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EFFECT OF PARTIAL REMOVAL AND READDITION OF A 23 KILODALTON PROTEIN ON OXYGEN YIELD AND FLASH-INDUCED ABSORBANCE CHANGES AT 320 nm OF INSIDE-OUT THYLAKOIDS

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Washing inside-out spinach thylakoids with 250 mM NaCl at pH 7.4 causes up to 75% inhibition of oxygen evolution which has been shown by reconstitution experiments to be due mainly to the removal of a 23 kDa protein (Åkerlund, H.E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10). Here we have found the same degree of inhibition and reconstitution of oxygen evolution measured either in flash light or in continuous light at different light intensities, which suggests that the salt-washing causes a complete block of electron transport in certain chains rather than a decreased rate in all chains. Flash-induced absorbance changes at 320 nm, reflecting mainly the electron transfer at the Photosystem-II acceptor side, from the primary to the secondary plastoquinone in their semiquinone form ($PQ_A^- PQ_B^- \rightarrow PQ_A PQ_B^{2-}$) and to minor extent the donor side of Photosystem II (Renger, G. and Weiss, W. (1983) Biochim. Biophys. Acta 722, 1–11), were measured under repetitive excitation conditions. Salt-washing the inside-out thylakoids caused a change in decay rate from 500–600 μs down to 200 μs, interpreted as an increased proportion of recombination between PQ_A^- and chlorophyll-a_{II}^+. Addition of the 23 kDa protein restored the kinetics up to some 400 μs. Measurements made on dark-adapted material gave evidence that salt-washing also affected S-state turnover. We conclude that the 23 kDa protein functions on the water-splitting side of Photosystem II and is indispensible for the photosynthetic water oxidation.

Introduction

Light energy captured by the antenna pigments of PS II is channelled into a reaction centre where it causes an oxidation of the primary donor Chl a_{II} and a reduction of a primary acceptor, possibly a pheophytin [1], which in turn reduces a primary plastoquinone (PQ_A). Charge stabilization at the reaction centre sufficient for water oxidation is achieved only after electron transfer to PQ_A [2]. Chl a_{II}^+ is rapidly reduced by electrons originating from water oxidation. The mechanism of oxygen evolution is, however, still far from understood. Attempts to isolate active protein components involved in this reaction have until recently been fruitless, although correlations between inhibitions and alteration or removal of certain proteins have provided possible candidates [3–5]. However, by combining the use of inside-out thylakoids, exposing the water-splitting system [6], with mild salt-washing to release electrostatically bound proteins from the membrane, it was possible to inhibit PS II-mediated electron transport reversibly [7–9]. Similar results have also been obtained on PS II particles prepared by detergent methods [9–11].

Abbreviations: Chl-a_{II}, chlorophyll-a_{II}; PQ, plastoquinone; PS II, Photosystem II.

By purification and reconstitution experiments, it has been shown that a 23 kDa protein was responsible for the effect [12]. Based on fluorescence-induction measurements, the functional site for the protein was inferred to be on the oxidizing side of the PS II. The highly purified and active protein did not, however, show any obvious prosthetic groups that could participate in redox reactions [12,13]. This finding and that salt-washing did not cause complete inhibition of oxygen evolution, but left 30–40% of the original activity, raises the question whether the 23 kDa protein is indispensible for the oxygen-evolving units or if it exerts regulatory control on a rate-limiting step in oxygen evolution.

A distinction between both alternatives should be possible by measurements of flash-induced oxygen evolution, combined with measurements of oxygen evolution in continuous light as a function of light intensity. Furthermore, in reaction centres with disconnected oxygen-evolving system Y, a cyclic electron flow from PQ_A^- to Chl a_{II}^+ is expected to take place under repetitive flash excitation. This reaction, which is characterized by a half-time of 100–200 μs, can be monitored by absorption changes at 320 nm [14–18]. In normal PS II, with functionally connected system Y, absorption changes at 320 nm reflect two processes [19,20]: reoxidation of PQ_A^- by the secondary plastoquinone in its semiquinone form PQ_B^- [21] and oxidation of state S_3 by the donor D_1^{ox} in system Y leading to oxygen evolution [22].

In the present work the effect of salt-washing and readdition of the purified 23 kDa protein has been studied by oxygen-evolution measurements in flash light and in continuous light as well as measurements of flash-induced absorbance changes at 320 nm. The results suggest that the 23 kDa protein is indispensible for the function of each water-splitting unit or for the connection to the reaction centre.

Materials and Methods

Inside-out thylakoids were obtained by mechanical disintegration of stacked spinach thylakoids followed by aqueous two-phase fractionation as described earlier [8,23]. For the salt-washing, the isolated inside-out thylakoids (20 μg chlorophyll/ml) were incubated in 250 mM NaCl/10 mM sodium phosphate (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.

Oxygen yield was measured with a Clark type electrode with the flash-polarography equipment described earlier [24]. The flash repetition rate was 2–10 Hz and the number of flashes given was 50–300. 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 20 μg chlorophyll/ml, when indicated, 26 μg 23 kDa protein/ml, and finally 0.2 mM phenyl-*p*-benzoquinone. The reconstitution was made by mixing 150 μl protein (1.6 mg/ml) with 100 μl salt-washed inside-out thylakoid membranes (1.8 mg chlorophyll/ml). This mixture was then added to buffer to yield the concentrations stated above. Oxygen evolution in continuous light was measured with a Clark type electrode using the same medium as for the oxygen yield measurements. Light intensities were varied by introducing metal nets in the light beam.

Absorbance changes at 320 nm were measured with a flash photometer [25] equipped with a pulsed measuring light beam switched on for 30 ms. The intensity of the measuring light was 50 $\mu W/cm^2$. The measurements were made in a medium with the same composition as for the oxygen yield measurements, except that phenyl-*p*-benzoquinone was omitted or exchanged for 0.1 mM ferricyanide. Excitation xenon flashes with 15 μs half-width were passed through a Schott RG1 filter. Optical pathlength, 10 mm; optical bandwidth on measuring light, 2 nm; electrical bandwidth, 200 kHz.

Results

The average oxygen yield per flash under repetitive excitation conditions for untreated inside-out thylakoids was found to be one O_2 per 1300–1800 chlorophylls. Upon washing inside-out thylakoids with 250 mM NaCl, a treatment known to release a 23 kDa protein from the membrane,

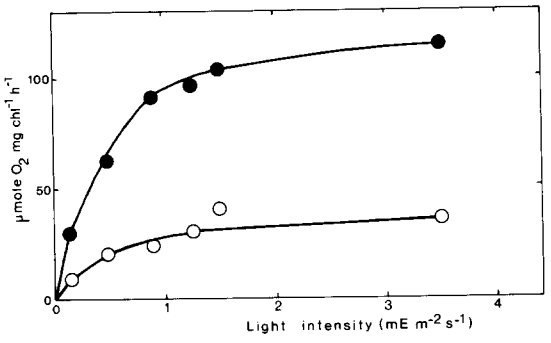

Fig. 1. Oxygen evolution of untreated and salt-washed inside-out thylakoids measured in continuous light as a function of light intensity; ●——●, control; ○——○, salt-washed.

the average oxygen yield per flash decreased by 60–70%. Upon readdition of the 23 kDa protein, up to 75% of the original level could be restored (data not shown). The same results were obtained regardless of the repetition rate (2–10 Hz), suggesting that the removal of the 23 kDa protein resulted in a complete block of electron transport, from water to the artificial electron acceptor phenyl-*p*-benzoquinone, in more than 50% of all chains, rather than a decreased rate in all centres.

Measurements on oxygen-evolution of untreated and salt-washed inside-out thylakoids in continuous light as a function of light intensity (Fig. 1) revealed the same degree of inhibition at all light intensities. This is what should be expected if salt-washing causes a complete block of the electron transport. If, however, the salt-inactivation had been due to a decreased rate of dark electron transport, the inhibition should have been less at low light intensities where the capture of light is the rate-limiting step. Furthermore, if damage to the antenna system was the cause of diminished oxygen evolution, the two curves would have differed in the intensity required for half-saturation. The data in Fig. 1 show that this was not observed.

In order to study the function of the 23 kDa protein in more detail, flash-induced absorbance changes at 320 nm were monitored under repetitive excitation conditions. The signal for untreated inside-out thylakoids showed an apparent decay half-time of 500–600 μs (Fig. 2). This value is similar to what is normally observed for unfractionated thylakoids [26]. The 500–600 μs relaxation has originally been interpreted as the PQ_A^- reoxidation [25]. However, more careful analysis indicates that it actually represents the overlapping of at least two processes: reoxidation of PQ_A^- by PQ_B^- [21], characterized by a half-time of 300–450 μs (Weiss and Renger, unpublished data), and oxidation of the water-splitting system Y in state S_3 by donor D_1^{ox} [22] taking place with a half-time of 1 ms.

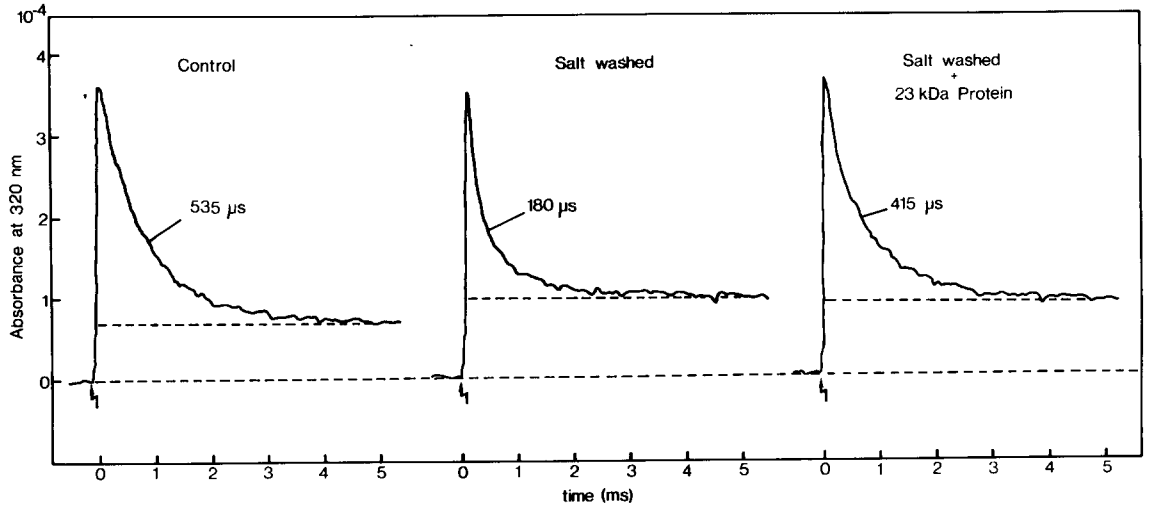

Fig. 2. Flash-induced absorbance changes measured at 320 nm under repetitive excitation condition, for untreated, salt-washed and reconstituted inside-out thylakoids. 512 signals were averaged with a repetition rate of 4 Hz.

Upon salt-washing of inside-out thylakoids, a markedly faster relaxation of the 320 nm absorption change was observed, giving an apparent half-time value of 150–200 μs. These faster kinetics suggest that a considerable part of PQ_A^- decays by direct back-reaction with Chl-a_{II}^+. Such a back-reaction has been suggested by several authors to occur when electron donation to Chl-a_{II}^+ is blocked [14–18]. This effect is mostly due to removal of the 23 kDa protein as demonstrated by the ability of the purified 23 kDa protein to reverse most of this effect (Fig. 2).

These results can be consistently explained by the assumption that the removal of the 23 kDa protein causes disconnection between the water-splitting enzyme system Y and the reaction centre, possibly by affecting the redox component D_1. This interpretation implies that the 320 nm absorption changes observed under repetitive excitation should not only reveal the characteristic kinetic modifications depicted in Fig. 2, but also the initial amplitudes would be expected to decrease in the salt-washed sample. However, the expected decrease would be maximally 15–20%. (The exact amplitude decrease depends at 320 nm on the extinction-coefficient difference due to the turnover of the donor components and Chl-a_{II}, respectively.) As shown in Fig. 2, the amplitudes of the absorbance changes were almost the same. However, due to some uncertainties in the dilution of the different samples and the small changes expected, no reasonable conclusions can be drawn from the amplitude data.

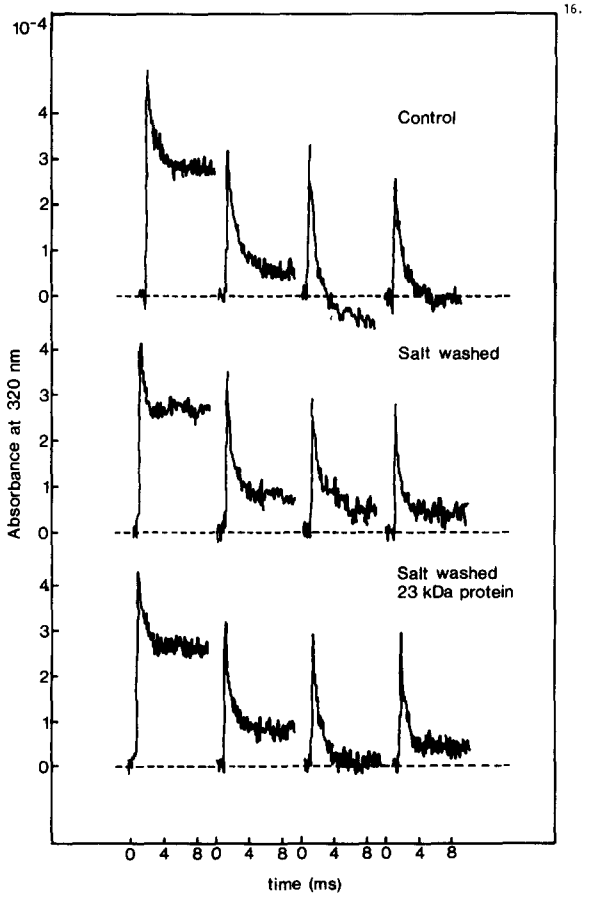

Fig. 3. Flash-induced absorbance changes measured at 320 nm on dark adapted (10–20 min) inside-out thylakoids. The time between flashes was 500 ms and 32 traces were averaged, with change of sample between each sequence.

In order to analyze the function of the 23 kDa protein further, dark-adapted inside-out thylakoids were illuminated by a train of four xenon flashes (half-time 10–15 μs) spaced by a dark-time of 500 ms. In this case the sample was renewed after each flash-train as described in Ref. 22. The results are depicted in Fig. 3. At the top is depicted the pattern of 320 nm absorption changes in normal inside-out thylakoids. It reveals a characteristic oscillation of the relaxation kinetics which is explicable by an overlapping of the binary pattern due to the transition $PQ_A^-PQ_B^- \rightarrow PQ_APQ_B^{2-}$ and the quarternary oscillation of the redox turnovers in system Y. The 1-ms relaxation was, as expected, especially pronounced after the third flash, which causes a maximum oxygen yield. Upon salt-induced release of the 23 kDa protein, the pattern was changed. The initial amplitude of the absorption changes due to the first flash was diminished by some 20% and the 1 ms relaxation of the absorption change due to the third flash largely disappeared. The latter effect is consistent with the assumed blockage of the system-Y turnover due to 23 kDa protein release. The decay of the signal at 320 nm after the first flash should give information on whether the donor D_1 is still in function after salt washing. The signal-to-noise level (Fig. 3) was unfortunately not sufficient to allow a kinetic comparison of the rapidly decaying component. However, the part of the signal decaying with half-life of more than 8 ms, representing the ex-

tremely slow reduction of PQ_A^-, was not significantly affected by the salt-washing. This suggests that Chl a_{II}^+ became reduced by D_1 rather than by PQ_A^- (100–200 μs kinetics) on the first flash also in the salt-washed material. Therefore, on the basis of these data, extraction of the 23 kDa protein is inferred to block the electron transfer from system Y to D_1. This conclusion is in line with previous findings which show that Tris-washing of inside-out thylakoids, leading to release of the 23 kDa- and 34 kDa-proteins [3], does not interrupt the functional connection between Chl-a_{II} and D_1, but only retards the electron transfer rate [27]. Direct measurements of 820 nm absorption changes described in the accompanying paper [28] also support this idea.

Discussion

The results obtained here provide different lines of evidence that the 23 kDa protein functions on the donor side of PS II. Measurements of 320 nm absorption changes under different flash excitation conditions reveal that after removal of the 23 kDa protein (a) the cyclic electron flow between PQ_A^- and Chl-a_{II}^+ ($t_{1/2} = 100$–200 μs) becomes switched on at the expense of the linear electron flow from the water-splitting enzyme system Y to Chl-a_{II}^+ under repetitive excitation and (b) the 1 ms relaxation kinetics reflecting the oxidation of state S_3 in system Y become suppressed. Both effects are partially reversed by reinsertion of the 23 kDa protein into the thylakoid membrane. The conclusion that the 23 kDa protein is acting on the oxidizing side of PS II is further corroborated by previous measurements of fluorescence induction curves [8] and by measurements of Chl a_{II}^+ kinetics via 820 nm absorbance changes, reported in the accompanying paper [28]. The measurements on oxygen production made here revealed the same degree of inhibition and reconstitution whether flash light excitation or saturating continuous light was used (Fig. 1, and also Ref. 8). This suggests that the removal of the 23 kDa protein causes a complete disconnection of water-splitting units from the reaction centres rather than introducing a new rate-limiting step on the oxidizing side of the reaction centre. The residual activity after salt-washing could than be due to a corresponding amount of the 23 kDa protein left on the membrane. This view has recently been supported by immunological studies [29]. Note, however, the work by Murata et al. [30].

The function of the 23 kDa protein is not yet elucidated. The protein cannot be the apoenzyme for the functional redox group of the primary donor to Chl a_{II}, referred to as D_1, since this donor is intact after Tris-washing of inside-out thylakoids, a treatment known to release the 23 kDa protein together with a 16 and 34 kDa protein, and the kinetics of electron transfer from D_1 to Chl a_{II}^+ are the same as in normal Tris-washed chloroplasts [27]. The absence of redox groups in the isolated 23 kDa protein favours the idea of a regulatory function rather than a direct participation in the redox reaction sequence. However, a possible function as apoenzyme for a functional redox group cannot totally be excluded if this redox group is assumed to remain attached to the membrane in an inactive state, from which the 23 kDa protein is released by the salt treatment. With respect to a regulatory function, different mechanisms should be taken into consideration. Switch on-off mechanism could arise if the 23 kDa protein is required to sustain the redox potential of the couple D_1/D_1^{ox} high enough for functional coupling between Chl a_{II}^+ and system Y. If the removal of the 23 kDa protein decreases the redox potential, the electron flow from system Y to Chl a_{II}^+ would be interrupted by an 'endergonic blockage' [31]. Another mechanism is a structural 'fit-together' of system Y with donor D_1. For the time being the role of the 23 kDa protein as an essential unit operating at the oxidizing side of system II appears to be well established, but the details of the mode of action still remain to be unravelled.

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