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Hydroxylamine Oxidoreductase from *Nitrosomonas*: Absorption Spectra and Content of Heme and Metal†

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ABSTRACT: Hydroxylamine oxidoreductase of *Nitrosomonas* accounted for 2.6% of the total cell protein. This soluble enzyme was purified 39-fold with a yield of 27% by ammonium sulfate precipitation and isoelectric focusing. The resulting fraction was 99% pure and had a turnover number of 2.3×10^4 or 9.5×10^3 mol min^{-1} (mol of enzyme)$^{-1}$ for hydroxylamine oxidized or nitrite produced, respectively. The enzyme also catalyzed the oxidation of pyrogallol. The value of isoelectric pH was 5.3. The absorption spectrum contained maxima at the following wavelengths (nm): oxidized 408, 534; dithionite reduced 418, 463, 524, 553 (558 shoulder). The enzyme was red in color and had no absorbancy at wavelengths greater than 600 nm. At 77 K the α region of the dithionite-reduced spectrum had maxima at 548 and 557 nm. The substrate NH_2OH caused reduction of 35% of the cytochrome with α maxima at 553 and 558 (548 and 557 at 77 K) but did not cause the appearance of the 460-nm maximum. All cytochromes were autoxidizable, but cytochrome P-460 was oxidized much more rapidly than the others. The 460-nm absorbancy was lost in the presence of CO. A small amount (5%) of the *c*-type heme bound CO. All absorbancy in the 500–560-nm region was accounted for by 18 *c*-type hemes/mol. The enzyme contained 20 mol of iron but no other metals. By difference, 2 mol of Fe was attributed to cytochrome P-460. Heme of P-460 is calculated to have an ϵ value of 76 mM^{-1} cm^{-1} at 460 nm. Cells contained 5.4 μmol of *c*-type heme/g of protein. All of the P-460 and 40% of the total cellular *c*-type heme was associated with hydroxylamine oxidoreductase.

Hydroxylamine oxidoreductase from the ammonia-oxidizing autotrophic bacterium, *Nitrosomonas europaea,* catalyzes the aerobic oxidation of hydroxylamine to nitrite in the presence of phenazine methosulfate (PMS).[1] The reaction involves the initial removal of two electrons from hydroxylamine and the subsequent net addition of an atom of oxygen to form nitrite. The enzyme has a particle weight of 200 000 (Rees, 1968) and contains cytochromes absorbing in the range 540–570 nm, which are 30% reducible by hydroxylamine (Falcone et al., 1963; Hooper and Nason, 1965; Rees, 1968; Ritchie and Nicholas, 1974). A CO-binding cytochrome P-460 containing a novel and uncharacterized heme (Rees and Nason, 1965; Erickson and Hooper, 1972; Ritchie and Nicholas, 1974) is unique to the ammonia-oxidizing bacteria and present in fractions containing hydroxylamine oxidoreductase. Treatment of the enzyme with H_2O_2 results in simultaneous loss of absorbancy due to P-460, substrate-reducibility of cytochromes, and hydroxylamine dehydrogenase activity, suggesting a role of P-460 in the action of the enzyme (Hooper and Terry, 1977). The present paper represents a procedure for purification of the enzyme to homogeneity with high yields and data on the spectral properties, amino acid composition, metal content, content of heme *c* and heme P-460, and the extinction coefficient of heme P-460.

Experimental Procedures

Chemical Assays. Nitrite, nitrate, hydroxylamine, and diethyl dithiocarbamate (DTC) were assayed as described (Hooper et al., 1977). Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. Oxygen utilization was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument, Yellow Springs, Ohio). Carbohydrate was estimated by reaction with phenol and sulfuric acid (Dubois et al., 1956). Using this assay, horseradish peroxidase (Worthington) was determined to contain 17.6% carbohydrate.

Metal analysis was carried out on enzyme samples (2.1 mg of protein) which had been dialyzed against two changes of 2 L of phosphate buffer (10 mM, pH 7.5), containing 10 μM sodium ethylenediaminetetraacetate. Samples were dried at 60 °C and digested with equal volumes of HNO_3, H_2SO_4, and $HClO_4$ (van de Bogart and Beinert, 1967). The white ash was dissolved in 2 mL of 1.1 N HCl and iron content determined

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[1] Abbreviations used are: PMS, phenazine methosulfate; DTC, diethyl dithiocarbamate; Tris, tris(hydroxymethyl)aminomethane; DCIP, 2,6-dichlorophenolindophenol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid.

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TABLE I: Purification of Cytochromes and Hydroxylamine Oxidoreductase of *Nitrosomonas*.

fraction	vol (mL)	protein concn (mg/mL)	hydroxylamine oxidoreductase total act. (units)	hydroxylamine oxidoreductase sp act. (units/mg)	cytochrome c^a (total μmol)	P-460[b] (total absorbancy)
(I) crude homogenate	400	27.3	7920	0.72	110	270
(III) 20 000*g* supernatant	440	12.1	6880	1.3	100	300
(IV) 1st ammonium sulfate fraction	65	11.6	5930	7.9		
(V) 2nd ammonium sulfate fraction	14.8	20.6	3960	13		
(VI) isoelectric focusing	7.2	9.4	1920	28	8.6	80

[a] Based on the pyridine ferrohemochrome. [b] In dithionite-reduced plus CO *minus* reduced difference spectra, $(\Delta A_{492} - \Delta A_{460}) \times$ volume (mL).

by atomic absorption, utilizing a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Content of 12 metals was determined on an identically treated sample (7.2 mg of protein; dissolved in 4 mL of 2 N HCl) with an optical emission spectrophotometer (Applied Research Laboratories, Sunland, Calif.). Values of metal content per mole of enzyme were calculated assuming a protein molecular weight of 189 000 and a measurement of the protein content by amino acid analysis (Table III). As an internal control for atomic absorption or plasma analysis, a parallel sample of mammalian cytochrome *c* (Sigma, Type III, 98% purity) was assayed, and the resulting iron determination was compared with the heme content determined from the pyridine ferrohemochrome.

Analytical discontinuous polyacrylamide gel electrophoresis was carried out with 4 to 80 μg of purified enzyme applied to 100 × 5.0 mm tubes containing 7% polyacrylamide with a 4-mm stacking layer of 2.5% polyacrylamide. Cathode buffer was 25 mM tris(hydroxymethyl)aminomethane (Tris)–glycine, pH 8.3; anode buffer was 25 mM Tris-HCl, pH 8.9. Gels were removed from the tubes and protein bands stained for 2 h with 0.2% brilliant blue G (Sigma) in 7% acetic acid. Excess stain was removed by washing with 7% acetic acid. Hydroxylamine oxidoreductase activity was located by incubating the gels overnight in 50 mM Tris-SO_4, pH 8.0, containing 100 μM PMS and 100 μg/mL 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide. The somewhat diffuse purple formazan band appeared in the presence of 500 μM hydroxylamine. Deposition was slow if PMS was omitted. Gels were scanned at 660 nm in a Beckman DU spectrophotometer, equipped with a Gilford Instruments Mode 2410-5 linear transport.

Amino acid composition of duplicate samples of enzyme (containing 20.6 nmol heme) was determined with a Beckman 12D automatic amino acid analyzer following hydrolysis for 24, 48 or 72 h in 1-mL volumes of 6 N HCl, 0.2% phenol, at 110 °C in tubes sealed in vacuo. The values of serine and threonine were corrected by extrapolation to zero time of the values obtained at 24, 48, and 72 h. Values for other amino acids were the average of the six determinations. Cysteine and methionine were estimated on one sample as cysteic acid and methionine sulfone after oxidation with performic acid (Hirs, 1967) and corrected using a recovery value of 72%, which was the average value of recovery following performate oxidation for all amino acids except tyrosine. The content of tryptophan was approximated in collaboration with Dr. Larry Vickery (Department of Physiology, University of California, Irvine, Calif.) from MCD measurement of the native enzyme using a value for the magnetic ellipticity of $\Delta\epsilon_{291} = 2.3$ (M cm T^{-1}).

Analysis for all amino acids except cysteine and tryptophan of a sample of horse heart cytochrome *c* containing 16 nmol of heme was identical to the published composition except for 1 extra aspartic acid. Analysis following 24-h hydrolysis of a sample containing the full amount of both cytochrome *c* and enzyme showed 90% or greater recovery of all amino acids except for tyrosine (74%), arginine (78%), and methionine (26%).

Enzyme Assays. All incubations were at 25 °C. A unit of activity is defined as the amount of enzyme which will catalyze a change in substrate or product of 1 μmol min^{-1}. Routine measurement of activity of hydroxylamine oxidoreductase was carried out in a standard reaction mixture containing 100 μM hydroxylamine, 5 μM PMS, and 3.6×10^{-10} M enzyme in 50 mM Tris sulfate solution, pH 8.0; total volume 2.5 to 15 mL. When utilized, DTC was present at a concentration of 10^{-4} M. The reaction was started by adding enzyme, and samples were withdrawn at intervals and assayed for nitrite and, as indicated, nitrate, hydroxylamine, and DTC. Hydroxylamine–cytochrome *c* reductase and nitrite reductase were assayed as described previously (Hooper and Nason, 1965; Hooper, 1968). Hydroxylamine–DCIP reductase was measured as the rate of decrease in absorbance at 600 nm of 100 μM 2,6-dichlorophenolindophenol, DCIP, in the presence of the enzyme, 100 μM NH_2OH, and 5 μM PMS in 0.05 M Tris, pH 8.0. Peroxidase and catalase (Hooper and Terry, 1977) and pyrogallol and *p*-phenylenediamine oxidase (Erickson and Hooper, 1972) were assayed as described previously.

Purification of Hydroxylamine Oxidoreductase (Table I). *Nitrosomonas europaea* (Schmidt strain) was grown and the crude homogenate (fraction I) and 20 000*g* supernatant fraction (fraction III) were prepared from 78 g of cells by freezing and thawing as previously described (Hooper et al., 1972). To prepare purified hydroxylamine oxidoreductase, solid ammonium sulfate was added to the 20 000*g* supernatant fraction, and the protein precipitating between 65 and 75% saturation was resuspended in 50 mM phosphate solution, pH 7.5, and retained (fraction IV). The ammonium sulfate precipitation was repeated, and the protein precipitating between 62.5 and 70% saturation was resuspended in phosphate solution and dialyzed overnight against two 1.0-L changes of 10 mM phosphate solution, pH 7.5 (fraction V). The protein was further purified by isoelectric focusing (LKB Application Note 198: "Preparative Flat Bed Electrofocusing in a Granulated Gel," LKB, Bromma, Sweden). For each run, approximately 80 mg of protein was applied to a 10 × 20 cm tray containing 4 g of Sephadex G-75-40 (Sigma Chemical Co., St. Louis, Mo.) in 2% Ampholyte 4-6 (LKB). Eight watts of power was

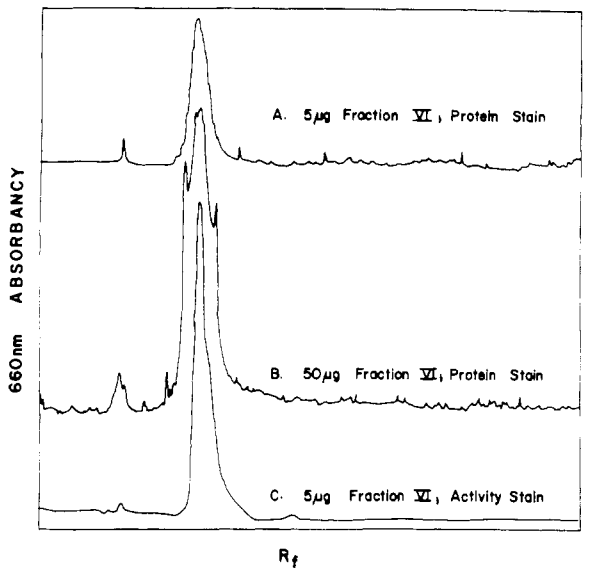

FIGURE 1: Polyacrylamide gel electrophoresis of hydroxylamine oxidoreductase (fraction VI) of *Nitrosomonas*. Procedures are described under Experimental Procedures: (A) 5 μg of protein, stained for protein; (B) 50 μg of protein, stained for protein; (C) 5 μg of protein, stained for enzyme activity.

applied, and the proteins were allowed to focus 8 to 9 h. The major red band, containing 90% or more of the recovered hydroxylamine oxidoreductase activity, was scraped from the plate and eluted from the gel with 50 mM phosphate solution, pH 7.5. To remove ampholyte, solid ammonium sulfate was added to a concentration of 85% of saturation. The precipitated protein was washed four times with 85% saturated ammonium sulfate, until the supernate showed no reaction of ampholyte in the protein assay. The final precipitate was resuspended in phosphate solution and dialyzed against three changes of 1 L of phosphate solution to yield fraction VI. The enzyme was divided into 1-mL aliquots and stored at −20 °C.

Spectra were measured at 25 °C with a 1-cm path length utilizing an Aminco DW-2 spectrophotometer in the split-beam mode. For derivative spectra, the dual-beam mode was utilized with 2-nm difference between monochromators and a 3-nm band-pass. For acidic acetone extraction of heme, a 0.2-mL sample was dried under a stream of nitrogen and suspended in 1 mL of 0.6 N HCl in acetone. After 1–2 h on ice, the precipitate was sedimented. To assay heme as pyridine ferrohemochrome, a sample dried under nitrogen was suspended in 2.1 mL of water. To that sample were mixed successively 0.5 mL of pyridine, 0.25 mL of 1 N NaOH, and a few grains of sodium dithionite. To quantify the pyridine ferrohemochrome of *c*-type heme, a ϵ mM value of 29.1 at 557 nm was employed (Drabkin, 1942). For low-temperature spectra, samples dissolved in 50% glycerol were devitrified in the cuvette by first cooling to the temperature of liquid nitrogen, warming to approximately −55 °C, and recooling to 77 K for measurement. The cuvette (0.1-cm light path) and unsilvered Dewar flask were parts of the Aminco low-temperature accessory.

Results and Discussion

Purification of Hydroxylamine Oxidoreductase. Cultures grow with a doubling time of 12 h and yield only 0.1 g wet weight of cells/L of culture. Hydroxylamine oxidoreductase is sensitive to inactivation by as little as 10^{-7} M H_2O_2 (Hooper and Terry, 1977). For those reasons, purification procedures giving high percent yields were favored. The results of a typical purification are shown in Table I. Inactivation of the enzyme did not occur during the cell breakage, centrifugation, or ammonium sulfate precipitation steps; essentially 100% of the enzyme activity was accounted for within each step. In contrast, 30% of the activity was unaccounted for following isoelectric focusing. When stored at −20 °C, activity has remained constant for 5 months.

As compared with fraction I, the enzyme of fraction VI was enriched 39-fold in specific activity with a recovery of 27%. In the standard reaction mixture, the activity [μmol min^{-1} (mg of $protein^{-1}$)] was 75 for hydroxylamine utilization and 30 for nitrite synthesis. In keeping with previous studies which utilized less pure samples of enzyme, the enzyme of fraction VI catalyzed the production of nitrate or oxidation of diethyl dithiocarbamate concomitant with hydroxylamine oxidation (Hooper et al., 1977), and Mn ion stimulated hydroxylamine oxidation and inhibited nitrite synthesis (Hooper and Terry, unpublished observations). Assuming a protein molecular weight of 189 000 (corrected for heme content) and a purity of 99%, the turnover numbers [mol min^{-1} (mol of $enzyme^{-1}$)] of hydroxylamine oxidoreductase in fraction VI were 2.3×10^4 for hydroxylamine utilization and 9.5×10^3 for nitrite synthesis.

In keeping with the great metabolic importance of hydroxylamine oxidoreductase to this autotropic bacterium, 2.6% of the total protein (5.2% of the soluble protein) was accounted for by the enzyme (calculated from increase in specific activity).

Homogeneity of Enzyme Fraction VI. As determined by analytical discontinuous polyacrylamide electrophoresis, 99% or more of the protein present in fraction VI migrated with the same R_f (0.224) as hydroxylamine oxidoreductase as shown by staining for hydroxylamine dehydrogenase activity (Figure 1). In the crude fraction II or the highly purified fraction VI, one major band of enzyme activity was observed. In fraction VI a minor band of lower R_f value (0.09), presumed to be aggregated enzyme, also had hydroxylamine dehydrogenase activity. Based on turnover number, 280-nm absorption, and analytical electrophoresis, the hydroxylamine oxidoreductase reported here is the purest reported to date and allows chemical analysis.

The following enzyme activities were found in the purified fraction VI [expressed as the change in substrate or product, μmol min^{-1} (mg of $protein^{-1}$)]: pyrogallol oxidase (pyrogallol oxidized), 0.008; *p*PD oxidase (*p*-phenylenediamine oxidized), 0.36. Activity of catalase or hydroxylamine–nitrite reductase was not detected in fraction VI. The percent recovery values of pyrogallol oxidase (20%) and hydroxylamine oxidoreductase (27%) were similar, suggesting that the two activities are catalyzed by the same enzyme. Less than 0.1% of the other contaminant enzyme activities were present in fraction VI.

Physical Properties. As determined by isoelectric focusing, the enzyme had an isoelectric pH value of 5.3. A particle weight of 200 000, which was determined by Rees (1965), is consistent with the low R_f value we observed on native gels. Preliminary experiments indicate that the protein consists of subunits of smaller molecular weight, although the protein is unusually resistant to dissociation by $NaDodSO_4$ and mercaptoethanol.

Chemical Composition. Carbohydrate was not detected (less than 0.3%) in fraction VI, although carbohydrate was present in large quantities (15%) in less purified fractions. The amino acid composition of hydroxylamine oxidoreductase is shown in Table II as the adjusted values of number of residues per heme. The sum of the mass of all amino acids and one heme was very close to $1/19$ of the enzyme particle weight of 200 000, but that calculation did not take into account the amide content

TABLE II: Amino Acid and Heme Composition of Hydroxylamine Oxidoreductase from *Nitrosomonas*.

component	residues/heme	residues/molecule[a]
Cys	2.62	47
Asx	10.73	193
Thr	5.56	100
Ser	3.65	66
Glx	9.83	177
Bro	3.87	70
Gly	6.04	109
Ala	7.27	131
Val	4.76	86
Met	2.56	33
Ile	2.51	45
Leu	5.27	95
Tyr	2.76	50
Phe	1.46	26
Lys	7.08	127
Arg	3.83	69
Try	4.03	73
total		1550
heme		18

[a] Values were calculated assuming a content of 18 hemes and a molecular weight of 200 000.

of glutamine and asparagine or possible poor recovery of certain amino acids such as were observed for arginine and tyrosine. Using a value of 18 hemes, the number of residues per molecule of hydroxylamine oxidoreductase was calculated (Table II). The resulting calculated molecular weight was 200 000 when adjustment was made for ammonia equal to ½ (Axs + Glx) and a loss of 20% of the tyrosine and arginine. The calculated protein molecular weight, corrected for 18 hemes, was 189 000. The protein assay by the Lowry (1951) procedure using bovine serum albumin as standard overestimated the protein content of hydroxylamine oxidoreductase or horse heart cytochrome *c* by factors of 1.6 or 1.5, respectively. Overestimation of cytochrome *c* by the Lowry procedure has been observed previously (Bradford, 1976).

Absorption Spectra. Fraction VI was deep red in color. Figure 2 shows the visible and UV absorption spectra of fraction VI. The spectrum of the oxidized resting enzyme included a protein band at 280 nm, a soret maximum at 408 nm, and a visible band at 534 nm, but little or no absorbancy in the 600–700-nm range. The ratio of absorbancy at 280 nm to absorbancy at 408 nm was 0.3. Within 1 min or less after the addition of dithionite to the enzyme solution, absorption maxima appeared at 418, 524, and 553 nm with a slight shoulder at 558 nm. After 8 min or more, the enzyme became fully reduced, revealing an absorption maximum at 463 nm and a slight increase in the maxima at 524, 553, and 558 nm but no change in absorption in the 600–700-nm range. When oxygen was reintroduced by inversion of the cuvette, the 463-nm peak immediately disappeared and, in the presence of an excess of dithionite, reappeared in approximately 8 min. In the absence of excess dithionite, the absorbancy at 524, 553, and 558 nm was diminished upon addition of oxygen by inversion of the cuvette. Subsequent addition of an excess of dithionite caused reappearance of the absorption maxima at 418, 463, 524, 553, and 558 nm. It therefore appears that P-460 of hydroxylamine oxidoreductase is reversibly and fairly rapidly autoxidizable. The oxidant may have been O_2 or H_2O_2 generated from $Na_2S_2O_4$ and O_2. In addition, the *c*-type cytochromes undergo slow and reversible oxidation. The passage of N_2 had no effect on the spectrum of the dithionite-reduced

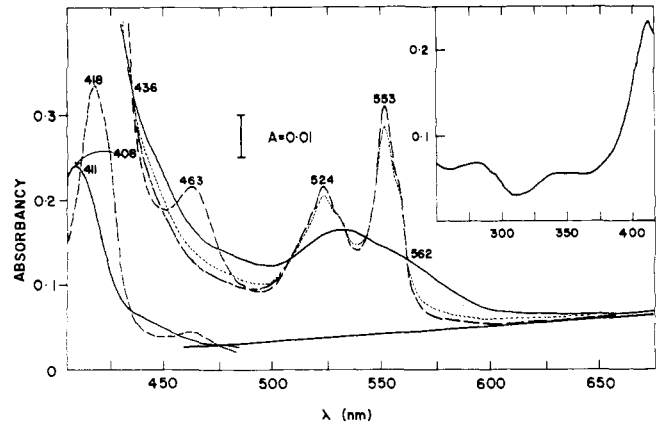

FIGURE 2: Absorption spectra of hydroxylamine oxidoreductase of *Nitrosomonas*. The absorption spectrum of 36 μg of protein/mL of fraction VI in 0.05 M phosphate, pH 7.5, was determined as described under Experimental Procedures and the text: (—) resting enzyme; (- - -) 1 min after the addition of dithionite; (– –) 8 min after the addition of dithionite; (- - -) after subsequently bubbling with CO for 30 s.

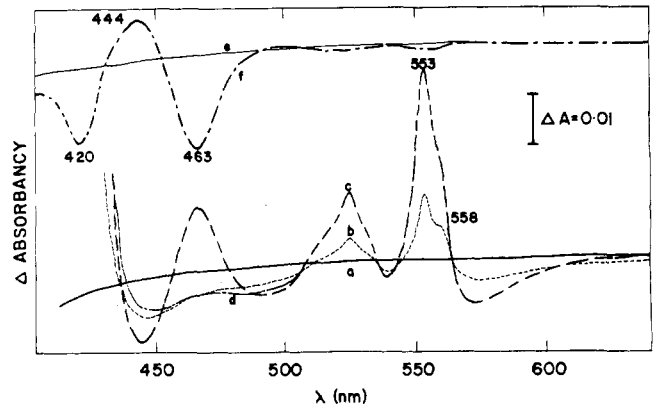

FIGURE 3: Difference spectra of hydroxylamine oxidoreductase. Observations were carried out as in Figure 2 on 36 μg of protein/mL of fraction VI: (a) oxidized *minus* oxidized; (b) NH_2OH (1 mL) *minus* oxidized; (c) dithionite reduced *minus* oxidized; (d) dithionite reduced plus CO *minus* oxidized; (e) dithionite reduced *minus* dithionite reduced; (f) dithionite reduced plus CO *minus* dithionite reduced.

enzyme.

The reduced *minus* oxidized spectrum (Figure 3c) contained maxima at 420, 463, 524, and 553 nm with a shoulder at 558 nm but no absorption maxima or minima in the 600–700-nm range. The presence of CO caused the 463-nm absorption maximum to disappear with little or no change in the 418, 524, 553, or 558 maxima (Figure 3d). The NH_2OH-reduced *minus* oxidized spectrum (Figure 3b) showed reduction of 35% of the cytochrome absorbing at 553 and 558 but no change in absorption at 463 nm. When dithionite was subsequently added to the NH_2OH-reduced sample, the *c*-type cytochromes became fully reduced but the 463 nm maximum did not appear. This is consistent with the observation of Erickson and Hooper (1972) that the spectra of P-460 reduced by dithionite in the presence of CO or NH_2OH are similar.

The reduced plus CO *minus* reduced spectrum (Figure 3f) contained a maximum at 444 nm and a minimum at 463 nm similar to the corresponding spectrum of purified P-460 (Erickson and Hooper, 1972). From a comparison of the dithionite-reduced *minus* oxidized spectrum of the enzyme in the presence and absence of CO (Figure 3c,d) or from examination of the reduced plus CO *minus* reduced spectrum (Figure 3f) a satisfactory estimate of the absorbancy due to P-460 was taken as the difference between the Δ absorbancy

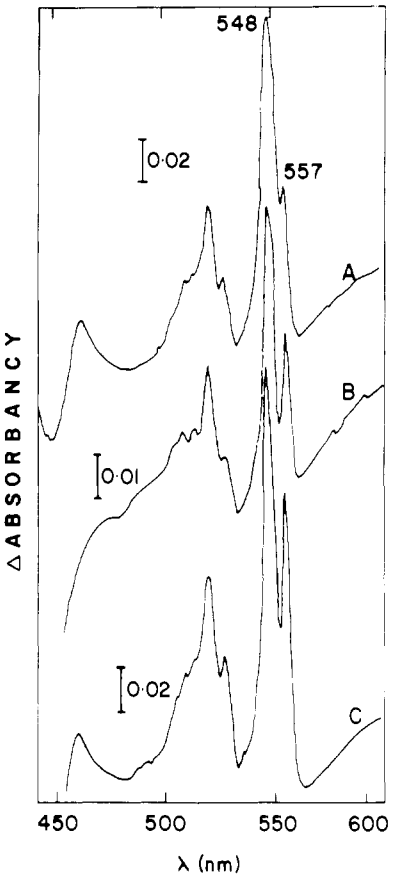

FIGURE 4: Reduced *minus* oxidized spectra of hydroxylamine oxidoreductase determined at 77 K. Observations were carried out as described under Experimental Procedures: (A) fraction III, 62 μg of protein/mL, reduced with dithionite; (B) fraction VI, 94 μg of protein/mL, reduced with 1 mM NH_2OH; (C) fraction VI, 94 μg of protein/mL, reduced with dithionite.

values at 460 and 492 nm. The reduced plus CO *minus* reduced spectrum also contained a maximum at approximately 409 nm and minima at 420 (major), 524 (minor) and 553 nm (minor). For a particular preparation of enzyme the portion of the CO-difference spectrum representative of P-460 was highly reproducible and did not change with time, whereas the 409-nm maximum and 420-nm minimum were variable in height and diminished with time. The latter absorption represents *c*-type cytochrome accounting for approximately 5% of *c*-type heme present in the enzyme which binds CO and is possibly oxidized during treatment with CO. This heme is of interest considering the possible role of an autoxidizable, CO-binding *c*-type cytochrome in bacterial methane oxidation (Tonge et al., 1974).

Extinction coefficients for hydroxylamine oxidoreductase are given in Table III. Cells of *Nitrosomonas* are red due to the unusually high content of cytochrome (5.4 μmol of *c*-type heme/g of protein). From a comparison of recovery of enzyme activity (27%), *c*-type cytochrome (7.5%), and P-460 (27%) in fraction VI (Table I), 40% of the total cellular *c*-type cytochrome and essentially 100% of the total P-460 is associated with hydroxylamine oxidoreductase. This is consistent with a role for P-460 in the action of the enzyme.

Peak heights of cytochrome absorption maxima were enhanced 15-fold at 77 K (Figure 4). The absorption spectrum of dithionite-reduced fraction III at 77 K (Figure 4A) contained the 460-nm peak and split peaks in the soret (418 and 422 nm) and visible regions (548 and 557 nm). The dithionite-reduced spectrum of fraction VI (Figure 4C) showed

TABLE III: Extinction Coefficients for Hydroxylamine Oxidoreductase of *Nitrosomonas*.[a]

spectrum	λ (nm) / ε (mM^{-1} cm^{-1})				
ox.	280 / 610	408 / 2000 (113)*		534 / 230	
red.		418 / 2800 (160)*	463 / 320	525 / 330	553 / 480 (27)*
red. − ox.		420 / 1400 (78)*	463 / 100 (46)†	525 / 100	553 / 300 (16)*
red. + CO − red.			463 / 170 (76)†		

[a] Values in parentheses are based on content of heme *c* (*) or heme P-460 (†).

greater separation of the two soret and visible peaks, suggesting that a heme species of intermediate wavelength had been removed during purification. As shown in Figure 4B, both the 548 and 557-nm maxima, but not the 463-nm maximum, appeared in the presence of hydroxylamine.

Heme Content. The absorption spectra of the dithionite-reduced alkaline pyridine derivative of hydroxylamine oxidoreductase indicated the presence of only *c*-type heme with an absorption maximum at 551 nm. The absorption at 449 or 433 nm of the oxidized or reduced forms, respectively, of the pyridine derivatives of free P-460 as reported by Erickson and Hooper (1972) was expected to account for approximately 10 or 50%, respectively, of the absorption in that region of the pyridine ferrohemochrome spectrum of hydroxylamine oxidoreductase. Corresponding peaks at 449 and 433 nm were not observed, suggesting that heme P-460 of hydroxylamine oxidoreductase was degraded under the conditions of formation of the pyridine ferrohemochrome.

A shoulder at 556 nm characteristic of the pyridine ferrohemochrome of protoheme IX was not detected in the direct spectrum or in the first derivative spectrum. Extraction of hydroxylamine oxidoreductase with acidic acetone removed less than 10% of the 551-nm absorption determined subsequently as the dithionite-reduced alkaline pyridine derivative of the material in the acidic acetone precipitate. The same result was obtained with a sample of horse heart cytochrome *c* with the same heme content. The ferrohemochrome of protoheme IX was not detected in the acidic acetone extract, whereas 99% of the protoheme IX was extracted from a sample of horseradish peroxidase. A difference spectrum comparing the pyridine ferrohemochrome of the native enzyme with that of the acidic acetone-extracted enzyme showed no significant absorption differences in the wavelength range 400–600 nm. We conclude that *c*-type heme accounts for all absorption maxima in the range 540–560 nm of hydroxylamine oxidoreductase.

The mM extinction coefficients per mol of heme *c* of hydroxylamine oxidoreductase were as follows: oxidized, 408 nm (113); reduced, 418 nm (160), 553 nm (27); reduced *minus* oxidized, 553 nm (16) (Table III). These values are about the same as the corresponding values for the cytochromes. For example, the mM extinction coefficients for cytochromes *c*-552 and *c*-554 from *Nitrosomonas* are 30.4 and 24.6, respectively (Yamanaka and Shinra, 1974). Although hydroxylamine oxidoreductase of *Nitrosomonas* is large by comparison with

other cytochromes, the ratio of protein molecular weight/*c*-type heme of 9940 is not unusual by comparison with a value of 12 000 for cytochrome *c*, for example.

Metal Content. Analysis of fraction VI by plasma emission analysis revealed the following content of metals (mol/mol of enzyme): >20 Fe, 2.3 Al, 1 Ca, 0.23 Cu, 0.38 Zn, and 0.42 Mg but undetectable amounts of Mo, Co, Mn, Ni, Cd, or Cr. Iron is apparently the only metal of catalytic significance to hydroxylamine oxidoreductase. Analysis by atomic absorption indicated the presence of 67.7 ± 2.03 nmol of Fe/mg of protein of fraction VI. The same sample of enzyme contained 60.2 ± 1.7 nmol of heme *c*/mg of protein. By difference 7.4 nmol of Fe/mg of protein was not in heme *c*. This corresponds to 2.2 (2 or 3) mol of Fe/mol of enzyme. In a parallel atomic absorption analysis of an aliquot of horse heart cytochrome *c* of the same concentration of heme as the enzyme, 98% of the iron was accounted for as heme *c*.

Identity of Non-Heme c-Fe. Although the enzyme sample analyzed for metal content had been dialyzed against two changes of 10^{-5} M EDTA for 4 h, it is still possible that small amounts of adventitious Fe was attached to the enzyme. Because of the lability of P-460 and enzyme activity (Hooper and Terry, 1977), it was difficult to dialyze under more stringent conditions without loss of activity and absorbancy due to P-460. Therefore, we cannot completely exclude the possibility that the enzyme contains Fe only in heme *c*. The odor of sulfide was not detected upon acidification of a sample of enzyme, suggesting that non-heme iron–sulfur moieties were not present.

Nature of P-460. P-460 has been considered as a derivative of an unknown heme on the basis of its dithionite reducibility and apparent ability to bind ligands, including CO (Rees and Nason, 1965), NH_2NH_2, NH_2OH, or KCN (Erickson and Hooper, 1972). The low value of non-heme *c*-Fe reported here raises the possibility that P-460 is, in fact, not an iron-containing pigment or that it is a *c*-type heme which is liganded so as to produce an unusual red shift in the soret region of the spectrum and to be autoxidizable and unusually labile to H_2O_2. If it is assumed that P-460 is a heme derivative accounting for 2 mol of Fe/mol of enzyme, the difference extinction coefficient at 460 nm (reduced *minus* oxidized) is 76 on the basis of the concentration of heme P-460. This value is similar to the corresponding value of 78 at 418 nm for *c*-type heme. Of the 18 *c*-type hemes of hydroxylamine oxidoreductase, 6.3 (35%) are reducible by NH_2OH. If P-460 functions in hydroxylamine dehydrogenase activity as suggested by Hooper and Terry (1977), each molecule of P-460 will promote the reduction of 3 mol of *c*-type heme on the enzyme. As shown here in spectra at room temperature (and 77 K), *c*-type hemes absorbing at both 553 (548) and 558 (557) nm were reduced in the presence of NH_2OH.

The absorption spectra of hydroxylamine oxidoreductase are somewhat similar to those of cytochrome *cd*, the cytochrome oxidase, and nitrite reductase of *Pseudomonas aeruginose* (Yamanaka and Okunuki, 1974). Both have a cytochrome *c*-type spectrum with a maximum at 553 nm and a shoulder at 558 nm (hydroxylamine oxidoreductase) or split maxima at 549 and 554 nm (cytochrome *cd*). In common with hydroxylamine oxidoreductase, cytochrome *cd* has a major shoulder at 460 nm, which is attributed to heme *cd*. Cytochrome *cd* also has major absorption maxima in the range 630–650 nm and is green in color. In contrast, hydroxylamine oxidoreductase is red in color and lacks absorbancy at wavelengths greater than 570 nm, so probably does not contain heme *d*. The catalytic capabilities of hydroxylamine oxidoreductase and cytochrome *cd*, which functions as a nitrite reductase and as a cytochrome oxidase, have interesting similarities: both enzymes (a) undergo reversible reduction by substrates; (b) will activate oxygen; and, (c) since one produces nitrite from a compound of the oxidation state of HNO and the other reduces nitrite to a compound of the oxidation of NHO, probably share common N-containing intermediates. H_2O_2 inactivates hydroxylamine dehydrogenase activity and destroys P-460 (Hooper and Terry, 1977). In analogous fashion, heme *d* and activity of cytochrome oxidase and nitrite reductase are also inactivated by H_2O_2 (Yamanaka et al., 1961).

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