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PURIFICATION AND CHARACTERIZATION OF CYTOCHROME C_3 (Mr 26,000) ISOLATED FROM *Desulfovibrio desulfuricans* NORWAY STRAIN

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An acidic cytochrome c (P_i = 4.8) has been purified from *Desulfovibrio desulfuricans* Norway. Its molecular weight was estimated to be 26,000 but a monomeric form of 13,500 molecular weight has been obtained. The comparison of its amino acid composition and N terminal sequence has characterized this cytochrome as a new cytochrome, different from cytochrome c_3 (Mr 13,000) and cytochrome $c_{553(550)}$ studied in the same organism. Its optical spectrum was similar to cytochrome c_3 (Mr 13,000) accordingly it has 4 haems per subunit. The absence of absorption at 695 nm indicates that two histidine residues are implicated as fifth and sixth ligand for haem iron. This new cytochrome is homologous to the cytochrome c_3 (Mr 26,000) previously described for *Desulfovibrio gigas* and *Desulfovibrio vulgaris*.

INTRODUCTION

In earlier publications (1,2), we reported the presence of three c-type cytochromes in *D. vulgaris* and *D. desulfuricans* El Agheila Z, namely cytochrome c_{553} cytochrome c_3 and cytochrome cc_3. The name cc_3 first proposed is not acceptable in the nomenclature of cytochromes and has been replaced by cytochrome c_3 (Mr 26,000). Cytochrome c_{553} is distinguishable from cytochrome c_3 in that it has a lower molecular weight (9,100), a single heme group (3,4) and a redox potential of + 10 mV (5). Cytochromes c_3 (Mr 13,000) are very different from eucaryotic c type cytochrome and constitutes a new class of haemoproteins characterized by a very low redox potential (in the region of - 300 mV) and the presence of four haems per molecule (of M.W. 13,000) as compared to mitochondrial cytochrome c with one haem per molecule.

The primary structures of cytochrome c_3 from different species of *Desulfovibrio* have been described (6-10).In addition,the three dimensional structure of the *D. desulfuricans* Norway strain cytochrome c_3 has been elucidated with a 2.5 Å resolution (11).

The sixth axial haem ligand has been demonstrated to be a nitrogen atom of a histidine residue instead of the sulfur atom of a methionine residue as in mitochondrial c type ctyochrome.

Cytochrome c_3 (Mr 26,000) from several *Desulfovibrio* species have been described. They have the required number of cysteins to bind eight haems. EPR studies

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have shown that each of the three c type cytochromes possesses characteristic absorption and g values (12,13,14).

The role of cytochrome c_3 (Mr 13,000) in the electron transport between hydrogenase and thiosulfate reductase in extracts of D. vulgaris has also been reported (15,16,17). In an other species of Desulfovibrio, D. gigas it has been shown by Hatchikian et al. (18) that cytochrome c_3 (Mr 26,000), flavodoxin or ferredoxin, but not cytochrome c_3 (Mr 13,000) stimulated thiosulfate reduction activity of cell extract containing thiosulfate reductase and hydrogenase but devoid of electron carriers (19).

The Norway strain of D. desulfuricans is characterized by an absence of desulfoviridin and flavodoxin (20). A monohaem cytochrome $c_{553(550)}$ has been purified in D. desulfuricans Norway (21) and D. desulfuricans Norway cytochrome c_3 (Mr 13,000) has been extensively studied (22,11,23).

D. desulfuricans Norway cytochrome c_3 (Mr 13,000) acts as a carrier in the coupling of hydrogenase and thiosulfate reductase in extracts of D. gigas and D. desulfuricans (Norway strain) in contrast to D. gigas cytochrome c_3 (Mr 13,000) (22). As the presence of a cytochrome c_3 (Mr 26,000) was not reported in D. desulfuricans Norway strain, it has been postulated that modifications take place in D. desulfuricans Norway, allowing the disappearance of flavodoxin and the exclusion of ferredoxin from the electron transfer chain between hydrogenase and thiosulfate reductase.

In this work, we report the purification of a new cytochrome c in D. desulfuricans Norway, which is homologous to the cytochromes c_3 (Mr 26,000) already described in two others Desulfovibrio species.

MATERIALS AND METHODS

Organism and growth conditions

D. desulfuricans strain Norway 4 (National Collection of Industrial Bacteria N° 8310) was grown in the medium of Starkey (24) and harvested as previously described (25).

Purification of D. desulfuricans Norway acidic cytochrome

All the purification steps were performed at + 4° C and all the buffers were at pH 7.6. The bacterial paste (1.7 kg wet weight) was suspended in 1 liter of 10 mM Tris HCl buffer. After addition of a few desoxyribonuclease crystals, the cell suspension was treated in a French pressure cell. The crude extract was centrifuged at 35,000 g for two hours. The pellet was discarded and the supernatant was stirred with 300 ml of silica gel (Baker) for three hours. The gel, with the cytochromes c_3 and $c_{553(550)}$ adsorbed, was separated by decantation. The unadsorbed proteins were stirred overnight with 600 ml of DEAE-cellulose. The DEAE-cellulose with acidic proteins adsorbed was decanted and washed several times with 10 mM Tris HCl buffer. The acidic proteins were eluted with 1 M Tris HCl buffer in a volume of 500 ml. This extract was dialyzed against 10 liters of distilled water. The acidic proteins were adsorbed on a column (5 x 30 cm) of DEAE-cellulose (DE-52), and eluted with a discontinuous gradient from 10 mM to 500 mM Tris-HCl buffer. A fraction containing desulforubidin, rubredoxin and the acidic cytochrome

was separated with 300 mM Tris HCl buffer in a volume of 190 ml. After dialysis against distilled water this solution was adsorbed on an other DEAE cellulose column (4,5 x 20 cm). The cytochrome was eluted with a fine discontinuous gradient of Tris-HCl buffer in 50 ml of 280 mM Tris-HCl buffer. The protein was then adsorbed on a calcina alumina column (2 x 10 cm) equilibrated with the same buffer. The cytochrome was eluted after a discontinuous gradient with 300 mM phosphate buffer in a final volume of 40 ml. The purity coefficient of the acidic cytochrome was :

$$\left[(A^{red}_{553} - A^{red}_{570})/A^{ox}_{280}\right] = 3.2$$

Acrylamide gel electrophoresis

Analytical gel electrophoresis was performed according to Davis (26) in 7 % polyacrylamide gel with Tris-HCl glycine buffer at pH 8.9.

Determination of molecular weight

The molecular weight was estimated by analytical gel electrophoresis on 10 % polyacrylamide gel at pH 8.8 in presence of sodium dodecyl sulfate (SDS) using the procedure of Weber and Osborn (7). The molecular weight standards were : Pseudomonas cytochrome c_{551} (9,100), horse heart cytochrome c (12,500), D. vulgaris flavodoxin (16,500), soja bean Trypsin inhibitor (20,100) and α chymotrypsin (25,000).

Spectrophotometric studies

Visible and ultraviolet absorption spectra were determined with a Kontron spectrophotometer Uvikon 810.

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

Determination of isoelectric point

The isoelectric point was determined by isoelectric focusing (28) on a LKB Multiphor apparatus.

Amino acid analysis

Amino acid analysis were performed on an LKB 3201 amino acid analyzer. Proteins samples were hydrolyzed in 6 N HCl at 110° C for 18 h, 24 h and 48 h according to the method of Moore and Stein (29). Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs (30).

Sequence determination

Sequence determination were performed in the Beckman protein sequencer. DMBA (N-N dimethyl-benzylamine) buffer was used. The phenylthiohydantoin derivatives were determined by HPLC (high pressure liquid chromatography Waters) as described by Bonicel et al. (31) and by amino acid analyzer after conversion to the parent amino acid by hydrolysis with hydriodic acid (32).

RESULTS

Homogeneity

The acidic cytochrome was judged to be pure by polyacrylamide gel electrophoresis at pH 8.8. The purity coefficient found was 3.2 and was similar to the

value obtained for others cytochrome c_3 (Mr 13,000) from Desulfovibrio.

Molecular weight and isoelectric point

The molecular weight of the purified acidic cytochrome c was estimated to be about 26,000 daltons by analytical gel electrophoresis on 10 % polyacrylamide gel in presence of sodium dodecyl sulfate. After preparation of the apocytochrome by removing the haem by mercuric chloride (33) the molecular weight determined was 13,500. It seems that D. desulfuricans Norway cytochrome c_3 (Mr 26,000) was a dimer of two 13,500 daltons subunits. Both subunits were strongly associated and the presence of denaturing agents such as 8 M urea or 6 M guanidine was not sufficient to dissociate the dimer (34). We have given the name cytochrome c_3 (Mr 26,000) to this acidic cytochrome c by comparison with the homologous cytochromes c_3 (Mr 26,000) already described in Desulfovibrio gigas and Desulfovibrio vulgaris (1). This cytochrome is distinct from the cytochrome c_3 (Mr 13,000) isolated from the same bacteria (D. desulfuricans Norway strain) (22).

The isoelectric point was determined by isoelectric focusing to be at pH 4.8.

Spectral properties

Absorption spectra of cytochrome c_3 (Mr 26,000) is similar to cytochrome c_3 spectra already described. The cytochrome was not reduced by sodium L(+) ascorbate but was completely reduced by sodium dithionite. Than this cytochrome has probably a low reduction potential as do the others cytochrome c_3 (Mr 26,00

Evidence has been presented (12) that the 695 nm band is due to an interaction of a methionine residue with haem iron. In contrast cytochromes c_3 (Mr 13,000) and cytochromes c_3 (Mr 26,000) lacks the 695 nm band and NMR (35) and cristallographic studies (11) indicate the 6th coordinative position is occupied by a histidine residue.

Millimolar extinction coefficients of the purified cytochrome are presented in Table I. The values around twice of the millimolar extinction coefficients of cytochrome c_3 (Mr 13,000) were taken to indicate the presence of 8 haems per molecule of cytochrome c_3 (Mr 26,000).

Comparison of optical spectra of cytochrome c_3 (Mr 26,000) from D. desulfuricans Norway and c_3 (Mr 26,000) from D. gigas shows some homologies in the ratios $A\gamma_{red}/A\gamma_{ox}$ (1.53 and 1.54) and $A\gamma_{red}/A\alpha_{ox}$ (13.8 and 14.4).

Amino acid composition

The amino acid composition of D. desulfuricans Norway cytochrome c_3 (Mr 26,000) is listed (Table I).

A comparison of the amino acid composition of the dimer (Mr 26,000) with cytochrome c_3 (Mr 26,000) of other Desulfovibrio species is presented (Table III) and it can be seen that it possesses the required number of cysteine (16 residues) to bind eight haems in dimer form.

Table I. Comparison of the physical characteristics of Desulfovibrio cytochrome c_3 (Mr 26,000)

Amino acid composition	D. desulfuricans Norway c_3 (Mr 26,000)	D. gigas c_3 (Mr 26,000)[a]	D. vulgaris c_3 (Mr 26,000)[a]
Lysine	16	19	29
Histidine	18	16	16
Arginine	10	11	11-12
Tryptophane	ND	ND	ND
Aspartic acid	22	21	23
Threonine	24	5	10-11
Serine	14	15	8
Glutamic acid	36	23	22
Proline	12	11	16
Glycine	12	20	18
Alanine	10	17	30
Cystine (Half)	16	16	16
Valine	16	11	9
Methionine	6	5	0
Isoleucine	12	8	9
Leucine	6	4	13
Tyrosine	8	4	1
Phenylalanine	12	8	6
Total residues	250	214	239

Spectral data

Molar extinction coefficients (M^{-1} cm^{-1}, at the indicated wavelenghts)

	λnm	
D. desulfuricans Norway cyt. c_3 (Mr 26,000)$_{ox}$	531	87,600
	410	1115,100
	349.5	210,200
D. desulfuricans Norway cyt c_3 (Mr 26,000)$_{red}$	553.5	245,200
	524	128,400
	421	1447,900

[a] Bruschi, M. et al. (1).

The D. desulfuricans Norway cytochrome c_3 (Mr 26,000) has more threonine, glutamic acid and phenylalanine and less glycine and alanine residues than cytochrome c_3 (Mr 26,000) from D. gigas and D. vulgaris. Comparison of amino acid compositions of cytochrome c_3 (Mr 26,000) with cytochrome c_3 (Mr 13,000) and $c_{553(550)}$ from the same organism (Table II) reveals important differencies, in particular in the number of histidine residues. Comparison of amino acid compo-

Table II. Comparison of amino acid composition and N terminal sequence of cytochrome c_3 (Mr 26,000) subunit with cytochrome c_3 (Mr 13,000) and cytochrome $c_{553(550)}$ from D. desulfuricans Norway

Amino acid composition

	Cytochrome c_3 (Mr 26,000) subunit	Cytochrome c_3 (Mr 13,000)$_a$	Cytochrome $c_{553(550)}$$_b$
Lysine	8	17	10
Histidine	9	8	2
Arginine	5	1	4
Tryptophane	ND	0	
Aspartic acid	11	12	6
Threonine	12	11	5
Serine	7	4	4
Glutamic acid	18	8	10
Proline	6	8	2
Glycine	6	9	9
Alanine	5	13	10
Cystine (Half)	8	8	2
Valine	8	6	3
Methionine	3	1	5
Isoleucine	6	3	3
Leucine	3	5	7
Tyrosine	4	1	3
Phenylalanine	6	3	1
Total residues	125	118	86

N terminal sequence

	1	5	10	15
Cyt c_3 (Mr 26,000)	Glu-Thr-Phe-Glu-Ile-Pro-Glu-Ser-( )-Thr-Met-Ser-Asx-Lys-Gln			
Cyt c_3 (a) (Mr 13,000)	Ala-Asp-Ala-Pro-Gly-Asp-Asp-Tyr-Val-Ile-Ser-Ala-Pro-Glu-Gly			
Cyt $c_{553(550)}$ (b)	Ser-Gly-Asp-Leu-Gly-Ala-Glu-( )-Tyr-Ala-Lys-Asp-( )-Lys----			

Brackets indicate that the residues in these positions could not be identified

a) Bruschi, M. (23)
b) Fauque, G. et al. (21)

sitions of the three cytochromes c isolated from D. desulfuricans Norway is consistent with the presence of three different c-type cytochromes in these bacteria.

N-terminal sequence

The N-terminal sequence was determined using the automatic sequencer, 250 nmoles of cytochrome c_3 (Mr 26,000) were degradated. The 15 first residues were identified (Table II)and compared with the N-terminal sequences of cytochrome c_3 (Mr 13,000) and cytochrome $c_{553(550)}$ from the same organism. These N-terminal sequences comparison shows no homologies between the different cytochromes from the same bacteria.

DISCUSSION

A cytochrome c_3 (Mr 26,000) has been characterized in D. desulfuricans Norway. Homologous cytochromes c have been isolated from D. gigas and D. vulgaris (1). Bruschi et al. were unable, by treatment with 8 M urea to obtain dissociation into subunits of these cytochromes. In the current study it was found that the same treatment does not dissociate the protein, on the other hand removal of the haems produced two identical monomers of 13,500 molecular weight. We thus postulate that the cytochrome c_3 (Mr 26,000) from D. gigas and D. vulgaris are dimers of identical subunits of 13,500 molecular weight. Although the monomeric form of cytochrome c_3 (Mr 26,000) has the same molecular weight as the cytochrome c_3 (Mr 13,000) isolated from the same bacteria, the amino acid composition and N terminal sequence confirm that we have characterized a new cytochrome. It has been pointed out by Margoliash (34) that cytochrome c often polymerized during purification of the protein. For cytochrome c_3 (Mr 26,000), only the dimer is purified and no monomeric form was detected.

The absorption spectrum of cytochrome c_3 (Mr 26,000) is similar to cytochrome c_3 (Mr 13,000) spectrum. The 695 nm band, due to the interaction of the sulfur of a methionine residue with the haem iron is not present in either cytochrome. By analogy the sixth ligand of cytochrome c_3 (Mr 26,000) would be a histidine residue as in cytochrome c_3 (Mr 13,000). The number of histidine and cysteine residues is the same in the monomer of the cytochrome c_3 (Mr 26,000) and in the cytochrome c_3 (Mr 13,000) and can be attributed to the presence of four haems.

The oxido reduction potentials of cytochrome c_3 (Mr 26,000) are probably around (- 350 mV) like the values attributed to three of the four haems of cytochrome c_3 (Mr 13,000) (36).

Comparison of the different c-type cytochromes in D. desulfuricans Norway has shown that cytochrome c_3 (Mr 26,000) is different from cytochrome $c_{553(550)}$ and c_3 (Mr 13,000). The existence of these different cytochromes has probably a physiological importance. The physiological electron donor and/or acceptor for cytochrome c_3 (Mr 13,000) is hydrogenase. However, the function of cytochrome $c_{553(550)}$ is still unknown. In D. gigas cytochrome c_3 (Mr 26,000) has been described as being implicated in the reduction of thiosulfate (1) which is an intermediate in the reduction of sulfite by these sulfate reducing bacteria. Hatchikian

(19) has reported the purification of D. gigas thiosulfate reductase and he has studied the electron transport between hydrogenase and thiosulfate reductase. With purified thiosulfate reductase and pure hydrogenase the most efficient restoration of activity was obtained in the presence of both cytochrome c_3 (Mr 13,000) and cytochrome c_3 (Mr 26,000). In D. desulfuricans Norway, cytochrome c_3 (Mr 13,000) is efficient in coupling of hydrogenase and thiosulfate reductase and can replace (cytochrome c_3 Mr 26,000) from D. gigas in testing the thiosulfate reductase activity in D. gigas.

We have tested the activation of electron transport between thiosulfate reductase and hydrogenase from D. desulfuricans Norway with cytochrome c_3 (Mr 26,000) and cytochrome c_3 (Mr 13,000). They present a similar activity but it is to be noted that natural electron carriers produced a weak activity as compared with the system containing methyl viologen (16 % in the best case) (19).

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