Identification of the primary electron donor in PS II of the Chl *d*-dominated cyanobacterium *Acaryochloris marina*

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Abstract The primary electron donor of photosystem (PS) II in the chlorophyll (Chl) *d*-dominated cyanobacterium *Acaryochloris marina* was confirmed by delayed fluorescence (DF) and further proved by pigment contents of cells grown under several light intensities. The DF was found only in the Chl *a* region, identical to *Synechocystis* sp. PCC 6803, and disappeared following heat treatment. Pigment analyses indicated that at least two Chl *a* molecules were present per each two pheophytin *a* molecules, and these Chl *a* molecules are assigned to P_{D1} and P_{D2}. These findings clearly indicate that Chl *a* is required for water oxidation in PS II.

Key words: Chlorophyll; Cyanobacterium; Photosynthesis; Reaction center; Time-resolved fluorescence spectroscopy; *Acaryochloris marina*

1. Introduction

Acaryochloris marina contains chlorophyll (Chl) *d* as a major Chl pigment, and a small amount of Chl *a* and a Chl *c*-like pigment, namely Mg-2,4-divinyl pheoporphyrin a_5 monomethylester (Mg-DVP), are also present [1,2]. Chl *a* is a minor component ranging from 1 to 5% of the total Chl, but it is always present [1–5]. In addition to these, there are two kinds of other pigments that are specifically associated with the reaction center, namely pheophytin (Phe) *a*, a demetalized Chl *a*, and Chl *d*′, an epimer of Chl *d* at the position 13^2 [3–5]. It has been postulated Phe *a* functions as the primary electron acceptor in photosystem (PS) II [3–5], and that Chl *d*′ is the primary electron donor of PS I by forming a heterodimer with Chl *d* [5]. By contrast, three kinds of functions of Chl *a* have been proposed: (1) it is the primary electron donor in PS II [3–7]; (2) it is the primary electron acceptor in PS I [8,9]; and (3) it is one of the antenna pigments [10]. Along with the interpretation of (2), the primary electron donor of PS II is considered to be Chl *d*, although the rationale for this is not well established and remains controversial. To elucidate the function of Chl *a* in *A. marina*, it is crucial to adopt a specific index for individual photosystems. Moreover, since the amount of Chl *a* is very small, an accurate method of analysis of the pigment content is necessary. To this end, we selected delayed fluorescence (DF) as an appropriate tool to monitor the PS II activity in vivo.

It is known that the DF originates from charge recombination between the primary electron donor and the primary electron acceptor of PS II, and the rate constant of this process is in the order of 10 ns [11]. A Becquerel-type phosphorometer is often used to detect DF, and its time resolution is usually in the range from about 10 μs to a few ms [12,13]. It is known that the DF in this time range is somehow related to the PS II activity [13]; however, it does not reflect the charge recombination. Therefore, we measured the DF in the ns time range as a direct index. The present study identified Chl *a* as the primary electron donor in the PS II of *A. marina* cells grown under three different light intensities based on the origin of the DF and the pigment stoichiometry.

2. Materials and methods

2.1. Algal culture

A. marina MBIC11017 was grown autotrophically in IMK medium (Nihon Pharmaceutical Co., Japan) [2,6,14] under the following three light intensities with an incandescent lamp: 5, 15, and 35 μE/(m^2 s). *Synechocystis* sp. PCC 6803 grown in BG11 medium with a light intensity of 15 μE/(m^2 s) was used as a control. The growth temperature was kept at 25 ± 1°C.

2.2. Measurement of DF

DF and time-resolved fluorescence spectra (TRFS) were measured by the time-correlated single-photon counting method at −196°C [6,11]. The time resolution was controlled to a range of between 2.6 and 51.2 ps/channel (ch) using a time-to-amplitude converter. The light source was a Ti:sapphire laser and the excitation wavelength was 398 nm, which brought about excitation of Chl *a* and Chl *d*. We obtained TRFS in a certain wavelength region and decay curves at particular wavelengths after subtracting stray light.

2.3. High-performance liquid chromatography (HPLC) analysis of pigments

Pigments were extracted and analyzed according to methods described elsewhere [3–5]. Key points for analyses were as follows. The temperature of the HPLC column was maintained at 4°C and an appropriate elution solvent was selected to avoid overlap of any two components including carotenoids, even though the elution time for one sample was approximately 5 h.

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Abbreviations: ch, channel; Chl, chlorophyll; DF, delayed fluorescence; Mg-DVP, Mg-2,4-divinyl pheoporphyrin a_5 monomethylester; Phe, pheophytin; PS, photosystem; TRFS, time-resolved fluorescence spectra

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3. Results

3.1. DF originates from Chl a, but not from Chl d

First, we measured the TRFS of *Synechocystis* sp. PCC 6803 at −196°C as a standard sample (Fig. 1a). We detected the 685, 696, and 729 nm fluorescence bands; the former two come from PS II and the last one from PS I. PS I fluorescence clearly showed a red-shift of the maximum with time. At times longer than 30.7 ns after excitation, only the 685-nm component was observed, indicating that the DF originated from PS II Chl *a*, therefore we assigned Chl *a* as the PS II primary electron donor. This assignment was consistent with the PS II crystal structure of the thermophilic cyanobacteria [15,16]. The lifetime of the DF was estimated on the decay curve at 685 nm by a convolution calculation. Four components were resolved and the longest one had a lifetime of 15.4 ns (a relative amplitude of 0.1%) (Fig. 1c), and this was consistent with that of spinach chloroplasts (approximately 20 ns) [11]. In the decay curve at 730 nm, three components were resolved and no such long-lived component was detected. These data were the standard for the following measurements.

We measured the TRFS at −196°C on *A. marina* cells grown at 5 μE/(m^2 s) (Fig. 1b), and confirmed our previous report [6], even though the light intensity for growth was different. We observed two fluorescence components at 683 and 730 nm even at 30.7 ns after the excitation (Fig. 1b); however, a long-lived component was detected only in the decay curve at 685 nm (a lifetime of 14.0 ns and a relative amplitude of 1.5%; Fig. 1d). This component was assigned to the DF and its lifetime was consistent with that of *Synechocystis* sp. PCC 6803 (15.4 ns). On the other hand, a decay curve at 725 nm gave no DF but the long-lived component had a lifetime of 4.8 ns, significantly longer than that of *Synechocystis* sp. PCC 6803 at 730 nm (1.7 ns). This was also the reason for the high fluorescence intensity at 725 nm in addition to a high Chl *d* content that was 65 times higher than that of Chl *a*. These results clearly indicated that the DF was observed only in the Chl *a* region.

To confirm the origin of the DF at 683 nm, cells were heat-treated at 55°C for 10 min, which inactivated the water oxidation system (data not shown). *Synechocystis* sp. PCC 6803 did not show any DF after heat treatment (data not shown). The decay curves of *A. marina* after heat treatment were fit by three components and showed no long-lived fluorescence components at 685 and 725 nm (Fig. 1e). The longest lifetime was 5.8 ns in both wavelengths, which is similar to that observed in organic solvents. These results clearly indicated that the DF was observed only under physiological conditions in the Chl *a* region, supporting our interpretation that the primary electron donor of PS II in *A. marina* was Chl *a*, not Chl *d*.

The TRFS and decay kinetics were measured on cells grown at 15 and 35 μE/(m^2 s) with essentially identical results (data not shown). The DF was observed in the Chl *a* region and the lifetime was almost the same (13.6 and 15.0 ns for cells grown at 15 and 35 μE/(m^2 s), respectively) as that observed on the cells grown at 5 μE/(m^2 s) (14.0 ns). These results support our interpretation of the nature of the primary electron donor of PS II in *A. marina*. One remarkable change in the TRFS was a shift of the PS I fluorescence from 760 nm for cells grown at 5 μE/(m^2 s) (Fig. 1b) to 750 nm for cells grown at 35 μE/(m^2 s) (data not shown).

Table 1
Pigment contents in *A. marina* grown under three different light intensities of incandescent light

Light intensity (μE/(m^2 s)):	5	15	35
Chl *d*′/Phe *a*	0.41 ± 0.04	0.51 ± 0.04	0.44 ± 0.06
Chl *d*/Chl *d*′	150 ± 10.8	130 ± 7.2	150 ± 20.9
Chl *d*/Phe *a*	65 ± 4.2	65 ± 3.4	65 ± 12.6
Chl *a*/Phe *a*	1.0 ± 0.03	1.5 ± 0.04	2.3 ± 0.25
Chl *a*/Chl *d*′	2.8 ± 0.16	3.0 ± 0.21	5.2 ± 0.49
Chl *d*/Chl *a*	65 ± 3.5	43 ± 1.3	29 ± 4.1

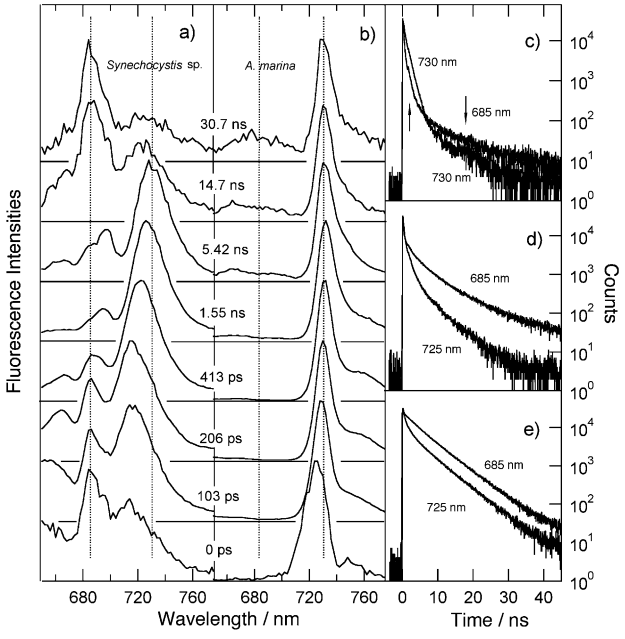

Fig. 1. TRFS and decay curves at −196°C. The excitation wavelength was 398 nm with a pulse width of 150 fs. The data acquisition interval was approximately 2 nm, and the time resolution was 51.2 ps/ch. The half-bandwidth of the monochromator was 6 nm. TRFS of *Synechocystis* sp. PCC 6803 (panel a), and of *A. marina* grown at 5 μE/(m^2 s) (panel b). Fluorescence intensities were normalized at the maxima of individual spectra. Decay curves of *Synechocystis* sp. PCC 6803 (panel c), of *A. marina* grown at 5 μE/(m^2 s) (panel d), and of *A. marina* grown at 5 μE/(m^2 s) after the heat treatment (panel e). Numbers in figures indicate wavelengths for monitoring of fluorescence. Vertical lines indicate the wavelengths for DF and PS I fluorescence.

3.2. Pigment analyses

We detected Chl *d*, Chl *d*′, Chl *a*, and Phe *a*, but neither Phe *d* nor Chl *a*′, on cells grown under the three light intensities (Table 1), and this composition was consistent with our previous reports [3–5]. We observed light-intensity-dependent and light-intensity-independent changes in pigment composition. The Chl *d*′/Phe *a* ratio was estimated to be in a range from 0.41 to 0.51 under the three light intensities, indicating that the PS I/PS II ratio was almost constant, i.e. in the range from 0.82 to 1.02 on the basis of Chl *d*′/PS I = 1 and Phe *a*/PS II = 2 [5]. The Chl *d*/Phe *a* and Chl *d*/Chl *d*′ ratios were 65 and approximately 150, respectively, under the three light intensities. These numbers gave rise to antenna sizes for PS I and PS II of approximately 100 and 30, respectively (cf. [5]). On the other hand, three indices relating to Chl *a* showed light-intensity-dependent changes. Under a high light intensity, the Chl *d*/Chl *a* ratio decreased from 65 to 29, indicating an increase in the relative content of Chl *a*. The Chl *a*/Phe *a* ratio

increased from 1.0 to 2.3, and that of Chl *a*/Chl *d*′ increased from 2.8 to 5.2 with increasing light intensities.

The Chl *a*/Phe *a* ratio approached 1 as the cells were grown at 5 μE/(m^2 s) (Table 1), and this was the minimum number in the composition among the three different cultures. Since two Phe *a* molecules function as the primary electron acceptors of PS II [3–5], this ratio indicates that two Chl *a* molecules were present per two Phe *a* molecules. When we estimated the pigment composition on cells grown up to 35 μE/(m^2 s), the Chl *a*/Phe *a* ratio exceeded 2.0 (Table 1). Furthermore, under a very high light intensity, the ratio became up to 4.0 (Kobayashi et al., unpublished data). Thus, the Chl *a* content was strongly dependent on the light intensity.

4. Discussion

4.1. Origin of the DF and the nature of the PS II primary electron donor

The DF was a crucial and reliable index for the PS II activity at the cell level. We found that the DF preferentially originated from the pigment responsible for the 685 nm fluorescence in *Synechocystis* sp. PCC 6803 (Fig. 1a) and for the 683 nm fluorescence in *A. marina* (Fig. 1b). Based on the optical properties of Chl *a* and Chl *d*, it is natural to conclude that the DF originated only from Chl *a*, confirming that the PS II primary electron donor of *A. marina* is Chl *a*. This also excludes the possibility that the primary electron donor is a heterodimer of Chl *a*/Chl *d*, because if this is the case, the DF would be expected to come from Chl *d*. This constitution is a common feature of oxygenic photosynthetic organisms. The amino acid sequences of PsaA/B were less conserved than those of PsbA/D (Miyashita, unpublished data) when compared with *Synechocystis* sp. PCC 6803. Based on these results, we inferred that Chl *a* cannot be replaced by other pigments for water oxidation; the oxidation potential of Chl *d* is lower by approximately 100 mV than that of Chl *a*, and this is very critical for the constitution of PS II in oxygenic photosynthetic organisms including *A. marina*.

4.2. Changes in pigment composition induced by light intensity

The Chl *a* content of *A. marina* is strongly dependent on light intensity for growth (Table 1). This point, however, has not been recognized and at least partially explains the controversy in the literature [3–10]. We showed that the minimum ratio of Chl *a*/Phe $a = 1$. The primary electron donor was assigned to Chl *a* by the DF, and the Chl *a*/Phe *a* ratio supported our assignment of the primary electron donor in PS II, namely a dimer of Chl *a* (P_{D1} and P_{D2} [15]). Under low light conditions, there was hence no other locus for Chl *a* except for the D_1–D_2 complex. The primary electron acceptor is assigned to Phe *a* ($Pheo_{D1}$ and $Pheo_{D2}$) [3–5], therefore the minimum ratio of Chl *a*/Phe $a = 2/2$ obtained here also indicates that accessory Chl (Chl_{D1} and Chl_{D2}) is not Chl *a* but Chl *d*, as shown in our model (Fig. 2).

Under high light conditions, additional Chl *a* molecules were present (Table 1); such Chl *a* molecules will be located in antenna, e.g. in CP43 or CP47 of PS II or in PS I; furthermore, it is located in the light-harvesting complex under high light conditions [10]. The function(s) of those additional Chl *a* is not certain; however, the possibility that Chl *a* molecule(s) is a quencher of Chl *d* under strong light cannot be excluded.

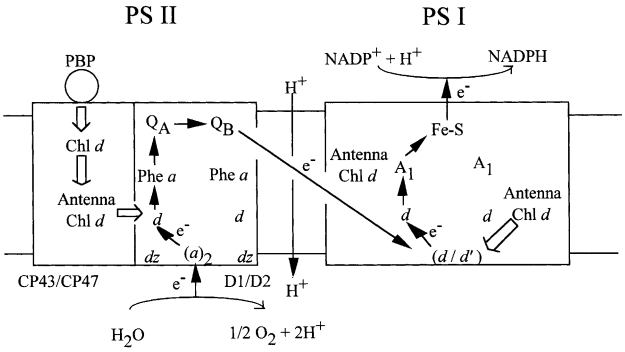

Fig. 2. A model for pigment arrangements in RC complexes of *A. marina*. The arrangement under weak light condition is shown. This is regarded as a basic constitution, and variation will be induced by light intensity. Open and closed arrows indicate flow of excitation energy and electron, respectively. A_1, secondary electron acceptor of PS I; PBP, phycobiliproteins; Q_A, Q_B, secondary electron acceptors (quinones) of PS II.

Recently, it was shown that the primary charge separation in PS II is initiated from the excitation of accessory Chl *a* (B) in the Chl *a*-type oxygenic organisms: $PB^* \rightarrow P^*B \rightarrow P^+B^-$ [17]. Our previous models demand the uphill energy transfer from antenna Chl *d* to accessory Chl *a* [3–5]. In the present model (Fig. 2), the primary charge separation initiated from accessory Chl *d* is theoretically also possible in the PS II RC of *A. marina*, after moderately uphill energy transfer from antenna Chl *d* to accessory Chl *d* (Sumi and Mukai, personal communication).

It is a remarkable feature of *A. marina* grown under incandescent light that the PS I/PS II ratio was close to 1, irrespective of the light intensities during growth. This phenomenon has never been reported in other cyanobacteria; usually the PS I/PS II ratio is higher than 1, and is changed by the light quality, light intensity, and nutrients [18]. This type of regulation is considered exceptional in all oxygenic photosynthetic organisms, while it is not clear whether either PS I or PS II is controlled, or whether both photosystems are controlled simultaneously. Further investigations are needed to better understand the Chl *d*-dominated cyanobacterium, and the oxygenic photosynthetic organisms in general.

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