Reconstitution of the B873 Light-Harvesting Complex of *Rhodospirillum rubrum* from the Separately Isolated α- and β-Polypeptides and Bacteriochlorophyll *a*†

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ABSTRACT: The light-harvesting complex of *Rhodospirillum rubrum* was reversibly dissociated into its component parts: bacteriochlorophyll and two 6-kilodalton polypeptides. The dissociation of the complex by *n*-octyl β-D-glucopyranoside was accompanied by a shift of the absorbance maximum from 873 to 820 nm (a stable intermediate form) and finally to 777 nm. In the latter state, bacteriochlorophyll was shown to be free from the protein. Complexes absorbing at 820 and 873 nm could be re-formed from the fully dissociated state with over 80% yield by dilution of the detergent. Absorbance and circular dichroism properties of the re-formed B820 complex were essentially identical with those of B820 formed from chromatophores. Phospholipids and higher concentrations of complex were required to obtain the in vivo circular dichroism spectrum for reassociated B873. Reconstitution of the light-harvesting complexes from separately isolated α- and β-polypeptides and bacteriochlorophyll was also demonstrated. Absorbance and circular dichroism spectra of these complexes were identical with those of complexes formed by the reassociation of the dissociated complex. Bacteriochlorophyll and the β-polypeptide alone formed a complex that had an absorbance at 820 nm, but an 873-nm complex could not be formed without addition of the α-polypeptide. The α-polypeptide alone with bacteriochlorophyll did not form any red-shifted complex. In preliminary structure–function studies, some analogues of bacteriochlorophyll were also tested for reconstitution.

Photons of excitation energy are absorbed and efficiently funneled by the light-harvesting (LH)[1] complexes of purple photosynthetic bacteria to reaction center complexes where the primary photochemical events occur (Okamura et al., 1982; van Grondelle, 1985; Thornber, 1986; Cogdell, 1986). Although many of the purple bacteria have more than one type of LH complex, *Rhodospirillum rubrum* contains only the B890 type (B881 for wild-type *R. rubrum*), which is closely associated with the reaction center.

The B881 LH complex of *R. rubrum* has been isolated in forms that retain their in vivo absorbance (Sauer & Austin, 1978; Cogdell et al., 1982; Picorel et al., 1983) and circular dichrosim (CD) spectral properties (Sauer & Austin, 1978; Kramer et al., 1984b; Cogdell & Scheer, 1985). The pigment–protein complexes in these preparations are large (M_r >100 000) (Thornber, 1986; Cogdell, 1986; Picorel et al., 1984) and contain two unique hydrophobic polypeptides of about 6 kDa each: B881-α and B881-β (Cogdell et al., 1982; Picorel et al., 1983). Both polypeptides are easily isolated by extraction with organic solvents (Tonn et al., 1977; Brunisholz et al., 1981, 1984; Gogel et al., 1983), and their amino acid (Brunisholz et al., 1981, 1984; Gogel et al., 1983) and gene sequences (Berard et al., 1986) have been determined. Bacteriochlorophyll *a* (BChl *a*) is noncovalently bound to the protein with a stoichiometry of 2 BChl/$\alpha\beta$ pair (Cogdell et al., 1982). In wild-type *R. rubrum*, one carotenoid molecule, spirilloxanthin, is also noncovalently bound to the complex per $\alpha\beta$ pair (Cogdell et al., 1982). The carotenoid is missing in the G-9 mutant of the species.

The unique environment of the complex effects a shift to lower energy of the red absorbance band of the BChl from 770 nm in organic solvents (e.g., methanol) to 881 nm in wild-type *R. rubrum*. The corresponding band is at 873 nm in the G-9 mutant or in the wild type after carotenoid has been extracted. Thus, the presence of carotenoid appears to cause a red shift of 8 nm from 873 to 881 nm. The red shift from 770 to 873 nm has been attributed to various pigment–pigment (Katz et al., 1977; Worcester et al., 1986; Loach et al., 1985; Scherz & Parson, 1984, 1986) and pigment–protein interactions (Thornber, 1986; Brunisholz et al., 1984; Ghosh et al., 1984; Drews, 1985; Zuber, 1985, 1986).

The minimal size of an isolated LH complex that is required for retention of its in vivo absorbance is unknown. Recently,

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[1] Abbreviations: BChl, bacteriochlorophyll; B881, the core light-harvesting complex of wild-type *R. rubrum* which has an absorbance maximum at 881 nm; B873, the core light-harvesting complex of the G-9 mutant (carotenoidless) of *R. rubrum* or the wild-type light-harvesting complex after benzene extraction (both with absorbance maxima at 873 nm); B873(chromatophores), the native B873 light-harvesting complex in chromatophores; B873(reassociated), the light-harvesting complex absorbing at 873 nm formed from a B820 or 777(dissociated) sample; B873(reconstituted), the B873 light-harvesting complex formed from a sample of B881-α, B881-β, and BChl in 4.5% OG; B820, the subunit form of B873 that has an absorbance maximum at 820 nm after treatment with OG; B820(chromatophores), B820 derived from chromatophores; B820(reassociated from 777), B820 formed from a 777(dissociated) sample; B820(reconstituted), B820 formed from a sample of B881-α, B881-β, and BChl in 4.5% OG; 777(dissociated), the material (most likely free BChl and polypeptides) formed after further addition of OG to B820; 777(reconstituted), separately purified B881-α, B881-β, and BChl in 4.5% OG; B800–850, the second type of light-harvesting complex found in *Rb. sphaeroides* and other bacteria; it is more peripheral to the reaction center than B881, and it has absorbance maxima at 800 and 850 nm; B881-α, the *R. rubrum* B881 polypeptide which contains one histidine; B881-β, the *R. rubrum* B881 polypeptide which contains two histidines; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); LDAO, lauryldimethylamine *N*-oxide; LH, light harvesting; OG, *n*-octyl β-D-glucopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BPh, bacteriopheophytin; BD, blue dextran; RC, reaction center.

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a stable B820 subunit of the LH complex of *R. rubrum* was isolated by using *n*-octyl β-D-glucopyranoside (OG) and gel filtration chromatography (Miller et al., 1987; Loach et al., 1985). It was shown to have the same 2:1 ratio of BChl to an αβ pair, but its pigment–protein–detergent micelle size was about 55 000 daltons, suggesting that the subunit was an $\alpha_2\beta_2$·4BChl complex, assuming that it bound an equal weight of OG. The far-red absorbance band of the subunit complex was blue-shifted from in vivo to 820 nm, but could be shifted back to near 873 nm by diluting or removing the OG. When the complex absorbed near 873 nm, its molecular weight was >100 000.

Our goal in studying these reversibly formed subunits of the LH complex has been to develop methodology for reconstituting the LH complex from its individual components, which would then allow the systematic exploration of basic structure–function questions through modification of the various components. Such reconstitution of pigment–protein complexes in photosynthetic systems from pure protein and pure BChl has not yet been reported. Previously, Clayton and Clayton (1981) developed methodology whereby the 800-nm component of the B800–850 LH complex of *Rhodobacter sphaeroides* could be depleted with lithium dodecyl sulfate and then restored by lauryldimethylamine *N*-oxide (LDAO) addition with or without addition of pure BChl (part of the 850-nm component was irreversibly lost in these experiments). That system has been characterized further by Kramer et al. (1984a). Also, Picorel et al. (1983) presented evidence that they possibly reconstituted a small portion of the 881-nm absorbance in their antenna holochrome preparation from *R. rubrum*.

We have previously reported that a smaller B777 complex could be formed from B820 and reversibly shifted back to 820 and 873 nm in quantitative yields (Parkes-Loach et al., 1987). The work reported here describes and further characterizes the conversion of B820 to a 777-nm-absorbing form [called 777(dissociated B820)] and the reconstitution of the 820- and 873-nm-absorbing complexes from separately isolated BChl *a*, B881-α, and B881-β. Also described are preliminary tests of reconstitution in which naturally occurring analogues of BChl (BChl *b*, bacteriopheophytin *a*, chlorophyll *a*, and chlorophyll *b*) were initially selected to test structural requirements for chromophore binding.

Materials and Methods

Isolation of B820. *R. rubrum* wild-type cultures were grown as described previously (Loach et al., 1963). Chromatophores were isolated by sonication of the whole cells followed by differential centrifugation (Loach et al., 1963). Preparation of the LH complex was carried out by isolating the B820 subunit form as described in Miller et al. (1987). Briefly, the chromaphores were washed with buffer containing EDTA and Triton X-100 to remove loosely bound proteins, leaving only the reaction center and LH complexes. The washed chromatophores were then lyophilized and extracted with benzene which removes carotenoid and ubiquinone. The carotenoid-depleted material was titrated with the detergent OG until the sample's far-red absorbance band had shifted from 873 to 820 nm. Reaction centers and any free BChl were separated from the LH complex by gel filtration on a Sephadex G-100 column. The resulting B820 complex was stable for at least 3–4 months if stored in the dark at 4 °C.

Preparation of 777(Dissociated B820). B820 was dissociated by further titration with OG at room temperature. The typical B820 sample had an absorbance at 820 nm of 0.5–1.2 cm^{-1} in 50 mM potassium phosphate/5 mM $MgSO_4$ (pH 7.5) containing 0.70–1.00% (w/v) OG. OG was added to the sample as a solid or as a 20% (w/v) solution in water. The rate of formation of 777(dissociated B820) was immediate, unlike the slow conversion of the last portion of B873 to B820 that required hours (Miller et al., 1987). The final OG concentration required to obtain over 90% conversion from 820 to 777 nm was usually 3.5–5.0%, but samples with higher 820-nm absorbances could require more OG (e.g., a sample with an A_{820nm} = 10 required addition of 8% OG for 85% conversion).

Isolation of B881-α and B881-β Polypeptides. B881-α was isolated by extracting chromatophores or whole cells with $CHCl_3/CH_3OH$ (1:1 v/v) and running the extract on a Sephadex LH-60 column equilibrated in $CHCl_3/CH_3OH$ (1:1 v/v) containing 0.1 M ammonium acetate to separate it from other protein, pigments, and lipid (Tonn et al., 1977; Brunisholz et al., 1981, 1984; Gogel et al., 1983). To isolate B881-β, the B881-α-depleted chromatophores or whole cells were first extracted with $CHCl_3/CH_3OH$ (1:1 v/v) containing 0.1 M ammonium acetate. Then the B881-β was extracted from the chromatophores or whole cells with $CHCl_3/CH_3OH$ (1:1 v/v) containing 0.1 M ammonium acetate and 10% acetic acid (Brunisholz et al., 1984). The intervening extraction step ($CHCl_3/CH_3OH$/ammonium acetate) improves the purity of the B881-β recovered in the subsequent extraction step with little apparent loss. The extracted B881-β was then passed through an LH-60 column equilibrated with $CHCl_3/CH_3OH$ (1:1 v/v) containing 0.1 M ammonium acetate and 10% acetic acid (Brunisholz et al., 1984). These procedures usually resulted in preparations of B881-α that contained less than 4% B881-β and preparations of B881-β that contained 20% or less contamination by B881-α, as judged by SDS–PAGE and reverse-phase HPLC (see below). No other polypeptides were found.

For purer preparations, and to check the purity of isolated B881-α or B881-β, the proteins were chromatographed on a reverse-phase HPLC system using a modification of previously described procedures (Miller et al., 1987; Tarr & Crabb, 1983). The B881-α or B881-β was dissolved in hexafluoroacetone trihydrate and injected onto either a Perkin-Elmer C18 column (HS-5 HCODS, 0.5 × 12 cm) or a Perkin-Elmer C8 column (HS-10, 0.5 × 25 cm). A gradient program was used to elute the protein. It employed 0.1% trifluoroacetic acid in water as the aqueous solvent and acetonitrile/2-propanol (2:1) containing 0.1% trifluoroacetic acid as the organic solvent (Tarr & Crabb, 1983). Both columns resolved B881-α and B881-β into distinct peaks. As seen in Figure 1, the C18 column often yielded two peaks of B881-α (confirmed by amino acid composition to be identical; unpublished data). The C8 column allowed better recovery of B881-β (>70% vs. <60% with the C18 column), but both B881-α and B881-β eluted as broad peaks with some tailing. Generally, where yields were not crucial, the C18 column was used.

BChl Addition to Bleached B820(Chromatophores) and to Bleached 777(Dissociated B820). B820(chromatophores) and 777(dissociated B820) were bleached by exposure to light from a tungsten projection lamp for 15–30 s (approximately 10^7 erg cm^{-2} s^{-1}). This photodegradation resulted in a loss of 95% or more of the near-infrared absorbance band at 820 or 777 nm. BChl *a* in CH_3OH was then added in an amount appropriate for the solution to have an A_{777nm} equivalent to that of the initial 777(dissociated B820) before bleaching, or, in the case of B820, an A_{777nm} that was 0.55 of the initial A_{820nm}. This ensured that the amount of BChl added was equivalent to the amount of original BChl. The absorbance spectra of the

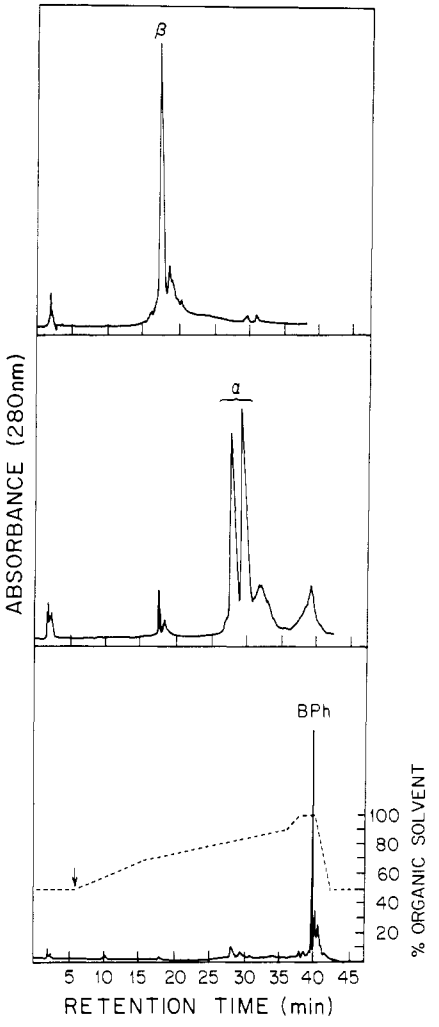

FIGURE 1: Reverse-phase HPLC chromatograms of B881-α, B881-β, and BChl. A C18 column and a three-stage linear gradient [bottom and (---)] were used. The gradient was started (arrow) 5 min after sample injection, and the flow rate was 0.7 mL/min (see text for details). Samples were detected by monitoring the absorbance at 280 nm. (Top) B881-β extracted from chromatophores and isolated by gel filtration on an LH-60 column. (Middle) B881-α isolated by gel filtration on an LH-60 column. (Bottom) BChl, which converts to bacteriopheophytin (BPh) under these HPLC conditions.

complexes were recorded after the percent OG was adjusted to that appropriate for either B820 or B873 formation as described under Reconstitution Assay.

Reconstitution of LH Complexes. Pure B881-α and B881-β were solubilized separately in hexafluoroacetone and their concentrations determined quantitatively using their UV absorbances at 290 nm and the molar absorption coefficient, ϵ_{290nm} = 10 200 M^{-1} cm^{-1}. This coefficient had been previously determined for B881-α in trifluoroethanol, 1% (w/v) SDS in water, and $CHCl_3/CH_3OH$ (1:1 v/v) by carefully weighing milligram amounts of purified B881-α and measuring the ultraviolet spectra of the solutions (Miller et al., 1987). Using B881-β obtained after purification on the HPLC column, we have determined its ϵ_{290nm} to also be 10 200 M^{-1} cm^{-1}. After the B881-α and B881-β solutions were prepared, they were either combined in a 1:1 ratio (moles of α/moles of β) and dried onto the sides of a round-bottom flask or separately dried onto the sides of two different round-bottom flasks by rotary evaporation. In each case, protein was dissolved in 3.5 mL of 50 mM potassium phosphate/5 mM $MgSO_4$ (pH 7.5) containing 4.5% (w/v) OG. The final protein concentration was about 10 μM. BChl *a* was dissolved in CH_3OH, and 25 μL was added to the protein solution. This resulted in a solution with an absorbance at 777 nm of 0.5–0.8 cm^{-1}. When BChl *b* or bacteriopheophytin was substituted for BChl, they were solubilized in acetone. Chlorophyll *a* and chlorophyll *b* were solubilized in methanol.

Reconstitution Assay. The solutions were tested for their ability to form 820-nm-absorbing and 873-nm-absorbing complexes (in the case of BChl *a*) or other red-shifted bands. To form an 820-nm-absorbing complex with BChl, the solution was diluted with buffer until the final OG concentration was 0.6–0.8%. The initial dilution was usually to 0.8%, and then the OG concentration was further reduced in approximately 0.05% increments until an optimal B820 spectrum was obtained. Finally, the percent OG was adjusted to 0.3–0.4% to form a complex absorbing near 873 nm. Controls of BChl alone in detergent solution were also diluted in the same manner.

Absorbance and CD Spectra. Absorbance spectra from 350 to 950 nm were recorded with a Shimadzu UV-160 spectrophotometer using opal glass after both the reference and sample cuvettes to compensate for scatter. For ultraviolet spectra of the polypeptides, the opal glass was removed. A Cary 14 recording spectrophotometer with scattered transmission attachment and a Varian 219 recording spectrophotometer were also used occasionally to verify absorbances and peak wavelengths. Data were stored on disk by interfacing the Shimadzu spectrophotometer with an Applie IIe microcomputer. The HP-Sciplot program of Interactive Microware, Inc., and a Hewlett-Packard 7470 printer were used to plot the data.

CD spectra were recorded with a Jasco J500-C spectropolarimeter. Samples had an absorbance of between 0.3 and 1.0 at their far-red absorbance band using either a 1-cm or a 10-cm cuvette. Absorbance spectra were taken before and after CD measurement to verify that the samples had not degraded or changed. A Hamamatsu R316 photomultiplier tube was used for detection from 950 to 550 nm, and a Hamamatsu R376 photomultiplier tube was used in the 700–250-nm range.

Size Estimation of the Subunits. Gel filtration chromatography and ultrafiltration through membranes with various pore sizes were used to estimate the sizes of 777(dissociated B820), B820(chromatophores), and B873(reassociated from B820) pigment–protein complexes. For gel filtration, a 2.5 × 100 cm Sephadex G-100-120 column was equilibrated with 50 mM potassium phosphate/5 mM $MgSO_4$ (pH 7.5) containing 0.85% OG. EDTA–Triton-washed, benzene-extracted chromatophores which had been titrated with OG to form B820 were applied to the column (17 mg in 11.3 mL of phosphate buffer containing 0.98% OG) and eluted with buffer containing 0.85% OG. The elution volumes of the 820-nm-absorbing material and reaction centers were determined. Blue dextran (3.9 mg) was solubilized in 2.6 mL of phosphate buffer containing 0.98% OG. BChl in ethanol (20 μL) was also added to this sample to a final A_{777nm} = 2.2 cm^{-1}. The sample was applied to the same G-100 column and eluted with phosphate buffer containing 0.85% OG, and the elution volumes of the BChl and blue dextran were determined.

A second set of gel filtration experiments was conducted using a 0.9 × 100 cm Sephadex G-100 column which was equilibrated with buffer containing 2.8% OG. Two samples, one of 777(dissociated B820) in 19.6% OG and one of blue dextran (6 mg/mL) and BChl in 19.6% OG, were separately applied to the column and eluted by using buffer with 2.8% OG. In additional experiments with this column, samples of

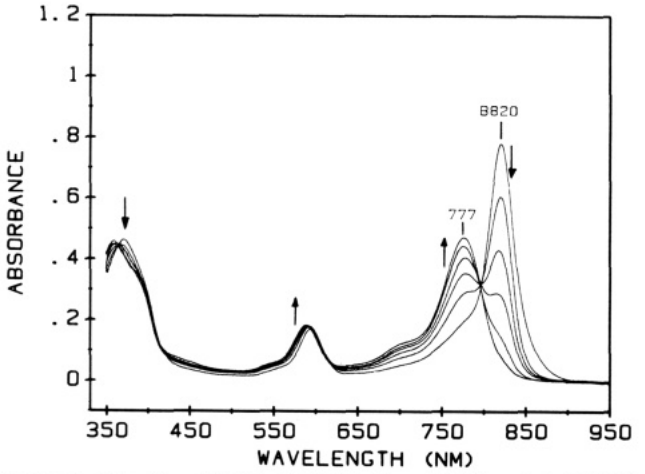

FIGURE 2: Titration of B820 with OG to form 777(dissociated B820). Absorbance spectra were recorded at OG concentrations of 0.8, 1.4, 2.2, 3.1, 4.3, and 5.9% (w/v). Arrows indicate change in absorbance with increasing OG concentration as 777(dissociated B820) is formed.

blue dextran and BChl in 2.8% OG, 777(dissociated B820) in 3.4% OG, and B881-α (2 mg/0.3 mL) in 2.8% OG were separately applied and eluted from the column by using buffer containing 2.8% OG. Similar experiments were conducted with Sephadex G-75-40 and G-50-80 columns.

For the ultrafiltration experiments, an Amicon ultrafiltration stirred cell (Model 202) with YM 10, YM 30, XM 50, and YM 100 membrane filters was used. The membranes had molecular weight cutoffs of 10000, 30000, 50000, and 100000, respectively. Samples were placed in the cell, and pressures of 25 psi were applied. Absorbance spectra were taken of the concentrates that were retained by the filter and filtrates that were able to pass through the filter.

Materials. *n*-Octyl β-D-glucopyranoside was purchased from Calbiochem and Pierce Chemical Co., hexafluoroacetone trihydrate (98%) was from Aldrich Chemical Co., and BChl *a* (isolated from *Rb. sphaeroides*) and chlorophyll *a* were from Sigma Chemical Co. Bacteriopheophytin *a* was a kind gift from Dr. Robert Uphaus, University of Nebraska, and BChl *b* and chlorophyll *b* were kind gifts of Dr. Tomasz J. Michalski, Argonne National Laboratory.

RESULTS

Preparation and Characterization of 777(Dissociated B820). 777(dissociated B820) was prepared by the addition of OG as shown in Figure 2. Since a set of isosbestic points is apparent, a single product was formed from a single reactant. The resulting absorbance spectrum was identical with that of free BChl *a* in 4.5% OG buffer from 950 to 350 nm, with the exception of a small residual 820-nm shoulder. OG in excess of 20% was required to completely shift the remaining shoulder.

777(dissociated B820) was quantitatively converted back to an 820-nm-absorbing form in which the 820-nm absorbance appeared immediately (30 s or less) upon dilution to the correct concentration of OG (Figure 3A). The re-formation of B820 occurs rapidly even at a concentration of 1 μM 777(dissociated B820). The similarity of the A_{777nm}:A_{820nm} ratios for the re-formed B820(reassociated from 777) and the B820 originally prepared from chromatophores indicates that at least 80% of the 777(dissociated B820) has been converted to B820. B873(reassociated from 777) was produced by further dilution of OG, but its formation was slower, as it was in earlier experiments involving formation from the B820(chromatophores) species (Miller et al., 1987). The initial shift to 860–870 nm

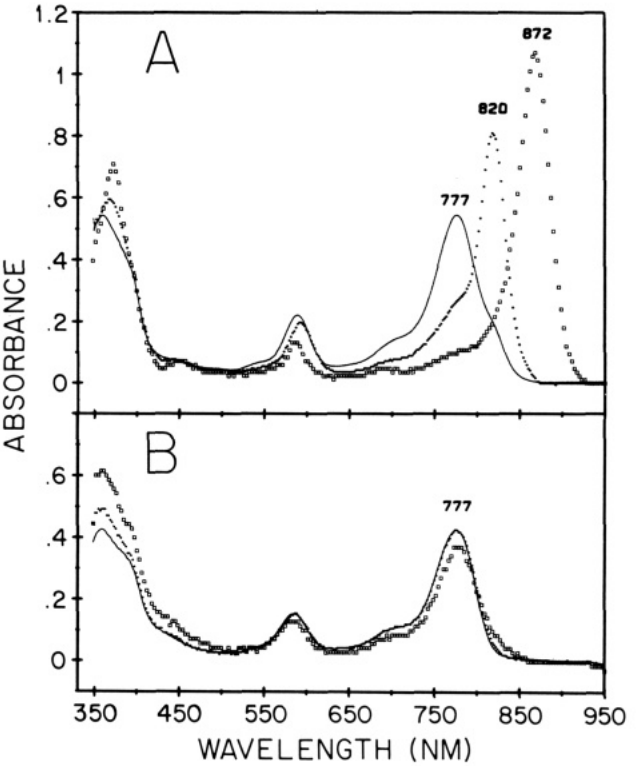

FIGURE 3: (A) Dilution of a 777(dissociated B820) sample to B820- and B873-forming conditions. Absorbance spectra are of 777(dissociated B820) at 4.5% OG (—), the same sample diluted with phosphate buffer to 0.72% OG to form B820(reassociated) (●), and the sample diluted to 0.36% OG to form B872(reassociated) (□). (B) Dilution of a sample of BChl in 4.5% OG to B820- and B873-forming conditions. BChl was dissolved in methanol, and 25 μL was added to 3.5 mL of phosphate buffer containing 4.5% OG (—). The sample was diluted with phosphate buffer to 0.63% OG (●) and then to 0.32% OG (□). For comparison purposes, the absorbance values for spectra of the samples at 0.72%, 0.63%, 0.36%, and 0.32% OG were multiplied by their respective dilution factors.

was immediate, but a gradual additional shift to 872 nm occurred over the next several hours. The B820(reassociated from 777) was converted to the long-wavelength form with little loss (Figure 3A). The yields of B820(reassociated from 777) and B873(reassociated from 777) were estimated to be greater than 80% on the basis of (1) the absence of significant free BChl (absorbing at 777 nm) in the B873 form and (2) the comparison of the relative absorbances of each of the original species (formed by dissociation of B873) to that of the subsequently reassociated forms.

Control experiments conducted with BChl alone are very important since BChl oligomers with long-wavelength λ_{max} values readily form in aqueous solution. The results of diluting a solution of BChl in 4.5% OG having a BChl concentration comparable to that in the reconstitution experiments are shown in Figure 3B. At higher concentrations of BChl, these controls often had a small, broad peak or shoulder at 848 nm.

The relative size of 777(dissociated B820) was determined by gel filtration chromatography and by ultrafiltration through different size-exclusion membranes. Neither BChl in 4.5% OG nor the pigment of 777(dissociated B820) in 4.5% OG would pass through a 10000 molecular weight cutoff filter. Both samples were partially concentrated with a 30000 molecular weight cutoff filter, with about half the 777-nm-absorbing material passing through the filter. This suggests that the BChl–OG micelles and the OG micelles of 777(dissociated B820) are about M_r 30000. This was supported by gel filtration on a Sephadex G-50 column (molecular weight exclusion limit = 30000). 777(dissociated B820) (including the

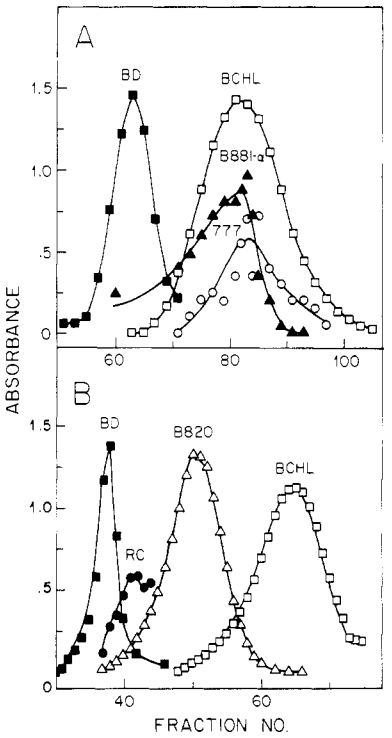

FIGURE 4: (A) Elution profiles for gel filtration chromatography of 777(dissociated B820), B881-α, BChl, and blue dextran (BD) on Sephadex G-100 using 2.8% OG in the elution buffer. Blue dextran (■) and BChl (□) were applied as one sample; B881-α (▲) and 777(dissociated B820) (○) were run separately. Absorbance was measured at 276 nm for B881-α, 640 nm for blue dextran, and 777 nm for BChl and 777(dissociated B820). For comparison purposes, the absorbances of the B881-α and 777(dissociated B820) fractions were multiplied by 3- and 5-fold, respectively. Each fraction contained 0.35 mL. (B) Elution profile for gel filtration chromatography of B820(chromatophores), BChl, blue dextran, and reaction centers (RC) on Sephadex G-100 using 0.80% OG in the elution buffer. B820 and the reaction centers were applied in the same sample, and BChl and blue dextran were applied together as a second sample. Absorbance was measured at 622 nm for blue dextran (■), 820 nm for B820-(chromatophores) (△), 800 nm for reaction centers (●), and 777 nm for BChl (□). The absorbances for fractions of blue dextran, BChl, and reaction centers were multiplied by 10 to facilitate comparison to B820(chromatophores). Fraction volumes were 4.4 mL.

protein) and BChl eluted near the void volume. BChl and 777(dissociated B820) were also retained to the same extent on a Sephadex G-100 column (equilibrated in 2.8% OG), whereas B820(chromatophores) eluted earlier than free BChl on a G-100 column equilibrated with 0.8% OG, indicating that the B820 subunit is larger than the 777(dissociated B820) (Figure 4). In control experiments, blue dextran and BChl eluted at the same respective volumes whether in 2.8% or 0.8% OG. Previously, the molecular weight of B820 had been determined to be about 55 000 (Miller et al., 1987).

Because the 777(dissociated B820) had an absorbance spectrum identical with that of free BChl and it had the same size as BChl in OG, it was of interest to us to determine whether 777(dissociated B820) was a loosely bound BChl–protein complex or whether BChl, B881-α, and B881-β were separately solubilized in OG. Unfortunately, on gel filtration columns, B881-α, free BChl, and 777(dissociated B820) all appeared at the same elution volume which did not allow us to distinguish between a complex and separate solubilization. However, CD spectra, fluorescence decay kinetics, fluorescence yields, and reconstitution experiments using separately isolated BChl and protein components (to be discussed later) support the conclusion that there are separately solubilized BChl and protein components in 777(dissociated B820). The CD spectra

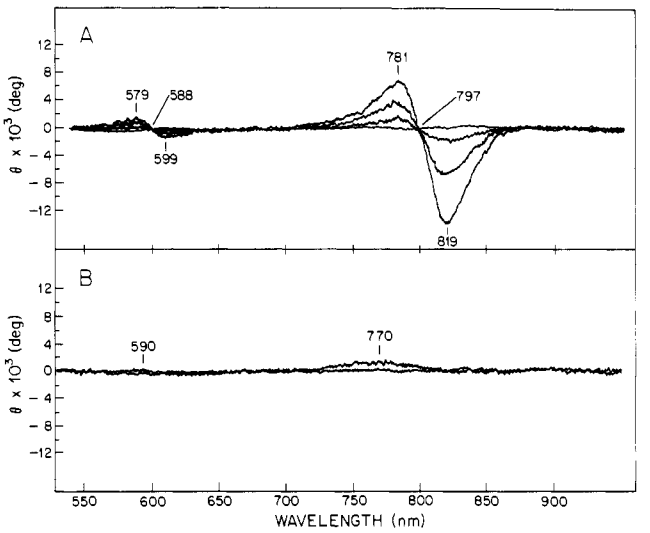

FIGURE 5: (A) CD spectra were recorded as B820 was titrated to 777(dissociated B820). The spectrum with the largest value of θ at 781 nm is that of B820 (A_{818nm} = 1.06 cm^{-1}) at 0.8% OG. The magnitude of θ decreased as the OG concentration was increased to 4.0% (middle line) and 7.4% [777(dissociated B820)]. The straightest line is the base line. At 7.4% OG, the 777(dissociated B820) had a spectrum similar to the 777(dissociated B820) in Figure 3A with a less pronounced 820-nm shoulder. (B) The CD spectrum was recorded for BChl in 5% OG. This sample had an absorbance at 777 nm of 1.08 cm^{-1}. A base line is also shown.

recorded during a B820 → 777 titration and a CD spectrum of free BChl in OG are shown in Figure 5. The CD spectrum of 777(dissociated B820) approximates that of BChl, allowing for residual B820 in the sample. Fluorescence yield and fluorescence decay kinetics were identical for 777(dissociated B820) in 4.5% OG and BChl in 4.5% OG (unpublished results). The fluorescence of both samples decayed exponentially with a lifetime of 2.0 ns.

Reconstitution of BChl into Bleached 777(Dissociated B820) and Bleached B820. If BChl in the 777(dissociated B820) state was indeed free from the protein, or at least so loosely associated with the protein that pigment–protein interactions did not alter its absorbance, CD, fluorescence yield, or fluorescence decay kinetics, it should be possible to incorporate added BChl into 777(dissociated B820) and then reform B820. Since the BChl in the B820 and 777 states is highly sensitive to light degradation, the samples were first exposed to illumination to completely degrade the native pigment. This allowed us to evaluate incorporation of subsequently added BChl. When a sample of 777(dissociated B820) was illuminated to degrade the BChl, over 95% of the absorbance at 777 nm disappeared within 15–30 s. Some small peaks due to BChl degradation products appeared at shorter wavelengths, and the sample became more scattering (unpublished data). BChl was added as a concentrated solution in CH_3OH instead of in an OG solution so that the BChl would presumably become incorporated into the same micelles as the protein. Reassociated B820 and B873 were formed under the appropriate conditions in about 50% yield with the other half of the BChl absorbance remaining at 777 nm (unpublished data).

In a similar manner, B820 was bleached by illumination and then pure BChl added. The BChl was again incorporated immediately into B820 (with a small shoulder at 777 nm), and B873 could be formed from the reconstituted B820. In controls with free BChl added to phosphate buffer containing OG in the same manner as above, an 820-nm peak did not form upon dilution of the OG concentration. The absorbance band re-

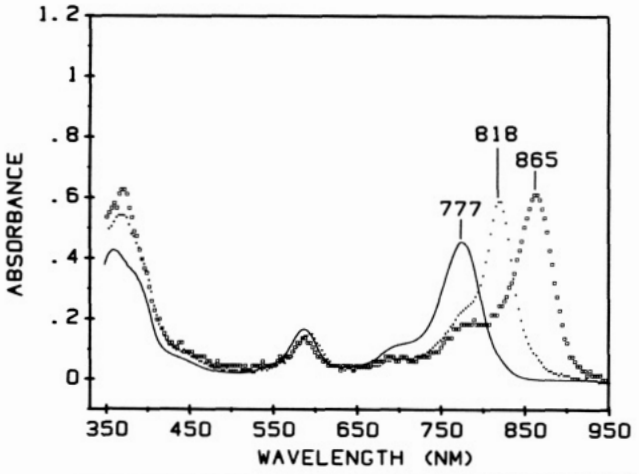

FIGURE 6: Reconstitution of B820 and B873 from separately isolated B881-α, B881-β, and BChl *a*. Absorbance spectra are of B881-α, B881-β, and BChl in 4.5% OG (—), diluted to B820-forming conditions at 0.67% OG (●), and diluted to B873-forming conditions at 0.34% OG (□). For comparison purposes, the absorbance values for spectra of the samples at 0.67% and 0.34% OG were multiplied by their respective dilution factors.

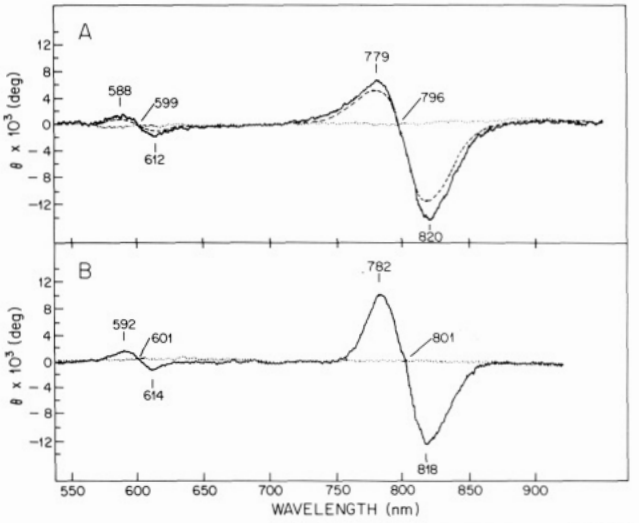

FIGURE 7: CD spectra of B820(chromatophores), B820 reconstituted with B881-α, B881-β, and BChl, and B820 reconstituted with B881-β and BChl. The spectra were adjusted for all samples to an absorbance at 820 nm of 1.09 cm^{-1} to facilitate comparison. (A) CD spectra of B820(chromatophores) (—) and B820(reconstituted) (---). The base line (···) is also shown. The B820(chromatophores) sample had an absorbance spectrum similar to B820 in Figure 2. The B820(reconstituted) sample had an absorbance spectrum similar to the sample in 0.67% OG in Figure 6. (B) CD spectrum of B820 reconstituted from B881-β and BChl. The sample had the absorbance spectrum shown in Figure 9 (●). The base line (···) is also shown.

mained at 777 nm with a small shoulder or small broad band at 848 nm sometimes appearing at the lowest OG concentration.

Reconstitution of the LH Complex with B881-α, B881-β, and BChl α. The reconstitution of the LH complex was attempted from the fundamental and independently isolated components: B881-α, B881-β, and BChl. B881-α and B881-β were isolated from chromatophores or whole cells using organic solvents, gel filtration, and reverse-phase HPLC (Tonn et al., 1977; Brunisholz et al., 1981, 1984; Gogel et al., 1983; Miller et al., 1987). Criteria used for their purity were a single band by SDS–PAGE, a single peak (or doublet in the case of B881-α) on reverse-phase HPLC, and an appropriate amino acid content. The lyophilized polypeptides were prepared in a 1:1 ratio (α:β) in phosphate buffer containing 4.5% OG (see Materials and Methods) after which BChl in CH_3OH was added and the absorbance spectrum of the resulting solution measured (Figure 6). Formation of B820 and B873 upon dilution of the OG concentration followed the same time course as for their formation from 777(dissociated B820). Most of the added BChl formed B820 and B873; about 20% remained at 777 nm. (In this experiment, the α:β:BChl mole ratio was approximately 1:1:0.5.)

The B820(reconstituted), formed from the reconstitution of B881-α, B881-β, and BChl *a*, was identical with that prepared directly from chromatophores with respect to its absorbance spectrum, its ability to be converted to the B873 form, and its stability as a B820 complex. The measurement of the CD properties of the reconstituted complex can be a more sensitive criterion than absorbance as to whether the structure of the original complex has been reconstituted (Sauer, 1975; Gregory, 1977). In Figure 7A, the CD spectrum of B820(reconstituted) is shown compared with that of B820(chromatophores). The structures of the two complexes are identical by this criterion. Upon further dilution of the sample to 0.33% OG, an 872-nm-absorbing species was formed (Figure 6). The CD spectrum of this B873 reassociated from reconstituted 777 (unpublished data) was identical with that measured when B820(chromatophores) at low absorbance (e.g., $\leq$0.1 cm^{-1}) was reassociated to form a species absorbing at 872 nm (Miller et al., 1987).

Reconstitution of B873. As indicated above, formation of a long-wavelength species with a λ_{max} near 873 nm occurred even at very low concentrations (<1 μM) of B820(reconstituted or reassociated). However, in the CD spectra of the resulting B873 complexes, the bands in the near-infrared were inverted relative to those of B873 in chromatophores (Miller et al., 1987). On the other hand, reassociation at higher concentrations (>5 μM) of B820(chromatophores) also gave rise to a long-wavelength λ_{max} approaching 873 nm, and the CD spectrum (Figure 8A) was more like the type seen in in vivo B873 (Figure 8B), although the CD trough is located at the absorbance λ_{max} and the crossover point and CD peak are both at shorter wavelengths. Also, the size of the CD peak is rather small compared with the size of the trough.

In order to more closely reconstitute the in vivo B873 CD properties, B820(chromatophores) at 50 μM concentration was added to liposomes composed of phospholipids of approximately the same composition as are found in chromatophores (Picorel et al., 1983; Snozzi & Bachofen, 1979). With phosphatidylethanolamine, diphosphatidylglycerol, and phosphatidylglycerol (2:1:1 w/w) present during dilution from 0.8% OG to 0.34% OG, not only does the long-wavelength λ_{max} quickly move to 873 nm but also the CD spectrum (Figure 8C) is nearly indistinguishable from that of chromatophores of *R. rubrum* (Figure 8B) if the CD features due to the reaction centers are excluded. Samples of B873 from B820(reconstituted) and B820(reassociated from 777) have not yet been prepared with phospholipid because of the high B820 concentrations required, but since these B820 preparations are, so far, indistinguishable from B820(chromatophores), it is expected that they will show similar in vivo like CD properties in the presence of phospholipid. Thus, according to these two criteria, absorbance and CD spectra, authentic B873 may be reconstituted from the separately isolated α- and β-polypeptides and BChl in the presence of phospholipid.

Interaction of BChl with the B881-α and B881-β Polypeptides. To determine whether BChl forms a specific complex with each polypeptide, BChl *a* was separately added to OG solutions of each polypeptide. Both polypeptides had been further purified by using HPLC methodology and were free

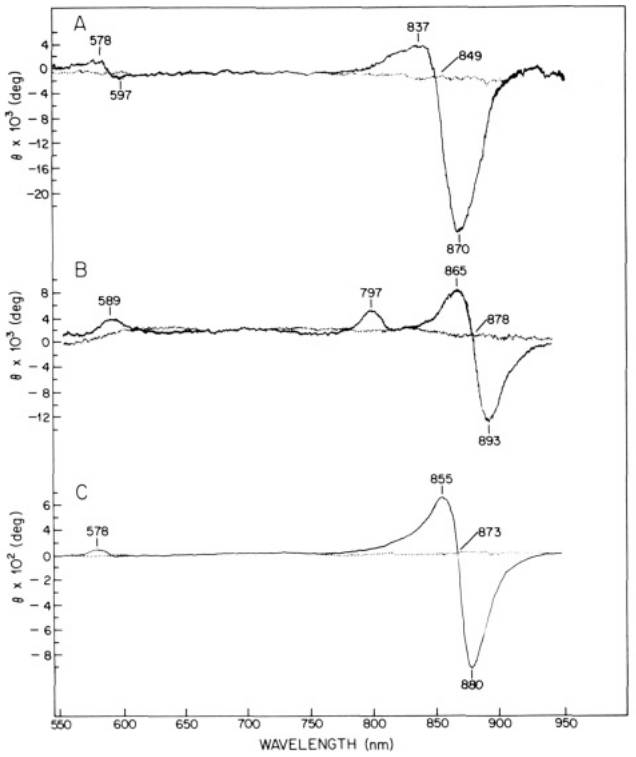

FIGURE 8: CD spectra of (A) B873(reassociated) in phosphate buffer containing 0.4% OG (λ_{max} = 866 nm, A_{866nm} = 0.60 cm^{-1}), (B) chromatophores from wild-type *R. rubrum* in phosphate buffer (λ_{max} = 881 nm, A_{881nm} = 0.75 cm^{-1}), and (C) B873(reassociated) prepared from B820(chromatophores) and phospholipids (λ_{max} = 873 nm, A_{873nm} = 2.15 cm^{-1}). Note that the CD minimum in (A) is very near the absorbance band maximum while in (C) the zero crossing is at the absorbance maximum, as it is in the in vivo system (B). The base line (···) is shown with all spectra.

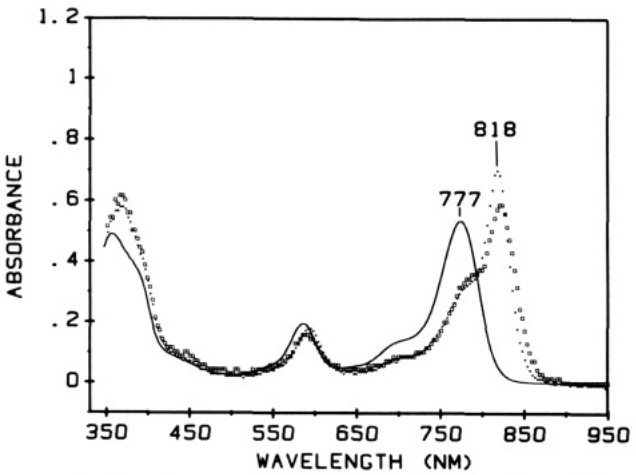

FIGURE 9: Absorbance spectra of B881-β and BChl under conditions for B820 and B873 formation. Absorbance spectra are of B881-β and BChl in 4.5% OG (—), diluted to B820-forming conditions at 0.63% OG (●), and diluted to B873-forming conditions at 0.32% OG (□). For comparison purposes, the absorbance values for spectra of the samples at 0.63% and 0.32% OG were multiplied by their respective dilution factors.

of contamination by the other (see Materials and Methods). When BChl was added to a solution of B881-α and diluted, no B820 or B873 was formed. In contrast, BChl and B881-β formed a complex with an 818-nm absorbance band (Figure 9). When this latter sample was diluted to B873-forming conditions, no significant red shift occurred, and the B820 became more unstable, substantially shifting within an hour to 777 nm. The CD of the B820 formed from interaction of BChl with B881-β showed the same major features as that of

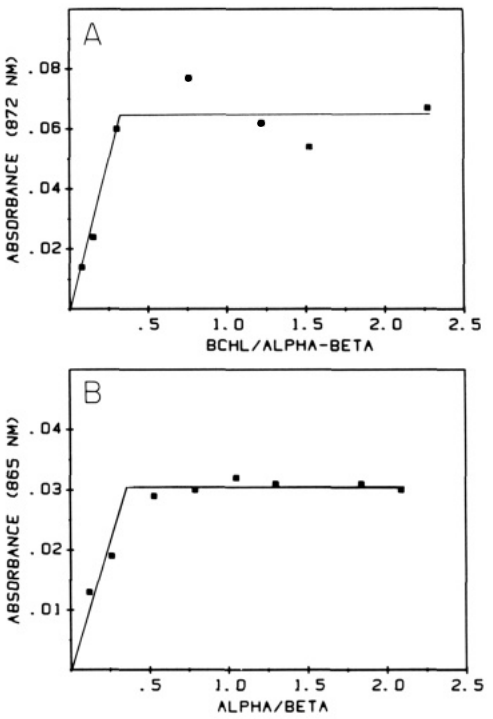

FIGURE 10: (A) Titration of a 1:1 mixture of B881-α and B881-β with BChl. BChl was initially added to a solution of B881-α and B881-β in 4.5% OG. The 873-nm absorbances of the samples at B873-forming conditions are plotted vs. the moles of BChl per moles of $\alpha\beta$ pair. For further details, see Materials and Methods. (B) Titration of the B881-β·BChl complex with B881-α. BChl was initially added to B881-β and varying amounts of B881-α in 4.5% OG. The absorbances at 865 nm were measured under B873-forming conditions. The x axis is the moles of B881-α per mole of B881-β. See Materials and Methods for further details.

B820 contained B881-α and B881-β, but the split peak crossing zero at 801 nm is more symmetric (Figure 7B). By addition of B881-α (in buffer with 0.63% OG) to the sample of B881-β and BChl at 0.63% OG, B873 was readily formed upon dilution of the detergent (data not shown).

Titration of B881-α and B881-β (1:1) with BChl. Although the data clearly indicated that B820 and B873 could be formed from the separately isolated α- and β-polypeptides and BChl, in preliminary experiments it seemed that at higher ratios of BChl to $\alpha\beta$ more pigment absorbance remained at 777 nm. This indicated that not all B881-α and B881-β may be active in the reconstitution assay. To evaluate this, we added increasing amounts of BChl to a solution of the α- and β-polypeptides (1:1) in 4.5% OG and then diluted to B820- and B873-forming conditions. The results are shown in Figure 10A. In B820(chromatophores), there are two BChl per $\alpha\beta$ pair. The results shown in Figure 10 indicate that there is less than 0.4 BChl/$\alpha\beta$ at saturation, implying that about 20% of the protein is involved in forming B820 or B873.

Titration of the B881-β·BChl Complex with B881-α. To evaluate which polypeptide's active form was limiting in the reconstitution assay, the "B820" formed from adding BChl to B881-β was titrated with increasing amounts of B881-α, and the samples were diluted to test for formation of B873. Such a titration was performed at a BChl:B881-β ratio of 0.5. The results of the data for this titration are shown in Figure 10B. Substantially less than 1 equiv of B881-α (0.35) was required for full effect. This result indicates that the limiting factor was the amount of active β-polypeptide present, assuming that the α-polypeptide associated with only the active β-polypeptide·BChl complex in a 1:1 stoichiometry and not with the inactive β-polypeptide.

Testing of BChl Analogues in Reconstituting the LH Complex. Initial experiments were conducted to study the structure–function relationships in the binding of BChl to the protein. Several chlorophylls related to BChl were added to a 1:1 mixture of B881-α and B881-β. Bacteriopheophytin, which varies from BChl only by the absence of the Mg^{2+} ion, did not show evidence of red shifts in the absorbance spectra. When chlorophyll *a* and chlorophyll *b* were tested, no evidence of red shifts was found although the ratio of peak heights of the red band to the Soret band for the chlorophyll *b* system decreased substantially. Those of controls without protein also changed similarly. On the other hand, when BChl *b* was added to the α- and β-polypeptides and the system diluted to conditions of B820 formation (0.7% OG), a narrow band at 853 nm was formed. Upon further dilution to 0.35% OG, the band red-shifted to 890 nm (unpublished data). Similar dilutions of BChl *b* alone in OG solution did not exhibit such shifts.

Discussion

Reversal of Dissociated Systems. Reports of reversible band shifts in antenna complexes of photosynthetic bacteria have been reviewed by Thornber et al. (1978). The 850-nm component of the B800–850 complex in two *Chromatium* species can be reversibly shifted to 830 nm by a variety of methods including lowering the pH (Thornber et al., 1978; Sinegub & Erokhin, 1971). A larger, reversible, pH-dependent wavelength shift was demonstrated recently with the antenna of two BChl *b* containing *Ectothiorhodospira* species (Steiner & Scheer, 1985). The far-red band shifted from 1018 to 964 nm, exhibiting a pK_a of 6.3–6.5. These reversible changes with pH may be due to a change in electrostatic interactions which may be important in chromophore or subunit interaction.

In contrast to shifts caused by pH changes, reversible band shifts due to detergent effects may result from the perturbation of the hydrophobic interaction between interacting BChl molecules or the proteins that bind them. Although there have been many reports of small, reversible band shifts due to detergent interaction (Thornber et al., 1978), most larger shifts observed were accompanied by an irreversible degradation of the complex, releasing free BChl. For example, addition of sodium dodecyl sulfate to broken whole cells of *Rhodopseudomonas viridis* caused an irreversible shift of the antenna from 1020 nm to a discrete form at 880 nm, followed by conversion to an 810-nm species and finally a 685-nm product (oxidized BChl *b*) (Thornber et al., 1978; Thornber, 1971). These results parallel our results obtained by treatment of the *R. rubrum* antenna to form a discrete B820 complex and then a 777-nm species except that our system is fully reversible.

Bachofen et al. (1987) have also recently reported reversible band shifts in adding detergent to *R. rubrum* G-9. In that system, the G-9 antenna can be shifted from 875 to 775 nm by adding LDAO (to 0.1%) and then reversed either by diluting the sample (immediately re-formed an 875-nm-absorbing material) or by adding 0.5–1.0% sodium cholate (slower reversal to 860 nm). With the slow reversal, discrete transitory states at 820–825 and 850 nm were observed. On the basis of sedimentation equilibrium experiments, they proposed that the intermediate spectral states are due to oligomers of intermediate size between the functional LH complex absorbing at 875 nm and the dimeric and monomeric (13 and 6 kDa) states absorbing at 775 nm.

In the work reported here, the rapid re-formation of B820 from 777(dissociated B820) initially led us to believe that the BChl may still be associated with the protein. However, absorbance and CD spectra, fluorescence yield, and fluorescence decay kinetics of 777(dissociated B820) were identical with those of BChl in OG solution, convincing us that the BChl had dissociated from the protein or was very weakly interacting with it. If BChl were, in fact, dissociated from the protein in 777(dissociated B820), then BChl added to a bleached 777 state should reconstitute to form B820 and B873. In accordance, we found that BChl could be added to bleached 777(dissociated B820) or bleached B820(chromatophores) and the appropriate complexes successfully reconstituted. It might seem surprising that BChl could be successfully added back to the protein of bleached B820, but the BChl binding site(s) is (are) apparently on, or accessible from, the surface of this complex, since its BChl readily interacts with $NaBH_4$ (Callahan et al., 1987).

Reconstitution from Separately Isolated BChl and Protein. In the experimental work reported here, reconstitution of B820 and B873 from separately isolated and pure B881-α, B881-β, and BChl has been demonstrated. This is the first report of reconstitution of a native chlorophyll– or bacteriochlorophyll–protein complex from pure components. Apparently, in spite of the relatively harsh conditions of lyophilization and solubilization in the organic solvents and acid used for isolation and purification of the polypeptides, a substantial portion of each can still readily return to or remain in its native state. Their small size and high α-helical content may be helpful in this regard. The polypeptides may be reconstituted into an LH complex either by solubilizing them together in an organic solvent, drying them onto the surface of a round-bottom flask, and then dissolving them in OG-containing buffer or by separately dissolving them from the flasks into OG-containing buffer and then mixing them together before or after adding BChl. It does not seem to matter whether the polypeptides and BChl are initially in separate or the same detergent micelles.

The reconstituted B820 complex is identical with that formed initially from chromatophores as shown by absorbance and CD measurements. The 873-nm-absorbing material formed from reconstituted B820 (at low concentration) is also identical with that formed from B820 prepared directly from chromatophores (at low concentration). B873 can therefore be reconstituted from the separately isolated α- and β-polypeptides and BChl. We have not attempted to prepare B873(reconstituted) with an in vivo B873 CD because of the large amount of pure B881-α and B881-β required. An in vivo like CD spectrum has been obtained for a dilution of a high concentration of B820 (chromatophores) when phospholipid was present (Figure 8C). It is expected that a high concentration of B820(reconstituted) and phospholipid would behave similarly since B820(reconstituted) is identical with B820-(chromatophores) in its spectroscopic and reassociation properties. Therefore, it is likely that the information necessary to form the in vivo B873 complex is built into the polypeptides so that, by providing them with a favorable environment of phospholipid, an appropriate structure will spontaneously form. It should be noted that the presence of reaction centers or carotenoid was not required.

B820 at both low and high concentrations without phospholipid forms a complex that absorbs near 873 nm. These aggregates of B820 have appropriate associations to cause the correct amount of red shift (to near 873 nm) and to result in large CD effects, but the shapes of the CD spectra differ substantially from each other and from in vivo B873. It seems that there are several different BChl orientations that result in the red shift of the absorbance band but only one (perhaps the one limited to two-dimensional aggregation) that results in the correct CD. This underscores the importance of

measuring the CD spectra as a more sensitive parameter in assaying for in vivo like reconstitution.

The ability to reconstitute a native complex from its fundamental components is a powerful tool for conducting structure–function studies. When pure B881-β is reconstituted with BChl, a B820-type complex is formed, but it is less stable than B820 formed when both α- and β-polypeptides are present. In support of this, the CD spectrum of B820 formed from B881-β only is similar to B820 containing both the α- and β-polypeptides, implying that the pigments are arranged similarly whether or not the α-polypeptide is present. (For both, the CD spectra indicate that two or more BChl interact in the complex.) The B881-β·BChl system can only form B873 when B881-α is added. These results suggest that B881-β more specifically and strongly binds the BChl than B881-α and that B881-α stabilizes the complex and is required to correctly associate to form B873.

The titration of separately isolated protein with BChl indicates that not all of the β-polypeptide appears to be functioning in the reconstitution assay. This is markedly different from the results of reversing the system in which B820-(chromatophores) is first dissociated to BChl and the two polypeptides. In the latter case, B820 and B873 were reformed typically with an overall yield of >80% (Figure 3A). It is possible that the 10% acetic acid used in the isolation procedure for the β-polypeptide causes some degradation of an important amino acid (such as Trp) or some other change in structure which is not readily reversible. The CD spectra of the isolated β-polypeptide in hexafluoroacetone or OG were identical with those of the isolated α-polypeptide in the same solvents (unpublished data). They were also nearly identical with earlier measurements from our laboratory reported for the purified α-polypeptide (Tonn, 1976). In each case, about 60% of the polypeptide was in an α-helical configuration. Although the CD spectrum of the α-polypeptide in OG was unchanged with time, that of the β-polypeptide showed substantial loss of α-helical content with time (unpublished results). It is hoped that further detailed studies will determine whether such a change in structure is responsible for the loss of activity of this polypeptide in reconstitution experiments.

During purification of the α-polypeptide by HPLC, the material separated into two polypeptides which were present in approximately equal amounts. From amino acid analyses, the two appear to be identical. Each was effective in the reconstitution assay. It is not known how the two samples differ in structure.

The reconstitution experiments were conducted using BChl isolated from *Rb. sphaeroides*. This BChl has a phytyl tail whereas *R. rubrum's* native BChl has a geranyl-geranyl tail (Katz et al., 1972). This change in tail structure does not seem to affect reconstitution. The Mg^{2+} ion, however, is apparently critical for appropriate BChl binding because addition of BPh *a* in place of BChl *a* did not result in any species with a red-shifted absorbance maximum. Attempted reconstitution with chlorophyll *a* and chlorophyll *b* did not give red-shifted absorbance bands. Of special interest are the preliminary results using BChl *b* in place of BChl *a*. The long-wavelength λ_{max} did red shift by 60 nm under B820-forming conditions and further red-shifted another 40 nm under B873-forming conditions. Although it did not red shift to the 1015-nm λ_{max} of the *Rps. viridis* antenna system, it is quite interesting that with the polypeptides isolated from *R. rubrum* the red shifts are very comparable to those seen with BChl *a*, suggesting that further red shifts in *Rps. viridis* may be explained by differences in amino acid sequences of the α- and β-polypeptides of the two species and not by the different BChl analogues. Specific amino acids may have an important interaction with the BChl molecules, or subtle changes in polypeptide structure may control the interaction of the BChl molecules with each other to cause the red shift.

Reconstitution studies using specifically modified BChl molecules, specifically modified polypeptides, and polypeptides from other bacteria are being studied to elucidate pigment–protein and protein–protein interaction in the LH complexes.

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Registry No. BChl *a*, 17499-98-8; Chl *a*, 479-61-8; BPh *a*, 17453-58-6; Chl *b*, 519-62-0.

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