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THE CYTOCHROMES OF *CHLOROBIUM THIOSULFATOPHILUM*

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SUMMARY

Three *c*-type cytochromes (*c*-551, *c*-553, *c*-555) have been isolated and characterized from a strain of the green photosynthetic bacterium *Chlorobium thiosulfatophilum*. These cytochromes are atypical when compared to horse heart cytochrome *c* in many properties, among them: oxidation–reduction potential at pH 7.0 (*c*-551, 135 mV; *c*-553, 98 mV; *c*-555, 145 mV), molecular weight (*c*-551, 45000–60000; *c*-553, 50000; *c*-555, 10000) and isolelectric point (*c*-551, 6.0; *c*-553, 6.7). No protoheme was detected in whole cells or cell-free extracts.

INTRODUCTION

Spectrophotometric techniques for determining the presence and function of cytochromes in the photosynthetic bacteria must be supplemented by isolation and characterization of the components to better understand their role in bacterial photometabolism. The emphasis, to date, has been on the soluble cytochrome components of the purple photosynthetic bacteria. Most of the facultative heterotrophic non-sulfur purple bacteria contain two readily soluble cytochromes of the c_2 and *cc'* types[1]. The obligately anaerobic purple sulfur bacteria, as represented by *Chromatium*, contain a complex *c*-type cytochrome with bound flavin, a *cc'* type, and a particle-bound cytochrome *c*-555, but no *b*-type cytochromes[1,2].

The green sulfur bacteria, as represented by Chlorobium have been reported by GIBSON[3] to contain two soluble *c*-type cytochromes but these have not been studied sufficiently to allow valid comparisons with other bacterial cytochromes.

This report describes an improved method of extraction and purification of the cytochromes of *Chlorobium thiosulfatophilum*, the further physical characterization of the known cytochromes, and the properties of a new cytochrome, *c*-551.

METHODS

Growth of cells

Chlorobium thiosulfatophilum (strain NCIB 8346, alternatively designated strain PM, a chlorophyll "660" strain), kindly supplied by Dr. JUNE LASCELLES, was grown on LARSEN'S[4] medium in magnetically stirred, 20-l carboys, which were illuminated with four 40-W showcase lamps (GE 40T8) per carboy. The medium was

filtered through a Millipore bacteriological filter into sterilized carboys and a 10% inoculum of bacteria added. The carboys were completely filled with sterile medium and closed with rubber stoppers which carried water-cooled stainless steel coils. The flow of cooling water was controlled by a thermostat inserted in one of the battery of carboys to maintain the culture temperature at 30° ± 3°. After 4–5 days of growth, the cells were harvested in a Sharples continuous centrifuge, and stored at −20°. Yields of about 0.5 g wet wt./l of growth medium were normally obtained. When the cells were grown with added sodium acetate (0.4 g/l), the yield more than doubled[5].

Isolation of cytochromes

100–300 g of cell paste were thawed and evenly suspended in 500 ml 0.1 M potassium phosphate buffer at pH 7.5 containing approx. 1 mg deoxyribonuclease (Worthington) and antifoam agent (Dow Corning Anti-foam AF Emulsion). The cells were broken with the aid of a Sorvall Ribi Cell Fractionator operated at 20000 lb/inch^2 and 20°. The resulting suspension was centrifuged at 30000 × *g* for 10 min; the green supernatant fraction was then centrifuged in a Spinco model L centrifuge at 100000 × *g* for 150 min. Ferredoxin and nucleic acid material were removed by passing the resulting red supernatant fraction through a DEAE-cellulose column (Brown Co., DEAE-Selectacel Standard), equilibrated with 0.1 M phosphate buffer at pH 7.5. The column was rinsed with the same buffer to remove non-adsorbed proteins. The eluate was desalted with the aid of a Sephadex G-25 column and then the buffer was changed to 0.02 M Tris at pH 7.8. The eluate was then charged onto a DEAE-cellulose (Brown Co., DEAE-Selectacel Type 20) column equilibrated with 0.02 M Tris–HCl at pH 7.8. Cytochromes *c*-553 and *c*-551 were eluted with NaCl at 0.02 M and 0.04 M, respectively, in the Tris buffer at 4°. The unadsorbed effluent from the DEAE-cellulose column was charged onto CM-cellulose (Brown Co., CM-Selectacel Standard), equilibrated with 0.02 M Tris buffer (pH 7.8), and cytochrome *c*-555 was eluted with 0.02 M NaCl in the Tris buffer at 4°. The isolated cytochromes were concentrated and re-chromatographed until the ratios of the Soret to protein (approx. 280 mμ) absorption peaks were constant.

Molecular weight

Approximate molecular weights were determined, based on single measurements and using the assumed $\bar{v} = 0.73$ in analogy with horse heart cytochrome *c*, for which the measured $\bar{v}$ is 0.728 (ref. 6).

A procedure based on the method of VAN HOLDE AND BALDWIN[7] for rapid sedimentation equilibrium was used for determining the molecular weight of cytochrome *c*-553 (ref. 8). The protein was dissolved (0.5%, w/v) in 50 mM of potassium phosphate (pH 7.0) and 100 mM NaCl for this measurement. The EHRENBERG[6] approach to sedimentation equilibrium was employed for cytochrome *c*-551, dissolved in 0.01 M potassium phosphate (pH 7.0). An estimation of the molecular weights of all three cytochromes was also made according to the method of MORRIS[9] and ANDREWS[10] using thin-layer plates of Sephadex G-200-SF, with bovine serum albumin and horse heart cytochrome *c* as markers and a moving phase consisting of 0.10 M NaCl in 0.01 M Tris–HCl (pH 7.8).

Amino acid analyses

Multiple amino acid analyses were carried out on purified cytochrome *c*-555 and a tentative composition was ascertained. About 1 mg of the cytochrome was hydrolyzed in 2 ml of 5.7 M HCl under anaerobic conditions at 102–106°. Tryptophan was determined after enzymatic hydrolysis, according the method of DUS *et al.*[11]. Cysteine was determined as cysteic acid after performic acid oxidation of the protein[12].

Heme content

Heme content and absorptivity values for each of the cytochromes were determined by using the value of 31.10 $mM^{-1} \cdot cm^{-1}$ as the absorptivity for the α peak of the reduced pyridine hemochrome of bovine heart cytochrome *c* (T. FLATMARK, personal communication), and using the protein concentration obtained from quantitative amino acid analyses. The concentration of cytochromes *c*-553 and *c*-551 was determined on the basis of a single amino acid analysis, using molecular weight values of 50000 and 45000, respectively. Pyridine hemochrome spectra were measured in 0.1 M NaOH, 25%, v/v, pyridine on a Cary 14R recording spectrophotometer[1].

Redox potentials

Oxidation–reduction potentials were measured according to the method of VELICK AND STRITTMATTER[13] using the iron–EDTA redox couple[14]. A solution of 20 μM cytochrome, 100 mM potassium phosphate or 100 mM Tris–HCl, 10 mM EDTA, and 1.0 mM ferric ammonium sulfate, was titrated anaerobically with a 100 mM ferrous ammonium sulfate solution at 20°.

Isoelectric point

The method of SVENSSON[15] for determining isoelectric points was employed at 0°, using a 1%, v/v, ampholyte solution (LKB-Produkter AB, Stockholm) and about 2 mg of each cytochrome[15,16].

RESULTS

Three major cytochromes were found in the soluble fraction of *C. thiosulfatophilum*. Pyridine hemochrome spectra of whole cells and of the particulate fraction after removal of chlorophyll with acetone–methanol (7:2, v/v), showed the presence of mesoheme only. Evidently *b*-type cytochrome does not occur in Chlorobium.

Absorptivity values for each of the cytochromes based on heme content are given in Table I.

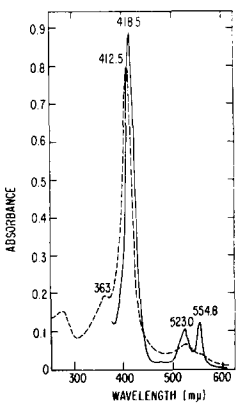
Cytochrome *c*-555 appears to be identical to the cytochrome *c*-554 found by GIBSON[3]. Its absorption spectrum is shown in Fig. 1. Chromatography on Sephadex G-200-SF, using horse heart cytochrome *c* as a marker, indicated a molecular weight near 12000 for cytochrome *c*-555. The cytochrome is very basic, the oxidized form having an isoelectric point of about 10.5 at 25°. The redox potential was found to decrease from 145 mV at pH 6.0 to 114 mV at pH 8.0. In contrast with most other *c*-type cytochromes, the absorption spectrum of cytochrome *c*-555 shows low α/β and α/γ ratios and the α peak is markedly assymmetric. At liquid-nitrogen temperature, in 50% glycerol, the α peak is split by 5 mμ (Fig. 2) with peaks at 548 and 553 mμ.

The protein contains 1 heme group and has the tentative amino acid composition

TABLE I

ABSORPTIVITY VALUES FOR *C. thiosulfatophilum* CYTOCHROMES *c*, BASED ON PYRIDINE HEMOCHROME SPECTRA

	c-555		*c*-553		*c*-551	
	λ_{max} ($m\mu$)	ε_{mM}/heme	λ_{max} ($m\mu$)	ε_{mM}/heme	λ_{max} ($m\mu$)	ε_{mM}/heme
Oxidized	412.5	166	410.0	129	410.5	85
	523	13.5	525	12	528	7.9
Reduced	418.5	184	416.7	156	416.0	114
	523.0	21.8	523.5	17.5	521.0	10.5
	554.8	26.2	553.5	30	551.0	17.6



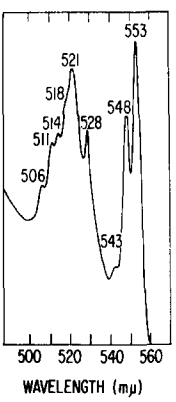


Fig. 1. Absorption spectra of *C. thiosulfatophilum* cytochrome *c*-555, oxidized (– – –) and dithionite reduced (———). The cytochrome concentration is 4.8 μM in 50 mM potassium phosphate buffer at pH 7.0.

Fig. 2. Absorption spectrum of *C. thiosulfatophilum* cytochrome *c*-555 at liquid-nitrogen temperature. The cytochrome concentration is 0.01 mM in 50%, v/v, glycerol, 10 mM potassium phosphate (pH 7.0). The spectrum was measured in a single-beam spectrophotometer with 0.5 $m\mu$ half-bandwidth which could be corrected for phototube response and lamp output.

given in Table II. This cytochrome differs from many other *c*-type cytochromes in having 8 methionine residues and no arginine or phenylalanine. From the amino acid composition and heme content, a formula weight of 9970 may be calculated.

Cytochrome *c*-553 was found to be a comparatively large cytochrome; chromatography on Sephadex G-200-SF indicated a molecular weight of approx. 50000, using bovine serum albumin as a marker. The modified method of VAN HOLDE AND BALDWIN[7] likewise yielded 50000 $\pm$ 2000, with no inhomogeneity apparent. The oxidized form of the protein showed a slightly acidic isoelectric point of 6.7 at 25°.

The cytochrome contains 1 heme and at least 1 flavin, as is apparent from examination of the spectrum in the 480-$m\mu$ region (Figs. 3 and 4). The flavin could not be removed by the usual acid ammonium sulfate or trichloroacetic acid treatments. The flavin was separated from the heme protein by saturating a desalted solution of cytochrome *c*-553 with urea, followed by chromatography on Sephadex G-25-F. The flavin

TABLE II

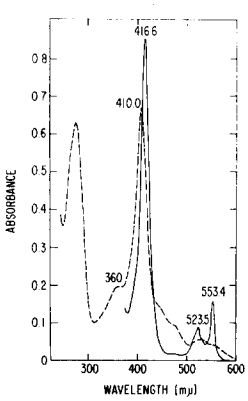
AMINO ACID COMPOSITION OF *C. thiosulfatophilum* CYTOCHROME *c*-555

Amino acids	*Number of amino acid residues relative to two histidines*			
	48-h hydrolysis	*72-h hydrolysis*	*Average****	*Integral number of residues*
Asp	8.88	8.54	8.71	9
Thr	4.05	3.90	4.40	4
Ser	2.80	2.60	3.10	3
Glu	2.35	2.35	2.35	2
Pro	4.90	4.50	4.70	5
Gly	12.9	13.1	13.0	13
Ala	17.4	17.0	17.2	17
Cys*			1.30	2
Val	6.60	6.71	6.65	7
Met	7.93	7.63	7.78	8
Ile	2.04	2.10	2.07	2
Leu	1.16	1.18	1.17	1
Tyr	4.05	4.08	4.06	4
Phe				0
His	2.00	2.00	2.00	2
Lys	11.5	12.0	11.7	12
Arg				0
Trp**			1.0	1
Total number of residues				92

* Cysteine was determined as cysteic acid after performic acid oxidation.

** Tryptophan was determined by enzymatic digestion followed by quantitative amino acid analysis.

*** Serine and threonine extrapolated to zero time.



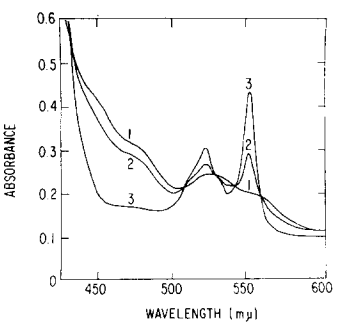


Fig. 3. Absorption spectra of *C. thiosulfatophilum* cytochrome *c*-553, oxidized (- - - -) and dithionite reduced (———). The cytochrome concentration is 5.2 μM in 50 mM potassium phosphate buffer at pH 7.0.

Fig. 4. Absorption spectra of *C. thiosulfatophilum* cytochrome *c*-553 measured during an anaerobic oxidation–reduction titration; (1) 15 μM oxidized cytochrome in 100 mM potassium phosphate (pH 6.0), 10 mM EDTA, and 1.0 mM ferric ammonium sulfate; (2) partially reduced cytochrome after adding 0.52 M ferrous ammonium sulfate to make ratio of $Fe^{2+}/Fe^{3+} = 1.04$ ($E_h = 110$ mV); (3) cytochrome completely reduced with an excess of dithionite.

fraction migrated on the column more slowly than the bulk of the heme protein and contained considerable amounts of denatured protein or peptide material, as well as small amounts of heme. Efforts to characterize the flavin as FAD or FMN by standard chromatographic procedures[17] were unsuccessful, although in one experiment FMN and riboflavin were identified in a chromatogram of the flavin isolated after bacterial protease (Calbiochem, Los Angeles, Calif.) digestion of the Sephadex-retarded material. The flavin has an abnormal absorption spectrum with peaks at 345 and 450 mμ, which are bleached on addition of sodium dithionite. The flavin may be bound to a protein subunit, or alternatively the flavin liberated by treatment with urea may have been trapped in a matrix of denatured protein. These possibilities are being investigated. The binding of flavin in *Chromatium* cytochrome *c*-552 is similar in some respects[18] as is the binding of flavin in Clostridial flavodoxin[19]. The oxidation–reduction potential of cytochrome *c*-553 was constant at 98 mV in the pH range 6–8. The potential of the flavin component of cytochrome *c*-553 was much lower than that of the heme, as indicated by the small extent of bleaching of the absorption band at 480 mμ when the heme appeared to be nearly one-half reduced (Fig. 4), but the value was not measured. The cytochrome *c*-553 isolated by GIBSON[3] was probably an altered form of the cytochrome reported here; the butanol purification step could have removed the flavin.

Cytochrome *c*-551 is also a large molecule, having 2 hemes per molecular weight of 45 000, as determined by the EHRENBERG[6] method. The Sephadex G-200-SF method gave a value of 60 000, using bovine serum albumin as a marker. The discrepancy between the two numbers may be due to departures from ideality in the shape of the cytochrome molecule. The isoelectric point was 6.0 for the oxidized protein at 25°. The oxidation–reduction potential was constant at 135 mV at pH 6–8. The absorption spectrum was normal for a *c*-type cytochrome, as shown in Fig. 5.

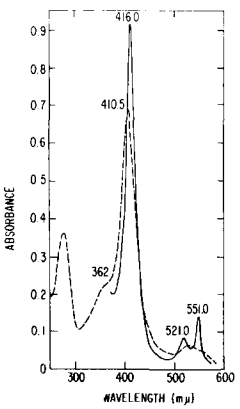


Fig. 5. Absorption spectra *C. thiosulfatophilum* cytochrome *c*-551, oxidized (– – – –) and dithionite reduced (————). The cytochrome concentration is 4.0 μM in 50 mM potassium phosphate buffer at pH 7.0.

DISCUSSION

The three cytochromes of *C. thiosulfatophilum* described in this paper show distinct differences from mammalian cytochrome *c*. However, the Chlorobium cyto-

chromes may be functional counterparts of the cytochromes of other photosynthetic bacteria, as indicated in the following comparisons: cytochrome *c*-555 has an absorption spectrum similar to cytochromes *c*-555 of *C. thiosulfatophilum* (strain NCIB 8327, also designated strain L, a chlorophyll "650" strain) and *Chloropseudomonas ethylicum* (J. OLSON AND E. STANTON, personal communication). The only soluble cytochrome of the purple photosynthetic bacteria which resembles cytochrome *c*-555 is *Rhodopseudomonas palustris* *c*-556 (T. HORIO AND R. G. BARTSCH, unpublished observations), for which the α/β and α/γ ratios are similar. At liquid-nitrogen temperature (Fig. 2), the Chlorobium cytochrome shows a 5-mμ splitting of the α absorption peak, whereas the *R. palustris* *c*-556 shows a splitting of 2.5 mμ. Chlorobium cytochrome *c*-555 is also spectroscopically similar to some algal cytochromes *f* (ref. 20).

The cytochromes *c*-555 of the photosynthetic bacteria may have similar functions. SYBESMA[21] has shown that cytochrome *c*-555 of *C. ethylicum* undergoes fast light-driven oxidation, is close to the active center chlorophyll, and may be part of a cyclic electron transport pathway[21]. The particle-bound cytochrome *c*-555 of *Chromatium* has been placed very close to the active center chlorophyll, and in a cyclic pathway[22–24]. OLSON AND NADLER[25] have shown that a cytochrome *c*-558 serves an analogous function in Rhodopseudomonas sp. NHTC 133.

Cytochrome *c*-553 was found to contain tightly bound flavin, which has not yet been positively identified. An identical cytochrome has been found in *C. thiosulfatophilum* NCIB 8327 but not in *C. ethylicum* (J. OLSON AND E. STANTON, personal communication). These cytochromes are similar to *Chromatium* cytochrome *c*-552 in that they all have bound flavin and equivalent absorption spectra. However, the *Chromatium* protein has 2 hemes per molecule, in the reduced state forms a complex with carbon monoxide, has a low standard redox potential, $E_{m,7} \simeq 0.01$ V, and differs from the Chlorobium cytochromes in circular dichroism properties[18].

Cytochrome *c*-551 also occurs in *C. thiosulfatophilum* NCIB 8327 and a spectroscopically similar cytochrome *c*-551.5 occurs in *C. ethylicum* (J. OLSON AND E. STANTON, personal communication). SYBESMA[21] puts the Chloropseudomonas cytochrome *c*-551.5 in a cyclic electron transport pathway. Further studies are required to show whether these three cytochromes are similar in structure and function.

C. thiosulfatophilum, *Chromatium* St. D., and *Thiobacillus x* utilize reduced sulfur compounds, including thiosulfate, for growth. A thiosulfate oxidizing enzyme has been isolated from all three organisms[26–28]. The enzyme from *Thiobacillus x* specifically reduces one cytochrome (*c*-553) of three soluble components[26], and preliminary evidence shows that the Chlorobium enzyme may be specific towards cytochrome *c*-551 (ref. 28). The specificity of the *Chromatium* enzyme is being checked; however, it is thought that cytochrome *c*-552 functions between reduced sulfur compounds and the photosystems[22,23].

It is of interest to note the similarity between *C. thiosulfatophilium* and *Chromatium* St. D. in that both lack a soluble high-potential *c*-type cytochrome with $E_{m,7} > 200$ mV and neither contain a *b*-type cytochrome. This is in contrast to the non-sulfur purple bacteria which yield at least one soluble high-potential *c*-type cytochrome[1] and also contain appreciable amounts of *b*-type cytochrome[1].

The near equivalence of the oxidation–reduction potentials of the Chlorobium cytochromes would argue against their functioning sequentially in an electron transport pathway, rather, parallel pathways through the same or different photo-

systems is suggested. Two and possibly three parallel pathways have been postulated for *Chromatium*[29, 22, 23].

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