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PRELIMINARY CHARACTERIZATION OF A VARIANT CO-BINDING HEME PROTEIN FROM *NITROSOMONAS*

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SUMMARY

A soluble pigment with a major absorption peak at 463 nm in the reduced-*minus*-oxidized absorption spectrum was found in the supernatant fraction resulting from centrifugation of extracts of *Nitrosomonas europaea*. It was purified by repeated ammonium sulfate fractionation, chromatography on Amberlite CG-50 and gel filtration with Sephadex G-100. The purified protein was essentially free of contaminating *a*-, *b*-, *c*- or *o*-type cytochromes but contained 30% RNA. Absorbance maxima of absolute spectra were as follows: oxidized, 435 nm with a broad shoulder at 490–580 nm; reduced, 460 nm; reduced-*plus*-CO, 446 nm. Small bands at 535 nm and 565 nm were seen in reduced-*minus*-oxidized spectra. The pigment was designated P-460. The prosthetic group was not extracted with acidic acetone. The ferrohemochrome of the pigment had absorption maxima at 433 nm, 530 nm and 560 nm. P-460 was slowly autooxidized but deteriorated to a form which did not absorb at 460 nm. Aging or treatment with cholate or ethanol caused conversion of the reduced or reduced-*plus*-CO P-460 to a 423-nm-absorbing form. Hydrazine, hydroxylamine, or cyanide caused a 7–12-nm red shift in the oxidized form of P-460 and a blue shift in the reduced form. Increasing pH in the range 6.5 to 8.5 caused a decrease in height of the 460-nm or 446-nm peaks of the reduced or reduced-*plus*-CO spectra, respectively, and a concomitant increase in the broad peak in the 530 region. Based on the preliminary spectral properties presented here, P-460 of *Nitrosomonas* appears to be a new heme protein.

INTRODUCTION

A soluble pigment with a reduced-*minus*-oxidized absorption maximum at 463–465 nm has been observed spectrophotometrically by a number of investigators in cell extracts of *Nitrosomonas europaea* and other ammonia-oxidizing nitrifying bacteria[1–3]. Rees and Nason[2] first showed that it bound carbon monoxide and proposed it to be a P-450-type cytochrome. It has also been tentatively identified as a soluble *a*-type[4] or *d*-type[5] cytochrome. Thus far, all observations have been carried out using crude extracts of *Nitrosomonas* which contained large amounts

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of *b*- and *c*-type cytochromes but in no instance has a purified preparation been characterized with respect to absolute absorption spectra.

This paper describes the purification and preliminary spectral characteristics of P-460 from *Nitrosomonas*. The most purified fraction was essentially free of *b*-, *c*- and *a*-type cytochromes and exhibited absorption maxima at 435 nm, 460 nm and 446 nm in the oxidized, reduced and reduced-*plus*-CO forms, respectively. It is similar in some aspects to the CO-binding cytochrome P-450, but it is shown here to differ in many important characteristics. We refer to this novel cytochrome as P-460.

METHODS

Spectra

Absorption spectra were measured at room temperature in a 1-ml volume (1 cm light path length) utilizing a Cary Model 15 or Model 14 Spectrophotometer. During purification, P-460 was measured as the absorbance difference between the value at 463 nm and a line connecting points at 447 nm and 488 nm in dithionite-reduced-*minus*-oxidized difference spectra. *c*-type cytochrome was measured as the absorbance difference between the value at 553 nm and a line connecting points at 540 nm and 564 nm.

For acid–acetone extraction of heme[2] 4 vol. of acetone and 1 vol. of 1.5 M HCl were added to 1 vol. of *Nitrosomonas* fraction and precipitated protein was removed by centrifugation at 10000 × *g* for 10 min. Pyridine derivatives were made by dissolving an untreated fraction of the acid–acetone precipitate in an appropriate volume of 2.1 M pyridine, 75 mM NaOH solution[6].

Pronase and ribonucleate digestion

Pronase and ribonuclease digestion of P-460 Fraction 10 were carried out by slightly modified procedures of Nomoto *et al.*[7] and Kalnitsky *et al.*[8], respectively. Fraction (110 μg of protein) and 1 μg of ribonuclease (3000 units/mg) in 1 ml of 50 mM phosphate buffer, pH 7.5, were incubated for 30 min at 30 °C. Pronase digestion was carried out at 30 °C for 24 h in 0.95 ml 50 mM phosphate buffer, pH 7.5, with 100 μg of pronase (45000 PUK/g), 110 μg of Fraction 10 and 0.05 ml ethanol. A control sample was incubated in the same reaction mixture *minus* pronase for 24 h. After incubation, the pronase, ribonuclease and control samples were brought to 95 % saturation with $(NH_4)_2SO_4$ (with the pH of the solutions maintained above 6.0) and stirred for 30 min at 4 °C. Each solution was centrifuged at 20000 × *g* for 10 min and the resulting pellet dissolved in 1 ml of 50 mM phosphate buffer, pH 7.5. The absorbance at 260 nm and 435 nm was measured in each pellet and supernatant solution.

Assays

All spectrophotometric assays were carried out at 25 ° using a Gilford Multiple sample Absorbance Recorder. Deoxyribonuclease used for standardization of the Sephadex column was assayed as the rate of increase in absorbance at 260 nm in a reaction mixture containing 0.5 ml of DNA substrate, 1 mM $MgCl_2$, enzyme and water to a total volume of 1 ml. To prepare the substrate, 1 mg DNA was allowed

to stand overnight at room temperature in 3 ml of distilled water. A 2 5 ml volume of 1.0 M acetate buffer, pH 5.0, was then added and the volume brought to 25 ml with water. Peroxidase was assayed[9] with *o*-dianisidine as electron donor. Phenazinemethosulfate-dependent nitrite synthetase[10], nitrite reductase[11] and hydroxylamine:cytochrome *c* reductase[1] activity and protein[1] were assayed as described previously. Reduced mammalian cytochrome *c* oxidase was assayed spectrophotometrically as the initial rate of oxidation of 60 μM reduced horse heart cytochrome *c* (reduced by titration with $Na_2S_2O_4$ followed by dialysis) in a 1-ml reaction mixture containing enzyme and 50 mM phosphate solution, pH 7.5. *p*-Phenylenediamine oxidase was assayed as the rate of oxygen utilization using a Yellow Springs Instrument oxygen analyzer. Enzyme was added to a 3-ml reaction mixture containing 10 mM *p*-phenylenediamine in 50 mM phosphate solution, pH 6.5. DNA was assayed colorimetrically by the indole reaction[12] and RNA by the orcinol assay[13].

Sources

Sodium cholate, *p*-phenylenediamine, *p*-hydroxymercuribenzoate, bathocuproine sulfonate, horseradish peroxidase, salmon DNA, bovine pancreas deoxyribonuclease, and Type II horse heart cytochrome *c* were purchased from Sigma Chemical Company (St. Louis, Mo.). Pronase was obtained from Calbiochem (Los Angeles, Calif.); bovine serum albumin from Mann Research Laboratories (New York); ribonuclease from Worthington (Freehold, N. J.); Sephadex G-100 and Blue Dextran 2000 from Pharmacia (Sweden); Amberlite CG-50 from Mallinckrodt Chemical Works (St. Louis, Mo.); and CO from Matheson Gas Products (East Rutherford, N.J.).

RESULTS

Purification

Unless otherwise indicated, all steps of the purification process summarized in Table I were carried out at 4 °C.

Batch cultures of *N. europaea* were grown and harvested as described previously[11]. To disrupt the cells by the freeze–thaw procedure[14] a 980-ml volume of a 0.2 g wet wt per ml suspension of *Nitrosomonas* in 50 mM phosphate solution, pH 7.5, containing a small amount of pancreatic deoxyribonuclease was frozen at −10 °C and thawed 3 times and the resulting homogenate designated the crude homogenate (Fraction 1). Fraction 1 was centrifuged for 20 min at 20000 × *g* and the resulting particulate fraction resuspended in phosphate solution to a concentration of 400 mg wet pellet per ml and sedimented for 20 min at 20000 × *g*. The two supernatants were combined and designated Fraction 2.

Fraction 2 was subjected to stepwise precipitation with $(NH_4)_2SO_4$ at 17, 26, 31, 42, 48, 54, 64, 70, 74, 78, 83, and 92 % saturation. Solid $(NH_4)_2SO_4$ was added at each step and the suspension stirred for 45 min. The suspension was centrifuged at 20000 × *g* for 15 min and the resulting precipitate dissolved in 50 mM phosphate buffer, pH 7.5. The resulting supernatant was then subjected to the next step in the $(NH_4)_2SO_4$ fractionation procedure. The fractions precipitating between 74 and 83 % saturation with $(NH_4)_2SO_4$ were pooled and designated Fraction 3.

For the next purification step a column (4 cm × 40 cm) was filled with Amber-

TABLE I

SUMMARY OF PURIFICATION OF P-460 FROM *Nitrosomonas*

Cytochrome P-460 (absorbance of the major peak at 463 nm) and cytochrome *c* (absorbance at 553 nm) were measured in reduced-*minus*-oxidized difference spectra as described in Methods.

Fraction	*Protein (mg/ml)*	*Ratio* $\frac{A_{463\ nm}}{A_{553\ nm}}$	*Ratio* $\frac{A_{463\ nm}}{mg\ protein}$	*Total* $A_{463\ nm}$
1 Crude homogenate	25.64	0.26	0.026	656
2. 20000 × *g* supernatant and wash supernatant	10.80	0.32	0.052	620
3. 74–83% $(NH_4)_2SO_4$ precipitate	7.30	0.34	0.112	52
4. Pooled eluate of 1st amberlite column	0.48	4.38	0.304	12
10 Sephadex eluate	2.27	1.21	0.638	3

lite CG-50 which had been equilibrated with 10 mM ammonium phosphate buffer (10 mM NH_4^+), pH 6.0, and the resin bed was stabilized by the passage of 2 vol. of the same buffer according to Horio *et al.*[15]. Fraction 3 was dialyzed for 6 h against 1 l (two changes) of the equilibrating buffer at a flow rate of 30 drops per min and collected in 15-ml fractions. A single protein band which contained none of the assayed enzyme activities was eluted. The first several tubes were green in color and were enriched with P-460 while the later tubes were red and contained *c*-type cytochromes.

Tubes containing P-460 were pooled (Fraction 4) and solid $(NH_4)_2SO_4$ was added to 78% saturation. The pH of the solution was maintained above 6.0 by titration of the $(NH_4)_2SO_4$-protein suspension with 50 mM phosphate buffer, pH 7.5. The suspension was stirred for 30 min and centrifuged for 15 min at 20000 × *g*. The green-brown pellet was redissolved in a minimum volume of 10 mM ammonium phosphate (10 mM NH_4^+) buffer, pH 6.0, and dialyzed twice against 500 ml of the same buffer for 3 h (Fraction 5).

A second Amberlite CG-50 column (2.5 cm × 41 cm) was packed and equilibrated as before with 10 mM ammonium phosphate buffer, pH 6.0. Fraction 5 was carefully layered on the column bed which was then developed with equilibrating buffer, collecting fractions in 5-ml volumes at a flow rate of 15 drops per min. Fractions containing P-460 were pooled to yield a green-colored Fraction 6 to which $(NH_4)_2SO_4$ was added slowly to 78% saturation. The suspension was stirred for 30 min, centrifuged for 15 min at 20000 × *g* and the resulting green pellet dissolved in a minimum volume of 50 mM phosphate buffer, pH 7.5, to a concentration of approximately 0.5 mg of protein per ml (Fraction 7). The same $(NH_4)_2SO_4$ fractionation procedure was repeated twice in Fraction 7 to yield Fractions 8 and 9.

A column of Sephadex G-100 was packed and equilibrated by passage of 3 vol. of 0.1 M NaCl–50 mM phosphate solution, pH 7.5, at a flow rate of 4 drops per min under 20 cm of hydrostatic pressure. Fraction 9 containing 5% (w/v) sucrose was layered on the column bed and eluted at a flow rate of 6 drops per min. Fractions containing P-560 were pooled and the pooled fraction was brought to 78% saturation with respect to $(NH_4)_2SO_4$ and centrifuged as before. The precipitate was dissolved in a minimum volume of 50 mM phosphate buffer, pH 7.5, and the clear green-yellow solution (Fraction 10) was used for further studies.

Approximately 8% of the total P-450 in Fraction 1 was present in Fraction

3 whereas 68 % of the total P-460 was recovered in the other ammonium sulfate fractions within the same purification step. However, the fractions making up Fraction 3 were chosen on the basis of *p*-phenylenediamine oxidase activity and not P-460 content because the enrichment in P-460 was not noted until after the relatively pure green Fraction 4 had eluted from the Amberlite column. As shown in Table I, P-460 of Fraction 10 was enriched 400-fold with respect to cytochrome *c*-553 and 12-fold with respect to protein.

While P-460 of Fraction 10 was stable for several months when frozen, several weeks of repeated freezing and thawing resulted in a progressive diminution in the 460 nm peak.

Molecular weight and purity

The molecular weight of P-460 was estimated to be approximately 52000 by Sephadex chromatography (Fig. 1) with reference to bovine albumin, mol. wt 67000[16]; horseradish peroxidase, mol. wt 40000[17]; deoxyribonuclease, mol. wt 31000[18]; horse heart cytochrome *c*, mol. wt 12400[16]. Fraction 10 contained 70 % protein and 30 % RNA by weight but no DNA. The large amount of RNA was first noted in Fraction 4, and remained in subsequent purification steps. Allowing for a 30 % RNA content by weight, the *maximum* apparent molecular weight of the protein moiety of P-460 was approximately 36000.

Fraction 10 did not contain *p*-phenylenediamine oxidase, mammalian cytochrome *c* oxidase, nitrite reductase or hydroxylamine:cytochrome *c* reductase activity.

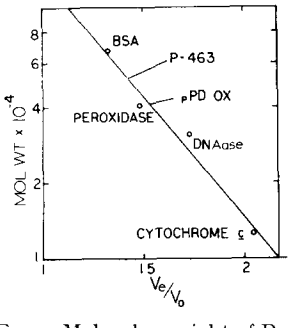


Fig 1. Molecular weight of P-460 as determined by gel filtration. V_e, elution volume of the applied material. V_0, void volume determined with Blue Dextran 2000. Molecular weights of the reference proteins are given in the text. BSA, bovine serum albumin; pPD OX., *p*-phenylenediamine oxidase.

Absorption spectra

As shown in Fig. 2 the absolute absorption spectrum of oxidized Fraction 10 displayed a peak at 435 nm with a broad shoulder extending from 490 to 580 nm. Upon reduction with dithionite, the peak sharpened and shifted to 460 nm with the concomitant disappearance of the broad 490–580-nm shoulder. When CO was bubbled through the reduced sample, the major peak shifted to 446 nm and a shoulder appeared at approximately 420 nm. The spectrum of oxidized P-460 showed no displacement of the 435-nm peak in the presence of CO although a slight increase in absorbance was noted in the 420-nm region. The spectrum of the oxidized

sample contained a large peak at 258 nm due to the presence of RNA. No change in the ultraviolet spectrum was observed when the sample was treated with $NaBH_4$.

A major absorption band at 462 nm and a trough in the 490–560-nm region were evident in the reduced-*minus*-oxidized difference spectrum (Fig. 3). Although not seen in Fig. 2 or 3, small peaks at approximately 535 nm and 565 nm occurred in reduced or reduced-*minus*-oxidized spectra. The reduced-*plus*-CO-*minus*-reduced

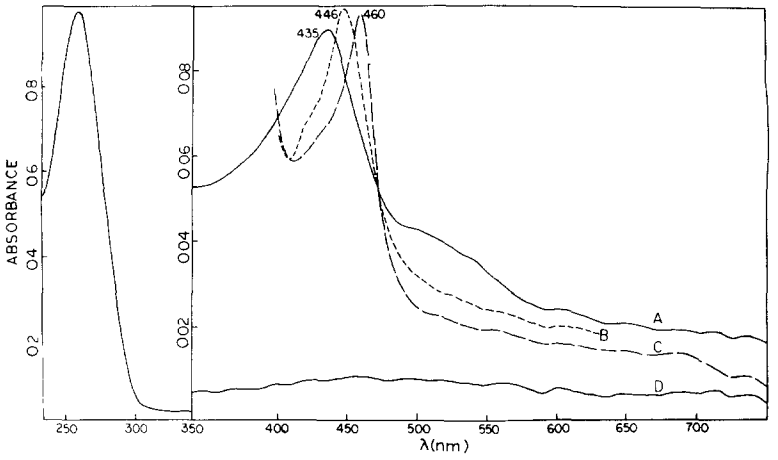


Fig. 2. Absolute absorption spectrum of P-460 from *Nitrosomonas*. An aliquot of Fraction 9 containing 68 μg of protein was diluted to 1 ml with 50 mM sodium–potassium phosphate buffer, pH 7.5. The oxidized spectrum (Curve A) was measured against a reference cuvette containing phosphate solution. After the addition of a few crystals of $Na_2S_2O_4$ to the sample, the reduced spectrum (Curve C) was measured. CO was then bubbled through the reduced sample for 1 min and the spectrum (Curve B) was recorded Curve D represents the baseline as measured with phosphate solution Inset: spectrum of the oxidized sample.

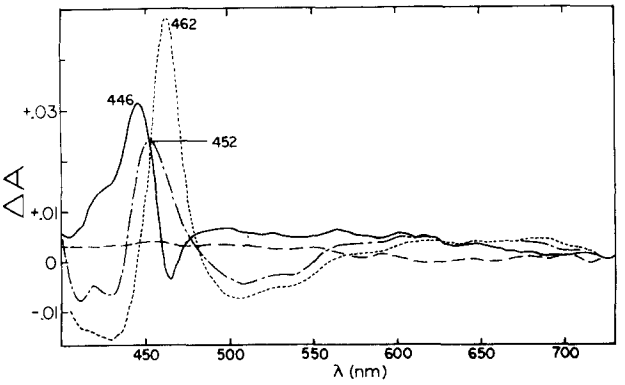


Fig. 3. Difference spectra of P-460 from *Nitrosomonas*. An aliquot of Fraction 9 containing 136 μg of protein was diluted to 2 ml with 50 mM sodium–potassium phosphate buffer, pH 7.5, divided between sample and reference cuvette and the baseline (— — —) recorded. The sample cuvette was reduced with a few crystals of $Na_2S_2O_4$ and the reduced-*minus*-oxidized difference spectrum (-------) measured. CO was then bubbled for 90 s through the sample cuvette and the reduced-*plus*-CO-*minus*-oxidized spectrum (– – – –) recorded The reduced-*plus*-CO-***minus***-reduced spectrum (———) was subsequently measured by reducing a new 2-ml volume of P-460 with dithionite, dividing into sample and reference, running a baseline and treating the sample with CO.

difference spectrum (Fig. 3) contained the major CO-induced band at 446 nm, a shoulder at 420 nm and a trough at 462 nm. The shoulder at 420 nm induced by reaction of CO with the reduced sample (Fig. 2) was evident as a peak in the reduced-*plus*-CO-*minus*-oxidized spectrum (Fig. 3). The latter spectrum consisted of a prominent peak at 452 nm and a trough in the 490–580-nm region.

Prosthetic group

The prosthetic group of P-460 in Fraction 10 was not extracted by treatment with acidic acetone. Absorption spectra of the ferrihemochrome of the protein in alkaline pyridine (Fig. 4A) exhibited a broad absorption peak at 449 nm and a shoulder at approximately 408 nm. The spectrum of dithionite-reduced ferrohemochrome contained a major band at 433 nm and small α and β bands at approximately 560 nm and 530 nm, respectively. Absorption peaks at 433 nm, approximately 530 nm and 560 nm and a trough in the 450–520-nm region were apparent in the

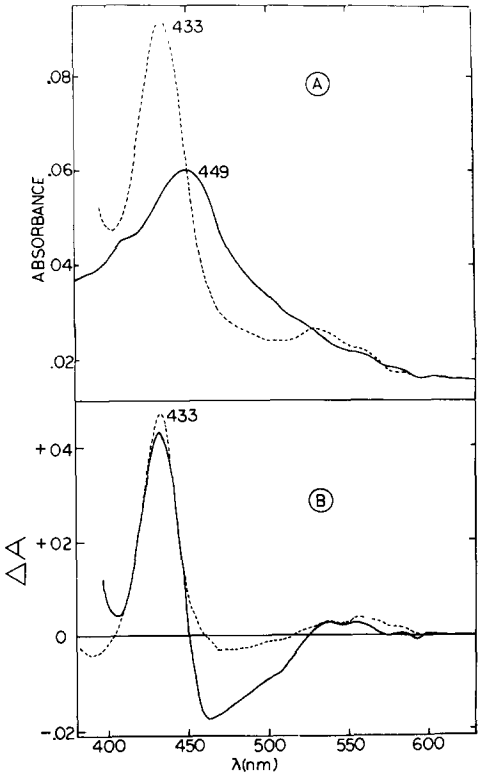


Fig. 4. Absorption spectrum of the pyridine derivative of *Nitrosomonas* P-460. (A) An aliquot of Fraction 9 containing 68 μg of protein was diluted to 1 ml with 2.1 M pyridine and 75 mM NaOH and the ferrihemochrome absorption spectrum (———) measured immediately. Following reduction of the oxidized sample with a few crystals of $Na_2S_2O_4$ the ferrohemochrome absorption spectrum (-------) was immediately recorded. In each case the reference cuvette contained pyridine–NaOH. (B) Two aliquots of Fraction 9 (68 μg of protein) were placed in a sample and reference cuvette and diluted to 1 ml with pyridine–NaOH. The sample cuvette was reduced with a few crystals of $Na_2S_2O_4$ and the spectrum (———) measured immediately. The reduced-*plus*-CO-*minus*-reduced difference spectrum of the pyridine derivative (-------) was then determined as described in Fig. 3.

reduced-*minus*-oxidized difference spectrum (Fig. 4B). With the addition of CO to the ferrohemochrome, increases occurred in the 433-nm and 560-nm absorption peaks and the 460-nm trough, as reflected in the reduced-*plus*-CO-*minus*-reduced difference spectrum (Fig. 4B).

Pronase and ribonuclease digestions

Table II illustrates the results of digestion of P-460 Fraction 10 with pronase and ribonuclease followed by precipitation of the protein with $(NH_4)_2SO_4$. Because of the low protein concentration of Fraction 10, not all of the P-460 was precipitated with $(NH_4)_2SO_4$ as the control figures show. A 10-fold enrichment of 435-nm absorbing pigment relative to 260-nm absorbance was obtained in the ribonuclease-treated pellet indicating separation of P-460 from ribonucleotides. In contrast, following treatment with pronase, the 435-nm-absorbing material was less precipitable than the control. Thus the prosthetic group of P-460 was apparently attached to protein rather than RNA.

TABLE II

DIGESTION OF FRACTION 10 WITH PRONASE AND RIBONUCLEASE

An aliquot of Fraction 10 containing 110 μg of protein was incubated with pronase or ribonuclease and treated with $(NH_4)_2SO_4$ as described in Methods. The pellet was dissolved in phosphate buffer and brought to the same volume as the supernatant. The absorbance was measured at 435 nm and 260 nm in each sample.

Treatment	$(NH_4)_2SO_4$ *fraction*	$A_{435\ nm} \times 10^3$	$A_{260\ nm} \times 10^3$	*Ratio* $\frac{A_{435\ nm}}{A_{260\ nm}}$
Control	Supernatant	5.2	1.07	4.9
	Precipitate	10	2.55	3.9
Ribonuclease	Supernatant	5.5	2.58	2.1
	Precipitate	10	0.274	37
Pronase	Supernatant	11	1.17	9.4
	Precipitate	3.8	1.99	1.9

Ligand effects

In the presence of hydrazine, the major peak of the absolute oxidized absorption spectrum shifted from 435 to 445 nm (Fig. 5A). Following the addition of a few crystals of $Na_2S_2O_4$ to the hydrazine-containing sample, the peak shifted to 457 nm (Fig. 5C) and the 535- and 565-nm peaks increased. With further addition of CO (Fig. 5B) the major peak shifted to 447 nm. In the presence of hydroxylamine (Fig. 5D) the 435-nm peak shifted to 442 nm. With the subsequent addition of dithionite the major absorption band shifted to 445 nm and small peaks appeared at 535 nm and 565 nm (Fig. 5E). The subsequent addition of CO resulted in no change in the hydroxylamine-*plus*-dithionite spectrum. In the presence of hydrazine or hydroxylamine, the oxidized form (Figs 5A and 5D) no longer exhibited as prominent a 490–580-nm shoulder as was observed in the absolute oxidized spectrum (Fig. 2A).

In the presence of KCN, the 435-nm-absorption peak in the oxidized spectrum (Fig. 6D) shifted to a broader peak at 447 nm (Fig. 6E) with a concomitant slight diminishing of the 490–580-nm shoulder. The oxidized-*plus*-cyanide-*minus*-oxidized difference spectrum (Fig. 6C) exhibited a trough at 413–427 nm and peak at 460 nm. Subsequent reduction with dithionite (Fig. 6F) resulted in the appearance of a 455-nm-absorption peak, a further decrease of the 490–580-nm shoulder and the appearance of small peaks at 535 nm and 565 nm. The absence of major cyanide-induced spectral shifts in the reduced form of P-460 were indicated by the reduced-*plus*-cyanide-*minus*-reduced difference spectrum (Fig. 6B).

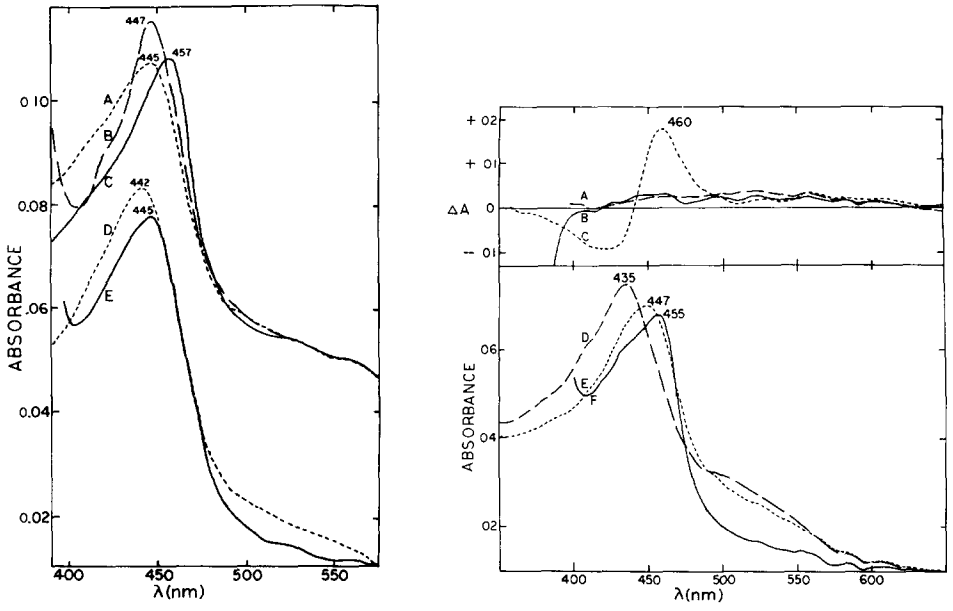


Fig. 5. Absolute absorption spectrum of P-460 from *Nitrosomonas* in the presence of hydrazine and hydroxylamine. An aliquot of Fraction 10 containing 68 μg of protein was diluted to 1 ml with 50 mM sodium–potassium phosphate buffer, pH 7.5. Hydrazine or hydroxylamine were present at a final concentration of 1 mM. The oxidized sample in the presence of hydrazine (Curve A) was treated with a few crystals of $Na_2S_2O_4$ and the spectrum (Curve C) recorded. Curve B represents the dithionite-reduced-*plus*-hydrazine sample after the addition of CO. Another oxidized sample of P-460 was treated with hydroxylamine (Curve D) and subsequently reduced with $Na_2S_2O_4$ (Curve E).

Fig. 6. Effect of cyanide on the absorption spectrum of P-460. An aliquot of Fraction 10 containing 68 μg of protein was added to a cuvette and diluted to 1 ml with sodium–potassium phosphate buffer, pH 7.5. The absolute oxidized spectrum was recorded before (Curve D) and after (Curve E) the addition of KCN (1 mM). A few crystals of $Na_2S_2O_4$ were then added to the cyanide-treated sample (Curve E). Curve A represents the baseline obtained with P-460 (68 μg protein) in sample and reference cuvettes. The oxidized-*plus*-cyanide-*minus*-oxidized difference spectrum is shown by Curve C, while the reduced-*plus*-cyanide-*minus*-reduced difference spectrum is demonstrated by Curve B.

In summary, spectral shifts in the Soret absorption band varying from 7 to 12 nm towards a longer wavelength and a slight diminution of the 450–580-nm shoulder were observed with the oxidized form of P-460 in the presence of hydrazine, hydroxylamine or cyanide. Each of the ligands prevented, to varying extents,

subsequent dithionite reduction of the pigment to a form with the characteristic 460-nm peak. Although the ligands caused a decrease in the 490–580-nm shoulder of the oxidized P-460, the appearance of the 535-nm- and 565-nm-absorption bands occurred only upon the addition of dithionite. NaN_3 or $NaNO_2$ (1 mM), had no effect on the oxidized or reduced spectrum of P-460.

Effect of pH on spectra

The pH of the buffer solution affected peak heights in the reduced (Fig. 7A) and reduced-*plus*-CO (Fig. 7B) spectra of P-460, but had no effect on the oxidized form. At pH 6.5 the 460-nm and the 446-nm peaks were considerably heightened as compared to a higher pH value of 8.5. Conversely, the peak in the 535-nm region was larger at the higher pH values.

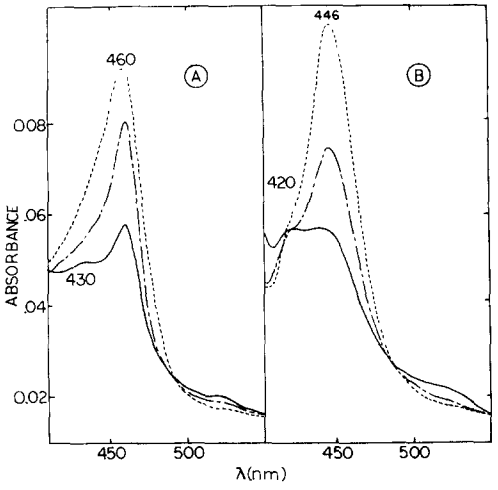


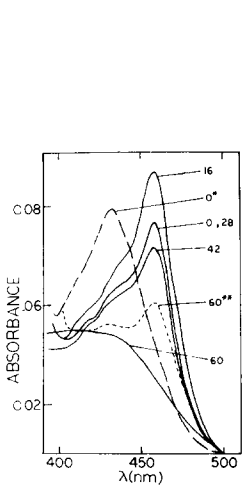
Fig. 7. Effect of pH on the absolute absorption spectrum of P-460. An aliquot of Fraction 10 containing 68 μg of protein was diluted to 1 ml with either 50 mM sodium–potassium phosphate buffer, pH 6.5 (-------), 50 mM sodium–potassium phosphate buffer, pH 7.5 (– – – –) or 50 mM Tris–HCl buffer, pH 8.5 (————). The sample was reduced with a few crystals of $Na_2S_2O_4$ and the absolute spectra (Inset A) measured. CO was then bubbled through the reduced samples and the resulting spectra (Inset B) recorded.

Autooxidizability

Fig. 8 shows the absorbance changes observed over time in a reduced sample of P-460. 16 min after the addition of dithionite the 460-nm peak had reached a maximum value and shoulders were apparent at approximately 430 nm and 420 nm. From 16 to 60 min, there was a gradual diminution of the 460-nm peak. After 60 min the 460-nm peak was replaced by a broad absorption peak in the 400–480 nm region with no substantial increase observed in the 435-nm peak. When dithionite was again added, in excess, to the 60-min sample, the size of the 460-nm peak was considerably less than that originally observed. Thus it appeared that P-460 slowly autooxidized and deteriorated.

Conversion to a 423-nm-absorbing form

In the presence of sodium cholate, the major absorption peak observed in the reduced-*plus*-CO absolute spectrum shifted to 423 nm (Fig. 9A). Initially peaks



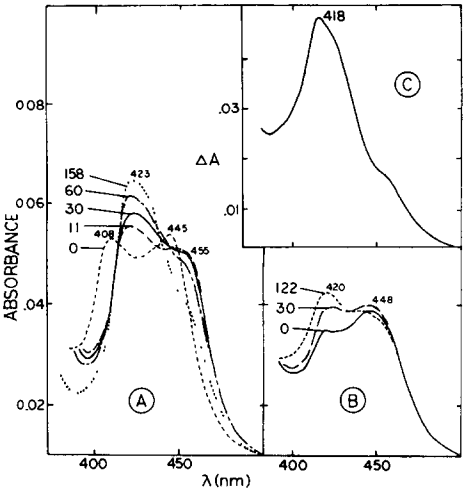


Fig. 8. Autooxidation of P-460 from *Nitrosomonas*. An aliquot of Fraction 10 containing 68 μg of protein was diluted to 1 ml with 50 mM phosphate buffer, pH 7.5. The sample was titrated with a small amount of $Na_2S_2O_4$ in order to just reduce the pigment and the absolute absorption spectrum was recorded at the times (min) indicated with repeated inversion of the cuvettes.

* Absolute oxidized absorption spectrum before the addition of $Na_2S_2O_4$.

** Absorption spectrum of the 60-min sample after the addition of an excess of $Na_2S_2O_4$.

Fig. 9. Conversion of P-460 to a 423-nm-absorbing form. Inset A: An aliquot of Fraction 10 containing 68 μg of protein was diluted to 1 ml with 50 mM phosphate buffer, pH 7.5, containing 3% (w/v) sodium cholate The sample was reduced with a few crystals of dithionite and treated with CO for 1 min. Absolute absorption spectra were recorded at the indicated times (min). Inset B: The sample (68 μg protein) was diluted to 1 ml with 50 mM phosphate solution, pH 7.5, reduced with dithionite and treated with CO. Absolute spectra were recorded at the indicated times (min). Inset C: The reduced-*plus*-cholate-*plus*-CO-*minus*-reduced-*plus*-CO difference spectrum after 160 min of incubation.

were present at 408 nm and 445 nm whereas at 11 min there were peaks at 423 nm and 455 nm. With time a gradual increase in the 423-nm peak occurred concomitant with a decrease in the 455-nm peak. After 2.5 h the 423-nm peak was predominant. A similar but less dramatic increase in a peak at 420 nm was revealed by the reduced-*plus*-CO absolute spectrum in the absence of cholate (Fig. 9B). Thus the reduced-*plus*-CO-*plus*-cholate-*minus*-reduced-*plus*-CO difference spectrum of a sample incubated for 160 min (Fig. 9C) exhibited a peak at 418 nm and a 455 nm shoulder. The sample used for the experiment shown in Fig. 9 had apparently undergone partial conversion during storage as indicated by the zero time spectrum of Fig. 9B.

In the presence of 25 % ethanol, 50 mM phosphate solution, pH 7.5, the oxidized absolute spectrum displayed a peak at 435 nm, and two peaks at 435 nm and 455 nm when reduced. Subsequent addition of CO to the reduced form resulted in a peak at 425 nm with a shoulder at 455 nm.

In the presence of 1.0 mM bathocuproine sulfonate only a small 418-nm peak was observed in a reduced-*plus*-CO-*plus*-bathocuproine-*minus*-reduced-*plus*-CO difference spectrum after 2.5 h of incubation. A spectral shift was not observed with 10^{-4} M *p*-hydroxymercuribenzoate after an equivalent length of time.

DISCUSSION

This paper describes a method of partial purification and the preliminary spectral characteristics of a new, soluble CO-binding protein from *Nitrosomonas* which had previously been observed by its 463-nm-absorption maximum in reduced-*minus*-oxidized difference spectra of intact cells or crude homogenates. Major absorption maxima were observed at 435 nm, 460 nm, 446 nm, and 462 nm in oxidized, dithionite-reduced and dithionite-reduced-*plus*-CO-absolute spectra and in reduced-*minus*-oxidized difference spectra, respectively. The pigment was thus designated P-460. The oxidized form of the protein had a broad absorption maximum in the 510–580-nm region which disappeared upon reduction with dithionite and remained absent with subsequent addition of CO. Small peaks which were consistently observed in the 530–535-nm and 555–565-nm regions are tentatively considered to be the α and β peaks, respectively, of a cytochrome P-460. We have not consistently observed absorption maxima or troughs in the 600–750-nm region of oxidized, reduced or reduced-*minus*-oxidized spectra which could have accounted for the green color of the protein.

Because the most highly purified fraction contained 30% RNA by weight, the protein may have existed in an aggregate containing several molecules of P-460 and the molecular weight of the protein may be less than 36000 as determined for the total protein in the aggregate.

In the purification reported here P-460 was enriched 400-fold with respect to 553-nm-absorbing material. The 408-nm peak observed in the oxidized absolute spectrum and the 420-nm, 530–535-nm and 555–565-nm peaks in the reduced absolute spectra may be attributed to the presence of a *b*-type cytochrome, cytochrome *o* or the 423-nm-absorbing form of P-460. The latter two possibilities are favored because the 420-nm peak was clearly affected by CO and the peaks at 420 nm, 535 nm and 565 nm were observed in the alkaline–pyridine ferrohemochrome of the acid–acetone-insoluble material. In addition, the absorbance peaks at 420 nm, 530–535 nm and 555–565 nm were affected by ligands such as CO, cyanide, hydrazine and hydroxylamine which also affected the 460-nm region of P-460.

The 435-nm-absorbing prosthetic group of P-460 was not dissociated by treatment with acidic acetone indicating a covalent form of bonding to the protein as is the case with *c*-type cytochromes[19]. Treatment here with pronase but not ribonuclease released the 435-nm-absorbing material into a form which was soluble in saturated $(NH_4)_2SO_4$ indicating that the primary association of the prosthetic group of P-460 was with protein rather than RNA. Although P-460 was present in dithionite-reduced-*minus*-oxidized spectra of intact cells, there remains the possibility that the prosthetic group was converted to a covalently bound form during extraction and purification. In some instances dilution of cell extracts effect a change from non-covalent to a covalent form of heme binding[20].

The alkaline–pyridine ferrohemochrome spectrum of P-460 did not precisely resemble the spectrum of any well characterized heme[21] although the α and β peaks were possibly indicative of the ferrohemochrome of protoporphyrin IX. Further studies are necessary to identify the heme of P-460 from *Nitrosomonas*.

The spectral properties of P-460 from *Nitrosomonas* are different from those of any previously-reported heme protein. It has been proposed that the *Nitro-*

somonas protein giving a reduced-*minus*-oxidized absorption peak at 463–465 nm was a form of cytochrome *a*[4], cytochrome *d*[5], or a cytochrome P-450[2]. P-460 has in common with those proteins a Soret peak at an unusually long wavelength and the fact that it binds CO. P-460 does not appear to be cytochrome *a* or *d* because, as reported here, α peaks which could be attributed to cytochromes *a*[22] or *d*[23,*] were not observed. Moreover, the hemes of cytochromes *a* and *d* are easily extracted by acidic acetone whereas the heme of P-460 was not.

As reported here, the absolute reduced-*plus*-CO spectra of P-460 with a peak at 446 nm strongly resembled the corresponding reduced-*plus*-CO compound of cytochrome P-450 from mammalian microsomes[24] or *Pseudomonas*[23]. The cyanide difference spectra of P-460 from *Nitrosomonas* closely resembled those reported for P-450 or *Rhizobium japonicum*[5]. Also, P-460 was slowly autooxidizable as is P-450[27]. Cytochrome P-450 is converted to a P-420 form by aging or with a variety of organic solvents and detergents[26,24], and we have shown a similar conversion of P-460 to a 423-nm-absorbing form in the presence of sodium cholate and ethanol. Bathocuproine sulfonate or *p*-hydroxymercuribenzoate are effective in conversion of P-450 to P-420 with microsomal[26] or bacterial[5] cytochrome P-450; however, bathocuproine sulfonate had only a very slight effect on P-460 of *Nitrosomonas* and *p*-hydroxymercuribenzoate had no effect.

Reduced P-460 and the CO derivative of *Nitrosomonas* have an interesting similarity to the corresponding ethyl isocyanide derivatives of cytochrome P-450 of liver microsomes. The latter have major absorption peaks at 455 nm and 445 nm, respectively[27].

The differences between P-460 of *Nitrosomonas* and P-450 of mammalian microsomes are perhaps more striking than the similarities. Cytochrome P-450 has Soret absorption maxima at 414 nm and 412 nm, in the oxidized and reduced forms, respectively, as compared with corresponding values of 435 nm and 460 nm for P-460. Cytochrome P-450 isolated from mammalian[24,28] and bacterial[23] sources is a *b*-type cytochrome with acid–acetone-extractable protoheme as the prosthetic group. In contrast, the prosthetic group P-460 of *Nitrosomonas* was not extracted with acidic acetone and may not be protoheme.

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