Reconstitution of photosynthetic charge accumulation and oxygen evolution in $CaCl_2$-treated PS II particles

II: EPR evidence for reactivation of the $S_1 \rightarrow S_2$ transition in $CaCl_2$-treated PS II particles with the 17-, 23-, and 34-kDa proteins

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Extraction of PS II particles with 1 M $CaCl_2$ caused complete disappearance of the light-induced signal of the possible Kok S_2 state of the water-splitting complex and total loss of the O_2 evolving activity, concomitant with perfect removal of the 17-, 23- and 34-kDa proteins from the particles. The recovery of the multiline signal in the $CaCl_2$-treated PS II was performed by reinserting the 34-kDa protein, when Cl^- was present in the solution for the EPR measurement. However, in the absence of Cl^-, besides the 34-kDa protein, the 17- and 23-kDa proteins were required for the recovery of the signal. These results are compared with the results on the recovery of the O_2 evolution in the reconstituted PS II to examine the role of these three proteins on the water splitting.

PS II particle *EPR multiline signal* *S_2 state reconstitution* *Water-splitting complex* *Manganese signal* *$CaCl_2$-treatment*

1. INTRODUCTION

It is widely believed that photosynthetic water oxidation requires the generation of 4 oxidizing equivalents to form one molecule of oxygen and this charge accumulation is performed through 5 intermediate oxidation states S_i (i = 0–4) of the water-splitting complex [1]. As mentioned in part I of this series [2], it has been found that the three proteins with molecular masses of 17, 23 and 34 kDa functionally associate with the water-splitting complex. However, the knowledge on the actual role of each protein is very limited [3,4], in particular, knowledge on which steps of the charge accumulation in the water-splitting complex each protein may function.

Recently, authors in [5] reported a flash-induced, multiline EPR signal in spinach chloroplasts. The signal intensity changed periodically with the number of flashes and the spectrum of the signal was similar to that obtained from a synthetic Mn(III)–Mn(IV) binuclear complex. From these results, they concluded that the Kok S_2 state of the water-splitting complex gives rise to this multiline signal. The same signal was observed in chloroplasts frozen in liquid N during continuous illumination [6]. Authors in [7] examined the temperature dependency of the formation and decay of this signal to find that the S_1 state can be fully advanced to the S_2 state at temperatures as low as 160 K and that the S_2 is capable of further advancement at temperatures above, but not below 210 K. A similar multiline signal has also been

Abbreviations: PS II, photosystem II; Mops, 3(*N*-morpholino)propanesulfonic acid; PBQ, phenylbenzoquinone; RPS II, reconstituted PS II; chl, chlorophyll

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observed in the PS II particles (unpublished) as well as in the intact chloroplasts illuminated with continuous light followed by immediate freezing in liquid N_2. The possibility that the S_2 state of the water-splitting complex could be quantitatively detectable from this EPR signal prompted us to examine the contribution of the 17-, 23- and 34-kDa proteins to the $S_1 \rightarrow S_2$ transition by using the reconstitution method presented in part I of this series [2]. We present here the experimental findings on the disappearance and recovery of the multiline EPR signal induced by the total removal and reinsertion of these three proteins in the PS II particles. Comparison of the results on the EPR signal and those obtained for the O_2 evolving activity leads to the conclusion that the 34-kDa protein participates in the step of $S_1 \rightarrow S_2$, and that, besides the 34-kDa protein, the 17- and 23-kDa proteins are required for this transition at a low level of Cl^-.

2. EXPERIMENTAL

PS II particles, the 17-, 23-, and 34-kDa proteins and lipids, were prepared as shown in part I of this series [2]. Depletion and reconstitution of PS II with regard to the three proteins were also carried out as shown in [2] with a slight modification at the final stage of the reconstitution; i.e., after diluting about 50 times with buffer solution A (0.2 M sucrose, 20 mM Mops, 10 vol. % glycerol, pH 7.0), the reconstituted particles were centrifuged at 30000 × *g* for 30 min. The pellet was resuspended in buffer solution B (0.2 M sucrose, 20 mM Mops, 50 vol. % glycerol of spectroscopic grade) to give a final (chl) of 4.0 mg/ml with 20 mM NaCl or without and submitted to the EPR and the O_2 evolving experiments.

For EPR measurements, the final suspension was transferred into a quartz tube of 3 mm in diameter. After dark adaptation for 60–90 min at 273 K, the EPR sample was equilibrated for 3 min at 195 K in the dark with solid CO_2 and methanol solution and then illuminated for 5 min, with light between 600 and 800 nm, provided by a 500 W tungsten lamp through a color glass filter (Kenko R1 or SR 60) and a water layer of 10 cm thickness. After illumination, the sample was immediately placed in liquid N_2 in the dark and stored until EPR measurement was carried out within 10 h. EPR spectra were recorded on a Varian E-109 system equipped with an Oxford EPR-900 continuous flow cryostat. All measurements were carried out at 8.0 ± 0.5 K.

Spectrometer conditions were: microwave frequency, 9.240 GHz; microwave power, 40 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G.

The assay of O_2 evolution was carried out by using a teflon-covered oxygen electrode under continuous illumination of saturating light between 600–800 nm through a 10 cm water layer. PBQ was used as an electron acceptor. Manganese contents in PS II particles and reconstituted PS II (RPS II) were determined by means of atomic absorption spectroscopy on a Perkin Elmer model 5000 with a carbon rod atomizer.

3. RESULTS AND DISCUSSION

A comparison of the protein patterns of the PS II and the $CaCl_2$-treated PS II in SDS-PAGE revealed that the three proteins were totally removed from the PS II by extraction with 1 M $CaCl_2$ as in [8]. The protein patterns of RPS II prepared with each of the three proteins together with the lipids clearly showed that each of these proteins was able to be reinserted into the $CaCl_2$-treated PS II by the reconstitution method shown in [2].

Fig.1 illustrates the light-induced multiline EPR signal of the possible Kok S_2 state observed at 8.0 ± 0.5 K with PS II and RPS II prepared under different conditions. As a control, a spectrum for the non-illuminated PS II (dark-adapted) is shown at the top of the figure together with a noise level (A). The multiline signal generated by continuous illumination at 195 K on our PS II samples was essentially identical to those observed in [5] using a single laser pulse, and in [6]. As mentioned previously, the signal strongly depended on the temperature where the spectrum was obtained. However, when the temperature and the chlorophyll concentration of the sample were fixed, the spectrum was reasonably reproducible both in line shape and intensity. Spectra C–F in fig.1 represent the recovery of the multiline signal in RPS II upon addition of the 3 proteins in the presence of Cl^- in each EPR sample solution. The multiline signal completely disappeared in $CaCl_2$-treated PS II and

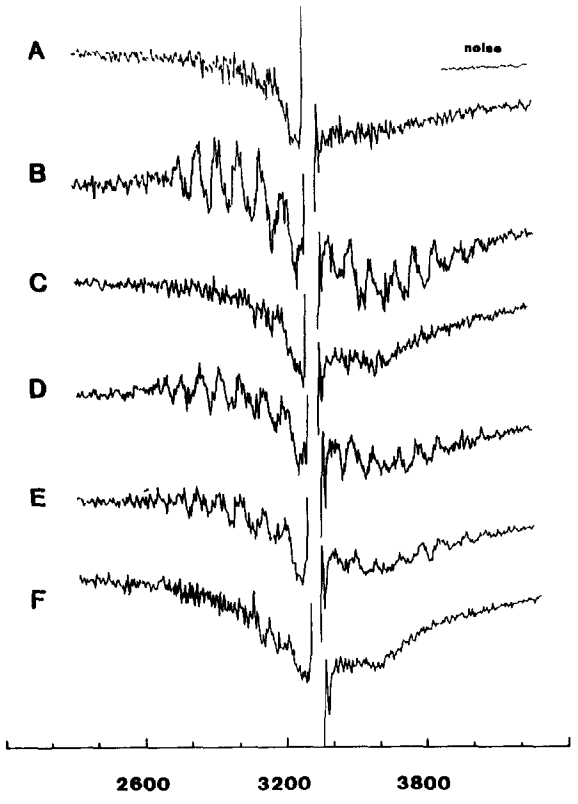

Fig.1. EPR spectra of the PS II and differently prepared RPS II samples in the presence of 20 mM Cl^-. (A) Dark-adapted PS II at 273 K and stored in liquid N_2, (B) PS II illuminated, (C) RPS II (with lipids) illuminated, (D) RPS II (with lipids and 17-, 23-, 34-kDa proteins) illuminated, (E) RPS II (with lipids and 34-kDa protein) illuminated, (F) RPS II (with lipids and 17-, 23-kDa proteins) illuminated. Illumination on each sample was done at 195 K for 5 min under the same conditions. EPR measurement conditions were: temperature, 8.0 ± 0.5 K; microwave frequency, 9.240 GHz; power, 40 mW; others as in text.

it was not recovered in the RPS II prepared with the lipids alone (C), but was recovered upon addition of the 34-kDa protein with the lipids to the incubation medium for reconstitution, at a protein/lipid/chl ratio of 0.6/3.0/1.0 by wt (E), when NaCl was contained at a level of 20 mM in the solution of EPR sample. The intensity of the multiline signal was enhanced by further addition of the 17- and 23-kDa proteins in the incubation medium, but the 17- and 34-kDa proteins by themselves had no effect on the recovery of the signal without the simultaneous addition of the 34-kDa protein. The corresponding results obtained with the samples which contained no NaCl are shown in fig.2. The intensity of the light-induced multiline signal of the PS II and RPS II prepared with the simultaneous addition of the 17-, 23- and 34-kDa proteins together with the lipids was reduced only by a factor of about 0.9 by the removal of NaCl from the sample solution. However, for the RPS II prepared with the 34-kDa protein and the lipids, removal of NaCl from the sample solution caused a complete disappearance of the multiline signal which was observed in the presence of 20 mM NaCl.

As shown in part I of this series, when the assay of the O_2 evolution for the reconstituted systems was carried out at a low level of NaCl (e.g. 0.1 mM), besides the 34-kDa protein, both the 17- and 23-kDa proteins were required for the O_2 evolution; but on the other hand, at a higher level of NaCl (e.g., 20 mM) these proteins were not required. To examine quantitatively the relationship between the multiline signal and the O_2 evolving activity for the same sample preparations, the

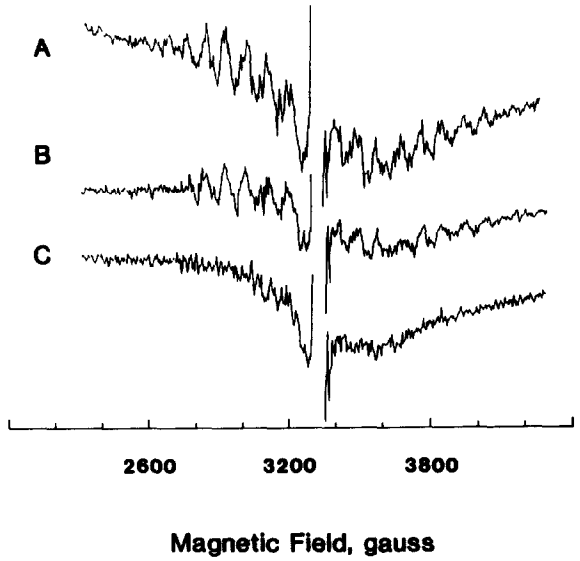

Fig.2. EPR spectra of the PS II and differently prepared RPS II samples in the absence of Cl^-. (A) PS II illuminated, (B) RPS II (with lipids and 17-, 23-, 34-kDa proteins) illuminated, (C) RPS II (with lipids and 34-kDa protein) illuminated. Illumination and EPR measurements were done as in fig.1.

signal intensity was estimated from the addition of peak to peak heights of three well-defined low field lines. In table 1 the results are summarized and compared with the O_2 evolution activity and manganese contents. The manganese content of the PS II used here was $9.8 \pm 0.5/400$ chl and it was reduced to $8.1 \pm 0.5/400$ chl by the $CaCl_2$-treatment. Despite the fact that more than 80% of the original level of manganese remained, the multiline signal, as well as the O_2 evolving activity, completely disappeared, and they were not recovered, unless the 17-, 23-, and 34-kDa proteins were simultaneously added to the incubation medium of the reconstitution, when the sample solutions did not contain any Cl^-. However, when the sample solutions contained Cl^- at a level of 20 mM, both the multiline signal and the O_2 evolving activity were recovered by the addition of the 34-kDa protein alone, although the signal intensity and the O_2 evolving activity were 0.5 and 0.4, respectively, of the corresponding value of the RPS II prepared with the 17-, 23-, and 34-kDa proteins. Qualitatively similar results on the recovery of the multiline signal in the RPS II prepared without the lipids were obtained, but the intensity of the signals was significantly smaller than that of the corresponding RPS II prepared with the lipids (not shown). This behavior concerning the effect of the lipids is similar to that of the O_2 evolution shown in [3].

The remarkable similarities in the protein requirements, the Cl^- dependency and the effect of the lipids between the light-induced multiline signal and the O_2 evolving activity gave extra support to the proposal [5] that the signal originates from one of the Kok states, presumably the S_2 state, of the water-splitting enzyme. Furthermore, the fact that the recovery of the multiline signal in each RPS II agrees fairly well with that of the O_2 evolution in the corresponding RPS II, provides a significant conclusion that the 17-, 23- and 34-kDa proteins participate in the $S_1 \rightarrow S_2$ transition rather than in other transitions, because the S_1 state is the most populated one in the dark, and at 195 K, where the EPR sample was illuminated, the S_1 state

Table 1

Comparison of the EPR multiline signal intensity and O_2 evolving activity in the PS II and differently prepared RPS II

Sample	Mn contents	In the presence of 20 mM Cl^-		In the absence of Cl^-	
		O_2 evolution	Relative intensity of multiline signal	O_2 evolution	Relative intensity of multiline signal
	molecules / 400 chl	μmol O_2 / mg chl·h		μmol O_2 / mg chl·h	
PS II	9.8 ± 0.5	380	100	133	85
RPS II (lipids)		0	0	0	0
RPS II (17-, 23- and 34-kDa proteins and lipids)		350	58	192	51
	8.1 ± 0.5				
RPS II (34-kDa proteins and lipids)		144	28	0	0
RPS II (17- and 23-kDa proteins and lipids)		0	0	0	0

The measurements of EPR and O_2 evolution were performed with the same preparation for each sample. The intensity of multiline signal for each sample was calculated relative to the value of the PS II in the presence of 20 mM Cl^-

can be fully advanced to the S_2 but further advancement cannot occur.

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