Bacteriochlorophyll–Protein Complexes from the Light-Harvesting Antenna of Photosynthetic Bacteria[†]

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ABSTRACT: Detergent-solubilized bacteriochlorophyll–protein complexes corresponding to the light-harvesting antenna are isolated from *Rhodopseudomonas sphaeroides*, strain 2.4.1 (wild-type) and R-26 (carotenoidless) mutant, and from *Rhodospirillum rubrum*, wild-type. Detailed studies of the complex derived from the R-26 mutant indicate that it contains two bacteriochlorophyll (BChl) molecules and two copies of a peptide of 8.5 kdaltons, together with about 20% phospholipid in a unit of about 22 kdaltons. Distinctive absorption and circular dichroism spectra indicate that this unit is present in whole cell chromatophores derived from the bacteria, in large aggregates obtained by fractionation using Triton X-100 and as a monomeric unit in the presence of 0.5% sodium dodecyl sulfate. A similar component derived from the wild-type *Rps. sphaeroides* contains three BChl, two peptides of about 10 kdaltons, carotenoid, and phospholipid. The additional BChl molecule appears to be associated with an absorption band at 800 nm that is present in addition to the 850-nm band seen in the R-26 mutant complex. A similar complex from *Rds. rubrum* was studied in less detail. The interactions of the BChl molecules within these complexes are interpreted in terms of intermediate exciton coupling. Additional weaker reactions between the complexes in aggregates or in the intracytoplasmic membranes of the bacteria are invoked to account for fluorescence depolarization and fluorescence yield observations. Excitation transfer within the light-harvesting antenna that leads to photochemical trapping in the reaction centers is probably a direct consequence of these interactions.

The bacteriochlorophyll of photosynthetic bacteria exists in two important but distinct situations. A small proportion, typically less than 5%, is incorporated in reaction centers where it participates directly in the primary photochemistry that initiates electron transport reactions (Clayton, 1973; Sauer, 1975; Parson & Cogdell, 1975; Dutton et al., 1977). The majority of the BChl[1] plays an essential but more passive role as a light-harvesting antenna, where incident light is absorbed and the resulting electronic excitation is transferred to the reaction centers. Detailed studies of the reaction centers, which can be separated and purified free of the antenna by using selective detergent treatment (Reed & Clayton, 1968), have led to rather detailed knowledge of their structure and function (Feher & Okamura, 1977; Loach, 1976).

Four BChl and two bacteriopheophytins (BPh) occur in a complex with three polypeptides (21, 23, and 28 kdaltons) along with two ubiquinones and one iron atom (Straley et al., 1973; Feher & Okamura, 1977). Such complexes exhibit photoactivity characteristic of the reaction centers regardless of whether associated cytochromes remain attached. The optical and EPR properties of these complexes reflect strong interactions among the pigment molecules (Philipson & Sauer, 1973a; Sauer, 1975; Norris et al., 1971; McElroy et al., 1972).

The antenna BChl complement has been studied in much less detail. Following mild detergent treatment and separation of reaction centers using fractionation methods, the non-re-

action center BChl usually appears in two forms: a low molecular weight component that behaves like solubilized pigment; and a fraction somewhat larger in amount that is associated with protein and phospholipid (Fraker & Kaplan, 1972; Clayton & Clayton, 1972). The protein contains peptides of low molecular weight, about 10 000. Because of the possibility of rearrangement induced by the detergent action, it remains to be demonstrated that the isolated BCl-proteins correspond to entities present in the intact intracytoplasmic membranes.

We have made use of the characteristic absorption and circular dichroism (CD) spectra of BChl in the intact membranes to monitor the retention of the native configuration during dissection of membranes derived from *Rps. sphaeroides* or *Rds. rubrum* using detergents. We find evidence that the repeating subunits of the BChl-protein from the antenna are relatively small complexes of about 20 kdaltons, containing two similar or identical peptides and either three BChl (wild-type organisms) or two BChl (R-26 mutant of *Rps. sphaeroides*). This latter complex is the smallest and simplest chlorophyll-protein that has been characterized to date. The spectroscopic properties suggest that the antenna BChl-proteins from the wild-type and the mutant organisms are related to one another in structure. Aggregates of 15 to 20 of these small BChl-proteins are obtained from the intracytoplasmic membranes using milder detergent treatment. These aggregates may represent the array of antenna BChl-protein associated with each reaction center. Differences in the extent of fluorescence depolarization and quenching exhibited by the different preparations may reflect important features of the mechanism of excitation transfer within the antenna and to the reaction centers.

Materials and Methods

The three strains of photosynthetic bacteria used in this research, *Rhodopseudomonas sphaeroides*, strain 2.4.1 (wild-type) and R-26 (carotenoidless) mutant, and *Rhodospirillum rubrum*, wild type, were grown anaerobically in 2-L

[1] Abbreviations used: BChl, bacteriochlorophyll a; BPh, bacteriopheophytin a; TX-100, Triton X-100; NaDodSO$_4$, sodium dodecyl sulfate; LDAO, lauryl dimethylamine oxide; LH, light harvesting.

bottles in a modified Hutner's medium under illumination from several 40-W tungsten lamps. (See Austin (1976) for details.) Cells were washed by centrifugation in 0.01 M Tris (pH 7.5) and usually lyophilized for storage. Fifteen grams of wet cells or 2 g of lyophilized cells was suspended in 40 mL of buffer (Tris, 0.01 M, pH 7.5) and passed three times through a precooled Aminco-French pressure cell at 1300 atm. Following repeated sedimentation (10 min at 20 000g) to remove unbroken cells and large fragments, the final supernatant containing pigmented intracytoplasmic membranes (chromatophores) was centrifuged at 200 000g for 90 min. The pellet was resuspended in buffer and washed twice by resedimentation at 200 000g. To remove ribosomes and small remaining amounts of cell wall material, a concentrated suspension ($A_{860nm} = 50$ cm^{-1}) in EDTA (0.01 M) was layered over a 0.9 M/1.2 M discontinuous sucrose gradient and centrifuged at 95 000g for 5 h. The chromatophore fraction was collected from the interface, dialyzed overnight at 4 °C in the dark against Tris buffer (0.01 M, pH 7.5), and collected as a pellet (200 000g, 1 h). A concentrated ($A_{860nm} = 50$ cm^{-1}) suspension of the chromatophores in buffer was treated dropwise with 0.1 volume of a 25% aqueous solution of the nonionic detergent Triton X-100 (TX-100) with stirring. The resulting solution was layered over a 0.6 M/1.2 M discontinuous sucrose gradient and centrifuged (Beckman SW 41 rotor) at 40 000 rev min^{-1} for 2 h. The lightly pigmented band above the 0.6 M sucrose solution exhibited spectral properties characteristic of the reaction center complex. A highly pigmented band at the 0.6 M/1.2 M sucrose interface showed no reversible light-induced bleaching and presumably contained the BChl antenna system. The nearly colorless pellet at the bottom of the tube contained unpigmented protein released from the chromatophore membranes. The antenna–BChl complexes (LH–TX100) were collected from the interface and served as the starting material for procedures described under Results.

BChl was assayed starting with extensively dialyzed or lyophilized samples, resuspended in Tris buffer (0.01 M, pH 7.5). The suspension (0.2 mL; $A_{860nm} = 50$ cm^{-1}) was diluted to 5.0 mL with a cold (-40 °C) acetone–methanol mixture (7:2 v/v) and centrifuged for 5 min at 200g. The extraction of BChl proved to be complete in one stage. The BChl in the supernatant was quantitatively converted to BPh by the addition of 0.02 volume of 5 N HCl, and its concentration was determined by the absorption at 525 nm, using the molar extinction coefficient, $\epsilon_{525} = 23.4$ mM^{-1} cm^{-1}, given by Straley et al. (1973).

The total protein content of samples precipitated and extracted as above was determined by the method of Lowry et al. (1951), with modifications to correct for the interference caused by Tris buffer and TX-100 (Ji, 1973; Bensadoun & Weinstein, 1976; ChandraRajan & Klein, 1975).

Total phosphorus was determined by the method of Bartlett (1959).

Molecular Weight Determinations. Gel filtration chromatography was carried out using either Sephadex G-50 (fine), G-100 (medium), or G-200 (medium), depending on the required fractionation range, in a water-jacketed 1.6 × 70 cm column (Pharmacia K 16/70) equipped with a flow adaptor (Pharmacia, type A16) to allow upward elution. The running buffer contained Tris (0.01 M, pH 7.5) and sodium dodecyl sulfate (NaDodSO$_4$; 1% w/v). A calibration curve was prepared for each gel by running four or more of the following proteins in the NaDodSO$_4$/buffer system: bovine serum albumin (67 kdaltons), ovalbumin (45 kdaltons), chymotrypsinogen (25 kdaltons), myoglobin (17.8 kdaltons), cytochrome

c (12.4 kdaltons) all from Schwarz/Mann; insulin (11.4 kdaltons), purified insulin A chain (2.3 kdaltons) and B chain (3.4 kdaltons) from Sigma; and α-bungarotoxin (8.0 kdaltons), courtesy of M. Hanley (Chemical Biodynamics Laboratory). The molecular weights used were those provided with the samples, except for α-bungarotoxin where the value obtained by Mebs et al. (1972) was used.

Gel electrophoresis experiments were run using an electrophoresis cell (Model 300 A) and precision power supply (Model 400) from Bio-Rad Laboratories. The sample buffer contained Tris (0.062 M, pH 6.8), NaDodSO$_4$ (1% w/v), and 2-mercaptoethanol (1% v/v). Details of the procedures, polyacrylamide gel preparation, staining, and destaining are given in Austin (1976).

Sedimentation velocity studies were run using an analytical ultracentrifuge (Beckman, Model E) equipped with a temperature control unit and a Schlieren optical system. Runs were performed at 20 °C at 59 780 rev min^{-1} in a rotor (AN-D) with a double sector quartz window cell employing an aluminum-filled Epon centerpiece. Schlieren patterns recorded using 15-s exposures at 32-min intervals on Kodak Metallographic plates were read on a microcomparator (Gaertner Scientific) equipped with a variable intensity light source and a rotating stage. Sedimentation coefficients were calculated from the migration rate of the maximum (ordinate) of each Schlieren peak.

Amino acid analysis of protein complexes was carried out following the removal of the pigment and lipids by three successive extractions with acetone/methanol (7:2, v/v). The residual protein was lyophilized and then hydrolyzed using constant-boiling hydrochloric acid (Pierce) for 20, 44, or 68 h at 110 °C in previously evacuated tubes. The results from the amino acid analysis (Beckman, Model 120C) were corrected for hydrolysis losses by extrapolation to zero time. Tyrosine/tryptophan ratios were determined from magnetic circular dichroism spectra by the method of Barth et al. (1972).

Spectrometric Measurements. Absorption spectra were determined using a Cary 14R spectrophotometer. Spectra of photooxidized reaction center fractions were obtained with the instrument in the IR 2 mode (Clayton, 1966). CD spectra were determined using either a JASCO J-20 spectropolarimeter with sensitivity extended to wavelengths of 1000 nm, or a CD/MCD instrument constructed especially in our laboratory (Sutherland et al., 1974).

Fluorescence measurements were made using a fluorescence spectrometer (Perkin-Elmer, MPF 2A) modified with an emission grating blazed for maximum efficiency at 1000 nm (Jobin-Yvon) and a long-wavelength sensitive photomultiplier (Hamamatsu R446). For polarization measurements a sheet polarizer (Polaroid) was used in the excitation beam and a Glan-Thompson prism in the emission beam. Instrument polarization corrections were made following the procedure of Houssier & Sauer (1969). Because of the small signals involved it was necessary to use a wide monochromator bandpass of 40 nm for the polarization measurements. Excitation wavelengths of 375 nm and 600 nm were studied; the emission monochromator was set for maximum signal for each sample.

Quantum yields of fluorescence emission were measured relative to that of pure BChl a in diethyl ether, for which the value of 0.25 was assumed (Tumerman & Rubin, 1962; Zankel et al., 1968). Samples were diluted so that the long wavelength absorbance was 0.2 cm^{-1} or less, and the path through the sample in the emission direction was only 2 mm to minimize distortions due to self-absorption. No significant concentration dependence was observed. A correction was made for the
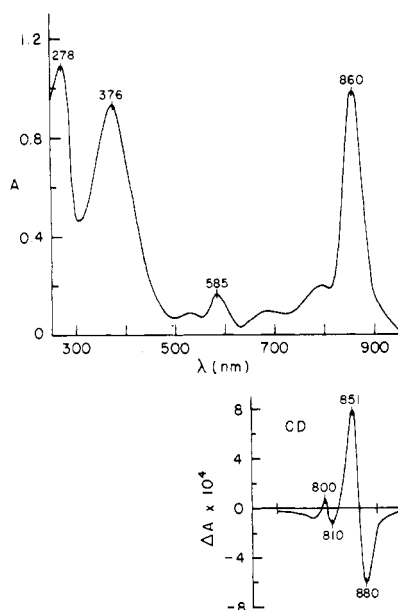
FIGURE 1: Chromatophores from *Rps. sphaeroides*, R-26 mutant. Absorption spectrum (top curve) and circular dichroism spectrum (bottom curve) in the long wavelength region; 1-cm path.
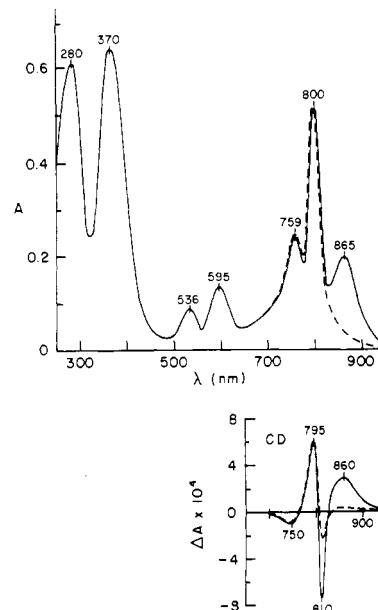
FIGURE 2: Reaction center particles from chromatophores of *Rps. sphaeroides*, R-26 mutant, isolated using Triton X-100. Absorption spectra (top curves) and CD spectra (bottom curves); 1-cm path. Dashed curves show the spectra of the photooxidized species in the long wavelength regions.

wavelength dependence of the instrument sensitivity over the range of fluorescence maxima encountered (750-900 nm) by a procedure described by Austin (1976). All samples were excited at 375 nm, and the signals were normalized with respect to the absorbance of each sample at that wavelength. Wavelength bandpass was typically 10 nm for these measurements.

Results

The absorption spectra of whole cells or chromatophores of the wild-type organism of *Rps. sphaeroides* show distinct differences from the corresponding spectra of the blue-green mutant, R26. In the wild-type spectrum there is a pronounced contribution in the 425-525-nm region from carotenoids that are missing in the mutant. In the long wavelength region of BChl absorption, the mutant is missing a sharp peak at 799 nm, and the 851-nm band appears at 860 nm. In the spectrum of *Rds. rubrum* the carotenoid bands are present in the 500-nm region as in the wild-type *Rps. sphaeroides,* but the long wavelength absorption occurs at 879 nm and the 800-nm band is relatively weak. When BChl a is extracted from any of these organisms into an organic solvent like diethyl ether, only a single absorption band at 770 nm is seen in the near infrared region.

Triton Complexes from Rps. sphaeroides, R-26 Mutant. For the *Rps. sphaeroides,* R-26 mutant, the CD spectrum in the long wavelength region for purified chromatophores is shown along with the absorption spectrum in Figure 1. Upon treatment with Triton X-100 the chromatophore membranes can be separated as described in Materials and Methods into a reaction center fraction containing the photochemically active sites and an antenna complex fraction. The absorption and CD spectra for the reduced and for the photooxidized reaction centers shown in Figure 2 are similar to those reported previously (Sauer et al., 1968; Philipson & Sauer, 1973a). The complex features in the long wavelength region (700-900 nm) have been interpreted in terms of excitonic coupling between closely associated BChl and BPh molecules in the reaction centers, and these undergo reversible changes upon illumination with actinic light.
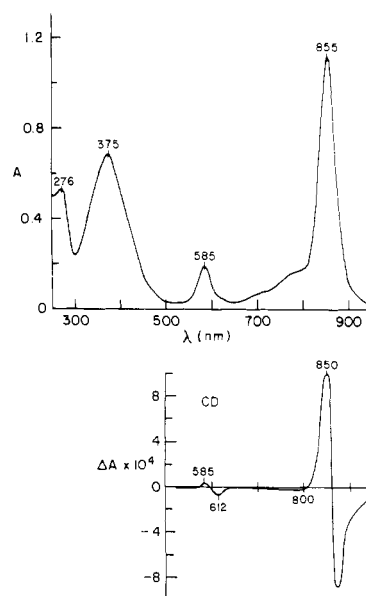
FIGURE 3: Light-harvesting BChl-protein complex (LH-TX100) isolated from Triton-solubilized chromatophores of *Rps. sphaeroides,* R-26 mutant. Absorption spectrum (top curve) and CD spectrum (bottom curve); 1-cm path.

The absorption and CD spectra for the antenna or light-harvesting BChl complexes, designated LH-TX100, isolated from the same treatment with Triton X-100 are shown in Figure 3. Considering that the reaction centers contain less than 5% and the LH-TX-100 complexes contain about two-thirds of the BChl of the chromatophores, the spectra shown in Figure 1 appear to represent the weighted sum of the components shown in Figures 2 and 3. The features around 800 nm appear to derive from the (reduced) reaction centers, and the longest wavelength absorption band and double CD (negative to long wavelength) derive from the light-harvesting BChl complex. There is a shift of this absorption maximum from 860
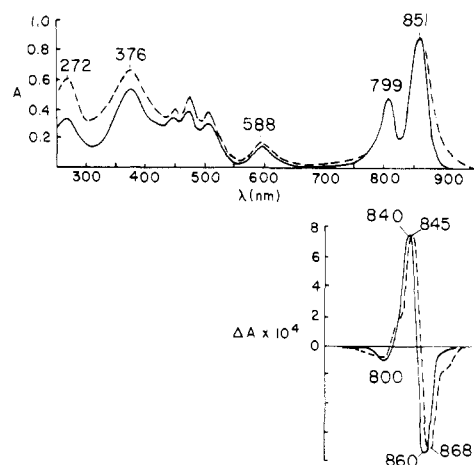
FIGURE 4: Chromatophores (dashed curves) and NaDodSO$_4$-solubilized, light-harvesting BChl-protein (LH–NaDodSO$_4$, solid curves) from *Rps. sphaeroides*, wild-type. Absorption spectra (top) and CD spectra (bottom); 1-cm path.
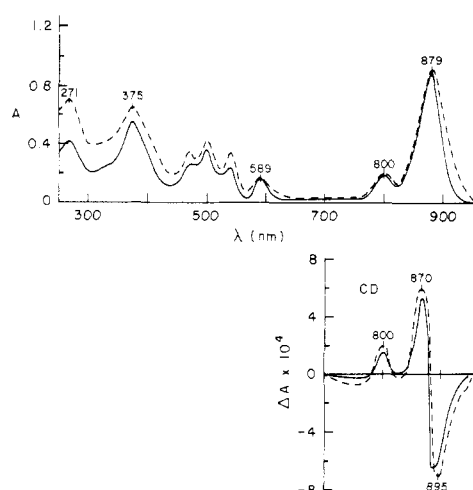
FIGURE 5: Chromatophores (dashed curves) and Triton-solubilized light-harvesting BChl-protein (LH–TX100, solid curves) from *Rds. rubrum*, wild-type. Absorption spectra (top) and CD spectra (bottom); 1-cm path.

TABLE I: Molecular Weights and BChl/Protein Ratios for Various BChl–Protein Complexes.

LH-TX100	Mol wt	Molecules of BChl per 10 kdaltons of protein (±0.2)
Rps. sp. (R-26)	360 000	1.2
Rps. sp. (wild-type)	400 000	1.7
Rds. rubrum (wild type)	420 000	1.7
LH-SDS-BChl		
Rps. sp. (R-26)	21 000	1.1
Rps. sp. (wild type)	23 000	1.6
Rds. rubrum (wild type)		
LH-SDS-BPh subunits		Molecules of BPh per 10 kdaltons of protein
Rps. sp. (R-26)	9 600	0.8
Rps. sp. (wild type)	9 800	1.2
Rds. rubrum (wild type)	10 000	1.3

nm in chromatophores to 855 nm in the detergent-solubilized complex, as had been noted previously (Clayton & Clayton, 1972).

The absorption and CD spectra of *Rps. sphaeroides*, wild-type chromatophores are nearly identical with those of the Triton-solubilized light-harvesting BChl complex fraction (LH-TX100). The spectra of a smaller complex obtained using NaDodSO$_4$ are compared with those of chromatophores in Figure 4. The 800-nm absorption and weak CD bands of the antenna obscure the contribution of the reaction centers in this region for the chromatophores. Again the antenna dominates in the long wavelength absorption and CD bands centered at about 850 nm. Apart from the loss of a long wavelength tail in the absorption spectrum of the chromatophores, probably owing to BChl that becomes solubilized in a free-running form by the detergent, the absorption and CD bands near 850 nm are similar for the wild-type and the R-26 mutant of *Rps. sphaeroides*.

For *Rds. rubrum* the absorption and CD spectra comparing chromatophores with LH-TX100 complexes appear in Figure 5. Again there are strong similarities between the two. We interpret these similarities in each case to indicate that Triton has separated out the major portion of the light-harvesting

BChl from the chromatophores in a configuration that closely resembles the native state. The CD spectra of all three BChl complexes in the long wavelength region are dominated by a pair of intense bands of opposite sign, negative at the longest wavelength and exhibiting a zero-crossing near the corresponding absorption maximum. By contrast, the single CD features near 800 nm are dissimilar: negative for *Rps. sphaeroides*, wild-type, zero for the R-26 mutant, and positive for *Rds. rubrum*.

We have characterized the LH-TX100 complexes in terms of size and composition as well as spectral properties. Upon examination using Sephadex gel filtration, they behave like relatively large particles. Although the sizes appear to change somewhat with growth conditions, nevertheless the complexes from each of the three organisms appear to be homogeneous and correspond to 400 ± 50 kdaltons. Analysis shows that they contain 10–15% BChl, over 50% protein, and 15–20% phospholipid, on a dry weight basis. When the LH-TX100 complexes are treated with NaDodSO$_4$ under strongly dissociating conditions (2.0% NaDodSO$_4$ and 2% mercaptoethanol in boiling water for 1 min), subsequent gel electrophoresis gives for each organism a single fast-moving polypeptide band at a position corresponding to 9.8 ± 0.4 kdaltons. Under these conditions the associated BChl is converted to BPh, but a majority of the pigment remains associated with the peptide during the course of gel electrophoresis. A summary of some of these results is included in Table I.

NaDodSO$_4$ Complexes from Rps. sphaeroides, R-26 Mutant. The Triton-solubilized antenna BChl-protein from the R-16 mutant of *Rps. sphaeroides* is a large complex that appears to contain about 40 BChl molecules, 40 copies of a small (9.6 kdaltons) peptide, and 21% phospholipid, assuming a lipid composition similar to that found by Gorchein (1968), Lascelles & Szilagyi (1965), and Haverkate et al. (1965) and a fatty acid composition like that reported by Hands & Bartley (1962) and Schmitz (1967). In a search for smaller subunits of this complex we found that treatment by dialysis overnight at 4 °C against Tris (0.01 M, pH 7.5) followed by addition of NaDodSO$_4$ to a final concentration of 1% (w/v) resulted in a substantial decrease in apparent molecular weight with little change in the characteristic spectroscopic properties. This smaller light-harvesting BChl-protein, designated LH–NaDodSO$_4$-BChl, was examined in some detail.

Absorption and CD spectra of the LH–NaDodSO₄–BChl-protein from the R-26 mutant are shown in Figure 6 (dashed curves). These are nearly identical with the corresponding spectra of the LH–TX100 complex shown in Figure 3. When the LH–NaDodSO₄–BChl complex is subjected to the strongly dissociating conditions for gel electrophoresis, a single peptide is observed migrating to a position corresponding to 10.2 kdaltons. The absorption and CD spectra are severely altered by these strongly denaturing conditions, as seen in Figure 6 (solid curves). The absorption spectrum is now characteristic of BPh and in the CD there remains only a very small signal characteristic of monomeric, "solubilized" pigment. Nevertheless, the BPh moves together with the peptide during the gel electrophoresis, and we designate it as a LH–NaDodSO₄–BPh subunit.

The pigment content of the LH–NaDodSO₄–BChl complex from the R-26 mutant is given in Table I, and it is essentially the same as for the Triton complex from which it is derived. The phospholipid is decreased to about 14% in the LH–NaDodSO₄–BChl complex and to about 13% in the LH–NaDodSO₄–BPh subunits.

Because of reports of the unreliability of molecular weight determinations of NaDodSO₄-solubilized membrane proteins, especially in the low molecular weight region (see Loach, 1976), we chose to examine the LH–BChl complexes by the additional methods of gel filtration, sedimentation velocity, chemical cross-linking, and amino acid analysis.

For gel filtration measurements we used columns of Sephadex G-50 or G-100, calibrated as described in Materials and Methods. The plot of mobility against logarithm of molecular weight was linear for the reference proteins including molecules as small as α-bungarotoxin (8.0 kdaltons). The reference proteins are all water soluble, however. Using gel filtration we found that the LH–NaDodSO₄–BChl protein migrates as a particle of 22 kdaltons, and the dissociated LH–NaDodSO₄–BPh subunit runs at a position corresponding to 9.8 kdaltons. In each case the sample eluted as a symmetrical peak. We feel that this result supports the idea that the LH–NaDodSO₄–BChl protein contains two copies of a 9-kdalton peptide.

Cross-linking of the peptide in the LH–TX100–BChl complex was achieved using the bifunctional reagent dimethyl adipimidate and the method of Davies & Stark (1970). The reaction was run at room temperature for 3 h using concentrations of imido ester and protein of 3 mg mL⁻¹ and in the presence of triethanolamine hydrochloride (0.1 M, pH 8.0). The reaction product was then subjected to strongly dissociating conditions (2% NaDodSO₄ and 2% 2-mercaptoethanol in boiling water for 1 min) prior to gel electrophoresis. Densitometer scans of the stained gels showed three observable protein bands. The fastest moving band moved to the same position as observed for the single subunit of an un-cross-linked control. A second band of about equal intensity appeared at a position corresponding to twice that molecular weight, but was not seen in the control. The majority of the staining occurred in a band that had migrated only a small distance (20% of that of the fast moving band) from the origin. Presumably this resulted from species with many peptide chains cross-linked together. While the presence of this latter material somewhat complicates the interpretation of this experiment, we feel that the result provides support for our picture involving two closely associated peptides in the LH–BChl-protein complexes; we found evidence of cross-linked dimers but no trimers or other small oligomers in the gel electrophoresis analysis following treatment with dimethyl adipimidate.

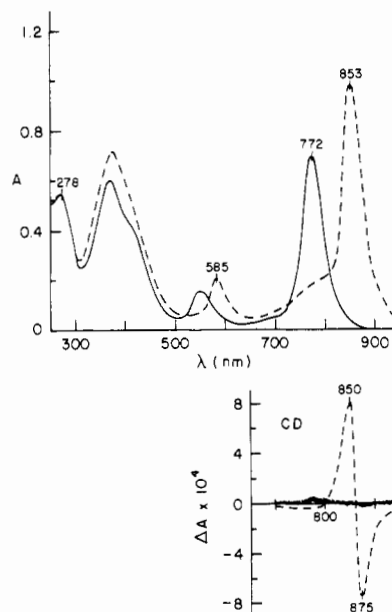Sedimentation velocity measurements were carried out on

FIGURE 6: NaDodSO₄-dissociated, light-harvesting BChl-protein (LH–NaDodSO₄–BChl, dashed curves) from *Rps. sphaeroides*, R-26 mutant, and dissociated peptides (LH–NaDodSO₄–BPh subunits, solid curves) from the same complex. Absorption spectra (top) and CD spectra (bottom); 1-cm path.

the LH–NaDodSO₄ subunit polypeptides following two successive extractions with acetone–methanol (7:2, v/v; see Materials and Methods) and resuspension in 0.5% NaDodSO₄. Two methods were used to estimate the subunit molecular weight (minus BChl and lipid). The first follows a procedure by Bais et al. (1974) and involves comparison of the sedimentation coefficient for the LH–NaDodSO₄ subunit, 1.4 S, with those for several standard reference NaDodSO₄–protein complexes. The molecular weight of 7700 obtained in this way is only approximate, because the sedimentation coefficients were not extrapolated to zero concentration and because the sedimentation coefficient of 1.4 S falls below that of the smallest molecular weight standard used (cytochrome c). In the second approach, gel filtration was used to estimate a value of 20 nm for the Stokes hydrodynamic radius of the LH–NaDodSO₄ subunits by comparison with several similarly treated standard proteins (Ackers, 1970). This value was then used in combination with the measured sedimentation coefficient and an estimated value of the partial specific volume (0.870 cm³ g⁻¹), following the method of Tanford et al. (1974), to calculate a molecular weight of 7600 (see Austin (1976) for details). The agreement between these values is good, and after adding the contributions of the phospholipids and BPh they compare quite favorably with the values determined from gel filtration or gel electrophoresis.

A summary of the amino acid analysis data for the extracted LH–NaDodSO₄ subunits is given in Table II. The amounts of serine and threonine were corrected for hydrolysis losses by extrapolation to zero time. The cystine and cysteine contents were found to be zero under HCl hydrolysis conditions and negligibly small when determined in a separate analysis as cysteic acid. Tryptophan was determined from the magnetic circular dichroism spectrum. Using the approach of Katz (1968), a least-squares fitting program (written by Susan Stanton, Chemical Biodynamics Laboratory) was used to calculate the most probable set of integral numbers for each amino acid from the measured mole percent values. These are tabulated in Table II and add to 84 amino acids, corresponding

TABLE II: Amino Acid Composition of Lipid-Extracted LH–NaDodSO₄ Peptide Subunits from *Rps. sphaeroides*. R-26 Mutant.

Amino acid	Mol %	No. of residues
Lys	3.58	3.0 (3)
His	2.05	1.7 (2)
Arg	2.08	1.8 (2)
Asx	5.89	5.0 (5)
Thr	7.26	6.1 (6)
Ser	5.97	5.0 (5)
Glx	8.14	6.8 (7)
Pro	4.94	4.1 (4)
Gly	9.77	8.2 (8)
Ala	17.99	15.1 (15)
Cys	0.0	0.0 (0)
Val	7.31	6.1 (6)
Met	2.36	2.0 (2)
Ile	3.39	2.9 (3)
Leu	10.73	9.0 (9)
Tyr	2.45	2.1 (2)
Phe	3.94	3.3 (3)
Trp	2.15	1.8 (2)
	Total	84

FIGURE 7: Triton-solubilized light-harvesting BChl–protein complex from *Rps. sphaeroides*, wild-type, after freeze-thaw cycles and strong illumination. Absorption spectrum (top) and CD spectrum (bottom); 1-cm path.

to a minimum molecular weight of 8700. As with other membrane proteins there is a high content of hydrophobic and neutral amino acids (65%), a relatively high proline content, very low cysteine, and a low level of charged amino acids.

It appears from the lines of evidence reported here that the peptide associated with the LH fractions from *Rps. sphaeroides*, R26 mutant, has a molecular weight between 8 and 9 × 10³. Two of these peptides plus two BChl molecules plus phospholipid gives about 22 kdaltons for the LH–NaDodSO₄-BChl protein, which is in good agreement with the value determined by gel filtration (Table I).

Triton and NaDodSO₄ Complexes from Rps. sphaeroides, Wild-Type. Both Triton-solubilized and NaDodSO₄-solubilized BChl–protein complexes were isolated from *Rps. sphaeroides*, wild-type. The LH–TX100-BChl complex behaves like a large aggregate (Table I) on Sephadex gel filtration columns, similar to the corresponding fraction from the R-26 mutant. The absorption and CD spectra of the wild-type LH–TX100 complexes closely resemble the corresponding spectra of the chromatophore fragments.

Dissociation of the BChl–protein using 1% NaDodSO₄ produces a LH–NaDodSO₄-BChl complex with about 23-kdaltons molecular weight (Table I) that exhibits spectra (Figure 4) essentially identical with those of chromatophores. Further dissociation using high NaDodSO₄ concentration and boiling produces a single fast-moving peptide corresponding to 10 kdaltons by gel electrophoresis. The pigment concentration of the LH–TX100 and LH–NaDodSO₄-BChl complexes (Table I) indicate about 50% more BChl per unit protein in the wild-type complex than in that from the R-26 mutant. The simplest stoichiometry for the wild-type complexes is, therefore, 3 BChl + 2 peptides, corresponding to 23 kdaltons.

We discovered fortuitously that the 800-nm absorption band of the wild-type LH complexes can be bleached apparently without affecting the 850-nm absorption or CD. A combination of freezing and thawing and exposure to illumination in the presence of 0.5% Triton X-100 converted material with spectra initially like those in Figure 4 to a product with the spectra shown in Figure 7. Apart from the carotenoid absorption, the
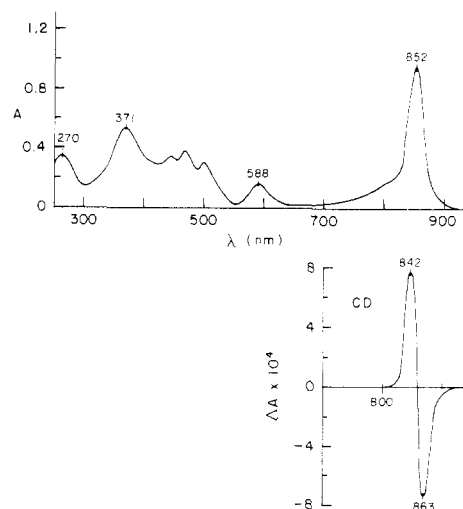
latter closely resemble the spectra of the R-26 mutant (Figure 3).

Triton Complexes from Rds. rubrum, Wild-Type. The spectral properties of the LH–TX100 fraction from *Rhodospirillum rubrum* are very similar to those of the chromatophores from which they were derived (Figure 5). The Triton complex corresponds to about 420 kdaltons (Table I). Addition of even small amounts of NaDodSO₄ led to denaturation of the LH–TX100 complex and pheophytinization of the pigment, and we could not isolate a LH–NaDodSO₄-BChl complex from *Rds. rubrum*. Under strongly dissociating conditons, gel electrophoresis resolved only a single peptide of about 10 kdaltons. Pigment analysis indicated the presence of 1.7 (±0.2) BChl per 10-kdalton protein for the LH–TX100 fraction.

Fluorescence of BChl–Protein Complexes. Measurements of fluorescence intensities and polarization ratios of the light-harvesting BChl–protein complexes provide additional information about the state of the BChl in them. The relative fluorescence yields and polarization values, *p*, are presented in Table III. The fluorescence yields, measured relative to that of BChl a in diethyl ether solution, are presented as approximate quantum yields. The value of 25% for the BChl solution emission is calculated from the observed fluorescence lifetime (Tumerman & Rubin, 1962) and a calculated natural lifetime (Zankel et al., 1968). For each of the organisms studied, there is a progressive increase in fluorescence yield as the chromatophores are dissociated first with Triton X-100 and then with NaDodSO₄.

For the fluorescence depolarization studies, the system was checked using BChl a dissolved in cyclohexanol, a viscous solvent that inhibits depolarization by rotational diffusion. Excitation was carried out either in the Q_x band near 600 nm or in the Soret band at 375 nm where, unfortunately, x and y polarized transitions overlap strongly. The negative value of *p* for Q_x excitation results from the fact that the emission oscillator lies predominantly in the y direction. The values obtained agree well with those reported by Goedheer (1957) and by Ebrey & Clayton (1969). The LH–TX100 complexes exhibit distinctly depolarized fluorescence relative to BChl monomers in cyclohexanol; however, the fluorescence of the LH–NaDodSO₄-BChl complexes is much less depolarized.

TABLE III: Fluorescence Quantum Yields and Polarization for BChl-Protein Complexes.[a]

	Quantum yield of fluorescence (%) [λ_{ex} (375 nm)]	Polarization, p	
		λ_{ex} (590 nm)	λ_{ex} (375 nm)
BChl a in diethyl ether	25.0		
BChl a in cyclohexanol		−0.17	+0.05
Chromatophores			
Rps. sp., R-26	3.2	−0.16,[b] −0.12[c]	
Rps. sp., wild-type	2.1	−0.06[c]	
Rds. rubrum, wild-type	1.9	−0.025,[d] −0.09[c]	
LH-TX100			
Rps. sp., R-26	7.0	−0.07	0.00
Rps. sp., wild-type	6.3	−0.10	+0.01
Rds. rubrum, wild-type	5.9	−0.05	+0.01
LH-NaDodSO$_4$-BChl			
Rps. sp., R-26	24.8	−0.15	+0.02
Rps. sp., wild-type	21.4	−0.14	+0.03

[a] Fluorescence quantum yields relative to that of BChl a in diethyl ether, assumed to be 25%. Fluorescence polarization, given by $p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, is corrected for instrument polarization artefacts. [b] Ebrey & Clayton (1969). [c] Ebrey (1971). [d] Goedheer (1957).

Discussion

In previous studies we showed that the principal features in the absorption and CD spectra of whole cells and intracytoplasmic membranes (chromatophores) of *Rps. sphaeroides* and *Rds. rubrum* are very similar (Dratz et al., 1967; Philipson & Sauer, 1973b). On the basis of studies presented here we conclude that the majority of these spectral features can be accounted for in terms of antenna BChl-protein complexes that can be separated out using detergents. Peptides attributed to the antenna BChl complex have been isolated and characterized from intracytoplasmic membranes of *Rps. sphaeroides*, wild-type (Fraker & Kaplan, 1972; Clayton & Haselkorn, 1972; Clayton & Clayton, 1972; Huang & Kaplan, 1973a,b; Loach, 1976; Niederman et al., 1976), from *Rps. sphaeroides*, R-26 mutant (Clayton & Haselkorn, 1972; Loach, 1976), and from *Rds rubrum* (Tonn et al., 1977; Loach, 1976; Cuendet & Zuber, 1977). There is some disagreement among the results, particularly with respect to the number of different low molecular weight polypeptides present, their molecular weights, and their amino acid composition. During the process of dissociation using detergents, the molecules are readily denatured and the BChl is pheophytinized, indicating that the pigment-protein association has been disrupted. Clayton & Clayton (1972) were the first to report the isolation of a complex that appears to be relatively intact: the absorption spectrum was little altered, it contained BChl (17% w/w), and it was associated with a single polypeptide of 9 kdaltons. This complex was isolated from *Rps. sphaeroides*, wild type, using the detergent LDAO. Because the properties of the BChl-protein complexes from the three organisms differ from one another, we shall discuss them separately.

BChl$_2$P$_2$ from Rps. sphaeroides, R-26 Mutant. The stoichiometry and polypeptide analysis for the LH-BChl protein from the R-26 mutant of *Rps. sphaeroides* suggests that it consists of two BChl molecules associated with two peptides of about 8.5 kdaltons each. Because the complex also has about 15% phospholipid associated with it, the total molecular weight is about 22 000. Although a fast-moving band corresponding to 10-11 kdaltons using polyacrylamide gel electrophoresis was reported by Clayton & Haselkorn (1972) and by Loach (1976) for the R-26 mutant, this complex has not previously been investigated in detail.

Our results show that the stoichiometry of 2 BChl per 2 peptides applies not only to the NaDodSO$_4$-solubilized complex which gives a molecular weight of 22 000 using gel filtration, but also to the Triton-solubilized LH fraction that behaves like a much larger particle of 360 kdaltons. When the peptides are separated by boiling with NaDodSO$_4$ at high concentration, the pigment (now BPh) to peptide ratio drops to 0.8.

The absorption and CD spectra of the LH-TX100 complex (Figure 3) or the LH-NaDodSO$_4$-BChl complex (Figure 6) from the R-26 mutant are consistent with the presence of 2 BChl molecules that are strongly interacting. The absorption spectrum in the long wavelength region appears to be a single band at 850 nm even at low temperature (however, see Cogdell & Crofts (1978) for evidence of splitting of this band at room temperature). Nevertheless, the conservative double CD with a zero-crossing near the absorption maximum is characteristic of exciton interaction between two BChl molecules in close association (Dratz et al., 1967). A similar double CD is found in the 600-nm region for the Q$_x$ transition of BChl (Figure 3). The fact that the absorption and CD of these bands for intact bacterial cells, for chromatophores, for LH-TX100 particles and for LH-NaDodSO$_4$-BChl particles are virtually identical, except for small band shifts, suggests that the arrangement of the BChl molecules and their environment in the particles is the same as that in the intact intracytoplasmic membranes.

The fluorescence polarization of the LH-NaDodSO$_4$-BChl complex is high, nearly as great as that of BChl a monomer in solution. One expects some depolarization from the dimer of BChl molecules proposed for the LH-NaDodSO$_4$-BChl protein, especially upon excitation in the Q$_x$ region where the perpendicularly related exciton components are strongly overlapping. (Our attempts to resolve these components at low temperature in absorption were unsuccessful.) It may be that one exciton component has much less intensity than the other, which would account for the high polarization of fluorescence as well.

The studies of fluorescence intensity and depolarization indicate that there are two levels of electronic interaction in the BChl-protein antenna. The pairwise interaction between the two BChl molecules in an individual protein complex, giving rise to the pronounced CD feature, is apparently not

altered by aggregation in the chromatophores or in the larger LH–TX100 complexes. However, the decreased fluorescence polarization demonstrates that additional excitation transfer can take place in these larger associations. The decreased fluorescence yield, particularly in the chromatophores where the presence of open reaction centers provides traps for the migrating excitation (Wang & Clayton, 1971), is additional evidence for the longer range energy transfer. The fate of the excitation that results in the decreased fluorescence yield of the LH–TX100 particles, where reaction centers are absent, is not clear. We do not find evidence of an unusually high quantum yield of fluorescence, as reported recently by Heathcote & Clayton (1977).

These results provide evidence to support a model for excitation transfer presented several years ago (Sauer, 1975), where excitation produced by an absorbed photon is first rapidly delocalized via exciton interaction among the closely coupled molecules in a small pigment–protein complex, and then transferred probably by a Förster mechanism involving weaker interactions from one such complex to the next until the reaction center or another trapping site in the membrane is reached.

BChl₃P₂ from Rps. sphaeroides, Wild Type. The wild-type strain of *Rps. sphaeroides* yields an antenna BChl protein that contains about 50% more BChl per 10 kdaltons of protein than does the R-26 mutant. The wild type also has an additional absorption band at 800 nm that has about half the intensity of the 850-nm band. The LH–NaDodSO₄–BChl complex behaves like a 23-kdalton particle by gel filtration, and it has absorption and CD spectra (Figure 4) essentially like those of whole cells, chromatophores (Sauer, 1972; Philipson & Sauer, 1973b), or the large (400 kdaltons) LH–TX100 complex from which it derives. We observed a single peptide of 9.8 kdaltons, in reasonable agreement with values reported by others (Fraker & Kaplan, 1972; Clayton & Haselkorn, 1972; Huang & Kaplan, 1973a,b). We do not find evidence of two different polypeptides in this fraction, as reported by Hall et al. (1973), however. Detailed analysis of the 10-kdalton peptide by Huang & Kaplan (1973b) shows that it differs distinctly from that of the R-26 complex reported here. Not only does the wild-type peptide have probably 105 to 107 amino acid residues, compared with 84 for the R-26 peptide, but the amino acid composition differs between the two in several significant respects. The wild-type peptide contains no His (2 in R-26) and 12 Val (6 in R-26), for example. Neither contains Cys, however, and the percent of nonpolar amino acids is very high in both cases.

The BChl analyses of Fraker & Kaplan (1972) and of Clayton & Clayton (1972) are both consistent with the ratio 3 BChl per 2 peptides for the wild-type complex. In a recent study, Cogdell & Crofts (1978) found that the carotenoid (spheroidene) seems to be present in a fixed stoichiometric ratio of 1 carotenoid per 3 BChl. Clayton & Clayton (1972) reported the presence of both the 800- and 850-nm absorption bands in the isolated BChl-protein. Studies by Aagaard & Sistrom (1972) and by Niederman et al. (1976) demonstrate that these two absorption components are linked to one another, despite variations in growth conditions or treatments that greatly alter the overall absorption spectrum. Furthermore, we find that the 850-nm absorption and CD components are very similar for the LH–BChl proteins of the wild-type and the R-26 mutant. We interpret this to reflect the presence of a strongly interacting pair of BChl molecules (a dimer) in both complexes, essentially in the same configuration in each case, and an additional BChl molecule (an associated monomer) in the wild-type complex only. This additional BChl molecule does not

interact strongly with the other two, hence its weak CD contribution; nor does it significantly perturb the interaction of the two molecules in the dimer with one another. The photolability of the monomeric BChl component (Figure 7) is additional evidence of its loose coupling to the BChl₂P₂ core. The fact that the peptides of the wild-type complex (Huang & Kaplan, 1973b; Loach, 1976) appear to have about 20 more amino acid residues than does the corresponding peptide from the R-26 mutant may reflect an additional segment of protein needed to encompass the monomeric BChl present.

The fluorescence studies show an increase in polarization and in fluorescence intensity upon dissociating the Triton-solubilized aggregates by adding NaDodSO₄. The behavior and, very likely, the explanation are similar to those invoked for the R-26 complexes. A more detailed study is required to determine the quantitative effect of the additional BChl present in the wild-type complex.

BChl₃P₂ from Rds. rubrum, Wild Type. The LH–BChl from *Rds. rubrum,* wild-type, bears some similarity to that from *Rps. sphaeroides,* wild-type. We find evidence of a 10-kdalton subunit peptide using polyacrylamide gel electrophoresis, a ratio of 3 BChl to 2 peptides, the presence of carotenoid in the complex, an absorption component at 800 nm associated with a weak CD (Figure 5), and evidence of a large aggregated form of about 400 kdaltons. The peptide size is in agreement with the value reported on the basis of electrophoretic mobility by Tonn et al. (1977); however, they felt that 19 kdaltons must be the correct minimum molecular weight based on amino acid analysis. We did not observe a significant discrepancy between the various methods of analysis when we applied them to the R-26 peptide, however. Recently Cuendet & Zuber (1977) reported that they get agreement between NaDodSO₄–polyacrylamide gel electrophoresis and amino acid analysis for the polypeptides derived from *Rds. rubrum;* their molecular weight of 14 000 is significantly larger than ours, however.

There are important differences as well between the LH–BChl-proteins derived from *Rds. rubrum* and those from *Rps. sphaeroides.* We have not been able to isolate a small LH–NaDodSO₄–BChl complex from *Rds. rubrum;* hence, we cannot characterize the simplest unit that corresponds to the stoichiometry. The long wavelength absorption band occurs at 880 nm, which is significantly longer than that seen for *Rps. sphaeroides;* however, the double CD with identical sign relation suggests the presence of a strongly coupled dimer of BChl in *Rds. rubrum* as well. The additional 800-nm absorption band appears to be too weak in *Rds. rubrum* to reflect an additional uncoupled (monomeric) molecule; it represents less than one-third of the total integrated absorption. At the same time, the 800-nm band represents too much absorption to reflect only the reaction center contribution; furthermore, we were not able to detect reaction center activity in the LH–BChl protein from *Rds. rubrum.* The contribution to the CD spectrum of the LH–BChl protein in this region is positive, which is opposite to that for *Rps. sphaeroides,* wild-type. In each case there appears to be a weak, single CD band centered at the wavelength of the corresponding absorption maximum. The sign may arise largely from the effect of the induced CD owing to the local protein environment.

Conclusions

The light-harvesting antenna BChl-proteins described here are an important element of the Pebble–Mosaic model of photosynthetic membrane structure (Sauer, 1975). In that view the membranes consist of an organized array of reaction center complexes, electron transport cofactors, and phosphorylation

coupling factors assembled in a two-dimensional arrangement with a complement of light-harvesting pigment. We feel that the LH-BChl-proteins characterized in the present study represent basic elements in this array for the purple photosynthetic bacteria. They consist of 2 or 3 BChl molecules in a complex with 2 peptides, phospholipids and, normally, with carotenoid in addition. These elements of the light-harvesting antenna, which are the smallest and simplest chlorophyll-proteins that have been isolated, constitute a major portion of the pigment array that absorbs photons and transfers the resulting electronic excitation to the reaction centers. The LH-BChl-proteins probably occur in larger aggregates of 15-20 units; perhaps this is a collection that functions to serve primarily a single reaction center. A model proposed arrangement for the components in the photosynthetic bacterial intracytoplasmic membranes is presented elsewhere (Sauer, 1978).

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