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Energy equilibration and primary charge separation in chlorophyll *d*-based photosystem I reaction center isolated from *Acaryochloris marina*

Shigeichi Kumazaki^{a,*}, Kazuki Abiko^a, Isamu Ikegami^b, Masayo Iwaki^c, Shigeru Itoh^{d,*}

^aSchool of Materials Science, Japan Advanced Institute of Science and Technology, Tatsunokuchi, Ishikawa 923-1292, Japan

^bFaculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan

^cDepartment of Biology, University College London, London WC1E 6BT, UK

^dDepartment of Physics, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan

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Abstract Primary photochemistry in photosystem I (PS I) reaction center complex from *Acaryochloris marina* that uses chlorophyll *d* instead of chlorophyll *a* has been studied with a femtosecond spectroscopy. Upon excitation at 630 nm, almost full excitation equilibration among antenna chlorophylls and 40% of the excitation quenching by the reaction center are completed with time constants of $0.6(\pm 0.1)$ and $4.9(\pm 0.6)$ ps, respectively. The rise and decay of the primary charge-separated state proceed with apparent time constants of $7.2(\pm 0.9)$ and $50(\pm 10)$ ps, suggesting the reduction of the primary electron acceptor chlorophyll (A_0) and its reoxidation by phyloquinone (A_1), respectively.

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Key words: Chlorophyll *d*; Photosystem I; Reaction center; Photosynthesis; Charge separation; *Acaryochloris marina*

1. Introduction

Acaryochloris marina, a marine cyanobacteria-like prokaryote [1], undergoes oxygenic photosynthesis with chlorophyll *d* (Chl *d*), which absorbs at 700–740 nm, as the major Chl species and Chl *a* which usually absorbs at 660–700 nm as a minor pigment of 3–9% content [2–4]. The discovery of the electron donor Chl *d* absorbing at 740 nm (P740) in photosystem I reaction center complex (PS I RC) of this organism [3] has altered a long-accepted idea that pigments other than Chl *a* contribute to oxygenic photosynthesis only as antenna to harvest light energy [5]. In the oxygenic photosynthesis of plants and cyanobacteria, solar energy is known to be converted to chemical energy by the function of a special pair of Chl *a* or its isomer. The primary electron donor Chls have been known to be a special pair of Chl *a*, named P680, in PS II RC, and a pair of Chl *a* and its epimer Chl *a'*, named P700 in PS I RC [6,7]. Only one exception for this rule known

before the discovery of P740 has been divinyl-Chl *a* found in PS I and II of *Prochlorococcus marina* that has spectral features essentially identical to that of Chl *a* [8].

The PS I RC complex of *A. marina* employs Chl *d* both as the major antenna pigment and as the primary electron donor P740 [3]. *A. marina* seems to benefit from the use of Chl *d* that can absorb far-red light that is normally out of reach of all the other oxygenic photosynthetic organisms. The comparison of *A. marina* PS I with cyanobacterial/plant PS I gives us a unique chance to see the effects of natural Chl exchange in the PS I RC complexes that are made up of highly homologous PsaA/PsaB polypeptides [4]. This will shed light on the evolution of oxygenic photosynthesis from more primitive anoxygenic one that uses bacteriochlorophylls absorbing mainly near-infrared light of 800–900 nm.

The pigment composition of intact cells of *A. marina* was shown to be four Chl *a*, two pheophytin *a*, and two Chl *d'* per 140 Chl *d* [4]. In the isolated PS I complex of *A. marina*, 145 ± 8 Chl *d* and only a trace amount (about 0.8) of Chl *a* were found per P740 [3]. As for PS II, situation is not clear yet. From the fluorescence properties of intact cells of *A. marina*, Mimuro et al. [9,10] indicated that Chl *d* works as the major antenna both in PS I and II, and assumed that PS II has a special pair of Chl *a* based on the rather long lifetime of fluorescence of Chl *a*-like pigment [9,10]. Determination of the chemical identity of the electron donor pigment in PS II, however, still waits for the isolation of PS II RC that is well characterized for pigment composition and photochemistry.

This paper reports the dynamics of the energy transfer and primary charge separation in the purified *A. marina* PS I with a femtosecond time resolution. Dependence of transient absorption signals on the redox conditions of P740 revealed the transient spectra of the pigments that function in the primary charge separation.

2. Materials and methods

Isolation of the PS I complex from *A. marina* was carried out as previously [3]. The isolated *A. marina* PS I complex contains 145 ± 8 Chl *d* and 0.8 Chl *a* per P740, core and peripheral polypeptides [3]. The isolated complex was suspended in a reaction medium containing 50 mM Tris buffer (pH 7.5), 50 mM NaCl and 0.01% Triton X-100 and stored at 77 K. The stock solution was further diluted by 20 mM phosphate buffer (pH 8.0) with 50 mM NaCl and used for the measurements as PS I RC sample. P740 in the isolated PS I, usually in the oxidized state (P740-preoxidized conditions) [3], was reduced by the

*Corresponding author. Faxes: (81)-761-51-1149; (81)-52-789-2883.

E-mail addresses: kumazaki@jaist.ac.jp (S. Kumazaki),

itoh@bio.phys.nagoya-u.ac.jp (S. Itoh).

Abbreviations: PS I, photosystem I; PS II, photosystem II; P700, electron donor chlorophyll in plant/cyanobacterial PS I; P740, electron donor chlorophyll in *A. marina* PS I; Chl, chlorophyll; RC, reaction center; FWHM, full width at half maximum; IRF, instrument-response function; DAS, decay-associated spectrum; PB/SE, photobleaching and/or stimulated emission

addition of sodium ascorbate and phenazine ethosulfate to give final concentrations of 10 mM and 30 μ M, respectively (P740-prereduced conditions). The temperature of the sample was maintained at 5–10°C during all the treatments.

Transient absorbance changes were measured as described previously [11]. A femtosecond laser operating at 1 kHz provided both the pump pulses at 630 nm with a full width at half maximum (FWHM) of 9–10 nm and white light continuum pulses to probe the absorbance changes over 660–775 nm region. An optical Kerr cross correlation gave the instrument-response function (IRF) with a FWHM of 0.23–0.28 ps and the zero time was set at the peak of the IRF. The magic angle (54.7°) was employed in the polarization conditions. The spectra were measured at delay times from –0.5 to 300 ps, where the interval between the nearest delay times was gradually increased in a constant manner. The data later than 0.2 ps were analyzed by a global curve-fitting program without convolution of the IRF, yielding decay-associated spectra (DAS). Sum of exponential functions and a non-decaying component was always assumed.

The PS I RC sample was introduced under nitrogen gas atmosphere into a gas-tight cylindrical cuvette with a path length of 2.5 mm. The cuvette was maintained at about 280 K and rotated at a sufficient speed so that each pair of the pump and probe pulses interrogated a new position of the sample. The peak absorbance at the Q_y band of the sample was 0.80–1.05. The shift of the peak wavelength after each measurement was at most 1.0 nm. The single pulse energy was 35 ± 5 or 80 ± 10 nJ for the pump, and < 10 nJ for the probe. The diameters of the pump and probe beams at the sample positions were 0.16 and 0.12 mm, respectively. The extinction coefficient of Chl *d* at the peak of the Q_y band was reported to be $983 \text{ mM}^{-1} \text{ cm}^{-1}$ in diethylether [12]. Because there is a peak shift of about 10–30 nm in the Q_y band between organic solvents and *A. marina* [2], we assumed that an average extinction coefficient of Chl *d* at 630 nm in *A. marina* is given by the extinction coefficient at 610 nm of Chl *d* in organic solvents, which is about $10 \text{ mM}^{-1} \text{ cm}^{-1}$. With these conditions, the pump energy of 35 nJ was estimated to correspond to excitation of 1 Chl among about 50. Singlet–singlet annihilation may be thus unavoidable in PS I with about 145 Chl *d* with the employed pump energies. However, our following discussion should not be seriously affected by such artifacts, because it is based primarily on the comparison between *A. marina* and Chl *a*-based PS I (including our own control experiments on spinach PS I and many previous studies on Chl *a*-based PS I with similar excitation conditions), or between the P740-prereduced and -preoxidized conditions.

3. Results

Fig. 1A,B shows the transient absorption spectra of *A. marina* PS I RC under the P740-prereduced conditions upon the excitation at 630 nm. The photobleaching signal and/or stimulated emission of the Chl *d* (PB/SE) are detected mainly between 690 and 740 nm. Note that the IRF-limited rise of a signal is estimated to reach the maximum intensity at 0.2 ps after the peak time (0.0 ps) of the IRF. The global curve fitting analysis applied to the data as in Fig. 1 yielded the DAS in Fig. 2. At least three time constants were necessary to fit the data that were obtained under the P740-prereduced (0.6, 4.8 and 54 ps) or -preoxidized conditions (0.7, 8.0 and 59 ps) in this time range (Table 1). The stronger excita-

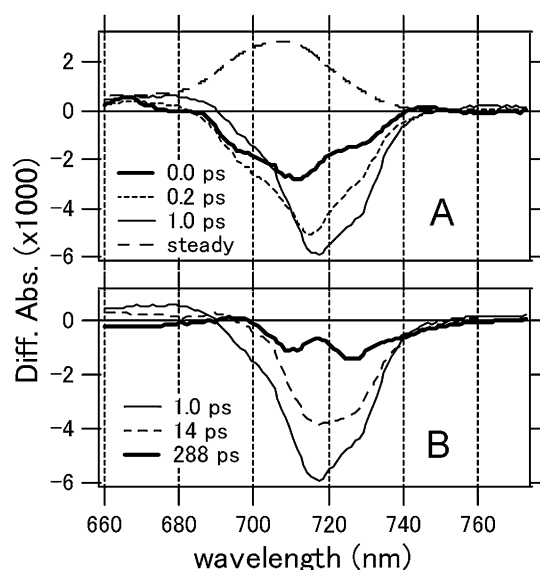


Fig. 1. Transient absorbance changes of *A. marina* PS I under the P740-prereduced conditions upon excitation at 630 nm at early (A) and late (B) delay times. The energy of the excitation pulse was 35 nJ. Steady state absorption spectrum with pre-reduced P740 after scaling by a factor of 1/330 is also shown in A.

tion pulse (80 nJ/pulse) that gave about twice larger amplitude of difference absorption changes induced 20–30% shortenings of the time constants of the slowest components presumably due to an annihilation quenching of the excitation as shown in Table 1 and slight change of the DAS features (not shown).

The difference spectrum of the transient spectra between the P740-prereduced and -preoxidized conditions, which we designate red-ox transient spectrum, should manifest the Chls involved in the electron transfer, as done in the past studies on cyanobacterial/plant PS I [13–16]. The red-ox transient spectra detected with the strong excitation pulses (80 nJ/pulse) are shown in Fig. 3A,B. The light induced reduced-minus-oxidized difference spectrum of P740 (P740⁺/P740 spectrum) obtained by a microsecond flash photolysis is also plotted in Fig. 3 [3]. The difference between the red-ox transient spectrum at 256 ps in Fig. 3B and the microsecond P740⁺/P740 spectrum is shown in Fig. 3C. The series of the red-ox transient spectra (as in Fig. 3A,B) was analyzed by the global curve fitting, which yielded at least two DASs with time constants of 6.3 ps and 40 ps plus one non-decaying component (Fig. 3D). The validity of the fitting was double-checked by calculating the difference between red-ox transient spectra at different delay times (Fig. 3E). The time dependence of the transient absorbance changes at 680 and 740 nm is shown in Fig. 4.

In the calculation of the red-ox transient spectra in Fig. 3,

Table 1
Time constants of the transient absorption changes obtained by the global curve analysis

Conditions of P740	Energy of pump pulse (nJ/pulse)	τ_1 (ps)	τ_2 (ps)	τ_3 (ps)
Prereduced	35	0.6 (± 0.1)	4.8 (± 0.3)	54 (± 4)
Preoxidized	35	0.7 (± 0.1)	5.1 (± 0.4)	57 (± 4)
Prereduced-minus-preoxidized	35		8.0 (± 0.6)	59 (± 6)
Prereduced	80	0.5 (± 0.1)	4.3 (± 0.3)	43 (± 3)
Preoxidized	80	0.7 (± 0.1)	5.5 (± 0.4)	46 (± 3)
Prereduced-minus-preoxidized	80		6.3 (± 0.6)	40 (± 5)

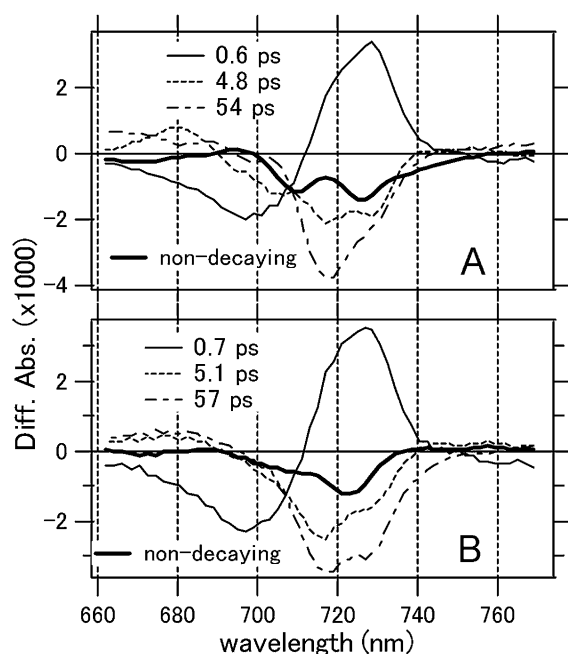


Fig. 2. DAS obtained for the *A. marina* PS I data as shown in Fig. 1. A: Under P740-prereduced conditions. B: Under P740-preoxidized conditions.

the difference between the P740-prereduced and -preoxidized samples was calculated after normalizing the amplitudes of the transient spectra at 719 nm and at 1.0 ps, which scaled the PB areas at 1.0 ps to be constant. This is rationalized by the observation that the area of the initial PB is proportional to the sufficiently weak excitation power and that there is little difference in the transient signals between the P740-prereduced and -preoxidized conditions on a subpicosecond time scale (Fig. 2).

4. Discussion

4.1. Fast spectral equilibration and trapping of excitation energy in *A. marina* PS I RC

The ground state absorption spectrum of *A. marina* PS I with reduced P740 shows the Q_y peak at 708 nm and a FWHM of 35 nm (Fig. 1A). The PB/SE signal in the transient spectrum at 0.0 ps shows a main peak at 711 nm and a FWHM of 34 nm (Fig. 1A). The similarity in the spectral width and the small peak difference suggest that all spectral forms of Chls are almost homogeneously excited and that most Chls are vibrationally relaxed at 0.0 ps with our time resolution. Excitation of some specific spectral forms of Chl is unlikely by the 630 nm excitation that excites rather flat vibrational progression of the Chl absorption. The peak difference of 3 nm seems to be explained by the SE of vibrationally relaxed Chls.

There is a dynamic red shift of the PB/SE peak from 711 nm to 717 nm from 0.0 ps to 1.0 ps (Fig. 1A) as also seen in the 0.6–0.7 ps DAS (Fig. 2). The 5.1 ps and 57 ps DASs under the P740-preoxidized conditions show similar spectral shapes. These results suggest that equilibration of excitation among different spectral forms is substantially completed with a time constant of $0.6(\pm 0.1)$ ps (Table 1). Single-step energy transfer between nearest Chls in the plant/cyanobacterial PS I core has

been estimated to require about 0.1–0.15 ps [17] so that only a few steps of energy transfer seem to be needed for the spectral equilibration in *A. marina* PS I RC. This feature is similar to those observed on the bulk antenna Chls in some cyanobacterial PS I core [17–19].

In the 4.8 ps and 5.1 ps DASs under the P740-prereduced

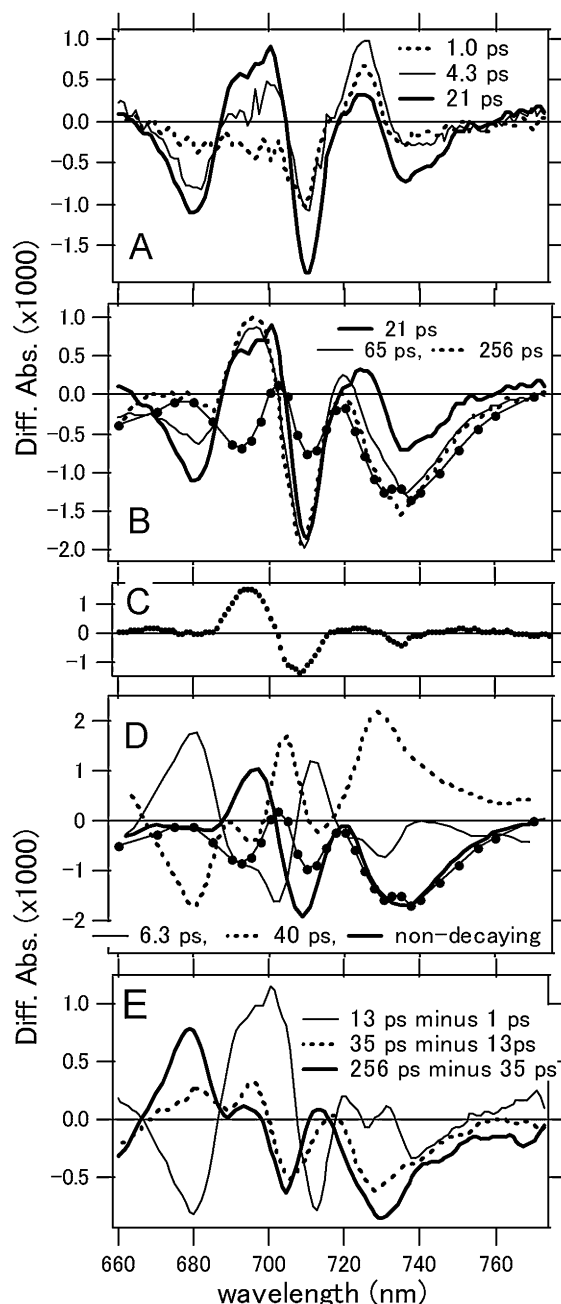


Fig. 3. A,B: Difference spectra between the transient spectra obtained under the P740-prereduced and -preoxidized conditions (pre-reduced-minus-preoxidized) in *A. marina* PS I at the relatively strong excitation energy (80 nJ/pulse). The light-induced reduced-minus-oxidized difference spectrum of $P740^+/P740$ obtained by microsecond flash photolysis is shown by a solid line connecting closed circles in B and D. C: Difference between the microsecond $P740^+/P740$ spectrum and the 256 ps spectrum in B. D: DAS based on the data as shown in A and B. E: Difference between the spectra at different delay times as in A and B.

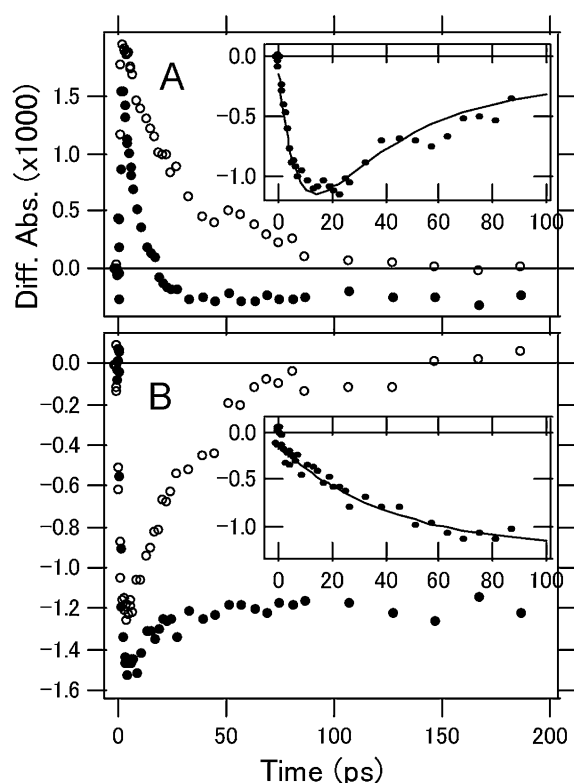


Fig. 4. Kinetic traces of the transient absorbance changes at fixed wavelengths. A: Probed at $680(\pm 3)$ nm. B: Probed at $740(\pm 3)$ nm. Closed and open circles represent the data under the P740-prerduced and -preoxidized conditions, respectively. Inserted graphs show differences between the two conditions (prerduced-minus-preoxidized) and the fitting curves by the global analysis. The energy of the excitation pulse was 80 nJ.

and -preoxidized conditions, respectively (Fig. 2), negative PB/SE components are predominant in the whole Q_y region (690–740 nm). This indicates a substantial quenching of excitation energy, presumably by the charge separation under the prerduced conditions or by $P740^+$ under the preoxidized conditions, on this time scale. Comparison of the negative PB area between 4.8 (5.1) ps DAS and 54 (57) ps DAS in Fig. 2 enables us to estimate that 39–44% of the excitation energy is quenched with the time constant of 4.8–5.1 ps.

The fast quenching of excitation makes a clear contrast to the situation in spinach PS I containing about 150 Chls/P700, on which transient absorption measurements under the same experimental conditions indicated that the DAS on this time scale consists of both negative and positive components with comparable areas (data not shown). This has been previously observed and attributed to the excitation equilibration between the bulk antenna Chls and the so-called long-wavelength form of Chls with absorption peaks redder than that of the electron donor Chl (P700) in plant/cyanobacterial PS I [18–23]. It can be concluded that the fast quenching of excitation energy within several picoseconds is more significant in Chl *d*-based *A. marina* PS I than in the Chl *a*-based PS I of plant and cyanobacteria.

The non-decaying component under the P740-preoxidized conditions carries 16% of the PB/SE area observed at 1.0 ps (Fig. 2B). This seems to be due to the same origin as the fluorescence component with a lifetime of 1.5–3.6 ns and an

amplitude of 10–20% of the initial amplitude (S. Itoh et al., unpublished data). Since this lifetime is typical for free Chls in organic solvents [24], the non-decaying component is tentatively attributed to minor uncoupled Chls in terms of energy transfer in our preparations.

4.2. Primary charge separation in Chl *d*-based *A. marina* PS I

The global analysis on the red-ox transient spectra in PS I RC of *A. marina* yields two decaying components (Fig. 3 and Table 1), which suggests that primary charge separation takes place with a time constant of 6.3–8.0 ($7.2(\pm 0.9)$) and/or 40–59 ($50(\pm 10)$) ps. Because there is a significant quenching of excitation energy with the 4.3–4.8 ps time constant with the prerduced P740 (Table 1), the $7.2(\pm 0.9)$ ps process can be estimated to represent a fast phase of the primary charge separation.

The apparent PB at 740 nm proceeds mainly with the time constant of $50(\pm 10)$ ps (Fig. 4B) and is not significant in the 6.3 ps DAS (Fig. 3D). Therefore, one idea may be that P740 is not directly involved in the first phase of the trapping. The other idea is that another component with a positive change around 730 nm, as seen in the 6.3 ps DAS in Fig. 3D, counterbalances the PB of P740 in the 6.3 ps DAS. The latter possibility cannot be totally eliminated if the oxidation rate of P740 is monitored at 710 nm, where the microsecond $P740^+/P740$ spectrum shows a sharp negative peak (Fig. 3B). In the red-ox transient spectra (Fig. 3), a sharp PB appears at 710 nm in the 6.3 ps DAS and remained with a slight blue shift. A major difficulty for this idea, however, is the identification of the component giving positive change at 730 nm so that detailed analysis is needed before a final conclusion. On the other hand, a component giving the PB at 680 nm (Fig. 3), which we designate C680, shows a rise and decay with time constants of $7.2(\pm 0.9)$ and $50(\pm 10)$ ps, respectively (Fig. 4A). C680 should be a Chl species directly involved in the primary charge separation.

A plausible candidate for C680 is a Chl *a* molecule that absorbs at 680 nm. *A. marina* PS I contains $145(\pm 8)$ Chl *d* molecules per RC and consequently about 0.8 Chl *a* molecule per P740 (average of eight preparations) [3]. C680 might be a Chl *a* molecule that functions as the electron acceptor A_0 . However, this assignment requires an asymmetric arrangement of the RC pigments by assuming Chl *a* as the electron acceptor A_0 and Chl *d* as its counterpart (A_0') at the C_2 symmetrical sites. The C_2 symmetrical arrangement of pigments as revealed in the 3D structure of Chl *a*-based cyanobacterial PS I [7] also seems to be rational in *A. marina* PS I judging from the amino acid sequence of PsaA/PsaB polypeptides (H. Miyashita, personal communication). The other candidate for C680 is a special form of Chl *d*. Although the Q_y peak of Chl *d* is at 697 nm in methanol [2] and typically at 700–730 nm in *A. marina* PS I, some interaction with protein and/or excitonic coupling between pigments might yield Chl *d* molecule(s) absorbing at 680 nm.

P740 becomes a stable cation radical showing a free-radical type electron paramagnetic resonance (EPR) spectrum of a 8 Gauss peak-to-peak width that is typical for a Chl dimer (Itoh et al., unpublished data). It probably consists of two Chl *d* (and/or Chl *d'*) molecules [3]. We may also assume another Chl molecule, designated A, which is located between P740 and A_0 . The pair of A and A_0 are expected to transfer an electron from P740 to phyloquinone according to the widely

accepted electron transfer scheme (i.e. P700–A–A₀–phylloquinone) in the Chl *a*-based PS I RC.

If the slow PB of P740 with the time constant of 50(±10) ps indicates that P740 is not directly involved in the primary charge separation, the 'A/A₀ pair made of Chl *d*' may be responsible for the primary charge separation, in analogy with the start of the primary charge separation from the excited state of the so-called 'accessory' bacteriochlorophyll in a purple bacterial RC [25]. The A/A₀ pair, then, may show absorption change at 680 nm by an excitonic coupling. The observed rapid trapping, however, may be achieved only by the function of Chl(s) with an absorption band at a long wavelength, by which excitation energy can be captured more efficiently than the other Chls. P740 can be an efficient collector of the excitation energy. On the other hand, even if the A/A₀ pair assumed above may form a non-parallel dimer with absorption bands at both 712 nm and 680 nm seen in the 6.3 ps DAS (Fig. 3D), the energy levels do not seem to be low enough to allow efficient energy transfer from P740. The role of P740 in the primary charge separation, thus, remains to be studied.

The red-ox transient spectrum at 256 ps is different from the microsecond P740⁺/P740 spectrum (Fig. 3B). The difference seems to represent a large shift-like feature centered at 702 nm (Fig. 3C). The electron transfer to the acceptor phylloquinone seems to proceed with the time constant of 50(±10) ps. The shift-like signal rises together with C680 and stays longer than C680 (Fig. 3A,B), which may suggest an electrochromic shift of a Chl *d* molecule nearby A₀ and/or phylloquinone.

In the case of spinach PS I with 150 Chl/P700, the situation is clearly different under the same experimental conditions. The (P700-prereduced-minus-preoxidized) transient spectra showed only a growth of the P700⁺/P700 difference spectrum without substantial signal of A₀[−]/A₀ at 685–690 nm (unpublished data). This is presumably due to the fast 13–30 ps reoxidation of A₀[−] that is comparable to or even faster than the trapping of excitation energy (22–69 ps) in PS I with large antenna sizes [13–16,26].

One critical argument against the above analysis of the charge separation kinetics based on the red-ox transient spectra may arise from possible dependence of the energy-transfer and trapping rate on the redox state of P740. *A. marina* PS I seems to lack the long-wavelength form of Chls that will collect excitation energy more efficiently than P740 (Fig. 1A). The lack might produce a pronounced change in the energy transfer process depending on the redox state of P740. This remains to be clarified by future studies.

The features of excitation transfer can be summarized as follows. Upon a rather homogeneous excitation at 630 nm, spectral equilibration is substantially completed with a time constant of 0.6(±0.1) ps, suggesting that only a few steps of excitation transfer are required for the equilibration. The primary charge separation induces a fast phase of the excitation trapping (4.9(±0.6) ps) that is apparently absent or much slower in most of Chl *a*-based PS I RC. These features are partially attributable to the lack of long-wavelength Chls in *A. marina* PS I.

The features of the charge separation can be summarized as follows. Chl(s) absorbing at 680 nm seem to be directly involved in the primary charge separation with a time constant of 7.2(±0.9) ps possibly as the primary acceptor Chl (A₀).

The PB of P740, monitored at 740 nm, apparently develops rather slowly with a time constants of 50(±10) ps. This may indicate either some interference with the P740 PB by another Chl(s) or a unique mechanism of primary charge separation in the A/A₀ pair. In the subsequent process with a time constant of 50(±10) ps, excitation energy seems to be fully trapped by the oxidation of P740 and reduction of the first non-Chl electron acceptor, presumably phylloquinone (A₁).

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