Interaction between the intermediary electron acceptor (pheophytin) and a possible plastoquinone–iron complex in photosystem II reaction centers

(photosynthesis/electron paramagnetic resonance)

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ABSTRACT Photoreduction of the intermediary electron acceptor, pheophytin (Pheo), in photosystem II reaction centers of spinach chloroplasts or subchloroplast particles (TSF-II and TSF-IIa) at 220 K and redox potential $E_h = -450$ mV produces an EPR doublet centered at $g = 2.00$ with a splitting of 52 G at 7 K in addition to a narrow signal attributed to Pheo $^{\cdot-}$ ($g = 2.0033$, $\Delta H \approx 13$ G). The doublet is eliminated after extraction of lyophilized TSF-II with hexane containing 0.13–0.16% methanol but is restored by reconstitution with plastoquinone A (alone or with β -carotene) although not with vitamin K $_1$. TSF-II and TSF-IIa are found to contain ≈ 2 nonheme Fe atoms per reaction center. Incubation with 0.55 M LiClO $_4$ plus 2.5 mM α -phenanthroline (but not with 0.55 M LiClO $_4$ alone) decreases this value to ≈ 0.6 and completely eliminates the EPR doublet, but photoreduction of Pheo is not significantly affected. Partial restoration of the doublet (about 25%) was achieved by subsequent incubation with 0.2 mM Fe $^{2+}$, but not with either Mn $^{2+}$ or Mg $^{2+}$. The Fe removal results in the development of a photoinduced EPR signal ($g = 2.0044 \pm 0.0003$, $\Delta H = 9.2 \pm 0.5$ G) at $E_h = 50$ mV, which is not observed after extraction with 0.16% methanol in hexane. It is ascribed to plastoquinone no longer coupled to Fe in photosystem II reaction centers. The results show that a complex of plastoquinone and Fe can act as the stable “primary” electron acceptor in photosystem II reaction centers and that the interaction of its singly reduced form with the reduced intermediary acceptor, Pheo $^{\cdot-}$, is responsible for the EPR doublet.

Recent reports (1–7) have shown that, in photosystem II (PS II) of green plants, pheophytin (Pheo), with a redox potential of -610 mV (4), acts as an intermediary electron acceptor between the primary donor, P680, probably either a dimer (see ref. 8) or a monomer (9) of chlorophyll (Chl), and the primary acceptor, Q, a special form of plastoquinone (PQ) (8–14). From measurements of nanosecond recombination luminescence from PS II, the lifetime of the state [P680 $^{\cdot+}$ Pheo $^{\cdot-}$] has been found to be ≈ 3 ns at 295 K [3]. After prior dark reduction of Q at redox potential $E_h < -200$ mV, Pheo $^{\cdot-}$ can be reversibly accumulated by continuous illumination at temperatures between 300 and 100 K (1) in subchloroplast particles (1–7) as well as in chloroplasts (4, 5), with concentrations approximating those of P680 (5, 7) and other PS II components (7). From the measurement of quantum yield of this photoreaction (1–3, 5) it has been suggested that photoaccumulation of the long-lived (> 1 s) state [P680 $^{\cdot+}$ Pheo $^{\cdot-}$] at $E_h < -200$ mV is possible because of the fast [≤ 1 - μ s (15–17)] reduction of P680 $^{\cdot+}$ by a secondary electron donor competing with charge recombination in [P680 $^{\cdot+}$ Pheo $^{\cdot-}$].

Phototrapping of Pheo $^{\cdot-}$ in PS II at 295 K is accompanied by the appearance of an EPR signal with $g \approx 2.0035$ and $\Delta H \approx 13$ G (1 G = 10^{-4} tesla) (6, 7), similar to that of the monomeric

anion radical of Pheo *in vitro* (18). After phototrapping of Pheo $^{\cdot-}$ at 220 K, however, an EPR “doublet” centered at $g = 2.00$ with a splitting of ≈ 52 G is also observed (7). In analogy with a similar observation in bacterial reaction centers (19–22), it was suggested that the doublet is probably the result of interaction of Pheo $^{\cdot-}$ with Q $^{\cdot-}$, which includes Fe or some other transition metal (7). Here we report data supporting the idea that a PQ-Fe complex acts as the stable “primary” electron acceptor in PS II reaction centers and that an exchange interaction of its singly reduced form with Pheo $^{\cdot-}$ accounts for the split EPR signal.

MATERIALS AND METHODS

Spinach chloroplasts and subchloroplast particles (Triton-fractionated subchloroplast fragments, TSF-II and TSF-IIa), highly enriched in PS II reaction center components and free of P700, were isolated as described (23–26). All EPR samples contained 0.05 M Tris-HCl (pH 8.0) and 50% (vol/vol) glycerol. Extraction and reconstitution of Q were carried out according to Knaff *et al.* (ref. 14 and references therein). Briefly, lyophilized PS II particles were extracted at 20°C for 2 hr in the dark in hexane (Mallinckrodt, analytical reagent grade) containing 0.1–0.2% methanol, collected by centrifugation ($40,000 \times g$ for 10 min), washed once with hexane, and dried under a stream of N $_2$. Reconstitution was performed by resuspension of extracted material in a small amount of hexane containing one or a combination of the following: 50 nmol of plastoquinone A (PQ-A), 50 nmol of vitamin K $_1$, or 100 μ g of β -carotene per mg of Chl.

Iron was extracted by incubating PS II preparations (Chl at 50 mg/ml) in 10 mM Tris-HCl (pH 8.0) containing 0.3–1 M LiClO $_4$ with or without 2.5 mM α -phenanthroline, for 2 hr at 2°C, followed by centrifugation ($200,000 \times g$ for 40 min) and dialysis (12 hr at 2°C) of the pellet against 10 mM Tris-HCl (pH 8.0)/50 mM NaCl/10 μ M EDTA (David Knaff, personal communication; also ref. 27). In the reconstitution experiments the extracted material was additionally dialyzed for 12 hr at 2°C under anaerobic conditions against 10 mM acetate buffer (pH 5.5) containing 0.2 M LiClO $_4$ and 1 μ M EDTA, with or without 0.2 mM Fe(NH $_4$) $_2$ (SO $_4$) $_2$, MnSO $_4$, or MgSO $_4$.

Nonheme iron content was determined by the bathophenanthroline method (28). Changes in absorbance and fluorescence yield, induced by actinic light (intensity ≈ 0.05 J $\cdot$ cm $^{-2}$ $\cdot$ s $^{-1}$) from a 1000-W incandescent lamp filtered by 5 cm of CuSO $_4$ solution, were measured in a phosphorescopic pho-

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Abbreviations: PS II, photosystem II; Pheo, pheophytin; Chl, chlorophyll; PQ, plastoquinone; TSF, Triton-fractionated subchloroplast fragment.

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tometer (7). EPR measurements were performed at 9.15 GHz as described (7).

RESULTS

Dark reduction of Q and phototrapping of Pheo[•] at 295 K in the EPR samples at $E_h \approx -450$ mV was monitored by characteristic changes (1–7) of Chl fluorescence yield at 295 K; at 220 K the photoreaction was monitored by the appearance of the narrow EPR signal of Pheo[•] with $g = 2.003$ and $\Delta H = 13$ G as described (6, 7).

At 7 K, the narrow EPR spectrum of Pheo[•], phototrapped in TSF-IIa at 295 K as well as 220 K, reached its maximal amplitude at a microwave power of ≈ 5 μ W and was almost unobservable at 50 mW due to saturation (Figs. 1 and 2). Phototrapping Pheo[•] at 220 K (but not at 295 K) produced a new EPR signal, a “doublet” centered at $g = 2.00$ with a splitting of 52 G, the ratio of the high- to low-field peaks being ≈ 1.7 . (Fig. 1, spectra C–E). It had a maximal amplitude in the 20- to 50-mW range of microwave power (Fig. 2) and was detectable only below 15 K. The doublet was also observed in TSF-II fragments and chloroplasts, but its amplitude was 1/2 and 1/14, respectively (Fig. 1, spectra C–E), consistent with the content of PS II reaction centers in these preparations. Neither the doublet nor the narrow signal of Pheo[•] was seen after illumination of TSF-II and TSF-IIa at $E_h = 50$ mV when Q was oxidized in the dark (data not shown).

FIG. 1. Dark (A) and light-induced (B, C) EPR spectra of TSF-IIa fragments after 1-min illumination at 295 K (B) and 220 K (C) at $E_h \approx -450$ mV, followed immediately by freezing in liquid nitrogen. D and E, light-induced EPR spectra of TSF-II fragments and chloroplasts, respectively, after illumination at $E_h \approx -450$ mV at 220 K. The light-induced spectra are the difference spectra between signals of separate, illuminated and unilluminated, samples prepared from the same material. Actinic light was as described in *Materials and Methods*. Microwave power, temperature, and gain are as shown. Modulation amplitude 7 G; Chl, 1.4 mg/ml.

FIG. 2. Effect of microwave power (P) on the amplitude of the photoinduced narrow (singlet) and the doublet EPR signals of TSF-IIa fragments after phototrapping of Pheo[•] at 295 K and 220 K, respectively (see Fig. 1 legend). Temperature 7 K; amplitude modulation 7 G.

After extraction of lyophilized TSF-II with hexane containing 0.1%, 0.13%, and 0.16–0.2% methanol, the EPR doublet was diminished to 75%, 45%, and $\approx 0\%$, respectively, of its initial value. On the other hand, photoaccumulation of the narrow signal of Pheo[•] was readily observed at 220 K as well as at 295 K. After addition of PQ-A, alone or with β -carotene, to TSF-II extracted with hexane containing 0.16% and 0.13% methanol, the EPR doublet was restored to approximately 65% and 100% of the initial value, respectively (Fig. 3). Addition of β -carotene alone also resulted in some reconstitution of the doublet; vitamin K₁ had no effect (Fig. 3).

Incubation of TSF-II with LiClO₄ at concentrations less than 0.6 M had no effect on the doublet, but further addition of 2.5 mM *o*-phenanthroline caused complete elimination of the split signal, although photoreduction of Pheo (as monitored by EPR or by characteristic absorbance changes) was not inhibited (Fig. 4 and Table 1). The analysis of Fe in TSF-II and TSF-IIa has

FIG. 3. Split EPR signal after phototrapping of Pheo[•] at 220 K (see Fig. 1 legend) in TSF-II fragments: lyophilized, extracted by hexane containing 0.13% (A) or 0.16% (B) methanol, reconstituted by adding PQ-A, β -carotene, PQ-A plus β -carotene, or vitamin K₁ plus β -carotene. Temperature 7 K; microwave power 50 mW, modulation amplitude 12 G; Chl, 0.75 mg/ml. Gain is equal to 4 (cf. Fig. 1).

FIG. 4. Effect of LiClO_4 and *o*-phenanthroline on light-induced EPR doublet signals of TSF-II fragments after phototrapping of Pheo⁺ at 220 K (see Fig. 1). (Upper) Magnitude of the doublet signal as a function of treatment with various concentrations of LiClO_4 in the presence (---) and absence (—) of 2.5 mM *o*-phenanthroline. Microwave power 50 mW, temperature 7 K. (Lower) Spectra after treatment with 0.4 M LiClO_4 (A) and with 0.4 M LiClO_4 + 2.5 mM *o*-phenanthroline (B); spectra C and D, same as B but with additional 12-hr incubation with 0.2 mM Fe^{2+} (C) or Mn^{2+} (D). Temperature and microwave power are: left column, 90 K and 50 μW ; right column, 7 K and 50 mW. Modulation amplitude 7 G; Chl 1.5 mg/ml.

shown that they contain ≈ 2 and 7–8 atoms, respectively, of nonheme Fe per 100 Chl molecules, or ≈ 2 Fe atoms per reaction center (Table 1). The iron content decreased to 0.6 Fe per reaction center upon treatment of TSF-II with 0.55 M LiClO_4 plus 2.5 mM *o*-phenanthroline (eliminating the EPR doublet) but was almost unchanged with 0.5 M LiClO_4 alone (Table 1). Incubation of TSF-II, depleted of Fe and completely lacking the EPR doublet, in a medium containing Fe^{2+} restored $\approx 25\%$ of this signal (Fig. 4, spectra B and C). In this case, the doublet appears together with the narrow signal of Pheo⁺ ($g = 2.0033$, $\Delta H = 13.5$ G). At 7 K, the maximal amplitude of the narrow signal was observed at a microwave power of ≈ 250 μW instead of ≈ 5 μW as previously noted (Fig. 2), possibly due to a weak coupling to added Fe. No restoration of the doublet signal by either Mn^{2+} (Fig. 4, spectra B and D) or Mg^{2+} (not shown) was observed.

Extraction of Fe and disappearance of the doublet EPR signal after treatment of TSF-II with LiClO_4 plus *o*-phenanthroline was accompanied by development of a new narrow EPR signal ($g = 2.0044 \pm 0.0003$, $\Delta H = 9.2 \pm 0.5$ G), which appeared as a result of illumination at 220 K and $E_h = 50$ mV (Fig. 5, spectra

Table 1. Effects of LiClO_4 treatment on iron content and signal amplitudes

Treatment	Fe/RC	Amplitude, %	
		Doublet signal	Narrow signal
Untreated	1.8 ± 0.2	100	100
0.5 M LiClO_4	1.5 ± 0.2	100	100
0.5 M LiClO_4 + <i>o</i> -phenanthroline	0.6 ± 0.2	0	120
0.75 M LiClO_4 + <i>o</i> -phenanthroline	<0.2	0	110

The number of nonheme iron atoms per PS II reaction center (Fe/RC) and the amplitudes of the EPR doublet at 7 K and the narrow EPR signal of Pheo⁺ at 90 K in TSF-II fragments were determined before and after treatment with LiClO_4 , alone or with 2.5 mM *o*-phenanthroline. For details on the measuring and treatment conditions see legend of Fig. 1. Concentration of PS II reaction centers in TSF-II (1 reaction center per 90–120 Chl molecules) was evaluated from photoinduced absorbance changes of Pheo reduction at 685 nm as described (7). $\pm$ indicates the range.

B). In untreated TSF-II there was also a narrow photoinduced EPR signal under the same conditions but its amplitude was 1/5 to 1/6. This signal was not observed (Fig. 5, spectra C) in TSF-II treated with hexane containing 0.16% methanol (with or without subsequent removal of Fe). In TSF-IIa the iron extraction increased the amplitude of the narrow photoinduced signal 1.5–2 times (Fig. 5, spectra D). This signal was not induced by illumination of TSF-IIa at 295 K at $E_h = 50$ mV (not shown) or by addition of dithionite in the dark (Fig. 5, spectrum E).

DISCUSSION

Data presented here show that the photoinduced split EPR signal of PS II at $E_h \approx -450$ mV arises from species on the reducing rather than on the oxidizing side of PS II, because the doublet (as well as the narrow EPR signal of Pheo⁺) is not ob-

FIG. 5. EPR spectra of PS II preparations at $E_h \approx +50$ mV before (left) and after (right) treatment with 0.55 M LiClO_4 and 2.5 mM *o*-phenanthroline. Spectra A, B, and C are for TSF-II particles: (A) dark; (B) light minus dark difference after 1-min illumination at 220 K; (C) same as B but after extraction with hexane containing 0.16% methanol (see Fig. 3). Spectra D and E, TSF-IIa particles: (D) light minus dark difference after 1-min illumination at 220 K ($E_h = +50$ mV); (E) dark signal in the presence of dithionite ($E_h \approx -450$ mV). Temperature 90 K; microwave power 50 μW ; modulation amplitude 3.8 G; gain $\times 4$. Chl 1.0 mg/ml for TSF-II and 0.7 mg/ml for TSF-IIa.

served after illumination at 220 K at $E_h = +50$ mV (data not shown), when Q is oxidized in the dark and redox conditions are more favorable for photoaccumulation of oxidized donors. Thus, photoaccumulation of the reduced intermediary electron acceptor of PS II, Pheo[•], appears to be essential for observation of the doublet. The other component involved in the formation of the split EPR signal is apparently the primary electron acceptor Q (which is reduced at $E_h = -450$ mV). In fact, the doublet was eliminated after treatment of TSF-II fragments with 0.1–0.2% methanol in hexane [i.e., under characteristic conditions for extraction of Q (14)]. Significantly, it could be restored by the addition of pure PQ-A (Fig. 3), an essential component of Q (8–11, 13, 14). Partial activation of the doublet with β -carotene (Fig. 3) is also in agreement with its requirement for reconstituting the C550 signal (12–14), which is apparently the result of a blue shift of an absorbance band of Pheo by reduced Q (1, 2, 10).

The EPR doublet in PS II is similar to that found in a number of enzyme-coenzyme B-12 systems and attributed to an exchange interaction between organic radicals and the Co(II)–B-12 complex (29). In this context, the magnitude of the splitting and the ratio of the high- to low-field peaks in the EPR doublet of PS II suggest that this signal results from an exchange interaction between Pheo[•] and a paramagnetic center with g value of ≈ 1.8 (cf. equations 2 and 3 in ref. 29). Thus, interaction of Pheo[•] with PQ alone [g value of which *in vitro* is 2.0 (30)] cannot produce the doublet. In bacterial reaction centers a complex of quinone with Fe^{2+} has an EPR signal with g value 1.82–1.87 (20–22, 31, 32), and its interaction with the reduced intermediary electron acceptor, bacteriopheophytin, produces a split EPR signal (19–22), similar to that reported here in PS II. In our preliminary experiments we have not been able to find such a high-field EPR signal in PS II, but we have found other evidence for the participation of a PQ–Fe complex in the formation of the doublet. One of them is effective elimination of the doublet (Fig. 4, spectra A and B) by treatment with *o*-phenanthroline, a strong chelating agent for Fe^{2+} and some other metals. Recently, nonheme Fe at a concentration of 3–4 Fe per P680 has been found in PS II preparations (14). TSF-II and TSF-IIa contain 2–3 nonheme iron atoms per reaction center (Table 1) but no iron-sulfur proteins (24). The treatment with $LiClO_4$ and *o*-phenanthroline, which eliminates the EPR doublet (Fig. 4 and Table 1), decreases the iron content to less than 0.6 Fe per reaction center. Restoration of the doublet after addition of Fe^{2+} (but not of Mn^{2+} or Mg^{2+}) to the Fe-depleted TSF-II supports the idea that a complex of PQ with Fe is involved in the production of this signal. Magnetic relaxation properties of the doublet in PS II (Fig. 2) are also characteristic of the iron-containing complexes (19–22, 31, 32).

Existence of a PQ–Fe complex in PS II reaction centers is also supported by the development of a photoinduced, narrow EPR signal (Fig. 5, spectra B), with characteristics ($g = 2.0044$, $\Delta H \approx 9.2$ G) close to those of the anion radical of PQ *in vitro* (30), after extraction of Fe and accompanying loss of the EPR doublet (Fig. 4, spectra B). We suggest that a singly reduced PQ, no longer coupled to Fe, is responsible for this narrow EPR signal. [In photosynthetic bacteria, a similar EPR signal of semiquinone with $g \approx 2.0045$ and $\Delta H \approx 8$ G was also found only after its association with Fe was disrupted (20, 21, 33).] The absence of this signal after Q has also been extracted from TSF-II (Fig. 5, spectra C) further supports our suggestion. Addition of dithionite (Fig. 5, spectra E) as well as illumination of Fe-depleted TSF-IIa at 295 K at $E_h = 50$ mV (data not shown) probably leads to accumulation of doubly reduced, diamagnetic PQ^{2-} . [Disruption of the quinone–Fe complex in bacteria facilitates the double reduction of quinone by di-

thionite (21).] Double reduction of PQ is probably responsible for the absence of the EPR doublet in PS II after photoaccumulation of Pheo[•] at 295 K (ref. 7; cf. ref. 22).

Thus, all the properties of the EPR doublet in PS II—namely, conditions for its appearance, its line shape, magnetic relaxation characteristics and temperature dependence, its disappearance and restoration under extraction and readdition of PQ as well as Fe^{2+} , appearance of an EPR signal assignable to $PQ^{•-}$ after Fe removal—are consistent with the idea that the primary electron acceptor of PS II is a PQ–Fe complex and that an exchange interaction of its singly reduced form with the reduced intermediary electron acceptor, Pheo[•], produces the EPR doublet signal.

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