Cytochromes of the Purple Sulfur Bacterium *Ectothiorhodospira shaposhnikovii*

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Two *c*-type cytochromes (a high spin cytochrome *c'* and a low spin cytochrome *c*-553(549) with asymmetrical α-band) and a low spin cytochrome *b*-558 from the purple sulfur bacterium *Ectothiorhodospira shaposhnikovii* were purified by ion exchange chromatography and gel filtration and characterized. Cytochrome *c'* has a molecular weight of 33000 (determined by sodium dodecylsulfate electrophoresis), an isoelectric point at pH 4.5 and a redox potential of +37 mV. Absorption spectra show in the oxidized state maxima at 404 nm and in the range of 635 nm, in the reduced form maxima at 426.5 nm, 549 nm and a shoulder at 435 nm. The best purity index obtained was 0.48 ($A_{280}/A_{426.5}$). Reduced cytochrome *c'* reacts with carbon monoxide.

Cytochrome *c*-553(549) has a molecular weight of 10400, an isoelectric point at pH 5.1 and a redox potential of +248 mV. The oxidized form shows the Soret-band at 410 nm. The reduced protein reveals an asymmetrical α-band at 553 nm with a shoulder at 549 nm, the β-band at 522 nm with a shoulder at 528 nm and the γ-band at 416 nm. The best purity index obtained was 0.18 (A_{280}/A_{416}). Both cytochromes could be isolated from the soluble fraction as well as from Triton X-100 treated membranes. Furthermore very low amounts of cytochromes *c*-553 and *c*-552.5 could be detected in detergent treated chromatophores.

Cytochrome *b*-558 – obtained from cells grown in the presence of reduced sulfur compounds in the medium – seems to be soluble or only weakly bound to the membrane. It has a molecular weight of 15800, an isoelectric point at pH 4.1 and a redox potential of −210 mV. The hemoprotein shows absorption maxima at 424.5 nm, 526.5 nm and 556.5 nm in the reduced form and at 416 nm in the oxidized state. The best purity index obtained was 0.26 ($A_{280}/A_{424.5}$). In addition, there were hints for the occurrence of a high spin cytochrome *b'*.

The cytochrome pattern as well as the amount of cytochromes were dependent on growth conditions.

Introduction

It was recently proposed to separate the genus *Ectothiorhodospira* from the Chromatiaceae and form the new family Ectothiorhodospiraceae with the only genus *Ectothiorhodospira* comprising extremely halophilic species (*e.g.*, *E. halophila*) and moderate halophilic species such as *E. shaposhnikovii* [1, 2]. Similar to the Chromatiaceae, *Ectothiorhodospira* species are able to use reduced sulfur compounds, *e.g.*, sulfide or thiosulfate as photosynthetic electron donors but deposit elemental sulfur during sulfide oxidation outside the cells [3]. During this process these sulfur compounds are oxidized anaerobically and electron carriers such as iron sulfur proteins or cytochromes function as redox mediators [4]. Electron transfer proteins of members of the Chromatiaceae have been described from *Chromatium vinosum* [5–9], *C. warmingii* [10, 11], *Thiocapsa pfennigii* [12] and *T. roseopersicina* [13–15]. In contrast, our knowledge about electron carrier proteins in *Ectothiorhodospira* species is quite fragmentary. Meyer [16] reported the presence of a cytochrome *c*-551 in the halophilic species *E. halochloris*, *E. abdelmalekii* and *E. halophila* (the latter contains also two HIPIPs and a cytochrome *c'*) which possibly acts as a sulfide: acceptor oxidoreductase in *E. abdelmalekii* [17]. According to Meyer [16] the moderate halophile *E. vacuolata* contains two HIPIPs, cytochrome *c'*, cytochrome *b'* and a cytochrome c_5-like protein. In the related *E. shaposhnikovii* two HIPIPs and a bacterial type ferredoxin could be identified [18].

In the following we report about the cytochromes of *E. shaposhnikovii*, their occurrence under varying growth conditions and some of their properties.

Abbreviations: HIPIP, high potential iron sulfur protein; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; APS, adenylylsulfate

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Materials and Methods

Ectothiorhodospira shaposhnikovii DSM 234 was grown photomixotrophically as previously described [18] and photoheterotrophically without reduced sulfur compounds in a medium containing in 1 l: 30 g NaCl, 1 g NH_4Cl, 1 g KH_2PO_4, 0.1 g $MgCl_2 \times 6\,H_2O$, 0.05 g $CaCl_2 \times 2\,H_2O$, 1 g yeast extract, 3 g Na-acetate, 3 g Na-malate and 1 ml trace element solution "SLA" [19]. Prior to autoclaving pH was adjusted to 8.7.

Redox determinations were carried out according to Steinmetz and Fischer [20]. The following redox mediators were added: 50 µM 1,2-naphthoquinone, 50 µM benzoquinone, 50 µM diaminodurene (cytochrome *c*-553(549)); 5.25 mM 2-methyl-3-phytyl-1,4-naphthoquinone, 50 µM duroquinone, 50 µM phenazine methosulfate, 0.5 mM $FeCl_3$ (cytochrome *c'*); 100 µM anthraquinone-1,5-disulfonate, 100 µM anthraquinone-2-sulfonate, 100 µM 1-OH-1,4-naphthoquinone, 100 µM glutathione and 100 µM lipoic acid (cytochrome *b*-558).

If not otherwise indicated all standard methods (purification of proteins, spectrophotometric determinations and other molecular properties) were carried out as described by Kusche and Trüper [18].

Results

Purification and yields of cytochromes

Cytochrome *c*-553(549), cytochrome *c'* and cytochrome *b*-558 from the purple sulfur bacterium

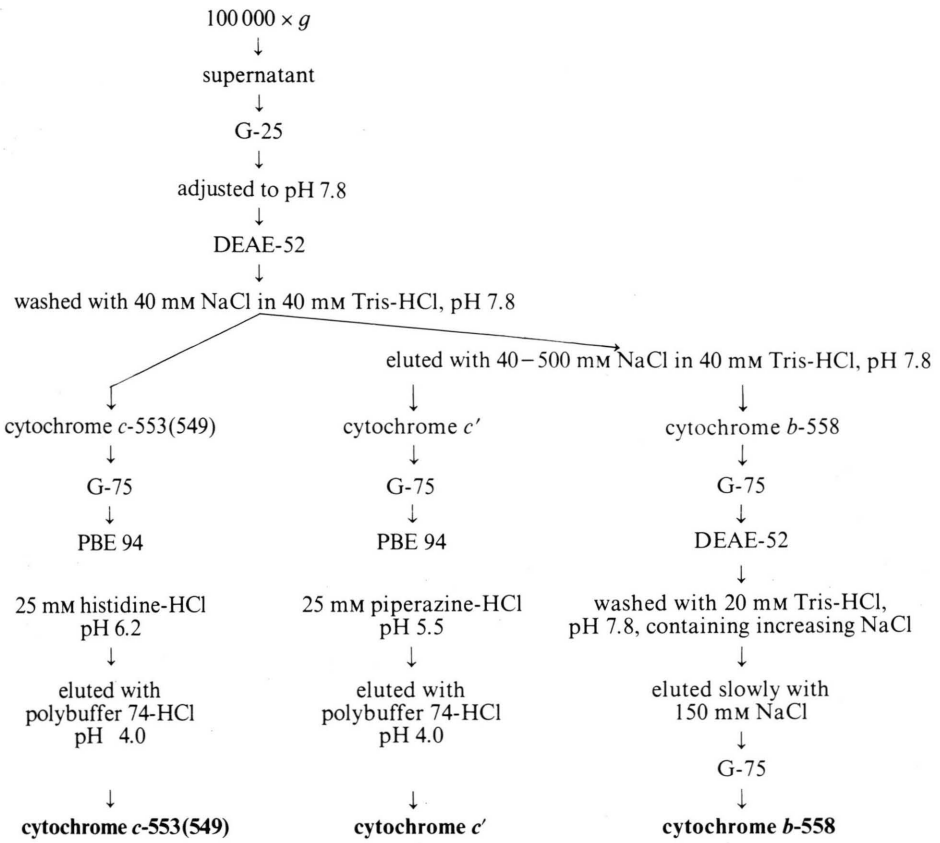

Fig. 1. Purification scheme for cytochrome *c*-553(549), cytochrome *c'* and cytochrome *b*-558 of *Ectothiorhodospira shaposhnikovii*. DEAE-52 cellulose was suspended in 2 mM Tris-HCl, pH 7.8. Sephadex G-25 was equilibrated in Aqua demin. and Sephadex G-75 in 50 mM Tris-HCl, pH 7.8, containing 100 mM NaCl. PBE™: polybuffer exchanger (Pharmacia, Sweden).

Ectothiorhodospira shaposhnikovii were highly purified. Fig. 1 shows a detailed purification scheme for these three proteins. For the detection of membrane-bound electron transfer proteins the 100000 × *g* sediment was suspended in 50 mM Tris-HCl, pH 7.8, containing 1% Triton X-100 and gently stirred for 30 min at 30 °C. This suspension was centrifugated again at 100000 × *g* for 3 h. The red-brown supernatant obtained was desalted (G-25), adsorbed on a DEAE-52 cellulose (equilibrated in 2 mM Tris-HCl, pH 7.8), washed with 40 mM NaCl in 40 mM Tris-HCl, pH 7.8, and eluted with a continuous NaCl gradient. Besides minor quantities of the above mentioned *c*-type cytochromes and two HIPIPs a small fraction of a cytochrome *c*-553 was obtained. Cytochrome *c*-552.5 was solubilized from chromatophores by a procedure covering repeated acetone: methanol (7:2, v/v) extraction, Triton X-100 treatment and $(NH_4)_2SO_4$ fractionation according to Wermter and Fischer [10].

Table I shows the yields of cytochromes and high potential iron sulfur proteins obtained from cells of *E. shaposhnikovii* grown in the presence and absence of sulfide and thiosulfate in the medium. Cytochrome *b*-558 was only present in cells grown photomixotrophically with the above mentioned sulfur compounds in the medium. In contrast, cytochrome *c*-553 could only be detected in photoheterotrophically grown cells (*cf.* Materials and Methods). The occurrence of cytochrome *c*-552.5 was independent of growth conditions, whereas the yields of cytochrome *c*-553(549), cytochrome *c'* and both HIPIPs were up to 4.5-times lower when reduced sulfur compounds were not available.

Purity and spectral properties

Fig. 2 shows the absorption spectra of the highly purified soluble cytochrome *c*-553(549) of *E. shaposhnikovii*. In the reduced form it reveals a γ-band at 416 nm (410 nm ox.), a β-band at 522 nm with a shoulder at 528 nm and an asymmetrical α-band at 553 nm with a shoulder at 549 nm resembling *f*-type cytochromes. The best purity index obtained was 0.18 (A_{280}/A_{416}).

Cytochrome *c'* was isolated in the oxidized form with absorption maxima at 404 nm and 635 nm. The reduced protein shows a broad maximum at 549 nm and a Soret-band at 426.5 nm with a characteristic shoulder at 435 nm (Fig. 3). The best purity index obtained was 0.48 ($A_{280}/A_{426.5}$). Reduced cytochrome *c'* reacted with carbon monoxide. Fig. 4 shows the typical spectrum of the dithionite reduced cytochrome *c'* after five-minutes treatment with CO revealing a distinct increase of the Soret-band which shifted to 419 nm. Only a slight reduction of absorbancy could be registered after keeping the cuvette in the dark for twelve hours. After exposure to light however the disintegration of the heme-CO-complex could be recorded within a few hours.

Cytochrome *c*-553 was eluted from the DEAE-52 cellulose column in the oxidized form. Its absorption spectra (Fig. 5) are characterized by maxima at 553 nm, 524 nm and 417 nm in the reduced form

Table I. Yield of Cytochromes and HIPIPs in *Ectothiorhodospira shaposhnikovii*.

Protein	Medium with reduced sulfur compounds		Medium without reduced sulfur compounds	
	Soluble	Membrane-b.	Soluble	Membrane-b.
c-553(549)	0.2	0.007	0.1	0.06
c'	0.94	0.14	0.17	0.07
c-553	–	–	–	+
c-552.5	–	+	–	+
b-558	0.023	–	–	–
b'	?	–	?	–
"early" HIPIP	4[a]	0.7	1	0.16
"late" HIPIP	5[a]	2.8	1.6	0.27

Yields are given in µmol per 100 g wet cell material and were calculated using the absorption coefficients for cytochromes *c* ε mM_{550} of 31.8 and for cytochromes *b* ε mM_{556} of 34.7 [21] and for HIPIPs ε mM_{388} of 16.1 for HIPIP of *Chromatium vinosum* [22].
[a] Data taken from Kusche and Trüper [18].
– not present; + present.

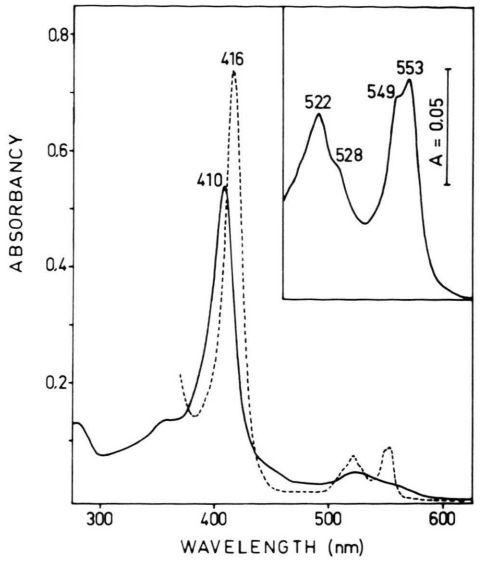

Fig. 2. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) cytochrome *c*-553(549) of *Ectothiorhodospira shaposhnikovii*. ——— oxidized; ------ reduced; insert shows absorption maxima of reduced α- and β-bands.

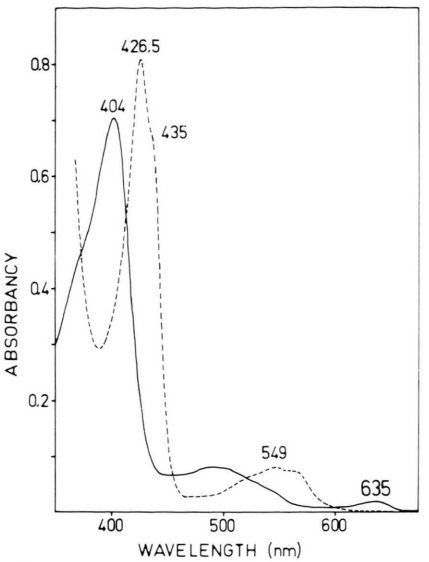

Fig. 3. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) cytochrome *c'* of *Ectothiorhodospira shaposhnikovii*. ——— oxidized; ------ reduced.

and at 409 nm in the oxidized state. Quite unusual is the fact that the reduced γ-band showed no higher absorbancy than the oxidized one. Furthermore it cannot be excluded that membrane bound cytochrome *c*-553 contained a flavin moiety (see bleaching between 460 nm and 490 nm in the spectrum of the reduced protein). To further clarify these two observations, investigations with a purified preparation of this protein will be necessary.

The difference spectrum (reduced-minus-oxidized) of membrane-bound cytochrome *c*-552.5 showed maxima at 552.5 nm, 523 nm and 428 nm (Fig. 6).

Cytochrome *b*-558 has absorption maxima at 424.5 nm, 526.5 nm and 557.5 nm in the reduced form and at 416 nm in the oxidized state (Fig. 7). The best purity index obtained was 0.26 ($A_{280}/A_{424.5}$). The heme-moiety of the protein could be extracted into methyl-ethyl-ketone and showed a maximum at 556.5 nm in alkaline pyridine (according to Bartsch [21]).

Molecular weight and isoelectric point

Electrophoresis on polyacrylamide gels in the presence of 0.1% SDS yielded molecular weights of

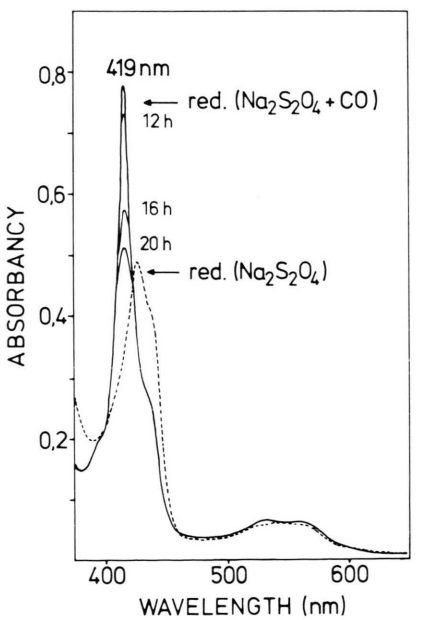

Fig. 4. Absorption spectra of reduced (by dithionite) and reduced carbon monoxide treated cytochrome *c'* of *Ectothiorhodospira shaposhnikovii*. CO was bubbled through the sample in the cells, sealed with a serum stopper. Absorption changes of the CO-treated cytochrome *c'* were followed with time. After 12 h the sample was exposed to light.

10400 for cytochrome *c*-553(549), of 33000 for cytochrome *c'*, and of 15800 for cytochrome *b*-558. Gel filtrations through Sephadex G-75 resulted in molecular weights of 10700 (cyt *c*-553(549)), 33500 (cyt *c'*) and 17800 (cyt *b*-558). The conformity of molecular weights for the two *c*-type cytochromes indicated i) that cytochrome *c*-553(549) contained

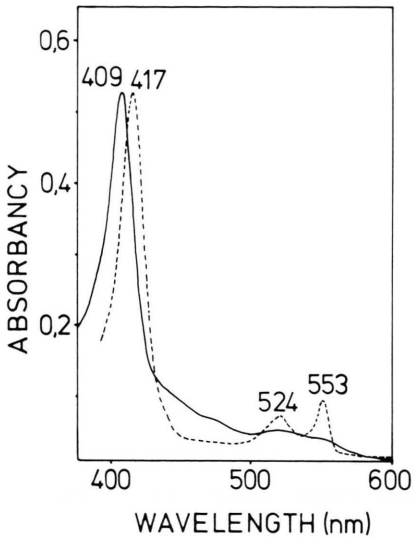

Fig. 5. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) cytochrome *c*-553 of *Ectothiorhodospira shaposhnikovii*. ——— oxidized; ------ reduced.

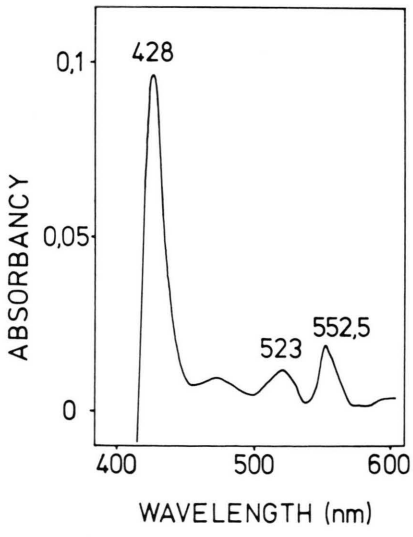

Fig. 6. Difference spectrum of solubilized cytochrome *c*-552.5 of *Ectothiorhodospira shaposhnikovii*, reduced (by dithionite) minus oxidized.

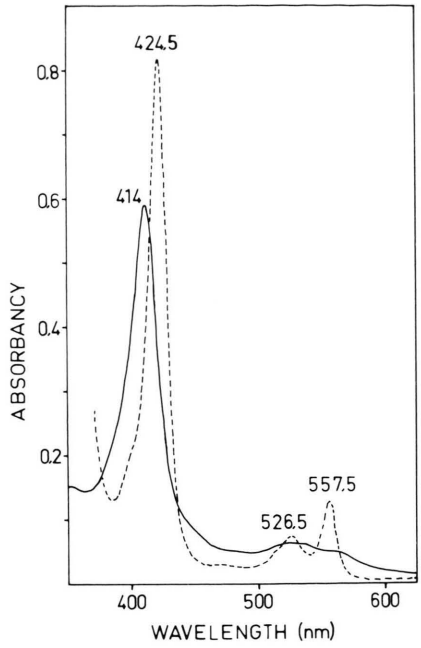

Fig. 7. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) cytochrome *b*-558 of *Ectothiorhodospira shaposhnikovii*. ——— oxidized. ------ reduced.

no flavin moiety and ii) that cytochrome *c'* was not an oligomer. Even after boiling cytochrome *c'* for 30 min in 1% SDS, no subunits of the protein could be detected.

The isoelectric points of the heme proteins – estimated by flat bed electrofocusing – were found at pH 5.1 for cytochrome *c*-553(549), at pH 4.5 for cytochrome *c'* and at pH 4.1 for cytochrome *b*-558.

Redox potentials

The midpoint oxidation-reduction potentials at pH 7.0 were +248 mV for cytochrome *c*-553(549), +37 mV for cytochrome *c'* and −210 mV for cytochrome *b*-558. Redox titrations were performed with 17 nmol of cytochrome *c*-553(549), 12 nmol of cytochrome *c'* and 15 nmol of cytochrome *b*-558. The ferricyanide titration curves were identical with theoretical values (calculated by the Nernst equation for a one-electron-transfer ($n = 1$) and the corresponding redox potentials (E'_0)), indicating that each protein molecule had only one heme group.

Discussion

In addition to the iron sulfur proteins previously described by Kusche and Trüper [18] the purple

sulfur bacterium *Ectothiorhodospira shaposhnikovii* contains four *c*-type cytochromes: cytochrome *c*-553(549) – which resembles *f*-type cytochromes –, high spin cytochrome *c'* and membrane-associated cytochromes *c*-553 and *c*-552.5. Additionally the organism contains at least one *b*-type cytochrome. Besides this cytochrome *b*-558 which could be clearly identified there were spectrophotometrical hints for the existence of a high spin cytochrome *b'*.

In contrast to high spin cytochromes *c'*, which have been found in many phototrophic purple bacteria [23], high potential cytochrome *c*-553 with a distinct shoulder in the α-band has been found only in *Chromatium vinosum* [6], *Thiocapsa pfennigii* [12] and *Rhodopseudomonas gelatinosa* [24]. Recently Meyer [16] reported that *E. vacuolata* contains a "cytochrome c_5-like protein" which may be similar to cytochrome *c*-553(549) we found in *E. shaposhnikovii*. Compared with the corresponding protein of *C. vinosum* (Table II), cytochrome *c*-553(549) of *E. shaposhnikovii* is less acidic (pI = 5.1) and has a lower redox potential ($E_{m,7} = +248$ mV). All other properties, such as purity index and molecular weight are in good accordance. Cytochrome *c*-553(549) was present in *E. shaposhnikovii* whether the culture medium contained a reduced sulfur compound or not. The protein was found only in low concentrations (*cf.* Table I). These two findings agree with the results of Cusanovich and Bartsch [6], who found only low amounts of cytochrome *c*-553(550) in *C. vinosum* under both photoautotrophic and photoheterotrophic conditions. Thus it seems unlikely that cytochrome *c*-553(549) participates in the anaerobic oxidation of reduced sulfur compounds.

Together with two high potential iron sulfur proteins [18] cytochrome *c'* is one of the major electron transfer proteins of *E. shaposhnikovii*. It could be obtained from the soluble fraction as well as from the chromatophores of cells grown in the presence and absence of reduced sulfur compounds (Table I). The yields, however, were about 4.5-times lower when cells were grown without oxidizable sulfur in the medium. Cytochrome *c'* was isolated in the oxidized state indicating a fairly low redox potential. With an $E_{m,7}$ of +37 mV, it is in the range of other high spin cytochromes *c'* that have been shown to possess redox potentials between −10 mV and +160 mV. Its molecular weight of 33500 (estimated by gel filtration through Sephadex G-75) and of 33000 (by SDS-PAGE), is comparably high (Table II). Other cytochromes *c'* so far known have been isolated as dimers with molecular weights of up to 37000 but disintegrate into monomers with molecular weights between 12000 and 14000 upon treatment with SDS. It is possible that cytochrome *c'* of *E. shaposhnikovii* also consists of subunits, but this seems unlikely as the protein even withstood boiling for 30 min in the presence of 1% SDS.

Besides the described electron transfer proteins, we were able to obtain a small fraction of cytochrome *c*-553 by detergent treatment of chromatophores from cells grown without reduced sulfur

Table II. Comparison of molecular properties of cytochrome *c*-553(549), cytochrome *c'* and cytochrome *b*-558 of *Ectothiorhodospira shaposhnikovii* with those of corresponding proteins of other phototrophic bacteria.

Property	*E. shaposhnikovii* Cyt *c*-553(549)	*C. vinosum* [a] Cyt *c*-553(550)	*E. shaposhnikovii* Cyt *c'*	*C. vinosum* [b] Cyt *c'*	*E. shaposhnikovii* Cyt *b*-558	*R. rubrum* [b] Cyt *b*-558
molecular weight	10400 (P)	12989 (A)	33000 (P)	37000 (S)	15800 (P)	23000
pI	5.1	4.38	4.5	4.6	4.1	4.6
$E_{m,7}$	+248 mV	+330 mV	+37 mV	−5 mV	−210 mV	−204 mV
maxima (red.)	416 522 553(549)	417.5 523 553(550)	426.5 549	426 547	424.5 526.5 557.5	425 – 557.5
maxima (ox.)	410	410	404, 635	399, 634	414	417
purity index (red.) ($A_{280}/A_{vis,max}$)	0.18	0.15	0.48	–	0.26	1.3 (ox.)

[a] Cusanovich and Bartsch [6].
[b] Data taken from Bartsch [24].
Molecular weights were estimated by polyacrylamide gel electrophoresis with SDS (P), molecular sieve size determination (S), amino acid composition (A).

source. This protein of which only absorption spectra could be recorded, had γ-bands (409 nm ox.; 417 nm red.) of equal absorbancy. This may be due to possible damaging during treatment with Triton X-100. The absorption spectrum of oxidized cytochrome *c*-553 shows a shoulder between 490 nm and 460 nm which is bleached out completely in the dithionite reduced spectrum. This indicates that the protein possibly contains a flavin moiety. As far as we know, flavocytochromes have been described for two members of the Chromatiaceae. Trüper and Rogers [13] found flavin and a *c*-type cytochrome as constituents of the APS-reductase of *T. roseopersicina*. Flavocytochrome *c*-552 of *C. vinosum* possesses sulfide: cytochrome-*c*-reductase activity [25, 15] and catalyzes the reduction of elemental sulfur to sulfide in the presence of a suitable electron donor [26]. In contrast, it is unlikely that membrane bound cytochrome *c*-553 of *E. shaposhnikovii* has similar properties, because this protein was present only in cells grown in the absence of a reduced sulfur compound.

The expression of membrane-bound cytochrome *c*-552.5 in *E. shaposhnikovii* did not depend on growth conditions. Because of this and its close attachment to the chromatophores, it may participate in cyclic electron transport. Pottosin and coworkers [27] reported that a *c*-type cytochrome with an $E_{m,7}$ of +290 mV was a component of the photosynthetic reaction center of the same organism. Further studies will have to show whether these two proteins are identical.

The occurrence of a cytochrome *b*-558 in *E. shaposhnikovii* was unexpected. For a long time, these protoheme IX containing cytochromes were thought to occur only in members of the *Rhodospirillaceae* but not in purple and green sulfur bacteria [28, 29]. First evidence for the existence of a *b*-type cytochrome in *Chlorobium limicola* and *Chl. thiosulfatophilum* was given by Fowler [30] and shortly afterwards by Knaff and Buchanan [31] who described chromatophore-associated *b*-type cytochromes from *Chromatium* and *Chlorobium* and postulated the participation of cytochrome *b* in sulfide oxidation. In contrast to these hemoproteins, cytochrome *b*-558 of *E. shaposhnikovii* was soluble or only loosely attached to the membranes. Its low redox potential of −210 mV and absorption chracteristics are similar to the corresponding protein of *Rhodospirillum rubrum* (Table II) whereas its molecular weight (15800) is much lower than those of other *b*-type cytochromes. Currently, only little is known about the possible functions of cytochromes *b*, except that they may function as electron donors in nitrate reducing organisms [32]. In *Thiobacillus thiooxidans* cytochrome *b* seems to be involved in the oxidation of sulfide to sulfate [33]. The fact that cytochrome *b*-558 was only found in cells of *E. shaposhnikovii* grown in the presence of reduced sulfur might indicate a possible participation in sulfur metabolism which we are currently investigating.

Acknowledgement

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