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Soluble cytochrome composition of the purple phototrophic bacterium, *Rhodopseudomonas sphaeroides* ATCC 17023

T.E. Meyer and M.A. Cusanovich *

Department of Biochemistry, University of Arizona, Tucson, AZ 85721 (U.S.A.)

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A detailed study of the soluble cytochrome composition of *Rhodopseudomonas sphaeroides* (ATCC 17023) indicates that there are five *c*-type cytochromes and one *b*-type cytochrome present. The molecular weights, heme contents, amino acid compositions, isoelectric points, and oxidation-reduction potentials were determined and the proteins were compared with those from other bacterial sources. Cytochromes c_2 and c' have previously been well characterized. Cytochrome *c*-551.5 is a diheme protein which has a very low redox potential, similar to certain purple bacterial and algal cytochromes. Cytochrome *c*-554 is an oligomer, which is spectrally similar to the low-spin isozyme of cytochrome c' found in other purple bacteria (e.g., *Rhodopseudomonas palustris* cytochrome *c*-556). An unusual high-spin *c*-type heme protein has also been isolated. It is spectrally distinguishable from cytochrome c' and binds a variety of heme ligands including oxygen. A large molecular-weight cytochrome *b*-558 is also present which appears related to a similar protein from *Rhodospirillum rubrum*, and the bacterioferritin from *Escherichia coli*. None of the soluble proteins appear to be related to the abundant membrane-bound *c*-type cytochrome in *Rps. sphaeroides* which has a larger subunit molecular weight similar to mitochondrial cytochrome c_1 and chloroplast cytochrome *f*.

Introduction

One of the most popular species for detailed biochemical and genetic studies of bacterial photosynthesis has been *Rhodopseudomonas sphaeroides* [1]. Cytochromes c_2 and c' are the two major soluble electron transfer components in many purple phototrophic bacteria [2] including *Rps. sphaeroides*, which was among the first to be studied [3]. Cytochrome c_2 (but not cytochrome c') has been directly implicated in photosynthetic electron transfer [4,5].

The amino acid sequences of *Rps. sphaeroides* cytochrome c_2 [6] and cytochrome c' [7] have been determined and the cytochrome c_2 has been extensively characterized. The oxidation-reduction potential of cytochrome c_2, measured as a function of pH, has been reported [8,9], and the kinetics of oxidation-reduction by inorganic reagents and oxidation by reaction centers have been examined [10,11]. Reaction center genes from *Rps.*

* To whom correspondence should be addressed.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FMN, flavin mononucleotide.

Supplementary data to this article are deposited with, and can be obtained from: Elsevier Science Publishers B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. References should be made to No. BBA/DD/310/807(1985)308. The supplementary information includes: absorption spectra of *sphaeroides* heme protein in Tris-Cl buffer; the pH titration of *sphaeroides* heme protein; plots of the absorbance changes in the preceding figure; absorption spectra of cytochrome c_2; absorption spectra of cytochrome c'; extinction coefficients of cytochromes; results of automated Edman degradation of *sphaeroides* heme protein

sphaeroides have been cloned and the reaction centers have been crystallized [12]. In addition to cytochromes c_2 and c', three other soluble cytochromes have been observed in *Rps. sphaeroides*, cytochrome *b*-559 [13], cytochrome *c*-553 [14] and cytochrome *c*-551.5 [15]. However, these have not been well characterized. Finally, it has been shown that membranes contain a light-reactive *c*-type cytochrome similar to plant and algal cytochrome *f* and also similar to mitochondrial cytochrome c_1 [16] as well as a *b*-type cytochrome involved in photosynthetic electron transfer [5]. Thus *Rps. sphaeroides* represents a major experimental organism to investigate photosynthetic electron transfer, having many properties in common with higher organisms yet amenable to genetic and biochemical characterization.

Our purpose in making this study was to characterize more fully the minor soluble cytochrome components of *Rps. sphaeroides* to show that they were unique gene products and not post-translationally derived from the major components, and to assure that they were not from contaminating organisms. It is anticipated that a more detailed understanding of the cytochrome composition and the properties of the various cytochromes present will be useful in fully characterizing the metabolism of the photosynthetic bacteria and will assist in properly interpreting genetic studies.

Materials and Methods

The native sizes of purified proteins were measured by the gel filtration technique [17] by comparison of the relative mobilities with horse mitochondrial cytochrome *c*, sperm whale myoglobin, chymotrypsinogen, *Chromatium vinosum* flavocytochrome *c*, and *Chlorobium thiosulfatophilum* cytochrome *c*-555. The proteins were chromatographed on a column of Sephadex G-75-F (1.4 × 50 cm, equilibrated with 0.1 M Tris-Cl (pH 7.3), plus 0.5 M sodium chloride).

Subunit sizes were determined using the SDS method for low-molecular-weight range proteins described by Bethesda Research Laboratories, Gaithersburg, Maryland. The procedure accompanied their protein standards kit, which included mitochondrial cytochrome *c*, lysozyme, chymotrypsinogen, bovine trypsin inhibitor, ovalbumin, beta-lactoglobulin, and insulin. Polyacrylamide gels were crosslinked 15% and contained 6 M urea, 0.1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol. Heme/porphyrin was located by the orange color and by greenish fluorescence [18].

The amino acid composition of purified cytochromes was determined on aliquots of known heme content using the Beckmann Spinco Amino Acid Analyzer, Model 119, following anaerobic hydrolysis in 6 M HCl at 110°C for 48 or 96 h. The amino acid sequence was determined with a Beckmann 890 M sequencer using the 0.1 M Quadrol program supplied by the manufacturer. Phenyl thiohydantoin derivatives were identified by HPLC (Beckmann System 334). The heme content was determined by using absorptivity values for the cytochromes, which were determined by comparing absorption spectra of aliquots in 0.1 M potassium phosphate buffer (pH 7.0) to those in 0.2 M sodium hydroxide, 25% v/v pyridine. The absorptivity value 31.18 $mM^{-1} \cdot cm^{-1}$ for the alpha peak of the alkaline pyridine complex of purified bovine mitochondrial cytochrome *c* was assumed for all the cytochromes *c* [19]. The formula weights were calculated from the tentative amino acid compositions.

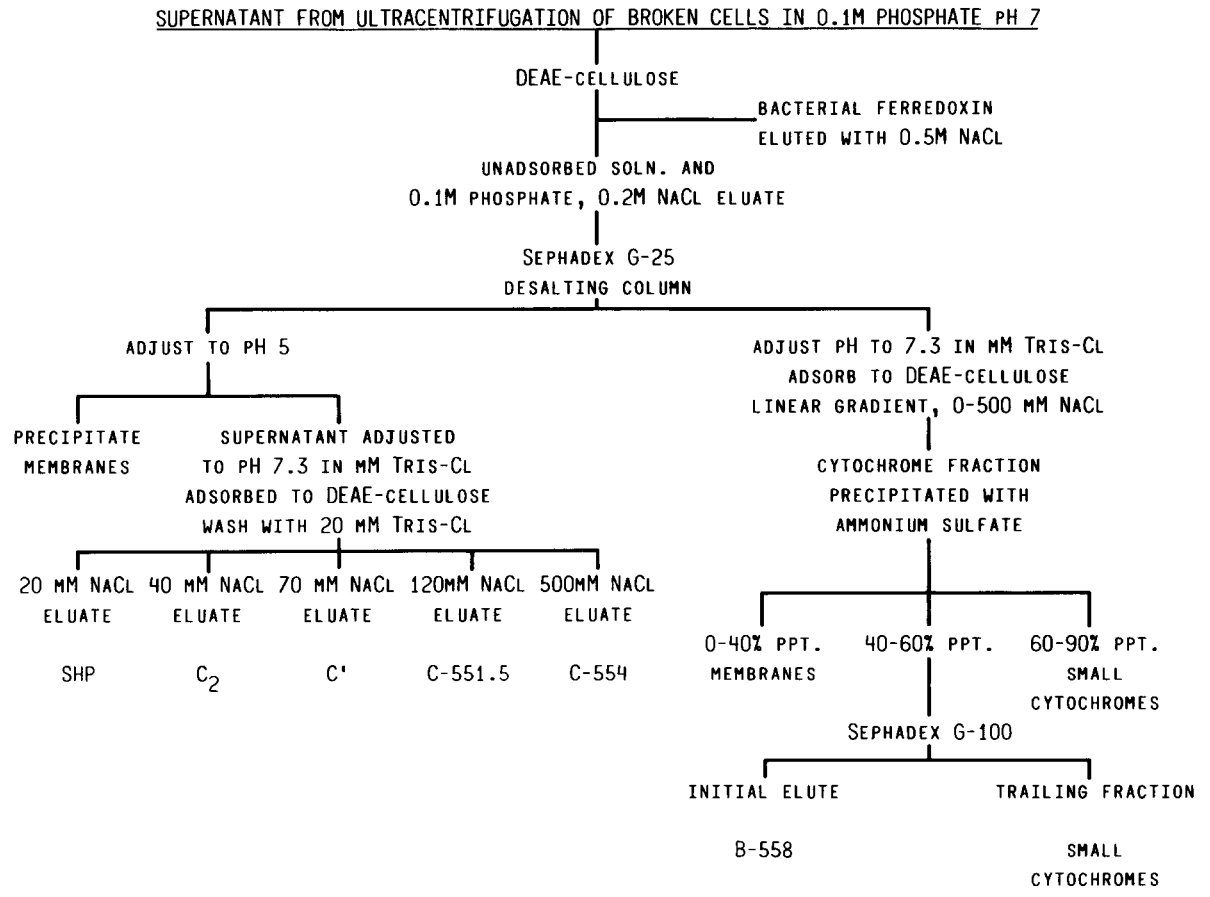
Isoelectric points were measured according to Vesterberg and Svensson [20] using 1% v/v solution of pH 3–10 carrier ampholytes (LKB Produktor AB, Stockholm) and about 1 mg of each cytochrome.

The oxidation-reduction potential of cytochrome *c*-551.5 was determined by anaerobically titrating a mixture of flavin mononucleotide (50 μM FMN) and cytochrome (15 μM) with sodium dithionite (50 mM $Na_2S_2O_4$) in 0.1 M potassium phosphate (pH 7.0). The ratio of oxidized-to-reduced cytochrome was calculated from the height of the alpha peak absorption, and the ratio of oxidized-to-reduced FMN was determined from the difference in absorption at 450 nm after the contribution due to cytochrome was subtracted. The reaction of FMN with small quantities of sulfite [21] produced in the titration was not considered to be a significant source of error. The midpoint oxidation-reduction potential was calculated assuming that for FMN, $E_{m,7} = -219$ mV [22]. The redox potentials of cytochrome c', and *sphaeroides* heme protein were determined as pre-

viously described [23]. For titrations of cytochrome *c'* and *sphaeroides* heme protein, a buffer consisting of 5 mM potassium oxalate, 100 μM indigotetrasulfonate, 100 μM ferric chloride, and 50 mM potassium phosphate (pH 7.0) was used for reductive titrations with 0.1 M dithionite. Oxidative titrations were performed on the same sample with 10 mM ferricyanide in 50 mM potassium phosphate (pH 7.0). The redox potential of cytochrome *c*-554 was measured with a spectroelectrochemical cell in which the potential was varied with a potentiostat. The buffer comprised 20 mM potassium phosphate/10 mM EDTA (pH 7). Mediators were methyl viologen, ferricyanide and Fe-EDTA.

Results

Rps. sphaeroides ATH 2.4.1 (ATCC 17023) was grown in a complex medium [24,25] in 1 l prescription bottles, illuminated by 40 W incandescent bulbs in a water bath maintained at 30°C. Cells were harvested after two days growth, yielding 6–8 g/l wet weight. A 20% suspension of cells (1 kg in 100 mM potassium phosphate, pH 7.5) was disrupted in the Sorvall Ribi Cell Fractionator (an automated French Press), operated at $1.4 \cdot 10^8$ Pa and about 25°C. The broken cell suspension was centrifuged in the Servall type SS-34 rotor (30000 $\times g$) for 20 min, and then in the Spinco type 42 rotor (205700 $\times g_{max}$) for 2 h. The Servall pellet was washed with 1 l 100 mM potassium phosphate (pH 7.0), and the washings combined with the original supernatant. Subsequent purification of cytochromes is illustrated by the flow diagram in Scheme I. The supernatant solutions were passed over a DEAE-cellulose column to remove bacterial ferredoxin. Unfortunately, the ferredoxin proved to be too labile for purification. The column was



Scheme I. Flow diagram for isolation of *Rps. sphaeroides* cytochromes. SHP, *sphaeroides* heme protein.

rinsed with 200 mM sodium chloride in 100 mM potassium phosphate (pH 7.0). The eluate was combined with the initial unabsorbed solution and desalted on a Sephadex G-25 coarse column. The solution was divided into two parts, one for purification of small cytochromes and the other for isolation of cytochrome *b*-558. The first portion was adjusted to pH 5 with dilute HCl and centrifuged in the Servall for 10 min to remove remaining chromatophore membranes. The supernatant solution was then made 1 mM in Tris-Cl, and the pH was adjusted to 7.3. The pH 5 precipitate was washed with 1 mM Tris-Cl (pH 7.3) and the washings added to the extract. The cytochromes were adsorbed onto a DEAE-cellulose column (Brown Co., Type 20) and were chromatographed using two stepwise gradients; first 1 to 20 mM Tris-Cl (pH 7.3) in 2 mM increments, and then 20 mM Tris-Cl plus sodium chloride 0–200 mM in 10 mM increments. The cytochromes were eluted in the sequence: *sphaeroides* heme protein, oxidized cytochrome c_2, reduced cytochrome c_2, cytochrome c', cytochrome *c*-551.5, and cytochrome *c*-554 (see below for details). The various cytochrome fractions were desalted, adsorbed to small DEAE-cellulose columns and eluted with 20 mM Tris-Cl (pH 7.3) plus 500 mM NaCl to concentrate.

The cytochromes were fractionated with ammonium sulfate using increments of 10% saturation (see Table I for individual precipitation conditions). Each concentrated cytochrome was then chromatographed on a G-100 Sephadex column, equilibrated with 200 mM NaCl in 20 mM Tris-Cl. The main band of each cytochrome was desalted on a Sephadex G-25 column, then chromatographed on a DEAE-cellulose column and eluted under the conditions described for each cytochrome in the following sections.

Sphaeroides heme protein

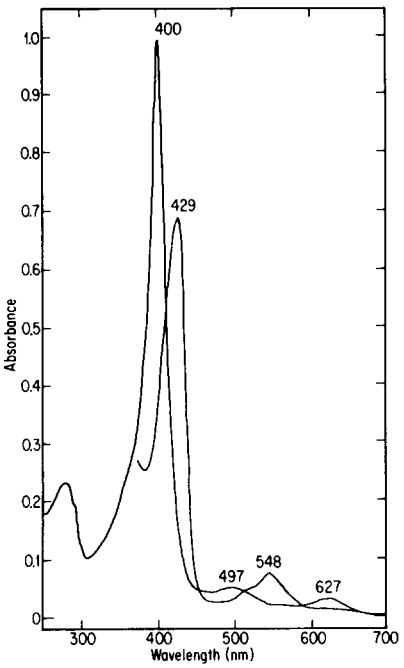
A green-colored heme protein was eluted from the initial DEAE-cellulose column with 20 mM Tris-Cl, plus 20 mM sodium chloride. A second DEAE-cellulose chromatogram of the protein resulted in material which had a constant ratio of 280–400 nm absorbance (0.13) for fractions throughout the elution. The absorption spectra for oxidized and reduced *sphaeroides* heme protein (Fig. 1) are characteristic of predominantly high-spin heme proteins and resemble those of myoglobin, except for the reversible decrease in absorption intensity of the Soret band on reduction. The alpha peak maximum of the alkaline pyridine complex (0.2 M NaOH, 25% pyridine) was at 550 nm, characteristic of *c*-type cytochromes. Extinction coefficients are given in Table A (BBA Data Bank). The native size of *sphaeroides* heme protein, estimated by comparison with other proteins on Sephadex G-75, was 13 kDa and the subunit size estimated by the SDS polyacrylamide gel method was also 13 kDa. The amino acid composition (Table II) was normalized to 121

TABLE I

SELECTED PROPERTIES OF *RHODOPSEUDOMONAS SPHAEROIDES* CYTOCHROMES *c*

Concerning the third column, this is the best ratio of 280 nm absorbance to oxidized Soret absorbance for sphaeroides heme protein, and of 280 nm absorbance to reduced Soret absorbance for the other four cytochromes. The formula weights were calculated from amino acid compositions, which for cytochromes c_2 and c' were based on amino acid sequences.

Protein	(μmol heme/kg w/w cells)	Purity A_{280}/A_s	Native size (kDa)	SDS size (kDa)	Formula weight (kDa)	Redox potential (mV)	p*I*	Percentage saturation ammonium sulfate to precipitate	Ferro-cytochrome carbon monoxide complex
Cytochrome c_2	15	0.23	13	13	14	356	5.5	70–100	no
Cytochrome c'	14	0.28	25	13	14	30	4.9	60–80	yes
Cytochrome *c*-551.5	4	0.08	21	16	8	−254	4.3	60–90	yes
Cytochrome *c*-554	1	0.35	44	14	14	203	4.1	80–100	yes
Heme protein	2	0.13	13	13	13	−22	4.4	60–80	yes
Cytochrome *b*-558	1	1.8	>100	16	17	very low	acidic	40–60	not determined



residues in order to approximate the molecular weight. There was 1.0 heme per protein containing 121 amino acid residues. Four cysteine residues were assumed based on recovery of 3.5 residues after performic acid oxidation. Glycine was the only end group revealed by dansylation of the whole protein. The results of the first 39 steps of Edman degradation (Table B, BBA Data Bank) are as follows: GDTSPAQLIAGAEAAAGA-PADAERGRALFLSTQTGGKPD... The redox potential ($E_{m,7}$) was found to be -22 mV by reductive titration and -54 mV by oxidative titration at pH 7.0. In both cases the redox curves gave a slope consistent with $N = 1$ from the Nernst equation, in spite of the observed hysteresis. The titration was sluggish, and it is likely that equi-

Fig. 1. Absorption of *sphaeroides* heme protein in 0.1 M potassium phosphate (pH 7.0). The reduced form was obtained by adding solid $Na_2S_2O_4$. Note the position of the reduced Soret maximum (429 nm).

TABLE II

AMINO ACID COMPOSITION OF *RHODOPSEUDOMONAS SPHAEROIDES* CYTOCHROMES

Numbers in parentheses are from the sequences of cytochromes c_2 and c', and are otherwise the average or extrapolated best integers for the other cytochromes. The composition of cytochrome c_1 is from Yu et al. [38].

	c_2	c'	*c*-551.5	*c*-554	*sphaeroides* heme protein	*b*-558	c_1
Asp	11.8 (12)	11.7 (11)	16.5 (17)	11.5 (12)	11.4 (12)	19.1 (19)	21
Thr	7.4 (9)	4.4 (5)	7.1 (7)	5.0 (5)	12.5 (13)	4.7 (5)	13
Ser	0.2 (1)	3.8 (5)	7.7 (8)	8.2 (9)	6.2 (7)	9.4 (9)	13
Glu	17.2 (16)	10.2 (10)	13.0 (13)	17.0 (17)	10.2 (10)	20.5 (20)	27
Pro	5.1 (4)	5.2 (5)	7.7 (8)	5.4 (6)	7.2 (7)	2.8 (3)	24
Gly	15.5 (15)	12.1 (12)	10.6 (11)	13.1 (13)	13.6 (14)	9.7 (10)	25
Ala	16.4 (16)	31.8 (31)	21.1 (21)	22.7 (23)	20.3 (20)	17.8 (18)	28
Cys	(2)	1.3 (2)	2.2 (4)	2.3 (3)	3.5 (4)	2.4 (3)	
Val	7.7 (8)	8.9 (9)	5.8 (6)	4.2 (4)	4.4 (5)	3.9 (4)	8
Met	2.0 (2)	2.6 (3)	3.5 (4)	4.9 (5)	0 (0)	4.0 (4)	8
Ile	2.9 (3)	1.2 (1)	2.8 (3)	4.2 (4)	2.9 (3)	8.2 (8)	7
Leu	5.1 (5)	8.7 (8)	13.5 (14)	11.0 (11)	9.0 (9)	18.2 (18)	20
Tyr	4.8 (5)	3.7 (4)	2.0 (2)	2.4 (3)	1.3 (1)	5.6 (6)	7
Phe	5.0 (5)	5.7 (6)	3.3 (3)	2.5 (3)	2.3 (2)	3.9 (4)	12
His	3.1 (3)	2.9 (3)	4.4 (5)	2.7 (3)	1.2 (1)	7.3 (7)	5
Lys	12.9 (13)	11.1 (11)	3.3 (4)	7.8 (8)	4.6 (4)	8.0 (8)	12
Arg	3.1 (3)	3.0 (3)	12.9 (134)	4.5 (5)	7.2 (7)	8.2 (8)	10
Trp	0.8 (2)	(1)	0 (0)	0 (0)	1.5 (2)	0.9 (1)	2
Total	124	130	(142)	(133)	(121)	(154)	(242)
Heme	1.1	1.0	1.8	1.1	1.0	0.41	(1)
F.W.	14100	14000	16400	14400	12700	17300	

librium was not fully established. Another possible explanation for the difference in the oxidative and reductive titrations may lie in the fact that *sphaeroides* heme protein has the apparent capacity for binding small molecules as ligands. For example, ultraviolet–visible absorption spectra in potassium-phosphate and in Tris-chloride buffer differ markedly for the reduced form (Fig. a, BBA Data Bank). That is, the reduced Soret peak is shifted to shorter wavelength, viz. 422 nm. Absorption spectra in sodium carbonate and sodium acetate buffers were similar to those in potassium phosphate, whereas spectra in Tris-phosphate, Tris-sulfate, Bistris-propane chloride, and sodium-Hepes were similar to those in Tris-chloride buffer. *Sphaeroides* heme protein apparently binds molecular oxygen, but the complex is unstable due to autooxidation. It was transiently generated either during slow ascorbate reduction or by shaking a dithionite reduced sample in air (Fig. 2). Ferro-*sphaeroides* heme protein binds carbon monoxide, while the ferri-form binds cyanide and azide. This

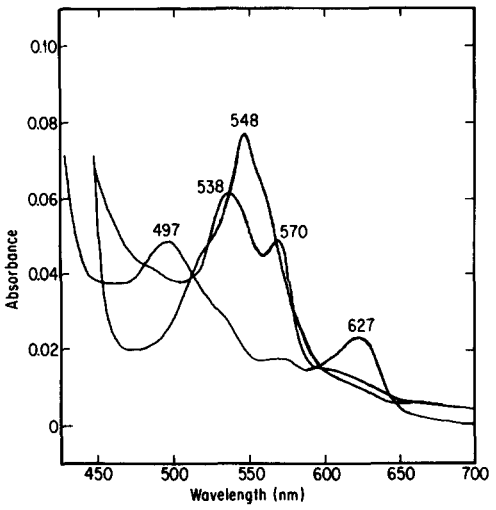


Fig. 2. Absorption spectra of oxidized and reduced forms, and oxygen complex of *sphaeroides* heme protein. Protein in 0.1 M Hepes buffer (pH 7.5) was reduced by solid dithionite and mixed with air until dithionite was just consumed. The spectrum of the oxygen complex (λ_{max}, 570 nm, 538 nm) was traced as the protein was slowly autooxidazed. Because *sphaeroides* heme protein was completely oxidized in less than 30 min, the relative peak intensities are not reliable. The very small 627 nm peak in the spectrum of the oxygen complex is due to the oxidized form and indicates that at least 80% of the protein was present as the O_2 complex when the spectrum was recorded.

is in sharp contrast to the cytochromes *c'* which bind only carbon monoxide, nitric oxide and ethyl isocyanide [26]. The oxidized form of *sphaeroides* heme protein undergoes a spectral transition to a predominantly low-spin form with a pK of 10.7 (Figs. b and c, BBA Data Bank). The transition gives a nonintegral value of n, thus is not consistent with a single proton ionization. Such a situation is typically seen with the cytochromes *c'* (Cusanovich, M.A., unpublished data). Other properties are summarized in Table I.

Cytochrome c_2

A pink-colored band composed mainly of reduced cytochrome c_2, was eluted after *sphaeroides* heme protein with 20 mM Tris-Cl, plus 30–40 mM sodium chloride in the initial DEAE-cellulose column. The absorption spectra of the oxidized and reduced forms (Fig. d and Table B, both in BBA Data Bank) were similar to those observed for other cytochromes c_2 [27] with the exception of a higher ratio of 280/417 nm absorbance due to higher tryptophan content. The amino acid sequence of *Rps. sphaeroides* cytochrome c_2 has been reported [6]. The amino acid composition (Table II) was normalized to 124 residues as indicated by the sequence. The heme content was 1.1 per 124 residues in agreement with the sequence which indicates a single heme binding site. The amino acid composition is presented in part to indicate the accuracy to be expected in those compositions for which there is no corresponding sequence data. In this instance there is 93% agreement between present experimental and known compositions. The oxidation-reduction potential of *Rps. sphaeroides* cytochrome c_2 and its pH dependence have been reported [8,9].

Cytochrome *c'*

Cytochrome *c'* was eluted from the initial DEAE-cellulose column as a brown band with 20 mM Tris-Cl, plus 50–80 mM sodium chloride just after cytochrome c_2. The absorption spectra of oxidized and reduced forms (Fig. 4 and Table A, both in BBA Data Bank) are similar to those of other cytochromes *c'* [27]. The amino acid composition (Table III) was normalized to 130 residues based on the known sequence [7] and the

heme content was found to be 1.0 per 130 residues. The composition is within 95% agreement with the known composition based on the sequence. The size of cytochrome *c'* determined by Sephadex chromatography, 25 kDa, is considerably higher than the known formula weight (14000) or SDS polyacrylamide gel electrophoresis size (13 kDa), which indicates dimerization and/or non-spherical shape. Sedimentation equilibrium studies in 20 mM Tris/500 mM sodium chloride gave a molecular weight of 23400 ± 800 [28], consistent with a dimer. The redox potential ($E_{m,7}$) of cytochrome *c'* was found to be 30 mV, with $N = 1$. The redox reaction was completely reversible. Other properties are summarized in Table I.

As is typical of the cytochromes *c'* [29], the oxidized form of *Rps. sphaeroides* cytochrome *c'* was found to undergo spectral transitions as a function of pH. These transitions are isosbestic, the first has a pK of 7.8 ($n = 1$) and the second has a pK of 11.0 (n, nonintegral). As the pH is raised, the Soret band of the oxidized cytochrome changes from an asymmetric peak centered at 400 nm to a peak at 405 nm with a distinct shoulder at 365 nm for the first transition. Further addition of base promotes the formation of a typically low-spin *c*-type cytochrome in both the oxidized and reduced form.

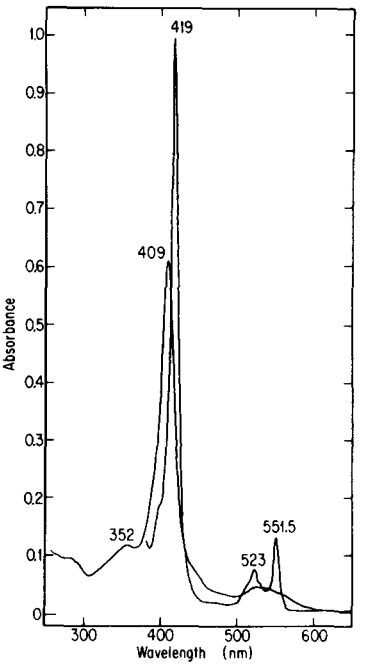


Fig. 3. Absorption spectra of *Rhodopseudomonas sphaeroides* cytochrome *c*-551.5 in 0.1 M potassium phosphate (pH 7.0). The spectrum of the reduced form was traced following addition of solid $Na_2S_2O_4$.

Cytochrome *c*-551.5

Cytochrome *c*-551.5 did not form a sharp band on DEAE-cellulose, but was for the most part eluted with 20 mM Tris-Cl (pH 7.3) plus 100–120 mM sodium chloride. The ultraviolet–visible absorption spectra of oxidized and reduced forms (Fig. 3, see also Table A in BBA Data Bank) were considerably different from those of cytochrome c_2, but similar to those of *Desulfovibrio vulgaris* cytochrome c_3 [15,30]. The low purity index ($A_{280}/A_{410} = 0.08$) and the fact that no bands other than those containing heme could be seen in SDS gels indicates that this protein is pure. The oxidation reduction potential was found to be −254 mV at pH 7, with $N = 1$. This is also similar to those of *Desulfovibrio* cytochromes c_3 [31]. The native size using the Sephadex G-75 method was 21 kDa. SDS polyacylamide gel electrophoresis of freshly prepared protein gave only a single heme-containing band at 16 kDa. However, pure protein which had been stored at −20° for 2 years yielded two heme-containing bands at 8 and 16 kDa of roughly equal intensity. Three end groups were observed on dansylation of the stored protein, glycine, alanine and phenylalanine, although the protein was pure by several criteria, which indicates that there was proteolytic cleavage of the peptide chain during long term storage. Storage did not affect the absorption spectra. The amino acid composition (Table II) indicates that there is only enough cysteine to bind the heme (1.8 hemes per 142 residues).

Cytochrome *c*-554

Cytochrome *c*-554 was isolated in the smallest quantities of the five *c*-type cytochromes observed (Table I). It was partially eluted from DEAE-cellulose with 20 mM Tris-Cl, 160 mM sodium chloride, but was still mixed with trailing cytochrome *c*-551.5. More of this cytochrome mixture could be obtained by soaking the DEAE-cellulose in 20

mM Tris-Cl and 500 mM sodium chloride overnight. Cytochrome *c*-554 was further purified by ammonium sulfate fractionation in which it precipitated at slightly higher concentrations than cytochrome *c*-551.5 (see Table I). The extent of separation was estimated by measuring the ratio of reduced to oxidized Soret peak absorbance. The ratio was 1.62 for purified cytochrome *c*-551.5 and 1.14 for cytochrome *c*-554. The absorption spectra of oxidized and reduced cytochrome *c*-554 (Fig. 4; see also Table A in BBA Data Bank) have wavelength maxima and relative intensities which are like the cytochromes *c*-556 from *R. palustris* and *Agrobacterium*. These latter proteins have been shown by amino acid sequence analysis to be low-spin homologues of cytochrome *c'*. SDS polyacrylamide gel electrophoresis of purified cytochrome *c*-554 indicated only minor contamination (less than 5%) of the heme-containing subunit of 14 kDa. The native size by the Sephadex method was 44 kDa which indicates that his protein is a trimer as isolated. Glycine was the only end group detected by the dansylation method and the heme

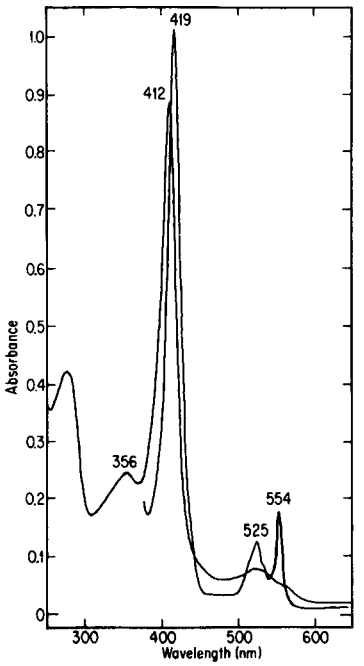


Fig. 4. Absorption spectra of *Rhodopseudomonas sphaeroides* cytochrome *c*-554 in 0.1 M potassium phosphate (pH 7.0). The spectrum of the reduced form was traced after addition of solid $Na_2S_2O_4$.

content was 1.1 per 133 amino acid residues. The amino acid composition (Table II) indicated more cysteine than required to bind the heme (cysteine recovery from *c*-type cytochromes is generally only about 60%) and the lack of tryptophan. Thus although pure by several criteria, the relatively large purity index (0.35) suggests the presence of some ultraviolet absorbing material, possibly nucleic acid which coelutes from DEAE-cellulose. The redox potential was found to be 203 mV at 23°C, 0.1 M ionic strength, and pH 7. The Nernst equation gave an N value of 1.0.

Cytochrome *b*-558

Cytochrome *b*-558 was found when an alternate cytochrome isolation procedure was followed (see Scheme I). A portion of crude desalted cell extract was adsorbed to DEAE-cellulose (without prior treatment at pH 5 to remove membranes) and a linear gradient from 0 to 500 mM sodium chloride in Tris-Cl buffer eluted all the cytochrome in a single band. The cytochrome eluate was fractionated with ammonium sulfate, 0–40%, 40–60% and 60–90% saturation. The 0–40% fraction contained primarily membranes. The 40–60% fraction was chromatographed on Sephadex G-100 and separated into two broad bands. Cytochrome *b*-558 eluted at the void volume and a *c*-type cytochrome mixture followed much later. The 60–90% fraction had no cytochrome *b*-558, but was enriched in cytochrome c_2 and cytochrome *c'*. The cytochrome *b*-558 was adsorbed to DEAE-cellulose and eluted with 260–300 mM sodium chloride in buffer. It was then chromatographed on Sephadex G-200, resulting in a preparation with a 280–418 nm ratio of 1.8. There was approx. 1 μmol (heme) isolated per kg cells. The cytochrome *b*-558 was only slowly reduced by dithionite (it took approx 1 h for spectra to stabilize). It was unaffected by mercaptoethanol or ascorbate. These observations suggest that the protein has a low redox potential and that the heme is relatively inaccessible to dithionite. In view of these properties, we have been unable to obtain a reliable oxidation reduction potential. The native size was not measured, but estimated by behavior on Sephadex to be greater than 100 kDa. SDS polyacrylamide gel electrophoresis indicated that the

subunit size was 16 kDa, and there was only a low degree of small molecular weight contamination (less than 5%). Dansylation revealed the presence of several end groups. Amino acid analysis indicated that there was only 0.41 heme per 154 amino acid residues totaling 17 kDa. The near homogeneity on SDS-polyacrylamide gel electrophoresis suggests pure protein, but the high 280–418 nm ratio, the low heme content and end group analysis suggest impure protein or other complications (see Discussion).

Discussion

In view of the importance of *Rps. sphaeroides* to research on photosynthesis and particularly with respect to electron transport, it is important to know how many heme proteins are synthesized and how they contribute to the functional integrity of the cell. Cytochromes c_2 and c' are well-known soluble constituents of many purple phototrophic bacteria, including *Rps. sphaeroides*. However, reports of minor soluble heme proteins from purple bacteria occasionally appear. In attempting to assess the role of these minor proteins, it is necessary to establish that they are not due to culture contamination, or that they are not proteolytic fragments or denatured forms of the principle components. Although our report concerns cytochromes of *Rps. sphaeroides* strain 2.4.1 (type strain), which we have cultivated over many years, with reproducible results, we have examined additional strains including SCJ, JB15, and R26, and have found the same proteins reported here. We feel that this effectively eliminates the possibility of culture contamination as the source of the minor components. In addition, the amino acid compositions of the soluble *Rps. sphaeroides* proteins studied indicates that none are derived from any other through proteolysis or other forms of posttranslational modification. Thus each of the soluble heme proteins appears to represent a separate gene product derived from *Rps. sphaeroides* which must be accommodated in the overall metabolic scheme.

None of the cytochromes we have isolated from the soluble fraction of *Rps. sphaeroides* is similar to the membrane-bound *c*-type cytochromes [16,32]. All have subunit sizes considerably lower than the 30 kDa subunit of the membrane cytochrome, and only cytochrome c_2 has a comparable redox potential. Yu et al. [33] completely purified the membrane cytochrome, which aggregates in the absence of detergents and is spectrally unlike any of the soluble proteins. Amino acid compositions exclude cytochrome *c*-551.5 as a possible fragment of the membrane cytochrome. However, we cannot exclude the possibility that *sphaeroides* heme protein and cytochrome *c*-554 are proteolytic fragments of the membrane bound protein.

Rps. sphaeroides cytochrome *b*-558 was first observed by Orlando [14] and subsequently a similar protein was recognized in *Rhodospirillum rubrum* and purified by Bartsch et al. [34]. The *R. rubrum* cytochrome has a molecular weight of 450 000, and has a subunit which is about 23 kDa. The heme has a low potential (−200 mV) and is relatively sluggish on reduction by dithionite. These properties are remarkably similar to those of the *Rps. sphaeroides* cytochrome, but a difference lies in the lower 280–417 nm ratio for the *R. rubrum* protein. The *R. rubrum* and *Rps. sphaeroides* cytochrome *b*-558 may be related to the bacterioferritins by virtue of similar native sizes, subunit structure and substoichiometric heme content. Both *Azotobacter* bacterioferritin [35] and *Rps. sphaeroides* cytochrome *b*-558 proteins have approx. one heme per two subunits. The difference in ratio of 280–417 nm absorbance in comparison of *Rps. sphaeroides* and *R. rubrum* proteins may be understood through comparison of *E. coli* bacterioferritin [36,37] fully charged with the ferric hydroxide core and the apobacterioferritin which have very different 280 nm to Soret ratios [36]. We consider it possible that all of the purple phototrophic bacteria produce a ferritin, but it may not bind heme in all species, and may therefore be more difficult to detect. The soluble bacterioferritin-like protein should not be confused with the membrane-bound *b*-type cytochrome of the bc_1 complex, which has a subunit size of 48 kDA [38].

The absorption spectra and redox potential of cytochrome *c*-551.5 are similar to the cytochromes c_3 from *Desulfovibrio* [15] suggesting similar out-of-plane ligands (histidine) [39]. However, cytochromes c_3 are generally 13 kDa tetraheme proteins, which is a very different situation from *Rps. sphaeroides* cytochrome *c*-551.5 (two hemes/16

kDa). Alternatively, *Rps. sphaeroides* cytochrome *c*-551.5 could be related to the cytochrome c_4 from *Azotobacter* and *Pseudomonas* [40] based on heme content and molecular weight, but cytochromes c_4 have high redox potentials consistent with histidine and methionine as the out-of-plane ligands. Interestingly, *Pseudomonas perfectomarinus* produces a diheme cytochrome *c*-552 which is similar in size to the cytochromes c_4 but one heme is high potential and the other low like the cytochromes c_3 [41]. Final classification of *Rps. sphaeroides* cytochrome *c*-551.5 will have to await amino acid sequence analysis. However, it is important to note that the presence of low redox potential cytochromes is not unusual among the phototrophs.

Rhodopseudomonas palustris, *Rhodospirillum tenue* [2] and *Chromatium vinosum* [42] as well as some blue-green bacteria and alga [43–47] all appear to produce a related protein based on molecular weight, redox potential and heme content. Interestingly, the blue-green algae low potential cytochrome was found to be most abundant during stationary phase of growth suggesting the possibility that it might serve as an electron donor to hydrogenase during dark, anaerobic fermentation such as in *Desulfovibrio* [48].

Rps. sphaeroides cytochrome *c*-554 appears to be the same as the cytochrome *c*-553 reported by Orlando [14]. The spectral properties are similar and they elute from DEAE at similar ionic strengths, suggesting the same isoelectric points. The redox potential of cytochrome *c*-554 is 203 mV. The native size of cytochrome *c*-554 is 44 000 and it dissociates on SDS polyacrylamide gel electrophoresis to 14 kDa subunits. Orlando reported a redox potential of 120 mV and a molecular weight of 25 000, but the subunit composition was not determined. The discrepancy in molecular weight and redox potential is probably not significant in view of different experimental conditions. Orlando felt that his protein might be the bacterial equivalent of cytochrome c_1 because aerobic cells produced four times as much as anaerobic cells. However, Wood [16] showed that *Rps. sphaeroides* has a membrane-bound cytochrome similar in minimal molecular weight to mitochondrial cytochrome c_1 (30 kDa). Thus, the subunit size of cytochrome *c*-554 indicates it is not related to cytochrome c_1. Although requiring an amino acid sequence for proof, the spectral and redox properties of *Rps. sphaeroides* cytochrome *c*-554 and *Rps. palustris* cytochrome *c*-556 are similar leaving open the possibility that cytochrome *c*-554 is a low-spin isozyme of cytochrome *c'* as is the *Rps. palustris* protein [2].

Rps. sphaeroides heme protein has been found in *Rps. sphaeroides* and *Rps. capsulata* [2] and a similar cytochrome was reported from *Chromatium vinosum* [49]. *Sphaeroides* heme protein is more like myoglobin than it is to cytochrome *c'* in spectral properties, and binds molecular oxygen. In oxidized cytochrome *c'*, there is a pH 7–9 transition from one high spin form to another high spin form followed by a very high pH transition to a low-spin form, but the *Rps. sphaeroides* heme protein is converted to a low spin form without an obvious intermediate transition. There is only one histidine and no methionine in *sphaeroides* heme protein, which leaves open the identity of the sixth heme ligand at high pH. Presumably there is no protein derived sixth ligand at neutral pH. The ultraviolet–visible absorption spectrum of the reduced form of sphaeroides heme protein varies depending on solvent, which suggests that the reduced form is able to bind buffer ions. The relatively large shift of the Soret band suggests that the exogenous ions bind directly to the heme iron. The hysteresis observed in redox titrations may also be the result of differential ligand binding. The ligand binding reactions are being pursued and will be the subject of a subsequent publication.

The sequence of the first 39 residues at the N-terminus of *sphaeroides* heme protein is unlike any known cytochrome. There is no heme-binding site in this sequence, which indicates that the covalently bound heme is somewhere in the C-terminal two-thirds of the protein. The heme is bound within 20 residues of the N-terminus in cytochrome c_2, but is within 12 residues of the C-terminus in cytochrome *c'*.

In summary, five *c*-type cytochromes and one *b*-type cytochrome can be solubilized from *Rps. sphaeroides*. With the exception of cytochrome c_2 and *c'*, these cytochromes are present in small amounts. Moreover, the minor components (*Rps. sphaeroides* heme protein, cytochrome *c*-551.5, cytochrome *c*-554 and the *b*-type cytochrome) are

similar to cytochromes found in other bacteria, but are not universally present. Although definitive functions cannot be assigned to the *Rps. sphaeroides* cytochromes, it is important to consider their possible participation in the metabolism and photosynthetic electron transport of *Rps. sphaeroides* as our understanding of the organism evolves.

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