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Two Plastoquinone A Molecules Are Required for Photosystem II Activity: Analysis in Hexane-Extracted Photosystem II Particles

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Prenylquinones were extracted with hexane from lyophilized oxygen-evolving photosystem II particles prepared from spinach chloroplasts. Determination by high performance liquid chromatography showed that two molecules of plastoquinone A remained per reaction center after the extraction, in contrast to the presence of three to four plastoquinone A molecules before the extraction. Electron transfer from water to phenyl-*p*-quinone was not inhibited by the extraction. Measurement of EPR signal II and microsecond chlorophyll fluorescence kinetics showed that hexane did not extract quinones which were acting as the secondary electron donor (Z) and the primary electron acceptor (Q_A) in photosystem II. These results, as well as the effect of quinone extraction on oxygen evolution, indicate that two molecules of plastoquinone A acting as Z and Q_A are essential for the activity of photosystem II. An artificial donor phenyl-*p*-quinone probably accepts electron from Q_A at the same site as the intrinsic secondary electron acceptor (Q_B). Q_A and Z seem to be surrounded by special microenvironments which differ from that of bulk quinones, and are resistant to hexane treatment.

Key words: Electron transfer — Oxygen evolution — Photosystem II — Plastoquinone — Spinach (*Spinacia oleracea*).

Several species of prenylquinones, plastoquinone A, plastoquinone B, plastoquinone C, tocopherolquinone and phylloquinone are known to exist in chloroplasts. Studies on the roles of quinones in photosynthetic reactions (Amesz 1973, Krogmann and Olivero 1962, Henninger and Crane 1966, Stiehl and Witt 1968, Kohl and Wood 1969, Amesz et al. 1972, Bensasson and Land 1973, Okayama 1974, Pulles et al. 1976, Sadewasser and Dilley 1978, Hales and Case 1981, Hales and Gupta 1981, O'Malley and Babcock 1984) have indicated the roles of quinones as electron carriers on both reducing and oxidizing sides of PS II. The secondary electron donor to PS II, which generates EPR signal II (Z), and the primary and the secondary electron acceptors (Q_A and Q_B) are assumed to be quinones. Z and Q_A are thought to be plastoquinone from EPR measurements (Kohl and Wood 1969, Hales and Case 1981, Hales and Gupta 1981, O'Malley and Babcock 1984) and light induced difference spectrum (Stiehl and Witt 1968, Bensasson and Land 1973), respectively. In addition, pool quinone molecules, which are located between PS II and the Cyt b_6-f complex, and the "Q_Z" type quinone species in the Cyt b_6-f complex function as electron and/or proton carriers.

Recently several groups (Berthold et al. 1981, Yamamoto et al. 1982, Kuwabara and Murata

1982, Yamamoto et al. 1984) developed methods of isolating oxygen-evolving PS II particles. Quantitative determination of such preparations showed that the content of quinones per PS II reaction center decreased during the isolation procedure (Murata et al. 1984, Yamamoto et al. 1984).

In this study, quinones were extracted from the oxygen-evolving PS II particles by a mild hexane treatment, and the characteristics of the extracted PS II particles were studied. Chemical determination of quinones and measurements of PS II activities indicated that two molecules of plastoquinone A were essential for the activity of PS II particles.

Materials and Methods

Preparation of oxygen-evolving PS II particles—Oxygen-evolving PS II particles were prepared from spinach thylakoids as described previously (Yamamoto et al. 1984). Broken chloroplasts prepared with a solution containing 0.33 M sorbitol, 120 mM NaCl and 10 mM MES (pH 6.5) were suspended in the same buffer solution at 0.5 mg Chl ml^{-1}. After addition of digitonin (0.2%, w/v), the chloroplast suspension was stirred for 20 min on ice. The suspension was then centrifuged at $20,000 \times g$ for 5 min and the pellet was homogenized with a teflon homogenizer and diluted to 0.5 mg Chl ml^{-1} with the same buffer. To this suspension Triton X-100 was added to make 0.85% and the suspension was kept at 0°C for 30 s. The Triton X-100 treated suspension, which contained subchloroplast particles, was then centrifuged at $35,000 \times g$ for 10 min. The pellet obtained was washed once with 10 mM MES (pH 6.5) and resuspended in the same buffer. This suspension was then lyophilized for 8 h.

PS II preparation used for the reconstitution experiment (Table 1) was prepared with 0.25% digitonin and 0.2% Triton X-100 according to Yamamoto et al. (1982).

Hexane extraction—The lyophilized PS II particles were extracted with the purest grade Na_2SO_4-dried hexane (10 ml hexane per mg Chl) for 10 min in a cold room followed by centrifugation at $3,000 \times g$ for 5 min. For reconstitution, the hexane extract was evaporated and redissolved in a small volume of hexane. The extracted pellet and the extract were combined to make the reconstituted particles. The extracted and reconstituted PS II particles were dried under a stream of dry nitrogen and suspended in a mixture of 0.33 M sorbitol, 5 mM $MgCl_2$ and 10 mM MES (pH 6.5). By the hexane extraction, 0.5% of total Chl was lost from the PS II particles.

Measurement of oxygen evolution—Oxygen evolution of the PS II particles was measured with a Rank type oxygen electrode at 20°C. The reaction mixtures are described under Tables 1 and 2. Red actinic light was provided from opposite sides by two 650 W slide projectors. The light was passed through a red glass filter (Toshiba VR 62).

Determination of quinones—Quinone was determined as described by Okayama (1984). After incubation for 10 min in 5 mM $MgCl_2$, 0.33 M sorbitol, 1 mM potassium ferricyanide and 10 mM MES (pH 6.5) at room temperature, the PS II particles (1 mg Chl) were extracted successively with 10 ml aqueous acetone (80%), 10 ml acetone, 10 ml diethylether and 10 ml petroleum benzin. Extracts were combined and washed with water in a separatory funnel. After removal of colorless water layer, the extracts were evaporated and redissolved in 5 ml petroleum benzin. The solution was adsorbed on an alumina column which contained 7.4% (w/w) water. Quinones were eluted with 30 ml of petroleum benzin–diethylether (7 : 3 v/v) mixture. The solvent was evaporated and quinones were redissolved in 1 ml ethanol. The ethanol solution was then analyzed by high-performance liquid chromatography with a Waters model 294 system equipped with a Radial-PAK C-18 column. The mobile phase was a mixture of ethanol and methanol (1 : 3 or 1 : 4, v/v) containing 50 mM $NaClO_4$. The separated quinones were determined polarographically using a Yanaco VMD-101 electrochemical detector.

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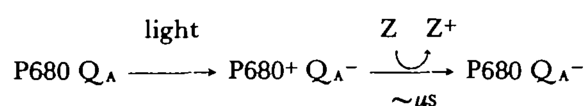
Determination of P700 and cytochromes—The content of P700 in the PS II particles was determined by ascorbate-minus-ferricyanide difference spectra in the 700 nm region (Marsho and Kok 1971). Cyt *f* was determined by the standard procedure of Bendall et al. (1971). Cyt *b*-559 and *b*-563 were assayed by the method of Rich and Bendall (1980).

Fluorescence measurement—Microsecond change in the flash-induced chlorophyll fluorescence yield was measured by a method similar to that described by Itoh et al. (1984). The PS II particles were excited by light from a xenon flash (Sugawara MS-230) passed through a Corning 4-96 filter. Fluorescence emitted was detected by a photomultiplier (Hamamatsu R374) covered with a 695-nm interference filter (Toshiba). The signal was stored in an Iwatsu DM950 wave memory (10 ns AD conversion per word, 8 bit × 1 k word) connected to a Hewlett-Packard 9885A computer, and the change of fluorescence yield calculated. To exclude the contribution of carotenoid triplet C^T on the fluorescence yield, the fluorescence profile from the dark-adapted PS II particles during flash excitation was divided by the reference fluorescence profile from the PS II particles under continuous background light in the presence of 5 μM DCMU and 0.1 mM NH_2OH. A train of eight flashes with 150 ms dark intervals were fired for each measurement. After calculation, it was possible to determine the change in fluorescence yield corresponding to the change of state of the reaction center from $P680^+ Q_A^-$ to $P680\ Q_A^-$ (reduction of $P680^+$ by the secondary electron donor, Z), so long as the formation of C^T under these two conditions is similar.



$$P680\ Q_A \xrightarrow{\text{light}} P680^+ Q_A^- \xrightarrow[\sim\mu s]{Z \to Z^+} P680\ Q_A^-$$

The increase in fluorescence yield in the microsecond range can therefore be interpreted as reflecting the reduction of $P680^+$. The flash intensity was set at a level that minimized quenching by C^T, but still allowed the formation of $P680^+ Q_A^-$ in the submicrosecond range. See further details in Itoh et al. (1984).

Results

Effect of the hexane extraction on oxygen evolution was examined in the PS II particles prepared with 0.25% digitonin and 0.2% Triton X-100 (Yamamoto et al. 1982) (Table 1). The hexane extraction decreased oxygen evolution to 36% and 69% when oxygen evolution was measured in the presence of ferricyanide alone, and both ferricyanide and dimethylquinone,

Table 1 Effect of hexane extraction on oxygen evolution of PS II particles prepared with 0.25% digitonin and 0.2% Triton X-100 (μmol O_2 evolved (mg Chl)$^{-1}$ h^{-1})

	Lyophilized	Extracted	Reconstituted
$H_2O \rightarrow$ ferricyanide	33 (100%)	12 (36%)	17 (51%)
$H_2O \rightarrow$ 2,5-dimethylquinone	62 (100%)	43 (69%)	57 (92%)
$H_2O \rightarrow$ phenyl-*p*-quinone	97 (100%)	88 (91%)	93 (96%)

Reaction mixture contained 0.33 M sorbitol, 5 mM $MgCl_2$, 5 mM NH_4Cl, 1 mM ferricyanide and PS II particles (20 μg Chl ml^{-1}) in 10 mM MES (pH 6.5). Where indicated, 1 mM 2,5-dimethyl-*p*-quinone and 0.1 mM phenyl-*p*-quinone were added.

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Table 2 *Effect of hexane extraction on oxygen evolution of PS II particles* prepared with 0.2% digitonin ,and 0.85% Triton X-100 (μmol O_2 evolved (mg Chl)$^{-1}$ h^{-1}

	Lyophilized	Extracted
$H_2O \rightarrow$ ferricyanide	67.7 (100%)	42.3 (62.5%)
$H_2O \rightarrow$ 2,5-dimethylquinone	147.9 (100%)	80.1 (54.2%)
$H_2O \rightarrow$ phenyl-*p*-quinone	166.5 (100%)	146.8 (88.2%)

Reaction mixture contained 5 mM $MgCl_2$, 5 mM NH_4Cl, 1 mM ferricyanide and PS II particles (10 μg Chl ml^{-1}) in 10 mM MES (pH 6.5). Where indicated, 1 mM 2,5-dimethylquinone and 0.1 mM phenyl-*p*-quinone were added.

respectively. However, the hexane extraction decreased oxygen evolution slightly when phenyl-*p*-quinone was added to the reaction mixture. Reconstitution with the hexane extract and the extracted PS II particles partially (H_2O to ferricyanine) or almost fully (H_2O to ferryicanide and dimethylquinone) restored oxygen evolution depending on the electron acceptor.

The rates of oxygen evolution of the lyophilized and the extracted PS II particles prepared in 0.2% digitonin and 0.85% Triton X-100 particles are also compared (Table 2). In lyophilized PS II particles, the activity of oxygen evolution was about 70–80% of the unlyophilized sample. As noted in Materials and Methods, only about 0.5% of total Chl was lost during the hexane extraction. In the presence of 1 mM potassium ferricyanide alone as the electron acceptor, a rather low rate of oxygen evolution was observed compared with the rate measured in the presence of 2,5-dimethyl-*p*-quinone or phenyl-*p*-quinone in both preparations. The hexane extraction decreased oxygen evolution to 62.4% and 54.1%, respectively, when oxygen evolution was measured in the presence of potassium ferricyanide alone or both potassium ferricyanide and dimethylquinone. However, the hexane extraction decreased oxygen evolution only slightly when phenyl-*p*-quinone was added to the reaction mixture. These oxygen evolution activities were inhibited in the presence of 10 μM DCMU.

Quinone content of the lyophilized and the hexane-treated PS II particles is shown in Table 3. The PS II particles had a high content of plastoquinone A, but low levels of plastoquinone C_{1-4}. Plastoquinone B and plastoquinone $C_{5,6}$ were not detected. The preparation had a trace amount of α-tocopherolquinone. Phylloquinone, which was concentrated in PS I fraction (Tevini and Lichtenthaler 1970), was not detected. Hexane extracted about

Table 3 Quinone content of lyophilized and hexane-extracted PS II particles (nmol (mg Chl)$^{-1}$)

	Lyophilized	Extracted
Plastoquinone A	16.1	7.4
Plastoquinone B	ND	ND
Plastoquinone C_{1-4}	1.0	0.3
Plastoquinone $C_{5,6}$	ND	ND
Phylloquinone	ND	ND
α-Tocopherolquinone	trace	ND

Quinones were determined by high performance liquid chromatography. ND, not detected.

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half of plastoquinone A and 70% of plastoquinone C_{1-4}. α-Tocopherolquinone was completely extracted by hexane. P700 and Cyt content in the PS II particles used for Table 2 were also determined. P700, Cyt *f* and Cyt *b*-563 were not detected. These results indicate that the PS II particles used in this study were free from PS I and Cyt b_6-*f* complex.

The ratio of Cyt *b*-559/chlorophyll was 1/125. According to Yamamoto et al. (1984) the ratio cytochrome *b*-559 to PS II reaction center in the preparation used can be assumed to be 2. Hence we assumed that the PS II antenna size was 250 in the preparation used for Table 2.

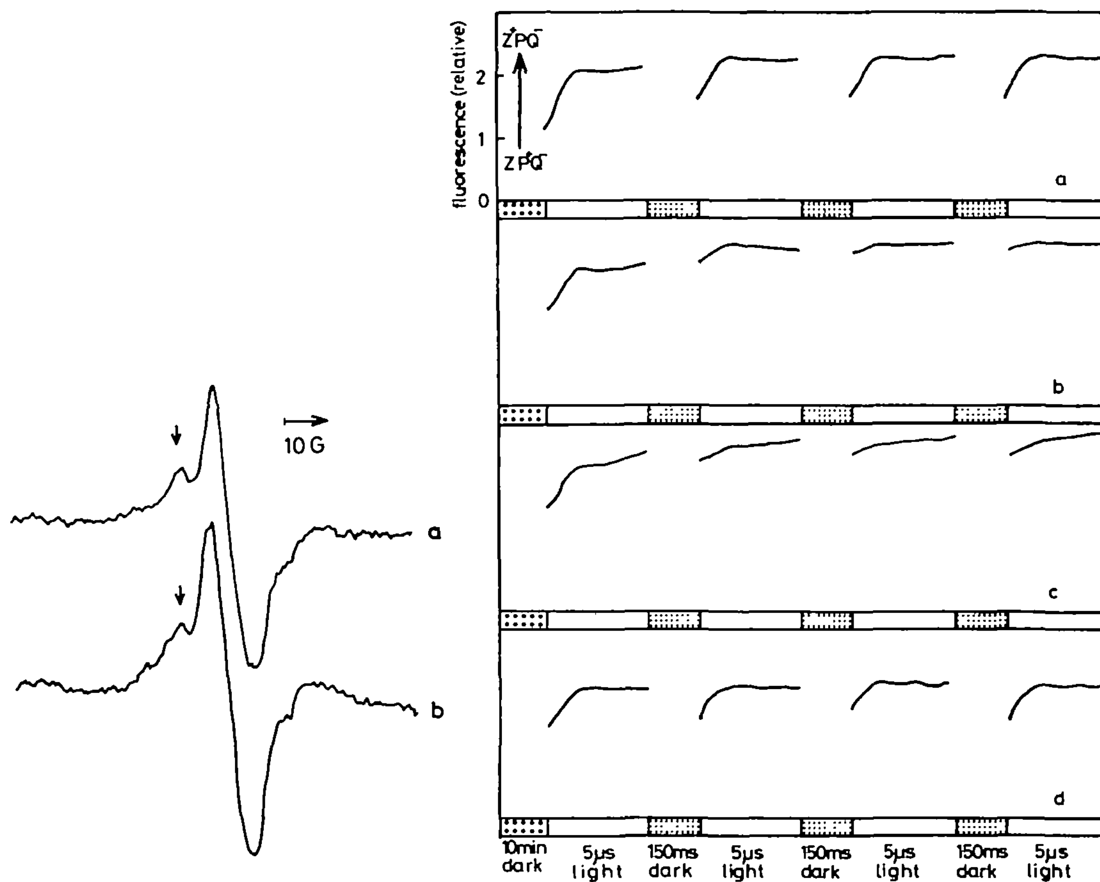

Fig. 1 Fig. 2

Fig. 1 EPR signal II of lyophilized (a) and hexane-extracted (b) PS II particles at 263 K under continuous illumination. Microwave power was set at 2 mW. Recorded in the X-band microwave region at 9.58 GHz. Modulation amplitude was 4 Gauss. Each sample contained PS II particles (1 mg Chl ml^{-1}), 0.33 M sorbitol, 100 µM phenyl-*p*-quinone, 120 mM NaCl and 10 mM MES, pH 6.5. Samples in 4 mm inner radius sample tubes were frozen in a cavity in the dark before illumination with saturating white light.

Fig. 2 Time courses of chlorophyll fluorescence transient in the microsecond time range at room temperature. Fluorescence yield change during each flash (5 µs) was recorded. Eight flashes were fired in a flash train with 150-ms intervals. Recordings during the first four flashes are shown in the figure. Reaction mixture contained PS II particles (5 µg Chl ml^{-1}), 0.4 M sucrose, 10 mM $MgCl_2$ and 50 mM Tricine, pH 7.8. a, Lyophilized PS II particles; b, lyophilized PS II particles in the presence of 5 µM DCMU; c, hexane-extracted PS II particles; d, extracted PS II particles in the presence of 6 µM phenyl-*p*-quinone. Samples were adapted in the dark for 10 min before each flash train.

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Taking this antenna size into account, 3.6 and 1.7 molecules of plastoquinone A per reaction center were estimated in the lyophilized and the extracted PS II particles, respectively. Okayama (1974) and Sadewasser and Dilley (1978) analyzed the effect of hexane extraction on electron transfer in isolated thylakoids. They concluded that quinones were functioning on both donor and acceptor sides of PS II. We examined the presence of quinones on the donor side of PS II by measuring the EPR signal II wihch is assumed to show the existence of stable quinone radicals on the donor side of PS II. Recordings of the EPR signal II of the lyophilized and hexane-extracted PS II particles are shown in Fig. 1. The magnitude of the typical signal II (estimated at the magnetic field indicated by the arrow) was not affected by the hexane extraction. The result indicated that the quinone functioning on the donor side of PS II was not significantly extracted by hexane under our experimental conditions.

Fig. 2 shows the kinetics of fluorescence transient, induced by repetitive flashes, in the microsecond time range in the lyophilized and the extracted PS II particles. Eight flashes were fired in one train of flashes with dark intervals of 150 ms. Traces during the first four flashes appear in the figure. The fluorescence transient of the lyophilized PS II particles is shown in Fig. 2a. The "induction" (rise in fluorescence yield in the first few microseconds) was observed during all the eight flashes (first four are shown). This indicated that $P680^+ Q_A^-$ changes to P680 Q_A^- by the electron donation from the secondary electron donor Z during each flash period and P680 Q_A^- changed to P680 Q_A in the 150 ms dark interval between the flash by the electron donation to the secondary electron acceptor Q_B. Fig. 2b shows the induction kinetics of the DCMU-poisoned lyophilized PS II particles. Time course with a full rise in the fluorescence yield was seen only during the first flash. After the flash, the lyophilized PS II particles were in a state of high fluorescence yield. DCMU inhibits the electron transfer from Q_A^- to Q_B. Thus Q_A^- cannot be reoxidized by Q_B in the presence of DCMU. As Q_A is a one-electron carrier (Cramer and Butler 1969) the Q_A^- state produced by the first flash is maintained as a single-electron-reduced state during the flash train with no clear rise after the flash. A result similar to Fig. 2b was obtained without DCMU in extracted PS II particles (Fig. 2c). Fluorescence transient with a rise in yield was observed only during the first flash. After the first flash, the extracted PS II particles had a high fluorescence yield state. The reduced primary electron acceptor, Q_A^-, was probably not reoxidized in 150 ms dark interval in this sample. Addition of 6 μM phenyl-*p*-quinone restored the induction kinetics of the hexane-extracted PS II particles to a pattern similar to that in the lyophilized particles (Fig. 2d).

Discussion

Prenylquinones function at least at four sites around PS II in chloroplasts (Amesz 1973, Krogmann and Olivero 1962, Henninger and Crane 1966, Stiehl and Witt 1968, Kohl and Wood 1969, Amesz et al. 1972, Bensasson and Land 1973, Okayama 1974, Pulles et al. 1976, Sadewasser and Dilley 1978, Hales and Case 1981, Hales and Gupta 1981, O'Malley and Babcock 1984). These are the primary (Q_A) and secondary (Q_B) stable electron acceptors, pool quinones and the secondary electron donor (Z).

Microsecond chlorophyll fluorescence analysis showed time courses with rising yield during all the repeated flashes in the lyophilized PS II particles. A full rising time course was observed only in the first flash in DCMU-poisoned lyophilized PS II particles. It indicated an inhibition of electron transfer from Q_A^- to Q_B. A similar result was obtained in the hexane-extracted PS II particles without DCMU. The kinetics with the rise in fluorescence yield in the first flash indicated the presence of electron-accepting Q_A and rapid reduction of $P680^+$ by Z. Absence of the rising time course during the following flashes suggested that Q_B is lost in the hexane-extracted PS II particles, though the possibility that Q_B still remained bound but had lost its

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reactivity cannot be excluded. Omata et al. (1984) showed two plastoquinone A molecules were present in the PS II reaction center complex. Our results essentially agree with theirs. They attributed one plastoquinone A molecule for Q_A. For the other plastoquinone A, they suggested two possible attributions, i.e., Z and Q_B. In the present study, we showed that two plastoquinone A molecules were left in the hexane-extracted PS II particles in which electron transfer from Q_A^- to Q_B was not observed.

The lyophilized and the extracted PS II particles contained 16.1 nmol and 7.4 nmol of plastoquinone A per mg Chl, respectively. Chlorophyll molecules extracted by the hexane treatment were negligible. When two molecules of Cyt *b*-559 are assumed as the components of a PS II reaction center (Yamamoto et al. 1984), three to four molecules of plastoquinone A can be estimated per reaction center in lyophilized PS II particles, and two molecules in extracted ones.

Hexane extraction of the PS II particles did not affect oxygen evolution when phenyl-*p*-quinone was used as an artificial electron acceptor. In the presence of phenyl-*p*-quinone, rapid reduction of $P680^+$ by Z was observed repeatedly after the hexane extraction. This indicates that Z was not affected by the hexane extraction. The EPR signal II was also unaffected by the hexane extraction. The signal II is assumed to be generated by the secondary electron donor of PS II which is probably plastoquinone (Kohl and Wood 1969, Hales and Case 1981, Hales and Gupta 1981, O'Malley and Babcock 1984). This indicates that only two molecules of plastoquinone A per reaction center, which are left after the hexane extraction of PS II particles, are sufficient for PS II activity. These essential quinone molecules probably correspond to those functioning as Z and Q_A. The secondary stable electron acceptor Q_B and pool quinones seem to be extracted with hexane.

Early workers (Henninger and Crane 1966, Sadewasser and Dilley 1978) have shown that plastoquinone C was more effective than plastoquinone A to restore the oxygen evolution activity of chloroplasts extracted with heptane or hexane. However, in the present study, only trace amounts of plastoquinone C_{1-4} per reaction center were detected after hexane extraction. Plastoquinone $C_{5,6}$ were not detected in both the lyophilized and the extracted PS II particles. The amount of plastoquinone C in the extracted PS II particles seems to be too low to account for the observed high oxygen evolving activity. Therefore, we may conclude that the quinone species functioning in PS II particles is primarily plastoquinone A.

Okayama (1974) and Sadewasser and Dilley (1978) showed that hexane extraction of chloroplasts inactivated the electron transfer from water to PS II. They restored the activity by addition of quinones extracted from lyophilized chloroplasts. In the present study, in which electron transfer from water to PS II was not inactivated by hexane treatment, quinones were extracted from the lyophilized PS II particles which were prepared with digitonin and Triton X-100. During the preparation of the PS II particles, it is possible that plastoquinone A probably acting as Z became resistant to the hexane extraction. The hexane treatment decreased the quinone content of the PS II particles in the first few minutes to the level of the extracted PS II particles indicated in Table 2, but a prolonged extraction (up to 15 min) did not remove quinones further. Thus we conclude that plastoquinone A molecules, Q_A and Z, are resistant to the hexane extraction in the PS II particles. It may be inferred that Q_A and Z exist in special microenvironments which block their accessibility to or extractability by organic solvents.

In the present paper, we showed that the addition of phenyl-*p*-quinone restored the pattern of microsecond fluorescence transient of the hexane-extracted PS II particles to that of the lyophilized PS II particles. Oxygen evolution was also not affected by the hexane extraction when phenyl-*p*-quinone was used as the electron acceptor. DCMU inhibited oxygen evolution of the extracted PS II particles in the presence of phenyl-*p*-quinone (also in the lyophilized

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particles, data not shown). These data indicate that phenyl-p-quinone accepts electrons from Q_A at the same site as the intrinsic secondary acceptor Q_B. 2,5-Dimethylquinone may partially accept electron from Q_A and partially from Q_B.

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