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Localization and concentration of hydroxylamine oxidoreductase and cytochromes *c*-552, *c*-554, c_m-553, c_m-552 and *a* in *Nitrosomonas europaea*

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Two membrane-associated cytochromes, cytochrome c_m-553 and cytochrome c_m-552, were derived from *Nitrosomonas europaea*. The major *c*-type cytochrome, cytochrome c_m-553, accounted for 92% of the *c* heme found in the membrane. It had absorption maxima at 410 nm in the oxidized form and at 417, 523 and 553 nm in the dithionite reduced form. Cytochrome c_m-552 possessed absorption maxima at 409 nm in the oxidized form, at 421, 522 and 552 in the dithionite reduced form, and at 418 in the dithionite reduced plus CO form. The concentration and cellular distribution of the two *c*-type membrane cytochromes, hydroxylamine oxidoreductase and cytochromes *c*-552, *c*-554, and *a* were determined. Over 95% of the soluble cytochromes (hydroxylamine oxidoreductase cytochromes and *c*-552 and *c*-554) were periplasmic, whereas cytochrome c_m-553, cytochrome c_m-552 and cytochrome *a* were associated with the cell membrane. The outer membrane and cytoplasm were devoid of cytochromes. The extracytoplasmic location of the proton-yielding hydroxylamine oxidizing system ($NH_2OH \rightarrow HNO + 2H^+ + 2e^-$) may contribute to an energy-linked proton gradient. The heme concentrations of hydroxylamine oxidoreductase and cytochromes *c*-552, *c*-554, c_m-553, c_m-552 and *a* were approx. 2.4, 1.2, 0.3, 1.3, 0.1 and 1.1 nmol/mg cell protein, respectively. The corresponding molar ratios of heme were 22:11:2.9:12:1.0:10. The enzyme or cytochrome concentrations for hydroxylamine oxidoreductase and cytochromes *c*-552, *c*-554, c_m-553, c_m-552 and *a* were approx. 0.13, 1.05, 0.09, 0.63, 0.055 and 0.56 nmol/mg cell protein, respectively. The corresponding molar ratios were 0.24:2.2:0.16:1.2:0.1:1.0.

Introduction

Nitrosomonas europaea is a gram negative chemolithotroph which utilizes the oxidation of ammonia to nitrite as its sole energy source. The soluble electron-transport components in this bacterium include hydroxylamine oxidoreductase [1,2,3], cytochrome *c*-554 [4], cytochrome *c*-552 [4], *P*-460 fragment [5,6], two CO-binding *c*-type cytochromes c_{CO}-550 and c_{CO}-552 [7], the high-potential cytochrome *c*-553 [7], and a non-heme Cu-Protein [8]. The insoluble or membrane-associated electron-transport components include an *a*-type cytochrome (decribed as cytochrome a_1) [9,10], cytochrome *c*-554 [11], cytochrome *b* [9,12] and ubiquinone-8 [9]. Little is known with certainty with respect to the role of the individual electron-transport components in ammonia oxidation and proton translocation. Yamanaka and Shira [4] propose the electron pathway hydroxylamine oxidoreductase → cytochrome *c*554 → cytochrome *c*-552, whereas Tsang and Suzuki [13] propose that cytochrome *c*-554 is the electron donor in the postulated ammonia monooxygenase. Olson and Hooper [14] have demonstrated the periplasmic location of hydroxylamine oxidoreductase and

proposed a mechanism for generation of a respiration-dependent proton gradient which includes: (a) periplasmic oxidation of NH_2OH with release of protons from substrate and water, (b) transmembrane transport of electrons through metal centers and (c) cytoplasmic coupling of oxygen, substrate electrons and cytoplasmic protons to form water. With this model in mind it is important to localize chrochrome *c*-554 and cytochrome *c*-552.

The present study describes the cytochrome composition of the cell membrane and demonstrates the presence of two *c*-type cytochromes (c_m) in the membrane. Further, it demonstrates the cellular distribution and concentration of hydroxylamine oxidoreductase, cytochrome *c*-552, cytochrome *c*-554, cytochrome c_m-553, cytochrome c_m-552 and cytochrome *a*.

A preliminary report of part of these observations has appeared elsewhere [15].

Materials and Methods

Bacteria

Nitrosomonas europaea (Schmidt strain) was grown and harvested as described previously [9].

Cell fractionation

All isolations were performed at 0–4°C. The outer membrane, periplasm, cell membrane and cytoplasm fractions were isolated from *N. europaea* as described previously [15]. The two membrane fractions were washed 5–7-times by suspension in 50 mM sodium potassium phosphate/100 mM KCl/2 mM sodium EDTA buffer (pH 7.5) (buffer A), stirred for 2–4 h followed by centrifugation at $144 \times g$ for 2 h at 4°C.

Quantitation of hydroxylamine oxidoreductase, cytochrome c-552 and cytochrome c-554

The soluble fractions (periplasm and cytoplasm) were dialyzed for 24 h against two changes of 10 mM sodium potassium phosphate buffer (pH 7.5) and two changes of buffer A. The samples were concentrated with a stirred cell (Diaflow, Amicon Corp.), using a YM5 filter and passed through a 2.5×49 cm Sephadex G-75 column equilibrated with buffer A.

For fractionation by ammonium sulfate, aliquots of the periplasmic space material from the stirred cell were brought to 90% saturation, incubated with stirring for 12 h and centrifuged at $13\,400 \times g$ for 20 min. The pellet was suspended in a minimal volume of the column buffer, dialyzed against three changes of buffer A and passed through a 2.5×49 cm Sephadex G-75 column, equilibrated with buffer A. The supernate from the 90% ammonium sulfate step was dialyzed as described above, concentrated with a stirred cell, and analyzed for content of cytochrome *c*-554.

Preparation of pure samples of hydroxylamine oxidoreductase, cytochrome c-552, and cytochrome c-554

Cytochrome *c*-552, cytochrome *c*-554, and hydroxylamine oxidoreductase were purified from a single batch of cells (26.4 g protein) by a modification of the methods of Hooper et al. [2] for hydroxylamine oxidoreductase and Yamanaka and Shinra [4] for cytochromes *c*-552 and *c*-554. Cytochrome *c*-554 was purified by chromatography on a column of Amberlite CG-50 (2.5×37 cm) [4] from the supernate of the soluble fraction, which had been brought to 80% saturation with ammonium sulfate as part of the isolation procedure for hydroxylamine oxidoreductase [2]. Cytochrome *c*-552 and hydroxylamine oxidoreductase were partially separated in the isoelectric focusing step used in the isolation of hydroxylamine oxidoreductase. Following isoelectric focusing, the fraction enriched in hydroxylamine oxidoreductase was passed through a column of Sephadex G-200 (2.5×37 cm) which had been equilibrated with buffer A. This procedure removed the protein with a molecular weight of 57 000 previously described as a contaminating protein [16]. Cytochrome *c*-552 was isolated as previously described [4] by two passes through a 2.5×23 cm DEAE-Cellulose column followed by two passes through a 2.5×37 cm Sephadex G-75 column equilibrated with buffer A. The additional steps employing the Sephadex G-75 column were necessary to remove low-molecular-weight contaminants. The purity of each sample was analysed by SDS-polyacrylamide gel electrophoresis.

Separation of cytochrome c_m-553 and cytochrome c_m-552

Washed cell membrane was resuspended in 50

mM sodium potassium phosphate/100 mM KCl/ 2 mM NaEDTA/5.0% triton X-100/1.0% sodium cholate solution (pH 8.5) (buffer B) to a final concentration of 12.5 mg protein/ml. The suspension was sonicated for 10 min, stirred for 12 h, and centrifgued at $144\,000 \times g$ for 2 h. The supernate was dialyzed against three changes 10 mM sodium potassium phosphate (pH 8.5) (buffer C) and loaded on a 5.0×30 cm DEAE-Cellulose (Bio-Rad, Cellex D) column equilibrated with 10 mM sodium potassium phosphate/0.5% triton X-100 buffer (pH 8.5) (buffer D). The column was washed sequentially with approx. 250 ml volumes of buffer D, 50 mM sodium potassium phosphate/0.2% triton X-100 buffer (pH 8.5) (buffer E), and 50 mM sodium potassium phosphate/100 mM KCl/ 2 mM sodium EDTA/0.2% triton X-100 buffer (pH 8.5) (buffer F). This resulted in the removal of most non-heme proteins. Cytochromes were eluted with 50 mM sodium potassium phosphate/300 mM KCl/2 mM sodium EDTA/0.2% triton X-100 buffer (pH 8.5) (buffer G) in three peaks each containing cytochrome c_m-553, cytochrome c_m-552 and cytochrome *a* in varying ratios. The cytochromes were dialyzed against three changes of buffer C and loaded onto a 2.5×33 cm DEAE-Sepharose CL-6B column equilibrated with buffer D. To remove residual non-heme protein the column was washed sequentially with approx. 150 ml volumes of buffer D, E and F. Cytochrome *a* and cytochrome c_m-553 were subsequently eluted with buffer F and cytochrome c_m-553 and cytochrome c_m-552 were eluted with buffer G.

Assays

The concentrations of protein, 2-keto-3-deoxyoctonic acid, and glutamate dehydrogenase were determined by the methods of Lowry et al. [17], Weissback and Hurwitz [18], and Hooper et al. [19], respectively. The rate of oxidation of hydroxylamine to nitrite was monitored as previously described [2] using phenazine methosulfate as the electron acceptor.

Electrophoresis

Slab gel electrophoresis in sodium dodecyl sulfate was performed by the Laemmli method [20] using horizontally split gels of 7% (2.5 cm) and 15% (7.5%). Gels were stained for total protein with Coomassie blue or by the diaminobenzidine method [21] for *c*-type cytochromes.

Spectroscopy

Absorbance spectroscopy was performed with an Aminco DW-2 split beam spectrophotometer. The concentrations of heme *a*, *b* and *c* were measured by the pyridine hemochromogen method [8] using an $\Delta\varepsilon$ (589 nm) of 21.7 $cm^{-1} \cdot mM^{-1}$ for heme *a* [22], a $\Delta\varepsilon$ (550 nm) of 29.1 $cm^{-1} \cdot mM^{-1}$ for heme *c* [23] and a $\Delta\varepsilon$ of 34.4 $cm^{-1} \cdot mM^{-1}$ for non-bound protoheme [23]. The concentration of HAO, cytochrome *c*-552, and cytochrome *c*-554 were measured using the $\Delta\varepsilon$ (553 nm) of 480 $cm^{-1} \cdot mM^{-1}$ for HAO [2], the $\Delta\varepsilon$ (552 nm) of 30.6 $cm^{-1} \cdot mM^{-1}$ for cytochrome *c*-552 [4] and the $\Delta\varepsilon$ (554 nm) of 49.2 $cm^{-1} \cdot mM^{-1}$ for cytochrome *c*-554 [4].

Electron paramagnetic resonance spectra were recorded at X-band on a Varian E-109 spectrophotometer equipped with an Oxford ESR-10 liquid helium cryostat. Temperature measurements and *g*-value calibrations were achieved as previously described [24].

Results

Cell fractionation

Table I summarizes the concentration and cellular distribution of heme *a*, heme *c*, the outer membrane marker 2-keto-3-deoxyoctonate [18,25, 26], the cytoplasmic marker glutamate dehydrogenase [14] and the periplasmic marker hydroxylamine oxidoreductase [14]. These values represent the average of five separate cell fractionations. As measured by release of glutamate dehydrogenase activity, an average of 22% lysis was observed during the removal of the outer membrane and periplasmic material. Hydroxylamine oxidase activity was present exclusively in the periplasm as observed by Olson and Hooper [14]. The hydroxylamine oxidoreductase activity in the cytoplasmic fraction (4.6%) is probably due to periplasmic contamination of the cytoplasmic fraction. The small percentage of 2-keto-3-deoxyoctonic (8.9%) in the cell membrane fraction and heme *a* (4.2%) in the outer membrane fraction suggests trace cross contamination of the two fractions. As expected the cell membrane fraction was the site of essen-

TABLE I

DISTRIBUTION OF PROTEIN, GLUTAMATE DEHYDROGENASE (GDH) ACTIVITY, HYDROXYLAMINE OXIDOREDUCTASE (HAO) ACTIVITY, 2-KETO-3-DEOXYOCTONATE (KDO), HEME *a* AND HEME *c* IN *N. EUROPAEA*

The values were adjusted to the concentration or activity obtained from 1 mg cell protein. The 100% value for protein was 1 mg. For GDH and HAO, activity and 100% values were 1.31 ± 0.17 and 0.9 ± 0.2 units, respectively. The 100% values for heme *a* and heme *c* were 1.11 ± 0.28 and 5.14 ± 0.06 nmol, respectively.

Fraction	Protein (%)	GDH activity (%)	HAO activity (%)	KDO (%)	Heme *a* (%)	Heme *c* (%)
Cells	100	100	100	100±	100	100
Outer membrane	9.4 ± 0.7	0	0	73 ± 8.3	4.5 ± 0.9	1.2 ± 0.1
Periplasm	10.2 ± 0.5	22.9 ± 3.1	78.8 ± 15.5	7 ± 2.2	0	76.3 ± 2.3
Cell membrane	45.5 ± 3.2	0	0	8 ± 1.6	84.2 ± 11.4	26.6 ± 5.4
Cytoplasm	43.7 ± 5.1	73.3 ± 8.4	4.4 ± 1.1	4 ± 2.6	0	2.3 ± 0.7
Total [a]	109	96	83	92	88	106

[a] Sum of cell fractions.

tially all heme *a* [9]. The cell membrane also accounted for 28% of the *c*-type heme. The latter is associated with two *c*-type membrane cytochromes and not with periplasmic contamination.

Cell membrane fraction

Absorption spectra of the washed cell membrane fraction showed maxima at 411 nm in the oxidized form and at 421, 524, 553 and 598 nm and 444 nm (shoulder) in the dithionite reduced form (Fig. 1). Dithionite-reduced minus oxidized absorption spectrum showed the presence of a *c*-type cytochrome with absorption maxima at 426, 522 and 553 nm, and an *a*-type cytochrome with absorption maxima at 444 and 598 nm (Fig. 2). Analysis of washed membrane by the pyridine hemochromogen method [23] also illustrated the presence of both *a*- and *c*-type cytochromes; absorption maxima at 415, 521, 551 and 587 nm (Fig. 1). The concentration of heme *a* and heme *c* in the cell membrane as measured by the pyridine

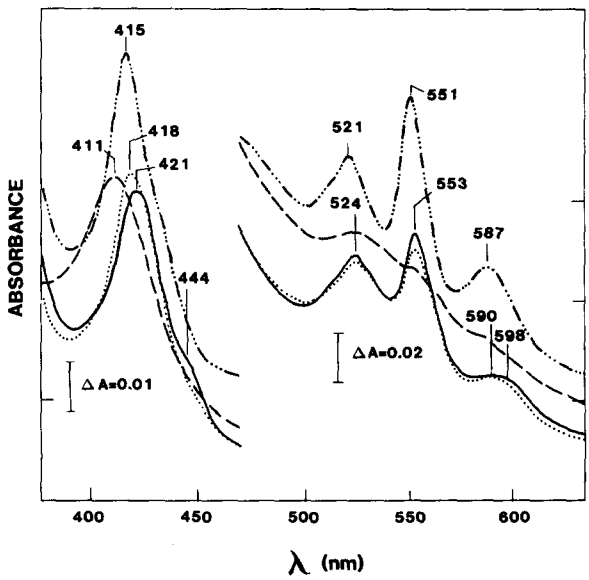

Fig. 1. Absorption spectra of washed cell membrane from *N. europaea* in 50 mM sodium potassium phosphate/100 mM KCl/2 mM EDTA (pH 7.5); (- - - - - -) oxidized, (———) reduced with dithionite, (· · · · · ·) reduced with dithionite plus CO, (— · · · —) reduced alkaline-pyridine.

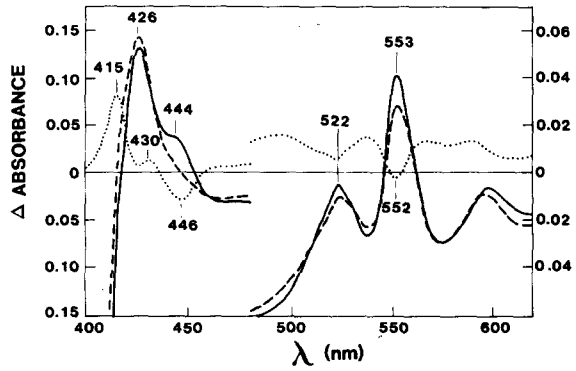

Fig. 2. Difference spectra of washed cell membrane from *N. europaea* in 50 mM sodium potassium phosphate/100 mM KCl/2 mM EDTA (pH 7.5); (———) reduced with dithionite minus oxidized (- - - - - -) reduced with dithionite plus CO minus oxidized, (· · · · · ·) reduced plus CO minus reduced.

hemochromogen method was 1.11 ± 0.28 and 1.37 ± 0.04 nmol/mg cell protein, respectively. Cytochrome *b* was present in such a low concentration as to be difficult to quantitate from the absorption spectrum of the pyridine derivative of the membrane fraction; the concentration was estimated to be less than 15% of the *c*-cytochrome. Dithionite-reduced plus CO absorption spectra indicated that binding of CO to reduced cytochromes caused an increase and shift in the soret peak to 418 nm, a small (approx. 7.7%) decrease in the 553 nm peak and disappearance of the shoulder at 444 nm suggesting reaction of CO with *c* and *a* hemes. The disappearance of the 444 nm shoulder and decrease in the 553 nm peak was also observed in the reduced plus CO minus oxidized difference spectrum (Fig. 2). The reduced plus CO minus reduced difference spectrum contained absorption maxima at 415 and 430 nm and minima at 446, 522 and 552 nm (Fig. 2).

Separation of cytochrome c_m-553 and cytochrome c_m-552

Fig. 3 illustrates the elution profile of a DEAE-Sepharose CL-6B column to which the DEAE-Cellulose column fraction (second peak) had been applied as described in Materials and Methods. Cytochrome *a* and c_m-553 were eluted together with buffer F in 4 peaks with an increasing ratio of cytochrome *a* to cytochrome c_m-553 in each peak. Cytochrome c_m-553 (peak number 5) and cytochrome c_m-552 (peak number 6) were eluted with buffer G. Cytochrome c_m-553, which accounted

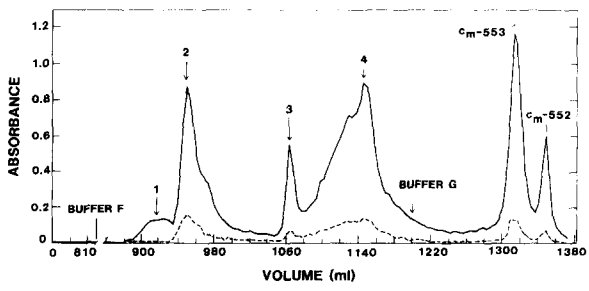

Fig. 3. Elution pattern of the third DEAE-Cellulose column fraction. Sample was applied to a 2.5 × 33 cm DEAE-Sepharose CL-6B column. Fractions were collected automatically and monitored at 280 (- - - - - -) and 410 nm (———).

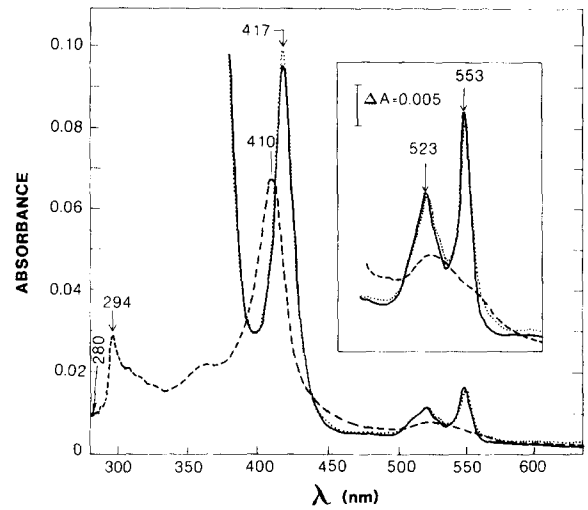

Fig. 4. Absorption spectra of cytochroem c_m-553 from *N. europaea* in 50 mM sodium potassium phosphate/0.2% triton X-100 (pH 8.5). Oxidized form (- - - - - -), reduced with dithionite (———), dithionite reduced plus CO (· · · · · ·).

for 92.3% of the absorption maximum at 410 nm, was characterized by an absorption maximum at 410 nm in the oxidized form and maxima at 417, 423 and 553 nm in the dithionite reduced form (Fig. 4). The 295 nm absorption maximum in Fig. 4 (and Fig. 5) is attributed to the presence of Triton X-100 in the sample buffer. The less concentrated membrane-associated cytochrome c_m-552 was characterized by an absorption maximum at 409 nm in the oxidized form, at 419, 523 and 552 nm in the dithionite-reduced form, and at 416 nm in the dithionite-reduced plus CO form (Fig. 5). Cytochrome c_m-552 accounted for 7.7% of membrane *c*-type cytochrome. The concentration of cytochrome c_m-552 was estimated from the decrease in absorbancy at 553 nm following addition of CO to the reduced membrane (Fig. 2). The remaining membrane-associated cytochrome *c* was associated with cytochrome c_m-553.

Concentration of periplasmic cytochromes

The relative concentrations of periplasmic cytochromes are listed in Table II. Prior to quantitation, pure samples of hydroxylamine oxidoreductase, cytochrome *c*-554 and cytochrome *c*-552 were prepared as described in Materials and Methods with a recovery of 27, 78 and 46%, respectively.

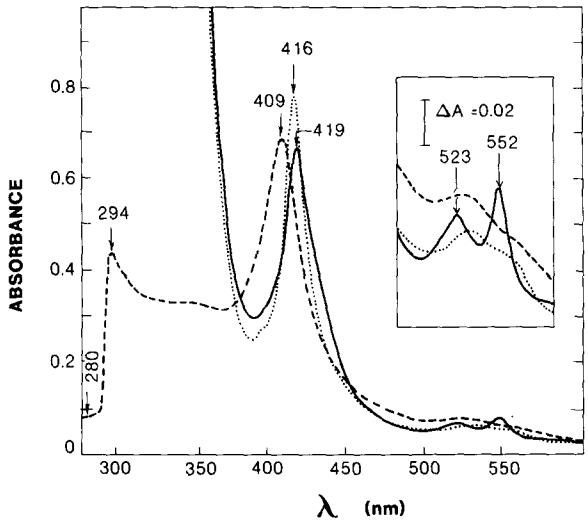

Fig. 5. Absorption spectra of cytochrome c_m-552 from *N. europaea* in 50 mM sodium potassium phosphate/0.2 ≠ triton X-100 (pH 8.5). Oxidized form (- - - - - -), reduced with dithionite (———), dithionite reduced plus CO (· · · · · ·).

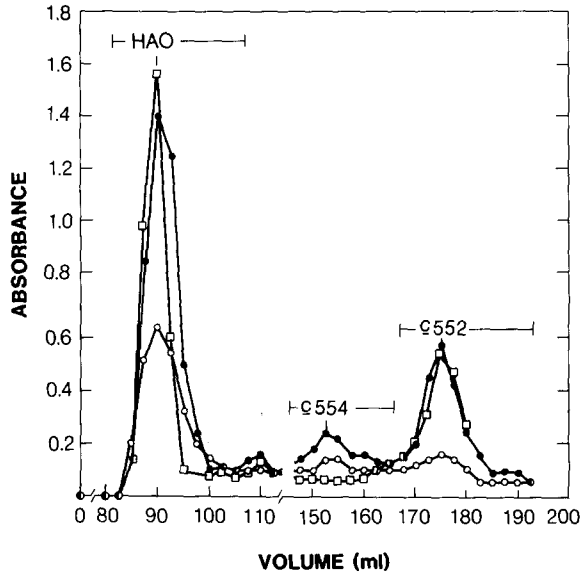

Fig. 6. Elution pattern of the periplasmic fraction applied to a 2.5×49 cm Sephadex G-75 column. Fractions were collected automatically and monitored at 280 (○) and 410 nm (●) before the ammonium sulfate (80%) fraction step and monitored at 410 nm (□) after the 80% ammonium sulfate fractionation step. HAO, hydroxylamine oxidoreductase.

The properties of these pure samples were used to estimate the concentration of the three components in less pure samples where the recovery was almost complete (or at least equal between components).

In order to measure the relative concentration of soluble cytochromes, samples of the periplasm were separated by gel filtration. Fig. 6 illustrates the elution profile of the periplasmic fraction passed through a 2.5 × 49 cm Sephadex G-75 column and demonstrates the separation of hydroxylamine oxidoreductase, cytochrome *c*-554 and cytochrome *c*-552. To improve the resolution of cytochrome *c*-554 from cytochrome *c*-552, a sample of the periplasm was first fractionated with 90% ammonium sulfate. The elution profile of the proteins precipitating in 90% ammonium sulfate lacked the cytochrome *c*-554 peak (Fig. 6) and allowed a clearer quantitation of cytochroem *c*-552.

Purity of Sephade G-75 column fractions

Each of the three Sephadex G-75 column fractions contained more than one species of protein as shown by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie blue. However, as analyzed for *c*-heme containing proteins with the diaminobenzidine stain [21], fractions enriched in cytochrome *c*-554 or *c*-552 stained identically to samples of the corresponding purified cytochrome (Fig. 7). The slowly migrating band seen in electrophoresis of cytochrome *c*-552 on SDS-gels appears to be due to aggregation of the protein, it is also observed in purified samples of cytochrome *c*-552 after freezing. The heme stain of the SDS gel bands of hydroxylamine oxidoreductase column fractions revealed the presence of an additional *c*-heme protein with an approximate molecular weight of 46000. Dithionite-reduced plus CO minus reduced difference absorption spectra of the periplasm and hydroxylamine oxidoreductase column fractions also revealed a small absorption maximum at 414 nm which is not associated with hydroxylamine oxidoreductase, cytochrome *c*-552 or cytochrome *c*-554. This 46 kDa cytochrome in the hydroxylamine oxidoreductase column fraction accounted for 6.3% of the total absorbancy at 410 nm on the unstained gel. A soluble CO-binding *c*-type cytochrome with a molecular weight of 32 kDa has been reported in

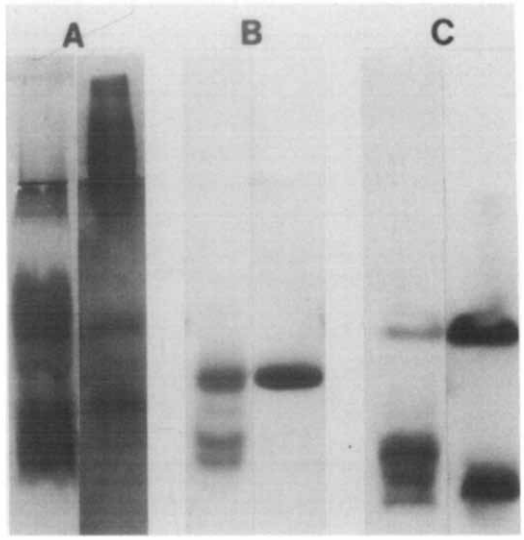

Fig. 7. Hydroxylamine oxidoreductase (a), cytochrome *c*-554 (b), and cytochrome *c*-552 (c) Sephadex G-75 column fractions electrophoresed on 7% (2.5 cm) and 15% (7.5 cm) SDS polyacrylamide gels and stained with Coomassie blue (left lanes) and diaminobenzidine (right lanes).

N. europaea [8,12] and termed cytochrome c_{CO}-550. We could not determine whether the cytochrome in the hydroxylamine fraction was actually cytochrome c_{CO}-550.

Absorption spectra of the column fractions at room temperature (Fig. 8) or 77 K indicated that cross contamination was not more than 15%. Dithionite-reduced cytochrome *c*-554 at pH 4.0 has been shown to bind CO [27], whereas cytochrome *c*-552 does not. The difference in reactivity to CO revealed a 5.4% contamination of the cytochrome *c*-554 fraction with cytochrome *c*-552 (Fig. 8). The concentrations of hydroxylamine oxidoreductase, cytochrome *c*-554 and cytochrome *c*-552 were corrected for the contaminations listed above; the 46 kDa cytochrome in the hydroxylamine oxidoreductase column fraction is listed as 'other cytochromes' in Table II.

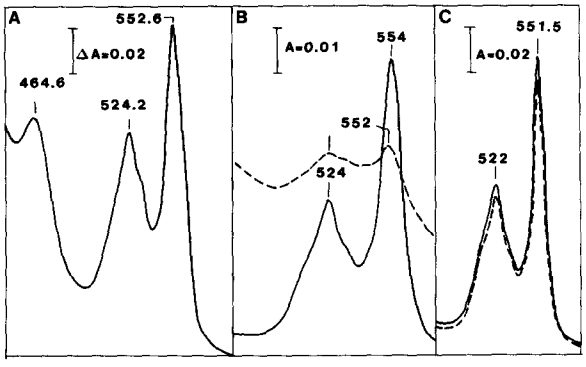

Fig. 8. Absorption spectra of the hydroxylamine oxidoreductase (A), cytochrome *c*-554 (B) and cytochrome *c*-552 (C) column fractions at room temperature after separation on a 2.5 × 49 cm Sephadex G-75 column. Dithionite-reduced (———), dithionite-reduced plus CO (· · · · · ·).

EPR spectrum of soluble fractions

The EPR spectrum at 10 K of the periplasmic fraction shows the presence of hydroxylamine oxidoreductase ($g = 3.06$, 2.99, 2.24 and 1.43) [28], cytochrome *c*-554 ($g = 4.18$ and 3.85) [27], and cytochrome *c*-552 ($g = 3.34$ and 1.67) (Fig. 9, graph A). An EPR spectrum was made of that part of the periplasm present in the included volume of a 2.5 × 47 cm Sephadex G-75 (thus lacking hydroxylamine oxidoreductase) and precipitating in 80% ammonium sulfate (thus also lacking cytochrome

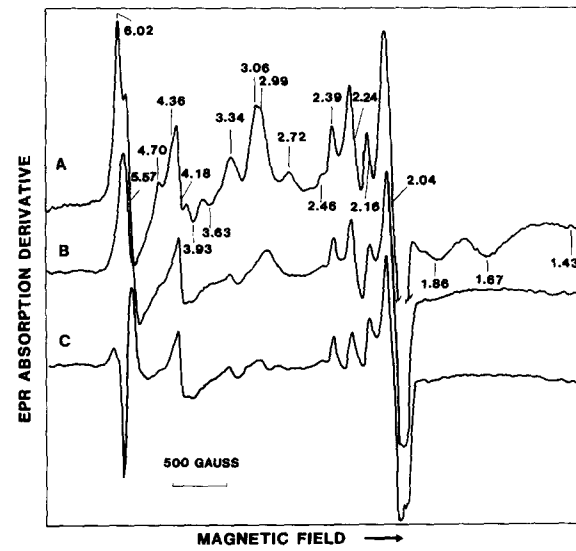

Fig. 9. EPR spectra of the periplasmic fraction in 50 mM sodium potassium phosphate (pH 7.5) at 10.3 K (A), after the removal of hydroxylamine oxidoreductase and cytochrome *c*-554 and the computor subtraction of cytochrome *c*-552 (B), and after the subtraction of the high-spin iron signals (C). Instrumental conditions were the following: microwave frequency, 9.22 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 kHz; microwave power, 0.2 mW.

c-554). When the EPR spectrum of cytochrome *c*-552 was then subtracted by computer, the remaining spectrum contained high spin iron signals ($g = 6.02$, 2.93, 2.24 and 1.47) and low spin copper signals ($g = 2.46$, 2.39, 2.16 and 2.04) (Fig. 9, graph B). Cytochrome *c*-554 at pH 4.0 has a similar spectrum to the high-spin iron signals [27]. Computer subtraction of the pH 4.0 cytochrome *c*-554 signals left an EPR spectrum containing copper signals similar to a type 1 'blue' copper protein [29] (Fig. 8C). The EPR spectrum of the cytoplasmic fraction (Fig. 10) contained rubridoxin like signals ($g = 9.3$ and 4.89) and a low spin copper signal ($g = 2.04$), but contained no heme signals.

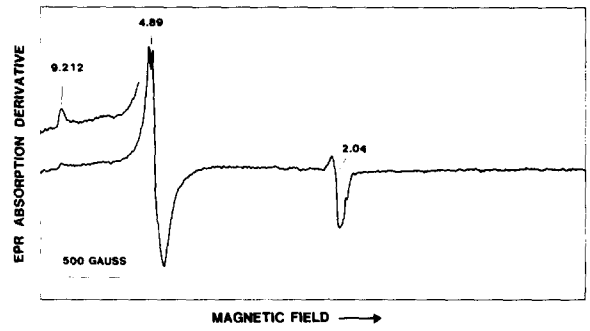

Fig. 10. EPR spectra of the cytoplasmic fraction in 50 mM sodium potassium phosphate buffer (pH 7.5). Instrumental conditions were the same as for Fig. 9.

Cellular distribution of electron-transport components

A summary of the concentration and cellular distribution of electron transport components is given in Table II. The values in Table II were adjusted to the concentration obtained from cells equivalent to 1 mg protein. The assumptions of two *c*-heme for cytochromes c_m-552 and c_m-553 and four for the 46 kDa cytochromes were used to estimate enzyme or cytochrome concentration in Table III. The distribution of electron-transport components appeared to follow a general scheme: the components involved in the oxidation of ammonia (i.e., hydroxylamine oxidoreductase, cytochrome *c*-552 and possibly cytochrome *c*-554) were observed only in the periplasm, whereas the com-

TABLE II

DISTRIBUTION, CONCENTRATION AND RATIO OF CYTOCHROMES IN *N. EUROPAEA*

Concentrations are given in nmol heme or nmol enzyme (cytochrome)/mg cell protein. The values were adjusted to the heme, cytochrome or enzyme concentration obtained from 1 mg cell protein.

Fraction	Enzyme/cytochrome	Heme (nmol/mg cell protein)	Cytochrome or enzyme (nmol/mg cell protein)	Ratios	
				Heme	Cytochrome or enzyme [a]
Cell membrane	Cytochrome *a*	1.11 ± 0.28	(0.56) [c]	10.1	5
	Cytochrome c_m-553	1.26 ± 0.04	(0.63) [d]	11.5	6
	Cytochrome c_m-552	0.11	(0.055) [d]	1.0	(0.5)
	Cytochrome *b*	(≤ 0.2)	(≤ 0.2)	(≤ 1.8)	≤ 2
	Ubiquinone-8 [b]	–	3.7	–	67
Periplasm	Hydroxylamine oxidoreductase	2.4 ± 0.06	0.13 ± 0.005	21.8	1.2
	Cytochrome *c*-552	1.17 ± 0.09	1.05 ± 0.08	10.6	11
	Cytochrome *c*-554	0.32 ± 0.07	0.09 ± 0.03	2.9	0.8 [e]
	Other *c*-type cytochromes	0.15	–	1.44	(0.4) [f]
	Cu-protein	–	–	–	–

[a] Normalized to 0.11.
[b] From Hooper, A.B. et al. (Ref. 9).
[c] Based on two hemes per molecule (DiSpirito, A.A., and Hooper, A.B., unpublished results).
[d] Based on two hemes per molecule (approx. 1 per 10 kDa).
[e] Based on four hemes per molecule (Andersson, K.K., and Hooper, A.B., unpublished results).
[f] Based on four hemes per molecule (approx. 1 per 10 kDa).

ponents which may be involved in the coupling of substrate electrons to O_2 (i.e., cytochrome *a* and possibly cytochrome *b*, cytochrome c_m-553 and cytochrome c_m-552) were observed only in the cell membrane. The periplasm also contains a 46 kDa cytochrome, a high-spin cytochrome and a low-spin copper protein. The outer membrane and cytoplasm were devoid of cytochromes.

Ratios of electron-transport components

On the basis of heme content, the electron-transport components observed in this study can be divided into three groups (Table II). The electron-transport components in high concentrations were hydroxylamine oxidoreductase, cytochrome *c*-552, cytochrome c_m-553 and cytochrome *a* in a ratio of 22 : 11 : 12 : 10, respectively. The components with intermediate concentrations were cytochrome *c*-554 and ubiquinone-8 in a ratio of 2.9 : 3.9. The components in lowest concentrations were the CO-binding cytochrome c_m-552 and the 46 kDa cytochrome in a ratio of 1.0 : 1.4.

An estimate of the molecular stoichiometry of electron-transport components is shown in Table II. The values in parentheses are for cytochromes of unknown heme content and assumed one heme per 10 kDa. In the periplasm, hydroxylamine oxidoreductase, cytochrome *c*-554, cytochrome *c*-552 and the 46 kDa cytochrome *c* are in a ratio of 1.2 : 0.8 : 11 : (0.4). In the membrane ubiquinone-8, cytochrome *b*, cytochrome c_m-552, cytochrome c_m-553 and an *a*-type cytochrome oxidase are in a ratio of 3.9 : 1.8 : 1.0 : 12 : 10.

Discussion

Membrane components

The cell membrane was shown to contain approximately 24% of the cellular heme *c*. Cytochrome c_m-553 (92%) and cytochrome c_m-552 (7.7%) was shown to account for most of the membrane cytochrome *c*. The 24 kDa membrane cytochrome *c*-554 ($E_0' = 170$ mV) reported by Miller and Wood [11] was not detected in this study; it is apparently present in relatively small amounts. The ratio of heme *b*/heme *c* in the cell membrane was determined to be less than 0.15 as the pyridine derivative. An earlier report on this ratio [9] is clearly incorrect, since the estimate involved the assumption that all cytochromes absorbing at 558 nm were of the *b*-type. A value of 0.5 for the heme *b*/heme *c* ratio in the membrane of *N. europaea* has been reported [12] together with indirect evidence for the existence of two types of *b*-cytochromes.

From the present work the membrane contains 46% of the cell protein and contains ubiquinone-8 [9], cytochrome *a*, cytochrome c_m-553, cytochrome c_m-552, and cytochrome *b* in concentrations of 8.0, 2.4, 2.8, 0.24 and 0.4 nmoles/mg membrane protein, respectively. The corresponding ratios as cytochromes or enzymes are 3.3 : 1.0 : 1.2 : 0.1 : 0.17. Roles for the various components are not established though the ratios suggest that ubiqunone, cytochrome oxidase and cytochrome c_m-553 might function separately from cytochrome c_m-552, cytochrome *b* and other minor *c*-type cytochromes.

Periplasmic components

In *N. europaea* the periplasm contains approximately 10% of the cellular protein, 18% of the soluble protein, and 76% of the *c*-heme. The majority of the periplasmic *c*-heme can be accounted for in hydroxylamine oxidoreductase (59%), cytochrome *c*-552 (29%) and cytochrome *c*-554 (8%). Evidence for four other soluble *c*-type cytochromes has been reported: (i) a 52 kDa cytochrome *c*-553 [11,30]; (ii) a 36 kDa cytochrome *c*-550 [11]; (iii) a 32 kDa CO-binding cytochrome *c*-550 [7]; (iv) and a 16 kDa CO-binding cytochrome *c*-552 [7]. The present data suggest that these four species are in small quantities. The three Sephadex G-75 column fractions of the periplasm contained only four *c*-heme-containing proteins as observed by polyacrylamide gel electrophoresis. The high-molecular-weight column fraction (hydroxylamine oxidoreductase fraction Fig. 7) contained bands characteristic of hydroxylamine oxidoreductase and a 46 kDa band accounting for approx. 4% of the *c*-heme as estimated by scanning the gels at 410 nm. By the same technique the only heme-staining bands in the middle ('cytochrome *c*-554' fraction) and lower ('cytochrome *c*-552' fraction) molecular-weight column fractions were accounted for by cytochrome *c*-554 and *c*-552, respectively. Thus, the periplasmic heme proteins were hydroxylamine oxidoreductase, cytochrome *c*-552, cytochrome *c*-554 and an uncharacterized

46 kDa *c*-type cytochrome in concentrations of 4.4, 2.2, 0.6 and 0.28 nmoles/mg periplasmic protein. The corresponding ratios of enzymes or cytochromes (relative to cytochrome oxidase) were 0.21 : 2.1 : 0.29 : 0.07, respectively. The ratios of cytochrome *c*-552, cytochrome c_m-553/cytochrome oxidase resemble values observed in other respiratory systems. In addition, the periplasm also contains hydroxylamine oxidoreductase and cytochrome *c*-554 in lower ratios although they account for 67% of the *c*-heme. Interestingly, the ratio of HAO P460/cytochrome oxidase is close to unity (approx. 0.7). As shown here, the periplasm also contains a copper protein and high-spin hemoprotein of unknown function.

Cytoplasmic components

The cytoplasm of *N. europaea* contains approx. 44% of the cellular protein (approx. 82% of the soluble protein), but little or no cytochrome. EPR data indicate the presence of a rubredoxin-like protein and a copper protein.

Significance to proton gradient

The periplasmic location of hydroxylamine oxidoreductase, cytochrome *c*-552 and cytochrome *c*-554 in the periplasmic space and the membrane location of the cytochromes involved in the coupling of electrons of O_2 has several energenic advantages. The release of protons from ammonia and water is in the extracellular compartment and aids in the generation of a proton gradient ($NH_3 + \frac{1}{2}O_2 + H_2O \rightarrow HNO_2 + 4H^+ + 4e^-$). Cytochromes involved in the reaction of electrons and protons with oxygen to form water are membrane bound and presumably face the cytoplasm; thus they also add to the generation of a proton gradient. The transport of NH_3 into the cell and NO_2^- out of the cell is not required. The location of electron transport components in the periplasmic space is not unique to *N. europaea.* It has been observed in a number of bacteria which utilize simple reductants [31–38].

Relevance to previous spheroplast work

The present paper also demonstrates that there is no NH_3 or NH_2OH oxidase activity (oxidation of substrate to NO_2^-) in the two membrane fractions or the cytoplasmic fraction of *N. europaea.* This observation is inconsistent with the NH_3 and NH_2OH oxidase activity which has been reported in spheroplasts of *N. europaea* [39,40]. The activity of the spheroplast preparations in these studies was less than 5% of that observed in whole cells. It would appear likely that this activity was due to the presence of intact cells or spheroplasts with trapped periplasmic cytochromes which were able to react with the membrane terminal oxidase.

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