Vol. 124, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1984 Pages 269-276

EPR STUDIES ON THE PHOTOSYSTEM II DONOR SIDE IN SALT-WASHED AND RECONSTITUTED INSIDE-OUT THYLAKOIDS

Christer Jansson[1,3], Örjan Hansson[2],
Hans-Erik Åkerlund[1] and Lars-Erik Andreasson[2]

[1]Department of Biochemistry,
University of Lund,
P.O.Box 124, S-22101 Lund, Sweden

[2]Department of Biochemistry and Biophysics,
Chalmers Institute of Technology and University of Göteborg,
S-41296 Göteborg, Sweden

Received August 29, 1984

EPR measurements on inside-out thylakoids revealed that salt-washing, known to inhibit oxygen evolution and release a 23 and a 16 kDa protein, induced a Signal II_f and decreased the EPR signal from state S_2. Readdition of the released 23 kDa protein restored the oxygen evolution and decreased the Signal II_f, but did not relieve the decrease in the state S_2 signal. It is suggested that salt-washing inhibits the electron transfer from the oxygen-evolving site to Z, the physiological donor to P680. In inhibited photosystem II units lacking Signal II_f, Z^+ is rapidly reduced, possibly by a modified S-cycle unable to evolve oxygen.

The 23 and 16 kDa proteins can be specifically removed from inside-out thylakoid vesicles or PS II detergent preparations by salt-washing (1-4) or from intact thylakoids by deoxycholate extraction (5), with a concomitant inhibition of oxygen evolution. Readdition of the released 23 kDa protein to salt-washed membranes can restore the oxygen evolution (2-4). Fluorescence analyses (2) and absorbance measurements on $P680^+$ reduction (6) showed that the functional site for the 23 kDa protein is along the electron pathway between water and P680. In the present work

[3]Present address and to whom correspondence should be sent: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824, USA
Abbreviations: chl, chlorophyll; PpBQ, phenyl-p-benzoquinone; PS II, photosystem II

0006-291X/84 $1.50

EPR measurements on salt-washed and reconstituted inside-out thylakoids were performed to investigate the role of the 23 kDa protein on the donor side of PS II.

The suggested immidiate donor to $P680^+$, Z, is EPR detectable in its oxidized state. The reduction of Z^+ is highly dependent on the integrity of the oxygen-evolving complex. In unaffected thylakoids the reduction is in the submillisecond region, typical of Signal II_{vf} (7). In PS II units with the oxygen evolution inhibited by e.g. Tris treatment, Z^+ is reduced slowly, with half-times between 100 ms and 1 s, characteristic for Signal II_f (8,9).

The results here show that salt-washing of inside-out thylakoids increased Signal II_f. The increase was reversed upon readdition of the purified 23 kDa protein. Furthermore, the multiline EPR signal, assumed to arise from the oxidation state S_2 in the oxygen-evolving complex (10-12), was irreversibly suppressed after salt-washing.

MATERIALS AND METHODS

Inside-out thylakoid vesicles were prepared and then salt-washed by incubation at pH 7.4 in 250 mM NaCl as described earlier (2). Untreated (control) and salt-washed inside-out vesicles (3.8-7.6 mg chl/ml) in 5 mM sodium phosphate pH 7.4, 3 mM NaCl, 500 mM sucrose and 5% dimethylsulfoxide were stored in small aliquots in liquid nitrogen until use. Reconstitution of oxygen evolution was achieved by addition of purified 23 kDa protein (13) to thawed, salt-washed vesicles. Samples for low-temperature EPR were prepared as in (11). Room-temperature EPR experiments were performed in 5 mM sodium phosphate pH 7.4, 3 mM NaCl, 25 mM $MgCl_2$, 0.1 mM EDTA (14), 500 mM sucrose, 5 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$ and 0.3 mM PpBQ as redox mediator. After the measurements, the samples were withdrawn from the cell, subjected to Tris incubation (0.8 M Tris pH 8.0 for 10 min at 0 °C and room light) and then reinjected into the cell for a repetition of the measurements.

EPR measurements were made with the equipment described in (11). Room-temperature experiments were made with a Varian cylindrical cavity working in the TM 110 mode. The cavity was flushed with a precooled nitrogen gas in order to maintain the temperature in the flat sample cell at 20.0 °C. Flash-induced Signal II kinetics was monitored at the position of the Signal II low field peak (8). Saturating white flashes, halfwidth 3 μs and spaced 4 s apart, were furnished from a

pulsed xenon light source. The flash-induced transient EPR signals were computer-averaged and then fitted to a sum of one rising and one decaying exponential component. Spin concentrations were estimated by comparisons with the signal obtained from 10 mM $VOSO_4$ in the sample cell, using standard methods (15). Signal II_{vf} was not observable in this work (instrument response time 30 ms).

Oxygen evolution was followed polarographically in the same medium as for the room-temperature EPR measurements except that 0.2 mM PpBQ and thylakoid material corresponding to 20 ug chl/ml was used.

RESULTS AND DISCUSSION

Dark-adapted oxygen-evolving inside-out thylakoid vesicles showed a stable EPR radical signal (Fig.1, inset) with properties typical of Signal II_u (8) and amounting to about one spin per 250 chl. This agrees with the estimated unit size (270-340 chl/PS II) in these vesicles (16,17). Upon illumination with brief, saturating light flashes there was a small transient increase (about 0.1 spins per PS II unit) of the Signal II amplitude (Fig. 1a), measured at $\underline{g}$=2.011 (Fig. 1, inset), where interference from the EPR signal from $P700^+$ (Signal I) is negligible. The half-time of the Signal II decay (about 300 ms) identified the transient as Signal II_f (8), which probably represented a fraction of PS II units inactivated during preparation. In addition, an even smaller light-induced transient was seen, probably Signal II_s (8), with a half-life of several minutes. Tris treatment resulted in a tenfold increase in Signal II_f with a decay half-time of about 1 s (Fig. 1b, Table 1).

Salt-washing of the inside-out thylakoids, which releases the 23 kDa protein (1,2), inhibited the oxygen evolution by 60% and doubled the Signal II_f amplitude and also increased the half-time of the decay to about 700 ms (Fig. 1c, Table 1). The other Signal II species were only slightly affected. These results demonstrate that removal of the 23 kDa protein inhibited the electron transfer from the oxygen evolving complex to Z. Also in

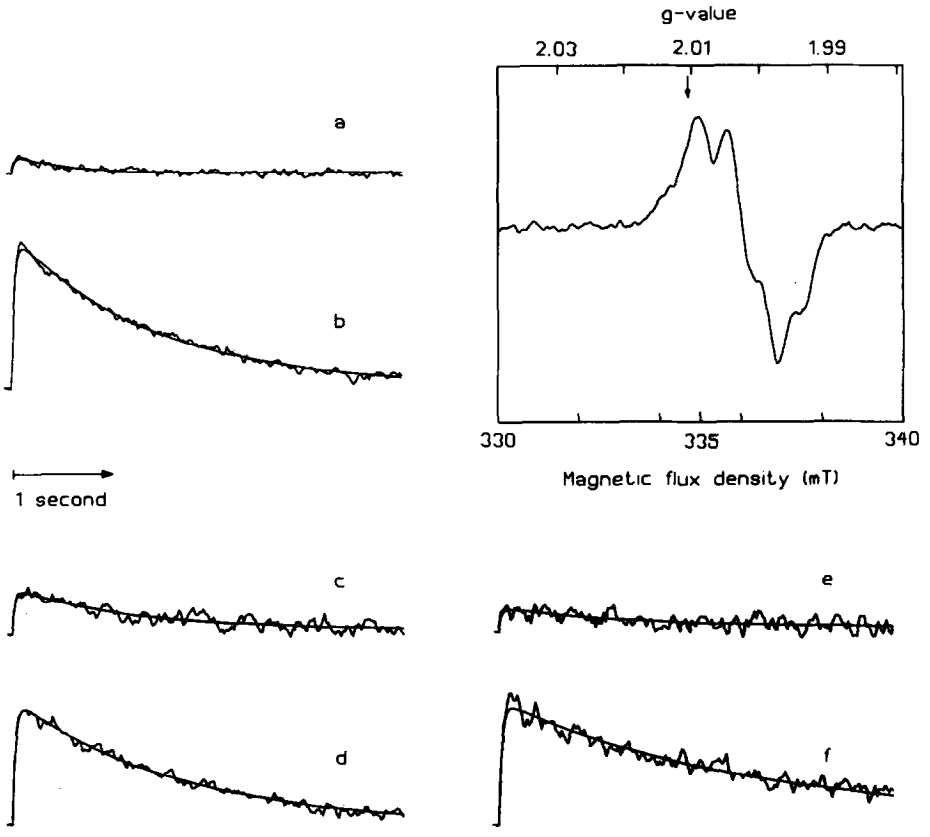

Fig. 1. Flash-induced EPR signals in inside-out thylakoids at room temperature. Trace a, flash-induced transients obtained from control inside-out thylakoids (3.6 mg chl/ml); b, sample a after Tris treatment (2.7 mg chl/ml); c, salt-washed inside-out thylakoids (3.7 mg chl/ml); d, sample c after Tris treatment (2.8 mg chl/ml); e, salt-washed thylakoids (3.2 mg chl/ml) reconstituted with the 23 kDa protein (0.9 mg protein/mg chl); f, sample e after Tris treatment (2.5 mg chl/ml). Traces are averages of 25-175 flash-induced transients. EPR conditions: microwave frequency, 9418 MHz; power, 2 mW; modulation amplitude, 0.33 mT; spectrometer time constant, 30 ms; temperature, 20.0 ± 0.5 °C.
Inset, room temperature EPR spectrum of dark-adapted control. EPR conditions as for a-f but with a magnetic field scan of 0.17 mT/s and a spectrometer time constant of 0.3 s. Gain, 0.4 relative to a-f. The arrow shows the magnetic field position (334.7 mT) at which the flash-induced Signal II was monitored.

PS II detergent preparations an increase in Signal II_f was observed after salt-washing (18). Restoration of the oxygen evolution by readdition of the 23 kDa protein to the depleted membranes almost fully reversed the increase in Signal II_f amplitude whereas the decay time was unaffected (Fig. 1e, Table 1). It should be mentioned, however, that the decay of these transients

TABLE 1

Effects on Signal II_f by salt-washing and reconstitution of inside-out thylakoid vesicles

	O_2-activity (%)	Signal II_f (spins/250 chl)	$t_{1/2}$ (ms)
Control	100	0.10	332 ± 27
Control + Tris	0	1.15	866 ± 12
Salt-washed	39	0.23	701 ± 63
Salt-washed + Tris	0	0.92	1014 ± 25
Reconstituted	97	0.13	775 ± 168
Reconstituted + Tris	0	0.98	1522 ± 56

Oxygen evolving activity was measured in the room-temperature EPR medium with PpBQ as PS II electron acceptor. The control activity was 40 μmoles O_2 per mg chl and h. [In normal assay-medium (2) the control activity was 110 μmoles O_2 per mg chl and h]. The Signal II_f measurements are described in the legend to Fig. 1. After salt-washing, 40% of the 23 kDa protein was retained in the membranes as determined by rocket immuno-electrophoresis.

can be fitted equally well to a linear combination of two components with half-times of 300 ms and 1 s.

Considering the inhibition in oxygen evolution, salt-washing would be expected to give rise to Signal II_f amounting to 60% of the PSII units. The low amount actually found (10%) suggests that Z^+ was still rapidly reduced (faster than 30 ms, the response time of the instrument) in most of the inhibited PS II units. The component responsible for this reduction of Z^+ might be a modified, partially functional S-cycle, uncapable of oxygen evolution, or cytochrome $\underline{b}_{559}$. In both cases a cyclic or exogenous re-reduction of this component is required. It has been shown that cytochrome $\underline{b}_{559}$ can be rapidly oxidized by PS II (19). However, at the redox potential (about 460 mV) in the present experiments, cytochrome $\underline{b}_{559}$ should initially be oxidized and has to be reduced before it can deliver electrons to Z^+, thus making this alternative less atractive. An alternative

explanation for the small Signal II_f amplitude in the salt-washed vesicles would be a complete block in the electron transfer between Z and P680, preventing the formation of Z^+. This is less probable, however, since Tris treatment of the salt-washed as well as the control vesicles induced a large Signal II_f (Table 1). Also other results indicate that this segment of the electron pathway remains operative after salt-washing (20).

The control inside-out thylakoids developed a multiline EPR signal when frozen in strong light (Fig. 2a). The positions of the lines and their relative amplitudes were similar to those found in chloroplasts (11) and in oxygen evolving PS II detergent preparations (Hansson, Andreasson, unpublished observations). It

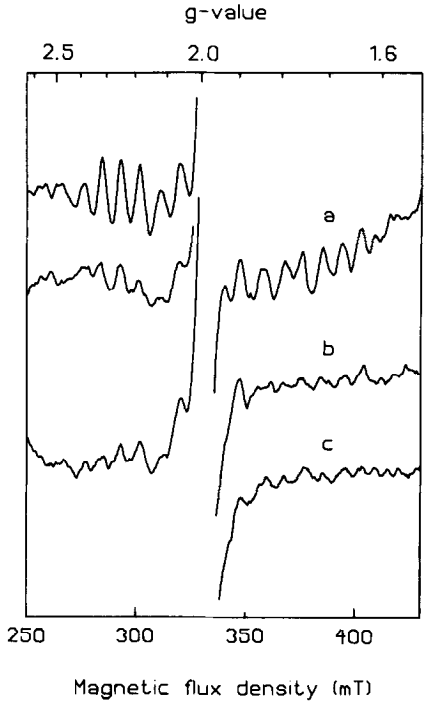

Fig. 2. Low-temperature EPR spectra of illuminated inside-out thylakoids. The samples were frozen in the presence of 4 mM PpBQ and 4 mM EDTA, either in the dark or during continuous illumination. Spectrum a, control inside out thylakoids (6.9 mg chl/ml); b, salt-washed inside-out thylakoids (4.9 mg chl/ml); c, salt-washed inside-out thylakoids (4.9 mg chl/ml) after addition of the 23 kDa protein (0.9 mg protein/mg chl). The spectra presented are differences between illuminated and dark-adapted samples. EPR conditions: microwave frequency, 9227 MHz; power, 126 mW; modulation amplitude 1.25 mT; temperature, 15 K.

has been suggested (10-12) that this multiline EPR signal arises from interacting manganese ions of the S_2 state. The amplitude of the multiline signal decreased considerably after salt treatment (to about 40% of control amplitude). This effect was not reversed after reconstitution of the oxygen evolution by readdition of the 23 kDa protein (Figs 2b and c, respectively). The reason for this is as yet not clear. However, when the oxygen evolving activity was assayed in the medium used for these low-temperature EPR measurements the reconstitution was only 50%. Thus, the expected increase in the multiline signal could be difficult to detect.

In short, our results indicate that removal of the 23 kDa protein may lead to deficiencies in the oxygen-evolving system such as to prevent specific S-state transitions, rather than shutting off the charge-accumulating process completely. However, most likely, the S_2-S_3 transition alone is not inhibited as this should lead to an accumulation of the S_2 state and an increase in the multiline EPR signal, contrary to our observations.

ACKNOWLEDGEMENTS

We thank professor Tore Vänngård for valuable discussions and helpful critizism and Mrs Ingun Sunden-Cullberg and Mrs Agneta Persson for skilful technical assistance. This work was supported by the Swedish Natural Science Research Council.

REFERENCES

1. Åkerlund, H.-E. (1981) in: Photosynthesis (Akoyunoglou, G. ed.) vol. II, pp 465-472, Balaban International Services, Philadelphia, Pa
2. Åkerlund, H.-E., Jansson, C. and Andersson,B. (1982) Biochim. Biophys. Acta 681, 1-10
3. Ljungberg, U., Jansson, C., Andersson, B. and Åkerlund, H.-E. (1983) Biochem. Biophys. Res. Commun. 113, 738-744
4. Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87-93

5. Franzén, L.-G. and Andréasson, L.-E. (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol. I, pp. 379-382, Martinus Nijhoff, The Hague
6. Åkerlund, H.-E. Brettel, K. and Witt, H.T. (1984) Biochim. Biophys. Acta 765, 7-11
7. Blankenship, R.E., Babcock, G.T., Warden, J.T. and Sauer, K. (1975) FEBS Lett. 51, 287-293
8. Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 329-344
9. Boska, M., Sauer, K., Buttner, W. and Babcock, G.T. (1983) Biochim. Biophys. Acta 722, 327-330
10. Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274-278
11. Hansson, Ö. and Andréasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261-268
12. Andréasson, L.-E., Hansson, Ö. and Vänngård, T. (1983) Chemica Scripta 21, 71-74
13. Jansson, C. in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol. I, pp 375-378, Martinus Nijhoff, The Hague
14. Babcock, G.T., Ghanotakis, D.F. Ke, B. and Diner, B.A. (1983) Biochim. Biophys. Acta 723, 276-286
15. Aasa, R. and Vänngård, T. (1975) J. Magn. Reson. 19, 308-315
16. Andersson, B. and Haehnel, W. (1982) FEBS Lett. 146, 13-17
17. Anderson, J.M. and Melis, A. (1983) Proc. Natl. Acad. Sci. USA 80, 745-749
18. Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127-130
19. Velthuys, B.R. (1981) FEBS Lett. 126, 272-276
20. Renger, G. and Voelker, M. (1982) FEBS Lett. 149, 203-207