BBA 46394

SOLUBILIZATION AND PURIFICATION OF CYTOCHROME a_1 FROM *NITROSOMONAS*

R. H. ERICKSON*, A. B. HOOPER AND K. R. TERRY

Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minn. 55101 (U.S.A.)

(Received May 23rd, 1972)

SUMMARY

Cytochrome a_1 was solubilized with Triton X-100 from a membrane–envelope preparation of *Nitrosomonas* and partially purified by repeated fractionation with $(NH_4)_2SO_4$. The purified fraction of cytochrome a_1 was enriched over the crude extract by a factor of 16 and 300 with respect to protein and *c*-type cytochrome, respectively. The cytochrome was characterized as cytochrome a_1 on the basis of (a) reduced absorption maxima at 444 nm and 595 nm, (b) acid acetone extractibility and ether solubility of the heme and (c) absorption maximum of 587 nm of the ferrohemochrome in alkaline pyridine. The α absorption band shifted from 600 nm to 595 nm upon solubilization of the cytochrome with Triton X-100. Spectral shifts were observed in the presence of cyanide and azide and the cytochrome changed with aging to a form with a reduced absorption band at 422 nm. Cytochrome a_1 was reduced anaerobically in the presence of reduced mammalian cytochrome *c* and was rapidly reoxidized in the presence of O_2. CO caused a shift in the soret peak of the reduced form but did not prevent reoxidation of cytochrome a_1 in the presence of $CO–O_2$ (95:5, v/v).

Cytochrome *c* oxidase activity found in the same fractions with cytochrome a_1 of *Nitrosomonas* was stimulated by 0.3 % cholate and inhibited by 10^{-5} M cyanide, 1 mM azide, 10^{-5} M sulfide, $5 \cdot 10^{-4}$ M hydroxylamine and 10^{-5} M diethyl dithiocarbamate. The activity was not inhibited in the presence of $CO–O_2$ (95:5, v/v). Hydroquinone, pyrogallol, *p*-phenylenediamine or 2,6-dichlorophenolindophenol did not serve as electron donors for oxidase activity.

INTRODUCTION

As indicated by spectrophotometric measurements, *a*-type cytochromes are present in a number of species of bacteria[1–3] but in no instance has a bacterial *a*-type

In keeping with the recommendations of the subcommittee of the International Union of Biochemistry[17] the cytochrome group is indicated by *a* based on the position of reduced absorption maxima. After more complete chemical identification in this work, the cytochrome was designated as cytochrome a_1.

Abbreviations: cytochrome *c* oxidase, reduced mammalian cytochrome *c* oxidoreductase; PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol.

* Present address: McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md. 21218, U.S.A.

cytochrome been solubilized and purified[1,3]. Reduced mammalian cytochrome *c* oxidoreductase (cytochrome *c* oxidase) activity has been described in *Nitrosomonas europaea* associated with particulate fractions [4–6] and an *a*-type cytochrome (absorption maximum in the 595–610-nm range) has been observed spectrophotometrically in whole cells reduced with dithionite, ammonia or hydroxylamine and in particulate fractions reduced with dithionite or hydroxylamine[5,7–9]. A membrane–envelope fraction has recently been isolated from *Nitrosomonas* and shown to contain most of the *a*-type cytochrome[10]. The present work describes the solubilization, partial purification and preliminary characterization of an *a*-type cytochrome from the membrane–envelope fraction of *Nitrosomonas*. It is characterized chemically as cytochrome a_1. Evidence is presented which bears on the possible involvement of cytochrome a_1 in the cytochrome *c* oxidase activity of the membrane–envelope fraction.

METHODS

Assay of enzyme activities

A unit of enzyme activity was defined as that amount of enzyme which catalyzed the reaction of 1 μmole of the assayed substrate or product per min at 25 °C. All spectrophotometric assays utilized a Gilford Multiple Sample Absorbance Recorder. To assay the oxidation or reduction of mammalian cytochrome *c* at 550 nm, a reduced *minus* oxidized extinction coefficient of 19.6 $mM^{-1} \cdot cm^{-1}$ (ref. 11) was employed.

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.

Other oxidase activities were assayed as the rate of utilization of O_2 utilizing a YSI oxygen analyzer. Enzyme was added to a 3-ml reaction mixture as follows: pyrogallol oxidase, 10 mM pyrogallol, 50 mM citrate phosphate solution, pH 6.0; *p*-phenylenediamine oxidase, 10 mM *p*-phenylenediamine, 50 mM phosphate solution, pH 6.5; hydroquinone oxidase, 10 mM hydroquinone, 50 mM phosphate, pH 7.1. Dichlorophenolindophenol (DCIP) oxidase was assayed in 50 mM citrate phosphate solution containing 1 mM DCIP and 3 mM pyrogallol. Enzymatic O_2 utilization coupled to pyrogallol oxidation was not observed at the pyrogallol and enzyme concentrations used in the DCIP oxidase assay. Glutamate dehydrogenase was assayed as the rate of reduction of $NADP^+$ (ref. 12); hydroxylamine cytochrome *c* reductase, as the rate of reduction of mammalian cytochrome *c*[5]. Nitrite reductase[13] and phenazine methosulfate(PMS)-dependent nitrite synthetase[14] were assayed as the rate of disappearance or appearance of nitrite, respectively, in the presence of hydroxylamine.

Spectra

Spectra were measured at room temperature utilizing a Cary Model 15 or Model 14 spectrophotometer. For reduced *minus* oxidized difference spectra during purification a sample oxidized with 50 μM $K_3Fe(CN)_6$ was compared with an equal concentration of sample reduced with a few crystals of $Na_2S_2O_4$. To effect complete reduction[11], cytochrome *a* preparations were allowed to stand ten minutes after the addition of dithionite. Cytochrome *a* was measured as the absorbance difference between the value at the absorption maximum of 595 or 600 nm and a line connecting

points at 583 and 615 nm; cytochromes *c* and *b* were estimated as the absorbance difference between the value at 553 or 558 nm, respectively, and a line connecting points at 540 and 564 nm; P463 was measured as the absorbance difference between the value at 463 nm and a line connecting points at 447 and 488 nm.

Heme extraction and pyridine derivatives

Acid acetone extraction of heme was carried out by a modified version of the procedure used by Rees and Nason[15]. 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. The supernatant was decanted, the heme extracted from the supernatant into 2 vol. of ether, and the ether extract evaporated to dryness under a stream of N_2. The ether residue was dissolved in an appropriate volume of 2.1 M pyridine and 75 mM NaOH solution as recommended by Paul *et al.*[16] for the study of pyridine derivatives.

Protein was assayed as described previously[5]. Changes in the gas atmosphere of reactions was accomplished in Thunberg cuvettes as described previously[13].

Sources

Triton X-100 was obtained from Rohm and Haas (Philadelphia, Pa.), Type II horse heart cytochrome *c*, sodium cholate and Type I pancreatic deoxyribonuclease from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Purification

Table I shows a summary of the purification of *a*-type cytochrome and cytochrome *c* oxidase from *Nitrosomonas*. Unless otherwise indicated, all steps of the purification process were carried out at 4 °C. Batch cultures of *Nitrosomonas europaea* were grown and harvested as described previously[13].

To disrupt the cells by the freeze–thaw procedure[10], a 980-ml volume of a 0.2-g wet weight/ml suspension of *Nitrosomonas* containing a small amount of pancreatic deoxyribonuclease was frozen at −10 °C and thawed 3 times and the resulting homogenate designated the crude lysate (Fraction 1). Fraction 1 was centrifuged for 20 min at 20000 × *g* and the resulting particulate fraction washed 6 times by successive resuspension in 50 mM phosphate solution, pH 7.5 (400 mg wet pellet/ml), agitation for 60 min and sedimentation for 20 min at 20000 × *g*. The resulting pellet was resuspended in the phosphate solution (400 mg wet pellet/ml) to yield the membrane–envelope complex (Fraction 2).

To reduce the contamination with *c*- and *b*-type cytochromes, Fraction 2 was first passed through a French pressure cell (Aminco) 3 times at 16000 lb/inch^2, and the resulting homogenate centrifuged for 20 min at 20000 × *g*. The cloudy brown supernatant (Fraction 3) was then centrifuged at 78000 × *g* for 2 h to yield a red, gelatinous pellet and a clear, yellow supernatant. The pellet (Fraction 4), was resuspended to 200 mg wet pellet per ml in 50 mM phosphate buffer, pH 7.5, which contained 5 % (w/v) KCl, stirred for 12 h and centrifuged at 78000 × *g* for 2 h to yield red supernatant and pellet fractions. Spectrophotometric examination of the supernatant fraction revealed that the KCl treatment had solubilized some

Biochim. Biophys. Acta, 283 (1972) 155–166

TABLE I

SUMMARY OF PURIFICATION OF CYTOCHROME *c* OXIDASE AND CYTOCHROME *a*

Cytochrome *a* (absorbance of the major peak at 600 nm or 595 nm), cytochrome *b* (absorbance at 558 nm) and cytochrome *c* (absorbance at 553 nm) were measured in reduced *minus* oxidized difference spectra as described in Methods.

Fraction	*Protein (mg/ml)*	*Mammalian cytochrome c oxidase activity*		*Cytochrome a***		*Ratios*		
		Spec. act. (10^2 × units/mg protein)	*Recovery (%)*	*Amount per mg protein (10^2 × absorbance/mg protein)*	*Recovery (%)*	*Cytochrome a: oxidase activity (absorbance/unit)*	*Cytochrome a: $A_{553\ nm}$*	*Cytochrome a: $A_{558\ nm}$*
1. Crude lysate	25.6	1.64	100	0.56*	100*	0.34	0.05	0.11
2. Membrane–envelope complex	40.7	2.01	55	0.78*	61*	0.38	0.13	0.20
5. KCl precipitate resuspended in Triton X-100	15.0	6.06	110	—	—	—	—	—
6. Pooled Triton X-100 supernatants	2.58	7.75	69	2.32+	61+	0.30	0.15	0.29
9. Oil from 35% $(NH_4)_2SO_4$ suspension	1.44	29.2	15	4.65+	7+	0.16	2.23	1.00
12. 35% $(NH_4)_2SO_4$ precipitate	2.80	10.3	1	9.04+	3+	0.88	15.8	7.68
7. First $(NH_4)_2SO_4$ precipitate	19.6	0.92	7	—	—	—	—	—
8. Oil from 27% $(NH_4)_2SO_4$ suspension	8.02	15.0	38	1.62+	12+	0.11	0.30	0.50

** Absorbance of the major peak at 600* or 595 nm+.

of the *c*- and *b*-type cytochromes while cytochrome *a* remained in the pellet fraction. This procedure was only partially effective even if repeated several times.

To solubilize cytochrome *a*, the KCl-treated pellet was resuspended to 200 mg wet pellet per ml in 50 mM phosphate buffer, pH 7.5, *plus* 0.5 % (v/v) Triton X-100 (Fraction 5). Fraction 5 was stirred for 12 h and centrifuged for 2 h at 78000 × *g*. The resulting precipitate was extracted 3 times with buffered Triton X-100 with stirring for 18, 19 and 30 h. The four Triton X-100 supernatants were pooled (Fraction 6) and stored at −15 °C. Fraction 6 was clear and brownish-green in color.

For purification of the solubilized cytochrome *a*, solid $(NH_4)_2SO_4$ was added to Fraction 6, with stirring, to 18 % saturation and the stirring was continued for 45 min. The suspension was centrifuged at 20000 × *g* for 15 min and the resulting brown pellet (Fraction 7) resuspended in a minimum volume of the buffered Triton solution and stored at −10 °C. The supernatant was subsequently brought to 27 % and then to 35 % saturation with $(NH_4)_2SO_4$ and centrifuged as before resulting in no precipitates but the presence of a thin brown oil on top of the aqueous phase. As the aqueous phase was removed by suction with a thin pipette passed carefully through the brown oil layer, the oil phase adhered to the sides of the centrifuge tube. The two oils resulting from fractionation at 27 % and 35 % $(NH_4)_2SO_4$ saturation were dissolved to a protein concentration of 5 mg/ml in buffered Triton solution and designated Fractions 8 and 9, respectively. Fraction 8 was stored at −10 °C. Fraction 9 was reduced with a small amount of $Na_2S_2O_4$, brought to 35 % saturation with solid $(NH_4)_2SO_4$ and stirred for 45 min. Centrifugation at 20000 × *g* for 15 min resulted in a brown oil which was collected as before and resuspended to 5 mg protein per ml in 50 mM phosphate buffer, pH 7.5, 2 % sodium cholate (Fraction 10). Fraction 10 was reduced with solid $Na_2S_2O_4$, $(NH_4)_2SO_4$ was added to 35 % saturation and the suspension was stirred and centrifuged as before. The resulting green pellet was resuspended to a protein concentration of 5 mg/ml in buffered cholate solution (Fraction 11), reduced with dithionite and precipitated with 35 % saturated $(NH_4)_2SO_4$ as before. The resulting green pellet was resuspended in a minimum volume of 50 mM phosphate, 2 % cholate solution (Fraction 12) and frozen.

On a protein basis a net 6-fold purification of cytochrome *c* oxidase and a 16-fold purification of 600 nm absorbing material was achieved in Fraction 12 (Table I) although the percent recovery of oxidase activity and cytochrome *a* was rather low. A greater recovery would have been expected had Fraction 8 been chosen for further purification rather than Fraction 9. Fraction 9 was used because it was considerably more enriched in cytochrome *c* relative to 553 nm and 558 nm absorbing material (Table I) and had twice the specific activity of cytochrome *c* oxidase when compared to Fraction 8.

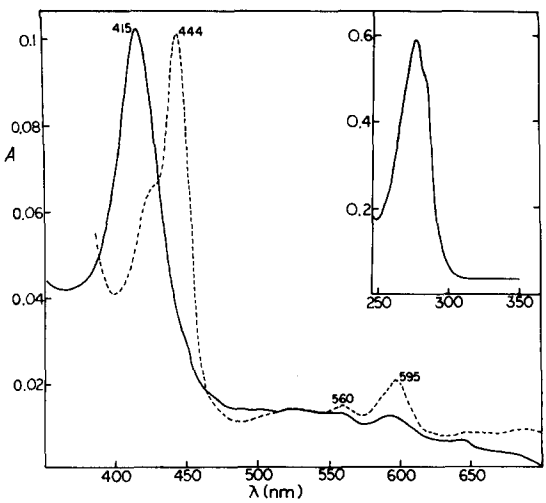
The major absorbance peak of cytochrome *a* was at 600 nm until solubilization with Triton X-100 (Fraction 5). In the 580–650-nm range the only major absorption maximum of Fraction 5 and all subsequent fractions was at 595 nm. Throughout the purification the ratios of cytochrome *a*:553 nm absorbance and cytochrome *a*: 558 nm absorbance increased so that Fraction 12 represented a 319 and 70-fold enrichment of cytochrome *a*, respectively. In Fraction 12 the increase in the ratios of cytochrome *a*:553 nm absorbance and cytochrome *a*:558 nm absorbance was higher than the increase in values of cytochrome *a*/mg protein or activity/mg protein indicating the selective removal of *c*- and *b*-type cytochromes during purification.

Stability

Nitrosomonas cytochrome *a* was completely soluble in the buffered cholate solution and was stable when frozen for several months. With repeated freezing and thawing, the cytochrome exhibited a progressive decrease in height of the reduced 444-nm peak with a concomitant increase in absorbance at 422 nm. This spectral change was seen if the spectrum was measured in the presence or absence of cholate. Cytochrome *c* oxidase activity was not substantially decreased after repeated freezing and thawing in buffered cholate solution.

Absorption spectra

The absolute oxidized absorption spectrum of Fraction 12 (Fig. 1) contained major absorption maxima at 415 nm and 595 nm with lesser maxima at approximately 558 nm and 640 nm and a single absorption maximum at 280 nm. After reduction with dithionite the 415-nm maximum shifted to 444 nm, the 595-nm and 558-nm maxima increased in height and a broad maximum at 530 nm and a shoulder at 426 nm appeared. The absorption maxima in the reduced form at 426 nm and 558 nm or 444 nm and 595 nm were indicative of a *b*-type cytochrome and cytochrome a_1, respectively. In the dithionite reduced *minus* oxidized difference spectrum (Fig. 2)



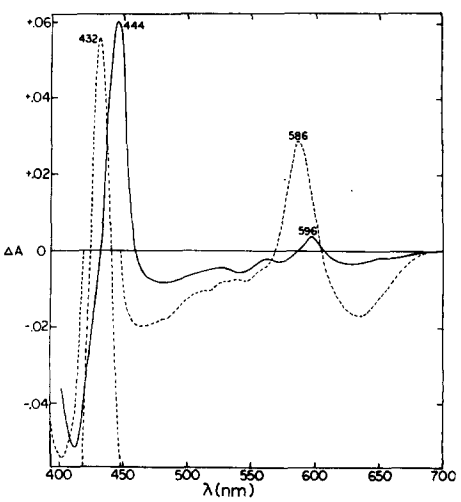


Fig. 1. Absolute absorption spectrum of purified *Nitrosomonas* cytochrome a_1. An aliquot of Fraction 12 containing 84 μg of protein was diluted to 1 ml with 50 mM phosphate buffer (sodium, potassium), pH 7.5. The oxidized spectrum (———) was measured against a reference cuvette containing phosphate solution. After the addition of a few crystals of sodium dithionite to the oxidized sample and a 10-min incubation period to effect complete reduction, the reduced spectrum (-------) was measured. Inset: ultraviolet spectrum of the oxidized sample.

Fig. 2. Reduced *minus* oxidized absorption spectrum of *Nitrosomonas* cytochrome a_1 and the pyridine derivative. An aliquot of Fraction 12 containing 84 μg of protein was added to a sample and reference cuvette and diluted to 1 ml with 50 mM phosphate buffer (sodium, potassium), pH 7.5. The sample cuvette was reduced with a few crystals of $Na_2S_2O_4$ and after an incubation period of 10 min, the reduced *minus* oxidized difference spectrum (———) was measured. The heme was extracted from a second sample of Fraction 12 with acid acetone and ether as described (Methods). The dried ether residue was dissolved in 2.1 M pyridine and 75 mM NaOH so that the amount of heme per ml was equivalent to that extracted from 230 μg of protein of Fraction 12. A 1-ml aliquot of the dissolved heme was added to sample and reference cuvettes, the sample cuvette was reduced with a few crystals of $Na_2S_2O_4$ and (-------) was measured immediately.

cytochrome a_1 was characterized by maxima at 596 nm and 444 nm. The *b*-type cytochrome contaminant was seen as a maximum at 558 nm and a shoulder in the 426-nm region.

Pyridine derivatives

The prosthetic group in Fraction 12 was extracted into acid acetone and the dried ether extract used to make the pyridine derivative. Fig. 3 shows the ferrihemochrome absorption spectrum in alkaline pyridine to contain absorption maxima at 406 nm and 587 nm. A broad maximum at 638 nm was also evident, along with a shoulder in the 425-nm region. Following reduction with dithionite the resulting ferrohemochrome showed a Soret peak at 432 nm and an α peak at 587 nm characteristic of heme *a*. A small maximum at approximately 560 nm was evident and the 638-nm peak seen in the ferrihemochrome was not observed. In a reduced *minus* oxidized difference spectrum (Fig. 2) peaks at 432 nm and 586 nm were observed along with a trough at 638 nm and smaller peaks in the 500–530-nm and 560-nm region.

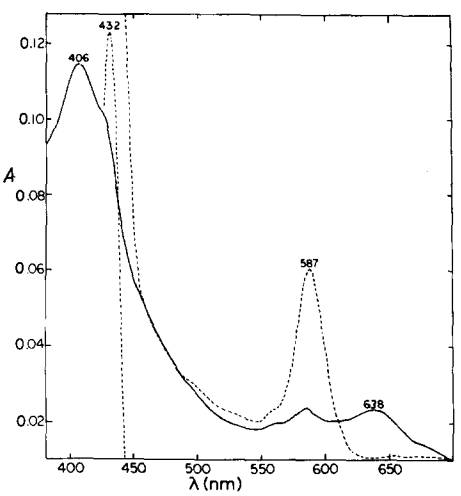


Fig. 3. Absolute absorption spectrum of the pyridine derivative of heme from *Nitrosomonas* cytochrome a_1. Heme was extracted from Fraction 12 by treatment with acid acetone as described (Methods). The dried ether residue was dissolved in 2.1 M pyridine and 75 mM NaOH so that the amount of heme per ml was equivalent to that extracted from 230 μg of protein of Fraction 12. The ferrihemochrome absorption spectrum (———) was measured immediately after addition of pyridine–NaOH to the ether residue. Following reduction of the oxidized sample with a few crystals of $Na_2S_2O_4$, the ferrohemochrome absorption spectrum (--------) was immediately measured. In each case the reference cuvette contained pyridine–NaOH.

Reduction by reduced mammalian cytochrome c and reoxidation

Under an atmosphere of N_2, a sample of cytochrome a_1 was completely reduced (an increase of 0.01 *A* at 595 nm) within 10 min in the presence of 5 μM reduced mammalian cytochrome *c*. When air was admitted to the sample cytochrome a_1 was reoxidized within 2 min. Essentially the same result was obtained when 33 μM $Na_2S_2O_4$ was used to effect reduction.

Ligand effects

Addition of CO to a reduced sample of cytochrome *a* completely shifted the 444-nm peak to 428 nm. No change was evident in the α peaks at 558 or 595 nm. CO had no effect on the oxidized spectrum. The reduced *plus* CO *minus* reduced difference spectrum consisted of a major peak at 430 nm and a trough at 444 mn. In an attempt to determine the reversibility of binding of CO a sample of cytochrome a_1 in an atmosphere of pure CO was treated with just enough dithionite to effect complete reduction. When the CO atmosphere was replaced by $CO–O_2$ (95:5, v/v) the 595-nm peak disappeared and the 428-nm peak shifted to 415 nm within 2 min. When dithionite was again added to effect reduction of the sample in the $CO–O_2$ (95:5, v/v) atmosphere the 428-nm and 595-nm peaks reappeared. When air was admitted to the vessel which previously contained reduced cytochrome a_1 in a pure CO atmosphere, the 595-nm peak disappeared and the 428-nm peak shifted to 415 nm within 2 min. When saturating amounts of dithionite were added after the vessel had been open to the air for 10 and 50 min, the Soret absorption maxima occurred at 428 and 444 nm, respectively. The spectrum at 50 min was indistinguishable from the spectra of freshly-reduced cytochrome a_1 as indicated by the absence of peaks in a difference spectrum (reduced at 50 min *minus* freshly reduced control). This CO readily dissociated from cytochrome a_1 and neither prevented reoxidation nor caused irreversible spectral changes in cytochrome a_1.

In the presence of 1 mM KCN or NaN_3 two peaks were apparent in the Soret region in spectra of dithionite-reduced samples: One at 441 nm and the other at 426 nm or 423 nm with cyanide or azide, respectively. The maximum at 441 nm was diminished in comparison with the normal reduced absorption maximum at 444 nm. No change was apparent in the reduced absorption maximum at 595 nm or at any wavelength in the oxidized spectrum with cyanide or azide.

Cytochrome c oxidase activity

Cytochrome *c* oxidase was routinely assayed during purification as indicated in Methods. However, proportionality was not obtained and the activity varied with the preparation: the cytochrome *c* oxidase activity of Fraction 2 was increased by 50 % after passage through the French pressure cell or after solubilization with Triton. Dilutions of Fraction 12 were made in 2 % buffered cholate solution to prevent loss of oxidase activity which otherwise occurred over a period of several hours if the dilutions were made in 50 μM phosphate solution, pH 7.5. Greater activity was obtained and proportionality improved in the presence of 0.15–0.3 % (w/v) sodium cholate in the final reaction mixture. After thawing the stored enzyme a 10-min incubation at room temperature was routinely carried out before storage on ice to eliminate a lag in the reaction which was observed when the enzyme was thawed at 0–4 °C and assayed immediately. All cytochrome *c* oxidase studies using Fraction 12 were carried out in a reaction mixture containing 50 mM phosphate buffer, pH 7.5, with 0.15–0.30 % sodium cholate. Under these conditions the time course of cytochrome *c* oxidation was linear for at least 5 min and the rate was proportional to enzyme concentration.

The activities of Fractions 1, 9 and 12 were 4–5 times the values reported in Table I when the above assay conditions were used. Thus the loss in specific activity in Fraction 12 was not compensated for by this stimulation phenomenon. The decrease

in specific activity of cytochrome *c* oxidase between purification steps 9 and 12 was reflected in a corresponding increase in the ratio of cytochrome *a* (595 nm) to cytochrome *c* oxidase activity. In contrast, the ratio of 553 nm or 558 nm absorbance to cytochrome oxidase activity was approximately the same in both Fractions 9 and 12 suggesting that the loss of cytochrome *c* oxidase specific activity may have been due to removal of *b*- or *c*-type cytochromes.

Other enzyme activities

Fraction 12 did not contain detectable glutamate dehydrogenase, hydroxylamine cytochrome *c* reductase, nitrite synthetase or nitrite or nitrate reductase nor did it catalyze the utilization of oxygen with pyrogallol, *p*-phenylenediamine, hydroquinone or DCIP as electron donors.

Inhibitors

Cytochrome *c* oxidase activity was inhibited by cyanide, azide, sulfide, diethyl dithiocarbamate, and hydroxylamine (Table II). A slight stimulation of activity was observed with 1 mM allylthiourea, α,α'-dipyridyl, hydrazine or nitrite.

TABLE II

EFFECT OF INHIBITORS ON CYTOCHROME *c* OXIDASE ACTIVITY

The enzymatic activity was measured by the standard procedure, except for the inclusion of the indicated inhibitors at the concentrations shown. The data are expressed as percentage of the uninhibited rate.

Compound	*Concn* (*M*)	*Inhibition* (%)
KCN	10^{-4}	89
	10^{-5}	53
NaN_3	10^{-2}	74
	10^{-3}	51
Na_2S	10^{-4}	100
	10^{-5}	67
NH_2OH	10^{-3}	73
	$5 \cdot 10^{-4}$	43
Diethyl dithiocarbamate	10^{-4}	85
	10^{-5}	13

Cytochrome *c* oxidase activity of a purified fraction was not inhibited in an atmosphere of CO–O_2 (95:5, v/v) as compared with a control reaction in an atmosphere of N_2–O_2 (95:5, v/v). Furthermore, a preparation of the enzyme in which the cytochrome a_1 had been reduced with dithionite in a pure CO atmosphere and reoxidized in a CO–O_2 (95:5, v/v) atmosphere had the same rate of cytochrome *c* oxidase activity as a control sample which had been reduced in a pure N_2 atmosphere and reoxidized in a N_2–O_2 (95:5, v/v) atmosphere.

DISCUSSION

This paper describes the solubilization, partial purification and preliminary characterization of a hemoprotein from *Nitrosomonas*. The protein is identified as a cytochrome *a* on the basis of the acid acetone extractability and ether solubility of the heme[17] and reduced α absorption maximum at 587 nm and absence of a β peak in the ferrohemochrome in alkaline pyridine[18] and specifically as cytochrome a_1 from the position of the reduced α absorption peak at 595 nm and Soret peak at 444 nm according to Kamen and Horio[3]. In addition to the 587-nm peak, the ferrihemochrome spectrum in alkaline pyridine exhibited an absorbance peak at 638 nm similar to that observed with heme a_2 in alkaline pyridine[19], or heme *a* in basic buffer[20] with oxidized peaks at 620 and 633 nm, respectively. However, the presence of a single peak at 587 nm in the alkaline pyridine ferrohemochrome spectrum was typical of heme *a*[18] and not heme a_2[19].

Purification of cytochrome a_1 from *Nitrosomonas* was greatly facilitated by the high cytochrome content of *Nitrosomonas* and the ease with which most of the *b* and *c*-type cytochromes and soluble enzymes were separated from the membrane–envelope complex following disruption by the freeze–thaw procedure[10]. The purification procedure employed here turned out to be similar in many respects to the procedure of Horie and Morrison[21] for the purification of mammalian cytochrome aa_3. The ratio of the absorbance at 444 nm (reduced):415 nm (oxidized) and 280 nm (oxidized):444 nm (reduced) were approximately 1.0 and 6.0, respectively, as compared to corresponding ratios of greater than 1.25 and less than 2.5, respectively, according to the standards of purity suggested by Yonetani for beef heart cytochrome oxidase[11]. Spectrophotometric examination of the most purified fraction revealed the presence of a 558-nm peak which was extracted into acid acetone indicating the likely presence of cytochrome *b* in the preparation. The 422-nm shoulder and the 558-nm band observed in the absolute reduced spectrum indicated the presence of cytochrome *b* or possibly an inactivated form of cytochrome a_1 by analogy with cytochrome aa_3 which is converted on storage to a form having reduced absorption bands at 430 nm and 590 nm[22].

As shown here, the major absorption maximum of reduced cytochrome a_1 shifted completely from 600 to 595 nm following extraction with Triton X-100 from the French pressed and salt-extracted membrane–envelope fraction suggesting an alteration of the cytochrome a_1 or of proteins or lipids associated with cytochrome a_1.

The spectral shift of the reduced Soret peak from 444 to 428 nm in the presence of CO observed here was the same as that observed with cytochrome a_1 of *Acetobacter pasteurianum*[23,24] and similar to a CO-induced reduced Soret blue shift of other *a*-type cytochromes[21,25]. Azide prevents reduction of mammalian cytochrome aa_3 and causes a shift of the oxidized Soret peak from 424 to 427 nm[26]. Cyanide affects the oxidized spectrum of cytochrome aa_3 in a similar manner and also causes a shift in the reduced Soret band from 443 to 439 nm[27]. With cytochrome a_1 of *Nitrosomonas* these investigations revealed a shift of the reduced Soret peak from 444 to 441 nm in the presence of cyanide or azide. The additional peaks observed in the absorption spectrum of partially purified *Nitrosomonas* cytochrome a_1 at 426 nm and 423 nm with cyanide or azide, respectively, may be due to ligand reactions with cytochrome *b* or inactivated cytochrome a_1.

The involvement of cytochrome a_1 in the cytochrome *c* oxidase activity from the membrane–envelope fraction of *Nitrosomonas* is suggested by the occurrence of the two in the same fractions during purification, the reducibility of cytochrome a_1 in the presence of reduced cytochrome *c*, the rapid autooxidizability of cytochrome a_1 and the fact that cyanide and azide bind cytochrome a_1 and inhibit cytochrome *c* oxidase activity. The action spectrum for relief of CO inhibition indicates involvement of cytochrome a_1 as terminal oxidase in *Acetobacter pasteurianum*[23]. Cytochromes a_1 and *c* 552 are associated with cytochrome *c* oxidase activity of respiratory particles from *Pseudomonas aeruginosa*[28].

Cyanide, azide and hydroxylamine were effective inhibitors of cytochrome *c* oxidase activity of *Nitrosomonas* in keeping with observations with known cytochrome *a*-type cytochrome *c* oxidase enzymes[26,27,29,30]. Inhibition with diethyl dithiocarbamate observed here suggests the possible involvement of copper with the enzyme. CO, a potent inhibitor of other cytochrome *a*-type cytochrome *c* oxidases and the cytochrome a_1-type oxidase of *Acetobacter*[23] and of *Pseudomonas* respiratory particles[28], did not inhibit cytochrome *c* oxidase activity of *Nitrosomonas* (CO–O_2 (95:5, v/v)). Spectral evidence reported here indicated that CO binds reversibly to reduced cytochrome a_1 of *Nitrosomonas* so as to shift the Soret peak from 444 to 428 nm. The binding was reversed in the presence of CO–O_2 (95:5, v/v) so that CO did not prevent rapid reoxidation of cytochrome a_1. It is possible that a decreased CO-binding ability accompanied the changes in cytochrome a_1 revealed by the 600-nm to 595-nm shift upon cholate solubilization.

Washed cells of *Nitrosomonas* or cells in logarithmic phase of growth catalyzed the utilization of 880 μmoles of O_2 $min^{-1} \cdot g^{-1}$ protein whereas the maximum rate of cytochrome *c* oxidase activity in the crude homogenate was equivalent to only approximately 20 μmoles O_2 $min^{-1} \cdot g^{-1}$ protein. Thus, utilizing the present assay system, the cytochrome *c* oxidase activity could not account for whole cell O_2 uptake. Further studies on stimulatory effects of various reagents and modified purification procedures are necessary to establish the maximum activity and significance of the oxidase to *Nitrosomonas* electron transport. Yamanaka and Okunuki have shown that the cytochrome *cd*-containing cytochrome oxidase of *Pseudomonas* has much greater activity with reduced bacterial cytochrome *c* as electron donor than with reduced mammalian cytochrome *c*[31]. The same may be true of cytochrome *c* oxidase of *Nitrosomonas*. This might explain the low specific activity observed here and the correlation between oxidase activity and the content of *b*- and *c*-type cytochromes in the most purified fractions.

ACKNOWLEDGEMENTS

We are grateful to Mike Allison and Rita Young for the growth of batch cultures of *Nitrosomonas* and to Drs Douglas Pratt and Rex Lovrien for the use of the Cary Spectrophotometers.

This investigation was supported by Research Grant GB 15101 from the National Science Foundation. One of us (R.H.E.) was supported by Public Health Service Predoctoral Fellowship 4 F01 GM 38352-02.

REFERENCES

1 R. G. Bartsch, *Annu. Rev. Microbiol.*, 22 (1968) 181.
2 N. S. Gel'man, M. A. Lukoyanova and D. N. Ostrovskii, *Respiration and Phosphorylation of Bacteria*, Plenum Press, New York, 1967, p. 92.
3 M. D. Kamen and T. Horio, *Annu. Rev. Biochem.*, 39 (1970) 673.
4 D. J. D. Nicholas and O. T. G. Jones, *Nature*, 185 (1960) 512.
5 A. B. Hooper and A. Nason, *J. Biol. Chem.*, 240 (1965) 4044.
6 R. S. Pokallus and D. Pramer, *Arch. Biochem. Biophys.*, 105 (1964) 208.
7 A. B. Falcone, A. L. Shug and D. J. D. Nicholas, *Biochim. Biophys. Acta*, 77 (1963) 199.
8 M. I. H. Aleem, *Biochim. Biophys. Acta*, 113 (1966) 216.
9 M. K. Rees, *Biochemistry*, 7 (1968) 353.
10 A. B. Hooper, R. H. Erickson and K. R. Terry, *J. Bacteriol.*, 110 (1972) 430.
11 T. Yonetani, in R. W. Estabrook and M. E. Pullman, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 332.
12 A. B. Hooper, J. Hansen and R. Bell, *J. Biol. Chem.*, 242 (1967) 288.
13 A. B. Hooper, *Biochim. Biophys. Acta*, 162 (1968) 49.
14 A. B. Hooper, *J. Bacteriol.*, 97 (1969) 776.
15 M. K. Rees and A. Nason, *Biochem. Biophys. Res. Commun.*, 21 (1965) 248.
16 K. G. Paul, H. Theorell and A. Akeson, *Acta Chem. Scand.*, 7 (1953) 1284.
17 M. Florkin and E. H. Stoltz, *Comprehensive Biochemistry*, Vol. 13, Elsevier, Amsterdam, 1964, p. 21.
18 J. E. Falk, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1964, p. 240.
19 T. Yamanaka and K. Okunuki, *Biochim. Biophys. Acta*, 67 (1963) 407.
20 T. E. King, C. F. Young and S. Takemori, in B. Chance, R. W. Estabrook and T. Yonetani, *Hemes and Hemoproteins*, Academic Press, New York, 1966, p. 83.
21 S. Horie and M. Morrison, *J. Biol. Chem.*, 238 (1963) 1855.
22 W. B. Elliot, W. C. Hülsmann and E. C. Slater, *Biochim. Biophys. Acta*, 33 (1959) 509.
23 L. N. Castor and B. Chance, *J. Biol. Chem.*, 217 (1955) 453.
24 B. Chance, *J. Biol. Chem.*, 202 (1953) 383.
25 K. Okunuki, I. Sekuzu, Y. Orii, T. Tsudzuki and Y. Matsumura, in K. Okunuki, M. D. Kamen and I. Sekuzu, *Structure and Function of Cytochromes*, Univ. Park Press, Baltimore, Md., 1968, p. 35.
26 A. O. Muijsers, E. C. Slater and K. J. H. van Buuren, in K. Okunuki, M. D. Kamen and I. Sekuzu, *Structure and Function of Cytochromes*, Univ. Park Press, Baltimore, Md., 1968, p. 129.
27 W. W. Waino, in T. E. King, H. S. Mason and M. Morrison, *Oxidases and Related Redox Systems*, Vol. 2, John Wiley and Sons, New York, 1965, p. 622.
28 E. Azoulay and Couchoud-Beaumont, *Biochim. Biophys. Acta*, 110 (1965) 301.
29 D. C. Wharton and A. Tzagoloff, in R. W. Estabrook and M. E. Pullman, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 245.
30 S. Yoshikawa and Y. Orii, *J. Biochem. (Tokyo)*, 68 (1970) 145.
31 T. Yamanaka and K. Okunuki, in K. Okunuki, M. D. Kamen and I. Sekuzu, *Structure and Function of Cytochromes*, Univ. Park Press, Baltimore, Md., 1968, p. 390.