Biochimica et Biophysica Acta, 765 (1984) 7–11
Elsevier

BBA 41475

REVERSIBLE ALTERATION OF NANOSECOND REDUCTION OF CHLOROPHYLL a_{II}^{+} IN INSIDE-OUT THYLAKOIDS CORRELATED TO INHIBITION AND RECONSTITUTION OF OXYGEN-EVOLVING ACTIVITY

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(Received August 12th, 1983)

Key words: Photosystem II; Chlorophyll a; Oxygen evolution; Electron transfer; Thylakoid membrane protein; (Spinach chloroplast)

The electron donation to Chl a_{II}^{+} has been studied by measurement of absorbance changes at 824 nm under repetitive excitation conditions. For untreated inside-out thylakoids the electron donation was dominated by 35 and 220 ns kinetics. After salt-washing, both oxygen-evolution and nanosecond phases decreased drastically with corresponding increase in the microsecond time range. On addition of a purified 23 kDa protein, a restoration of the nanosecond phases up to 75% of the orginal level was obtained concomitant with a corresponding restoration of oxygen evolution. The results are consistent with a function of the 23 kDa protein at the oxidizing side of Photosystem II and that the nanosecond donation to Chl-a_{II}^{+} is coupled to the natural path of electrons from water.

Introduction

The primary reactions at the Photosystem II reaction centre lead to light-induced oxidation of a primary donor, the chlorophyll a_{II} protein (Chl-a_{II}), also named P-680 [1,2] and to reduction of the first stable acceptor, a plastoquinone named PQ_A or X-320 [3,4]. A transient intermediate between Chl-a_{II} and PQ_A is presumably a pheophytin [5]. In subsequent reactions Chl a_{II}^{+} is reduced by the secondary electron donor, D_1, which is then reduced by electrons from the oxygen-evolving complex. The protein components involved in these reactions and the mechanism of oxygen evolution are still far from resolved. One of the problems has been that the components needed for water oxidation appear to be located at the lumenal side of the membrane [6,7]. The isolation of inside-out thylakoids with retained PS II activity [8,9] have, however, opened up new possibilities for more direct studies of the water oxidation system. Thus, by washing inside-out thylakoids with 250 mM NaCl it was possible to release a few polypeptides concomitant with an inhibition of water oxidation [10,11]. Similar results have been obtained on PS II particles isolated by detergent methods [12,14]. One of these polypeptides was a 23 kDa polypeptide. This polypeptide has been purified and shown to be able to rebind to the inner membrane surface under low-ionic strength concomitant with a reconstitution of oxygen evolution [11,15,16]. The 23 kDa protein could also rebind to salt-washed PS II detergent particles [13,16,17]. The isolated protein did not show any prosthetic group, and the function of the protein remains unresolved. The functional site for this protein was inferred to be on the water-splitting side of PS II, based on the effect on variable fluorescence [11,13].

Abbreviations: Chl, chlorophyll; PQ, plastoquinone; PS II, Photosystem II; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

A more direct way to locate the functional site of the protein would be to measure the primary reactions of PS II.

The primary donor of PS II was detected spectrophotometrically by Döring et al. [1] and showed characteristic maxima in flash-induced absorbance changes at 680 and 430 nm [2]. Measurements at 680 nm are difficult due to the high background absorption of chlorophyll and the high level of fluorescence. This is not the case in the 820 nm absorption band of oxidized Chl-a_{II} [18,19]. The redox reaction of Chl-a_{II} was investigated under repetitive-excitation conditions as well as under single-flash conditions. At 820 nm it was found that after a single flash given to dark-adapted chloroplasts, Chl-a_{II}^+ is reduced for the most part with a half-life time of 25–45 ns [18]. Under repetitive illumination, however, a multiphasic reduction was observed at 680 nm as well as 820 nm which could be described by half-life times of 30 ns (50%), 250 ns (20%) and further phases in the microsecond range (30%) [19,20]. After inhibition of the oxygen-evolving system, the fast electron transfer to Chl a_{II}^+ disappeared and Chl a_{II}^+ was reduced exclusively in the microsecond range [18–20].

In the present work we have used nanosecond time-resolved absorption spectroscopy to study the PS II reaction centre in inside-out thylakoids after inhibition of oxygen evolution by salt-washing and subsequent restoration by addition of the 23 kDa protein. The results are consistent with a localization of the functional site for the 23 kDa protein at the oxidizing side of PS II and that the nanosecond electron donation to Chl a_{II}^+ is coupled to the natural path of electrons from water.

Materials and Methods

Inside-out thylakoids were obtained by mechanical disintegration of stacked thylakoids followed by aqueous two-phase fractionation as described earlier [9,11]. For the salt-washing, the isolated inside-out thylakoids (20 μg Chl/ml) were incubated in 250 mM NaCl/10 mM sodium phosphate buffer (pH 7.4) on ice for 30 min. After centrifugation (100000 × *g*; 30 min) the membranes were suspended in 500 mM sucrose/5 mM sodium phosphate (pH 7.4)/2.5 mM NaCl/5% dimethylsulphoxide and stored in liquid nitrogen until use.

Flash-induced absorbance changes were measured using equipment recently described [19]. Excitation light of 532 nm was provided by a frequency-doubled Nd/YAG laser with a half-width pulse duration of 3 ns. The measuring light source was a laser diode (CQL12 from AEG), emitting about 8 mW at 824 nm, halfwidth 5 nm. This wavelength lies within the infrared absorption band of Chl-a_{II}^+ around 820 nm [21]. The light passed through a cuvette with optical pathlength of 30 nm and was detected by a photodiode preamplifier module (C30847E from RCA). The signal was amplified, digitized and averaged as described earlier [19]. The electrical bandwidth of the whole detection system was about 0.3 Hz–30 MHz. The increase in the signal-to-noise ratio compared to earlier measurements [19] is mainly due to the application of a laser diode with lower intrinsic noise. The fluorescence artifact was negligible in all measurements reported in this paper.

Measurements were made at room temperature in a medium composed of 30 mM sodium phosphate buffer (pH 6.5), 3 mM NaCl, 70 mM sucrose, thylakoid material corresponding to 42 μg chlorophyll/ml, when indicated, 26 μg 23 kDa protein/ml, and finally 0.2 mM phenyl-*p*-benzoquinone. The 23 kDa protein, prepared according to a recently described procedure [15], was a generous gift from C. Jansson.

Results

Upon excitation of inside-out thylakoids with 3 ns laser flashes under repetitive conditions, a rapid increase of absorbance at 824 nm was observed (Fig. 1a). The signal showed multiphasic decay kinetics in the time-range from nanoseconds up to milliseconds. The very fast decay in the range up to 10 ns (above the dashed line) probably reflects another event than the physiological electron donation to Chl-a_{II}^+ [20]. It may reflect the decay of excited singlet states or triplet states in the antennae or an electron back transfer from reduced pheophytin to Chl a_{II}^+. In the following evaluation this part of the signal will not be considered. For the further decay, half-life times of 30 ns, 250 ns, 5 μs, 35 μs, 250 μs and 1 ms have been

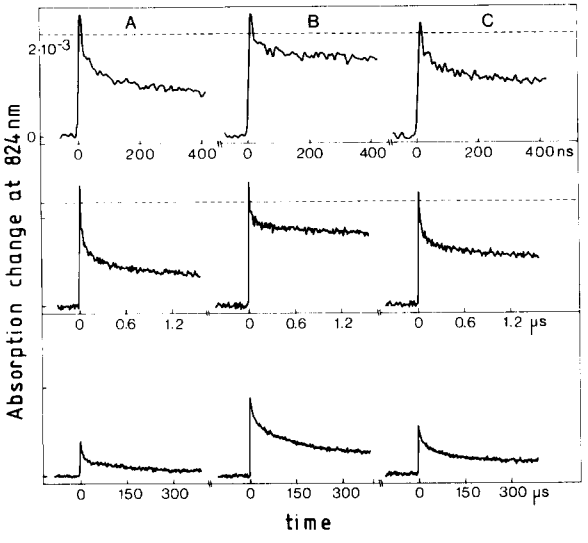

Fig. 1. Absorbance changes at 824 nm induced by 3-ns laser flashes ($\lambda = 532$ nm, excitation energy density per flash 10 J/m^2, repetition rate 5 Hz) as a function of time. 512 signals have been averaged. (A) Untreated inside-out thylakoids; (B) Salt-washed inside-out thylakoids; (C) Salt-washed inside-out thylakoids with added 23 kDa protein. The signal above the dashed line is not considered to reflect the reduction of Chl a_{II}^+, see text.

evaluated (assuming exponential decays) (see Table I). The 30 and 250 ns phases, which together contribute to about 60% of the total signal in untreated inside-out thylakoids, are characteristic for the electron transfer to Chl-a_{II}^+ via the natural pathway from water [18,19]. The half-times of the microsecond phases are also in line with the half-life times observed for the Chl-a_{II}^+ reduction in right-side-out thylakoids. Under the experimental conditions of the present study, it is possible that also Chl-a_I^+ (P-700$^+$) reduction contributes in part to the microsecond phases.

The remainder of the signal, decaying on a millisecond time scale, is probably due to Chl a_I^+ reduction. This is supported by the finding that addition of DCMU abolishes all signals except those below 5 ns and above 1 ms (not shown). That the contribution from Chl a_I^+ was small is partly due to the low content of Photosystem I in these inside-out thylakoids [9] and partly due to the high repetition rate used (changing the repetition rate from 5 to 0.2 Hz doubled the 1 ms component without changing the other components significantly).

TABLE I

HALF LIFE-TIMES AND RELATIVE AMPLITUDES OF THE DECAY PHASES AND RELATIVE OXYGEN-EVOLUTION

The half-life times and amplitudes, respectively, were evaluated from the curves in Fig. 1 assuming multiphasic exponential decay. The very fast decay during the first 10 ns was not considered.

Half-life times	Untreated (%)	Salt-washed (%)	Salt-washed + 23 kDa protein (%)
30 ± 10 ns	37 ± 10	17 ± 7	32 ± 10
250 ± 100 ns	25 ± 7	8 ± 4	15 ± 5
5–250 µs	34 ± 5	62 ± 9	44 ± 6
(> 1 ms)	(5 ± 2)	(13 ± 3)	(9 ± 2)
Oxygen yield [a]	100	35	75

[a] Relative oxygen yield per flash, taken from Ref. 22.

Upon washing inside-out thylakoids with 250 mM NaCl, a treatment known to release a 23 kDa protein from the membranes concomitant with an inhibition of oxygen evolution [11], a drastic change in the decay kinetics was observed (Fig. 1b). These signals can be adapted using the same half-life times as for signals in Fig. 1a, but strongly changed amplitudes for the individual phases. No significant change was observed, however, for the total amplitude of the signal. Thus, the amplitude of the nanosecond phases dropped to two-fifths of the value for untreated inside-out material, while the sum of the microsecond phases was almost doubled. The increase of the microsecond reduction at the expense of the nanosecond kinetics corresponds qualitatively to observations after inhibition of oxygen evolution with NH_2OH in right-side-out thylakoids [18–20]. These results clearly demonstrate that the inhibition of oxygen evolution caused by salt-washing is actually on the oxidizing side of Chl-a_{II}^+.

Upon readdition of the purified 23 kDa protein to the salt-washed inside-out thylakoids, the dominating part of the Chl a_{II}^+ reduction was again found in the nanosecond time range (Fig. 1c). Nearly 80% of the original level of the nanosecond phases (30 and 250 ns) was recovered. This means that the main effect of salt-washing on electron

donation to Chl-a_{II}^+ is due to the removal of the 23 kDa protein.

In an attempt to characterize further the donor capacity of the salt-washed inside-out thylakoids, an artificial electron donor, hydroquinone, was added instead of the protein. The hydroquinone accelerated the microsecond decay of Chl a_{II}^+ to a single phase of a half-life time of 5 μs, but could not restore the nanosecond phases.

Discussion

Salt-washing has been shown to cause an inhibition of PS-II-mediated electron transport, specifically in inside-out thylakoids. The effect was shown by reconstitution experiments to be mainly due to the removal of a 23 kDa protein. The decreased rate of electron donation to Chl a_{II}^+ observed in this work after salt-washing and the reversing effect of the added 23 kDa protein reveal that the functional site for the 23 kDa protein is on the oxidizing side of PS II. Note that the total amplitude was not changed by the salt treatment, suggesting that the reaction centre itself was not damaged. The same conclusions were reached from measurements on the first stable acceptor, PQ_A [22] and also more indirectly from measurements of fluorescence induction [11].

The proportion of the nanosecond components that was converted to slower components (65%) and the reconversion by addition of the 23 kDa protein correspond well to the degree of inhibition and reconstitution of oxygen evolution seen in continuous light (Table I and Ref. 11). The same degree of inhibition and reconstitution was also seen on measuring oxygen yield in single-turnover flashes under repetitive conditions [22]. Furthermore, preliminary immunological studies reveal that the type of salt-washing used here released some 50–70% of the 23 kDa protein (not shown). These results taken together strongly argue for an absolute requirement for the 23 kDa protein for oxygen-evolution, either in the oxygen-evolving complex or for the transfer of electrons to the reaction centre. Note, however, the work by Murata et al. [17].

The inability of the artificial electron donor, hydroquinone, to restore the nanosecond donation to Chl-a_{II}^+ can be interpreted in two ways.

(a) Removal of the 23 kDa protein inhibits electron transfer from the natural secondary donor, D_1, to Chl a_{II}^+; this would imply that there exists another donor parallel to D_1 reducing Chl a_{II}^+ in about 5 μs. This donor is subsequently reduced by hydroquinone. In principle, the same conclusion was drawn by Conjeaud et al. [23] from measurements on Tris- or NH_2OH-treated thylakoids, with reduced phenylenediamine as electron donor.

(b) Removal of the 23 kDa protein inhibits electron transfer from H_2O to D_1^+ and also slows down the rate of electron transfer from D_1 to Chl a_{II}^+ to about 5 μs. In this case, hydroquinone would donate an electron to D_1^+. The ability for the 23 kDa protein, in contrast to hydroquinone, to restore the very fast electron donation to Chl a_{II}^+ supports that the nanosecond electron donation to Chl a_{II}^+ is coupled to the natural path of electrons from water.

The exact function of the 23 kDa protein is not yet clear but the apparent lack of prosthetic groups in the protein [15] suggests some regulatory or structural role rather than direct involvement as redox component. This, however, does not exclude the possibility that the protein is facilitating electron-transfer reactions or is involved in proton-exchange reactions.

The reversibility of the effects of salt-washing on the extremely rapid electron donation to Chl a_{II}^+ in inside-out thylakoids should make the system suitable for further studies on the electron transport at the oxidizing side of Chl a_{II}^+.

Acknowledgements

This work was supported by the Swedish Natural Science Research Council and the European Molecular Biology Organization as well as the Deutsche Forschungsgemeinschaft.

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