Notes

Detection of Chlorophyll *d′* and Pheophytin *a* in a Chlorophyll *d*-Dominating Oxygenic Photosynthetic Prokaryote *Acaryochloris marina*

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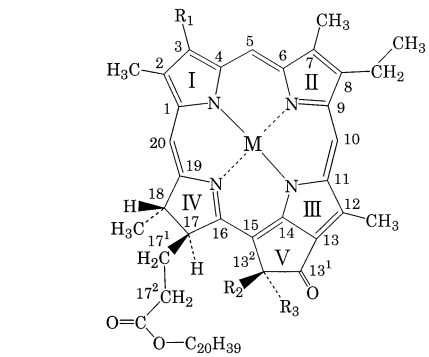
Sunlight is the ultimate energy source for the earth, and photosynthesis is essential for maintaining almost all life. The primary process of photosynthesis is initiated by photoexcitation and subsequent charge separation at the so-called reaction center (RC). Oxygenic photosynthetic organisms (higher plants, algae and cyanobacteria) have two types of photosystems (PS), according to the nature of the component molecules of the electron acceptor side.[1,2] One is called PS1, possessing Fe-S centers; another is PS2, having quinones.

Chlorophyll (Chl) *a* (Fig. 1) is an essential pigment for oxygenic photosynthesis. However, charge separation is driven by a few special chlorophyll molecules in each RC of PS1 and PS2. Upon excitation, the primary donor pigments reduce the primary acceptor pigments. The primary donors exhibit sharp absorption changes associated with transient oxidation by flash excitation; they are named P700 in PS1 and P680 in PS2, referring to the characteristic peak wavelengths of the absorbance decrease. The primary donor of PS1 has been speculated to be Chl *a′*, the 13^2-epimer of Chl *a* (Fig. 1);[3] the primary acceptor of PS2 is a metal-free Chl *a*, namely, Phe *a* (Fig. 1).[4] In contrast, both the primary donor of PS2 and the primary acceptor of PS1 are apparently normal-type Chl, namely, Chl *a*.

In 1996, a novel oxygenic alga *Acaryochloris marina* was isolated from a species of colonial ascidians.[5] Although in all previously known organisms capable of oxygenic photosynthesis the dominating chlorophyll is Chl *a*, the dominating pigment of *A. marina* is Chl *d* (Fig. 1). The Chl *a* content in *A. marina* is very low, *ca*. 3% of the Chl *d* content.[5-7] Phycobiliproteins (PBP) are present as antenna in relatively small amounts only.[8,9]

Such an unusual pigment composition results in a unique constitution of the photosystems of *A. marina*. Because isolated PS1 particles showed a flash-induced absorbance loss maximum at *ca*. 740 nm, the primary donor of PS1 was named P740,[10] corresponding to P700 in other oxygenic photosynthetic organisms. P740 was assumed to consist of Chl *d*, and not Chl *a′*.[10] The nature of the primary donor of PS2, P680, is still under debate, because it is hard to detect unambiguously the characteristic spectral signal. In comparison with P700 or P740, the data on P680 still remain scanty. Recently, time-resolved florescence spectroscopy in the ps and ns time range on the *A. marina* cells showed that a delayed fluorescence peak from PS2 was in the Chl *a* emission range, and not in the wavelength region of Chl *d* emission;[11] hence, the electron donor of PS2 in *A. marina* was most probably identical with that of other oxygenic photosynthetic organisms, namely, P680 consisting of Chl *a*. In contrast, information on the primary electron acceptors of PS1 and PS2 in *A. marina* is so far quite limited.

In this paper, we report on the results of a pigment composition analysis of *A. marina* cells by silica normal-phase HPLC. Chl *d′* and Phe *a* were detected as minor pigments in



	M	R_1	R_2	R_3
Chl *a*	Mg	$CHCH_2$	H	$COOCH_3$
Chl *a′*	Mg	$CHCH_2$	$COOCH_3$	H
Phe *a*	2H	$CHCH_2$	H	$COOCH_3$
Chl *d*	Mg	CHO	H	$COOCH_3$
Chl *d′*	Mg	CHO	$COOCH_3$	H
Phe *d*	2H	CHO	H	$COOCH_3$

Fig. 1 Molecular structure and carbon numbering of Chl *a*, Chl *a′*, Phe *a*, Chl *d*, Chl *d′* and Phe *d*, according to the IUPAC numbering system.

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addition to the major pigment, Chl *d*. No Chl *a*′ and Phe *d* were detected. The molar ratios were Chl *a*/Phe *a* = 2, Phe *a*/Chl *d*′ = 1 and Chl *d*/Chl *d*′ = 70, suggesting that the RC of PS2 consists of 4Chls *a* and 2Phes *a*, and that the primary donor, P740, consists of two Chl *d*′ molecules.

Materials and Methods

Algal culture

A. marina was cultured in a K + ESM medium[6,12] at 301 K and pH 8 with gentle aeration, as described elsewhere.[6]

Pigment preparation

Chl *d* was extracted with methanol at 277 K from dried cells of *A. marina*. The extract was applied to a preparative-scale HPLC (Senshupak 5251-N, 250 mm length × 20 mm i.d.) and eluted with hexane/2-propanol/methanol (100/2/0.3, v/v) at a flow rate of 7 mL min^{-1} at 277 K, as described elsewhere.[13] Chl *a* was extracted from parsley (*Petroselinum crispum* Nym.) and purified by the same method as for Chl *d*. Other authentic pigments (Chl *a*′, Chl *d*′, Phe *a* and Phe *d*) were prepared by the epimerization and pheophytinization of Chl *a* and Chl *d*, as described elsewhere.[14]

Pigment analysis

Pigments were extracted from a cell suspension (*ca*. 10 μL) by sonication in a *ca*. 300-fold volume of acetone/methanol (7/3, v/v) mixture for 2 min in the dark at room temperature. The extract was filtered and dried *in vacuo*. The whole procedure was completed within 5 min. The thus-obtained solid material was immediately dissolved in 10 μL of chloroform, and injected into a silica HPLC column (Senshupak 1251N, 250 mm length × 4.6 mm i.d.) cooled to 277 K in an ice-water bath. The pigments were eluted isocratically with degassed hexane/2-propanol/methanol (100/0.7/0.3, v/v) at a flow rate of 1.40 mL min^{-1}, and were monitored with a JASCO UV-970 detector (λ = 670 nm) and a JASCO Multiwavelength MD-915 detector (λ = 300 – 800 nm) in series.

The Chl *d*/Chl *d*′ molar ratio was calculated directly from their HPLC peak-area ratio with an absorbance detector (670 nm), because they have the same absorption spectra. The Chl *a*/Phe *a* and Chl *a*/Chl *d*′ ratios were calculated from their HPLC peak-area ratios, which had been calibrated repeatedly by injecting a standard solution containing known amounts of authentic Chl *a* (>99.98% in purity), Phe *a* (99.9%) and Chl *d* (>99.95%), based on the molar extinction coefficient of each pigment.[14,15]

Results and Discussion

A typical HPLC trace for acetone/methanol extract from cells of *A. marina* is shown in Fig. 2A. Large amounts of Chl *d*, as well as Chl *a* and Phe *a*, were detected. A minor pigment eluting at 35.5 min, between Chl *a* and Chl *d*, is Chl *d*′ (Fig. 1), the 13^2-epimer of Chl *d*, which was identified by comparing its elution time with that of Chl *d*′ prepared by the epimerization of Chl *d* (Fig. 2B). In contrast, Chl *a*′ constituting P700 and Phe *d* (*t* = 18 min, peak is not seen in Fig. 2) either, was not detected in *A. marina*. Taking the apparatus sensitivity into account, we estimate that the contents of Chl *a*′ and Phe *d* were less than 0.01% of Chl *d*, which is too small to regard these two pigments as essential components of the photosynthetic apparatus.

The possibility that the detected Chl *d*′ is an experimental artifact is excluded on the basis of the following observations: (1) We have already demonstrated the integrity of Chl *a* during extraction and chromatography;[3] Chl *a* → Chl *a*′ conversion was not observed in the present work, neither (see Fig. 2A). The integrity of more unstable BChl *g* was also demonstrated.[13] (2) The rates of Chl *d* → Chl *d*′ measured in several organic solvents were similar to those of Chl *a* (unpublished result). (3) The purification by HPLC led to a Chl *d* sample with 99.95% or higher purity.

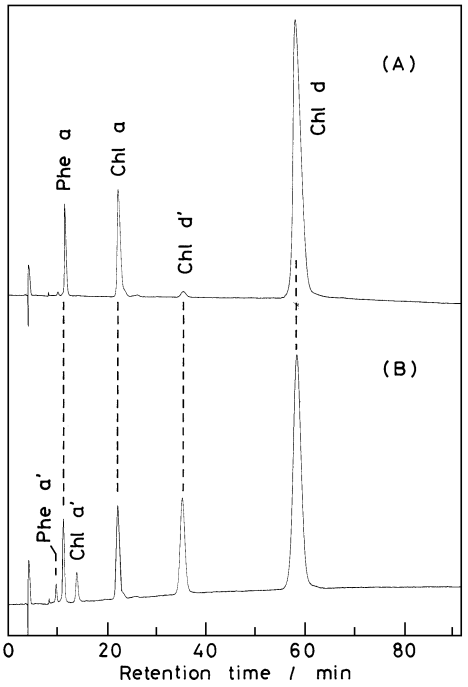

Fig. 2 HPLC elution profiles for (A) an acetone/methanol extract of *A. marina* and (B) a mixture of Phe *a*′, Phe *a*, Chl *a*′, Chl *a*, Chl *d*′ and Chl *d*. The detection wavelength is 670 nm.

The pigment molar ratios in the cells of *A. marina* were found to be Chl *a*/Phe *a* = *ca*. 2, Phe *a*/Chl *d*′ = *ca*. 1 and Chl *d*/Chl *d*′ = *ca*. 70. The ratio of Chl *a*/Phe *a* = 4/2 found in *A. marina* is the same as that of bacteriochlorophyll/bacteriopheophytin (BChl/BPhe) = 4/2 in the purple bacterial RC,[16,17] and slightly smaller than that of Chl *a*/Phe *a* = 6/2 in D1/D2/cyt *b*559 RC complexes of PS2 in other oxygenic organisms.[18-21] The function of pheophytins appears to be limited to electron accepting in RC2.[2] In view of these observations, Chl *a* and Phe *a* found in *A. marina* are most probably present exclusively in the core of PS2. The ratio of Chl *a*/Phe *a* = 4/2 in *A. marina* indicates that the RC of PS2 in *A. marina* may be at an intermediate stage in the evolution from the purple bacterial RC (BChl/BPhe = 4/2) to the RC of PS2 in higher plants, cyanobacteria and other algae (Chl *a*/Phe *a* = 6/2).

The molar ratio of Chl *d*/Chl *d*′ = *ca*. 70 in the cells of *A. marina* was in a range similar to that of Chl *a*/Chl *a*′ in cyanobacterial cells.[3] The ratio of Chl *d*/P740 = *ca*. 140 in the PS1 complex of *A. marina* was reported to be in a range similar to that of Chl *a*/P700 = 100 – 150 in PS1 of higher plants, algae and cyanobacteria.[10] The longer wavelength of P740 in *A. marina* than that of P700 in other PS1 was ascribed to the red-shift of the Q_Y absorption band of Chl *d* as compared to Chl *a*.[10] However, Chl *d*′ is not distinguishable from Chl *d* by the absorption spectra. Moreover, the role of Chl *a* molecules should be limited in PS2 as mentioned above, and Chl *a*′

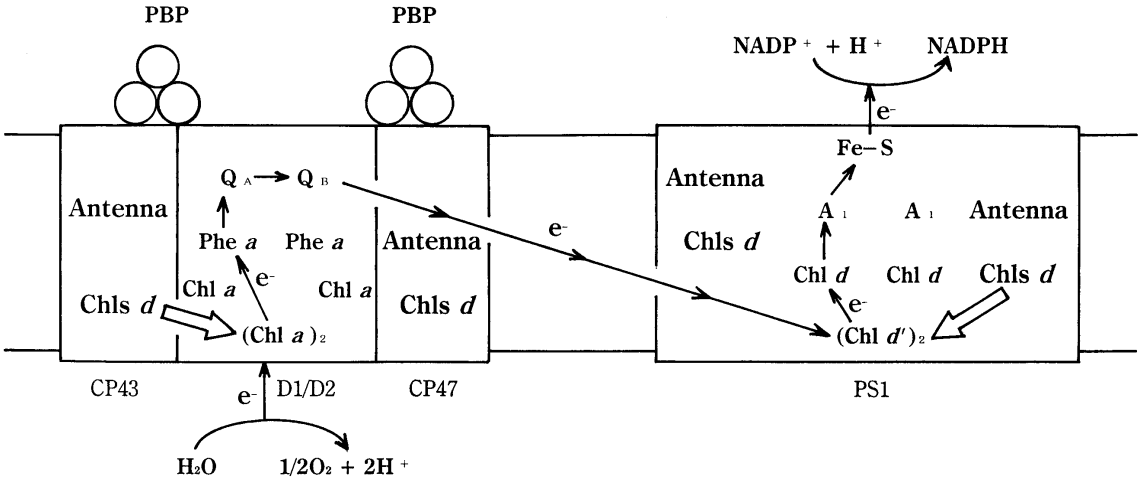

Fig. 3 Proposed pigment arrangement in photosystem of *A. marina*. A_1, secondary electron acceptor of PS1; PBP, phycobiliproteins; Q_A, Q_B, secondary acceptors (quinones) of PS2.

functions as P700 and Chl *a* as the primary electron acceptor of PS1 of other oxygenic photosystems. Based on these considerations, we suppose that P740 consists of Chl *d*′, and that the primary electron acceptor of PS1 is Chl *d*, in *A. marina*.

This hypothesis could be supported at least in part by the amino acid sequences of photosystem proteins in *A. marina* (data will be published elsewhere). The amino acid sequences of PS1 RC proteins, PsaA and PsaB, in *A. marina* were 75 – 76% homologous to those of general cyanobacteria. The sequences of PS2 core antenna proteins, CP47 (PsbB) and CP43 (PsbC), in *A. marina* were 73 – 68% and 72 – 68% homologous to those of general cyanobacteria, respectively. These homologies were *ca*. 10% lower than those among the cyanobacteria. The lower homology should be due to substitution of the pigment associated with these proteins in *A. marina* from Chl *a* to Chl *d*. However, the homologies of D1(PsbA) and D2(PsbD) were 86 – 83% and 88 – 87%, respectively. This level of the homologies was also confirmed within cyanobacteria. This suggests that amino acid substitution in D1 and D2 proteins in *A. marina* resulted from the same constraint as that in other cyanobacteria. Probably the D1 and D2 proteins in *A. marina* had to use Chl *a* even after the antenna molecules had been switched to Chl *d*.

A proposed model of the pigment arrangement in *A. marina* is displayed in Fig. 3. In PS1, the primary donor and acceptor are Chl *d*′ and Chl *d*, respectively, with Chl *a* lacking. This feature renders *A. marina* to the sole exception among the oxygenic photosynthetic organisms studied to date. In PS2, the primary donor and acceptor are respectively Chl *a* and Phe *a*, with accessory pigments being Chl *a*; this is essentially identical to other oxygenic photosynthetic organisms. However, this model has a problem with the PS2 part, because the antenna pigment, Chl *d*, has a lower electronic energy level (*ca*. 1.7 eV) *in vivo* than the primary electron donor, Chl *a* (*ca*. 1.8 eV). Thus, an uphill energy transfer should be required of PS2 in *A. marina*. Energy transfer from Chl *d* to Chl *a* in an organic solvent did not occur.[22] Quite recently, however, Mimuro *et al*. detected the occurrence of an uphill energy transfer in PS2 of *A. marina*;[22] this may be in line with the assumptions mentioned above. However, further work is needed to finally elucidate the RC photoprocesses taking place within this newly found photosynthetic organism.

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