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Characterization of reaction center/antenna complexes from bacteriochlorophyll *a* containing *Ectothiorhodospiraceae*

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Pigment-protein complexes have been isolated from three bacteriochlorophyll-*a*-containing *Ectothiorhodospira* species: *E. mobilis*, *E. shaposhnikovii* and *E. halophila*, using detergent solubilisation of photosynthetic membranes and sucrose-density gradient centrifugation. This isolation procedure yields pure LHII complexes and reaction centers with either one or both antenna complexes attached (i.e., RC/LHI and RC/LHI/LHII complexes). The RC/LHI/LHII complexes consist of three reaction center polypeptides, H (35 kDa), M (34 kDa) and L (26.5 kDa), plus two LHI and two LHII antenna polypeptides. In photosynthetic membranes of the three *Ectothiorhodospira* species two major types of heme *c* are present, of low (25 mV) and high (297 mV) midpoint potential, respectively. These heme groups belong to separate cytochromes. During solubilisation and purification, no loss of the H subunit from the reaction centers is observed. However, the reaction centers do lose their *c*-type cytochromes. The high-potential cytochrome *c* is completely lost during solubilisation of RC complexes from photosynthetic membranes, while the low-potential cytochrome is largely lost during isolation of RC/LHI complexes. The residual amount of this low-potential cytochrome, associated with the RC/LHI complexes, varies with the salt concentration used during the isolation, but remains below 0.2 mol heme *c* per mol reaction center. This is less than 20% of the amount originally present in photosynthetic membranes.

Introduction

Research on anoxygenic photosynthesis is focused on a small number of closely related proteobacteria like: *Rhodobacter sphaeroides*, *Rhodopseudomonas viridis*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum* [1]. Much less is known about the molecular details of photosynthesis in representatives of the *Ectothiorhodospiraceae* [2]. These bacteria carry out anoxygenic photosynthesis, using reduced sulfur compounds as electron donors [3]. In this process sulfur is deposited extracellularly, as an intermediate in the oxidation process. Characteristic for this group of bacteria is their optimal growth in saline and alkaline media [4]. Among the *Ectothiorhodospiraceae* are two groups that contain either bacteriochlorophyll *a* or *b*. In parallel with this distinction these groups display physiological differences, like the (in)ability to grow autotrophically with sulfide as an electron donor [3]. The light-harvesting (LH) complexes and reaction centers (RCs) in the *Ectothiorhodospiraceae* are embedded in intracytoplasmic lamellar membrane stacks, which form a continuum with the cytoplasmic membrane [5–7].

Our interest in photosynthesis in *Ectothiorhodospiraceae* was raised by the capacity of these organisms to grow under extremophilic conditions [4]. This brings up questions about generation, size and maintenance of ion gradients across their cytoplasmic membrane. A cyclic electron transfer chain, supposedly composed of a reaction center and a cytochrome *b/c* complex [3], is primarily responsible for the conversion of light energy into these ion gradients. A number of

Abbreviations: E_0', midpoint potential at (pH 7.0); kDa, kilodalton; LHI or LHII, light harvesting complexes I or II; octylglucoside, *n*-octyl β-D-glucopyranoside; RC(s), reaction center(s); SDS-PAA, sodium dodecyl sulfate polyacrylamide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane.

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reports have appeared on the properties of RCs from *Ectothiorhodospiraceae* [8–12]. As a representative of the bacteriochlorophyll *b* containing species, *E. halochloris* has been characterized in considerable detail with respect to its reaction center and antenna complex [10–13]. In the bacteriochlorophyll *a* containing group most work on this aspect has been performed in *E. shaposhnikovii* [14,15] and an unidentified *Ectothiorhodospira* sp. [8,9]. The RCs of the *Ectothiorhodospiraceae*, however, have been described to have several uncommon characteristics: a bacteriochlorophyll *a* containing *Ectothiorhodospira* sp. was reported to contain a heme staining H subunit [8]. Recently, however, this heme staining H subunit has been re-characterized as a cytochrome and in this latter report the H subunit was described to be lost from the RCs [9]. Loss of the H subunit was also reported for the bacteriochlorophyll *b* containing *E. halochloris*. In this organism the H subunit was preferentially isolated with the antennae [13]. *E. shaposhnikovii* was reported to contain RC associated high- and low-potential cytochromes [14,15]. A fourth (cytochrome) subunit in addition to the three polypeptides routinely found in the RCs of purple bacteria [16] has, however, been clearly demonstrated only in *Rps. viridis*, *Chromatium vinosum* and *Rps. acidophila* [17–20].

In this paper we report on the isolation and characterization of pigment-protein complexes of three well-characterized species of the bacteriochlorophyll *a* containing *Ectothiorhodospiraceae*. We used the isolation procedure essentially as described by Varga and Staehelin [21] and slightly modified by Molenaar et al. [22], so as to avoid the use of harsh detergents, which may lead to loss of RC components. The RCs are characterized by absorption spectroscopy, sodium dodecyl sulfate gel electrophoresis (SDS-PAGE), Western blotting and redox difference spectroscopy.

Materials and Methods

Strains and growth conditions

Ectothiorhodospira mobilis strain BN9903 and *E. halophila* strain BN9630 were obtained from J.F. Imhoff (Rheinische Friedrich-Wilhelms-Universität, Bonn, F.R.G.). *E. shaposhnikovii* strain M3 [23] was obtained from H. van Gemerden (University of Groningen, The Netherlands). Cells were grown anaerobically under low light intensity at their optimal salt concentration and temperature [4] in the medium described by Imhoff and Trüper [24], slightly modified by replacing sodium succinate by sodium acetate and by omitting the vitamin solution.

Isolation of intracellular membranes

Cells at the end of exponential growth were harvested, washed twice in 50 mM sodium phosphate buffer (pH 8.0), supplemented with 5 mM $MgCl_2$, 6.5 mM Na_2S, 10 mM KCl and sucrose or NaCl and resuspended in the same buffer. For the experiments presented in Figs. 2 to 4, 10% sucrose was added to this buffer. For the other experiments NaCl was used: 0.5 M for *E. mobilis* and *E. shaposhnikovii* and 2.2 M for *E. halophila*. These salt concentrations are the optimal concentrations for growth. Cells were broken by two successive passages through a French pressure cell (250 MPa, 4°C). Debris was removed by low-speed centrifugation (30 min, $17000 \times g$ at 4°C). Intracellular membranes were collected by ultracentrifugation (1.5 h, $140000 \times g$ at 4°C) and resuspended in the buffer described above at a concentration of 1 mM bacteriochlorophyll. These intracellular membranes ('chromatophores') were stored in liquid nitrogen until further use.

Isolation of RC complexes

RC complexes were isolated according to a procedure modified from Varga and Staehelin [21,22]. The complexes were extracted from chromatophores in the buffer described above, supplemented with 20 mM EDTA and either 30 mM (RC/LHI/LHII, 20 min at 0°C) or a mixture of 30 mM *n*-octyl β-D-glucopyranoside (octylglucoside; Boehringer-Mannheim, F.R.G.) and 19 mM sodium deoxycholate (RC/LHI, 60 min at 0°C). During solubilisation the preparation was whirlmixed every 5 min. Next, 0.35 ml was layered on top of a 9 ml linear sucrose gradient (10–55% sucrose (w/v) in 50 mM sodium phosphate (pH 8.0) supplemented with 6.5 mM Na_2S and either 30 mM octylglucoside or a mixture of 30 mM octylglucoside and 19 mM sodium deoxycholate). For the experimental data presented in Table I, 300 mM NaCl was added to the sucrose gradients. The gradients were centrifuged at $200000 \times g$ for 19 h at 4°C. Pigmented bands were recovered from the gradient, analyzed spectrophotometrically and stored on ice when used within 2 days. For longer periods samples were stored in liquid nitrogen.

Analytical gel electrophoresis

Analytical gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to Laemmli [25] in a Bio-Rad (Richmond, U.S.A.) mini slab gel apparatus. Preceding electrophoresis, the samples were incubated with SDS at 80°C for 1 min. The gels were stained with Coomassie brilliant blue G250. Phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) were used as molecular mass markers (Pharmacia, Uppsala, Sweden).

In order to detect heme-dependent peroxidase activity, intact cells in 50 mM Tris (pH 7.0) supplemented with 10% sucrose, were sonicated (six times, 30 s at 0°C) and mixed with an equal volume of ice-cold di-

ethyl ether/ethanol (50:50, v/v). Proteins were recovered from the buffer/ethanol phase and dialysed against 50 mM Tris (pH 7.0) (1 h, 4°C). The samples were immediately incubated with SDS at room temperature for 1 h and electrophoresed. Staining for heme-dependent peroxidase activity was performed according to Thomas et al. [26]. Subsequent Coomassie staining of these gels was carried out according to Fairbanks et al. [27].

Immunodetection

Western blotting was performed as described by Towbin et al. [28]. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad. Polyclonal antibodies, directed against *Rhodobacter sphaeroides* RCs [29] were a gift from G. Feher (UCSD at La Jolla, U.S.A.). Prior to incubation with antibodies the nitrocellulose sheet was stained for proteins with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid.

Redox potentiometry

Redox titrations were performed essentially as described by Wilson [30] and Dutton [31] in an Aminco DW2000 spectrophotometer, equipped with a magnetic stirrer. A 3 ml glass cuvette was fitted with a teflon stopper with gaps for a platinum electrode, an Ag/AgCl reference electrode, an argon gas line and a gap for additions. The platinum electrode was cleaned with hydrochloric acid after each titration to maintain a fast response. Before and after each titration, the set-up was calibrated with saturated solutions of quinhydrone in 500 mM sodium phosphate at a range of pH values [32]. Measurements were performed in 100 mM sodium Hepes (pH 7.0), supplemented with 2 mM $MgCl_2$. Oxygen was excluded from the cuvette by flushing with argon (oxygen content: ≤ 0.5 ppm). Reductive titrations were performed by stepwise addition of a solution of sodium ascorbate (200 mM) or sodium dithionite (1 M), while oxidative titrations were performed with potassium ferricyanide (200 mM).

For a fast exchange and to stabilize the redox potential, titrations were carried out in the presence of the following mediators: 50 μM 1,4-benzoquinone ($E_0' = +293$ mV), 100 μM hydroquinone ($E_0' = +280$ mV), 100 μM ubiquinone$_0$ ($E_0' = +100$ mV), 40 μM *N*-methylphenazonium methosulfate ($E_0' = +85$ mV), 40 μM *N*-ethyldibenzopyrazine ethyl sulfate ($E_0' = +65$ mV), 100 μM tetramethyl-*p*-benzoquinone ($E_0' = +5$ mV), 100 μM 2-OH-1,4-naphthoquinone ($E_0' = -139$ mV), 50 μM anthraquinone-1,5-disulfonate ($E_0' = -184$ mV), 50 μM anthraquinone-2-sulfonate ($E_0' = -225$ mV) and 50 μM 1,1'-dimethyl-4,4'-bipyridylium dichloride ($E_0' = -440$ mV). Titration data were transformed logarithmically and best-fitting curves were determined by means of linear-least-squares analysis.

Analytical procedures

Protein was determined according to Bradford [33] using bovine serum albumin as a standard. Bacteriochlorophyll *a* was determined by the method of Clayton [34] using an extinction coefficient at 771 nm of 65.3 $mM^{-1}\,cm^{-1}$ [35]. For calculations of cytochrome *c* concentrations an extinction coefficient of 20 $mM^{-1}\,cm^{-1}$ was used [8]. The RC concentration was calculated from redox difference spectra at 887 nm with an extinction coefficient of 114 $mM^{-1}\,cm^{-1}$ [8].

Results

Isolation of reaction center complexes

We have applied the method of Varga and Staehelin [21,22] to isolate RC complexes from *Ectothiorhodospira*, with the aim of isolation of functionally intact RCs. To accomplish this, cells were grown at relatively low light intensity, in order to have a maximal content of antenna pigments present. From these cells two different RC complexes were solubilised separately, to optimise the yield of both RC/LHI and RC/LHI/LHII. The solubilisation with only octylglucoside and subsequent centrifugation on a sucrose gradient yielded pure RC/LHI/LHII complexes for all three *Ectothiorhodospira* species (Fig. 1). These complexes were recovered at 1.13 g cm^{-3}. When a combination of octylglucoside and deoxycholate was used, a separation of RC/LHI and LHII complexes was achieved, with hardly any RC/LHI/LHII remaining (with these detergents at 1.17 g cm^{-3}). RC/LHI complexes were recovered at 1.12 g cm^{-3} and LHII complexes at 1.09 g cm^{-3}. The RC/LHI/LHII complexes of the three strains display approximately the same absorption characteristics as their corresponding chromatophores, only the LHII over LHI ratio differs slightly. The spectral contamination of RC/LHI with LHII is very small, particularly for *E. mobilis* (Fig. 1A). In the spectra of these complexes isolated from *E. halophila* (Fig. 1C) a small shoulder, indicating LHII contamination, can be seen at approx. 840 nm. This is most likely due to overlapping bands in the sucrose-density gradient.

The infrared bacteriochlorophyll absorption maximum at 850 nm of isolated LHII complexes has shifted slightly to the blue (to 840 nm, Fig. 1A, C). In *E. shaposhnikovii* this shift already occurs during solubilisation of RC/LHI/LHII complexes from chromatophores (Fig. 1B). The absorption maxima of LHI and carotenoids do not shift upon the addition of detergents. The carotenoid over bacteriochlorophyll ratio, particularly for LHII, appears to be slightly higher for *E. mobilis* (Fig. 1B).

Analysis of the polypeptide composition of the RC complexes

The polypeptide composition of isolated RC and antenna complexes was analyzed by SDS-PAGE. Care

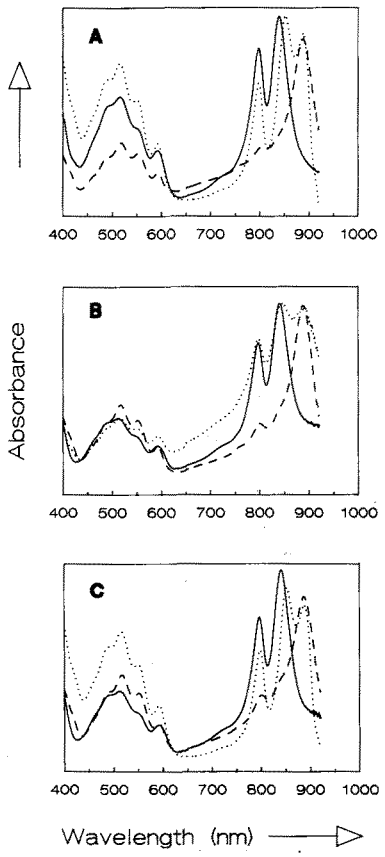

Fig. 1. Absorption spectra of RC/LHI/LHII (·····), RC/LHI (— — —) and LHII (———) complexes isolated from *E. mobilis*, *E. shaposhnikovii* and *E. halophila*, respectively. Samples of sucrose gradients were diluted 30-fold in distilled water. (A) *E. mobilis*; full-scale absorption of the LHII complexes equals 0.7, while that of the RC complexes equals 0.35. (B) *E. shaposhnikovii*; full-scale absorption of the RC/LHI/LHII complexes equals 0.2, while that of the other complexes equals 0.4. (C) *E. halophila*; full-scale absorption of the RC/LHI/LHII complexes equals 0.4, while that of the other complexes equals 0.5.

was taken to prevent aggregation of polypeptides during preincubation of the proteins with SDS. Therefore, incubation was performed at 80°C for only 1 min. Longer incubation times and/or higher incubation temperatures caused aggregation of the RC subunits. As shown in Fig. 2, RCs in RC/LHI complexes isolated from both *E. mobilis* and *E. shaposhnikovii* (lanes 2 and 4) consist of four subunits. We tentatively assign these to a cytochrome and the H, M and L subunit of the RC, with molecular masses of 42, 35, 34 and 26.5 kilodalton (kDa), respectively. The difference in size between the H and M subunit is very small; therefore, these two components often appear as one on a 15% SDS-PAA gel. On gels with a lower percentage of polyacrylamide, however, the light harvesting polypeptides are not separated from the front of the gel. Therefore, the 15% SDS-PAA gel has been selected for presentation. Significant variations in the apparent molecular weight of the H, M and L subunits, as a function of the percentage polyacrylamide, were not observed. The apparent molecular masses as determined with gels of 12%, 13%, 14% and 15% polyacrylamide, were within 1 kDa. The apparent molecular weight of the cytochrome subunit ranged from 39 to 45 kDa. RC complexes of *E. halophila* also showed the presence of four components with nearly the same molecular weight as described for *E. mobilis* and *E. shaposhnikovii*. However, the RCs of this organism showed extensive aggregation in our SDS-PAGE system; a presentation of these RCs in the form of a Coomassie stained gel is not included.

Fig. 2 demonstrates that polypeptides of LHI and LHII are present as diffuse bands in the low molecular weight region of the gel. In RC/LHI complexes of both *E. mobilis* and *E. shaposhnikovii* (lanes 2 and 4) two small polypeptides of LHI are present. The LHII complexes of both organisms (lanes 3 and 5) show the presence of two additional small polypeptides. The LHII complexes also show the presence of a very pronounced band between the cytochrome and the H subunit. This band is only observed in LHII (lanes 3 and 5) and RC/LHI/LHII (lane 7) complexes when these samples, prior to electrophoresis, are incubated at elevated temperatures (i.e., like 80°C). This band is most likely due to aggregation of the LHII polypeptides since this band is absent when the samples are incubated with SDS at room temperature. However, when the RC complexes are incubated with SDS at room temperature as described for *Ectothiorhodospira* sp. [9] extensive aggregation of the light harvesting polypeptides of both LHI and LHII is observed in the upper part of the gel. In RC/LHI/LHII complexes this additional band is rather dominant; for this reason RC/LHI/LHII complexes

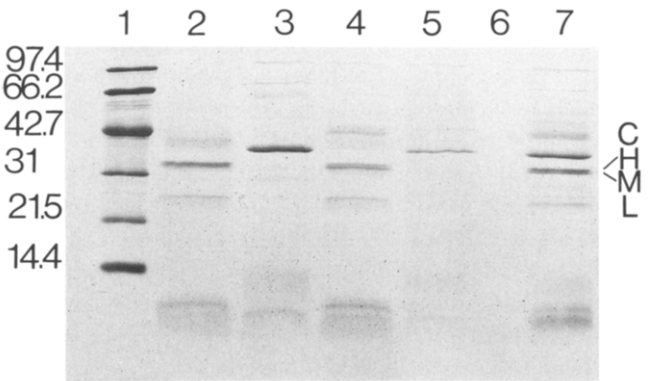

Fig. 2. Coomassie blue staining of a 15% polyacrylamide gel showing purified RC complexes of *E. mobilis* and *E. shaposhnikovii*. Lane 1, marker proteins (1 μg); the molecular masses are indicated in the margin in kDa. Lanes 2 and 3 contain RC/LHI and LHII complexes of *E. mobilis*. Lanes 4 and 5 contain the corresponding complexes of *E. shaposhnikovii*. Lane 7, RC/LHI/LHII complexes of *E. mobilis*. No protein was applied to lane 6. The amount of protein applied to lanes 2 to 5 as well as to lane 7 was 5 μg. The complexes were isolated without addition of NaCl as described in Materials and Methods.

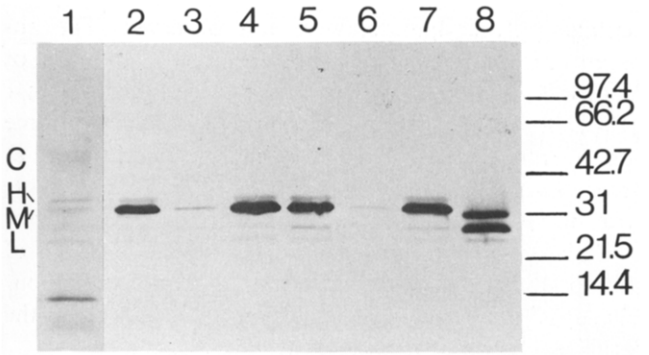

Fig. 3. Western blot of a 12% polyacrylamide gel with various samples of *E. mobilis*, *E. shaposhnikovii* and *Rb. sphaeroides*. The blot was stained for protein with Ponceau S and subsequently incubated with antibodies directed against *Rb. sphaeroides* RCs. Lane 1 contains protein-stained RC/LHI complexes of *E. mobilis*. Lanes 2 to 4 contain RC/LHI/LHII, LHII and RC/LHI complexes of *E. mobilis*, respectively. Lanes 5 to 7 contain the corresponding complexes of *E. shaposhnikovii*. Lane 8 contains RC/LHI complexes of *Rb. sphaeroides* (0.5 μg). The position of the marker proteins is indicated in the right margin (in kDa). The protein content of lanes 1 to 7 was 2 μg. The RC complexes of both *Ectothiorhodospira* species are isolated without addition of NaCl as described in Materials and Methods.

are presented on a separated lane of the gel (lane 7), only for *E. mobilis*.

Immunodetection of RC subunits

Cross reaction of RC complexes from either *E. mobilis*, *E. shaposhnikovii* or *Rb. sphaeroides* with antibodies directed against *Rb. sphaeroides* RCs are shown in Fig. 3. RC/LHI complexes of *Rb. sphaeroides* (lane 8) clearly show cross reaction with the antibodies at the position of the H and M subunit. Cross reaction with the L subunit is much weaker. Both the RC/LH/LHII (lanes 2 and 5) and the RC/LHI (lanes 4 and 7) complexes of *E. mobilis* as well as of *E. shaposhnikovii* clearly show cross reaction with the antibodies at the position of their M subunit. Cross reaction with the H subunit is weaker, while a very weak staining of the L subunit is observed in these RC complexes. LHII complexes of both organisms (lanes 3 and 6) are slightly contaminated with RC complexes, as is evident from the very weak cross reaction at the position of the M subunit.

The results presented above support the conclusion that RCs from *E. mobilis* and *E. shaposhnikovii* do contain an H, M and L subunit. No loss of the H subunit from the RCs [9,13] is observed under the conditions applied here. The RC subunits of both species show similarity with the corresponding subunits of RCs from *Rb. sphaeroides*. The presence of a cytochrome subunit will be discussed in the next section.

RCs of bacteriochlorophyll a containing Ectothiorhodospira species do not contain a bound cytochrome subunit

Two different cytochromes *c* are present in chromatophores of *E. shaposhnikovii*; one cytochrome *c* with a high and one with a low midpoint potential [14,15]. Elsewhere [8,9,12], it has been concluded that *Ectothiorhodospiraceae* contain a 4-heme *c*-type cytochrome, tightly bound to the reaction centers. In order to determine whether the RCs from *Ectothiorhodospira* do or do not contain a bound cytochrome, SDS-PAA gels were stained for heme-containing proteins. Care was taken to prevent apparent heme staining by bacteriochlorophyll *a*. Therefore, samples were first mixed with diethyl ether/ethanol to extract bacteriochlorophyll. As shown in Fig. 4, cells of both *E. shaposhnikovii* and *E. mobilis* (lanes 2 and 3) contain two heme *c* staining bands of approx. 42 and 73 kDa. Chromatophores also contain these two heme-staining bands. Compared to the 42 kDa heme-staining band of *E. mobilis* and *E. shaposhnikovii*, the corresponding band in cells of *E. halophila* (lane 1) has a slightly higher molecular mass. These cells show the presence of an additional heme *c* staining band of high molecular mass. The heme-staining band at the front of the gel may be due either to the presence of a small cytochrome *c* or to free heme *c* or other porphyrins. RC/LHI/LHII complexes of *E. mobilis* (lane 4) exclusively show the presence of the 42 kDa heme-staining band. A protein

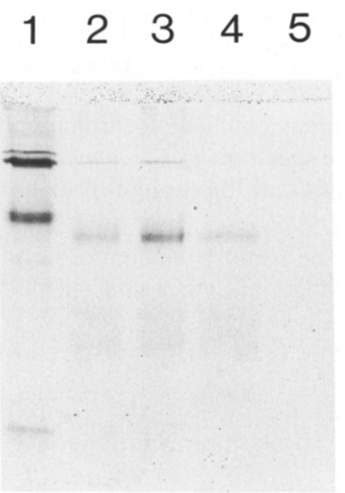

Fig. 4. Heme staining of a 12% polyacrylamide gel containing cells and/or RC complexes of *E. mobilis*, *E. shaposhnikovii* and *E. halophila*. Lanes 1 to 3, sonicated cells of *E. halophila*, *E. shaposhnikovii* and *E. mobilis*, respectively. Lane 4, RC/LHI/LHII complexes of *E. mobilis*. These complexes were isolated without addition of NaCl. Lane 5 contains RC/LHI complexes of *E. mobilis* which were isolated and subsequently washed (once) at high ionic strength (500 mM NaCl). The amount of protein applied in lanes 1 to 3 was approx. 15, 7 and 10 μg, respectively. The protein content of lanes 4 and 5 was 5 μg.

of similar molecular mass was already observed in RC complexes of both *E. mobilis* and *E. shaposhnikovii* (Fig. 2). In RC/LHI complexes of *E. mobilis* which are isolated and subsequently washed (once) at high ionic strength (500 mM NaCl) no heme *c* staining band is observed (lane 5, Fig. 4).

The results presented above indicate that cells of the three investigated *Ectothiorhodospira* species contain at least two different *c*-type cytochromes. In cells of *E. halophila* possibly two additional *c*-type cytochromes are present. Except for the 42 kDa component, these cytochromes are lost during the isolation of RC complexes (see also below). To characterize these cytochromes further, we performed a redox-titration of chromatophores of *E. mobilis*, which is presented in Fig. 5. The amplitude of the absorption changes in a reductive titration reaches its maximum within the range of −50 to −100 mV. Above this redox potential, the absorption changes biphasically to a minimum at approx. 400 mV. Transformation of the data to a logarithmic scale and subsequent fitting of the curves gives the best result with $n = 1$ and two midpoint potentials of 25 and 297 mV, respectively. Compared to the high-potential heme *c*, the low-potential heme *c* of *E. mobilis* as well as *E. halophila* absorbs at a slightly shorter wavelength (555 vs 553 nm, see Fig. 6); therefore the redox titration was performed at 554 nm.

The redox difference spectrum of chromatophores of *E. mobilis* (Fig. 6A) shows that approx. 50% of the total amount of heme *c* can be reduced with ascorbate, whereas the remainder can only be reduced by dithionite. This indicates that in chromatophores of *E. mobilis* approx. 50% of the total amount of heme *c* is of high-midpoint potential. This ratio is slightly different from the one in Fig. 5 and reflects the differences between different batches of chromatophores. The redox difference spectrum of *E. halophila* chromatophores (Fig. 6B) shows that the content of high-potential heme

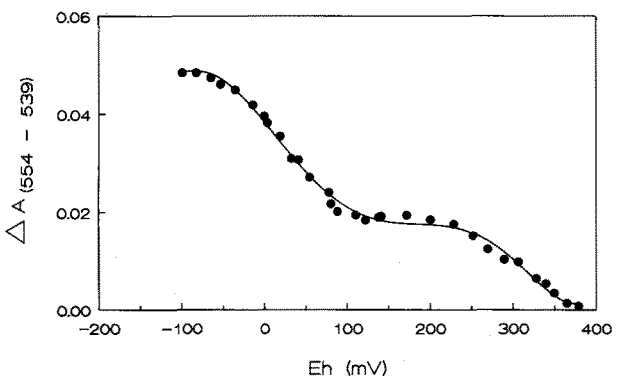

Fig. 5. Redox titration of absorption changes (554–539 nm) of *c*-type cytochromes from chromatophores of *E. mobilis*. The chromatophores were isolated in the presence of 0.5 M NaCl. The RC concentration of the sample was 95 μM. Measurements were performed as described in Materials and Methods.

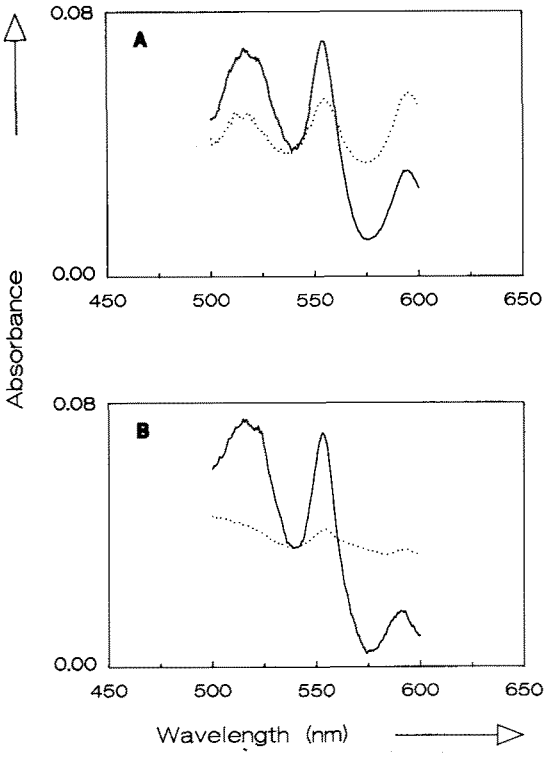

Fig. 6. Redox spectra of cytochromes in chromatophores of *E. mobilis* (A) and *E. halophila* (B). The samples were oxidized with 2 mM potassium ferricyanide and reduced with 10 mM sodium dithionite (———) or 5 mM sodium ascorbate (· · · · · ·), respectively. (A) The chromatophore suspension, prepared in the presence of 0.5 M NaCl, was diluted with 100 mM Hepes (pH 7.0) and 2 mM $MgCl_2$ to a final RC concentration of 1.1 μM. (B) The chromatophores were isolated in the presence of 2.2 M NaCl. Final RC concentration was 3.3 μM.

c in this sample is only 20% of the total amount of heme *c*.

The results presented in Figs. 5 and 6 clearly show that chromatophores of *E. mobilis* contain two different types of heme *c*, i.e., of low and high midpoint potential. These two types of heme *c* belong to two different cytochromes as is supported by the results presented in Fig. 4.

To investigate whether part of the cytochrome *c* is lost during the isolation procedure, as is suggested by the low content of high-potential cytochrome *c* in chromatophores of *E. halophila* (Fig. 6B) and the presence of only one cytochrome *c* in RC/LHI/LHII complexes of *E. mobilis* (Fig. 4), the cytochrome *c* content of various samples was measured (Table I). Chromatophores of *E. mobilis* and *E. halophila* contain 0.7 and 0.08 mol high-potential heme *c* per mol RC, respectively. Strikingly, a complete loss of the high-potential cytochrome *c* does take place during solubilisation of RC complexes from chromatophores of both species. Furthermore, the low-potential cytochrome *c* is largely lost during the isolation of RC/LHI complexes. The residual amount of low-potential heme *c* of these complexes is less than 20% of the amount present in chro-

TABLE I

Heme c content of various RC preparations of E. mobilis and E. halophila

The measurements were performed as described at Fig. 6. The values are the average of two independent experiments. The RC complexes are isolated in the presence of NaCl as described in Materials and Methods.

fraction	Heme c_L/RC *	Heme c_H/RC
E. mobilis		
chromatophores	1.36±0.22	0.70±0.07
RC/LHI/LHII	1.17±0.04	–
RC/LHI	0.19±0.01	–
E. halophila		
chromatophores	0.42±0.01	0.08±0.01
RC/LHI/LHII	0.10±0.02	–
RC/LHI	0.08±0.01	–

* mol low- or high-potential heme *c* per mol reaction center. –, not detectable.

matophores; this is equivalent to less than 0.2 mol heme *c* per RC. When RC complexes are isolated and washed several times at a higher ionic strength, even more of the low-potential cytochrome *c* is lost.

From these results the conclusion can be drawn that the high-potential cytochrome *c*, which is only found in whole cells and chromatophores, is merely a contaminant of the RCs. The results further indicate that the low-potential cytochrome *c* is not an integral part of the RCs from the bacteriochlorophyll *a* containing *Ectothiorhodospira*. It is largely lost during preparation of the isolated core-unit of anoxygenic photosynthesis, the RC/LHI complexes.

Discussion

In this paper we describe the isolation and characterization of RCs from three strains of the well-characterized bacteriochlorophyll *a* containing *Ectothiorhodospira*: *E. mobilis* BN9903, *E. halophila* BN9630 and *E. shaposhnikovii* M3. With the isolation procedure presented here, pure RC/LHI/LHII, RC/LHI and LHII complexes can be obtained from these species. A striking feature of these RC complexes is the presence of an H subunit, as is shown by SDS-PAGE and cross reaction with antibodies against *Rb. sphaeroides* RCs (Figs. 2 and 3, respectively). This is in contrast to the previously reported loss of the H subunit from RCs of *E. halochloris* [13] and *Ectothiorhodospira* sp. [9]. It is probably due to the more gentle isolation procedure applied in this investigation. Loss of the H subunit may lead to changes in the binding site for the secondary quinone [36] and thereby alter functional characteristics of the RCs.

In LHII complexes of both *E. mobilis* and *E. shaposhnikovii* a weak cross reaction with antibodies against the *Rb. sphaeroides* RCs is visible, although no RC subunits can be observed in these complexes in Fig. 2. This is probably due to the much higher sensitivity of Western blotting compared to staining with Coomassie blue. These results suggest that contamination of LHII complexes with RC subunits is very minor.

In RC complexes of both *E. mobilis* and *E. shaposhnikovii* weak staining is observed at the position between the M and L subunit after protein staining as well as incubation with antibodies in Fig. 3. This band is also visible in SDS-PAA gels with preparations of *Ectothiorhodospira* sp. [9]. The absence of this additional band in the same complexes (with more protein applied) presented in Fig. 2 suggests that a very weak proteolysis of either the H or M subunit takes place. This could be due to storage of the dialysed samples for several days (at 4°C) before they were used for Western blotting.

Cells of both *E. mobilis* and *E. shaposhnikovii* contain two major types of cytochrome *c* as can be concluded from the results presented in Figs. 4 and 5. Cells of *E. halophila* contain possibly two additional *c*-type cytochromes. It is surprising that heme staining of cells of both *E. mobilis* and *E. shaposhnikovii* did not reveal additional *c*-type cytochromes. However, it is important to note in this respect that in a sample with roughly equal amounts of two different types of heme *c*, as determined with absorption spectroscopy, the intensity of heme staining on the gel differs significantly (Fig. 4, lane 3). The absence of additional *c*-type cytochromes in the two moderately halophilic strains may be due to this phenomenon or to differences in their relative abundance.

With respect to the *c*-type cytochromes, the strains investigated in this study differ from *Rps. viridis*. In *Ectothiorhodospira*, the high- and low-potential types of heme *c* that can be detected in chromatophores, do belong to different cytochromes. Furthermore, the association of the low-potential *c*-type cytochrome to the RCs is of ionic nature, as this cytochrome is largely lost during isolation of RC/LHI complexes. Incubation of RC complexes from either *E. mobilis*, *E. shaposhnikovii* or *E. halophila* with antibodies against the cytochrome subunit of *Rps. viridis* did not reveal an immunological cross reaction (not shown). These results underline the differences between the *c*-type cytochromes of the investigated *Ectothiorhodospira* species and the well known 4-heme *c*-type cytochrome of *Rps. viridis*. A 4-heme *c*-type cytochrome, firmly bound to the RC has until now only clearly been demonstrated in *Rps. viridis* [17,18], *Chr. vinosum* [19] and *Rps. acidophila* [20], whereas such a cytochrome may also be present in *Thiocapsa pfennigii* [38], *Rhodomicrobium vannielli* [39], *Erythrobacter* sp. (OCh 114) [40] and possibly some *Ectothiorhodospiraceae* [8,9,12,14,15]. In *Rhodocyclus gelatinosus* the 4-heme *c*-type cytochrome is easily dis-

sociated from RC/LHI complexes by washing with detergent [41]. Importantly, the latter cytochrome can not be washed off from the RC/LHI complexes with high concentrations of sodium chloride.

Although quantitative interpretation of our observations (Table I) is dependent on an assumption for the extinction coefficient of the cytochromes [8], we conclude that no cytochrome remains bound to the RC/LHI complexes of *E. halophila* and *E. mobilis* when these complexes are isolated in the presence of NaCl. Given the high similarity of *E. mobilis* and *E. shaposhnikovii* according to DNA-DNA hybridization [42], we consider it likely that the RCs from the latter organism also do not contain a cytochrome subunit in their RCs. The conclusion that the RCs of the three investigated strains do not contain a cytochrome *c* as an integral part of the RCs contradicts results reported previously for *Ectothiorhodospira* sp. [8,9], *E. halochloris* DSM 1059 [12] and *E. shaposhnikovii* (strain unspecified) [14,15]. This difference can be due to the use of different isolation procedures or different strains. However, for the strains mentioned above, it is important to confirm the presence of an RC-bound cytochrome through a redox titration of isolated RCs. Future studies in other organisms, focusing on binding of a (4-heme) *c*-type cytochrome to purified RCs or RC/LHI complexes, should include an assay of the ionic nature of this binding. Redox titrations or heme staining of SDS-PAA gels of chromatophores only may lead to an erroneous conclusion.

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