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Time-Resolved Spectroscopy of Chlorophyll-*a* like Electron Acceptor in the Reaction Center Complex of the Green Sulfur Bacterium *Chlorobium tepidum*

Masayo Iwaki [1], Shigeru Itoh [1], Shoichiro Kamei [2], Hiroshi Matsubara [3] and Hirozo Oh-oka [2]

[1] *National Institute for Basic Biology, Okazaki, 444-8585 Japan*
[2] *Department of Biology, Graduate School of Science, Osaka University, Osaka, 560-0043 Japan*
[3] *Department of Biochemistry, Faculty of Science, Okayama University of Science, Okayama, 700-0005 Japan*

The absorption changes of chlorophyll (Chl) *a*-like pigments (C670) were studied by ns-ms laser spectroscopy at 77 K in the untreated and urea-treated homodimeric reaction center (RC) complex of the green sulfur bacterium *Chlorobium tepidum*. The untreated RC complex contained 9 molecules of C670 in addition to 41 molecules of Bchl *a* and 0.9 molecules of menaquinone-7 per one primary electron donor Bchl *a* dimer (P840). Upon photo-oxidation of P840, C670 showed an absorption change of a red-shift with an isosbestic wavelength at 668 nm. The absorption change of P840 decayed with time constants ($t_{1/e}$) of 55 and 37 ms at 283 and 77 K, respectively, and was assigned to represent the charge recombination between $P840^+$ and FeS^-. In the urea-treated RC complex, a bleach peaking at 670 nm with a shoulder peak at 662 nm, which is ascribable to the reduced primary electron acceptor A_0^-, was detected after the laser excitation in addition to the shift at 668 nm indicating the formation of the $P840^+A_0^-$ state. The $P840^+A_0^-$ state decayed with a $t_{1/e}$ of 43 ns at 77 K and produced a triplet state $P840^T$ due to the suppression of the forward electron transfer. These results indicate the two different types of C670 species in the RC complex; the one peaking at 670 nm functions as A_0, while the other peaking at 668 nm shows the electrochromic shift, which presumably functions as the accessory pigment located in the close vicinity of P840.

Key words: Chlorophyll — Electron transfer — Green sulfur bacteria— Iron-sulfur cluster — Reaction center.

In photosynthetic organisms, solar energy is converted into electrochemical energy in the membrane-spanning reaction center (RC) pigment protein complexes. The RC complex of the green sulfur bacteria (*Chlorobiaceae*) shares common structural features with the photosystem (PS) I RC complex of plants and cyanobacteria or the heliobacterial RC complex (Schubert et al. 1998). These RC complexes, which are called FeS-type RCs or Type-I RCs, contain three 4Fe4S clusters as the terminal electron acceptors that mediate electron transfer to $NADP^+$ (see reviews by Feiler and Hauska 1995, Amesz 1995, Brettel 1997). Although the entire homology in the amino acid sequence of the PscA RC core protein of the green sulfur bacteria to that of the PsaA or PsaB RC core protein in PSI is relatively low (about 14–15%), the regions holding the 4Fe4S (F_X) clusters in these polypeptides exhibit high similarity (75%) to each other (Büttner et al. 1992). The RC complexes of green sulfur bacteria have a homodimeric structure, which is made of two identical PscA polypeptides (Büttner et al. 1992). In the case of the heliobacteria, two PshA polypeptides make up the homodimeric RC complex (Liebl et al. 1993). This provides a clear contrast to all the other RC complexes that are heterodimers of partially homologous polypeptides; PsaA/PsaB in PSI, PsbA/PsbD in PSII, and PufM/PufL in purple photosynthetic bacteria (Feiler and Hauska 1995, Amesz 1995).

The purified RC core complex of the *Chlorobium* is made of two PscA proteins that bind about 40–60 bacteriochlorophyll *a* (Bchl *a*) molecules, one F_X-type cluster, 6–9 molecules of C670, and two cytochromes c_z (Oh-oka et al. 1995a, b). "C670" in this paper denotes an isomer of chlorophyll *a* (Chl *a*), which has a Qy absorption band at 670 nm in vivo (van de Meent et al. 1992, Feiler et al. 1994). The total number of pigments contained in the *Chlorobium* RC core are smaller than that in the PSI PsaA/PsaB complex that binds about 90 Chl *a* molecules with two phylloquinones and one FeS cluster (F_X). Two additional 4Fe4S clusters exist on the peripheral PscB polypeptide in an analogous way to the F_A and F_B clusters in the PsaC polypeptide of PSI. We studied the electron transfer in the isolated RC complex of the thermophilic green sulfur bacterium *Chlorobium tepidum* (Oh-oka et al. 1995b) which contains five different polypeptides: two core polypeptides (PscA), an F_A/F_B protein (PscB), two cytochromes c_z (PscC), a 17–18 kDa protein (PscD) whose function is not yet known (Hager-Braun et al. 1995), and two or three antenna "FMO" proteins (Olson 1978, Li et al. 1997). Each FMO protein carries 7 Bchl *a* molecules that transfer light energy to the RC core. The RC preparation used in this study was confirmed to contain one molecule of menaqui-

Abbreviations: (B)Chl, (bacterio)chlorophyll; P840, the primary electron donor in the green sulfur bacterial reaction center; PS, photosystem; RC, reaction center.

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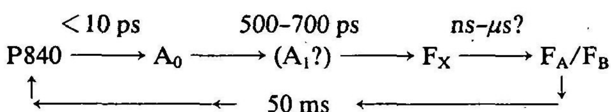
none-7 (Takaichi and Oh-oka 1999).

In the RC complex of green sulfur bacteria, an electron is sequentially transferred after the excitation of the primary donor P840 in a way analogous to that in PSI (Oh-oka et al. 1997, Kusumoto et al. 1994, 1995, Kjaer et al. 1994, Feiler and Hauska 1995, Brettel 1997) (see Fig. 1). When the electron transfer from cytochrome c_z to $P840^+$ is suppressed, the reduced $(F_A/F_B)^-$ re-reduces $P840^+$.



P840 is a special pair of Bchl *a* molecules (Sybesma and Vredenberg 1963) and the C670 species functions as the primary acceptor A_0 (van de Meent et al. 1992, Feiler et al. 1994). No evidence has been obtained as to the turnover of A_1 that might function if the electron transfer occurs as in PSI. The observed oxidation rate of A_0^- was 550–700 ps (Nuijs et al. 1985, Shuvalov et al. 1986) that is significantly slower than the 23 ps reaction time between A_0^- and A_1-phylloquione in the PSI RC (Kumazaki et al. 1994, Hastings et al. 1994). The slow reaction time and the pulse EPR analysis (van der Est et al. 1998) suggested that F_X is directly reduced by A_0^-. The purified RC preparations from *Chl. limicola* (Oh-oka et al. 1993) and from *Chl. tepidum* (Frankenberg et al. 1996), which seemed to lack quinones, were reported to be photoactive. These results provide a clear contrast to the situation in PSI in which the forward electron transfer from A_0^- to the FeS clusters was abolished by the extraction of phylloquinone (Itoh et al. 1987, Biggins and Mathis 1988) and fully recovered by the reconstitution of phylloquinone or its analogs (Iwaki and Itoh 1989). A possibility that a menaquinone-7 molecule functions as A_1 still seems to remain since a semiquinone-type EPR signal can be detected after photoaccumulation at low temperature in the RC complex of *Chl. vibrioforme* that contains about 2 molecules of menaquinone-7 (Kjaer et al. 1998).

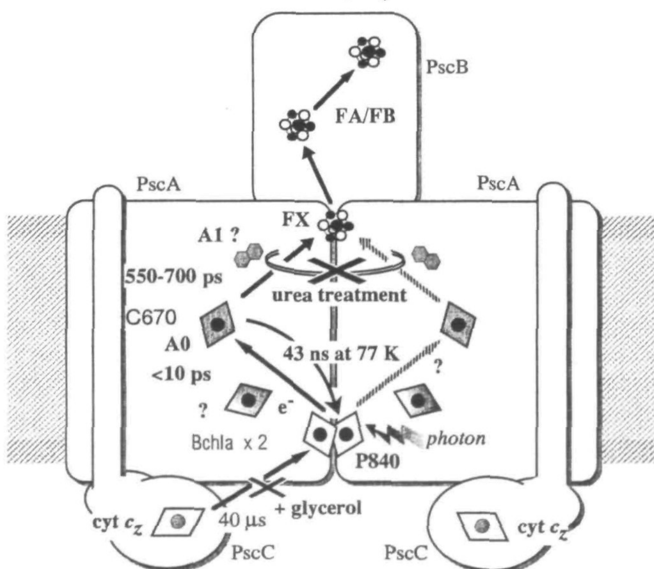


Fig. 1 Electron transfer scheme in the *Chlorobium* RC complex. Arrows indicate the putative electron transfer pathway with reported time constant ($t_{1/e}$) values (see Oh-oka et al. 1997, 1998 and a review by Feiler and Hauska 1995). The 43-ns reaction time at 77 K of A_0^- with $P840^+$ was obtained in this study. Cofactors inside RC are schematically drawn based on those in PSI RC (Schubert et al. 1997). Crosses represent the suppression of the electron transfer from A_0^- to FeS induced by the 7 M urea treatment and that from cytochrome c_z to $P840^+$ in the presence of glycerol. It is not clear whether the electron is transferred via cofactors on the left or right side, or on both sides between P840 and F_X (see text).

The question is how an electron is transferred in the homodimeric RC complexes. If we assume its structure to be analogous to that of PSI RC (Schubert et al. 1997), the RC is expected to have a symmetrical arrangement of cofactors; a pair of Bchl *a* molecules (P840), two branches of the accessory and acceptor Chls and presumably menaquinone-7. These cofactors as well as the protein environments in both branches will be identical in the homodimeric RC (see Fig. 1). It is not yet known whether the two branches of the cofactor lines are evenly active in the electron transfer or one branch is more active through some asymmetry produced by the asymmetrical arrangement of peripheral polypeptides or cofactors inside the complex. The precise determination of the electron transfer process through A_0 will tell us whether one or two A_0 molecule(s) function in this RC.

We studied the effect of anaerobic urea treatment on the electron transfer reactions of P840 and A_0 in the RC complex of *Chl. tepidum*. The F_A/F_B clusters in PSI have been shown to be selectively destroyed by the treatment with 6 M urea (Parrett et al. 1989) or by the 50°C-heat treatment with 50% ethylene glycol (Hoshina et al. 1990) and then the photo-reduced F_X^- reduces $P700^+$ with a 1 ms reaction time. The urea treatment in the presence of ferricyanide (Parrett et al. 1990) or the 60°C-heat-ethylene-glycol treatment (Hoshina et al. 1990) further destroys F_X and then the photo-produced $P700^+A_1^-$ state decays with a 0.1 ms decay time. The urea treatment prolonged the lifetime of A_0^- due to the removal of FeS clusters in the *Chl. tepidum* RC complex and allowed an analysis of the spectral features of A_0 and the other C670 molecules.

Materials and Methods

Sample preparation—The RC complex of *Chl. tepidum* was isolated as previously described (Oh-oka et al. 1995b). The RC complex contained 9 molecules of C670 in addition to 41 molec-

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ules of Bchl *a*, 7 molecules of carotenoids and 0.9 molecules of menaquinone-7 per one P840 (Takaichi and Oh-oka 1999). Two PscA, one PscB, two cytochromes c_z (PscC), two or three antenna "FMO" proteins, and one 17–18 kDa protein were contained in the RC complex. For the urea treatment, the stock solution of urea was added to the RC solution which had been concentrated by an ultrafiltration method (Millipore, UFC3THK00, 100000 MW-cut) to a final concentration of A_{810}=300–350. The stock solution of urea (9 M) was prepared by dissolving urea into the reaction medium (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM sucrose monolaurate and 2 mM dithiothreitol). After a 5-min incubation at various concentrations of urea, each reaction mixture was diluted about 50–200 times by the addition of the urea-free reaction medium containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM sucrose monolaurate, 2 mM dithiothreitol and 60% (v/v) glycerol. The sample solution was then transferred into an air-tight cuvette. Absorption spectra and the concentrations of Bchl *a* and C670 in the RC preparations were almost unchanged before and after the urea treatment. All procedures were performed at room temperature under anaerobic conditions.

Optical measurements—The transient absorption spectrum was measured with 3.5-ns time resolution by the apparatus that has a spectrograph made of a Jobin-Ybon 320 mm monochrometer with a gatable image intensifier-photodiode array detector (Princeton Instruments). The sample was excited by a 532-nm, 10-ns (FWHM) pulse light from a Nd-YAG laser (Quanta Ray, DCR-2-10) at a 0.05 Hz repetition rate. The probing light was obtained from a xenon flash of 3-μs duration through suitable band pass filters. The time sequence of the excitation flash, probing flash and the gating of the image-intensifier was controlled by a delay pulse generator (Stanford Research, DG5351). 64–256 signals from the diode array were averaged. Fluorescence from the sample excited by the actinic laser flash was also detected at the same geometry without a probing flash and was subtracted in the calculation of the time-resolved difference spectrum. Absorption and fluorescence emission spectra under the steady state were measured by the same apparatus as described above except that a continuous light from a tungsten-iodine lamp through appropriate glass filters was used as the probing and excitation light sources.

Transient absorption changes at selected wavelengths in the μs-ms time range were measured with a split-beam spectrometer with the probing light obtained by a combination of two monochrometers, a mechanical shutter and a 100 W tungsten-iodine lamp. The 532-nm excitation pulse from the Nd-YAG laser as stated above was also used. The sample in a cuvette with a 10-mm light path was placed either in a cryostat (Oxford DN904) at 77 K or in a temperature-controlled cuvette holder at 283 K during the measurements.

Results

Absorption and fluorescence spectra of C670 at 77 K—An absorption spectrum of the purified RC complex of *Chl. tepidum* at 77 K is shown in Fig. 2. Multiple peaks at 800–850 nm represent the Qy absorption bands of Bchl *a*. The primary electron donor, P840, partially contributes to the 840-nm peak. The Chl *a*-like pigment, C670, showed a Qy absorption band at 668 nm. A fluorescence emission spectrum of C670 excited by a continuous blue illumination light showed a peak at 673 nm with a shoulder at 669 nm and with a bandwidth wider than that of the absorption band. These results suggest the existence of multiple spectral forms for C670.

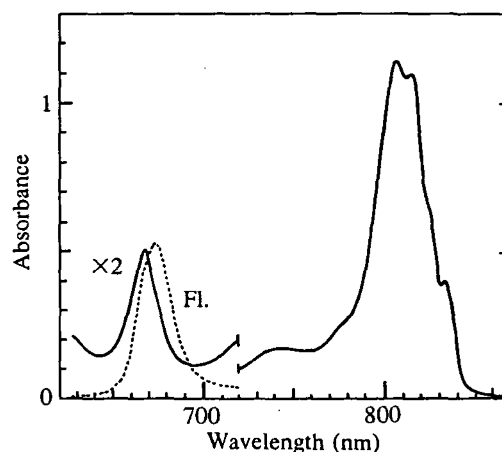

Fig. 2 Absorption spectrum of *Chl. tepidum* RC complex at 77 K. The amplitude of absorbance at 630–720 nm is twice expanded compared to that beyond 720 nm. The fluorescence emission spectrum at 630–720 nm at 77 K is also shown as a dotted line with an arbitrary unit. Fluorescence was measured by excitation with a continuous blue light from a tungsten-iodine-lamp through a glass filter (Corning 4-96). The fluorescence spectrum was not corrected for the spectral response of the detecting system.

Inactivation of the FeS clusters by urea treatment—In the isolated RC complex, laser excitation at 288 K induced a bleach at 830 nm due to the photo-oxidation of P840. The bleach recovered with a time constant ($t_{1/e}$) of 55 ms (Fig. 3A) when measured with an instrument response of 50 μs in the presence of 60% (v/v) glycerol. In the presence of glycerol, the electron donation from the bound cytochrome c_z to P840$^+$ was almost suppressed (Oh-oka et al. 1997) so that the 55-ms phase can be attributed to the reduction of P840$^+$ by the reduced F_A/F_B clusters. The extent of the 55-ms decay phase decreased to about 40% by the anaerobic 5 M urea treatment for 5 min and completely decreased by the 7 M urea treatment (Fig. 3A). When measured with faster 5-μs time resolution in the 5 M urea treated RC at 77 K (Fig. 3B), the faster decay phase with a $t_{1/e}$ value of 290 μs and the slower 37 ms phase were observed at 840 nm.

Difference spectra of the 290 μs and 37 ms decay phases at 77 K are shown in Fig. 3C. The slow phase showed the depletion of P840 ground state bands at 840 nm and at 600 nm. It is accompanied by shift-like absorption changes of Bchl *a* at 790–810 nm, and C670 at 660–680 and 430–460 nm. The 37-ms decay seems to reflect the recombination between P840$^+$ and the reduced acceptor such as F_X^- judging from the spectrum (Swarthoff and Amesz 1979) and the decay time (Oh-oka et al. 1997). We also detected a 37-ms decay phase of the EPR signal attributable to FeS$^-$ under similar conditions (unpublished result).

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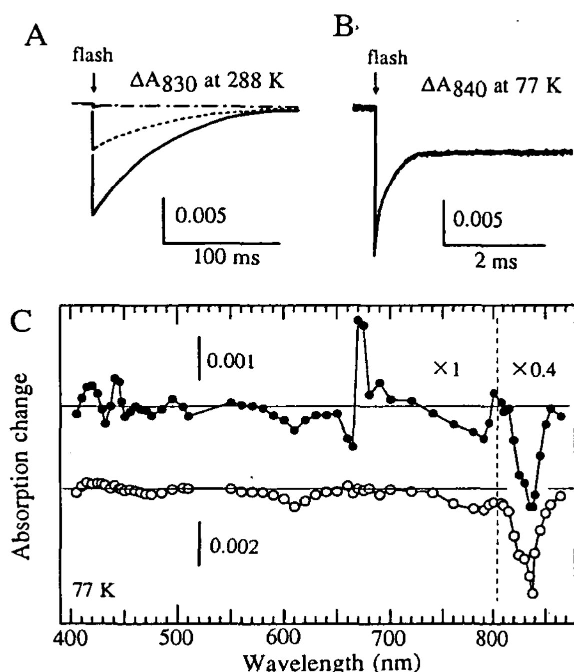

Fig. 3 Effects of urea-treatment on the electron transfer reactions in the RC complex of *Chl. tepidum.* (A) Time course of absorption change at 830 nm at 288 K measured with a 50-μs time resolution. Samples were untreated (solid line) and treated with 5 M (dotted line) or 7 M urea (dashed-and-dotted line), respectively. (B) Time course of absorption change at 840 nm at 77 K measured with a 5-μs time resolution in the 5 M urea treated RC complex. (C) Difference spectra of the slow ($t_{1/e}$=37 ms) and the fast ($t_{1/e}$=290 μs) decay phases in the 5 M urea treated RC complex at 77 K. Extents of the 37 ms and 290 μs phases were calculated from the measurements similar to those in B and expressed by the closed and open circles, respectively. Note that the vertical scale is different in each spectrum. The magnitudes of the spectra above 800 nm are reduced to 40%. The sample concentrations were adjusted to be A_{810}=1.5 at room temperature.

The 290-μs decay phase showed bleaches at 840 nm and 610 nm and lacked the shift-like signals. This phase can, therefore, be assigned to the decay of the triplet state ($P840^T$) produced in the charge recombination between $P840^+$ and A_0^- based on its decay time and the spectral features since the obtained $P840^+$/P840 and $P840^T$/P840 difference spectra resemble those measured earlier in a crude RC preparation isolated from *Prosthecochloris aestaurii* (Swarthoff and Amesz 1979). A larger $P840^T$ extent was detected in the 7 M urea treated sample that gave almost no slow decay phase in 830 nm at 288 K. Therefore, it is suggested that the urea treatment destroyed all the FeS clusters (and presumably the quinone-type acceptor, if it exists) and increased the charge recombination between $P840^+$ and A_0^- that produces $P840^T$ in less than a microsecond as will be shown in the next section.

Nanosecond time-resolved difference absorption spectrum at 77 K—The difference spectra of C670 in the Qy region in the nano to millisecond time ranges were further studied with a time resolution of 3.5 ns after an excitation with the 532-nm, 10-ns laser flash of almost saturating intensity (Fig. 4). In the untreated RC complex, the laser excitation induced a shift-like difference spectrum with negative and positive peaks at 666 nm and 670 nm, respectively (Fig. 4B). The amplitude of the absorption change decreased by 20% with a $t_{1/e}$ of 7 ns after the laser (see Fig. 5). The decrease is due to the decay of the overlapping bleach around 660 nm as seen from the comparison of the 15 and 40 ns spectra. The component with a rapid decay ($t_{1/e}$ of 7 ns) of the bleach can be assigned to the excited state of C670 that is responsible for the fluorescence with a 7-ns decay time measured at 77 K (Oh-oka et al. 1995a). The function of such C670 molecules that appear to be inactive in light harvesting remains to be studied. The spectral shape did not significantly change after 40 ns. The difference spectrum ((c) in Fig. 4B) can, therefore, be interpreted as an electrochromic shift of the C670 species responding to $P840^+$ that gives a bleach at 840 nm (not shown). It is thus estimated that forward electron transfer from A_0^- to the adjacent acceptor molecule was almost completed within the instrument response time as expected from the reported reaction time of 550–700 ps (Nuijs et al. 1985, Shuvalov et al. 1986).

In the 7 M urea treated RC complex, a broad bleach at 662 nm and a shift in the 666–671 nm region were generated by the flash and they decreased within 100 ns without a significant change in the spectral shape (Fig. 4B, 5). This indicates that formation of the $P840^+FeS^-$ (or $P840^+A_1^-$) state was suppressed in the urea-treated RC complex (see Fig. 1). A steep slope at 666–670 nm in the difference spectra suggests that these spectra also contained the $P840^+$-associated shift of C670.

Kinetics in the ns-ms time range—The time course of the absorption changes at 666–671 nm in the *Chl. tepidum* RC complex at 77 K is shown in Fig. 5. The difference between ΔA671 and ΔA666 (absorption changes at the positive and negative peaks of the shift) was plotted versus time. The extent in the untreated RC complex decayed by 20% with a time constant of 7 ns, but remained almost constant in the following 10 ms, and then decayed (with a $t_{1/e}$ of 37 ms as detected in Fig. 3). The first 7-ns decay component showed a broad bleach at 660 nm (see (b) in Fig. 4B) and, therefore, seems to represent the decay of the excited state of C670 that gives fluorescence with the 7-ns decay time at 77 K (Oh-oka et al. 1995a). The 37-ms phase showed a clear shift-like signal (see Fig. 3C, 4B) indicating its association with the re-reduction of $P840^+$.

In the 7 M urea treated RC complex, the amplitude of ΔA671–ΔA666 monotonously decayed mainly with a $t_{1/e}$ of 43 ns (Fig. 5). This time constant seems to represent the charge recombination between $P840^+$ and A_0^-. The spec-

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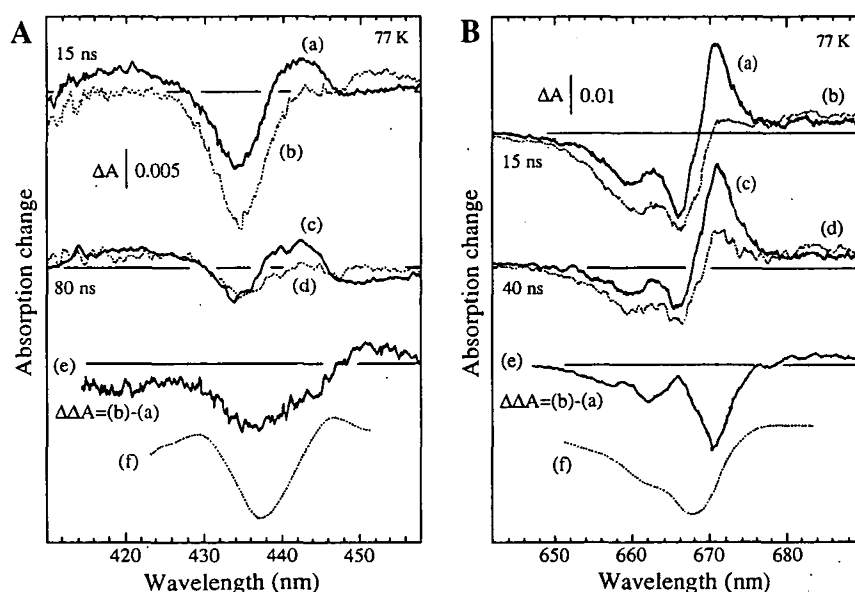

Fig. 4 Time-resolved difference absorption spectra of the untreated and 7 M urea treated *Chl. tepidum* RC complex at 77 K. A, in the Soret region. (a) and (b) at 15 ns in the untreated and urea-treated samples, respectively. (c) and (d), at 80 ns in the untreated and urea-treated samples, respectively. (e), difference between (a) and (b). (f), integration of spectrum (c). B, in Qy region. (a), and (b), at 15 ns in the untreated and urea-treated samples, respectively. (c) and (d), at 40 ns in the untreated and urea-treated samples, respectively. (e), difference between (a) and (b). (f), integration of spectrum (c). The vertical bars indicate the scales of ΔA and the horizontal lines indicate the zero levels. Integrated spectra were represented in arbitrary units. Indicated times are expressed as that from the peak of the 10-ns laser pulse. The sample concentrations were adjusted to be $A_{810}=6.5$ at room temperature.

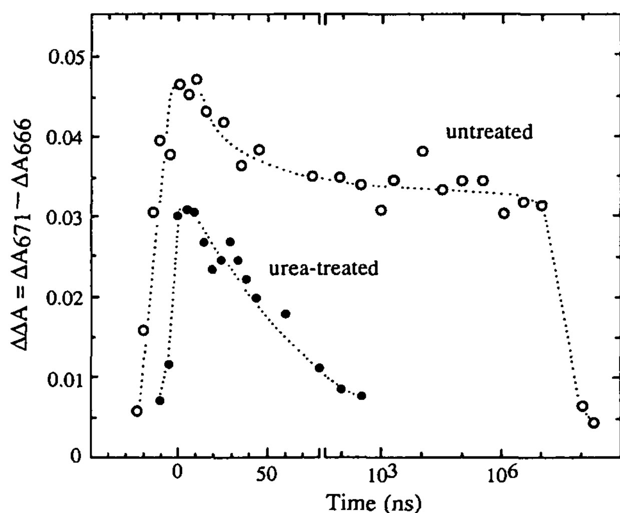

Fig. 5 Time courses of the absorption change of C670 at 77 K in the *Chl. tepidum* RC complex. The amplitudes were calculated as the difference between ΔA at 666 nm and ΔA at 671 nm to extract the time course of the red-shift or the bleach of C670 species. Open and closed circles represent the extents in the untreated and 7 M urea treated RC complexes, respectively. The original data were obtained from the experiments as in Fig. 4. The sample concentrations were adjusted to be $A_{810}=6.5$ at room temperature. Dotted lines are drawn simply as visual guides. Note that the horizontal time scale is changed from linear to logarithmic at 80 ns.

tra at 15 and 40 ns ((b) and (d) in Fig. 4B) are thus assumed to represent the $P840^+A_0^-$ state. The spectrum at 40 ns ((d) in Fig. 4B) was almost free from the contributions of fluorescence and bleach of the excited states. Its spectral feature resembles that of the absorption change measured at 130 ps at room temperature in the crude RC preparation of *Prosthecochloris aestaurii* (Nuijs et al. 1985) that also was assumed to represent the $P840^+A_0^-$ state. The width of the difference absorption bands is, however, significantly narrower in the present spectrum that was measured at low temperature with the wavelength accuracy of 1 nm.

Absorption change with ns-time resolution in the Soret region of C670—The time-resolved difference absorption spectra in the blue region were also measured after the ns laser excitation at 77 K. In the untreated RC complex, the spectrum at 15 ns showed a red-shift with negative and positive peaks at around 434 and 442 nm, respectively (Fig. 4A). It did not significantly change its shape and amplitude until 10 ms and decayed afterwards as seen in the Qy region of C670 (not shown). The shift can be attributed to the electrochromic shift of C670 associated with the $P840^+$ formation. In the 7 M urea treated RC complex, the shift appears to be overlapped with a broader bleach with a peak at 434 nm. Without significant change in the spectral shape, the amplitude decayed within 100 ns. These results confirmed the observation in the Qy region (Fig. 4B) and suggest that the urea treatment suppresses the stable

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charge separation between P840 and FeS, and elongates the decay time of A_0^- that gives a broader bleach at 434 nm.

Discussion

Upon the laser excitation of the RC complex of *Chl. tepidum*, electrons are expected to move from P840 to the F_A/F_B-type FeS clusters through A_0, (A_1), F_X and then come back to $P840^+$ in a cyclic manner when the rapid reduction of $P840^+$ by cytochrome c_z is suppressed in the presence of 60% (v/v) glycerol (Oh-oka et al. 1997, see Fig. 1). The $t_{1/e}$ value of the $P840^+$ re-reduction by FeS clusters was 55 ms at 283 K and 37 ms at 77 K in the *Chl. tepidum* RC complex. This is different from the situation in PSI RC in which A_1^- (phylloquinone$^-$) does not efficiently reduce F_X below 200 K and directly reduces $P700^+$ with a 200 μs $t_{1/e}$ (Itoh and Iwaki 1992, Schlodder et al. 1995).

In the urea treated RC, the photo-induced $P840^+A_0^-$ state decayed with a $t_{1/e}$ of 43 ns forming $P840^T$ that decayed with a $t_{1/e}$ of 290 μs at 77 K. The high yield of $P840^T$ as well as the long lifetime of A_0^- indicates the suppression of the electron transfer beyond A_0. It is different from the situations in PSI RC and the membrane of heliobacteria, in which simple urea treatment removes only F_A and F_B (Parrett et al. 1990, Kleinherenbrink et al. 1994). In the PSI RC, severer urea-ferricyanide treatment inactivates the A_1-phylloquinone and F_X (Parrett et al. 1990). The effect of urea treatment resembles that of NaI treatment performed in air on the *Chl. vibrioforme* membranes that removed FeS clusters and accelerated the recovery of the absorption change of P840 ($P840^+$ or $P840^T$) to give a $t_{1/2}$ value of 80 μs at 77 K from that of 20 ms in the untreated membranes (Miller et al. 1992). However, FeS clusters were only partially destroyed by the anaerobic treatments with a high concentration of NaI in *Chl. tepidum* reaction center (unpublished results).

The spectral red-shift measured at 15 ns in the Qy region in the untreated RC ((a) in Fig. 4B) can be assigned to the difference in the $P840^+FeS^-/P840FeS$ couple. The one at 15 ns in the 7 M urea treated sample ((b) in Fig. 4B) showed a bleach at 662 nm in addition to the similar red-shift at 670 nm, and can be assigned to the difference spectrum of the $P840^+A_0^-/P840A_0$ couple. Both spectra seemed to also contain a small amount of the excited states of C670 around 660 nm. Therefore, the difference between these two spectra should give the difference spectrum of the $P840^+A_0^-/P840^+A_0$ couple since FeS clusters will give no absorption changes around 670 nm. The difference spectrum thus calculated gave a peak at 670 nm with a shoulder at 662 nm (line (e) in Fig. 4B). A narrow 7-nm bandwidth at the half-height of the spectrum suggests that A_0 is a Chl *a*-like pigment in a monomeric form. An extinction coefficient of $P840^+A_0^-/P840^+A_0$ can be estimated to be 50–60 cm^{-1} mM^{-1} at 670 nm by assuming the extinction coefficient of P840 at room temperature to be 100 mM^{-1} (Olson et al. 1973). A similar calculation in the Soret region gave a peak at 435 nm for $P840^+A_0^-/P840^+A_0$ (line (e) in Fig. 4A).

The RC complex of *Chl. tepidum* in this preparation contains about 9 molecules of C670 (Takaichi and Oh-oka 1999). Therefore, the C670 molecules other than A_0 might also be responsible for the red-shift associated with $P840^+$. To obtain the spectral feature of the C670 species, the difference spectrum at 40 ns (line (c) in Fig. 4B) obtained in the untreated sample was integrated. The spectrum showed a peak at 668 nm as expected from the isosbestic wavelength of the shift, and a shoulder at 661 nm (line (f) in Fig. 4B). In the Soret region, an integration of the difference spectrum at 80 ns ((c) in Fig. 4A) gave a peak at 437 nm ((f) in Fig. 4B). The Chl molecules with peaks at 668 and 661 nm seem to sense the change in the local electric field induced by the positive charge on $P840^+$. The spectral shapes ((f) in Fig. 4A, B) are clearly different from that of $P840^+A_0^-/P840^+A_0$ ((e) in Fig. 4A, B) both in the Qy and the Soret regions. These results indicate that the *Chl. tepidum* RC complex contains two or three types of C670 species nearby P840, the one functions as A_0 with a peak at 670 nm and the other shows the red shift in response to $P840^+$. The $P840^+A_0^-/P840^+A_0$ difference spectrum in Fig. 4B might be a little different from the A_0^-/A_0 spectrum itself due to the contribution of the shift in the A_0 spectrum at the $P840^+A_0$ state.

In the RC complex of purple bacteria, accessory Bchls and bacteriopheophytins both in the active and inactive branches exhibit spectral shifts in response to the oxidation of the special pair P860. A similar pigment arrangement was shown in the core moiety of PSI RC (Schubert et al. 1997, 1998) and can be assumed in the core of *Chl. tepidum* RC (see Fig.1). The positive charge on $P840^+$ is, then, expected to induce the spectral shifts of the nearby pigments. If the pigments in *Chl. tepidum* RC are arranged at exactly symmetrical sites and two pigments in each pair have the same spectral and functional features, A_0 can be detected as a mixture of two C670 molecules peaking at 670 nm. The C670 molecule with a peak at 668 or 661 nm might then bind at the sites for the accessory pigments. If the RC is somewhat asymmetrical, we can assume the 670-nm form at the A_0 site in the active branch and the 668- or 661-nm form in the site for the accessory pigment or the counterpart of A_0 in the inactive branch. However, it is also noted that Bchls that show a shift in the 790–810 nm region in the difference spectrum of $P840^+/P840$ (see Fig. 3C) might exist nearby P840.

The results from this study indicate that the multiple forms of the C670 species exist in the *Chl. tepidum* RC, and one of them functions at the A_0 site and some at the sites for the accessory pigments. Photo-reduced A_0^- can be

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more stabilized by the urea treatment which suppresses the electron transfer from A_0 to F_X (see Fig. 1). The use of Chl *a* or its homologs as the primary acceptor is a common feature shared by the FeS-type RC complexes of green sulfur bacteria, heliobacteria (van de Meent et al. 1991) and PSI, although the electric field sensitive spectral shift of Chls has not been well separated from the absorption change of P700 in PSI. Determination of the locations and roles of C670 species in the homodimeric RCs of the green sulfur and heliobacterial photosynthesis will provide a key to understanding the evolution of photosynthesis.

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References

Amesz, J. (1995) The antenna-reaction center complex of heliobacteria. *In* Anoxygenic Photosynthetic Bacteria. Edited by Blankenship, R.E. pp. 687–697. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Biggins, J. and Mathis, P. (1988) Functional role for vitamin K1 in photosystem I of the cyanobacterium *Synechocystis* 6803. *Biochemistry* 27: 1494–1500.

Brettel, K. (1997) Electron transfer and arrangement of the redox cofactors in photosystem I. *Biochim. Biophys. Acta* 1318: 322–373.

Büttner, M., Xie, D.-L., Nelson, H., Pinther, W., Hauska, G. and Nelson, N. (1992) Photosynthetic reaction center genes in green sulfur bacteria and in photosystem 1 are related. *Proc. Natl. Acad. Sci. USA* 89: 8135–8139.

Feiler, U., Albouy, D., Pourcet, C., Mattioli, T., Lutz, M. and Blankenship, R.E. (1994) Structure and binding site of the primary electron acceptor in the reaction center of *Chlorobium*. *Biochemistry* 33: 7594–7599.

Feiler, U. and Hauska, G. (1995) The reaction center from green sulfur bacteria. *In* Anoxygenic Photosynthetic Bacteria. Edited by Blankenship, R.E. pp. 665–685. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Frankenberg, N., Hager-Braun, C., Feiler, U., Fuhrmann, M., Rogl, H., Schneebauer, N., Nelson, N. and Hauska, G. (1996) P840-reaction centers from *Chlorobium tepidum*-quinone analysis and functional reconstitution into lipid vesicles. *Photochem. Photobiol.* 64: 14–19.

Hager-Braun, C., Xie, D.-L., Jarosch, U., Herold, E., Büttner, M., Zimmermann, R., Deutzmann, R., Hauska, G. and Nelson, N. (1995) Stable photo-bleaching of P840 in Chlorobium reaction center preparation: presence of the 42-kDa bacteriochlorophyll *a* protein and a 17-kDa polypeptide. *Biochemistry* 34: 9617–9624.

Hastings, G., Kleinherenbrink, F.A.M., Lin, S. and Blankenship, R.E. (1994) Time resolved fluorescence and absorption spectroscopy of Photosystem I. *Biochemistry* 33: 3193–3200.

Hoshina, A., Sakurai, R., Kunishima, N., Wada, K. and Itoh, S. (1990) Selective destruction of iron-sulfur centers by heat/ethylene glycol treatment and isolation of photosystem I core complex. *Biochim. Biophys. Acta* 1015: 61–68.

Itoh, S., Iwaki, M. and Ikegami, I. (1987) Extraction of vitamin K1 from photosystem I particles by treatment with diethyl ether and its effects on the A_1^--EPR signal and system I photochemistry. *Biochim. Biophys. Acta* 893: 508–516.

Itoh, S. and Iwaki, M. (1992) Exchange of the acceptor phylloquinone by artificial quinones and fluorenones in green plant photosystem I photosynthetic reaction center. *In* Dynamics and Mechanisms of Photoinduced Electron Transfer and Related Phenomena. Edited by Mataga, N., Okada, T. and Masuhara, H. pp. 527–541. Elsevier, Amsterdam, The Netherlands.

Iwaki, M. and Itoh, S. (1989) Electron transfer in spinach photosystem I reaction enter containing benzo-, naphtho- and anthraquinones in place of phylloquinone. *FEBS Lett.* 256: 11–16.

Kjaer, B., Jung, Y.-S., Yu, L., Golbeck, J.H. and Scheller, H.V. (1994) Iron-sulfur centers in the photosynthetic reaction center complex from *Chlorobium vibrioforme*. Differences from and similarities to the iron-sulfur centers in Photosystem I. *Photosynth. Res.* 41: 105–114.

Kjaer, B., Frigaard, N.-U., Yang, F., Zybailov, B., Miller, M., Golbeck, J.H. and Scheller, H.V. (1998) Menaquinone-7 in the reaction center complex of the green sulfur bacterium *Chlorobium vibrioforme* functions as the electron acceptor A_1. *Biochemistry* 37: 3237–3242.

Kleinherenbrink, F.A.M., Chou, H.-C., LoBrutto, R. and Blankenship, R.E. (1994) Spectroscopic evidence for the presence of an iron-sulfur center similar to F_X of Photosystem I in *Heliobacillus mobilis*. *Photosynth. Res.* 41: 115–123.

Kumazaki, S., Iwaki, M., Ikegami, I., Kandori, H., Yoshihara, K. and Itoh, S. (1994) Rates of primary electron transfer reactions in Photosystem I reaction center reconstituted with different quinones as the secondary acceptor. *J. Phys. Chem.* 98: 11220–11225.

Kusumoto, N., Inoue, K., Nasu, H. and Sakurai, H. (1994) Preparation of a photoactive reaction center complex containing photoreducible Fe-S centers and photooxidizable cytochrome *c* from the green sulfur bacterium *Chlorobium tepidum*. *Plant Cell Physiol.* 35: 17–25.

Kusumoto, N., Inoue, K. and Sakurai, H. (1995) Spectroscopic studies of bound cytochrome *c* and an iron-sulfur center in a purified reaction enter complex from the green sulfur bacterium *Chlorobium tepidum*. *Photosynth Res.* 43: 107–112.

Li, Y., Zhou, W., Blankenship, R.E. and Allen, J.P. (1997) Crystal Structure of the bacteriochlorophyll *a* protein from *Chlorobium tepidum*. *J. Mol. Biol.* 271: 456–471.

Liebl, U., Mockensturn-W., M., Trost, J.T., Brune, D.C., Blankenship, R.E. and Vermaas, W. (1993) Single core polypeptide in the reaction center of the photosynthetic bacterium *Heliobacillus mobilis* structural implications and relations to other photosystems. *Proc. Acad. Sci. USA* 90: 7124–7128.

Miller, M., Liu, X., Snyder, S.W., Thurnauer, M.C. and Biggins, J. (1992) Photosynthetic electron-transfer reactions in the green sulfur bacterium *Chlorobium vibrioforme*: evidence for the functional involvement of iron-sulfur redox centers on the acceptor side of the reaction center. *Biochemistry* 31: 4354–4363.

Nuijs, A.M., Vasmel, H., Joppe, H.L.P., Duysens, L.N.M. and Amesz, J. (1985) Excited states and primary charge separation in the pigment system of the green photosynthetic bacterium *Prosthcochloris aestaurii* as studied by picosecond absorbance difference spectroscopy. *Biochim. Biophys. Acta* 807: 24–34.

Oh-oka, H., Kakutani, S., Matsubara, H., Malkin, R. and Itoh, S. (1993) Isolation of the photoactive reaction center complex that contains three types of Fe-S centers and a cytochrome *c* subunit from the green sulfur bacterium *Chlorobium limicola f. thiosulfatophilum*, strain Larsen. *Plant Cell Physiol.* 34: 93–101.

Oh-oka, H., Kakutani, S., Kamei, S., Matsubara, H., Iwaki, M. and Itoh, S. (1995a) Highly purified photosynthetic reaction center (PscA/cytochrome c_{551})$_2$ complex of the green sulfur bacterium *Chlorobium limicola*. *Biochemistry* 34: 13091–13097.

Oh-oka, H., Kamei, S., Matsubara, H., Iwaki, M. and Itoh, S. (1995b) 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*. *FEBS Lett.* 365: 30–34.

Oh-oka, H., Iwaki, M. and Itoh, S. (1997) Viscosity dependence of the electron transfer rate from bound cytochrome *c* to P840 in the photosynthetic reaction center of the green sulfur bacterium *Chlorobium tepidum*. *Biochemistry* 36: 9267–9272.

Oh-oka, H., Iwaki, M. and Itoh, S. (1998) Membrane-bound cytochrome c_z to couples quinol oxidoreductase to the P840 reaction center complex in isolated membranes of the green sulfur bacterium *Chlorobium tepidum*. *Biochemistry* 37: 12293–12300.

Olson, J.M., Philipson, K.D. and Sauer, K. (1973) Circular dichroism and absorption spectra of bacteriochlorophyll-protein and reaction center complexes from *Chlorobium thiosulfatophilium*. *Biochim. Biophys.*

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Acta 292: 206–217.

Olson, J.M. (1978) Bacteriochlorophyll *a*-proteins from green bacteria. *In* The Photosynthetic Bacteria. Edited by Clayton, R.K. and Sistrom, W.R. pp. 161–178. Plenum Press, New York.

Parrett, K.G., Mehari, T., Warren, P.G. and Golbeck, J.H. (1989) Purification and properties of the intact P-700 and F_X-containing Photosystem I core protein. *Biochim. Biophys. Acta* 973: 324–332.

Parrett, K.G., Mehari, T. and Golbeck, J.H. (1990) Resolution and reconstitution of the cyanobacterial photosystem I complex. *Biochim. Biophys. Acta* 1015: 341–352.

Schlodder, E., Brettel, K., Falkenberg, K. and Gergeleit, M. (1995) Temperature dependence of the re-oxidation kinetics of A_1^- in PSI. *In* Photosynthesis: form Light to Biosphere. Edited by Mathis, P. vol. II. pp. 107–110. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schubert, W.-D., Klukas, O., Krauß, N., Saenger, W., Fromme, P. and Witt, H.T. (1997) Photosystem I of *Synechococcus elongatus* at 4 Å resolution: comprehensive structure analysis. *J. Mol. Biol.* 272: 741–769.

Schubert, W.-D., Klukas, O., Saenger, W., Witt, H.T., Fromme, P. and Krauß, N. (1998) A common ancestor for oxygenic and anoxygenic photosynthetic systems: a comparison based on the structural model of photosystem I. *J. Mol. Biol.* 280: 297–314.

Shuvalov, V.A., Amesz, J. and Duysens, L.N., M. (1986) Picosecond spectroscopy of isolated membranes of the photosynthetic green sulfur bacterium *Prosthecochloris aestaurii* upon selective excitation of the primary electron donor. *Biochim. Biophys. Acta* 851: 1–5.

Swarthoff, T. and Amesz, J. (1979) Photochemically active pigment-protein complexes from the green photosynthetic bacterium *Prosthecochloris aestaurii. Biochim. Biophys. Acta* 548: 427–432.

Sybesma, C. and Vredenberg, W.J. (1963) Evidence for a reaction center P840 in the green photosynthetic bacterium *Chloropseudomonas ethylicum. Biochim. Biophys. Acta* 75: 439–441.

Takaichi, S. and Oh-oka, H. (1999) Pigment composition in the reaction center complex from the thermophilic green sulfur bacterium, *Chlorobium tepidum*: carotenoid glucoside esters, menaquinone and chlorophylls. *Plant Cell Physiol.* 40: 691–694.

Van der Est, A., Scheller, H.V., Hager-Braun, C., Hauska, G. and Stehlik, D. (1998) Forward electron transfer in *Chlorobium* reaction centers studied by transient EPR. *In* Photosynthesis: Mechanisms and Effects. Vol. I. pp. 547–550. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Van de Meent, E.J., Kobayashi, M., Erkelens, C., van Veelen, P.A., Amesz, J. and Watanabe, T. (1991) Identification of 8′-hydroxychlorophyll *a* as a functional reaction center pigment in heliobacteria. *Biochim. Biophys. Acta* 1058: 356–362.

Van de Meent, E.J., Kobayashi, M., Erkelens, C., van Veelen, P.A., Otte, S.C.M., Inoue, K., Watanabe, T. and Amesz, J. (1992) The nature of the primary electron acceptor in green sulfur bacteria. *Biochim. Biophys. Acta* 1102: 371–378.

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