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THE ROLE OF PLASTOQUINONE AND β-CAROTENE IN THE PRIMARY REACTION OF PLANT PHOTOSYSTEM II

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SUMMARY

Extraction of Triton Photosystem II chloroplast fragments with 0.2 % methanol in hexane for 3 h results in the removal of 90 to 95 % of the plastoquinone in the original preparation. The extracted fragments (chlorophyll : plastoquinone ratio, 900 : 1) showed no *P*-680 photooxidation at 15 K after a single laser flash. The extracted fragments also showed no light-induced C-550 absorbance change at 77 K. Reconstitution of the primary reaction of Photosystem II, as evidenced by restoration of low-temperature photooxidation of *P*-680, could be obtained by the addition of plastoquinone A but not by the addition of β-carotene. The addition of β-carotene plus plastoquinone A restored the C-550 absorbance change. These results indicate that plastoquinone functions as the primary electron acceptor of Photosystem II and that β-carotene does not play a direct role in the primary photochemistry but is required for the C-550 absorbance change.

INTRODUCTION

The effect of extraction with non-polar solvents on the primary photochemical reactions of plant Photosystem II and the delineation of the components required for Photosystem II reactions at cryogenic temperatures have been the subject of two recent investigations [1, 2]. These studies provided conflicting evidence concerning the roles of two chloroplast components, plastoquinone A and β-carotene. These reports and recent spectral evidence that plastoquinone A may function as the primary electron acceptor of Photosystem II [3–7] prompted us to investigate the effect of extraction and reconstitution on the primary reaction of Photosystem II, as measured by *P*-680 photooxidation and C-550 photoreduction at cryogenic temperature. Our results provide direct evidence for a role of plastoquinone as the primary electron acceptor of Photosystem II and indicate that β-carotene has no direct role in the primary photochemistry.

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METHODS

Photosystem II chloroplast fragments were prepared from greenhouse-grown spinach with the detergent Triton X-100 as described previously [8] except that they were resuspended in distilled water rather than in the blending medium. Reinvestigation of the properties of fragments prepared in this way showed that they contain *P*-700 but only at a residual level of one *P*-700 molecule per 2000–3000 chlorophyll molecules.

After lyophilization for 18 to 20 h, the fragments were extracted by suspension in the indicated solvent (40 ml/mg chlorophyll) and stirring in the dark at room temperature. [Extractions not performed in darkness resulted in the solubilization of substantial amounts of chlorophyll; the extract obtained (by centrifugation at $35\,000 \times g$ for 2 min) from extractions performed in darkness was yellow and contained only traces of chlorophyll.] The extracted fragments were collected by centrifugation ($35\,000 \times g$ for 2 min), washed once with hexane (Mallinckrodt, analytical reagent grade), and dried under a stream of nitrogen. Precautions were taken to insure that the reaction mixture contained no water during any of these steps. The extracted fragments were reconstituted by resuspension in a small amount of hexane containing 100 μg of β-carotene per mg of chlorophyll and/or 30 nmol of plastoquinone per mg of chlorophyll. This amount of plastoquinone is approximately a 2-fold excess over the amount of plastoquinone in the lyophilized control. A crude preparation that contained β-carotene and plastoquinone A was obtained by extracting unfractionated spinach chloroplasts with hexane, according to the method of Cox and Bendall [2].

P-680 photooxidation was measured at 15 K by the recently described method [9] using the reversible electron paramagnetic resonance signal of $P\text{-}680^+$. Samples were flash-activated with a 680-nm laser flash (0.2 μs full-width at half-maximum) and the kinetics were recorded at the high-field peak of the first-derivative EPR signal. C-550 photoreduction at low temperature [10, 11] and chemical difference spectra at low temperature [12] were measured as described previously on samples suspended in 58 % glycerol. Plastoquinone was measured by a modification of the method of Redfearn and Friend [13]; potassium ferricyanide was added to insure that all of the plastoquinone was oxidized prior to methanol treatment and pyrogallol was omitted. The amount of plastoquinone was calculated on the basis of the absorbance difference between 255 nm and 276 nm with a difference extinction coefficient of 14.8 $mM^{-1} \cdot cm^{-1}$. The oxidized minus reduced difference spectrum of plastoquinone was measured in an Aminco DW-2 spectrophotometer and it was possible to detect as little as 0.3 nmol of plastoquinone in each sample. Non-heme iron was measured by the method of Miller and Massey [14]. Chlorophyll concentrations were determined as previously described [15].

Triton X-100 and β-carotene (Type I) were purchased from the Sigma Chemical Company. Pure plastoquinone A was donated by Prof. N. I. Bishop of Oregon State University, Prof. W. A. Cramer of Purdue University, and the Hoffmann-LaRoche Co. of Basel, Switzerland.

RESULTS

Photosystem II fragments (prepared by Triton treatment) that contained no

more than 10 % contamination from Photosystem I were used in all experiments (rather than untreated chloroplasts) so that the large pool of plastoquinone and carotene normally associated with Photosystem I [16, 17] need not be considered in interpreting the results of this study. An additional advantage of these fragments is that the $P\text{-}680^+$ EPR signal can be monitored with little interference from the EPR signal of $P\text{-}700^+$ that is seen in preparations that contain Photosystem I [9]. Although these Photosystem II fragments retain no more than 10 % of the Photosystem I activity of the chloroplasts from which they are prepared (measured by *P*-700 content) they are not highly enriched in photochemically active Photosystem II reaction centers. The magnitude of the light-induced C-550 absorbance change at 77 K, which can be used as a measure of the concentration of Photosystem II reaction centers [18], is only 15 % larger in these fragments than in the starting material (on an equal chlorophyll basis), and the amplitude of the $P\text{-}680^+$ EPR signal is approximately equal to that found in untreated chloroplasts.

The Triton Photosystem II fragments were found to have a chlorophyll : plastoquinone ratio of 70 ± 15 : 1. This corresponds to approximately 5 molecules of plastoquinone per Photosystem II reaction center (see Discussion). Extraction of the Photosystem II fragments with pure hexane or with 0.10 % methanol in hexane for as long as 4 h results in the removal of approximately 85 % of the plastoquinone and decreases the chlorophyll : plastoquinone ratio to 400–450 : 1 after extraction. To obtain more complete removal of plastoquinone, fragments were extracted with 0.15 % or 0.20 % methanol in hexane for 3 to 3.5 h. In this case, the chlorophyll : plastoquinone ratio after extraction was 900 ± 100 : 1, or a removal of 93 % of the original plastoquinone. On occasions, extraction with 0.20 % methanol in hexane resulted in fragments with even lower plastoquinone content (chlorophyll : plastoquinone greater than 1300 : 1). These fragments showed no *P*-680 photooxidation after reconstitution. Photosystem II fragments with chlorophyll : plastoquinone ratios between 500 and 600 : 1 were prepared by extraction for 1 h with 0.02 % methanol in hexane. Washing the Photosystem II fragments with distilled water prior to extraction or the use of methanol at concentrations higher than 0.02 % resulted in an almost complete loss of chlorophyll and these treatments were not pursued.

Fig. 1 shows the effect of extraction and reconstitution with plastoquinone, as measured by *P*-680 photooxidation at 15 K after a single laser flash. The initial, rapid increase in signal amplitude, seen with the lyophilized fragments, represents the photooxidation of *P*-680; the decay after the flash represents a back-reaction between $P\text{-}680^+$ and the reduced Photosystem II primary electron acceptor [7, 9, 19, 20]. The irreversible component of the EPR signal [21] does not arise from $P\text{-}680^+$ but is from an as yet unidentified component [9]. The extracted fragments (chlorophyll : plastoquinone = 950 : 1) show no detectable *P*-680 photooxidation, as was found for all extracted preparations with chlorophyll : plastoquinone ratios in the range from 750 to 1250 : 1.

As may be seen in Fig. 1, reconstitution with β-carotene did not result in any restoration of *P*-680 photooxidation, as evidenced by the lack of a reversible EPR signal; some irreversible component is formed after flash activation of this sample. However, reconstitution with pure plastoquinone resulted in restoration of 65 % of the *P*-680 photooxidation (measured by the extent of the reversible EPR signal). In four experiments an average of 60 % restoration of *P*-680 photooxidation was obtained with pure plastoquinone, but no restoration was observed with pure β-carotene.

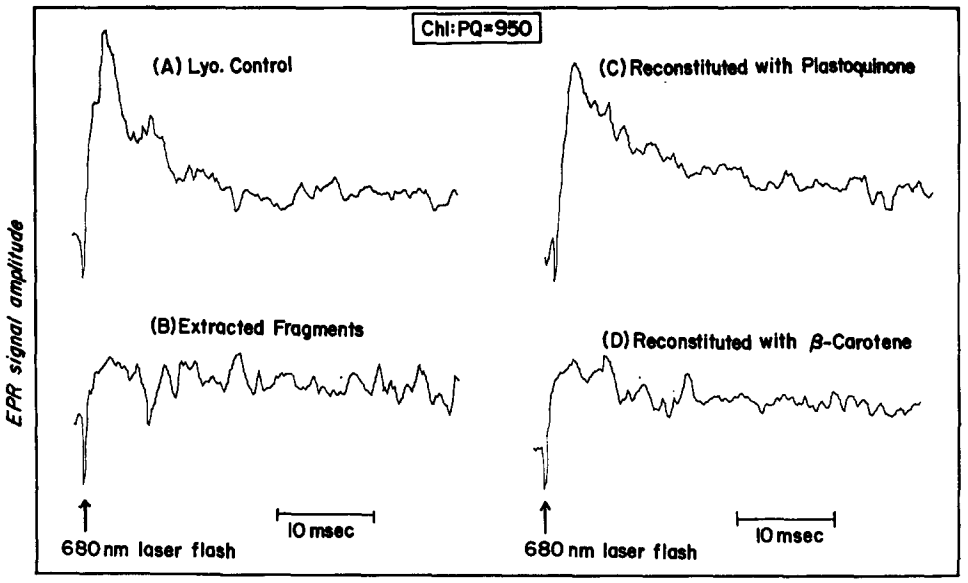

Fig. 1. Effect of extraction and reconstitution on *P*-680 photooxidation at 15 K. The chlorophyll : plastoquinone ratio of the extracted sample was 950 : 1. The reaction mixture contained the indicated chloroplast fragments at a chlorophyll concentration of 250 μM, 50 mM potassium phosphate buffer (pH 7.6), and 0.5 mM potassium ferricyanide. Samples were incubated in the dark at 4 C prior to freezing to 77 K. EPR conditions: frequency, 9.226 GHz, modulation amplitude 10 G; microwave power, 2 mW; temperature, 15 K; response time, 1 ms. The *P*-680 response was monitored after a 680-nm laser flash at the high-field peak of the derivative EPR signal. Each trace is the result of a single laser flash. The samples were prepared as described in the Methods. (A) Lyophilized (lyo) Triton Photosystem II fragments; (B) Extracted fragments; (C) Fragments reconstituted with plastoquinone A; (D) Fragments reconstituted with β-carotene.

The back-reaction between *P*-680$^+$ and the reduced primary electron acceptor exhibits first-order decay kinetics. Fig. 2 shows a first-order rate plot of the back-reaction at 15 K in both the lyophilized (lyo) fragments and the extracted fragments that were reconstituted with plastoquinone (PQ). The kinetics are similar and in several preparations half-times of 3.7 ± 1.3 ms (seven determinations) for the lyophilized fragments and 3.3 ± 0.4 ms (four determinations) for the plastoquinone- reconstituted fragments have been measured. These half-times are similar to values for the back-reaction in untreated chloroplasts of 3.0 to 5.0 ms, measured by a number of techniques and at various low temperatures [7, 9, 19, 20].

In addition to investigating the effects of extraction and reconstitution on *P*-680 photooxidation, we have examined the effects of these treatments on the low-temperature C-550 absorbance change. This absorbance change [1, 2, 11, 12, 19] has been associated with the reduction of the primary electron acceptor of Photosystem II [22, 23]. Fig. 3 shows the effect of extraction and reconstitution on the light-induced C-550 absorbance change at 77 K. These results are typical of those obtained in extracted preparations with chlorophyll : plastoquinone ratios between 750 and 1250 : 1.

No C-550 absorbance change is observed in the preparations that were reconstituted with plastoquinone (PQ) alone, although these preparations do show

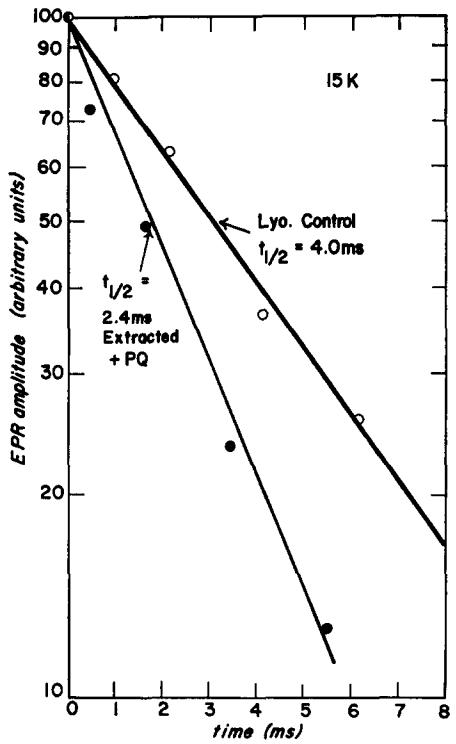

Fig. 2. Kinetics of *P*-680$^+$ reduction at 15 K following a laser flash in control and reconstituted fragments. Data taken from Fig. 1.

substantial *P*-680 photooxidation (see Fig. 1). Similarly, the addition of β-carotene alone has no effect. However, the addition of β-carotene plus plastoquinone restored 80% of the C-550 photoreduction observed in the lyophilized (lyo) control. Addition of crude extract (containing β-carotene and plastoquinone) has the same effect as does the addition of β-carotene plus plastoquinone. These results are similar to those of Cox and Bendall [2] but differ from the results of Okayama and Butler [1] who reported that C-550 photoreduction at low temperature could be restored by the addition of β-carotene alone and that plastoquinone had no effect on the reaction.

A possible explanation for the conflicting results on the requirements for plastoquinone and/or β-carotene for C-550 photoreduction is that the extracted preparations used by Okayama and Butler [1] and by Cox and Bendall [2] may have contained different amounts of plastoquinone [2]. To investigate this possibility we extracted our Photosystem II fragments for one h instead of 3.5 h to obtain fragments with higher plastoquinone content (chlorophyll : plastoquinone = 500-600 : 1).

Fig. 4 shows that, in contrast to the preparation with less plastoquinone (see Fig. 1), fragments extracted for one h retain 75% of the *P*-680 photooxidation of the lyophilized control. Reconstitution with crude extract (containing β-carotene and plastoquinone) completely restored the original *P*-680 photooxidation. In these preparations, the pattern of C-550 photoreduction at low temperature was quite different from that observed with fragments containing less plastoquinone. Fig. 5

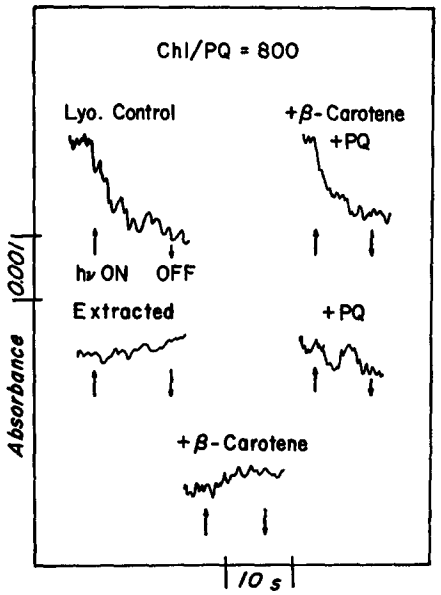

Fig. 3. Effect of extraction and reconstitution on C-550 photoreduction at 77 K. C-550 photoreduction was monitored at 547 nm minus 542 nm. The reaction mixture contained 58 % glycerol, 50 mM potassium phosphate buffer (pH 7.6), 0.5 mM potassium ferricyanide, and the indicated Photosystem II fragments at a chlorophyll concentration of 100 μM. The chlorophyll : plastoquinone ratio of the extracted sample was 800 : 1. Optical pathlength, 2 mm. Actinic light, 664 nm.

shows that in this preparation considerable restoration of C-550 photoreduction is obtained after the addition of β-carotene alone. Addition of plastoquinone alone has no effect. These results, which are similar to those of Okayama and Butler [1], are summarized in Table I. With a preparation containing a chlorophyll : plastoquinone ratio of 580 : 1 the percentage of restoration of C-550 photoreduction observed on reconstitution with β-carotene alone (82 % of the control) agreed well with the percentage of *P*-680 photooxidation retained in the extracted fragments (75 % of the control).

DISCUSSION

Optical light minus dark difference spectra attributable to the reduced minus oxidized difference spectrum of the primary electron acceptor of Photosystem II [3, 5–7, 24] resemble the difference spectrum obtained when plastoquinone is chemically reduced to its unprotonated semiquinone anion [4]. These observations have led to the proposal that the primary electron acceptor of Photosystem II is plastoquinone [4–6]. If plastoquinone is the primary acceptor of Photosystem II, removal of this plastoquinone molecule should result in the loss of *P*-680 photooxidation at low temperatures.

Our data indicate that when the plastoquinone content of Photosystem II fragments is lowered below one plastoquinone molecule per *P*-680 molecule the extent of *P*-680 photooxidation at low temperature declines. Fragments with chlorophyll : plastoquinone ratios of more than 450 (plastoquinone: *P*-680 $\leqslant$ 0.7, see below) show

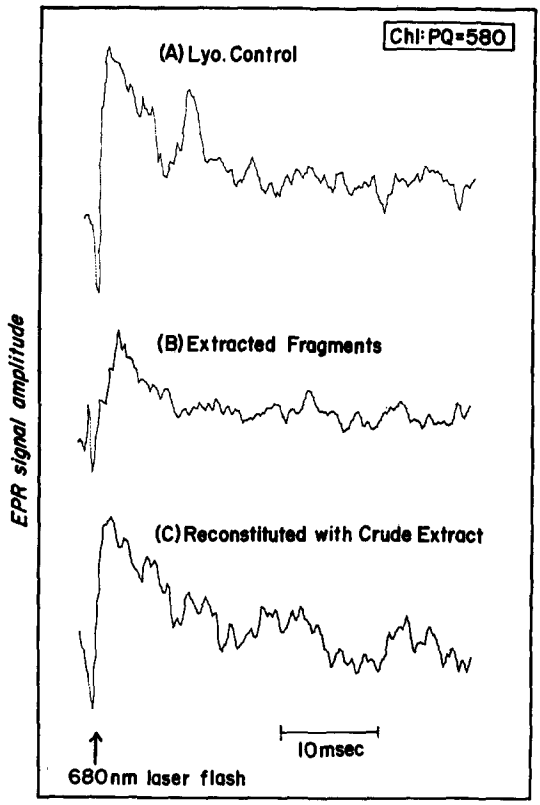

Fig. 4. Effect of extraction and reconstitution on *P*-680 photooxidation at 15 K. The chlorophyll : plastoquinone ratio of the extracted sample was 580 : 1. Other conditions as in Fig. 1.

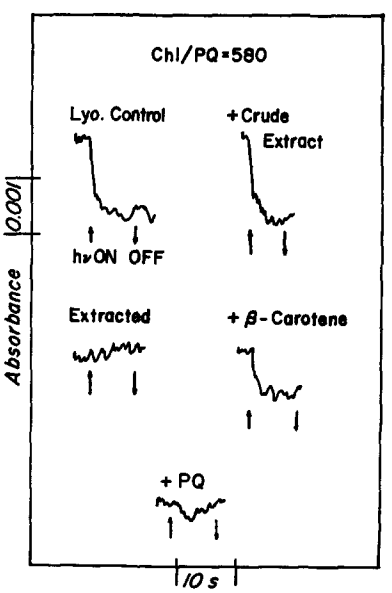

Fig. 5. Effect of extraction and reconstitution on C-550 photoreduction at 77 K. The chlorophyll : plastoquinone ratio of the extracted sample was 580 : 1. Other conditions as in Fig. 3.

TABLE I

EFFECT OF EXTRACTION AND RECONSTITUTION ON *P*-680 PHOTOOXIDATION AND C-550 PHOTOREDUCTION

Chlorophyll : plastoquinone ratio = 580 : 1. *P*-680 photooxidation was measured at 15 K by the EPR method. C-550 was measured at 77 K. (Data taken from Figs. 4 and 5).

	(% of extent in lyophilized control)	
	P-680 photooxidation	C-550 photoreduction
Lyophilized control	100	100
Extracted	74	0
Extracted + β-carotene	–	82
Extracted + crude extract	85	109

a 20–30 % decrease in *P*-680 photooxidation, compared to the lyophilized control. More completely extracted fragments (chlorophyll : plastoquinone = 900 : 1, which corresponds to approximately 0.3 plastoquinone per *P*-680) show no *P*-680 photooxidation. (The plastoquinone : *P*-680 ratio is calculated by assuming that untreated chloroplasts contain one Photosystem II reaction center per 360 chlorophyll molecules [25] and that (based on the magnitude of the C-550 absorbance change) the Triton Photosystem II fragments are 15 percent enriched in *P*-680 compared to untreated chloroplasts.) The residual *P*-680 photooxidation (30 %) that might be expected on the basis of the residual quinone content is barely above the detection level of the EPR technique used to measure *P*-680 photooxidation. Furthermore, plastoquinone is known to exist in several pools in chloroplasts [26] and this residual plastoquinone may belong to a pool other than that which functions as the Photosystem II primary electron acceptor.

Not only is *P*-680 photooxidation activity lost when the plastoquinone : *P*-680 ratio falls below 1.0; more importantly, low-temperature *P*-680 photooxidation can be restored by the addition of pure plastoquinone. These results strongly suggest that plastoquinone functions as the primary acceptor of Photosystem II. Furthermore, the observation that the half-time for the back-reaction between $P\text{-}680^+$ and the reduced acceptor is identical in plastoquinone-reconstituted and control fragments argues that the reaction center is not significantly altered in the restored preparation, compared to the starting material [12]. The finding that β-carotene has no effect on *P*-680 photooxidation suggests that β-carotene plays no direct role in the Photosystem II primary reaction.

It appears likely that plastoquinone is the only component extracted by nonpolar solvents that restores *P*-680 photooxidation when added to the extracted fragments. We interpret the observations that not more than 70 % of the *P*-680 photooxidation can be restored by the addition of plastoquinone in fragments with chlorophyll : plastoquinone ratios near 900 : 1 and that *P*-680 photooxidation cannot be restored by the addition of plastoquinone to fragments with chlorophyll : plastoquinone ratios greater than 1250 : 1 as the result of some irreversible damage to the system caused by the severe conditions needed to obtain complete removal of plastoquinone.

Figs. 1 and 3 show that one can obtain photochemically active Photosystem II

fragments (extracted fragments reconstituted with plastoquinone) that show no light-induced C-550 absorbance change. These results are in agreement with our earlier observation with K_2IrCl_6-treated preparations [12]. Unfortunately, we were unable to determine unambiguously if extracted fragments reconstituted with plastoquinone contain no C-550 because chemically reduced minus chemically oxidized C-550 difference spectra were made unreliable by spectral interference from the large amount of low-potential cytochrome *b*-559 in the fragments [8].

Although we are unable to conclude with certainty that extracted Triton Photosystem II fragments contain no C-550, our data do permit a reconciliation of the apparent contradiction between the results of Cox and Bendall [2] and those of Okayama and Butler [1]. Under all conditions, β-carotene is essential for the light-induced C-550 absorbance change. In preparations with substantial residual plastoquinone, the addition of β-carotene alone resulted in the restoration of the C-550 absorbance change (see Fig. 5), as was found by Okayama and Butler [1]. In preparations with little plastoquinone, no photochemistry results and no C-550 absorbance change is seen after the addition of β-carotene (see Fig. 3). Restoration of photochemistry after the addition of plastoquinone makes possible the C-550 absorbance change after β-carotene is added. In these instances, β-carotene plus plastoquinone is required for C-550 photoreduction, as reported by Cox and Bendall [2]. As may be seen in Table I, a quantitative relationship exists between the ability of β-carotene alone to restore C-550 photoreduction and the extent of *P*-680 photooxidation remaining after extraction which, in turn, is related to the residual plastoquinone content.

Although β-carotene is clearly required for the C-550 absorbance change, our data do not allow us to decide whether the C-550 absorbance change is a β-carotene band shift or whether the presence of β-carotene makes possible an electrochromic shift of some other membrane pigment. From the spectral characteristics of the C-550 absorbance change [27], the latter possibility seems more likely. It appears that β-carotene can have secondary effects, as indicated by the observation [1, 2, 28] that it is needed for the low-temperature photooxidation of chloroplast cytochrome *b*-559.

In conclusion, our results strongly support the hypothesis that plastoquinone A functions as the primary electron acceptor of Photosystem II. Photosystem II fragments prepared by treatment with Triton X-100 contain approximately 5 plastoquinone molecules per *P*-680 molecule, and this plastoquinone complement appears to be divided into two pools. One pool of 4 plastoquinones can be extracted relatively easily (it is removed by extraction with pure hexane or by brief extraction with 0.20 % methanol in hexane) and does not appear to be involved in the primary reaction of Photosystem II (*P*-680 photooxidation is little affected by removal of 80 % of the plastoquinone). The second pool consists of a single, more tightly bound plastoquinone (prolonged extraction with 0.20 % methanol in hexane is required to remove it) which appears to function as the Photosystem II primary electron acceptor.

It is of interest to note that as recent evidence has been accumulating to support the role of plastoquinone as the primary electron acceptor of plant Photosystem II similar evidence has led to the conclusion that a related quinone, ubiquinone, functions as the primary electron acceptor in the purple non-sulfur photosynthetic bacteria [29–32]. It has been suggested that in these bacteria the primary acceptor, ubiquinone, is in the form of a quinone-iron complex [30]. Other similarities between the primary

acceptors in plant Photosystem II and in bacteria [33–35] raise the possibility of a similar quinone-iron complex in Photosystem II. Preliminary investigations in our laboratory indicate that Triton Photosystem II fragments (dialyzed against EDTA to remove adventitious iron) contain one tightly bound non-heme iron per 100 chlorophyll molecules (3–4 Fe per *P*-680) and that this iron is not in the form of an iron-sulfur protein. The possible role of this iron in the primary photochemistry of Photosystem II is currently under investigation.

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