Reconstitution of a Functional Photosynthetic Receptor Complex with Isolated Subunits of Core Light-Harvesting Complex and Reaction Centers†

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ABSTRACT: The B820 subunit form of the core light-harvesting complex LHI, isolated from the photosynthetic bacterium *Rhodospirillum rubrum*, was combined in a reassociation assay with the reaction center (RC) isolated from the same or related bacteria. This reassociation produced a photoreceptor complex (PRC) which appeared, by absorption spectroscopy, circular dichroism measurements, and kinetic absorption spectroscopy measuring transient photochanges, as analogous to the PRC in the intact bacteria. Energy transfer between the LHI and reaction center progressed with almost 100% efficiency and indicated a cooperative pattern of transfer. Treatment of the RC with proteinase K resulted in peptide cleavages of all three polypeptides of the RC but did not alter the light-induced charge separation in the RC or prevent the reassociation of the LHI and modified RC. Energy transfer efficiency from LHI to RC still approached 100% but the cooperative behavior seen in reconstitutions with the intact RC was not observed. Initial experiments using interspecies reassociations (LHI from *Rhodobacter sphaeroides* and RC from *Rs. rubrum*) showed a low efficiency of energy transfer from LHI to RC. Possible association domains for the LHI−RC interaction based on considerations of the comparative amino acid sequences of the RC of each bacteria and the most feasible remaining residues in the proteinase K treated RC are considered.

The photosynthetic bacteria contain large amounts of bacteriochlorophyll organized by protein into antenna complexes, the function of which is to collect and funnel light energy into the reaction center (RC)[1] where this energy is trapped as a charge-separated state. Bacteria contain two principal kinds of these antenna or light-harvesting complexes. The core light-harvesting complex, referred to as LHI or B880, has been found in all photosynthetic bacteria, and is intimately associated with and is present in a constant ratio to the reaction center. Accessory LH complexes such as B800−850, referred to as LHII, are also present in many photosynthetic bacteria (*e.g., Rhodobacter sphaeroides, Rhodobacter capsulatus*), are physically distant from the RC, and occur in variable amounts depending on growth conditions. For a recent review of photosynthetic light-harvesting complexes, see Zuber (1993).

The core light-harvesting complex displays a marked similarity through many bacterial species, and a common structural subunit of LHI has been isolated from several photosynthetic bacteria (Miller et al., 1987; Chang et al., 1990b; Heller & Loach, 1990; Jirsakova, et al., 1992; Kerfeld et al., 1992; Meckenstock et al., 1992). These subunits can be reassociated to re-form *in vivo*-like LHI complexes (Miller et al., 1987) and can also be reversibly dissociated into individual polypeptides and BChl as well as reconstituted from separately isolated BChl and protein (Parkes-Loach et al., 1988; Loach et al., 1994).

The reaction center has been isolated from many bacteria and has been well characterized [for reviews, see Okamura et al. (1982), Parson (1987), and Rees et al. (1989)]. Amino acid sequences of the three polypeptides of the reaction center have been determined for several bacteria (Williams et al., 1983, 1984, 1986; Michel et al., 1986b; Belanger et al., 1988; Berard & Gingras, 1990; Youvan et al., 1984), and crystal structural studies have defined polypeptide and pigment locations within the RC for both *Rhodopseudomonas viridis* (Michel et al., 1986a; Deisenhofer & Michel, 1989, 1993) and *Rb. sphaeroides* (Allen & Feher, 1984; Allen et al., 1987a,b; Yeates et al., 1987; Chang et al., 1986, 1991).

Light energy absorbed by the LHII complex is very efficiently transferred to LHI (Duysens, 1951; Monger & Parson, 1977). Excitation energy absorbed in the LHI complex is then transferred with almost 100% efficiency to the RC, the site of the first stable charge separation (Vredenberg & Duysens, 1963; Loach & Sekura, 1968). Many mechanistic details of electron transfer from the special pair bacteriochlorophyll to the secondary quinone have been established [for reviews of electron and proton transfer in reaction centers, see Kirmaier and Holten (1987), Friesner and Won (1989), and Okamura and Feher (1992)].

The nature of the interaction between the RC and LHI (and LHII) is less well-documented than are the characteristics of the individual components, however, but it is likely that all photosynthetic bacteria contain a distinct supra-

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[1] Abbreviations: RC, reaction center or reaction centers; LH, light-harvesting complex; LHI, core light-harvesting complex; LHII, accessory light-harvesting complex; BChl, bacteriochlorophyll; PRC, photoreceptor complex consisting of RC and LHI; OG, *n*-octyl β-D-glucopyranoside; B820, subunit form of the core light-harvesting complex isolated from *Rs. rubrum*; B825, subunit form of the core light-harvesting complex isolated from *Rb. sphaeroides*; w.t., wild-type; LM fraction, reaction center preparation containing the L- and M-polypeptides but lacking the H-polypeptide; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; LDAO, *N,N*-dimethyldodecylamine *N*-oxide; [RC]/[LH], concentration of reaction centers divided by the concentration of light-harvesting complexes assuming 24 BChl per LHI; TX-100, Triton X-100; SDS−PAGE, polyacrylamide gel electrophoresis using the detergent sodium dodecyl sulfate.

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molecular organization, a photoreceptor complex (PRC), consisting of the RC and tightly coupled LHI [for a review of supramolecular membrane protein assemblies, see Joliot et al. (1993)]. The existence, in a single operon, of the structural genes coding for the L- and M-polypeptides of the RC as well as the α- and β-polypeptides of LHI (Youvan et al., 1984; Kiley et al., 1987) also indicates the importance of orchestrating the biosynthesis of RC and LHI. The demonstration of a strong association between LHI and RC was confirmed through the biochemical isolation of a PRC (Loach et al., 1970a,b) in *Rhodospirillum rubrum* and *Rb. sphaeroides*. Detergent fractionation and chemical cross-linking studies indicate the same strong associations in *Rb. capsulatus* (Peters et al., 1983).

On a larger scale, PRC have been shown to interact with each other to form a large mosaic where light energy absorbed anywhere in the complex may be shared and find its way to 1 of 50 or more available RC (Bakker et al., 1983; Vos et al., 1988). This cooperative state seems to be reversibly convertible to a noncooperative state, perhaps under physiological control (Zebrower & Loach, 1981, 1982). The PRC organization may be further extended by evidence which implies that the RC may be organized as dimers in the *in vivo* state (Loach, 1976; Joliot et al., 1990).

In this paper, we describe the results of reassociating the LHI structural subunit of *Rs. rubrum* with its RC to re-form the PRC. The integrity of the reassociation was determined by absorption spectra, circular dichroism measurements, and the measurement of the quantum yield for energy transfer from the antenna to the RC. Probes of the nature of the interaction have been initiated by proteolytic modification of the RC. Initial experiments testing conditions of inter-species reassociations have also been performed.

MATERIALS AND METHODS

Materials. Bacteriochlorophyll (BChl), *n*-octyl β-D-glucopyranoside (OG), Sephadex G-100, Sephadex LH-60, proteinase K (type XI-A) attached to 4% cross-linked beaded agarose, diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol were obtained from Sigma Chemical Co. Benzene was high-purity liquid chromatography grade solvent which was purchased from Aldrich. *Rs. rubrum* strains S.1 and G-9 were grown and chromatophores prepared as previously described (Miller et al., 1987) except that chromatophores used for photochemical measurements were not washed with deionized water, but stored at high concentration in potassium phosphate buffer at 4 °C until used.

Isolation of Rs. rubrum LHI Subunits (B820) and Column Reaction Centers. B820 was isolated as described in Miller et al. (1987). As a slight modification of that procedure, these LH-60 columns used for subunit (and in some cases, RC) isolation were run with OG concentrations not exceeding 0.75% w/v. Strict adherence to use of fresh materials was followed; i.e., bacteria were harvested in log phase, and chromatophores were prepared and washed, followed by lyophilization. After 2 weeks storage at 0 °C, the chromatophores were extracted with benzene and titrated with OG, and the isolated subunit was separated on the Sephadex G-100 column from reaction centers and free BChl within 30 h. Chromatophores obtained from carotenoidless mutants were lyophilized and immediately titrated with OG to form B820. Reaction centers preceded elution of B820 on the Sephadex G-100 column and were dialyzed against deionized water for 4 h in the dark at room temperature before being stored. All column fractions were stored dark at 4 °C until use.

Preparation of PUC705-BA LHI Subunits (B825) and Column Reaction Centers. B825 and reaction centers from the *Rb. sphaeroides* LHII$^-$ mutant PUC705-BA were prepared according to the procedure of Chang et al. (1990b), with two exceptions. The amount of the detergent OG used in the buffer for the G-100 column separation was 0.85% w/v, and the titration was performed on 60 mg of benzene-extracted, freshly prepared chromatophores from PUC705-BA and required a final concentration of 2.6% OG to convert all of the LHI to B825 for application to the G-100 column. The column RC were collected, dialyzed 4 h at room temperature in the dark, and stored at 4 °C until use.

Preparation of Rs. rubrum Reaction Centers. Reaction centers from G-9 were prepared by the procedure of Vadeboncoeur et al. (1979). The reaction centers were dialyzed to remove salt and resuspended in 10 mM Tris buffer, pH 8, without additional detergent before being stored cold and dark until use. *Rs. rubrum* w.t. reaction centers were also prepared by the Vadeboncoeur et al. (1979) procedure for wild-type cells.

Removal of H-Polypeptide from RC. The H-polypeptide was removed from the RC by the lithium perchlorate/cholate procedure of Debus et al. (1985). This procedure was developed for removal of H from preparations of RC from R-26 but was also successful in this study with G-9 reaction centers although somewhat lower yields were obtained. The procedure was used unmodified, and the resulting LM preparation was usually utilized immediately. Longer storage times were at liquid nitrogen temperatures. The purity of the LM fraction was confirmed by SDS−PAGE conducted on a Pharmacia PhastSystem using PhastGel 8−25 gels (8−25% acrylamide gradient with 2% cross-linking). The gels were stained with PhastGel Blue R Coomassie stain and destained with 30% methanol/10% acetic acid in deionized water.

Proteinase K Digestion of the RC. The nonspecific protease, proteinase K, was used to digest the RC polypeptides, especially the H-polypeptide as reported by Wiemken (1984). Proteinase K attached to beaded agarose having 2.5 units (54 mg) of activity was washed with deionized water and then twice with buffer containing 0.1% w/v cholate, 10 mM Tris, and 1 mM EDTA at pH 8. After the initial washing, the beads were used without further preparation. When w.t. RC, 2 mL $Abs_{800} = 2.0$, were treated with the protease, L- and M-polypeptides were also cleaved in addition to the H-polypeptide. Most incubations were conducted with the same amount of RC (about 0.2 mg of RC protein) and 0.25−0.6 unit of activity of proteinase K for incubation times varying from 5 to 60 minutes at room temperature in the dark. The protease reaction was stopped by a 10 min centrifugation in a tabletop centrifuge at room temperature. The RC in the supernatant was removed from the beaded proteinase K and used immediately for assay.

Vesicle Preparation. A mixture of 2:1:1 phosphatidylethanolamine/phosphatidylglycerol/diphosphatidylglycerol was chosen for the lipid composition of vesicles prepared because these lipids are the major components in the membranes of chromatophores of *Rs. rubrum* and occur in the ratio given

(Picorel et al., 1983). The lipids were dissolved in chloroform, and the solvent was removed by rotoevaporation at 37 °C followed by high-vacuum drying on a Schlenk line for 45 min. Potassium phosphate buffer, 0.05 M, pH 7.5, was added to make the final lipid concentration 2 mg/mL. The round-bottom flask was stoppered, and the lipids were hydrated for 45 min at 35−37 °C in a water bath. Sonication of the sample was carried out with a Branson Model 200 probe sonicator under nitrogen flow for 10 min on 50% duty cycle in a 3 dram vial, and a clear solution was produced. The vesicles were centrifuged at 105000*g* for 30 min to monitor an appropriate size distribution of the vesicles. No visible lipid but only black, metallic flakes from the probe of the sonicator were found in the pellet. These vesicles were stored at 4 °C under nitrogen until used.

Reassociation Procedure. Isolated B820 which was stored at 4 °C was mixed and warmed to room temperature until the maximum spectral shift to 820 had occurred. Often additional octyl glucoside, usually enough to increase its concentration by 0.05% to a final OG concentration of 0.80%, was added to ensure complete dissociation of LHI to the subunit (B820) state. Vesicles were added to B820 in an amount designed to result in a 2:1 lipid:bacteriochlorophyll (w/w) ratio, which approximates the *in vivo* ratio. This was usually achieved by adding 12 μL of vesicles to 1.3 mL of B820, $Abs_{820} = 1.0$. Reaction centers were added in various amounts to this B820 and lipid to produce initial RC:LHI complex ratios of 0.1−5.0 (0.1−5 RC combined with 1 LHI complex assumed to consist of 24 BChl and 12 α,β pairs). These mixed samples were then chilled 20−96 h at 4 °C until visible settling of the samples occurred. The colorless supernatant above the settling residue was carefully removed, and an equivalent amount of 0.05 M potassium phosphate buffer, pH 7.5, without OG was added. This removal of half or more of the starting volume ensured stabilization of the LHI state by dropping the OG concentration to 0.4% or less, while maintaining the original sample volume. The sample could then be warmed to room temperature and its spectral properties and quantum yield measured. Those samples which would not aggregate and settle were diluted 1:1 with cold buffer. The LHI was stabilized in this fashion, but its concentration was lower than that obtained by the method which did not introduce a dilution.

Determination of Reaction Center and LHI Concentrations. Because of variations in solubilization and degradation behavior of RC material at different concentrations, the RC:LH ratios were not always those expected based on the ratios of mixed components. Therefore, the actual [RC]:[LH] ratios in the resulting complexes were determined experimentally. RC concentrations were determined by CD. The characteristic ellipticity at 790 and 804 nm of the reassociated system was measured and compared to the size of the CD bands at the same wavelengths in LDAO-isolated G-9 RC. The absolute size of this peak and trough CD change was shown to be directly proportional to the absorbance measured at 800 nm. In our measurements, freshly prepared LDAO-isolated G-9 RC usually exhibited 49−52 $mdeg/OD_{800} = 1$. From this comparison, an absorbance at 800 could be calculated, and using an extinction coefficient of 288 mM^{-1} cm^{-1} at 800 nm (Straley et al., 1973), the molar concentration of RC could be determined even in reassociated LHI plus RC samples where the RC absorbance is obscured by absorbance due to BChl in LHI. A small correction for the overlap of the LHI CD, usually near 873 nm, into the 804 CD of the RC was made before RC concentration was calculated. This amount was defined as 10% of the positive lobe of the LHI CD and was added to the 804 CD of the *Rs. rubrum* RC. A similar correction was made for the *Rb. sphaeroides* RC, but in this instance, the quantity was subtracted from the 804 lobe to account for the opposite orientation of the *Rb. sphaeroides* LHI CD signal.

LHI concentrations were determined directly from the absorbance at 873 nm using an extinction coefficient per bacteriochlorophyll of 120 mM^{-1} cm^{-1} (Chang et al., 1990a). The *in vivo* ratio of 24 LHI BChl/RC in *Rs. rubrum* (Loach, 1976; Borisov, 1990) was also applied in order to determine the presumed molarity of the LHI complex.

Absorbance and Circular Dichroism Spectra. Absorbance spectra were recorded with a Shimadzu UV-160 spectrophotometer interfaced to an Apple IIe computer, and the data were stored on disk. For wavelengths between 350 and 1000 nm, opal glass was used to minimize the effects of scattered light. CD spectra were recorded with a Jasco J500C spectropolarimeter and the data manipulated using a Leading Edge microcomputer with the Jasco IF500 interface and software program. Hamamatsu R316 and R376 photomultiplier tubes were used for measurements in the spectral ranges of 700−1000 and 200−700 nm, respectively.

Absorbance Changes and Quantum Yield Measurements. Both measurements were made using a custom-built spectrophotometer (Loach & Loyd, 1966), with modifications as described by Woodle et al. (1984). For PRC quantum yield measurements, exciting light was restricted to the Q_y band of LH antenna at either 880 nm or 890 nm by use of narrow-band-pass filters (Baird-Atomic, B-9, filters with maximum transmission at 881.0 and 886.5 nm). *Rs. rubrum* chromatophores were used as the actinometer for quantum yield measurements, assuming $\Phi = 1.0$ (Loach & Sekura, 1968). Absorbances of samples at their long-wavelength maxima were adjusted to 0.50 ± 0.03. To take into account small differences in the location of the long-wavelength absorbance bands of reassociated samples (876 nm) relative to chromatophores (881 nm), a correction was made for the quantity of exciting light absorbed. The transmittance spectrum of the narrow-band-pass filter was superimposed on the spectrum of the sample being measured. The integrated area of the absorbance band of the measured sample that was within that transmittance window was compared to the integrated area of the absorbance band of the corresponding *Rs. rubrum* chromatophore control that fell within the same window. The difference in integrated areas was taken to be the difference in absorbed light of the control chromatophore sample compared with the reassociated sample. This correction, which rectified differences in absorbance as well as differences in location of the long-wavelength absorbance maxima, varied between values of 0 and 20% for the reassociated samples, with the most common correction being 9%. Use of G-9 chromatophores for the control system eliminated the need for a wavelength correction since the reassociated PRC and the chromatophores had the same long-wavelength absorbance maxima. However, quantitative measurements of the quantum yield have only been determined with wild-type whole cells and chromatophores and not specifically with G-9. For these experiments, results with either the G-9 or the wild-type reference systems were in close agreement.

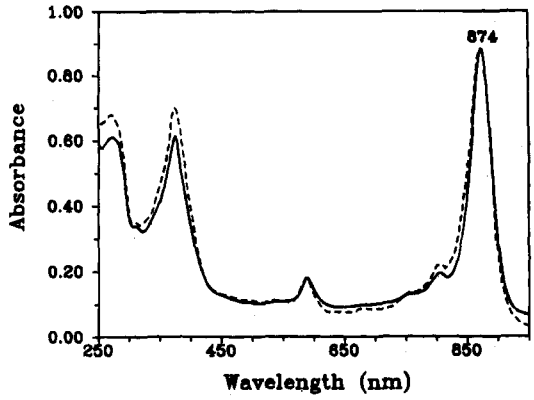

FIGURE 1: Absorption spectra of *Rs. rubrum* G-9 chromatophores freshly diluted into deionized water (—) and PRC reassociated from B820 subunits and reaction centers prepared from wild-type *Rs. rubrum* (- - -). The spectra were normalized at the absorbance maximum, which for both samples was 874 nm. The quantum yield of the reassociated PRC was 0.92, and the [RC]/[LH] was 0.53.

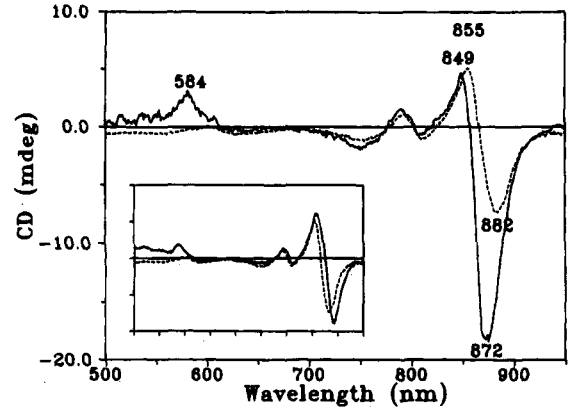

FIGURE 2: CD of *Rs. rubrum* G-9 (- - -) chromatophores and PRC (—) prepared from G-9 LDAO isolated RC and w.t. B820. The chromatophores were diluted into 0.05 M potassium phosphate buffer, pH 7.5, and 0.01 mM phenazine methosulfate and 1 mM sodium ascorbate were added. The [RC]:[LH] ratio for the reassociated sample was 0.39, and Φ was 0.58. The chromatophore traces were a composite of nine scans in the near-IR spectral region and four scans in the visible region. The reassociated PRC was a single scan in the 950−500 nm range. The traces were normalized to the same RC concentration as expressed by the CD (see Materials and Methods). The inset compares G-9 chromatophores (- - -) to wild-type chromatophores (—) measured under exactly the same conditions.

Exciting light was provided by pulses from a Sunpak 600 photoflash. The intensity was reduced until absorbance changes of chromatophores were 10−25% of saturation values. Absorbance changes of reassociated samples were measured at 605 nm and compared to those of chromatophores for the same incident light and integrated absorbance area.

Relative quantum yields of G-9 reaction centers were measured to ensure that the RC were fully active. First, the sample was excited by light filtered through a Baird-Atomic, B-9 narrow-band-pass filter with maximum transmission at 800 nm. Then, the resulting absorbance changes at 605 nm were measured and compared to LDAO-prepared R-26 reaction centers of the same concentration which were assumed to have a quantum yield of 1.0 (Bolton et al., 1969). Each reaction center preparation was characterized by the extent of photochanges, quantum yield, and CD, and all reaction center preparations used were fully active by those three criteria.

RESULTS

Absorption Spectra. Reassociation of B820, the subunit form of LHI, and RC from *Rs. rubrum* resulted in a reassociated PRC with an absorption spectrum which was nearly identical to that of G-9 chromatophores (see Figure 1). The absorbance maxima of the Q_y band of the reassociated LHI shifts red from 820 to 874 nm, the location of λ_{max} in benzene-extracted w.t. chromatophores and in the mutant carotenoidless G-9 chromatophores. The 800 and 760 nm absorbances of reaction center components retain their characteristic locations. However, it should be noted that the red-shift of the antenna complex does not require a close interaction of B873 with either lipids or RC since the characteristic 873 nm absorbance can be obtained by reassociation of B820 without the presence of either component (Parkes-Loach et al., 1988; Chang et al., 1990a).

CD Measurements. Many features of the CD spectrum of a reassociated PRC resemble those of chromatophores (Figure 2). The CD changes attributable to the RC between 700 and 830 nm are also the same whether the RC is alone or combined in a PRC. There are no band shifts of the 795−810 nm maxima and minima, and the ratio of the two bands rule out random oxidation of the trap (controls using 1×10^{-3} M ascorbate with 1×10^{-5} M phenazine methosulfate showed no difference) resulting from the lack of secondary acceptors or from the methods used for the combination of the RC and LHI. In the near-infrared region of the spectra, both the reassociated PRC and the chromatophores exhibit the same qualitative features attributable mainly to LHI: a maximum of lesser ellipticity located about 850 nm and a minimum of moderately strong ellipticity located near the λ_{max} of the long-wavelength spectral absorbance band.

However, in reassociated PRC, the location of the LHI CD band for the Q_y transition is approximately the same as the long-wavelength absorbance maximum, whereas the location of the LHI CD in both wild-type and G-9 chromatophores is red-shifted about 10 nm from their respective absorption spectrum maxima. In addition, the Q_x band more closely resembles that of the wild-type band although the reassociated system does not contain carotenoid (Figure 2, inset). The differences in location of the CD bands of the Q_x and Q_y transitions in wild-type and G-9 chromatophores can probably be ascribed to the interactions of the carotenoid with the bacteriochlorophyll and, or, protein.

The interval between the long-wavelength minima and maxima of the reassociated PRC CD is noticeably narrower than that of w.t. chromatophores; average 23 nm for PRC compared to 33 nm for w.t. chromatophores. Interestingly, the corresponding bands of G-9 chromatophores, 26 nm, are also narrower than the wild type. Thus, the broadening of the CD band locations observed in wild-type chromatophores may be similarly linked to the presence of carotenoids as is the spectral absorbance shift that occurs in wild-type chromatophores.

CD spectra of the reassociated LHI show substantial variation in molar ellipticity depending on the initial concentration of B820, the presence or absence of lipid, the method used for removal of OG, or the temperature (Parkes-Loach et al., 1988; Chang et al., 1990a). Increasing

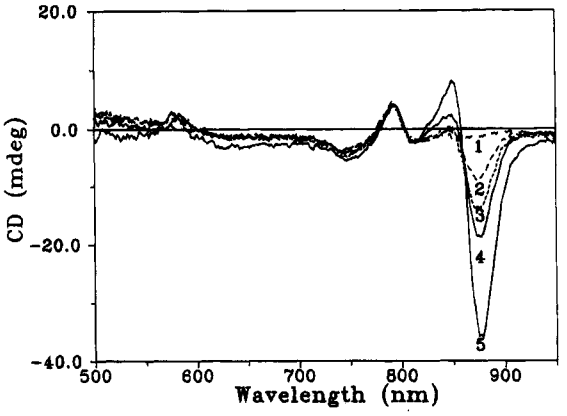

FIGURE 3: CD of PRC prepared with w.t. column RC and w.t. B820 in the presence of 2:1 lipid/BChl. The RC concentration was held constant at 4.7×10^{-4} mM, and the [LH] varied from 2.2×10^{-4} to 4.9×10^{-4} mM. 1, [RC]/[LH] = 2.0; 2, [RC]/[LH] = 1.3; 3, [RC]/[LH] = 1.03; 4, [RC]/[LH] = 0.96; 5, [RC]/[LH] = 0.60.

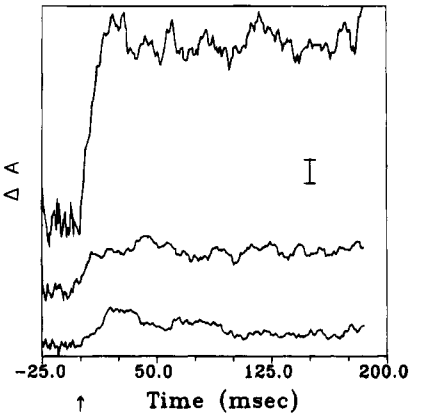

FIGURE 4: Absorbance changes in wild-type *Rs. rubrum* chromatophores, $OD_{880} = 0.43$. A small amount of ferrocyanide was added to ensure complete trap reduction. The top trace shows the fully light-saturated changes for the chromatophores while the middle trace shows chromatophores at a lower light intensity which represents 22% of the saturation value. This lower light intensity at 880 nm was used for the quantum yield measurements. The interval between light events was 32 s for chromatophores to allow the decay to complete. The bottom trace shows the low-intensity absorbance change for a reassociated PRC sample prepared from w.t. B820 and w.t. RC combined in a [RC]:[LH] ratio of 0.29. The quantum yield of this PRC sample was 0.92. The marker on the figure represents an absorbance change of 0.0002 at 605 nm. The chromatophore full saturation signal represents 32 light events at a display of 256 while the low-light and PRC are 64 events at a display of 512. All traces were normalized to 64 events at display 512.

quantities of lipid (about 20 lipid/BChl or 500 lipid/LHI complex) result in an inverted or greatly diminished CD signal (data not shown). When the RC is also present with LHI, further variability in the resulting CD spectrum of the antenna complex can be observed as in Figure 3. At low [RC]:[LH] ratios, 0.05−0.5 (1 RC for 48−480 antenna BChl), the LHI CD spectrum seems essentially unperturbed by the presence of the RC (Figure 3, trace 5). However, at high [RC]:[LH] ratios such as exist *in vivo* of 0.8−2.0 (1 RC for 12−30 antenna BChl) the 849 nm peak and 875 nm trough diminish in size to the extent that they can disappear entirely (Figure 3, trace 1), similar to the phenomenon that occurs with lipid additions. The absorbance spectrum maximum location and extinction do not change in these samples even though the CD varies substantially. In contrast, variation in LHI concentration had no effect on the size or the location of the RC CD.

Quantum Yield. Because recovery of BChl in the reassociated B820 (B873) was quantitative, evaluation of the LHI content of reassociated systems could be determined either from the amount of B820 added to the reassociation mixture or from the subsequent absorbance at 873 nm using an extinction coefficient of 120 mM^{-1} cm^{-1} per BChl (Chang et al., 1990a) and assuming the *Rs. rubrum in vivo* ratio of 24 BChl/B873 complex (Loach, 1976; Borisov, 1990). However, a substantial variation in the amount of RC recovered in the reassociated PRC complex prevented use of the amount of added RC to indicate its final concentration. Several methods were evaluated for determining the final concentration of RC. The fact that the sum of the absolute values of the ellipticities at 790 and 804 nm in the CD spectrum of the RC was easily measurable and was found to be linearly proportional to the RC concentration as measured by the absorbance at 800 nm (data not shown) led to the use of this measurement as the quantitative indicator for the amount of RC present.

The efficiency of light utilization was determined by measuring the absorbance change at 605 nm caused by a low-intensity pulse of irradiation at 880 or 890 nm which is principally absorbed by the BChl of the LHI complex. The resulting absorbance change at 605 nm is characteristic of the oxidation of the primary electron donor in the RC and was compared with that of a sample of chromatophores having the same absorbance at 873 nm (see Figure 4). The quantum yield, Φ, for the chromatophore system has been previously determined to be 0.95 ± 0.05 (Loach & Sekura, 1968). For the purposes of this paper, a Φ of 1.0 was assigned to the chromatophore control system. In Figure 4, the decay kinetics are faster for the reassociated system than chromatophores due to the lack of the secondary quinones which were removed during RC isolation procedures. However, when the curves are extrapolated back to the time of the flash, the magnitude of the absorbance change for the reassociated PRC was nearly identical to that found in the chromatophore sample. A quantum efficiency of 0.96 was determined for this particular sample. Control experiments with RC only varying in concentration from 2.4×10^{-7} to 5.3×10^{-7} M without other additions showed absorbance changes at 605 nm of 5−15% of the size obtained with the same RC concentration when LHI was also present. Corrections for a background amount of RC were not made because, except in reassociations where the [RC]:[LH] ratios were very high, the absorbance at 880 nm from LHI was overwhelming relative to the absorbance of the RC. For example, in the 1:1 [RC]:[LH] ratio at 2.4×10^{-4} M, the RC would contribute an absorbance at 880 nm of approximately 0.025 while the LHI complex would contribute 0.63, again assuming the molecularity of 24 BChl in the LHI complex. The likelihood of a significant absorbance change at 605 nm due to direct excitation of the RC under these circumstances is minimal.

The inherent problem with these quantum yield measurements is not the accuracy of the measured absorbance changes; these vary by less than 10%. The basic inconsistencies of the measurements arise from the difficulties in assessing the [RC]/[LH]. Final RC concentrations were not strictly predictable because absorbance sometimes decreased

Table 1: PRC Reassociation[a]

sample	no.	[RC]:[LH] ratio	Φ	*E*
(A) *Rs. rubrum* w.t. or G-9 B820 + *Rs. rubrum* w.t. or G-9 RC	1	0.13	0.26	2.00
	4	0.28[b]	0.30 ± 0.08	1.07
	2	0.41[b]	0.53 ± 0.01	1.29
	4	0.48[b]	0.76 ± 0.23	1.58
	3	0.56 ± 0.03	0.70 ± 0.12	1.25
	3	0.75 ± 0.04	0.93 ± 0.35	1.24
	1	0.93	0.79	0.85
(B) *Rs. rubrum* w.t. or G-9 B820 + *Rs. rubrum* G-9 proteinase K-modified RC	2	0.38 ± 0.02	0.34 ± 0.04	0.89
	2	0.50 ± 0.03	0.44 ± 0.06	0.88
	1	0.59	0.66	1.12
	3	0.68 ± 0.05	0.62 ± 0.26	0.91
	2	0.82 ± 0.02	0.84 ± 0.11	1.02
	2	0.97 ± 0.02	0.83 ± 0.20	0.86
(C) *Rb. sphaeroides* w.t. or PUC705-BA B825 + *Rs. rubrum* G-9 RC	1	0.33	0.46	1.39
	2	0.58 ± 0.05	0.17 ± 0.17	0.29
	2	0.75 ± 0.04	0.21 ± 0.09	0.28
	2	0.88 ± 0.02	0.30 ± 0.05	0.34
	1	1.1	0.0	0.00
	2	1.25 ± 0.05	0.21 ± 0.08	0.17

[a] no. refers to the number of experiments conducted with the given [RC]:[LH] ratio. Φ refers to the quantum yield measured for the samples. *E* is the quantum yield divided by the [RC]:[LH] ratio. [b] All data points in this set had the same [RC]:[LH] ratio.

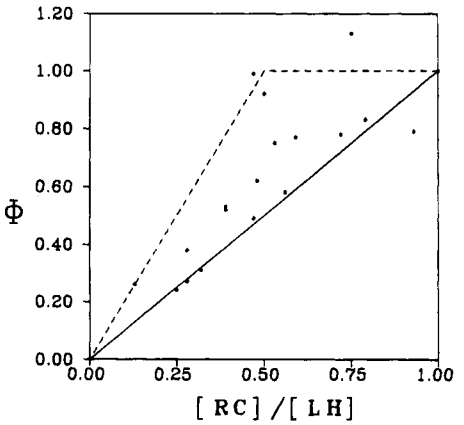

FIGURE 5: Quantum yield measurements of energy transfer from the BChl of LHI antenna to the RC of reassociated PRC formed from B820 and isolated RC. The 45° solid line represents the theoretical, noncooperative behavior, where a maximal quantum yield occurs at the *in vivo* ratio of 1 RC for every 1 LHI complex. The dashed line indicates the theoretical curve where 2 LHI transfer energy to a single RC. The wavelength of excitation for these measurements was 880 nm.

through degradation. The extent to which LHI self-associates is also unknown and may affect interaction with RC.

A summary of the experimentally determined quantum yields obtained with the reassociated LHI and RC systems is given in Figure 5. The solid line represents the quantum yield expected if each RC was associated with 1, and only 1, LHI complex when the LHI complex is defined as containing 24 BChl. The dashed line indicates the theoretical data for a situation where two LHI complexes are linked to each RC. Most of the data points fall in the area between these two lines. Table 1A shows the number of experiments performed and the spread of the data points.

Controls using RC mixed with B820 subunits in 0.80% OG (conditions under which RC and LHI were dissociated) were excited using an 825 nm narrow-band-pass color filter and did not show absorbance changes at 605 nm greater than that expected for direct RC excitation. Therefore, the subunit state is not able to effectively transfer significant amounts of excitation energy to the RC when dispersed in 0.80% OG.

Reassociation of B820 with Modified RC. Initial experiments in which B820 was reassociated with RC isolated concomitantly on the LH-60 column produced varied results. One common feature of those systems which displayed low efficiency was the apparent lack of the intact H-polypeptide in the RC as determined by SDS−PAGE. The function of the H-polypeptide of the RC, although not known with certainty, has been connected to the organization and stability of the RC (Chory et al., 1984; Drews, 1985; Deisenhofer et al., 1985). It seemed possible then that the H-polypeptide might function in orchestrating the LH and RC interaction. This possibility was explored in two ways. First, the H-polypeptide was removed from isolated RC by the method of Debus et al. (1985). The second procedure used proteinase K, a nonspecific protease, to digest the RC. These modified RC, lacking an intact H-polypeptide, were then used in reassociation with B820 to re-form PRC.

RC modified by the removal of H by cholate disruption produced a resulting fraction which showed on SDS−PAGE only the remaining L and M bands (data not shown). The absorbance and CD spectra, the photoinduced absorbance changes, and the quantum yield of those photochanges were unchanged relative to RC containing the H-polypeptide. However, quantum yields determined for reassociated PRC using these RC containing only the L- and M-polypeptides were low (≤10−25% of the predicted values). It was found that this inactivity actually reflected the instability of the modified RC in 0.80% OG. RC prepared by the same procedure as used for B820 isolation, which after prolonged storage lacked the H-polypeptide, also exhibited, at times, extreme instability in the higher detergent concentration used for the reassociations. The relative stabilities of the various RC preparations used were as follows: *Rb. sphaeroides* R-26(LDAO prep) ≫ *Rs. rubrum* G-9(LDAO prep) ≫ *Rs. rubrum* w.t. or G-9 G-100 column RC(LMH) > *Rs. rubrum* w.t. RC(TX-100) > G-9 LM(cholate) >>> *Rs. rubrum* w.t. LM(cholate). Therefore, these experiments using LM generated by proteinase K activity were carried out without delay promptly after preparation of LM to maximize its stability. Proteinase K-produced LM could retain 96% of its activity for over an hour in 0.80% OG while cholate-produced LM

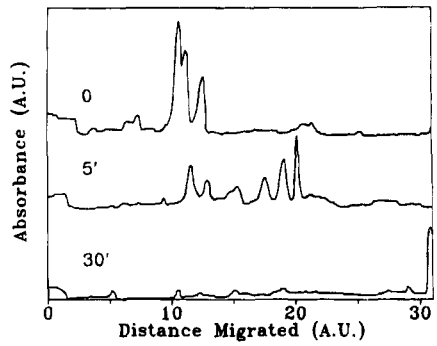

FIGURE 6: Laser scan traces of Phast-Gel SDS−PAGE of G-9 RC incubated for 0, 5, and 30 min with proteinase K. All traces represent the same quantity of protein loaded onto the gel. All samples show spectral integrity equal to the control G-9 RC unreacted with proteinase K. At 0 time, the H-polypeptide has moved the least while the L-polypeptide has moved the furthest. M is in the middle.

lost most of its activity in minutes. The experiments using LM generated by cholate treatment were disregarded because of the rapidity of the degradation of the LM in OG detergent when LH was added.

Use of proteinase K resulted in a rapid digestion of the H-polypeptide, followed by the M- and L-polypeptides, and showed the appearance of many additional bands of smaller molecular weight (Figure 6). The absorption and CD spectra of proteinase K-modified RC were identical to those of untreated RC even for the samples treated for up to 60 min. In addition, the PRC formed from the reassociation of the protease-treated RC and B820 gave equivalent absorption spectra compared to reassociated PRC formed from intact RC. Table 1B summarizes the results of the measurements of quantum yield in reassociated PRC formed from B820 and the protease-treated RC. Note that most of the E values are distributed around the value $E = 1$, indicating ideal energy transfer from one LHI complex to one RC.

Reassociations of Reconstituted B820 with RC. The fact that RC combine with LHI to provide a PRC that resembles spectrophotometrically and energetically an intact membrane preparation is an important result both for understanding the supramolecular complex *in vivo* and also for providing an effective method for elucidating structure−function relationships. Although SDS−PAGE and HPLC analyses of the protein components of B820 preparations indicated that only the two major polypeptides, α and β, were present, the possibility still existed that other comigrating components or a minor polypeptide might be present which assist in the association of the RC and LH. Because B820 can be reconstituted solely from separately isolated α- and β-polypeptides and BChl, it was of interest to determine whether reconstituted B820 would associate with the RC to form the PRC.

Therefore, α- and β-polypeptides of *Rs. rubrum* were separately isolated by organic solvent extraction, and their purity was verified on HPLC as in Parkes-Loach et al. (1988). These pure polypeptides were combined with BChl *a* to form reconstituted B820 which was subsequently separated from any unreacted bacteriochlorophyll or polypeptides by purification on a Sephadex G-100 column like that used for B820 preparation from LHI. As is the case with B820 prepared from LHI, the B820 eluted from the column followed by unbound protein and free BChl. This column-purified, reconstituted B820 was then combined with G-9 RC. Because it is very difficult to obtain large quantities of reconstituted, column-purified B820, the absorbance of the resulting reconstituted B820 was lower than was usually used in reassociation experiments. The amount of RC was also lowered to maintain the same ratio of the two components. The resulting PRC settled to the bottom of the tube on standing in the dark at 4 °C as is usually the case for reassociation experiments. An interesting feature of this reassociation was the formation of a PRC CD spectrum which closely resembled *in vivo Rs. rubrum* chromatophore CD (data not shown). LHI reconstitutions with the α- and β-polypeptides and BChl *a* have exhibited inverted CD bands in the near-infrared region unless *Rs. rubrum* BChl *a* with a geranylgeranyl tail was used to reconstitute B820 at very high protein concentrations (Parkes-Loach et al., 1990). In the experiment reported here, the BChl *a* contained a phytyl tail, as is typical with BChl *a* isolated from *Rb. sphaeroides*. Even so, when the PRC was re-formed with RC, a *Rs. rubrum in vivo* LHI CD was produced. The Φ of the resulting reconstituted PRC was 0.49 for a sample having an [RC]/[LH] = 0.68. This corresponds to an overall efficiency of 75% for energy transfer from BChl of LHI to RC (compare this result with E values reported for other PRC reassociations, Table 1). Thus, B820 containing only BChl and well-defined α- and β-polypeptides provides reassociation with the RC to form an energy-efficient PRC.

Hybrid Reassociations. Reassociations involving LHI from *Rb. sphaeroides* wild type and the PUC705-BA mutant have provided an insight into the effectiveness of the assay as a probe into the structural requirements for the specific association of the RC with LHI. B825 isolated from *Rb. sphaeroides* w.t. or PUC705-BA was combined with RC from G-9 *Rs. rubrum*, and the absorbance and CD spectra as well as the quantum yield of the resulting complexes were measured. Because the hybrid samples would not settle out of solution upon standing a reasonable length of time, these reassociations were conducted by a modification in which the mixed sample was diluted 2-fold instead of allowing the re-formed PRC to settle out of solution. Control experiments conducted with the *Rs. rubrum* system using this technique produced quantum yields indicating 100% efficiency of energy transfer of reassociated PRC. The dilution of the sample does not provide as high a concentrtion of the PRC as does the other technique, and in some instances, the resulting CD spectrum is affected by showing smaller and less red-shifted antenna CD. However, PRC formed using *Rb. sphaeroides* B825 and *Rs. rubrum* G-9 RC did produce absorbance and CD spectra that are similar to those of *Rb. sphaeroides* PUC705-BA chromatophores. The absorbance spectra of the reassociated PRC were not as red-shifted, 874 nm compared to 880 nm, as expected when comparing systems without and with carotenoid, but the CD spectrum was oriented similarly to the LHI of PUC705-BA chromatophores, the inverse of *Rs. rubrum* LHI (Figure 7). The RC component (*Rs. rubrum* RC) has the same location as usual when mixed with its own proteins, and the LH component (*Rb. sphaeroides* LHI) is blue-shifted from that of the PUC705-BA chromatophores, but the orientation of both components mirrors those of the control.

As the results in Table 1C indicate, the combination of the two systems did not result in a high efficiency of energy transfer from the antenna to the RC. Except for one

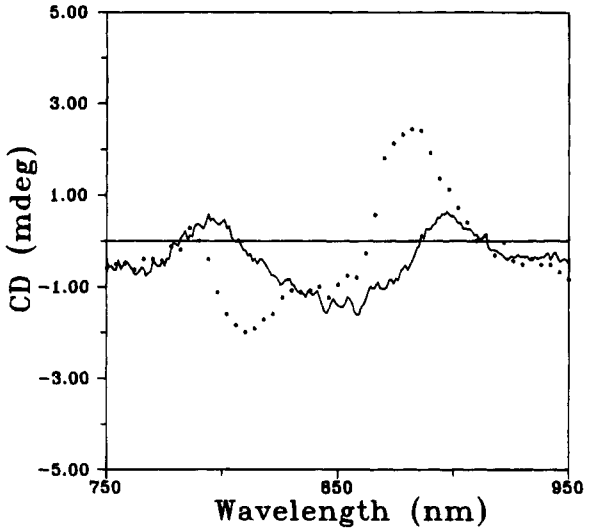

FIGURE 7: CD spectra of *Rb. sphaeroides* PUC705-BA chromatophores (—) and a hybrid reassociated PRC composed of B825 prepared from *Rb. sphaeroides* PUC705-BA and RC prepared from *Rs. rubrum* RC (•••). The PUC705-BA spectrum was two sweeps averaged and the reassociated PRC a single scan.

experiment, regardless of the ratio of [RC]/[LH], the energy transfer efficiency was only about 24%. This would seem to indicate that only one in four incident light quanta absorbed by the antenna found a RC.

DISCUSSION

The reassociated system described in this report demonstrates that the PRC can be assembled from LHI and RC components to reconstruct a system which appears spectroscopically and energetically like the PRC in the *in vivo* membrane. Reassembly of the RC and LHI does not change the characteristics of either component; the absorption and CD spectra are essentially additive. Energetically, the reassociated PRC is capable of utilizing light absorbed by the BChl of LHI and transferring the excited singlet state energy to the RC with near 100% efficiency and in some cases in a cooperative manner. Importantly, although this does not rule out the possibility of the involvement of other components *in vivo*, successful use of reconstituted B820 (purified α, β, and BChl *a*) with RC indicates that no other biochemical entity is required for PRC formation or for efficient energy transfer in *Rs. rubrum*.

The specificity of the reassociation of LHI and RC is emphasized most dramatically by the high quantum yield of energy transfer from LHI to RC. The LHI structure has been suggested to be as small as a dimer of the αβ·2BChl B820 subunit (Ghosh et al., 1988; Braun & Scherz, 1991; van Mourik et al., 1992) to as large as a hexamer or dodecamer of this basic subunit structure (Bachofen et al., 1987; Boonstra et al., 1993; Nunn et al., 1992). The results of the studies reported here require that a specific complex involving at least 24 LHI BChl/RC (as is the stoichiometry of *in vivo Rs. rubrum* PRC) is formed or the highly efficient energy transfer indicated by the experimental data (Figure 4) could not have been obtained. However, whether the size of the LHI formed is larger than that found *in vivo* is unknown.

Further support for the specificity of reassociation of LHI and RC emerged from the results with attempted hybrid reassociations between B825 isolated from *Rb. sphaeroides* and RC isolated from *Rs. rubrum*. It seems apparent that sites of recognition must exist between homologous LHI and RC that facilitate the formation of an energy-efficient PRC; the hybrid reassociation components lack specific binding elements or have steric problems in associating properly which result in a low quantum yield. Here, too, the size of the LHI complex might affect results.

Digestion of the RC by proteinase K results in a number of cleavages of the L-, M-, and H-polypeptides and generated three distinct observations. First, the digestion of the H-polypeptide does not hinder the association of LH and RC to form an energy-efficient PRC. The H-polypeptide is thoroughly digested with no remaining recognizable protein sequences (Wiemken & Bachofen, 1982, 1984). Although not visible on SDS−PAGE, the single membrane span of the polypeptide may still exist *in situ* and be responsible for the superior stability of the protease-treated LM relative to the LM produced by the perchlorate/cholate-disruption method of Debus et al. (1985). Nevertheless, the intact H-polypeptide, which has been suggested to play a role in PRC organization (Chory et al., 1984; Drews, 1985), is apparently unnecessary for effective interaction between the remaining two polypeptides of the RC and LH in this *in vitro* reassociation.

Second, the changes in the L- and M-polypeptides as measured by SDS−PAGE indicate that at least some of the connecting loops which join the membrane-spanning segments of the polypeptide were cleaved by the protease. It is to be expected that proteinase K cleavages might occur on both sides of a RC in detergent solution, but there are no documented cleavages from the periplasmic side of the membrane because, except for the work of Wiemken and Bachofen (1984), all of the enzyme cleavages were carried out on chromatophores. However, earlier studies showed three additional cleavages on the cytoplasmic side of the RC beyond the loss of the H-polypeptide when proteinase K was used (see Figure 8). There are usually 15−27 amino acids cleaved from the N-terminus of the L-polypeptide, 46−48 cleaved from the N-terminus of the M-polypeptide, and often an additional cleavage that allows isolation of the M−E membrane span (Brunisholz et al., 1984; with *Rs. rubrum*). As was the case with the removal of the H-polypeptide, the absorbance, the CD spectra, and the relative quantum yield of the enzyme-modified RC were identical to the untreated RC. Since association of this more radically modified RC with LHI produced a reassociated PRC in which energy could be transferred also with 100% efficiency, significant parts of the N-termini of the L- and M-polypeptides appear to be uninvolved in binding for the RC/LH reassociation.

The third observation is that although the quantum yield for the reassociation of proteinase K-cleaved RC and LHI remains ideally efficient, no evidence was found for the LHI cooperativity that was often observed in the reassociated PRC formed with intact RC. A very extensive cooperative state for energy transfer has been demonstrated in whole cells (Clayton, 1966; Monger & Parson, 1977; Bakker et al., 1983) but this cooperativity was lost in preparing membrane vesicles (chromatophores) or by exposing the whole cells to certain other treatments including darkness or loss of Mg^{2+} (Zebrower & Loach, 1981). The simplest explanation might be that the portions of the RC affected by the proteinase K cleavages are the portions involved in cooperative behavior (the H-polypeptide or the N-terminal sequences of L and

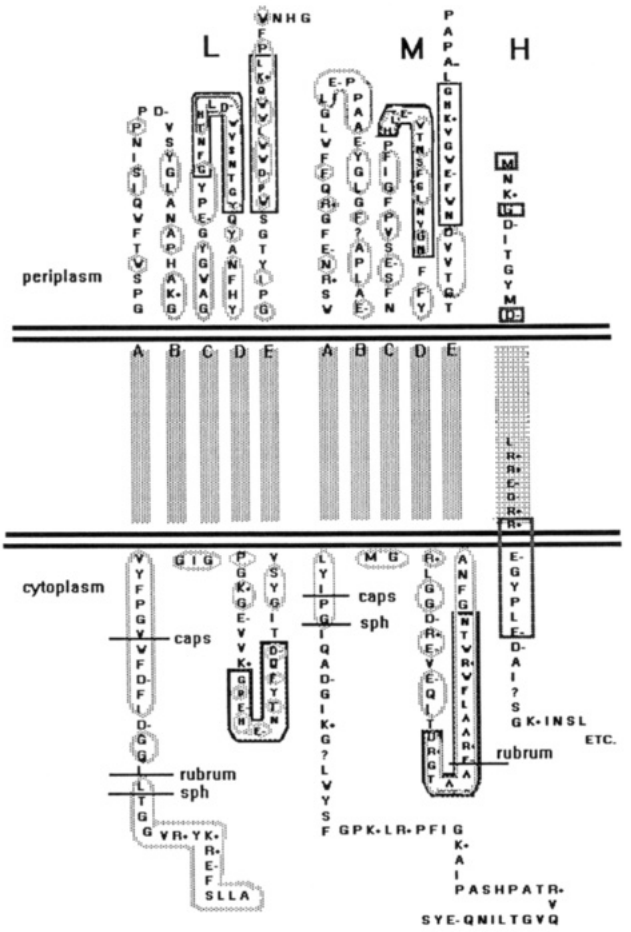

FIGURE 8: Representation of the RC sequences of *Rs. rubrum* as compared to *Rb. sphaeroides* as reported by Belanger et al. (1988) and Williams et al. (1986). The basic RC structure illustrated here is that of *Rs. rubrum*. Amino acids that are circled in the L- and M-polypeptides in light gray are the conserved amino acids in the two bacterial sequences. Those which are in a black box are presumed helical segments. In the portion of the H-polypeptide depicted, the conserved amino acids are boxed in gray. For the L- and M-polypeptides, the N-term is on the cytoplasmic side while for the H-polypeptide the N-term is on the periplasmic side. Black lines on the cytoplasmic side of the diagram labeled with abbreviated designations indicate the documented proteinase K cleavages for *Rb. capsulatus* (caps), *Rs. rubrum* (rubrum), and *Rb. sphaeroides* (sph). L-polypeptide cleavages reported are 15 aa (Tadros et al., 1988) with *Rb. sphaeroides*, 16 aa (Wiemken & Bachofen, 1984) with *Rs. rubrum* G-9, 16 aa (Brunisholz et al., 1984) with *Rs. rubrum* G-9, 26 aa (Tadros et al., 1987) with *Rb. capsulatus*, and 27 aa (Tadros et al., 1986) with *Rb. capsulatus* Ala+. M-polypeptide cleavages are 46 aa (Tadros et al., 1988) with *Rb. sphaeroides* and 48 aa (Tadros et al., 1987) with *Rb. capsulatus*. Note the letters labeling the α-helical membrane spans in the L- and M-polypeptides.

M). Other possibilities include the suggestion that the RC may have two binding sites for LHI, perhaps reflecting the 2-fold symmetry that exists in RC (Michel et al., 1986). Protease K treatment of the RC then might result in the inability or decreased ability of the RC to interact with one of these sites or a somewhat decreased affinity at both sites. Alternatively, evidence also exists suggesting that an associated, possibly dimeric, state of RC exists *in vivo* (Joliot et al., 1990). Earlier experimental results from this laboratory were best interpreted by assuming the close interaction of two RC (Loach, 1976). It is unclear how dimeric RC might interact with multiple LHI complexes, but the data might indicate the loss, through protease activity, of the site of association for dimeric RC and thus the disruption of multiple LH complexes.

Because RC with proteinase K cleavages can still reassociate with LHI and allow complete energy transfer while reassociating *Rs. rubrum* RC with *Rb. sphaeroides* LHI subunits shows little energy transfer, it seems most worthwhile to compare relative amino acid sequences between the two bacteria in those domains most likely to be involved in association. On the cytoplasmic side of membrane, there are few heterologies adjacent to the membrane surface except in the L-DE loop (which is probably helical and in the interior of the RC volume involved in the four-helix DE bundle) and the M N-terminus sequence (which is probably cleaved in the proteinase K-cleaved systems). Because representations of the RC of *Rb. sphaeroides* from crystal studies (Yeates et al., 1987) show that on the periplasmic side the connecting loops of the L- and M-polypeptides are flattened against the hydrophobic membrane core (Allen et al., 1987b), areas of interaction between RC and LH, if they exist on this side of the membrane, are probably limited to areas around the periphery of the RC, the L-AB and M-AB loops and the L and M C-termini. In fact (see Figure 8), homology between the sequences of *Rs. rubrum* and *Rb. sphaeroides* is least in these very regions which are the most accessible on the periphery of the RC. The C-terminal regions of the α- and β-polypeptides of LHI also show little homology on the periplasmic side of the membrane, and if this region is important for binding to the RC, they may be responsible for the lack of complex formation with LHI from *Rb. sphaeroides* and with RC from *Rs. rubrum*.

Crystallization of the RC has opened new avenues of exploring the molecular structure of the RC, and the same opportunities may soon exist for LHI (Nunn et al., 1992). Such structural information, combined with the use of the methodology of reconstitution, could be used to explore a more complicated level of structure−function relationships. For example, the binding site for BChl and recognition sites for reassembly of B820 into a functional LHI are being probed by (a) using α- and β-polypeptides from different bacterial strains, (b) using chemically modified polypeptides, (c) using site-directed mutants containing specifically modified polypeptides, and (d) synthesizing polypeptides with more subtle modification of only an atom or a small moiety of interest (Loach & Parkes-Loach, 1994). LHI complexes reconstituted from these modified polypeptides can then be associated with the RC to assess the interaction between these complexes. Further questions regarding the sites of recognition on the RC might be explored by using mutant bacterial strains or different interspecies RC or RC which have been modified by site-directed mutagenesis. The existence of methodology that can investigate not only LHI structure but also the interaction of the LHI and RC complexes should be quite useful in studying supramolecular interactions.

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