Biochimica et Biophysica Acta, 637 (1981) 18–27
Elsevier/North-Holland Biomedical Press

BBA 48086

ISOLATION AND PROPERTIES OF CYTOCHROME *c*-553, CYTOCHROME *c*-550, AND CYTOCHROME *c*-549, 554 FROM *NITROBACTER AGILIS*

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(Received September 3rd, 1980)
(Revised manuscript received March 23rd, 1981)

Key words: Cytochrome c; Nitrifying bacteria; Respiratory chain; (N. agilis)

Three *c*-type cytochromes isolated from *Nitrobacter agilis* were purified to apparent homogeneity: cytochrome *c*-553, cytochrome *c*-550 and cytochrome *c*-549, 554. Their amino acid composition and other properties were studied. Cytochrome *c*-553 was isolated as a partially reduced form and could not be oxidized by ferricyanide. The completely reduced form of the cytochrome had absorption maxima at 419, 524 and 553 nm. It had a molecular weight of 25 000 and dissociated into two polypeptides of equal size of 11 500 during SDS gel electrophoresis. The isoelectric point of cytochrome *c*-553 was pH 6.8. The ferricytochrome *c*-550 exhibited an absorption peak at 410 nm and the ferrocytochrome *c* showed peaks at 416, 521 and 550 nm. The molecular weight of the cytochrome estimated by gel filtration and by SDS gel electrophoresis was 12 500. It had an $E_{m(7)}$ value of 0.27 V and isoelectric point pH 8.51. The N-terminal sequence of cytochrome *c*-550 showed a clear homology with the corresponding portions of the sequences of other *c*-type cytochromes. Cytochrome *c*-549, 554 possessed atypical absorption spectra with absorption peaks at 402 nm as oxidized form and at 419, 523, 549 and 554 nm when reduced with $Na_2S_2O_4$. Its molecular weight estimated by gel filtration and SDS polyacrylamide gel electrophoresis was 90 000 and 46 000, respectively. The cytochrome had an isoelectric point of pH 5.6. Cytochrome *c*-549, 554 was highly autoxidizable.

Introduction

Nitrobacter, a nitrifying bacterium that oxidizes nitrite to nitrate, was first described by Winogradsky [1]. A cytochrome *c* was shown to be involved in the respiratory chain of this bacterium [2].

The spectral properties and redox potentials of a partially purified as well as a purified cytochrome *c* from *Nitrobacter* [3,4] were similar to horse heart cytochrome *c*. In view of the redox potential of cytochrome *c* ($E_{m(7)} = 0.270$ V) [3–6] compared with the nitrite/nitrate couple ($E_{m(7)} = 0.43$ V) [7], the direct reduction of cytochrome *c* by nitrite was believed to be impossible [8] and to require input of energy [9,10]. Studies with inhibitors and uncouplers [11,12] supported the energy-dependent reversal of electron flow from cytochrome a_1 to *c* and the entry site of nitrite at cytochrome a_1 level rather than cytochrome *c*. O'Kelley et al. [13], on the other hand, reported the energy-independent reduction of horse heart cytochrome *c* with nitrite in the presence of nitrite : cytochrome *c* reductase.

On the basis of their spectrophotometric studies Van Gool and Laudelout [14] reported that the α absorption peak of *Nitrobacter* cytochrome *c* was at 554 nm rather than 550–551 nm as reported by other workers [3,4].

These controversial findings together with a recent report of an electron transfer component with low redox potential and absorption maximum in the α region of *c*-type cytochrome [15] have prompted

us to reinvestigate the *c*-type cytochrome(s) of *Nitrobacter*.

This paper reports the isolation of three *c*-type cytochromes; cytochrome *c*-553, cytochrome *c*-550 and cytochrome *c*-549, 554 and describes some of their properties.

Materials and Methods

Growth of bacteria. Mass cultivation of *Nitrobacter agilis* was performed in several 20 l carboys each containing 16 l sterilized medium [16] at 28°C in the dark. Nitrite concentration was gradually increased [2] such that the highest application used was 65 mM NO_2^-. Oxygen requirements of the growing organism were met by supplying air through two glass spargers. Sterilization of the air was ensured by allowing air to pass through a sterilized air filter before entering the spargers. The pH of the growing culture was maintained by a Radiometer pH stat. Actively growing cells (about to complete oxidation of the final aliquot of added nitrite judged by using Griess-Ilosvay reagents) were harvested in a Sharples centrifuge (with a flow rate of 15 l/h). Such cultures yielded 0.1 g wet cells/l. The cell paste was washed twice with 10 mM potassium phosphate buffer, pH 7.8 containing 1 mM EDTA, 1 mM GSH, 5 mM $MgCl_2$ and 300 mM sucrose. The cells were then suspended in the same buffer (1 g wet cells/5 ml) and stored at 0°C no longer than 1 week.

Isolation of cytochromes. All the isolation and purification procedures were performed at 0–4°C. A *Nitrobacter* cell suspension of 350 ml (approx. 70 g) was homogenized (in a 'MSK' Mechanical Cell Homogenizer, Bronwill Scientific, Rochester, NY) at 0°C by a similar method that was reported by Kiesow [10] and Cobley [15] except that the homogenizer treatment consisted of eight 15-s pulses separated by 10-s intervals. The cell homogenate was centrifuged at 15 000 × *g* for 15 min. The supernatant obtained was further centrifuged at 144 000 × *g* for 4 h. The supernatant (F) was applied to a DEAE-cellulose (Whatman DE-32) column (2.5 × 40 cm), previously equilibrated with the buffer used for cell washing. The column was washed with the same buffer until the effluent became colorless. The red fractions thus obtained were pooled (F_1), and used for the isolation of cytochrome *c*-550 and cytochrome *c*-549, 554 (Scheme I).

To isolate cytochrome *c*-553, the DEAE-cellulose column was first washed with 20 mM potassium phosphate buffer, pH 7.8 and then with 50 mM potassium phosphate buffer, pH 7.5. Cytochrome *c*-553 was eluted as a single red band with 100 mM potassium phosphate buffer, pH 7.5.

Purification of cytochrome c-553. The fractions containing cytochrome *c*-553 were combined and ammonium sulfate was added to a concentration of 40% saturation. After centrifugation at 15 000 × *g* for 10 min, the pellet was discarded and the ammonium sulfate concentration was raised to 80% of saturation in the supernatant. After 1 h, the supernatant was centrifuged as above and the pellet containing cytochrome *c*-553 was dissolved in a minimum amount of 5 mM Tris-HCl, pH 8.5. This fraction was dialyzed overnight against the same buffer.

The dialyzed fraction was then applied to another DEAE-cellulose column (1 × 6 cm) pre-equilibrated with the 5 mM Tris buffer. The column was washed with 50 mM Tris-HCl, pH 7.5 and the cytochrome *c*-553 was eluted with the same buffer containing 50 mM KCl. The colored fractions were pooled further and purified and concentrated by ultrafiltration (Millipore Immersible CX, single use Ultrafiltration Unit-NMWL 10 000).

This concentrated fraction was chromatographed on a Sephadex G-100 column (2.5 × 40 cm) in 50 mM Tris-HCl buffer, pH 7.5. The red fractions were again combined, concentrated as above and passed through a second Sephadex G-100 column in 50 mM Tris-HCl/100 mM KCl, pH 7.8. Cytochrome *c*-553 thus obtained was used for further studies.

Purification of cytochrome c-550. The fraction F_1 from the DEAE-cellulose column (see Scheme I) was dialyzed overnight against 5 mM potassium phosphate buffer, pH 6.5 and loaded on a CM-cellulose (Whatman CM-52) column (4 × 6 cm) equilibrated with 5 mM potassium phosphate buffer, pH 6.5. Cytochrome *c*-550 was adsorbed on top of the column, while the fraction eluted with the same buffer contained a hemoprotein with anomalous spectra (F_2). The CM-cellulose column was then washed extensively with 10 mM potassium phosphate buffer, pH 7.5. When the concentration of buffer was increased to 20 mM phosphate, the adsorbed cytochrome *c*-550 started moving down slowly. When

the red band reached the middle of the column, the cytochrome *c*-550 was eluted as a single dark red band with 30 mM phosphate buffer, pH 7.8. The cytochrome *c*-550 containing fractions were diluted 3-fold with distilled water and adjusted to pH 6.5 with dilute HCl. This combined fraction was then loaded on a CM-Sephadex column (2 × 6 cm). The column was extensively washed with 20 mM phosphate buffer, pH 7.5. With the increase in buffer concentration to 50 mM phosphate, a red band of cytochrome *c*-550 started moving down the column; however, the elution rate was quite slow. When the red band was in the middle of the column, it was finally eluted with 100 mM phosphate buffer, pH 7.5. This fraction was chromatographed on a Sephadex G-100 column (2.5 × 80 cm) in 100 mM phosphate buffer, pH 7.5. The cytochrome *c*-containing red colored fractions eluted from Sephadex G-100 were concentrated by ammonium sulfate precipitation. The fraction obtained between 45–95% saturation of ammonium sulfate was desalted by passing through a Sephadex G-25 column in 50 mM Tris-HCl, pH 7.5/100 mM KCl. Cytochrome *c*-550 fraction was finally passed through a Sephadex G-75 column (2.5 × 40 cm) in the same buffer and used as a purified fraction.

Purification of cytochrome c-549, 554. The fraction that passed through the CM-cellulose column (F_2) was adjusted to pH 8.5 with a dilute alkali and applied to a DEAE-Sephadex column (2.5 × 6 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 8.5 (see Scheme I). The hemoprotein (cytochrome *c*-549, 554) was adsorbed on the DEAE-Sephadex. The column was washed with 50 mM phosphate buffer, pH 7.8 and a yellow brown band of the cytochrome *c*-549, 554 was eluted with 100 mM potassium phosphate buffer (pH 7.0). The yellow brown fraction gave a spectrum with a flat-top peak at 549–555 nm. The fraction was treated with ammonium sulfate (40% saturation) and was centrifuged at 15 000 × *g* for 10 min. The supernatant was adjusted to 90% saturation of ammonium sulfate and recentrifuged. The pellet containing cytochrome *c*-549, 554 was suspended in 50 mM Tris-HCl, pH 7.5 and dialyzed against the same buffer. The dialyzed fraction was chromatographed on a Sephadex G-150 column (2.5 × 40 cm) in the Tris-HCl buffer. The fractions displaying 402 nm absorption were combined and concentrated by ultrafiltration as described for cytochrome *c*-553.

The concentrated cytochrome fraction was finally passed through a Sephadex G-100 column (2.5 × 40 cm) in 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl. The cytochrome obtained from the eluate of this column was used as a purified fraction.

Analytical procedures. Absorption spectra were recorded at room temperature on a Shimadzu MPS-50L or a Unicam SP700 spectrophotometer using a cell with 1 cm lightpath. A Gilford spectrophotometer was also routinely used during the course of protein purification.

The pyridine hemochrome derivatives of purified cytochromes were prepared according to Rieske [17].

The midpoint redox potential at pH 7 ($E_{m(7)}$) of cytochrome *c*-550 was determined by titration with a $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox system [18] under aerobic conditions.

Molecular weight determinations of the isolated cytochromes were done by gel filtration on superfine Sephadex G-100 or G-75 [19] and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) [20]. The gel concentration used was 12.5%. After electrophoresis the gels were fixed in 50% trichloroacetic acid for 4 h and destained in 10% acetic acid/20% ethanol (v/v). The marker proteins used were from Pharmacia Fine Chemicals, Uppsala, Sweden (calibration kit for molecular weight determination) or Sigma Chemical Co., St. Louis, MO (SDS Molecular Weight Marker Kit).

Isoelectric focusing was performed by the procedure of O'Farrell [21] using carrier ampholytes (LKB, Sweden or Brinkmann Instrument Inc. Westbury, NY.). Isoelectric points were estimated by comparing a stained gel after isoelectric focusing with the pH gradient developed during isoelectric focusing in a blank gel run parallel to the sample gel.

Protein content was determined according to Lowry et al. [22]. Bovine serum albumin was used as a standard.

Amino acid analyses were performed by Technicon NC-2P single column amino acid analyzer. The samples were hydrolyzed for 20 h in vacuo at 110°C in constant boiling HCl [23]. Cysteine

was determined as cysteic acid after performic acid oxidation [24].

Amino acid sequence determination. Edman degradations were performed automatically in a Beckman 890C Sequencer by standard methods [25]. The programme used was Beckman Catalogue No. 072172C, with 1 M Quadrol as buffer. The butyl chloride contained 0.1% ethylmercaptan or 15 mg/l Cleland's reagent to protect the thiazolinones of serine and threonine. Butyl chloride extracts were divided into two roughly equal proportions. One-half was reduced to dryness with a stream of nitrogen, heated 10 min at 80°C in 1 M HCl to convert the thiazolinones to phenylthiohydantoins, and the aqueous acid extracted twice with 0.7 ml portion of ethyl acetate. The ethyl acetate extracts were again taken to dryness, and the residues examined for phenylthiohydantoins by gas chromatography [26], and thin-layer chromatography on Whatman silica gel plates, type LK6DF, using the system of Jeppsson and Sjöquist [27]. The other half of each butyl chloride extract was taken to dryness in a hydrolysis tube, covered with 1.0 ml of a freshly prepared solution of $SnCl_2$ (1 mg/ml) in constant boiling HCl, and the tube evacuated and sealed. After the hydrolysis of thiazolinones for 4.0 h at 150°C, the tubes were opened and dried in vacuo over solid NaOH. The residues, which contained free amino acids derived from the thiazolinones, were subjected to amino acid analysis.

A known amount of the phenylthiohydantoin of norleucine (about 50 nmol) was added to each tube of the Sequencer fraction collector before beginning a run, and the recovery of norleucine in the amino acid analyzer was used to calculate the yield of the amino acid obtained at each cycle of the Edman degradation.

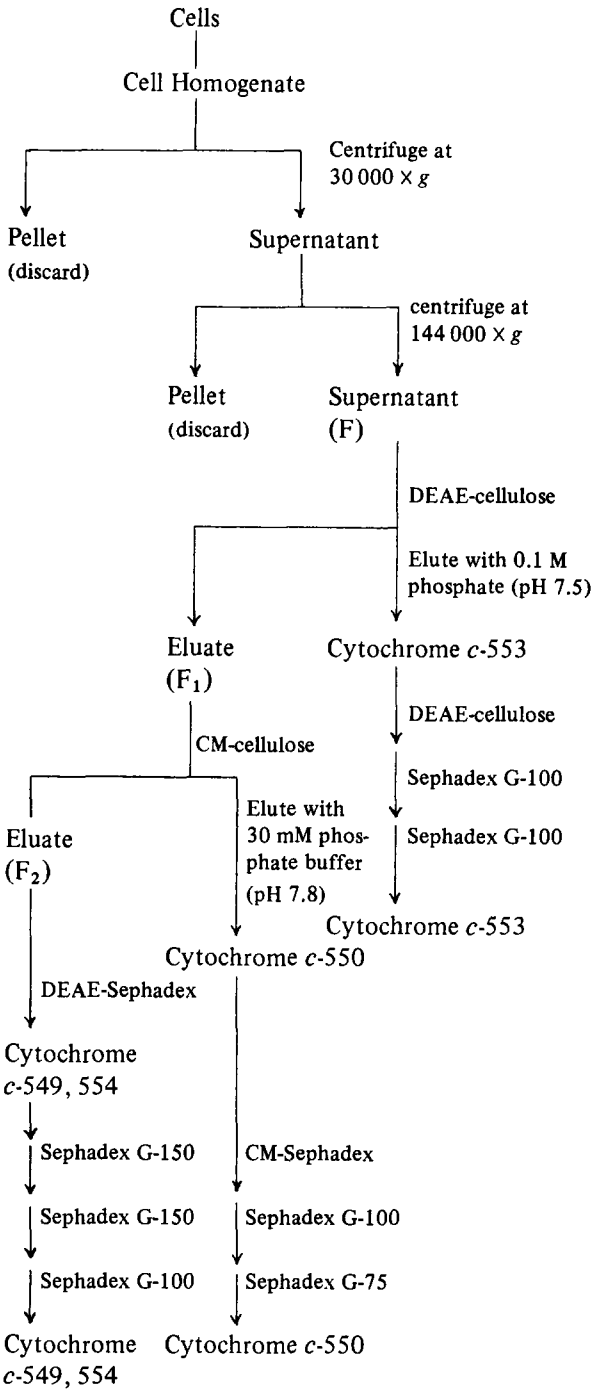
All chemicals used in the Sequencer were Sequencer grade, from Beckman. The standard phenylthiohydantoins were from Pierce.

Results

Purification of cytochromes

A summary of purification procedures of three *c*-type cytochromes is shown in Scheme I.

In several preparations, the isolatin of cytochrome *c*-550 and cytochrome *c*-549, 554 was reproducible



Scheme I. Purification scheme for *c*-type cytochromes from *Nitrobacter agilis*.

by the procedures described in Materials and Methods. Isolation of cytochrome *c*-553, however, was found to be more difficult. In fact, the buffer used for washing the first DEAE-cellulose column was found to be very critical in removing other proteins which interfered in its isolation.

Cytochrome *c*-550 was always present in larger amounts than other cytochromes in the 144 000 × *g* supernatant and its purification was the easiest, yielding 15 mg protein per 70 g wet cells. Results of many preparation trials gave average quantity ratios of 20 : 3 : 1 for cytochrome *c*-550/cytochrome *c*-549, 554/cytochrome *c*-553 in the supernatant fraction (F). Their estimation in the intact cells, cell homogenate or pellet fractions was not possible. The total cytochrome *c* content estimated from absorption at 550 nm upon reduction gave the approximate distribution ratios of 15 : 60 : 25 for 30 000 × *g* pellet/144 000 × *g* pellet/144 000 × *g* supernatant on the basis of 100 for cell homogenate.

In view of difficulties in obtaining *Nitrobacter* cells in large quantities, the procedure used for the isolation of these cytochromes was devised in order to utilize the cell materials fully for the isolation of other enzymes and proteins as well. For example, cytochrome *b* [28] and some oxidoreductases were isolated from the fraction adsorbed on the first DEAE-cellulose column, and cytochrome oxidase was isolated from a pellet fraction [29].

Properties of cytochrome c-553

Absorption spectra. The isolated cytochrome *c*-553 was found to be partially reduced with absorption maxima at 410, 524, and 553 nm. The completely reduced form of cytochrome *c*-553 showed a shift of the Soret band to 419 nm, whereas the α and β bands remained at the same wavelengths, although they were more intense (spectra not shown). Ferricyanide at 10–50 μM could not oxidize the partially reduced cytochrome *c*-553, it however, converted ferrocytochrome back to the partially reduced state. Pyridine hemochrome prepared from cytochrome *c*-553 gave a spectrum typical of heme *c*. The absorption maxima of reduced cytochrome *c*-553 were similar to those reported by Van Gool and Laudelout [14] with their crude cell-free extracts.

Molecular weight. A molecular weight of 24 000 was obtained for cytochrome *c*-553 preparations by gel filtration on Sephadex G-100. SDS-polyacrylamide gel electrophoresis performed on the purified preparations showed a molecular weight of 11 500. This indicates that cytochrome *c*-553 is a dimer, a conclusion in agreement with the estimate of two hemes per molecule of 24 000.

Isoelectric point. The isolated cytochrome *c*-553 was subjected to isoelectric focusing using a mixture of carrier ampholytes with pH ranges 5–8 and 3.5–10 in the ratio 4 : 1. A sharp red band in the isoelectrically focused gels corresponded to a protein band upon staining with Coomassie blue. A p*I* value of 6.8 was measured for the red band of cytochrome *c*-553.

Amino acid composition. Results of amino acid analyses are given in Table I, which differentiate cytochrome *c*-553 from the other two *c*-type cytochromes isolated from *Nitrobacter*. This cytochrome has higher amounts of leucine, methionine and arginine but less lysine residues compared with cytochrome *c*-550.

Properties of cytochrome c-550

Absorption spectra. The oxidized form of cytochrome *c*-550 showed an absorption maximum at 411 nm, and the reduced form maxima at 416, 521 and 550 nm (Fig. 1). Although the ratio between 280 and 550 nm absorption was high, the results presented below indicate that the cytochrome preparations were electrophoretically pure. Previously purified cytochrome *c*-550 was also shown to exhibit similar absorption spectra [4]. From the absorbance at the α peak of the pyridine ferrohemochrome, the ϵ_{mM} at the α peak of cytochrome *c*-550 was determined to be 30.0, assuming the ϵ_{mM} at the α-peak of pyridine ferrohemochrome of heme *c* to be 29.1 [30].

Molecular weight. Gel filtration on Sephadex G-75 yielded a molecular weight of 12 500 for cytochrome *c*-550. It moved as a single band during polyacrylamide gel electrophoresis in the presence of SDS. The results obtained by gel electrophoresis gave the same molecular weight as that obtained by the gel filtration method indicating that this molecule is a monomer.

Isoelectric point. Isoelectric focusing of cyto-

TABLE I

AMINO ACID COMPOSITION OF CYTOCHROME *c*-550, *c*-553 AND *c*-549, 554 ISOLATED FROM *NITROBACTER AGILIS*

To determine cysteine, samples were oxidized with performic acid prior to hydrolysis. Tryptophan estimations were not made.

Amino acid	No. of residues per mol of cytochrome		
	c-550	*c*-553	*c*-549, 554
Aspartic acid	14	12	48
Threonine	5	8	29
Serine	7	7	26
Glutamic acid	13	10	45
Proline	7	7	26
Glycine	15	10	41
Alanine	15	13	59
Valine	7	8	21
Methionine	1	2	3
Cysteine	2	not measured	9
Isoleucine	4	3	13
Leucine	7	11	37
Tyrosine	2	2	7
Phenylalanine	4	3	18
Histidine	3	2	5
Lysine	13	7	25
Arginine	2	4	20
Total residues	121	109	432
Molecular weight *	12 500	11 500	46 000
Molecular weight **	12 500	24 000	90 000

* Estimated by SDS polyacrylamide gel electrophoresis.
** Estimated by gel filtration.

chrome *c*-550 was carried out by standard procedures using carrier ampholytes of pH ranges 5–8, 3.5–10 and 2–11 in the ratio 2 : 2 : 1. The isoelectric point determined was 8.51. Isoelectric focusing of horse heart cytochrome *c* performed by the same procedure gave a p*I* value of 10.55.

Oxidation-reduction potential. The midpoint redox potential ($E_{m(7)}$) of cytochrome *c*-550 was measured by titration with ferricyanide/ferrocyanide redox system under aerobic conditions. The $E_{m(7)}$ value obtained was 0.271, similar to that reported previously [3–6].

Amino acid composition. The amino acid analysis of the purified preparations of cytochrome *c*-550

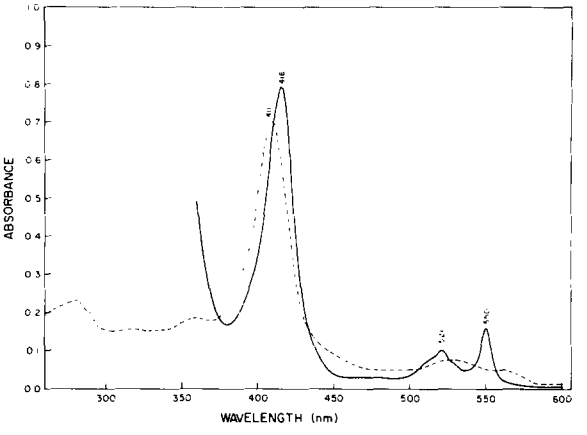


Fig. 1. Absorption spectra of cytochrome *c*-550 in 50 mM potassium phosphate, pH 7.8. The concentration of the cytochrome used was 4.4 μM. Oxidized form (------); reduced (———) with $Na_2S_2O_4$.

(Table I) indicates that *Nitrobacter* cytochrome *c*-550 is more closely related to the so-called 'L' group (long) of *c*-type cytochromes [31]. Although tryptophan determination was not carried out, the high absorption at 280 nm is indicative of high content of this residue in the cytochrome *c*-550.

Amino acid sequence. The N-terminal sequence of *Nitrobacter* cytochrome *c*-550 is shown in Table II, along with the corresponding portions of the sequences of the *c*-type cytochromes from a few selected organisms.

The identification of serines at position 8 and 9 are tentative, due to low recoveries of alanine (45% of expected values) after back hydrolysis to the free amino acids. The free acid forms were assigned at position 2 and 4 from the thin-layer method. Not enough sample was available for the thin-layer method at position 11, and so the position is assigned as Asx. No residue could be identified at positions 13 or 16; by analogy to other *c*-type cytochromes, these are probably the two cysteines to which the heme is attached (Table II). Because so little sample was available, we did not attempt to remove the heme and to derivatize cysteine for easier identification, before running the protein in the Sequencer. The evidence for histidine at position 17, and for the other residues, was unequivocal.

TABLE II

COMPARISON OF N-TERMINAL AMINO ACID SEQUENCES OF FOUR *c*-TYPE CYTOCHROMES WITH THAT OF *NITROBACTER* CYTOCHROME *c*-550

Sequence information for lines 1, 2, 4 and 5, and alignments with proposed deletions or insertions, are from Dayhoff [32]. A, horse heart cytochrome *c*; B, *Paracoccus denitrificans* cytochrome *c*-550; C, *Nitrobacter agilis* cytochrome *c*-550; D, *Pseudomonas denitrificans* cytochrome *c*-551; E, *Monochrysis lutheri* cytochrome c_6.

A			Gly-	Asp-	Val-	Glu-	Lys-	Gly-	Lys-	Lys-	Ile-	Phe-	Val-	Gln-	Lys-		-Cys-	Ala-	Gln-	Cys-	His-	Thr-	Val-
B	Asn-	Glu-	Gly-	Asp-	Ala-	Ala-	Lys-	Gly-	Glu-	Lys-	Glu-	Phe-	Asn-		-Lys-		-Cys-	Lys-	Ala-	Cys-	His-	Met-	Ile-
C			Gly-	Asp-	Val-	Glu-	Ala-	Gly-	Lys-	(Ser)-	(Ser)-	Phe-	Asx-		-Lys-		-x	-Lys	-Ala	-x	-His-	-x	-x-
D						Ser-	Thr-	Gly-	Glu-	Glu-	Leu-	Phe-	Lys-	Ala-	Lys-	Ala-	Cys-	Val-	Ala-	Cys-	His-	Ser-	Val-
E			Gly-	Asp-	Ile-	Ala-	Asn-	Gly-	Glu-	Gln-	Val-	Phe-	Thr-	Gly-	Asn-		-Cys-	Ala-	Ala-	Cys-	His-	Ser-	

The initial coupling in the sequencer run was 36.0% and the repetitive yield was 89.8%, based on the recoveries of the residues at positions 1, 5, 6, 10, and 15.

Properties of cytochrome c-549, 554

Absorption spectra. The oxidized form of cytochrome *c*-549, 554 had an absorption maximum at 402 nm (Fig. 2). The Soret peak is at an unusually low wavelength compared to most *c*-type cytochromes. The reduced form showed absorption peaks at 419, 523, 549 and 554 nm (Fig. 2). The Soret region also has a shoulder at 430 nm. Cytochrome *c*-554 purified from another nitrifying bacterium, *Nitrosomonas europaea* [33] has also been reported to possess a shoulder at 430 nm in the reduced form, but only a single peak at the α region. The pyridine ferrohemochrome prepared from the purified preparations possessed a heme *c* spectrum with absorption peaks at 415, 521 and 551 nm. Therefore, the cytochrome *c*-549, 554 may be regarded as a *c*-type cytochrome. The cytochrome was reduced with dithionite, but not with ascorbate, sodium borohydride, or potassium ferrocyanide. The cytochrome was found to be highly autoxidizable.

Effect of pH. Fig. 3 shows the reduced spectra of cytochrome *c*-549, 554 in buffers of different pH. It is apparent that the reduced peaks were more fully developed at a higher pH. At pH 6.0, there were no apparent reduced α, β, and γ peaks, rather the spectrum was greatly distorted. Ferricytochrome spectra were also affected by pH. In

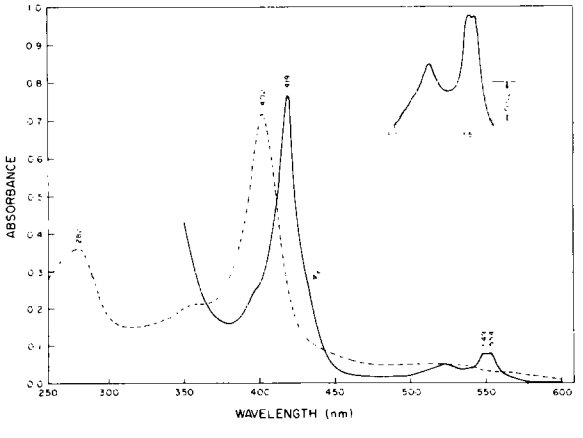


Fig. 2. Absorption spectra of cytochrome *c*-549, 554 in 50 mM potassium phosphate, pH 7.5. The concentration of the cytochrome used was 3.9 μM. Oxidized form (- - - - -); reduced (———) with $Na_2S_2O_4$.

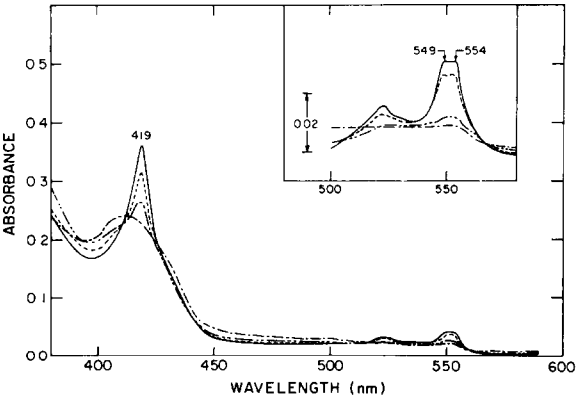


Fig. 3. Absorption spectra of reduced cytochrome *c*-549, 554 at different pH values. The concentration of the cytochrome used was 1.9 μM. The cytochrome was reduced with $Na_2S_2O_4$ and the spectra were recorded when no further reduction was observed. The spectra at pH 7.5 and 10.0 were identical. pH 7.5 (———); pH 7.0 (- - - - -); pH 6.5 (– - –); pH 6.0 (– - –).

0.1 M NaOH, the cytochrome spectra were converted into typical *c*-type cytochrome spectra (not shown) with reduced α, β, and γ peaks at 551, 522 and 417 nm and an oxidized Soret peak at 410 nm. These pH effects were reversible. Absorption spectra of an anomalous class of cytochrome *c* have been reported to be influenced similarly by pH [34,35].

Molecular weight. The purified preparations of cytochrome *c*-549, 554 yielded a molecular weight of 90 000 by gel filtration on Sephadex G-100.

The molecular weight estimated by SDS-gel electrophoresis was 46 000. Thus, the native cytochrome existed probably as a dimer. A similar high molecular weight cytochrome *c*-552, 558 from *Pseudomonas stutzeri*, a denitrifying bacterium, has been reported [36].

Heme content. The pyridine hemochrome prepared from cytochrome *c*-549, 554 indicated that it contained two hemes (*c*-type) per molecule assuming the molecular weight of 46 000 (as estimated by SDS-gel electrophoresis).

Isoelectric point. Purity of the preparations was also judged by a single protein band upon isoelectric focusing of the cytochrome. The isoelectric point determined was pH 5.6, value much lower than the other two cytochromes.

Amino acid composition. Results of the amino acid analysis of the purified cytochrome *c*-549, 554 preparation are shown in Table I.

Discussion

Ever since the discovery of a *c*-type cytochrome in *Nitrobacter* [2], its exact function in the nitrite oxidation has remained a matter of great controversy [8,10,11,13,37]. Although in the controversy only one type of cytochrome *c* has been assumed to be present in *Nitrobacter* a critical examination of the spectrophotometric studies of *Nitrobacter* cytochrome *c* (Table III) shows differences in the absorption maxima [2–6,14,15,38,39], which are quite unlikely if there were only one cytochrome *c* present in *Nitrobacter*. Our investigation has not only succeeded in isolating two typical *c*-type cytochromes (*c*-550 and *c*-553) but also demonstrated a variant type of cytochrome *c* in this organism.

The spectral properties of cytochrome *c*-553 (Table III) are similar to those observed by Van Gool and Laudelout [14] and its isoelectric point (6.80) is close to that reported by Butt and Lees

TABLE III

COMPARISON OF ABSORPTION MAXIMA OF *NITROBACTER* *c*-TYPE CYTOCHROME

ET particles, electron-transport particles.

Refs.	Reduced			Oxidized	$E_{m(7)}$	*p*I	Materials used
	α	β	γ	γ			
2	551	520	–	–	–	–	Cell suspensions
3	550	521	416	–	0.25	6.5	Extracts
38	552	–	–	–	–	–	Cell suspensions
39	550	520	415	–	–	–	Extracts
14	554	523	419	–	–	–	Cell suspensions and extracts
	552.5	523	416	411	–	–	Soluble fractions
4	550	521	417	411	0.282	–	Purified cytochrome
5	–	–	–	–	0.274	–	Extracts
6	–	–	–	–	0.270	–	ET particles
15	–	–	–	–	0.270	–	ET particles
	552.5	–	–	–	−0.110	–	ET particles
This report	550	521	416	411	0.271	8.51	Purified cytochromes
	553	524	419	410	–	6.80	
	549, 554	523	419	402	–	5.60	

[3]. Cytochrome *c*-553 was reduced by ascorbate, ferrocyanide, sodium borohydride and dithionite, but NH_4^+, NH_2OH or NO_2^- had no effect. The observation that the isolated cytochrome *c*-553 (partially reduced) could not be oxidized by ferricyanide indicates that it has a high redox potential.

Cytochrome *c*-550 exhibited absorption spectra with peaks at 411 nm for the oxidized form and at 416, 521 and 550 nm for the reduced form and so it is similar to that purified by Ketchum et al. [4]. The molecular weight of 12 500 of cytochrome *c*-550 was close to that of horse heart cytochrome *c* as judged by gel filtration and polyacrylamide gel electrophoresis. These cytochromes were different, however, in their p*I* values and amino acid compositions (Table I). The isoelectric point of cytochrome *c*-550 was also different from the p*I* value reported earlier for cytochrome *c* of *Nitrobacter* [3]. In this earlier work, isoelectric point determinations were carried out using a crude extract and a red band with a p*I* value of 6.5 was regarded as cytochrome *c*. In view of the present findings, the red band might have been cytochrome *c*-553 purified here, which had a p*I* value 6.8.

The N-terminal sequence of *Nitrobacter* cytochrome *c*-550 shows a clear homology with the corresponding portions of the sequences of other *c*-type cytochromes. This homology is readily seen in Table II. The four other *c*-type cytochromes shown are simply examples, chosen to be representatives of their classes. The N-terminal region of *c*-type cytochromes is strongly conserved, and *Nitrobacter* cytochrome *c*-550 is identical with the four sequences shown at positions 1, 2, 6, 10, 12 and 17 (and presumably 13 and 16, where the heme cysteines are expected to be, by analogy with the other sequences).

The *c*-type cytochromes of non-photosynthetic bacteria may be placed in two evolutionary groups on the basis of sequence data: one which contains *c*-550 and the c_2 cytochromes derived from a number of *Rhodospirillum* and *Rhodopseudomonas* species; and another which contains *c*-551 and a few miscellaneous cytochromes c_2 [32]. The N-terminal sequence of *Nitrobacter* cytochrome *c*-550 resembles the first group more closely than the second, since it has only one residue between the constant residues Phe-10 and Lys-12 (*Nitrobacter* numbering), whereas all other groups of *c*-type cytochrome have two residues; and in having four amino acids between Lys-12 and His-17, where cytochromes *c*-551 have five. The great length of *Nitrobacter* cytochrome chain (approximately 121 amino acids, see Table I) is also consistent with its placement in the first group, since it is only in that group that *c*-type cytochromes as long as 130 amino acids are found [32]. The final assignment into the group, however, will require the complete sequence analysis.

The novel hemoprotein isolated from *Nitrobacter* and designated here as cytochrome *c*-549, 554 had characteristically atypical spectra. It had a high molecular weight not only as a native protein (90 000) but also as a monomer (46 000) obtained by denaturation with SDS. Preliminary studies indicate that cytochrome *c*-549, 554 had a low redox potential. It was highly autoxidizable and appeared to bind carbon monoxide, cyanide and nitrite (results not shown). Its absorption spectra were characteristically affected by the pH of the buffers. Many of these properties were similar to the properties of anomalous *c*-type cytochromes [34–36,40], but a more detailed study is required for its identification.

Although the present investigation extends our knowledge on the electron transport components of *Nitrobacter*, the exact physiological function of the isolated cytochromes remains to be resolved.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada. We thank Wayne D. Buchannon for assistance with the determination of the N-terminal sequence of cytochrome *c*-550.

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