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Topography of the Photosynthetic Apparatus of *Chloroflexus aurantiacus*†

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ABSTRACT: The isolation and identification of reaction center, antenna bacteriochlorophyll *a* (Bchl *a*), and chlorosome antenna Bchl *c* polypeptides of *Chloroflexus aurantiacus* have been described. Subsequently, the localization of the chlorosome- and pigment-specific polypeptides was probed in two subcellular fractions. Reaction centers, located in the cytoplasmic membrane, were isolated according to the method of Pierson and Thornber [Pierson, B. K., & Thornber, J. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 80–84]. The reaction center preparation contained a major polypeptide with an apparent molecular weight of 26 000. The antenna Bchl *a* (B808–866) complex, also a cytoplasmic membrane constituent, was isolated by hydrophobic interaction chromatography on phenyl-Sepharose. In fractions with the highest purity and still native absorption properties, only one polypeptide of M_r 5300 was found. Purified chlorosomes contained three major proteins (M_r 18 000, 11 000, and 3700), and a fourth polypeptide (M_r 5800) was present in minor quantities. The M_r 3700 polypeptide was found to be associated with the antenna Bchl *c*. The two larger chlorosome proteins were accessible to proteinase K proteolyses and could additionally be modified with the membrane surface specific photolabel 3-azido-2,7-naphthalenedisulfonate. From the resistance against proteolytic cleavage and the lack of photolabeling, we surmised that the antenna Bchl *c* polypeptide (M_r 3700) is located in the chlorosome interior. Upon chemical cross-linking with dimethyl 3,3′-dithiobis(propionimidate), only dimers of the M_r 3700 protein were formed in high yield. According to our calculations, this dimer was comprised of 2 copies of the M_r 3700 protein and 10–16 molecules of Bchl *c*. The cross-linking pattern of the B808–866 antenna Bchl *a* protein (M_r 5300) indicated a quaternary structure of a trimer, which should be part of a penta- or hexameric in vivo aggregate. Under the experimental conditions used, the M_r 5300 antenna protein was never photolabeled and together with the reaction center protein resisted any proteolytic attack, indicating a more sequestered localization in the cytoplasmic membrane.

The photosynthetic apparatus of the thermophilic, facultative aerobic green bacterium *Chloroflexus aurantiacus* is located in two cytologically distinct compartments. This arrangement and the presence of two bacteriochlorophyll (Bchl)[1] types, Bchl *a* and species-dependent Bchl *c*, *d*, or *e*, are characteristic for all green bacteria (*Chlorobiaceaece*). In *C. aurantiacus*, the photochemical reaction centers and a Bchl *a* containing light-harvesting complex with absorption maxima at 866 and 808 nm are located in the cytoplasmic membrane (CM).

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; LDAO, lauryldimethylamine oxide; NaDodSO$_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Bchl, bacteriochlorophyll; DTBP, dimethyl 3,3′-dithiobis(propionimidate); CM, cytoplasmic membrane; Bpheo, bacteriopheophytin; RC, reaction center; ANDS, 3-azido-2,7-naphthalenedisulfonate; DSP, dithiobis(succinimidyl propionate).

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Recently, a method has been described for the isolation of purified CM, completely devoid of Bchl *c* and chlorosomes (Feick et al., 1982). The amount of light-harvesting Bchl *a* per reaction center (RC) in the CM remained constant [Bchl *a*:RC = (20–25):1] regardless of the light conditions during growth. Further characterization of isolated CM by light-induced absorption spectroscopy, redox potentiometry, and electron spin resonance spectroscopy revealed that the reaction center of *C. aurantiacus* possesses a Bchl *a* dimer as a primary electron donor (absorbing at 865 nm with an $E_{m,8.1}$ = +360 mV) and a two menaquinone acceptor system [$E_{m,8.1}$ of first menaquinone acceptor = −50 mV (Bruce et al., 1982; Hale et al., 1983)].

The recent success in preparing spectroscopically pure reaction centers (Pierson & Thornber, 1983) allowed a more detailed investigation of the early photochemical events by picosecond transient absorption spectrometry. The identification of bacteriopheophytin *a* (Bpheo *a*) as an early-intermediate electron acceptor and the 300-ps time constant for quinone reduction further substantiate the close resemblance between the reaction centers of purple bacteria and *C. aurantiacus* (Kirmaier et al., 1983; Blankenship et al., 1983).

The major Bchl type in *C. aurantiacus*, Bchl *c*, is located and organized in chlorosomes and functions solely as a light-harvesting pigment (Schmidt, 1980; Sprague et al., 1981a; Feick et al., 1982). Chlorosomes are oblong bodies, approximately 100 nm long and 30 nm wide, and are firmly attached to the inner side of the cytoplasmic membrane. The chlorosome envelope consists of a 3–5-nm-thick single-layered membrane (Staehelin et al., 1978). Besides the bulk of antenna Bchl *c* which absorbs in vivo at 740 nm, a small but distinct amount of Bchl *a*, absorbing at 790 nm (B790), has been observed in chlorosomes and, hence, appeared to be an element of the chlorosome structure. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE) of isolated chlorosomes initially revealed only three major polypeptides with molecular weights previously estimated to be 15000, 12000, and 6000 (Feick et al., 1982). The function of the chlorosome-specific B790 Bchl *a* became clear when fluorescence spectroscopic measurements of intact *Chloroflexus* cells showed that this pigment component actively facilitated energy migration from chlorosomes to the CM (Betti et al., 1982). The results of this study were summarized in a model describing the transition dipole orientation of Bchl *c* and Bchl *a* (B790) in the chlorosomes, and this model additionally presented a pathway of energy transfer from the chlorosome pigment to the reaction center.

On the basis of electron microscopic investigation, Staehelin et al. (1978) proposed that chlorosomes are attached via a specific structural connection, a paracrystalline base plate, to the CM. Since efficient energy transfer should require a specific functional connection, this B790 Bchl *a* may well be organized in, or be part of, this base plate, providing the energetic linkage between the chlorosome and the CM.

These findings obtained by freeze–fracture electron microscopy led to a model describing the supramolecular architecture of chlorosomes and their respective binding sites to the CM (Staehelin et al., 1978).

Since no biochemical data are yet available to verify this model, the aim of our present work is to survey the topographical arrangement of chlorosome- and pigment-specific proteins in two subcellular fractions, isolated chlorosomes and cytoplasmic membranes with attached chlorosomes. We attempted to identify pigment-specific polypeptides and use this information to evaluate the results obtained from chemical modification of the two subcellular fractions.

Materials and Methods

Growth Conditions and Membrane Isolation. Cultivation of *C. aurantiacus* J-10-fl and isolation of the "whole membrane fraction", containing CM and chlorosomes, were performed as previously reported (Feick et al., 1982) except that 2 mM PMSF was added immediately after cell breakage. Unless noted elsewhere, 10 mM Tris-HCl, pH 8.0, was used throughout all isolation procedures. For topographical studies, e.g., proteolytic treatment photolabeling and cross-linking experiments, the whole membrane fraction was further purified by isopycnic gradient centrifugation. Usually 5 mL of material with an in vivo absorbance at 866 nm of ~1, corresponding to 1 mg of protein/mL, was applied on top of a two-step sucrose gradient (10 mL of each, 30% and 55% w/v) and centrifuged for 15 h at 145000*g* (35000 rpm; Ti50.2, Beckman) at 4 °C. In the following, this is designated as 15-h gradient centrifugation. The upper and lower pigmented bands were enriched in "free" chlorosomes and "free" CM, respectively. The middle band contained chlorosomes attached to the CM and will be referred to as "purified membranes".

Chlorosome Isolation. A modified procedure of Feick et al. (1982) was used for chlorosome isolation. The whole membrane fraction was adjusted to an optical density at 866 nm of ~2, and Miranol S2M-SF was added to a final concentration of 0.30% (w/v). The suspension was stirred for 30 min at room temperature, applied on top of a two-layered sucrose gradient (23–45% w/v) containing 0.2% Miranol, and subjected to 15-h gradient centrifugation. Chlorosomes banding in the middle of the gradient were collected, dialyzed against buffer, and resedimented (120 min, 145000*g*). The sediment was resuspended in the same volume of buffer containing 0.1% Miranol and subjected to a second 15-h gradient centrifugation in the presence of 0.1% Miranol.

Preparation of Pigment–Protein Complexes. Reaction centers of *C. aurantiacus* were prepared as described by Pierson & Thornber (1983), except that the whole membrane fraction was used as starting material and the incubation time with LDAO was shortened to 30 min.

For isolation of the antenna Bchl *a* (B808–865) complex, a suspension of whole membranes with an A_{866} = 1.5–2 was adjusted to 0.5% Miranol–0.08% LDAO and stirred for 30 min at room temperature. This solution was subjected to a 15-h sucrose-gradient centrifugation (0.2–0.5% M sucrose) in the presence of 0.3% Miranol, 0.04% LDAO, and 0.1% Triton. The pigmented fraction banding in the upper third of the gradient was collected and dialyzed for 2–4 h against Tris buffer. The dialyzed pigmented fraction was brought to 4 M NaCl concentration and subsequently loaded on a phenyl-Sepharose column (27 × 1 cm) previously equilibrated with Tris buffer. After the column was washed with approximately 100 mL of 20 mM Tris–0.2% Miranol, pH 8.0, the detergent composition was changed to 0.4% Miranol, 0.1% LDAO, and 0.1% Triton X-100, which allowed the elution of antenna Bchl *a* (B808–866)–protein complexes.

For identification of chlorosome-specific antenna Bchl *c* protein and Bchl *a* B790 pigment–protein complexes, isolated chlorosomes (1.5 mg/mL) were subjected to trypsin and chymotrypsin digestion (40 μg of each protease/mg of chlorosome protein) for 30 min at 33 °C. The reaction was terminated by adding PMSF, Miranol, and Triton X-100 to final concentrations of 5 mM, 0.4%, and 0.2%, respectively. Subsequently, the material was applied on a continuous sucrose gradient (15–35% w/v) supplemented with 0.3% Miranol, 0.2% Triton X-100, and 1 mM PMSF and centrifuged at

145000*g* for 36 h at 4 °C. The desired pigment–protein fraction banded at ~20% (w/v) sucrose concentration. For further analysis, this fraction was dialyzed against buffer and concentrated against polyethylene glycol (M_r 20000).

Proteolytic Treatment. Purified whole membrane or isolated chlorosomes were resuspended in Tris buffer to a concentration of 2 mg of protein/mL and 1:2 (v/v) diluted with proteinase K solution (specific activity 20 units/mg) to concentrations as indicated. The reaction was carried out at 33 °C and terminated at the indicated times by addition of a 4-fold volume of termination buffer containing 20 mM glycylglycine, 5% ethanol, and 3 mg/mL PMSF, pH 6.5. The digested membranes were sedimented at 180000*g* for 120 min and washed twice with termination buffer and once with tris buffer.

Photolabeling. Purified membranes and isolated chlorosomes washed once in 50 mM sodium phosphate buffer, pH 8.0, were resuspended in the same buffer to a protein concentration of 1 mg/mL. The samples were diluted 1:2 (v/v) with a freshly prepared 3-azido-2,7-naphthalenedisulfonate (ANDS) solution (0.5 mg/mL) in the dark. Immediately after being mixed, the suspension was spread out in a petri dish and irradiated for 5 min from the top and bottom with long-wavelength UV light (~370 nm). During illumination, the temperature was maintained at 4 °C. After photolysis, excess label was removed by two ultracentrifugations at 145000*g* for 120 min. After $NaDodSO_4$–PAGE, labeled proteins were visualized by irradiating the gel with long UV-wavelength light (340 nm). The fluorescence of ANDS-labeled polypeptides (~400 nm; Dockter, 1979) was photographed on Kodak (technical pan) film while the film was protected from UV light by a filter combination (Kodak 2B, 2E, 64).

Chemical Cross-Linking. For chemical cross-linking studies, purified membranes were resuspended to 1 mg of protein/mL of 180 mM triethanolamine hydrochloride, pH 9.2, and treated with 8 mM *N*-ethylmaleimide for 20 min at 30 °C. Subsequently, an equal volume of freshly prepared dimethyl 3,3′-dithiobis(propionimidate) (DTBP) solution (1 mg/mL) was added and incubated at 33 °C. At various time intervals, the reaction was terminated by the addition of 0.2 mL of 1 M glycine in 1 M Tris-HCl, pH 8.0. The cross-linked membranes were sedimented by ultracentrifugation (145000*g*, 120 min) and resuspended in Tris buffer. Prior to the addition of solubilization buffer, the sample was treated with 16 mM *N*-ethylmaleimide.

Polyacrylamide Gel Electrophoresis (PAGE). One- and two-dimensional $NaDodSO_4$–PAGE was done according to Laemmli with the following modifications. A 12.5–16.5% polyacrylamide gradient for both the first and second dimensions was stabilized by an accompanying sucrose gradient (4–18% w/v). The separation gel buffer was adjusted to pH 8.7, and the electrode buffer contained 64.6 g of glycine/L. Before electrophoresis, samples were diluted 1:2 (v/v) with solubilization buffer containing 3.3% $NaDodSO_4$, 0.125 M Tris, 5% mercaptoethanol, and 20% glycerol, pH 6.8, and heated for 30 min at 65 °C. Samples with cross-linked material were solubilized in the absence of mercaptoethanol. For two-dimensional gel electrophoresis, the first dimension (1-mm-thick slab gel) was sliced into its respective lanes and incubated for 75 min in 62.5 mM Tris, 0.1% $NaDodSO_4$, and 30 mM DTT, pH 8.6 at room temperature. Usually, two or three lanes were horizontally affixed with 1.5% agarose (in Tris–DTT buffer) on the top of the second-dimension gel (0.7 mm thick).

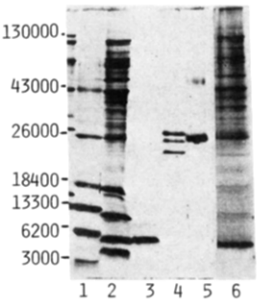

FIGURE 1: Polypeptide profiles of subcellular fractions of *C. aurantiacus* after $NaDodSO_4$–PAGE in a continuous 12.5–16.5% gel: lane 1, marker proteins; lane 2; purified membranes; lane 3, isolated B808–866 antenna Bchl *a* complex; lane 4, isolated reaction centers from *Rps. sphaeroides*; lane 5, isolated reaction centers from *C. aurantiacus*; lane 6, isolated cytoplasmic membranes.

The following water-soluble proteins were employed for molecular weight determination: insulin (3000), aprotinin (6200), lysozyme/cytochrome *c* (13300), myoglobin (17200), β-lactoglobulin (18400), α-chymotrypsinogen (25700), carbonic anhydrase (29000), ovalbumin (43000), bovine serum albumin (68000), phosphorylase *b* (92500), and β-galactosidase (130000). Low molecular weight markers were purchased from Bethesda Research Laboratories.

Protein concentration was estimated according to Peterson (1977) except that prior to the addition of Lowry reagent A, samples were treated with 2.5% $NaDodSO_4$ for 45 min at 60 °C. Pigment content was determined as described previously (Feick et al., 1982).

Chemicals. Miranol S2M-SF concentrate was a gift from the Miranol Chemical Co., Irvington, NJ; proteinase K and thermolysin were obtained from Boehringer Mannheim; DTBP and ANDS were purchased from Pierce Chemical Co., Rockford, IL.

Results

Identification of Pigment Binding Proteins. The absorption spectrum of isolated reaction centers was identical with that previously reported (Pierson & Thornber, 1983). For a more detailed spectroscopic analysis, picosecond absorption spectroscopy, and pigment composition, see Kirmaier et al. (1983) and Blankenship et al. (1983). Polyacrylamide gel electrophoresis of purified *C. aurantiacus* reaction centers revealed one dominant polypeptide with an apparent molecular weight of 26000 (Figure 1, lane 5), which is similar in size to the middle protein band of *Rhodopseudomonas* (*Rps.*) *sphaeroides* reaction centers (Figure 1, lane 4). The presence of small amounts of a polypeptide having a molecular weight of 52000 seemed to be a result of incomplete sample denaturation. If less harsh solubilization conditions were used (e.g., 30 min at room temperature), a photochemically active reaction center pigment–protein complex of M_r 52000 could be prepared in high yield. A reelectrophoresis of the extracted and denatured pigment–protein band revealed the M_r 26000 polypeptides to be a protein constituent of the M_r 52000 pigment–protein moiety. It has yet to be demonstrated whether this complex is composed of two copies of the same polypeptide or whether there are two different polypeptides present having identical size or electrophoretic mobilities.

The antenna Bchl *a* (B808–866) complex of *C. aurantiacus* was isolated by hydrophobic interaction chromatography. Fractions collected during the column elution contained reaction center and antenna Bchl *a* complexes in varying relative amounts; fractions collected later contained much more enriched or purified antenna complexes. Isolated B808–866

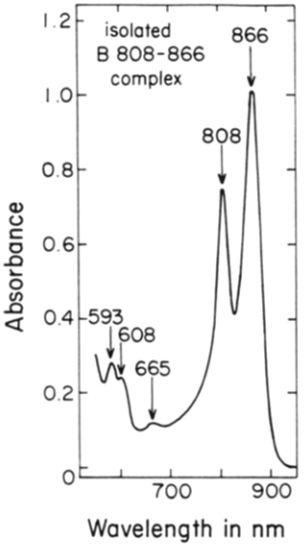

FIGURE 2: Absorption spectrum of the B808–866 antenna Bchl *a* complex isolated by hydrophobic interaction chromatography.

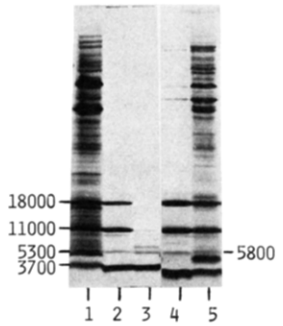

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of (lane 1) purified membranes, (lane 2) isolated chlorosomes, and (lane 3) the antenna Bchl *c* B790 fraction. Samples in lanes 2 and 3 were adjusted to the same 740-nm absorption intensity. Note the same amount of M_r 3700 polypeptides present in both lanes. Lane 4 shows the chlorosome-enriched fraction. Lane 5 shows purified membranes as in lane 1 but with better resolution to show the M_r 5800 polypeptide.

complexes exhibited absorption bands at 808 and 866 nm in a constant ratio of 1.6:1 (Figure 2). The absence of any reaction center contamination was indicated by the lack of reversible light-induced bleaching of the 866-nm band. The two absorption maxima at 608 and 593 nm reflected a rather unique Bchl *a* absorption feature in the Q_x region. The isolated B808–866 complex contained only a single polypeptide with an apparent molecular weight of 5300 (Figure 1, lane 3). The same polypeptide was the dominant protein in the isolated CM (Figure 1, lane 6) and was previously estimated to have a molecular weight of 8000 (Feick et al., 1982).

Purified chlorosomes (for the absorption spectrum, see Figure 4B) were comprised of three major polypeptides which can easily be detected by $NaDodSO_4$–PAGE. However, if sufficient material was subjected to gel electrophoretic analysis, a fourth polypeptide became apparent (Figure 3, lane 2). The molecular weights of those four chlorosome-specific proteins were determined in a $NaDodSO_4$–PAGE system which could resolve molecular weights down to 3000 (Figure 1, lane 1). Using water-soluble proteins as calibration standards, we estimated the molecular weights of the three dominant chlorosome proteins to be 18 000 (18 060 ± 160, n = 8), 11 000 (11 210 ± 600), and 3700 (3650 ± 258) and that of the fourth polypeptide to be 5800 (5840 ± 300). Due to the presence and amount of M_r 5300 antenna Bchl *a*–protein, the detection of the M_r 5800 polypeptide in whole membranes was not an

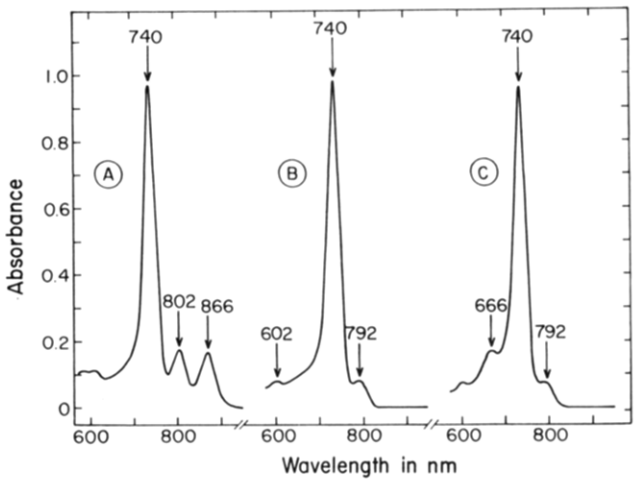

FIGURE 4: Near-infrared absorption spectra of (A) purified membranes, (B) isolated chlorosomes, and (C) the chlorosomes antenna Bchl *c*–Bchl *a* (B790) fraction. The small shoulder at 666 nm represents the monomeric Bchl *c* and/or Bpheo *c* absorptions which might have been caused by the presence of detergent and 5% ethanol during preparation.

easy task. Only under optimal electrophoretic conditions (of particular importance was the pH of the separation buffer) could those two proteins be separated (compare lanes 1 and 5 of Figure 3).

A Bchl *c*/Bchl *a* (B790) pigment–protein complex having unaltered absorption characteristics (Figure 4C) could be prepared by treating isolated chlorosomes with trypsin and chymotrypsin followed by an isopycnic gradient centrifugation in the presence of 0.3% Miranol and 0.2% Triton X-100. While the relative amount of the M_r 3700 and 5800 proteins remained unaffected, only a few minor degradation products of the M_r 18 000 and 11 000 proteins were present after proteolysis and subsequent fractionation (Figure 3, lane 3). More severe digestive treatment caused a concomitant decrease of the Bchl *c* 740-nm intensity and loss of the M_r 3700 polypeptide (see below). The decrease of the B790 band seemed to parallel the disappearance of the M_r 5800 protein band. Further support for a B790–M_r 5800 polypeptide association could be drawn by comparing polypeptide profiles of chlorosomes isolated from "high- and "low-light-grown" *Chloroflexus* cells. On a Bchl *c* basis, high-light chlorosomes were 4-fold enriched in Bchl *a* (B790) and contained 3.5–4.5 times more M_r 5800 protein than found in low-light chlorosomes. These correlations indicate an association of Bchl *c* with the M_r 3700 polypeptide whereas B790 (Bchl *a*) seemed to be associated with the M_r 5800 protein.

Subsequently, we attempted to elucidate the stoichiometric relationship between Bchl *c* molecules and the M_r 3700 polypeptide by applying the following analytical data obtained with isolated chlorosomes: (1) a specific Bchl *c* content of 500 nmol of Bchl *c*/mg of protein; (2) an M_r 3700 polypeptide, on the basis of densitometry. Since an underestimation of the amount of this protein had to be considered (see Discussion and Conclusions for reasons), we based the estimation on M_r 3700 and 5500, respectively, which will account for a 50% underestimation of the molecular weight. Depending on these molecular weight values, we determined that a minimal stoichiometric unit would be comprised of 5.3–7.8 (5–8) Bchl *c* molecules per polypeptide of M_r 3700 or 5500, respectively.

Proteolytic Modification. The susceptibility of exposed proteins in purified membranes and isolated chlorosomes was probed by proteolytic treatment with proteinase K, trypsin, and chymotrypsin. Due to the "mesophilic incubation

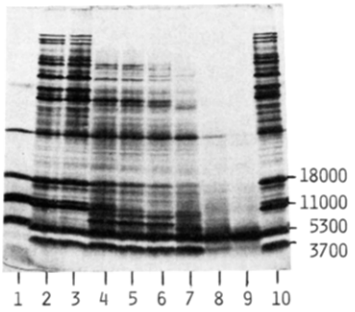

FIGURE 5: Effect of proteinase K on the polypeptide pattern of purified membranes after the NaDodSO$_4$–PAGE: lane 1, marker proteins; lanes 2 and 10, control without proteinase K, incubated at 33 °C for 40 min; lane 3, zero-time control (200 μg of proteinase K/mg of membrane protein for 0 min); lane 4, 2μg, 5 min; lane 5, 2 μg, 10 min; lane 6, 10 μg, 15 min; lane 7, 40 μg, 20 min; lane 8, 200 μg, 20 min; lane 9, 1000 μg, 35 min.

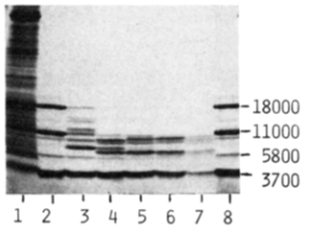

FIGURE 6: Polypeptide profiles after NaDodSO$_4$–PAGE of isolated chlorosomes treated with proteinase K: lane 1, purified membranes; lanes 2 and 8, zero-time control (50 μg of proteinase K/mg of chlorosome protein for 40 min); lane 3, 5 βg, 5 min; lane 4, 10 μg, 5 min; lane 5, 20 μg, 10 min; lane 6, 50 μg, 20 min; lane 7, 200 μg, 30 min.

temperature" at which these enzyme reactions were performed, a change in the fluidity of the thermophilic *Chloroflexus* membranes and thus an alteration in the membrane topography have to be considered. Hence, we additionally used thermolysin to perform the proteolysis at 55 °C. Since all four enzymes revealed essentially the same result, only the data of proteinase K modification are presented. As can be seen from Figures 5 and 6, low proteinase K concentrations (2–40 μg/mg of membrane protein) caused the nearly complete disappearance of the M_r 18 000 and 11 000 chlorosome proteins in both fractions within 20 min. After a more severe digestive treatment, the relative amount of the M_r 3700 chlorosome polypeptide also decreased, along with the parallel loss of Bchl *c* absorption intensity at 740 nm. In samples where the 740-nm absorption was "degraded" to 65% and 45% of its orginal intensities, a corresponding decrease in the relative amounts of M_r 3700 polypeptide to 58% and 39%, respectively, could be observed. The proteolysis of the M_r 5800 protein was only observed when isolated chlorosomes were digested. This polypeptide seemed to be more susceptible to proteinase K degradation than the M_r 3700 constituent. The amount of the M_r 26 000 reaction center protein decreased significantly only after treatment with 200 μg of proteinase K for 20 min (Figure 5, lane 8).

The B808–866 antenna–M_r 5300 polypeptide showed no signs of proteolytic degradation, consistent with the observation of an unchanged absorption intensity at 866 nm.

Photolabeling. The membrane-impermeable probe ANDS was also used to identify exposed membrane surface proteins. Upon light activation, this reagent forms a highly reactive nitrene capable of nonspecific covalent modification of hydrophilic membrane surface regions (Dockter 1979). The photolabeled proteins can be identified by their fluorescence after NaDodSO$_4$–PAGE. The results of ANDS photolabeling

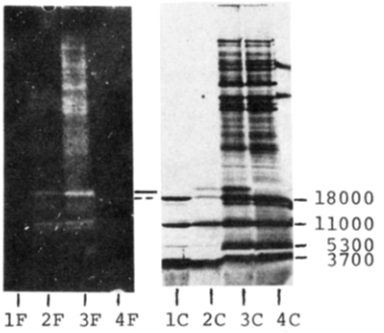

FIGURE 7: Protein pattern of ANDS-photolabeled purified membranes and isolated chlorosomes after NaDodSO$_4$–PAGE. F is fluorescence of ANDS-labeled polypeptides prior to Coomassie stain, and C is Coomassie Blue stained polypeptides: lanes 1, isolated chlorosomes not ANDS treated; lanes 2, isolated chlorosomes ANDS labeled; lanes 3, purified membranes ANDS labeled; lanes 4, purified membranes not ANDS treated.

of isolated chlorosomes and purified membranes are shown in Figure 7. The M_r 11 000 chlorosome polypeptide could be labeled in both membrane fractions as indicated by the fluorescence (Figure 7, lanes 2 and 3). While the ANDS binding to this protein did not change its relative amount and electrophoretic mobility, the M_r 18 000 protein was affected in two ways by the photomodification: (a) the relative amount of that protein diminished (e.g., the amount of stained protein band decreased); and (b) a new fluorescing band at the M_r 19 500 position appeared. In addition, if the amount of M_r 19 500 labeling product (quantified by densitometry) was added to the amount of unmodified M_r 18 000 polypeptide, a relative content approaching that observed in the controls was obtained. Thus, the fluorescing M_r 19 500 band should be a product of the photolabeled M_r 18 000 protein. The slower electrophoretic mobility might be a result of multiple binding of ANDS molecules (binding of four ANDS molecules per molecule of M_r 18 000 protein could account for a 1450 molecular weight increase) or a change in the net charge of the M_r 18 000 protein and hence a different NaDodSO$_4$ binding capacity. The M_r 3700 chlorosome polypeptide and the M_r 5300–antenna B808–866 protein were not labeled, which agreed well with results of proteolytic modification studies. It further substantiated the less accessible location of those two membrane constituents.

Chemical Cross-Linking. Chemical cross-linking with the homobifunctional, cleavable imido ester DTBP was used to elucidate nearest-neighbor protein–protein interactions in purified membranes of *C. aurantiacus*. This reagent contained a mercaptan-cleavable disulfide bridge and spanned an effective distance of ~12 Å. The individual components of cross-linked products were separated and identified by two-dimensional NaDodSO$_4$–PAGE (first dimension in the absence and second dimension in the presence of DTT) in which they appear as "off-diagonal spots". To prevent possible SH–SS interchange between DTBP and available sulfhydryl groups of proteins during the cross-linking reaction and subsequent sample solubilization for NaDodSO$_4$–PAGE, *N*-ethylmaleimide was present during both treatments.

Preliminary experiments have shown that on a qualitative basis, the pattern of cross-linked products remained the same and independent of (a) the membrane concentration and (b) the ratio of membrane protein concentration to DTBP concentration. All subsequent studies were performed at a reasonable low concentration of membrane protein (0.5 mg/mL) to minimize the random collision of different membrane fragments during the cross-linking reaction. A time course

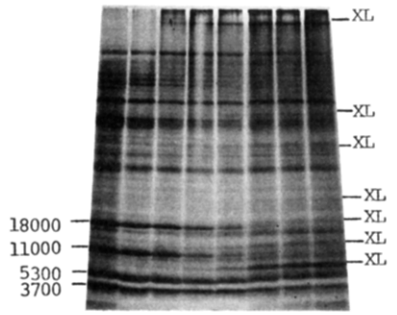

FIGURE 8: $NaDodSO_4$–PAGE of purified membranes after treatment with DTBP (0.5 mg/mg of membrane protein). Electrophoresis is in the absence of reducing agent. XL = cross-linked product. (From left to right) lane 1; control, no DTBP treatment, lane 2; zero-time control; lane 3; 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 40 min; lane 8, 60 min.

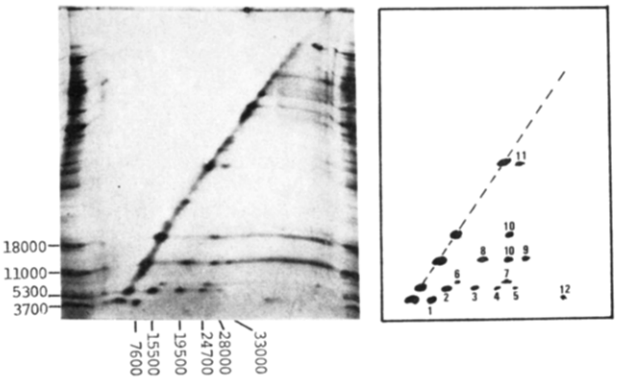

FIGURE 9: Second dimension of DTBP-treated purified membranes. The sample's polypeptide profile after the first dimension is shown in lane 8. Lane 8 was incubated with DTT and horizontally applied on top of a second $NaDodSO_4$–PAGE gel as described under Materials and Methods.

experiment in which the concentration of DTBP per membrane protein was held constant is presented in Figure 8. Due to the high yield in formation, several cross-linked products became readily visible after the first $NaDodSO_4$–PAGE dimension. The four major low molecular weight polypeptides were most obviously affected by the DTBP treatment.

As presented in Table I, the summed molecular weights of individual components were generally within 4% (and never more than 8%) of the total molecular weight of the cross-linked product, indicating the proper assignment of polypeptides to products.

The results of a two-dimensional analysis of DTBP-treated, purified membranes are shown in Figure 9 (see Figure 8, lane 8, for the polypeptide profile after the first dimension). A major cross-linked product, having a molecular weight of 7600, represented a homodimer (spot 1) of the M_r 3700 chlorosome polypeptide. Spots 2, 3, 4, and 5 reflected the formation of trimers, tetramers, pentamers, and possibly hexamers of the B808–866 Bchl *a*–M_r 5300 antenna polypeptide. The M_r 5800 protein was cross-linked to a dimer and a pentamer (spots 6 and 7). In addition to dimers (spot 8) and trimers (spot 9) of the M_r 11 000 protein, heterodimer formation (spot 10) between the M_r 11 000 and 18 000 chlorosome proteins revealed the close proximity between these two molecules. Spot 11 was result of cross-linking the M_r 26 000 reaction center protein to a component with an approximate molecular weight of 6000. On the basis of this molecular weight, either the M_r 5800–chlorosome Bchl *a* or the M_r 5300–antenna Bchl *a* polypeptide might have been cross-linked to the reaction center protein. The origin of the protein which caused spot 12 is not

Table I: Assignment of Cross-Linked Products and Their Respective Individual Constituents after Two-Dimensional $NaDodSO_4$–PAGE

spot no.	M_r of cross-linked product	origin of cross-linked product	expected M_r of cross-linked product
1	7 600	dimer of M_r 3700 antenna *c* protein	7 400
2	15 500	trimer of M_r 5300 antenna B808–866 protein	15 900
3	19 500	tetramer of M_r 5300 antenna B808–866 protein	21 200
4	25 000	pentamer of M_r 5300 antenna B808–866 protein	26 500
5	30 000	hexamer(?) of M_r 5300 antenna B808–866 protein	31 800
6	18 700	trimer of M_r 5800 chlorosome protein	17 400
7	28 000	pentamer of M_r 5800 chlorosome protein	20 000
8	23 000	dimer of M_r 11 000 chlorosome protein	22 000
9	33 000	trimer of M_r 11 000 chlorosome protein	33 000
10	29 000	heterodimer of M_r 18 000 and 11 000 chlorosome proteins	29 000
11	30 000	heterodimer of M_r 26 000 RC protein and M_r ≈6000 polypeptide	
12	54 000	heterodimer(?) of M_r 3700 antenna Bchl *c* protein and M_r ≈50 000 protein?	

clear yet. The high yield of "3700 dimer" (spot 1) and the absence of any intermediate oligomeric form seem to indicate linkage to an M_r 50 000 protein rather than formation of a homooligomer (pentadecamer) of the M_r 3700 chlorosome polypeptide.

Discussion and Conclusions

The isolation of the antenna Bchl *a* complex allowed the conclusive identification of the polypeptide (M_r 5300) associated with pigment. The polypeptide profile of that fraction revealed a contaminating protein, representing less than 2% of the total protein. Beside the two characteristic near-infrared absorption bands at 866 and 808 nm, which were present in an always constant ratio of 866:808 = 1.6:1, the two maxima at 608 and 593 nm represented a rather uncommon Bchl *a* absorption feature in the Q_X region. The possible implications of these two maxima regarding Bchl *a* organization and its interaction with its environment are currently not clear. Studies on isolated B800–850 complexes from *Rps. capsulata* (Feick & Drews, 1978, 1979; Shiozawa et al., 1980, 1982) and *Rps. sphaeroides* (Sauer & Austin, 1978; Broglie et al., 1980; Cogdell et al., 1980) have shown that the 800- and 850-nm absorption bands reflect the absorbance of "different" Bchl molecules associated with two different polypeptides (Feick & Drews, 1979). Although the near-infrared absorption characteristics of the *Chloroflexus* B808–866 antenna complex appeared to be analogous to those of the B800–850 complex of *Rhodopseudomonas* species, it is unclear whether the 808- and 866-nm bands can be attributed to two different Bchl *a* molecules of to two transitions of excitonically interacting Bchl *a* molecules.

The experimental data emphasized the relationship of the M_r 3700 chlorosome polypeptide to the Bchl *c* in vivo absorbance at 740 nm. A fraction could be isolated which had the same ratio of 740-nm absorption to M_r 3700 polypeptide quantity (determined by densitometry) as observed in isolated chlorosomes. The M_r 3700 polypeptide made up 90% of the

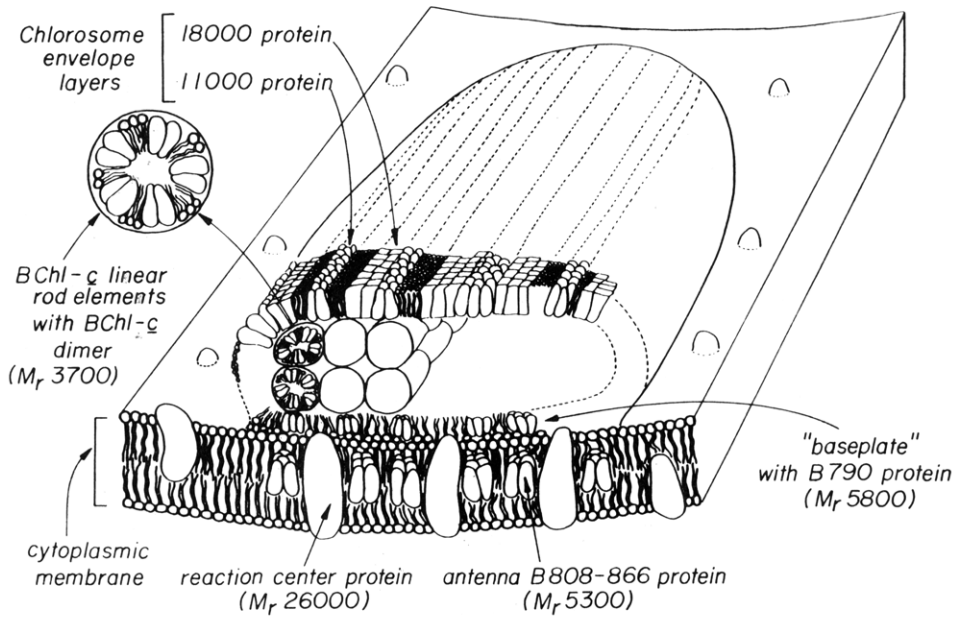

FIGURE 10: Proposed model of a chlorosome attached to the cytoplasmic membrane of *C. aurantiacus*.

total protein present in this fraction. When the relative intensity of the 740-nm band was decreased by proteolytic treatment, a parallel loss of the M_r 3700 polypeptide was also observed.

As described under Results, we attempted to assess the molar Bchl *c*–M_r 3700 polypeptide stoichiometry. Depending on the molecular weight of the protein moiety, we calculated a ratio of 5–8 Bchl *c* molecules per polypeptide of M_r 3700 or 5500, respectively. It is noteworthy that the 5 Bchl *c* molecules per M_r 3700 moiety should represent the very minimal number of Bchl *c* molecules. When the uncertain molecular weight (see below for reasons) is considered in this calculation, the real number is more likely to be 7 or 8. Therefore, the mass of Bchl *c* molecules at least equals or even exceeds the size of the protein portion.

In a comparison of the Bchl *a*:protein stoichiometry (2:1) in antenna complexes of purple bacteria [for reviews, see Drews & Oelze (1981) and Thornber & Markwell (1981)] to the number of Bchl *c* molecules per M_r 3700 polypeptide in *Chloroflexus* chlorosomes, the higher number might be indicative of a fundamental difference in the Bchl *c* arrangement. This is not to say that the protein constituent is superfluous. The sensitivity of the in vivo Bchl *c* absorbance against proteolytic digestion and the insensitivity against lipase treatment (results not shown) clearly emphasized the importance of the protein moiety, but Bchl *c* in chlorosomes might be arranged in a much more aggregated form compared to Bchl *a* in the purple bacteria antenna system. This notion is further prevailed by results of cross-linking experiments, where treatment with either DTBP or dithiobis(succinimidyl propionate) (DSP) (data not shown) caused only dimer formation of the M_r 3700 polypeptide in a yield of up to 60% (DTBP) and 90% (DSP). Thus, in chlorosomes, Bchl *c* and the M_r 3700 polypeptide most likely are organized as a dimeric complex comprised of 10–16 Bchl *c* molecules per pair of M_r 3700 proteins. Due to the large number and mass of Bchl *c* molecules relative to the protein portion, the pigment could well be organized in a more aggregated form within this dimeric entity. This situation might be analogous to different states of Bchl and chlorophyllid aggregates observed in colloidal, microcrystalline, and crystalline suspensions [for reviews, see Sauer (1975); Norris & Kato (1978); and Seeley (1977)]. Very recently, Smith et al. (1983) compared the line width of oxidized Bchl *c* in methylene chloride–methanol (8.4 G) to the line width of chlorosome-bound Bchl *c* (23 G; Betti et al., 1982). From the narrowing factor of the line width, they estimated that in vivo the unpaired electron is shared by 13 Bchl *c* molecules. This finding is in complete accord with our results of 10–16 Bchl *c* molecules per 2 M_r 3700 polypeptides.

Although our NaDodSO_4–PAGE system provides a very good resolution of low molecular weight proteins, and additionally shows in that range a linear relationship between relative mobility and log molecular weight, the size of the M_r 3700, 5300, and 5800 proteins appeared to be underestimated. By comparing the polypeptide profiles of *Chloroflexus* membranes to that of the isolated B800–850 complex from *Rps. sphaeroides* (a kind gift from Dr. Niedermann, Rutgers University), we estimated the two B800–850 polypeptides to have molecular weights of 3900 and 4300, compared to molecular weights of 5700 and 6400 according to amino acid analysis (Cogdell et al., 1980).

The validity of the results of our chemical cross-linking experiments was substantiated by the following experimental observations: (1) the relative high yield of cross-linked products, readily detected by Coomassie staining; (2) the formation of mostly protein homooligomers; and (3) the incubation temperature used during the cross-linking reaction. Since the treatment was performed at a "mesophilic" temperature, the fluidity of the "thermophilic" *Chloroflexus* membranes should have been greatly decreased. These were important prerequisites to assure that the observed cross-linking pattern was a result of static, long-term protein–protein interactions (rather than coincidental collision events) and hence truely reflects the protein organization in the membrane.

The nearest-neighbor relationship between the M_r 18 000 and 11 000 chlorosome proteins was substantiated by their hterodimer formation and was additionally indicated by two horizontal streaks in the second dimension. If two different protein species are present in an aggregated form and within close range, the number of possible cross-linked products between these two proteins can be so high that consequently a smearing streak might be observed. From the susceptibility to proteolytic cleavage and to ANDS photolabeling, it seemed logical to propose that the M_r 18 000 and 11 000 proteins are part of the chlorosome envelope layer. As described earlier, the M_r 3700 polypeptide could be arranged in the chlorosome

interior in a dimeric aggregate with 10–16 Bchl *c* molecules. This dimer might possibly be part of the 5-nm rod-shaped elements in the chlorosome interior as observed in electron micrographs of chlorosomes (Staehelin et al., 1980).

The cross-linking pattern of the B808–866 antenna protein (M_r 5300) indicated a quaternary substructure, which should be part of a larger aggregate such as a pentamer or hexamer (spot 5, much better visualized with a silver stain). However, it is currently not yet clear whether spot 5 can really be assigned to the formation of an M_r 5300 hexamer or whether it represented a reaction center (M_r 26 000)–M_r 5300 hexamer or whether it represented a reaction center (M_r 26 000)–M_r 5300 protein cross-link. The resistance of the M_r 5300 protein against proteolysis, particularly against proteinase K which is well-known for its aggressive cleavage of native and denatured proteins, was most remarkable. We attribute this resistance partially to the unaccessible location, and additionally, if this protein is indeed embedded in the hydrophobic membrane core, a lipid environment might provide some shielding effect against proteolytic cleavage.

A tentative arrangement of the proteins investigated in this study is presented in Figure 10. This model is an extension of the original proposed by Staehelin et al. (1978), which was based on their data from freeze–fracture electron microscopy. Additionally, it takes into consideration the results of fluorescence spectroscopy by Betti et al. (1982), who determined the transfer of excitation energy as follows: chlorosome antenna 740-nm Bchl *c* → base-plate 790-nm Bchl *a* → CM antenna B808–866 complex → reaction center.

Acknowledgments

We thank Prof. Robert Blankenship for his collaboration and for very valuable discussions. Once again, the help of Dr. Judith Shiozawa is acknowledged.

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