van der Meer et al., 1987). Recent data, however, suggest that it may not be true PQQ but a unique *o*-quinone species which is structurally very similar to PQQ (Vellieux et al., 1989).[2] We have previously characterized the spectral properties of the three redox states of this enzyme (Husain et al., 1987) as well as many of its kinetic (Davidson, 1989; Davidson & Kumar, 1990) and physical (Husain & Davidson, 1987; Davidson & Neher, 1987; Chen et al., 1988) properties. The physiological electron acceptor for methylamine dehydrogenase is a type I copper protein, amicyanin (Husain & Davidson, 1985; Husain et al., 1986; Gray et al., 1986). These two proteins form a weakly associated complex which is necessary to allow electron transfer to the membrane-bound respiratory chain (Gray et al., 1988; Chen et al., 1988). Chemical cross-linking studies (Kumar & Davidson, 1990) have shown that the association between these proteins is stabilized by a combination of electrostatic and hydrophobic interactions. Contact between the large subunit of methylamine dehydrogenase and amicyanin is stabilized by hydrophobic interactions, whereas contact between the small subunit and amicyanin is stabilized by electrostatic interactions.

Questions as yet unanswered concern the role of the semiquinone in catalysis and electron transfer by methylamine dehydrogenase, and the mechanism by which this enzyme

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[1] Abbreviations: PQQ, pyrroloquinolinequinone; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; PES, phenazine ethosulfate; DCIP; 2,6-dichlorophenolindophenol; NME, norleucine methyl ester.

[2] L. Chen, F. S. Mathews, and V. L. Davidson, unpublished results.

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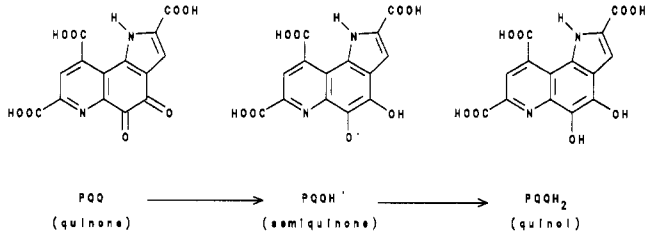

FIGURE 1: Proposed structure of the three redox forms of PQQ. The structure of oxidized PQQ is that deduced by Salisbury et al. (1979) from a crystalline derivative of PQQ.

which possesses a four-electron capacity (two cofactors) donates electrons to a one-electron carrier, amicyanin. This report describes an unusual mechanism of semiquinone formation by methylamine dehydrogenase from *P. denitrificans* which involves intermolecular electron transfer between reduced and oxidized cofactors. It also provides evidence that a common hydrophobic region on the surface of the enzyme is important in stabilizing protein–protein interactions that allow intermolecular electron transfer both between methylamine dehydrogenase molecules and from methylamine dehydrogenase to amicyanin. The physiological relevance of the semiquinone in catalysis and energy metabolism is also discussed.

Experimental Procedures

Methylamine dehydrogenase, amicyanin, and cytochrome *c*-551i were purified from *P. denitrificans* (ATCC 13543) as described previously (Husain & Davidson, 1985, 1986, 1987). Protein concentrations were calculated from previously determined extinction coefficients (Husain & Davidson, 1985, 1986; Husain et al., 1987). Antibodies to amicyanin were elicited from female New Zealand White rabbits which were immunized subcutaneously at 2-week intervals, with 1 mg of pure protein, until a sufficiently high titer of antibodies was attained. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was purchased from Pierce. Methylamine, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO), norleucine methyl ester (NME), phenazine ethosulfate (PES), and 2,6-dichlorophenolindophenol (DCIP) were obtained from Sigma.

Absorbance spectra were recorded with a Kontron Uvicon 810 spectrophotometer. Methylamine dehydrogenase activity was measured spectrophotometrically with a dye-linked assay in which the oxidation of methylamine was coupled to a change in the absorbance of a redox-sensitive dye, DCIP (Davidson, 1989). The ability of methylamine dehydrogenase and amicyanin to catalyze the methylamine-dependent reduction of cytochrome *c*-551i was assayed as described previously (Husain & Davidson, 1986). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli and Favre (1973) except for the inclusion of 0.5 M urea in the resolving and stacking gel, and 4 M urea and 4% SDS in the final sample buffer. Western blotting with alkaline phosphatase conjugated IgG as a secondary antibody was performed with Bio-Rad reagents and equipment according to the instructions of the manufacturer. Molecular weights were determined by comparison with Bio-Rad prestained low molecular weight standards.

In chemical modification experiments, protein samples were incubated for 60 min at room temperature with a 500-fold molar excess of NME and 5 mM EDC in 10 mM potassium phosphate buffer, pH 6.5. Two control incubations were run simultaneously. One control contained EDC but no NME, and the other contained NME but no EDC. After modification, free EDC and NME were separated from the protein by passage over a small Ultrogel AcA 202 (IBF Biotechnics) desalting column. Resolution of the large and small subunits of methylamine dehydrogenase was as previously described (Husain & Davidson, 1987). The extent of modification was quantitated from the amount of norleucine which was incorporated into the protein. This was determined by amino acid analysis of acid hydrolysates of the modified holoenzyme or subunits, which do not normally contain norleucine. Amino acid analysis was performed with a Beckman 119CL amino acid analyzer. Chemical cross-linking experiments with EDC were performed as described previously (Kumar & Davidson, 1990).

Results

pH-Dependent Semiquinone Formation. When methylamine was added to methylamine dehydrogenase at pH 7.5 in a 1:1 ratio (0.5:1 per cofactor), rapid changes in the visible absorbance spectrum were observed which corresponded to complete reduction of 50% of the enzyme-bound cofactor (Figure 2A). Addition of a second molar equivalent of methylamine caused rapid spectral changes which were indicative of complete reduction of the enzyme. No changes in these absorbance spectra were observed on standing for several hours under aerobic conditions. At pH 9.0, addition of methylamine to methylamine dehydrogenase in a 1:1 ratio also caused rapid reduction of 50% of the enzyme-bound cofactor. At this pH, however, the reduced species was not stable, and after standing for 15 min, an absorbance spectrum indicative of 100% of the semiquinone form of the enzyme was observed (Figure 2B). Addition of a second molar equivalent of methylamine caused little immediate change in the absorbance spectrum. Several hours were required to observe complete reduction of the semiquinone (Figure 2C). If, at pH 9.0, methylamine was initially added to methylamine dehydrogenase in a 2:1 ratio, the absorbance spectrum corresponding to the fully reduced form of the enzyme was immediately observed (Figure 2D). In contrast to what was observed at pH 7.5, both the semiquinone and fully reduced forms of the enzyme exhibited slow reoxidation under aerobic conditions at pH 9.0 (data not shown).

The rate of semiquinone formation was quantitated by measuring either the increase in absorbance with time at 425 nm or the decrease in absorbance with time at 325 nm. The time courses for the conversion of methylamine dehydrogenase from the 50% reduced plus 50% oxidized to 100% semiquinone form at pH values greater than 7.5 are shown in Figure 3. The rapid initial decrease in absorbance at 425 nm which was observed after addition of methylamine reflects the rapid reduction of 50% of the enzyme-bound cofactor. The subsequent increase in absorbance reflects the rate of semiquinone formation. At each pH, the initial reduction occurs instantly. The initial rate of semiquinone formation, however, increased with increasing pH values. Under these conditions at pH values of 7.5 and below, no semiquinone formation was observed. Identical results were obtained regardless of whether rates were determined from quantitation of changes in the complete absorbance spectra or from continuous measurements. To confirm that these phenomena were truly pH dependent, these experiments were also performed using NaAMPSO buffer in place of potassium phosphate buffer. Essentially identical results regarding the pH dependence of semiquinone formation were also obtained in this buffer system. Since the extinction coefficients of the major peaks in the absorbance spectrum of methylamine dehydrogenase varied with pH (discussed below), the initial rates in all figures are

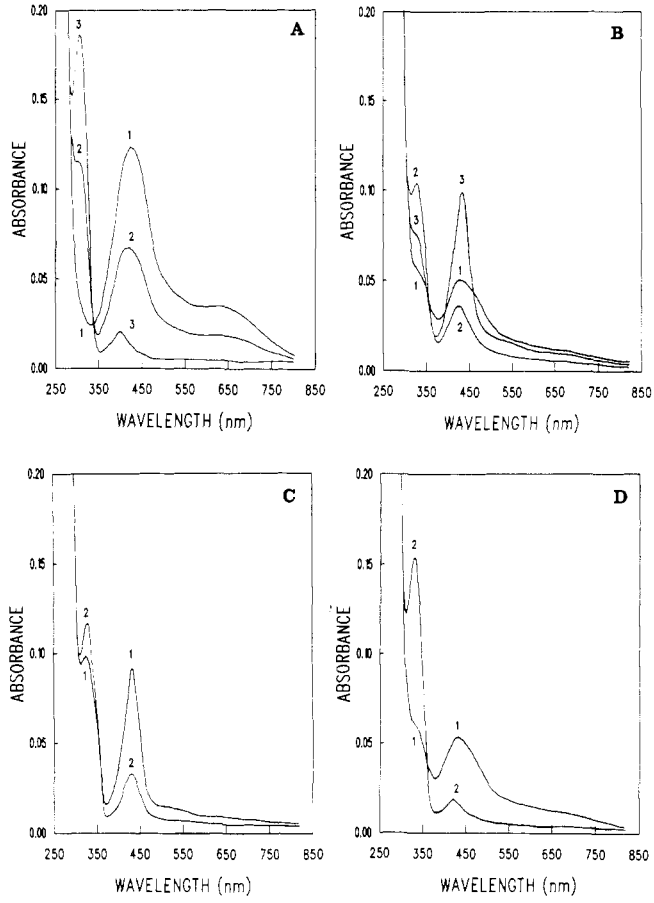

FIGURE 2: Changes in redox state caused by addition of methylamine to methylamine dehydrogenase. (A) Methylamine dehydrogenase (4.3 μM) was present in 50 mM potassium phosphate, pH 7.5. Spectra were recorded of oxidized methylamine dehydrogenase before treatment (1), 1 min after addition of 4.3 μM methylamine (2), and 1 min after a second addition of 4.3 μM methylamine (3). (B) Methylamine dehydrogenase (3.2 μM) was present in 50 mM potassium phosphate, pH 9.0. Spectra were recorded of oxidized methylamine dehydrogenase before treatment (1), 1 min after addition of 3.2 μM methylamine (2), and 15 min later with no subsequent additions (3). (C) A second molar equivalent of methylamine was added to the final sample shown in (B). Spectra were recorded 1 min after addition of 3.2 μM methylamine (1) and 180 min later with no subsequent additions (2). (D) Methylamine dehydrogenase (3.2 μM) was present in 50 mM potassium phosphate, pH 9.0. Spectra were recorded of oxidized methylamine dehydrogenase before treatment (1) and 1 min after addition of 6.4 μM methylamine (2).

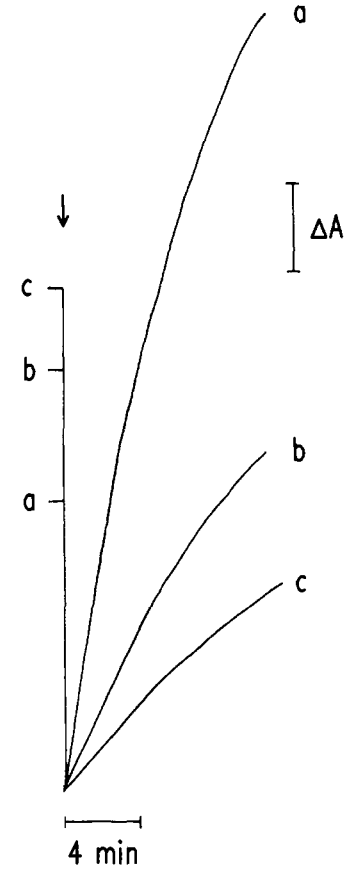

FIGURE 3: Time course of semiquinone formation by methylamine dehydrogenase at different values of pH. The assay mixtures contained methylamine dehydrogenase (3.6 μM) in 50 mM potassium phosphate, pH 9.0 (a), pH 8.5 (b), and pH 8.0 (c). In each experiment, the reaction was initiated by addition of 3.6 μM methylamine as indicated by the arrow. Changes in absorbance were monitored at 425 nm.

Table I: Absorption Properties of Methylamine Dehydrogenase

redox state	pH	extinction coefficient[a] (mM^{-1} cm^{-1}) at 330 nm	428 nm	440 nm
fully oxidized	7.5[b]	20.6	25.2	26.2
	9.0	13.6	14.5	14.2
semiquinone	7.5	25.2	50.4	32.4
	9.0	23.0	29.0	21.2
fully reduced	7.5	56.4	1.8	1.2
	9.0	41.6	3.8	3.0

[a] One mole of methylamine dehydrogenase refers to the 124-kDa complex which contains two cofactors per holoenzyme (Husain & Davidson, 1987). [b] Values for pH 7.5 are taken from Husain et al. (1987).

expressed as the percent change per minute, with 100% corresponding to the difference in absorbance at 425 nm observed between the 50% reduced plus 50% oxidized and 100% semiquinone forms of the enzyme.

Experiments were performed to ascertain whether pH was influencing the stability of the semiquinone form of methylamine dehydrogenase as well as the rate of its formation from the mixture of reduced and oxidized species. After generation of the 100% semiquinone form of the enzyme at pH 9.0, the pH of the solution was adjusted to 7.5. After adjustment of the pH, no change in the spectrum of the 100% semiquinone form of the enzyme was observed.

It was previously reported that the visible absorbance spectrum of oxidized methylamine dehydrogenase was perturbed at pH values greater than 7.5 (Davidson, 1989). The most notable changes were a decrease in intensity and slight shift in position of the broad peak centered at 440 nm. Analysis of the semiquinone and reduced forms of this enzyme at pH 9.0 indicated that for each redox state the general features of the absorbance spectrum of the protein-bound cofactor are essentially unchanged but in each case the intensities of the major peaks were reduced at higher pH (Table I).

To ensure that incubation of the enzyme at pH 9.0 had not irreversibly modified methylamine dehydrogenase, the 100% semiquinone form of the enzyme which was generated at that pH was assayed for activity at pH 7.5 by two steady-state kinetic methods. The rates were measured of methylamine-dependent reduction of DCIP by methylamine dehydrogenase and PES, and of methylamine-dependent reduction of cytochrome *c*-551i by methylamine dehydrogenase and amicyanin. The activities of the treated enzyme with either these nonphysiological or these physiological electron acceptors were indistinguishable from untreated controls.

Evidence for Intermolecular Electron Transfer. To ascertain whether the redistribution of electrons between the

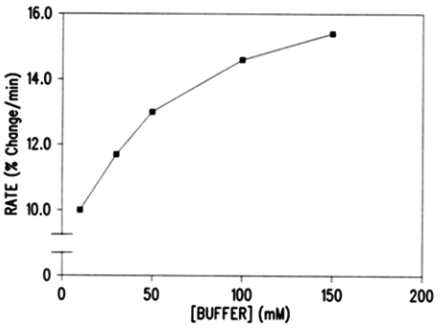

FIGURE 4: Dependence upon buffer concentration of the rate of semiquinone formation by methylamine dehydrogenase. Initial rates of semiquinone formation were determined from experiments similar to that shown in Figure 3. Each assay mixture contained methylamine dehydrogenase (3.2 μM) in potassium phosphate buffer, pH 9.0, at the indicated concentration of buffer. 100% corresponds to the difference in absorbance at 425 nm between the 50% reduced and 100% semiquinone species.

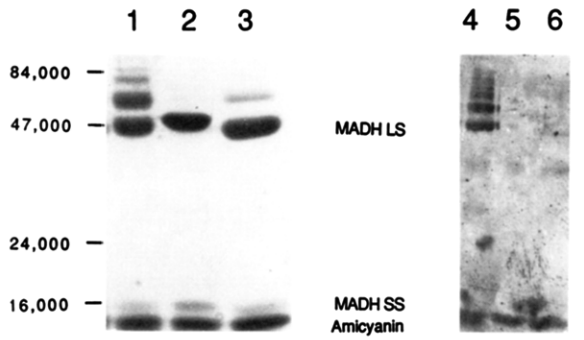

FIGURE 5: Inhibition by NME modification of cross-linking between methylamine dehydrogenase and amicyanin. EDC-treated mixtures of proteins were analyzed by SDS–PAGE (lanes 1–3) and Western blot (lanes 4–6) with antibody specific for amicyanin. Lanes 1 and 4 contained samples of methylamine dehydrogenase plus amicyanin which had been incubated at pH 6.5 in the presence of 5 mM EDC. Lanes 2 and 5 contained samples of NME-modified methylamine dehydrogenase and amicyanin which were incubated in the absence of EDC. Lanes 3 and 6 contained NME-modified methylamine dehydrogenase and amicyanin which were incubated in the presence of 5 mM EDC. The positions of molecular weight standards are indicated on the left, and the positions of migration of amicyanin and the large subunit (LS) and small subunit (SS) of methylamine dehydrogenase (MADH) are indicated.

quinone cofactors of methylamine dehydrogenase was intramolecular or intermolecular, the rate of semiquinone formation after addition of methylamine at a 1:1 ratio at pH 9.0 was measured with different concentrations of the enzyme. A linear relationship was observed between the rate of semiquinone formation and the concentration of methylamine dehydrogenase (data not shown), indicating that this phenomenon was a bimolecular process.

Experiments were performed to explore the possibility that either electrostatic or hydrophobic interactions between protein molecules might facilitate the apparent intermolecular electron transfer between cofactors. The dependence of this process on ionic strength was determined by measuring the rate of semiquinone formation at a fixed concentration of methylamine dehydrogenase at pH 9.0 in different concentrations of buffer. A decrease in rate with decreasing ionic strength was observed (Figure 4), suggesting a role for hydrophobic protein–protein interactions in this bimolecular process.

NME Modification of Methylamine Dehydrogenase. It had been shown previously (Kumar & Davidson, 1990) that the large subunit of methylamine dehydrogenase and amicyanin could be covalently cross-linked in the presence of EDC and that this association was stabilized at least in part by hydrophobic interactions between the proteins. This cross-linked species was formed by generating amide bonds between one or more carboxylic amino acid residues of one protein with one or more positively charged residues of the other protein. These charged groups were apparently located in close proximity to the hydrophobic regions that stabilized this protein–protein interaction. To further characterize this domain of the protein, those carboxylic acid residues on methylamine dehydrogenase which participated in the cross-linking with amicyanin were modified with NME. The process is initiated by reaction of an accessible carboxylic amino acid residue with EDC to form an acylisourea intermediate (Carraway & Koshland, 1972). In the presence of an excess of a reactive nucleophile, in this case the amino nitrogen of NME, the activated carboxyl group further reacts to form a covalent amide bond. This amide bond will be cleaved during acid hydrolysis of the protein and yield 1 mol of norleucine for each mole of amino acid which has been modified, and as such the extent of modification can be determined by amino acid analysis of the hydrolysate.

NME modification of methylamine dehydrogenase completely inhibited the ability to subsequently cross-link this enzyme to amicyanin in the presence of EDC (Figure 5). This was evident from the greatly reduced intensity of the species at approximately 60 kDa in the Coomassie blue stained gel, and from the absence of a cross-reactive species of that molecular weight in the Western blot. Analogous treatment of amicyanin alone with EDC and NME had no effect on the ability to subsequently cross-link this protein to methylamine dehydrogenase. Amino acid analysis of the modified methylamine dehydrogenase indicated that, under the conditions used, approximately 5 mol of norleucine was incorporated into each mole of the holoenzyme. Analysis of the individual subunits of methylamine dehydrogenase which had been separated after modification of the holoenzyme indicated that approximately 2.2 and 0.3 mol of norleucine had been incorporated, respectively, into the large and small subunits of the enzyme. Thus, it appears likely that two carboxylic amino acid residues of the large subunit of methylamine dehydrogenase, which are located near the site of interaction with amicyanin, are responsible for the EDC-activated cross-linking of the large subunit to amicyanin.

Redox and Kinetic Properties of NME-Modified Methylamine Dehydrogenase. The absorbance spectra of the oxidized, reduced, and semiquinone forms of NME-modified methylamine dehydrogenase were identical with those of the unmodified enzyme. When methylamine was added to NME-modified methylamine dehydrogenase at pH 9.0 in a 1:1 ratio, rapid reduction of 50% of the enzyme-bound cofactor was observed. These spectral changes were identical with that observed for the unmodified enzyme in Figure 2B. However, in contrast to what was seen with unmodified methylamine dehydrogenase, no immediate change in this spectrum indicative of semiquinone formation was observed. The rates of semiquinone formation of the native and NME-modified methylamine dehydrogenase were measured as in Figure 3. The observed rate of semiquinone formation by the modified enzyme was approximately 2.5% of that of the native enzyme. From these data, it is evident that the intermolecular redistribution of electrons was greatly inhibited by the NME modification of the enzyme.

To ensure that NME modification had not caused a general nonspecific alteration of the enzyme, the catalytic and electron-transfer activities of NME-modified methylamine dehydrogenase were assayed by two steady-state kinetic methods.

The initial rates of methylamine-dependent reduction of DCIP by methylamine dehydrogenase and PES, which reflect the catalytic competence of the enzyme, were essentially unaffected by the modification (data not shown). The rates of methylamine-dependent reduction of cytochrome *c*-551i by methylamine dehydrogenase and amicyanin, which further reflect the ability of the enzyme to donate electrons to its physiological redox partner, were inhibited by the modification. To determine the nature of this effect, initial rates of this activity were measured at varying concentrations of amicyanin in the presence of saturating levels of methylamine and cytochrome *c*-551i. Analysis of double-reciprocal plots of these data indicated that NME modification had little effect on the K_{cat} for this process but caused approximately a 3-fold increase in the K_m of amicyanin. These results are consistent with the cross-linking experiment described above in suggesting that modification of key carboxyl residues of methylamine dehydrogenase has inhibited its ability to interact with amicyanin, as well as the intermolecular transfer of electrons between quinone cofactors.

Discussion

Previous studies of the redox behavior of *P. denitrificans* methylamine dehydrogenase (Husain et al., 1987), which were performed at pH 7.5, described a monophasic redox titration with substrate from the fully oxidized to fully reduced form of the enzyme. The semiquinone form was only observed during a redox titration with sodium dithionite, and reduction of the semiquinone during the second half of that titration was quite slow. These data indicated that the semiquinone was stable once formed and suggested a kinetic barrier to further reduction. The data presented here are completely consistent with that study and provide new evidence that at higher pH an unusual redistribution of electrons occurs between quinone cofactors. These data suggest that electron transfer occurs between an oxidized cofactor of one enzyme and a reduced cofactor of another enzyme to generate two stable semiquinone forms of the enzyme-bound cofactor. This could occur by one of two scenarios: reaction of one molecule each of fully oxidized and fully reduced methylamine dehydrogenase (eq 1) or reaction of two molecules of enzyme each of which possesses one oxidized and one reduced cofactor (eq 2). In each case,

$$Q\text{–}E\text{–}Q + QH_2\text{–}E\text{–}QH_2 \rightarrow Q\text{–}E\text{–}QH^{\bullet} + {}^{\bullet}QH\text{–}E\text{–}QH_2 \rightarrow 2\,{}^{\bullet}QH\text{–}E\text{–}QH^{\bullet} \quad (1)$$

$$Q\text{–}E\text{–}QH_2 + Q\text{–}E\text{–}QH_2 \rightarrow Q\text{–}E\text{–}QH^{\bullet} + {}^{\bullet}QH\text{–}E\text{–}QH_2 \rightarrow 2\,{}^{\bullet}QH\text{–}E\text{–}QH^{\bullet} \quad (2)$$

two molecules of the semiquinone are generated. The data described herein clearly indicated that ionizable groups on the structural protein play a critical role in the intermolecular redistribution of electrons and stabilization of the semiquinone species. The pronounced effect of pH on the spectral properties of the enzyme (Table I) further suggests that pH-dependent changes in the cofactor may also contribute to the phenomena which were observed.

The suggestion that electron transfer between cofactors is an intermolecular rather than an intramolecular process is reasonable in light of recent structural data obtained from X-ray crystallographic studies of methylamine dehydrogenases[2] (Vellieux et al., 1989). The active site and covalently bound cofactor are located near the surface of the enzyme at an interface region between one large and one small subunit of this $\alpha_2\beta_2$ oligomer. The intramolecular distance between the cofactors of this symmetrical molecule is approximately 45 Å, whereas the distance between the cofactor and the surface of the enzyme is only about 5 Å.[2] Our laboratory has been studying the mechanism of intermolecular electron transfer between methylamine dehydrogenase and another soluble redox protein, its physiological electron acceptor, amicyanin (Gray et al., 1988). Chemical cross-linking studies of these proteins indicated that complex formation was stabilized in part by hydrophobic interactions between the large subunit of methylamine dehydrogenase and amicyanin (Kumar & Davidson, 1990). The observed ionic strength dependence for semiquinone formation suggests that such interactions also play a role in stabilizing the complex formation between methylamine dehydrogenase molecules which allows intermolecular electron transfer between cofactors. The chemical modification studies further suggest the possibility that a common hydrophobic region of the large subunit of the enzyme may be responsible for stabilizing each of these protein–protein interactions. The data do not, however, completely rule out the possibility that different domains could be involved.

These data may also resolve certain inconsistencies in the literature regarding another methylamine dehydrogenase, that from bacterium W3A1. Kenny and McIntire (1983) reported reduction by substrate of this enzyme to the semiquinone form and proposed a catalytically relevant intramolecular comproportionation of reduced and oxidized cofactors. Much of the data shown in that paper was obtained at pH 9.0. Subsequent studies of the same enzyme by McWhirter and Klapper (1987, 1989) yielded no evidence for rapid semiquinone formation or for a catalytically relevant role for the semiquinone in the reductive half-reaction of this enzyme. The data presented here clearly show that for the *P. denitrificans* enzyme semiquinone formation is a bimolecular process.

The observed dependence of semiquinone formation on high pH raises questions as to the physiological relevance of this reaction. Methylamine dehydrogenase is a periplasmic enzyme. The actual pH of the periplasmic space has never been measured. *P. denitrificans* is a soil bacterium and in the laboratory is grown in a minimal medium which is weakly buffered at neutral pH by potassium phosphate. One would assume that the phosphate ion which is accessible to the periplasm via porins would buffer that space as well. However, given the influence on the process of semiquinone formation of enzyme concentration, pH, and ionic strength, one cannot rule out the possibility that these in vitro requirements are mimicking some in vivo localized environment or conformation of the enzyme which encourages semiquinone formation under physiological conditions. That methylamine does not readily reduce the semiquinone strongly suggests an important role for this redox form in the oxidative half-reaction of methylamine dehydrogenase with amicyanin, rather than the reductive half-reaction with methylamine.

By virtue of its interaction with the copper protein amicyanin, methylamine dehydrogenase has already proven to be a unique and powerful subject for the study of the phenomenon of intermolecular electron transfer between proteins. Preliminary crystallographic data are available for methylamine dehydrogenase (Chen et al., 1988), for amicyanin (Lim et al., 1986), and for a complex of methylamine dehydrogenase and amicyanin (Chen et al., 1988). In the near future, the precise structures of these proteins free and in complex should be known, and it will be possible to make direct correlations between structure and function. The phenomenon described here of intermolecular electron transfer between the quinone cofactors of methylamine dehydrogenase provides another opportunity to use this enzyme to define the factors which allow long-range electron transfer between protein-bound redox

centers, and which influence interactions between soluble proteins.

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Stabilization of Clathrin Coats by the Core of the Clathrin-Associated Protein Complex AP-2†

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ABSTRACT: AP-2 is the class of clathrin-associated protein complex found in coated vesicles derived from the plasma membrane of eukaryotic cells. We demonstrate here, using a chemical method, that an AP-2 complex is an asymmetric structure consisting of one large α chain, one large β chain, one medium AP50 chain, and one small AP17 chain. The complex has been shown to contain a core and two appendages. The AP core includes the small AP17 and the medium AP50 chains together with the amino-terminal domains of the large α and β chains. One appendage corresponds to the carboxy-terminal domain of the β chain. We find that as in the case of the β chains, the carboxy-terminal portion of the α chains is an independently folded domain corresponding to the second appendage. We use limited tryptic proteolysis of clathrin/AP-2 coats to show the release of the appendages from the interior of the coats and the retention of the AP core by the remaining clathrin lattice. In addition, we find that the AP core stabilizes the coat and prevents its depolymerization. These results are consistent with the proposal that the AP core contains the binding site(s) for clathrin, while the α- and β-chain appendages interact with membrane components of coated pits and coated vesicles.

Clathrin-coated pits and coated vesicles are organelles that serve to concentrate membrane proteins and ligands destined for vesicular membrane traffic [reviewed in Goldstein et al. (1985) and Pfeffer and Rothman (1987)]. The major constituents of the coat are clathrin and its associated protein complexes (APs), which together represent about 90% of the protein content. It seems likely that these components act as a molecular trap or filter, to recruit and retain proteins selectively in the coated pit (Pearse & Bretsher, 1981). In addition, it is thought that assembly of the clathrin lattice actually drives the vesiculation step, transforming a coated pit into a coated vesicle (Harrison & Kirchhausen, 1983). It is not yet known which of the components of the coat allow the uptake to be selective but the properties of the AP complexes

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