Redox Properties of the Quinoprotein Methylamine Dehydrogenase from *Paracoccus denitrificans*†

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ABSTRACT: *Paracoccus denitrificans* synthesizes a methylamine dehydrogenase that contains a covalently bound form of pyrroloquinoline quinone as a prosthetic group [Husain, M., & Davidson, V. L. (1987) *J. Bacteriol. 169*, 1712–1717]. Anaerobic reductive titration of this enzyme with dithionite proceeded through a semiquinone intermediate with spectral properties quite distinct from those of the oxidized and reduced species. From these data the molar extinction coefficients were calculated at various wavelengths for the three redox states of this enzyme. The semiquinone was slowly reoxidized under aerobic conditions. The fully reduced enzyme was stable in the presence of oxygen and slowly reoxidized by ferricyanide. Reductive titration of methylamine dehydrogenase with methylamine proceeded directly to the fully reduced form of the enzyme without detectable formation of the semiquinone. Electrochemical titrations of the enzyme yielded an overall midpoint potential value for the two-electron couple (fully oxidized/fully reduced) of 100 ± 4 mV and an n value of 2.15 ± 0.15.

It has been established recently that certain oxidoreductases from a variety of sources contain PQQ[1] as a prosthetic group (Duine & Frank, 1981; Ameyama et al., 1981; Anthony, 1982; Duine et al., 1986). These quinoproteins include bacterial methanol (Duine & Frank, 1979) and glucose (Duine et al., 1979) dehydrogenases, which possess noncovalently associated PQQ, and bacterial methylamine dehydrogenase, which contains a covalently bound form of PQQ (De Beer et al., 1980; Kenny & McIntire, 1983; Husain & Davidson, 1987). Mammalian plasma amine oxidase (Lobenstein-Verbeek et al., 1986; Moog et al., 1986), lysyl oxidase (van der Meer & Duine, 1986; Williamson et al. 1986), and choline dehydrogenase (Ameyama et al., 1985) also possess covalently attached PQQ. Very little is known about the catalytic mechanisms of these quinoproteins and their interaction with other redox proteins. While several quinoproteins have now been isolated, information concerning oxidation–reduction properties has thus far been obtained only for one PQQ-containing enzyme, the methylamine dehydrogenase of bacterium W3A1 (Kenny & McIntire, 1983). While the oxidation–reduction potentials for free PQQ and some analogues of PQQ have been measured (Duine et al., 1981; Eckert et al., 1982; Sleath et al., 1985; Faraggi et al., 1986), no E_m values of PQQ-containing proteins have previously been reported.

When grown on methylamine as a sole source of carbon and energy, *Paracoccus denitrificans* synthesizes a methylamine dehydrogenase (Husain & Davidson, 1987) which functions in the periplasm of this gram negative bacterium and donates electrons to a periplasmic Type I blue copper protein, amicyanin (Husain & Davidson, 1985). This enzyme was isolated as a complex of two 47-kDa subunits and two 15-kDa subunits that possessed covalently bound PQQ. We have previously characterized the properties of methylamine dehydrogenase (Husain & Davidson, 1987), amicyanin (Husain & Davidson, 1985; Husain et al., 1986; Lim et al., 1986), and periplasmic c-type cytochromes (Husain & Davidson, 1986) from *P. denitrificans* and have measured the oxidation–reduction potentials of amicyanin and c-type cytochromes that accept electrons from methylamine dehydrogenase via amicyanin (Gray et al., 1986). To gain further insight into the mechanism of intermolecular electron transfer between methylamine dehydrogenase and amicyanin and to better understand the redox properties of quinoproteins in general, we have characterized spectrally the fully oxidized, semiquinone, and fully reduced forms of *P. denitrificans* methylamine dehydrogenase and have measured the E_m value of the fully oxidized/fully reduced couple of this representative quinoprotein.

Experimental Procedures

Methylamine dehydrogenase was purified from *P. denitrificans* (ATCC 13543) as described previously (Husain & Davidson, 1987). Anaerobic reductive titrations were performed according to Williams et al. (1979) in a Thunberg-type cuvette equipped with a gastight syringe. Absorption spectra were recorded with a Cary 219 spectrophotometer. The absorption spectra were calibrated for wavelength accuracy with horse heart cytochrome c, which exhibited an α-band maximum at 550.0 nm. Electrochemical titrations were performed as previously described (Smith et al., 1981) by using an optically transparent gold electrode in a thin-layer cell. Absorption spectra for the electrochemical titrations were recorded in 50 mM potassium phosphate buffer, pH 7.5, at 5

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[1] Abbreviations: PQQ, pyrroloquinoline quinone; E_m, midpoint potential; EDTA, ethylenediaminetetraacetic acid.

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Table I: Absorption Properties of Methylamine Dehydrogenase

redox state	extinction coefficient[a] (mM^{-1} cm^{-1}) at 330 nm	364 nm	428 nm	440 nm	460 nm
fully oxidized	20.6	6.4	25.2	26.2	24.4
semiquinone	25.2	6.0	50.4	32.4	11.2
fully reduced	56.4	6.0	1.8	1.2	0.8

[a] One mole of methylamine dehydrogenase refers to the 124-kDa complex that contains 2 mol of PQQ (Husain & Davidson, 1987).

°C with an Aminco DW-2a UV–vis spectrophotometer. Methylamine dehydrogenase (30 mg/mL) was titrated in the presence of 10 μM duroquinone, 10 μM 1,2-naphthoquinone, 20 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride, 5 μM phenazine methosulfate, and 5 μM phenazine ethosulfate. The oxidation states of the protein were determined by monitoring a ΔA at two wavelengths, 444 and 360 nm, using the isosbestic point at 360 nm as a reference. Control titrations, performed with horse heart cytochrome *c*, methylviologen, and spinach ferredoxin, gave E_m values within 5 mV of literature values. Protein concentrations were determined by the method of Bradford (1976) with Pierce protein assay reagent using bovine serum albumin as a standard.

Results

Reductive Titrations of P. denitrificans Methylamine Dehydrogenase. The visible absorption spectrum of the fully oxidized form of methylamine dehydrogenase exhibited a peak centered at 440 nm, a shoulder at 326 nm, and significant absorbance between 500 and 800 nm. The titration of this enzyme with dithionite proceeded through two phases (Figure 1). The first phase, which consumed 0.99 mol of dithionite per mole of enzyme, resulted in the formation of a spectrally distinct half-reduced semiquinone intermediate. Formation of the semiquinone was essentially quantitative as judged by the preservation of isosbestic points at 380, 444, and 756 nm (Figure 1A) and by the linear relationship between the absorbance changes and the amount of dithionite added (Figure 1C). The transition from the fully oxidized enzyme to the radical form was characterized by a significant increase in absorbance of the major peak and concomitant shift to an absorbance maximum at 428 nm (Figure 1A). Approximately half of the absorbance at longer wavelengths was lost, and a slight increse in absorbance at 326 nm was observed. During the first phase of the titration, changes in absorbance caused by the addition of each increment of dithionite occurred very rapidly. The spectra shown in Figure 1A were recorded immediately after dithionite addition, and no additional changes were detected after prolonged incubation. The second phase of the dithionite titration (Figure 1B), which consumed an additional 1.06 mol of dithionite per mole of enzyme, resulted in formation of the fully reduced species. An isosbestic point was maintained at 364 nm (Figure 1B), and absorbance changes were again linear with respect to the amount of dithionite added (Figure 1C). The second phase of the titration proceeded much more slowly than the first. Progressively longer times were required to observe maximum changes in absorbance after each addition of dithionite. These times ranged from approximately 10 min for the first addition to 45 min for the last addition. The reduction of the radical form to the fully reduced species was characterized by the loss of most of the absorbance at 428 nm, loss of the remaining absorbance at longer wavelengths, and significant increase in absorbance at 326 nm. The complete titration consumed 2.05 mol of dithionite. The molar extinction coefficients, calculated

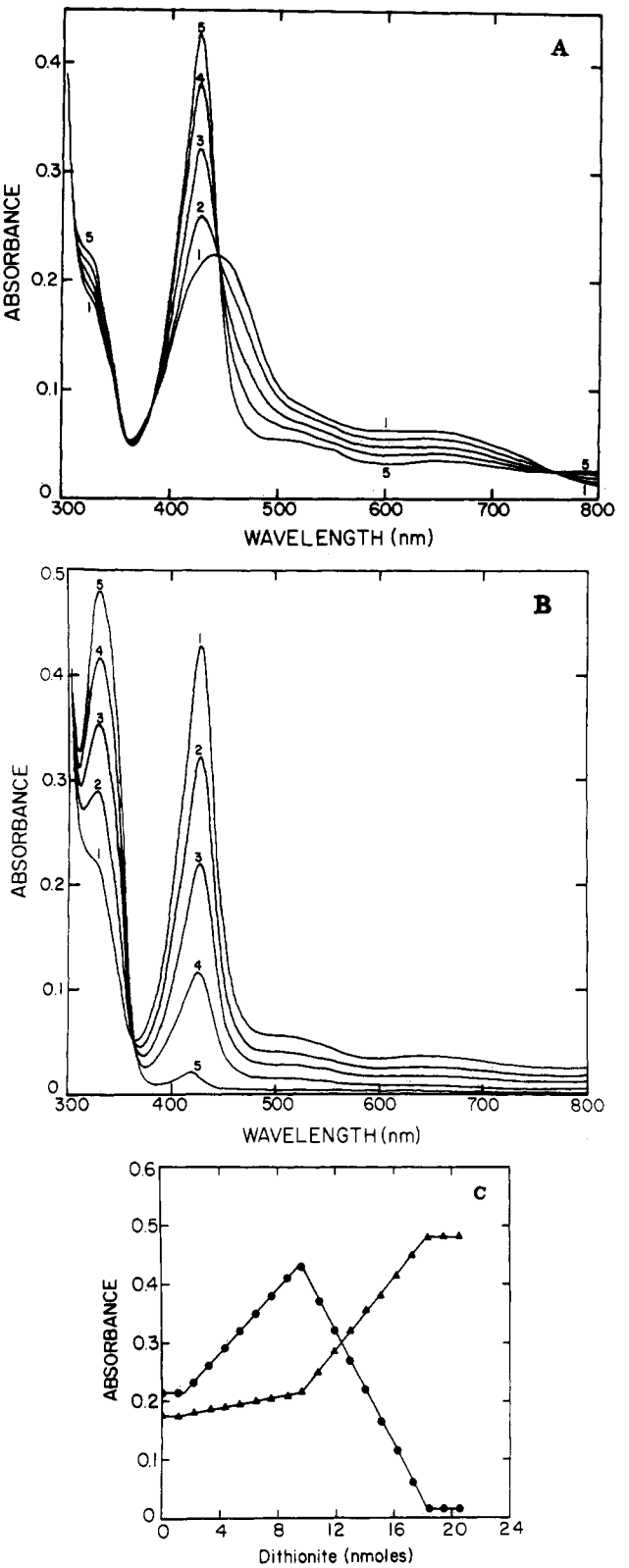

FIGURE 1: Dithionite titration of methylamine dehydrogenase. Methylamine dehydrogenase (8.3 nmol in 1 mL) in 50 mM potassium phosphate, pH 7.5, was deoxygenated and titrated with an anaerobic solution of 0.54 mM sodium dithionite at 25 °C. (A) Quantitative reduction of oxidized methylamine dehydrogenase to the semiquinone required 8.2 nmol of dithionite. Spectra were recorded after addition of (curve 1) 0, (curve 2) 3.2, (curve 3) 5.4, (curve 4) 7.6, and (curve 5) 9.7 nmol of dithionite. (B) Quantitative reduction of the semiquinone to fully reduced methylamine dehydrogenase required an additional 8.8 nmol of dithionite. Spectra were recorded after addition of (curve 1) 9.7, (curve 2) 11.9, (curve 3) 14.0, (curve 4) 16.2, and (curve 5) 18.4 nmol of dithionite. (C) Data for the complete titrations at (●) 428 nm and (▲) 330 nm are given.

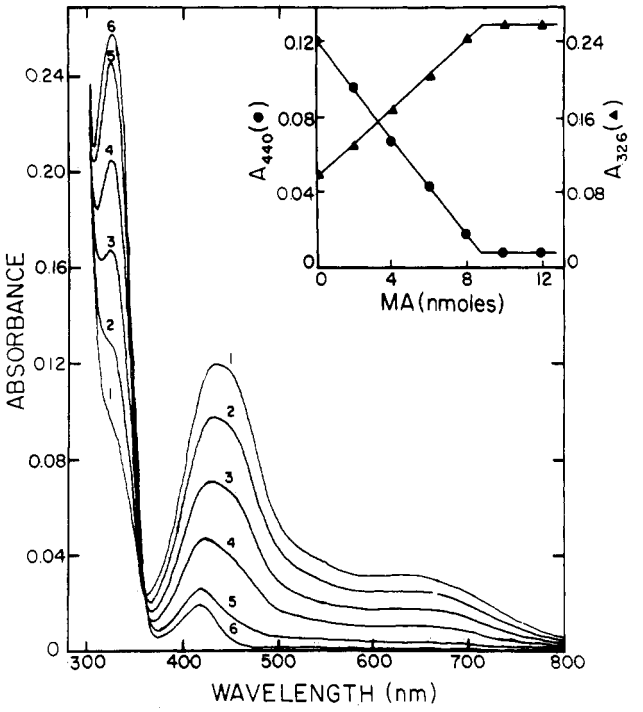

FIGURE 2: Substrate titration of methylamine dehydrogenase. Methylamine dehydrogenase (4.6 nmol in 1 mL) in 50 mM potassium phosphate, pH 7.5, was deoxygenated and titrated with an anaerobic solution of 1.0 mM methylamine hydrogen chloride at 25 °C. Spectra were recorded after addition of (curve 1) 0, (curve 2) 2.0, (curve 3) 4.0, (curve 4) 6.0, (curve 5) 8.0, and (curve 6) 10.0 nmol of methylamine hydrogen chloride. Data for the titration are shown in the inset. In the complete reduction, 8.8 nmol of methylamine hydrogen chloride were consumed in the complete reduction.

from the data obtained during these titrations, at selected wavelengths for each of the three redox states of the enzyme are given in Table I.

Whereas the titration of methylamine dehydrogenase with dithionite proceeded through a distinct semiquinone intermediate, the titration of this enzyme with methylamine proceeded directly from the fully oxidized to the fully reduced species (Figure 2). Addition of substoichiometric amounts of methylamine (Figure 2) to methylamine dehydrogenase caused a progressive bleaching of absorbance beyond 500 nm, an increase in absorbance at 326 nm, and a bleaching of absorbance at 440 nm. The latter effect was accompanied by a shift to lower wavelength of the absorbance maximum of that peak. The final spectrum obtained from the methylamine titration was nearly identical with that obtained in the dithionite titration. The maximum change in absorbance caused by the addition of each increment of methylamine occurred very rapidly. Spectra were recorded immediately after each addition. No further absorbance changes occurred after prolonged incubation. An isosbestic point was maintained at 360 nm, and absorbance changes were linear throughout the titration (Figure 2). Identical results were obtained for titrations performed under aerobic and anaerobic conditions. The complete titration consumed 1.9 mol of methylamine per mole of enzyme. The data obtained from the dithionite and substrate titrations of *P. denitrificans* methylamine dehydrogenase confirm the presence of 2 mol of PQQ per mole of enzyme.

The fully reduced form of methylamine dehydrogenase was extremely stable against reoxidation. No reoxidation of the fully reduced enzyme was observed under aerobic conditions. Addition of ferricyanide to the reduced enzyme caused a very slow reoxidation of the enzyme to the fully oxidized species

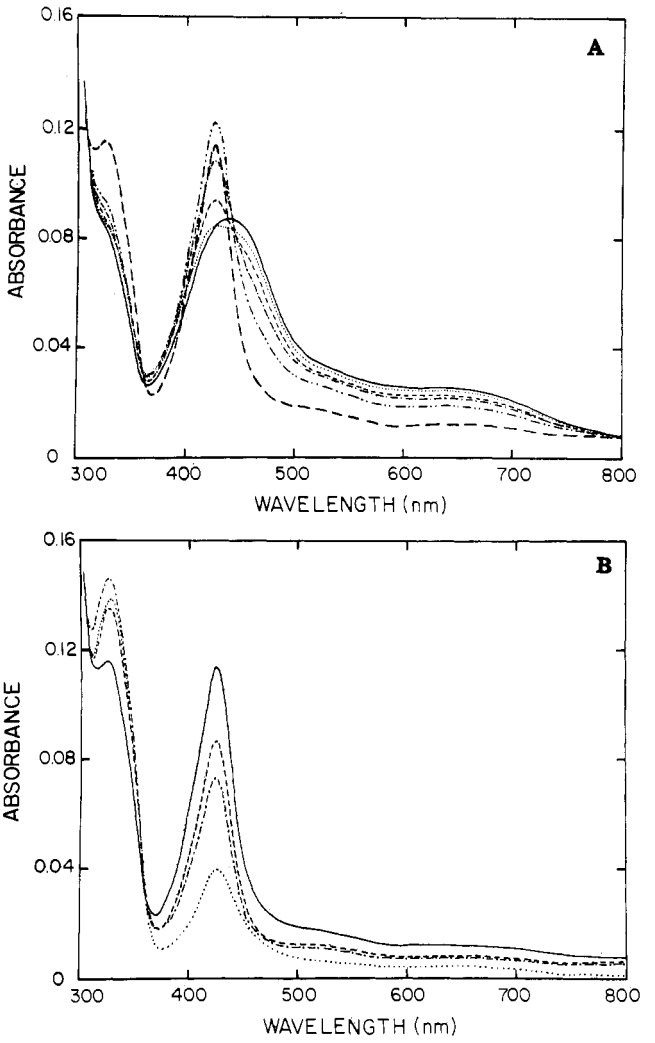

FIGURE 3: Photochemical reduction of methylamine dehydrogenase. Methylamine dehydrogenase (3.2 μM) was incubated anaerobically with 15 mM EDTA and 1 μM 5-deazariboflavin in 1 mL of 50 mM potassium phosphate, pH 7.0. The sample was cooled to 10 °C and exposed to light from a 650-W tungsten–halogen lamp, which was placed approximately 10 cm from the sample. (A) The absorption spectra of oxidized methylamine dehydrogenase recorded before (—) and after (···) addition of 5-deazariboflavin and after exposure to light for 5 s (---), 10 s (—), 15 s (—·), and 25 s (——). (B) Absorption spectra recorded after exposure to light for 25 s (—), 45 s (---), 4 min (—·—), and 10 min (···).

without formation of the semiquinone as an intermediate (data not shown). Maximum changes in absorbance caused by the addition of ferricyanide required 1–4 h. The semiquinone form of the enzyme was slowly reoxidized on exposure to air. Approximately 50% of the semiquinone species was reoxidized after exposure to air for 6 h.

Since the reductive titrations of this enzyme by substrate and dithionite proceeded via different routes, the redox properties of methylamine dehydrogenase were further examined by performing a photochemical reduction of the enzyme in the presence of EDTA and 5-deazariboflavin, a system which has been successfully used to fully reduce many redox proteins (Massey & Hemmerich, 1978). It is apparent from the spectral changes observed (Figure 3) that some semiquinone was formed. However, before the radical species was generated in quantitative amounts, it underwent further reduction to the fully reduced form, as indicated by the loss of the isosbestic point at 444 nm and the significant increase in absorbance at 326 nm (Figure 3A). Continued irradiation (Figure 3B) caused a substantial decrease in absorbance at

428 nm, consistant with the formation of the fully reduced enzyme. The expected concomitant increase in absorbance at 326 nm was, however, not observed. Instead of a steady increase, the absorbance at 326 nm first increased and then decreased with longer times of irradiation. This behavior was most likely the result of a photochemical reaction that occurred in addition to the photochemical reduction after long periods of irradiation.

Measurement of the Oxidation–Reduction Potentials of Methylamine Dehydrogenase. The electrochemical titration of *P. denitrificans* methylamine dehydrogenase was fully reversible. Identical E_m values were obtained over a 2-fold range of mediator concentrations, and these values were independent of the mediators used. The average E_m value calculated from five independent titrations for the two-electron couple (fully oxidized/fully reduced) was 100 ± 4 mV. The average n value for these titrations was 2.15 ± 0.15. The spectral changes observed during the reductive electrochemical titrations were essentially identical with those in Figure 2. As in the substrate titration of methylamine dehydrogenase, the semiquinone form of the enzyme was not observed during either the reductive or oxidative titrations.

Discussion

Prior to this work, the methylamine dehydrogenase of bacterium W3A1 was the only quinoprotein with which reductive titrations had been performed (Kenny & McIntire, 1983). The data obtained in substrate and dithionite titrations of the *P. denitrificans* enzyme (Figures 1 and 2), while similar in many respects, reflect significant differences between the two enzymes. The most striking difference is that for the bacterium W3A1 enzyme: reductions by dithionite and methylamine were each relatively slow and each proceeded through a stable semiquinone intermediate. That the semiquinone was formed during reduction by a two-electron donor, methylamine, was unexpected and explained by a scheme involving comproportionation between one fully reduced and one fully oxidized PQQ to form the stable radicals (Kenny & McIntire, 1983). Conversely, reduction of the *P. denitrificans* enzyme by methylamine was rapid and monophasic, and isosbestic points were maintained throughout the titration. This indicates that the two PQQ cofactors in the holoenzyme are in identical or very similar environments and that comproportionation does not occur as the fully oxidized enzyme was converted directly to the fully reduced form without detectable formation of the semiquinone form.

The formation of a stable semiquinone intermediate during the dithionite titration and the relatively slow rate of reduction of the semiquinone during the second phase of this titration indicated that a barrier existed to the transfer of the second electron. The measured redox midpoint potential of 100 mV, which is much more electropositive than that of dithionite, suggests that the barrier is kinetic rather than thermodynamic. One possible reason that the semiquinone species was not detected during electrochemical titrations is that several mediators were present which could facilitate the transfer of the second electron. To examine this possibility, catalytic amounts of mediator dyes were added to the semiquinone that had been generated by titration with dithionite. Consistent with the above-mentioned hypothesis, no semiquinone was detected after addition of the mediators (data not shown). Partial, rather than quantitative, formation of the semiquinone during photoreduction of the enzyme could also be explained by the presence of free flavin, which could act as a mediator to facilitate transfer of the second electron and overcome the kinetic barrier.

The E_m value for the oxidized/reduced couple of *P. denitrificans* methylamine dehydrogenase of 100 mV is very close to the value of 90 mV reported (Duine et al., 1981) for free PQQ at neutral pH in aqueous solution. Similar values have also been obtained for synthetic structural analogues of PQQ at neutral pH (Eckert et al., 1982; Sleath et al., 1985). The redox potentials of free PQQ and PQQ analogues measured in aprotic solvents are quite different from those measured in aqueous solution (Eckert et al., 1982). That the redox potential for the protein-bound PQQ of methylamine dehydrogenase is so similar to that of the free cofactor suggets that (i) the protein environment that surrounds PQQ does not appreciably affect its redox potential and (ii) that the PQQ moiety resides in a relatively hydrophilic environment. The latter suggestion is quite reasonable in view of the fact that the electron acceptor for methylamine dehydrogenase is another soluble redox protein, amicyanin (Husain & Davidson, 1985). thermodynamically, the measured E_m value of 100 mV for methylamine dehydrogenase is also consistant with its role as an electron donor to amicyanin, which has an E_m value of 296 mV (Gray et al., 1986). As no detectable semiquinone was formed during the electrochemical titrations, it was not possible to measure the potentials of the two one-electron transfers, oxidized/semiquinone and semiquinone/reduced. It should also be noted that, whereas the fully reduced form of free PQQ is rapidly reoxidized in solution, the reduced and semiquinone forms of PQQ bound to methylamine dehydrogenase are quite stable against reoxidation by air. Thus, the protein environment of PQQ may play a significant role in stabilizing the semiquinone or reduced forms of PQQ or both.

Recent work by McIntire and Stults (1986) indicated that the exact structure of the covalently attached cofactor of the methylamine dehydrogenase of bacterium W3A1 was not identical with that of free PQQ, which is secreted by methylotrophic bacteria and found noncovalently associated in methanol dehydrogenases. Specifically, three carboxyl groups, which are present on the tricyclic PQQ nucleus of free PQQ, are apparently absent in the covalently bound form of PQQ in the bacterium W3A1 enzyme. This may also be true for the covalently bound PQQ of the *P. denitrificans* enzyme. If so, the data presented here would suggest that the presence of absence of those carboxyl groups does not significantly affect the redox potential of the PQQ cofactor.

The realization that PQQ is the cofactor of a large number of prokaryotic and eukaryotic oxidoreductases makes it very important to understand the mechanisms by which this family of enzymes functions. Methylamine dehydrogenase from *P. denitrificans* is a particularly good subject for the study of the redox properties of quinoproteins in that (i) it can be easily purified in large quantities (Husain & Davidson, 1987); (ii) its physiological role is well-defined (Anthony, 1982); and (iii) the properties of its natural electron acceptor, amicyanin, have been characterized in great detail (Husain & Davidson, 1985; Husain et al., 1986; Lim et al., 1986). Recent studies which raised the possibility that methylamine dehydrogenase and amicyanin may interact in a manner which significantly affects the redox potential of amicyanin (Gray et al., 1986) are particularly intriguing in that mammalian PQQ-containing amine oxidases also possess bound copper. Now that the redox properties of *P. denitrificans* methylamine dehydrogenase have been described it should be possible to perform potentiometric titrations of the individual redox centers in the putative methylamine dehydrogenase–amicyanin complex and to examine the mechanism by which electrons are transferred from methylamine dehydrogenase, which possesses two PQQ cofactors

and a four-electron capacity, to a one-electron carrier, amicyanin.

Registry No. PQQ, 72909-34-3; methylamine dehydrogenase, 60496-14-2.

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Site-Directed Mutagenesis in the Effector Site of *Escherichia coli* Phosphofructokinase

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ABSTRACT: A new vector for the expression of phosphofructokinase (pfk-1) was constructed with pEMBL, which allows reliable, inducible, high-expression, and facile mutagenesis of the gene. Two mutants in the effector site of the enzyme were produced by site-specific mutagenesis of residue Tyr-55 to assess the role of its side chain in binding an allosteric inhibitor, phosphoenolpyruvate (PEP), and an activator, guanosine 5′-diphosphate (GDP): Tyr-55 → Phe-55 and Tyr-55 → Gly-55. The dissociation constant of PEP from the T state is unaffected by the mutations. Mutation of Tyr-55 → Phe-55 only slightly increases the dissociation constant of GDP from the R state, indicating a minimal involvement of the hydroxyl group in binding. A 5.5-fold increase in the dissociation constant of GDP on the mutation of Tyr-55 → Gly-55 suggests a small hydrophobic interaction of the aromatic ring of the tyrosine residue with guanine of GDP.

Phosphofructokinase catalyzes the phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate. One of the two such enzymes in *Escherichia coli*, pfk-1, is a key regulatory element in the glycolytic pathway. The kinetic properties of *E. coli* pfk-1 have been studied extensively by Blangy et al. (1968) and Blangy (1971). This enzyme exhibits strong cooperative interactions with respect to fructose 6-phosphate and shows cooperative kinetics with respect to allosteric control, being inhibited by phosphenolpyruvate (PEP) and activated by ADP or GDP. The crystal structure of the active conformation of the enzyme obtained from *Bacillus*

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