Function of a PscD Subunit in a Homodimeric Reaction Center Complex of the Photosynthetic Green Sulfur Bacterium Chlorobium tepidum Studied by Insertional Gene Inactivation

REGULATION OF ENERGY TRANSFER AND FERREDOXIN-MEDIATED NADP⁺ REDUCTION ON THE CYTOPLASMIC SIDE

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Yusuke Tsukatani‡§, Ryo Miyamoto†, Shigeru Itoh¶, and Hiroya Oh-oka‡

From the ‡Department of Biology, Graduate School of Science, Osaka University, Machikaneyama 1-1, Toyonaka, Osaka 560-0043, Japan and the ¶School of Material Science (Physics), Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan

The PscD subunit in the homodimeric “type I” photosynthetic reaction center (RC) complex of the green sulfur bacterium Chlorobium tepidum was disrupted by insertional mutagenesis of its relevant pscD gene. This is the first report on the use of the direct mutagenic approach into the RC-related genes in green sulfur bacteria. The RC complex of C. tepidum is supposed to form a homodimer of two identical PscA subunits together with three other subunits: PscB (F₅₇/F₇₅-containing protein), PscC (cytochrome c₆), and PscD. PscD shows a relatively low but significant similarity in its amino acid sequence to PsaD in the photosystem I of plants and cyanobacteria. We studied the biochemical and spectroscopic properties of a mutant lacking PscD in order to elucidate its unknown function. 1) The RC complex isolated from the mutant cells showed no band corresponding to PscD on SDS-PAGE analysis. 2) The growth rate of the PscD-less mutant was slower than that of the wild-type cells at low light intensities. 3) Time-resolved fluorescence spectra at 77 K revealed prolonged decay times of the fluorescence from bacteriochlorophyll c on the antenna chlorosome and from bacteriochlorophyll a on the Fenna-Matthews-Olson antenna protein in the mutant cells. The loss of PscD led to a much slower energy transfer from the antenna pigments to the special pair bacteriochlorophyll a (P840). 4) The mutant strain exhibited slightly less activity of ferredoxin-mediated NADP⁺ photoreduction compared with that in the wild-type strain. The extent of suppression, however, was less significant than that reported in the PsaD-less mutants of cyanobacterial photosystem I. The evolutionary relationship between PscD and PsaD was also discussed based on a structural homology modeling of the former.

Photosynthetic organisms convert light energy into electrochemical free energy by carrying out a series of light-driven electron transfer reactions. This process, which is fundamental for life, is mediated by reaction center (RC) complexes. The RCs are primarily grouped into two types based on their terminal electron acceptors, type I (FeS-type) RCs and type II (quinone-type) RCs. Purple photosynthetic bacteria contain only type II RCs, which do not evolve oxygen, whereas oxygenic cyanobacteria and plants utilize both type I (photosystem I) and type II (photosystem II) RCs, which are connected in-line through the b₆f complex. Green sulfur bacteria and heliobacteria have unique type I RCs, so-called “homodimeric” RCs, which are made of two identical core polypeptides.

The “heterodimeric” type I and II RCs, which are found in all photosynthetic organisms other than green sulfur bacteria and heliobacteria, consist of a set of two partially different core polypeptides that produce a slightly asymmetric arrangement of cofactors. A three-dimensional structure of the heterodimeric RC was first obtained from the type II RC of the purple bacterium Blastochloris viridis in 1985 (1). Recently, those of photosystem I and II RCs from the thermophilic cyanobacterium Synechococcus elongatus were determined at high resolutions (2, 3). These structures have clarified that the folding motifs of membrane-spanning α-helices within core proteins as well as the spatial configurations of the electron transfer components are essentially identical in type I and II RCs, suggesting that they have a common origin. Various methods of gene manipulation have been applied to pursue research on heterodimeric RCs on the molecular level, and the results have led us to a comprehensive understanding of the electron transfer mechanisms and molecular architectures as well. The electron transfer reactions within these heterodimeric RCs occur via one of the two pathways that contain almost symmetrically arranged cofactors.

On the other hand, homodimeric type I RCs have never been successful targets for crystal structural analysis and mutagenic approach due to the obligate anaerobic and photosynthetic nature of green sulfur bacteria and heliobacteria. In fact, the RC complexes isolated from them are very fragile when exposed to oxygen, and many RC-related genes are essential. However, a study on the molecular level of the homodimeric RCs, which have a simple architecture and still retain the features of an ancestral RC, will offer valuable information to understand the mechanism and evolution of photosynthetic apparatuses.

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‡ To whom correspondence should be addressed. Tel.: 81-6-8185-5423; Fax: 81-6-8185-5425; E-mail: tsuka@bio.sci.osaka-u.ac.jp.

§ The abbreviations used are: RC, reaction center; BChl, bacteriochlorophyll; Chl, chlorophyll; Fd, ferredoxin; FMO, Fenna-Matthews-Olson; FNR, ferredoxin:NADP⁺ oxidoreductase; MOPS, 4-morpholinepropane-sulfonic acid.
The RC complex of the green sulfur bacterium Chlorobium tepidum consists of four subunits, PscA, PscB, PscC, and PscD with an antenna size of about 30 bacteriochlorophyll (BChl) molecules, whereas the heterodimeric photosystem I complex is made of 12 polypeptides with a much larger antenna size of nearly 100 chlorophyll (Chl) molecules (4, 5). The functions of PscA, PscB, and PscC have been studied intensively using biochemical and spectroscopic methods. A pair of PscA forms a core protein, which is supposed to make up a homodimeric (PscA)2 structure, unlike a PsaAPsaB heterodimeric one in photosystem I (6). This core protein contains a special pair, P840, a primary electron acceptor, Chl a-670 (A1), and an iron-sulfur center, FX, although the existence of the acceptor quinone (A2) remains controversial (4). PscB ligates two iron-sulfur clusters that are comparable with the F3 and F4 centers in PsaC of photosystem I but these proteins show almost no significant similarity to each other in their amino acid sequences (6, 7). PscC is the monoheme-type cytochrome c59, which serves as a direct electron donor to the special pair of BChl a, P840 (8–10), and has no counterpart in photosystem I. This cytochrome c59 mediates the electron transfer between the cytochrome bc and RC complexes without participation of other soluble cytochromes (10).

However, the function of PscD has been unknown because of the absence of any cofactors within it. Three-dimensional imaging analysis with the electron microscopic technique (STEM) suggested that PscD was in contact with the Fenna-Matthews-Olson (FMO) protein on the cytoplasmic side (11). Although PscD has been assumed to function in the same way as PsAD in photosystem I, as judged from their sequence similarities (12), there is no definite evidence for this speculation. The PsAD of photosystem I has been demonstrated to stabilize F3/F4-containing PsAC and facilitate the electron transfer reaction to ferredoxin (Fd). The Chlorobium RC complex also donates electrons to Fd (13), and NADPH is finally produced by Fd:NADP+ oxidoreductase (FNR) (13, 14).

Furthermore, the Chlorobium RC complex is associated with antenna protein, the FMO protein. The FMO protein is watersoluble and contains seven molecules of BChl a. X-ray crystallographic analyses conducted by two research groups have shown that this protein forms a trimeric structure (15, 16). It is estimated that one or two FMO trimers have been included in the RC preparations so far reported, probably depending on the chemical properties of the detergents used in their isolation procedures (4, 17, 18).

In the photosynthesis of green sulfur bacteria, light energy is captured by a supramolecular antenna complex, so-called chlorosome, which contains self-aggregates formed by large amounts of BChl c, d, or e depending on the species (e.g., the chlorosomes of C. tepidum contain BChl c aggregates) (19, 20). The BChl aggregates in chlorosomes make rod structures that are surrounded by a lipid monolayer envelop attached to the cytoplasmic surface of the inner membrane. The light energy absorbed by the BChl c aggregates in chlorosomes is transferred to the baseplate, which contains a small amount of BChl a, and then to the FMO protein, which seems to be located between chlorosomes and the RC complex and finally to the RC complex.

In this study, we constructed a C. tepidum mutant lacking PscD with the recently established natural transformation system in this organism (21). PscD was shown to be involved in the efficient energy transfer from chlorosomes to PS40, probably by allocating FMO proteins properly, and to enhance the NADP+ photoreduction via Fd. PscD was suggested to have an important role in the survival of green sulfur bacteria, even in the light-limited environment in which they are found in nature.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions**—The strain of C. tepidum used in this study was WT2321 (22). For liquid cultivation and plating incubation, CL and CP media were prepared, respectively, as previously described (21). The growth temperature was set at 40 °C during the present work. The anaerobic growth on CP plates was carried out in an anaerobic jar (model G, grade HP31; OXOID, Ltd.) equipped with a palladium catalyst and a H2/CO2-generating gas pack (BBL Gas Pack; BD Biosciences) and supplemented with 0.1 g of thioacetamide to generate H2S by adding 1 ml of 0.5 M HCl.

**Plasmid Construction and Transformation of C. tepidum**—A 1.2-kb fragment containing the pscD gene of C. tepidum was amplified by PCR, using a pscD primer (5′-CTGATTCTGCTACAGAAAGCCCATCC) and an internal primer (5′-GACACGCCGTTGCGCGACG). The fragment obtained with the above primers was cloned into the EcoRI site of pUC18, yielding plasmid pCD1. A 2.0-kb fragment containing the aadA gene, which was produced by Smal digestion of pH451 (23), was inserted into the Nael site of pCD1, yielding plasmid pCD4 (Fig. 1). Plasmid pCD4 was then prepared in large amounts by using a MIDI-prep kit (Invitrogen). About 1 μg of Adh-digested pCD4 was used for the transformation. A natural transformation method was performed as described previously by Frigaard and Bryant (21). Transformants grown on selective (Sm/Amp) CP plates were restreaked three times onto selective CP plates. Each single colony on the third CP plate was inoculated into a fresh liquid CL medium containing selective antibiotics. The cultures to start growth were cultivated two or three times and used for further investigations.

**Preparation of Genomic DNA and Hybridization**—Cell lysates were prepared essentially as previously reported (24). An equal volume of phenol was added to the cell lysate and centrifuged for 3 min at 15,000 × g, and the supernatant was then mixed with an equal volume of chloroform. After recentrifugation, nucleic acids in the aqueous phase were precipitated with 99.5% (v/v) ethanol supplemented with 300 mM sodium acetate, washed with 70% (v/v) ethanol, and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNase was added to a final concentration of 50 μg/ml, and the mixture was incubated for 30 min at 37 °C. The suspension was mixed with 3 times the volume of the premixed glycol solution (13% (w/v) polyethylene glycol, 1.8 M NaCl) and incubated for 30 min on ice. Genomic DNA was precipitated, washed, and suspended in TE buffer.

One microgram of genomic DNA was digested by HincII and used for hybridization analysis. Southern hybridization was carried out according to a manual on molecular cloning (25) or instructions supplied by the manufacturer. PCR analysis was also used as a convenient means to confirm the insertional inactivation of the targeted gene. DNA fragments containing the pscD gene were amplified by the same primers as those used for cloning. The pscD marker in the mutant genome was detected by using the primers aadA946 and aadA947 (26).

**Preparation of the RC Complex and SDS-PAGE**—The RC complex was isolated from the wild-type and mutant cells as described previously by Oh-oka et al. (27). All procedures for the preparation were carried out under anaerobic conditions in a chamber (Coy Laboratory Products, Ann Arbor, MI). SDS-PAGE was performed according to Laemmli (28). After electrophoresis, the separated protein bands were stained with Coomassie Brilliant Blue. A protein marker kit for the molecular mass estimation was purchased from New England Biolabs Inc.

**Spectral Analysis**—The steady-state fluorescence emission spectra and absorption spectra were measured with a spectrofluorophotometer RF-1500 (Shimadzu) and spectrophotometer UV-3101PC (Shimadzu), respectively. The cells were suspended in 40 mM Mops buffer (pH 7.0) in an anaerobic chamber and placed in an air-tight anaerobic cuvette. The concentrations of the samples were adjusted to give an absorbance of 0.5 at 750 nm when measuring the fluorescence spectra. The time-resolved fluorescence spectra and kinetics were measured by a streak scope (Hamamatsu Photonics, Hamamatsu) with an excitation flash from a laser diode of 50-ps full width at half-maximum intensity operated at 1 MHz (Hamamatsu Photonics, Hamamatsu). The fluorescence excited by a laser beam was focused onto the entrance slit of the 30-cm monochrometer and dispersed depending on the wavelength inside the monochrometer along the horizontal axis. An image of the spectrum of the fluorescence at the exit slit of the monochrometer then excited the image intensifier at the entrance of the streak camera. A number of electrons produced by each photon in the image intensifier was biased by a time-dependent electric field along the vertical axis with respect to the delay of arrival and made an image on the phosphor plate. The images of each photon, whose location was biased due to

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were purified by Cui et al. their previous study (13). The ferredoxin and FNR from Sakurai (Waseda University) (13). This ferredoxin was named FdC in C. tepidum from chlorosomes were prepared as previously described (10). The compositions reported by two research groups (13, 14). Membranes free n filter with a bandwidth of 10 nm.

wavelength along the x axis and the arrival time along the y axis, were captured by a CCD camera. The location of the trace of each photon on each image of CCD was counted and accumulated in the memory of a computer as shown in Fig. 6A.

The ESR spectra were recorded using a Bruker ESP-300 EPR spectrometer equipped with a standard resonator (TE102). A gas flow temperature control system (CF935; Oxford Instruments) was used. Samples for measurements were chlorosome-free membranes prepared by disruption of cells and subsequent centrifugations before solubilization with detergents (10).

NADP\(^+\) Photoreduction—The rates of ferredoxin-mediated NADP\(^+\) photoreduction were measured using membrane preparations (A\(_{450} = 0.7\), corresponding to ~3 \(\mu\)g of BChl a/ml) in a 1-ml reaction mixture containing 50 mm Tris-HCl (pH 7.8), 10 mm MgCl\(_2\), 8 mm sodium ascorbate, 100 \(\mu\)M 2,6-dichloroindophenol, 50 \(\mu\)M 2,3,5,6-tetramethyl-p-phenylenediamine, 0.05% n-dodecyl-\(\beta\)-maltoside, 5 mm glucose, 2 units of glucose oxidase, 20 units of catalase, 0.5 mm NADP\(^+\), 0.2 \(\mu\)M FNR, and various concentrations of ferredoxin, basically according to the compositions reported by two research groups (13, 14). Membranes free from chlorosomes were prepared as previously described (10). The C. tepidum ferredoxin was a generous gift of Dr. D. Seo and Prof. H. Sakurai (Waseda University) (13). This ferredoxin was named FdC in their previous study (13). The ferredoxin and FNR from Spirulina sp. were purified by Cui et al. (29). Reduction of NADP\(^+\) was measured by monitoring absorption changes at 340 nm with a UV-3101PC spectrophotometer (Shimadzu) and a combination of appropriate filters. The samples were illuminated by actinic light with wavelengths longer than 490 nm. The photomultiplier was protected by a 340-nm interference filter with a bandwidth of 10 nm.

RESULTS

Construction of the PscD-less Mutant—The pscD gene was inactivated by insertion of the aadA streptomycin/spectinomycin resistance cassette (Fig. 1) as described in Ref. 27. Southern hybridization analysis verified the insertion of a 2.0-kb aadA marker into the genome of the mutant (Fig. 2). A pscD probe hybridized with a 2.2-kb fragment in the wild-type and with a 4.2-kb fragment in the mutant. In addition, an aadA probe hybridized with a 4.2-kb fragment in the mutant but not with the wild-type genomic DNA. PCR analysis using pscDF and pscDR primers (Fig. 1) also exhibited a 2.0 kb longer fragment in the mutant than in the wild type (data not shown). These results indicated that the aadA cassette was introduced correctly into the targeted pscD gene.

The RC complexes were isolated from the mutant and wild-type strains according to the method described previously (27). The SDS-PAGE analysis (Fig. 3) clearly indicated the absence of PscD in the mutant strain. It is characteristic that no other subunits appeared to be affected prominently in their contents due to the deletion of PscD, suggesting that PscD would be loosely associated with and/or functionally independent of other subunits within the Chlorobium RC complex.

Growth of the \(\Delta\)pscD Strain—Growth rates were measured under high and low light conditions (Fig. 4). At a high light intensity of 164 \(\mu\)mol photons/m\(^2\)/h, both the wild-type and mutant cells showed the same rates of photosynthetic growth. On the other hand, at a low light intensity of 2.5 \(\mu\)mol photons/m\(^2\)/h, the growth rate of the \(\Delta\)pscD mutant strain was almost the same as that of the wild-type strain until their culture turbidity, measured at 660 nm (OD\(_{660}\)), reached 0.5. However, the former growth rate declined to about one-third beyond OD\(_{660}\) = 0.5, whereas the latter continued to grow at the same rate. A possible explanation of this rate transition during growth is that the light intensity penetrating into the interior portion of the culture would decrease far below 2.5 \(\mu\)mol photons/m\(^2\)/h after the culture finally became turbid. Therefore, these results suggested that the \(\Delta\)pscD mutant strain was incapable of collecting extremely low light energy at full efficiency, probably due to some damage in its antenna system. Another possibility might be that a subtle difference in some metabolic activity might be amplified during their growth, resulting in the delayed growth of the \(\Delta\)pscD mutant strain compared with the normal one of the wild-type strain.

Absorption and Fluorescence Spectra at Room Temperature—Absorption spectra of the wild-type and mutant cells are compared with the normal one of the wild-type strain. On the other hand, at a low light intensity of 2.5 \(\mu\)mol photons/m\(^2\)/h, the growth rate of the \(\Delta\)pscD mutant strain was almost the same as that of the wild-type strain until their culture turbidity, measured at 660 nm (OD\(_{660}\)), reached 0.5. However, the former growth rate declined to about one-third beyond OD\(_{660}\) = 0.5, whereas the latter continued to grow at the same rate. A possible explanation of this rate transition during growth is that the light intensity penetrating into the interior portion of the culture would decrease far below 2.5 \(\mu\)mol photons/m\(^2\)/h after the culture finally became turbid. Therefore, these results suggested that the \(\Delta\)pscD mutant strain was incapable of collecting extremely low light energy at full efficiency, probably due to some damage in its antenna system. Another possibility might be that a subtle difference in some metabolic activity might be amplified during their growth, resulting in the delayed growth of the \(\Delta\)pscD mutant strain compared with the normal one of the wild-type strain.

Absorption and Fluorescence Spectra at Room Temperature—Absorption spectra of the wild-type and mutant cells are

![Fig. 1. Construction of a PscD-less mutant of C. tepidum.](image)

![Fig. 2. Southern hybridization analysis of HincII-digested genomic DNAs isolated from the wild-type (lanes 1 and 3) and mutant (lanes 2 and 4) strains of C. tepidum.](image)

![Fig. 3. SDS-PAGE analysis of the isolated RC complexes stained by Coomassie Brilliant Blue. Lanes M, WT, and D represent molecular weight markers, RC isolated from the wild type strain, and RC isolated from the PscD-less mutant strain, respectively.](image)
shown in Fig. 5A. They showed similar absorption spectra with high amounts of chlorosome BChl c pigments, although the absorption peak of chlorosome in the mutant cells was blue-shifted only by 3–4 nm. The contents of BChl c and BChl a did not show a significant change in the wild-type and mutant cells.

Steady-state fluorescence emission spectra were measured in the wild-type and mutant cells at room temperature (Fig. 5B). Both types of cells exhibited emission peaks at 768 and 807 nm, which were derived from BChl c and BChl a, respectively. The spectrum with the high 807-nm BChl a peak in Fig. 5B was somewhat different from that reported in the isolated chlorosomes, which gave higher BChl c fluorescence compared with that of BChl a in the baseplates (19). The major portion of the 807-nm BChl a peak was thus ascribable to the fluorescence derived from BChl a in FMO protein instead of the baseplate BChl a in chlorosomes. On the other hand, the 768-nm peak was ascribable to that derived from BChl c aggregates in chlorosomes. In the ΔpseD cells, the emission peaks of both BChl c and a were larger than those in the wild type when measured at the same turbidity of OD$_{750} = 0.5$. The ratio of the 768- to 807-nm fluorescence intensity was also larger in the ΔpseD strain. These results implied that the light energy absorbed by chlorosomes and FMO proteins was transferred to P840 less efficiently in the mutant.

Time-resolved Analysis of Fluorescence Spectra at 77 K—The time-resolved fluorescence spectra were measured in the picosecond time scale at 77 K in the presence of excess dithionite to determine the efficiency of the energy transfer from chlorosomes to the RC. Fig. 6A shows a two-dimensional (wavelength-time) image of the fluorescence decay in the wild-type cells of C. tepidum. The emission peaks detected at 785 and 825–829 nm (points a–d in Fig. 6A) at 77 K were ascribable to those of BChl c in chlorosomes and BChl a in the baseplate and FMO protein, respectively. The fluorescence peak in FMO protein is known to shift to the longer-wavelength side upon cooling to 77 K; therefore, the 829-nm peak was ascribable to the emission from FMO protein (19). After the selective excitation of BChl c in chlorosomes with a 645-nm laser flash of 50-ps width, the BChl c emission band at 785 nm rose first (point a in Fig. 6A), and then the BChl a one at 825 nm increased, indicating the energy transfer to the baseplate, as shown by point b (the fastest energy migration inside BChl c aggregates was not resolved with the present experimental time resolution). Fig. 6A further shows that the BChl a peak at 825 nm then shifted to 829 nm within 100–300 ps (point c), showing the energy transfer to the longer wavelength BChl a molecules in FMO protein. The fluorescence from FMO protein decayed slowly, presumably because of its equilibrium with the slow energy dissipation in the RC (point d). These measurements were performed in the presence of excess dithionite, whose condition suppressed the fluorescence quenching by quinones in chlorosomes (30) and allowed only a primary electron transfer process, such as the reversible reduction of a primary acceptor $A_A$ by $P840$ in the RC. The time-resolved spectra in Fig. 6B, calculated from slices along the horizontal wavelength axis of the two-dimensional image in Fig. 6A, clearly indicate the energy transfer processes mentioned above. The decay kinetics of the fluorescence at 785 and 830 nm were also calculated from slices along the vertical axis of the two-dimensional image in Fig. 6A (see Fig. 6C). The kinetics exhibited the fast decay of the BChl c fluorescence and the slow rise and decay of the BChl a fluorescence. All of these results indicate the fast energy transfer from BChl c aggregates to the RC via BChl a molecules in chlorosome baseplate and FMO protein, as has been reported elsewhere (19).

On the other hand, in the ΔpseD cells, the energy transfer process occurred in a similar sequence, but both the fluorescence from BChl c and BChl a gave slower decay kinetics than those in the wild-type cells (Fig. 6D). After the fast rise of the fluorescence at 785 nm (point a), the BChl c fluorescence decayed more slowly. The rise of the BChl a fluorescence was
biphasic, with a fast rise of the 825-nm fluorescence (point b) and a subsequent slow increase of the 829-nm band (point c), followed by a very slow decay (point d). The shift of the peak from 825 to 829 nm with a slight increase of intensity is clearly observable. The BChl α fluorescence band at 829 nm remained even at 800 ps, as shown in the time-resolved spectrum (Fig. 6). The decay kinetics at 830 nm (Fig. 6F) also indicates clearly the biphasic rise and the slower decay of the BChl α emission in the ΔpscD cells.

Fig. 6F shows that the emission intensity at 785 nm (BChl c) in the ΔpscD strain decayed biexponentially with time constants of 0.27 and 0.61 ns (with relative intensities at time 0 of 2.86 and 0.74, respectively, as shown in Table I). These time constants were slower than those of their counterparts in the wild type, which were estimated to be 0.14 and 0.35 ns with initial intensities of 2.82 and 0.35, respectively. In the same way, the emission intensity at 830 nm (BChl α) in the ΔpscD strain decayed with a time constant of 1.87 ns with a relative intensity of 0.65, which was also slower than the corresponding ones in the wild type. The 830-nm fluorescence from the wild type showed biphasic decay with time constants of 0.34 and 1.39 ns with relative intensities of 0.35 and 0.20, respectively. These results clearly indicated that the energy transfer from FMO protein to the RC in the mutant cells became slower, as judged from the slower decay rate of the BChl α emission intensity. The longer decay times of BChl c in the mutant cells, on the other hand, were rather strange, since the deletion of PscD was not expected to affect the energy transfer process in the interior of chlorosomes.

The difference in the kinetic features of the fluorescence in the ΔpscD strain was rather difficult to demonstrate with only the data obtained by the conventional steady-state fluorescence measurement system, as shown in Fig. 5B. When all of the fluorescence components detected within 5 ns in Fig. 6 were summed up, the sum gave almost the same heights of 785- and 830-nm fluorescence in both wild-type and mutant cells, with a slightly stronger integrated intensity in the mutant sample, as seen at room temperature in Fig. 5B (data not shown). The time-resolved fluorescence measurements at low temperature provide useful information with regard to the efficiency of the energy transfer.

Effects on the NADP⁺ Photoreduction Rate by Deletion of PscD—The rates of ferredoxin-mediated NADP⁺ photoreduction were measured with chlorosome-depleted membranes prepared from the wild-type and ΔpscD cells (Fig. 7). The deduced $V_{\text{max}}$ values of the reactions using the wild-type and ΔpscD membranes were estimated to be 633 and 503 μmol/mg BChl α/h, respectively. The affinities of C. tepidum Fd for the wild-type and mutant RC were also estimated to be $K_m = 0.39$ and 0.44 μM, respectively. Therefore, the PscD-less RC exhibited a small decline in NADP⁺ photoreduction activity in comparison with the wild-type one. Four Fds have been isolated from C. tepidum (FdA–FdD) (13). In our heterologous assay systems, various concentrations of C. tepidum FdC and an almost saturating amount of FNR from the cyanobacterium Spirulina sp. were used. Seo et al. compared the activity of the C. tepidum Fds with that of spinach Fd by using isolated Chlorobium RC and spinach FNR and demonstrated that even heterogeneous Fds could be substituted for authentic ones when measured in the in vitro assay system of NADP⁺ photoreduction using the Chlorobium RC (13). In fact, we also measured the activity of Spirulina Fd and found that the Spirulina Fd also showed an activity similar to that of Chlorobium FdC (data not shown).

Properties of Iron-Sulfur Centers $F_A$ and $F_B$—The effect of the absence of PscD on the resonance parameters of centers $F_A$ and $F_B$ was investigated by measuring low temperature ESR spectra using membranes isolated from the wild-type and ΔpscD cells. The magnetically interacting signals between centers $F_A$ and $F_B$ were detected after illumination during freezing to a cryogenic temperature as shown in Fig. 8A. The obtained spectral shapes were basically identical to those reported previously in C. tepidum, C. viciosiforme, and C. limicola as well (31–34). Although the signals in the resonance region at around $g_z = 2.07$ were extremely noisy, the interacting spectra detected at a high magnetic field were clear enough for a comparison of the wild-type and mutant strains. Their $g$ values
as well as line widths were thus found to be completely consistent with each other regardless of the deletion of the intrinsic PscD subunit from the RC complex.

On the other hand, the profiles of the signal intensities when the temperature was changed were somewhat different between the wild-type and mutant strains (Fig. 8B; normalized at 26.6 K). The signal intensity at 1.89 observed in the wild-type strain was maximal at 8 K, whereas the temperature in the case of the mutant strain was found to shift slightly to a higher temperature, namely, at 12 K. Therefore, these results indicate that the absence of PscD never induced any conformational change and/or distortion of centers FA and FB in PscB but slightly altered the environments surrounding their centers to give different relaxation rates.

**DISCUSSION**

*Functions of the PscD Subunit—*Genetic manipulation has never been conducted on the homodimeric type I RC of green sulfur bacteria, in contrast to the large numbers of mutagenesis reports on the heterodimeric RCs. *C. tepidum* has recently been demonstrated to be naturally transformable and available for a homologous recombination method (21). We here generated a mutant strain lacking the PscD subunit by inserting the aadA cassette into the pscD gene. This accomplishment will be expected to become an initial step for a study on the energy conversion mechanism in the homodimeric RC. Since the amino acid sequences of PscD have been shown to retain a weak similarity to those of the PsaD of photosystem I, the former function had been speculated to stabilize the *Chlorobium* RC (12). However, the absence of PscD did not raise any alteration in the contents of other constituent subunits of the RC complex (Fig. 3). The low temperature ESR measurements also proved that there were no changes in the structures of iron-sulfur clusters FA and FB, because their respective g values were identical in the wild-type and mutant strains. Instead, a slight modification of environments surrounding FA/FB clusters was suggested, as judged from the different temperature dependence of their signal intensities (Fig. 8). These results indicate that PscD resides in the vicinity of PscB, which

**TABLE I**

Comparison of fluorescence decay components measured at 785 nm (BChl c) and 830 nm (BChl a) at 77 K between the wild-type and ΔpscD mutant cells of *C. tepidum*

Fluorescence decay times and relative amplitudes at indicated wavelengths were calculated from the data obtained in Fig. 6. The values in parentheses are the fluorescence decay times.

| Cells                  | Relative amplitude (fluorescence decay times) |
|------------------------|---------------------------------------------|
| Wild-type cells        |                                             |
| 785 nm (BChl c)        | 2.82 (136 ps)                               |
| 830 nm (BChl a)        | 0.35 (347 ps)                               |
| ΔpscD mutant cells     |                                             |
| 785 nm (BChl c)        | 2.86 (271 ps)                               |
| 830 nm (BChl a)        | 0.74 (612 ps)                               |

**FIG. 7.** Activity of NADP⁺ photoreduction mediated by various concentrations of ferredoxins. Membrane preparations from the wild-type (circles) and mutant (triangles) cells were used. The reduction of NADP⁺ was measured by monitoring absorption changes at 340 nm. See “Experimental Procedures” for the details of the measurements.

**FIG. 8.** A, low temperature ESR spectra of membranes isolated from wild-type (WT; solid line) and PscD-less mutant (broken line) cells. Samples were once frozen to 4 K after illumination during freezing. B, temperature dependence of the signal intensities at g = 1.89 of the wild-type (filled squares) and mutant (open circles) membranes.
contains FA/FB clusters on the cytoplasmic side of RC, and that this topology appears to be similar to the case of PsaD in photosystem I.

A time-resolved fluorescence measurement demonstrated that the excited state remained longer on BChl \( \alpha \) in FMO in the \( \Delta \)pscD cells (Fig. 6). PscD is therefore essential for the efficient energy transfer from chlorosomes to the RC complex via FMO protein. This interpretation is in agreement with the previous results obtained by an STEM imaging analysis, which suggests the contact of PscD with FMO protein (11). The slower decay of the BChl \( \alpha \) fluorescence represents the slower energy transfer rate from FMO protein to RC and thus implies some modification of the interface between them. The slower decay kinetics of the BChl \( \alpha \) fluorescence in the mutant seems to be the indirect effect of the energy equilibration between the BChl \( \alpha \) molecules on the baseplates and FMO proteins. A rational explanation would be that the slower energy dissipation from FMO protein slowed down the emission decay of baseplate BChl \( \alpha \), resulting in the apparently slower decay of BChl \( c \). Another probable explanation might be that the energy transfer rate between the baseplate and FMO protein was also slowed down by some physical distortion in them, which was induced secondarily by the mutation affecting the allocation and/or orientation of FMO protein on the surface of the RC. A preliminary electron microscopic observation, however, revealed that the wild-type and mutant cells had chlorosomes with the same size and structure (data not shown).

The suppression of the energy transfer between FMO and RC was partial, allowing for an almost normal growth rate of the mutant cells under high light conditions (Fig. 4). Contrary to this, the inefficient energy transfer exhibited in the PscD-less mutant became obvious, showing a much slower growth rate under low light intensities. This situation is almost the same as that reported in mutants deficient in BChl \( c \) biosynthesis (26,

### Table II
Comparison of antenna sizes of the RC and membrane preparations from green sulfur bacteria

| Preparations         | BChl a/RC (BChl a/P840) | FMO/RC | Reference/source |
|----------------------|--------------------------|--------|-----------------|
| C. tepidum RC       | 47                       | 4.4    | Ref. 37         |
| C. tepidum RC       | 50                       | 4.9    | Ref. 12         |
| C. tepidum membranes| 50                       | 4.9    | Ref. 38         |
| C. tepidum membranes| 49                       | 4.7    | This work       |
| \( \Delta \)pscD membranes | 43               | 3.9    | This work       |
| C. tepidum RC-core  | 16                       | 4.7    | Ref. 38         |
| C. tepidum RC-core  | 16                       | 4.7    | Ref. 39         |
| P. aestuarii RC-core| 16                       | 4.7    | Ref. 39         |

* The number of FMO monomers per RC (P840) is obtained by dividing the excess over 16 by 7.

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Fig. 9. A, alignment of the amino acid sequences of PsaD and PscD. Identical amino acid residues are marked by asterisks. *Tepidum*, PscD of *Chl. tepidum*; *limicola*, PscD of *Chl. limicola*; *PCC6803*, PsaD of *Synechocystis* sp. *PCC 6803*; *vulcanus*, PsaD of *Synechococcus vulcanus* and *S. elongatus*; *Nostoc*, PsaD of *Nostoc* sp. *PCC 8009*. B, structural homology modeling of PscD of *C. tepidum*. The modeling was conducted using GeneMine software (41) with the coordinate data of the structure of PsaD in photosystem I RC of *S. elongatus* (2). The predicted structure of PscD was represented by a green ribbon. PsaA and PsaB were colored in white and gray, respectively. PsaC and PsaD were represented by purple and cyan ribbons, respectively.
35). Green sulfur bacteria are obligate anaerobes and can therefore thrive in natural habitats such as sulfide-rich lakes or sediments, where the light intensity is limited to almost below 0.1% compared with that in the water surface. In such environments, the huge antenna system, chlorosome, is very important to collect light energy enough to sustain their growth. PscD is thus essential for their optimal growth in their habitats due to its obligatory requirement for the efficient energy transfer from chlorosomes to RC.

In the present study, the PscD-less mutant also exhibited only partially decreased rates of ferredoxin-mediated NADP⁺ photoreduction when measured using membranes under saturated light conditions (Fig. 7). However, the role for PscD in the electron transfer reaction toFd appears to be additive and not too serious, even under dim light conditions.

STEM imaging analysis has suggested the contact of PscD with a trimer of FMO protein (11), which binds seven BCHλα molecules in each monomer (16). In this study, therefore, the deletion of PscD was expected to slightly affect the binding of FMO protein to the RC. Chlorosome-free membranes were prepared, and the molar ratio of BCHλα to P840 was estimated on the basis of the oxidized minus reduced difference spectra, as summarized in Table II. The ratio of BCHλα/P840 in the membranes of wild-type cells was estimated to be 49, in agreement with previous reports (12, 36, 37), whereas the ratio obtained in the ΔpscD membranes was 43. These results suggest that the FMO proteins were partially detached from the mutant membranes during the preparation and that the low efficiency in the energy transfer between FMO protein and RC would reflect some distorted organization of FMO protein. The PscD subunit seems to be beneficial to a proper orientation of FMO protein against the surface of the RC.

**Evolutionary Implication of PscD in Chlorobium**—The amino acid sequences of the PscD subunit in Chlorobium exhibit relatively low but significant similarities as a whole to those of the PsAD subunit in photosystem I (Fig. 9A). Their polypeptides have almost the same numbers of amino acid residues (e.g. PscD of *C. tepidum*, 143 residues; PsAD of *Synechocystis* sp. PCC 6803, 141 residues) and share similar distributions of charged basic residues, as previously mentioned by Hager-Braun et al. (12). This is in clear contrast to the case that the amino acid sequences of F₈₄/F₈₃ and core proteins show more divergent aspects between *Chlorobium* RC and photosystem I. The F₈₄/F₈₃-containing PsB of *C. tepidum* consists of 231 amino acid residues, i.e. nearly 3 times the number of residues in its cyanobacterial counterpart, PsA (namely 81 residues in that of *Synechocystis* sp. PCC 6803). The former has an N-terminal extension of 130 residues, which is highly positive and shows a characteristic repetition of two consecutive alanine residues. Although they exhibit only 20% similarity to each other in their C-terminal region, which holds two [4Fe-4S] clusters, their evolutionary relationship is not clear at present. The sequence similarity between the core proteins of PsC and PsAv/PsaB is rather poor, being estimated at 14–15%. Moreover, their sequence alignment requires many insertions and deletions (not shown). On the other hand, PscC is a monoheme-type cytochrome c₅ that contains three membrane-spanning α-helices in its N-terminal moiety. This subunit neither has counterparts in photosystem I nor exhibits any homologies to c-type cytochromes so far reported.

Therefore, the appreciable similarities among the amino acid sequences of PsC and PsA/Psd, as indicated in Fig. 9A, suggest the interesting concept that they may be counterparts and thus evolutionarily related. The results of the present study seem to be partially in agreement with this idea, although the functions of PscD are not completely equivalent to those of PsA (i.e. the stabilization of F₈₄/F₈₃-containing PsC and the facilitation of electron transfer reaction to Fd). The absence of PscD did not destabilize the *Chlorobium* RC complex, including the F₈₄/F₈₃-containing PsB, but slightly decreased the NADP⁺ photoreduction activity compared with the almost complete inactivation of its activity in the PsA-less mutant of *Synechocystis* sp. PCC 6803 (36), as mentioned above. With the use of the coordinate data concerning the three-dimensional structure of the photosystem I RC complex, the homodimeric structure of the *Chlorobium* RC complex could be roughly depicted. The structural homology modeling of PscD, which is shown in Fig. 9B, was conducted on the basis of sequence alignment with PsAD of *Synechococcus elongatus* (2). The predicted structure of PscD in Fig. 9B indicates that its C-terminal portion containing a conserved lysine residue (corresponding to lysine 106 in PsA), which was considered to be involved in the direct interaction with Fd in photosystem I, surrounds the surface of the F₈₄/F₈₃ protein and that its exposed surface to solvent could be easily accessible by Fd. Since the C-terminal portion of PscD is also rich in charged amino acid residues, it seems nevertheless to regulate and/or enhance the reactivity with Fd. The N-terminal portion of PscD, on the other hand, would bind a trimer of FMO protein, although the structural information of F₈₄/F₈₃-containing PscB is still lacking.

Plants have no stromal antennae apparatus, but rather, they have peripheral light-harvesting membrane proteins (light-harvesting complexes I and II). Therefore, it is clear that PsAD, which locates on the stromal side of photosystem I, is not involved in the energy transfer process. In contrast, the PscD of *Chlorobium* RC was demonstrated in this study to serve mainly as a proper allocation and/or orientation of the FMO protein to mediate the efficient energy transfer between chlorosome and RC in addition to slightly enhancing the reactivity with Fd. These primitive functions of PscD might have been specialized exclusively to facilitate the electron transfer from RC to Fd along with a loss of the cytосolic antenna apparatus. This conversion would be related to the adaptation to higher light conditions and to the oxygenic atmosphere as well, which occurred in the course of evolution from the anaerobic homodimeric to aerobic heterodimeric type I RCs.

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PscD-less Mutant of C. tepidum