Function of Chlorophyll *d* in Reaction Centers of Photosystems I and II of the Oxygenic Photosynthesis of *Acaryochloris marina*†

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ABSTRACT: Reaction center chlorophylls (Chls) in photosystems II and I were studied in the isolated thylakoid membranes of a cyanobacterium, *Acaryochloris marina*, which contains Chls *d* and *a* as the major and minor pigments, respectively. The membranes contained PS I and II complexes at a 1.8:1 molar ratio on the basis of the spin densities on the tyrosine D radical and the photo-oxidized PS I primary donor ($P740^+$). In the presence of ferricyanide, laser excitation induced bleach at 725 nm that recovered with time constants of 25 μs and 1.2 ms. The signal, designated P725, was suppressed by PS II inhibitors DCMU and hydroxylamine. The P725 spectrum was tentatively assigned to the absorption changes of the special pair Chl *d*, the accessory Chl *d*, and the acceptor pheophytin *a* in PS II. The addition of ascorbate induced the additional signal with a slow decay time constant of 4.5 ms. This signal showed a broad bleach at 740 nm and shift-type absorption changes at around 707 and 685 nm, which were assigned to the absorption changes of PS I special pair of Chl *d* (P740), the accessory Chl *d*, and the primary acceptor Chl *a* (A_0), respectively. Mechanisms and the evolution of the Chl-*d* based reaction centers using far-red light are discussed together with the amino acid sequences of PS II D1 and D2 proteins.

Acaryochloris marina is a newly found unicellular cyanobacterium that contains chlorophylls (Chls[1]) *d* and *a* as the major (95%) and minor (5%) pigments, respectively (*1*−*3*). The organism was isolated from colonial ascidians collected in Palau in the Pacific Ocean (*1*−*3*) and grows photoautotrophically in an inorganic medium. *Acaryochloris* species were also collected from red algae in Japan (*4*) or as a free-living organism in a salt lake in the USA (*5*). All these species reside in cyanobacterial lineage based on the genes of the ribosomal RNA (*3*), tRNA (*5*), and antenna pigment (*6*−*9*) and proteins (*10*). It is not yet clear whether all of them have Chl *d* in their photosystems I and II (PS I and II) reaction centers (RCs).

A. marina has a PS I RC that contains a Chl *d* dimer P740, which was named after its peak wavelength, as the primary electron donor (*11*). Until the discovery of P740, solar energy conversions in oxygenic photosynthesis in plants and cyanobacteria are known to be driven by the special pair of Chl *a* (*12*, *13*) (or its analogues, divinyl Chl *a* (*14*) or Chl *a*′ (*15*)) named P680 and P700, respectively, in the RC complexes of PS I and II. The quantum energies of 680−700 nm red light absorbed by P680 and P700 are larger than those of 800−860 nm far red light absorbed by the primary donor bacteriochlorophylls (Bchls) in anoxygenic photosynthetic bacteria (*12*, *13*). The high quantum energy of the light absorbed by Chl *a* has been assumed to be essential for the function of PS II that oxidizes water into molecular oxygen and reduces plastoquinone at the same time. However, *A. marina* undergoes oxygenic photosynthesis even with Chl *d* that absorbs 700−730 nm near-far-red light (*1*).

Chl *d* has a formyl group on the C13 atom of the chlorin macrocycle ring and absorbs light above 700 nm *in vivo*, in contrast to Chl *a*, which has a vinyl group at the C13 position (*2*, *16*). The chemical properties of Chl *d* are different from those of Chl *a*, as revealed by chromatography (*2*, *17*) and Raman studies (*18*, *19*). *A. marina* contains Chl *d*, phycocyanin, Chl *a*, and a trace amount of Chl *c* as the photosynthetic pigments (*2*, *20*−*22*). Chl *d* binds to both PS II and PS I proteins (*9*, *11*) and to the 30−38 kDa antenna polypeptides homologous to *pcB* (*8*, *10*). The PS I RC purified from *A. marina* contained 140 Chl *d* and at least one Chl *a* per one flash-oxidizable P740 (*11*). P740, therefore, is most likely a special pair of Chl *d*, judging from its optical difference absorption spectra (*11*), FTIR difference spectra (*24*, *25*), and ENDOR features (*26*). Chl *a* seems to function as the primary acceptor A_0 (*23*). The function of P740 in the intact untreated membranes, however, has not been reported yet.

In PS II, Chl *d* functions as the major antenna, as shown by studies of the fluorescence induction (*6*) and action spectrum of oxygen evolution in intact cells (*27*, *28*). We reported the absorption change at 720 nm but not at 680 nm in the ferricyanide-oxidized thylakoid membranes and as-

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[1] Abbreviations: Bchl, bacteriochlorophyll; Chl, chlorophyll; P740 and P725, special pair of PS I and PS II in *Acaryochloris marina*, respectively; RC, reaction center.

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sumed the function of Chl *d* in PS II (*29*). Mimuro et al., on the other hand, assumed the primary donor of PS II to be P680, based on the picosecond fluorescence kinetics in whole cells (*30*−*32*). They assigned a minor 680 nm fluorescence band that decays slowly after laser excitation as the delayed fluorescence, and assumed the uphill energy transfer from the antenna Chl *d* to P680 (*30*−*32*). The other groups, on the other hand, demonstrated almost no uphill energy transfer from Chl *d* to Chl *a* (*33*), and the emission of millisecond delayed fluorescence from Chl *d* (*34*). Chen et al. recently demonstrated the photoinduced reduction of pheophytin *a* in the crude PS II core complex that contains about 20 Chl *d* per 2 pheophytin *a* (*35*). Normal function of tyrosine Z was also shown (*36*). Akiyama et al. showed that the thylakoid membranes of *A. marina* contained small amounts of pheophytin *a* and Chl *a* at a molar ratio of 1:2 and assumed them to be in PS II RC (*8*, *17*).

Very recently Tomo et al. reported the light induced changes in the FTIR and difference absorption spectrum in the isolated PS II reaction center that contains D1, D2, CP43, CP47, and several small polypeptides together with 3.0 Chl *a*/55 Chl *d* (*37*). Schlodder et al. also reported the absorption changes in the PS II enriched membranes that contained Chl *d* and *a* as the major and minor components (*38*). Both studies treated thylakoid membranes with *n*-dodecyl-*β*-D-maltoside to obtain the solubilized materials. Tomo et al. (*37*) assigned the special pair of PS II to be a dimer of Chl *d* based on the light-induced 713 nm bleach and a 842 nm positive absorption change, and the FTIR spectral changes ascribable to formyl groups. The conclusion of the paper denied the former strong proposal of this group that the chemical identity of the special pair is P680 made of Chl *a* (*30*−*32*). They also offered a new assumption that Chl *a* function as the accessory Chls that give rise to the 670 nm long-lived fluorescence. Schlodder et al. (*38*), on the other hand, detected the absorption decrease and increase at 435 nm and 820 nm, respectively, and assumed the special pair to be either a Chl *a* pair in agreement with the former proposal of Mimuro et al. (*30*−*32*) or a heterodimer of Chl *a*/Chl *d*. They showed strong electrochromic shift of Chl *d*, which they assumed to represent accessory Chls nearby the special pair and assumed one of them to function as the initial electron donor, based on the absorption changes at room temperature and at 77 K. These two papers are contradictory as for the conclusion, and the light-induced difference absorption spectra are significantly different from each other. Most of the data presented were not complementary too except the FTIR data. The chemical identity of the electron donor Chl as well as the function of Chl *a*, in PS II of *A. marina*, thus, is still under debate. It is not clear whether the discrepancies between the publications (*37*, *38*) might come either from the different measurement systems or from the different preparations.

In this paper, laser-induced absorption changes were measured in the suspensions of *A. marina* thylakoid membranes, which were isolated without detergent treatment, poised at different redox potentials to identify the pigment species that function in PS I and II RCs.

MATERIALS AND METHODS

A. marina cells were grown under white fluorescent light at 20 μmol/cm^2 for 4 days as described previously (*11*) and collected by centrifugation at 3000*g* for 20 min. Pellets of the cells were then dispersed in a medium containing a 50 mM Tris-Cl buffer (pH 7.5) and 10 mM NaCl and washed with the same buffer again. The cells were broken in a bead beater for 20 min (Biospec, Bartlesville) with 0.2 mm glass beads. After removing the cell debris and glass beads by centrifugation at 3000*g* for 20 min, the supernatant obtained was centrifuged at 10000*g* for 30 min. The precipitates that contained thylakoid membranes were resuspended and washed again with the same medium to remove phycobilisomes. The membranes were dispersed in the same medium and stocked on ice or stored at −80 °C until use. The membranes that were frozen and thawed showed low activity of oxygen evolution.

The absorption spectra of membranes at 77 and 290 K were measured with a double beam double-monochromator spectrophotometer (UV-3100PC, Shimadzu, Kyoto) with a liquid nitrogen cryostat (DN90, Oxford Inst, Oxford).

Laser flash-induced absorption changes were measured with a home-built split-beam double-monochromator spectrophotometer with a 10 ns, 532 nm Nd:YAG excitation laser flash (Quanta-Ray LAB-130-10TH; Spectra-Physics, California) at 15 °C with a time constant of 5 μs as described previously (*12*). The excitations were performed at a low repetition rate of 0.25 Hz to attain the full oxidation/reduction of photoproducts in each dark interval between laser flashes. Samples in a glass sample cuvette (1 cm light path) were illuminated by a measuring beam from a tungsten lamp through a 15 cm monochromator (MC15, Ritsu, Asaka, Japan) for 0.2 s during the measurement of the absorption change after each 3.8 s dark interval. The laser-induced Chl fluorescence from the sample was eliminated by optical filters and a second 30 cm monochromator (MC30, Ritsu, Asaka, Japan) that was set at a 2 nm wavelength resolution and placed before a detection photomultiplier. The signals were accumulated for 32−256 times, as was required in each measurement to increase the signal/noise ratio. The thylakoid membranes were dispersed in a reaction medium containing a 50 mM Tris-Cl buffer (pH 7.5) and 20 mM NaCl to give an absorbance of 1.7 at 705 nm.

The ESR signals of $P740^+$ of PS I and the tyrosine D radical (Tyr-D signal) of PS II were measured with an X-band CW ESR spectrometer (ESR 200, Bruker, Germany) at 150 K as described elsewhere (*26*). The Tyr-D signal was measured in the dark-adapted membranes, and the $P740^+$ signal was obtained as the light-minus-dark difference signal. Measurements of the ESR signals were conducted at modulation amplitude of 2 G and microwave power of 0.02 mW at 150 K.

DNA sequencing of psbA gene was carried out using a DYEnamicET Terminator Cycle Sequencing Kit (GE Healthcare) and an automated DNA sequencer (ABI373A, Applied Biosystems) and translated into amino acid sequence. We obtained the psbA gene sequence from H. Miyashita (personal communication) first and determined by ourselves from *A. marina* genome (T.U. in DDBJ with an accession No. AB303650). The psbA and psbD gene sequences can be also available by BLAST searches in "Phototrophic Prokaryotes Sequencing Project" (http://genomes.tgen.org/blast.php).

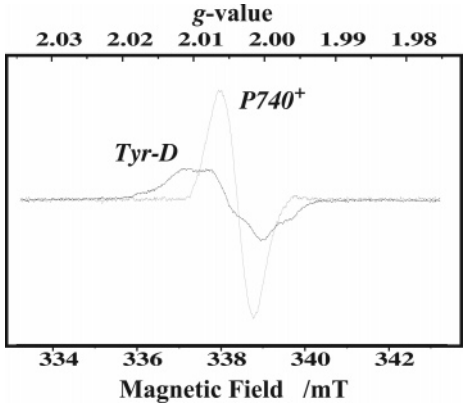

FIGURE 1: Electron spin resonance spectra of $P740^+$ and the tyrosine-D radical in isolated thylakoid membranes of *A. marina*. The Tyr-D signal was measured in dark-adapted membranes. The $P740^+$ signal was obtained as the additional signal induced by illumination at 150 K and is shown as the light-minus-dark difference spectrum. The reaction medium contained a 50 mM Tris-HCl buffer (pH 7.5). Conditions for the measurement of ESR: modulation amplitude, 2 G; microwave power, 0.02 mW at 150 K.

RESULTS

ESR Signals of PS II Tyrosine D Radical and PS I $P740^+$ in Thylakoid Membranes of A. marina. The isolated thylakoid membranes of *A. marina* showed typical ESR signals of the tyrosine D radical in PS II in the dark, as well as the light-induced $P740^+$ radical on PS I at 150 K (Figure 1). Both signals were measured at the nonsaturating microwave power to correctly estimate their contents. Spin density on each component was calculated by the integration of each signal. The membranes are calculated to contain PS I and PS II complexes at a molar ratio of 1.8:1. The membranes contained Chl *d* as a major pigment as seen in the absorption spectrum measured at 290 and 77 K (see Figure 6D). The absorption spectrum at 77 K showed a main peak at 713 nm and shoulders at 703 and 730 nm due to Chl *d* absorption bands, and showed no specific peak of Chl *a* at around 670−680 nm.

Kinetics of Light-Induced Absorption Changes. The absorption changes induced by the excitation of a 10 ns, 532 nm Nd:YAG laser flash were measured in *A. marina* thylakoid membranes (Figure 2). The absorption changes were first measured in the presence of ferricyanide, which preoxidizes P740 and suppresses its flash-induced absorption change. The photoreaction detected under this condition, then, could be mainly due to PS II, since the oxidant has almost no effect on the photoreaction of PS II primary donor, which should have an extremely positive E_m of around +1000 mV. Triplet states of antenna Chl *d* or carotenoids might also give ferricyanide-insensitive absorption change. The time courses of absorption change at typical wavelengths are shown in Figure 2. The laser excitation induced large negative and positive absorption changes at 725 (−) and 715 (+) nm, respectively, while almost no changes were observed at 680 and 740 nm (Figure 2A). In the traces, the contribution from the laser-induced fluorescence, which gave negative spikes within 7 μs under present experimental conditions, was omitted. The kinetics of signal recovery at 725 nm was composed of fast and slow phases with time constants of 25 μs and 1.2 ms, with relative initial extents of 2.1 and 1.0, respectively.

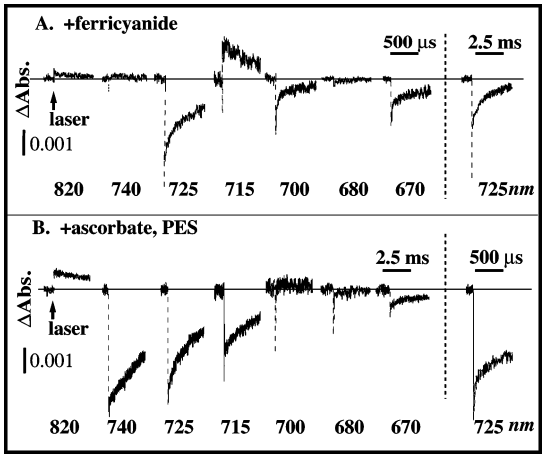

FIGURE 2: Time courses of the flash-induced absorption change at different wavelengths in the thylakoid membranes of *A. marina* under oxidizing (A) and reducing conditions (B). A. In the presence of 1 mM potassium ferricyanide. B. Upon further additions of 10 mM sodium ascorbate and 1 μM phenazine ethosulfate. Signals were induced by a 532 nm, 10 ns laser flash given at 0.25 Hz. The reaction medium contained a 50 mM Tris-HCl buffer (pH 7.5). Measurements were conducted as described in Materials and Methods at 10 °C.

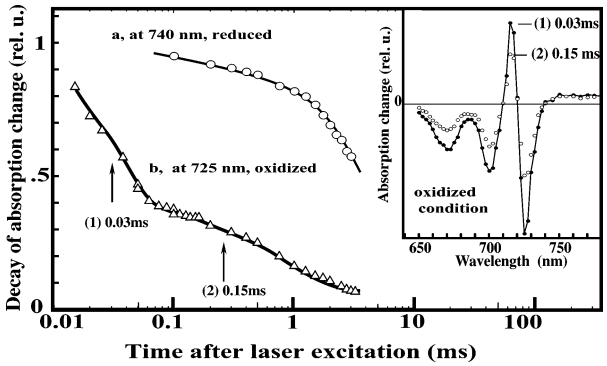

FIGURE 3: Plots of the flash-induced absorption changes at 740 nm and at 725 nm versus logarithms of time after the flash excitation: (a) at 740 nm measured in the presence of 10 mM sodium ascorbate and 1 μM phenazine ethosulfate; (b) at 725 nm measured in the presence of 1 mM potassium ferricyanide. Note that the amplitudes of the negative absorption changes were shown in positive directions to show the decay kinetics clearly in (a) and (b). Inset: difference absorption spectra measured at (1) 0.03 and (2) 0.15 ms after the flash excitation in the presence of 1 mM potassium ferricyanide.

To confirm the PS I activity in the same thylakoid membrane, absorption changes were measured after addition of 10 mM ascorbate as a reductant and 1 μM phenazine ethosulfate (PES) as a mediator in addition to ferricyanide. Under this condition, photoreactions in both PS I and II could be observed since the primary donors of PS I (P740) and II were in the active prereduced forms. The laser excitation induced a new signal with a large negative change at 740 nm (Figure 2B), which was recovered in a single decay phase with a time constant of 6.3 ms. Note that the time axis in Figure 2B (with a 2.5 ms bar) is five times longer than that of Figure 2A to cover the slow kinetics. (See Figure 3 for the comparison of kinetics at 740 and 725 nm.) The bleach and recovery at 740 nm indicates the photo-oxidation and rereduction of P740, as reported in the isolated PS I complex of this organism (*11*). The rereduction rate became slower at the lower PES concentrations to around 30 ms (not shown).

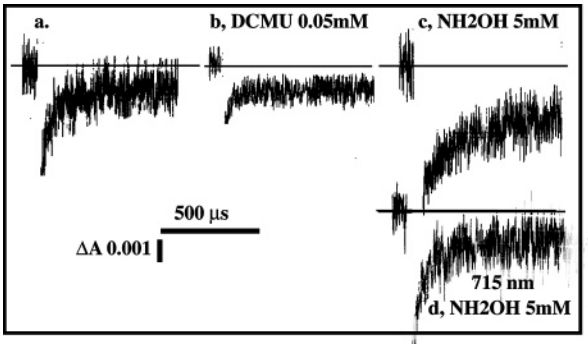

FIGURE 4: Effects of DCMU and hydroxylamine on the absorption change at 725 nm (a−c) and at 715 nm (d) in thylakoid membranes of *A. marina* under the oxidizing condition: a, no addition; b, with 0.05 mM DCMU; c and d, with 5 mM hydroxylamine at 725 and 715 nm, respectively. The reaction medium contained a 50 mM Tris-HCl buffer (pH 7.5) and 1 mM ferricyanide. Other conditions were similar to those in Figure 2.

At 715 nm, the positive change, which was detected with ferricyanide alone, was replaced by the negative change. At 725 nm, the amplitude was increased and the recovery rate became slower compared to those detected in the ferricyanide-oxidized condition. The 725 nm recovery showed an additional slow 6 ms phase together with the fast 1.2 ms phase at relative amplitudes of 1.0 and 0.64, respectively. The faster component with a 25 μs time constant was also seen in the expanded time scale as shown in the right side panel. The faster two components were, thus, almost comparable to those detected in the presence of ferricyanide alone. Therefore, the absorption change detected in the reduced condition also includes the one detected in the presence of ferricyanide.

We compared the decay kinetics at 740 nm detected under the ascorbate-reduced condition with that at 725 nm measured under the ferricyanide-oxidized condition in more detail in Figure 3. The former showed only a slow decay with a time constant of 6 ms with no fast decay phase, while the latter showed two decay phases with time constants of 25 μs and 1.2 ms as described above. The plot of the extents at 30 μs in the latter against wavelength showed complex spectra with negative and positive peaks (Figure 3 inset). The difference spectrum essentially confirms the one previously measured under similar conditions (*29*). The plot of the extents at 150 μs showed a similar spectrum indicating the major function of the same component, though with a little lower relative intensity of positive peak at 715 nm.

Effects of PS II Inhibitors on the Kinetics at 725 nm. In order to see the relation of the absorption change detected in the ferricyanide-oxidized condition to the PS II activity, we tested the effects of PS II inhibitor DCMU on the 725 nm kinetics (Figure 4). Fresh thylakoid membranes showed fast decay kinetics at 725 nm. Addition of 0.05 mM DCMU decreased the extent of the 725 nm absorption change to be almost a half without significant changes in the kinetics. The result is as expected because DCMU is expected to inhibit the reoxidation of photoreduced electron acceptor plastoquinone Q_A and to decrease the extent of photoactive PS II under the repetitive flash excitations.

Figure 4 also shows the effect of hydroxylamine. This treatment eliminated the fast decay phase and increased the extent of absorption decrease at 725 nm in the slower decay

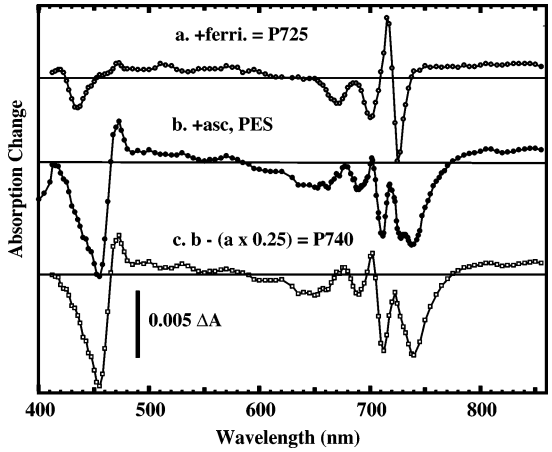

FIGURE 5: Difference spectra of flash-induced absorption changes in thylakoid membranes of *A. marina* under oxidizing (a) and reducing conditions (b): a, difference absorption spectrum of P725 measured at 0.1 ms after laser excitation in the presence of 1 mM potassium ferricyanide; b, difference absorption spectrum measured at 3 ms in the presence of 10 mM sodium ascorbate, 1 μM phenazine ethosulfate, and 1 mM potassium ferricyanide. c: Difference absorption spectrum of P740 calculated as (a) − (0.25 × b). Data points were calculated from experiments as in Figure 2.

phase. This is opposite to the expectation because hydroxylamine is known to inhibit the electron donation to PS II by destroying manganese cluster and to make the rereduction of the PS II electron donor too fast to be detected being as a reductant by itself (*39*). Under this condition we, however, detected only a broad bleach at 700−740 nm as the slow phase component and detected only the negative absorption change even at 715 nm (trace d). The slow phase enhanced by hydroxylamine was DCMU-insensitive. The addition of hydroxylamine, therefore, seemed to have enhanced the flash-induced bleaching of bulk Chl *d*.

The fast absorption changes with the negative and positive changes at 725 and 715 nm, respectively, seem to be associated with the donor side of PS II function, namely, the oxidation of electron donor in PS II RC. It is also suggested that the damages on the PS II donor side affect the kinetics and extent of the 725 nm absorption change too. The rapid absorption change at 725 nm was rather unstable and became smaller after the long period of measurement even at 15 °C.

Difference Spectra of P740 and P725. The extents of the absorption changes at 0.1 ms after the laser excitation measured under the ferricyanide-oxidized condition were plotted against wavelength in a wider wavelength range (Figure 5a). Samples were replaced each 0.5−1 h to avoid photodamage during the measurement. The difference spectrum gave peaks at 671 (−), 684 (+), 700 (−), 715(+), and 725(−) nm and small broad positive changes at 740−860 nm with a slight hump at around 840 nm. The data points below 500 nm showed 430 (−) and 475 (+) small shifts although the peak heights were less accurate due to the effects of overlapped ferricyanide absorption. We designated this spectral component as P725, which could represent the absorption changes of the electron donor pigment of PS II RC in *A. marina*.

Absorption changes measured in the presence of ascorbate and PES were also plotted (Figure 5b). The slow decay phase could be due to P740. The extent at 3 ms was taken as data

point to minimize the contribution of P725 that decays faster. The difference spectrum gave peaks at 455 (−), 473 (+), 678 (+), 690 (−), 702.5 (+), 713 (−), 718 (+), 728 (−), and 740 (−) and a broad positive change above 776 nm with a slight hump at around 840 nm. The spectrum almost agreed with that of P740, which has been reported in the isolated PS I RC complex (*11*), except for a sharp shoulder peak at 728 nm. The 728 nm feature was more prominent at a shorter time range, which suggested it to be contributed by the absorption change of P725. In order to eliminate the contribution of residual P725 signal, we estimated its contribution based on the ratio of extents at 0.1 ms to that at 3 ms of P725 signal in Figure 3, and then the 25% amplitude of spectrum (a) was subtracted from spectrum (b), to give spectrum (c). In the spectrum thus calculated, the 728 nm shoulder was lost and the shift at 713 (−)/718 (+) became more prominent showing the characteristic of the $P740^+$/P740 spectrum.

A comparison of the P740 difference spectra (see Figure 6B and C) with the absorption spectra of the thylakoid membranes measured at 290 and 77 K indicates that the pigment species of P740 is one of the longest wavelength-absorbing Chl *d* among all the Chl *d* molecules in the thylakoid membranes. In the P725 difference spectrum (see Figure 6A), on the other hand, the pigments longer than 740 nm were not involved.

DISCUSSION

Absorption Changes in Thylakoid Membranes of A. marina. Isolated thylakoid membranes of *A. marina* contained PS I and II complexes at a molar ratio of 1.8:1 as determined by ESR measurement. In the presence of ferricyanide, the laser flash induced an immediate bleach at 725 nm that was followed by a biphasic recovery with time constants of 25 μs and 1.2 ms. The signal intensity was decreased to about a half by adding DCMU, which is known to bind at the secondary plastoquinone (Q_B) binding site and to suppress the PS II charge separation under repetitive flash excitation, as done in the present study, due to the accumulation of Q_A^-. On the other hand, hydroxylamine, which is known to destroy the manganese complex, depleted the fast decay phase and increased the slow recovery phase that was insensitive to DCMU. These results suggest that P725 difference spectrum measured as the extent of the 25 μs phase represents the absorption change associated with PS II, namely, the rereduction of the electron donor Chl by tyrosine Z.

When P740 was prereduced by the external reductants, an additional slow (6 ms) kinetic phase appeared due to P740. The photo-oxidized $P740^+$ was rereduced by PES most likely, rather than by FeS clusters under the present experimental condition because the slower recovery was detected at 740 nm in the absence of PES. In isolated PS I complex of this organism, $P740^+$ was rereduced by the iron sulfur centers F_A/F_B with a time constant longer than 20 ms (*11*, *25*).

Difference Spectrum of P725. It can be argued that the difference spectrum of P725 might also contain nonspecific absorption changes, such as the bleaching of antenna pigment and/or the formation of triplet state. The major 25 μs decaying absorption change, however, seems to be attributed

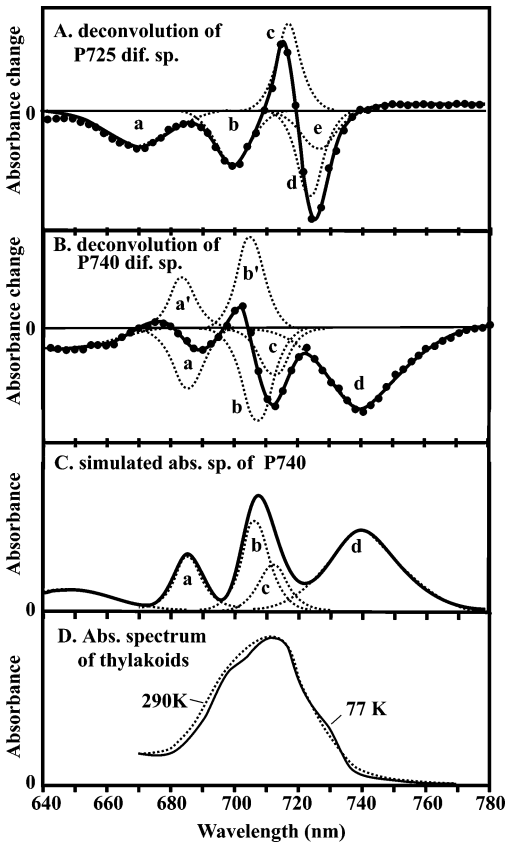

FIGURE 6: Analysis of the difference spectra of P725 (A), P740 (B and C), and the absorption spectra of the thylakoid membrane measured at 290 and 77 K (D): A, deconvolution of the difference spectrum of P725 obtained in Figure 5a (solid line) with the simulated component Gaussian curves (dotted lines); B, deconvolution of the difference spectrum of P740 (closed circles and a solid line) obtained in Figure 5c as the sum of the component Gaussian curves (dotted lines); C, simulated absorption spectra of the core pigments (a solid line) in PS I as the sum of the negative bands (dotted lines) estimated in B, which are estimated to represent P740 (d, c), accessory pigments (b), and electron acceptor pigments A_0 (a); D, absorption spectra of isolated thylakoid membranes of *A. marina* measured at 290 (dotted line) and 77 K (solid line). In the measurement of the absorption spectra, glycerol was added to give a final concentration of 60% to the reaction medium containing Tris-HCl 50 mM (pH 7.5). The parameters of each Gaussian component (peak wavelength in nm, peak height in relative unit, bandwidth in cm^{-1}) are as follows. In A, a (669.3 nm, −33, 668 cm^{-1}), b (700.3 nm, −49, 326 cm^{-1}), c (717.5 nm, 78, 222 cm^{-1}), d (724.1 nm, −78, 222 cm^{-1}), and e (726.8 nm, −35, 289 cm^{-1}). In B, a (685.2 nm, −54.5, 282 cm^{-1}), a′ (682.8 nm, +44, 261 cm^{-1}), b (707.4 nm, −100, 265 cm^{-1}), b′ (705.0 nm, +98, 241 cm^{-1}), c (712.0 nm, −45, 250 cm^{-1}), and d (739.7 nm, −82, 582 cm^{-1}). In C, a (685.0 nm, +54.5, 270 cm^{-1}), b (707.4 nm, +100, 265 cm^{-1}), c (712.0 nm, +45, 250 cm^{-1}), and d (739.7 nm, +82, 582 cm^{-1}).

to the oxidized electron donor, because (1) a slight increase above 740 nm with a slight hump at around 840 nm suggests formation of a Chl cation, (2) the Q_B site inhibitors decreased the amplitude with little effects on the kinetics, (3) the signal amplitudes saturated at a moderate laser power, and (4) hydroxylamine abolished the extent of fast phase and induced the flash-induced bleaching. We, therefore, assume that P725 represents the reaction of PS II electron donor that is either the special pair Chls or the accessory Chls adjacent to the special pair. It has been known that P680 in PS II is rereduced by Tyr-Z rapidly with a time constant of 23−260 ns depending on the S states (*40*) and mainly with time constants of 1.4 and 32 μs in the Mn-depleted PS II (*39*). It

seems that we measured a slow rereduction phase of PS II electron donor Chl in the inactivated PS II in the thylakoid in the present study because the membranes stored frozen had very low oxygen-evolving activity. The bleaching induced by hydroxylamine may indicate either the triplet formation or oxidation of Chl *d* although it still remains to be studied.

To examine the spectral features of special pair and nearby pigments in PS II of *A. marina*, the difference spectrum of P725 was deconvoluted into some Gaussian spectral components (Figure 6A). The deconvolution process was done in analogy to that in spinach PS I RC (*13*). The calculated components were negative bands at 725 (trace e), 701 (trace b), and 669 (trace a) nm and a shift at 717 (+)/725 (−) nm (traces c and d). The bands above 700 nm seem to be assigned to Chl *d*, and those below 700 nm to either Chl *a* or pheophytin *a*. The shift at 717/724 nm, then, might be assigned to the electrochromic shift of accessory Chl *d*. We tentatively assigned the negative bands at 725 nm and/or 701 nm together with a broad positive change at around 840 nm to the special pair Chl *d* dimer and the band at 670 nm to the acceptor pheophytin *a*. The assignments of special pair and the accessory Chl *d* might be opposite because the red-most Chl *a* in PS II was proposed to the accessory Chl *a* in *Synechocystis* (*41*). We still lack additional evidence for it. The broad 670 nm band might also contain a mixture of side bands of longer-wavelength Chl *d* molecules. The possibility of function of P680 (Chl *a* pair) is rather unlikely because the amplitude of 670 nm band is low, although this possibility cannot be totally eliminated. It seems more probable that this band reflects the absorption change (electrochromic shift) of the acceptor pheophytin *a* that is usually unclear because of overlap of P680 change in Chl *a*-based PS I. Another less probable possibility may be that the special pair is made of a heterodimer of 725 nm Chl *d* and 701 nm Chl *a*. However, if a Chl *a* exhibits the absorption peak at 701 nm, it should have a strong interaction with another Chl molecule as seen with Chl *a* molecules in P700. A Chl *a* in the binding site almost homologous to that of P680 will not show the 701 nm long wavelength peak because the Chl *a* molecules in P680 are assumed to be weakly coupled each other (*41*).

The P725 spectrum resembles that reported by Schlodder et al. (*38*) measured in the detergent-treated PS II enriched fraction at room temperature or at 77 K, although the peak positions and relative peak heights are somewhat different. Schlodder et al. (*38*) assumed the PS II special pair to be made of a Chl *a* dimer or a Chl *a*/Chl *d* heterodimer based on the results that the absorption change shows a small bleach at 430 nm as shown in Figure 6A and the cation peak at around 820 nm but not at 840−850 nm that is expected for a Chl *d* dimer as seen in the absorption change of P740 in PS I. They assigned the shift type spectrum centered at 723 nm as seen in the present study to be accessory Chl *d* that accumulates excitation energy and functions as the electron donor in the primary charge separation. The difference spectrum of P725 in Figure 6A also shows a small peak at 430 nm too. However, the absorption changes at around 660−680 nm, at which wavelength Chl *a* is expected to give significant contribution, is rather low in Figure 6A. Therefore, it seems rather difficult to assume Chl *a* pair to be the special pair. Figure 6A shows the positive absorption changes elongated to 850 nm in contrast to the result by Schlodder et al. (*38*) and shown rather low negative changes at 670−680 nm. On the other hand, the difference spectrum obtained by Tomo et al. (*37*) showed positive changes until 842 nm and a bleaching peak at 713 nm only with little shift type nature. An absorption change below 690 nm was not shown (*37*). Their result is, therefore, also somewhat different from the one in Figure 6A. The light-induced FTIR difference spectrum in this preparation suggested the band shifts of formyl groups in support of the view that Chl *d* dimer to be the PS II special pair. Then it might be assumed that the spectral shape (*37*) different from the one in Figure 6A is caused by the action of detergent treatment. The 725 nm bleach peak in Figure 6A might be shifted to the one at 713 nm by the severe detergent treatment (*37*). Tomo et al. (*37*) also proposed the contribution of Chl *a* as the accessory pigments,. However, the P725 spectrum in Figure 6A gives low contribution of Chl *a* in the red Qy region. It should be also noted that the millisecond delayed fluorescence is emitted only from Chl *d* but not from Chl *a* in the intact cells of *A. marina* (*34*) contradictory to the assignment of (delayed) fluorescence from Chl *a* (*37*).

As a consensus of previous work (*35*, *37*, *38*) and the present study, pheophytin *a* is concluded to function as the electron acceptor. It seems also clear that the PS II of *A. marina* uses Chl *d* as the major antenna pigment and uses it either in the special pair (this work and (*37*)) or as the accessory pigment (*38*), which functions as the primary electron donor (*38*). The role of Chl *a*, however, has not been clearly determined on concrete evidence yet because the result in the present study and those reported (*37*, *38*) show some but rather low contributions of Chl *a* in the difference absorption spectrum. It is clear that PS II in this organism is very different from those of the other oxygenic organisms that use Chl *a*.

We studied the amino acid sequences and structure of PS II core polypeptides D1 and D2 of this organism to consider the roles and binding sites of Chls *a* and *d*. The sequences of *A. marina* (see Appendix) keep high homologies with those in the other cyanobacteria. All the specific amino acid residues that are the ligands for or in contact with the cofactors are well conserved except for the modification of the highly conserved D1 phenylalanine 206 that is in contact with pheophytin *a* to leucine in *A. marina* as shown in Figure 7. The modification suggests some effects on the pheophytin redox properties as suggested in (*37*). The results suggest that Chl *d* can bind to most of the Chl *a* binding site rather easily without significant modification of amino acid residues. The serious discrepancy in the literature (*37*, *38*) might partially come from the detergent treatment that modifies the pigment situations.

Analysis of the Difference Spectrum of P740. The calculated difference spectrum of $P740^+$/P740 in thylakoid membranes showed a bleach at 740 (−) nm and blue shifts at 705 and 685 nm (Figure 6B), which are consistent with the difference spectrum of $P740^+$/P740 measured in the isolated PS I complex (*11*). The spectrum could be contributed from absorption bands of the special pair, accessory, and primary acceptor Chls. The overall spectrum was also deconvoluted into Gaussian components as done in the $P725^+$/P725 spectrum (Figure 6A). The spectrum was deconvoluted into a major negative band at 740 (−) nm (Figure 6B, trace d), two blue shifts at 705 (+)/707 (−)

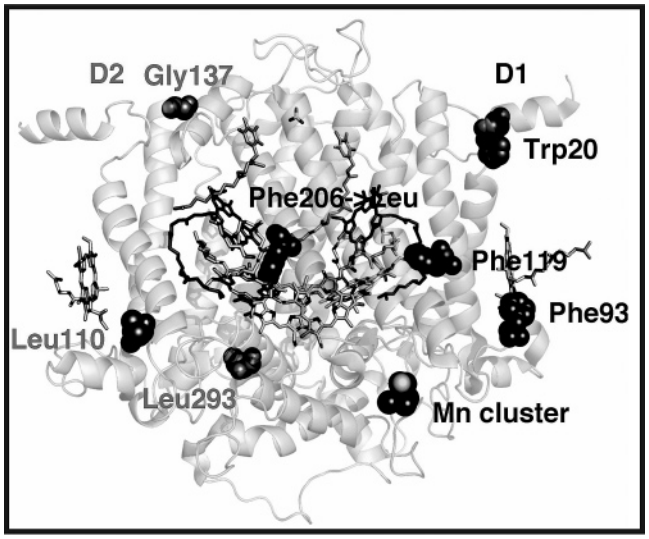

FIGURE 7: Amino acid residues unique for *A. marina* D1 and D2 sequences. 7 residues that are conserved in other cyanobacteria are shown in space filling models superposed on the structure of PS II of *Thermosynechococcus elongatus*.| See Appendix for the sequences of *A. marina* D1 and D2 polypeptides.

(traces b′, b) and 683 (+)/685 (−) (traces a′, a) nm and a minor negative band at 712 (−) nm (trace c). The inverse of sum of all negative bands (traces a, b, c, d), then, will produce the absorption spectrum of photoactive pigments at the ground state (Figure 6C).

The spectral patterns of the light-induced difference spectrum and the ground state spectrum of P740 resemble the corresponding spectra of P860 in RC of purple photosynthetic bacteria, respectively (*42*). The difference spectrum of P860 is known to be composed of the bleach of a special pair at 860 nm, the blue shifts of accessory BChls at 800 nm, and the red shift of the primary acceptor bacteriopheophytin at 750 nm (*42*). In analogy to P860, the difference spectrum of P740 may be interpreted as the bleach of special pair at 740 nm, the shift of accessory Chl at 707 nm, and the shift of the acceptor Chl at 685 nm. The special pair can be made of Chl *d* dimer with a slight hump at around 740 nm, as has been assigned (*11*, *23*, *25*) (or a dimer of Chl *d* and Chl *d*′, in analogy to P700 as proposed (*7*)). We also propose that the 707 nm band represents the accessory Chl *d* and that the 685 nm band represents the acceptor Chl *a* (A_0). The role of Chl *a* as A_0 was suggested based on the results that one Chl *a* was found per P740 in the isolated PS I complex (*11*) and that the fast rise and decay of the 680 nm pigment was assigned to A_0 in the picosecond absorption spectroscopy (*23*). A comparison of the P740 spectra in Figures 6B and C with the absorption spectra of thylakoid membranes in Figure 6D indicates that P740 is the longest wavelength-absorbing Chl *d* species in this organism. It is interesting that the Chl *d* in PS I RC and in the antenna give absorption bands at wavelengths longer than those of PS II in this organism and that P740 appears to be the longest-wavelength-absorbing Chl *d* in PS I of this organism. It interprets no long-wavelength fluorescence in the isolated PS I RC complex (*43*).

Mechanism of Photoreactions in A. marina. The results in the present study suggest that the PS II RC of *Acaryochloris* contains the electron donor made of a dimer of Chl *d* (or a heterodimer of Chl *d* and Chl *d*′) with a peak at 725

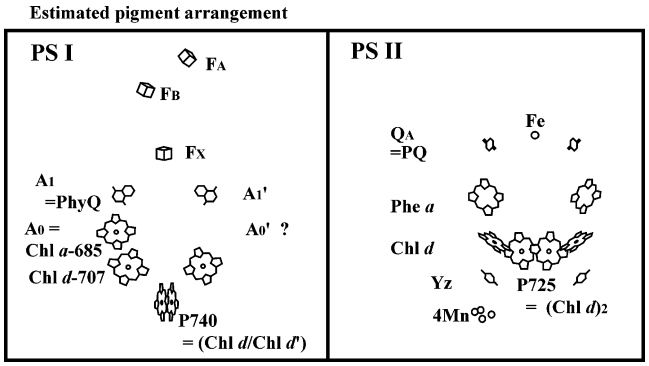

FIGURE 8: Tentative estimations of pigment arrangements in the central cores of PS I and PS II reaction centers of *Acaryochloris marina* based on the results of simulation in Figure 6. It is also noted that the assignment of special pair and accessory Chls in PS II still is a matter of argument as seen in refs (*37*, *38*).

and/or 701 nm, accessory Chl *d* molecules with a peak at 720 nm, and the electron acceptor pheophytin *a* with a peak at 670 nm. It seems also probable to assign the red-most 725 nm peak to the accessory Chl *d* as was the case in the other Chl *a*-based PS II (*41*). On the other hand, the PS I RC seems to contain a dimer of Chl *d* as P740, accessory Chl *d* molecules with peaks at around 707 nm, and Chl *a* with a peak at 685 nm as the electron acceptor A_0. Figure 8 shows schematic drawings of the pigment arrangements we propose, although the estimations are still tentative as for PS II and require more confirmations.

In *A. marina* the light energy captured by antenna Chl *d* molecules is funneled into P725 or P740. The quantum energy of photons at 725−740 nm is 7−10% lower than those at 680−700 nm, which are absorbed by P680 and P700 in all the other oxygenic photosynthetic systems. In the *A. marina* PS I, the low energy level of P740* was shown to be compensated by the 100 mV negative shift of E_m of P740 compared to that of P700 (*11*). In PS II, if the E_m of P725 is more negative than that of P680, then, P725* will be producing the reducing power equivalent to that of P680*. However, it will decrease the energy gap between the redox potentials of tyrosine Z and P725, and will slow down the reduction rate of $P725^+$ and might lead to the lower oxygen evolution activity as argued previously (*29*−*32*). The oxidation rate of tyrosine Z, however, seems to be almost the same as that of the Chl *a*-type PS II (*36*). On the other hand, if the E_m of P725 is almost equal to that of P680, the effective redox potential of P725* should be more positive than that of P680*. This may result in the smaller driving force for the electron transfer to the acceptor pheophytin *a*, and may lead to the higher probability of the charge recombination reaction that leads to the higher loss of quantum energy. Some modifications in the residues nearby the acceptor pheophytin, as shown in the mutagenesis studies in PS II (*44*, *45*) or in Figure 7 that shows modifications of amino acid residues unique in *A. marina*, might also work to modify the redox levels of the cofactors, though the other parts of amino acid sequences of D1 and D2 polypeptides of *A. marina* are very similar to those in other cyanobacteria. We detected the Chl *d* delayed fluorescence from this organism at an intensity almost comparable to that from ordinary cyanobacteria, suggesting the high quantum efficiency (*34*). Further study to determine the redox potential of each

component is necessary to understand the energetics in PS II RC of *A. marina*.

Chlorophyll d and the Evolution of Oxygenic Photosynthesis. The quantum energy of light absorbed by Chl *d*-type RCs (P740 and P725) is 1.6−1.7 eV and is an intermediate between those of light absorbed by Chl *a*-type RCs (1.80 and 1.77 eV in PS II and PS I, respectively) and BChl *g*- or BChl *a*-type RCs in anoxygenic photosynthetic bacteria (1.6−1.4 eV). Although the Chl *d*-supported photosynthesis might be taken as a missing link in the evolution from anoxygenic to oxygenic photosynthesis judging from the near-far-red absorption band of Chl *d* (*1*−*3*, *11*−*13*), gene analysis put this organism to be amid of cyanobacteria (*3*). It can be speculated that the immediate ancestor of *A. marina*, which had Chl *a*-type oxygenic photosynthesis, created a biosynthesis pathway of Chl *d* first, and then, the Chl *a* was replaced by Chl *d* in both PS I and PS II followed by some adaptive optimization through some slight modifications of pigment proteins.

The successful use of Chl *d* in *A. marina* must be attained through a unique molecular feature of Chl *d*, which not only has high affinities to the pre-existing Chl *a*-binding sites in the RCs (*46*) and antenna proteins (*8*−*10*) but also has appropriate optical and redox properties suitable to replace the functions of Chl *a*, especially as the energy sinks/special pairs to accumulate the excitation energy from the other pigments. It makes a clear contrast to the situation of Chl *b*, which is more widely distributed among plants and cyanobacteria but has never been used as the special pair in the natural as well as artificial systems such as that in the cyanobacterial mutant that was transformed to oversynthesize Chl *b* (*47*). Chl *b* neither functions as the energy sink for Chl *a* and phycocyanin nor has the high affinities to the Chl *a*-binding sites on the RC proteins. The Chl-*d*-based PS I and PS II of *A. marina* also indicate the high flexibility/adaptability of oxygenic photosynthesis.

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APPENDIX: AMINO ACID SEQUENCES OF D1 AND D2 PROTEIN IN *A. MARINA*

Predicted helix regions are shown by underlines. Bold characters indicate unique residue modification sites shown in Figure 7. Numbers and residues in brackets represent consensus sequences found in the other 40 (D1) or 20 (D2) species of cyanobacteria. Numbers correspond to those in *T. elongatus* sequence.

An amino acid sequence of D1 protein in *A. marina:*
MTTVLQRRESASAWERFCS**F(W20)**ITSTNNRLYIGWF-GVLMIPTLLTAVTCFVIAFIGAPPVDIDGIREPVAGSL-LYGNNIITGAVVPSSNAIGLH**L(F93)**YPIWEAASLDEWL-YNGGPYQLIIFH**Y(F119)**MIGCICYLGRQWEYSYRLGMRP-WICVAYSAPLAATYSVFLIYPLGQGSFSDGMPLGISG-TFNFMFVFQAEHNILMHPFHMFGVAGV**L(F206)**GGS-LFAAMHGSLVSSTLVRETTEGESANYGYKFGQEEETY-NIVAAHGYFGRLIFQYASFSNSRSLHFFLGAWPVVCIW-LTAMGISTMAFNLNGFNFNHSIVDSQGNVVNTWADV-LNRANLGFEVMHERNAHNFPLDLAAGESAPVALTAPVING.

We obtained this sequence from H. Miyashita (personal communication) first and determined by ourselves from *A. marina* genome (T.U. in DDBJ with accession No. AB303650). The sequence can be also available by a BLAST search in "Phototrophic Prokaryotes Sequencing Project" (http://genomes.tgen.org/blast.php).

An amino acid sequence of D2 protein in *A. marina:*
MTVALGRVQERGWFDVLDDWLKRDRFVFIGWSGLL-LFPCAFLSIGGWFTGTTFVTSWYTHGLASSYLEGCNF-LTAAVSTPADSMGHSLLLLWGPEARGDFTRWCQLG-G**M(L110)**WNFVTLHGAFGLIGFMLRQFEIARLVN**(G137)**VRPY-NAVAFSGPIAVFVSVFLMYPLGQSSWFFAPSWGVAS-IFRFLLFVQGFHNLTLNPFHMMGVAGILGGALLCAIHGATVE-NTLFEDTKDANTFSGFSPTQSEETYSMVTANRFWSQI-FGIAFSNKRWLHFFMLFVPVTGLWASAIGLVGIALN**M(L293)**-RAYDFVSQEIRAAEDPEFETFYTKNILLNEGLRAWMA-PQDQIHENFVFPEEVLPRGNAL.

The sequence was obtained by a BLAST search in "Phototrophic Prokaryotes Sequencing Project" (http://genomes.tgen.org/blast.php).

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