Characterization of Highly Purified Photosystem I Complexes from the Chlorophyll d-dominated Cyanobacterium Acaryochloris marina MBIC 11017*†

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Photochemically active photosystem (PS) I complexes were purified from the chlorophyll (Chl) d-dominated cyanobacterium Acaryochloris marina MBIC 11017, and several of their properties were characterized. PS I complexes consist of 11 subunits, including PsaK1 and PsaK2; a new small subunit was identified and named Psa27. The new subunit might replace the function of Psa1 that is absent in A. marina. The amounts of pigments per one molecule of Chl d were 97.0 ± 11.0 Chl d, 19 ± 0.5 Chl a, 25.2 ± 2.4 α-carotene, and two phylloquinone molecules. The light-induced Fourier transform infrared difference spectroscopy and light-induced difference absorption spectra reconfirmed that the primary electron donor of PS I (P740) was the Chl d dimer. In addition to P740, the difference spectrum contained an additional band at 728 nm. The redox potentials of P740 were estimated to be 439 mV by spectroelectrochemistry; this value was comparable with the potential of P700 in other cyanobacteria and higher plants. This suggests that the overall energetics of the PS I reaction were adjusted to the electron acceptor side to utilize the lower light energy gained by P740. The distribution of charge in P740 was estimated by a density functional theory calculation, and a partial localization of charge was predicted to P1 Chl (special pair Chl on PsaA). Based on differences in the protein matrix and optical properties of P740, construction of the PS I core in A. marina was discussed.

In all oxygenic photosynthetic organisms, two photosystems (PSs) cooperatively function to drive photochemical reactions; both consist of many subunits and pigments, forming supramolecular complexes (for reviews see Refs. 1, 2). PS I catalyzes the light-driven electron transfer from the plastocyanin in the lumenal side of the thylakoid membranes to ferredoxin in the stromal side (for review, see Ref. 3). The PS I complex has been isolated from cyanobacteria as a trimer with three identical monomers. The crystal structure of the PS I isolated from the cyanobacterium Thermosynechococcus elongatus has been determined, with a resolution of 2.5 Å (4). The results revealed the presence of 12 protein subunits and 127 cofactors, including 96 chlorophylls (Chls), 2 phylloquinones, 3 FeS4 clusters, 22 carotenoids, and 4 lipids. The PsA and PsB bind Chl a/Chl a′ as the primary electron donor (P700), and A0, A1, and Fx as the electron acceptors (5). Recent studies have reported that the chlorophyll network was distributed within PsA and PsB, but this point is still under debate (6–10).

Acaryochloris spp. are unique cyanobacteria that differ from the majority of photosynthetic organisms by having Chl d as the major pigment (>95%) (11–14). The type strain Acaryochloris marina MBIC 11017 has been well characterized, and its complete genome sequences were determined recently (15). Chl a is always present as a minor component, but its content varies according to the light conditions (16). The absorption maximum of Chl d in organic solvents is located at a wavelength ~30 nm longer than that of Chl a (17, 18). In vivo, the absorption maximum of A. marina cells is found at ~715 nm, significantly longer than that of Chl a. According to Miyachi et al. (19), Chl d functions as an efficient antenna for both PSs; thus the energy transfer to the individual reaction centers should be very efficient.

In photosynthetic organisms, pigments function in two roles as follows: as light-harvesting components and as electron

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** The abbreviations used are: PS, photosystem; Chl, chlorophyll; DM, dodecyl β-d-maltoside; FTIR, Fourier-transformed infrared; OTTLE, optically transparent thin layer electrode; Pheo, phaeophytin; TRFS, time-resolved fluorescence spectrum; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.

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Characterization of PS I in A. marina

EXPERIMENTAL PROCEDURES

Isolation of PS I Complexes—A. marina MBIC 11017 was cultured in a 20-liter culture bottle of IMK medium under continuous illumination from an incandescent light (15 μmol photons m⁻² s⁻¹) at 298 K. Air was continuously supplied through a filter (Millipore Millex FG-50). On each occasion, two separate bottles were used for the preparation of samples. Isolation was conducted at 277 K unless otherwise indicated. Cells were harvested by a membrane filter method with a Millipore Pellicon2 and washed once with growth medium and then with a buffer solution (50 mM MES, pH 6.5) containing 25% (w/v) glycerol, 10 mM MgCl₂, and 5 mM CaCl₂. Cells were broken twice with a French press at a pressure of 150 MPa, and cell debris was removed by centrifugation (2,000 × g, 5 min). Thylakoid membranes were recovered by centrifugation (37,000 × g, 20 min) and stored at 193 K until use.

PS I complexes were solubilized from thylakoid membranes by 1% dodecyl β-D-maltoside (DM) in the dark. The sample solution was gently stirred with a magnetic stirrer for 20 min and then centrifuged at 37,000 × g for 30 min. Supernatants were purified by column chromatography. The first step was purification on a DEAЕ-Toyopearl 650S column. Fractions containing PS I complexes were subjected to sucrose density gradient centrifugation to remove contaminating PS II and the small amount of monomeric PS I. Finally, the PS I sample was purified by passage over a UnoQ column. A trimer form of the PS I complex was obtained, and its subunit composition was analyzed using SDS-PAGE according to the method of Ikeuchi and Inoue (30). The stacking gel was 4.5%, and the running gel was 16–22% acrylamide. Gels were stained with 0.1% Coomassie Brilliant Blue R-250.

Determination of N-terminal Amino Acid Sequences—The low molecular weight subunits were separated by electrophoresis, and the proteins on the gel were transferred onto a polyvinylidene difluoride membrane (Millipore MA), stained with 0.1% Coomassie Brilliant Blue R-250, and de-stained with a tyrosine membrane (Millipore MA). Samples were injected onto an Inertsil SIL100 column (100 × 4.6 mm, GL Sciences Inc., Tokyo, Japan) after filtration (0.2 μm). The mobile phase was hexane/2-propanol/methanol (100: 0.8:0.4) with a flow rate of 1.0 ml/min (32, 33). Pigments were detected by a photodiode-array detector (JASCO, MD-915, Tokyo, Japan). A standard solution containing known amounts of authentic α-carotene, Chl a, Chl d, and pheophytin (Pheo) a

Transfer components. Changes in pigment composition affect pigment function, the reaction environment, and the modified proteins that accommodate the pigments. Electron transfer reactions are organized as a sequence of absolute redox potentials. In contrast, energy transfer processes are governed by the energy levels, orientations, and distances between donors and acceptors. An absolute excitation energy of a pigment is not significant, but the energy levels relative to that of the reaction center are important. For this reason, it is of particular interest to understand the effects of pigment changes on the electrochemical properties of the system and not to rely solely on changes in the light-harvesting properties.

PS I complexes of A. marina have been isolated by many groups, and many aspects of their structure and function have been characterized, but inconsistencies in these data remain. Hu et al. (20) isolated PS I particles and detected a flash-induced difference maximum at ~740 nm (P740). Sivakumar et al. (21) showed a light-induced P740+/P740 Fourier transform infrared (FTIR) difference spectrum that involved characteristic CH stretching bands arising from the 3-formyl group of Chl d. Theses data indicate that the primary electron donor of PS I in A. marina is a Chl d dimer. Hu et al. (25) reported that the redox potential of P740 was 335 mV. This is significantly lower than that of the T. elongatus PS I (+423 mV) (22, 23) and of PS I from other species (24), and it is inconsistent with recent studies of P740 in A. marina (25, 26).

With regard to the protein composition of PS I, Hu et al. (20) reported the presence of PsA, PsaB, PsaC, PsaD, PsaE, PsaF, PsaL, PsaK, and two unidentified polypeptides with apparent molecular masses below 6 kDa. One of these two might be PsaL, a common cyanobacterial PS I. However, the complete genome sequence of A. marina MBIC 11017 indicates that psaL is absent (15). The crystal structure of the T. elongatus PS I shows that Psal localizes to the attaching site of individual monomers (4). However, almost all particles of PS I isolated from A. marina were trimers. Therefore, it is necessary to identify the A. marina protein that leads to stabilization of the trimer structure.

The major pigment in A. marina is Chl d. Chl a is a minor component but is not absent (16, 27). In PS II, at least three Chl a molecules are present in the core complex (28). In the case of PS I, it has been reported that nearly one Chl a is present per unit of PS I (20). However, Chl d’, a constituent of P740, was not detected in that study, and the Chl number was higher than that observed in other cyanobacteria (4, 24). Kumazaki et al. (29) suggested that the role of Chl a in PS I was as the primary electron acceptor. However, the role and localization of Chl a in PS I remain controversial.

To facilitate further analysis, highly purified and chemically stable samples are required. In this study, we obtained highly purified PS I complexes from A. marina by a combination of detergent treatment, column chromatography, and sucrose density gradient centrifugation. The PS I activity of the resulting sample was comparable with that of other cyanobacteria, and the sample was further characterized by absorption, steady-state fluorescence, time-resolved fluorescence, FTIR spectroscopy, redox chemistry, and computational chemistry. We found several essential differences from those properties reported earlier. Based on our new findings, we discuss the overall energetics in PS I of A. marina and the general features of PS I, including other oxygenic photosynthetic organisms.
Characterization of PS I in A. marina

was used for quantitative calibration. We used published molar extinction coefficients for individual pigments (34–36).

The secondary electron acceptor was determined by HPLC analysis. Quinones were extracted from lyophilized samples with an acetone/methanol mixture, dried in vacuo, and analyzed by HPLC using a polyetherether ketone column packed with ODS (Senshupak, Senshu Science, Tokyo, Japan) (37). An authentic sample of quinone purchased from Wako Chemicals Co. Ltd. (Osaka, Japan) was used as a control.

Absorption and Fluorescence Spectra—Absorption spectra were measured at 283 K and 80 K using a Cary 500 spectrophotometer with a cryostat for liquid nitrogen temperature (OptistatDN, Oxford Institute, Oxford, UK) and an Oxford ITC-601PT controller. Fluorescence spectra at 77 K were measured using a Hitachi 850 spectrofluorometer (Hitachi, Japan) with a custom-made Dewar system (38). Polyethylene glycol (average molecular weight 3350, final concentration 15% (w/v)) was added to obtain homogeneous ice at 77 K. The spectral sensitivity of the fluorometer was corrected by using a standard lamp with a known radiation profile (Hitachi, Japan).

Savitzky–Golay derivative spectra were obtained by the method of Savitzky and Golay (39).

Measurement of Photosynthetic Electron Transport Activity—PS I-mediated electron transport rates were measured as oxygen uptake by the Mehler reaction, using 1 mM ascorbate, 2 mM diaminodurene, and 2 mM methyl viologen and a Teflon-coated oxygen electrode (Rank Brothers, UK) (40). Light (intensity of 1,800 µmol photons m⁻² s⁻¹) from a halogen lamp was guided via a glass fiber (Moritex, Japan). The reaction mixture contained 10% glycerol, 20 mM NaCl, 40 mM HEPES-NaOH (pH 7.5) and ~15 µg of Chl d.

Measurements of FTIR Spectra—FTIR spectra of the P740⁺/P740 and P700⁺/P700 were recorded on a Brucker IFS-66/S spectrophotometer equipped with an MCT detector (D313-L) as described previously (41). An aliquot (5.5 µl) of a suspension of the PS I core complexes (~5.7 mg Chl/ml) in a Tris-HCl buffer (10 mM Tris and 0.05% DM, pH 7.0), with 4 µl of 100 mM sodium ascorbate (adjusted to pH 7.0 by NaOH) and 0.7 µl of benzylviologen, was loaded onto a BaF₂ plate (13 mm in diameter). The sample was then lightly dried under N₂ gas flow and sandwiched with another BaF₂ plate and 0.7 µl of water. The sample temperature was adjusted to 275 K in a liquid N₂ cryostat (Oxford DN1704) using an Oxford ITC-5 controller. Spectral measurements were taken for 5 s under both dark and illuminated conditions and were repeated 1000 times. The spectra were averaged to calculate light-minus-dark difference spectra. Light illumination was performed with continuous red light (~700 µmol photons m⁻² s⁻¹ at the sample point) from a halogen lamp (Hoya-Schott HL150) equipped with a red cutoff filter (>600 nm, Toshiba, Japan). The spectral resolution was 4 cm⁻¹.

Measurements of the Redox Potential and Light-induced Absorption Change—The P740 redox potential was determined spectroelectrochemically using an optically transparent thin layer electrode (OTTLE) cell by monitoring redox-induced absorbance changes at several wavelengths (22, 23, 42). The assembly of the OTTLE cell (optical path length, ~180 µm), comprised of a gold mesh working electrode, a platinum wire counter electrode, and an Ag-AgCl (saturated KCl) reference electrode, has been described previously (42). The electrode potential is hereafter reported against the standard hydrogen electrode (+199 mV versus Ag-AgCl in saturated KCl).

For measurement of the light-minus-dark spectra, samples were suspended in 0.03% DM, 1 mM ascorbate, 200 µM methyl viologen, 25 µM N,N',N''-tetramethyl-p-phenylenediamine, and 50 mM Tris-HCl (pH 8.0) and were illuminated with white light. Light-induced absorbance changes were saturated under our measuring conditions, and the actinic effect of the measuring light and interference of fluorescence was not observed (43).

Time-resolved Fluorescence Spectra (TRFS) and Decay Curves—TRFS were measured by the time-correlated single photon counting method at 77 K (38, 44) using a custom-made Dewar bottle. The light source was the second harmonic of a titanium:sapphire laser with an excitation wavelength of 425 nm, which gave preferential excitation of the Soret bands of Chl d and Chl a. The time interval of data acquisition was set to 24.4 ps/channel, using a time-to-amplitude converter. Excitation pulses were detected as a pulse with a certain time width, typically 30 ps. Time zero was set as the time when the amplitude of the excitation beam was highest on the time-to-amplitude converter. TRFS was measured in a given wavelength region, and decay curves were measured at several discrete wavelengths. Fluorescence lifetimes were estimated using a convolution calculation (45).

Estimation of Charge Distribution by the Density Functional Theory Calculation—All calculations were performed in GAUSSIAN 03 (46). The coordinates of P700 were adopted from the PS I of T. elongatus (Protein Data Bank 1B0), and those of the P740 were estimated with replacement of a vinyl group at the C-3 position of Chl a to a formyl group. The phyl groups of Chl a and Chl d were replaced with hydrogen atoms. The positions of the hydrogen atoms were optimized at HF/6-31G(d) level. Final electronic energies with natural population analysis were calculated at the B3LYP/6-31G(d) level for the optimized structures. These calculations were performed in solvents approximated with IEF-PCM (PCM calculation using the integrated equation formalism model) (47, 48).

RESULTS

Subunit, Pigment, and Co-factor Composition—PS I complexes of A. marina consist of 11 subunits as shown by SDS-PAGE (Fig. 1). Based on the molecular weight sizes on PAGE, the high molecular weight bands were identified as PsA, PsB, PsD, PsE, PsF, and PsL, respectively. PsA/B were also identified by the Western blot analysis (data not shown). Using N-terminal sequence analysis, two subunits (PsE and PsA) were identified (Table 1, co-migrating to the same position. PsA (band 9), PsA (band 11), and a newly identified subunit (band 10) were formylated at their N terminals. As predicted by the genome analysis (15), Psal was not detected. However, a new subunit with a sequence of MISDILPAMTPLYW (Table 1) was detected in a low molecular mass region (band 10 in Fig. 1). The new subunit was only poorly stained with Coomassie Brilliant Blue, but an equimolar amount of amino acids was detected by sequence analysis, indicating that this subunit is a real constit-
In conjunction with the genome information, a novel open reading frame encoding a polypeptide with the sequence MISDILPAIMTPLVVLIGGGAAMTAFFYYVEREG was identified. The calculated molecular mass of this formylated subunit was 3675.34. The new subunit was designated Psa27. It is known to PsbW, which stains poorly with Coomassie Blue on PAGE (49). Like PsbW, Psa27 lacks cysteine, histidine, and lysine residues, allowing it to be only poorly stained (49). PsaJ and PsaM contain lysine residues, which might account for differences in the degree of stainability.

Two PsaK subunits were observed in *A. marina*, with a protein identity of 36% (15). A previous report demonstrated the existence of two unidentified polypeptides with apparent molecular masses of ~6 kDa and less than 6 kDa (20). Our sequence analysis suggests that the unidentified polypeptide of ~6 kDa is PsaK2. The quantity of PsaK2 detected was similar to that of PsaK1; a relative content of PsaK1 to PsaK2 was 0.99 by sequence analysis. In *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*), the amount of PsaK2 was higher when grown under high light conditions (50). However, this increase in the amount of PsaK2 was not observed in *A. marina*, as determined by comparison of the high light (150 μmol photons m⁻² s⁻¹) and normal light (15 μmol photons m⁻² s⁻¹) conditions (data not shown).

The complexes were found to contain four kinds of pigment, Chl *d*, Chl *d*′, Chl *a*, and α-carotene; Pheo *a* was detected as a trace component (the presence of Chl *d*′ in *A. marina* had been first described in Ref. 32). The amounts of individual pigments per one molecule of Chl *d*′ were 97.0 ± 11.0 Chl *d*, 1.9 ± 0.5 Chl *a*, 25.2 ± 2.4 α-carotene, and 0.3 ± 0.2 Pheo *a* (n = 6). According to the amount present, Pheo *a* is unlikely to be stoichiometrically bound to the complexes but might be converted from Chl *a*. A previous report showed the number of Chl *a* were 0.8 per one P740, i.e. one Chl *a* per 180 Chl *d* (20), inconsistent with our results. When cells were grown under the high light condition (150 μmol photons m⁻² s⁻¹) or under the far-red light (>700 nm), the amount of Chl *a* was increased to three or four per one molecule of Chl *d*′ in the isolated PS I complexes (data not shown).

### Characterization of PS I in *A. marina*

**TABLE 1**

| Polypeptide number | N-terminal amino acid sequences | Identification | Deduced molecular mass |
|--------------------|---------------------------------|----------------|-----------------------|
| 1                  | PsaA/B                          | 83399.63      |
| 2                  | PsaA/B                          | 82129.51      |
| 3                  | PsaD                            | 15095.51      |
| 4                  | PsaD                            | 15095.51      |
| 5                  | PsaL                            | 15339.47      |
| 6                  | VQRGSKV                         | PsaE          | 9416.45               |
| 6                  | SHTVKIY                         | PsaE          | 9416.45               |
| 7                  | TTFESHPTW                       | PsaK2         | 8594.69               |
| 8                  | TAVRAVNW                       | PsaK1         | 8032.36               |
| 9                  | MFFSTAPVIALVFF                | PsaJ          | 4349.10               |
| 10                 | MISDILPAIMTPLUV                | Psa27         | 3675.34               |
| 11                 | MEISLQTVIALVVA                 | PsaM          | 3207.34               |

* The molecular mass of PsaA is indicated.
* The molecular mass of PsaB is indicated.
* The formylated N terminus is indicated.
* The formylated N terminus of amino acids weight plus formylation mass is indicated.
Characterization of PS I in A. marina

The secondary electron acceptor of PS I in A. marina was identified as phylloquinone, by comparing the retention time on HPLC and the absorption spectra with those of an authentic compound. The number of phylloquinone was approximately two per 100 molecules of Chl d.

Absorption and Fluorescence Spectra—At physiological temperature, the absorption maximum of the Qy band was located at 708.5 nm (Fig. 2A). The spectral shape was similar to that reported previously (20, 51). At cryogenic temperature, several Chl d bands were observed (Fig. 2B). The main band was located at 711 nm. In the long wavelength region of the maximum, there were at least two bands (728 and 740 nm), and in the Soret-band region, at least three bands were discernible at 398, 430, and 460 nm. Carotenoid showed its maximum at 492 nm, but its other peak(s) was not clearly detected because of an overlap with the Soret band of Chl d.

The fluorescence spectra at 77 K consisted of three bands at 703, 732, and 760 nm on excitation at 457 nm (Fig. 2C). The band at 703 nm was wide, similar to the absorption spectra of Chl d in organic solvents. When excited at 435 nm, an additional peak at 675 nm was resolved. When α-carotene was excited at 490 nm, the fluorescence intensity at 703 nm was drastically decreased, and the 675-nm component was not resolved. Fluorescence excitation spectra indicated the contribution of individual pigments to fluorescence bands. When monitored at 730 and 756 nm, an excitation spectrum clearly showed that Chl d and α-carotene both contributed to Chl d emission (Fig. 2D, dotted and dash-dotted line). On the other hand, when monitored at 702 nm, Chl d was still a major contributor, but α-carotene scarcely transferred energy to this form of Chl d (Fig. 2D, dash-dotted line). With monitoring at 670 nm, two bands were detected at 415 and 435 nm (Fig. 2D, solid line), corresponding to the spectrum shape and peak positions of Chl a. These results clearly indicate that the fluorescence bands in the wavelength region from 670 to 690 nm were attributable to Chl a. Even though the presence of Chl a was not evident in the absorption spectra, it was unambiguously demonstrated by the fluorescence excitation spectra.

Photosynthetic Activity of PS I Complexes—Our PSI complexes were photochemically active, showing an oxygen uptake (Mehler) reaction of 544 ± 18 μmol O2 (mg of Chl)−1 h−1. The observed level of activity was sufficient for further analysis; for comparison, PS I complexes isolated from Synechocystis and Gloeobacter violaceus showed activity of 750 ± 35 and 360 ± 35 μmol O2 (mg of Chl)−1 h−1, respectively (52).

FTIR Spectra—The special pair of PS I in A. marina was assigned to a Chl d dimer (20, 21) and further to a Chl d/Chl d′ heterodimer in consideration of the pigment content (32) and the x-ray crystallographic structure of T. elongatus PS I (4). This hypothesis was confirmed by the cation-minus-neutral FTIR spectra (Fig. 3). The spectral region at 1800–1100 cm−1 involves the C=O stretching and chlorine ring vibrations of Chls (53, 54). The overall spectral features of the P740+/P740 spectrum of A. marina (Fig. 3A, a) were very similar to those of the P700+/P700 spectrum of spinach (Fig. 3A, b). Prominent positive/negative peaks at 1717/1702–1701 and 1659–1654/1643–1638 cm−1 can be assigned to the 131-keto C=O vibrations of the cation/neutral forms of P2 (special pair Chl on PsAB) and P1 (special pair Chl on PsA), respectively (55). Also, minor peaks at 1754–1750/1748–1745 and 1741–1740/1735–1729 cm−1 were ascribed to the 132-ester C=O vibrations of P2 and P1, respectively (55). The 3-formyl C=O bands might be present in the structures at 1690–1660 cm−1 in the P740+/P740 spectrum of A. marina (Fig. 3A, a), but accurate assignments were hampered by the overlap of the amide I bands of the protein backbones in the same region. Medium or weak bands in the regions of 1610–1605, 1560–1500, 1350–1280, and 1200–1150 cm−1 were because of the chlorine ring vibrations. Because these vibrations are sensitive to differences in the chemical structure of Chl (53), they are useful as “fingerprints” to discriminate between Chl species. Most of the ring vibration peaks in A. marina were shifted by a few wave numbers from those of spinach (Fig. 3A), indicating that Chl species were different between P740 and P700. The CH stretching bands of the...
3-formyl group were also observed at 2726 and 2717 cm⁻¹ in our P740⁺/P740 difference spectra of *A. marina* (Fig. 3B, a), whereas no peaks were observed in the P700⁺/P700 spectra of spinach (Fig. 3B, b), consistent with the previous results reported by Sivakumar et al. (21). Thus, the FTIR spectra clearly indicate that P740 of *A. marina* consists of two Chl d molecules. Because one Chl d⁺ was detected per ~97 Chl d molecules, the special pair was most probably assigned to a Chl d/Chl d⁺ heterodimer, as had been first suggested (56) and substantiated (57) previously.

**The Redox Potential of the Special Pair**—The redox potential of the P740 was initially reported to be +335 mV by Hu *et al.* (20), which is lower by as much as 100 mV than that of P700. However, this value is not consistent with recent reports by a few groups (25, 26). Even the reported value of the redox potential of P700 in spinach exhibits a heavy scatter from +430 to +525 mV (23, 24). One possible cause for such a scatter is a low accuracy of chemical titration using potassium ferricyanide as the redox mediator or for imposing the redox potential. To overcome this drawback, we measured the P700 redox potentials of *A. marina* by spectroelectrochemistry with an OTTLE cell, in which equilibration was very rapid and the error range was very small, i.e. a few millivolts (42). Fig. 4, A and B, shows the light-induced absorption change and the spectroelectrochemical oxidized (+650 mV-)minus-reduced (+50 mV) difference spectrum of *A. marina*, respectively. The peak positions and spectral shapes of these spectra were almost identical. The peaks were located at 692, 710, 728, and 740 nm. The broad peak located at ~810 nm was assigned to a cation band of the special pair. Fig. 4C shows the difference spectrum (20). To examine the spectral features of the special pair and the additional band, the difference spectrum of P740 was deconvoluted into two Gaussian spectral components (Fig. 5). The peaks of the resolved components were located at 726.2 nm and 738.2 nm. The latter band is assigned to a dimeric Chl d based on the area (P740) and the former to a monomeric Chl d.

In the difference spectrum of P740 in *A. marina*, multiple bands were clearly detected, and the redox potentials of all peaks at 692, 710, 728, 740, and 820 nm were measured by OTTLE cells. Fig. 6A shows the redox-dependent absorbance changes at 740 nm. P740 was first reduced at E° = +50 mV and then oxidized by application of a varying anodic electrode potential. ΔAₕ₋₄ decreased rapidly and reached a steady state within 120 s, for any electrode potential applied. When the electrode potential was returned to E° = +50 mV, the ΔAₕ₋₄ value increased rapidly to zero, indicating that the redox reaction of P740 was fully reversible. The reversibility of the redox reaction was maintained over several hours of experiments. Complete oxidation and re-reduction of P740 at E° = +650 and +50 mV were confirmed by application of higher and lower potentials. A molar ratio of P740⁺ was plotted against the applied voltage, and the curve was simulated by a one-electron Nernst curve (Fig. 6B). When the potential value was plotted against the logarithmic fraction of P740⁺, a slope of 60.2 mV/decade (Fig. 6C) was derived; because this is very close to 59 mV/decade for an ideal one electron redox reaction, our measurement was sufficiently accurate to allow estimation of a redox potential. Both plots (Fig. 6, B and C) indicated that the redox potential of P740 was +439 mV (±2 mV for five independent samples). The same measurements were performed on all peaks, and the redox potentials were estimated to be +438, +441, +436, and +439 mV at 692, 710, 728, and 820 nm, respectively; all values could be regarded as containing the same degree of experimental error. At 710 nm, part of the irreversible component was noted during measurement. This was probably

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3 Y. Kato, A. Nakamura, and T. Watanabe, unpublished data.
Characerization of PS I in A. marina

**Figure 4. The difference absorption spectra of P740.** A, light-induced difference absorption spectrum; B, oxidized (+650 mV)-minus-reduced (+50 mV) difference absorption spectrum measured by spectroelectrochemistry; C, potassium ferricyanide-minus-sodium ascorbate difference absorption spectrum. Chl d concentration was adjusted to 1.2 mM. The potential of potassium ferricyanide is +433 mV and that of sodium ascorbate is +58 mV.

due to overlap of electrochromic shift, as described above (Fig. 4C). These results clearly demonstrate that all the peaks have an identical redox potential.

**Charge Distribution in the Special Pair**—P740 was compared and characterized by the charge density on the cation state, i.e. P700⁺ and P740⁺. We used a density functional theory calculation with natural population analysis at the B3LYP/6–31G(d) level by Gaussian03 (46). Selection of a correlation function and a basis set was performed according to the method described by Yamasaki et al. (58). When applied to the bacterial reaction center, this analysis confirmed the asymmetry of the charge distribution of the special pair (58). The results on P700⁺ and P740⁺ are summarized in Table 2. When a dielectric constant (ε) was taken in a range from 2.25 to 4.34, a charge of P700⁺ was almost delocalized. On the contrary, a charge of P740⁺ was partially localized on the Chl d' in PsaA (P1 Chl) in benzene (ε = 2.247), and this tendency was pronounced to approximately two-thirds in ether (ε = 4.335). These results show that P740 differs significantly from P700.

**TRFS**—TRFS of the A. marina PS I are shown in Fig. 7A. A short lived component was observed at 730 nm soon after the excitation, and this shifted into the red, up to 760 nm, with a distinct intermediary component in less than 1 ns. The 760 nm band disappeared in 3 ns, indicating its short lifetime. These phenomena were consistent with the observation made in intact cells (16). At 675 nm, a component with an intermediary lifetime was observed, and a long lived component was clearly observed at 706 nm. A fluorescence decay curve revealed the nature of those long lived components (Fig. 7, B and C). At individual wavelengths, a short lived component and a long lived component were clearly resolved. The lifetimes of the short lived components were 55 and 49 ps at 675 and 706 nm, respectively, but TRFS did not reveal the presence of such a short lived component at 675 nm. Because the observed lifetimes were close to one another, and the 706 nm band was wide, it is reasonable to assign the short lived component detected at 675 nm to an edge of a short lived component detected at 706 nm. On the other hand, long lived components were resolved to 5.60 and 5.93 ns at 675 and 706 nm, respectively, by the convolution calculation. The lifetime of uncoupled Chl has been reported to be around 6 ns (59–61); therefore, the long lived components detected at 675 and 706 nm were assigned to uncoupled pigments. The four spectral components observed at 675, 703, 730, and 760 nm were consistent with a steady-state fluorescence spectrum (Fig. 2C), even though their relative
intensities differed. Delayed fluorescence was not observed in isolated PS I complexes. In intact cells of A. marina, it was found at 685 nm with a lifetime of 15 ns (38), but this originates from the special pair or the accessory Chl of PS II (62).

**DISCUSSION**

**Subunit Compositions**—We identified 11 subunits in the PS I complex of A. marina, including a new subunit Psa27. A corresponding novel open reading frame was also identified in the genome of A. marina MBIC 11017 (15). The secondary structure prediction analysis shows that this peptide contains one helix region. Its sequence is moderately similar to plant-type Psal and PsaZ of G. violaceus (63) as shown via phylogenetic analysis (supplemental Fig. 1). These two subunits are assigned to a stabilizer of the trimer structure of PS I complexes. A crystal structure of PS I from T. elongatus showed that Psal is localized in the attaching site of individual monomers. In the case of G. violaceus, which is considered to be the most primordial cyanobacterium, three small subunits, Psal, Psb, and Psak, are missing (63); instead a new subunit PsaZ was associated with PS I (64), and might serve to stabilize the trimer structure. The sequence homology of Psa27 with Psal or PsaZ suggests that the Psa27 functions as a stabilizer of the trimer structure. Actually, we found that almost all subunits of PS I were recovered as trimers in A. marina (see “Experimental Procedures”).

It is reported that PsaK2 is involved in the energy transfer from phycobilisome to PS I under the high light condition in Synechocystis (50). However, in A. marina PS I, the amount of PsaK2 was constant and did not increase even under the high light condition (data not shown). These results seem to be consistent with the observation that A. marina does not form phycobilisomes but instead accumulates rod-shaped phycobiliprotein complexes (65).

The localization of PsaK1 and PsaK2 was unclear. In the crystal structure of T. elongatus, Psak was localized on the outside of the trimer. Because only psak2-type was present in T. elongatus genome, the localization site of Psak in T. elongatus will be applicable to A. marina. The localization of PsaK1 was not clear, but by analogy to Psag in higher plants, its site could be inferred. Fujimori et al. (50) suggested that the cyanobacterial psak seems to be the common ancestor of psaK/pasag in higher plants. The localization of Psak in higher plants seems to match that of PsaK2 in T. elongatus (66). In contrast, Psag was localized to a site that was vacant in cyanobacteria. Therefore, it is probable that Psak1 associates with the core at the PsaG site in higher plants and that this will be applicable to A. marina. However, further studies will be necessary to confirm these possibilities.

**Redox State of P740**—A redox potential of P740 in A. marina is one of the key properties of PS I; the initially reported value of +335 mV (20) is inconsistent with later reports (25, 26). Speculating that the main cause for the discrepancy is the low accuracy of chemical titration (22, 23, 42), the spectroelectrochemical method was adopted in this study. The redox potential values were deduced from the five bands seen in the difference spectra, at 692, 710, 728, 740, and 820 nm (Fig. 4, A and B), and
Characterization of PS I in A. marina

![Graphs showing fluorescence spectra and decay curves](image)

**FIGURE 7.** Time-resolved fluorescence spectra and fluorescence decay curves at 77 K. The excitation wavelength was 425 nm with a pulse width of 150 fs. The data acquisition interval was 2 ns, and the acquisition time was 24.4 ps/channel. The half bandwidth of the monochrometer was 6 nm. Fluorescence intensities were normalized at the maxima of individual spectra (A). The lifetimes (τ) of the longest components were presented in B and C.

All bands were found to give a potential of +439 mV, with only minute experimental errors. This result is consistent with recent reports (25, 26) and is well within the reported P700 values of other organisms. A photon energy captured at 740 nm corresponds to 1.68 eV, which is lower by 0.09 eV than that absorbed by P700 (1.77 eV). Thus, the regulation of redox potential in the electron transfer chain in individual PSs is probably adjusted at the level of the primary electron acceptor. The same manner for adjustment of the potential is known in PS II of A. marina (Fig. 8) (33, 67). The main reason for an unchanged redox potential of PS I in A. marina is probably because of a relationship with plastocyanin.

The redox potential of plastocyanin has been reported to be between +360 and +389 mV (68–72), and is affected both by the amino acid that provides a ligand to the active center (copper) of plastocyanin, and by other amino acids in close vicinity to a ligand. These amino acids are also responsible for binding to PS I and were highly conserved among A. marina, other cyanobacteria, and higher plants (NCBI data base). Sigfridsson et al. (71) demonstrated that the T83H mutation, which is adjacent to an acidic patch on plastocyanin, caused an upward shift in the redox potential of plastocyanin in spinach; however, Thr-83 and the amino acids in the close vicinity were also conserved in A. marina. Therefore, it is reasonable to assume that the redox potential of plastocyanin in A. marina is similar in other cyanobacteria. As a result, the potential of P740 is maintained in other photosynthetic organisms and is higher than the redox potential of plastocyanin.

The difference absorption spectra of P740 (Fig. 4, A and B) clearly indicate the presence of an additional band at 728 nm, which was ascribed to a monomeric Chl d (Fig. 5). Because the corresponding band was not detected in the difference spectrum of P700, identification of an origin of the 728 nm band provides information on the constitution of the core pigments of PS I. In the case of P700+ (4), an FTIR spectrum indicated that the charge distribution of P700+ was delocalized between P1 and P2 Chls with a ratio ranging from 1:1 to 1:2 (55, 73). In contrast, the ESR study suggests that 85% of the spin density is localized on one of the two Chl molecules of P700 (probably P2) (74). The discrepancy between the two types of techniques, charge and spin, is still under debate. Charge localization has important effects on the chemical properties of the constituent pigments and on their interactions with the protein environments in PS I of A. marina (75). In the case of P740+, no experimental data on the charge distribution were available; however, the calculation suggests that a charge is partially localized to P1 (Table 2), whereas P700+ was shown to be delocalized. This difference may arise from the chemical properties of the pigments and from pigment-pigment interactions.

The sequence homology of PsaA between A. marina and other cyanobacteria was highly conserved. However, there were significant differences in a few amino acids near the special pair, especially near the P1 Chl (Fig. 9). We constructed a model structure of A. marina PS I using the Swiss model comparative protein modeling server (76). We confirmed that a hydrogen bond to the special pair was formed only in the P1 Chl d; no hydrogen bond exists at the P2 Chl d (4). In the P740+/P740 FTIR difference spectrum of A. marina (Fig. 3A, a), the 132-ester C=O peaks at 1750–1729 cm⁻¹ were lower by 1–6 cm⁻¹ than those of spinach, and the 1659/1643 cm⁻¹ peaks, which likely arose from the hydrogen-bonded 131-keto C=O of P1, were found to be 5 cm⁻¹ higher than the corresponding peaks of spinach (Fig. 3A, b).
Isolated PS I complexes contained one molecule of Chl d' per ~100 Chl d molecules; this Chl content was comparable with P700 in other cyanobacteria and suggests that Chl d' is a constituent in reaction center, similar to Chl a' in P700. Chl d' is not distinguishable from Chl d by spectroscopy; therefore, our spectral analyses did not provide a definitive conclusion on the localization of Chl d' in the special pair. Giving consideration to the crystal structure of *T. elongatus*, Chl d' was tentatively assigned to P1. However, this assignment, together with the bleach of the band at 728 nm, should be studied further.

Chl *a* in PS I—Chl *a* is always present in intact cells of *A. marina* grown under any light intensity conditions (16). PS I complexes isolated from cells grown at 15 μmol photons m⁻² s⁻¹ contained two Chl *a* molecules per Chl d' molecule in this study. The Chl *a* number increased under the high light culture conditions, even in isolated PS I complexes (150 μmol photons m⁻² s⁻¹), consistent with previous observations in intact cells (16). Hu et al. (20) reported that the Chl *a* number in PS I particles was approximately one per P740. Consequently, it appears that the Chl *a* number in PS I varied depending on the light conditions. Kumazaki et al. (29) performed laser photolysis measurements on partially purified PS I particles and found that the primary electron acceptor (A₀) was possibly Chl *a*, based on the decay-associated spectra at 680 nm; the time constant of the disappearance of the A₀ signal was reported to be 50 ps. In contrast, the lifetime of A₀ in *Synechocystis* was estimated to be 20–30 ps (77). In a recent analysis of flash-induced spectra of the thylakoid membrane of *A. marina*, Itoh et al. (78) showed that the 685 nm spectrum change could be assigned to the primary electron acceptor of PS I. We identified three kinetic components in the fluorescence decay curve at 675 nm: 55 ps (68.5%), 1.25 ns (12.3%) and 5.60 ns (19.2%). However, TRFS did not yield a clear component corresponded to the disappearance of A₀ reported earlier. Thus, we did not assign the short lived component observed at 675 nm to Chl *a* but assigned the long lived component to uncoupled Chl *a*. Furthermore, our PS I complexes showed a high photochemical activity (Mehler reaction) of ~540 μmol of O₂ (mg of Chl)⁻¹ h⁻¹. If an uncoupled Chl *a* functions as the primary electron acceptor, it is unlikely that such a high photochemical activity could be achieved. Consequently, we did not assign Chl *a* to an active electron transfer component in PS I of *A. marina*. We estimated the redox potential of the 690 nm band to be 438 mV. If this change was because of the primary electron acceptor, the redox potential was inconsistent with the value of A₀. As such, the location and role of Chl *a* in PS I of *A. marina* merit further investigation.

These observations might be attributable to the fact that pigment species and amino acid residues surrounding the pigments differ between P700 and P740. This might also induce an additional difference in the properties of the P740, such as an additional band observed at 728 nm.
Characterization of PS I in A. marina

Conclusions—We isolated highly purified and photochemically active PS I complexes from A. marina, and a new subunit, Psa27, was identified, along with Psak1 and Psak2. Electron transfer components such as Chl $d'$ and phylloquinone are stoichiometrically bound. The primary electron donor in PS I of A. marina was confirmed to be a Chl d dimer (probably a heterodimer of Chl d and Chl d') and keeps the redox potential close to that of P700, even though the pigment species and surrounding amino acids differ. The 728 nm band, the specific band found in the difference spectrum of P700, might be evidence of a significant difference between P700 and P740, but this has not yet been completely resolved. The overall energetics of the photochemical reaction in PS I are adjusted on the acceptor side, similar to the case of PS II in A. marina (28). The lower gain of light energy of P740, relative to that of P700, might be compensated for by the acceptor, whose potential is higher than that of P700. However this has not yet been confirmed. Chl $a$ was associated with the isolated complexes but was uncoupled from other pigments. The function and localization of Chl $a$ remain controversial, and further study will be necessary to reach a final conclusion.

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