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Formation and flash-dependent oscillation of the S_2-state multiline EPR signal in an oxygen-evolving Photosystem-II preparation lacking the three extrinsic proteins in the oxygen-evolving system

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In PS-II-enriched membranes lacking the three extrinsic water-soluble proteins in the oxygen-evolving system (18, 24 and 33 kDa), but still evolving oxygen to some extent, the formation of the multiline EPR signal originating from the S_2-state is dependent on the concentration of Cl^-. In 200 mM Cl^- the multiline signal was observed after the first flash and oscillated with the flash number with a period of four. At 20 mM Cl^- no signal could be observed in this material. These results suggest that the extrinsic proteins are not necessary for multiline signal formation and that complete advancement through the S-states can occur in their absence when sufficient Cl^- is present.

Introduction

Photosystem II is a membrane-bound multicomponent enzyme that catalyzes the reduction of plastoquinone and the light-driven oxidation of H_2O to O_2 in plants, algae and cyanobacteria. The molecular organisation of the oxygen-evolving system has been extensively studied in recent years, and biochemical analysis has revealed the importance of four manganese atoms and three extrinsic water-soluble proteins (of 33, 24 and 18 kDa, respectively) in the oxygen evolution [1]. These extrinsic proteins can be selectively extracted from PS II by various treatments [1] and it has been suggested that they play an obligatory role in oxygen evolution [2,3]. However, it is now clear that, under optimal ionic conditions, oxygen evolution can still proceed (at least to some extent) in the absence of these proteins [4,5]. Instead, the role of the extrinsic proteins seems to be to increase the affinity for Cl^- and Ca^{2+}, and these ions are essential for oxygen evolution [1]. The presence of the 24 kDa protein provides a high-affinity site for Ca^{2+} [6]. The 18 and 24 kDa proteins lower the Cl^- requirement for optimal oxygen evolution from 30 mM in their absence to less than 1 mM in their presence [7], while removal of the 33 kDa protein drastically changes the Cl^- requirement of the system and, in its absence, as much as 200 mM Cl^- is necessary for maximal oxygen evolution [7,8].

Four successive charge separations in the PS II reaction center create the oxidizing equivalents which are necessary for the formation of one molecule of oxygen from two molecules of water (for a recent review, see Ref. 9). These positive

Abbreviations: PS II, Photosystem II; Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid; PPBQ, phenylbenzoquinone.

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charges are stored, possibly on the manganese cluster in the oxygen-evolving system, which exists in five intermediary oxidation states: S_0–S_4 [10]. The chemistry of the S-states remains in many respects a matter of speculation. One probe to the structure of the S_2-state and to the S-state turnover is an unusual multiline EPR signal which originates from the S_2-state [11–14]. Power saturation studies [15], computer simulations of the signal shape [16] and comparisons to model systems [3] have provided evidence that the signal originates from a cluster of two or possibly four manganese atoms in a mixed-valent state. So far the multiline signal has been reported from all oxygen-producing materials that have been investigated, while many treatments that inhibit oxygen evolution also inhibit the formation of the multiline signal [3].

A number of studies have correlated the removal of the extrinsic proteins with inability to form the multiline signal from the S_2-state [3,17–20]. However, as with many studies of oxygen evolution it seems likely that these results are due to the use of nonoptimal concentrations of Cl^- and/or Ca^{2+}, since removal of the extrinsic proteins results in much lowered affinity for these ions (for a discussion, see Refs. 21 and 22). Despite this, it was claimed in a recent study of the role of Cl^- and the extrinsic proteins in the oxygen-evolving system that the 33 kDa protein was required for formation of the multiline signal [17].

In this communication we have attempted to resolve some of the anomalous EPR observations and to bring them in line with the current knowledge of the function of the extrinsic proteins and Cl^-. We demonstrate that in the absence of the three water-soluble proteins the S_2-state exhibits an essentially normal multiline EPR signal, which oscillates with a period of 4 with the maximum amplitude after the first flash. It is also shown that the absence of the multiline signal reported by Imaoka et al. [17] is due to insufficient Cl^- in their study.

Materials and Methods

PS-II-enriched membranes were prepared from spinach chloroplasts according to Kuwabara and Murata [23]. These membranes showed an oxygen-evolving activity in 10 mM Cl^- of 440 μmol O_2/mg Chl per h with 0.3 mM PPBQ as electron acceptor. These membranes (hereafter called 'intact material') were treated with either 1 M NaCl or 2.6 M urea in combination with 0.2 M NaCl. The treatment with 1 M NaCl specifically removes the 18 and 24 kDa proteins from PS II (the material is called 'NaCl-washed') [23], while the treatment with urea + NaCl removes all three extrinsic proteins while the Mn-atoms are still bound to the PS II centers (this material is called 'urea + NaCl-treated') [8]. Less than 5% of the 33 kDa protein remained bound to the membranes as judged from Coomassie brilliant blue staining of SDS-polyacrylamide gels using purified 33 kDa protein an as internal standard. These preparations showed oxygen-evolving activity of 350 μmol O_2/mg Chl per h for NaCl-washed membranes (measured in 10 mM Ca^{2+} and 20 mM Cl^-) and 170 μmol O_2/mg Chl per h for urea + NaCl-treated membranes (measured at 10 mM Ca^{2+} and 200 mM Cl^-). The urea + NaCl-treated membranes showed no oxygen evolution when measured in 20 mM Cl^-. Purification of the 33 kDa protein and reconstitution of the urea + NaCl-treated membranes with the purified protein was performed as described in Ref. 24. The activity of this material (called 'urea + NaCl-treated + 33 kDa') was 253 μmol O_2/mg Chl per h. The preparations were frozen in liquid nitrogen and could be stored for several months at −80°C. The buffer used throughout the study was 20 mM Mes-NaOH at pH 6.5 containing 300 mM sucrose, 10 mM $CaCl_2$, 30% ethylene glycol (v/v/v) and varied concentrations of Cl^- (added as NaCl). Ca^{2+} was present at 10 mM in all experiments to saturate the requirement for this ion [1].

EPR samples in calibrated quartz tubes were dark-adapted for 10 min at 20°C before being illuminated at 4°C with saturating flashes [14] from a Nd-YAG laser (15 ns, 100 mJ, 530 nm). The time between flashes in a flash-train was 1 s. In most experiments the samples were given a preflash, after which they were allowed to relax at 20°C in absolute darkness. After 10 min, 20 mM PPBQ in dimethylsulfoxide was added as an acceptor to a final concentration of 0.5 mM. After addition of PPBQ the sample was illuminated with the required number of flashes. The illuminated

samples were rapidly frozen at 200 K and subsequently stored at 77 K. EPR spectra were recorded at liquid-helium temperatures with a Bruker ER-200t-X-band EPR spectrometer or a Bruker ER200D-SRC spectrometer, equipped with an Oxford Instruments cryostat. A Tracor-Northern 1710A apparatus was used for subtraction of spectra. All sample handling was performed in near darkness.

Results and Discussion

In Fig. 1A is shown the high-field part of the multiline signal obtained from the urea + NaCl-treated material illuminated with various numbers of flashes (for the complete spectrum, see Fig. 3C). The multiline signal was formed after the first flash. From the amplitude of the three well-defined lines at high field we estimate that the amplitude of the signal after one flash in the urea + NaCl-treated PS-II-centers was approx. 50% of the multiline signal formed after one flash in the intact material (Fig. 1B). The formation of the multiline signal was dependent on the flash number. The signal amplitude was maximal after the first and fifth flashes. Thus it oscillated with a period of 4 and reflects the S_2-state. The oscillation pattern (Fig. 1A, right) is similar to that observed in the intact material (Fig. 1B, right) and to that earlier observed in PS-II-enriched membranes [14,18] prepared according to Berthold et al. [25].

It was sometimes observed that in the urea + NaCl-treated membranes the first flash resulted in simultaneous formation of the multiline signal and an increased amount of EPR-Signal II_{slow} (S II_s). This signal is thought to be due to a cationic quinone radical on the donor side in PS II [26] which, when present in its reduced form, can donate an electron to the S_2-state [27], thus reducing the yield of any multiline signal formed. S II_s formation on the first flash indicates that it was present in the reduced form in the dark in this material. This is not normally the case in PS-II-enriched membranes. However, because of its sensitivity to light the urea + NaCl-treated material was prepared under very low light conditions [8] which might have allowed some decay of S II_s in

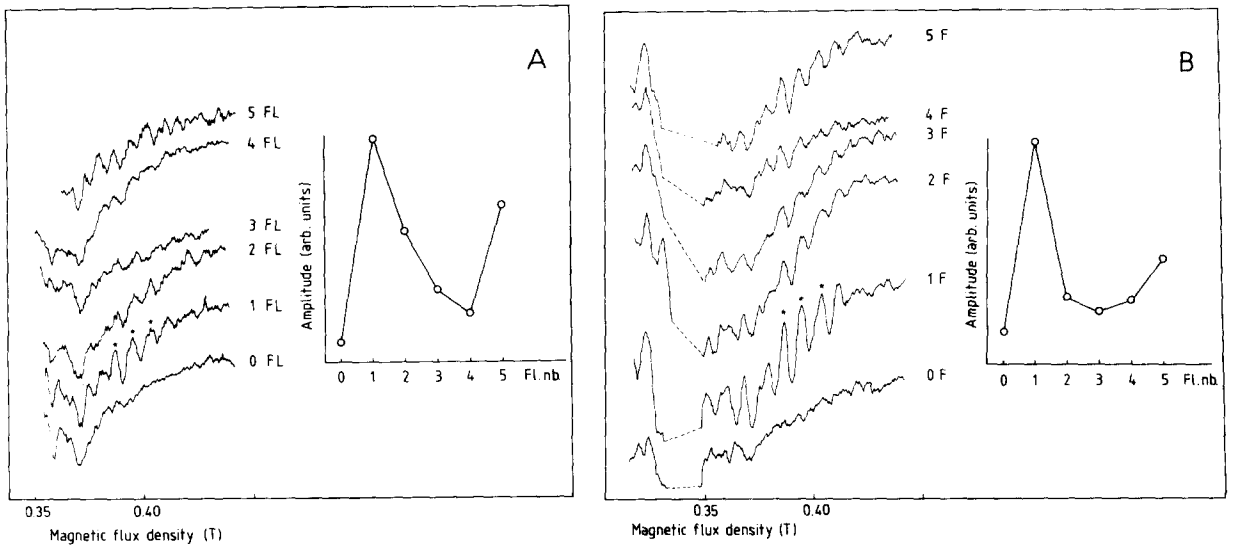

Fig. 1. Flash-dependent oscillation of the multiline EPR signal from the S_2-state. (A) Urea + NaCl-treated membranes; (B) intact membranes. The high-field part of the EPR spectra is shown for samples given a preflash and, 10 min thereafter, a different number of flashes as described in the text. The concentration of the membranes was 2.9 mg Chl/ml and 2.7 mg Chl/ml in (A) and (B), respectively. The respective concentrations of Cl^- were 200 and 20 mM. The amplitude of the multiline signal as a function of the number of flashes given is shown at the right side. The amplitude was calculated from the added amplitudes of the three easily distinguished high-field peaks marked with asterisks in the figure. Microwave frequency, 9.43 GHz; temperature, 8 K; modulation amplitude, 3.2 mT; microwave power, 32 mW; spectrometer gain, $4 \cdot 10^5$.

the dark. The simultaneous formation of the multiline signal and S II_s would result in strong damping of the flash-dependent oscillation of the multiline signal. To avoid this, we used one preflash to oxidise S II_s. This preflash also resulted in the formation of some multiline signal. It has recently been shown [28], that the S_2-state has a half-time for the deactivation of 2.5 min in the dark at 20°C in the urea + NaCl-treated material, and thus 10 min dark adaptation after the preflash was needed to allow essentially complete deactivation of the S_2-state. S II_s remained oxidized during this time. The preflash treatment was used in all experiments. It should be noted that the Mn cluster in the urea + NaCl-treated membranes is more labile than in the intact membranes. This results in some loss of manganese from the membranes which gives an EPR-spectrum from nonfunctional Mn^{2+} which overlaps the multiline spectrum around $g = 2$. Lines originating from this Mn^{2+} are for example seen in the spectrum after zero flash in Fig. 1A. It seems likely that the loss of manganese atoms from the PS II centers in the urea + NaCl-treated membranes at least partly explains the lower amount of the multiline signal observed in this material.

Thus it is possible to obtain high yields of an oscillating multiline signal from a PS-II-preparation lacking the three water-soluble proteins. This result is clearly different from those obtained by Imaoka et al. [17] who were unable to observe the multiline signal from the S_2-state in PS-II-enriched membranes washed with 1 M $CaCl_2$. This material lacks the three extrinsic, water-soluble proteins of 33, 24 and 18 kDa and is known to evolve oxygen only at elevated concentrations of Cl^- [5]. It is thus very similar to the urea + NaCl-treated membranes used in our study [4]. However, their experiments were performed at 20 mM Cl^-, which is too low to allow oxygen evolution from PS-II centers lacking the three water-soluble proteins. This suggests that it was not the absence of the 33 kDa protein that was directly responsible for the inability to form the multiline signal (in contrast to the suggestion by Imaoka et al. [17]). Instead, this inability can probably be explained by the low concentration of Cl^- used.

To test this we performed an experiment under comparable conditions (20 mM Cl^-) (Fig. 2). Urea + NaCl-treated membranes, stored in 200 mM Cl^-, were diluted 10 times in a buffer containing 20 mM Cl^-, allowed to equilibrate on ice for 10 min and then pelleted by centrifugation. The membranes were resuspended in a buffer containing 20 mM Cl^- and divided into two samples. To one was added 2 M NaCl to a final concentration of 200 mM Cl^- and to the other a similar volume of 20 mM NaCl. EPR spectra were recorded after one saturating flash. From Fig. 2A and B it is clear that high yield of the multiline signal was observed in the sample containing 200 mM Cl^-, while no multiline signal was observed at 20 mM Cl^-. Thus it seems clear that it is the concentration of Cl^- that governs the formation of the multiline signal.

It was therefore tested whether the change in Cl^- requirement caused by the removal of the 33 kDa protein was reversible with regard to the

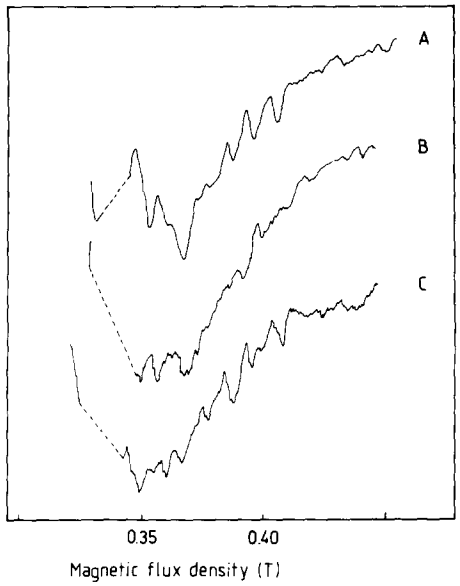

Fig. 2. Effect of the chloride concentration and the 33 kDa protein on the formation of the multiline EPR signal. (A, B) Samples of urea + NaCl-treated membranes prepared as described in the text and given one saturating flash in the presence of 200 mM Cl^- (A) and 20 mM Cl^- (B); the concentration of Chl was identical: 3.0 mg Chl/ml in either sample. (C) Urea + NaCl-treated membranes (3.0 mg Chl/ml) reconstituted with the 33 kDa protein and given one flash in the presence of 20 mM Cl^-. Instrumental settings; microwave frequency, 9.46 GHz; temperature, 8 K; microwave power, 32 mW; modulation amplitude, 2.2 mT; spectrometer gains; $2.5 \cdot 10^5$ (A), $4 \cdot 10^5$ (B) and $4 \cdot 10^5$ (C).

formation of the multiline signal. Such a reversibility has been shown for oxygen-evolution [4,17]. In Fig. 2C it is shown that urea + NaCl-treated membranes which were reconstituted with the 33 kDa protein exhibited appreciable amounts of the multiline signal after one flash. This experiment was performed at 20 mM Cl^-, which indicates that rebinding of the 33 kDa protein increased the Cl^- affinity by more than 10-fold.

NaCl-washed PS-II-enriched membranes have a high oxygen-evolving activity at 20 mM Cl^- [7] and a high yield of the multiline signal was obtained after one flash in this material at this concentration of Cl^- (Fig. 3B). The formation of large amounts of the multiline signal in the absence of the 24 and 18 kDa proteins is in contrast to earlier experiments [18,20] which were performed in low (1 mM, Ref. 18) or nonsaturating (10 mM, Ref. 20) concentrations of Cl^-. However, the result is in agreement with the earlier observations of the multiline signal in this type of material in 20 mM Cl^- [17,19]. Thus the formation of the multiline signal is dependent on the concentration of Cl^- in the surrounding medium in a manner similar to the Cl^- dependence of the oxygen evolution in this material also.

In Fig. 3 are shown the EPR spectra of the multiline signal formed after one flash in four different PS-II-enriched materials. The spectra from the intact material (Fig. 3A) and from the NaCl-washed material (lacking the 18 and 24 kDa proteins) (Fig. 3B) are essentially similar. The peaks are similarly situated and the relative amplitudes of the peaks are comparable. Thus the removal of the two smaller proteins does not lead to any major changes in the configuration of the Mn cluster in the S_2-state.

Removal of the 33 kDa protein by the urea + NaCl-treatment or by $CaCl_2$-washing renders the Mn cluster more labile and at low concentrations of Cl^-, two Mn atoms are easily released [5,8]. This suggests that the environment of the Mn cluster is changed when the 33 kDa protein is released. Alterations of the Mn cluster are also revealed by the multiline signal (Fig. 3C). Due to lower concentration of the multiline signal the spectrum from the urea + NaCl-treated membranes is weaker, and the presence of nonfunctional manganese complicates the subtraction of

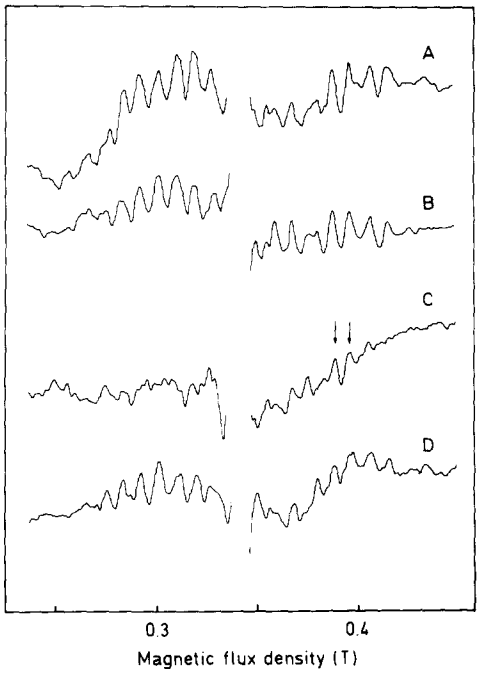

Fig. 3. Comparison of the multiline EPR signal originating from the S_2-state in PS-II-enriched membranes with different protein composition. (A) Intact material (3 mg Chl/ml; 20 mM Cl^-); (B) NaCl-washed material (lacking the 18 and 24 kDa proteins) (3 mg Chl/ml; 20 mM Cl^-); (c) Urea + NaCl-treated material (lacking the 18, 24 and 33 kDa proteins) (3 mg Chl/ml; 200 mM Cl^-); (D) Urea + NaCl-treated material reconstituted with the 33 kDa protein (lacking the 18 and 24 kDa proteins) (3 mg Chl/ml; 20 mM Cl^-). The figure shows the spectra obtained after subtraction of the dark spectrum from the spectrum recorded after one flash in the same sample. First the spectrum was recorded after one flash, thereafter the sample was thawed and allowed to equilibrate at 20°C in complete darkness. After 10 min the sample was frozen and the dark spectrum was recorded. This procedure allowed near complete relaxation of the S_2-state, but also resulted in some release of manganese from the urea + NaCl-treated membranes (C). Instrumental settings: modulation amplitude, 1.25 mT; spectrometer gain, $2.5 \cdot 10^5$; other settings as in Fig. 2.

the dark-spectrum from the spectrum recorded after one flash. This is especially prominent on the low-field side near $g = 2$. Despite these problems it is clear that some peaks (marked with arrows in the spectra) are present in positions similar to those in the intact membranes (Fig. 3A) or in the reconstituted material (Fig. 3D), while in other regions (particularly at low-field values) there is little correspondance with the other spectra. The changes suggest that removal of the 33 kDa pro-

tein results in a changed coupling between the Mn atoms giving rise to the multiline signal.

In Fig. 3D is shown the multiline signal obtained with the urea + NaCl-treated membranes reconstituted with the 33 kDa protein. This spectrum is different from the spectrum recorded in the absence of the 33 kDa protein (Fig. 3C). It is similar, but not identical, to that recorded for the NaCl-washed material (Fig. 3B), which has the same composition in terms of the extrinsic proteins. The slight differences between spectra 3D and 3B, if significant, could be due to incomplete restoration of the configuration of the Mn cluster, even though oxygen evolution was restored to 80%.

Summarizing we conclude that the 33 kDa protein can be replaced by Cl^- for both multiline-signal formation and oxygen evolution. However, the absence of this protein modifies the Mn cluster leading to an altered multiline signal from the S_2-state. Cl^- can also replace the 24 and 18 kDa proteins with respect to oxygen evolution [1] and multiline formation (Refs. 17 and 19; see also this work, Fig. 3B) when Ca^{2+} is present. Thus Cl^- is very important for the formation of the multiline signal from the S_2-state, and it seems clear that suboptimal concentrations of Cl^- results in inability to form the multiline signal in intact [21], NaCl-washed (Refs. 18 and 19; this work, Fig. 3B) and urea + NaCl-treated membranes (this work, Figs. 1A and 2). However, in contrast to earlier suggestions [17] it is not necessarily the S_1–S_2 transition that is inhibited at very low concentrations of Cl^-. Instead, it was reported recently that the S_1–S_2-state transition functions at low Cl^- concentrations forming an S_2-state not showing the multiline signal and that Cl^- depletion inhibits the S_2–S_3 transition [21].

Finally, from the observation in this report of the flash-dependent oscillation of the multiline signal (Fig. 1A) and from earlier activity measurements [5,8] it is clear that the water-soluble proteins are not necessary for oxygen evolution. Furthermore, the observation of the multiline signal in the absence of the extrinsic proteins (Fig. 3) suggests that their presence or absence does not cause major changes in the mechanism for oxygen evolution, provided sufficient Cl^- is present in the medium.

After the submission of this manuscript the formation of the multiline signal after illumination at 200 K was reported in $CaCl_2$-washed material lacking the three extrinsic proteins. The formation of the multiline signal in this material was dependent on the concentration of Ca^{2+}, provided sufficient Cl^- (200 mM) was present in the medium (Miller, A.-F., De Paula, J.C. and Brudvig, G.W. (1987) in Proceedings of the VIIth International Congress on Photosynthesis (Biggins, J., ed.), Martinus Nijhoff, Dordrecht, The Netherlands, in the press).

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References

1 Murata, N. and Miyao, M. (1985) Trends Biochem. Sci. 10, 122–124
2 Åkerlund, H.-E. (1983) in The oxygen evolving system of photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 201–208, Academic Press Japan, Tokyo
3 Dismukes, G.C. (1986) Photochem. Photobiol. 43, 99–115
4 Kuwabara, T., Miyao, M., Murata, T. and Murata, N. (1985) Biochim. Biophys. Acta 806, 283–289
5 Ono, T.A. and Inoue, Y. (1984) FEBS Lett. 168, 281–286
6 Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173
7 Miyao, M. and Murata, N. (1985) FEBS Lett. 180, 303–308
8 Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354
9 Renger, G. and Govindjee (1985) Photosynth. Res. 6, 33–55
10 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
11 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278
12 Hansson, Ö. and Andréasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261–268
13 Brudvig, G.W., Casey, J.L. and Sauer, K. (1983) Biochim. Biophys. Acta 723, 361–371
14 Zimmermann, J.L. and Rutherford, A.W. (1984) Biochim. Biophys. Acta 767, 160–167
15 De Paula, J.C. and Brudvig, G.W. (1985) J. Am. Chem. Soc. 107, 2643–2648
16 Hansson, Ö., Andréasson, L.-E. and Vänngård, T. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.),

vol. I, pp. 307–310, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
17 Imaoka, A., Akabori, K., Yanagi, M., Izumi, K., Toyoshima, T., Kawamori, A., Nakayama, H. and Sato, J. (1986) Biochim. Biophys. Acta 848, 201–211
18 Franzén, L.-G., Hansson, Ö. and Andréasson, L.-E. (1985) Biochim. Biophys. Acta 808, 171–179
19 Toyoshima, Y., Akabori, K., Imaoka, A., Nakayama, H., Ohkouchi, N. and Kawamori, A. (1984) FEBS Lett. 176, 346–350
20 Blough, N.V. and Sauer, K. (1984) Biochim. Biophys. Acta 767, 377–381
21 Ono, T., Zimmermann, J.L., Inoue, Y. and Rutherford, A.W. (1986) Biochim. Biophys. Acta 851, 193–201
22 Zimmermann, J.L. and Rutherford, A.W. (1985) Physiol. Vég. 23, 425–434
23 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539
24 Kuwabara, T., Murata, T., Miyao, M. and Murata, N. (1986) Biochim. Biophys. Acta, in the press
25 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
26 O'Malley, P.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 765, 370–379
27 Velthuys, B.R. and Visser, J.W.M. (1975) FEBS Lett. 55, 109–112
28 Miyao, M., Murata, N., Lavorel, J., Maison-Peteri, B., Boussac, A. and Etienne, A.-L. (1987) Biochim. Biophys. Acta 890, issue 2, in the press