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Biochimica et Biophysica Acta 1364 (1998) 390–402

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Reconstitution of the B800 bacteriochlorophylls in the peripheral light harvesting complex B800–850 of *Rhodobacter sphaeroides* 2.4.1 with BChl *a* and modified (bacterio-)chlorophylls

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Received 7 January 1998; revised 4 March 1998; accepted 18 March 1998

Abstract

A method is described for reversibly removing bacteriochlorophyll from the B800-site of the B850–850 antenna complex from *Rhodobacter sphaeroides*. This method uses the oligosaccharidic detergent Triton BG-10, together with an incubation at pH 5.0. Reconstitution at the B800-site has been successfully achieved for a range of modified bacteriochlorophylls.

Keywords: Photosynthesis; Light harvesting complex; B800–850; B850; Bacteriochlorophyll; Pigment modification; Pigment extraction; Pigment exchange; Reconstitution; Reversible dissociation; Circular dichroism

1. Introduction

The purple photosynthetic bacterium *Rhodobacter* (*Rb.*) *sphaeroides* contains two light-harvesting complexes, called B875 (or LH1) and B800–850 (or LH2) according to their absorption maxima in the near-infrared (NIR) spectral region [1]. Light energy absorbed by these antenna complexes is transferred quickly and efficiently to the reaction centre (RC) [2]. These antenna complexes are arranged hierarchically, with LH1 closely associated with the RC and LH2 located more peripherally. This organisation allows the energy transfer to be directed towards the reaction centre.

Both types of antenna complexes are oligomers of two kinds of small, hydrophobic apoproteins (α and β) [1]. LH1 and LH2 complexes have been prepared from many different species of purple bacteria and extensively studied, both biophysically [2] and by site-directed mutagenesis [3]. The crystal structure of LH2 from *Rhodopseudomonas* (*Rp.*) *acidophila* has

Abbreviations: BChl = bacteriochlorophyll; BPhe = bacteriopheophytin; B*xxx* = pigment complement absorbing at *xxx* nm (viz. B800); CD = circular dichroism; Chl = chlorophyll; DEAE = diethylaminoethyl; EIRS = environment-induced red shift; LDAO = dodecyl-dimethyl-aminoxid; LDS = lithium dodecyl sulphate; LH = light harvesting complex; NIR = near infrared; *Rb.* = *Rhodobacter*; RC = reaction center; *Rp.* = *Rhodopseudomonas*; *Rs.* = *Rhodospirillum*; SDS = sodium dodecyl sulphate; TBG = Triton BG-10; Tris = Tris–hydroxymethyl-aminomethane; TL (*c*/pH), TD (*c*/pH) and TB (*c*/pH) = Tris–HCl buffer (20 mM) containing LDAO, LDS and TBG, respectively, with the detergent concentration *c* in % (w/v) and the pH shown in brackets; TX = Triton X-100

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0005-2728/98/$19.00
PII S0005-2728(98)00086-3

recently been solved to a resolution of 2.5 Å [4]. It consists of a nonameric torus $(\alpha\beta)_9$. In the $\alpha\beta$ protomer of this LH2 complex, two bacteriochlorophyll (BChl) *a* molecules absorbing at 850 nm are non-covalently bound to the apoproteins by ligation of their Mg^{2+} ions to histidine residues. The third, 800 nm absorbing BChl *a* molecule present per $\alpha\beta$ protomer has its Mg^{2+} ion liganded by the N-terminal methionine from the α-apoprotein. In the intact oligomer there are 18 closely spaced BChl *a* absorbing at 850 nm which lie parallel to the transmembrane α-helices, and nine more separated 800 nm absorbing BChl *a*, which lie within the plane of the membrane in a rather peripheral position between the β-apoprotein helices. The protomer also contains two carotenoids in close proximity to the BChl [5]. Spectroscopic studies on a variety of LH2 complexes have shown that in general they all have rather similar properties [1,6]. However, more recently the crystal structure of a second LH2 complex, from *Rhodospirillum* (*Rs.*) *molischianum*, has been determined [7]. In this case it is an octamer $(\alpha\beta)_8$, so clearly the size of the LH2 'ring' can vary.

The spectral properties of the bound pigments are strongly modulated by interactions with other pigments and with amino acids in their binding sites. The latter have been well documented by site-directed mutagenesis studies, where for example changing aromatic residues from Tyr and/or Trp to Phe and/or Leu shifts the B850 absorption band in LH2 of *Rb. sphaeroides* from 850 nm to 826 nm [3]. Similar H-bond induced shifts have recently been studied also for the monomeric BChl-B800 [8]. These studies showed the importance H-bonding in modulating position and intensity of the Q_y absorption band of the ring of tightly coupled BChl *a* molecules. Other sources of the environment-induced red-shifts (EIRS) are currently less understood. They involve bulk and local changes in the dielectric properties of the environment [8,9], deviations of the tetrapyrrole macrocycle from planarity [4,7,10–12], accessibility to water [8] and excitonic coupling among the pigments [13–16].

An alternative approach to probing the structure and function of pigment–protein complexes is to exchange the native pigments for chemically modified ones with altered (photo)chemical and -physical properties. This approach has been used to investigate purple bacterial reaction centres [11] and LH1 [17], as well as the higher plant LHCII [18]. Here, we present data on the development of a system for exchanging the B800-BChl *a* molecules in LH2 from *Rb. sphaeroides*. Modification of the B800 pigment complement in B800–850 provides a means to study the interactions responsible for spectral shifts. These pigments are H-bonded by their C3-acetyl-group [19] and are in situ clearly non-planar [4,7], but are less densely packed than the B850 pigments and therefore assumed to be free from significant excitonic interactions [13,14,16]. This is supported by the current study. Previous work had already shown that BChl *a*-B800 is quite labile and can be readily removed from its binding site [20–23], which has been explored here for an exchange procedure.

2. Materials and methods

2.1. Rb. sphaeroides 2.4.1

Rb. sphaeroides 2.4.1 was grown in the light at 30° in modified *Rs.*-medium [24] with malate (1.5 g per liter) instead of succinate (Table 1). Following harvesting the cells were stored at −20°C. Chromatophores were isolated by French-press (Amicon) disruption of cells at 133 MPa, followed by centrifugation at $15\,000 \times g$ and pelleting at $240\,000 \times g$.

2.2. Detergents

Dodecyl-dimethyl-aminoxid (LDAO) (Fluka, 30% solution) and lithium dodecyl sulfate (LDS) (Sigma) were used as provided. The composition of the oligosaccharidic detergent Triton BG-10 (TBG, 70% solution in water, Union Carbide) is variable and not well defined. It was purified before use by dilution to 10% with bidistilled water, a first filtration over a paper filter and a second one over a column of 1/20 volume of diethylaminoethyl-cellulose (DE52, Whatman). The detergents (LDAO, LDS and TBG) were dissolved in Tris/HCl-buffer (20 mM); the resulting buffers were termed TL, TD and TB, respectively, with the detergent concentration *c* [% w/v] and pH shown in brackets.

2.3. Modified pigments

[3-acetyl]-Chl a was prepared according to Smith and Calvin [25]. All other pigments were prepared as

Table 1
Absorption and emission maxima [nm] of B800–850 from *R. sphaeroides* 2.4.1, and of the different complexes derived thereof

Sample	Buffer system	Absorption					Absorption ratio ($A_{800/850}$)	Emission[a]
		Soret-band	Carotenoids	Q_x	Q_y-BChl800	Q_y-B850		
B800–850	TL (0.03–2.0/8.0) TB (0.5–0.7/8.0)[b]	375	511	589	798	848	0.69	858
B800–850 (type I)	TL (0.5–0.1/8.0)	375	511	589	798	848	> 0.65	858
B800–850 (type II)	TL (0.03–1.0/8.0)[c] TB (0.5–0.7/8.0)	375	509	591	797	849	0.4	861
B850	TB (0.7/5.0–8.0)	376	506	593	–	851	≈ 0.1	864

[a] Fluorescence of the B850 complement.
[b] Small spectral variations possible, depending on buffer conditions and incubation time.
[c] Labile complex (see Section 3).

described by Struck et al. [26]. Extinction coefficients were taken from the literature [25–31]. For conversion to other solvents, a small amount of the desired pigment was dissolved in ether, and aliquots were dried under a stream of argon. These aliquots were then redissolved in identical volumes of the solvent in which ε was known, and of a solvent in which ε was to be determined, and the absorption spectra recorded. The values reported were derived from > 3 parallel experiments.

2.4. B800–850 in LDAO-buffer

Chromatophores ($OD_{850\ nm} = 50\ cm^{-1}$) were treated repeatedly with TL (0.2/8.0) with subsequent pelleting of the undissociated remaining membrane fractions at $240\,000 \times g$, until B800–850 started to appear in the supernatant. The B800–850 complex was then solubilized with TL (2.5/8.0), followed by centrifugation at $240\,000 \times g$. The supernatant was chromatographed on DEAE-cellulose (DE52, Whatman) in TL (0.1/8.0). Free pigments and other photosynthetic complexes were removed by elution with a NaCl-gradient (50–150 mM) in TL (0.1/8.0). B800–850 eluted at 150-180 mM NaCl. The chromatography was repeated first with TL (1.0/8.0) and then again with TL (0.1/8.0). The purified B800–850 was stored at −20°C.

2.5. Detergent exchange from LDAO to TBG

Concentrated solutions of B800–850 in TL (0.1/8.0) were diluted 50–100 times with TB of the desired concentration and pH. For complete removal of LDAO, the diluted solutions B800–850 in TL (0.1/8.0) were adsorbed on DEAE-cellulose, washed with the appropriate TB, and then desorbed with TB containing NaCl (180 mM).

2.6. B850 in Triton BG10-buffer

B800–850 in TB (0.7–1/8.0) containing ascorbic acid (5 mM) was acidified with acetic acid to pH 5.0. For spectral analysis NaCl (0.5–1 M) was added in order to obtain an optically clear solution. Dissociated BChl *a* was removed by chromatography on DEAE-cellulose in TB (0.7/5.0) at low (< 150 mM) NaCl. The B850 was then eluted with > 150 mM NaCl. Generally, this step must be repeated to remove all free BChl *a*, which is seen in the absorption spectra at 770–790 nm (see Section 3, Fig. 2). B850 was stored at −20°C in the elution buffer. The complete removal of the B800 pigment complement from the preparation was tested by adding NaOH (10%) to pH 8.0: there should be no absorption increase at 800 nm due to reconstitution of B800 with free pigments.

2.7. Reconstitution with BChl a and modified pigments—general procedure

BChl *a* or modified pigments (3–30 fold molar excess with respect to the original B800 content) were dissolved in methanol such that the volume of the methanolic solution was 1/20 of the sample-volume. These pigment solutions were then added to

B850, either at pH 5 and titrated back to pH 8, or at pH 8.0. After incubation, complexes were purified in the corresponding buffers by chromatography on DEAE cellulose. Different protocols and their specific advantages and disadvantages are specified in Section 3.

2.8. Pigment analysis

Samples were loaded to small DEAE-cellulose columns (packed in Tris–HCl (20 mM, pH 8.0) into Pasteur-pipettes) and washed with distilled water (10 ml). Excess water was pushed out gently with Ar, avoiding to dry the material. Pigments were eluted by consecutive extractions with methanol containing Na-ascorbate (0.2 g l^{-1}), acetone and diethyl-ether. The organic extracts were combined, dried in a stream of nitrogen or in the vacuum, and stored (if necessary) in vacuum over $CaCl_2$. Chromatography on silicagel was done as described by Struck [30] and Hartwich [27] with the following program: solvent A = toluene:isopropanol:methanol 100:0.05:0.05 (v/v), solvent B = toluene:isopropanol:methanol 100:1.25:12 (v/v); 2 min A, linear gradients from 2 to 8 min to 20% B, from 8 to 9 min to 30% B, from 9 to 10 min to 50% B and from 10 to 13 min to 100% B. Reverse phase HPLC was performed isocratically on RP8-columns (4.6∗100 mm Rosil, Alltech) with methanol/water (9:1) as eluent.

SDS-PAGE was done according to Schägger and von Jagov [32] with 18% acrylamide (96:4 acrylamide: bis-acrylamide) in the sample gel and 5% acrylamide in the stacking gel. If necessary, samples were concentrated by membrane filtration (10 kDa pores, Centricon) or lyophilisation prior to electrophoresis.

2.9. Spectroscopy

Absorption spectra were measured with a Lambda 2 spectrophotometer (Perkin-Elmer). For scattering correction, a set of spectra was generated from TBG solutions (0.7% in water) containing varying concentrations of NaCl. Fluorescence spectra were measured with a Fluorolog 221 (Spex), equipped with a NIR sensitive photomultiplier R316 (Hamamatsu) and a detection system produced by SMT (Seefeld, Germany). They were corrected for the photomultiplier response and the Xe-lamp spectra as described in the work of Bandilla [33] and further processed by a program written by A. Pazur and M. Bandilla. The excitation spectra show some noise because of incomplete correction of the Xe-lamp emission bands. Typical slit widths were 5 nm (excitation) and 12–15 nm (emission), and no polarization filters were used. Yields of excitation energy transfer of modified complexes were determined from the excitation spectra after normation of the B850-band to 1, and relative to unmodified complexes, for which we assumed 100% energy transfer [2,22,34]. Circular dichroism (CD) spectra were measured with a CD6 Dichrograph with an extended spectral range from 170–1100 nm (ISA-Jobin/Yvon). Wavelength deviations at $\lambda > 700$ nm were corrected by simultaneous recording of the absorption spectra.

3. Results

3.1. B800–850

The spectral properties of the purified B800–850 complex in TL (0.1/8.0; here and in the following the first number stands for the detergent concentration, the second for the pH, see abbreviations) (Fig. 1) and in TB (0.5–0.7/8.0) are similar. They are characteristic of the native complex and were used to provide a standard with which to compare changes during pigment removal/exchange. The ratio of A_{800}:A_{850} is 0.69 ± 0.01, which compares well with previously published data [21]. The CD spectrum of the isolated complex shows an intense, conservative (−/+) CD signal in the NIR, with the zero crossing 5 nm to the red of the B850 absorption maximum. At 800 nm, a weak but distinct conservative (−/+) CD signal is seen, which is overlayed by a broad negative band. CD signals < 700 nm are assigned to the Q_x and Soret-absorptions of BChl *a* and to the carotenoids [6].

3.2. Acid-induced dissociation

Upon acidification of B800–850 in TB (0.7/8.0) to pH 5.0, a selective loss of the absorbance at 798 nm occurs. This is accompanied by an increase at 775 nm and a loss of the small conservative CD-feature at

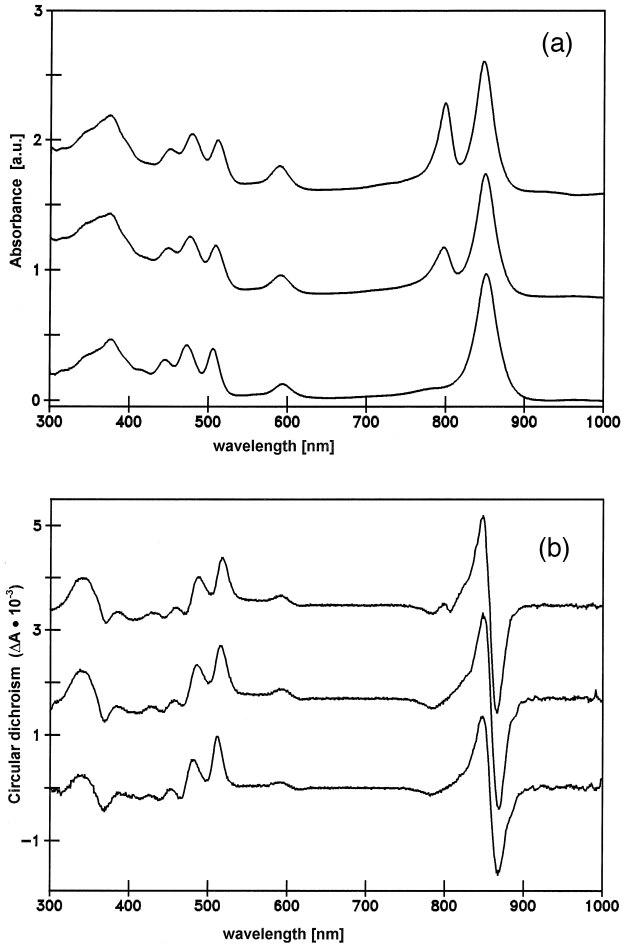

Fig. 1. Absorption (A) and CD-spectra (B) of B800–850 in TL (0.1/8.0) (top), reconstituted B800–850 (type II) in TL (0.1/8.0) (center), and of B850 in TB (0.7/5.5) (bottom). All complexes were purified by chromatography on DEAE-cellulose. The CD-spectra were normalized to an absorption of $A_{850} = 1$.

800 nm. The other VIS/NIR absorptions show only minor changes. There is a small, but distinct red shift of the 848 nm band to 851 nm and a 5 nm blue shift of each of the carotenoid absorption bands (Fig. 1). The corresponding CD bands are shifted in the same way. The pattern around 850 nm remains unchanged, but reduced in intensity by $\approx$ 20%. A comparison of the fluorescence excitation spectra of B800–850 before and after the acidification reveals that the efficiency of the singlet–singlet energy transfer from the carotenoids to the B850 is reduced to about 75% when the 798 nm absorption band has been lost. Concomitantly, a new emission band appears at 795 nm, which is not sensitized by light absorbed by the carotenoids (Fig. 2). This clearly represents a pool of uncoupled BChl *a*.

The spectral changes induced by acid are fully (> 95%) reversible. The sample shown in Fig. 4 was titrated back to pH 8.0 with NaOH, kept for 3 h at RT, and then subjected to a detergent exchange with LDAO. Best results were obtained by treatment first with TL (0.05/8.0) for 30 min, then with TL (0.1/8.0) and finally TL (0.2/8.0), followed by a freeze/thaw cycle, and subsequent chromatography on DEAE-cellulose. The resulting preparation is referred to below as type I-complex.

The energetically uncoupled 775 nm absorbing BChl *a* produced at low pH can be removed by chromatography on DEAE cellulose. This leads to a B800 depleted complex which has been termed B850. The absorption, fluorescence and CD spectra of B850 in TB at pH 8.0 are identical to those at pH 5.5 shown in Fig. 1, thus demonstrating that the TBG-solubilized B850 is stable over a wide pH range, and that the spectral shifts in the carotenoid and Q_y-region are pH independent and thus caused by the loss of BChl-B800.

B850 is unstable in LDAO. Addition of LDAO to a solution of B850 at pH 8 results in a decrease of absorption at 850 nm and a concomitant increase at 800 nm. Obviously, LDAO dissociates part of the BChl *a*-B850, which subsequently binds to empty B800 sites, yielding the more stable B800–850.

3.3. Reconstitution of pigment into the B800-site

The B850 complex described above was used for a series of reconstitution experiments. Basically, the solubilized complex was incubated with small volumes of a methanolic solution containing an excess of the desired pigment. Various strategies were tried. In particular, concentration, pH and detergent were varied to optimize the conditions, as well as the subsequent workup protocol. This screening was done with two pigments. Generally, it was conducted with BChl *a*, because the B800-site has evolved for this pigment and no complications arising from improper matches are expected. In cases where a distinction from endogeneous BChl *a* was necessary, as is the case with LDAO under the conditions described above, the exogeneously added pigment was [3-(1-

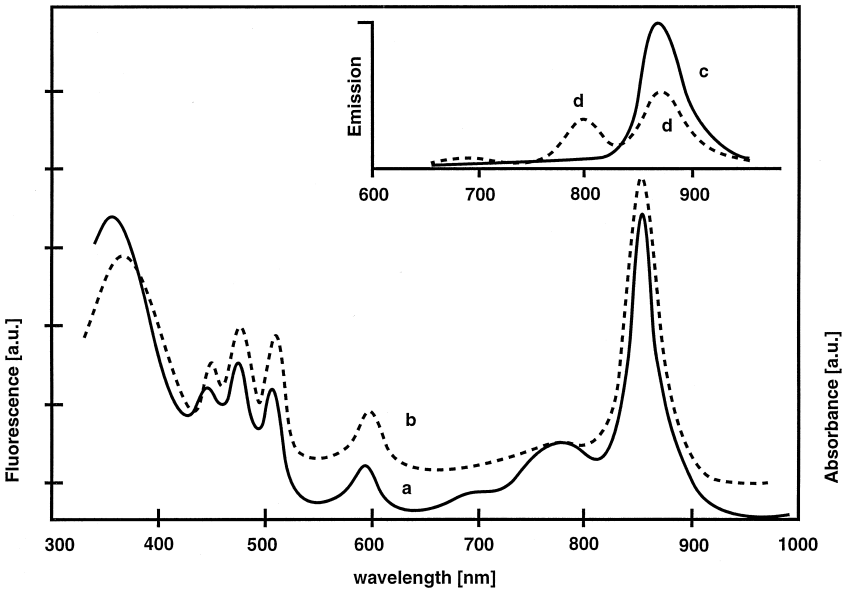

Fig. 2. Normalized absorption (a) and fluorescence excitation spectra (b, $\lambda_{em} = 875$ nm) of B800–850 in TB (0.7/5.5), obtained by titration of B800–850 with acid without further purification. Inset: Fluorescence emission spectra excited in the carotenoid region at 510 (c) and in the BChl Q_x-region at 590 nm (d).

hydroxyethyl)]-BChl *a* ([3^1-OH]-BChl *a*). Pigments with this modified C-3 substituent absorb at considerably shorter wavelengths than the native ones containing a 3-acetyl substituent instead, yet are accepted equally well by $B_{A,B}$ and the $H_{A,B}$ binding sites of purple bacterial RC [30]. As will be shown below, this is also true for the B800 site.

In the first protocol, the exogenous pigment is added directly to crude B850, as obtained by acid treatment of B800–850 in TBG-buffer. A methanolic solution was added just before retitration to pH 8.0. Under such conditions, the exogenous pigment competes with the one derived from the B800–850 complex, and therefore the protocol has been optimized with [3^1-OH]-BChl *a*. It yields B800–850 complexes (termed type I, see Fig. 4) which contain a mixture of BChl *a* and [3^1-OH]-BChl *a* in the B800 site, with a total occupancy at this site $> 95\%$. The maximum ratio of [3^1-OH]-BChl *a* over BChl *a* was only $\approx 1{:}2$, even when a large excess of [3^1-OH]-BChl *a* is added. This method is therefore useful only in those cases where a mixed occupancy is desired.

In the second protocol, B850 in TB was brought back to pH 8.0 and then incubated with exogenous pigment. The sample was kept for 30–60 min at RT, and then subjected to one or two freeze/thaw cycles. This reconstitution relies on the stability of B850 in TBG-buffer at higher pH (see above). An example of such a reconstitution is shown in Fig. 5. This protocol yielded complexes (termed type II) in which the B800 site is uniformly occupied by the exogeneously added pigment only. However, reconstitution with [3^1-OH]-BChl *a* was limited to 50%, a limit observed also with BChl *a* (see below).The third protocol uses LDAO-buffer, but as detailed above reconstitution has to be done in this case at carefully controled conditions: it works best if the transition to high pH is done as quickly as possible. Best results were obtained if B850 was dialysed against Tris/acetate (20 mM, pH 5.5), containing ascorbic acid (10 mM). The modified pigments were added in 5–30 fold molar excess in methanol, followed immediately by addition of a 3–10 fold volume TL (0.02/8) in which the buffering capacity was increased to 50 mM Tris/HCl and which contained additionally sodium ascorbate (10 mM) and NaOH in a concentration necessary to bring the pH of the solution after mixing to 8.0. This reconstitution mixture was kept for 30 min at RT, then the LDAO-concentration was raised to 0.05%. Finally, the sample was subjected to one or

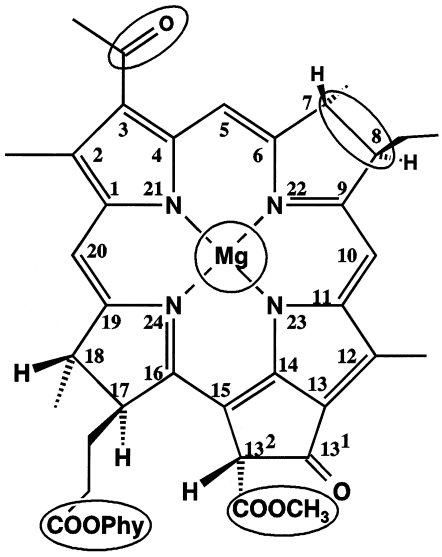

Fig. 3. Structural formula of BChl *a*. Ellipses indicate those functional groups which were modified to probe the B800 binding site (see Table 3).

two freeze/thaw cycles. As all protocols using LDAO, mixed populations at the B800-site and labile complexes are obtained.

The spectra of type II complexes, which were partially reconstituted with BChl *a*, were largely as expected: the 800 nm band was restored to 60% (corresponding to 50% occupancy) as compared to B800–850, and all shifts of the BChl *a*-B850 and the carotenoids were intermediate between the native and the B850 complexes. There is one minor but significant difference in the CD: the small conservative band at 800 nm was not restored in either of the two protocols, indicating that this feature arises from coupling among pigments in neighboring B800 sites (Fig. 6). Such couplings are much less likely if this site has only 50% occupancy, even if the distribution is random. Since both protocols resulted reproducibly in quite well defined preparations with a ratio of $A_{800}:A_{850}$ of 0.4 ± 0.01, there may even be a non-random (e.g., alternate) occupancy at these sites, but this has not been followed in more detail in this study.

3.4. Structural requirements of pigments for reconstitution

A variety of modified (bacterio-)chlorophylls were tried for reconstitution into the B800 site (Fig. 3, Table 2). [3^1-OH]-BChl *a* was found to be the best suited one to optimize the reconstitution conditions and shall therefore be discussed in detail. It binds as well as BChl *a*, but its absorption bands are well separated from those of the latter. Its maximum occupancy of the B800-sites in the reconstituted com-

Table 2
Functional side-groups of modified (bacterio-)chlorophylls used in the experiments (see Fig. 3 for numbering)

Pigment[a]	C-3	$C13^2$	C-7/8	$C\text{-}17^{4a}$	Central ion	$\varepsilon_{relativ}$(LM)[c]
BChl *a*	Acetyl	$COOCH_3$/H	dihydro	p	Mg^{2+}	1.0 (T,E)
BChl a_{gg}	Acetyl	$COOCH_3$/H	dihydro	gg	Mg^{2+}	1.0 (T)
[3^1-OH]-BChl *a*	α-Hydroxyethyl	$COOCH_3$/H	dihydro	p	Mg^{2+}	0.6 (T)
[3-Vinyl]-BChl *a*	Vinyl	$COOCH_3$/H	dihydro	p	Mg^{2+}	0.75 (T)
Chl *a*	Vinyl	$COOCH_3$/H	double bond	p	Mg^{2+}	0.94 (T)
[3-Acetyl]-Chl *a*	Acetyl	$COOCH_3$/H	double bond	p	Mg^{2+}	0.82 (T)
[3^1-OH]-Chl *a*[b]	α-Hydroxyethyl	$COOCH_3$/H	double bond	p	Mg^{2+}	0.8[b] (T)
Pyro-BChl *a*	Acetyl	H/H	dihydro	p	Mg^{2+}	1.0 (T)
13^2-OH-BChl *a*	Acetyl	$COOCH_3$/OH	dihydro	p	Mg^{2+}	1.0 (T)
BPhe *a*	Acetyl	$COOCH_3$/H	dihydro	p	$2H^+$	0.74 (E)
[Pd]-BChl *a*	Acetyl	$COOCH_3$/H	dihydro	p	Pd^{2+}	0.42 (E)
[Ni]-BChl *a*	Acetyl	$COOCH_3$/H	dihydro	p	Ni^{2+}	0.70 (E)

[a] p = Phytol, gg = geranylgeraniol. Pigment without subscript in their names are esterified with p.
[b] [3^1-OH]-Chl *a* contaminant in [3^1-OH]-BChl *a*, structure assumed in analogy to formation of [3-acetyl]-Chl *a* from BChl *a*. The extinction coefficient from the spectroscopically very similar BChl *c* was used.
[c] Relative extinction coefficients of the Q_y-transitions of modified pigments to that of BChl *a* in solution (T = toluene, E = diethylether), which were used for quantitation of modified pigments bound to B800 binding sites.

plex was 50% if judged from the absorption spectrum, the excitation spectrum and HPLC analysis. The first criterion to ascertain a successful integration of the modified pigment was the observation of an EIRS. In B800–850 reconstituted according to protocols 2 and 3 with [3^1-OH]-BChl *a*, the EIRS amounts to 400 cm^{-1} for the Q_y-band (Table 3, also Fig. 5). However, this criterion is by itself not very reliable. (Bacterio-)chlorophylls can form red-shifted aggregates [35–37] and bind unspecifically to proteins.

Four additional criteria were therefore used to ascertain a successful reconstitution: (1) There is efficient energy transfer from the modified pigment to B850, which was determined from the florescence excitation spectrum (Figs. 4 and 5). (2) There is also an increase in the efficiency of the carotenoid-to-BChl *a* energy transfer, similar to the one observed for the corresponding complexes reconstituted with BChl *a* (Figs. 4 and 5). (3) Incorporation of the modified pigments yields the same spectral features of the B850 and the carotenoid bands as B800–850 reconstituted with BChl *a* under the same conditions. (4) The modified pigment is protected from photodestruction after integration into the B800 binding site, due to the presence of the carotenoids. Destruction of free pigments by light is rapid and can occur already while recording the fluorescence spectrum. An example is shown in Fig. 5 by the bleaching of the broad band around 720 nm which does not contribute to B850 fluorescence. By contrast, both the

Table 3
Absorption maxima [nm] of the modified pigments solubilized in 0.05%–0.1% LDAO-buffer (TL) or 0.7% TBG-buffer (TB) after equilibration(P) and bound in the B800-binding site of the reconstituted complexes B800–850 (type I) and B800–850 (type II) from *R. sphaeroides*

Pigment	Type of complex	λ_{max} [nm] Soret/Q_x/Q_y-band	EIRS [cm^{-1}][h] Q_y-band	Occupancy of B800 site[b,c]
BChl *a*	P	362/585/775		
	I	364[g]/585[g]/798	370	100%
	II	364[g]/585[g]/798	370	50% (TB), 50%(TL)
[3^1-OH]-BChl *a*	P	344/563/722[i]		
	I	345/563/749	500[i]	30%[d]
	II	345/561/743	400[i]	50% (TB)[d]/33% (TL)[d]
[3-Vinyl]-BChl *a*	P	350/565/742[i]		
	II	349/564/746[i]	≈ 90[i]	7% (TL)
[3-Acetyl]-Chl *a*	P	443/−/685		
	II	443/−/689	70	10% (TL)
[3^1-OH]-Chl *a*[e]	P	408/−/657		
	II	n.d./−/657	0	7% (TB)[e]/+ (TL)
Chl *a*	P	434/−/668		
	II	434/−/668	0	n.d. (TB)/7% (TL)
Pyro-BChl *a*	P	365(392)[a]/593/782(890)[a]	−	n.d.
13^2-OH-BChl *a*	P	363/584/775	−	n.d.
BPhe *a*	P	357/525/758(845)[a]	−	n.d.
Ni(II)-BPhe *a*	P	338/540/780[i]	−	n.d.[f]
Pd(II)-BPhe *a*	P	343/537/761(> 880)[a]	−	n.d.[f]

[a]Absorptions of pigment aggregates in brackets.
[b]In type II complexes, total occupancy (modified pigment plus BChl *a*) is always ≈ 50%.
[c]In type I complexes, 100% total occupancy (modified pigment plus BChl *a*).
[d]Sum of [3^1-OH]-BChl *a* plus [3^1-OH]-Chl *a*.
[e]Contaminant of [3^1-OH]-BChl *a* (≤ 15%).
[f]Judged by absorption, pigments are not or too weakly fluorescent for fluorescence analysis [31].
[g]From difference spectra B800–850 minus B850.
[h]EIRS relative to the absorption maximum of the free pigment in the same buffer system.
[i]Peak position not very accurately determinable.
n.d.: No reconstitution detectable by absorption or fluorescence spectroscopy.
See Fig. 3 for structures of pigments.

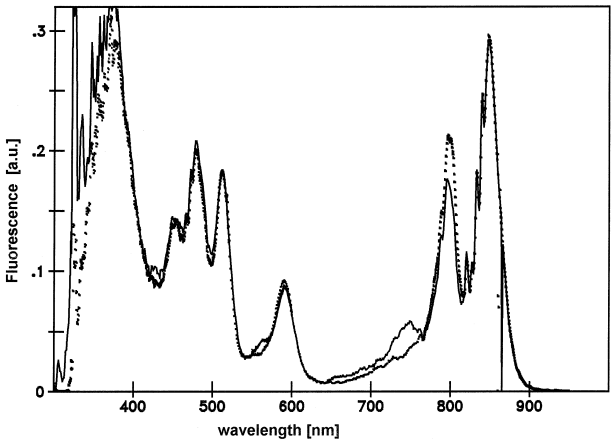

Fig. 4. Fluorescence excitation spectra ($\lambda_{em} = 875$ nm) of B850 reconstituted with [3^1-OH]-BChl *a* (solid line), and with BChl *a* (dashed line), both in TL (0.1/8.0). Reconstitution with [3^1-OH]-BChl *a* was done according to the first protocol (see text for details), with a three-fold molar excess in the mixture, and without subsequent purification on DEAE-cellulose.

bands at 743 (= [3^1-OH]-BChl *a* at B800 site) and 850 nm (= BChl *a* at B850 site) bleach very slowly and with the same kinetics, if they are irradiated with a strong incandescent light-source (not shown).

The basic spectral features of the modified samples produced by all three protocols are similar, if one takes into account the different occupancies of the B800 site. The Q_y-absorption of [3^1-OH]-BChl *a* is red-shifted in the reconstituted complexes as compared to a solution of the pigment in the detergents. The EIRS is of the same magnitude (400/500 cm^{-1}) as that of BChl *a* in the B800 binding site (370 cm^{-1}), but somewhat different for the different protocols. These shifts are reproducible and independent of using TBG and LDAO, and therefore characteristic for the distinction between type I and type II complexes. For type II-complexes, the spectral shifts are independent on whether the modified pigment is occupying all 50% (as the sole pigment in B800 binding site) or only 15%–30%. A further distinction of the type I and type II complexes was, that in the latter there was in no case a CD-signal detectable of the reconstituted pigment (Fig. 6).

Experiments with other modified pigments are summarized in Table 3, and the absorption spectra of several modified complexes are shown in Fig. 7. In all cases, the fluorescence excitation spectra follow closely the absorptions (not shown). Binding is possible for several pigments modified at rings A or B. Using protocol 3, none of them could, however, be incorporated to a comparable degree as [3^1-OH]-BChl *a*. Notably, the B800 site also accepts Chl, viz. pigments with an unsaturated ring B. This was verified by all criteria given above for [3^1-OH]-BChl *a*. It is noteworthy, that the EIRS is considerably reduced,

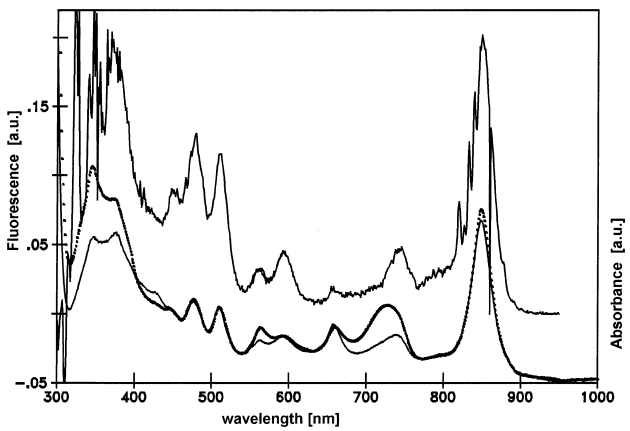

Fig. 5. Fluorescence excitation spectrum ($\lambda_{em} = 870$ nm) of B850 reconstituted according to the second protocol (see text) with [3^1-OH]-BChl *a*, recorded in TB (0.7/8.0) without purification in the reconstitution mixture (upper trace), and the absorption spectra (lower traces) of this sample before (dotted heavy trace) and after the fluorescence measurement (thin trace).

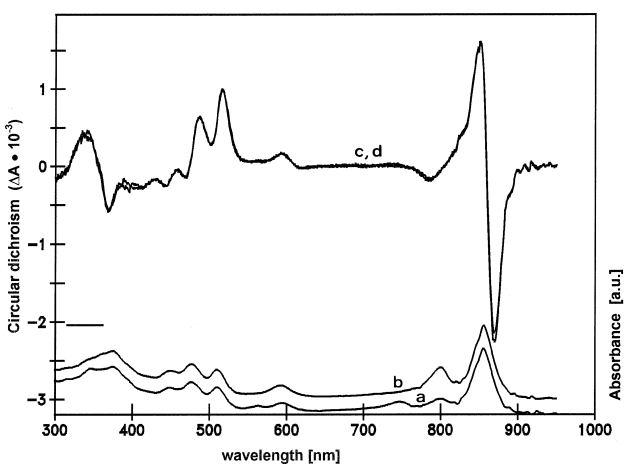

Fig. 6. CD-spectra (upper traces) and absorption spectra (bottom traces) of B800–850 reconstituted according to the third protocol (see text) with [3^1-OH]-BChl *a* (a, c) and BChl *a* (b, d) in TL (0.05/8.0).

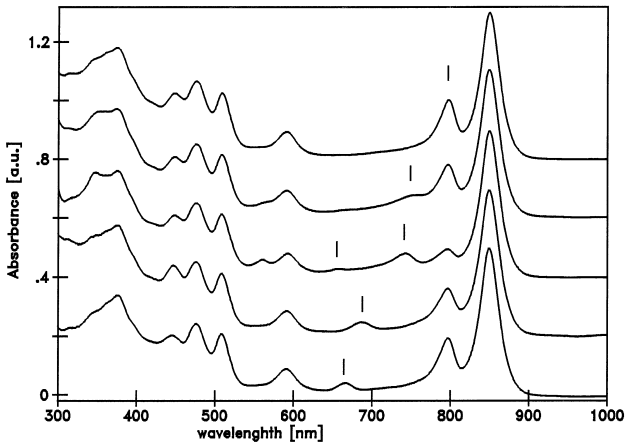

Fig. 7. Absorption spectra of modified complexes. The Q_y-bands of the modified pigments are indicated. Samples were prepared according to protocol 3 and subsequently purified over DEAE cellulose. The pigments contained were, from top to bottom: BChl *a*, [3-vinyl]-BChl *a*, [3^1-OH]-BChl *a* (together with [3^1-OH]-Chl *a* as a contaminant absorbing at 657 nm), [3-acetyl]-Chl *a* and Chl *a*.

or even absent, in the chlorins. By contrast, we were unable to incorporate pigments in which Ring E was modified or the central Mg replaced by Ni or Pd.

Reconstitution in buffers containing TBG or LDAO normally gave identical results, differing only by partial integration of BChl *a* into the B800 binding site of complexes prepared in LDAO-buffer (cf. Figs. 5 and 7 and see above). However, reconstitution with Chl *a* clearly indicates, that the two buffer systems are not totally equivalent: Modified complexes with Chl *a* could only be prepared in TL, not in TB.

4. Discussion

Unlike the previous methods which have been used to remove the BChl *a*-B800 from its binding site in the B800–850 complex from *Rb. sphaeroides* [20–23], the combination of the detergent, TBG, with the acid treatment allows this process to become reversible. This provided the basis to study the reconstitution and the altered photochemical properties of complexes with modified pigments in the B800 binding site. Although it has not yet been possible to achieve full reconstitution with modified pigments, the usefulness of this procedure has been clearly demonstrated.

Two distinct types of complexes could be characterized after reconstitution, which differ by the occupancy of the B800 site: Type I complexes are obtained, if the original BChl *a* is left in the mixture together with the added, modified pigment. In this case, the occupancy is 100%, but the pigment content is heterogeneous, viz. BChl *a* and the respective modified pigment in the B800 site. (2) Type II complexes are obtained, if the BChl *a* originating from the B800 site is removed completely from the reaction mixture prior to reconstitution. In this case the occupancy is reduced to $\leq 50\%$, but the pigment composition at the B800-site is homogeneous (in TBG, see below). There are several arguments that these are two distinct types of complexes: (1) The CD-spectra cannot be reproduced by averaging the spectra of the 100% reconstituted complex and B850 (a detailed discussion of the CD-spectral analysis will be published separately), and (2) reconstitution with [3^1-OH]-BChl *a* results in distinct EIRS for the two types of complexes, which indicates different environmental conditions in their binding sites. For exchange experiments, both types of complexes can have certain advantages. Presently, we consider type II the more promising one for future applications and improvements. Even though the occupancy is presently limited to $\leq 50\%$, it contains no residual BChl *a* at the B800 site if TBG is used as the detergent. Reconstitution experiments in LDAO lead to partial dissociation of BChl *a* from the B850 sites and re-incorporation into the B800 site, and therefore always to contamination and mixed-pigment composition of the latter.

It is not clear from looking at the X-ray data of LH2 from *Rp. acidophila* [5] and *Rs. molischianum* [7], why there is only such a reduced reconstitution yield in the type II complexes. In both structures, there is no heterogeneity discernible in the B800 binding sites, and in the nonameric complex an alternating occupancy also presents conceptual problems. The 50% occupancy may then indicate, that some other component is removed together with the BChl-B800, which then is missing in the reconstitution mixture.

The pH dependent dissociation of B800 could be reproduced with complexes of several *Rp. acidophila* species, but not with *Rs. molischianum.* The X-ray structures of these complexes are known and show

small, but distinct differences of the BChl-B800 binding sites [5,7]. The acid pH necessary for the removal, could indicate an influence of His-β(-18), which is conserved in both species as well as in *Rb. sphaeroides* (for which no X-ray structure is known). Mutagenesis has shown that it is involved in binding BChl-B800 [38].

The present reconstitution experiments show, with the modified pigments listed in Table 3, that the B800 site has unique 'selection rules', which differ from the well studied BChl *a* binding sites, viz. B870 in LH1 [17] and B800 in RC [11]. These selection rules can be summarized as follows:

(1) The central Mg can be neither replaced with Ni or Pd complexes, nor can the metal-free bacteriopheophytin *a* (BPhe *a*) be introduced. Discrimination between BChl and BPhe is a common feature of all BChl binding sites, but lack of binding for [Ni]-BChl indicates more stringent selection rules for the center of the macrocycle than has been found for the B800-site in RC and the B875 site(s) in LH1.

(2) The tolerance to alterations of the C-3 substituent is similar to that of the BChl-B800 in RC from *Rb. sphaeroides*, while such pigments are tolerated only in the 'subunit-type' complexes of LH1 and not in the more highly aggregated B875 [17]. This low discrimination of the B800-site is somewhat unexpected, because Raman-data of several LH2-complexes indicate H-bonding to the C3-acetyl-group of BChl-B800, and it is believed that this contributes considerably to the binding energy [3,19,39,40].

(3) Unlike the sites of RC and LH1, the B800 site of LH2 tolerates no changes to ring V, but rather accepts Chl, which are dihydroporphyrins. No such behavior has been found in any other BChl *a*-binding site, but it should be noted that the BPhe *a*-binding sites in RC accept pheophytin *a*, which also is a dihydroporphyrin [31].

A long standing question in the spectroscopy of (bacterio-)chlorophyll proteins is the origin of the EIRS, which for BChl *a* varies considerably (500–1800 cm^{-1}) among the different pigment binding sites. Pigment–pigment interactions, as well as specific interactions with the neighboring amino-acid residues via e.g., H-bonding, have been recognized as important mechanisms [3,8,39–41]. Additional effects, like e.g., distortions of the macrocycle, have so far been studied in less detail with BChls [10–12], but are obvious in all available high-resolution X-ray structures [5,7,42–46]. Introduction of modified pigments into the monomeric B800-sites of both RC (see Ref. [11]) and LH2, is a way (complementary to site-directed mutagenesis [3,8]) towards quantitation of the different contributions without the complication of pigment–pigment interactions. All available evidence including the results of this work shows, that BChl-B800 has no strong interaction with BChl-B850 which is discernible by absorption or CD-spectroscopy. In the series of BChls modified at C-3, the EIRS is related to the H-bonding capacity of the substituent, which in highly reduced form is also discernible in the Chl-series. Conjugation with the macrocyclic π-system can not be responsible for this effect: it is not possible for the α-hydroxy-ethyl-group of [3^1-OH]-BChl *a*, yet this pigment shows an even larger EIRS than BChl *a*.

The different EIRS in Chls and BChls could reflect the different flexibilities of the dihydro (= chlorin) and tetrahydroporphyrin (= bacteriochlorin) systems, resulting in different distortions from planarity. None of the BChl molecules in the structurally characterized binding sites is planar [5,7,42–46]. In the porphyrins [12,47] and the BChl *c*, and *e* which are chlorins, the influence of these deviations from planarity has been shown to result in considerable red shifts of the Q_y-bands. It is also experimentaly well established, that chlorins sterically hindered by a C-20 substituent, are red-shifted as compared to the unsubstituted analogs [48].

A noteworthy feature in the CD-spectrum of B850 is the broad, negative band centered around 785 nm (Fig. 1), which remains unchanged even after the complete removal of any free pigment, and which is discernible also in complexes containing modified pigments in the B800 site (Fig. 6). This CD-band must therefore be related to BChl-B850. It is intriguing to assign it to higher excitonic state(s) of the closely spaced and strongly interacting BChl-B850. A similar conclusion may be derived from mutagenesis results. Fowler et al. [41] show in their Fig. 4 an (undiscussed) shift of this broad CD-band together with Q_y, and bands in this region have also been predicted from calculations based on the X-ray structure of *Rp. acidophila* [14,16]. In view of this structure, it is particularly intriguing that exchanges are possible, too, in LH2 from *Rp. acidophila* (see

above). This line of research, on which first results are to be published shortly, is currently pursued with emphasis on higher yields of reconstitution and optimizing for preparations of stable complexes at concentrations high enough for structure analysis.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 143: 'Elementarprozesse der Photosynthese'), the British Council and the Deutscher Akademischer Austauschdienst. M.B. and B.Ü. are grateful for stipends from the Hans-Fischer-Gesellschaft, München. Pigments were provided by Ingrid Katheder, Gerhard Hartwich, Robert Porra, Bernhard Gall and Markus Fischer (all from the Botanisches Institut der Universität München). We are indebted to the Bayrol (Martinsried near München) for the gifts of Triton BG-10.

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