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Isolation of reaction center and antenna complexes from the halophilic purple bacterium *Rhodospirillum salexigens*. Crystallization and spectroscopic investigation of the B800-850 complex

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Intracytoplasmic membranes were isolated from the halophilic phototrophic bacterium *Rhodospirillum salexigens*. The membrane fraction was solubilized in lauryldimethylamineoxide (LDAO) and the reaction center (RC) and antenna complexes were separated by sucrose gradient centrifugation and column chromatography on DEAE cellulose. The RC preparation contains three polypeptides of M_r similar to that of the RC of *Rhodobacter sphaeroides*. Two polypeptides, of apparent M_r 12000 and 10000, are associated with the B870 complex. The B800-850 complex absorbing at 800 and 837 nm, seems to contain two low-M_r polypeptides, of about M_r 9000. Crystals grown of the B800-850 complex showed X-ray reflections to 0.8 nm. Crystals of 10 × 30 μm edge length were used for microspectroscopy with polarized light. All absorption bands showed a strong dichroism in the plane of the rectangular platelet. The dichroism of the 800 nm band was stronger than that of the 837 nm band.

Introduction

The photosynthetic apparatus of most purple sulfur and nonsulfur bacteria (*Rhodospirillales* [1]) is bound to intracytoplasmic membranes and contains two different antenna complexes which funnel the absorbed light energy to the photochemical reaction center (RC). The antenna complexes are named by their near-infrared absorption bands B870 and B800-850, although the individual wavelengths of absorption maxima vary, dependent on the strain and the isolation procedure [2]. *Rhodospirillum (Rsp.) salexigens* [3] is an obligatory salt-dependent purple bacterium which seems not to be closely related to other halophilic purple bacteria [4–9]. It contains bacteriochlorophyll *a* (BChl). The three sharp in vivo absorption bands at 800, 840 and 870 nm suggest that this bacterium contains two antenna complexes, namely B870 (LHI) and B800-850 (LHII) [2]. As the pigment-protein complexes of the bacterial photosynthetic apparatus are conservative structures [10,11], we are interested to learn how the pigment-protein complexes of *Rsp. salexigens*, a species not related to other Rhodaspirillaceae, are organized. In this

Abbreviations: BChl, bacteriochlorophyll; LDAO, lauryldimethylamineoxide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; RC, reaction center; *Rb.*, *Rhodobacter*; *Rsp.*, *Rhodospirillum*.

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article we describe the isolation and partial characterization of the RC and the two antenna complexes B870 and B800-850 and the crystallization and spectroscopic investigation with polarized light of the crystals of the B800-850 complex.

Materials and Methods

Cultivation of the bacteria. *Rhodospirillum salexigens,* type strain WS68, DSM 2132 [3], was cultivated in a synthetic medium [9]. For mass culture, a 12 liter New Brunswick Biotronic benchtop batch fermenter was inoculated with 100 ml of a preculture in a screw-cap bottle. The cells were grown phototrophically for 48 h under anaerobic conditions at 40 °C.

Membrane isolation. The cells were harvested late in the exponential growth phase by centrifugation in a Padberg continuous flow centrifuge (Lahr, F.R.G.) and washed in a salt solution containing per liter: 90 g NaCl, 5 g $MgSO_4 \cdot 7\ H_2O$, 0.2 g $CaCl_2$, 4 g KCl (pH 7.5). The cells were resuspended in the same salt solution under addition of phenyl-methylsulfonyl fluoride (2 mM PMSF) and DNAase (1 mg/10 ml) and disrupted in a French pressure cell. Cell debris were removed by centrifugation in a Sorvall refrigeration centrifuge RC5B (20 min, 12000 × *g*). The supernatant was layered on a discontinuous gradient of 0.6, 1.0, 1.2 and 1.5 M sucrose in 50 mM Tris-HCl buffer/(pH 8.0)/0.05 M KCl/2 mM $MgCl_2$. After centrifugation for 16 h at 4 °C and 90000 × *g* (Beckmann centrifuge L65, rotor Ti 60) the pigmented band was removed with a Pasteur pipette and washed with 50 mM Tris-HCl buffer (pH 8.0).

Isolation of pigment-protein complexes. The intracytoplasmic membrane fraction was resuspended in Tris-HCl buffer to a density of 420 μg bacteriochlorophyll (BChl)/ml and mixed dropwise with an equal volume of 1.2% (w/v) LDAO in Tris-HCl buffer at room temperature in darkness. After 30 min the solution was layered on a discontinuous sucrose gradient (0.3, 0.6, 1.2 M sucrose in Tris-HCl buffer plus 0.25% LDAO). After a run of 16 h at 130000 × *g* (rotor Ti 60) at 4 °C, the pigmented bands were removed, their absorption spectrum was measured and the isolated fractions were dialysed overnight at 4 °C against 20 mM Tris-HCl buffer (pH 8.0).

Purification of pigment-protein complexes by column chromatography. The fractions isolated by the sucrose gradient were further purified by chromatography on DEAE-cellulose (DE-52, Whatman, U.K.). The columns (2.5 × 30 cm) were equilibrated with 50 mM Tris − HCl buffer (pH 8.0)/0.05% LDAO. The RC-B870-enriched fraction was applied to the preequilibrated DEAE column and eluted with Tris-HCl buffer (pH 8.0)/0.1% LDAO/0.12 M NaCl. The B800-850-enriched fraction was applied afterwards and the column was washed with the equilibration buffer + 0.06 M NaCl. The B800-850 complex was eluted with 50 mM Tris-HCl buffer (pH 8.0)/0.25 M NaCl/0.1% LDAO. For crystallization, the protein solution was dialysed against Tris-HCl buffer/0.05% LDAO and chromatofocused as described in Ref. 12 with the exception that the pH gradient was formed with 25 mM histidine-HCl (pH 4.0). The B800-850 complex eluted at pH 5.5.

Analytical procedures. The absorption spectra were measured with a Kontron Uvikon spectrophotometer 860 and with a Perkin Elmer Lamda 5. Protein bands eluted from the DEAE column, the chromatofocusing column and crystals were washed by resuspension with mother solution (see below) and concentrated by centrifugation. The complexes of the isolated complexes were analysed by SDS-PAGE) using a 11.5–16.5% linear polyacrylamide gradient. The buffer system was that of Laemmli [13]. Protein concentration were determined by the method of Lowry et al. [14]. In isolated pigment-protein complexes the protein content was determined by amino acid analysis. BChl was extracted from cells and membranes in acetone/methanol (7 : 2 v/v) and the concentration was calculated using the absorption coefficient of 76 $mM^{-1} \cdot cm^{-1}$ at 770 nm [15].

Crystallization and preliminary X-ray studies of crystals of the B800-850 complex. Crystals could be obtained by using either ammonium sulfate or poly(ethylene glycol) (PEG) 1000 (Merck, Darmstadt, F.R.G.) as precipitating agents.

For crystallization with ammonium sulfate, the B800-850 complex eluted from the chromatofocusing column was concentrated to a protein concentration of roughly 15 mg/ml with a Amicon ultrafiltration cell equipped with an XM-50 membrane (Amicon, Witten, F.R.G.) and dialysed

against 10 mM Tris-HCl buffer (pH 8.0)/0.04% LDAO/3 mM NaN_3/2 M $(NH4)_2SO_4$. The amorphous precipitate was removed by centrifugation in a bench-top centrifuge. A presaturated protein solution was prepared by addition of saturated ammonium sulfate in a concentrated phosphate buffer solution of the desired pH and a concentrated solution of heptanetriol (Oxyl, Bobingen, F.R.G.) to obtain a final concentration of 3.2 M ammonium sulfate, 50 mM phosphate buffer, 5 mg/ml of protein and 0.02% LDAO and 2% heptanetriol. The presaturated solution was equilibrated with a reservoir solution containing 4.0 M ammonium sulfate via the vapor phase.

For crystallization with PEG 1000, the protein was concentrated as above and dialysed against 10 mM Tris-HCl buffer (pH 8)/0.04% LDAO/5 mM NaN_3 to a final concentration of 10 mg/ml of protein. A presaturated protein solution was prepared by addition of concentrated PEG 1000 solution containing a concentrated phosphate buffer of the desired pH and a concentrated solution of heptanetriol to final concentrations of 5 mg protein/ml, 15% (w/v) PEG and 3% (w/v) heptanetriol. The presaturated solution was equilibrated with a reservoir containing 20–23% (w/v) PEG 1000 in 25 mM Tris-HCl (pH 8.0) and 5 mM NaN_3 via the vapor phase.

All crystallization solutions were kept at room temperature. The crystals were mounted for diffraction experiments in 1.0 mm glass capillaries and fixed in a precession camera (STOE, reciprocal lattice explorer, Darmstadt, F.R.G.). The capillary was kept at 10 °C all the time by a stream of cooled air.

Microspectrophotometry of crystals of the B800-850 complex. Thin crystals of suitable optical density were obtained by spreading 5 µl of protein solution presaturated with PEG 1000 on a cover slide as a thin film and placing the latter into a closed box containing the reservoir solution. The linear dichroism spectra of single crystals were measured in a home-made microspectrophotometer as described in Ref. 12.

Results

Purification of pigment-protein complexes

The reaction center and antenna complexes of *Rsp. salexigens* were solubilized from the membrane after treatment with LDAO (LDAO : BChl = 28.5 : 1, w/w) in the dark at room temperature. The following sucrose density centrifugation resulted in three bands enriched in material having absorption maxima at 800 and 865 nm (top layer), enriched in B800-850 complex (middle layer) and in material having absorption bands at 800, 838 and 865 nm and the maximum absorption at 875 nm (lower layer). The three fractions were removed from the gradient, dialysed and applied to chromatography on DE-52 as described under Materials and Methods. The three resulting fractions were strongly enriched in RC, B870 and B800-850, but not free from contaminating proteins. Further purification of the RC and B878 complexes were accompanied by changes of the spectroscopic features.

In some cases, ammonium sulfate precipitation of the B800-850 complex led to partial loss of the carotenoids, but with no change in the near-infrared spectral range (carotenoid-poor complex). Additional chromatofocusing of the B800-850 complex removed further contaminating protein, resulting in a highly purified B800-850 preparation.

Absorption spectra and protein patterns of the enriched pigment-protein complexes

The RC preparation showed absorption maxima at 370, 385, 535, 592, 754, 798 and 850 nm (Fig. 1). The content of carotenoids was low compared with the antenna pigment complexes. The SDS polyacrylamide gels showed three bands,

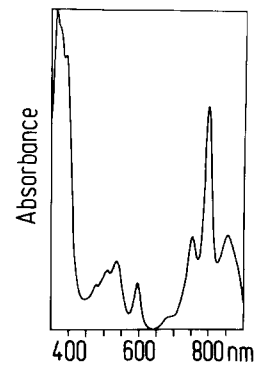

Fig. 1. Absorption spectrum of reaction center preparation of *Rsp. salexigens.*

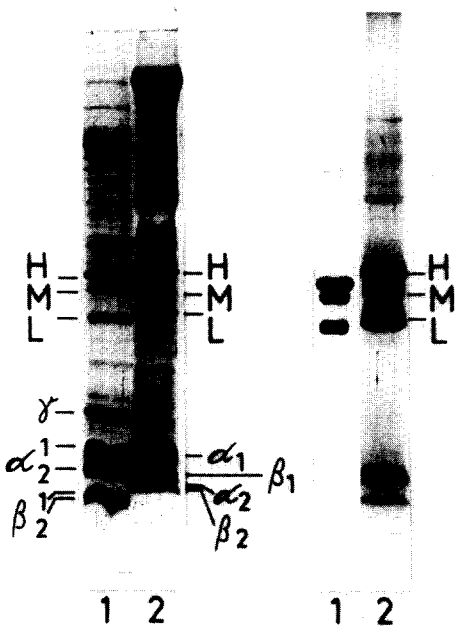

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified membranes isolated from *Rb. capsulatus* 37b4 (wild-type strain), left part of figure, lane 1, and *Rsp. salexigens* lane 2; Right panel: reaction center preparation of *Rb. capsulatus* (lane 1) and *Rsp. salexigens* (lane 2). H, L, M are the subunits of reaction center and α and β the polypeptides of antenna-pigment protein complexes [2]; the subscripts 1 and 2 refer to the B870 and to the B800-850 complex, respectively.

which ran to positions of higher apparent M_r compared with the respective subunits of the RC of *Rb. capsulatus* (Fig. 2). The bands of the enriched RC appear as double bands under all preparation conditions and the M band was faintly stained. No band was heme-stained. There is no evidence for a RC-bound cytochrome as in *Rps. viridis*. After excitation the reversible bleaching of the 852 nm band and the blue shift of the 800 nm band were observed (Fig. 3).

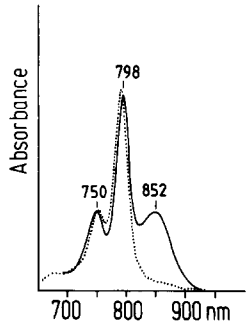

Fig. 3. Reaction center of *Rsp. salexigens* in the reduced (full line) and light-oxidized state (dotted line).

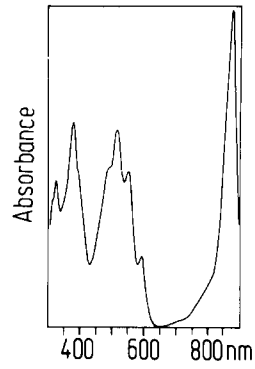

Fig. 4. Absorption spectrum of the B870 complex isolated from membranes of *Rsp. salexigens*

The B870 preparation showed a strong single near infrared absorption band at 878 nm besides the Soret band at 375 nm and Q_x absorption at 594 nm (Fig. 4). The carotenoids absorbed at 514,

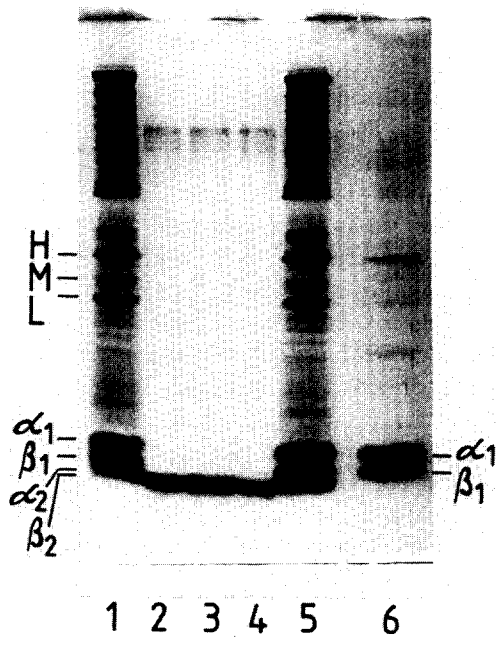

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified membranes (lanes 1 and 5) and isolated pigment-protein complexes (B800-850) lanes 2–4; B870 lane 6) from *Rsp. salexigens*. Abbreviations as in Fig. 2.

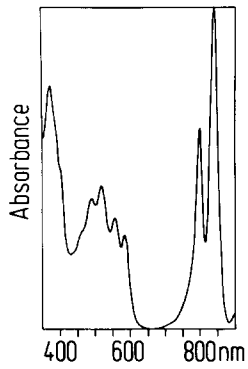

Fig. 6. Absorption spectrum of the isolated B800-850 complex from *Rsp. salexigens.*

536 and 548 nm. The SDS-polyacrylamide gel electrophoretic patterns showed two major bands at apparent M_r of about 12000 and 10000 (Fig. 5, lane 6).

The B800-850 (LHII) fraction showed two major near infrared absorption maxima at 800 and 837 nm (Fig. 6). The carotenoids absorbed at 488, 516 and 556 nm (Fig. 6). The pigments seem to be bound to two polypeptides, which ran on SDS-PAGE to a position of apparent M_r of about 9000 (Fig. 2 and 5). They appeared as one band on SDS-polyacrylamide gels (Fig. 5) and after electrofocusing of FPLC chromatography (not shown). The BChl content of the preparation was determined to be 137.8 μg BChl/mg protein (determined as amino acids). On the basis of 2 mol polypeptides of apparent M_r 8000 and 9000, the LHII preparation would contain 2.6 mol BChl per 2 polypeptides. It is tentatively concluded that the B800-850 complex contains 3 mol BChl per smallest subunit as in other B800-850 complexes [10]. The polypeptides of the antennae and RC complexes were blotted on nitrocellulose sheets and treated with antibodies against RC and antennae complexes from *Rb. capsulatus*. No cross-reaction was observed (not shown).

Crystallization and measurement of linear dichroism

With ammonium sulfate or poly(ethylene glycol) 1000 as a precipitant and at pH 8.0, crystals of the B800-850 complex with a length of 0.1 mm in each direction were obtained after 2 weeks. No crystals could be obtained from the preparation with low carotenoid content. X-ray reflections of both types of crystal were observed to 0.8 nM. The space group was not determined, but it should be different from the $C222_1$ symmetry observed in the corresponding crystals of *Rps. palustris* [16].

Thin crystals grown for microspectrophotometry had a rectangular shape with edge lengths of approx. 10 μm $\times$ 30 μm. The spectra obtained with linear polarized light along the long edge (A_{vert}) and the short edge (A_{hor}) of the rectangle show strong dichroism of all absorption bands in the plane of the rectangular platelet. The dichro-

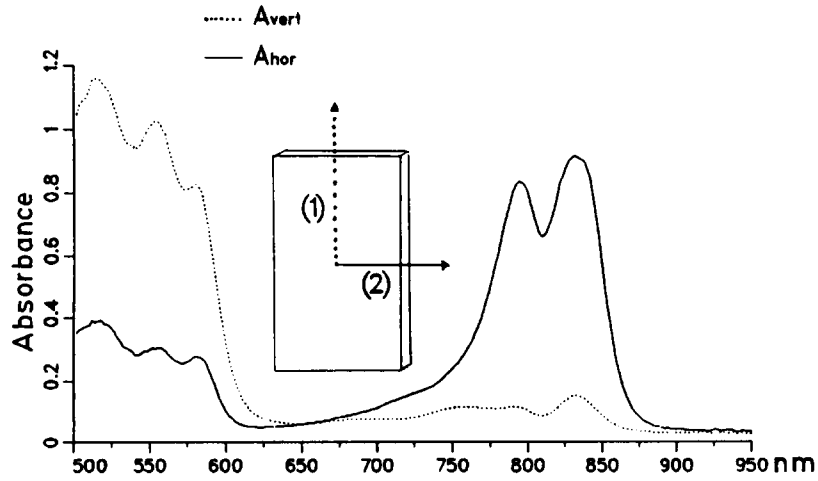

Fig. 7. absorption spectra of crystals of the B800-850 antenna complex from *Rsp. salexigens,* strain WS 68, measured with vertical and horizontal polarized light. For details see text.

ism of the 800 nm band is stronger than that of the B840 nm band (Fig. 7).

The absorption, A, of a light beam polarized along the unit vector, $\vec{p}$, when passing through an arrangement of transition moments $\vec{t}_i$ is given by the following formula:

$$A = k \cdot (\vec{p} \cdot \Sigma \vec{t}_i)^2$$

If one plots the square root of A in a suitable system of coordinates (principal axes) as a polar diagram of the vector $\vec{v}$ with $\vec{v} = (a \cdot p_x,\ b \cdot p_y,\ c \cdot p_z)$, it has the form of an ellipsoid surface with three orthogonal half-axes a, b, c into the direction of the three coordinate axes. The vector $\vec{v}$ in general is not parallel to $\vec{p}$, but for the directions of the three half-axes, $\vec{v}$ and $\vec{p}$ are parallel. An ellipsoid can be attributed to each arrangement of transition moments absorbing at the same wavelength, i.e., those of a single B800-850 complex, those of the asymmetric unit as well as those of the unit cell of a crystal. The bigger the differences between the three half-axes of the ellipsoid, the more dichroic is the arrangement. In a crystal, the ellipsoid is formed by applying all point symmetry operations to the transition moments of the asymmetric unit and superimposing all resulting images. The transition moments of the asymmetric unit in turn are an unknown superposition of the transition moments of single B800-850 complexes. Each of these superpositions may result in a less dichroic ellipse than the one of the single B800-850 complex. The observed dichroism thus represents a lower limit of dichroism for the single complex. As the absorption of the Q_y transitions of the 800 and 840 BChl *a–s* is small along the vertical edge of the crystal, the Q_y transition moments of the single complex must be distributed in good approximation within a plane orthogonal to this edge. The spectra also show that the two carotenoid bands at 516 nm and at 556 nm have dichroic behavior similar to that of the Q_x transition moments at 595 nm. Both carotenoid transitions moments thus may be oriented in a similar manner in the complex.

Discussion

In a first view, the isolated pigment-protein complexes from *Rsp. salexigens* are similar to other RCs, B870 and B800-850 complexes containing BChl *a* and isolated from other *Rhodospirillum, Rhodobacter* and *Rhodopseudomonas* species [10]. In contrast to the respective complexes of *Rb. capsulatus* and *Rb. sphaeroides*, the B850 and B870 absorption bands of *Rsp. salexigens* are clearly separated from each other (Fig. 8). One fluorescence emission band appears in *Rsp. salexigens* membranes at 903.5 nm (not shown here). Thus, there is enough overlap for efficient energy transfer. Quantitative measurements have not been done. While the polypeptide pattern of the RC is very similar to that of other RC preparations, the polypeptide pattern of light-harvesting complexes is different (Figs. 2, 5). While the polypeptides of the B870 complex have apparent M_r similar to those of B870 in *Rb. capsulatus*, the preparation of the B800-850 complex from *Rsp. salexigens* contains only one major band in SDS-polyacrylamide gels (Fig. 5), which we could not resolve in two bands by three methods. As all pigment-protein complexes from various bacterial photosynthetic apparatus contain two different pigment binding polypeptides, It is believed that the same is true for the B800-850 complex of *Rsp. salexigens*. The sequence studies which are in progress will clarify this point.

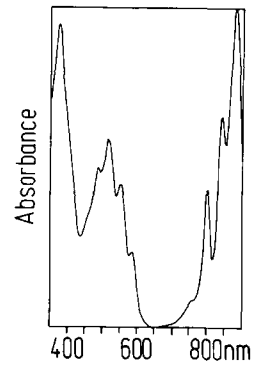

Fig. 8. Absorption spectrum of purified membranes from *Rsp. salexigens.*

The *Rsp. salexigens* B800-850 complex was stabile at high ionic strength and could be crystallized with ammonium sulfate in contrast to the respective complexes from *Rb. capsulatus* and *Rps. palustris*, which were denatured by high ammonium sulfate concentrations (unpublished observation). It is possible that the high stability of

the former complex is due to the salt-dependence of *Rsp. salexigens*.

The dichroism of the *Rsp. salexigens* pigment absorption bands (Fig. 7) is much higher than the dichroism in the spectra of *Rps. palustris* [16], but similar to spectra of crystals of the B800-850 complex of *Rps. acidophila* (Fig. 6 in Ref. 17). We propose that the B800-850 complexes from *Rsp. salexigens* and *Rps. acidophila* crystallize in a space group different from the orthorhombic space group found in the *Rps. palustris* crystals. This is also supported by X-ray results although the space groups of the *Rsp. salexigens* and *Rps. acidophila* crystals are unknown as yet. The high dichroic ratio of the BChl 800 and BChl 840 Q_y transitions in the two spectra sets a lower limit to the dichroism of single B800-850 complexes. Fluorescence polarization studies indicated a circular degeneracy of the Q_y transition moments of the 800 and the 850 BChl-*s* within a plane [18,19]. The ellipsoid of a single complex thus should have a discoidal shape with two identical half-axes and the third axis being small compared to the other two. The linear dichroism spectra presented in Fig. 7 and those of *Rps. acidophila* are in accordance with these observations.

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