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Oxidation of Hydroxylamine by Cytochrome P-460 of the Obligate Methylotroph *Methylococcus capsulatus* Bath

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An enzyme capable of the oxidation of hydroxylamine to nitrite was isolated from the obligate methylotroph *Methylococcus capsulatus* Bath. The absorption spectra in cell extracts, electron paramagnetic resonance spectra, molecular weight, covalent attachment of heme group to polypeptide, and enzymatic activities suggest that the enzyme is similar to cytochrome P-460, a novel iron-containing protein previously observed only in *Nitrosomonas europaea*. The native and subunit molecular masses of the *M. capsulatus* Bath protein were 38,900 and 16,390 Da, respectively; the isoelectric point was 6.98. The enzyme has approximately one iron and one copper atom per subunit. The electron paramagnetic resonance spectrum of the protein showed evidence for a high-spin ferric heme. In contrast to the enzyme from *N. europaea*, a 13-nm blue shift in the soret band of the ferrocytochrome (463 nm in cell extracts to 450 nm in the final sample) occurred during purification. The amino acid composition and N-terminal amino acid sequence of the enzyme from *M. capsulatus* Bath was similar but not identical to those of cytochrome P-460 of *N. europaea*. In cell extracts, the identity of the biological electron acceptor is as yet unestablished. Cytochrome *c*-555 is able to accept electrons from cytochrome P-460, although the purified enzyme required phenazine methosulfate for maximum hydroxylamine oxidation activity (specific activity, 366 mol of O_2 per s per mol of enzyme). Hydroxylamine oxidation rates were stimulated approximately 2-fold by 1 mM cyanide and 1.5-fold by 0.1 mM 8-hydroxyquinoline.

Methanotrophs and ammonia-oxidizing bacteria show a number of morphological, physiological, and biochemical similarities (6). In methanotrophs, as in the nitrifiers, the oxidation of ammonia is a two-step process (Fig. 1). The first step is the energy-dependent oxidation of ammonia to hydroxylamine, catalyzed by the methane monooxygenase (MMO) (9, 11). Both forms of the MMO, soluble MMO (sMMO) and membrane-associated MMO (pMMO), have been shown to catalyze this reaction (9, 11, 46, 56). The second step involves the four-electron oxidation of hydroxylamine to nitrite (11, 54). Little is known about the enzyme that catalyzes this reaction. In cell extracts, hydroxylamine oxidation rates are stimulated by cyanide, 8-hydroxyquinoline, and phenazine methosulfate (11). Inhibitor and cell fractionation studies have shown that hydroxylamine oxidation activity is separate from MMO and methanol dehydrogenase activities (11, 54). This activity has been partially purified from *Methylococcus thermophilus* and identified as a hydroxylamine oxidoreductase-like enzyme, although no evidence was given for this conclusion (54).

In ammonia-oxidizing bacteria, two different enzymes which oxidize hydroxylamine to nitrite, hydroxylamine oxidoreductase (HAO) and cytochrome P-460, have been found (18, 22, 23, 43). HAO is the periplasmic enzyme responsible for the energy-yielding step in ammonia oxidation (16, 23, 44, 65). The physiological role of cytochrome P-460 in nitrifiers has not been resolved, but it may serve to protect the cell from this toxic and mutagenic intermediate (20a). Both enzymes contain a novel chromophore with an absorption maximum at 463 nm (2, 3, 18, 23, 40). In *Nitrosomonas europaea*, 95% of the chromophore absorbing at 463 nm is associated with hydroxylamine oxidoreductase (18). Each subunit of HAO contains seven *c*-type hemes and one P-460 heme group (4). Arciero et al. (5) recently determined the structure of heme P-460 in HAO to be an iron protoporphyrin IX bound to the peptide by two thioether bridges and by a covalent bond between a meso heme carbon and an aromatic ring carbon on a tyrosyl residue. All available evidence indicates that the P-460 heme of HAO is involved in the catalytic function of the enzyme (25). During hydroxylamine oxidation, electrons pass from hydroxylamine through the P-460 chromophore to the *c*-hemes of HAO (5, 21, 25, 26).

The second chromophore with an absorbance at 463 nm in *N. europaea* is associated with a low-molecular-weight (M_r 17,000 to 18,400) protein called cytochrome P-460 (20). Initially believed to be a solubilized heme P-460-containing fragment of HAO and called the P460 fragment, cytochrome P-460 has been shown to be a different polypeptide from HAO (38, 50). The absorption maximum of the ferrocytochrome, resonance Raman spectroscopy, and hydroxylamine oxidation activity suggest that the chromophore in cytochrome P-460 is structurally very similar to heme P-460 in HAO (3). In addition, as observed with the heme P-460 in HAO, the heme group is not extracted with acid acetone, ethyl acetate, Ag_2SO_4, *o*-nitrophenylsulfenyl chloride, or sodium dodecyl sulfate (SDS)-urea-mercaptoethanol, which suggests additional covalent linkages beyond thioether bonds (5, 18, 43). However, other spectral evidence (Mössbauer, electron paramagnetic resonance [EPR], and optical) and redox properties have indicated structural differences in the chromophore in HAO and cytochrome P-460 (2, 5, 10, 35). The structure of the 463-nm chromophore in cytochrome P-460 has not been determined.

The present report describes the first purification of a hydroxylamine-oxidizing enzyme from a methylotroph. The enzyme is similar but not identical to cytochrome P-460 from *N. europaea*. We describe a purification procedure, molecular

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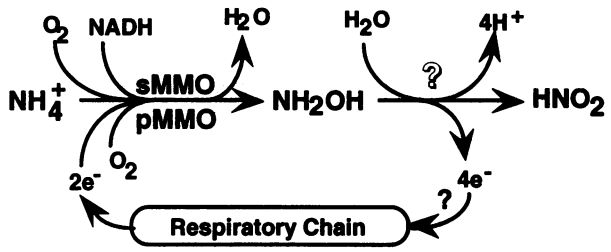

FIG. 1. Proposed mechanism of ammonia oxidation by methanotrophs. Abbreviations: sMMO, soluble methane monooxygenase; pMMO, membrane-associated methane monooxygenase.

weight, subunit composition, metal composition, N-terminal amino acid sequence, optical absorption, and EPR spectra. The electron donor and acceptor specificities of the enzyme are also considered.

MATERIALS AND METHODS

Culture conditions. *Methylococcus capsulatus* Bath was grown in nitrate mineral salts medium (63) plus 5 μM $CuSO_4$ and a vitamin mixture (34) at 37°C under an atmosphere of 40% methane and 60% (vol/vol) air. Cells grown in a 12-liter fermentor were sparged at flow rates of 80 to 150 ml of methane and 2,000 to 2,500 ml of air per min at 42°C. Cells were harvested by centrifugation at 13,000 × *g* for 15 min at 4°C, resuspended (1:5, wt/vol) in a buffer containing 10 mM Tris-HCl (pH 8.2) (buffer A), and centrifuged at 13,000 × *g* for 15 min at 4°C. The cell pellet was resuspended (1:1, wt/vol) in buffer A containing 1 μg of DNase (Sigma) per ml, frozen in liquid nitrogen, and stored at −80°C.

Isolation of cytochrome P-460. All procedures were performed at 4°C. Freeze-thawed cells were passed through a French pressure cell three times at 15,000 lb/in^2. The cell suspension was centrifuged at 13,000 × *g* for 15 min to remove unlysed cells and debris. The supernate (cell extract) was centrifuged at 155,000 × *g* for 1.5 h. The supernate (soluble fraction I) was saved, and the pellet was resuspended with a Dounce homogenizer in buffer A and 500 mM KCl. The sample was centrifuged at 155,000 × *g* for 1.5 h, and the supernate (soluble fraction II) was pooled with soluble fraction I. The combined soluble fractions were dialyzed for 6 h against three changes of buffer A and loaded on a DEAE-cellulose column (5.0 by 30 cm) equilibrated with buffer A. The sample did not bind to DEAE-cellulose and eluted with buffer A. This fraction was brought to 40% saturation with ammonium sulfate, stirred for 1 h, and centrifuged at 13,000 × *g* for 30 min. The pellet was discarded, and the concentration of ammonium sulfate in the supernatant was raised to 60% saturation. The solution was then stirred for 1 h and centrifuged at 13,000 × *g* for 30 min. The pellet (40 to 60% ammonium sulfate fraction) was resuspended in a minimal volume of buffer A and dialyzed against three changes of buffer A. Following dialysis, the sample was concentrated on a stirred cell (YM10 filter) and loaded on a preparative isoelectric focusing bed (15 by 30 cm) containing 4% Ultrodex and 2% ampholyte (pH 5 to 8) and electrophoresed for 16 h at 4°C. The cytochrome P-460 fraction appeared as a green band focusing at pH 6.98. The band was eluted from the Ultradex with buffer A, concentrated with a stirred cell (YM10 filter), and loaded on a Sephadex G-75 column (2.5 by 96 cm) equilibrated with 25 mM Tris-HCl (pH 8.2)–100 mM KCl buffer (buffer B). The 25,000- to 45,000-Da fractions were pooled and loaded on a phenyl-Sepharose CL-4B column (1.25 by 20 cm) equilibrated with buffer B. The sample was washed with 2 bed volumes of buffer B and eluted with 10 mM Tris-HCl–25 mM KCl buffer (pH 8.2). The sample was dialyzed with three changes of buffer A and then loaded on a DEAE-Sepharose CL-6B column (1.25 by 60 cm) equilibrated with buffer A. Cytochrome P-460 appeared as an intense green band on the top of the column. The column was washed with 4 bed volumes of buffer A, and the purified cytochrome P-460 was slowly eluted with approximately 4 bed volumes of 10 mM Tris-HCl (pH 8.2)–25 mM KCl buffer. The sample was concentrated with a stirred cell (YM10 filter), and dodecyl-β-D-maltoside or dodecyl-β-D-glycopyranoside was added to a final concentration of 100 μM.

Electrophoresis. SDS-polyacrylamide slab gel electrophoresis was carried out by the Laemmli method on 10 to 16% polyacrylamide gels (33). Gels were stained for total protein with Coomassie brilliant blue R or by the silver stain method of Nielsen and Brown (42). Proteins with peroxidative activity in SDS-polyacrylamide gels were stained by the diaminobenzidine method (37).

Mass spectrometry. The molecular mass of cytochrome P-460 was determined by time-of-flight mass spectrometry on a Kratos matrix-assisted laser desorption ionization (MALDI III) mass spectrometer with a sinapinic acid matrix and a laser power setting of 39 (Kratos Analytical, Ramsey, N.J.).

Enzyme activity. Whole-cell ammonia oxidation was measured by the rate of oxygen utilization (Clark oxygen electrode) or nitrite production at 37°C. Reaction mixtures (3 ml) contained 1 to 5 mg of cell protein per ml, 2 mM ammonium sulfate, and 5 mM sodium formate in 10 mM Tris-HCl buffer, pH 8.0. The electrode was calibrated by the method of Robinson and Cooper (48). Nitrite was determined by the sulphanilamide *N*-(1-naphthyl)ethylenediamine hydrochloride method of Nicholas and Nason (41) and measured at 540 nm. Spectral assays were performed on 1-ml samples containing 5 mM hydroxylamine hydrochloride (pH 8), 10 mM Tris (pH 8), and approximately 50 μg of sample protein. Reactions were initiated by the addition of phenazine methosulfate (PMS), cytochrome *c*-554, cytochrome *c*-555, cytochrome *c*-557, and/or cytochrome *c'* from *M. capsulatus* Bath.

Hydroxylamine oxidation was measured as the rate of oxygen utilization or nitrite production in the presence of phenazine methylsulfate as described by Dalton (11). Assays were run in 10 mM Tris-HCl buffer, pH 8.0, or 10 mM NaK-phosphate buffer, pH 7.0, containing 5 mM hydroxylamine and 100 μM PMS or one or more of the soluble ferricytochromes from *M. capsulatus* Bath (cytochrome *c*-554, cytochrome *c*-555, cytochrome *c'*, or cytochrome *c*-557).

Spectroscopy. Optical absorption spectroscopy was performed with an SLM Aminco DW-2000 spectrophotometer in the split-beam mode.

EPR spectra were recorded at X-band on a Bruker ER 200D EPR spectrometer equipped with an Oxford Instruments ESR-900 liquid helium cryostat. Operating parameters were as follows: modulation frequency, 100 kHz; modulation amplitude, 4 G; microwave frequency, 9.422 GHz; microwave power, 20 dB; time constant, 100 ms; and sweep time, 50 s. Samples were maintained at 8 K during spectrum acquisition.

Heme and protein determination. The optical extinction coefficient values for cytochrome P-460 were quantified by using the absorption maximum of the soret band and protein determinations by the method of Lowry et al. (36) with bovine serum albumin as a standard. Heme composition was determined by the pyridine ferrohemochrome method (12). The

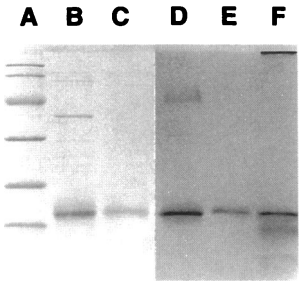

FIG. 2. SDS-polyacrylamide slab gel electrophoresis (A) of molecular mass standards, 92, 66.2, 45, 31, 21.5, and 14.4 kDa; (B and D) following chromatography on Sephadex G-75 (8.3 μg of protein); (C and E) of purified cytochrome P-460 (2.8 μg of protein); and (F) of heme-stained standards: cytochrome *c'*, 16.7 kDa (2.5 μg of protein); cytochrome *c*-555, 14.1 kDa (1.7 μg of protein); and cytochrome *c*-554, 10.7 kDa (2.0 μg of protein). Samples in lanes A to C were heated at 100°C for 1 min before being loaded and stained with Coomassie blue R-250. Samples in lanes D to F were not heat treated and were stained with diaminobenzidine.

acid-acetone method was used to determine covalent linkage of the prosthetic groups to the polypeptide (19).

Metal analysis. Protein samples were dialyzed for 18 h against five changes of 5 mM Na-EDTA. Protein (10 to 25 nmol) samples were analyzed for zinc, copper, and iron with a Perkin Elmer model 5100 graphite furnace atomic absorption spectrophotometer.

Amino acid sequence and analysis. Amino acid analysis was carried out with an Applied Biosystems 420A derivatizer coupled to an Applied Biosystems 130A separation system. Samples were hydrolyzed in 6 M HCl plus trace amounts of phenol in HCl vapors for 1 h and then in a vacuum at 150°C. After hydrolysis, norleucine was added as an internal standard.

The N-terminal amino acid sequence of cytochrome P-460 was analyzed by Edmond degradation with an Applied Biosystems 477A protein sequencer/120A analyzer.

Other methods. Tetraacetyl-α-D-glucopyranosyl bromide was synthesized by the method of Jeremias et al. (30) and used in the synthesis of dodecyl-β-D-glycopyranoside. Dodecyl-β-D-glycopyranoside was synthesized as described previously (62).

Cytochrome *c*-557 was isolated by the method of DiSpirito et al. (15).

Cytochrome *c'* was separated from cytochrome P-450 on the Phenyl-Sepharose CL-4B column (see above under isolation of cytochrome P-460). Cytochrome *c'* remained bound to the column as a visible brown-red band following elution of cytochrome P-460 and was eluted with 6 column volumes of buffer A. This fraction was electrophoretically homogeneous and had an apparent molecular mass of 16,700 Da on SDS-polyacrylamide gels and a pI of 7.0. The optical spectra and ligand-binding properties of cytochrome *c'* were similar to those of other *c'*-type cytochromes (32, 39, 51).

Cytochrome *c*-554, named cytochrome *c*-555 by Ambler et al. (1), was isolated from the soluble cell fraction by precipitation with ammonium sulfate at a 50 to 70% saturation. The pellet was resuspended in a minimal volume of buffer A and dialyzed against three changes of buffer A. Following dialysis, the sample was concentrated by ultrafiltration and loaded on a preparative isoelectric focusing bed with 4% Ultradex (Pharmacia) and 2% ampholine (pH 3.5 to 10) and electrophoresed for 16 h at 4°C. Cytochrome *c*-554 appeared as a light pink band with a pI of 8.83. The cytochrome migrated on SDS denaturing gels with an apparent molecular mass of 10.7 kDa and a ferrocytochrome alpha maximum at 553.5 nm.

TABLE 1. Amino acid composition of cytochrome P-460 from *M. capsulatus* Bath and *N. europaea*[a]

Amino acid	*M. capsulatus* Bath P-460		*N. europaea* P-460[b]	
	mol%	No. of residues/Fe atom	mol%	No. of residues/Fe atom
Asx	10.3	15	12.2	15
Ser	5.1	7	4.1	5
Glx	11.1	16	10.6	13
Pro	7.4	10	7.3	9
Gly	11.8	17	9.8	12
Ala	15.5	22	12.2	15
Cys	ND[c]		1.6	2
Val	5.7	8	6.5	8
Met	0.9	1	1.6	2
Ile	4.6	6	0.8	1
Leu	7.2	10	5.7	7
Tyr	0.8	1	4.1	5
Phe	3.6	5	4.9	6
His	2.1	3	1.6	2
Lys	7.4	10	7.3	9
Arg	2.9	4	3.3	4
Thr	3.6	5	6.5	8
Trp	ND		ND	

[a] The minimum molecular weight of the enzymes from *M. capsulatus* and *N. europaea* is 14,345 and 13,200, respectively, based on iron concentration, assuming one iron atom per subunit.
[b] Data are from reference 43.
[c] ND, not determined.

Cytochrome *c*-555 was isolated from the soluble 70% ammonium sulfate fraction as described previously for the isolation of cytochrome *c*-554. The sample was applied to a Phenyl-Sepharose column (2.5 by 20 cm) equilibrated in 70% ammonium sulfate and washed with 1 column volume of 70% ammonium sulfate. Cytochrome *c*-555 was eluted from the column with 6 column volumes of 35% ammonium sulfate and dialyzed against three changes of buffer A. Cytochrome *c*-555 migrated on SDS denaturing gels with an apparent molecular mass of 14.1 kDa, a pI of 6.0, and a ferrocytochrome alpha maximum at 555 nm.

RESULTS

Purification. Hydroxylamine oxidation activity in cells lysed in 10 mM Tris-HCl was distributed evenly between the soluble and membrane fractions. A high-salt (500 mM KCl) wash was required to solubilize the membrane-associated activity. Lower-salt washes of 10 mM Tris-HCl buffers containing 100 or 250 mM KCl solubilized only a small fraction of the membrane-associated hydroxylamine oxidation activity. Once solubilized, the cytochrome P-460 in the soluble fraction and in the sample solubilized in the high-salt washes behaved similarly during purification.

An unusual feature of the purification was a 13-nm blue shift in the absorption maximum of the soret band of ferrocytochrome P-460 during the final purification steps. The major spectral shift (463 to 452 nm) was observed following gel filtration on Sephadex G-75. Electrophoretic and spectral analysis of proteins separated at this step indicated the separation of two minor polypeptides with subunit molecular masses of 61,200 Da and 39,700 Da and cytochrome *c'* (16,700 Da) from cytochrome P-460. In non-heat-treated samples, the 61,200-Da polypeptide migrated with a molecular mass of 47,000 Da and stained positive with diaminobenzidine (Fig. 2, lanes B and D). When assayed together on SDS

TABLE 2. Properties of purified cytochrome P-460 from *M. capsulatus* Bath and *N. europaea*

Property	*M. capsulatus* Bath P-460	*N. europaea* P-460
Molecular mass (Da)		
Enzyme[a]	38,900 ± 6,100	36,000–60,000[b,c,d]
Subunit		
SDS-polyacrylamide gel electrophoresis	16,800 ± 100	17,000–18,500[b,c,d]
Mass spectrum	16,390	ND[e]
Amino acid analysis	14,345	13,200
Isoelectric point	6.98	ND
Metal concentration[f] (mol/mol enzyme)		
Fe	2.4	2.6[b]
Cu	2.4	ND
Absorption maxima (nm)		
Oxidized	419	435[c,d]
Plus NaN_3	432	ND
Plus KCN	435	447[d]
Reduced	450, 653	460, 688[d]
Plus CO	435	448[d]
Plus NaN_3	437	ND
Plus KCN	441	455[c]
Plus $NaNO_2$		454[b]
Ferrohemochromagen	420, 528, 556	433, 530, 560
Optical extinction coefficients (cm^{-1} mM^{-1})		
Reduced: 450–600 nm	75.6	ND
Resting: 419–600 nm	78.5	ND
EPR (*g* values)	6.19, 5.70, 2.0	5.92, 5.63, 1.99[b]; 6.15, 5.70, 2.0[d]
Hydroxylamine oxidase[g] (mol of O_2/s/mol of enzyme)	366	6
Plus 1 mM cyanide	770	ND
Plus 100 μM 8-hydroxyquinoline	514	ND

[a] Determined by gel filtration on Sephadex G-75.
[b] From reference 43.
[c] From reference 18.
[d] From reference 2.
[e] ND, not determined.
[f] Assuming a molecular weight of 33,600 for the *M. capsulatus* Bath enzyme and 34,600 for the *N. europaea* enzyme.
[g] PMS and cytochrome *c*-552 were used as the electron acceptors for the enzymes from *M. capsulatus* and *N. europaea*, respectively.

denaturing gels, cytochrome *c'* could not be distinguished from cytochrome P-460. The concentration of cytochrome *c'* in these samples was estimated from absorption and EPR spectra.

In an attempt to stabilize the spectral characteristics of cytochrome P-460, three different purification procedures were developed. The results from the different purification procedures associated the spectral shifts with the separation of cytochrome *c'* and the 61,200-Da peroxidase-staining polypeptide from cytochrome P-460. Reconstitution experiments with proteins separated at this step, alone and in different combinations, did not alter the spectral or enzymatic properties of the cytochrome P-460. Although the isolated ferrocytochrome P-460 showed an absorption maximum at 450 nm, we will refer to the sample as cytochrome P-460 (see Discussion).

Cytochrome P-460 also became labile with the separation of cytochrome *c'* and the 61,200-Da polypeptide. The sample lost distinguishing spectral characteristics and PMS-dependent hydroxylamine oxidase activity within 48 h of purification. The spectral properties and enzyme activity in this sample could be stabilized by the addition of 100 μM dodecyl-β-D-maltoside or dodecyl-β-D-glucopyranoside to the purified enzyme. The enzyme could also be stabilized by the addition of the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM) and *N*-ethylmaleimide (0.5 mM) to the lysis and purification buffers. However, regardless of the purification procedure or stabilizing agent, separation of cytochrome *c'* from cytochrome P-460 always resulted in a spectral shift in the soret band of the ferrocytochrome to 454 to 450 nm.

Purified Triton X-100 (Boehringer Mannheim Biochemicals) was also tested as a stabilizing agent on cytochrome P-460, but this detergent caused a rapid loss of hydroxylamine oxidation activity and spectral properties of the enzyme.

Properties of cytochrome P-460. The properties of cytochrome P-460 are summarized in Tables 1 and 2 and Fig. 3.

Molecular mass. The molecular mass of cytochrome P-460 was determined to be 38,900 ± 6,100 Da on a Sephadex G-75 column (2.5 by 96 cm). Reference proteins were horse heart cytochrome *c* (M_r 12,400), lysozyme (M_r 14,300), horse heart myoglobin (M_r 18,800), carbonic anhydrase (M_r 29,000), and bovine hemoglobin (M_r 64,500). In SDS-polyacrylamide gels, cytochrome P-460 migrated as a single band corresponding to a molecular mass of 16,800 ± 100 Da (Fig. 2, lanes C and E). The results suggest that the enzyme is a dimer composed of two identical subunits. The sample did not require β-mercaptoethanol or heat treatment before loading on SDS-polyacrylamide gels for subunit separation (results not shown). Thus, the interaction between subunits appears to be noncovalent.

The molecular mass of the purified sample was also analyzed by mass spectroscopy and showed major *M/e* peaks at 16,391 ($[M + 1H]^+$) and 8,151 ($[M + 2H]^{2+}$), which was similar to the

N.europaea Cytochrome P-460: Ala-Glu-Val-Ala-Glu-Phe-Asn-Asp-Ile-Gly
1 2 3 4 5 6 7 8 9 10

M.capsulatus Bath Cytochrome P-460: Glu-Pro- ? -Ala-Ala-Pro- Asn-Gly-Ile-Ser
1 2 3 4 5 6 7 8 9 10

FIG. 3. N-terminal amino acid sequence of cytochrome P-460 from *M. capsulatus* Bath and *N. europaea* (43).

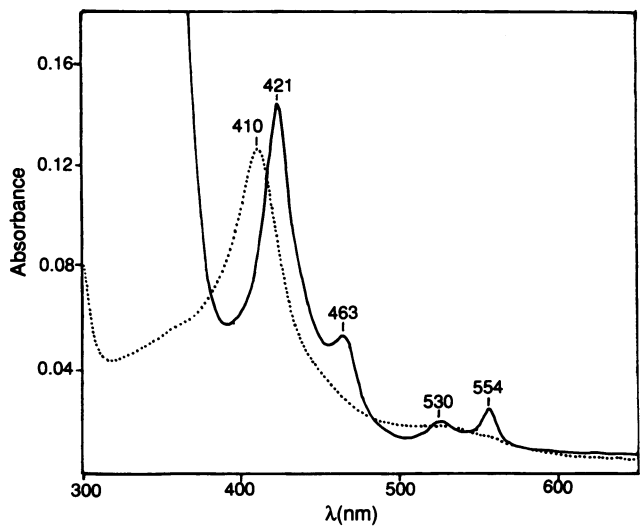

FIG. 4. Absorption spectra of the cytochrome P-460-containing fraction from 40 to 6% ammonium sulfate fraction in 25 mM Tris-HCl–100 mM KCl buffer (pH 8.2). ····, absorption of the resting enzyme; ——, absorption following reduction with dithionite.

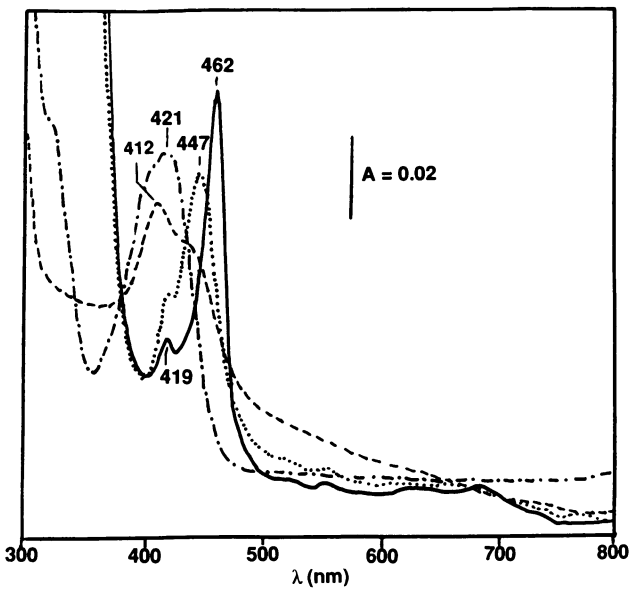

FIG. 5. Absorption spectra of the final step in the purification of cytochrome P-460 in the presence of dodecyl-β-D-glycopyranoside. Absorption of the resting enzyme (– – – –), following oxidation with ferricyanide (–·–·–·–), following reduction with dithionite (——), and dithionite reduced plus CO (····).

subunit molecular mass determined on the SDS-polyacrylamide gel.

Amino acid analysis and sequence. The minimum molecular mass determined from the amino acid composition and iron concentration was similar to that determined by SDS-polyacrylamide gels or by mass spectroscopy (Table 1). The amino acid composition and N-terminal amino acid sequence presented in Table 1 and Fig. 3 indicate that the cytochrome P-460 from *M. capsulatus* Bath is similar but not identical to the enzyme from *N. europaea*.

Heme and metal components. As reported for the cytochrome P-460 from *N. europaea* (18), the prosthetic group of cytochrome P-460 was very sensitive to H_2O_2 and not extracted with acid-acetone. The heme also remained associated with the polypeptide in SDS-polyacrylamide gels in samples incubated in sample buffers containing 1.5% β-mercaptoethanol at room temperature and stained weakly with diaminobenzidine (37). The absorption spectrum of the pyridine ferrohemochrome of cytochrome P-460 was similar but not identical to that of the enzyme from *N. europaea*, with absorption maxima at 420, 528, and 556 nm (Table 2).

As determined by atomic absorption in conjunction with the protein determination (36), with bovine serum albumin as a standard, the content of iron and copper of the purified enzyme was 51.2 and 51.4 nmol/mg of protein, respectively. The concentration of iron and copper of the purified enzyme based on the amino acid composition was 71.6 and 71.9 nmol/mg of protein, respectively. Based on the molecular mass determined by SDS-gel electrophoresis (16,800 Da) or by mass spectroscopy (16,390 Da), a protein containing one iron atom should have a metal content of 59.5 or 61 nmol/mg of protein, respectively. The lower value of copper and iron per milligram of protein is the consequence of the method of Lowry et al. (36), which overestimates the protein content of cytochrome P-460. Comparison of protein concentration by that method with bovine serum albumin as the standard and amino acid analysis indicates that the Lowry method overestimates the protein concentration by a factor of 1.4.

The *M. capsulatus* Bath enzyme contained less than 7 mol% zinc.

Spectral properties. Cytochrome P-460 was first detected by absorption spectroscopy in the 40 to 60% ammonium sulfate fraction (Fig. 4). As described by Sokolov et al. (54), the optical spectrum of this fraction is similar to that of HAO (Fig. 4) (22); however, unlike HAO, the *c*-type cytochromes could be separated from cytochrome P-460.

The spectral properties of the dithionite-reduced and reduced-plus-CO samples isolated in the presence of cytochrome *c'* and the 61,200-Da polypeptide were similar to those of the sample isolated from *N. europaea* (Fig. 5) (43). The absorption maximum at 418 nm was associated with contaminating cytochrome *c'* in this sample. In the presence of these two polypeptides, the resting spectrum of cytochrome P-460 showed a significant population in the ferrous form. Whether this spectrum is the result of interactions between heme P-460 and the chromophores in the 61,200-Da polypeptide and cytochrome *c'* or of a difference in the heme environment has not been determined.

Separation of the 61,200-Da polypeptide and cytochrome *c'* from cytochrome P-460 resulted in a 13-nm spectral shift in the soret band of the ferrocytochrome spectrum (Fig. 4 to 6). In the absence of dodecyl-β-D-maltoside or dodecyl-β-D-glycopyranoside, reduction of the purified sample by $Na_2S_2O_3$ in the presence of oxygen lowered the absorbance by 70 to 80% compared with the absorbance observed under anaerobic conditions (data not shown). Reduction under anaerobic conditions or in the presence of dodecyl-β-D-maltoside or dodecyl-β-D-glycopyranoside protected the enzyme from destruction by $Na_2S_2O_3$ and O_2. The addition of carbon monoxide, cyanide, or azide before the addition of dithionite also protected the enzyme from the effects of $Na_2S_2O_4$ and O_2, as was observed for cytochrome aa_3 in *N. europaea* (14).

The absorption spectrum of the purified cytochrome P-460 from *M. capsulatus* Bath differed from that of the enzyme of *N. europaea* in several ways. First, the ferricytochrome from *M. capsulatus* Bath lacked the 535-nm absorption maximum ob-

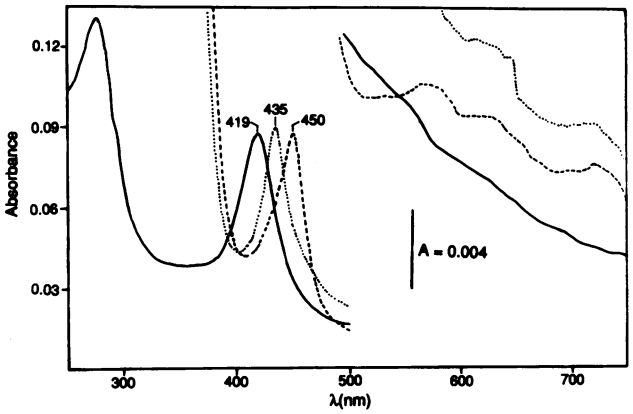

FIG. 6. Absorption spectra of cytochrome P-460, 26 μg of protein per ml, in 10 mM Tris-HCl (pH 8.2). Absorption of the resting enzyme (····), dithionite reduced (——), and dithionite reduced plus CO (– – – –).

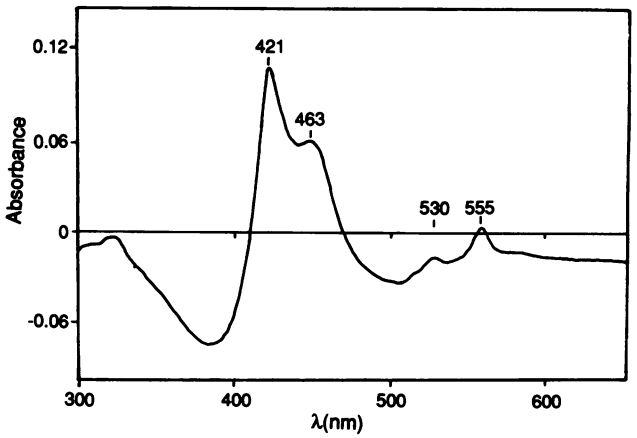

FIG. 8. Hydroxylamine reduced minus resting absorption spectra of the cytochrome P-460-containing fraction from the Sephadex G-75 column in 25 mM Tris-HCl–100 mM KCl buffer (pH 8.2).

served for the *N. europaea* enzyme (43). Second, the cytochrome from *M. capsulatus* Bath showed complete reduction with dithionite in less than 30 s. This is in contrast to the cytochrome from *N. europaea*, which requires approximately 16 min for complete reduction (18, 43). Lastly, the soret band of the ferricytochromes of the two purified enzymes differed by 10 to 13 nm.

EPR spectrum. The low-temperature X-band EPR spectra of the resting cytochrome P-460 in the presence and absence of cytochrome *c'* and the 61,200-Da polypeptide are shown in Fig. 7. The purified cytochrome P-460 was typical of a high-spin ferric heme (g = 6.19) and showed a marked similarity to cytochrome P-460 from *N. europaea* (2, 43) (Fig. 7B). In contrast to the spectral changes observed in the absorption spectra, the separation of cytochrome *c'* and the 61,200-Da polypeptide had little effect on the EPR spectra of cytochrome P-460 (Fig. 7A). The second high-spin ferric heme signal at g = 6.0 is associated with cytochrome *c'*. The copper associated with cytochrome P-460 is apparently EPR silent. Future studies will examine the nature of the copper in this enzyme.

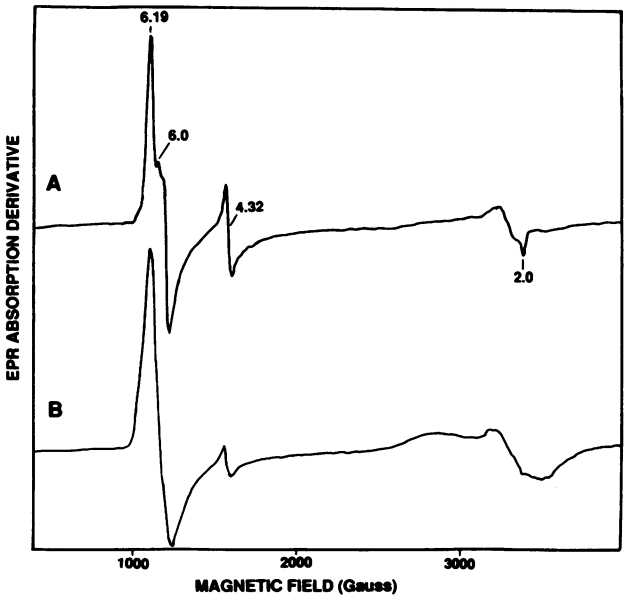

FIG. 7. EPR spectrum of (A) sample following chromatography on phenyl-Sepharose (84 mg of protein per ml) and (B) purified cytochrome P-460 (180 μM) in 10 mM Tris-HCl (pH 8.2) at 8 K. Instrumental conditions were as follows: modulation frequency, 100 kHz; modulation amplitude, 4 G; microwave frequency, 9.422 GHz; time constant, 100 ms.

Enzyme activity. As described by Dalton (11), cell extracts of *M. capsulatus* Bath oxidized hydroxylamine to nitrite at rates of 0.6 ± 0.1 nmol of nitrite produced per min per mg of protein and was stimulated more than 20-fold by the addition of PMS. As shown in Fig. 8, the addition of hydroxylamine to the cell extracts resulted in the reduction of cytochrome P-460 and cytochrome *c*-555. However, in the absence of an additional electron acceptor such as PMS, no detectable nitrite is formed.

The purified enzyme was active with PMS as an electron acceptor (Table 2) but inactive with cytochrome *c*-555, cytochrome *c*-554, cytochrome *c*-557 (15), and cytochrome *c'* from *M. capsulatus* Bath. The turnover rates (Table 2) were higher than observed with the enzyme from *N. europaea* and were surprisingly close to the turnover rates observed with HAO (26). The product of hydroxylamine oxidation by cytochrome P-460 was nitrite; the stoichiometry of hydroxylamine oxidized to nitrite produced was 1:0.85. As in cell extracts (11), the hydroxylamine oxidation activity by the purified enzyme was stimulated 2-fold by low concentrations of cyanide (0.7 mM) and 1.5-fold by 8-hydroxyquinoline (100 μM) (Table 2). Higher concentrations (10 mM) of cyanide inhibited hydroxylamine oxidation by 90%.

DISCUSSION

The relative ecological roles of the methanotrophs and nitrifiers in the oxidation of methane and ammonia have yet to be resolved. A problem in field studies is separating the contribution of ammonia-oxidizing and methane-oxidizing bacteria in the global carbon and nitrogen cycles (6). The similarities in the membrane-associated MMO (pMMO) and the ammonia monooxygenase (AMO) have made the development of a simple procedure for differentiating between the two groups of microorganisms impossible. Although neither enzyme has been purified, all available evidence indicates that both enzymes are copper proteins, are coupled to the membrane component of the electron transport chain, and may

have similar polypeptides (8, 13, 17, 24, 25, 28, 38, 46, 47, 52, 55, 57, 58). In addition, both AMO and pMMO will catalyze the energy-dependent oxidation of ammonia to hydroxylamine and methane to methanol. After the initial oxidation step, the similarities in ammonia and methane oxidation between these two groups of bacteria end. High concentrations (up to 500 μM) of methanol accumulate during methane oxidation by nitrifiers. The methanol is slowly oxidized to CO_2, with formaldehyde and formate as intermediates (29, 31, 60, 61). The enzyme(s) responsible for the oxidation of methanol, formate, and formaldehyde in whole-cell studies may prove to be the AMO, but this has yet to be determined. During ammonia oxidation by methanotrophs, hydroxylamine accumulates and is subsequently oxidized to nitrite (11). The results presented in this paper identify, for the first time, an enzyme capable of hydroxylamine oxidation from a methanotroph and show that the enzyme, cytochrome P-460, is different from the major enzyme responsible for in vivo oxidation in nitrifiers. This information may provide the needed method for separating the contributions of methanotrophs and nitrifiers in field studies.

Hydroxylamine oxidation to nitrite by methanotrophs has been examined by several authors; the activity has been described as insensitive to the MMO inhibitor acetylene and stimulated by PMS, cyanide, and 8-hydroxyquinoline (11, 27, 45, 54, 64). In keeping with earlier observations, the purified enzyme presented in this study is insensitive to acetylene and is stimulated by PMS, cyanide, and 8-hydroxyquinoline. The results presented in this study indicate that the enzyme catalyzing this activity appears to belong in the cytochrome P-460 class of enzymes. This is the first report of a P-460-type enzyme outside of the ammonia-oxidizing bacteria.

In crude cell extracts, the optical absorption spectra of cytochrome P-460 from *M. capsulatus* Bath were similar to those of the enzyme observed in *N. europaea* (2, 43). The enzyme was also similar in molecular mass (M_r 38,000), subunit composition (α_2), ligand binding, and EPR spectra to cytochrome P-460 from *N. europaea*. The two enzymes also had similar amino acid compositions and N-terminal sequences. However, the optical spectrum and metal composition of the purified enzymes from *M. capsulatus* and *N. europaea* differed. In addition to iron, the enzyme from *M. capsulatus* Bath also contained equimolar levels of copper. Copper analysis of cytochrome P-460 from *N. europaea* has either not been done or not been published. The EPR spectrum of cytochrome P-460 from *N. europaea* indicates that copper either is not present or, like the enzyme from *M. capsulatus* Bath, is EPR silent (3, 43). Also in contrast to the enzyme from *N. europaea*, cytochrome P-460 from *M. capsulatus* Bath was very unstable in the absence of detergents or protease inhibitors. The optical spectrum of cytochrome P-460 was also stabilized by the addition of these detergents or protease inhibitors during purification.

The spectral shift in the soret band from 463 to 450 nm makes the nomenclature of the hydroxylamine-oxidizing enzyme from *M. capsulatus* Bath problematic. Cytochrome P-450, which has a protoporphyrin IX as a prosthetic group, has an absorption maximum at 450 nm in the reduced-plus-CO form (7, 20). The purified cytochrome P-460 from *M. capsulatus* Bath has an absorption maximum at 450 nm, but it is observed in the absence of carbon monoxide. Furthermore, in contrast to cytochrome P-450, the heme group in cytochrome P-460 is not extracted with acid-acetone (19). The enzyme also shows other molecular and spectral differences (optical and EPR) from bacterial cytochrome P-450s (20, 49, 53). The enzyme from *M. capsulatus* Bath is similar to cytochrome P-460 from *N. europaea* (Table 2) and has few properties in common with the cytochrome P-450 class of enzymes. Thus, the enzyme from *M. capsulatus* Bath is tentatively called cytochrome P-460.

The enzyme functions well as a hydroxylamine oxidoreductase and probably functions to protect the cell from this toxic compound. The cellular concentration of cytochrome P-460 from *M. capsulatus* Bath is similar to the levels of the enzyme observed in *N. europaea* (18). The function may also prove to be similar in both groups of microorganisms. The cellular location of cytochrome P-460 in *N. europaea* is unknown, but a cytoplasmic location would support a protective function.

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