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A photosystem I reaction center driven by chlorophyll *d* in oxygenic photosynthesis

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ABSTRACT **A far-red type of oxygenic photosynthesis was discovered in *Acaryochloris marina*, a recently found marine prokaryote that produces an atypical pigment chlorophyll *d* (Chl *d*). The purified photosystem I reaction center complex of *A. marina* contained 180 Chl *d* per 1 Chl *a* with PsaA–F, -L, -K, and two extra polypeptides. Laser excitation induced absorption changes of reaction center Chl *d* that was named P740 after its peak wavelength. A midpoint oxidation reduction potential of P740 was determined to be +335 mV. P740 uses light of significantly low quantum energy (740 nm = 1.68 eV) but generates a reducing power almost equivalent to that produced by a special pair of Chl *a* (P700) that absorbs red light at 700 nm (1.77 eV) in photosystem I of plants and cyanobacteria. The oxygenic photosynthesis based on Chl *d* might either be an acclimation to the far-red light environments or an evolutionary intermediate between the red-absorbing oxygenic and the far-red absorbing anoxygenic photosynthesis that uses bacteriochlorophylls.**

The conversion of solar energy by a red-light-absorbing pigment, chlorophyll *a* (Chl *a*), has been recognized as a key to current oxygenic photosynthesis. Special dimeric forms of Chl *a* molecules, P700 and P680, that were named after their absorption wavelengths (1, 2), function as the primary electron donors in the reaction center pigment–protein complexes of photosystems I and II (PS I and II), respectively. Ultimately, PS I generates a strong reducing power to produce NADPH with electrons supplied from PS II that oxidizes water to molecular oxygen.

Neither the light-harvesting antenna pigments in the oxygenic organisms such as Chl *b* and *c*, phycobilins, or carotenoids, nor the bacteriochlorophylls in anoxygenic photosynthetic bacteria have been known to replace the roles of Chl *a* in the electron transfer in PS I and II reaction centers. One exception has been divinyl-Chl *a* (see Fig. 1) found in a cyanobacteria-like oxygenic organism *Prochlorococcus marina* that shows a red absorption band similar to that of Chl *a* (3). Thus the machinery that can use red light with the high quantum energy might have been required before the evolution of oxygenic photosynthesis from the anoxygenic one that uses low quantum energy of 800- to 900-nm far-red light in the photochemical reaction.

A cyanobacteria-like photosynthetic prokaryote was recently isolated from a species of colonial ascidians and named *Acaryochloris marina* (4). Cells of *A. marina* grow photoautotrophically (5) and exhibit high oxygen evolving activity even under far-red light of 712 nm (6). The cells contained Chl *d* and Chl *a* in a molar ratio of ≈30:1, together with a trace amount of phycobilins (4, 7), and exhibited component absorption peaks at 694, 714, 726, and 740 nm at 77 K (8). The chemical structure of Chl *d*, which has a formyl group instead of a vinyl group of Chl *a*, at C-3 position of the macrocycle chlorin ring (Fig. 1) according to structural analysis by NMR (5) interprets these far-red Qy absorption peaks.

In this work, we studied the PS I reaction center complex of *A. marina*. PS I reaction centers of cyanobacteria and plants are known to consist of 11 protein subunits (PsaA–F and I–M), about 100 Chl *a* molecules, and 20 β-caroten and catalyzes oxidation of plastocyanin/cytochrome *c* at the luminal side and reduces ferredoxin at the stromal side (9). The primary donor P700 (Chl *a* dimer) situated at the luminal side transfers electron to the electron acceptor A0 (Chl *a* monomer), A1 (phylloquinone), and then to the 4Fe4S cluster F_X on the reaction center PsaA/B proteins and then to the two 4Fe4S clusters F_A and F_B on the peripheral PsaC protein (see a review in ref. 9 and Fig. 6). We purified the PS I reaction center complex from thylakoid membranes of *A. marina* and studied the function of chlorophylls. The evolution of the PS I-type of photosynthesis also is discussed.

MATERIALS AND METHODS

Isolation of PS I Complex. *Acaryochloris marina* was grown in K+SM medium (5, 10) at 28°C and pH 8.0 with gentle aeration as described (5). Ten-liter batch cultures were illuminated by fluorescent light at the light intensity of 25 μmol m^2 s^{-1}. Cells at the late exponential phase were harvested and suspended in 20 mM Bis-Tris buffer (pH 7.0) containing 20% (wt/vol) glycerol, 10 mM $CaCl_2$, 10 mM NaCl, 2 mM EDTA-Na_2, 2 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride. Cells were disrupted by a Bead-Beater (Johanna Otto GmbH, Hechingen, Germany) at 4°C by using 0.2-mm glass beads. Unbroken cells were separated from the thylakoid membranes by pelleting at 6,600 × *g* for 10 min in a Hitachi RP83T rotor. The supernatant was centrifuged at 165,000 × *g* for 40 min, and the resulting pellets of thylakoid membrane were resuspended in Bis-Tris buffer as described above.

Thylakoid membranes at 1 mg Chl *d* per ml^{-1} were stirred on ice with 0.8% (wt/vol) β-dodecylmaltoside for 1 h in the dark. The extracts were separated from insoluble membranes by centrifugation at 165,000 × *g* for 10 min and then layered onto 10–30% (wt/vol) linear sucrose density gradients. The gradients were centrifuged overnight at 198,600 × *g* in a

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Abbreviations: ABS, absorbance; Chl, chlorophyll; P700, primary donor chlorophyll *a* in photosystem I of plants and cyanobacteria; P740, primary donor chlorophyll of *Acaryochloris marina* photosystem I; PS, photosystem.

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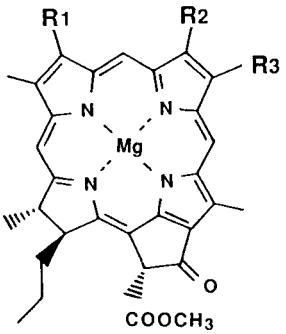

	R1	R2	R3
Chlorophyll *d*	CHO	CH_3	CH_2CH_3
Chlorophyll *a*	$CHCH_2$	CH_3	CH_2CH_3
Chlorophyll *b*	$CHCH_2$	CHO	CH_2CH_3
Divinyl Chlorophyll *a*	$CHCH_2$	CH_3	$CHCH_2$
Divinyl Chlorophyll *b*	$CHCH_2$	CHO	$CHCH_2$

FIG. 1. Chemical structure of chlorophylls in oxygenic photosynthesis of plants and cyanobacteria.

Hitachi P40ST swing-out rotor at 4°C. Three well-separated green bands were resolved on the gradients. The lowest green band rich in PS I, which later turned out to be trimeric form of PS I reaction center complexes, was collected. The PS I complex were further purified on an anion exchange column (Mono Q HR 5/5 column; Pharmacia Biotech). Polypeptide composition of the complex was determined by a 16–22% linear gradient SDS/PAGE as described by Ikeuchi and Inoue (11). Samples were preincubated with 1% (wt/vol) SDS, 10% (wt/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 62 mM Tris (pH 6.8) for about 20 min at 80°C. Gels were run at room temperature and visualized by Coomassie blue staining.

Pigments were determined by a HPLC system with a reversed-phase column (TSKgel ODS-80TM, Tosoh, Tokyo) eluted with a methanol/water mixture, after extraction of cells or PS I complexes with cold methanol (4°C) for 1 min as described (5).

Measurements. Absorption changes in the PS I complex upon the excitation by a 10-ns, 532-nm Neodymium-yttrium/aluminum garnet (YAG) laser flash (Quanta Ray, Mountain View, CA; DCR-2-10) were measured by a photomultiplier with a split beam spectrophotometer. Experimental resolution of time was 3 μs. A total of 10–64 scans at 0.3–1 Hz were averaged to increase the signal/noise ratio. Temperatures of a sample in a 10-mm light-path acrylic cuvette were maintained at 15°C by circulating water. Redox titration was done chemically as described (12). Flash-induced absorption changes at 740 nm were monitored at different ambient redox potentials which were set by adding solutions of dithionite or ferricyanide in a medium containing 50 mM Tris·HCl (pH 7.5) and 10 mM $MgCl_2$. Oxidation-reduction potential of the medium was monitored with a Pt electrode with a calibrated Ag/AgCl electrode as the standard.

RESULTS AND DISCUSSION

Isolation of PS I Complex. The PS I complex was isolated from the *A. marina* membranes solubilized with β-dodecyl maltoside followed by a sucrose density gradient centrifugation. This process yielded three pigment-containing fractions as shown in Fig. 2*A*. Analyses by spectroscopy, SDS/PAGE, and Western blotting indicated that the bottom green band (band 3 in Fig. 2*A*) was enriched in PS I complex. Bands 1 and 2 were a Chl *d*/carotenoids/protein complex and a mixture of PS I and II complexes, respectively.

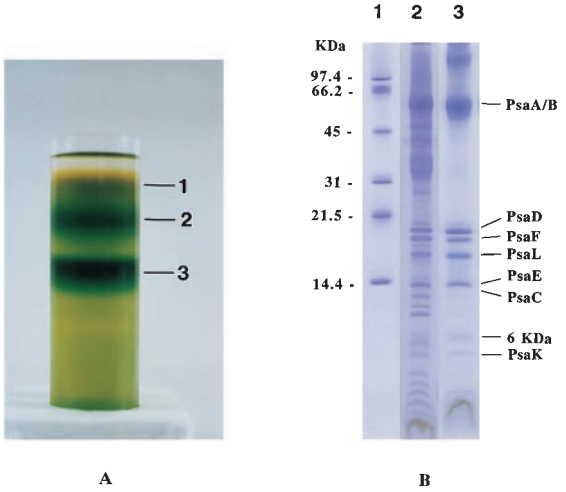

FIG. 2. (*A*) Separation of the pigment–protein complexes from detergent-solubilized thylakoid membranes of *A. marina* by sucrose density gradient centrifugation. The membranes were solubilized at 4°C for 30 min with 0.8% β-dodecyl maltoside, 20 mM Bis-Tris (pH 7.0), 10% glycerol, 10 mM NaCl, 10 mM $CaCl_2$ and 2 mM EDTA-Na_2. The sucrose gradient centrifugation (198,600 $\times$ *g* for 16 h) yielded three pigment-protein bands (1–3 on the right). (*B*) Polypeptide composition of the thylakoids and purified PS I complex from *A. marina*. Materials were treated with SDS and resolved on a 16–22% SDS/urea-PAGE. Lanes: 1, molecular weight standards; 2, thylakoid membranes; 3, PS I reaction center complex. The gel was stained with Coomassie blue. Sizes of molecular weight standards are indicated on the left, and subunit identifications based on N-terminal amino acid sequencing analysis are indicated on the right.

The bottom fraction containing PS I complex was further purified on anion-exchange columns, and polypeptide composition of the complex was determined by SDS/PAGE and by N-terminal amino acid sequence analysis. SDS/PAGE showed that the thylakoid membranes have more than 30 polypeptides, ranging in molecular masses from 60 to less than 2 kDa. SDS/PAGE of the purified PS I complex (Fig. 2*B*) resembled that of reported cyanobacterial PS I complexes (9, 11). The PsaA, PsaB, PsaC, PsaD, PsaE, PsaF, PsaL, and PsaK proteins were present as well as two unidentified polypeptides with apparent molecular masses below 6 kDa (Fig. 2*B*). These subunits were identified from their molecular weights and N-terminal amino acid sequences that showed high homologies to the corresponding polypeptides of PS I complexes of cyanobacteria or plants.

The purified PS I complex contained about 180 Chl *d* molecules per Chl *a* together with α-caroten on analysis by HPLC (Table 1). The Chl *d*/Chl *a* ratio was six times higher than that in intact cells, suggesting the loss of Chl *a* during the isolation procedure. The absorption spectrum (ABS) of the PS I complex is shown in Fig. 3*A* (solid line). It showed a red maximum at 708 nm and a Soret maximum at 456 nm. These peaks seem to originate from the Qy and Soret peaks of Chl *d*, respectively, because extracted Chl *d* gives peaks at around 690 and 450 nm in organic solvents (4, 5). The shoulder around 490 nm can be ascribed to the absorption bands of the

Table 1. Molar ratios of major pigments in intact cells and isolated PS I complex of *A. marina*

Preparation	Chl *d*/α-car*	Chl *d*/Chl *a**	Chl *d*/P740†
Intact cells	5.8 ± 0.6	30 ± 4	
PS I complex	6.0 ± 0.5	180 ± 10	145 ± 8

Pigment contents were determined as described in *Materials and Methods*.
*Average of eight experiments.
†Average of four experiments.

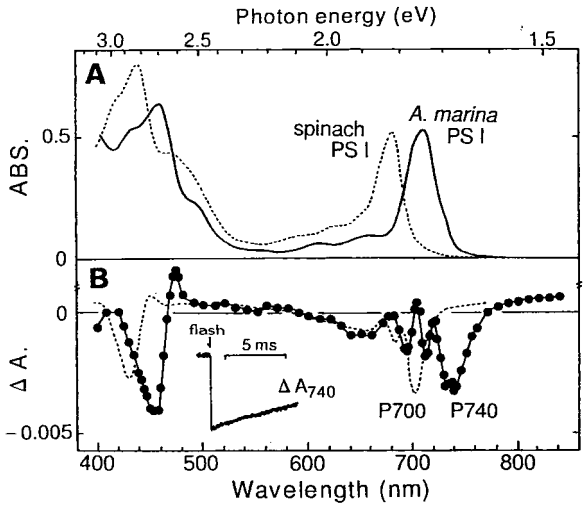

FIG. 3. (*A*) Absorption (ABS) spectrum (*Upper*) and (*B*) laser flash-induced difference absorption (ΔA) spectrum (*Lower*) at 15°C of *A. marina* PS I complex (solid line, filled circles). The PS I complexes (band 3 in Fig. 2*A*) was suspended in Tris·HCl buffer (pH 7.5) containing 100 mM NaCl and 0.3 mM sodium ascorbate. Absorption and difference absorption spectra of P700 of spinach PS I (dashed line) are also shown.

α-caroten and zeaxanthin that were identified recently (5). The spectrum differs significantly from the spectrum of PS I reaction center complexes of plant or cyanobacteria as typically represented by that of spinach in Fig. 3*A* (dashed line) which gives peaks at 680 and 430 nm due to Chl *a*.

Primary Donor P740. Excitation of the PS I complex of *A. marina* by a 10-ns, 532-nm green laser flash induced a fast absorption change, followed by a decay with a time constant ($t_{1/e}$) of 40 ms at 15°C (*Inset* in Fig. 3*B*). A point-by-point difference spectrum of the absorption change revealed negative peaks at 740, 710, 690, 650, and 455 nm (Fig. 3*B*, filled circles). The positive changes at 475 nm and that above 765 nm with a flat peak around 850 nm suggest the formation of chlorophyll cation radical. The wide bandwidth of the 740-nm peak and the complex absorption changes in the shorter wavelength side suggest the contribution of multiple chlorophyll molecules to the difference spectrum. The spectrum has only a small change at 700 nm and significantly differs from that of the primary electron donor Chl *a* (P700) of spinach, which gives negative peaks at 700 and 430 nm (Fig. 3*B*, dashed line). P700 is known to show only a few nm variation in the peak wavelengths among various PS I preparations (9, 12).

The bleaching at 740 nm can be estimated to represent the oxidation of the primary donor because its extent varied in response to the change of medium ambient redox potentials and its decay was accelerated by the addition of reductant such as ascorbate or phenazine ethosulfate. Redox titration of the extent of flash-induced bleach at 740 nm was performed in the presence of appropriate mediators according to ref. 12. The titration gave a redox midpoint potential (E_m) value of +335 mV versus normal hydrogen electrode (NHE) (Fig. 4). The extent of narrow shift-type absorption changes detected at 670–720 nm also varied in parallel with that at 740 nm.

We named the donor P740 after its red peak wavelength. The spectral feature of P740 seemed to be interpreted if Chl *d* functions as the primary electron donor. Another possibility which remains to be tested is that P740 represents a minor component Chl *a*, because some antenna Chl *a* is known to give a peak around 740 nm in cyanobacterial PS I, although it does not show stable absorption changes (13). The Soret band at 455 nm of P740, however, seems to be rather difficult to be interpreted by Chl *a* that ordinarily gives a peak around 430 nm.

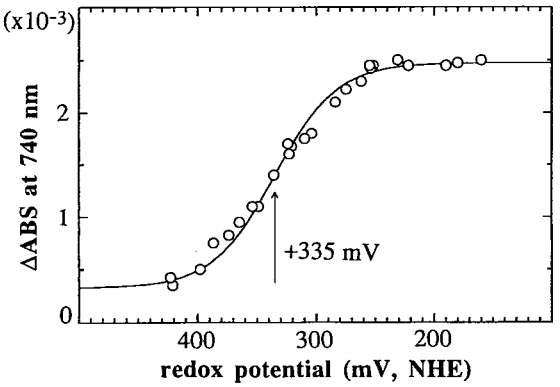

FIG. 4. Redox titration of the primary donor (P740) of *A. marina* PS I. The initial extent of the absorption change (ΔABS) at 740 nm after a laser flash excitation was plotted against the redox potential of the medium. The midpoint redox potential value was estimated to be +335 mV as shown by a fitting with an one-electron Nernst's curve by assuming 5% irreversible absorption change (solid line).

Effects of various reagents that are known to affect the reaction kinetics of P700 in ordinary PS I also were examined in *A. marina* PS I (Fig. 5). In the presence of ascorbate alone, excitation with the laser flash induced a rapid bleach at 740 nm followed by a recovery with a 40 ms $t_{1/e}$ (Fig. 5, trace a). In the presence of an electron acceptor methyl viologen ($E_0' = -446$ mV, trace b), the overall recovery rate became slower. On the other hand, addition of dithionite in the presence of methyl viologen shortened the recovery time to 1.4 ms (Fig. 5, trace c) with almost no change of spectral shape (data not shown). We also monitored the reduction of Safranin ($E_0' = -290$ mV) at 518 nm that also is known to function as the electron acceptor to F_A/F_B as methyl viologen does (14). This reagent added at 40 μM showed reduction and reoxidation after the flash with $t_{1/e}$ values of 1.8 and 26 ms, respectively (Fig. 5, trace e). The $t_{1/e}$ values became shorter at the higher concentration of Safranin.

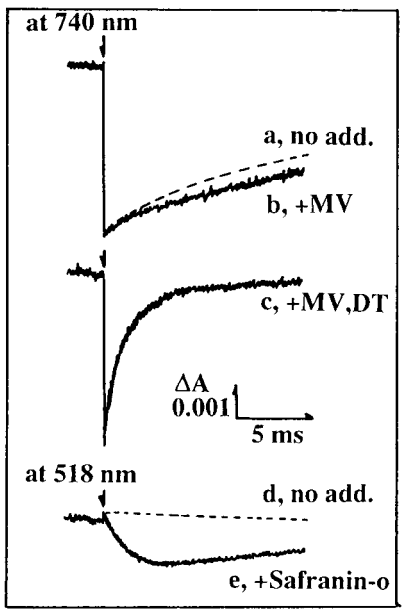

FIG. 5. Absorption changes of P740 and Safranin-o after laser flash excitation in *A. marina* PS I complex. Absorption changes were monitored at 740 nm in traces a–c and at 518 nm in traces d and e. Traces: a, no addition; b, with 300 μM methyl viologen; c, with 300 μM methyl viologen and 2 mM dithionite; d, no addition; e, with 40 μM Safranin-o. Other experimental conditions were similar to those in Fig. 3.

The effects of methyl viologen and Safranin seemed to be well interpreted by the reaction scheme (Fig. 6) established in the PS I reaction centers of plants and cyanobacteria (9, 15). In the P700-type PS I reaction centers, methyl viologen or Safranin is known to rapidly oxidize the photo-reduced F_A/F_B iron sulfur centers that otherwise reduce $P700^+$ with a $t_{1/e}$ of 30 ms and prevent the re-reduction of $P700^+$ (14). The results in Fig. 5 suggest that this was also the case with P740-type reaction center. Reduction of F_A/F_B centers with dithionite before the flash excitation, on the other hand, is expected to increase the charge recombination between F_X^- and $P700^+$ that has about 1 ms $t_{1/e}$ and to accelerate the re-reduction rate of $P700^+$ (9, 15). This mechanism clearly interprets the acceleration of $P740^+$ decay by dithionite. These results suggest the activities of F_X-, F_A-, and F_B-type iron sulfur centers in the *A. marina* PS I complex. This finding is consistent with the results of polypeptide analysis in Fig. 1 that indicated PsaC (F_A/F_B protein) as well as PsaA and PsaB reaction center proteins that contain F_X.

The contents of P740, Chl *d*, and Chl *a* were compared in PS I complex of *A. marina*. By comparing the amplitude of light-induced absorption decrease at 740 nm of P740 and that at 518 nm of peak of Safranin as done in Fig. 5 at varied concentrations of the latter, we estimated the extinction coefficient of P740 at 740 nm to be 90 mM^{-1} cm^{-1} according to Hiyama and Ke (14). This value is 1.4 times larger than the extinction coefficient of P700 at 700 nm, which is 64 mM^{-1} cm^{-1} (14). With this extinction coefficient, the content of photo-oxidizable P740 in the reaction center complex was determined to be 1 per 144 Chl *d* molecules (Table 1). The total content of P740 might be a little larger because the method counts P740 only with fully active electron acceptors. Another approach for the precise determination of the P740 content also was attempted by measuring the chemically induced absorption change of P740; however, it was difficult in the present PS I preparation because of the large overlapping bleaching of antenna chlorophylls upon the oxidation with ferricyanide. The estimated P740 content was in a range similar to or a little lower than that of P700 in plant and cyanobacterial PS I preparations (around 1 per 100–150 Chl *a*) (9, 15).

The nature of P740 can be summarized as follows. (*i*) The amount of Chl *a* contained in the PS I complex of *A. marina* was 0.8/P740. If we assume P740 to be a pair of chlorophyll molecules as is the case of P700 that is a pair of Chl *a* (16), the Chl *a* content is insufficient to constitute a dimer. (*ii*) The difference spectrum of P740 with negative Qy and Soret peaks at 740 and 455 nm, respectively, and a positive cation radical peak around 850 nm seems to be more easily interpreted if it is a dimer of Chl *d*. (*iii*) The difference extinction coefficient of Qy peak of P740 was 1.4 times larger than that of P700. The high value also might be interpreted if P740 is made of Chl *d* that has a Qy extinction coefficient 1.3 times higher than that of Chl *a* in ethanol (5). Based on *i–iii* above, we concluded that P740 is a special pair of Chl *d*. Another possibility might be that P740 is a heterodimer made of Chl *d* and Chl *a*.

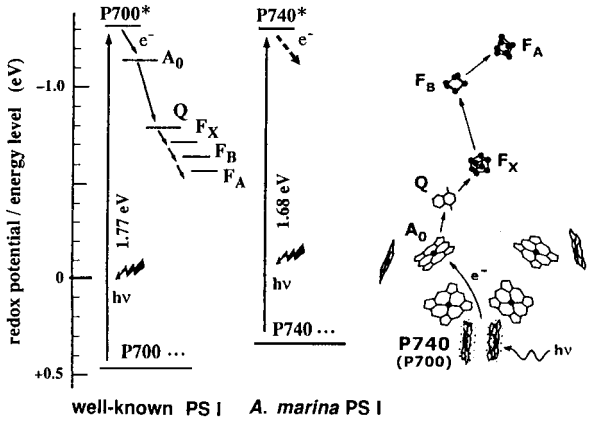

FIG. 6. Electron transfer in the PS I reaction center. (*Left*) Electron transfer pathways in PS I of plants, cyanobacteria, and *A. marina*. Light induces the electron transfer from the donor (P700 in plants or cyanobacteria, and P740 in *A. marina*) to the acceptor chlorophyll (A_0), quinone (Q), and the 4Fe4S clusters (F_X, F_B, and F_A). The acceptor side of *A. marina* PS I is not known. (*Right*) Schematic representation of the central portion of PS I reaction center complex modified from the x-ray structure of cyanobacteria (16). In plants and cyanobacteria the chlorophylls including P700 and A_0 are Chl *a* (9, 15, 16). In *A. marina*, P740 can be estimated to be a special pair of Chl *d*.

The E_m value of P740 is by 120–150 mV more negative than that (+450 to +480 mV) of P700 in literature (9, 15). A photon energy at 740 nm is calculated at 1.68 eV, which is 0.1 eV lower than that absorbed by P700 (1.77 eV). Thus, it is likely that the reducing power of P740 is almost similar to that of P700 because a more negative E_m of P740 apparently compensates for its lower excitation energy (see Fig. 6 *Left*). Effects of methyl viologen and dithionite shown in Fig. 5, on the other hand, suggest the existence of iron-sulfur centers homologous to (F_A/F_B) and Fx. The function of quinone also was suggested by the preliminary experiments of the extraction and reconstitution of phylloquinone according to ref. 17.

It is suggested that the reducing side of the *A. marina* PS I reaction center complex is almost homologous to that of the ordinary PS I that uses Chl *a* for P700 (15, 17), as schematically shown in Fig. 6. The identification of the other electron carriers including that of electron acceptor chlorophyll (A_0) in PS I, and the function of Chl *d* in the PS II reaction center of *A. marina*, are under investigation.

Evolution of Oxygenic Photosynthesis. It has long been recognized that the pigments (Chl *b* and *c*, phycobilins, and carotenoids) other than Chl *a* contribute to oxygenic photosynthesis only through the harvesting of light energy (18). Chl *a*, or its epimer Chl *a'* that has almost identical spectral feature (19), seems to make up the reaction center chlorophylls. Divinyl-Chl *a* found in *P. marina* has been an exception for this rule. However, the pigment has a spectral feature nearly identical to that of Chl *a*, showing a Qy peak at 2–3 nm shorter wavelengths and a Soret peak at about 10 nm longer wavelengths than those of Chl *a* (3). The issue is now altered by the discovery of a PS I reaction center driven by Chl *d* (P740) that absorbs far-red light. Contents of Chl *d* and *a* epimers in the PS I complex have not been determined yet.

A. marina must be benefited by the use of Chl *d* by absorbing the far-red light that is out of reach of photosynthesis by other plants and cyanobacteria. This finding indicates the wide divergency of photosynthesis and leads to an idea that the invention of Chl *a* might not be a prerequisite for the establishment of oxygenic photosynthesis. The quantum energy absorbed by P740 of *A. marina* is just at intermediate between those of the 800- to 900-nm far-red light absorbed by the primary donor bacteriochlorophylls of anoxygenic bacterial photosynthesis and the 680- to 700-nm red light absorbed by P680 and P700 of PS I and II reaction centers. Thus the oxygenic photosynthesis in *A. marina* might remain a feature of photosynthesis intermediate between the anoxygenic bacterial photosynthesis and the oxygenic plant/cyanobacterial photosynthesis.

The other attractive idea is that *A. marina* constitutes one of the new varieties evolved after establishment of Chl *a*-type photosynthesis in acclimation to the abundance of far-red light. The organism contains α-caroten (5), which is unique among the oxygenic prokaryotes, the primitive phycobiliprotein aggregates (8), and appreciable amount of Chl *c*-like pigment (5) that is known to be a precursor of Chl *a* (20). The evolutionary relationship with *Prochlorococcus* that also produces α-caroten as well as divinyl-chlorophylls *a* and *b* (3) remains to be studied.

The Chl *d*-based photosynthesis in *A. marina* adapted to far-red light also reminds us of a recent finding of the purple bacterial-type photosynthesis that is adapted to extremely acidic growth conditions by the use of Zn-containing pigment in an aerobic bacterium *Acidiphillium rubrum* (21). The bacterium uses Zn instead of Mg as a central metal of bacteriochlorophyll *a*, and produces the fully functional light-harvesting and reaction center complexes (22).

Recent phylogenic analyses using the gene sequences of 16S rRNA and rbc L filled *A. marina* in the cyanophycean lineage (23). Further analysis of genes of photosynthetic proteins as well as the functional features of this organism are now being studied. Proliferation of the oxygenic as well as anoxygenic photosynthetic organisms on the Earth might have been enabled by the unexpectedly high flexibility of their photosynthetic apparatus.

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