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Purification and Properties of Methylamine Dehydrogenase from *Paracoccus denitrificans*

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Methylamine dehydrogenase from *Paracoccus denitrificans* was purified to homogeneity in two steps from the periplasmic fraction of methylamine-grown cells. The enzyme exhibited a pI value of 4.3 and was composed of two 46,700-dalton subunits and two 15,500-dalton subunits. Each small subunit possessed a covalently bound pyrrolo-quinoline quinone prosthetic group. The amino acid compositions of the large and small subunits are very similar to those of other methylamine dehydrogenases which have been isolated from taxonomically different sources. The enzyme was able to catalyze the oxidation of a wide variety of primary aliphatic amines and diamines, but it did not react with secondary, tertiary, or aromatic amines. The enzyme exhibited optimal activity at pH 7.5, with K_m values of 12.5 μM for methylamine and 156 μM for phenazine ethosulfate and a V_{max} of 16.9 μmol/min per mg of protein. No loss of enzyme activity was observed after incubation for 48 h at pH values ranging from 3.0 to 10.5, and the enzyme was very stable to thermal denaturation. Enzyme activity and immunological detection of each subunit were only observed with cells which had been grown on methylamine as a carbon source.

It has been established recently that certain oxidoreductases from a variety of sources contain pyrrolo-quinoline quinone (PQQ) as a prosthetic group (2, 4, 10, 11). These quinoproteins include bacterial methanol (9) and glucose dehydrogenases (12), which possess noncovalently associated PQQ, and bacterial methylamine dehydrogenase, which contains a covalently bound form of PQQ (8, 21). Mammalian plasma amine oxidase (26) and choline dehydrogenase (3) also possess covalently attached PQQ. When grown on methylamine as a sole source of carbon and energy, *Paracoccus denitrificans* synthesizes a methylamine dehydrogenase which functions in the periplasm of this gram-negative bacterium and donates electrons to a periplasmic type I blue copper protein, amicyanin (16). We have previously reported the partial purification of methylamine dehydrogenase from *P. denitrificans* (16) and have characterized the physical and redox properties of amicyanin (14, 16, 18, 25) and periplasmic *c*-type cytochromes (14, 17) which accept electrons from methylamine dehydrogenase via amicyanin. This paper reports a relatively simple procedure by which methylamine dehydrogenase was purified to homogeneity from *P. denitrificans* and describes several of the physical and kinetic properties of this enzyme.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. denitrificans* (ATCC 13543) was grown aerobically at 30°C in the medium of Kornberg and Morris (23), supplemented with 0.05% $NaHCO_3$, 0.01% yeast extract, and 0.5% methylamine, 0.5% methanol, or 0.3% succinate.

Preparation of proteins. Methylamine dehydrogenase was purified from the periplasmic fraction of methylamine-grown cells, which was prepared by the method of Alefounder and Ferguson (1). Routinely, 15 to 20 g (wet weight) of cells was fractionated at a time, and four such preparations were pooled, concentrated by ultrafiltration over an Amicon YM5 membrane (Amicon Corp., Lexington, Mass.), dialyzed against 20 mM potassium phosphate (pH 7.2), and applied to a DEAE-cellulose column (3.5 by 30 cm) which had been equilibrated in the same buffer that was used for dialysis. This column was eluted with a linear gradient (2.0 liters) of 0 to 400 mM NaCl in the starting buffer. Fractions containing methylamine dehydrogenase were pooled, concentrated by ultrafiltration over an Amicon PM 30 membrane, dialyzed against 20 mM potassium phosphate (pH 7.2), and applied to a DEAE-Trisacryl (LKB Instruments, Inc., Rockville, Md.) column (3 by 30 cm) which had been equilibrated in the same buffer. After being washed with 200 ml of the starting buffer, the column was eluted with a linear gradient (2.0 liters) of 0 to 400 mM NaCl in the equilibration buffer. Yellowish-green fractions exhibiting methylamine dehydrogenase activity eluted between 200 and 250 mM NaCl. These fractions were pooled, concentrated, and stored frozen in 10% ethylene glycol for future use.

The large and small subunits of methylamine dehydrogenase were prepared by incubation of the holoenzyme overnight at 25°C in 6 M guanidine hydrochloride, followed by gel filtration with Sephadex G-100 which had been equilibrated with the incubation buffer.

Assay methods. Methylamine dehydrogenase activity was assayed spectrophotometrically as described by Eady and Large (13), except that KCN was omitted and 4 μmol of phenazine ethosulfate (PES) and 10 μmol of methylamine hydrochloride were present in a 3-ml assay mixture. PES was used rather than phenazine methosulfate because, unlike phenazine methosulfate, PES gave essentially no blank rate. To quantitate the rates of reaction at the different pH values, the respective extinction coefficients of 2,6-dichloroindophenol at those pH values were calculated from the data of Armstrong (5).

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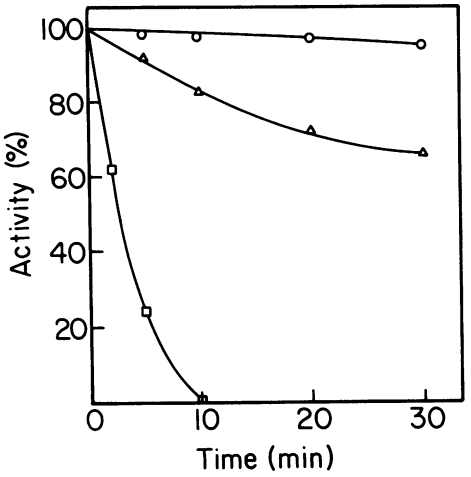

FIG. 1. Thermal stability of methylamine dehydrogenase. Methylamine dehydrogenase (0.125 mg/ml) was incubated in 100 mM potassium phosphate (pH 7.5) at 70°C (○), 80°C (△), and 83°C (□). Samples (10 μl) were withdrawn at the indicated times and assayed for activity as described in Materials and Methods.

Analytical techniques. Native molecular weight was determined by the approach-to-equilibrium method (34) with a Beckman model E ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) equipped with an RTIC temperature control unit and Rayleigh interference optics and an An-D rotor (Beckman) with double-sector centerpiece and sapphire windows. The protein (0.5 mg/ml) was run in 50 mM potassium phosphate (pH 7.5) against a buffer blank at 16,200 rpm at 26°C for 77 h. Data were collected by using Kodak spectrographic plates (Eastman Kodak Co., Rochester, N.Y.), and molecular weights were calculated by the method of Kahlon et al. (20), assuming a partial specific volume of 0.74.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli and Favre (24), except for the inclusion of 0.5 M urea in the resolving and stacking gels and 4 M urea and 4% SDS in the final sample buffer. The M_r standards used were bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (22,000), and lysozyme (14,000). Western blots, with alkaline phosphatase-conjugated immunoglobulin G (Promega Biotec, Madison, Wis.) as a second antibody, were performed with Bio-Rad reagents and equipment by the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Isoelectric focusing was performed by the instructions of the manufacturers with agarose (FMC Corp., Philadelphia, Pa.) gels and a Pharmacia FBE 3000 flatbed apparatus (Pharmacia, Inc., Piscataway, N.J.). Absorption spectra were recorded with a Cary 219 spectrophotometer (Varian, Sunnyvale, Calif.). The absorption spectra were calibrated for wavelength accuracy with horse heart cytochrome *c*, which exhibited an α-band maximum at 550.0 nm. Amino acid analysis was performed as described previously (17).

RESULTS

Purification. The purification procedure described in Materials and Methods is simpler and more effective than that described previously (16), in which the active fractions obtained from DEAE-cellulose chromatography are subjected to further chromatography with hydroxyapatite and gel filtration with Sephadex G-100. That procedure does not completely resolve methylamine dehydrogenase from methanol dehydrogenase. The current procedure consisted of two successive ion-exchange chromatography steps and used larger and shallower elution gradients than previously used. The specific activity of the purified enzyme was 17 μmol/min per mg, 60-fold greater than that of the crude periplasmic fraction. The final yield was approximately 65%.

The methylamine dehydrogenase preparation was judged to be pure by several criteria. When subjected to SDS-PAGE, the preparation exhibited two bands with M_r values of 46,700 and 15,500. A single band was observed when the preparation was subjected to nondenaturing PAGE and isoelectric focusing, and a single symmetrical peak was observed on gel filtration by high-performance liquid chromatography. Analysis by analytical ultracentrifugation indicated a homogenous preparation with a molecular weight of 124,000. Western blots of crude cell extracts, when probed with antiserum which was raised against this preparation, exhibited two bands corresponding to the positions of migration of the two subunits of the purified enzyme. No reaction with the antiserum was observed with crude extracts of *P. denitrificans* cells which were not synthesizing methylamine dehydrogenase.

Thermal and pH stability. The thermal stability of methylamine dehydrogenase was examined by incubating the enzyme at high temperatures and assaying its activity as a function of time (Fig. 1). The enzyme was very resistant to thermal denaturation, retaining most of its activity after a 30-min incubation at 70°C and approximately 65% of its activity after incubation at 80°C. At temperatures above 80°C, denaturation occurred more readily.

The effect of pH on the stability of methylamine dehydrogenase was examined by incubating the enzyme at 30°C in buffers with pH values ranging from 3.0 to 10.5. The enzyme was extremely stable under these conditions. No decrease in activity was observed at any of the pH values after incubation times as long as 48 h.

Kinetic properties. Methylamine dehydrogenase exhibited a pH optimum for activity at pH 7.5 (Fig. 2). With PES as an electron acceptor and concentrations of methylamine which

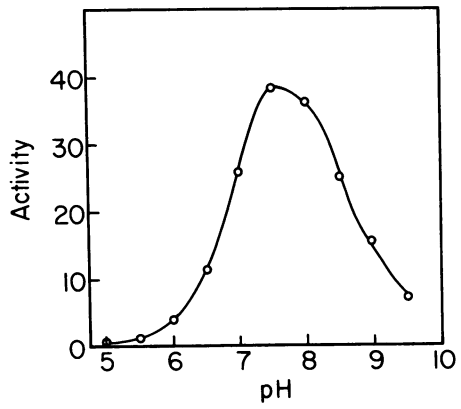

FIG. 2. Effect of pH on the activity of methylamine dehydrogenase. Assay conditions were as described in Materials and Methods, with the following buffers: pH below 6.0, 100 mM sodium acetate; pH 6.0 to 8.0, 100 mM potassium phosphate; and pH above 8.0, 100 mM sodium glycine. The amount of enzyme used in each assay was 2.4 μg. The units of activity are micromoles per minute per milligram of protein.

ranged from 2.5 to 30 μM, the enzyme exhibited a K_m for methylamine of 12.5 μM and a V_{max} of 16.9 μmol/min per mg of protein (Fig. 3A). The K_m for PES was 156 μM, and an identical value for V_{max} was observed (Fig. 3B).

Substrate specificity. The abilities of various other amines to act as substrates for this enzyme were examined (Table 1). Each of the primary aliphatic monoamines and diamines, histamine, and ethanolamine were oxidized at a rate which was comparable to that observed with methylamine. The enzyme did not react with aromatic, secondary, or tertiary amines, or with amino acids.

Physical properties. The native and subunit molecular weights and isoelectric point (pI) of *P. denitrificans* methylamine dehydrogenase were determined and compared with those properties of methylamine dehydrogenases isolated from other sources (Table 2). Each of the enzymes is composed of two large and two small subunits, and the enzymes which have been characterized thus far exhibit a wide range of pI values. The PQQ prosthetic group was covalently attached to the small subunit of *P. denitrificans* methylamine dehydrogenase, as it was not released from the protein during incubation with trichloroacetic acid or 6 M guanidine hydrochloride, and comigrated with the small subunit during chromatography on Sephadex G-100 in the presence of 6 M guanidine hydrochloride. The absorption spectra of the oxidized and reduced forms of *P. denitrificans* methylamine dehydrogenase are shown in Fig. 4. The oxidized enzyme exhibited a peak centered at 438 nm, a shoulder at 326 nm, and significant absorbance between 600 and 800 nm. On reduction by methylamine, most of the A_{438} was lost and the remaining peak was centered at 416 nm. In addition, the A_{326} was substantially increased. The absorption spectrum of the isolated small subunit of methylamine dehydrogenase (Fig. 5) did not exhibit the shoulder at 326 nm or the broad peak between 600 and 800 nm, and the major peak was centered at 420 nm. The isolated small subunit was not reduced by methylamine. Incubation of the small subunit with 6 M guanidine hydrochloride (Fig. 5) caused a slight increase in absorbance and a shift to a maximum at 428 nm.

The amino acid compositions of *P. denitrificans* methylamine dehydrogenase and its individual subunits are given in Table 3 and compared with the compositions of three other methylamine dehydrogenases for which data are available. The compositions of the PQQ-bearing small subunits of the three enzymes are nearly identical. Each enzyme possesses an unusually high number of cysteine and proline residues in its small subunit and few if any cysteines in its large subunit.

TABLE 1. Substrate specificity of *P. denitrificans* methylamine dehydrogenase

Compound	% of rate with methylamine[a]
Methylamine	100
Ethylamine	62
Propylamine	80
Butylamine	76
1,3-Diaminopropane	104
1,4-Diaminobutane	112
Histamine	88
Ethanolamine	64
Dimethylamine	0
Trimethylamine	0
Benzylamine	0
Alanine	0
Lysine	0

[a] Enzyme activity was assayed as described in Materials and Methods, except that methylamine hydrochloride was replaced by the indicated compounds, which were each added to a final concentration of 10 mM. The rate with methylamine, which corresponds to 100%, was 17.2 μmol/min per mg of protein.

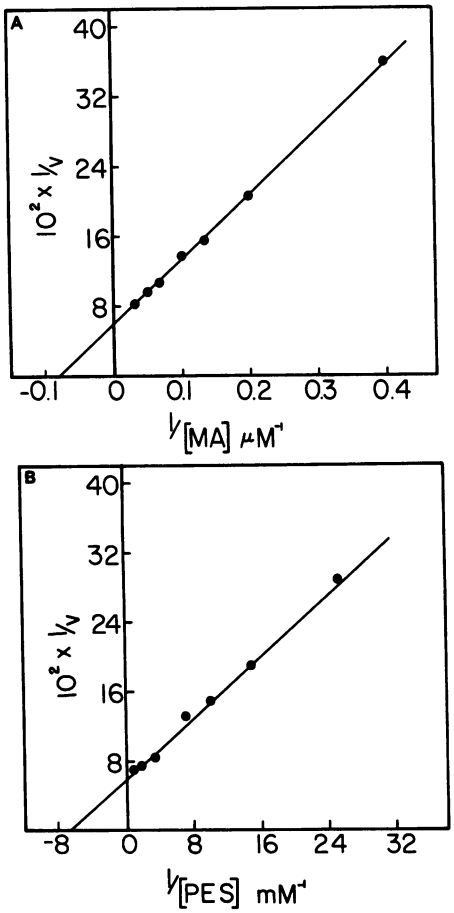

FIG. 3. Double-reciprocal plots for the reaction catalyzed by methylamine dehydrogenase with methylamine (A) and PES (B) as the variable substrates. The 3-ml reaction mixture contained 0.3 mmol of potassium phosphate (pH 7.5), 0.17 μmol of dichloroindophenol, and 1.2 μg of enzyme. The fixed concentrations of methylamine (A) and PES (B) were, respectively, 170 μM and 1.33 mM. Reduction of dichloroindophenol was followed spectrophotometrically at 600 nm at 30°C. The units of activity are micromoles per minute per milligram of protein.

TABLE 2. Properties of methylamine dehydrogenases from various sources

Source of enzyme	M_r (10^3)			pI	Reference
	Native enzyme	Large subunit	Small subunit		
P. denitrificans	124	46.7	15.5	4.3	
Bacterium W3A1	127	45	15.5	ND[a]	21
Methylomonas sp. strain J	105	40	13	9.0	27
M. methylotrophus	ND	42.7	15.9	ND	15
Pseudomonas sp. strain AM1	105	40	13	5.2	31
T. versutus	123.5	47.5	12.9	3.9	32

[a] ND, Not determined.

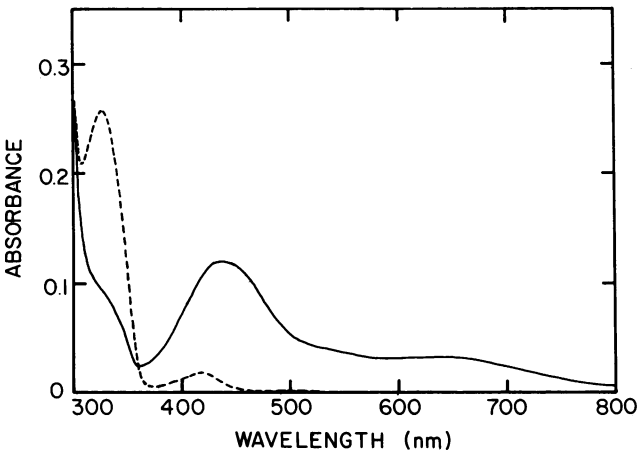

FIG. 4. Absorption spectra of the oxidized and reduced forms of *P. denitrificans* methylamine dehydrogenase. Absorption spectra, recorded in 50 mM potassium phosphate (pH 7.5), are of 0.74 mg of oxidized methylamine dehydrogenase per ml before (——) and immediately after (- - - -) the addition of 50 μM methylamine.

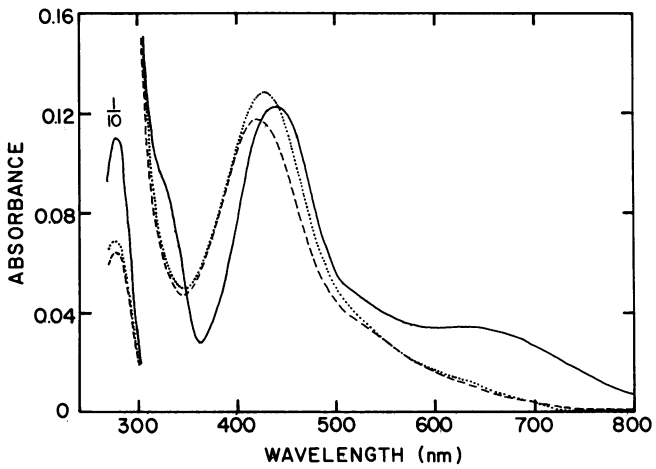

FIG. 5. Absorption spectra of *P. denitrificans* methylamine dehydrogenase and its small subunit. Absorption spectra, recorded in 50 mM potassium phosphate (pH 7.5), are of methylamine dehydrogenase (0.74 mg/ml) (——), its isolated small subunit (0.14 mg/ml) (- - - -), and the small subunit (0.14 mg/ml) (· · · ·) which had been incubated overnight in buffer containing 6 M guanidine hydrochloride.

Similarities in the compositions of the large subunits of the four methylamine dehydrogenases are also apparent.

Regulation of enzyme expression by carbon source. *P. denitrificans* was grown with methylamine, methanol, or succinate as a source of carbon, and extracts of those cells were assayed for the presence of methylamine dehydrogenase. The specific activity of methylamine dehydrogenase in crude extracts of sonicated methylamine-grown cells was 0.4 μmol/min per mg of protein. No methylamine dehydrogenase activity was detected in the sonic extracts of cells which were grown on either of the other carbon sources. To determine whether the small and large subunits of the enzyme were present in cells which did not show activity, total-cell extracts were subjected to SDS-PAGE and Western blotting with antibodies specific for the two subunits of methylamine dehydrogenase (Fig. 6). Each of the subunits was present only in the extracts of methylamine-grown cells, and each exhibited a reaction which was identical to that observed with the purified enzyme.

DISCUSSION

The facultative autotrophic bacterium *P. denitrificans* is taxonomically quite different (19) from the other bacteria from which methylamine dehydrogenases have been isolated. These enzymes exhibit a wide range (3.9 to 9.0) of pI values. However, the molecular weights, subunit compositions, and amino acid compositions of the methylamine dehydrogenases thus far isolated are very similar, indicating

TABLE 3. Amino acid compositions of methylamine dehydrogenases[a]

Amino acid	No. of amino acid residues in methylamine dehydrogenase of:								
	P. denitrificans			Bacterium W3A1		*Pseudomonas* sp. strain AM1		*Methylomonas* sp. strain J	
	Native enzyme	Large subunit	Small subunit	Large subunit	Small subunit	Large subunit	Small subunit	Large subunit	Small subunit
Asx	120	40	18	46	22	40	17	37	17
Thr	68	26	7	22	12	20	6	19	10
Ser	48	20	10	28	11	25	15	22	13
Glx	109	46	8	35	9	33	9	37	8
Pro	70	27	9	20	11	23	8	19	9
Gly	85	35	12	23	14	29	13	27	13
Ala	127	50	14	34	7	40	9	28	7
Cys	28	2	10	0	13	0	9	2	10
Val	60	26	5	23	5	23	5	29	5
Met	15	5	1	9	1	4	1	9	1
Ile	44	15	6	21	5	20	5	12	5
Leu	66	28	5	34	8	25	5	37	6
Tyr	36	11	6	12	6	11	5	10	5
Phe	43	19	2	25	4	21	2	19	3
Trp	ND[b]	ND	ND	4	1	4	2	4	1
Lys	29	12	3	36	5	26	4	32	5
His	32	13	3	12	2	9	2	9	2
Arg	57	22	5	17	6	16	5	15	5

[a] Comparison compositions are from references 22, 28, and 31.
[b] ND, Not determined.

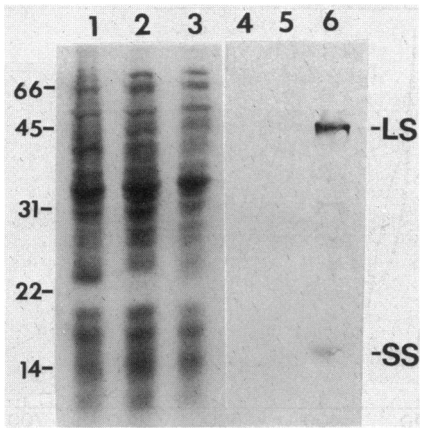

FIG. 6. SDS-PAGE and Western blot of *P. denitrificans* cell extracts. Lanes 1 to 6 contain total-cell extracts of *P. denitrificans* which were grown on succinate (lanes 1 and 4), methanol (lanes 2 and 5), or methylamine (lanes 3 and 6) as a carbon source. Lanes 1 to 3 represent an SDS-polyacrylamide gel which was stained for protein with Coomassie blue R-250. Lanes 4 to 6 represent a Western blot of an identical SDS-polyacrylamide gel which was probed with antiserum specific for methylamine dehydrogenase. The positions and molecular weights of the protein standards (in thousands) are indicated on the left. The positions of migration of the large (LS) and small (SS) subunits of methylamine dehydrogenase are indicated on the right.

a high degree of conservation among this family of proteins. The amino acid compositions of the small PQQ-bearing subunits, which are rather unusual in their high cysteine and proline contents, are nearly identical. Methylamine dehydrogenases are also very similar in stability against denaturation by high temperature and high and low pH. Retention of significant activity after exposure to temperatures up to 70°C has also been observed for the enzymes that were isolated from *Pseudomonas* sp. strain AM1 (31), *Methylophilus methylotrophus* (15), and *Thiobacillis versutus* (15). Stabilities against exposure to pH values ranging from 4 to 10 have also been reported for the enzymes of *Pseudomonas* sp. strain AM1 (31) and *Methylomonas* sp. strain J (27). The high degree of stability of the *P. denitrificans* enzyme against extremes of both temperature and pH indicates that it may be the most stable of the methylamine dehydrogenases isolated thus far. The pH optimum for activity and K_m value for methylamine which are exhibited by the *P. denitrificans* enzyme are comparable to those observed for other methylamine dehydrogenases (13, 21, 27). However, the specific activity of the purified *P. denitrificans* enzyme is four- to sevenfold greater than those observed for most of the previously described methylamine dehydrogenases. The substrate specificity of the *P. denitrificans* enzyme is similar to that of the enzyme from *Pseudomonas* sp. strain AM1, a facultative methylotroph which also oxidizes a wide variety of primary aliphatic amines and diamines (13), but different from that of the enzyme from the obligate methylotroph *Methylomonas* sp. strain J, which oxidizes a limited range of primary amines (27).

The absorption spectrum of the oxidized form of methylamine dehydrogenase from *P. denitrificans* (Fig. 4) is similar to those of other methylamine dehydrogenases (21, 32) and quite different from those of methanol and glucose dehydrogenases (9, 12) which contain noncovalently associated PQQ. The spectrum of the native small subunit was significantly different from that of the holoenzyme, suggesting that the protein environment surrounding the PQQ prosthetic group is influenced either directly by the large subunit or by interactions between the large and small subunits.

The coinduction of groups of proteins that are involved in the oxidation of C_1 compounds appears to be a characteristic trait of bacteria which are capable of growth on these compounds (4, 6, 7, 29, 30, 33). In methylotrophic bacteria, methylamine dehydrogenase is only synthesized during growth on methylamines (4, 6). In *P. denitrificans*, amicyanin, the natural electron acceptor for methylamine dehydrogenase, is only present during growth on methylamine (16). The observation that the methylamine dehydrogenase of *P. denitrificans* was also only detected during growth on methylamine (Fig. 6) is consistent with the above-mentioned observations and suggests that the mechanisms which regulate the expression of the proteins involved in methylamine-dependent respiration in methylotrophs may also operate in the facultative autotroph *P. denitrificans*.

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