Probing the Bacteriochlorophyll Binding Site by Reconstitution of the Light-Harvesting Complex of *Rhodospirillum rubrum* with Bacteriochlorophyll *a* Analogues†

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ABSTRACT: Structural features of bacteriochlorophyll (BChl) *a* that are required for binding to the light-harvesting proteins of *Rhodospirillum rubrum* were determined by testing for reconstitution of the B873 or B820 (structural subunit of B873) light-harvesting complexes with BChl *a* analogues. The results indicate that the binding site is very specific; of the analogues tested, only derivatives of BChl *a* with ethyl, phytyl, and geranylgeranyl esterifying alcohols and BChl *b* (phytyl) successfully reconstituted to form B820- and B873-type complexes. BChl analogues lacking magnesium, the C-3 acetyl group, or the C-13^2 carbomethoxy group did not reconstitute to form B820 or B873. Also unreactive were 13^2-hydroxyBChl *a* and 3-acetylchlorophyll *a*. Competition experiments showed that several of these nonreconstituting analogues significantly slowed BChl *a* binding to form B820 and blocked BChl *a*–B873 formation, indicating that the analogues may competitively bind to the protein even though they do not form red-shifted complexes. With the *R. rubrum* polypeptides, BChl *b* formed complexes that were further red-shifted than those of BChl *a*; however, the energies of the red shifts, binding behavior, and circular dichroism (CD) spectra were similar. B873 complexes reconstituted with the geranylgeranyl BChl *a* derivative, which contains the native esterifying alcohol for *R. rubrum*, showed in-vivo-like CD features, but the phytyl and ethyl B873 complexes showed inverted CD features in the near infrared. The B820 complex with the ethyl derivative was about 30-fold less stable than the two longer esterifying alcohol derivatives, but all formed stable B873 complexes.

In photosynthetic bacteria, light-harvesting (LH)[1] complexes absorb light energy and funnel it to reaction centers where it is converted to chemical potential (van Grondelle, 1985; Parson & Ke, 1982). The LH systems are organized into bacteriochlorophyll (BChl)–protein complexes in the intracytoplasmic membranes of these bacteria (Drews, 1985; Thornber, 1986; Cogdell, 1986). Some bacteria contain only the core LH complex (B875), which is closely associated with the reaction center and is in constant ratio to it, while other bacteria contain, along with the core complex, one or more types of peripheral LH complexes (for example, B800–850, B800–820). These peripheral complexes deliver the absorbed light energy to the core B875 complex, and their abundance in the membrane varies with environmental conditions such as light intensity. The various LH complexes are named and classified by their near-infrared absorption maxima (BChl Q_y), hence the core B875 class and the more peripheral B800–850 and B800–820 classes. Within the classes there are variations in wavelength; for instance, the core LH complex of *Rhodospirillum rubrum* wild type is at 881 nm, that of the carotenoidless mutant, *R. rubrum* G-9, is at 873 nm, and that of the R-26 mutant of *Rhodobacter sphaeroides* is at 870 nm (Thornber et al., 1978). Each type of LH complex is composed of small (5–7 kDa), hydrophobic polypeptides, BChl, and carotenoid. The amino acid sequences of many of the polypeptides are known (Zuber, 1986, 1987), and they show much similarity to each other, each having a hydrophobic (probably membrane-spanning) region and conserved amino acids.

In LH complexes containing BChl *a*, even though the polypeptides are similar and the chromophores identical, the near-infrared absorption maxima vary over a wide range, from 800 to 920 nm. Not enough is known about the structures of these complexes to explain what determines the absorption maximum in each case. Another important characteristic of the LH system in these bacteria is the greater than 95% efficiency of transfer of the absorbed light energy to the reaction center. Little is understood at the molecular level about how the LH system is organized to give such a high efficiency of energy transfer. To begin to understand how the LH system functions, it is important to determine how the BChl molecules are bound to the protein and how they are arranged in the membrane.

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[1] Abbreviations: BChl, bacteriochlorophyll; BChl a_e, BChl *a* esterified with ethyl alcohol; BChl a_{gg}, BChl *a* esterified with geranylgeranyl alcohol (the native BChl in *R. rubrum*); BChl a_p, BChl *a* esterified with phytyl alcohol (the native BChl in *Rb. sphaeroides*); BChl b_p, BChl *b* esterified with phytyl alcohol (the native BChl in *Rps. viridis*); BChl g_f, BChl *g* esterified with farnesyl alcohol (the native BChl in *H. chlorum*); pyroBChl a_e, pyroBChl *a* with a 2-hydroxyethyl esterifying alcohol; CD, circular dichroism; B881, the core light-harvesting complex of wild-type *R. rubrum* which has an absorbance at 881 nm; B873, the core light-harvesting complex of the G-9 mutant (carotenoidless) of *R. rubrum* or of the wild-type light-harvesting complex after benzene extraction (both with absorption maxima at 873 nm); B820, the subunit form of B873 that has an absorption maximum at 820 nm; 777-nm-absorbing form, BChl *a* with the α- and β-polypeptides in 4.5% octyl glucoside (absorption is the same as that of free BChl); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); LH, light harvesting; PDMS, plasma desorption mass spectrometry.

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R. rubrum has only the core type of LH complex (Picorel et al., 1983), which absorbs at 881 nm and contains two 6-kDa polypeptides (α and β), in a 1:1 ratio, binding two BChl *a* molecules and one carotenoid (spirilloxanthin) molecule (Cogdell et al., 1982). There are about 12 copies of each polypeptide and 24 BChl molecules per reaction center (Loach & Sekura, 1968). Recently, our laboratory developed methodology to isolate a structural subunit, B820, of this LH complex (Loach et al., 1985; Miller et al., 1987). It was shown that the complex could be completely dissociated where the BChl was free from the protein and then reassociated to reform B820 and in-vivo-like B873 (the absorbance is shifted from 881 to 873 nm in the wild-type complex when the carotenoid is removed; it is also the native absorbance maximum of the LH complex from *R. rubrum* G-9, the carotenoidless mutant) (Parkes-Loach et al., 1988). It was also found that B820 and B873 could be reconstituted from separately isolated α- and β-polypeptides and BChl *a*. This reconstitution methodology allows us to investigate the structure–function relationships of the BChl and protein components of the LH complex. In this paper we report the results of the reconstitution of the α- and β-polypeptides from *R. rubrum* with various analogues of BChl *a* as probes of the BChl binding site in the LH complex. Some of these studies have been presented in preliminary form (Loach et al., 1990a,b).

Materials and Methods

Materials. *n*-Octyl β-D-glucopyranoside (octyl glucoside) was obtained from Sigma Chemical Co., solvents were of HPLC grade, and Triton X-100 and hexafluoroacetone trihydrate were purchased from Aldrich Chemical Co.

Protein Preparation. Whole cells or chromatophores of *R. rubrum* wild type [grown or prepared, respectively, as in Loach et al. (1963)] were washed twice in deionized water and centrifuged, and the pellet was lyophilized to dryness. Either the lyophilized whole cells or chromatophores were used for isolating the α- and β-polypeptides. The lyophilized material was extracted with chloroform/methanol (1:1 v/v) to isolate the α-polypeptide (Tonn et al., 1977), and it was separated from larger polypeptides, pigments, and lipid by gel filtration of the extract on a Sephadex LH-60 column developed with chloroform/methanol (1:1) containing 0.1 M ammonium acetate (Brunisholz et al., 1981; Gogel et al., 1983). To isolate the β-polypeptide, the chloroform/methanol-extracted residue was extracted with chloroform/methanol containing 0.1 M ammonium acetate to remove the remaining α-polypeptide. The residue was then extracted with chloroform/methanol containing 0.1 M ammonium acetate and 10% acetic acid, and the β-polypeptide solubilized in this final extraction was then purified by gel filtration on a Sephadex LH-60 column (Brunisholz et al., 1984; Parkes-Loach et al., 1988). Each polypeptide was further purified by HPLC when required (Parkes-Loach et al., 1988). Both polypeptides were dialyzed against deionized water, lyophilized, and stored at −20 °C until they were used.

BChl Analogue Preparation. The BChl analogues used for reconstitution were prepared as described below and their identities confirmed by NMR, ^{252}Cf plasma desorption mass spectrometry (PDMS), HPLC, absorption spectra, and comparison, when possible, with authentic samples.

BChl a Esterified with Phytyl Alcohol and BChl a Esterified with Geranylgeranyl Alcohol. BChl *a* with a phytyl tail (BChl a_p) from *Rb. sphaeroides* was obtained from Sigma Chemical Co., or, when a pure sample free of 13^2-hydroxyBChl was required, it was prepared as in Michalski et al. (1988), modified as described below. BChl *a* with the geranylgeranyl tail (BChl a_{gg}) was isolated from *R. rubrum* according to the procedure of Michalski et al. (1988) with the following change. Freeze-dried bacteria (0.5 g) were extracted with 3 × 3 mL of acetone to remove the carotenoids, and then the solid residue was extracted with 3 × 3 mL of methanol or acetone/ethanol/methylene chloride/pyridine (70:15:10:5 v/v). BChl a_p preparative HPLC: flow rate, 6 mL/min; retention time, 27–29 min. BChl a_p analytical HPLC (4.6 mm × 25 cm column): flow rate, 1 mL/min; retention time, 6.8 min. BChl a_p ^{252}Cf-PDMS: $[M]^+$, 911.53 (calcd), 910.7 (obsd). BChl a_{gg} preparative HPLC: flow rate, 6 mL/min; retention time, 18–19 min. BChl a_{gg} analytical HPLC: flow rate, 1 mL/min; retention time, 4.5 min. BChl a_{gg} ^{252}Cf-PDMS: $[M]^+$, 905.48 (calcd), 904.8 (obsd).

Bacteriopheophytin a. Bacteriopheophytin *a* was prepared from *R. rubrum* by the method of Strain and Svec (1966) and was kindly supplied by Dr. R. Uphaus of the Iowa State University.

PyroBChl a. PyroBChl *a* was isolated as described in Michalski et al. (1988). Preparative HPLC: flow rate, 7 mL/min; retention time, 30 min. Analytical HPLC: flow rate, 1 mL/min; retention time, 9.2 min.

13^2-HydroxyBChl a_p. 13^2-HydroxyBChl a_p was isolated as a frequently present contaminant of aged BChl *a* preparations that had been purposely exposed to long contact with solvents such as methanol at room temperature and that were not protected from light and oxygen. It was also formed during prolonged development with acetone of the DEAE-Sepharose CL-6B column. Preparative HPLC: MeOH/EtCO$_2$Me/MeCN (87:8:5 v/v); flow rate, 5 mL/min; retention time (13^2-hydroxyBChl *a*), 28–29 min; retention time (BChl *a*), 33–36 min. Analytical HPLC (1 cm × 25 cm column): flow rate, 3 mL/min; retention time (13^2-hydroxyBChl *a*), 10.2 min; retention time (BChl *a*), 11.8 min. Absorption maxima (pyridine): 13^2-hydroxyBChl *a*, 374 nm (vs), 606 (w), 780 (vs); BChl *a*, 374 nm (vs), 610 (w), 780 (vs). ^{252}Cf-PDMS (13^2-hydroxyBChl *a*): $[M]^+$, 927.53 (calcd), 928.1 (obsd); $[M - (phytyl)]^+$, 647.9 (calcd), 647.3 (obsd).

BChl b. Chromatophores of *Rhodopseudomonas viridis* were prepared from whole cells suspended in 10 mM Tris/1 mM EDTA buffer, pH 7.8, by using a French press at 15000 psi at 8 °C in the dark. Unbroken cells and large debris were removed by centrifugation at 17900*g* for 15 min, and the chromatophores were isolated as a pellet by centrifugation at 160900*g* for 90 min. The pellet was homogenized in buffer and slowly added to liquid nitrogen. The chromatophores were stored in this frozen form at −80 °C for up to 6 weeks without change in their absorption spectrum. Frozen pellets were extracted at room temperature with acetone/ethanol/methylene chloride/pyridine (70:15:10:5 v/v). After the solvents were removed, the sample was solubilized in 1.5 mL of HPLC solvent, filtered, and purified by using preparative HPLC. Preparative HPLC: flow rate, 4 mL/min; retention time, 41–44 min. Analytical HPLC (4.6 mm × 25 cm column): flow rate, 1 mL/min; retention time, 7.10 min. Absorption maxima (acetone): 370 nm (vs), 406 (vs), 582 (s), 715 (vw), 794 (vs). ^{252}Cf-PDMS: $[M]^+$, 907.5 (calcd), 908.3 (obsd); $[M - (phytyl)]^+$, 627.9 (calcd), 629.8 (obsd).

BChl g. Chromatophores of *Heliobacterium chlorum* were prepared and stored as described above for *R. viridis* (BChl *b* section). Chromatophores from *H. chlorum* showed a very weak absorption band at 670 nm, probably due to partial oxidation of BChl *g*. All operations in the isolation of BChl *g* were performed under strict exclusion of light. The chromatophore pellets were extracted and solvents removed by

using the same procedure as for BChl *b*. The sample was then solubilized in 2 mL of acetone/ethanol (1:1 v/v), filtered, and either utilized directly or further purified by preparative HPLC. Preparative HPLC: flow rate, 4 mL/min; retention time, 30–32 min. Absorption maxima (acetone): 364 nm (vs), 404 (vs), 566 (w), 696 (vw), 760 (vs). ^{252}Cf-PDMS: $[M]^+$, 819.34 (calcd), 818.7 (obsd); $[M - (farnesyl)]^+$, 615.9 (calcd), 615.4 (obsd). In addition, the identity of the structure was confirmed by conversion of BChl *g* to bacteriopheophytin *g* and characterization of bacteriopheophytin *g* as described previously (Michalski et al., 1987b). The purified BChl *g* could be stored in a dry form at −24 °C for 9 days with less than 10% decomposition.

Chlorophyll a. Chlorophyll *a* was purchased from Sigma Chemical Co. and used without further purification.

Chlorophyll b. Chlorophyll *b* free of 13^2-hydroxy impurities was prepared from freeze-dried *Scenedesmus obliguus* by extraction of the pigments with acetone/ethanol (1:1 v/v) followed by their repetitive precipitation with water/dioxane (10:1 v/v) and separation of chlorophyll *a* and chlorophyll *b* by preparative HPLC. Preparative HPLC: flow rate, 7 mL/min; retention time (chlorophyll *a*) 36–37 min; retention time (chlorophyll *b*) 19–20 min.

3-Devinyl-3-acetylchlorophyll a. 3-Devinyl-3-acetylchlorophyll *a* (3-acetylchlorophyll *a*) was prepared according to the method of Lindsay and Calvin (1966) with a modification which retards partial conversion of the crude reaction mixture to its 13^2-hydroxy derivative. A solution of BChl a_p (10^{-5} M) in acetone was titrated with a 10^{-5} M solution of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). The reaction was terminated when the 770-nm absorption band of BChl a_p had nearly disappeared by adding 1 μL of nonyl mercaptan to stabilize the product. After removal of the solvent, the crude product was solubilized in 1.5 mL of HPLC solvent (MeOH/$EtCO_2Me$/MeCN, 87:8:5 v/v), filtered, and purified by using preparative HPLC. Preparative HPLC: flow rate, 5 mL/min; retention time, 36–38 min. Absorption maxima (ethyl ether): 394 nm (s), 438 (vs), 678 (vs). ^{252}Cf-PDMS: $[M]^+$, 909.5 (calcd), 910.4 (obsd); $[M - (phytyl)]^+$, 631 (calcd), 631.6 (obsd).

Ethyl Bacteriochlorophyllide a and 2-Hydroxyethyl Pyrobacteriochlorophyllide a. Ethyl bacteriochlorophyllide *a* (BChl a_e) and 2-hydroxyethyl pyrobacteriochlorophyllide *a* (pyroBChl a_e) were prepared by following this general description of the enzymatic transesterification procedure. Chlorophyllase acetone powder was prepared from chloroplasts of *Ailanthus altissima* leaves as described previously (Michalski et al., 1987a). The pigments to be modified were dissolved in acetone and/or pyridine and were added to the chlorophyllase acetone powder which was suspended in a mixture of water, acetone, and alcohol or diol. Reaction progress was monitored by analytical HPLC by following changes in concentration of BChl a_p and pyroBChl a_p. At the end of the reaction, the enzyme was removed by filtration, and the filtrate was diluted with an amount of cold water sufficient to precipitate the pigment products. The green suspension was filtered through a 30–40 μm C-18 glass column (1 cm × 3 cm), and the products were absorbed on the top layer. The column was washed with 50 mL of water under suction, and BChl pigments and their ester derivatives were eluted with 25 mL of acetone followed by 25 mL of acetone/ethanol (1:1 v/v) and passed through a DEAE-Sepharose CL-6B column (2.5 cm × 15 cm) with acetone. Bacteriochlorophyllides and pyrobacteriochlorophyllides were retained on the column. After concentration of the fractions containing the desired BChl esters, the latter were purified by preparative HPLC.

An alternate procedure was also used for isolation of transesterification products. After the reaction, solvents were removed under reduced pressure and the esters were extracted with ethyl ether containing 5% benzene. The extract was then washed with water, and the pigments were concentrated and purified by repetitive preparative HPLC.

For preparation of BChl a_e, 10 mg of BChl a_p in 1 mL of acetone/pyridine (1:1 v/v) was added to 1 g of chlorophyllase in 2.7 mL of water, 10 mL of acetone, and 15 mL of ethanol. After 8 h, 32% of the original ester had changed. Preparative HPLC: flow rate, 4 mL/min; retention time, 17–18 min; yield, 1.6 mg. Absorption maxima (ethyl ether): 358 nm (s), 392 (s), 578 (w), 770 (vs). ^{1}H NMR (300 MHz, $CDCl_3$/acetone-d_6/pyridine-d_5, 1:1:1 v/v): δ 9.09, 8.43, 8.25 (each s, α-, β-, and δ-meso H), 6.23 (s, H), 3.96 (q, $COOCH_2$), 3.75 (s, CO_2CH_3), 3.55 (s, 2^1-CH_3), 3.51 (s, 12^1-CH_3), 3.09 (s, 3^2-CH_3), 1.69 (d, 7^1-CH_3), 1.48 (d, 18^1-CH_3), 1.30 (t, 8^2-CH_3), 1.03 (t, CH_3). ^{252}Cf-PDMS: $[M]^+$, 661.0 (calcd), 660.4 (obsd); $[M - (CH_2CH_3)]^+$, 632.0 (calcd), 631.6 (obsd).

For preparation of pyroBChl a_e, 10 mg of pyroBChl a_p in 1 mL of acetone/pyridine (1:4 v/v) was added to 1.0 g of chlorophyllase in 2.5 mL of water, 10 mL of acetone, and 12 mL of ethylene glycol. After 10 h, 80% of the original ester had changed. Preparative HPLC: flow rate, 4 mL/min; retention time, 18–19 min; yield, 3.4 mg. Absorption maxima (ethyl ether): 358 nm (s), 392 (s), 576 (w), 770 (vs). ^{1}H NMR (200 MHz, $CDCl_3$/acetone-d_6/pyridine-d_5, 1:1:1 v/v): δ 9.18, 8.57 (each s, α-, and β-meso H), 8.46 (s, δ-meso H), 5.07, 4.98 (ABq, J = 19.9 Hz, 10-CH_2), 4.16 (t, $COOCH_2$), 3.72 (t, OCH_2), 3.56 (s, 2^1-CH_3), 3.50 (s, 12^1-CH_3), 3.07 (s, 3^2-CH_3), 1.69 (d, 7^1-CH_3, J = 7.1 Hz), 1.63 (d, 18^1-CH_3, J = 7.1 Hz), 1.30 (t, 8^2-CH_3). ^{252}Cf-PDMS: $[M]^+$, 619.02 (calcd), 619.17 (obsd); $[M - (CH_2)_2OH]^+$, 572.95 (calcd), 574.25 (obsd).

HPLC Procedures for Pigment Preparation. Analytical HPLC determinations were carried out with a dual pump Beckman 110A system equipped with a Hewlett-Packard 8451A detector. The LC Survey (Hewlett-Packard) program was used for data reduction. Development of the chromatogram on an Ultrasphere ODS, 5 μm, 4.6 mm × 25 cm and/or 10 mm × 25 cm column was followed simultaneously at 11 different wavelengths, and absorption spectra from 350 to 550 nm or from 600 to 800 nm were recorded every 2 s. Preparative scale separations were carried out on a Du Pont Zorbax column (21.2 mm × 25 cm) with an Altex Model 156 refractive index detector. The mobile phase consisted of acetone/ethanol/water (72:20:8 v/v) unless otherwise indicated.

Reconstitution Assay. Two different procedures were used for the reconstitution assay. For both procedures the α- and β-polypeptides were dissolved in hexafluoroacetone and then proportioned such that each sample had equal amounts of each (usually 0.1–0.25 mg each). A film of the polypeptides was dried onto the sides of a flask by rotary evaporation.

An aerobic dilution procedure was the method preferred when it was important to start at 777-forming conditions, when samples needed to be handled quickly, or when it was desired to use very low concentrations of pigment and protein. For this procedure, 3.5 mL of 4.5% octyl glucoside (w/v) in 50 mM potassium phosphate buffer, pH 7.5, containing 5 mM $MgSO_4$ were added to a flask containing the dried α- and β-polypeptides and the contents swirled to dissolve the protein. To this solution BChl *a* (or an analogue) in acetone (20–50 μL) was added to an A_{777nm} = 0.2–0.5 cm^{-1}. These were the 777-forming conditions. To adjust the sample to B820-forming

conditions, it was diluted with 18 mL of phosphate buffer to 0.73% octyl glucoside. Additional buffer in 0.5–1.0-mL increments was added to maximize the A_{820nm}/A_{777nm} ratio. For B873-forming conditions, either the B820 sample was diluted 1:1 with phosphate buffer at room temperature or the sample was chilled (4 °C, 1 h–overnight). To record its spectrum, the chilled sample was either kept cold or diluted with cold buffer and then warmed to room temperature. Chilling the sample often produced more quantitative reconstitutions than dilution.

The anaerobic cold procedure was used when the BChl analogues degraded quickly (i.e., BChl *b* and BChl *g*), when a sample with higher absorbance and protein concentration was desired, or to form B820 more quantitatively. Octyl glucoside (4.5%) in phosphate buffer (0.5 mL) was added to a flask containing the dried α- and β-polypeptides, and the mixture was diluted with 2.0 mL of phosphate buffer to 0.9% octyl glucoside. The sample was transferred to a plastic cuvette, a stirring bar was added, and the cuvette was sealed with a rubber septum. Two syringe needles were used, one for nitrogen flow into and one for flow out of the cuvette. Nitrogen flowed over the top of the stirred solution and was not bubbled into it because it would cause excessive foaming. The sample was degassed in this manner for 20–30 min with constant stirring. BChl or an analogue in degassed acetone (20–50 μL) was added under nitrogen to an absorbance of 0.15–0.35 cm^{-1}. With BChl *a*, B820 formed under these conditions. To improve the A_{820nm}/A_{777nm} ratio, the sample was cooled to 4 °C or diluted with 0.1-mL increments of degassed buffer. B873 was formed either by diluting the octyl glucoside in the sample to between 0.65% and 0.75% and then cooling the sample to 4 °C overnight or by diluting the sample at room temperature with an equal volume of degassed buffer.

In control experiments, each analogue was added to octyl glucoside buffer without protein and reconstituted in the same manner as with protein to determine whether a red-shifted absorbance resulted due to aggregation of the analogue. For the reconstitution assays and the controls, the BChl analogues were used at relatively low concentrations to minimize formation of aggregates. The analogues were usually added to the octyl glucoside buffer in acetone, but methanol or pyridine was also used if it dissolved the analogue better. No difference was observed with BChl *a* reconstitution when BChl *a* was initially dissolved in acetone, methanol, or pyridine. Pyridine was used with analogues such as bacteriopheophytin to minimize aggregation and to increase solubility in the concentrated solution before the analogue was added to the octyl glucoside buffer. The BChl analogue was usually added in a mole ratio of about 1:5 to the β-polypeptide in the reconstitution assays.

Competition Assay. Samples containing 0.1 mg each of the α- and β-polypeptides were dissolved in 1.25 mL of 4.50% octyl glucoside buffer and then diluted to 2.25% octyl glucoside with phosphate buffer. To one sample was added an amount of chlorophyll *a* in excess of 2 chlorophyll/$\alpha\beta$ pair ($A_{670nm} > 1.3$ cm^{-1}), to another, a similar excess of chlorophyll *b* ($A_{680nm} > 0.7$ cm^{-1}), and to a third, an excess of 3-acetylchlorophyll *a* ($A_{670nm} > 1.3$ cm^{-1}). To each of these samples, and to a fourth (control) sample, was added BChl *a* to an $A_{777nm} = 0.35$ cm^{-1}. All four samples were assayed for formation of B820 and B873 with BChl *a* by diluting the samples with 5.5 mL of phosphate buffer (0.70% octyl glucoside, B820 conditions) and then cooling to 4 °C overnight (B873 conditions).

Absorption and Circular Dichroism (CD) Spectra. Absorption spectra were taken by using a Shimadzu UV-160 spectrophotometer that was interfaced with an Apple IIe microcomputer. Opal glass was used to reduce the light scatter.

The CD data were taken on a Jasco J500C spectropolarimeter with an IF-500 interface and a Leading Edge microcomputer. Samples had absorbances of less than 0.8 in 1- or 2-cm cuvettes. The absorption spectrum of the sample was monitored after each scan to ensure the sample had not changed significantly.

NMR and Mass Spectrometry. ^1H NMR spectra were recorded on Bruker AM (300 MHz) and Nicolet NT (200 MHz) spectrometers. ^1H NMR samples were dried by azeotropic removal of water with benzene and then $CDCl_3$ and, finally, CCl_4. Samples were prepared in $CDCl_3$ (TMS) and acetone-d_6 and diluted with up to 50% pyridine-d_5 when required to prevent aggregation. Average molecular weights of the analogues were determined by ^{252}Cf-PDMS (Hunt & Michalski, 1989) on an instrument built at Argonne.

Results

Reconstitution of BChl Analogues. The standard procedure for the reconstitution assay involved combining the individual components (α- and β-polypeptides of *R. rubrum* and BChl a_{gg} or BChl a_p) under B820-forming conditions and then adjusting to B873-forming conditions (Parkes-Loach et al., 1988). In testing the BChl analogues, a reconstitution was considered successful if the analogue Q_y absorption band red shifted upon adjustment of the octyl glucoside concentration to that for B820 formation and if it red shifted again upon dilution to B873-forming conditions (see Figure 1 for two examples of successful reconstitution). If control samples of the BChl analogues without the α- and β-polypeptides showed the same red shifts that appeared with the polypeptides, it was assumed that the red-shifted absorbances were due to BChl analogue aggregates and not due to binding of the analogue to the protein. Also, it was expected that red-shifted absorption bands resulting from formation of protein complexes would be narrow and specifically located, similar in character to those of B820 and B873, and not the broad peaks often observed when BChl aggregates in octyl glucoside solutions. CD measurements were also taken of any red-shifted species that were formed with BChl analogues, and the spectra were compared with those for B820 and B873.

The results of *R. rubrum* B881-α and B881-β reconstitution with various BChl analogues are presented in Table I. The analogues were chosen to determine the importance of different structural features of BChl *a* (Figure 2). Bacteriopheophytin tested the importance of the magnesium atom, pyroBChl *a* and 13^2-hydroxyBChl *a* tested changes around the C-13^2 carbon atom, and chlorophyll *a*, chlorophyll *b*, BChl *b*, BChl *g*, and 3-acetylchlorophyll *a* tested the importance of the C-3 carbonyl and the oxidation or reduction of ring II. The three BChl *a* analogues with different esterifying alcohols (tails) were tested for the importance of the tail in binding. Of all the analogues tested, only BChl *a* with phytyl, geranylgeranyl, or ethyl tails and BChl *b* were successfully reconstituted.

The control experiments for two of the BChl analogues (pyroBChl a_p and bacteriopheophytin *a*) showed red-shifted species. Bacteriopheophytin *a* (without protein) exhibited a red-shifted absorbance at B820 conditions, but did not further red shift at B873 conditions. The same behavior was observed when the protein was present, so it was concluded that the red shift was due to bacteriopheophytin aggregation and not due to formation of a pigment–protein complex. PyroBChl a_e (without protein) exhibited a red-shifted absorbance at B873-forming conditions (but not at B820-forming conditions) and, again, the same behavior was also observed in the presence

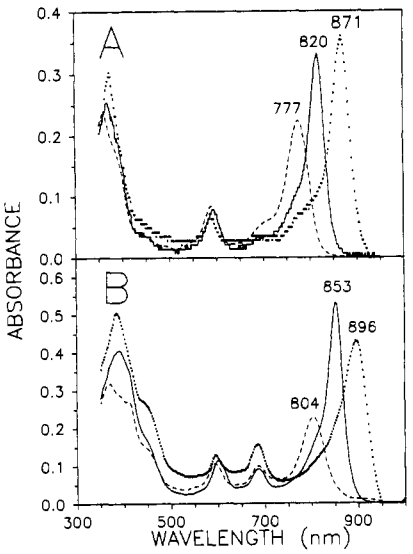

FIGURE 1: Absorption spectra of reconstituted samples of BChl a_p (A) and BChl *b* (B) with the α- and β-polypeptides from *R. rubrum*. (A) For the BChl a_p complexes, BChl *a* in acetone was added to a solution containing 0.25 mg each of the α- and β-polypeptides in 4.5% octyl glucoside buffer (---), and the aerobic dilution procedure was followed. To form B820 (—), the sample was diluted 7-fold to 0.64% octyl glucoside. B873 (···) was formed by storing the B820 (0.64% octyl glucoside) at 4 °C overnight. The absorbances for B820 and B873 were multiplied by the dilution factor for comparison to the starting 4.5% octyl glucoside solution. Initial BChl *a* concentration of the sample in 4.5% octyl glucoside was 4.1 μM. Spectra were taken by using 1-cm cuvettes. (B) The anaerobic procedure was used to form the BChl *b* complexes. BChl *b* in acetone was added under nitrogen to a solution of 0.25 mg each of the α- and β-polypeptides in degassed buffer containing 0.9% octyl glucoside (B820 conditions) to form B853 (—). The sample was then diluted to 0.75% octyl glucoside and stored at 4 °C overnight (B873 conditions) to form B896 (···). For the sample whose absorption band maximum is at 804 nm (---), an amount of BChl *b* equivalent to that added above to form B853 was added to 0.25 mg each of the α- and β-polypeptides in buffer containing 4.5% octyl glucoside. The latter spectrum is the same as that of BChl *b* dissolved in 4.5% octyl glucoside without protein. The absorption peaks at 695 nm and the shoulders at 450 nm in the spectra in (B) are due to a degradation product of BChl *b*. The absorbance of B896 was corrected for dilution. The initial BChl *b* concentration of the sample in 4.5% octyl glucoside was 4.0 μM (using ϵ_{804nm} = 55 mM^{-1} cm^{-1} for BChl *b* in phosphate buffer containing 4.5% octyl glucoside). Spectra were taken by using 1-cm cuvettes.

of protein. In the chlorophyll *a* reconstitution assay, no red shift occurred, but the extinction coefficient of the Q_y absorption band decreased relative to that of the Soret band as B873 conditions were reached. This was true for both the control without protein and in the sample with protein. The control systems for the other analogues did not show any absorption changes at B820 and B873 conditions.

Comparison of BChl a and BChl b Reconstitutions. The in vivo, long-wavelength absorbance of BChl *a* in *R. rubrum* wild-type LH complexes is 881 nm compared with 771 nm for BChl *a* in ethyl ether. For BChl *b* in *Rps. viridis* the in vivo LH absorbance is 1015 nm compared with 791 nm for BChl *b* in ethyl ether. It is not known whether BChl *b* or the protein is responsible for the larger red shift in *Rps. viridis*. Because of this, it was especially interesting to determine the extent of the red shift when BChl *b* was added to the *R. rubrum* LH polypeptides in the reconstitution assay. The spectra of the complexes formed from the BChl *a* and BChl *b* reconstitutions are shown in Figure 1. The absorbances of the BChl *b* complexes were red-shifted from those of BChl *a*, but not to the extent expected for native *Rps. viridis*. When reconstituted with the *R. rubrum* polypeptides to form B873, both analogues showed similar changes in energy for the red shifts: 1415 cm^{-1} for BChl *a* and 1388 cm^{-1} for BChl *b*. The energy of the red shift for BChl *b* in vivo in *Rps. viridis* from BChl *b* in ethyl ether is 2790 cm^{-1}, twice that seen with the *R. rubrum* polypeptides. Apparently, the *Rps. viridis* polypeptides or another unknown component is required for the appropriate interaction to give the additional red shift.

Table I: Reconstitution of Various BChl *a* Analogues with the α- and β-Polypeptides of *R. rubrum*

analogue	successful reconstitution	absorption maxima (nm) at 777 conditions	B820 conditions	B873 conditions
BChl a_{gg}	yes	777	818–820	865–872
BChl a_p	yes	777	818–820	865–872
BChl a_e	yes	774	814–817	868–874
bacteriopheophytin	no	751	751, 851	751–754, 851[a]
pyroBChl a_p	no	782	782	783, 881[b]
pyroBChl *a*, ethylene glycol	no	778	777	779
13^2-hydroxy-BChl *a*	no	778	781	781
BChl *b*	yes	804	851–853	890–902
BChl *g*	no		764–771	765–774
3-acetyl-chlorophyll *a*	no	688	688–701	688–695
chlorophyll *a*	no	668	669	668
chlorophyll *b*	no	651	651	651

[a] The 851-nm absorbance was less than a third of the 751-nm absorbance. Since it was also seen in the controls without protein, it was assumed to be a bacteriopheophytin aggregate. [b] A small 881-nm peak appeared at B873 conditions, but this was also seen in the controls without protein and was therefore considered an aggregate of pyroBChl a_p.

It has been previously shown that BChl *a* and the β-polypeptide form a B820 complex, but cannot form a B873 complex without the addition of the α-polypeptide (Parkes-Loach et al., 1988). BChl *b* also reconstituted with the β-polypeptide, forming a complex at B820 conditions (at 853 nm), but was unable to be further red-shifted under B873 conditions unless the α-polypeptide was also present. Like with BChl *a*, BChl *b* and the α-polypeptide alone did not form red-shifted complexes at B820 or B873 conditions. BChl *b*, therefore, has a binding behavior similar to that of BChl *a* with the *R. rubrum* polypeptides.

The similarity of the structures in the BChl *a*- and BChl *b*-reconstituted complexes was investigated by CD spectroscopy. These CD spectra are shown in Figure 3A,B. The spectra of the complexes formed at B820 conditions are similar in that the CD minima occur close to the absorption maxima. However, the CD peak at 775 nm for BChl *a* is missing in the corresponding BChl *b* CD spectrum. The CD spectra of BChl a_p and BChl *b* reconstituted at B873 conditions are very similar, so the structures of these complexes are probably quite similar.

BChl a Analogues Substituted with Different Esterifying Alcohols. The function of the esterifying alcohol of BChl in binding to the protein was investigated by reconstitution of three different BChl *a* derivatives. The geranylgeranyl tail, with 4 double bonds in its 20-carbon chain, is the natural form found in *R. rubrum*. The phytyl tail, with 1 double bond in its 20-carbon chain, is found in *Rb. sphaeroides*. The third derivative has a very short esterifying alcohol, replacing the 20-carbon chain with an ethyl group. All three of these BChl *a* analogues were reconstituted, giving similar red shifts of their absorption maxima when forming the complexes, the ethyl derivative being about 3 nm to the blue of the other two at 777- and B820-forming conditions. The CD spectra of the

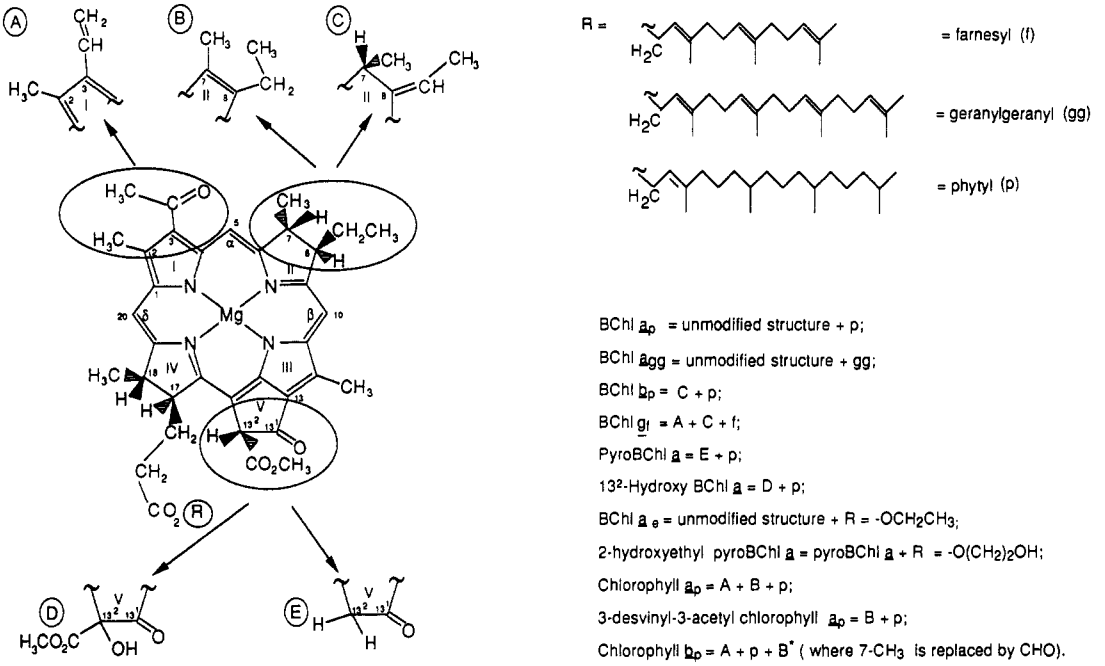

FIGURE 2: Structures of BChl *a* and its analogues used in these experiments.

B820 and B873 ethyl complexes (not shown) are identical with those of the geranylgeranyl B820 and phytyl B873 shown in Figure 3, indicating that the structures are similar and not affected by this variation in esterifying alcohol. With the phytyl esterifying alcohol, the B820 complex also had a CD spectrum identical with that of the ethyl and geranylgeranyl B820 complexes (Parkes-Loach et al., 1988), indicating that the three B820 complexes have similar structures. However, the geranylgeranyl B873 complex showed a CD inverted in the near-infrared region relative to that of the phytyl and ethyl complexes (Figure 3C), but identical with that of in vivo B873 (Dratz et al., 1967). This is the first time that we have been able to reconstitute the correct in-vivo-like CD spectrum for reconstituted B873 [see Parkes-Loach et al. (1988) for previous attempts and conditions]. Previously the phytyl derivative had been used for the reconstitutions.

The stabilities of the complexes formed with BChl a_e, BChl a_p, or BChl a_{gg} (how long the absorbance of the complexes remained at 820 nm) were also compared. B820 with BChl a_p had a stability equivalent to that of B820 prepared with BChl a_{gg}. B820 made with BChl a_e was much less stable and reverted to a 777-nm-absorbing species in 1.5 h in the dark at room temperature, whereas B820 made with BChl a_{gg} had decreased in absorbance at 820 nm only 20% after 5 h under identical conditions (data not shown). Since B820 formed quickly with BChl a_e, the subsequent reversion to a 777-nm-absorbing form must be the result of a reaction in which either the protein or the BChl is changed. Because the protein does not seem to show similar instability in B820 complexes formed with BChl a_p or BChl a_{gg}, the instability observed would appear to be due to BChl a_e modification. BChl a_e is much more rapidly degraded (to material absorbing at 690 nm and then to colorless products) in detergent solution than the BChl derivatives with longer tails. For example, BChl a_p in 0.9% octyl glucoside buffer at room temperature in air in the dark was approximately 60 times more stable than BChl a_e. Protection of the longer tail derivatives from degradation could be a result of the tail itself or the type of detergent micelle that forms around the long-tail derivatives. A similar type of degradation may also occur while the BChl is bound to the B820 complex, but for the most part, the complex of each derivative appeared to dissociate to 777-nm-absorbing material first. One of the chemical changes that occurs in octyl glucoside solutions of BChl is the formation of a 13^2-hydroxy derivative which still has an absorption band at 777 nm but will not reconstitute with the protein. Formation of such a product would result in a depletion of BChl a_e in the free form, offsetting the equilibrium and causing B820 to dissociate.

The B873 complexes of all three analogues were stable with half-lives longer than 20 h at room temperature in air, with the ethyl derivative being at least as stable as the other two.

Another way to compare the BChl binding in the complexes is to determine the amount of B820 initially formed with each derivative at identical conditions (the starting protein, analogue, and octyl glucoside concentrations are identical). At the conditions we selected for this experiment, much less B820 was formed with BChl a_e compared to the amount of B820 formed with BChl a_{gg} (B820 formed with BChl a_e was <15% of that formed with BChl a_{gg}). Thus, the B820 association constants for the longer tail derivatives are much greater than for the ethyl-tail derivative.

Competition Experiments. When the control reconstitution systems (no protein), in which a BChl analogue aggregated to form a red-shifted species, were compared with protein-containing systems for the same analogue, the analogue often did not aggregate, or aggregated to a lesser extent, when the polypeptides were present. The α-polypeptide also seemed to prevent aggregate formation with higher concentrations of BChl a_p. This indicated to us that the analogues could be binding to the polypeptides but were not able to form the specific red-shifted complexes tested for in the reconstitution assay. To determine whether the analogues were binding to the polypeptides, the polypeptides were reconstituted with a concentration of an analogue greater than twice the concentration of the β-polypeptide (or greater than the combined concentrations of α- and β-polypeptides when both were present) and a small amount of BChl a_p. Since two BChl bind per $\alpha\beta$ pair, this concentration of analogue could saturate the BChl *a* binding sites. By this procedure, it was only possible to test analogues with absorbances well separated from the BChl *a* absorbance. The presence of an excess of chlorophyll *a* (relative to protein) decreased the rate of formation of the

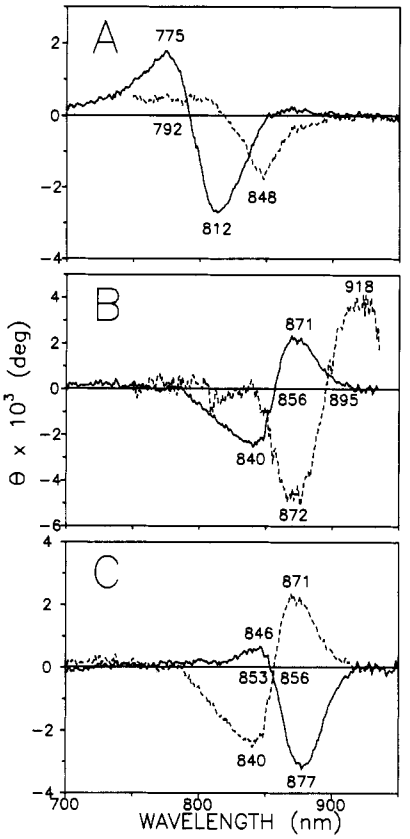

FIGURE 3: CD spectra of complexes reconstituted with the α- and β-polypeptides of *R. rubrum*. (A) Comparison of complexes formed with BChl a_{gg} (—) or BChl b_p (---) at B820 conditions. The B820 containing BChl *a* was in 0.7% octyl glucoside buffer and was formed by using the anaerobic procedure. The B853 containing BChl *b* was in 0.83% octyl glucoside and was also formed by using the anaerobic procedure. The spectra of the two samples were normalized to the same absorbance at their far-red absorption bands ($A_{820nm} = A_{853nm} = 0.28$ cm^{-1}). The actual absorbances of the samples in a 1-cm cuvette were 0.28 and 0.41, respectively. The BChl *b* spectrum was taken with one scan using a 1-cm cuvette, and the BChl *a* spectrum was the average of five scans using a 2-cm cuvette. The latter spectrum was normalized to a 1-cm path length. (B) Comparison of complexes formed with BChl a_p (—) or BChl *b* (---) at B873 conditions. The B873 sample containing BChl *a* contained 0.25% octyl glucoside and was formed by cooling the B820 sample to 4 °C overnight and then diluting the sample with cold buffer. The B896 sample containing BChl *b* was formed by using the same procedure and contained 0.37% octyl glucoside. The spectra of the two samples were normalized to the same absorbance at their far-red absorption bands ($A_{871nm} = A_{896nm} = 0.25$ cm^{-1}). The actual absorbances of the samples in 1-cm cuvettes were 0.25 and 0.17, respectively. Five scans were averaged for the BChl *a* spectrum, and one scan was taken for the BChl *b* spectrum. Both CD spectra are of samples in 2-cm cuvettes and have not been corrected to 1 cm. (C) Comparison of B873 complexes formed with BChl a_{gg} (—) or BChl a_p (---) under identical conditions. Both were formed by using the anaerobic cold procedure and are in phosphate buffer containing 0.25% octyl glucoside. Five scans were averaged for each spectrum. The spectra of these samples were normalized to the same absorbance at their far-red absorption bands ($A_{871nm} = A_{867nm} = 0.25$ cm^{-1}). Actual absorbances of the samples in 1-cm cuvettes were 0.41 and 0.25, respectively. Both CD spectra are of samples in 2-cm cuvettes and have not been corrected to 1 cm.

BChl *a*–B820 complex. Normally this complex is formed in less than 20 s, but when chlorophyll *a* was also present, 5–10 min was required for the complex's formation. Similar results were obtained with 3-acetylchlorophyll *a*. In both of these latter experiments, although B820 was more slowly formed, the amount eventually observed was the same as in control experiments with no analogue present. With chlorophyll *b*, even after 4 days, only a fraction of the normal amount of B820 had formed (Figure 4). Whereas BChl *a* control samples formed B873 overnight in the cold, the presence of an excess of these chlorophyll analogues prevented B873 formation, even after 7 days. The latter samples retained the same absorption spectra that they had at B820 conditions.

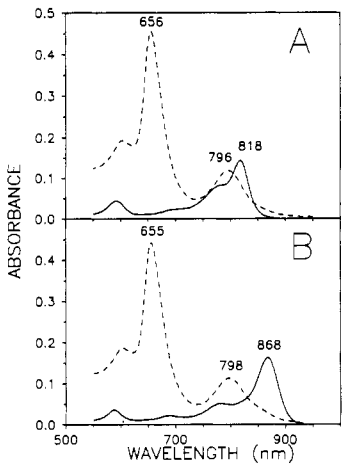

FIGURE 4: (A) Competition experiment comparing BChl *a*–B820 formation with (---) and without (—) the presence of excess chlorophyll *b*. All other conditions were identical for the two samples (see Materials and Methods). The peak at 796 nm is a combination of the absorbance at 777 nm of free BChl and B820 absorbance. (B) BChl *a*–B873 formation, with (---) and without (—) the presence of excess chlorophyll *b*, obtained by cooling the samples used for (A). BChl *a* concentrations for both samples at 777-forming conditions were 6.6 μM. Chlorophyll *b* concentration at 777-forming conditions was approximately 40 μM [using the molar extinction coefficient of chlorophyll *b* in ethanol (Svec, 1978); the value in octyl glucoside was not determined]. Spectra were taken by using 1-cm cuvettes.

DISCUSSION

Structural Requirements for BChl Binding. The importance of various aspects of the BChl *a* structure for binding to the protein was determined by testing for reconstitution with BChl *a* analogues to form B820 and B873 complexes. One would propose that structural features of BChl *a* that have potential binding interactions with the protein [e.g., ligand coordination, hydrogen-bond acceptors, aromatic interactions, and unique packing such as may occur between the methyl groups ("knobs") of the isoprenoid tail and the "holes" (Dunker & Jones, 1978)] in the α-helical part of the polypeptides would be important for formation of the red-shifted complexes. Since bacteriopheophytin, which differs from BChl *a* only in the replacement of the magnesium atom with two hydrogen atoms, did not reconstitute, it is concluded that the magnesium atom is required for binding to this protein. This is consistent with resonance Raman data that suggest that the magnesium is 5-coordinate in B820 and B873 (Chang et al., 1990) and that, in chromatophores, histidine may provide the coordinated ligand (Robert & Lutz, 1985).

The preservation of the structure around the C-13^2 carbon atom was also found to be important for binding. The ability to properly bind is lost even with small changes, such as replacing the C-13^2 hydrogen with a hydroxyl group as with 13^2-hydroxyBChl *a* or replacing the carbomethoxy group with a hydrogen as with pyroBChl *a*. The 13^2-hydroxyBChl *a* may not bind because of steric problems, changes due to adding a hydrogen bond donor group (hydroxyl), or possibly because, in its formation, the carbomethoxy group might have been relocated to the epimeric position which might substantially change its availability for hydrogen bonding. With the loss of the carbomethoxy group in pyroBChl *a*, two possible hydrogen-bond acceptors are lost, which may explain the lack of binding with that analogue. It is interesting, in this regard, that the carbomethoxy groups of the four BChl and two

bacteriopheophytins in the reaction center are often involved in hydrogen bonding to the protein (Deisenhofer et al., 1984; Michel et al., 1986; Allen et al., 1986, 1988).

Resonance Raman data have also suggested that the C-3 carbonyl of the BChl *a* is hydrogen-bonded in the LH complex, more strongly in B873 than in B820 (Chang et al., 1990). BChl *b*, which has the C-3 carbonyl, binds to the protein, but BChl *g*, which differs from BChl *b* by the vinyl group in the C-3 position instead of an acetyl group, does not form B820 and B873 complexes. Therefore, it is concluded that the C-3 carbonyl is important in binding. (BChl *g* also has a farnesyl tail instead of a phytyl tail, but since more widely different tails did not seem to prevent B820 and B873 formation, this difference is probably not the reason for the inability of BChl *g* to bind.) Chlorophyll *a* and chlorophyll *b*, which also do not have C-3 carbonyls, do not red shift at B820- and B873-forming conditions.

The oxidation state of ring II also affects analogue binding. BChl *a* has the bacteriochlorin oxidation state. In the BChl *b* structure, ring II has one less hydrogen and a double bond is made to the α-carbon of the side chain (see Figure 2), but it still reconstitutes with the protein to form B820- and B873-type complexes. Thus, this change in structure from that of BChl *a* seems to be acceptable for binding. 3-Acetylchlorophyll *a*, made from BChl *a*, has the same structure as BChl *a* except that ring II has been oxidized to the chlorin oxidation state. This analogue and the other analogues with chlorin oxidation states (chlorophyll *a* and chlorophyll *b*) do not reconstitute with the protein to form red-shifted species. It is known that the flexibility of the macrocyclic ring decreases when it changes oxidation state from a bacteriochlorin to a chlorin (Hanson, 1988). Perhaps the more flexible bacteriochlorin oxidation state is important for appropriate binding to the protein. It is interesting that a decrease in planarity due to binding may cause some red shift in the Q_y band (Barkigia et al., 1988).

Function of the Esterifying Alcohol in Binding. The function of the BChl *a* esterifying alcohol in the LH complex is intriguing. The esterifying alcohol does not give the chromophore its major absorption characteristics, nor does it have hydrogen-bond donors and acceptors to help stabilize packing. It does, however, have a long, hydrophobic chain with four methyl side groups that could pack well along the polypeptide α-helices. Carotenoids have a similar element in their structure because of also being constructed from isoprenoid units. The phytyl, geranylgeranyl, and ethyl esterifying alcohols that were tested all reconstituted, but the ethyl analogue formed less B820 and the B820 that formed was less stable. So the longer tail has a function in stabilizing this structural subunit. The B873 complexes of these three analogues were stable, and since they are more aggregated than the B820 complexes, their increased stability may be derived more from this more highly associated state. It is also known that the BChl in B820 is exposed to the aqueous environment and can be attacked by reagents in aqueous solution (e.g., sodium borohydride), whereas in B873 complexes the BChl is prevented from reacting with such reagents (Callahan et al., 1987). This suggests a structure of the B820 complex where the BChl molecules are on the outside surface of the protein and the BChl–protein interactions are very important in holding the complex together. With the B873 complexes, the subunits have associated so that the BChl molecules are surrounded and protected by protein. The additional protein–protein interactions are perhaps then of greater importance in holding this associated complex together.

The three BChl *a* tail derivatives showed that BChl–protein interactions are important for the stability and formation of B820 but did not show similar importance in the stability and formation of B873. This is consistent with the finding that the analogue structures required to form B820 were the same as those required to form B873. (All analogues that formed B820 also formed B873.) If there were different or additional structural requirements for BChl binding to form B873, then it should be possible to find analogues that form only B820 and not B873. So far, none have been found.

The BChl tail analogues did have an effect on B873 structure, however, as was evident from their CD spectra. To reconstitute a B873 complex that had an in-vivo-like CD, BChl a_{gg} was required. BChl a_p, BChl a_e, and BChl b_p gave inverted near-infrared spectra under identical reconstitution conditions. Possibly the three extra double bonds in the geranylgeranyl tail affect the packing of the B820 complex and the way it associates to form B873. However, it should be noted that BChl a_p is not always associated wth an inverted B875 CD spectra in vivo. For example, in *Rhodopseudomonas acidophila* 7750 (Cogdell & Scheer, 1985) and the MW 442 B800–850$^-$ mutant of *Rb. capsulatus* (unpublished results) the B875 complexes, which also contain BChl a_p, display CD features that are similar in shape to those of *R. rubrum*. However, in the R26 mutant of *Rb. sphaeroides* (Kramer et al., 1984) and the puc705-BA B800–850$^-$ mutant of *Rb. sphaeroides* (unpublished results), B875 has an inverted CD in the Q_y region relative to that of *R. rubrum*. Thus, CD measurements provide a sensitive structural probe for a given in vivo complex, showing structural variations even when absorption spectra are similar. It must be noted, however, that there is a substantial variation in CD spectra of in vivo core complexes, but all of these complexes apparently function well with a high quantum yield for delivering excitation energy to the reaction center.

Analogue Interaction with Protein. A limitation to the reconstitution assay experiments for specific binding is that they are performed under selected conditions. If there is binding, but the binding constant is much smaller than that of BChl *a*, reconstitution would not be observed. Also, the assay does not indicate when an analogue interacts with the protein without causing a red shift. The competititon experiments were designed to test for this latter type of binding. In these experiments, the chlorophyll analogues slowed or prevented formation of a BChl *a*–B820 complex and BChl *a*–B873 formation did not occur even after several days. The most likely explanation of these results is that the chlorophyll analogues are sufficiently competitive with BChl *a* to bind to both the α- and β- polypeptides. Perhaps they coordinate to the imidazole groups of the histidine amino acids. Since these polypeptides presumably evolved to bind BChl, it would be expected that close analogues would also bind, but probably not as strongly as BChl *a*. If the analogues bind to the α-polypeptide and, as has been shown (Parkes-Loach et al, 1988), the α-polypeptide is required for B820 association to form B873, this may explain why the analogues prevent B873 formation in the competition experiment.

Another possible explanation of the competition results is that the chlorophyll analogues could be forming a complex with the BChl *a*, preventing it from interacting with the protein to form B820 and B873. This is not as likely an explanation for two reasons: (1) In the experiments with chlorophyll *a* and with 3-acetylchlorophyll *a*, BChl *a*–B820 formed to the same extent as in the control experiments with BChl *a* and no analogues; it just formed more slowly. Thus, the BChl *a*

is in a form that is available to interact with the protein. (2) No unique absorbance bands attributable to a BChl *a*–chlorophyll analogue complex were observed.

Relevance of in Vitro Reconstitution to the in Vivo Structure. The specificity exhibited by the α- and β-polypeptides in this reconstitution assay is quite high, as only a very restricted set of structural changes in the BChl *a* molecule were accepted. Also, the association constant for B820 formation is very large (B820 forms readily in 0.7% octyl glucoside with <0.3 μM concentrations of BChl and each of the polypeptides). These data indicate to us that the polypeptides, even in the B820 complex, are nearly native in structure. High specificity and large association constants would not be expected for more random binding. No additional factors, even the reaction center, seem to be required. But are these conclusions on the BChl structural requirements valid for the in vivo LH complex? The reconstituted B873 material has been found to have spectroscopic properties (absorbance, CD, resonance Raman, fluorescence) that closely reproduce those in the in vivo complex, implying that the structure of reconstituted B873 and in vivo B873 are the same. Thus, it seems likely that the structural requirements for binding BChl in B873 are the same in vitro and in vivo.

One might also address the question as to whether the in vitro reconstitution process differs in some way from the in vivo assembly process. On the one hand, in vivo conditions and interactions to form B873 may require specific lipid or protein components or membrane-formation geometry that is not present in the reconstituted systems. But conversely, since the polypeptides are small with over 50% α-helical structure that seems to be maintained in a variety of environments, since they span the bilayer only once, and since they form B820 and B873 by association of these small units, the folding and mechanisms of complex formation may be rather similar. In vivo, however, the initially translated polypeptides in *R. rubrum* are larger than those isolated from the mature complex (Berard et al., 1986), and there is at least one gene product intimately related to the LH α and β genes whose function has yet to be determined (Donohue et al., 1988). Further biochemical characterization of the formation of this membrane complex may clarify the mechanism of its incorporation into the membrane.

Reconstituting Membrane Protein Complexes. In the area of research on integral membrane proteins containing prosthetic groups, there has as yet been little success in reconstituting a functional complex from the isolated components. Other than bacterial LH complexes, only bacteriorhodopsin and rhodopsin have been reconstituted with their chromophores and shown to be fully functional (Oesterhelt, 1982). The demonstration that conditions were found for proper association of integral membrane complexes such as the LH systems of photosynthetic bacteria offers hope that the methodology used might be successfully applied to other complex systems such as the photosynthetic reaction center or the bc_1 complex. If such complex systems could be reconstituted from their isolated components, a new level of understanding of how these important bioenergetic systems work could be achieved.

The reconstitution experiments reported here have provided some initial information about how BChl *a* is bound to the protein in the B881 LH complex of *R. rubrum*. Additional structural features of BChl *a* will be tested as analogues become available. Also, experiments involving reconstitution with α- and β-polypeptides isolated from bacteria other than *R. rubrum* will address the similarity of the BChl binding site(s) in other photosynthetic bacteria. In a parallel approach, modification of specific amino acid side chains of the protein is in progress to evaluate their role in binding.

Acknowledgments

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Control of Electric Field Induced Cell Membrane Permeabilization by Membrane Order

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ABSTRACT: Cells can be made temporarily permeable if pulsed by high-intensity short-duration electric fields. The molecular mechanisms underlying this electropermeabilization are still unknown. The kinetic events may be described by four successive steps: induction, expansion, stabilization, and resealing. On one hand, cell electropermeabilization is detected only under more stringent conditions when cells have been treated by ethanol. On the other hand, lysolecithin is observed to facilitate cell electropermeabilization. More precisely, these molecules that modify membrane order, when used in concentrations compatible with cell viability, are shown to affect only the expansion and resealing steps. Electropermeabilization is inducing a transition in the membrane organization. Membrane order is modulating the energy barrier needed to evoke this membrane transition which occurs when cells are submitted to a field larger than a characteristic threshold (expansion step). Less order would increase the magnitude of this energy barrier; more order would decrease it.

The cytoplasmic content of cells is protected from exogenous molecules by the selective permeability of the plasma membrane. This barrier function can be temporarily removed by applying short high-intensity electric pulses to the cell suspension (Neumann & Rosenheck, 1972). Such "electropermeabilization" is very easy to handle and is now routinely used in cell biotechnology to introduce plasmids into the host cell genome (electrotransformation) for genetic manipulation (Neuman et al., 1982; Potter, 1988). This approach is valid for almost any cell system [mammalian cells, plant, yeast, and bacterial protoplasts (Neumann et al., 1982; Shillito et al., 1985; Shivarova et al., 1983)] and was recently shown to be operative with walled systems (Taketo, 1988). Another property of electropermeabilization is the creation of a fusogenic state in the cell membrane. It has been known since 1980 that pulsation of cells held in close contact would cause them

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