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Evidence for the interaction of the Photosystem I secondary electron acceptor *X* with chlorophyll *a* in spinach Photosystem I particles

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Ether extraction of antenna pigments from PS I particles (Ikegami, I. and Katoh, S. (1975) Biochim. Biophys. Acta 376, 588–592) led to the change of EPR signal of the PS I secondary electron acceptor '*X*'. The g_x value of the EPR signal of *X*, which was 1.77 in the PS I particles, remained unchanged as far as the pigment extracted was less than 50%. However, further extraction of pigments shifted it to the higher value; it came up to 1.80 in the particles containing about 5% chlorophyll *a*. g_y and g_z values of the EPR signal of *X* were less sensitive to the pigment extraction. The g_x signal intensity of the EPR signal of *X* remained almost constant through the pigment extraction. The re-incorporation of the purified chlorophyll *a* to the pigment-extracted particles resulted in a partial recovery of the g_x value. On the other hand, vitamin K-1 had no significant effect on the recovery of the g_x value. The results suggest the close location of the component *X* to chlorophyll *a* in the vicinity of PS I reaction center.

Introduction

EPR studies on PS I complex of higher plants reveal the presence of at least three membrane-bound iron-sulfur centers, designated as *X*, centers A and B, which function as electron acceptors in PS I [1–3]. Both the centers A and B are chemically reduced by dithionite in the dark [4], whereas the reduction of the component *X* is achieved only by illumination of the sample after chemical reduction of centers A and B [5,6]. Therefore, the component *X* is assumed to have a more negative midpoint potential [7,8] and function as an earlier electron acceptor than centers A and B [1–3]. The identity of *X* is somewhat uncertain, though a chemical assay of iron and sulfur in the PS I reaction center [9] and a Mössbauer study [10] suggests that it is an Fe-S protein similar to the centers A and B. An unusual EPR *g*-value, temperature and power dependency [5,6,11], however, have given alternative interpretation on the identity of *X*; i.e., a quinone-iron complex [3,12], or a chlorophyll anion radical interacting with an iron atom [13]. Moreover, the interaction between $X(A_2)$ and chlorophyll *a* molecule(s) is suggested from the presence of the electrochromic shift due to chlorophyll *a* in the reduced-minus-oxidized difference absorption spectrum of $X(A_2)$ [14–16].

Ikegami and Katoh previously found that most antenna pigments can be extracted without any loss of P-700 activity by the treatment of PS I particles with wet diethyl ether [17]. Thus, the

Abbreviations: PS, Photosystem; EPR, electron paramagnetic resonance; Chl, chlorophyll.

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P-700 content of the particle obtained can be as high as P-700 in 6–9 chlorophyll molecules. This preparation still contains about 12 atoms each of Fe and S per P-700 [16], suggesting the presence of three iron-sulfur acceptors, each probably with 4Fe-4S center. On the other hand, this extreme pigment depletion is expected to affect the organization of the Fe-S proteins, especially, of the component *X*, because *X* is likely to have some interaction with chlorophyll *a* as pointed out [14–16].

This paper shows that the g_x value in the EPR signal of the component *X* increased by the extraction of antenna pigments from PS-I particles. The re-incorporation of purified chlorophyll *a* to the pigment-depleted particles resulted in a partial recovery of this shift, indicating that the unusual g_x value of *X* arises, at least partly, from the interaction of *X* with Chl *a*.

Materials and Methods

PS I particles containing various amounts of antenna chlorophyll were prepared by the ether extraction of lyophilized PS I particles as described previously [17,18]. The extent of pigment extraction was dependent on the water content in diethyl ether; a higher water saturation in ether resulted in more pigment extraction [17]. For example, 40% of the total Chl *a* in the PS I particles was extracted with dry ether, and the 97% with water-saturated ether. The Chl *a*/P-700 ratios were 140 in the original PS I particles and 8 in the particles having the lowest content of chlorophyll *a* (i.e., the P-700-enriched particles). The characteristics of the particles used in the experiment were summarized in Table I.

Pigments and/or lipids were re-incorporated into the P-700-enriched particles as described previously [19]. Monogalactosyldiacylglycerol and vitamin K-1 were purchased from Funakosi Ltd Co. and Wako Ltd Co., respectively. Chl *a* was purified from ether extracts of PS I particles by a column of powdered sugar with the elution solvent of petroleum ether containing *n*-propanol (0.5%, v/v) [20].

EPR spectra were determined with an X-band EPR spectrometer (Model ER200, Bruker, Germany), equipped with a liquid-helium cryostat (Model ESR-900, Oxford Instruments, England). Samples were suspended anaerobically in 0.1 M glycine-NaOH buffer (pH 10) containing 10 mM sodium dithionite. To trap the reduced state of the component *X*, samples in 3 mm i.d. EPR sample tubes were illuminated at 4°C with white light supplied by a projector lamp for about 1 min and then frozen to 77 K under the same light illumination. Sample concentrations were all adjusted to 5 mg Chl/ml before pigment extraction.

Chlorophyll was measured by the method of Arnon [21].

TABLE I

THE CHARACTERISTICS OF THE PHOTOSYSTEM I PARTICLES CONTAINING VARIOUS AMOUNTS OF ANTENNA CHLOROPHYLL

For details for the preparation of the ether-extracted particles, see Materials and Methods, and Refs. 17 and 18.

Particles	H_2O content in ether (%)	Chl *a* (%)	Chl *a*/P-700 (mol/mol)	Recovery of P-700 (%)
PS I particles	–	100	142	100
Ether-extracted particles	0	60	68	115
	50	15	18	118
	80	5	8	88

Results

Fig. 1 shows the EPR spectra, determined at 10 K, of the PS I particles containing various amount of antenna chlorophyll *a*, in which the reduced state of the component *X* was trapped by illumination during freezing in the presence of dithionite (see Materials and Methods). The original PS I particles have a typical EPR profile due to the reduced component *X* with a characteristic trough at $g = 1.77$, in addition to the signals due to the reduced centers A and B which were in an interacting form [22]. This profile changed after the extractions of antenna pigments. The most significant feature is the shift of g_x value of *X*. The value shifted stepwise with the increase in the pigment extracted, from 1.77 in the original PS I particles to 1.80 in the particles having 5% Chl-*a*

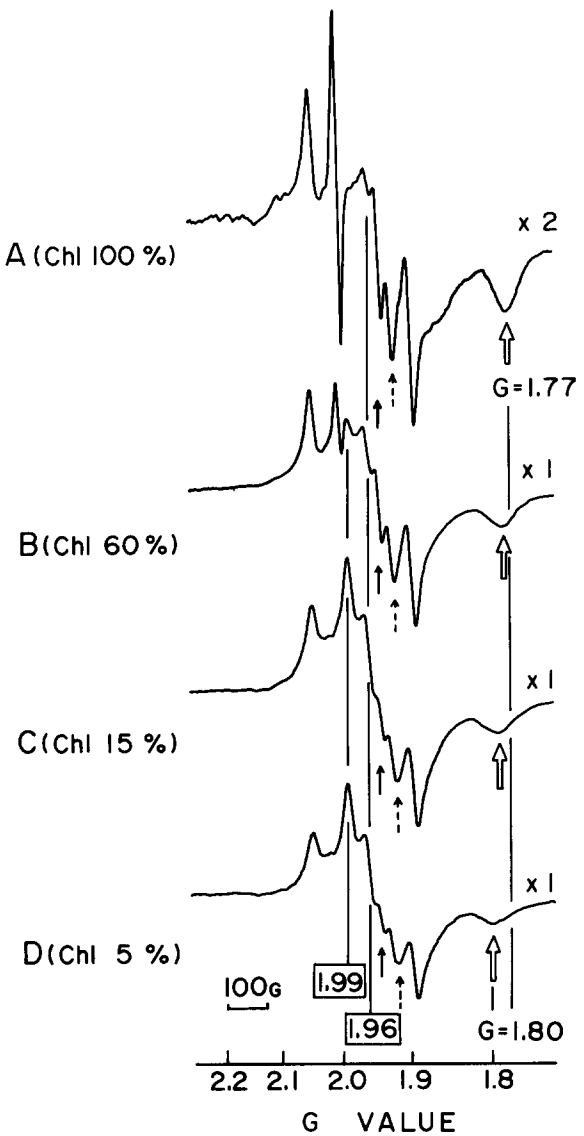

Fig. 1. Representative EPR spectra determined at the reducing level of X before (A) and after (B–D) the pigment-extraction. The Chl a contents in the particles were presented in the figure as % of that in the unextracted PS I particles. The characteristics of samples are given in Table I. EPR experimental conditions: temperature 10 K; microwave power 200 mW; modulation amplitude 10 G; microwave frequency 9.55 GHz; scan speed 20 G/s; time constant 200 ms.

unextracted. The g_x signal intensity of X remained almost constant as far as the pigment extraction was not beyond 90% of the total Chl a. Further extraction of pigments resulted in the reduction of the g_x signal intensity to about 60–80%, which was in parallel to the recovery of P-700 in the particle (data not shown). These observations suggest that the ether-treatment does not destroy the component X, but may change its organization in the membrane.

Fig. 2 shows the relationship between the g_x value of X and the extent of the pigment extraction. Only a small shift of the g_x value was observed when the pigment extracted was less than about 50% of that in the original PS I particles. On the other hand, a significant shift occurred proportional to the pigment extraction after 70% of the pigments was extracted. These observations suggest the interaction of X with the antenna chlorophylls, which are more resistant to the ether extraction and are therefore probably located near the PS I reaction center.

Fig. 3 shows the EPR spectrum of X obtained in the particles having 5% Chl a unextracted in which the shift of the g_x value was maximum (see the figure legend for details). The profile was similar to those reported for the EPR spectrum of X [5,6,11], except for its high g_x value. This g_x value is close to those of ferredoxins, so that its profile somewhat resembled the EPR spectrum of the normal Fe-S center [23]. These results suggest that X is a Fe-S protein and its unusual g_x value

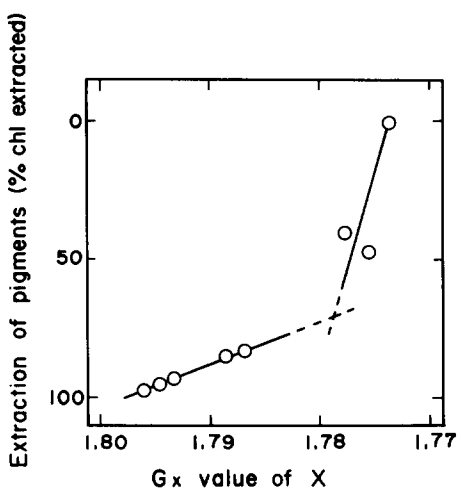

Fig. 2. The g_x value of X plotted as a function of the extent of pigment extraction. Data were taken from EPR spectra in several sets of the experiments as in Fig. 1. EPR experimental conditions were the same as in Fig. 1.

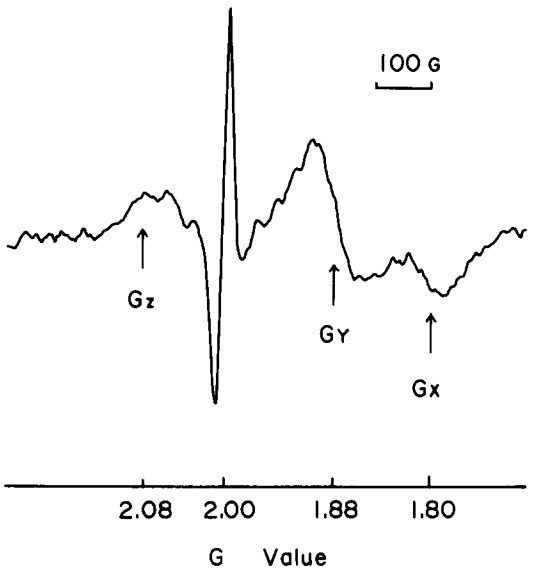

Fig. 3. EPR spectrum of X with the maximum g_x value. The Chl a content in the particles used was 5% of that in the original PS I particles. The spectrum was determined by a difference between two spectra; one obtained with the sample frozen during illumination in the presence of dithionite (10 mM) (reflecting the redox state of X^- center B^- center A^-) and the other, frozen in the dark in the presence of dithionite (10 mM) and methyl viologen (50 μM) (reflecting the redox state of X center B^- center A^-). Other experimental conditions were the same as in Fig. 1.

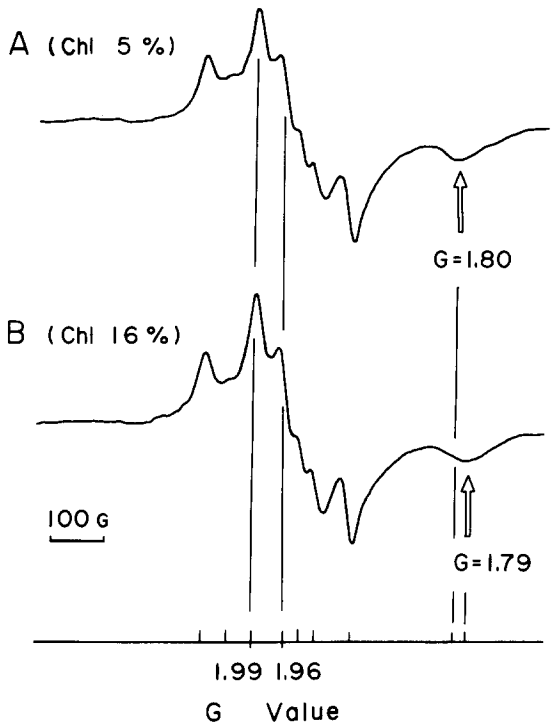

Fig. 4. EPR spectra determined before (A) and after (B) the pigment reconstitution. Pigment reconstitution was performed with ether extract as in Ref. 19. The Chl a contents in the particles obtained before and after pigment reconstitution were 5 and 16%, respectively, of that in the original PS I particles. More details, see legends for Table II.

reflects, at least partly, the interaction of the protein with chlorophyll a molecules around X. Evidence supporting this is given in Fig. 4. As Ikegami reported previously [19], the antenna pigments can be added back to the pigment-extracted particles as effective PS I light-harvesting pigments. The g_x value of X was partially reversed after the reconstitution of antenna pigments. The partial recovery of the g_x value may reflect that the amount of pigments incorporated was partial. The further incorporation of pigments was possible, but with no more recovery of g_x value. This is consistent with no more increase in the light-harvesting efficiency of the pigments associated under these conditions as reported previously [19].

In the experiments above, total ether extract, which includes both pigments and lipids [19], was used for reconstitution. Therefore, some lipids, instead of or in addition to chlorophyll a, may be effective in the recovery of the g_x value. However, it was not the case. Table II shows that the reconstitution with purified Chl a gave the same recovery of the g_x value as with the ether extract. It is noteworthy that incorporation of only a small number of Chl a (5–10 molecules per P-700) was sufficient to its recovery, suggesting that active binding sites of Chl a locate close to the component X in the PS I reaction center. A typical lipid in the photosynthetic membrane, monogalactosyl diacylglycerol, had no additive effect on the recovery of the g_x value in the Chl a-reconstituted particles. The presence of vitamin K-1 in or near PS I reaction center with the ratio of 1–2 to one P-700 has been reported by Takahashi et al. [24] and, Schoeder and Lockau [25]. However, the addition of vitamin K-1 with or without chlorophyll a has no significant effect on the recovery of the g_x value.

The microwave power and the temperature de-

TABLE II

CHANGE IN THE g_x VALUE OF THE COMPONENT *X* BY RECONSTITUTION WITH Chl *a* AND/OR LIPIDS

EPR parameters were the same as in Fig. 1. P-700 contents were determined from the ferricyanide (0.2 mM)-oxidized minus ascorbate (5 mM)-reduced absorbance changes at 696 nm with the difference molar extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ by Hiyama et al. [26]. The amount of Chl *a* incorporated into the P-700-enriched particles was dependent on the concentration of pigments (Chl *a*) in the reconstitution-medium. The concentrations of ether extract and purified Chl *a* used here were adjusted to 1 mg and 0.5 mg Chl *a*, respectively, per 20 mg lyophilized particles (in 20 ml of suspension), which corresponded to a half and a quater of Chl *a*, respectively, originally present in the unextracted particles. The lipid/Chl *a* ratios in the reconstitution medium were about 10 (w/w). MGDG, monogalactosyldiacylglycerol; Other experimental conditions, see Ref. 19.

Sample	Chl (%)	Chl/P-700 (mol/mol)	Recovery of P-700	g_x value of *X*
PS I particles	100	142	100	1.770 ± 0.002
P-700-enriched particles	5.0	9	79	1.800 ± 0.004
REconstituted particles with				
ether extract	16.0	31	73	1.788 ± 0.004
Chl a	7.3	14	74	1.787 ± 0.005
Chl a + vitamin K-1	7.9	15	75	1.782 ± 0.003
Chl a + MGDG	7.0	14	71	1.791 ± 0.005
Vitamin K-1	5.0	9	79	1.800 ± 0.004

pendencies of the EPR signal intensities of *X* did not change by the pigment extraction (data not shown), suggesting only a slight change in the spin state of the Fe-S core of *X* through this ether extraction.

Another change of the EPR spectrum induced by the ether extraction was the appearance of new EPR signals which had *g* values of 1.96 and 1.99, respectively (Fig. 1). The $g = 1.96$ signal observed here seems to be the same signal as has been found by Ke [27], Dimukes and Sauer [28], Rupp et al. [13] and Hiyama et al. [29]. This signal has been detected only when the redox potential became much lower than those of centers A and B. In this experiment, however, the reducing conditions were almost the same in all the samples. Furthermore, the $g = 1.96$ signal developed as the $g = 1.94$ signal which originates from center A, became small. These observations suggest that this signal is not due to a new Fe-S protein. The appearance of this signal probably reflects some conformational change of center A (and center B) induced by, for example, the ether extraction of lipids and/or antenna pigments.

Further extraction of pigments caused the appearance of the $g = 1.99$ signal. There are few reports (cf. Ref. 13) on $g = 1.99$ signal probably due to a large $g = 2.00$ radical signal overlapping to this signal. After the pigment extraction, $g =$ 2.00 signal, most of which probably reflects a radical signal from antenna chlorophyll, became less significant so that $g = 1.99$ signal became more obvious. This signal also seems to originate from centers A and B (and the component X), probably through their some structural changes. Its power and temperature dependency were quite similar to those of the component *X*; i.e., this signal became more recognizable at the lower temperature (approx. 10 K) and at the higher microwave power (approx. 100 mW) (data not shown) (cf. Ref. 13). These signals were also weakly affected by the pigment reconstitution.

Discussion

Correlations between the kinetic behaviors of A_2 designated by flash kinetic studies [1,3,30,31], and the EPR component '*X*', have been established [14,32,33], suggesting that A_2 is identical with the component *X*. In fact, the reduced-minus-oxidized difference absorption of A_2 was interpreted as originating possibly from an iron-sulfur center because of its broad absorption decrease in the 400–500 nm region [14–16,34]. This spectrum always accompanied an electrochromic shift of Chl *a* [14–16], suggesting a close location of A_2 to Chl *a*. On the other hand, the interaction of *X* with Chl *a* was similarly confirmed by the

EPR study described here. The gradual shift of its g_x value by the gradual extraction of Chl *a* (Figs. 1 and 2) suggests that the component *X* interacts with not only one Chl *a*, but several Chl *a* molecules around *X*. Furthermore, these Chl *a* seem to locate in the vicinity of the reaction center, since the shift of the g_x value was mostly induced by the extraction of the Chl *a* near the reaction center (Fig. 2). These results suggest that the component *X* is embedded in the reaction-center site of the chlorophyll-containing large subunit (P-700-Chl *a* protein), or alternatively, that *X* is a constituent of P-700-Chl *a* protein [cf. Refs. 35 and 36].

Of special interest is that the extracted Chl *a* can be re-associated around *X* and induces a partial recovery of its g_x value (Fig. 4, Table II). As previously shown [19], a several number of Chl *a* binding sites are present on the structural protein of pigment-extracted particles and Chl *a* which binds to these sites can act as efficient light-harvesting pigments. The structure around the reaction center remains probably undamaged even after the pigment extraction and some active Chl *a* binding sites may locate close to the component *X*.

Recently, we have found that all the vitamin K-1 in the PS I particles (about 2 vitamin K-1 per one P-700) can be removed by the ether extraction of the lyophilized PS I particles [37], which indicates that each of the ether-extracted particles used in the present work has no vitamin K-1, independently of the amount of pigments extracted. These vitamin K-1-depleted particles show, after a flash excitation, the very rapid decay of $P\text{-}700^+$, possibly reflecting the back electron flow from A_0^- to $P\text{-}700^+$ [37], which agrees with the recent proposal of vitamin K-1 to A_1 [38,39]. However, we were still able to observe the photoreduction of *X* (and centers A and B) even in the absence of vitamin K-1 when we used the continuous illumination as the excitation light (Fig. 1, Ref. 37). We can explain this discrepancy by assuming the by-pass electron flow from A_0 to A_2 (*X*), by which electrons can gradually accumulate on *X* (and centers A and B) during prolonged illumination (cf. Ref. 37).

The presence of the EPR signal of *X* in these ether-extracted particles (Fig. 1) seems to exclude the possibility that *X* is a quinone (vitamin K-1)-iron complex (cf. Refs. 3 and 12). No significant interaction of *X* with vitamin K-1 was also evidenced by the reconstitution of ether-extracted particles with vitamin K-1 which showed little effect on the EPR signal of *X* (Table II). Thus, we conclude that the component *X* is an iron-sulfur protein (cf. Fig. 3) and its unusual characteristics originate probably from the spatial organization of Chl *a* around *X*.

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