Two molecules of cytochrome *c* function as the electron donors to P840 in the reaction center complex isolated from a green sulfur bacterium, *Chlorobium tepidum*

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Abstract **A photoactive reaction center complex was isolated from a thermophilic green sulfur bacterium, *Chlorobium tepidum* under anaerobic conditions. The electron transfer occurred from heme *c* to the photo-oxidized reaction center chlorophyll, $P840^+$, with a half time ($t_{1/2}$) of 110 or 340 μs at 24 or 12°C, respectively. Optical measurements under multiflash excitations indicated that two hemes function as the immediate electron donors to $P840^+$. SDS-PAGE analysis of the RC complex in combination with the N-terminal amino acid sequence analyses revealed five subunit bands; a core protein (65 kDa), the light harvesting bacteriochlorophyll *a* protein (41 kDa), a protein with 2[4Fe-4S] clusters (31 kDa), monoheme cytochrome *c* (22 kDa), and a 18-kDa protein whose function is unknown. The reaction center complex, thus, contains two molecules of cytochrome *c* per P840.**

Key words: Cytochrome *c*; Electron transfer; Green sulfur bacteria; Photosynthesis; Reaction center; Iron-sulfur center

1. Introduction

Photosynthetic reaction center (RC) of green sulfur bacteria as well as heliobacteria can be considered to be an evolutionary ancestral type to that of photosystem I (PS I) in oxygenic organisms, judging from the following evidences. (i) A pair of PscA core protein, that shows homology in amino acid sequences to both of PsaA and PsaB core proteins of PS I, seem to make up the so-called 'homodimer' RC in contrast to the asymmetric 'heterodimer' RC of other photosynthetic organisms [1]. (ii) The RC contains three iron-sulfur clusters that resemble F_X, F_A and F_B clusters in PS I RC [2–6]. The binding motifs for F_X- and F_A/F_B-like clusters reside in the PscA core and the PscB proteins, respectively, in *C. limicola* [1,7]. (iii) Purified RC complex have only 6–7 polypeptides compared with more than 15 subunits in PS I RC complex [4–6,8]. This type of RC complex seems to conserve the feature of the ancestral RC complex that evolved under anaerobic environment in the primitive earth. It is interesting that all of the RC preparations, so far reported, contain 1–4 molecules of tightly-bound *c*-type heme(s) that never exist in PS I RC [4–6,8].

In the RC complex of green sulfur bacteria, the light-induced electron transfer sequence has been assumed to be as follows in analogy with PS I:

$$\text{cytochrome } c \xrightarrow{h\nu} \text{P840} \rightarrow \text{A(BChl-671)} \rightarrow [\text{quinone?}] \rightarrow \text{iron-sulfur centers}$$

where BChl-671 is the electron acceptor bacteriochlorophyll with a peak at 671 nm that has a macrocycle isomeric to chlorophyll *a* [9]. The evidence for the secondary acceptor quinone is still scarce. Photo-oxidized P840 is reduced by cytochrome *c* [3,5,10,11]. The gene encoding the cytochrome *c* identified in *C. vibrioforme* has a single heme binding moiety in a conserved motif, Cys-X-X-Cys-His [8]. However, the ratio of bound heme to P840 varies in different RC preparations; 0.9–1.2 in *C. vibrioforme* [8], 1.2–1.7 in *C. tepidum* [6], 2 in *C. limicola* [5], 3–4 in *C. limicola* [4], 0.5–1.5 in *Prothecochloris aestuarii* [11], and 2 (or 3) in the membrane preparation of *C. tepidum* [12]. The number of bound heme *c*, thus, has been a matter of strong arguments. The controversy has arisen in part from the instability and low activity of the isolated RC preparations.

We isolated the photoactive RC complex from a thermophilic bacterium, *C. tepidum*, under anaerobic conditions. The purified RC complex is stable and photoactive, and is shown to contain two hemes that directly reduce $P840^+$.

2. Materials and methods

C. tepidum cells were grown according to Wahlund et al. [13] in 1.2-liter bottles for 2 days under tungsten lamps at 43°C. The subsequent procedures were carried out under anaerobic conditions in a chamber (Coy Laboratory Products, Ann Arbor, MI, USA) at room temperature unless otherwise indicated. Cysteine (Cys) or dithiothreitol (DTT) were added to buffers that were fully degassed and flushed with N_2 gas repeatedly. The crude RC complex was prepared as described previously [5] with modifications as follows: (i) all buffers contained the protease inhibitors; 1 mM EDTA, 1 mM *p*-aminobenzamidine, 1 mM 6-amino-n-caproic acid, 0.2 mM PMSF, 2 μM E-64, 1 μM pepstatine; (ii) the cells (about 20-g wet wt.) were disrupted by passage through a French pressure cell under a stream of N_2 gas at 4°C; (iii) the glucose oxidase/catalase-system solution (at a final concentration of 2 U/ml glucose oxidase, 20 U/ml catalase, 20 mM glucose) was added to suspensions during the cell disruption and membrane collection; (4) after solubilization, the precipitate sandwiched between 40 and 55% saturated ammonium sulfate ($(NH_4)_2SO_4$) was collected and suspended in 16 ml of Buffer A (50 mM Tris-HCl (pH 8.0), 2 mM DTT, 2 mM sucrose monolaurate and the inhibitors including 40% saturated $(NH_4)_2SO_4$), followed by centrifugation to get the supernatant rich in

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Abbreviations: BChl, bacteriochlorophyll; RC, reaction center; PS, photosystem; P840, primary electron donor; PMSF, phenylmethanesulfonyl fluoride; Cys, cysteine; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; N-, amino-; TMBZ, 3,3′,5,5′-tetramethylbenzidine; PscA and PscB, core and iron-sulfur proteins encoded by *pscA* and *pscB* genes, respectively, in *Chlorobium*.

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RC complex. A 100% saturated $(NH_4)_2SO_4$ solution was prepared at 4°C and diluted at room temperature before use. The crude RC complex was loaded onto a column (1.5 × 3 cm) of Toyopearl HW-65 (Tosoh, Japan) equilibrated with Buffer A. After the column was washed with one column volume of Buffer A, a green-colored fraction was eluted by decreasing saturated $(NH_4)_2SO_4$ concentration from 40 to 30%. $(NH_4)_2SO_4$ in the eluate was removed by passage through a column (1.1 × 26 cm) of Sephadex G-25 (Pharmacia) equilibrated with Buffer B (50 mM Tris-HCl (pH 8.0), 2 mM DTT, 2 mM sucrose monolaurate and the inhibitors). The fraction was then loaded onto a column (1.5 × 4 cm) of DEAE Bio-Gel A (Bio Lad) equilibrated with Buffer B. The purified RC complex flowed out through the column.

Pigment contents (BChl *a* and *c*) were estimated after extraction with acetone/methanol (7:2, V/V) by the method of Feick et al. [14]. SDS-PAGE was performed by the method of Laemmli [15]. Heme staining was done according to the method of Thomas et al. [16]. The N-terminal sequences were determined by a gas-phase sequencer (Applied Biosystems, model 473A) after blotting on PVDF-membranes.

Absorption spectra were measured as reported previously [5]. Laser-flash photolysis was performed as described previously [17]. The probing light was detected by a photomultiplier or a gatable multichannel photodiode array through a monochrometer after an excitation by a 10-ns, 532-nm Nd-YAG laser flash [17]. Repetition rate of laser was less than 0.05 Hz except otherwise mentioned. Samples were suspended in Buffer B with the glucose/catalase system in an air-tight cuvette.

3. Results

The absorption spectrum of the purified RC complex of *C. tepidum* under the DTT-reduced condition and its chemically oxidized-minus-reduced difference spectrum are shown in Fig. 1. Both spectra were almost identical to those of the RC complex of *C. limicola* obtained by similar treatments with cholate/octyl glucoside and subsequent sucrose monolaurate [5] or of *C. tepidum* by Triton X-100 treatment [6]. The α-peak wavelength of reduced cytochrome *c* was at 552 nm. The peak wavelength is a little different from 551 nm measured in the RC complex of *C. limicola* [5] or 553 nm in the membrane preparation of *C. tepidum* [12]. The discrepancy may come from some structural modification during the solubilization procedures. The *C. tepidum* RC complex contained 1.9–2.2 hemes per P840 as calculated from the difference spectrum with estimated

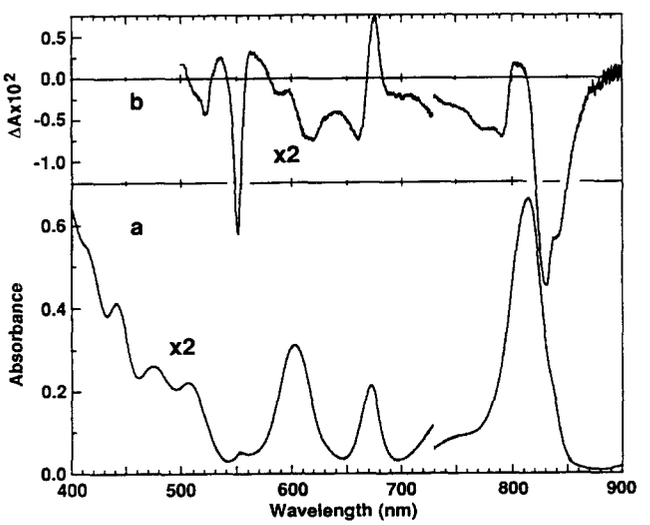

Fig. 1. Absorption spectrum (a) and chemically oxidized-minus-reduced difference spectrum (b) of the RC complex at room temperature. The desalted sample (A_{810} = 1.0) was oxidized by addition of a small amount of ferricyanide and then reduced by an excess amount of ascorbate.

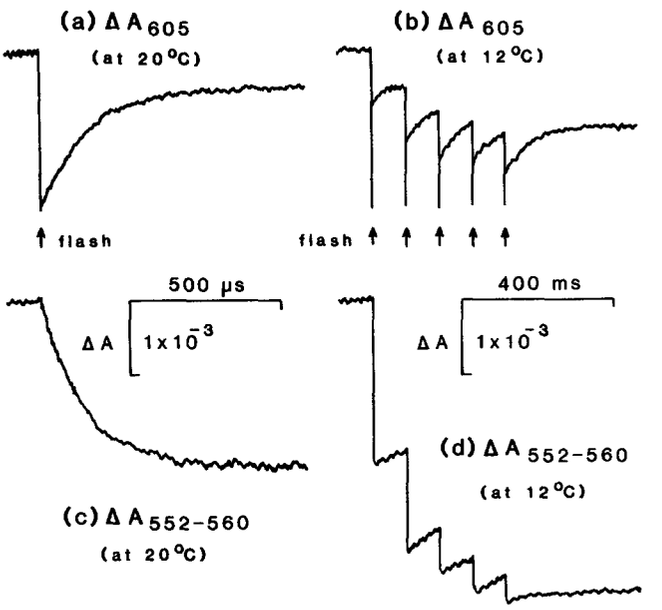

Fig. 2. Flash-induced absorption kinetics of P840 and cytochrome *c* in the RC complex. (a) and (b), P840 monitored as ΔA_{605}. (c) and (d), cytochrome *c* monitored as $\Delta A_{552\text{-}560}$. (a) and (c), single-flash-induced absorption changes at 20°C. 32 scans were averaged in each case. (b) and (d), multiflash-induced (90-ms interval) absorption changes at 12°C. A single scan signal was shown in each case. Samples were suspended in Buffer B (A_{810} = 1.5). Arrows indicate the time for the firing of flash.

difference extinction coefficients of $\Delta\varepsilon_{830}$ = 100 $mM^{-1}\cdot cm^{-1}$ for P840 and $\Delta\varepsilon_{552\text{-}540}$ = 20 $mM^{-1}\cdot cm^{-1}$ for heme *c*.

The flash-induced kinetics of P840 and heme *c* was monitored at 20°C as the absorbance change at 605 nm (ΔA_{605}) and as $\Delta A_{552\text{-}560}$, respectively (Fig. 2). The heme *c* was oxidized in a single phase with a half time ($t_{1/2}$) of 110 μs at 20°C (Fig. 2c). About 80% of flash-induced $P840^+$ was reduced also with a $t_{1/2}$ of 110 μs and the rest with a $t_{1/2}$ longer than 150 ms (Fig. 2a). The fast phase, thus, corresponds to the reduction of $P840^+$ by heme *c*. The reaction time of 110 μs is almost comparable to that previously measured in the membrane of *C. vibrioforme* [3] or the RC complex of *C. limicola* [5]. The difference extinction coefficient of P840 at 605 nm can be calculated to be 14.5 $mM^{-1}cm^{-1}$ from Fig. 2 in agreement with Fig. 1b. The magnitudes of flash-induced oxidations of P840 and heme *c* in Fig. 2a and 2c correspond to 30 and 15% of their chemically-determined contents in Fig. 1b, respectively.

Photo-oxidation of heme *c* was also measured with multi-flash (Fig. 2d). Temperature was decreased to 12°C to slow the re-reduction rate of heme *c*. The extent after the fifth flash is about twice of that after the first flash, indicating that two hemes reduce one $P840^+$. The total extent of flash-induced heme *c* corresponds to 30% of the chemically-determined extent. P840 was oxidized to almost the same extent just after each flash. $P840^+$ formed was reduced rapidly after the first flash, while unreduced $P840^+$ increased as the flash number (Fig. 2b). The incomplete reduction of $P840^+$ after each flash indicates a small difference between the redox midpoint potentials (ΔE_m) of P840 and heme *c*.

If two hemes are connected to P840, the single-flash experiments in Fig. 2a and 2c suggest that 19% of P840 and 41% of

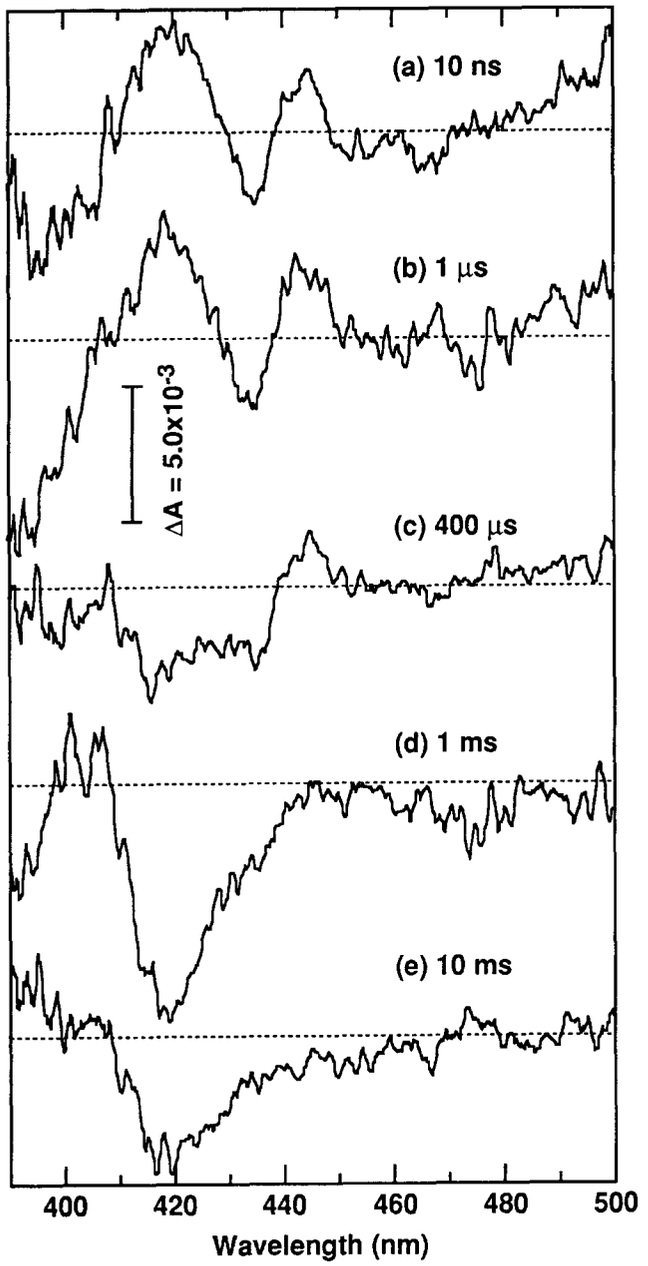

Fig. 3. Transient difference absorption spectra at (a) 10 ns, (b) 1 μs, (c) 400 μs, (d) 1 ms and (e) 10 ms after excitation with a laser flash. Averages of 32–64 measurements. The samples were suspended in Buffer B (A_{810} = 1.5); the temperature was 12°C.

hemes in photoactive RC complexes are in oxidized states at 800 μs after the flash, respectively. A simple calculation (Eqn. **(1)** below) with the extents of oxidized (ox.) and reduced (red.) forms of heme and P840 gives an ΔE_m value of about 28 mV, provided that two hemes and P840 attained an equilibrium by this time.

$$\Delta Em = RT/F \ln \frac{(\text{heme red.})}{(\text{heme ox.})} \frac{(\text{P840 ox.})}{(\text{P840 red.})} \qquad \text{Eqn. (1)}$$

This value almost agrees with the ΔE_m value of 50–20 mV that can be calculated from the reported midpoint potentials (E_ms) of +230mV and +180 mV (or apparent E_m value of +210 mV) for P840 and *c*-type heme, respectively, in the membrane of this organism [12]. On the other hand, if we assume that only one heme is connected to P840, 19% of P840 and 81% of heme are calculated to be in oxidized states and then the larger ΔE_m value of 76 mV, that does not agree with the reported E_m values, is obtained. Calculations using the extents of oxidized P840 and hemes at 5 ms after the first and the third flashes in Fig. 2b and 2d give similar ΔE_ms of 21 and 35 mV, respectively. Even if we assume that 10% of P840 lacks the active hemes in this preparation, similar ΔE_ms of 30 and 39 mV were obtained, respectively. These calculations suggest that each RC has two hemes, both of which have similar (or identical) E_ms more negtive than that of P840 (ΔE_ms = 20–40 mV) and rapidly reduce $P840^+$.

Following the rapid reduction by the heme, $P840^+$ was reduced in the slower 43-ms and the longer hundreds-ms phases after each flash, especially after the second to the fourth flashes (Fig. 2b). This indicates that $P840^+$ was reduced by the components other than the reduced hemes since the re-reduction of the oxidized hemes were also observed in parallel with the $P840^+$ reduction. The $t_{1/2}$ of 43 ms seems to represent the reduction time of $P840^+$ by the photo-reduced iron-sulfur center(s) as seen in the isolated PS I RC [18]. This $t_{1/2}$ is somewhat longer than the estimated $t_{1/2}$ of 50 μs or 7 ms in the reaction between $P840^+$ and F_X- or F_A/F_B-type center, respectively, in the membrane of non-thermophilic green sulfur bacterium, *C. vibrioforme* measured at room temperature [3].

Time-resolved difference spectra were measured after excitation with a 10-ns, 532-nm laser flash at 12°C (Fig. 3). At 10 ns after the peak time of laser flash, absorption decreased around 400, 435 and 465 nm and increased around 420, 445 and 500 nm. The spectral shape resembles that of the $P840^+$/P840 difference spectrum measured in a μs time range [19] and seems to be ascribable to the shift of carotenoids and Soret-band of BChl-671 accompanied with the oxidation of P840. The spectrum at 10 ns did not change until 1 μs, and then decreased in

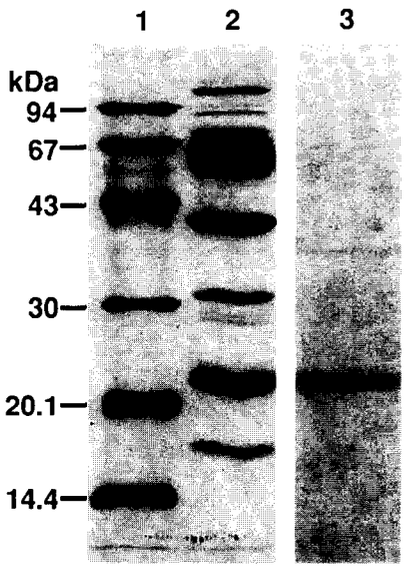

Fig. 4. Subunit composition of the purified RC complex from *C. tepidum* analyzed by SDS-PAGE. Lanes 1 and 2 were stained with Coomassie brilliant blue, and lane 3 by TMBZ. Lane 1, molecular-weight markers; phosphorylase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), trypsin inhibitor (20.1 K) and lysozyme (14.4 K). Lanes 2 and 3, the purified RC complex.

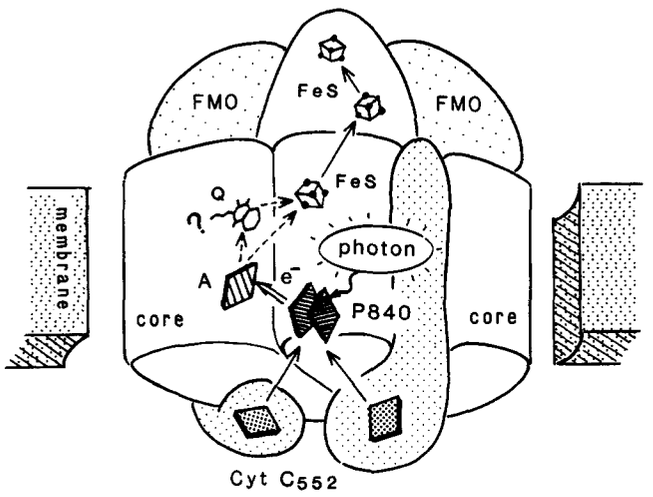

Fig. 5. Schematic representation of *C. tepidum* reaction center complex projected from the donor side (see text for the details). The α-peak wavelength of cytochrome *c* is 552 nm in the present RC preparation instead of 553 nm in the membrane (also see text).

parallel with the oxidation of heme *c* (e.g. at 400 μs in Fig. 3). Rise and decay of a Soret-peak of heme *c* at 418 nm with a shoulder at 435 nm was detected between 400 μs and 10 ms, giving the same time constants of 340 μs for the oxidation of heme *c* and the reduction of $P840^+$. These results indicate that $P840^+$ formed in less than 10 ns after the laser excitation does not decay until 1 μs before being reduced by heme *c*. The turn over of iron-sulfur centers in this RC will be reported elsewhere.

SDS-PAGE analysis of the purified RC complex revealed five subunits with apparent molecular masses of 65, 41, 31, 22, and 18 kDa (Fig. 4). No low-molecular-weight subunit was detected (not shown), although Kjær et al. [20] reported additional 9-kDa subunit in their photoactive RC preparation of *C. vibrioforme*.

A pair of 65-kDa subunit (PscA core protein) seem to form the homodimeric core part of the RC that carry P840, electron acceptors and bacteriochlorophylls [1]. This protein with a calculated molecular weight of 82.2 K is known to show a diffuse band at 65–68 kDa on SDS-PAGE analysis [1], as do the PsaA and PsaB core proteins in PS I. N-terminal sequences of the 41- and 31-kDa subunits were also analyzed. The sequence of the 41-kDa subunit was completely the same up to the 15th residue as that of the light-harvesting bacteriochlorophyll *a* protein (FMO-protein) of *C. tepidum* [21], and that of 31-kDa one showed almost identical alignment up to the 10th residue (AEP(V)ENKNQA-) with that of the PscB iron-sulfur protein of *C. limicola* [1]. The 22-kDa subunit is cytochrome *c* which is stained with TMBZ as indicated previously in the *C. limicola* RC [5]. The function of 18-kDa subunit is unknown. If the cytochrome *c* subunit binds only one heme, as expected from its DNA sequence in *C. vibrioforme* [8], it is highly likely that the RC complex contains two molecules of cytochrome *c* per one P840 as schematically shown in Fig. 5.

4. Discussion

The isolated RC complex of *C. tepidum* has five subunits and showed stoichiometric reduction of $P840^+$ by two hemes. This complex resembles the ones from the other *Chlorobium* species [4,5,8], but shows the higher and more stable photoactivity as the one from the same organism prepared with Triton X-100 [6]. The isolated complex is active for more than two weeks under anaerobic conditions below 4°C. A preliminary EPR study of the complex also indicated the signals of multiple iron-sulfur centers (Itoh et al., unpublished results).

The extent of photo-oxidized P840 was 30% after a single laser flash of saturated intensity (Fig. 2), and 60% under the steady-state illumination (data not shown). The reason for the low flash yield is not clear yet. Kusumoto et al. [6] reported the oxidation of 60% heme *c* by a flash as well as by continuous illumination. Their RC preparation, however, showed irreversible bleaching of bacteriochlorophylls by ferricyanide oxidation. Precise check of the quantum yield of our preparation is now undergoing under carefully controlled experimental conditions.

Feiler et al. [4] reported tetraheme cytochrome *c* with about 32 kDa molecular mass in their RC complex from *C. limicola*. The ratio of heme per P840 has been estimated to be 1–2 in the other *Chlorobium* RC preparations [5,6,8] or 2 (or 3) in the membrane preparation of *C. tepidum* [12]. Multiflash experiment in this study indicates the ratio to be exactly 2. The ratio determined here is more reliable since the method does not depend on the extinction coefficients of P840 and heme *c* that have not been determined yet.

The RC core complex has been considered to be a 'homodimer' made up of a pair of PscA core protein [1]. Mid-point potentials of the two *c*-type hemes seem to be almost identical [12] and their α-peak wavelengths are at the same position even at 77K (Oh-oka et al., unpublished results). It is, therefore, suggested that the two identical molecules of cytochrome *c* are bound to the 'homodimer' RC core complex and reduce $P840^+$ with the similar rates.

The heme *c* reduced $P840^+$ with a $t_{1/2}$ of 110 and 340 μs at 20 and 12°C, respectively. The rate might be even faster at the growth temperature (43–47°C) of this organism. A similar rate was also observed in *C. vibrioforme* and *C. limicola* [3,5]. The reaction rates are comparable to those between plastocyanin/cytochrome *c* and P700 in PS I [18] or that between cytochrome c_2 and P860 in purple bacterial RC [22]. By the use of the empirical distance-rate relationship of the intraprotein electron transfer proposed by Moser et al. [23], we can roughly estimate the distance between the heme *c* and P840 to be around 19 Å. The distance might be even smaller since this reaction does not seem to be fully optimized as for the free energy gap, judging from the small ΔE_m of 20–40 mV estimated previously [12] or in this work. In combination of this distance and the proposed structure of the cytochrome *c* that has three membrane spanning α-helices and a heme-containing hydrophilic domain [8], we can draw a schematic picture of this RC complex as in Fig. 5 by analogy with the structure of PS I RC proposed by the X-ray crystallography [24]. Further studies in the present *Chlorobium* RC complex will give more precise information.

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