Direct evidence for cooperation of the 17- and 23-kDa proteins in the recovery of oxygen evolution in cholate-treated thylakoids

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Repeated extractions of spinach thylakoid membranes with a solution containing 50 mM sodium cholate, 1 M NaCl, 3 mM $MgCl_2$, 0.2 M sucrose and 20 mM tricine at pH 8.4 for 15 min perfectly inhibited the O_2 evolution of the thylakoids, concomitant with a complete release of the 17- and 23-kDa proteins and partial release of many other proteins from the thylakoid membranes. Recovery of O_2 evolution in the cholate-treated thylakoids was achieved up to about 40% of that in the original thylakoids by the simultaneous reinsertion of the 17- and 23-kDa proteins, but not by the reinsertion of one of them only. The recovery of O_2 evolution induced by the reinsertion of the 17- and 23-kDa proteins was enhanced by the further addition of a certain fraction of the crude thylakoid extract up to about 70% of the non-depleted control, suggesting that in addition to the 17- and 23-kDa proteins, one or more unknown component(s) released partially from the thylakoids upon cholate treatment is (are) also (a) constituent(s) of the O_2 evolving apparatus. The purified 34-kDa protein did not replace the unknown component.

Reconstitution, of oxygen evolution *Photosynthesis* *Water splitting* *Thylakoid protein* *Photosystem II*

1. INTRODUCTION

Three proteins (17-, 23- and 34- kDa) which are located on the inner surface of thylakoid membranes have drawn particular attention of investigators in connection with the photosynthetic O_2 evolution. Results of recent experiments involving the reconstitution of the O_2 evolving apparatus have suggested that either all, or part of the 3 proteins are involved in the water splitting process, presumably as constituents of the enzyme complex [1–5] responsible for O_2 evolution. However, some discrepancies have arisen in the results obtained with different methods of reconstitution or different PS II particles. A partial inhibition of the O_2 evolution in inside-out thylakoids obtained by washing the thylakoids with a buffer solution containing 0.25 M NaCl at pH 7.4 was accompanied by a release of the 17- and 23-kDa proteins. Readdition of the 23-kDa protein to the washed inside-out thylakoids under low ionic strength restored 60% of the lost activity [1,3]. Similar results were obtained with a PS II detergent particle preparation [4]. However, the results obtained with the inside-out thylakoids were contradictory as to the relationship between the inhibition of the O_2 evolution and the loss of the 23-kDa protein. The 17-kDa protein was reported to be more effective than the 23-kDa protein in restoring the O_2 evolution in a cholate-depleted thylakoid preparation [2]. No evidence for the cooperation of the 17- and

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Abbreviations: tricine, *N*-tris(hydroxymethyl)methylglycine; MOPS, 3-(*N*-morpholino)-propane-sulfonic acid; PBQ, phenyl-*p*-benzoquinone; kDa, kilodalton; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; chl, chlorophyll; PS II, photosystem II

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34-kDa proteins in the reconstitution of the O_2 evolving apparatus has yet been obtained, even though the 34-kDa protein can rebind to the depleted thylakoids. Thus, the role of these proteins in O_2 evolution is not yet clear.

Here, we provide direct evidence for the cooperation of the 17- and 23-kDa proteins in the photosynthetic water-splitting process, by improving our method used for the reconstitution of the O_2 evolving apparatus in cholate-depleted thylakoids [2].

2. MATERIALS AND METHODS

Broken thylakoid membranes, named UP-10, were prepared from spinach class 2 chloroplasts as in [2]. In order to obtain the 17-, 23- and 34-kDa proteins, UP-10 was incubated in buffer solution A (0.2 M sucrose, 3 mM $MgCl_2$, and 20 mM tricine at pH 8.4) containing, in addition, 50 mM sodium cholate, 1 M NaCl, and stirred for 2 h on ice at a concentration of 2 mg chl/ml. The mixture was centrifuged at 255000 × *g*. The supernatant was dialyzed overnight against buffer solution A, concentrated, and applied to a DEAE-Sephacel (Pharmacia Fine Chemicals) column equilibrated with buffer solution A containing 10 vol.% glycerol. After the components not bound to DEAE-Sephacel at pH 8.4 were thoroughly eluted, proteins were eluted with buffer solution B (0.2 M sucrose, 3 mM $MgCl_2$, 20 mM MOPS and 10 vol.% glycerol at pH 6.2), the ionic strength of which was changed by varying the NaCl from 0 to 0.3 M. Since the 17-kDa protein was eluted in the void volume fraction (P-1) of the column, this fraction was applied again to a carboxymethyl (CM)-Sephadex column and eluted with buffer solution B varying the NaCl from 0 to 0.5 M.

Depletion and reconstitution of UP-10 were performed as follows. UP-10 was dispersed in buffer solution A containing 50 mM sodium cholate, 1 M NaCl, and stirred on ice for 15 min, followed by centrifugation at 144000 × *g*. The pellet was resuspended in the same solution to repeat the extraction, if necessary. The final pellet was washed by buffer solution C (0.2 M sucrose, 3 mM $MgCl_2$, 20 mM MOPS at pH 7.0) containing 20 mM sodium cholate and suspended in the same solution. To this suspension, either crude cholate extract, or one of the purified 17-, 23- and 34-kDa proteins, or some other components, or their combinations, were added together with glycerol at a final concentration of 25 vol.%. After incubation for 1 h at 4°C, the mixture was diluted 50-times with buffer solution C and centrifuged. The pellet, named RUP-10, was resuspended in buffer solution D (0.2 M sucrose, 20 mM MOPS at pH 7.0) to give a final [chl] of 8.3 μg/ml. Control RUP-10 was also prepared by the same procedure, without the addition of any protein to the medium of reconstitution. The measurement of O_2 evolution was carried out with UP-10, RUP-10 and control RUP-10 preparations by using a teflon-covered oxygen electrode (Bionics Instrument) at 25°C. Continuous illumination between 500 and 750 nm at saturation light intensity was provided by a 500 W Xenon lamp through a pair of color glass filters (Toshiba, Y-50 and IRA-25S) and a 10-cm water layer.

3. RESULTS AND DISCUSSION

Fig.1 shows the elution profiles of column chromatography on DEAE-Sephacel and CM-Sephadex columns, monitored at 280 nm. The fractions marked with bars in the figures were collected as the 17-, 23- and 34-kDa protein preparations, respectively, and submitted to SDS–PAGE and reconstitution experiments. Fig.2 shows SDS–PAGE of the 3 protein preparations, UP-10, cholate extracted UP-10 and RUP-10 prepared under different conditions. Each preparation of the 17- and 23-kDa proteins gave a single band on PAGE, indicating that they are free from contamination by other proteins. The 34-kDa protein preparation was contaminated with a small amount of 40-kDa protein and traces of other proteins. A comparison of the protein patterns of the UP-10 and the cholate-extracted UP-10 preparations reveals that the 17- and 23-kDa proteins were thoroughly removed from UP-10 by extraction twice with 50 mM cholate, while the 34-kDa protein was retained to a considerable extent. The protein patterns of RUP-10 prepared with one of the 17-, 23- and 34-kDa proteins clearly show that all of these proteins were rebound to the reconstituted membranes.

Fig.3 shows the recovery of O_2 evolution in RUP-10 upon addition of the crude, cholate extract. The O_2-evolving activity of control RUP-10

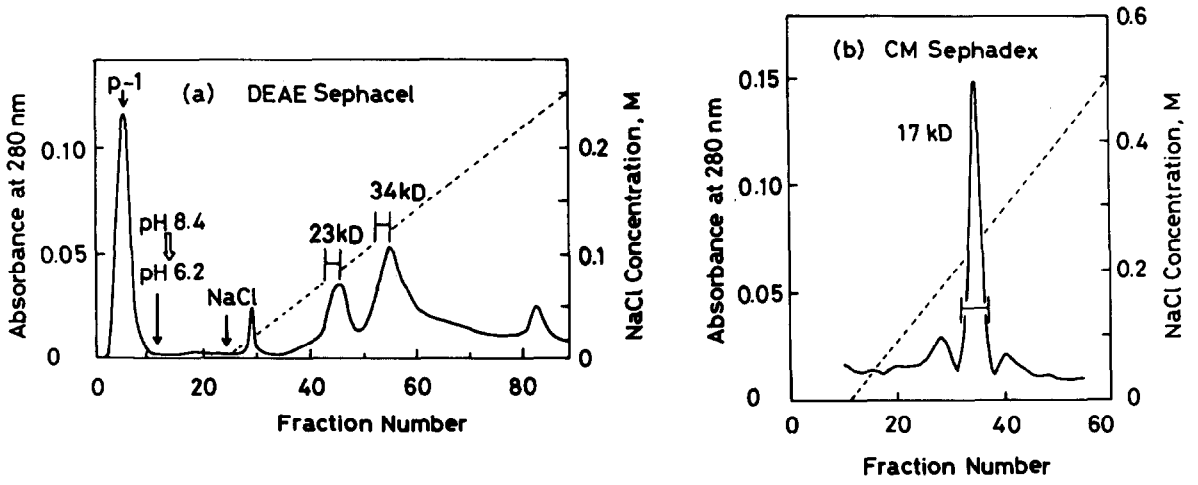

Fig.1. Separation of cholate-extracted proteins from UP-10 on a DEAE-Sephacel (a) and a CM-Sephadex (b) column. The volume of each fraction was 5 ml. The eluent for the DEAE-Sephacel column was changed from buffer solution A to B at the point indicated by the arrow.

prepared from UP-10 (extracted twice with cholate) was zero without the addition of any cholate extract, but O_2 evolution was recovered to about 70% of that of the original UP-10 upon addition of crude cholate extract to the incubation medium used for reconstitution, at a protein/chl ratio of 2.0 (w/w). Effects of the purified 17- and 23-kDa proteins on the recovery of O_2 evolution in RUP-10 prepared from UP-10 extracted twice with

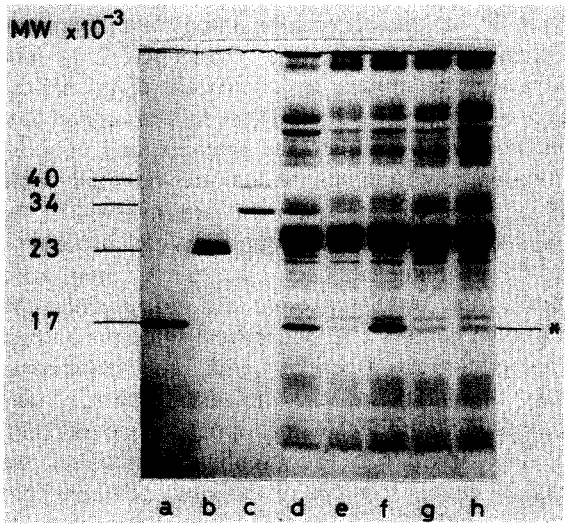

Fig.2. SDS–PAGE of the proteins obtained from UP-10 upon various treatments: () 17-kDa protein; (b) 23-kDa protein; (c) 34-kDa protein; (d) UP-10; (e) cholate-extracted UP-10 (144000 × *g* pellet after the extraction of UP-10 twice with cholate; (f,g,h) RUP-10 prepared by incubating with the 17-, 23- and 34-kDa proteins, respectively of an UP-10 preparation extracted twice with cholate; (*) a protein just below the 17-kDa protein.

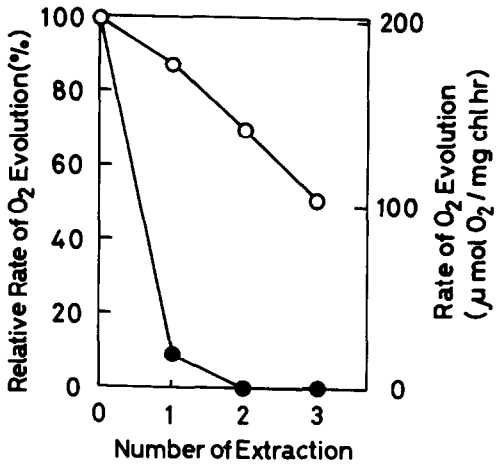

Fig.3. Effect of the number of cholate extractions on the O_2 evolving capacity of RUP-10 (○) and control RUP-10 (●) prepared by incubation with and without the crude cholate extract of UP-10, respectively. The reaction mixture for the assay of O_2 evolution contained 0.2 M sucrose, 20 mM MOPS (pH 7.0), 0.3 mM PBQ and 8 μg chl/ml.

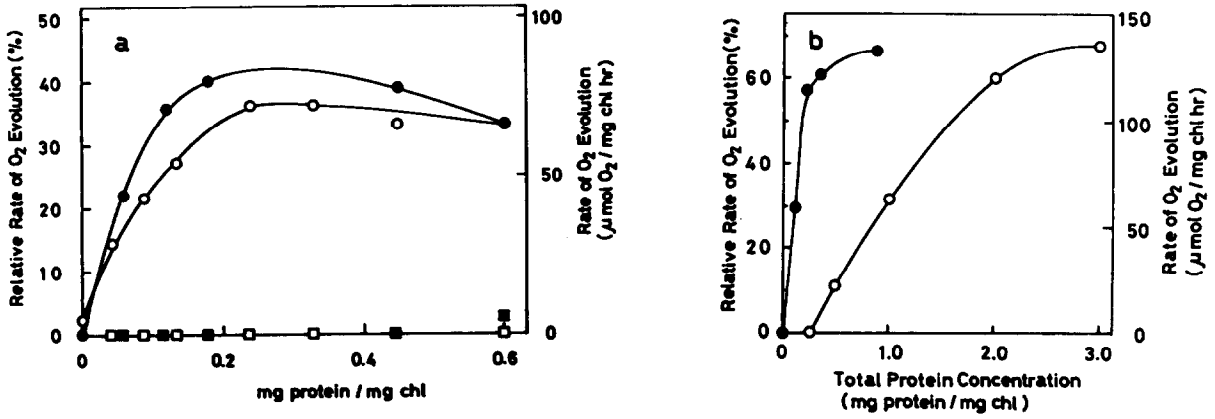

Fig.4. Effect of the purified 17- and 23-kDa proteins, the crude cholate extract of UP-10 and the filtrate on the O_2 evolution in RUP-10. (a) Effect of the purified 17- and 23-kDa proteins: (□) 17-kDa protein; (■) 23-kDa protein; (○) various amounts of the 17-kDa protein in the presence of a constant amount of the 23-kDa protein at a 23-kDa protein/chl ratio of 0.6 (w/w); (●) various amounts of the 23-kDa protein in the presence of a constant amount of the 17-kDa protein at a 17-kDa protein/chl ratio of 0.6 (w/w). (b) Effect of the crude cholate extract of UP-10 and that of the filtrate: (○) crude cholate extract of UP-10; (●) various amounts of the 17- and 23-kDa proteins at a molar ratio of 1:1, in the presence of sufficient amounts of the filtrate.

cholate are shown in fig.4a. It may be seen that neither the 17- nor 23-kDa protein alone restored the O_2-evolving capacity in RUP-10, as shown by the open and filled squares, respectively. The open circles show the data obtained by changing the amount of the 17-kDa protein in the presence of constant 23-kDa protein concentration at a 23-kDa protein/chl ratio of 0.6 (w/w). The filled circles show the results of an experiment in which the concentration of the 17-kDa protein was kept constant and that of the 23-kDa protein was varied. The data show that the recovery of the O_2 evolution in RUP-10 requires the presence of both proteins, strongly suggesting a cooperation of both proteins in the photosynthetic water splitting process. Although the 34-kDa protein was rebound to RUP-10, it had no effect on the O_2 evolution, whether the 17- and 23-kDa proteins were present or not. The recovery of O_2 evolution induced by the simultaneous addition of the 17- and 23-kDa proteins increased when increasing the amount of the proteins, but did not exceed a level of 40% of the original UP-10. The effect of the crude cholate extract was stronger. By using whole cholate extracts the O_2 production was about 70% of that of UP-10, as shown in fig.4b. Furthermore, fig.4b shows that the recovery of the O_2 evolution induced by the addition of the 17- and 23-kDa proteins was enhanced up to about 70% of that of UP-10 by further addition of a filtrate, of the supernatant obtained after cholate extraction of UP-10, through an ultrafiltration membrane (Amicon Ultrafiltration Membrane Corns CF-25). The filtrate alone showed no significant effect on the recovery of O_2 evolution. These results suggest that besides the 17- and 23-kDa proteins, one or more unknown components which are released from UP-10 upon cholate treatment are involved in the water-splitting process, probably as constituents of the O_2 evolving enzyme complex. As mentioned above, the 34-kDa protein did not replace the unknown component. Moreover, since the filtrate scarcely contained proteins with larger molecular mass than 15 kDa, the molecular mass of the unknown component was supposed to be less than 15 kDa. Apart from the unknown component, we may conclude from the results obtained here that the 17- and 23-kDa proteins are essential components of the photosynthetic oxygen evolution process in the thylakoid membranes.

ACKNOWLEDGEMENT

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