Biochimica et Biophysica Acta 848 (1986) 359–363
Elsevier

BBA 41964

H_2O_2 accessibility to the Photosystem II donor side in protein-depleted inside-out thylakoids measured as flash-induced oxygen production

Wolfgang P. Schröder and Hans-Erik Åkerlund

Department of Biochemistry, University of Lund, Box 124, 221 00 Lund (Sweden)

(Received October 31st, 1985)

Key words: Photosystem II; Oxygen evolution; H_2O_2; Electron transport; (Spinach chloroplast)

The oxygen yield pattern from Photosystem II-enriched inside-out vesicles depleted of the 16 and 23 kDa polypeptides was studied. Two changes were observed. Firstly, there was as expected a decrease in the average amplitude due to the overall inhibition of oxygen-evolving capacity. Secondly, a signal was observed already at the first flash. This latter change in oscillation pattern was found to be caused by H_2O_2 and weakly bound manganese present in the material. Thus, catalase, EDTA and high salt concentrations inhibited the signal on the first flash, while addition of H_2O_2 or $MnCl_2$ increased the signal. The interpretation of these results is that removal of the 16 and 23 kDa proteins modifies the structure of the oxygen-evolving complex in such a way that it exposes the water-splitting site and makes it possible for H_2O_2 to act as an electron donor to Photosystem II even at low concentrations.

Introduction

The photosynthetic oxygen-evolving process is still not resolved with respect to its mechanism and the identity and number of participating proteins. A widely accepted model that constitutes the framework for our current picture of the photosynthetic oxygen evolution is that proposed by Kok et al. [1]. According to this model all water-splitting units work independently from each other and each is able to accumulate successively four positive charges before a molecule of oxygen is produced. The model is based on the oxygen yield pattern obtained from dark-adapted algae or chloroplasts upon illumination by a train of short saturating flashes of light. Important information about the stability of accumulated charges and kinetics of individual steps can be obtained from the oxygen-yield pattern simply by varying the dark-adaptation time and the time between flashes. For an early review, see Joliot and Kok [2].

In recent years much attention has been paid to the protein components of the oxygen-evolving system. The progress in this field was greatly facilitated by the isolation of inside-out thylakoids [3] and Photosystem II particles prepared by the use of detergents [4]. The first protein shown to be associated with the oxygen-evolving process, by direct reconstitution experiments, had a molecular weight of 23 kDa [5]. Later, direct-reconstitution experiments have established a role also for a 33 kDa protein [6,7] and a 16 kDa protein [7]. The function of these proteins is still not completely understood but they appear to facilitate the utilization of chloride and calcium ions by Photosystem II [7–10]. Thus after removal of the 23 and 16 kDa protein by washing the membranes with high concentrations of NaCl or removal of the 33, 23 and 16 kDa proteins by $CaCl_2$ washing, the oxygen-evolving activity was dependent either on

Abbreviations: Mes, 4-morpholineethanesulphonic acid; a.u., arbitrary unit; ABTS, 2,2′-azinodi(3-ethylbenzthiazoline-6-sulphonic acid).

the readdition of these proteins, or alternatively on the addition of extra chloride or calcium.

An interesting question is how the removal of one or a number of these proteins influence the electron-transfer steps and the accumulation of charges on the oxidizing side of Photosystem II. In a preliminary study on inside-out thylakoids deprived of the 23 and 16 kDa proteins, it was found that the oxygen yield pattern was indeed changed [11]. The treatment not only caused a decrease in average amplitude, but also the appearance of oxygen already at the first flash. A similar pattern has been reported for untreated Photosystem II particles [12], but could not be repeated in a later work [13].

The present study was carried out to elucidate the mechanism behind the changed oscillation pattern. The results suggest that the presence of small amounts of hydrogen peroxide, produced by the thylakoid material upon illumination, can act as an electron donor on the oxidizing side of Photosystem II and thereby produce oxygen.

Materials and Methods

Inside-out thylakoids were obtained by mechanical disintegration of stacked spinach thylakoids followed by phase partition as previously described [14]. For the salt washing, the isolated inside-out thylakoids were incubated in 250 mM NaCl, 10 mM sodium phosphate (pH 7.4) (20 μg chlorophyll/ml) on ice in room light for 30 min. After centrifugation (65 000 $\times$ g, 30 min) the membranes were suspended in 500 mM sucrose/5 mM sodium phosphate (pH 7.4)/2.5 mM NaCl, and either used directly or stored in liquid nitrogen after addition of 5% dimethylsulfoxide until use.

Oxygen yields from sequences of 1.5 μs xenon light flashes, spaced 0.7 s, utilizing a PRA Model 610C (London, Ontario) as source, were measured at room temperature (20–23°C). A bare platinum electrode polarized 0.6 V vs. the Ag|AgCl reference electrode, was essentially as described by Joliot and Joliot [15]. The flow medium used was 30 mM Mes (pH 6.5) and either $CaCl_2$ or KCl with a concentration as indicated. The samples (1 mg chlorophyll/ml) were kept in the dark on ice and applied to the electrode in weak green light, and further dark adapted for 5 min on the electrode. The flash-induced oxygen yields (Y_n) were measured and normalized to the average of the yield obtained on flash 3, 4, 5 and 6, here defined as the steady-state value, Y_{ss}.

The hydrogen peroxide concentration in thylakoid samples was determined by use of the lactoperoxidase-catalysed reaction with 2,2'-azinodi(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) [16]. After removal of thylakoid membranes by centrifugation, 250 μl of sample were added to a system containing 500 μl ABTS (2 mg/ml) and 250 μl lactoperoxidase (3.2 units/ml) and the absorbance was measured at 420 nm, and compared to a standard curve.

Results

The oxygen yield pattern obtained from dark-adapted Photosystem II-enriched inside-out vesicles, when subjected to a train of flashes, is shown in Fig. 1. The pattern is similar to that obtained from intact thylakoids. Thus, no oxygen was produced on the first flash, a maximum amount at the third, followed by a dampened oscillation with a period of four. Changing the KCl concentration in the range from 100 mM down to 2 mM did not influence this pattern appreciably.

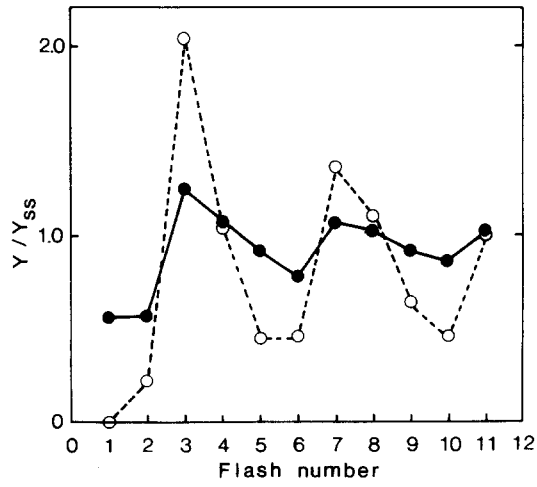

Fig. 1. Oxygen-yield pattern of untreated inside-out thylakoids (○, Y_{ss} = 100 a.u.), and salt-washed inside-out thylakoids (●, Y_{ss} = 65 a.u.). The flow medium contained 2 mM KCl and 30 mM Mes, pH 6.5. The time between flashes was 0.7 s.

TABLE I

OXYGEN YIELD ON THE FIRST FLASH AS A FUNCTION OF MEDIA COMPOSITION FOR INSIDE-OUT THYLAKOIDS

Values are in percentage of the steady-state value. All additions were made to the flow medium, except for catalase, which was added directly to the thylakoid samples.

Additions	Y_1/Y_{ss} (%) Inside-out thylakoids	
	Untreated	Salt-washed
2 mM KCl	0	57
10 mM $CaCl_2$	0	12
2 mM KCl + catalase	0	0
2 mM KCl + 1 mM H_2O_2	47	117
2 mM KCl + 0.25 mM H_2O_2	21	119
2 mM KCl + 0.05 mM H_2O_2	0	113
2 mM KCl + 1 mM EDTA	0	0
2 mM KCl + 100 μM $MnCl_2$	0	88

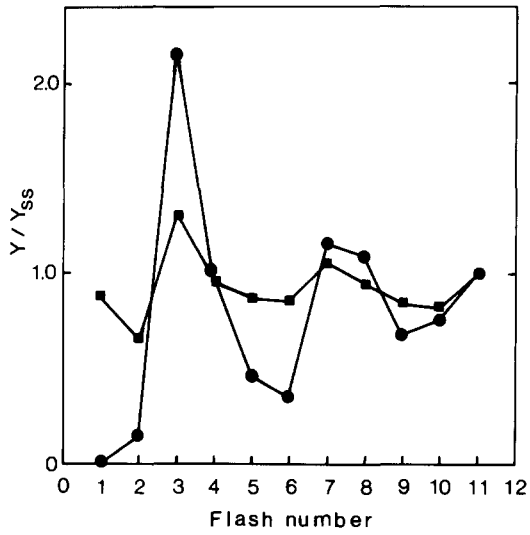

Fig. 2. Oxygen-yield pattern of salt-washed inside-out thylakoids, with catalase (●, Y_{ss} = 25 a.u.), and with 100 μM $MnCl_2$ (■, Y_{ss} = 50 a.u.).

Upon salt washing of the inside-out thylakoids, two changes in the oxygen-yield pattern were observed at low salt concentrations (Fig. 1). First, there was a 35% decrease in the average amplitude, and secondly a signal was observed already at the first flash. Moreover, the oxygen yield at the first flash was dependent on the salt concentration and was more pronounced at lower concentrations (Table I).

In a preliminary study [11] it was suggested that the signal at the first flash originates from oxygen produced when hydrogen peroxide is oxidized by Photosystem II. The hydrogen peroxide in turn was thought to be produced by the electrode. To test whether hydrogen peroxide could be involved, catalase was added to the samples in a ratio of 2 : 1 (mg chlorophyll/mg catalase). Indeed, for the salt-washed inside-out thylakoids the signal at the first flash disappeared completely and the oscillation became more pronounced (Fig. 2, filled circles).

When small amounts (less than 0.05 mM) of hydrogen peroxide were added to the flow medium, the oxygen yield for salt-washed inside-out thylakoids at the first flash increased, while the untreated inside-out thylakoids were unaffected. At higher concentrations of H_2O_2 it was possible to induce a yield at the first flash also in untreated inside-out thylakoids (Table I and Fig. 3). Furthermore, an oscillation with a period of two rather than four was observed. At this high concentration of H_2O_2 the salt-washed inside-out thylakoids showed a maximum at the first flash and the oscillation was no longer seen (Fig. 3). These results show that the oxygen yield pattern from salt-washed inside-out thylakoids can be strongly influenced by the presence of hydrogen peroxide.

In order to determine if the electrode could produce hydrogen peroxide, pure buffer were applied to the electrode, withdrawn at different time intervals and analysed for hydrogen peroxide content. In contrast to an earlier suggestion [11] the electrode did not produce any detectable amount of hydrogen peroxide. On the contrary, the electrode consumed hydrogen peroxide when added.

Thus the origin of the hydrogen peroxide must be the thylakoid material. It was found that both untreated and salt-washed inside-out thylakoid fractions contained small amounts of hydrogen peroxide (Fig. 4, time zero). Illumination of the thylakoid material caused a linear increase in the hydrogen peroxide concentration. This light-induced hydrogen peroxide production by the thylakoid material is in accordance with earlier observations (e.g., Ref. 17). Therefore it is likely that the hydrogen peroxide present in the thylakoid

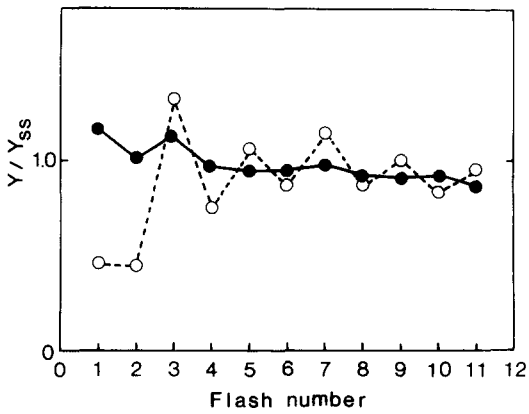

Fig. 3. Oxygen-yield pattern of untreated (○, $Y_{ss} = 60$ a.u.) and salt washed (●, $Y_{ss} = 100$ a.u.) inside-out thylakoids in the presence of 1 mM H_2O_2.

samples has been produced by the light absorbed during the isolation and pretreatment of the sample. The difference in hydrogen peroxide production seen between untreated and salt-washed material has been reproducible. However, the origin of this difference is not obvious, but an interesting possibility that is under current invstigation is that hydrogen peroxide, as an intermediate in the oxygen-evolving process, leaks out more easy from the salt-treated thylakoids which lack the 23 and 16 kDa proteins.

It is known that hydrogen peroxide can donate electrons to Photosystem II. However, as shown by Velthuys [18], hydrogen peroxide cannot donate directly to Photosystem II, but requires the presence of Mn^{2+}. To establish the role of free Mn^{2+} in our system either 1 mM EDTA or 100 μM $MnCl_2$ was added to the flow medium. EDTA addition caused a total disappearance of the signal on the first flash (Table I) and a pronounced oscillation with a period of four was observed. Addition of $MnCl_2$ to salt-washed thylakoids normally caused only a minor increase in the oxygen yield on the first flash (Fig. 2). However, in some samples of salt-washed inside-out thylakoids, where the yield obtained at the first flash was low, it was increased to essentially the same final level by addition of $MnCl_2$. Thus, free Mn appears to play an essential role for the generation of the signal observed on the first flash.

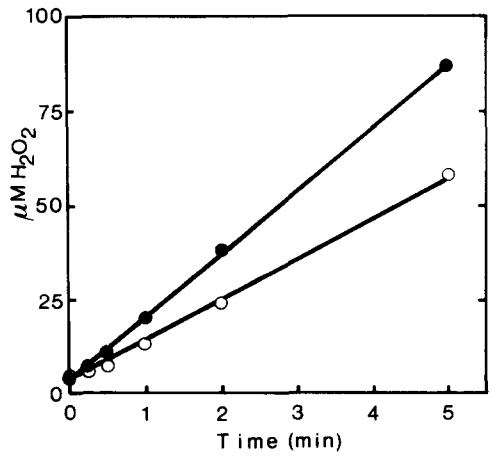

Fig. 4. H_2O_2 production as a function of illumination time for untreated inside-out thylakoids (○), and salt-washed inside-out thylakoids (●).

Discussion

The results show that the altered oxygen yield pattern observed for slat-washed inside-out thylakoids is due to the presence of hydrogen peroxide and free manganese ions. The hydrogen peroxide was found to originate from the thylakoid material, rather than from the electrode as was proposed in the preliminary study [11].

It is interesting to note that the untreated inside-out thylakoids did not show this oxygen yield pattern, despite their production of hydrogen peroxide. The difference seen between the untreated and salt-washed material should therefore be related to either a difference in the free Mn content or due to an increased accessibility for the hydrogen peroxide donation site in the material depleted in the 16 and 23 kDa proteins. Since addition of Mn to the untreated inside-out thylakoids did not induce a signal on the first flash (Table I), the results suggest that the 23 and 16 kDa proteins have a shielding effect.

This interpretation is supported by the results of Ghanotakis et al. [19], using Photosystem II particles. They found that after removal of the 23 and 16 kDa proteins, reducing agents like NH_2OH and H_2O_2 more easily caused a destruction of the Mn complex measured as a release of Mn and inhibition of oxygen-evolving capacity. From studies on Zn-induced Mn release, Miller [20] also suggested that the 16 and 23 kDa proteins shield the Mn site.

The anomalous oxygen yield pattern reported by Lavorel [12] for Photosystem II particles was explained in terms of an increased stability of state S_3. An alternative explanation is that the anomalous oxygen production was due to H_2O_2 acting as electron donor.

It has been shown by several workers that high Cl concentration attenuates the effect of removal of the 16 and 23 kDa proteins. At higher salt-concentration the oxygen yield on the first flash also decreases (Table I). It is not clear whether this means that Ca/Cl also has shielding effects. Another possibility is that weakly bound manganese is more efficiently removed by the flow medium at higher ionic strength. The salt dependence may explain why such a signal was not seen for salt-washed Photosystem II particles in the recent study by Boussac et al. [21].

The oscillation with a period of two seen in Fig. 3 for untreated inside-out thylakoids in the presence of high concentrations of hydrogen peroxide is similar to that observed by Velthuys [22], and was explained by a direct two-electron donation by hydrogen peroxide. This may also be the case in the experiments presented here where high concentrations of hydrogen peroxide was used, as addition of EDTA in this case did not completely restore the period of four oscillation (not shown).

To obtain a reliable oscillation pattern for protein-depleted samples at low ionic strength without contribution from hydrogen peroxide, catalase should be added.

In conclusion, removal of 16 and 23 kDa proteins modifies the structure of the oxygen-evolving complex in such a way that the reaction site becomes exposed and accessible to H_2O_2.

Acknowledgements

We thank Dr. B. Maison-Peteri for providing information about equipment for oxygen-yield measurements, and Mr. G. Carlsson for building the device. This work was supported by the Swedish Natural Science Research Council and the Magnus Bergvall Foundation.

References

1 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
2 Joliot, P. and Kok, B. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 388–412, Academic Press, New York
3 Andersson, B. and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 503, 462–472
4 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
5 Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10
6 Ono, T.-A. and Inoue, Y. (1983) FEBS Lett. 164, 255–260
7 Imaoka, A., Yanagi, M., Akabori, K. and Toyoshima, Y. (1984) FEBS Lett. 176, 341–345
8 Andersson, B., Critchley, C., Ryrie, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. 168, 113–117
9 Akabori, K., Imaoka, A. and Toyoshima, Y. (1984) FEBS Lett. 173, 36–40
10 Ghanotakis, D.F., Topper, J.N., Babcock, G.T., Yocum, C.F. (1984) FEBS Lett. 170, 169–173
11 Åkerlund, H.-E. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 391–394, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
12 Lavorel, J. and Seibert, M. (1982) FEBS Lett. 144, 101–103
13 Seibert, M. and Lavorel, J. (1983) Biochim. Biophys. Acta 723, 160–168
14 Åkerlund, H.-E. and Andersson, B. (1983) Biochim. Biophys. Acta 725, 34–40
15 Joliot, P. and Joliot, A. (1968) Biochim. Biophys. Acta 153, 625–634
16 Olsson, B. (1986) Microchem. Acta, in the press
17 Sayre, R.T., and Homann, P.H. (1979) Arch. Biochem. Biophys. 196, 525–533
18 Velthuys, B. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 83–90, Academic Press, Japan, Tokyo
19 Ghanotakis, D.F., Topper, J.N. and Yocum, C.F. (1984) Biochim. Biophys. Acta 767, 524–531
20 Miller, M. (1985) FEBS Lett. 189, 355–360
21 Boussac, A./, Maison-Peteri, B., Vernotte, C. and Etienne, A.-L. (1985) Biochim. Biophys. Acta 808, 225–230
22 Velthuys, B. and Kok, B. (1978) Biochim. Biophys. Acta 502, 211–221