Highly Purified Photosynthetic Reaction Center (PscA/Cytochrome c_{551})$_2$ Complex of the Green Sulfur Bacterium *Chlorobium limicola*†

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ABSTRACT: The photosynthetic reaction center (RC) complex that forms a homodimer of core and cytochrome *c* subunits was isolated from *Chlorobium limicola* f. *thiosulfatophilum*, strain Larsen. The complex showed only two subunit bands at 68 (PscA core) and 21 kDa (cytochrome c_{551}) on SDS−PAGE analysis, indicating the complete deletion of the light-harvesting bacteriochlorophyll *a* (BChl *a*) protein as well as the iron−sulfur protein. It contained 27 ± 3 molecules of BChl *a*, 7 ± 1 Chl-670, 3 ± 1 carotenoids, and 1.6 ± 0.1 *c*-type hemes per the primary electron donor P840. The complex showed a light-induced charge separation and recombination between P840 and the acceptor Chl-670 at 77 K as follows: P840*Chl-670 → P840$^+$Chl-670$^-$ → P840^{T}Chl-670 → P840 Chl-670. Pigment compositions and their function in the (PscA/cytochrome c_{551})$_2$ complex were studied by absorption, circular dichroism, and fluorescence spectroscopy.

In the photosynthesis of plants and bacteria, solar energy is converted into chemical energy in the reaction center (RC)[1] complexes. The RC complexes, so far known, can be grouped into essentially four types: (i) the RCs of photosystem (PS) I and (ii) PS II of plants and cyanobacteria; (iii) the RCs of purple bacteria; and (iv) the RCs of green sulfur bacteria and heliobacteria (Blankenship, 1992). The RC complexes of the former three types contain a set of two different core subunits, which resemble each other (designated PsaA and PsaB in PS I, D1 and D2 in PS II, and L and M in the purple bacteria), and thus are "heterodimer" complexes. The two different core subunits produce asymmetry in the arrangements and energy levels of the prosthetic groups and seem to provide a basis for the efficient unidirectional electron transfer (Deisenhofer et al., 1985).

The amino acid sequence of the RC core protein (PscA) of the green sulfur bacterium *Chlorobium limicola* f. *thiosulfatophilum* (Büttner et al., 1992) shows homology to that of *Heliobacillus mobilis* (Liebl et al., 1993) or to those of the PsaA and PsaB core proteins of PS I, especially in a region that is assumed to form the iron−sulfur center similar to F_X in PS I. However, only one type of gene can be assumed to encode the RC core protein of *C. limicola* or *H. mobilis*. This strongly suggests that these RC complexes are so-called "homodimer" complexes made up of a set of two identical core subunits that provide the four cysteine ligands necessary to hold the F_X-like iron−sulfur cluster (Büttner et al., 1992; Lieble et al., 1993).

Various photoactive preparations of RC complexes have recently been purified from green sulfur bacteria. Feiler et al. (1992) reported the RC complex made of 6−7 subunits, about 40 bacteriochlorophyll *a* (BChl *a*), 3 types of iron−sulfur centers, and 4 *c*-type hemes per P840. Okkels et al. (1992), Oh-oka et al. (1993), and Kusumoto et al. (1994) prepared similar RC complexes that, however, contained only 1−2 *c*-type hemes. All these preparations contain the light-harvesting BChl *a* protein (also known as the FMO protein after Fenna, Mattews, and Olson), in addition to the RC core protein, and functionally resemble the photosystem protein (PP) complex of *Prosthecochloris aestuarii* isolated by Swarthoff and Amesz (1979) and Swarthoff et al. (1981). The PP complex, however, contained larger numbers of protein subunits. Electron transfer sequences in these RC complexes have been estimated to be as follows in analogy with PS I RC:

P840 → A (Chl-670) → (quinone?) → Fe-S (F_X-type) → Fe-S (F_A/F_B-type)

although no firm evidence for the function of quinone has ever been obtained.

In this paper, we report the structure and photochemistry of the highly purified *C. limicola* RC complex (designated CRC-2), which is made up of only PscA core and cytochrome c_{551} subunits and gives firm evidence for the "homodimer" RC complex.

MATERIALS AND METHODS

Isolation of Reaction Center Complex. The photoactive RC complex with the iron−sulfur centers (designated as the

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[1] Abbreviations: (B)Chl, (bacterio)chlorophyll; RC, reaction center; CRC, *Chlorobium* reaction center; PS, photosystem; P840, the primary electron donor in green sulfur bacterial reaction center; CD, circular dichroism; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TMBZ, 3,3′,5,5′-tetramethylbenzidine; N-, amino-; C-, carboxyl-; ORF, open reading frame.

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crude RC complex in this study) was prepared as reported previously (Oh-oka et al., 1993). The crude RC complex was further purified as follows. The fraction was loaded onto a column (2 × 3.2 cm) of hydroxyapatite (Nakarai Tesque) equilibrated with 50 mM potassium phosphate (pH 6.8), 2 mM sucrose monolaurate, 10 mM cysteine, 1 mM EDTA, and 0.2 mM PMSF (designated "50-KSCEP" to represent the millimolar concentration of potassium phosphate). The column was washed with 2 column volumes of 50-KSCEP and then with 3 column volumes of 50-KSCEP including 0.5 M NaCl. After equilibration of the column again with 2 column volumes of 50-KSCEP, a blue-colored fraction containing FMO protein was eluted with 300-KSCEP. The fraction containing the RC complex was obtained by elution with 100-KSCEP containing 20% saturated ammonium sulfate. The eluate concentrated with Centricon-100 (Aminco) was applied to a column (1.6 × 93 cm) of Sephacryl S-300 (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0), 2 mM sucrose monolaurate, 5 mM dithiothreitol, 1 mM EDTA, 0.2 mM PMSF, and 200 mM NaCl (designated "TSDEPN-200"). After dialysis, the RC fraction was again loaded onto a column (2.2 × 3 cm) of Q-Sepharose equilibrated with TSCEPN-0 containing 10 mM cysteine, instead of 5 mM dithiothreitol. The column was then washed with 2 column volumes of TSCEPN-10. The finally purified RC complex was obtained by elution with TSCEPN-80. All the above procedures were performed at 0−4 °C except for the hydroxyapatite chromatography step, which was done at 22−24 °C, a temperature critical for complete removal of FMO protein from the RC complex.

Various Assays. Contents of BChl *a* and Chl-670 were estimated after extraction with acetone/methanol (7:2, v/v) by the method of Feick et al. (1982). The values obtained were consistent with the results of HPLC analysis by Kobayashi et al. (1992).

SDS−PAGE was performed by the method of Schägger et al. (1986), followed by Coomassie brilliant blue staining. A kit of low-molecular-weight markers (MW-SDS-17) was purchased from Sigma. Heme staining was done according to the method of Thomas et al. (1976).

BrCN cleavage and enzymatic digestion on PVDF membranes were performed according to the manual by Applied Biosystems. The peptides recovered were purified with a Waters HPLC system (600E with 991J detector). The N-terminal sequences were determined by a gas-phase sequencer (Applied Biosystems, Model 473A).

Measurement of Absorption, Fluorescence, and Circular Dichroism Spectra. Absorption and circular dichroism (CD) spectra at room temperature were measured with a Shimadzu spectrophotometer (Model UV-3101PC) and a Jasco J-200B spectropolarimeter, respectively.

Absorption and fluorescence spectra at low temperature were measured using a spectrograph with an image-intensified multichannel photodiode array (Itoh & Iwaki, 1988) and a measuring beam or fluorescence excitation beam from a tungsten−iodine lamp through glass or interference filters (10-nm half-bandwidth, Nihon Shinkukagaku). Samples were placed in a 1 cm (light path) x 1 cm x 4 cm plastic or quartz cuvette in an Oxford DN-704 cryostat. Time-resolved transient absorption and fluorescence spectra were measured after excitation with a 532-nm, 10-ns hfwm actinic flash from a second harmonic of a Nd-YAG laser (Quanta Ray DCR-2-10) and a weak-probing xenon flash. The image intensifier

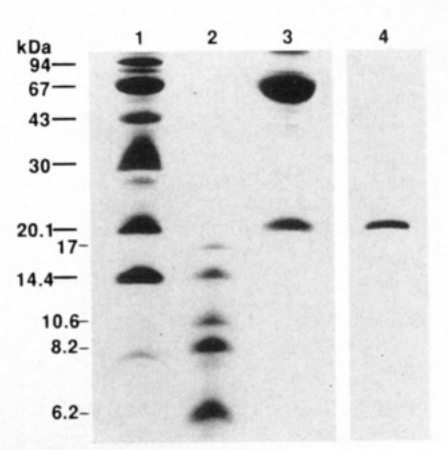

FIGURE 1: Analysis of the CRC-2 complex by SDS−PAGE. Lanes 1−3 were stained with Coomassie brilliant blue, and lane 4 by TMBZ. Lane 1, molecular weight (MW) markers, namely, phosphorylase *b* (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20.1K), and lysozyme (14.4K); lane 2, low-MW markers, namely, myoglobin (17K) and its peptide fragments I+II (14.4K), I+III (10.6K), I (8.2K), and II (6.2K); lanes 3 and 4, the CRC-2 complex.

was activated for 3.5 ns at varied times after the laser flash. Usually, 64−1024 signals were accumulated to obtain each difference spectrum as required. Fluorescence emission spectra were corrected as for the detector sensitivity.

The reaction mixture for the measurements contained 60% (v/v) glycerol in 50 mM Tris-HCl (pH 8.0), 2 mM sucrose monolaurate, 10 mM cysteine, and 1 mM EDTA with a glucose oxidase/catalase system (2 units/mL glucose oxidase, 20 units/mL catalase, and 20 mM glucose). To oxidize P840 in the RC complex, a few grains of potassium ferricyanide were added after dialysis against a buffer without cysteine. Extinction coefficients of $\Delta\epsilon_{830} = 100$ mM^{-1} cm^{-1} for P840$^+$/P840 (Olson et al., 1973) and $\Delta\epsilon_{551-540} = 20$ mM^{-1} cm^{-1} for cytochrome c^+/c (Meyer et al., 1968) were used.

RESULTS

Protein Subunits of the Chlorobium Reaction Center Complex (CRC-2). The isolated RC complex showed only two bands at 68 and 21 kDa on SDS−PAGE (Figure 1). The 68-kDa band is assigned to the PscA core protein with a calculated molecular weight of 82.2K, which is known to give a diffuse band at 65−68 kDa (Büttner et al., 1992). The 21-kDa band was stainable with TMBZ, and thus is cytochrome *c* as reported previously in the RC preparation of this organism (Oh-oka et al., 1993). Neither a band for the light-harvesting BChl *a* protein (FMO protein) that is known to give a band at about 40 kDa (Olson, 1978) nor low-molecular-weight subunits like those present in the PS I complex were detected (Golbeck & Bryant, 1991).

The 21-kDa band contained only the cytochrome *c* subunit and did not contain the other proteins. The N-terminal sequence, which was determined after blotting on a PVDF membrane, was identical up to the 25th residue with that of the cytochrome c_{551} subunit of *C. vibrioforme* reported by Okkels et al. (1992). No other sequence was obtained even after pretreatment of the PVDF membrane with 1 N HCl, which deblocks the N-terminal formyl group. Several peptide fragments recovered by HPLC after BrCN cleavage or lysylendopeptidase digestion on the PVDF membrane also corresponded to the fragments of cytochrome c_{551} (not

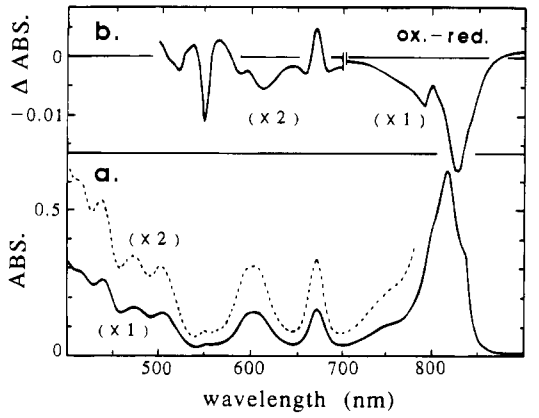

FIGURE 2: Absorption spectrum (a) and chemically oxidized-minus-reduced difference spectrum (b) of the CRC-2 complex at 293 K. The desalted sample (A_{810} = 0.56) was oxidized by addition of a small amount of ferricyanide and then reduced by an excess amount of ascorbate.

shown). These results indicated that the RC complex is made up of only PscA core and cytochrome c_{551} subunits.

Absorption Spectrum of the CRC-2 Complex. The absorption spectrum of the CRC-2 complex reduced by 10 mM cysteine showed a Q_y band of BChl *a* at 817 nm with shoulders at 800 and 830 nm, and a broad Q_x band around 600 nm (Figure 2a) at room temperature. The 670.5-nm peak and a portion of the 438-nm peak can be assigned to the Q_y and Soret bands of Chl-670, respectively. This Chl, which was originally designated BChl 663 by Braumann et al. (1986), has a chlorin macrocycle as Chl *a* does (Van de Meent et al., 1992) and is referred to here as Chl-670 to indicate its peak wavelength in the complex. The spectrum also shows the 551.5-nm α-peak of reduced cytochrome *c* and the 472- and 503-nm peaks of carotenoids.

A ferricyanide-oxidized minus ascorbate-reduced difference spectrum showed absorption decreases at 830 and 612 nm due to P840, and at 551 and 530 nm due to cytochrome c_{551} and the shiftlike absorption change around 670 nm due to Chl-670 (Figure 2b). Using difference extinction coefficients of $\Delta\epsilon_{830}$ = 100 mM^{-1} cm^{-1} for $P840^+$/P840 (Olson et al., 1973) and $\Delta\epsilon_{551-540}$ = 20 mM^{-1} cm^{-1} for cytochrome c^+/c, the CRC-2 complex is estimated to contain 27 ± 3 Bchl *a*, 7 ± 1 Chl-670, 3 ± 1 carotenoids (determined by HPLC by Kobayashi and Oh-oka, unpublished results), and 1.6 ± 0.1 *c*-type hemes per P840, respectively. Therefore, the present CRC-2 complex will be expressed as a (PscA/cytochrome $c_{551})_2$ complex. P840 contributes about 6% to the total absorbance at 830 nm. One molecule of Chl-670 seems to function as the primary electron acceptor (Van Bochove et al., 1984).

Circular Dichroism Spectrum of the CRC Complex. A CD spectrum of the CRC-2 complex was measured at room temperature (Figure 3). BChl *a* showed a 593(+)-nm Q_x peak, and 792(+)-, 809(−)-, and 834(+)-nm Q_y peaks. The 593(+)-nm CD band seems to originate from the light-harvesting BChl *a* since its peak wavelength is shorter than the 602-nm absorption peak or the 612-nm peak of the $P840^+$/P840 difference spectrum. The 668(+)- and 672(−)nm CD peaks of Chl-670 may reflect the interacting and/or symmetrical arrangement of these molecules. The positive CD signals around 430 nm may also arise from Chl-670. The CD spectrum is different from that of the crude RC complex (dashed line in Figure 3) which was previously

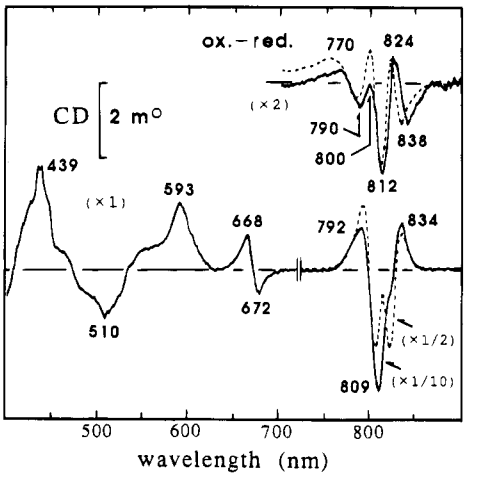

FIGURE 3: (Lower panel) Circular dichroism spectra of the CRC-2 complex (solid line) and the crude RC complex (dashed line, not shown at wavelengths shorter than 720 nm) at 293 K. Conditions were similar to those in Figure 2 except that the sample concentrations were adjusted to give an absorbance at 815 nm of 0.75 in the CRC-2 complex and at 810 nm of 0.25 in the crude RC complex, respectively. (Upper panel) Oxidized-minus-reduced circular dichroism difference spectra of the CRC-2 complex (solid line) and the crude RC complex (dashed line). Peak wavelengths in the CRC-2 complex are indicated by numbers.

obtained from this organism and contains 6−7 subunits (Oh-oka et al., 1993) or of the bacteriochlorophyll−reaction center complex demonstrated by Olson et al. (1973).

The CD spectrum of the crude RC preparation seems to be interpreted as a mixture of the signals of the core protein and of the FMO protein that is known to give 781(−)-, 800(+)-, 810(+)-, and 823(−)-nm peaks in the far-red region (Olson et al., 1973). In fact, the core complex isolated from *P. aestuarii* (Vasmel et al., 1983), which was almost completely depleted of FMO protein, showed a CD spectrum very similar to that of the CRC-2 complex. CD peaks of carotenoids at 470(+), 480(−) and 510(−) nm suggested their special orientation.

The oxidized-minus-reduced difference CD spectrum was rather complex with 790(−)-, 800(+)-, 812(−)-, 824(+)-, and 838(−)-nm peaks (Figure 3, inset). The 838(−)- and presumably 824(+)-nm peaks arise from P840, and the other peaks at shorter wavelengths from the Bchl *a* interacting with P840 in its vicinity. The difference spectrum resembles the one obtained in the crude RC complex.

Absorption Spectrum at 77 K. The spectra of the CRC-2 and crude RC complexes were measured at 77 K. The spectrum of the CRC-2 complex gave smaller contributions of the 800−830-nm bands compared to that of the crude RC complex when normalized at the 832−837-nm band (Figure 4a). The difference between these two spectra gave a spectrum with peaks at 806 and 825 nm and a shoulder at 814 nm. It was almost identical to the spectrum of FMO protein purified from this organism (Olson et al., 1973). The area of this difference spectrum was almost the same (101%) as that of the CRC-2 complex when integrated in a wavelength range between 790 and 850 nm. Relative contents of FMO protein varied a little from preparation to preparation, and the spectrum in Figure 4a is one with a high content of this protein. The crude RC complex thus is concluded to contain 3 or 4 molecules of FMO protein on the assumption that the FMO protein and the CRC-2 complex

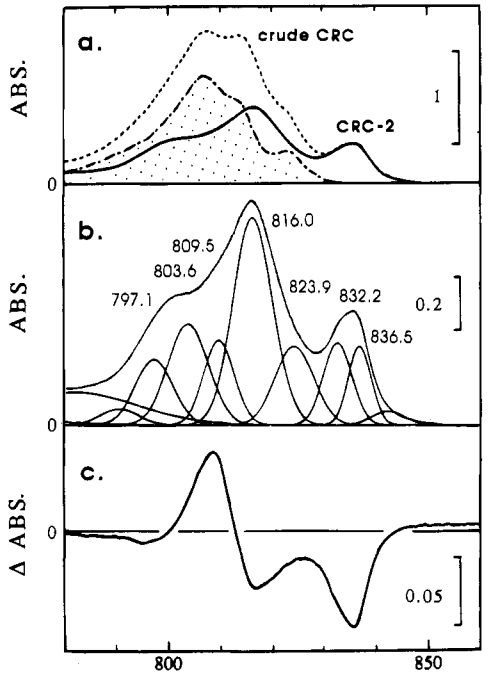

FIGURE 4: (a) Absorption spectra of the CRC-2 complex and the crude RC complex at 77 K. Absorption spectra of the CRC-2 complex (solid line) and the crude RC complex (dashed line) were normalized at the 830−840-nm region. The difference between these two spectra is shown by the dashed and dotted lines. (b) Analysis of the BChl forms in the spectrum at 77 K of the CRC-2 complex. Peak wavelength of each Gaussian curve is shown in nanometers. See text for details. (c) Light-induced absorption change in the CRC-2 complex at 77 K. The spectrum was calculated as the difference between the spectra obtained before and after illumination for 30 min with a blue light from a slide projector through filters that pass wavelengths between 400 and 530 nm. The reaction mixture contained 60% (v/v) glycerol in 50 mM Tris-HCl (pH 8.0), 2 mM sucrose monolaurate, 10 mM cysteine, and 1 mM EDTA with the glucose/catalase system.

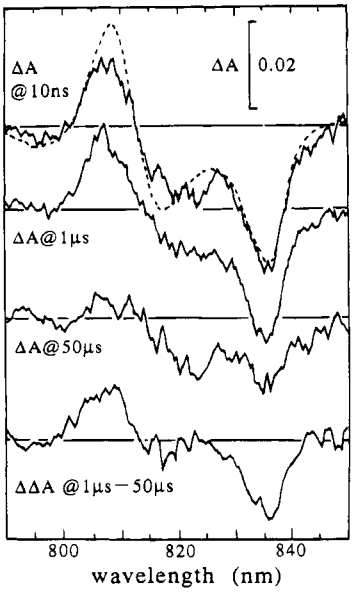

FIGURE 5: Time-resolved difference absorption spectrum of the CRC-2 complex at 10 ns, 1 μs, and 50 μs after the 10-ns, 532-nm laser flash at 77 K and the difference between the 1- and 50-μs spectra. The base line of each spectrum was shifted to avoid overlaps. The difference spectrum obtained in Figure 4c, which was normalized at 836 nm, is superimposed onto the spectrum at 10 ns (dashed line).

contain 7 (Olson et al., 1973) and 27 ± 3 (this study) BChl *a* molecules, respectively.

The spectrum of the CRC-2 complex at 77 K was analyzed into the sum of Gaussian curves (Figure 4b). The peak wavelengths were estimated from the second-derivative peaks, and then each curve was calculated to give the best fit to the data (Iwaki et al., 1992). The data points were well fitted with BChl forms at 797.1, 803.6, 809.5, 816.0, 823.9, 832.2, and 836.5 nm. P840 contributes to about one-third of the 836.5-nm band (see below). The other two-thirds of this band is contributed by BChls (designated as BChl-837 afterwards) other than P840. There was no band with a peak at 805−806 nm in the deconvoluted spectrum, indicating the loss of FMO protein that shows a main peak at 805−806 nm as seen in Figure 4a.

The spectrum of the CRC-2 complex resembled that of a "reaction center pigment−protein (RCPP)" complex isolated from *P. aestuarii* that contained 6 subunits including the FMO protein subunit (Swarthoff & Amesz, 1979). The RCPP complex showed an A_{815}/A_{834} ratio of 2.4 at 77 K and clear peaks at 834 and 837 nm at 4.2 K, whereas the CRC-2 complex showed a lower A_{815}/A_{834} ratio of 2.0, due to the complete loss of FMO protein. On the other hand, the putative spectrum of the RC core part was calculated by subtracting the contribution of the FMO protein from the spectrum of the FMO−RC complex (Miller et al., 1994). The spectrum thus obtained is somewhat similar to that of the CRC-2 complex except for a little larger absorption around 795−815 nm in the latter.

Long (30 min) illumination at 77 K with continuous light induced a stable absorption change with negative peaks at 837 and 820 nm and a positive peak around 810 nm (Figure 4c). This signal disappeared above 250 K and can be photoinduced again on cooling the sample to 77 K. The spectrum resembled that of $P840^+/P840$ reported previously (Swarthof et al., 1981) but had a larger positive change at 808 nm. No absorption change of cytochrome c_{551} was seen either at 77 K or at room temperature (not shown).

Transient Absorption Change of the CRC-2 Complex. Time-resolved difference absorption spectra were measured after the excitation with a 532-nm, 10-ns laser flash at 77 K. At 10 ns after the laser peak, a narrow bleach at 836 nm with a shoulder around 820−830 nm and a positive change at 808 nm were detected (Figure 5). The spectrum resembled that accumulated by the continuous light in Figure 4c and was about 25−30% of the latter in its magnitude. The extent and spectral shape did not significantly change until 1 μs. The spectrum afterward showed a relatively larger 825-nm band with a smaller change around 800−810 nm as seen at 50 μs and resembled that of the $P840^+/P840$ difference spectrum measured at 80 K in the PP or RCPP complex (Swarthoff et al., 1981).

The difference spectrum between 1 and 50 μs, that mainly reflects a component with a time constant ($t_{1/e}$) of 30 μs (see below), showed large 808(+)- and 836(−)-nm peaks with a very small 820-nm bleach. This spectrum resembled that assigned to the triplet-minus-singlet (T−S) spectrum of BChl-837 (note that it has a very small 820-nm shoulder) measured by the absorbance-detected magnetic resonance (ADMR) in the RCPP complex by Hoff et al. (1988). Bleaches of Chl-670 and the Q_x band of P840 around 615 nm were also detected at 10 ns, and the former almost disappeared at 1 μs (not shown).

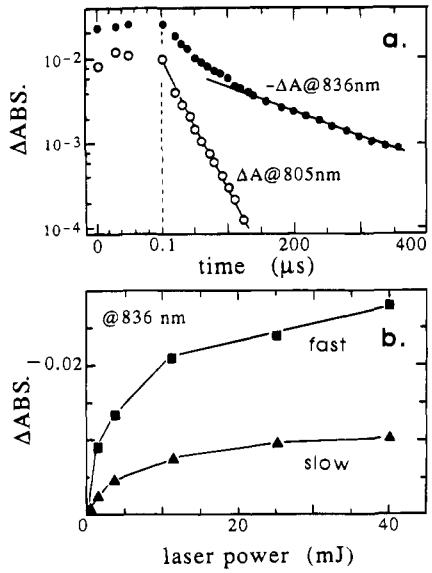

FIGURE 6: (a) Time courses of the absorption changes in the CRC-2 complex at 805 and 836 nm at 77 K. The extents of absorption changes measured as in Figure 5 (within 1 μs) and with the split beam spectrophotometer (between 1 and 400 μs) were plotted against time after the laser excitation. Straight lines indicate the 30- and 150-μs decays, respectively. (b) Dependence of the extents of absorption changes on the excitation laser power. Extents of the fast (30-μs decay) and the slow (150-μs decay) phases are shown by closed squares and triangles, respectively.

The bleach at 836 nm increased slightly up to 100 ns after the flash and then decayed in two phases with time constants ($t_{1/e}$) of 30 and 150 μs, respectively (Figure 6a). The 30-μs decay component (BChl-837^T) contributed to about 60% at 836 nm and 100% at 805 nm. The 150-μs decay component showed a prominent shoulder at 825 nm and smaller changes around 800−810 nm. The spectrum resembled the T−S spectrum of P840 shown by Hoff et al. (1988). It is thus suggested that the P840$^+$Chl-670$^-$ biradical state was formed within a few nanoseconds and that the charge recombination between P840$^+$ and Chl-670$^-$ produced the triplet state of P840 (P840^T) within 100 ns. Both the extents of P840^T (150-μs decay phase) and BChl-837^T (30-μs decay phase) almost saturated at a laser intensity of about 10 mJ, where 2−5 photons/P840 were absorbed by the carotenoids and BChls in the complex (Figure 6b). It seems that both P840 and BChl-837 are connected to similar numbers of light-harvesting BChls. The flash-induced absorption change gradually decreased after excess flash excitations in parallel with the accumulation of the irreversible signal shown in Figure 4c.

Fluorescence at 77 K. Time-resolved fluorescence spectra were measured at 0−50 ns after the laser flash. The emission peaks were observed at 670 nm (this peak wavelength became shorter with the increase in the laser intensity, suggesting the heterogeneity of the fluorescing pigments) and 837 nm with a shoulder at 828 nm (Figure 7). The intensity at 837 nm decayed in a single phase with a time constant ($t_{1/e}$) of 3.9 ns (a little slower than the 3.5-ns time resolution) until 1/10000th of the peak intensity (Figure 8a). The 670-nm fluorescence showed a time constant ($t_{1/e}$) of 6.8 ns, suggesting that some Chl-670 shows low efficiency in the excitation transfer to the other pigments and fluoresces by itself.

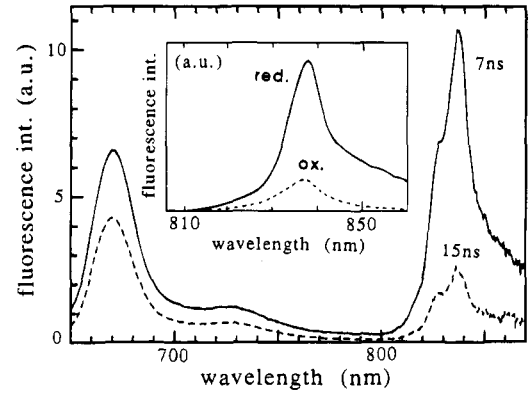

FIGURE 7: Time-resolved fluorescence spectra of the CRC-2 complex at 77 K. Measured after laser excitation at 45 mJ with 3.5-ns time resolution at the times indicated. Inset: effects of ferricyanide on the fluorescence of the CRC-2 complex at 77 K. Fluorescence was induced by continuous 600-nm excitation light under the reduced (red.) and oxidized (ox.) conditions: the fluorescence spectra were measured in the presence and absence of ferricyanide.

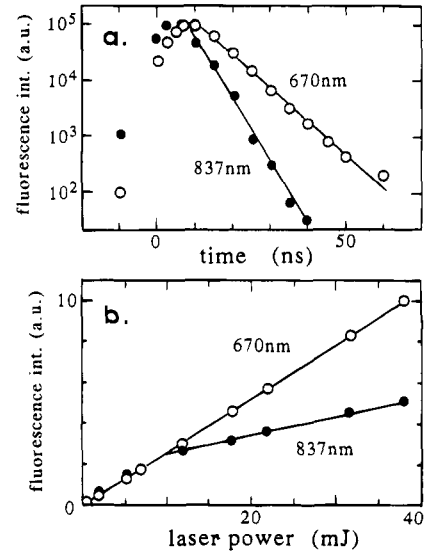

FIGURE 8: (a) Decay kinetics of fluorescence intensity at 670 and 837 nm, respectively, at 77 K. Peak intensities were normalized to each other. (b) Dependence of the intensities of 670- and 837-nm fluorescence on the excitation laser power. The intensities were normalized to give a similar slope at the low laser power. The other experimental conditions were similar to those in Figure 7.

Fluorescence excited with a weak continuous 600-nm light was also measured at 77 K (Figure 7, inset). Almost the same fluorescence spectra were obtained with excitation at 672 nm (Chl-670) or at 500 nm (carotenoids) (data not shown), indicating excitation energy transfer among the pigments. With an increase in temperature, the relative intensities of the shorter-wavelength fluorescence bands increased and the total fluorescence yield decreased (not shown). Oxidation by ferricyanide decreased the fluorescence intensity to 22% at 77 K (Figure 7 inset) or to 30% at 293 K with little change in the spectral shape.

The effect of ferricyanide suggests that the redox state of P840 (or of some other redox components) affects the fluorescence intensity. This point was further studied by checking the excitation laser power. The integrated fluorescence intensity at 837 nm after the flash in the reduced condition showed saturation at about 10 mJ (Figure 8b). This saturation pattern resembles that of the flash-induced extent

of ΔA_{836} in Figure 6b. On the other hand, the integrated intensity at 670 nm increased almost linearly with the increase in the excitation power.

DISCUSSION

Subunits and Prosthetic Groups of the CRC-2 Complex. The CRC-2 complex is composed of only the core and cytochrome c_{551} subunits. Okkels et al. (1992) demonstrated that the RC complex of *C. vibrioforme* contains a monoheme-type cytochrome with a calculated molecular weight of 22.9K (18K on SDS−PAGE), judging from its DNA sequence analysis. The tight association between the core and cytochrome c_{551} subunits in the CRC-2 complex indicates that the monoheme-type cytochrome, similar to that reported by Okkels et al. (1992), is a component of the RC and functions as the immediate electron donor to P840, which has been clarified recently in a more intact RC preparation of *C. tepidum* (Oh-oka et al., 1995). This conclusion disagrees with that by Feiler et al. (1992), who reported that a tetraheme-type cytochrome with an apparent molecular mass of 32 kDa on SDS−PAGE functions as the direct electron donor to P840 in their RC preparation. The reason for the discrepancy is not yet clear.

The CRC-2 complex is estimated to contain about 27 BChl *a*, 7 Chl-670, 3 carotenoids, 1.6 hemes, no menaquinone, and probably no iron−sulfur center on the assumption of $\Delta\epsilon_{830} = 100\ mM^{-1}\ cm^{-1}$ for $P840^+/P840$ (Olson et al., 1973) and $\Delta\epsilon_{551-540} = 20\ mM^{-1}\ cm^{-1}$ for cytochrome c^+/c (Meyer et al., 1968), respectively. Although these extinction coefficients are still somewhat tentative, association of two molecules of cytochrome c_{551} per one RC complex has recently been indicated by kinetic analysis (Oh-oka et al., 1995). The CRC-2 complex, thus, is concluded to contain two sets of the core and cytochrome c_{551} subunits, and can be expressed as a (PscA/cytochrome c_{551})$_2$ complex provided that one F_X-like iron−sulfur center is constructed between the two identical core subunits.

The 30−38 molecules of BChl *a* and Chl-670 per P840 bind to the CRC-2 complex. This number is about twice that of histidine residues in a single core polypeptide. Histidines can function as ligands for the Mg atoms of BChls. The RC core subunit(s) of *C. limicola*, *H. mobilis*, or the maize PS I (PsaA/PsaB) has (have) 20 (Büttner et al., 1992), 26 (Liebl et al., 1993), or 42/37 (Fish et al., 1985) histidine residues. These complexes contain 30−38 (this work) or 24−40 (Trost & Blankenship, 1989; Van de Meent et al., 1990) BChls or 80−100 (Golbeck & Bryant, 1991) Chls per RC, respectively. The correlation suggests that each RC complex contains two core subunits. The RC complex of PS I has acquired the larger number of antenna Chls in its interior during the evolution. This is in marked contrast to the evolutionary strategy of the PS II or purple bacterial-type RC complex that has increased the number of light-harvesting Chl proteins but has not increased Chls in the RC complexes themselves.

Function of the CRC-2 Complex. The CRC-2 complex shows a chemically-induced difference spectrum that is typical for $P840^+/P840$. The stable charge separation was not detected at room temperature. The flash-induced absorption change at 77 K indicates the formation of the $P840^+$-$Chl\text{-}670^-$ state and the following recombination reaction between $P840^+$ and $Chl\text{-}670^-$ within 100 ns that forms $P840^T$. A transient absorption change in Chl-670 was also observed (Oh-oka et al., unpublished data). The charge recombination time and the triplet formation are similar to those reported in the phylloquinone-extracted PS I RC complex (Itoh & Iwaki, 1989). Chl-670 emits fluorescence and shifts its emission band with the oxidation of P840 in the CRC-2 complex. Specific hydrogen bonds between Chl-670 and the RC protein were also reported (Feiler et al., 1994).

The isolated CRC-2 complex undergoes only the primary charge separation between P840 and the acceptor Chl-670. Addition of menaquinone did not recover the stable charge separation (Oh-oka et al., unpublished results). Menaquinone is known to replace the function of the acceptor phylloquinone in PS I (Itoh & Iwaki, 1989). Therefore, no evidence for the function of quinone has been obtained in the CRC-2 complex as in the situation in the RC complex of *H. mobilis* (Trost et al., 1992; Kleinherenbrink et al., 1993). Addition of benzyl viologen that is an efficient acceptor to iron−sulfur centers in PS I had no effect. The complex is thus concluded to lack the electron acceptor to $Chl\text{-}670^-$. The chemical identity of the secondary electron acceptor, which can be presumed to be either the F_X-like iron−sulfur center or the quinone-like acceptor, still remains to be studied.

The fluorescence study suggests that both BChl-837 and P840 function as an energy sink and transfer energy to each other. The rapid excitation energy transfer among the light-harvesting BChls in the CRC-2 complex can be estimated because the shorter-wavelength (higher energy level) BChls emitted more fluorescence at the higher temperature. The saturation curve in Figure 8b indicates that some Chl-670 fluoresces by itself without energy transfer while the 837-nm fluorescence saturates at the laser power at which $P840^T$ also saturates. One interpretation may be that a portion of the 837-nm fluorescence is emitted as the delayed fluorescence from some metastable state of the $P840^+Chl\text{-}670^-$ biradical state, although the time resolution in this study is not sufficient to resolve the faster phases of the fluorescence.

The CRC-2 complex produced no delayed fluorescence of 50−100 ns lifetimes, although such delayed fluorescences are known to be produced in the charge recombination reactions in the PS I RC lacking phylloquinone as well as in purple bacterial and PS II RCs lacking the Q_A-quinones (Itoh & Iwaki, 1988). The discrepancy will reflect either the very large energy gap between $P840^*$ and the relaxed $P840^+Chl\text{-}670^-$ biradical state or some other situation.

Is Asymmetry Necessary for the Function of the RC Complex? The CRC-2 complex is expected to be a "homodimer" and to have *C2* symmetry for the arrangements of the subunits and prosthetic groups. P840 seems to be a dimer of BChl *a*, judging from its difference absorption and CD-active nature. Chl-670 showed a dimer-like CD signal so that some of the 6−8 Chl-670 molecules within the CRC-2 complex may be arranged in a symmetrical way. This apparently is in contrast to the situation in the heterodimer PS I RC complex that has the CD-inactive Chl *a*-690 as the electron acceptor (Ikegami & Itoh, 1986). It is to be determined whether the CD signal arises from the acceptor Chl-670 or from the other Chl-670 molecules.

The purple bacterial RC contains two almost symmetrical but slightly different L and M branches to the prosthetic groups. The partial difference in amino acid sequences

between the L and M subunits seems to give priority to the L branch as the electron transfer pathway (McDowell et al., 1991). The "homodimer CRC-2 complex" leads us to expect either the two symmetrical electron transfer pathways from P840 to the F_X-type center or only a single pathway along the *C2* symmetrical axis. Our results for the CRC-2 complex indicate that the asymmetrical RC is not necessary, at least for the primary charge separation.

The possibility cannot be excluded that the peripheral protein, such as the FMO protein or the iron−sulfur protein, induces asymmetry in the native complex. The cytochrome c_{551} subunit, however, does not seem to be a candidate because of its even stoichiometry to the core subunit as demonstrated recently (Oh-oka et al., 1995).

The feature of the CRC-2 complex indicates that the minimal requirement for the photoactive RC complex of *C. limicola* is the 2 identical core subunits that bind 30−38 pigments. The study of the primary electron transfer in the (PscA/cytochrome c_{551})$_2$ complex will give us a key to solve the puzzle of the "homodimer" RC complex.

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