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PURIFICATION AND SOME PROPERTIES OF CYTOCHROME $C_{553(550)}$ ISOLATED FROM DESULFOVIBRIO DESULFURICANS NORWAY

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SUMMARY

A new c-type cytochrome containing a single heme group, cytochrome $c_{553(550)}$ has been purified from Desulfovibrio desulfuricans (Norway strain) and some of its properties have been investigated. It has an isoelectric point of 6.6 and a higher redox potential than cytochrome c_3 isolated from the same bacteria. Its molecular weight was estimated to be 9,200 by gel filtration. The main absorption peaks are at 553, 522.5 and 417 nm in the reduced form and at 690, 529, 411, 357 and 280 nm in the oxidized form. The asymmetric α band of the reduced state is similar to the one reported for so-called "split α" cytochromes c. The cytochrome contains 86 amino acid residues with 5 methionine, two cysteine and two histidine residues. The N terminal sequence of D. desulfuricans Norway cytochrome $c_{553(550)}$ presents no evident homology with that of Desulfovibrio vulgaris Hildenborough cytochrome c_{553}.

INTRODUCTION

Sulfate reducing bacteria have been classified into two genuses, Desulfotomaculum and Desulfovibrio (1). In contrast to Desulfotomaculum, Desulfovibrio species contain cytochrome c_3 and do not sporulate. The bisulfite reductase of Desulfovibrio can be either desulfoviridin (2) or desulforubidin as in the Norway 4 strain of D. desulfuricans (3, 4).

Recently the purification and the physicochemical properties of several electron carrier proteins from the Norway strain have been reported (5). During the first step of purification of cytochrome c_3 another cytochrome with a smaller molecular weight was separated. This cytochrome presented a "split α" band upon reduction with ascorbic acid.

In the present communication we report the purification and some properties of the cytochrome $c_{553(550)}$ isolated from D. desulfuricans (strain Norway 4).

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MATERIALS AND METHODS

Organism and growth conditions : D. desulfuricans (strain Norway 4 NCIB n° 8310) was cultivated in the medium of Starkey (6) and harvested as previously described (7).

Acrylamide gel electrophoresis : Analytical gel electrophoresis was performed according to Davis (8) in 7 per cent polyacrylamide gel with Tris-HCl glycine buffer at pH 8.9.

Determination of molecular weight : The molecular weight of purified cytochrome was determined by gel filtration on a Sephadex G-50 column following the procedure of Whitaker (9).

Determination of isoelectric point : The isoelectric point was determined by isoelectric focusing (10) on a LKB Multiphor apparatus.

Spectrophotometric studies : The visible and ultraviolet absorption spectra were determined with a Cary 14 recording spectrophotometer.

Molar extinction coefficients : The molar extinction coefficients of the cytochrome were obtained by measuring the values of the optical densities of their absorption maxima of a solution of known protein concentration calculated from amino acid analysis.

Amino acid analysis : Amino acid analysis were carried out on a Beckman Multichrom amino acid analyzer. Protein samples were hydrolyzed in 6 M HCl at 110° C for 20 h in evacuated, sealed tubes according to the method of Moore and Stein (11). Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs (12).

Sequence determination : Sequence determination was performed on a Beckman Protein Sequencer. DMBA (N-N-dimethyl-benzylamine) buffer is used instead of quadrol as for other low molecular weight proteins. The concentration of the PTH derivatives was determined by comparison with the known amounts of the appropriate standards on gas chromatography (Beckman gas chromatograph GC45 using SP400 as stationary phase) according to the technique of Pisano et al. (13) An analysis of the silylated PTH derivatives of the amino acids was always performed. PTH derivatives were also analyzed by thin layer chromatography on silica gel containing an ultra-violet fluorescent indicator (Silica Gel GF254, Merck) as described by Edman (14, 15) particularly for the identification of lysine, Glu/Gln and Asp/Asn residues (16). PTH derivatives have been characterized by amino acid analysis after conversion to the parent amino acid by hydrolysis with chlorhydric acid (17) or hydriodic acid (18).

Purification of cytochrome $c_{553(550)}$: All the purification steps were performed at + 4° C and all the buffers were at pH 7.6. To 2.8 Kg wet weight cells about 2 l of 10 mM Tris-HCl buffer were added. After addition of a few desoxyribonuclease crystals, the cell suspension was treated in a French pressure cell. The resulting extract was centrifuged at 35,000 x g for 1 h and the pellet was discarded. The supernatant was stirred overnight with 500 ml of silica gel Baker, and the gel, with the cytochromes adsorbed, was separated by decantation. The silica gel was washed several times with 50 mM Tris-HCl buffer and the cytochromes were eluted from the gel with 1 M K_2HPO_4 containing 1 M NaCl. After dialysis overnight against 10 liters of distilled water the dialyzed solution containing the cytochromes was passed through two DEAE-cellulose (DE-52) column (4.5 x 30 cm) equilibrated with 10 mM Tris-HCl

buffer. All the cytochrome c_3 was absorbed on DE-52. The cytochrome fraction that was not retained was concentrated and filtered through a Sephadex G-50 column (4.5 x 100 cm) equilibrated with 10 mM Tris-HCl buffer. Two cytochromes bands were separated : the first (with the largest molecular weight) contained a cytochrome presenting a "split α" band upon reduction with sodium ascorbate ; the second contained a cytochrome, partly reduced (in very weak amount) and was not purified further.

Cytochrome $c_{553(550)}$ was adsorbed onto an alumina column Merck (2 x 7.5 cm) equilibrated with 10 mM Tris-HCl buffer and eluted by a non-linear gradient of potassium phosphate buffer (10-50 mM). After dialysis against distilled water, cytochrome $c_{553(550)}$ was adsorbed onto a hydroxylapatite (Bio-Gel HTP) column (2 x 5 cm) equilibrated with 10 mM Tris-HCl buffer and eluted with 10 mM-50 mM potassium phosphate buffer. The last step of this purification procedure consists of a second filtration on a Sephadex G-50 column (2.5 x 90 cm) equilibrated with 10 mM Tris-HCl buffer. The purity coefficient of this "split α" cytochrome defined as [A 553 nm (reduced) - A 570 nm (reduced)] / A 280 nm (oxidized) was of 1.18. Finally the cytochrome $c_{553(550)}$ was dialyzed against distilled water and lyophilized giving 9 mg of cytochrome. The purification of cytochrome $c_{553(550)}$ from the Norway strain is summarized in Table I.

RESULTS

Homogeneity : The cytochrome $c_{553(550)}$ was judged to be pure by polyacrylamide gel electrophoresis at pH 8.9. The elution diagram, obtained after second filtration on Sephadex G-50, exhibited a symmetrical peak. The highest purity coefficient found for cytochrome $c_{553(550)}$ is 1.18, and is similar to the value found for mitochondrial cytochrome c.

Molecular weight and isoelectric point : The molecular weight of purified cytochrome $c_{553(550)}$ was estimated to be 9,200 daltons by gel filtration on Sephadex G-50. This value is in good agreement with the minimum molecular weight value of 9,381 calculated for the apoprotein from amino-acid composition. The isoelectric point was determined to be at pH 6.6 by isoelectric focusing.

Spectral properties : The absorption spectra of the oxidized and reduced forms of the purified cytochrome $c_{553(550)}$ are presented on figure 1. The characteristic feature of the spectrum is the asymmetric α band in the reduced state with an absorption maximum at 553 nm and a prominent shoulder at 550 nm. In contrast with cytochromes c_3 and c_7 from *Desulfuromonas acetoxidans* (19) the cytochrome $c_{553(550)}$ spectrum shows in the oxidized form a peak in the 280 nm region and a peak at 690 nm. The millimolar extinction

TABLE I - Purification of cytochrome $c_{553(550)}$ from D. desulfuricans Norway

Purification steps	Amount (mg)	Purity coefficient*	Yield (%)
First Sephadex G-50 filtration	24	0.41	100
Elution from Alumina column	16.8	0.59	70
Elution from Hydroxylapatite column	10.9	1.01	45.4
Second Sephadex G-50 filtration	9	1.18	37.5

*The purity coefficient is defined as (A 553 nm (reduced) - A 570 nm (reduced)) / A 280 nm (oxidized).

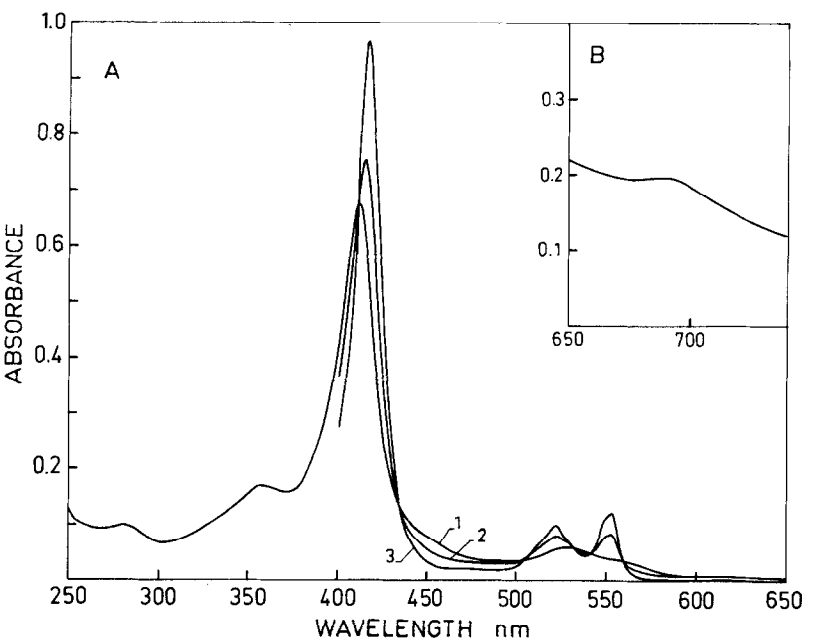

Figure 1 - Absorption spectra of D. desulfuricans Norway cytochrome $c_{553(550)}$. A - 1. The oxidized spectrum of the cytochrome (2.6 μM) in 50 mM Tris HCl, pH 7.6 ; 2. Solution 1 after reduction with sodium L (+) ascorbate ; 3. Solution 1 after reduction with slight excess of sodium dithionite. B - Near IR spectrum of D. desulfuricans Norway ferricytochrome $c_{553(550)}$ (82.9 μM).

coefficients of the purified cytochorme are 1.99 at 690 nm in the oxidized state and 47.3 at 553 nm in the reduced form. The absorbance ratio of A_γ (reduced)/A_α (reduced) equals 7.66 and in contrast with most other c-type cytochromes, the cytochrome $c_{553(550)}$ shows low ratio α/β (= 1.22) in the reduced state.

Redox potential : The cytochrome is partly reduced by sodium L (+) ascorbate and completely by sodium dithionite showing a "split α" band in the two cases. It has a higher redox potential (50 mV $\pm$ 20 mV; J.J.G. Moura and A.V. Xavier, unpublished results) than cytochrome c_3 which is not reduced by ascorbic acid.

Amino acid composition : The amino acid composition of D. desulfuricans strain Norway cytochrome $c_{553(550)}$ is presented in Table II and compared with that obtained for D. vulgaris Hildenborough cytochrome c_{553} (20). Tryptophan was not determined and the serine and threonine values were corrected for partial destruction during hydrolysis. The main characteristics of this cytochrome are a high number of methionine residues (5) and the presence of only two residues of cysteine, just sufficient to link a single heme group to the apoprotein. The cytochrome $c_{553(550)}$ contains two histidine residues while cytochrome c_{553} from D. vulgaris Hildenborough has only one histidine. The ratio Asx + Glx/Lys + Arg is 1.14 and agrees with the value of 6.6 found for the isoelectric point.

N terminal sequence determined by using the automatic sequencer : Before Edman degradation of the cytochrome, the heme was removed according to the method of Ambler (21). The protein (25 mg/ml) was dissolved in 0.1 M HCL-8 M Urea. $HgCl_2$ (50 mg/ml) was added and the mixture was incubated with shaking for 15 h at 37° C. The apoprotein was isolated by gel filtration through Sephadex G-25 equilibrated with 5 % formic acid. The apoprotein is then oxidized according to Hirs (12) and subjected to sequential Edman degradation on the automatic sequencer. The sequence of the first 14 residues is reported on figure 2. No evident homology is observed when comparing the

TABLE II - Amino-acid composition of D. desulfuricans Norway strain cytochrome $c_{553(550)}$ and comparison with cytochrome c_{553} from D. vulgaris Hildenborough

Amino acid	D. desulfuricans Norway cytochrome $c_{553(550)}$	D. vulgaris Hildenborough cytochrome c_{553}[a]
Lysine	10	12
Histidine	2	1
Arginine	4	1
Tryptophan	n.d.[b]	0
Aspartic acid	6	6
Threonine	5	1
Serine	4	6
Glutamic acid	10	6
Proline	2	1
Glycine	9	12
Alanine	10	14
Cystine (half)*	2	2
Valine	3	2
Methionine*	5	6
Isoleucine	3	1
Leucine	7	5
Tyrosine	3	6
Phenylalanine	1	0
Total residues	86	82
Molecular weight	9 381**	8 566**
	9 200***	9 100***

[a] From Bruschi and Le Gall (20)
[b] n.d., not determined.
* Calculated after performic acid oxidation
** Minimum molecular weight calculated from amino acid composition
*** Molecular weight estimated by gel filtration

N terminal sequence of D. desulfuricans Norway cytochrome $c_{553(550)}$ with the D. vulgaris Hildenborough cytochrome c_{553} or with the N terminal of D. desulfuricans Norway cytochrome c_3 (Bruschi, M., unpublished results).

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ser-Gly-Asp-Leu-Gly-Ala-Glu-( )-Tyr-Ala-Lys-Asp-( )-Lys-

Figure 2 - N terminal sequence of D. desulfuricans Norway cytochrome $c_{553(550)}$. Parentheses indicate that the residues in these positions could not be identified.

DISCUSSION

Three c-type cytochromes, namely c_{553}, c_3 (MW 13,000) and c_3 (MW 26,000) have been isolated from sulfate reducing bacteria of the genus Desulfovibrio. Cytochrome c_3 (MW 13,000) was the first c-type cytochrome found in sulfate reducing bacteria in 1954 (22, 23). It is present in all desulfovibriones and contains four hemes per molecule.

Cytochrome c_{553} isolated from D. vulgaris Hildenborough (24) is distinguished from cytochrome c_3 (MW 13,000) by a single heme group, a lower molecular weight (9,100) and a less negative redox potential.

Cytochrome c_3 (MW 26,000) isolated from D. gigas (25) contains eight hemes per molecule and acts as an electron carrier in the electron transport system of thiosulfate reduction (26).

The D. desulfuricans (strain Norway 4) cytochrome $c_{553(550)}$ is the first type of cytochrome isolated from sulfate reducing bacteria presenting a "split α" band at room temperature in the reduced state. "Split α" cytochromes have been found in several photosynthetic bacteria : cytochrome c_{555} from Chlorobium thiosulfatophilum (27), cytochrome $c_{553(550)}$ from Chromatium Strain D (28) and cytochrome c_{555} from Prosthecochloris aestuarii (29) isolated in "Chloropseudomonas ethylica" strain 2 K (30). The origin of this splitting has been attributed to some peculiar arrangement of the iron ligands and such monoheme cytochromes might be categorized as a separate class of bacterial cytochromes (31).

In contrast with cytochromes c_3 (MW 13,000), c_3 (MW 26,000) and $c_{551.5}$ (so-called cytochrome c_7 from Desulfuromonas acetoxidans) the Desulfovibrio cytochromes c_{553} and $c_{553(550)}$ spectra present two peaks at about 280 and

TABLE III - Properties of mono-hemic cytochromes c of sulfate reducing bacteria

	D. desulfuricans Norway cytochrome $c_{553(550)}$	D. vulgaris Hildenborough cytochrome c_{553}
Histidine	2	1
Methionine	5	6
Phenylalanine	1	0
Isoelectric point	6.6	basic (> 8.6)
Millimolar extinction coefficient (at 553 nm in the reduced state)	47.3	26
"Split α" band upon reduction	+	-
Amino terminal residue	Ser	Ala

690 nm in the oxidized form. The presence of an absorption maximum at 695 nm has been attribuated to an interaction of methionine with heme iron (32). Very recently, methionine has been identified as the sixth axial heme iron ligand in Prosthecochloris aestuarii cytochrome c_{555} (33). NMR studies indicate that in D. desulfuricans Norway cytochrome $c_{553(550)}$, methionine is liganded at the sixth coordinate position of the heme iron (J.J.G. Moura and A.V. Xavier, unpublished results).

A comparison of D. vulgaris Hildenborough cytochrome c_{553} and D. desulfuricans Norway cytochrome $c_{553(550)}$ is given in Table III. The physiological functions of these two mono-heme c-type cytochromes are still unknown. D. vulgaris cytochrome c_{553} shows some amino acid sequence homologies with other small cytochromes c such as eukaryotic cytochrome c, Pseudomonas aeruginosa cytochrome c_{551} and Chlorobium thiosulfatophilum cytochrome c_{555}. These cytochromes appear to represent one uniform class of evolutionarily related molecules (34, 35). The determination of the primary structure of D. desulfuricans Norway cytochrome $c_{553(550)}$ and comparison with that of other small

cytochromes c will perhaps allow the possible creation of a new type of monohemic cytochrome c in sulfate reducing bacteria.

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