ABSTRACT: The core light-harvesting LH1 complex of Rhodospirillum rubrum consists of an assembly of membrane-spanning α and β polypeptides, each of which binds one bacteriochlorophyll (BChl) a molecule. In this work, we describe a technique that allows the replacement of the natural, Mg BChl a cofactors present in this protein by Zn–bacteriopheophytin (Zn–Bpheo). This technique makes use of the well-characterized, reversible dissociation of LH1 induced by the detergent β-octylglucoside. Incubation of partially dissociated LH1 with exogeneous pigments induces an equilibrium between the protein-bound BChl and the exogeneous pigment. This results in the binding of chemically modified pigments to LH1, in amounts which depend on the pigment composition and concentration of the exchange buffer. This method can yield information on the relative affinities of the LH1 protein-binding sites for the different pigments BChl and Zn–Bpheo and can also be used to obtain fully reassociated LH1 proteins, with a variable content of modified pigment, which may be precisely monitored. Absorption and FT-Raman spectroscopy indicate that this exchange procedure leads to LH1 proteins containing modified pigments, but retaining a binding site structure identical to that of native LH1. Furthermore, examination of the binding curves suggests that there are two distinguishable binding sites, probably corresponding to the two polypeptides, with very different properties. One of these two binding sites shows a marked preference for Zn–Bpheo over BChl, while the other binding site appears to prefer BChl.

In purple photosynthetic bacteria, light energy is collected by the light-harvesting (LH) 1 pigment–protein complexes, which ensure an efficient funneling of excitation energy toward the photochemical reaction centers, where its transduction into chemical potential energy takes place. In purple photosynthetic bacteria, the reaction center is generally associated with a so-called “core” antenna (or LH1), frequently an additional light-harvesting system, the “peripheral” antenna (or LH2), also exists in most species. These different types of antenna complexes both have the same basic structural arrangement, they are multimers of a minimal unit containing two small, transmembrane, polypeptides, α and β, to which are bound the pigments, bacteriochlorophyll (BChl) and carotenoid. In the “core” antennae of Rhodospirillum rubrum, each polypeptide binds a single BChl a molecule, and the pigments on the different polypeptides in the multimer interact with each other and the protein environment so as to exhibit a lower energy singlet absorption transition at ca. 880 nm. The structures of two related LH2 complexes have been resolved, that of the peripheral antenna complexes of Rhodopseudomonas acidophila (1) and Rhodospirillum molischianum (2) and have been shown to consist of a ring of heterodimeric subunits. Within this annular structure, the α polypeptides form the internal protein ring, while the β polypeptides form the external ring, and two of the three BChl molecules of each protein are located between these two protein rings. To date, no atomic resolution structure for a core antenna protein has been determined; however, projection structures obtained from two-dimensional crystals of the core antenna from Rsp. rubrum (3, 4) indicated that this protein forms a larger ring than the peripheral antennae, containing 16 heterodimeric subunits and 32 intercalated chromophores. This ring is large enough to contain a reaction center that has been proposed to be located within the LH1 ring (4). Recent investigations of two-dimensional crystals of core complexes, LH1 with reaction center, from Rsp. Rubrum and Rhodobacter sphaeroides (5, 6), have confirmed this possibility, though the general existence of such 16-membered, closed rings in intact membranes is still an open question.

Besides its role in the photosynthetic process, the LH1 protein is a particularly interesting membrane protein as it can be reversibly dissociated by detergent treatment to yield a subunit form (7–11). In LH1 isolated from the carotenoid-less strain of Rsp. rubrum called Gα+, treatment with the detergent n-octyl-β-D-glucopyranoside (βOG) results in formation of a small subunit form (B820) and a concomitant shift of the absorption maximum from 873 to 820 nm (11,
Treatment of this B820 with additional βOG results in a further shift in the absorbance to 777 nm (12) associated with a further disaggregation of the protein. This stepwise formation of the 777 nm-ab sorbing form (B777) is reversible, and upon removal of the detergent B820 and then intact LH1 (B873) can be formed (12). It has been shown, by FTIR spectroscopy that neither of these dissociation steps affects the secondary structure of the polypeptides (13). It has been further demonstrated that B777 consists of isolated α-helical α and β polypeptides, still retaining their bound BCHl molecule (13, 14) and that B820 exhibits the spectroscopic properties of a dimer (15, 16), although it frequently appears to behave as a tetramer from a biochemical point of view (8, 11). The exact nature of B820 is thus still unclear. An adventitious association of two αβ subunits, interacting through their N-terminal ends, could account for the apparently dual dimer—tetramer properties. The possibility of reversibly dissociating the LH1 has been used in particular to evaluate the thermodynamic parameters associated with the B820 formation (13) as well as the influence of helix—helix association on the dynamics of the polypeptide backbone (17).

In addition to the reversible dissociation of LH1, it is possible to reconstitute B873-type complexes starting from isolated α and β polypeptides and BCHl molecules (12). Such reconstitution has been attempted in the presence of a wide variety of modified BCHl molecules (18, 19) and has led to the formation of LH1 complexes containing modified BCHl. This reconstitution technique provides an opportunity to understand the structural features involved in the interaction between BCHl and its protein-binding sites as well as the physics underlying the absorption properties of LH1. Indeed, theories developed in order to account both for the 1600 cm⁻¹ downshift of the Qy transitions of LH1-bound BCHl molecules, as well as for the 400 cm⁻¹ bandwidth of this transition, could be tested on these newly engineered complexes. However, although the development of this method represents a breakthrough for studying LH proteins, it also possesses a number of severe drawbacks. The absorption properties of complexes reconstituted with BCHl a are slightly different than those of native LH1 proteins (absorbing in the near-infrared most often at 868 nm and not at 873 nm (19)). Moreover, complexes obtained after 2% OG dissociation exhibit multiple transitions in the 770–880 nm range (18, 19), possibly indicating a proportion of misfolded or mis-associated protein (see ref 19 and spectra therein). Moreover, it is clear that when starting from isolated polypeptides and pigments obtaining reconstituted complexes depends not only on the affinity of the modified BCHl molecules for the protein-binding sites but also on every step involved in the reconstitution of this 32 subunit protein. Any failure may thus be attributed to a variety of different mechanisms. In this paper, we describe a different, alternative method for obtaining LH1 containing modified pigments. Our method of pigment exchange makes use of the well-characterized LH1/B820/B777 reversible dissociation process. This method (i) allows the estimation of the relative affinities of modified BCHl vs BCHl a molecules for the binding site(s), thus yielding to a quantitative picture of how the BCHl—polypeptide interactions are affected by the molecular changes, and (ii) yields spectroscopically pure reconstituted complexes in which the amount of modified pigments may be varied. The new complexes formed by this process, containing both normal BCHl and modified BCHl molecules in variable amounts, will help in testing the current theories about the absorption of LH1 proteins.

**MATERIAL AND METHODS**

**Protein Purification.** LH1 proteins were prepared as described in Sturgis and Robert (13). Briefly, reaction centers are first solubilized by treating twice *Rps. rubrum* chromatophores (concentration adjusted at OD735 = 50) with 0.35% LDAO. Membranes are then solubilized in the presence of 2% lauryl maltoside. After ultracentrifugation (50000 rpm for 50 min at 4 °C), the supernatant is loaded onto an FPLC anion exchange column (Ressource Q, Pharmacia) and eluted by a NaCl gradient.

For pigment exchanges, B820 were purified as follows. *Rps. rubrum* Go⁺ chromatophores (OD735 = 35) were solubilized by 2% β-octylglucopyranoside (OG) at room temperature for 30 min. After ultracentrifugation (50000 rpm, 50 min, 4 °C), the supernatant was loaded onto an anion exchange column (DEAE cellulose, Whatman, 52), pre-equilibrated in the presence of 10 mM Tris-Cl, pH 8.0, 0.8% β-OG, and eluted by a NaCl-step gradient. A small amount of B820 subunits, formed during membrane solubilization with β-OG are eluted at [NaCl] = 0. At [NaCl] = 250 mM, pure LH1–RC complexes are eluted, as controlled by gel electrophoresis (SDS-PAGE, followed by staining either with Coomassie blue or silver (BioRad kit, as recommended by the manufacturer)). After overnight dialysis to remove NaCl, LH1 were dissociated in their B820 form in the presence of 2% β-OG (3 h, room temperature) and loaded onto a FPLC anion exchange column (Ressource Q, Pharmacia) pre-equilibrated with 10 mM Tris-Cl, pH8.0, 2% β-OG, 15 mM NaCl solution. Elution was performed with a 15 mM–1 M NaCl linear gradient. Fractions containing pure B820 (as controlled by SDS-PAGE) were collected around [NaCl] = 200 mM and stored at –20 °C until further use. Typically, fractions collected after the FPLC exhibit an OD820 of ca. 20.

**Pigment Preparation.** Two different types of BCHl a molecules are present in *Rps. rubrum*, which differ in the chemical nature of the isoprenoid alcohol esterified to the C153 carboxyl group, namely, a phytol or a geranyl-geraniol. The phytol pigment is exclusively associated with the reaction centers and was removed from the pigments used in these studies. After pigment extraction from *Rps. rubrum* (20) with methanol, the geranyl-geranyl BCHl a was purified on DEAE cellulose (Whatman) in chloroform (21). The magnesium ion of BCHl a was exchanged for a Zn ion by transmetalation by way of the labile cadmium complex (22, 23). Geranyl-geranyl Zn—BPheo was then purified on a reverse-phase column (LiChroprep RP-18, Merck) in acetone–HEPES (25 mM, pH 7.85) (80:20 v/v) (24). The purity of the material was checked by visible—near-infrared absorption spectroscopy, reverse-phase high performance thin-layer chromatography (HPTLC, RP-8, Merck, acetone–HEPES (25 mM, pH 7.85) (80:20 v/v)) and silica thin-layer chromatography (60F254, Merck, toluene/acetone (90:10 v/v) (23)).

**Pigment Exchange.** β-OG was solubilized in a 10 mM Tris-Cl (pH 8.0)—acetone solution (1:80 (v/v)) to a
concentration of 10% (wt/vol). Pigments (Zn-Bpheo, BChl, or mixtures of these two pigments) dissolved in diethyl ether were added to this solution and dried under argon. This procedure allows an easy transfer of pigments to aqueous solutions. The pigments and detergent were then taken up in 50 mM ammonium bicarbonate, pH 8.0, to a final β-OG concentration of 2%. B820, also in 2% β-OG, 50 mM ammonium bicarbonate, pH 8.0, was then added to a protein/extraneous exogenous pigments ratio of 1:10. This mixture was then incubated at 30 °C for an hour. Under our conditions (typically 2% β-OG and 0.3 x 10^-8 mol pigment), B820 is partially dissociated at this temperature and both B820 and B777 are present in the solution. The temperature is a particularly critical parameter for the experiment, at lower temperatures the pigment exchange becomes very slow, and the equilibrium is thus difficult to reach, while at higher temperatures the reconstitution yield was much lower. This reduction in reconstitution yield is probably caused by irreversible processes occurring during this step. The sample is then 2-fold diluted with ammonium bicarbonate buffer equilibrated at room temperature to obtain a final, 1% β-OG solution. It is then loaded onto an anion exchange column (DEAE cellulose, Whatman, 52) preequilibrated in the presence of 50 mM ammonium bicarbonate, pH 8.0. 1% β-OG, which retains the free pigments. The fact that, at this step, Zn-Bpheo is found associated with the polypeptide thus corresponds to an exchange procedure, occurring while the sample is maintained at high temperature. The B820 fractions containing exchanged pigments are then collected and diluted with 50 mM ammonium bicarbonate to a final β-OG concentration of 0.8%. This triggers the reconstitution of the B820 into forms exhibiting lower energy absorption transitions. Completion of the reconstitution procedure was achieved following the method of Davis et al. (19), i.e., letting the sample stand overnight in the cold (4 °C). To measure the relative affinity of BChl and Zn-Bpheo to the dissociated LH1, these pigments were mixed before their transfer to aqueous solutions, so that the total amount of added pigment was kept identical and only the relative concentrations of BChl and Zn-Bpheo were modified.

The relative amount of pigment bound was determined as follows. LH1 proteins were bound to an anion exchange column (DEAE cellulose, Whatman, 52) preequilibrated with 50 mM ammonium bicarbonate pH 8.0. The column was then dried with a stream of Argon and washed with acetone/methanol (7:2 (v/v)) to elute the pigments. The eluted pigments were dried under Argon and taken up in an acetone–HEPES buffer (25 mM, pH 7.85, 75:25 (v/v)). The pigment mixtures were then analyzed by high-performance liquid chromatography, using an isocratic gradient acetone–HEPES buffer (25 mM, pH 7.85, 75:25 (v/v)), on a C8 nucleosil column (150 × 4.6 mm, Sigma/Aldrich) and detecting the eluted pigments by their absorption at 360 nm. The molar ratios between the different pigments were determined from the chromatogram after calibrating the detection system for the different pigments.

Sedimentation Chromatography Experiments. The samples (native LH1 and LH1–50% Zn-Bpheo) were disposed on a linear sucrose gradient from 1.2 to 0.2 M and ultracentrifuged (20000 rpm for 20–25 h at 4 °C).

Spectroscopy. Absorption spectra were recorded on a Cary 5 double-beam spectrophotometer (Varian plc, Sydney).

FIGURE 1: Absorption spectra (250–950 nm) of (a) Purified LH1 proteins (dashed curve), (b) LH1 protein after incubation in the presence of BChl a molecules (dotted curve), and (c) LH1 proteins after incubation in the presence of Zn–Bpheo molecules, containing 90% of Zn–Bpheo (see text for details).

Circular dichroism (CD) spectra were recorded on a dichrographe III spectrophotometer (I. S. A., France) coupled to an infrared photomultiplier. FT-Raman spectra were recorded at 4 cm⁻¹ resolution using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module, equipped with a continuous Nd:YAG laser (providing excitation at 1064 nm) as described in Mattioli et al. (25). Spectra are the result of 10 000 co-added interferograms.

RESULTS

Electronic Absorption Spectroscopy of the LH1 and LH1 Subunits after Pigment Exchange. Figure 1 displays the absorption spectra of LH1 complexes reconstituted after exchange, containing 0% (control), or 90% Zn–Bpheo, compared to the absorption spectrum of purified LH1. The control sample here was exposed for the same time to the same concentration of exogenous pigment molecules, as the sample exchanged with 90% Zn–Bpheo. The absorption of the control sample is extremely similar to that of isolated LH1. In particular, the position of the Qy transition is the same (±1 nm) in both the isolated and reconstituted complexes. Depending on the temperature of the water bath (more than 30 °C) where the exchange sample was incubated and on the concentration of the detergent in the anion exchange column used to retain the free pigments, an additional transition around 786 nm is sometimes present, probably reflecting a small amount of mis-associated polypeptide, or a small amount of BChl a bound unspecifically to a secondary site on one of the polypeptides. In the spectra of
the complexes containing 90% Zn–BPheo, the Q_y transition is blue-shifted to 854 nm. Such a blue-shift is expected for these complexes, as the Q_y transition of isolated Zn–BPheo in diethyl ether is 8 nm blue-shifted relative to that of isolated BChl in the same solvent. Its bandwidth is similar to that of the Q_y bands of the two other spectra, indicating the absence of partially associated complexes. However, there is a weak transition at 780 nm, accounting for about 10% of the total area of the absorption in the near-infrared, presumably equivalent to the transition at 786 nm sometimes observed during control samples after reconstitution.

For a series of other pigment compositions, we will focus on the near-infrared region (800–925 nm) of the absorption spectra of LH1 complexes (Figure 2) containing 10, 25, 50, 75, and 90% Zn–BPheo. The position of the Q_y transition of these complexes varies according to the amount of Zn–BPheo they contain (see Figure 2, inset), and this dependence of absorption wavelength on the proportion of Zn–BPheo may be reasonably fitted (correlation coefficient 0.97; χ^2 = 1.46) by a linear function with a slope of 0.24 nm/% Zn–BPheo. However, a slightly better fit may be obtained by fitting these data to a sigmoid curve (see Figure 2; χ^2 = 1.39).

It is nearly impossible to precisely evaluate the quaternary structure of LH1 in solution, and evaluating that of reconstituted complexes is equally difficult. Sedimentation experiments were performed in which the samples were disposed on a linear sucrose gradient from 1.2 to 0.2M, and ultracentrifuged (20000 rpm for 20–25 h at 4 °C). Native LH1 and LH1 reconstituted with Zn–BPheo migrated on the gradient at the same depth (data not shown). This indicates that the native LH1 and LH1 reconstituted with Zn–BPheo have the same density. CD spectroscopy is sometimes used to assess the quality of LH1 preparations. Indeed, CD spectra of LH1 exhibit a characteristic signal constituted by a nonconservative peak at 877 nm (II). CD spectra of LH1 containing 25% Zn–BPheo also exhibit a non conservative peak, shifted at 887 nm (data not shown). Presence of such a nonconservative signal may be considered as an indication that the structure of reconstituted complexes is closely related to that of native LH1.

The LH1 complexes enriched in Zn–BPheo can be dissociated into the sub-complexes B820 and B777 (by addition of 2 and 5% βOG, respectively) (Figure 3) derived from LH1 complexes containing different amounts of Zn–BPheo. It should be noted that the Q_y transition of a Zn–BPheo-containing B820 subunit form is located at 813 nm, and that of a B820 form containing both Zn–BPheo and BChl is located at 815 nm. The width of these transition is similar in these three spectra. Absorption of Zn–BPheo when
bound to the α and/or β polypeptides is at 771 nm (Figure 3). In addition to the shifts in the Q transition, it should be noted that in these spectra the Q transition of Zn–Bpheo is shifted from 558 nm (seen in diethyl ether) to 589 nm, indicative of a strong interaction between the central Zn atom and one axial ligand (26, see discussion).

**FT-Raman Spectroscopy.** FT-Raman spectroscopy in pre-resonance with the long-wavelength Q transition of BChl molecules provides a sensitive technique for probing the BChl-binding site and is particularly sensitive to the hydrogen bonding interactions of the conjugated carbonyl groups of the BChl macrocycle as well as the conformation of the BChl in its protein-binding sites (27). This spectroscopic technique has been used to obtain information on both LH2 (28–32) and LH1 (33–36) apoproteins and has been shown to be extremely sensitive to perturbations of the BChl-binding sites. To assess, at a molecular level, the quality of reconstitution of LH1, preresonance FT-Raman spectra of BChl- and of Zn–Bpheo-containing LH1 were thus measured. In Figure 4, we show the carbonyl stretching region of the FT-Raman spectra of native LH1, compared with that of reconstituted complexes, obtained after exchange with either BChl or a large excess of Zn–Bpheo. FT-Raman spectra of native LH1 proteins are characterized by three resolved bands between 1600 and 1700 cm$^{-1}$ (33, 36). The band at 1607 cm$^{-1}$ arises from skeletal stretching modes, involving in particular the methine bridges of the bacteriochlorins, and it is sensitive to the conformation of the BChl molecules (37). For isolated Zn–Bpheo in diethyl ether, this band is observed at 1616 cm$^{-1}$, i.e., about 7 cm$^{-1}$ blue-shifted than for 5-coordinated BChl in the same solvent, and this shift was attributed to differences in the macrocycle conformation induced by the change of the size of the central ion between these two molecules (37). The frequency of this band in Zn–Bpheo-containing LH1 at 1616 cm$^{-1}$ thus constitutes an internal control that the Zn–Bpheo molecules are responsible for the Raman signal observed in the spectra. As discussed in Lapouge et al. 1999 (39), binding BChl to LH1 results in a particular conformation of the bacteriochlorin macrocycle, which may be characterized by the analysis of a limited number of Raman bands. Table 1 displays the frequencies of these Raman bands, for both the BChl, Zn–Bpheo, and native LH1 and LH1 reconstituted with 90% Zn–Bpheo. It may be seen that most of the shifts observed in the Raman spectra of LH1-bound BChl vs isolated BChl α are observed also for the bound Zn–Bpheo. It can thus be concluded that the conformation of the bacteriochlorin macrocycle are similar in BChl and Zn–Bpheo when these molecules are bound to the LH1 apoprotein.

The band at 1643 cm$^{-1}$ in Raman spectra of LH1-bound BChl was attributed to the stretching modes of the acetyl carbonyl groups of both BChl molecules bound to the αβ subunit. Each one is interacting with a neighboring tryptophan, Trp $\alpha_{43}$ and Trp $\beta_{25}$, respectively (36). The band at 1663 cm$^{-1}$ has been attributed to the stretching modes of the keto carbonyl groups of these BChl molecules, each one interacting with the nitrogen atom of the histidine involved in the liganding of the Mg ion of the other BChl, through its other, protonated, nitrogen atom (35). These bands are observed at 1642 and 1665 cm$^{-1}$, respectively, in spectra of Zn–Bpheo containing LH1. To relate these frequencies to the strength of the H-bonds these groups are involved, it is necessary to take into account the frequencies of these groups observed for isolated molecules in organic solvents. The strength of the H-bonds is indeed related not to the absolute carbonyl frequency, but to the downshift in this frequency

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**Table 1: Frequencies of the Main Bands of FT-Raman Spectra of BChl α and Zn–Bpheo α in diethyl ether Reconstituted LH1 with BChl Molecules and Reconstituted LH1 with 90% Zn–Bpheo Molecules**

<table>
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<tr>
<th>BChl in ether</th>
<th>Zn–Bpheo in ether</th>
<th>Reconstituted LH1 with BChl molecules</th>
<th>Reconstituted LH1 with 90% Zn–Bpheo molecules</th>
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*Observed at room temperature, with a 1064 nm excitation."
Two binding sites for the Zn to Bpheo incorporated into LH1 increased only slowly from large differences in their relative affinities toward Zn and BChl. This model is taking into account the binding site for these molecules. With more than 20% high amounts of Zn-7 binding sites on the 

\[
\alpha{\text{BChl}} + \text{Zn} - \text{Bpheo} \iff \alpha{\text{Zn}} - \text{Bpheo} + \text{BChl}
\]

\[
\beta{\text{BChl}} + \text{Zn} - \text{Bpheo} \iff \beta{\text{Zn}} - \text{Bpheo} + \text{BChl}
\]

The equation of the model is as follow:

\[
y = 1/2 \left[ \frac{Rx}{1 + (R - 1)x} + \frac{Sx}{1 + (S - 1)x} \right]
\]

with: \(y = \text{Zn} - \text{Bpheo}-\text{contained in LH1 after exchange}, x = \text{Zn} - \text{Bpheo}-\text{contained in the exchange solution}, R = \text{ratio of the affinity constant of } \alpha \text{ or } \beta \text{ LH1-polypeptides for } \text{Zn} - \text{Bpheo} \text{ and } \text{BChl} (K_{\text{Zn} - \text{Bpheo}} / K_{\text{BChl}}), \text{ and } S = \text{ratio of the affinity constant of } \beta \text{ or } \alpha \text{ LH1-polypeptides for } \text{Zn} - \text{Bpheo} \text{ and } \text{BChl} (K_{\text{Zn} - \text{Bpheo}} / K_{\text{BChl}}). \) One of these sites possessing a ca. 25 times higher affinity for Zn-Bpheo than for BChl, and the other one having a slightly (ca. twice) higher affinity for BChl than for Zn-Bpheo.

**DISCUSSION**

In this work, we describe a technique allowing the exchange of the natural, BChl, cofactors of LH1 for chemically modified ones. This technique is mainly based on the exposure of the dissociated LH1 proteins to exogenous pigments, which results in an exchange of the BChl molecules bound to the monomeric \(\alpha\) and \(\beta\) polypeptides for modified pigments. Neither the warming of the B820, nor the presence of additional BChl molecules in the solution (nor, in all probability, the exchange of these exogenous cofactors with the polypeptide-bound pigments) impairs the ability of LH1 polypeptides to reassociate into the native form. Indeed, when the whole procedure is conducted with BChl, the position of the Q absorption transition of the reconstituted LH1 is at 873 nm, similar to that of isolated LH1. For comparison, the positions of this transition have been reported at 870 nm after B820 reconstitution (12) or at 868 nm when starting from isolated pigments and polypeptides (19). This absorption is identical to that of the reconstituted LH1 after LDAO solubilization, which were
used for 2D-crystallography experiments (3). We thus conclude that the reconstituted LH1 after our control procedure must possess a structure very close, if not identical, to that of native, purified LH1. Also, the width of this transition is identical (±1 nm) to that of purified LH1, indicating the absence of only partially reassociated proteins. A higher energy transition at 786 nm can sometimes be observed, the intensity of which is strongly dependent on how the first and final step of the reconstitution is performed. When present, we have not been able to separate this pigment from the reconstituted LH1, by ultracentrifugation on sucrose density gradients, by anion exchange chromatography, or by size exclusion chromatography. We thus conclude that this transition arises from the pigment molecules bound to the reconstituted LH1. As this transition is not observed when LH1 are reconstituted from B777 and/or B820 forms in the absence of additional, isolated, pigments, it seems likely that it corresponds to BChl molecules bound to a secondary site on either the α or on the β polypeptide.

When dissociated LH1 complexes are incubated in the presence of Zn−Bpheo, an exchange between these molecules and the protein-bound pigments is observed, allowing the purification and reconstitution of LH1 containing variable proportions of these two types of chromophores. FT-Raman spectra of Zn−Bpheo-containing LH1 indicate that the binding state of the carboxyl groups of these molecules are very similar to those of the BChl in LH1 proteins. As discussed above, the downshift of the band arising from the keto carbonyl stretching modes upon LH1 binding is exactly the same for Zn−Bpheo and BChl. This indicates that these groups are involved in H-bonds of nearly identical strength and geometry. The downshift experienced by the bands arising from the acetyl carbonyl stretching frequencies is slightly different for Zn−Bpheo and BChl (Δν = 3 cm⁻¹). Though this difference is spectrally relevant, it is not larger than that observed between LH1 complexes from different species, or containing different types of carotenoid molecules. For example, this band is observed at 1641 cm⁻¹ in the spectra of LH1 extracted from *Rhodobacter sphaeroides* (33, 36), and at 1643 cm⁻¹ in those of *Rsp. rubrum*. These frequencies thus provide evidence that both the carbonyl groups of LH1-bound Zn−Bpheo are interacting with the same partner molecules with the same geometry than those of LH1-bound BChl molecules, and thus that Zn−Bpheo binds to LH1 precisely in the BChl protein-binding pocket with the same geometry. These experiments thus show that exposing dissociated LH1 to Zn−Bpheo induces a spontaneous exchange between these molecules and the natural cofactors of LH1 proteins.

Pigment exchange does not require, at any point the unfolding of the α or β polypeptides. Indeed, as reported by Sturgis et al. (13), reversible dissociation of LH1 never induces drastic changes in their secondary structure. It is thus likely that this method is less constraining for reconstituting LH1 with modified cofactors than previously reported protocols involving reconstitution from isolated α and β polypeptides. Raman results clearly indicate that it leads to substituted LH1 possessing a structure that is nearly identical to BChl-containing LH1. Additional evidence for this method to be less constraining than full protein reconstitution, comes from exchange experiments performed in the presence of unmodified BChl. In these experiments where exogenous BChl is added to partially dissociated LH1 complexes, an exchange must occur between these molecules and the LH1-bound ones. Nevertheless, the LH1 obtained after such procedure exhibit an absorption at 873 nm, identical to that of native LH1, while those obtained after full reconstitution from isolated polypeptides and BChl molecules absorb at shorter wavelength.

In 1996, Davis et al. (19) studied the reconstitution of LH1 from isolated polypeptides, in the presence of various modified (bacterio)chlorin molecules, including Zn−Bpheo. When using the α and β polypeptides from *Rsp. rubrum*, these authors observed the formation of a subcomplex, equivalent to the B820 form, absorbing at 805 nm, and could reconstitute a LH1-like complex absorbing at 852 nm. When using the α and β polypeptides from *Rb. sphaeroides*, they obtained a B820 form absorbing at 813 nm. However, they were unable to reconstitute a proper, far-red absorbing LH1-like complex from this intermediate form. The pigment they used for performing these reconstitution experiments possessed a phytly chain esterified to the C17° carboxylate group, i.e., it was the pigment found in the LH1 of *Rb. sphaeroides*; however, that found in *Rsp. rubrum* is esterified instead with geranyl-geraniol. So is the Zn−Bpheo used here. The absorption transition in the near-infrared of the B820 form obtained after Zn−Bpheo exchange was observed at 813 nm, and under no conditions we could observe contributions at 805 nm. However, we were perfectly able, upon detergent removal, to obtain Zn−Bpheo-containing LH1 absorbing at 853 nm. In light of these result, the data reported by Davis et al. (19) appear particularly interesting. They indeed suggest that the 805 nm-absorbing species obtained from the *Rsp. rubrum* polypeptide is not properly associated, though it is able to lead to Zn−Bpheo-containing LH1, exhibiting spectral properties close to those we observe, upon detergent removal. In contrast, when starting from the *Rb. sphaeroides* polypeptides, although the absorption found for the partially associated complex is the same as that which we report here; this form is unable to lead to the properly associated LH1 protein. This situation can be interpreted in two different ways, either the structural requirements for reconstituting LH1 proteins are different when starting from isolated polypeptides, and when exchanging pigments in situ or the chemical nature of the hydrophobic chain esterified at position C17° influences the correct reconstitution of the B820 form. In the latter case with the *Rsp. rubrum* polypeptides, it would seem that the full reassociation of the LH1 brings enough energy into the system, so that the incorrect association in the subunit B820 can be rectified, and a reorganization leads to the fully and correctly associated protein.

The pigment exchange procedure constitutes an invaluable method for evaluating the affinity of the modified cofactors to the LH1 polypeptides, relative to that of BChl. The data obtained clearly indicate the presence of two different binding sites, one exhibiting a 25-fold higher, the other only half the affinity toward Zn−Bpheo than toward BChl, respectively. As there are two different binding sites present in LH1, the simplest explanation is that each of these binding sites exhibit a different affinity toward Zn−Bpheo. However, the exchange is performed in the presence of a mixture of the B777 and B820 forms, i.e., of a mixture of isolated (B777) and partially associated polypeptides (B820), which
are highly bound to pigments. It is thus possible that each of the α and β polypeptides, when isolated, exhibit different affinities toward Zn–Bpheo. It is also possible that the observed difference in affinity arises from the difference of the binding sites in the B820 form. Indeed, even if the kinetics of pigment exchange are more rapid in the B777 form, the presence of the Zn–Bpheo on α (or β) could favor the formation of B820, thus leading to an accumulation of B820 with Zn–Bpheo in one of the two possible binding sites. We thus cannot, at present, determine precisely which spectral form is responsible for the observed difference in affinity. Structurally, such a difference may be explained by slight differences in constraints on pigment conformation for polypeptide binding and/or B820 formation. Indeed, in the crystallographic structure of LH2 from Rps. acidophila (38), a clear difference in the pigment conformation appears between the BCHl molecules bound to the α- and β-polypeptides. More recently, the conformation of LH-bound BCHl molecules has been systematically addressed by using Raman spectroscopy (39). That study showed in particular that the conformation of LH2- and LH1-bound BCHl’s were closely and pair wise related. A similar difference in conformation between α- and β-bound BCHl molecules is thus to be expected in LH1 proteins. The relaxed conformation of Zn–Bpheo is slightly different from that of BCHl, the bacteriochlorin macrocycle of Zn–Bpheo being slightly more distorted than that of BCHl (37). Binding Zn–Bpheo to one of the BCHl-binding sites could thus be less energetically costly, if this molecule presents, in its relaxed conformation, a deformation corresponding to that imposed by the protein environment. It is worth noting at this point that bacteria naturally inserting Zn–Bpheo in their LH1 proteins have recently been characterized (40).

In summary, exchanging pigments in LH1 proteins constitutes a new, less constraining method, for modifying the chemical nature of the LH1 cofactors. It allows in particular the engineering of core antenna proteins containing mixtures of chemically different cofactors, the study of which, either in their fully reconstituted, LH1, form or in their B820 form, will open a novel route for performing experiments to elucidate the pigment–pigment and pigment–polypeptide interactions in these complexes. Modeling the variations of the electronic properties of the reconstituted LH1, according to their Zn content, may help in particular in evaluating the influence of molecular disorder on the absorption properties of these proteins, the coupling between pigments, as well as the degree of excitation delocalization in antenna proteins. Such studies are currently being performed in our laboratory.

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REFERENCES