Biochimica et Biophysica Acta, 460 (1977) 58–64

BBA 47274

CYTOCHROME *c*-551.5 (c_7) FROM *DESULFUROMONAS ACETOXIDANS*★

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(Received October 4th, 1976)

SUMMARY

Cytochrome *c*-551.5 of the anaerobic sulfur-reducing bacterium *Desulfuromonas acetoxidans* has been purified to homogeneity and characterized. It elicits absorption bands at 551.5, 522.5 and 418 nm in the reduced form; the absorptivity ratio $A_{\alpha\,(red)}/A_{280\,nm\,(ox)}$ equals 3.8 for the pure preparation. The molecular weight was estimated to be 9800 by gel filtration.

Determination of the amino acid composition and analysis of the N-terminal amino acid sequence showed the cytochrome to be identical with the threehaem cytochrome *c*-551.5 (c_7) isolated from the syntrophic mixed culture *Chloropseudomonas ethylica* strain 2K. The occurrence of multihaem cytochromes *c* in bacteria is discussed.

INTRODUCTION

Gray et al. [1] first demonstrated that *Chloropseudomonas ethylica* constituted a syntrophic mixed culture of the phototrophic green sulfur bacterium *Chlorobium* together with a metabolically unidentified chemoorganotrophic bacterium. Recently Pfennig and Biebl [2] described a new genus of anaerobic acetate-oxidizing, sulfur-reducing bacteria, *Desulfuromonas acetoxidans*. These authors also showed that the unknown chemoorganotrophic companions of the *Chloropseudomonas* cultures 2K and N2 belong to this new species. *D. acetoxidans* is a strict anaerobic rod-shaped bacterium, unable to ferment organic substances; it obtains energy for growth by anaerobic sulfur respiration. Acetate or ethanol serve as carbon and energy sources; their oxidation to CO_2 is stoichiometrically linked to the reduction of elemental sulfur to sulfide [2]. Cell suspensions, pink in colour, exhibit absorption spectra of C-type cytochromes, which by a preliminary redox titration experiment were shown to have a midpoint redox potential as low as cytochrome c_3 of *Desulfovibrio*.

C-type cytochromes of the *Chloropseudomonas* culture 2K have long been the subject of study. Cytochrome *c*-551.5 isolated by Olson and Shaw [3] and Shioi et al. [4] was further purified and characterized as a low potential threehaem cytochrome by Meyer et al. [5]. The amino acid sequence analysis carried out by Ambler

★ A preliminary report has been presented at the International Symposium on Electron Transfer Systems in Microorganisms, in Marseille.

[6] also showed the cytochrome to be a threehaem cytochrome *c*. It was the first representative of a new and structually distinct type of cytochrome *c*, and was given the trivial name of cytochrome c_7.

As *Chloropseudomonas* was shown to be a syntrophic mixed culture, it seemed likely that *D. acetoxidans*, with its large quantities of low potential cytochrome *c* might be the actual source of cytochrome "c_7". This work was, therefore, pursued with the intention of purifying and characterizing the cytochrome *c* of *Desulfuromonas*. We will present evidence that the cytochrome *c*-551.5 from *D. acetoxidans* and the cytochrome *c*-551.5 from the *Chloropseudomonas* culture 2K are identical.

METHODS

Growth of organism. D. acetoxidans strain 5071, isolated from the anaerobic green photosynthetic culture designated *C. ethylica* strain 2K (kindly provided by Prof. Takamiya, Fukuoka, Japan), was cultured as recently described by Pfennig and Biebl [2]. The basal salt medium contained 0.05 % ethanol (carbon and energy source) and 0.2 % DL-sodium malate (electron acceptor). Cells were grown in a 200 l fermenter (Chemap) starting with an inoculum of 40 l of fresh culture. The maximal absorbance of $A = 0.37$–0.4 (436 nm) was reached after 18–20 h of growth. The cell yield could be enhanced by a second addition of ethanol and malate when these two substrates were exhausted in the medium. Cells were harvested by centrifugation and stored at −20 °C until use.

Spectrophotometry. Cytochrome spectra were recorded at room temperature on a Beckman split-beam photometer (model 25) using cuvettes of 1-cm light path. Dilutions of the samples were made in 100 mM Tris (pH 7.6); the reduced spectra were obtained by adding a few grains of solid dithionite.

Electrophoresis. Analytical disk gel electrophoresis was performed according to Davis [7] at 3 mA/tube for 2 h, using gels containing 7 % (w/v) final concentration of polyacrylamide (pH 8.8).

Molecular weight determinations. Cytochrome: The molecular weight of the purified cytochrome was determined by gel filtration through Sephadex G-50 (1 × 100 cm) equilibrated with 20 mM phosphate buffer (pH 7.6) and 1 M NaCl according to the method of Whitaker [8].

Apoprotein: The haems were separated from the apoprotein by the method of M. Morrison (quoted in ref. 9). 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 agitation for 15 h at 37 °C. The apoprotein was isolated by filtration through Sephadex G-25 gel, equilibrated with 5 % formic acid. The molecular weight was determined by gel filtration through Sephadex G-25 (1 × 150 cm) equilibrated with 5 % formic acid. Standard proteins used for calibration: cytochrome *c*-553 from *Desulfovibrio vulgaris* ($M_r = 9\,100$); cytochrome *c* (horse heart) from Sigma ($M_r = 12\,500$); soya bean trypsin inhibitor from Sigma ($M_r = 20\,100$).

Protein determination. Protein was determined by the method of Lowry et al. [10].

Determination of amino acid composition. The hydrolysis of the cytochrome was carried out in 100 µl of 6 M HCl at 110 °C for 18 h. After hydrolysis the acid was evaporated under vacuum. The amino acid composition was determined with an

amino acid analyzer Beckman (model Multichrom). Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively [11].

Determination of the N-terminal sequence. Sequence analysis by automated Edman degradation was performed with a Socosi Sequencer (P.S. 100). Sequencer chemicals were further purified. Dimethyl benzylamine appeared to be suitable for low molecular weight proteins. The phenylthiohydantoin derivatives were identified by thin-layer chromatography on fluorescent silica gel and by Beckman gas chromatography GC 45 using D.C. 560 stationary phase according to the techniques of Pisano et al. [12].

Quantitative determination of the phenylthiohydantoin derivatives was done relative to known amounts of the appropriate standards. To each step of the degradation the phenylthiohydantoin amino acids were identified as unsilylated phenylthiohydantoin amino acids and silylated phenylthiohydantoin amino acids and also by thin-layer chromatography as described by Edman [13–15].

RESULTS

Isolation and purification of cytochrome c-551.5

180 g cells (wet weight), the cell yield of 500 l culture medium, were suspended in 100 ml 0.5 M Tris · HCl (pH 7.6), stirred for 10 min and centrifuged at 25 000 × *g* for 10 min to separate the cell pellet from the wash liquid. This procedure was repeated and the combined wash liquids centrifuged at 120 000 × *g* for 60 min. The supernatant, referred to as wash supernatant was dialysed against 10 mM Tris · HCl (pH 7.6) overnight. The extracted cells were resuspended in 0.5 M Tris (pH 7.6), DNAase was added and the mixture passed once through a French pressure cell at 1500 kP/cm^2. The disrupted cell suspension was fractionated into particulate and supernatant fractions by centrifugation at 120 000 × *g* for 2 h and the supernatant, referred to as cytoplasmic fraction, was dialysed against 10 mM Tris (pH 7.6).

Wash supernatant. 250 ml were fractionated on a DEAE-cellulose column (3 × 14 cm) equilibrated with 10 mM Tris · HCl (pH 7.6). The column was washed with the same buffer to remove unabsorbed proteins and cytochrome *c*-551.5 was eluted with 0.1 M Tris · HCl (pH 7.6). For this cytochrome fraction, an absorption ratio of $A_{\alpha\,(red)}/A_{280\,nm\,(ox)} = 1.1$ was obtained ($A_{280\,nm\,(ox)}/A_{\gamma\,(red)} = 0.13$), which indicates a very high degree of purification after this first chromatographic step. The cytochrome was dialyzed against 10 mM Tris · HCl (pH 7.6) and the desalted solution then chromatographed on a DEAE-Sephadex A-50 column (5 × 31 cm) equilibrated with 0.1 M Tris · HCl (pH 7.6). The column was washed with 150 ml of the equilibration buffer and the cytochrome was eluted with 0.15 M Tris · HCl (pH 7.6). The eluate was absorbed on an aluminium oxide column (Laboratoire du Bois de Boulogne, Paris; 2.5 × 19.5 cm) equilibrated with 10 mM Tris · HCl (pH 7.6). The column was washed with 0.2 and 0.4 M potassium phosphate buffer (pH 7.0) to remove contaminating protein, and the tightly bound cytochrome was eluted with 0.7 M phosphate buffer and dialyzed against 10 mM Tris · HCl (pH 7.6).

Cytoplasmic fraction. More cytochrome was obtained from the cytoplasmic fraction. The purification was carried out using essentially the same chromatographic steps as described for the wash supernatant. The following differences need to be

mentioned: The cytoplasma once freed from most cytochrome *c*-551.5 by chromatography on DEAE-cellulose (4.5 × 22 cm), was desalted and charged once more on the DEAE column. An additional fraction of the cytochrome could be eluted with 0.1 M Tris · HCl (pH 7.6). The combined cytochrome fractions were dialyzed against 10 mM Tris, concentrated on DEAE and purified on DEAE-Sephadex A-50 and two successive aluminium oxide column as described above. This cytochrome was identical in all properties to the cytochrome obtained from the wash supernatant. The purified cytochrome was homogeneous in electrophoresis. The $A_{\alpha\,(red)}/A_{280\,(ox)}$ ratio was constant throughout a zone eluted from the column at the last step of purification. For the purified preparation an absorptivity ratio $A_{\alpha\,(red)}/A_{280\,nm\,(ox)}$ of 3.8 was obtained. The cytochrome tends to precipitate slowly when stored frozen in 10 mM Tris buffer. This can be prevented by using 100 mM Tris; the precipitate can be easily redissolved in 1 M Tris.

Spectral properties

The absorption spectra of the oxidized and reduced form of the purified cytochrome are shown in Fig. 1. The characteristic feature of the spectra is the very low absorbance at 280 nm, attributed to aromatic amino acid residues in proteins, and the high $A_{\gamma\,(red)}/A_{\gamma\,(ox)}$ ratio of 1.60. The absorbance ratio of $A_{\gamma\,(red)}/A_{\alpha\,(red)}$ equals 6.42.

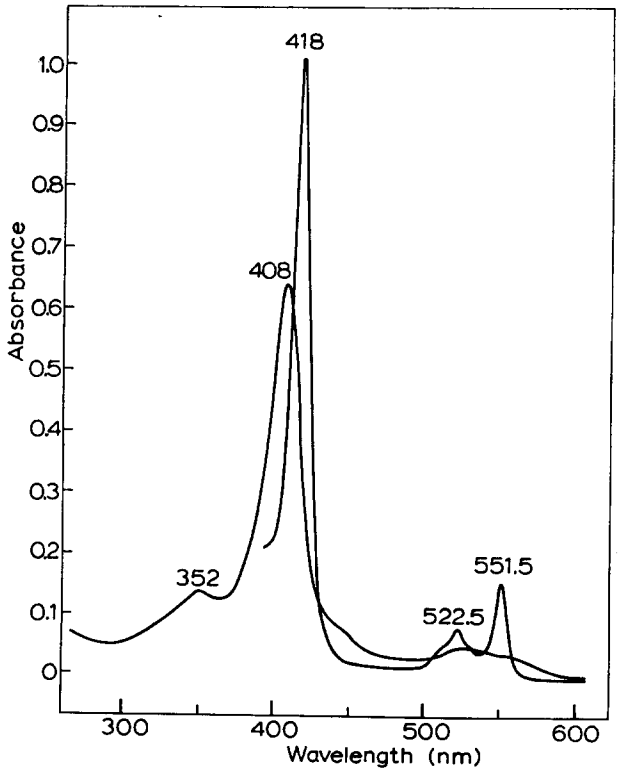

Fig. 1. Absorption spectra of *D. acetoxidans* cytochrome *c*-551.5 (1.7 μM) in 0.1 M Tris–HCl buffer (pH 7.6). The baseline was adjusted to $A = 0$.

TABLE I

AMINO ACID COMPOSITION OF CYTOCHROME *c*-551.5

Amino acid	Number of residues	
	This study	From Ambler [6]
Lysine	12	12
Histidine	6	6
Arginine	0	0
Tryptophan	n.d.	0
Aspartic acid	9	9
Threonine	5	5
Serine	2.3	2
Glutamic acid	3	3
Proline	2	2
Glycine	6	6
Alanine	8	8
Cysteine*	5–6	6
Valine	3	3
Methionine*	0	0
Isoleucine	3	3
Leucine	1	1
Tyrosine	1	1
Phenylalanine	1	1

n.d., not determined.

* Analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively.

Both values are in good agreement with those reported previously [4, 5] for cytochrome *c*-551.5 from *Chloropseudomonas*. The cytochrome can be reduced by excess quantities of sodium L-(+) ascorbate (100 mM); reduction occurs very slowly under anaerobic conditions within 2 h. CO has no effect on the spectrum of the reduced cytochrome.

Molecular weight

By the gel filtration method the molecular weights of the cytochrome and the apoprotein were estimated to be 9800 and 9600, respectively. These values are in good agreement with the molecular weight value of 8473, calculated for the apoprotein on the basis of the sequence analysis data given by Ambler [6].

Amino acid composition and N-terminal amino acid sequence

The amino acid composition is given in Table I. The serine and threonine values were corrected for partial destruction during hydrolysis; tryptophan was not

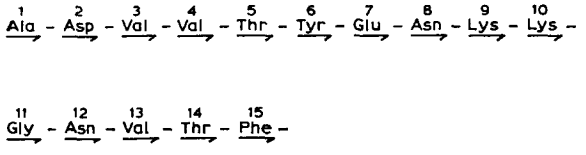

Fig. 2. Amino acid sequence of the N-terminus of cytochrome *c*-551.5.

determined. The values are identical to those reported by Meyer et al. [5] and to sequence analysis data given by Ambler [6] for cytochrome *c*-551.5 from *Chloropseudomonas*. Finally, to further support the hypothesis that the two cytochromes are identical, an amino acid sequence analysis of the N-terminus was undertaken. After removal of the haem, 15 steps of the degradation of the protein were sequenced (Fig. 2.) The sequence is identical to that determined by Ambler [6].

Based upon these results we feel certain that cytochrome *c*-551.5 isolated from the syntrophic mixed culture *Chloropseudomonas* 2K actually is an electron carrier of the sulfur-oxidizing companion *D. acetoxidans*.

DISCUSSION

Low potential multihaem cytochromes c_3 have first been found in the sulfate-reducing bacteria of the genus *Desulfovibrio* [16, 17]. The isolation of low potential cytochromes *c*, similar in absorption spectra to cytochrome c_3, from photosynthetic bacteria, pointed out that this special type of cytochrome *c* might not be a unique feature of sulfate-reducing bacteria. At present, low potential cytochromes *c* as major soluble cytochromes have been found only in the aerobic photosynthetic blue-green bacterium *Anacystis nidulans* and in the anaerobic photosynthetic *C. ethylica* culture. The cytochrome occurs at only low concentrations in but two of six photosynthetic bacteria examined [5]. Cytochrome c_3 of *Desulfovibrio* species has been implicated in various low redox potential electron transport systems and functions in the dissimilatory sulfate reduction [18–21]. In contrast, the function of the c_3-analogues in photosynthetic bacteria is not clear. Sybesma [22], however, observed the slow light-induced oxidation and rapid dark reduction of a cytochrome *c*-551 in intact cells of the *Chloropseudomonas* culture and tentatively placed the cytochrome in a cyclic electron transport pathway.

Cytochrome *c*-551.5 of the *Chloropseudomonas* culture 2K has been isolated and described by two different research groups. The two preparations had identical spectral properties (absorptivity values); molecular weight and redox potential measurements were in good agreement. However, Meyer et al. [5] described the cytochrome as a threehaem protein with an isoelectric point of 4.1, whereas Shioi et al. [4] found an isoelectric point of 7.77 and described the cytochrome to contain two haems. A sequence analysis has only been carried out with the cytochrome preparation of Meyer et al.. [5]. *D. acetoxidans* used in this study, was isolated from the strain 2K, which has been studied by Shioi et al. [4]; the green photosynthetic companion in this 2K culture was identified as *Prosthecochloris aestuarii* [2]; the green sulfur bacterium present in the *Chloropseudomonas* culture of Dr. Olson that was used by Meyer et al. [5], has also been identified as *P. aestuarii*. Our results demonstrate the identity of cytochrome *c*-551.5 from *D. acetoxidans* with cytochrome *c*-551.5 isolated from the *Chloropseudomonas* strain 2K by Meyer et al. [5]. The presence of low potential *C*-type cytochrome(s) with the reduced absorption band at 551.5 nm in the *Desulfuromonas* strain 11070 [2] suggests, however, that cytochromes similar to cytochrome *c*-551.5 (c_7) might generally occur in desulfuromonads, as strain 11070 was isolated from black mud of the antarctic ocean and not from a *Chloropseudomonas* culture.

A clear distinction between low potential and multihaem cytochromes *c* should

be made. We propose that the term c_3 should be restricted to low potential multihaem cytochromes *c* with two histidines as iron ligands. Until now, cytochromes *c* which satisfy this demand, have only been found in chemoorganotrophic sulfate- or sulfur-reducing bacteria; this, however, does not mean that these cytochromes are unique for this type of metabolism. Anyhow, the possibility that multihaem cytochromes *c* are obligate electron carriers in the reduction of sulfur compounds (dissimilatory type) is a challenging hypothesis.

Furthermore, it has been known for a long time that cytochrome c_3 from *D. vulgaris* can reduce S° to H_2S [23] although this strain will not grow in the presence of colloidal sulfur as terminal electron acceptor. It is then tempting to postulate that the important structural variation between cytochrome c_3 and cytochrome $c_{551.5}$ allowes the latter to function as a terminal oxidase in *D. acetoxidans*.

ACKNOWLEDGEMENTS

We thank Dr. Monique Scandellari and M. Raymond Bourrelli for cultivation of 500 l *Desulfuromonas*. The technical assistance of Mme Nicole Forget for the purification of the protein and also of Mme Gisèle Bovier-Lapierre, M. Jacques Bonicel and Mme Paule Couchoud for sequence analysis is gratefully acknowledged.

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