Selective Release, Removal, and Reconstitution of Bacteriochlorophyll *a* Molecules into the B800 Sites of LH2 Complexes from *Rhodopseudomonas acidophila* 10050[†]

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Received April 6, 1999; Revised Manuscript Received May 24, 1999

ABSTRACT: A method is described which allows the selective release and removal of the Bchl*a*-B800 molecules from the LH2 complex of *Rhodopseudomonas acidophila* 10050. This procedure also allows reconstitution of ∼80% of the empty binding sites with native Bchl*a*. As shown by circular dichroism spectroscopy, the overall structures of the B850-only and reconstituted complexes are not affected by the pigment-exchange procedure. The pigments reconstituted into the B800 sites can also efficiently transfer excitation energy to the Bchl*a*-B850 molecules.

In the primary events of purple bacterial photosynthesis, light energy absorbed by antenna light-harvesting (LH)[1] complexes is rapidly and efficiently transferred to reaction centers (RC) where it is "trapped" as chemical potential (*1*). In most species of purple bacteria, there are two types of antenna complexes (*2*−*4*). The first type, LH1, is intimately associated with RC in forming the so-called "core" complex. Arranged more peripherally to this, and present in variable amounts, is the second type of antenna complex called LH2. The spectral properties of these antenna complexes are precisely tuned to ensure the efficient funneling of excitation energy toward the RC (*5*).

In both types of LH complexes, the light-harvesting pigments (Bchl*a* and carotenoid molecules) are noncovalently attached to a protein scaffold. The crystal structure of the LH2 complex from *Rhodopseudomonas* (*Rps.*) *acidophila* 10050 has recently been determined to a resolution of 2.5 Å (*6*). It has a nonameric ring structure. Each monomeric unit contains an α and *β* apoprotein, three Bchl*a*, and one carotenoid (rhodopin glucoside) molecule. The nine α apoproteins form a hollow cylinder with the nine *β* apoproteins arranged radially outside. The complex contains two discrete pools of Bchl*a* molecules. Eighteen of the Bchl*a* molecules are sandwiched between the α and *β* apoproteins and form a continuous overlapping ring. They are each coordinated to a conserved His on either an α or *β* apoprotein via their central Mg^{2+} ion. These molecules absorb at ∼850 nm. We call them the Bchl*a*-B850 molecules. The other nine Bchl*a* molecules lie toward the cytoplasmic side of the membrane and are located between the transmembrane helices of the *β* apoproteins. They are ligated to an extension of the N-terminal methionine of the α apoprotein. These Bchl*a* molecules absorb at 800 nm and are denoted Bchl*a*-B800. Since then, the crystal structure of the LH2 complex from *Rhodospirillum* (*Rs.*) *molischianum* has been determined (*7*). It has essentially the same structure as that of *Rps. acidophila* except that the complex is an octamer, the Bchl*a*-B800 molecules are ligated to an Asp residue at position 6 on the α apoprotein, and the bacteriochlorin macrocycle of the Bchl*a*-B800 molecules is twisted by 90° and its plane tilted by 20° with respect to that in *Rps. acidophila*.

From these structures, the distances between neighboring pigments and the relative orientations of their transition dipoles have now been identified. This structural information has allowed the tentative assignment of a likely mechanism to each of the energy transfer processes in LH2. For example, the distances between and the relative orientations of the Q_y transition dipoles of the Bchl*a*-B800 and -B850 molecules suggests that both B800 → B800 and B800 → B850 energy transfer may occur by Förster's dipole−dipole, weak interaction mechanism (*8*). Such insights into the mechanisms of energy transfer from the structure alone are at best speculative. Nonetheless, the structure can be used to design critical experiments for probing the mechanisms of energy transfer within the complex.

We are particularly interested in trying to understand the mechanism of energy transfer between the Bchl*a*-B800 and -B850 molecules. Generally, it has been thought to occur by the Förster dipole−dipole, weak interaction mechanism (*1*). Here, the rate of energy transfer between donor (D) and acceptor (A) pigments, k_{DA}, can be expressed as

[†] We acknowledge generous financial support from the BBSRC (R.J.C. and P.J.D.), the Gatsby Charitable Trust (N.J.F.), the DFG (H.S.), and the Hans-Fischer Gesellschat (B.Ü.).

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[1] Abbreviations: A_x, absorption at *x* nm; AU, absorption units; B800 → B850, energy transfer from the Bchl*a*-B800 to the -B850 molecules; Bchl*a*, bacteriochlorophyll *a*; Bchl*a*-B800 and -B850, Bchl*a* molecule in the B800 and B850 binding pockets, respectively; *β*OG, *β*-octyl glucoside; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Chl*a*, chlorophyll *a*; LDAO, *N*-lauryl-*N*,*N*′-dimethylamine *N*-oxide; LH, light-harvesting; LM, *n*-dodecyl *β*-D-maltoside; mdeg, millidegrees; *Rb.*, *Rhodobacter*; RC, reaction center; *Rps.*, *Rhodopseudomonas*; *Rs.*, *Rhodospirillum*.

10.1021/bi990796i CCC: $18.00
Published on Web 07/02/1999

$$k_{DA} = \frac{\kappa^2 J}{n^4 (R_{DA})^6} \frac{\phi_D}{\tau_D}$$

where κ is the geometric factor, J is the overlap integral between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor pigments, n is the refractive index of the medium, R_{DA} is the distance between the chromatophores, and ϕ_D and τ_D are the fluorescence quantum yield and lifetime of the donor excited state, respectively (*9*). To test whether B800 → B850 energy transfer occurs by this mechanism, it is necessary to systematically alter one of the above parameters (keeping the other parameters constant) and then compare the effect of this alteration on the actual rate of energy transfer with that predicted by the theory. The variable which can be altered most easily is the spectral overlap function. It can be manipulated in a variety of ways. For example, LH2 complexes in which the Q_y absorption maximum of either the Bchl*a*-B800 or -B850 molecules is blue-shifted have been created by site-directed mutagenesis (*10*, *11*). The spectral separation between the Bchl*a*-B800 and -B850 Q_y transitions can also be increased by either increasing the pressure (*12*) or lowering the temperature (*13*). On cooling, the spectral overlap between the Bchl*a*-B800 and -B850 Q_y transitions decreases due to narrowing of both and the pronounced red shift in the Bchl*a*-B850 band. The extent of spectral overlap variability using these techniques is, however, rather limited.

In contrast, the photochemical properties of RC and LH complexes can be radically altered by selectively exchanging certain native pigments with modified tetrapyrrole molecules. Depending upon which modified pigment is exchanged, it is possible to alter the wavelength at which the pigment absorbs or the lifetime of its first excited state. Consequently, these reconstitution techniques have the potential to form modified complexes with spectral properties which cannot be created using conventional methods. For example, a diverse range of modified pigments can be incorporated into specific sites in the RC (*14*−*17*). These complexes have provided considerable insight into the singlet−singlet (*18*) and triplet−triplet energy transfer (*19*, *20*) as well as into the electron transfer (*21*) processes which occur in the RC. A more limited range of modified tetrapyrrole pigments can be incorporated into LH1 complexes (*22*−*24*). Unfortunately, in this case, those bacteriochlorin molecules which do undergo exchange have spectroscopic properties similar to those of the native pigments. In addition, as LH1 has only one pool of Bchl*a* molecules, no site selective exchange is possible. For these reasons, this approach has only had limited usefulness in improving our understanding of the energy transfer processes within LH1.

At present, techniques which allow the selective exchange of modified (bacterio)chlorin molecules into LH2 are rather limited. Existing work has focused upon the reversible binding of the Bchl*a*-B800 molecules. In 1981, it was shown that the 800 nm absorption peak can be attenuated by treating LH2 with lithium dodecyl sulfate (LDS) (*25*). On dialysis into buffer containing LDAO, the absorption peak was reinstated. Originally, this effect was interpreted as the reversible binding of the Bchl*a*-B800 molecules to the complex. It was later shown that the absorption changes are actually due to changes in the conformation of the protein (*26*, *27*). Recently, a method was reported which allows the reversible binding of the Bchl*a*-B800 molecules to the LH2 complex from *Rhodobacter* (*Rb.*) *sphaeroides* (*28*). In this approach, the Bchl*a*-B800 molecules can be released from their binding pockets by an acid treatment using buffer containing the novel Triton detergent TBG10. B850-only complexes can then be purified by ion exchange chromatography. The B800 sites can later be reconstituted with a limited number of modified (bacterio)chlorin molecules, including Bchl*a* (an estimated occupancy of 50%), 3^1OH Bchl*a* (50%), 3^1vinyl Bchl*a* (7%), and acetyl Chl*a* (7%). Two possible reasons were given for these low levels of reconstitution. First, a component essential for Bchl*a*-B800 binding may have been removed during the pigment release process. If so, its absence would have hindered the subsequent reconstitution reaction. Alternatively, and more likely, the conditions for reconstitution were not fully optimized. For example, the choice of detergent used for reconstitution appeared to be crucial. Reconstitution experiments performed using the detergent LDAO resulted in denaturation of some of the LH complexes. The Bchl*a* which was released was reincorporated into the empty B800 binding sites of intact, neighboring complexes. This meant that the B800 population in the reconstituted complexes had a mixed composition. In contrast, reconstitution experiments performed using the detergent TBG10 gave complexes with a homogeneous B800 population. The potential of this approach, however, was not fully explored.

In the study presented here, we have adopted the approach of Bandilla et al. (*28*) and optimized it for LH2 complexes from *Rps. acidophila* 10050. We describe a protocol which allows the B800 binding sites to be completely depleted. Approximately 80% of the empty binding pockets can later be reconstituted with the native pigment using the detergent *n*-dodecyl β-D-maltoside (LM). As judged by CD spectroscopy, the overall structures of the B850-only and Bchl*a*-B800 reconstituted complexes are not affected by the pigment exchange procedure. We also show that the reconstituted pigments can efficiently transfer excitation energy to the Bchl*a*-B850 molecules.

MATERIALS AND METHODS

Isolation of LH2 Complexes

Liquid cultures of *Rps. acidophila* 10050 were grown anaerobically in light at 30 °C using Pfenning's medium (*29*). Cells were harvested by centrifugation. LH2 complexes were prepared as described by Hawthornthwaite et al. (*2*). The method is now briefly summarized. Photosynthetic membranes were isolated by rupturing the whole cells in a French press at 15 000 psi. The membranes were diluted with 20 mM Tris-HCl (pH 8.0) to an A_{859} of 75 before they were treated with 2% (v/v) LDAO for 4 h.Insoluble material was removed by centrifugation prior to application of the supernatant to a sucrose gradient consisting of four discrete layers [0.8, 0.6, 0.4, and 0.2 M sucrose in 20 mM Tris-HCl (pH 8.0) and 0.2% (v/v) LDAO]. The sucrose gradients were spun in an ultracentrifuge at 150000*g* at 4 °C for 16 h overnight. The upper band containing the LH2 complexes was removed from the tubes and further purified by gel filtration chromatography using a Superdex G200 column. LH2 fractions with an A_{859}/A_{280} ratio of >3 were collected.

Purified LH2 was stored at 4 °C for up to 1 month prior to its use in the pigment exchange experiments.

Optimization of the Conditions for the Release of the Bchla-B800 Molecules

Release of the Bchl*a*-B800 molecules from their binding pockets was optimized with respect to protein concentration, and the pH and temperature of incubation. For each set of conditions, purified LH2 was diluted 1:10 with 20 mM Tris-HCl (pH 8.0) and 1% (v/v) TBG10 such that the sample had the desired protein concentration. The release reaction was started by adjusting the pH with acetic acid. When this was done, the sample turned cloudy. When TBG10 was gradually added from a 10% (v/v) stock solution, however, the sample became clear again. At regular time intervals, the absorption spectrum of the sample was recorded and the A_{800} noted. Once the A_{800} had reached a steady minimum value, the reaction was complete and the length of time for complete Bchl*a*-B800 release determined. A graph of A_{800} versus time was also plotted. The initial rate of reaction was calculated by measuring the tangent to this curve at time zero. The temperature of incubation was controlled by immersing the sample in a water bath.

Time Course of the Reconstitution Reaction

The reconstitution reaction was followed by determining the B800 site occupancy every 10 s over a 2 h period by measuring the yield of fluorescence ($\lambda_{emission} = 875$ nm) from the Bchl*a*-B850 molecules after exciting the incubation mixture at 800 nm. Initially, the cuvette contained B850-only complexes with an A_{859} of 0.1 and a 3-fold molar excess of Bchl*a*. The reaction was started by adjusting the pH to 8 with KOH. The excitation and emission slit widths were both 5 nm.

Optimizing the Conditions for Reconstitution with Bchla

The conditions for reconstitution were optimized with respect to the choice of detergent, the concentration of exogenous Bchl*a* in the reaction mixture, and the temperature of incubation. B850-only complexes ($A_{859} = 17$) were diluted 1:10 with 20 mM potassium phosphate (pH 4.75) containing either 0.1% (w/v) LM, 1% (w/v) βOG, or 0.05% (v/v) Triton X-100. Bchl*a* was resuspended in methanol and its concentration determined by absorption spectroscopy ($\epsilon_{770} = 42$ mM^{-1} cm^{-1}) (*30*). An aliquot was then added to the B850-only sample such that its final concentration was 15 μM. The pH of the sample was adjusted to 8 with KOH before it was gently shaken for 2 h at room temperature. The incubation mixture was then loaded onto a DE52 column in 20 mM Tris-HCl (pH 8.0). Excess, unbound Bchl*a* was removed by washing the column with 20 mM Tris-HCl (pH 8.0) and 0.05% (w/v) LM. The reconstituted complexes were later eluted with 300 mM NaCl in 20 mM Tris-HCl (pH 8.0) and 0.05% (w/v) LM. In the reconstituted complexes, the B800 site occupancies were determined according to their A_{800}/A_{859} ratio relative to that in the native (complete occupancy) and B850-only (no occupancy) complexes. The effect of temperature was investigated by comparing the B800 site occupancy in a reconstituted sample made at room temperature (20−22 °C) with that at 30 °C.

Optimized Pigment Exchange Procedure

Pigment Removal. Purified LH2 was loaded onto a DE52 ion exchange column in 20 mM Tris-HCl (pH 8.0). The sample was washed with 5 column volumes of 20 mM Tris-HCl (pH 8.0) followed by 5 column volumes of 20 mM Tris-HCl (pH 8.0) and 1% (v/v) TBG10 before it was eluted using 300 mM NaCl in 100 mM sodium acetate (pH 4.75) and 1% (v/v) TBG10. The Bchl*a*-B800 molecules were released by incubating the sample under the optimum conditions as described in the Results. Afterward, the incubation mixture was loaded onto a phosphocellulose column in 20 mM potassium phosphate (pH 4.75). Any "free" or weakly bound Bchl*a* molecules were removed by washing the column with 20 mM potassium phosphate (4.75) and 1% (v/v) TBG10. The column was washed until the eluant had an A_{770} of <0.01. The B850-only complexes were then exchanged into the detergent dodecyl β-D-maltoside (LM) by washing with 5 column volumes of 20 mM potassium phosphate (pH 4.75) followed by 20 mM potassium phosphate (pH 4.75) and 0.1% (w/v) LM until the eluant had an A_{770} of <0.01. Finally, the B850-only complexes were eluted with 500 mM potassium phosphate (pH 4.75) and 0.1% (w/v) LM. The B850-only complexes were further purified by application to a Sephadex 200 column in 500 mM potassium phosphate (pH 4.75) and 0.1% (w/v) LM. One milliliter fractions were collected, their absorption spectra checked, and the best fractions pooled. B850-only complexes were stored for up to 1 month at 4 °C prior to their use in reconstitution experiments.

Reconstitution of the B800 Site. The B850-only complexes were diluted with 20 mM potassium phosphate (pH 4.75) and 0.1% (w/v) LM such that they had a Bchl*a* concentration of 9 μM. An appropriate volume of the desired pigment resuspended in methanol was added such that its final concentration was 15 μM. The pH of the reconstitution cocktail was adjusted to 8 with KOH before the sample was gently shaken for 2 h at room temperature. The sample was concentrated under nitrogen using an Amicon stirred flow cell and then applied to a Sephadex 200 column in 500 mM sodium chloride, 20 mM Tris-HCl (pH 8.0), and 0.05% LM. As the reconstituted complexes emerged from the column, 1 mL fractions were collected and their absorption spectra recorded. The best fractions were pooled and concentrated using Amicon centricon concentrators (50 kDa cutoff) in a Sigma 3K20 benchtop centrifuge. Reconstituted complexes were stored at −20 °C.

Spectroscopic Techniques

Absorption Measurements. Absorption spectra were measured using a Shimadzu UV-PC2101 spectrophotometer. All samples had an A_{859} of 0.5 in a cuvette with a 1 cm path length.

Circular Dichroism Measurements. All circular dichroism measurements were taken using a JASCO J-600 spectropolarimeter at the Scottish Circular Dichroism Facility (University of Stirling, Stirling, U.K.). All samples had an A_{859} of 0.6 in a 1 cm cell. Spectra in the visible and NIR regions were recorded from 300 to 650 and 630 and 950 nm, respectively. Baseline corrections were made using an appropriate buffer solution.

Fluorescence Emission Measurements. All steady-state fluorescence measurements were taken using a Spex Fluo-

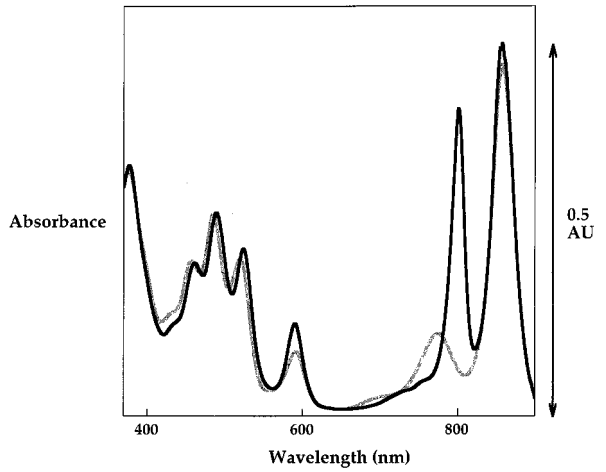

FIGURE 1: Changes in absorption which occur on complete release of the Bchl*a*-B800 molecules from LH2. The pigments were released by incubating a LH2 sample in buffer containing the detergent Triton TBG10 at pH 5.3: black, before pigment release; gray, after pigment release.

rolog 2. All samples had an A_{859} of 0.02 in a cuvette with a path length of 1 cm. B800 → B850 energy transfer in the native and Bchl*a*-B800 reconstituted complexes was probed by measuring the sensitized fluorescence from the Bchl*a*-B850 molecules after exciting the Bchl*a*-B800 molecules in their Q_y transition at 785 nm. An emission spectrum was recorded in the region of 800−950 nm. The excitation and emission slit widths were both 5 nm.

RESULTS

Optimizing the Conditions for Bchla-B800 Release

Acidification of LH2 complexes from *Rps. acidophila* 10050 to pH 5.3 in buffer containing the detergent Triton TBG10 results in a decrease in its A_{800} (corresponding to bound Bchl*a*-B800) with a concurrent rise in the A_{770} (corresponding to free Bchl*a*) (Figure 1). This spectral change is due to release of the Bchl*a*-B800 molecules from their binding pockets. The conditions for rapid Bchl*a*-B800 release were optimized with respect to protein concentration, and the pH and temperature of incubation.

The length of time necessary for complete release of the Bchl*a*-B800 molecules decreases with LH2 concentration (Table 1a). At a LH2 concentration of 0.7 μM, rapid release occurs without involving excessively large sample volumes.

Bchl*a*-B800 release is also very sensitive to pH (Table 1b). Above pH 5.5, the Bchl*a*-B800 molecules are released very slowly, if at all. On reduction of the pH of incubation from 5.5 to 3.75, however, the initial rate of pigment release increases by a factor of 4 and the length of time for complete release decreases from an estimated 8 h to 30 min (Table 1b). At pH 4.75, rapid pigment release occurs without exposing the protein to unnecessarily extreme acidic conditions.

Finally, the effect of temperature on Bchl*a*-B800 release was investigated (Table 1c). Below 20 °C, release of the Bchl*a*-B800 molecules occurs very slowly. In contrast, on the temperature of incubation being increased from 20 to 35 °C, the initial rate of Bchl*a*-B800 release increases by a factor of 6 and the length of time for complete release decreases from 5 h to 25 min (Table 1c). At 30 °C, the Bchl*a*-B800 molecules are released swiftly without causing significant LH2 denaturation.

Table 1: Effects of (a) Protein Concentration, (b) pH, and (c) Temperature of Incubation on the Overall Length of Time for and the Kinetics of Bchl*a*-B800 Release

(a)

protein concentration (μM)	time for Bchl*a*-B800 release (min)
0.4	60
0.7	90
1.5	75% of molecules released after 5 h
3.7	50% of molecules released after 6 h

(b)

pH	time for Bchl*a*-B800 release (min)	inital rate of Bchl*a*-B800 release (AU min^{-1})
3.7	30	2.33×10^{-2}
4.0	45	1.33×10^{-2}
4.2	60	1.23×10^{-2}
4.5	90	8.08×10^{-3}
4.7	90	6.60×10^{-3}
5.0	90	5.60×10^{-3}
5.5	75% of molecules released after 5 h	3.70×10^{-3}
8	no reaction	0

(c)

temperature (°C)	time for Bchl*a*-B800 release (min)	initial rate of Bchla-B800 release (AU min^{-1})
0	no reaction	0
10	75% of molecules released after 6 h	6.56×10^{-3}
20	5 h	1.79×10^{-2}
25	90	4.05×10^{-2}
30	45	6.40×10^{-2}
35	25	1.09×10^{-1}

In short, all of the Bchl*a* molecules can be released from the B800 binding sites by incubating a LH2 sample with a concentration of 0.7 μM in buffer containing the Triton detergent TBG10 at a pH of 4.75 at 30 °C for 1 h. These conditions were routinely used in all subsequent preparations of B850-only complexes.

Purifying B850-Only Complexes

After optimization of the release of Bchl*a*-B800 molecules from their binding sites, it was necessary to develop a method for separating the LH complexes from the free Bchl*a*. Bandilla et al. (*28*) were able to remove the free Bchl*a* by binding the LH complexes to a DE52 anion exchange column at pH 5.3 and then washing with buffer containing a low concentration of salt and detergent. Purified B850-only complexes were later eluted with high salt. Under these conditions, LH2 complexes from *Rps. acidophila* 10050 do not bind to DE52, suggesting that they have a net positive charge at pH 4.75. This was confirmed by its binding to the negatively charged cation exchange medium phosphocellulose. The free Bchl*a* was removed by extensive washing with buffer containing TBG10. Once the eluant had an A_{770} of <0.01, the B850-only complexes were said to be "pure". While it was possible to elute B850-only complexes from the column in TBG10 using high salt, it was first preferable to exchange the sample into the optimal detergent for reconstitution, dodecyl β-D-maltoside (LM). On the column

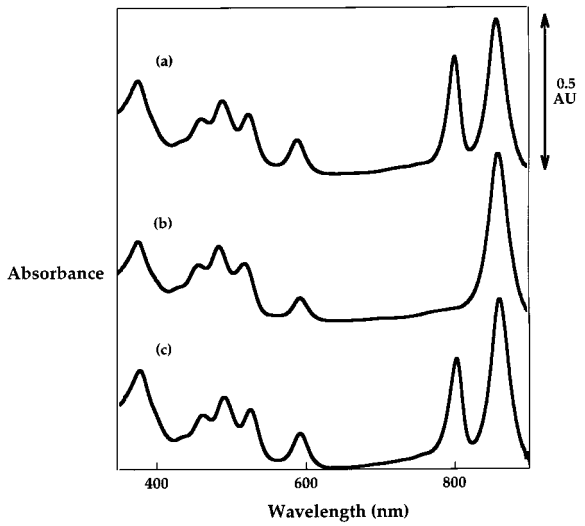

FIGURE 2: Absorption spectra of the (a) native, (b) B850-only, and (c) Bchl*a*-B800 reconstituted complexes. The B850-only and Bchl*a*-B800 reconstituted complexes were prepared as described in Materials and Methods. The wavelengths of the absorption maxima in each complex are given in Table 2.

Table 2: Wavelengths of the Pigment Absorption Maxima in Native, B850-Only, and Bchl*a*-B800 Reconstituted Complexes

	wavelength of maximum absorption (nm)		
electronic transition	LH2	B850-only	Bchl*a*-B800 reconstituted complex
Bchl*a*-B850 Q_y	858	859	859
Bchl*a*-B800 Q_y	802	n/a	802
Bchl*a* Q_x	590	594	591
carotenoid (S_0-S_2)	524, 490, 461	520, 485, 458	524, 490, 461
Bchl*a* Soret	377	377	377

being washed with buffer containing LM, however, a fraction of the B850-only complexes denatured. This material was removed by extensive washing with buffer containing LM. Intact B850-only complexes were then eluted with high salt.

Except for the loss of the absorption peak at 800 nm, the absorption spectra of the native and B850-only complexes are rather similar (Figure 2 and Table 2). In the native complex, the Q_y transitions of the Bchl*a*-B800 and -B850 molecules are at 802 and 858 nm, and their combined Q_x and Soret transitions are at 590 and 377 nm, respectively. The peaks at 461, 490, and 524 nm belong to rhodopin glucoside. In the B850-only complex, the Q_y absorption band of the Bchl*a*-B850 molecules is red-shifted by 1 nm relative to that in the native complex. A broad absorption shoulder can be seen in the region of 780−790 nm. This can be attributed to either the upper excitonic component of or vibrational energy levels associated with the Bchl*a*-B850 molecules (*23*, *24*). The Bchl*a* Q_x absorption maximum is red-shifted by 3−4 nm whereas, the carotenoid maxima are blue-shifted by 3−5 nm. In general, these are rather minor spectral changes.

Optimizing the Conditions for Reconstitution

(a) Time Course of the Reconstitution Reaction. The reconstitution reaction was followed by determining the B800 site occupancy of the reconstituted complex every 10 s during a 2 h period (Figure 3). B800 site occupancy was determined

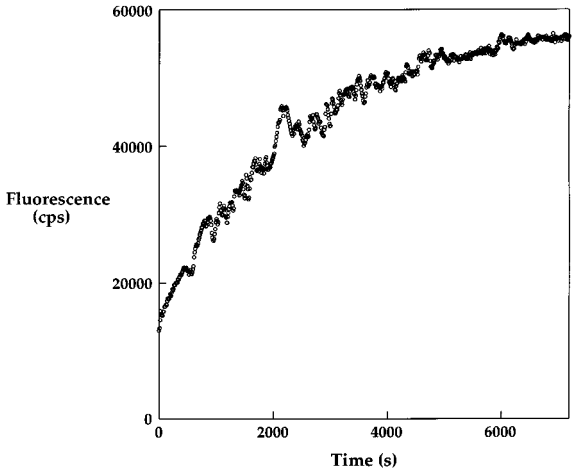

FIGURE 3: Time course of the reconstitution reaction. B800 site occupancy was determined indirectly at 10 s intervals during a 2 h period by measuring fluorescence emission from the Bchl*a*-B850 molecules after Q_y excitation of those pigments bound correctly within the reconstituted complex ($\lambda_{excitation}$ and $\lambda_{emission}$ were 800 and 875 nm, respectively).

indirectly by measuring the yield of fluorescence from the Bchl*a*-B850 molecules after Q_y excitation ($\lambda_{excitation} = 800$ nm) of those properly bound Bchl*a*-B800 molecules. At time zero, a basal level of fluorescence (14 000 cps) was observed. This is due to direct excitation of the Bchl*a*-B850 molecules. Fluorescence increases rapidly during the first hour (3600 s) before increasing more gradually during the next hour. Maximal fluorescence occurs at times of ≥2 h. This shows that the reconstitution reaction is complete after an incubation period of 2 h.

(b) Effects of Detergent, the Concentration of Exogenous Bchla in the Reconstitution Mixture, and the Temperature of Incubation on B800 Site Occupancy. (1) Choice of Detergent. Reconstitution reactions in which the detergents CHAPS, Brij 98, βOG, and LM were used were performed as described in Materials and Methods. After being incubated, the reconstituted complexes were purified by ion exchange chromatography and their absorption spectra recorded (results not shown). With Brij 98 and CHAPS, the reconstituted complexes were irreversibly adsorbed onto the DE52 column which precluded the further use of both detergents. The B800 site occupancies in those complexes reconstituted using LM and βOG were both ∼80%. This is significantly better then the 50% occupancy which was reported by Bandilla et al. (*28*) for *Rb. sphaeroides*. LM was used in all subsequent reconstitution experiments.

(2) Bchla Concentration. The B800 site occupancy rises sharply with increasing concentrations of exogenous Bchl*a* and quickly reaches a maximum of ∼80% when the incubation mixture contains a 3-fold excess of Bchl*a* (results not shown). At higher concentrations of Bchl*a*, the occupancy of the B800 sites decreases slightly. This is likely due to the nonspecific aggregation of Bchl*a* molecules in solution which would have prevented their incorporation into the empty B800 sites. A similar effect has been reported in RC (*31*).

(3) Temperature. Reconstitution cocktails containing a 3-fold excess of Bchl*a* were incubated for 2 h at either 22 or 30 °C. The complexes were later purified and their absorption spectra recorded (results not shown). A B800 site occupancy of ∼80% was obtained at both temperatures. The

absorption spectrum of control samples which had no exogenous Bchl*a* did not change under the same incubation conditions, showing that minimal, if any, LH2 denaturation had occurred.

(4) Optimum Conditions. The B800 binding sites can be optimally reconstituted by incubating a B850-only sample with a 3-fold excess of Bchl*a* for 2 h at pH 8 and room temperature.

The absorption spectrum of the Bchl*a*-B800 reconstituted complex is essentially the same as that of native LH2 (compare traces a and c of Figure 2; Table 2). The Q_y absorption band associated with the Bchl*a*-B800 molecules is restored. By comparing the height of this peak with that in the native complex, we estimate that the average occupancy of the B800 sites in the reconstituted complex is ~80%. In addition, the blue shift of the carotenoid absorption and the red shift of the Bchl*a* Q_x transition, which occur upon removal of the Bchl*a*-B800 molecules, are reversed in the reconstituted complex (Table 2). This effect has also been observed in *Rb. sphaeorides* and was interpreted as correct pigment binding (*28*).

A Comparison of the Overall Structures of the Native, B850-Only, and Bchla-B800 Reconstituted Complexes

Given the acidic conditions necessary for the release of the Bchl*a*-B800 molecules and their considerable bulk, it is quite possible that their removal affects the overall structure of LH2. For these reasons, it was important to investigate whether the pigment exchange procedure had affected the quaternary structure of the LH complex. In LH2, both the Bchl*a* and carotenoid molecules have very pronounced CD signals which are due to their precise conformation and arrangement within the complex (*32*). Putative changes in the arrangement and position of the chromatophores in the B850-only and reconstituted complexes were assessed by comparing the shape of their CD signals in the NIR and visible regions with that in native LH2.

The CD spectra of the LH2, B850, and Bchl*a*-B800 reconstituted complexes in the NIR are shown in Figure 4. The CD signal associated with the Q_y band of the Bchl*a*-B850 molecules (region of 820−920 nm) has the same shape in all three complexes. It consists of a positive band with a maximum at 858 nm and a negative band with a minimum at 880 nm. This signal is due to excitonic coupling of the Bchl*a*-B850 molecules (*32*−*37*). In the B850-only complex, this signal is red-shifted by 6 nm with respect to that in LH2. In the Bchl*a*-B800 reconstituted complex, this red shift is partially reversed by 2 nm. This may suggest that the interactions between the Bchl*a*-B800 and -B850 molecules in the reconstituted complex are subtly different from those in the native complex. Alternatively, this effect may be due to incomplete occupancy of the B800 sites. As the signal has the same shape in all three complexes, however, it suggests that the removal and reconstitution of Bchl*a* molecules into the B800 binding pockets do not affect the structure of the B850 ring. In native LH2, the negative CD signal with a minimum value at 798 nm arises from the Bchl*a*-B800 molecules. In the B850-only complex, this large, negative signal is absent. Instead, a very weak, broad negative CD signal which has a minimum value at 777 nm is observed. A similar signal has recently been reported by

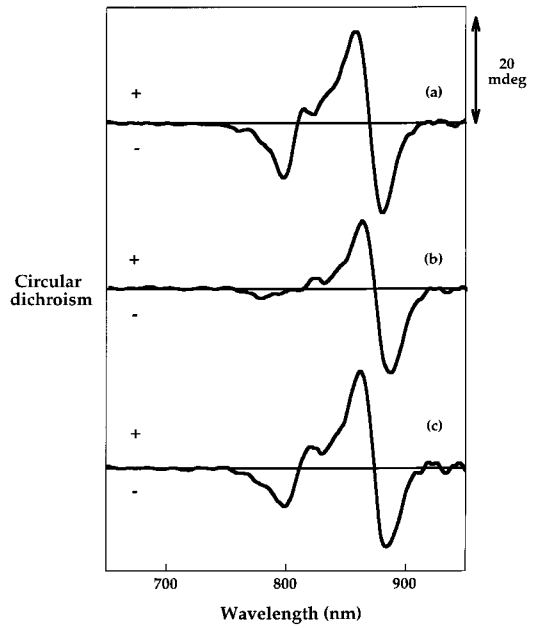

FIGURE 4: CD spectra of the native, B850-only, and Bchl*a*-B800 reconstituted complexes in the NIR. The CD signals associated with the Bchl*a*-B850 molecules (region of 820−920 nm) have a similar shape in all three complexes. This shows that removal and reconstitution of Bchl*a* molecules into the B800 binding sites do not affect the ring structure of the Bchl*a*-B850 molecules. The negative CD signal which is associated with the Bchl*a*-B800 molecules (region of 760−810 nm) is absent in the B850-only but is largely restored in the reconstituted complex. This suggests that the reconstituted pigments have an orientation and arrangement similar to those in native LH2.

Koolhass et al. (*37*) in a low-temperature CD study of a B850-only mutant from *Rb. sphaeroides*. This signal was attributed to the upper excitonic component of the B850 absorption. In the Bchl*a*-B800 reconstituted complex, the negative CD signal at 798 nm is largely restored. The return of this CD signal suggests that the orientation and arrangement of the Bchl*a*-B800 molecules in the reconstituted complex are similar to those in native LH2.

The CD signal associated with the carotenoids (region 450−550 nm) has the same shape in all three complexes also (Figure 5). In the native complex, this signal has maxima at 470, 498, and 531 nm and minima at 480 and 514 nm. As in the absorption spectrum, these maxima and minima are blue shifted by 4 nm in the B850-only complex. In the Bchl*a*-B800 reconstituted complex, this blue shift is completely reversed. The carotenoid CD signal is due to the twisted conformation which they adopt within LH2 and to their interactions with proximal pigments (*32*). As the carotenoid CD signal has the same shape in all three complexes, it suggests that the conformation of the carotenoids is not affected by either the removal or reconstitution of the Bchl*a* molecules into the B800 sites. In addition, the similarity of the CD signals suggests that the carotenoid−carotenoid and carotenoid−Bchl*a* interactions are also the same. This is further evidence which suggests that the native, B850-only, and Bchl*a*-B800 reconstituted complexes have the same overall structure. In the native complex, the CD signal associated with the Bchl*a* Q_x transition is positive and has a maximum value at 590 nm (Figure 5a). This signal has the same shape in the B850-only and Bchl*a*-B800 reconstituted complexes. In the B850-only complex, however, this maxi-

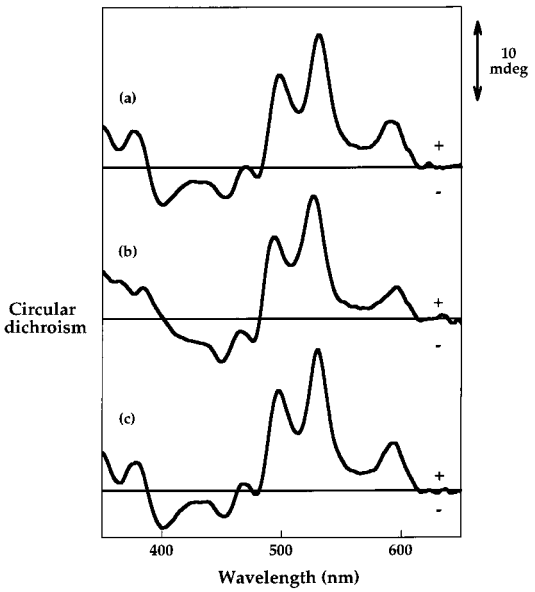

FIGURE 5: CD spectra of the native, B850-only, and Bchl*a*-B800 reconstituted complexes in the visible region. The CD signals associated with the carotenoid molecules (region of 450−550 nm) have a similar shape in all three complexes, suggesting that they all have a similar asymmetric conformation.

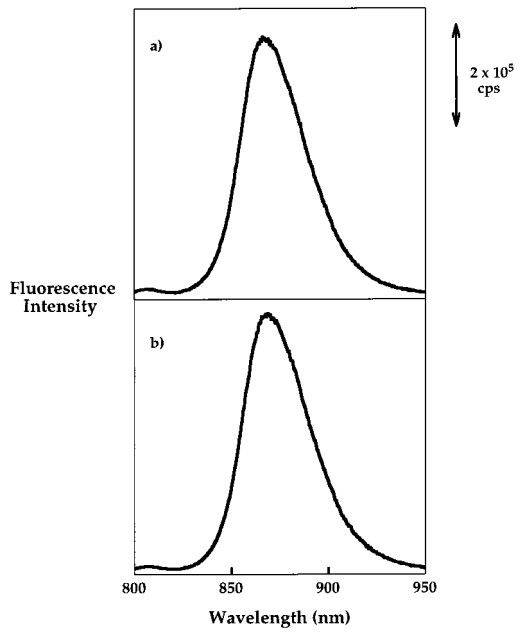

FIGURE 6: Fluorescence emission spectra of the native and Bchl*a*-B800 reconstituted complexes. In both complexes, the Bchl*a*-B800 molecules were excited on the blue edge of their Q_y absorption band at 785 nm. Both spectra have a small peak at 805 nm and a large peak at 875 nm which are due to fluorescence from the Bchl*a*-B800 and -B850 molecules, respectively. In both complexes, as the majority of emission occurs from the Bchl*a*-B850 molecules, it can be concluded that B800 → B850 energy transfer occurs with high efficiency.

mum is red-shifted by 6 nm. This red shift is reversed by 3 nm in the Bchl*a*-B800 reconstituted complex. In the native complex, the CD signal associated with the Bchl*a* Soret transition (region 300−450 nm) has a well-defined character with maxima at 350, 376, and 432 nm and minima at 364 and 400 nm (Figure 5a). In the B850-only complex, the Soret CD signal has a poorly defined shape (Figure 5b). In the Bchl*a*-B800 reconstituted complex, the CD signal is essentially the same as that in native LH2 with maxima at 350, 378, and 432 nm and minima at 364 and 400 nm (Figure 5c). The restoration of the CD signal associated with the Bchl*a* Soret transition in the Bchl*a*-B800 reconstituted complex suggests that the orientation and arrangement of the Bchl*a*-B800 molecules in the native and reconstituted complexes are similar.

The Reconstituted Pigments Participate in Efficient B800 → B850 Energy Transfer

Previously, the reconstitution reaction was assessed by measuring the yield of fluorescence emission from the Bchl*a*-B850 molecules after excitation of the Bchl*a*-B800 molecules. This experiment did not, however, give any insight into the efficiency of B800 → B850 energy transfer. Here, the efficiency of B800 → B850 energy transfer in the reconstituted complex was assessed qualitatively by comparing the yields of fluorescence from the Bchl*a*-B800 and -B850 molecules after Q_y excitation of the Bchl*a*-B800 molecules. If the reconstituted pigments had limited ability to transfer their excitation energy, and were not otherwise quenched, fluorescence would occur directly from them. Otherwise, predominantly all of the fluorescence would come from the Bchl*a*-B850 molecules. The relative contributions of fluorescence from the Bchl*a*-B800 and -B850 molecules are then a sensitive measure of the energetic coupling between them. The spectra of both the native and Bchl*a*-B800 reconstituted complexes have a minor fluorescence emission band at 805 nm and a large fluorescence emission peak at 875 nm (Figure 6). These are due to fluorescence from the Bchl*a*-B800 and -B850 molecules, respectively. In both complexes, predominantly all of the excitation energy which is absorbed by the Bchl*a*-B800 molecules is emitted as fluorescence by the Bchl*a*-B850 molecules. This shows that the efficiencies of B800 → B850 energy transfer in both the native and reconstituted complexes are very high.

DISCUSSION

The effect of spectral overlap on the rate of B800 → B850 energy transfer can be investigated in LH2 complexes where the energy gap between the Q_y absorption bands of the Bchl*a*-B800 and -B850 molecules is altered. Previously, this has been achieved using a range of techniques, including site-directed mutagenesis (*10*, *11*), pressure (*12*), and temperature (*13*). The spectral properties of LH2 can also be altered using pigment exchange methods. Recently, Bandilla et al. (*28*) have shown that the Bchl*a*-B800 molecules can be selectively removed from LH2 complexes of *Rb. sphaeroides* and later replaced with native and a limited range of modified (bacterio)chlorin molecules. Here, we have built upon this method and optimized it for LH2 complexes from *Rps. acidophila* 10050. The pigment exchange procedure has three main stages. First, the Bchl*a*-B800 molecules can be released from their binding pockets by an acid treatment using buffer containing the detergent Triton TBG10. B850-only complexes can then be purified by ion exchange chromatography using phosphocellulose. Finally, ∼80% of the empty binding sites can be reconstituted with Bchl*a*. Using their method, Bandilla et al. (*28*) were able to reconstitute ∼50% of the empty B800 binding sites with Bchl*a*. The increase in B800 site occupancy observed here can be attributed to the

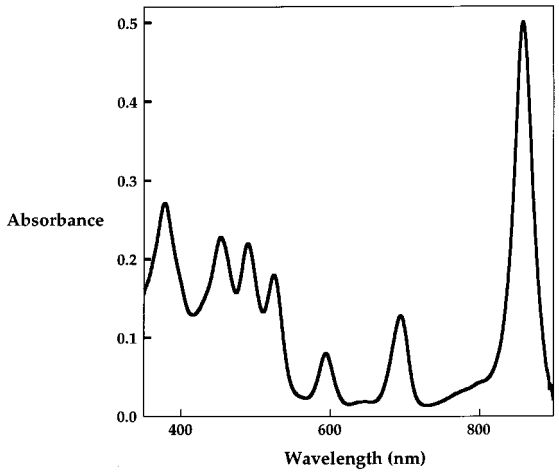

FIGURE 7: Absorption spectrum of B850-only complexes reconstituted with 3-acetyl Chl*a*.

detergent used in the reconstitution reaction. The detergents LM and βOG gave significantly higher B800 site occupancies than any of the other detergents that were tested. The reason for this is not known. Even using these detergents, however, it is not possible to reconstitute all of the B800 sites. This can be explained in one of two ways. First, the conditions for reconstitution have yet to be fully optimized. Alternatively, limited damage to a fraction of the B800 sites during pigment release (such as acid hydrolysis of the extension to the N-terminal Met on the α apoprotein) would prevent subsequent reconstitution of these sites.

Although the B800 binding pockets are located on the periphery of LH2, it is quite possible that the removal and reconstitution of Bchl*a* molecules into them may affect the overall structure of the complex. In LH2, the ring of Bchl*a*-B850 molecules is sandwiched between the α and β apoproteins and the carotenoids adopt an extended conformation across the whole complex (*6*, *7*, *38*). As both the Bchl*a*-B850 and carotenoid molecules are central to the LH2 structure, they are good determinants of complex structural integrity. As judged by CD spectroscopy, the conformations adopted by and the pigment−pitment interactions of the Bchl*a*-B850 and carotenoid molecules in the native, B850-only, and Bchl*a*-B800 reconstituted complexes are similar. This suggests that the overall structure of the LH complex is not affected by the pigment exchange procedure. CD can also be used to check the binding of the Bchl*a*-B800 molecules in the reconstituted complex. The negative CD signal associated with the Q_y transition of the Bchl*a*-B800 molecules (region of 760−810 nm) is absent in the B850-only complex but is restored in the reconstituted complex (Figure 4). This suggests that the orientation and arrangement of the Bchl*a*-B800 molecules in the reconstituted complex are similar to those in native LH2. Bchl*a*−protein interactions can be probed more sensitively by resonance Raman spectroscopy. Elsewhere, we have shown that the acetyl groups of the Bchl*a*-B800 molecules in the reconstituted complex are hydrogen bonded (*39*). Together, these results show that the reconstituted pigments are correctly bound within the B800 binding pockets.

As shown by steady-state fluorescence measurements, the Bchl*a*-B800 molecules in the reconstituted complex participate in efficient B800 → B850 energy transfer. This measurement does not, however, give any indication of the rate of B800 → B850 energy transfer, merely that it occurs significantly quicker than the fluorescence lifetime of the donor pigments. To determine whether the time constants for B800 → B850 energy transfer in the native and Bchl*a*-B800 reconstituted complexes are the same, kinetic measurements will have to be performed.

Finally, the protocol which is presented here can be used with a range of modified (bacterio)chlorin molecules to create a series of LH2 complexes in which the Q_y absorption band of the (B)Chl-B800 molecules is progressively blue-shifted. As an example, Figure 7 shows the absorption spectrum of B850-only complexes reconstituted with 3-acetyl Chl*a*. In this complex, the 3-acetyl Chl*a*-B800 Q_y absorption maximum occurs at 693 nm. The full range of modified complexes that can be made will be described in a forthcoming paper.

ACKNOWLEDGMENT

We are indebted to Drs. Sharon Kelly and Nick Price at the Scottish Circular Dichroism Facility (University of Stirling) who performed all of the CD measurements.

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BI990796I