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The light-harvesting core-complex and the B820-subunit from *Rhodopseudomonas marina*. Part I. Purification and characterisation

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The BChla-containing B880-complex (core-complex) of *Rhodopseudomonas marina* (*Rhodospirillaceae*) was isolated with a new purification method. The isolation of the B880-complex was performed by solubilisation of the photosynthetic membranes with the detergent LDAO and subsequent fractionated ammonium-sulfate precipitation with about 50% recovery. The B880-complex retained its original spectral properties as revealed with absorption, fluorescence and circular dichroism spectroscopy. Furthermore, we dissociated the B880-complex with the detergent *n*-octyl-β-glucoside (OG) and purified the developed subcomplex by the method of Miller et al. [1], which showed an absorption maximum at 820 nm (B820). The α- to β-polypeptide ratio and the α- or β-polypeptide to BChla ratio, respectively, were estimated to be 1:1 in both complexes. The molecular weights of the B880 and the B820-complexes, determined by gel filtration chromatography, were 181 and 32 kDa, respectively. Thus, it appears that the B880-complex of *Rp. marina* consists of 24 polypeptides and the B820-complex of four polypeptides. Six B820-complexes or possible subunits could form the B880-complex. On the basis of these data we propose a model for the structure of BChla containing core-complexes.

Rhodospirillaceae; Light-harvesting complex; Non-sulfur purple bacteria; Bacteriochlorophyll *a*; B880; B820

1. INTRODUCTION

The structures of the antenna complexes of the various photosynthetic bacteria have been of special interest during the last decade. In contrast to the bacterial reaction center of *Rp. viridis* and *Rp. sphaeroides* [2–4], X-ray diffraction data with high resolution of antenna complexes have not been available so far and most information about their organisation is gathered from biochemical and spectroscopic studies.

It is common to most antenna complexes of purple bacteria that they consist of two types of small polypeptides with 40–60 amino acid residues (α and β), carotenoids and either BChla or BChlb. As many of the primary structures of these antenna polypeptides have been determined it has become clear that they have common structural principles [5,6]. The antenna polypeptides aggregate to larger complexes of, for example, twelve polypeptides in the case of the peripheral B800–850-complex of *Rp. acidophila* as revealed with X-ray diffraction structure analysis [7]. For the various core-complexes (e.g. B880) ratios of BChla to the reaction center of between 21:1 and 41:1 have been found [8]. It is possible to dissociate the core-complex with detergents into smaller subcomplexes which then change their absorption maximum. One of these, the B820-complex, which was first described by Cuendet and Zuber [9], shows an absorption maximum at 820 nm, and it is possible to reassociate the complex to an aggregate which absorbs at the original wavelength [1,10]. Because this complex was isolated from at least three species of purple bacteria, it was postulated that it could be in general a structural subunit of the core-complex [11,12]. The size of the B820-complex was postulated to be one-sixth of the core-complex [1,13]. Until now, a number of core complexes of some *Rhodospirillaceae* have been isolated. The main disadvantages of these core complex preparations are that they are not as stable as, for example, the peripheral complexes and that the total separation from the RC polypeptides is not always achieved [14]. Thus, an extensive characterisation has been very difficult if not impossible in many cases.

An extensive characterisation of the B880- and the B820-complex would have answered the questions of how many B820-complexes form the B880-complex, of how many polypeptides the complexes consist of, and what the stoichiometry of the antenna polypeptides and the BChl is likely to be.

Rp. marina was first described by Imhoff [15]. During

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Abbreviations: B880, core-antenna complex with an absorption maximum at 880 nm; B820, subcomplex with an absorption maximum at 820 nm; B873, core-antenna without carotenoids and an absorption maximum at 873 nm; B860, core-complex which was reassociated from B820 and has its absorption maximum at 860 nm; RC, reaction center; CD, circular dichroism; NIR, near infra-red; OG, *n*-octyl-β-glucoside; LDAO, lauryl-*N*,*N*-dimethyl-amine-*N*-oxide; LDS, lithium dodecyl sulfate; Tris, tris[hydroxymethyl]aminomethane; BChl, bacteriochlorophyll; *Rp.*, *Rhodopseudomonas*; kDa, kiloDalton; PAGE, polyacrylamide gel electrophoresis.

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our work we found that the B880-complex of *Rp. marina* is very stable and seems to be suitable to investigate its structure. The amino acid sequences of the α- and β-polypeptides of *Rp. marina* show the common features of those of other *Rhodospirillaceae* [16]. The α-polypeptide is about 75% homologous and the β-polypeptide about 53% homologous to the corresponding polypeptides of *Rhodospirillum rubrum*.

In this paper we present a new purification method for the B880-complex of *Rp. marina* and demonstrate that it is possible to isolate a B820-subcomplex. Both complexes were extensively characterised.

2. MATERIALS AND METHODS

2.1. *Strains and growth conditions*

Rhodopseudomonas marina DSM strain 2698 was purchased from the German collection of microorganisms (Göttingen). One-litre cultures were grown under anaerobic conditions on DSM-27 *Rhodospirillaceae* medium, which was supplemented with 3% NaCl [15]. Cells were harvested by centrifugation and stored at −20°C.

2.2. *B880 preparation*

Frozen cells were thawed and the cell suspension adjusted to an OD_{880} of 240. After addition of DNase and $MgCl_2$, the cells were broken twice with a french press at 1200 psi. Cell debris was spun down at 8000 × *g* for 10 min, and the supernatant was centrifuged at 65,000 × *g* for 30 min to collect the photosynthetic membranes. The pellet was resuspended with a buffer containing 0.25% LDAO (Calbiochem) and 50 mM potassium phosphate, pH 8.0, and was adjusted to an OD_{880} of 120. After a 30-min incubation on ice, the photosynthetic membranes were centrifuged a second time. The pellet was resuspended in 50 mM potassium phosphate pH 8.0, 1.0% LDAO to yield an OD_{880} of 240. Immediately after the solubilisation of the membranes, the solution was diluted 1:1 with 50 mM potassium phosphate, pH 8.0. After 30 min, the solution was centrifuged at 150,000 × *g* for one hour. The supernatant was mixed in a volume ratio of 1:1 with a 3.5 M $(NH_4)_2SO_4$ solution. After two hours the precipitated material was spun down at 65,000 × *g*. The pellet contained the RC and cytochromes. The B880 containing supernatant was at once diluted 1:1 with 50 mM potassium phosphate, pH 8.0, dialysed overnight against 0.001% LDAO, 50 mM potassium phosphate, pH 8.0 and concentrated by a 20-h centrifugation at 150,000 × *g*. The purified B880-complex was stored at 4°C.

2.3. *B820 preparation*

The B820-complex was isolated as described by Miller et al. [1]. A solution of solubilized membranes containing B880, RC, cytochromes and 0.5% LDAO was dialysed against 50 mM potassium phosphate, pH 8.0, and subsequently lyophylised. The dry material was extracted several times with benzene to remove the carotenoids and dried in a desiccator. Twenty mg carotenoid-free material was resuspended in 1 ml of 1% OG (Novabiochem), 5 mM $KHPO_4$, pH 8.0, and loaded on a Sephadex G-100 superfine column. The 150 cm column was equilibrated and run with 0.8% OG, 5 mM $KHPO_4$ pH 8.0.

2.4. *Calculation of the protein/BChla ratio*

Aliquotes of the B880 or the B820 preparations were lyophylised and extracted five times with a mixture of methylene chloride/methanol/acetic acid (9:9:2) 0.1% ammonium acetate. The solution was loaded on a Pharmacia LH-60 gel filtration column equilibrated with methylene chloride/methanol/acetic acid (4:4:2) to remove the pigments and salts from the polypeptides. The fractions which contained the antenna polypeptides were pooled and aliquots were dried in a speed vac apparatus. The dry material was hydrolysed in 6 N HCl for 120 h at 110°Celsius and its amino acid content was analysed. For calculation of the protein concentration, we quantified the amount of arginine and tyrosine of which the content per polypeptide is known from the amino acid sequences of the α- and β-polypeptide [16]. Other aliquots were concentrated on a Rotavap vacuum rotator and loaded on a Macherey-Nagel HPLC reversed phase column (C_4, 1000 Å;). The two α- and β-polypeptides were separated with a linear 40% to 100% acetonitrile gradient to calculate the α;β ratio either with amino acid analysis or photometrically by measuring the tryptophan absorbance at 280 nm.

To establish the BChla content of the aliquots, 50 μl were extracted with 5 μl of ice-cold acetone/methanol (7:2) and the absorption spectra were taken (see below).

2.5. *Electrophoresis*

Polyacrylamide gels were run on a Bio-Rad Mini-Gel system with the tricine-buffer system from Schägger and Jagow [17].

2.6. *Spectra*

Absorption spectra were taken either on a Perkin-Elmer λ5 or on a Perkin-Elmer λ array 3840 spectrophotometer. We used the extinction coefficients of $\varepsilon_{772}=76\ (mM)^{-1}$ to calculate the BChla content [18] and $\varepsilon_{280}=5600\ cm^{-1}\ M^{-1}$ [19] to calculate the tryptophan content.

Fluorescence measurements were performed on a Spex Fluorolog 1680. For emission spectra we excited at 605 nm in case of the B880-complex and at 590, 770 or 820 nm in the case of the B820-complex.

CD spectra were taken on a Jasco J-710 Spectropolarimeter with a Hamamatsu R316 photomultiplier for the NIR range. The curves were usually smoothed with a noise reduction program of the J-700 system software. For CD spectra in the NIR-range we used a pathlength of 2 mm with an OD_{880} of 6 cm^{-1}.

2.7. *Determination of molecular weights*

The determination of the molecular weights of B880 and B820 was performed on a Pharmacia FPLC system with a Superose12 gel filtration column. The elution solvent was 0.8% OG 20 mM Tris-HCl, pH 8.0. The column was calibrated with the seven combithek calibration proteins from 12,500 to 450,000 Da (cytochrome *c*, chymotrypsinogen A, hen egg albumin, bovine albumin, aldolase, katalase, ferritin) (Boehringer Mannheim).

3. RESULTS

3.1. *Purification of the B880-complex*

The procedure to solubilise the photosynthetic membranes of *Rp. marina*, containing the B880-complex, is similar to the procedures used in most antenna preparations of *Rhodospirillaceae* [8]. The recovery of the complex upon solubilisation of the photosynthetic membranes was about 80%. In Fig. 1a), the absorption spectrum shows a peak in the near-infrared region at 880 nm and two peaks at 796 nm and 750 nm which belong to the RC. In addition, there is a shoulder at 405 nm visible, which belongs to cytochromes. There are no traces of free BChla, which would exhibit a peak at 777 nm. After the ammonium-sulfate precipitation step the core-complex was obtained with a yield of 60% calculated on the basis of the absorption at 880 nm (total recovery of 50%). There are no RC (complex or polypeptides) or cytochromes detectable neither in the absorption spectrum (Fig. 1a) nor with other analytical methods like LDS page (Fig. 2). Similarly, there is no free BChla in this preparation as deduced from the absorption spectrum. The ratio of the optical densities

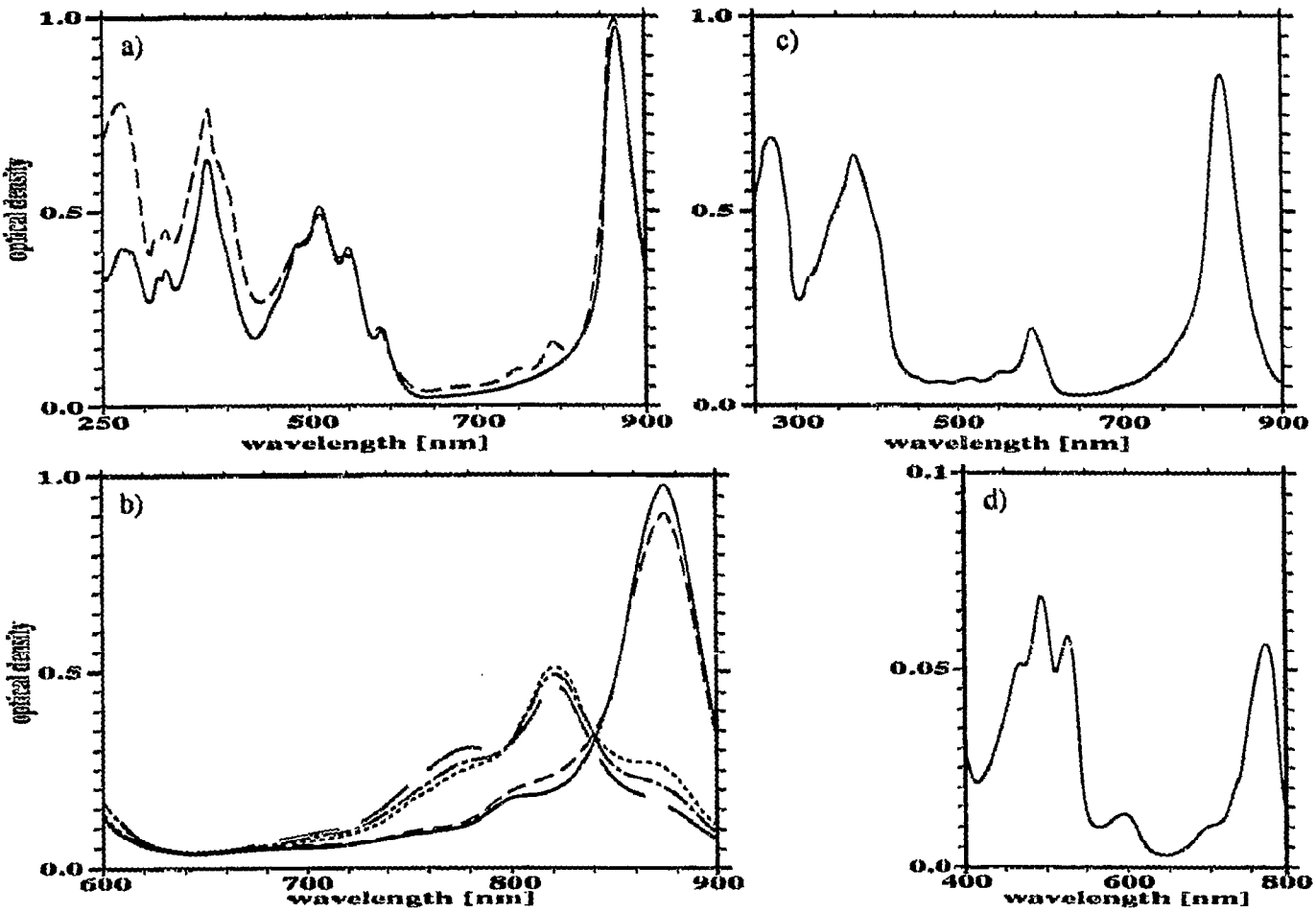

Fig. 1. (a) Absorption spectra of solubilised membranes (—) and purified B880-complex (—). (b) Purified B820-complex. (c) Carotenoid-free B873-complex titrated with increasing concentrations of the detergent OG. 0.6% OG (—), 0.7% OG (—), 0.8% OG (···), 0.9% (-·-) and 1% OG (————). (d) BChla extracted with a mixture of acetone/methanol=7:2.

OD_{880}/OD_{280} indicating the purity of the sample increased from 1:1 (solubilised membranes) to 1:0.6 (isolated B880-complex) upon this purification step. In Superose 12 gel filtration chromatography the protein eluted in a single peak (data not shown).

The B880-complex is stable at 4°C for several months after dialysis and concentration by ultracentrifugation.

3.2. *Purification of the B820-complex*

Depending on the protein concentration we titrated a solution of carotenoid-free B873-complex containing RC and cytochromes with increasing OG-concentrations to form the B820-complex or furthermore to form the 777 nm absorbing free BChla (Fig. 1b). The transition between the carotenoid free B873 and the B820-complex seems to be stoichiometric as the absorption spectra of the different titration concentrations show an isospestic point. Upon dilution with 50 mM $KHPO_4$ to 0.8% OG the B820 portion increased indicating a reassociation from the 777 nm absorbing form. Further dilution to concentrations below the critical micellar concentration of OG (0.7%) resulted in the formation of a reassociated B860-complex which showed an absorption maximum at 860 nm (data not shown).

The B820-complex was purified by Sephadex G100 chromatography and showed two distinct peaks in the elution profile. RC and cytochromes corresponded to the first peak while the second peak included the B820-complex (data not shown). In the purest B820-fractions the ratio of the OD_{820}/OD_{280} was 1:0.6 (Fig. 1c). At 4°C the B820-complex was stable for at least two months.

3.3. *Stoichiometry of the α,β antenna polypeptides and BChla*

As described in section 2.4. the amino acid content of the LHP-containing fractions was determined and the ratio of the α- to β-polypeptide as well as the absolute amount of each polypeptide was calculated from the amounts of the amino acids tyrosine and arginine. According to the sequence tyrosine appears only once in the β-polypeptide and arginine three times in the α- as well as in the β-polypeptide [16]. In our measurements

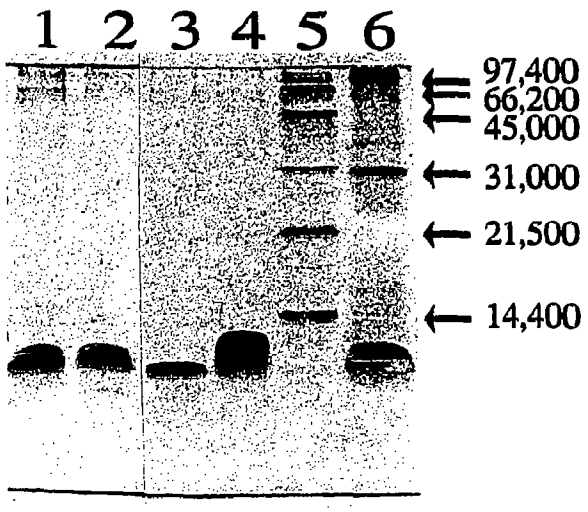

Fig. 2. LDS-page with 12% polyacrylamide. (Lane 1) Purified B880-complex. (Lane 2) Purified B820-complex. (Lane 3) Purified α-polypeptide. (Lane 4) Purified β-polypeptide. (Lane 5) Protein standard (Bio-rad low molecular weight). (Lane 6) LDAO solubilised photosynthetic membranes.

the amount of tyrosine was exactly one sixth of the amount of arginine (data not shown). This shows that the ratio of α:β is 1:1 as reported in [16]. The stoichiometry of 1:1 fits also very well for the other amino acids. Furthermore, the fractions of the reversed phase HPLC chromatography containing the α- or β-polypeptide were either submitted to amino acid analysis or the OD_{280} was measured to calculate the protein concentrations (section 2.4.). These methods showed the same 1:1 ratio of α and β as the previous one (data not shown). Using the same procedures the same results were obtained with the B820-complex. The amount of BChla determined in the acetone/methanol extract of an aliquot of the B880-solution together with the amount of polypeptides (section 2.4.) resulted in a ratio of 0.85 BChla molecules per polypeptide. The absorption spectrum of the extracted BChla shows that at least 10% of the BChla was oxidized to a species absorbing at 690 nm (Fig. 1d). Thus, if we add 10% BChla to the BChla to protein ratio of 0.85, the result is about a one-to-one ratio of BChla per polypeptide. The same procedure was performed with the B820-complex. Because of the missing carotenoids of the B820-complex the oxidation of BChla was higher as compared to the B880-complex. The calculation of BChla to protein resulted in a ratio of 0.7 BChla molecules per polypeptide. If we add at least 10% of oxidised BChla the result is a ratio of 0.8 BChla per polypeptide. In addition, we show in Fig. 1b that upon titration of the carotenoid-free B880-complex with OG there is no free BChla visible in the absorption spectrum indicating that the BChla:protein ratio does not change between the B880- and the B820-complex. Thus the ratio of BChla per polypeptide in the B820-complex should be 1:1 as in the B880-complex.

Table I

Determined molecular weights of the B880 and the B820-complex (column 1). In column 2 the data from column 1 were divided by the calculated MW of the potential minimal functional unit of α+β+2BChla (14,181). The calculated MW in column 3 was obtained by a multiplication of the integer values of column 2 (12 and 2, respectively) with the MW of the minimal unit (14,181). Column 4 shows the calculated number of polypeptides per protein complex

	determined MW	multiple of (α+β+2BChla)	calculated MW	calculated number of pp
B880	181,000	12.7	170,172	24
B820	32,000	2.3	28,362	4
(α+β+2BChla)	(calculated)	1.0	14,181	2

3.4. *Molecular weight of the B880 and the B820-complexes*

Extensively dialysed B880 samples were dissolved in 0.8% OG buffer and submitted to gel filtration on a Superose 12 column (section 2.7.). The determined molecular weight was 181 kDa for the B880-complex and 32 kDa for the B820-complex (Table I). Thus the molecular weight of the B880-complex is 5.7 or, about six times larger than that of the B820-complex.

Because the ratio of α:β:BChla is 1:1:2 in both complexes the sum of the molecular weights of the α- and the β-polypeptide (6096 and 6262 Da) and of two BChla molecules (2 × 911.5 Da) results in a total molecular weight of 14,181 Da of a minimal functional unit (heterodimer). Hence, the estimated molecular weight of the B880-complex and of the B820-complex correspond to 12 or 2 times the molecular weight of the minimal functional unit respectively (Table I). This means that the B880-complex consists of 24 polypeptides and the B820-complex of 4 polypeptides.

3.5. *Electrophoresis*

The purified B880- and the B820-complex showed in the 12% LDS page only two bands at the positions of the α- and the β-polypeptide (Fig. 2, lanes 1 and 2). The preparation of solubilised membranes shows the same polypeptide pattern and additional bands around 30,000 Da which probably belong to the RC polypeptides (lane 6).

3.6. *CD-measurements*

The CD-spectrum in the NIR-range of photosynthetic membranes shows two conservative lobes (Fig. 3a). The first lobe with a zero crossing at around 800 nm belongs to the accessory pigments of the RC. The second lobe with a zero crossing at around 880 nm is related to the B880-complex and the special pair of the RC [20]. In comparison with the CD-spectrum of the solubilised membranes, the lobe at 880 nm is too small. This is probably due to the reduced sensitivity of the photomultiplier at longer wavelengths and the turbidity

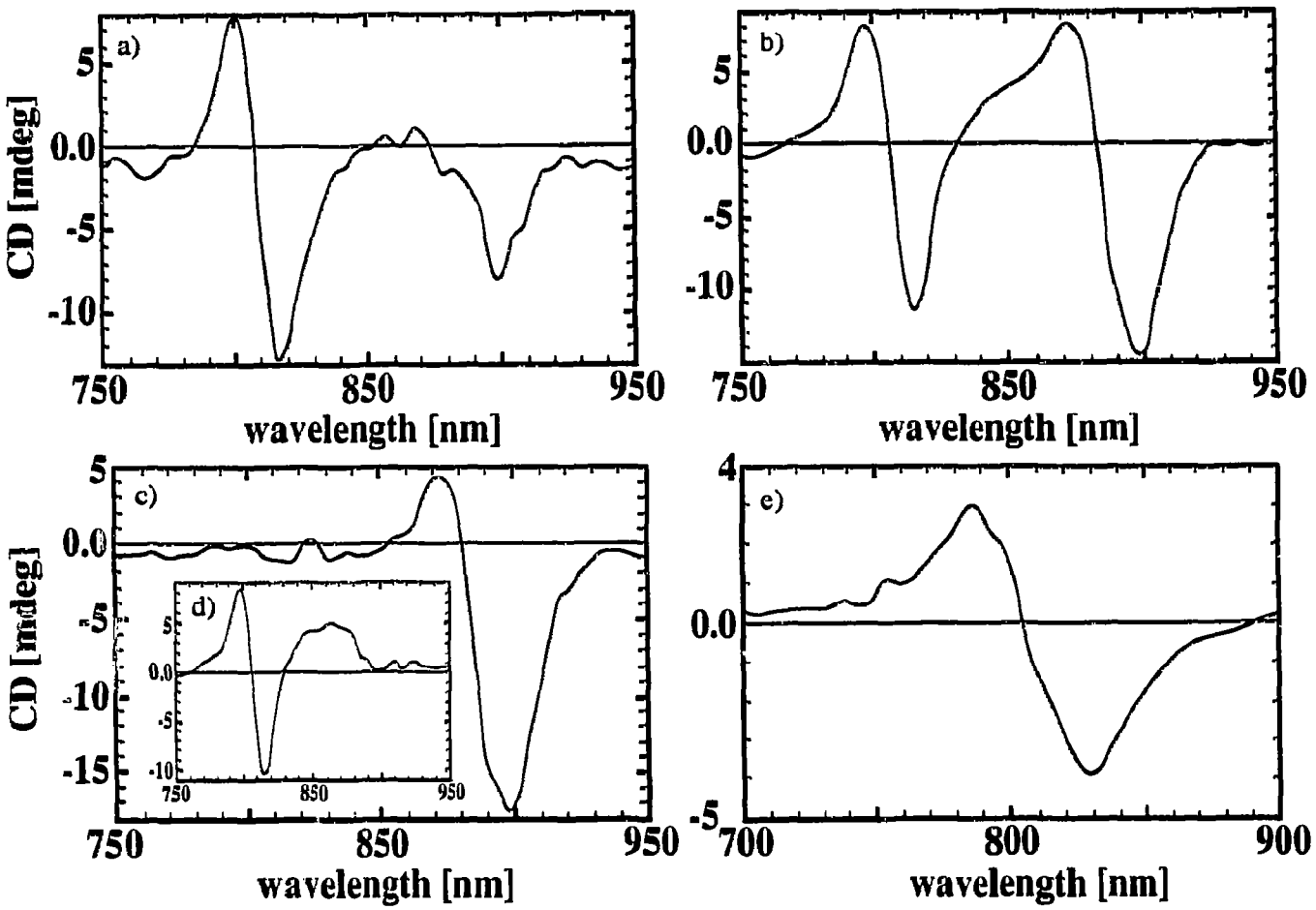

Fig. 3. NIR CD-spectra of (a) photosynthetic membranes, (b) solubilised membranes, (c) purified B880-complex, (d) insert: reduced RC. Difference spectra of (b) and (c), (e) purified B820-complex.

of the sample which make a proper quantification of the signals impossible.

As the solubilised-membrane preparation is not turbid these difficulties did not occur. The two peaks have nearly the same size with regard to the upper curve while the lower curve of the 880-lobe is a little larger (Fig. 3b). In additon, the 880-peak has a large shoulder in front of its upper lobe which belongs to the special pair of the RC. The CD-spectrum of the purified B880-complex lacks the lobe at 800 nm of the accessory pigments of the RC (Fig. 3c). Furthermore it lacks the large shoulder in front of the upper lobe at 880 nm. Thus, the upper lobe at 880 nm decreases to approximately one-third of the lower lobe. The zero crossing point and the lobe positions do not change upon purification. The CD-difference spectrum of the solubilised membranes and the purified B880-complex corresponds to the spectrum of the reduced RC of *Rsp. rubrum* (Fig. 3d) [20].

Fig. 3e shows the NIR CD-spectrum of purified B820-complex. The CD-signal is conservative with a zero-crossing point at around 800 nm which is similar to the results of Miller et al. [1].

3.7. *Fluorescence measurements*

Fluorescence measurements were performed to analyse changes in the pigment organisation of the B880-complex during purification. The fluorescence maximum at around 910 nm does not significantly change between the several purification steps (Fig. 4a). However, the relative sharp fluorescence peak of the photosynthetic membranes broadens upon solubilisation of the membranes. There is no difference visible in the fluorescence spectrum between the solubilised membranes and the purified B880-complex. Therefore the pigment organisation of the BChla molecules should not have been changed upon the purification of the B880-complex. The B820-complex shows a fluorescence maximum at around 835 nm after excitation at 820 nm (Fig. 4b). If the B820-complex is excited at 590 nm, two fluorescence peaks appear: the first at 800 nm and the second at 835 nm (Fig. 4b). Even in the purest B820-preparations, where no free BChla is detectable in the absorption spectrum (Fig. 1c), at least 50% of the emitted counts appear at a wavelength around 800 nm. This is in agreement with the fluorescence measurements of

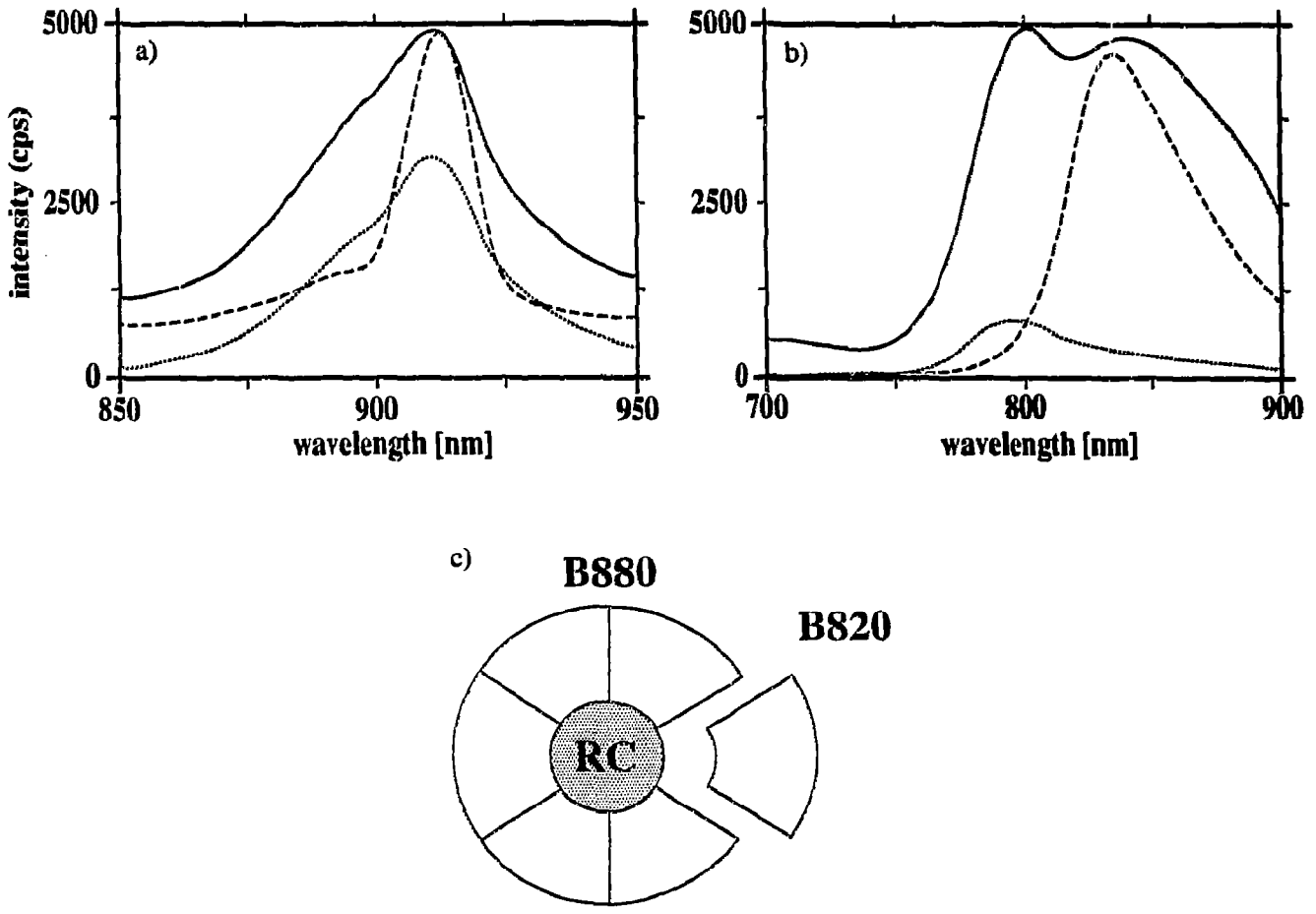

Fig. 4. Fluorescence emission spectra of (a) photosynthetic membranes (dashed line), solubilised membranes (solid line) and purified B880-complex (dotted line). The excitation wavelength was 605 nm. (b) B820-complex. Excitation wavelengths were 590 nm (solid line), 770 (dotted line) and 820 nm (dashed line) respectively. (c) Possible arrangements of the photoreceptor units within the membrane. Each B880-complex is drawn as a ring consisting of six identical subunits or B820-complexes.

Visschers et al. [21], who observed in B820 preparations from three different bacteria that a fluorescence peak at around 800 nm is as large as the 850 nm peak.

The 800 nm peak seems to belong to the 770 nm absorbing, free BChla in the solution, as it is the only emission peak upon excitation at 770 nm (Fig. 4b). In addition, this indicates that there is no energy transfer from the 770 nm absorbing BChla to the 820 nm absorbing BChla. The large 800 nm peak after excitation at 590 nm in comparison to the small 800 nm peak upon excitation at 770 nm seems to be caused by the emission characteristics of the xenon lamp.

4. DISCUSSION

In this paper we were able to demonstrate that the B880-complex of *Rp. marina*, which is isolated with a recovery of 50%, shows the characteristics of the complex in situ. The isolated core-complex was essentially free of RC and other polypeptides like cytochromes. The molecular weight of the B880-complex was determined by gel filtration chromatography. It has been postulated that the globular calibration proteins and the membrane proteins could bind different amounts of detergent due to their different hydrophilic surface. We studied this problem in calibrating the Superose 12 column with various buffer systems with and without detergent. The retention times of the calibration proteins showed a 5% increase upon addition of 0.8% OG to the buffer system. This leaves the question open, to which extent the binding of the detergent influences the estimated molecular weight of the membrane proteins or whether the calibration proteins bind the same amount of detergent as the membrane proteins.

The B820- and the B880-complex are composed of the same polypeptides, and the main difference in the surface of the two complexes, where detergent could bind, is the interaction sites of the B820-complexes

which are covered by the neighbouring subunits when the B820-complex aggregates to form the B880-complex. The interaction sites are probably only a small part of the surface of the B820 complex. Thus, the contribution of these variable interaction sites should be within the errors of the measurement and the two complexes should bind about the same relative amount of detergent. Consequently, the determined ratio of the molecular weights of the B880- and the B820-complex (MW_{B880}:MW_{B820}=6:1) should be accurate. The accuracy of the estimation of the molecular weight of the B880- and the B820-complex can be proven by calculating the possible molecular weights of the complex based on the stoichiometry of α/β/BChla and the ratio of the molecular weights of the two complexes (6:1) including the possible effect of detergent binding. As the estimated stoichiometry of α:β:BChl=1:1:2 yields a molecular weight of 14,181 Da for the minimal functional unit of the antenna complexes the calculated molecular weight of the B880-complex should be a multiple of six times 14,181, i.e. 85,086 Da (factor 1), 170,172 Da (factor 2) and 235,258 Da (factor 3). To calculate the maximal influence of detergent binding, we can add the average molecular weight of an OG-micelle, which is about 24,000 Da [22], to the calculated molecular weights of each factor. The best fit with our data (181,000 for the B880-complex) is obtained with factor 2 (170,172+24,000=194,172 Da). These conclusions suggest that the measured molecular weight of 181,000 Da for the B880-complex represents the molecular weight of the core-complex. Thus, the B880-complex of *Rp. marina* consists of 24 polypeptides ($6 \times 2 \times [\alpha+\beta]$) and the B820-complex of 4 polypeptides ($2 \times [\alpha+\beta]$). Nevertheless, it is still open whether the isolated B820-complex (a_2, b_2) is really a structural subcomplex in situ or just a rearrangement product of the dissociated B880-complex. The fact, however, that the NIR CD-signals of B820-complexes from *Rp. marina* and of a number of other bacteria are more or less similar supports the assumption that the B820-complex is a structural subunit of the core-complex. The substructure of the core-complex with 24 polypeptides could be a further hint to the arrangement of the BChls around the RC in BChla-containing bacteria. Probably the antenna consists of six subunits (α_2,β_2) which surround the RC (see the following paper: Part II). On the basis of the presented data and conclusions we propose that each B880-complex of *Rp. marina* consists of 24 polypeptides which are arranged in six identical subunits (α_2,β_2) (Fig. 4c). These subunits (B820-complexes) aggregate to a ring-like structure of the B880-complex, similar to the model systems described in [5,6].

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