Characterization of Two Soluble Basic Cytochromes Isolated from the Anoxygenic Phototrophic Sulfur Bacterium *Chlorobium phaeobacteroides*

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Chlorobium phaeobacteroides contains two soluble basic *c*-type cytochromes, a flavocytochrome *c*-552 and a small cytochrome *c*-555. Both electron transfer proteins were highly purified by ion exchange chromatography and gel filtration.

The flavocytochrome *c*-552 exhibits maxima at 552 nm, 523 nm and 416 nm in the reduced state and at 409.5 nm with two shoulders at 440 nm and 480 nm in the oxidized form. The best purity index (A_{280}/A_{416}) obtained was 0.65. The molecular properties of this flavocytochrome are as follows: isoelectric point, pH 9.5–10; redox potential, +63 mV; molecular weight, 56,000.

Cytochrome *c*-555 is a small basic hemoprotein with an isoelectric point of pH 9.5–10, a molecular weight of 9,500 and a midpoint redox potential of +105 mV. The best purity index (A_{280}/A_{418}) obtained was 0.176. The oxidized form of this cytochrome has a maximum at 411.5 nm, while the reduced state shows three maxima (α-band at 554.5 nm; β-band at 523 nm, and γ-band at 418 nm). The α-band is asymmetrical with a typical shoulder at 551 nm.

Introduction

The brown-coloured, non-thiosulfate-utilizing phototrophic sulfur bacterium *Chlorobium phaeobacteroides* belongs to the family Chlorobiaceae. The predominant pigments of this anoxygenic phototrophic bacterium are the carotenoids isorenieratene and β-isorenieratene, and bacteriochlorophyll e as light harvesting pigments located in chlorosomes. The photosynthetic reaction centers contain bacteriochlorophyll a, localized in the cytoplasmic membrane. The organism is obligately phototrophic and strictly anaerobic, which enables it to perform an anoxygenic photosynthesis [see 1–3]. Because Anoxyphotobacteria lack photosystem II, they cannot use water as electron donor for their photosynthesis and never evolve oxygen in the light. Therefore, reduced sulfur compounds such as sulfide or elemental sulfur serve as electron donors for their anoxygenic photosynthesis and carbon dioxide reduction [4]. In contrast to purple sulfur bacteria, Chlorobiaceae fix CO_2 *via* a reductive carboxylic acid cycle and not *via* the Calvin cycle [5]. During anaerobic sulfide oxidation by Chlorobiaceae, elemental sulfur appears in form of globules as an intermediate product in the medium before it is further oxidized to sulfate [6–8]. It is generally known that cytochromes are centrally involved in the oxidation of reduced sulfur compounds in Anoxyphotobacteria [9]. The participation of cytochromes in the dissimilatory sulfur metabolism of Anoxyphotobacteria has been reviewed by Fischer [9] and in addition, the distribution, occurrence and molecular properties of soluble *c*-type cytochromes in Chlorobiaceae have been compiled recently by the same author [10]. While the thiosulfate-utilizing subspecies of Chlorobiaceae like *Chl. limicola f. thiosulfatophilum* and *Chl. vibrioforme f. thiosulfatophilum* contain three soluble *c*-type cytochromes (cytochrome *c*-551, a flavocytochrome and a small cytochrome *c*-555), the non-thiosulfate-utilizing Chlorobiaceae species lack cytochrome *c*-551 [see 10]. This cytochrome is involved in thiosulfate oxidation and functions as the only endogenous electron acceptor for the thiosulfate oxidizing enzyme [7, 10, 11]. On the other hand, flavocytochromes of purple and green sulfur bacteria themselves possess enzymatic activity and act as sulfide:cytochrome c reductases or as adenylylsulfate reductases [6, 7, 11–13].

The small cytochrome *c*-555 may function as an "electron mediator" between other cytochromes and bacteriochlorophyll. This cytochrome serves as positive effector for the thiosulfate:cytochrome *c*-551

Abbreviations: Chl., *Chlorobium;* cyt., cytochrome.

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oxidoreductase and as electron acceptor for the sulfide:cytochrome *c* reductase, the above-mentioned flavocytochrome [11, 14, 15]. A direct photooxidation in green sulfur bacteria was only demonstrated for a membrane-bound cytochrome *c*-555 but not for the soluble one [16].

It was the aim of this study to isolate the soluble cytochromes of *Chl. phaeobacteroides*, to study their molecular properties and to compare them with those of other non-thiosulfate-utilizing species of Chlorobiaceae.

Materials and Methods

Chlorobium phaeobacteroides was kindly provided by Dr. L. J. Stal, University of Amsterdam, The Netherlands, and was grown photolithoautotrophically at 30 °C at 1,500 Lux in 20 l carboys in Pfennig's medium as described by Steinmetz and Fischer [6, 8]. To get a better cell yield, the culture was fed once with 1 l of a solution containing 15 g $Na_2S \times 9\ H_2O$ and 10 g Na_2CO_3, neutralized by the addition of 40 ml of a sterilized 2 M H_2SO_4 solution. Cells were harvested by continuous flow centrifugation at 10,000 rpm in a Christ Labofuge 15,000.

Cells of *Chl. phaeobacteroides* were disrupted by sonification [6] and the cytochromes were prepared by the methods described by Steinmetz and Fischer [6, 8], but with the following modification: the supernatant of the ultracentrifugation (100,000 × *g* for 150 min) was desalted on Sephadex G-25, adjusted to pH 7.8, and chromatographed on a DEAE-52 cellulose column (equilibrated in 20 mM Tris-HCl, pH 7.8). Instead of ammoniumsulfate fractionation, the unadsorbed cytochrome – containing fractions were pooled, concentrated by ultrafiltration using a UM 2 membrane (Amicon Corp.) and then applied to a Sephadex G-75 column (equilibrated in 20 mM Tris-HCl, pH 7.3, containing 100 mM NaCl). The two cytochromes were eluted with the same buffer and for further purification separately chromatographed on CM-52 cellulose columns [6, 8].

If not otherwise indicated, all standard methods (estimation of protein, redox potential, isoelectric point and molecular weight) were carried out as previously described by Steinmetz and Fischer [6–8]. Ultrogel AcA 44 was obtained from LkB, Stockholm, Sweden. All other chemicals were purchased as described by Steinmetz and Fischer [6, 8].

Results and Discussion

Like other non-thiosulfate-utilizing green sulfur bacteria, cells of *Chlorobium phaeobacteroides* contain only two soluble *c*-type cytochromes, namely a flavocytochrome *c*-552 and a small cytochrome *c*-555. Both cytochromes are basic and are easily purified by ion exchange chromatography on CM-cellulose and gel filtration through Sephadex G-75. The elution diagram of a Sephadex G-75 gel filtration of the unadsorbed protein extract of *Chl. phaeobacteroides* after DEAE-52 cellulose column chromatography is shown in Fig. 1. As can clearly be

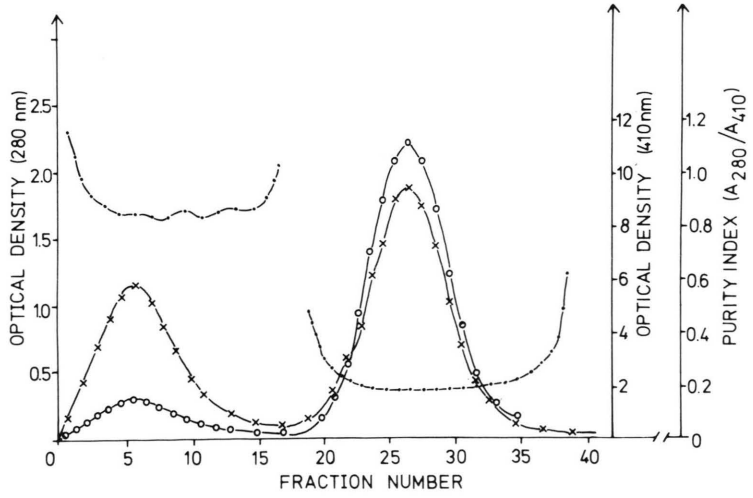

Fig. 1. Filtration and separation of *Chlorobium phaeobacteroides* flavocytochrome *c*-552 (first band) and cytochrome *c*-555 (second band) through Sephadex G-75. The gel was equilibrated in 20 mM Tris-HCl, pH 7.0 containing 100 mM NaCl and was eluted with the same buffer. Fractions with 2 ml were collected. x——x Optical density at 280 nm; ○——○ Optical density at 410 nm; ●——● Purity index ($A_{280\ nm}/A_{410\ nm}$).

seen, both cytochromes are distinctly separated with a high degree of purity by this procedure. The first band contained the flavocytochrome *c*-552 and the highest purity index obtained (A_{280}/A_{416}) was 0.65. The small cytochrome *c*-555 was found in the second band and the highest purity index obtained (A_{280}/A_{418}) was 0.176. Both purity values lie in the order of magnitude found for the comparable cytochromes of other non-thiosulfate-utilizing species of Chlorobiaceae (see Table I). The typical absorption spectra of the flavocytochrome *c*-552 are shown in Fig. 2 and those of cytochrome *c*-555 in Fig. 3. The oxidized form of the flavocytochrome exhibited only the γ-band at 409.5 nm and two shoulders at 440 nm and 480 nm indicating the flavin component of this cytochrome. When reduced by the addition of sodium dithionite these shoulders were completely bleached out, the γ-band shifted to 416 nm and additionally the α-band (552 nm) and β-band (523 nm) appeared. The native spectrum of cytochrome *c*-555 showed maxima at 554.5 nm (α-band), 523 nm (β-band), and 418 nm (γ-band) in the reduced form, while the γ-band of the oxidized form was found at 411.5 nm. The α-band of the reduced state is asymmetrical with a characteristic shoulder at 551 nm. Such a shoulder was also found for all cytochromes *c*-555 so far isolated from other Chlorobiaceae (see Table I) and is also typical of the comparable plant *f*-type cytochromes [7, 19].

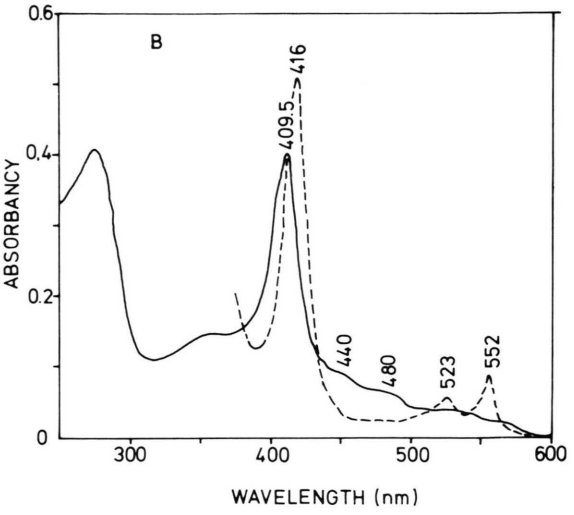

Fig. 2. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) flavocytochrome *c*-552 of *Chlorobium phaeobacteroides* in 20 mM Tris-HCl, pH 7.8. 1 cm cells contained 3.3 nmol of flavocytochrome *c*-552 (calculated using E_{mM} of 156; Bartsch [17]). —— oxidized; – – – reduced.

In molecular weight (Fig. 4), redox potential (Fig. 5), isoelectric point and also in the spectral properties (Fig. 2), flavocytochrome *c*-552 of *Chl. phaeobacteroides* resembles very much the corresponding hemoprotein of *Chl. limicola*, but differs

Table I. Comparison of molecular properties of soluble *c*-type cytochromes of non-thiosulfate-utilizing species of Chlorobiaceae (cytochrome *c*-551 is absent in all organisms).

Organism and cytochromes	*Chl. phaeobacteroides*[1]		*Chl. limicola*[2]		*P. luteolum*[3]		*Chl. vibrioforme*[4]	*Pr. aestuarii*[5]
Molecular properties	*c*-552	*c*-555	*c*-553	*c*-555	*c*-553	*c*-555	*c*-555	*c*-555
Molecular weight	56,000	9,500	56,000	10,000	10,700	9,500	11,500	12,000
Redox potential [mV]	+63	+105	+65	+140	+220	+160	+80	+103
Flavin group	+	–	+	–	–	–	–	–
Isoelectric point	9.5–10	9.5–10	9.0	9.5–10	10.2	10.5	7.3	4.65
Purity index ($A_{280}/A_{\gamma\text{-band}}$)	0.65	0.176	0.96	0.13_{ox}	0.18	0.17	0.15	0.18
Maxima [nm]								
Oxidized (γ-band)	409.5	411.5	410	416	413	413	412.5	412
Reduced (α-band	552	554.5	553.5	555	553	555	555	555
and		(551)		(551)		(551)	(551)	(550)
γ-band)	416	418	417	417.5	417	418	418	417.5

Data taken from: [1] own results; [2] Steinmetz and Fischer [6]; [3] Steinmetz and Fischer [8]; [4] Steinmetz *et al.* [15] and [5] Shioi *et al.* [18]. ox = oxidized; P. = *Pelodictyon;* Pr. = *Prosthecochloris.*

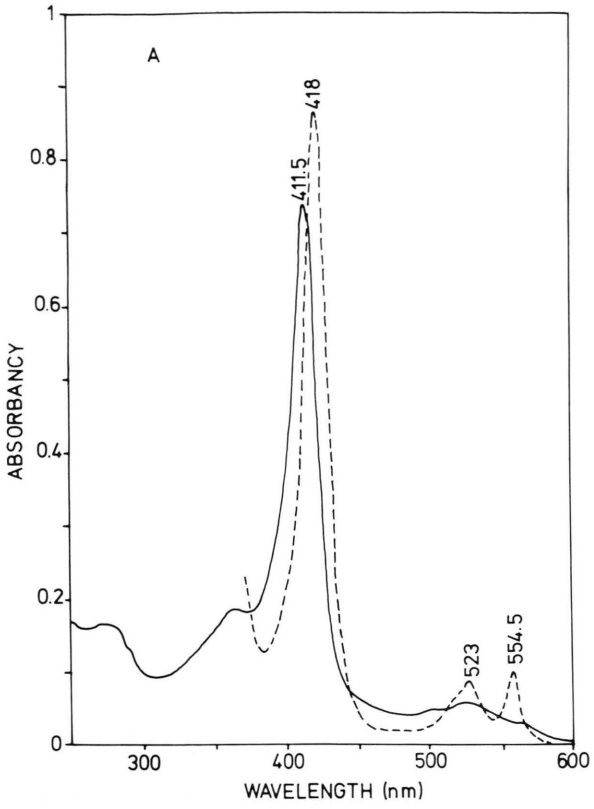

Fig. 3. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) small cytochrome *c*-555 of *Chlorobium phaeobacteroides* in 20 mM Tris-HCl, pH 7.8. 1 cm cells contained 5.7 nmol of cytochrome *c*-555 (calculated using E_{mM} of 153; Bartsch [17]). —— oxidized; – – – reduced.

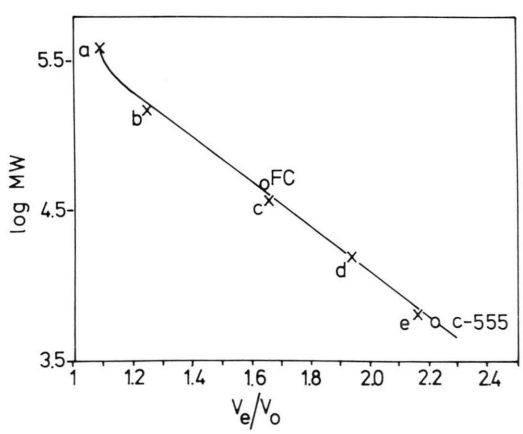

Fig. 4. Estimation of the molecular weights of flavocytochrome *c*-552 and cytochrome *c*-555 of *Chlorobium phaeobacteroides* by gel filtration through Ultrogel AcA44. The gel was equilibrated in 50 mM potassium-phosphate-buffer, pH 7.4 and was eluted with the same buffer. The elution volumes of *Chl. phaeobacteroides* cytochromes (V_e) were compared with those of ferritin (a; MW: 450,000), catalase from bovine liver (b; MW: 240,000), hen egg albumin (c; MW: 45,000), chymotrypsinogen A from bovine pancreas (d; MW: 25,000) and horse heart cytochrome *c* (e; MW: 12,500). The void volume (V_o) was determined with dextran blue. Fractions with 1 ml were collected.

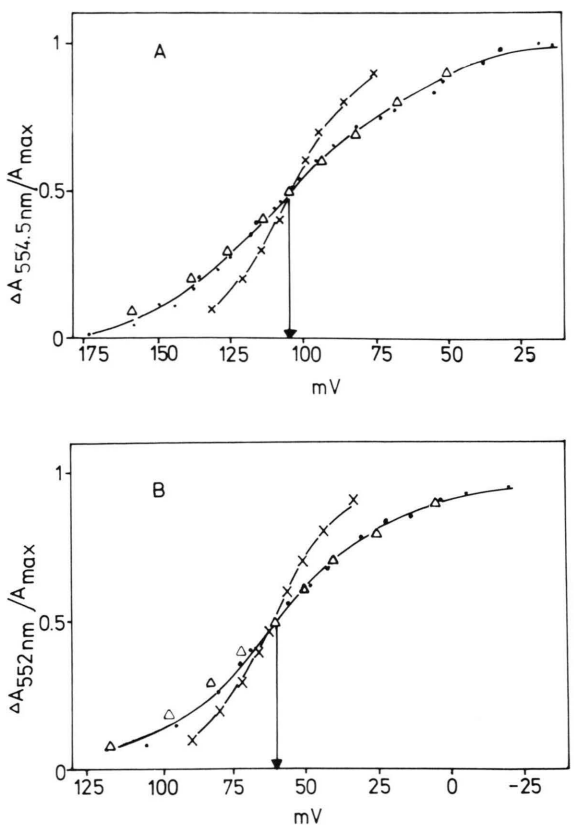

Fig. 5. Redox titrations of the *Chlorobium phaeobacteroides* cytochrome *c*-555 (A) and flavocytochrome *c*-552 (B). The redox potentials of both cytochromes were determined anaerobically at pH 7.0 and 23 °C by the stepwise addition of 50 mM $K_3[Fe(CN)]_6$ solution and a 100 mM $Na_2S_2O_4$ solution as described by Steinmetz and Fischer [7]. ●——● Ferricyanide titration curves of cytochrome *c*-555 (A) and flavocytochrome *c*-552 (B) of *Chl. phaeobacteroides;* △——△ Theoretical curves according to the Nernst equation for $n=1$ and midpoint potentials of 105 mV (cyt. *c*-555; A) or 63 mV (flavocyt. *c*-552; B), respectively; x——x Theoretical curves according to the Nernst equation for $n=2$ and midpoint potentials of 105 mV (cyt. *c*-555; A) or 63 mV (flavocyt. *c*-552; B), respectively.

from cytochrome *c*-553 of *Pelodictyon luteolum* which contains no flavin group, has a lower molecular weight and a higher positive redox potential (see Table I and [6, 8]). The other non-thiosulfate-utilizing species *Chl. vibrioforme* and *Prosthecochloris aestuarii* do not contain such a flavocytochrome or an equivalent cytochrome. These organisms possess only the small cytochrome *c*-555. In addition, it is necessary to mention that the thiosulfate-utilizing subspecies *Chl. limicola f. thiosulfatophilum* [17, 20] and *Chl. vibrioforme f. thiosulfatophilum* [7] also contain flavocytochromes. In comparison to the basic flavocytochromes of the non-thiosulfate-utilizing species of Chlorobiaceae these flavocytochromes are weakly acidic (pH 6−6.3) and have higher redox potentials (+90 − +98 mV) (for review see [2, 10]). Flavocytochromes consist of two subunits where the flavin component is covalently bound to the larger subunit with a molecular weight of about 40,000, while the smaller subunit (MW: 11,000−21,000) contains the heme group [see 17, 21].

Flavocytochromes of Anoxyphotobacteria are involved in their anaerobic sulfide − and sulfite oxidation (for review see chapter 3 in [22]). The *Thiocapsa roseopersicina* flavocytochrome with a molecular weight of 180,000 has adenylylsulfate reductase activity [13]. On the other hand and so far examined, all flavocytochromes of Chlorobiaceae and of the purple sulfur bacterium *Chromatium vinosum* possess sulfide:cytochrome *c* reductase activities, catalyzing the oxidation of sulfide [6, 7, 12, 23, 24]. In addition, it was also reported that the flavocytochrome of *C. vinosum* can act as an elemental sulfur reductase by reducing elemental sulfur to sulfide [25]. Such an activity could not be confirmed for the corresponding hemoprotein of *Chl. vibrioforme f. thiosulfatophilum* [7].

The existence of reduced sulfur compounds in the culture medium has an influence on the formation of cytochrome patterns. When *Chl. limicola f. thiosulfatophilum* is grown on sulfide as the sole electron donor, this organism contains more flavocytochrome than after cultivation with thiosulfate [11]. This finding could not be confirmed for *Chl. vibrioforme f. thiosulfatophilum*. In this organism, the ratio of cytochrome contents is constant and is independent of the sulfur source offered [7]. On the other hand, it has clearly been proved that the absence or occurrence of cytochromes in *Ectothiorhodospira shaposhnikovii* is regulated by sulfur compounds. When sulfur compounds were available, this organism synthesized a soluble *b*-type cytochrome. Without any reduced sulfur compound in the medium, it only contained a membrane-bound cytochrome *c*-553 [26]. A regulation of cytochrome concentration in *Rhodobacter sulfidophilus* has also been reported [27].

Cytochrome *c*-555 of *Chl. phaeobacteroides* is a small, monomeric, soluble and basic cytochrome (see Table I). Concerning their spectral properties, cytochromes *c*-555 and *f*-type cytochromes have some characteristics in common. Both types of cytochromes exhibit an asymmetrical α-band in the reduced state with a maximum at 554 (±1) nm and a shoulder at around 550 nm (see Fig. 3).

With respect to its molecular properties the *Chl. phaeobacteroides* cytochrome *c*-555 resembles the corresponding hemoproteins studied from other Chlorobiaceae, which have molecular weights of between 9,500−12,000. The redox potentials of these *f*-type-like cytochromes range from +80 mV to +160 mV and are distinctly lower than those of the corresponding *f*-type cytochromes from other Anoxyphotobacteria, cyanobacteria, algae and higher plants (+220 mV−+390 mV) (see [2, 21, 22]). While the *f*-type cytochromes of the latter organisms exhibit acidic isoelectric points (see [2, 17]), most Chlorobiaceae cytochromes *c*-555 have isoelectric points around pH 10, with the exception of *Chl. vibrioforme* and *Prosthecochloris aestuarii,* where the isoelectric points were determined at pH 7.3 and pH 4.65, respectively (see Table I). The latter organisms do not contain any other soluble cytochrome than the small cytochrome *c*-555.

Cytochrome *c*-555 of Chlorobiaceae serves as positive effector for the thiosulfate:cytochrome *c*-551 oxidoreductase and as electron acceptor for the flavocytochrome, a sulfide:cytochrome *c*-reductase, before the electrons flow to oxidized bacteriochlorophyll a (see [2, 10]). The first function is realized only in the thiosulfate-utilizing Chlorobiaceae species where cytochrome *c*-551 is always present, but it was never found in the non-thiosulfate-utilizing green sulfur bacteria [10]. The absence of this endogenous electron acceptor in thiosulfate oxidation might be an explanation why these organisms are unable to use thiosulfate as additional electron source. The statement that the thiosulfate-utilizing Chlorobiaceae contain always three soluble *c*-type cytochromes (cyt. *c*-551, flavocytochrome and

cytochrome *c*-555) (see [10]) and the non-thiosulfate-utilizing species only two or even one (see Table I) is confirmed by the results obtained with *Chl. phaeobacteroides*.

Recently, Davidson *et al.* [28, 29] reported the formation of a complex of cytochrome *c*-555 with flavocytochrome *c*-553 and cytochrome *c*-551 of *Chl. limicola f. thiosulfatophilum*, using affinity chromatography. The binding site of cytochrome *c*-555 on the flavocytochrome *c*-553 is located on the heme containing subunit. A similar cytochrome complex was described for the cytochromes of *Chromatium vinosum*. This complex oxidizes sulfide to elemental sulfur [30].

It must be pointed out that a direct photo-oxidation in Chlorobiaceae was demonstrated only for a membrane-bound cytochrome *c*-555 but not for the soluble one [16]. These membrane-bound cytochromes *c*-555 have redox potentials between +165 and +220 mV and are not identical with the soluble cytochrome *c*-555 (see [17, 21]). In contrast to the cytochromes c_2 of Rhodospirillaceae, cytochromes *c*-555 of Chlorobiaceae do not serve as direct electron donors for reaction center bacteriochlorophyll. One might therefore assume that the soluble cytochrome *c*-555 leads the electrons obtained during anaerobic sulfide or thiosulfate oxidation *via* the membrane-bound cytochrome *c*-555 to the reaction center [2, 17]. Regarding the redox potentials, such an electron flow must be possible. Further studies on the participation of soluble and membrane-bound cytochromes in sulfur metabolism as well as in the photosynthetic electron transport chain in Chlorobiaceae must help to clarify this question.

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