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L protein, encoded by *psbL*, restores normal functioning of the primary quinone acceptor, Q_A, in isolated D1/D2/CP47/Cytb-559/I photosystem II reaction center core complex

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Abstract Plastoquinone-9 (PQ-9)-depleted PSII reaction center core complex, consisting of CP47/D1/D2/Cytb-559/I, was isolated from spinach PSII particles. PQ-9, lipids and several proteins were extracted from the original PSII particles and separated by several steps of chromatography to be reconstituted into the isolated complex. PQ-9 reconstituted in the complex with the help of thylakoid lipids (digalactosyldiglyceride) did not function as Q_A by itself. However, PQ-9 simultaneously reconstituted with L protein and the thylakoid lipids successfully functioned as Q_A in the complex. Other proteins of PSII origin, such as CP43, H, K, nuclear encoded 4.1 and 5.0 kDa proteins, are unable to restore the Q_A activity in the complex.

Key words: Photosystem II; Q_A; *psbL*; L protein; Plastoquinone-9

1. Introduction

The PSII reaction center (RC) of oxygenic photosynthetic organisms contains two plastoquinone-9 (PQ-9) molecules which function as the primary (Q_A) and secondary (Q_B) stable electron acceptors. PQ-9 at the Q_A site undergoes one-electron reduction and is much more tightly bound to RC than that at the Q_B site which carries out two-electron reduction. However, an RC complex consisting of D1/D2/Cytb-559/I proteins, isolated by Nanba and Satoh [1], lacks PQ-9 in both the quinone sites. Several groups have reported reconstitution of this type of RC with exogenous quinones. Decylplastoquinone elicits the reduction of Cytb-559 with DPC [2]. 2,5-Dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) suppresses the charge recombination between pheophytin$^-$ (Pheo$^-$) and P680$^+$ and mediates electron transfer to Cytb-559 [3]. It was also shown that besides DBMIB, various kinds of 1,4-benzoquinones and 1,4-naphtoquinones, particularly halogenated quinones, partially stabilize P680$^+$ in the millisecond time range by suppressing nanosecond charge recombination between P680$^+$ and Pheo$^-$ [3,4]. PQ-9 was also found to support the reduction of Cytb-559 and catalyze the photoreduction of 2,6-dichlorophenolindophenol (DCPIP) in the presence of exogenous electron donor under continuous illumination. In all of these cases, however, recovery of the quinone-mediated electron transport required rather high concentrations of quinone relative to the RC, such as more than 10 molecules of quinone per RC. Even then, recovery of Q_A function was far from the original level.

Recently, we reported that Q_A function is restored in a PQ-9-depleted RC core complex, consisting of CP47/D1/D2/Cytb-559/I proteins, to up to 40% of the original level by reconstituting it with PQ-9 together with a protein fraction containing H, L and nuclear encoded 4.1 kDa proteins of PSII origin, but not without the protein fraction [5]. The addition of two molecules of PQ-9 per reaction center in the reconstitution medium is sufficient to reach the saturation level. Here we report that among the three candidate proteins only L protein, encoded by *psbL*, supports Q_A function in the PSII complex.

2. Materials and methods

PSII reaction center core complex (original RC) that on average retains 1.7 PQ-9 per RC consists of CP47/CP43/D1/D2/ Cytb-559/H/I/L/K/n-5.0/n-4.1 proteins, was prepared from fresh spinach leaves as described in [5], modifying the method of Dekker et al. [6] (here n-5.0 and n-4.1 denote the nuclear encoded proteins of 5.0 and 4.1 kDa, respectively). Depletion of CP43, H, L, K, n-5.0 and n-4.1 proteins from the original RC was performed by using a mixed detergent system (17.5 mM *n*-octyl-β-D-glucopyranoside (OG) and 10 mM *n*-octyl-β-D-thioglucopyranoside (OTG)) as in [5]. PQ-9 per RC in the complexes after treatment with the mixed detergents (depleted RC) was decreased on average to less than 0.1, concomitant with the loss of the Q_A activity. The total components were solubilized into the detergent solution and pooled, and were denoted as crude extract.

The protein compositions in the original, depleted and reconstituted RCs were examined by SDS-PAGE (16–22% polyacrylamide gel with 7.5 M urea) according to Ikeuchi et al. [7]. PQ-9 and Cytb-559 were analyzed as described in [8] and [9], respectively. The concentration of RC in each RC preparation was determined on the basis of Cytb-559, assuming Cytb-559 per RC = 1 [9].

PQ-9 to be used for reconstitution experiments was isolated from spinach thylakoid membranes as in [10]. Digalactosyl diglyceride (DGDG) was purchased from Serdary Research Laboratories Inc.

Chromatographic separation of the protein components in the crude extract was performed as follows. Crude extract was dialyzed against solution A (5 mM Tris-HCl, pH 7.5) for 3 h at 4°C and loaded on to a DEAE-Sephacel column equilibrated with solution A additionally containing 10 mM OG and 5 mM OTG. After extensive washing with solution B (17.5 mM OG and 10 mM OTG, 5 mM Tris-HCl, pH 7.5) to elute the lipids and PQ-9, the column was subjected to stepwise elution with solution B additionally containing 10, 15 or 120 mM NaCl.

Reconstitution of the depleted RC with different combinations of PQ-9, lipids (or DGDG) and the protein components fractionated from crude extract were carried out as in [5]. As required, the reconstituted

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Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; Cytb-559, cytochrome b_{559}; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCPIP, 2,6-dichlorophenol-indophenol; DGDG, digalactosyldiglyceride; DM, *n*-dodecyl-β-D-maltopyranoside; DPC, diphenylcarbazide; MES, 2-(n-morpholino)-ethanesulfonic acid; OG, *n*-octyl-β-D-glucopyranoside; OTG, *n*-octyl-β-D-thioglucopyranoside; PQ-9, plastoquinone-9; PSII, photosystem II; Q_A, primary quinone acceptor of photosystem II; Q_B, secondary quinone acceptor of photosystem II; RC, reaction center complex of photosystem II; SDS-PAGE, SDS polyacrylamide gel electrophoresis; Tricine, tris(hydroxymethyl)methylglycine.

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RC were collected as a pellet in the final step centrifugation in [5], resuspended in a small volume of solution C (20 mM NaCl, 10 mM $MgCl_2$, 0.05% *n*-dodecyl-β-D-maltopyranoside (DM), and 20 mM Bis-Tris, pH 6.5), and subjected to sucrose gradient (10–30% w/v) centrifugation at 40,000 × *g* for 10 h at 4°C in the presence of 0.05% DM to separate the reconstituted RC from the components which had not assembled.

The Q_A activity of the RC was mainly assayed by measuring the photoreduction rate of dichlorophenolindophenol (DCPIP) in the presence of diphenylcarbazide (DPC) as an electron donor. The photoreduction rate of DCPIP was determined spectrophotometrically by following the change in absorbance at 605 nm upon illumination with continuous red light provided by a tungsten lamp of 1 kW through a pair of glass filters (Toshiba IRA-25 aand R-65) and a 10 cm water layer as described in [11]. Flash photolysis and the measuring of FTIR spectra were also carried out in parallel as described in [5].

3. Results and discussion

Fig. 1 shows the recovery of the Q_A activity in the reconstituted RC with crude extract. The Q_A activities of the reconstituted RC determined from the transient absorbance change at 325 nm upon a laser flash illumination and from the photoinduced electron transport from DPC to DCPIP, are plotted against the amount of crude extract and compared with the corresponding values determined from the light-induced absorbance change at 1,478 cm^{-1} observed in the FTIR difference spectra. The values obtained from three different assays were within the experimental error.

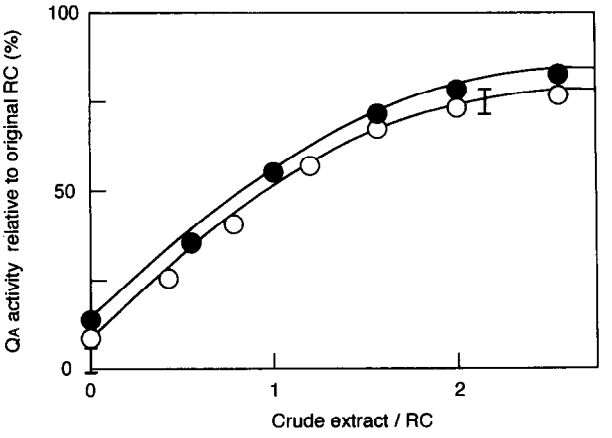

Fig. 1. Q_A activity in the reconstituted RC with crude extract. ○ and ● represent the data determined from the transient absorbance change at 325 nm upon a laser flash illumination and from the photoinduced electron transport from DPC to DCPIP, respectively. Error bars represent the value determined from the amplitude of the light-induced absorbance change at 1,478 cm^{-1} observed in the FTIR difference spectra. The abscissa indicates the amount of crude extract added to the reconstitution medium relative to the amount of RC.

Fig. 2 shows the chromatographic separation of the proteins contained in crude extract on a DEAE-Sephacel column. Identification of the H, L, K and n-5.0 proteins was performed by determining their N-terminal amino acid sequences, as in [12].

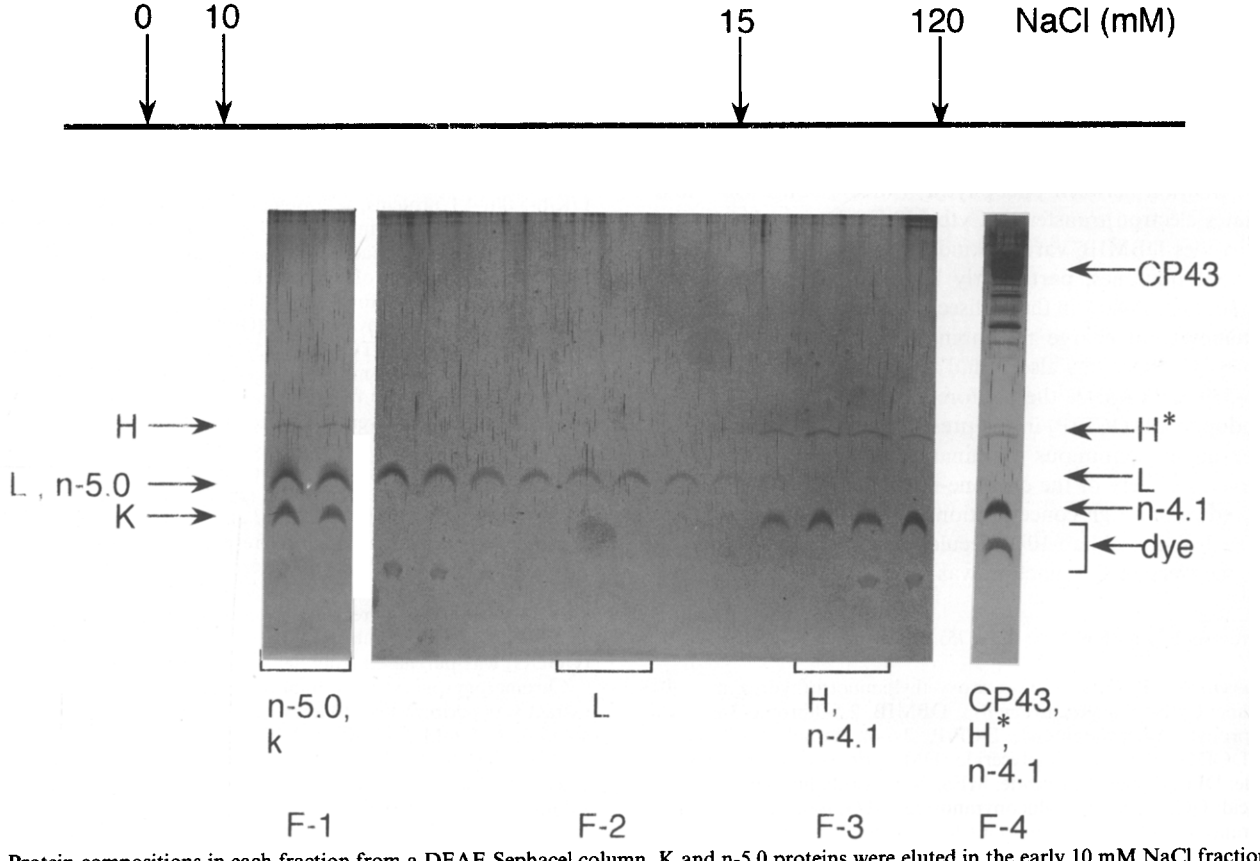

Fig. 2. Protein compositions in each fraction from a DEAE-Sephacel column. K and n-5.0 proteins were eluted in the early 10 mM NaCl fractions, and L protein in later fractions. It should be mentioned that the pooled fraction designated as F-2 contains only L protein. Non-phosphorylated H protein was eluted with 15 mM NaCl together with some n-4.1 protein. Phosphorylated H protein (H*), CP43 and n-4.1 proteins were eluted with 120 mM NaCl. F-1 to F-4 were submitted to reconstitution experiments.

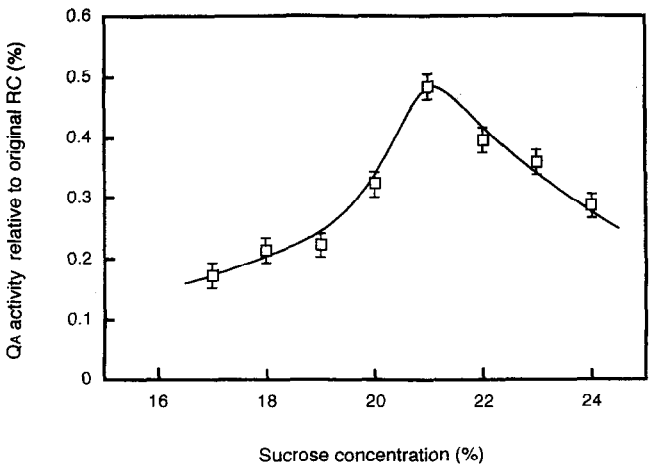

Fig. 3. Profile of the Q_A activities after sucrose gradient centrifugation. Q_A activity was determined from photoinduced electron transport from DPC to DCPIP.

A part of the K protein was eluted by washing the column with solution B containing no NaCl, and the rest was eluted in the early fractions of 10 mM NaCl elution together with the 5.0 kDa protein, as shown in the first two lanes of Fig. 2. After the disappearence of the K protein band, a single band was detected at 5 kDa on SDS-PAGE with the 10 mM NaCl elution fractions. However, the colour of the 5 kDa band upon silver staining changed from black to brown in successive fractions, suggesting at least two proteins of nearly same molecular size eluting with different retention times with 10 mM NaCl. N-Terminal sequencing of the proteins obtained from the 5.0 kDa band on SDS-PAGE followed by electroblotting revealed that the early 10 mM NaCl elution fractions contained n-5.0 protein and the later fractions mainly L protein: intermediate fractions gave a pair of the amino acid signals from both the n-5.0 and L proteins. The amino acid signals of the 5.0 kDa bands from the pooled fractions, designated as F-1 and F-2 in Fig. 2, matched the sequences of n-5.0 and L proteins, respectively: the signal from the other protein was not detected. Non-phosphorylated H protein was eluted in the 15 mM NaCl fraction, while phosphorylated H protein was eluted in the 120 mM NaCl fraction together with CP43. Four pooled fractions, designated F-1 to F-4 in Fig. 2, were submitted to the reconstitution experiments. Reconstitution of the CP47/D1/D2/Cytb-559/I complex was carried out with PQ-9 and the isolated lipids (or DGDG) together with different combinations of four fractions from the DEAE-Sephacel column. The amounts of PQ-9 and the isolated lipids (or DGDG) in the reconstitution medium were fixed at 2 mol of PQ-9 and 300 g lipids per 1 mol of RC, at which the recovery of the Q_A activity in the complex reconstituted with total proteins from crude extract had been confirmed to be optimal in our recent work [5]. Taking into account the results shown in Fig. 1, the amount of test proteins added to the reconstitution medium was roughly adjusted to be twice that of the RC by molar ratio.

The Q_A activities of the resulting particles determined from the rate of photoinduced electron transfer from DPC to DCPIP and the transient absorbance change at 325 nm upon illumination are summarized in Table 1. Significant recovery of the Q_A activity was observed only when the L protein fraction was added to the reconstitution medium: any protein combination without L protein was unable to restore the Q_A activity in the core complex. If the L protein fraction was treated with a non-specific proteinase, subtiricine, before the reconstitution, its ability to restore the Q_A activity was lost. Comparison of lines 3 and 4 in Table 1 demonstrates that DGDG alone, and not specific lipids in thylakoid membranes, are required for Q_A activity. These results strongly suggest that L protein is an essential component for normal functioning of Q_A in PSII complexes.

The Q_A activity restored in the reconstituted complex increased with increasing amounts of L protein added to the reconstitution medium, but it became saturated at ca. 35% of the activity of the original RC, presumably because a large amount of the RC complexes do not assmble with L protein

Table 1
Recovery of Q_A activity in the RCs reconstituted with different components

Reconstituted components							Recovery of Q_A activity (%)	
total lipids	DGDG	F-1 n-5.0/K	F-2 L	F-3 H/n-4.1	F-4 CP43/ H*/n-4.1	total proteins	DPC → DCPIP	ΔA_{325}
○							7 ± 1	3 ± 1
						○	6 ± 1	4 ± 1
○						○	72 ± 5	70 ± 5
	○					○	60 ± 5	62 ± 5
	○	○					6 ± 1	4 ± 1
	○		○				37 ± 5	33 ± 5
	○			○			6 ± 1	n. m.
	○				○		5 ± 1	3 ± 1
	○		○		○		37 ± 5	n. m.

Open circle indicates the components added in the reconstitution medium. n.m. indicates not measured

even after reconstitution. To separate out the unassembled complexes, the reconstituted RC were subjected to sucrose gradient centrifugation in the presence of 0.05% DM, and the Q_A activity on the basis of Cytb-559 was measured for several fractions after centrifugation. The results are shown in Fig. 3. The profile of the Q_A activity exhibited a peak at 21% sucrose with a value of about 50% of the original level. Analysis of the protein composition by SDS-PAGE also indicated that the RC complexes associated with L protein distributed at around 21% sucrose. A technical limitation of sucrose gradient centrifugation may be the cause of the limited recovery of the apparent Q_A activity in the peak fraction. These results may indicate that, apart from the difficulty of insertion of L protein alone into the D1/D2/CP47/Cytb-559/I complex, the RC complexes actually assembled with PQ-9, DGDG and L protein have normal activity in Q_A function.

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