A protein essential for recovering oxygen evolution in cholate-treated chloroplasts

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Received 13 October 1982

A novel method for the reconstitution of oxygen evolution in cholate-extracted spinach thylakoid membranes was established and a protein essential for the reconstitution was purified from cholate extracts. Purification of the protein was accomplished by chromatography on a DEAE-Sephacel column. This protein (M_r 17000) was reinserted into vesicular membranes reconstituted from cholate-extracted thylakoids in the presence of 25% glycerol to reactivate oxygen evolution.

Pigment system 2 *Reconstitution of oxygen evolution* *Reconstituted membrane* *Spinach chloroplast* *Thylakoid membrane* *Vesicle*

1. INTRODUCTION

The biochemical aspects of photosynthetic oxygen evolution are still speculative. The majority of the present knowledge has been limited to concepts based on kinetic data, including the well-known 'S-state model' for storage of oxidizing equivalents [1–3]. A manganese protein (M_r 65000) has been isolated from spinach chloroplasts and reconstituted oxygen evolution activity by the reinsertion of this protein, in liposomes containing cholate-extracted chloroplasts. In [6] a heme-protein (M_r 58000) extracted from spinach thylakoids brought about an enhancement of oxygen evolution when incorporated into liposomes prepared using the procedure in [4,5]. Other attempts to isolate the catalyst for phtotsynthetic oxidation of water have failed. However, many investigators have been unable to reproduce the work of Spector and Winget (see [7]). Evidence for a protein-dependent, organic solvent-induced reconstitution of oxygen evolution in cholate–asolectine-extracted chloroplasts was presented in [8]. Although the nature of the protein essential for the reconstitution has not been clarified, the M_r 65000 and 58000 proteins were suggested not to be required for the reconstitution of oxygen evolution. Independently, we have reported [9] a method for the reconstitution of oxygen evolution in cholate-extracted chloroplasts using glycerol, but no asolectine. Here, we present the novel method and the purification of a protein (M_r 17000) essential for the reconstitution of oxygen evolution in cholate-extracted thylakoid membranes.

Abbreviations: DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenol indophenol

2. MATERIALS AND METHODS

Class 2 chloroplasts isolated from fresh spinach leaves by a standard method were suspended in buffer solution A (15 mM NaCl, 30 mM tricine, pH 7.5) and immediately sonicated for 1 min with 2 min incubation in ice. After unbroken chloroplasts and undispersed particles were removed by centrifugation at $2000 \times g$, the supernatant was centrifuged at $10000 \times g$. The pellet (UP-10) was collected as the starting material for our reconstitution experiments.

Specific proteins were extracted from UP-10 by incubating it in a solution of 50 mM sodium cho-

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

late, 0.2 M sucrose, 3 mM $MgCl_2$ and 20 mM tricine at pH 8.0 with stirring for 1 h on ice at 2 mg chl/ml, The mixture was centrifuged at 144000 × *g*. The pellet was kept for further experiments. The supernatant was concentrated and applied to a Sephadex G-50 column to remove the sodium cholate, followed by centrifugation at 20000 × *g*. Separation of the proteins contained in this 20000 × *g* supernatant was carried out by chromatography on a DEAE-Sephacel (Pharmacia Fine Chemicals) column. Buffer solution B (0.2 M sucrose, 3 mM $MgCl_2$, 20 mM tricine (pH 8.0)) was used as an eluent, the ionic strength of which was linearly changed by varying [NaCl] from 0–0.3 M.

The reconstitution of UP-10 was done 1 hr after incubation of the extracted UP-10 (the pellet after cholate extraction) with total cholate extracts or each one of the peak fractions of the DEAE-Sephacel column or, without any additive, in buffer solution B containing, additionally, 20 mM sodium cholate and 25% glycerol by volume. After incubation, the mixture was applied for gel filtration on a Sephadex G-25 column to remove the sodium cholate. The green fraction eluted as a single band was collected as reconstituted UP-10 (RUP-10).

The assay for photoinduced oxygen evolution was performed by using a Teflon-coated oxygen electrode (Bionics Instruments) connected to a high speed electrometer (417 High speed picoammeter, Keithley Instruments). Temperature of the sample chamber with the electrode was controlled at 25.0 ± 0.1°C by circulating thermostatted water. Illumination with light of 680 nm at 2.1 mW/cm^2

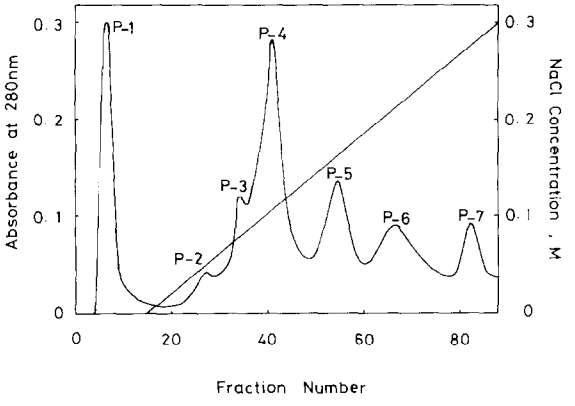

Fig. 1. Separation of cholate-extracted proteins from UP-10 on a DEAE-Sephacel column. The volume of each fraction was 3.0 ml. P-1 fraction eluted at the void volume of the column, suggesting that the protein contained in this fraction is not adsorbed on the DEAE-Sephacel gel at pH 8.0.

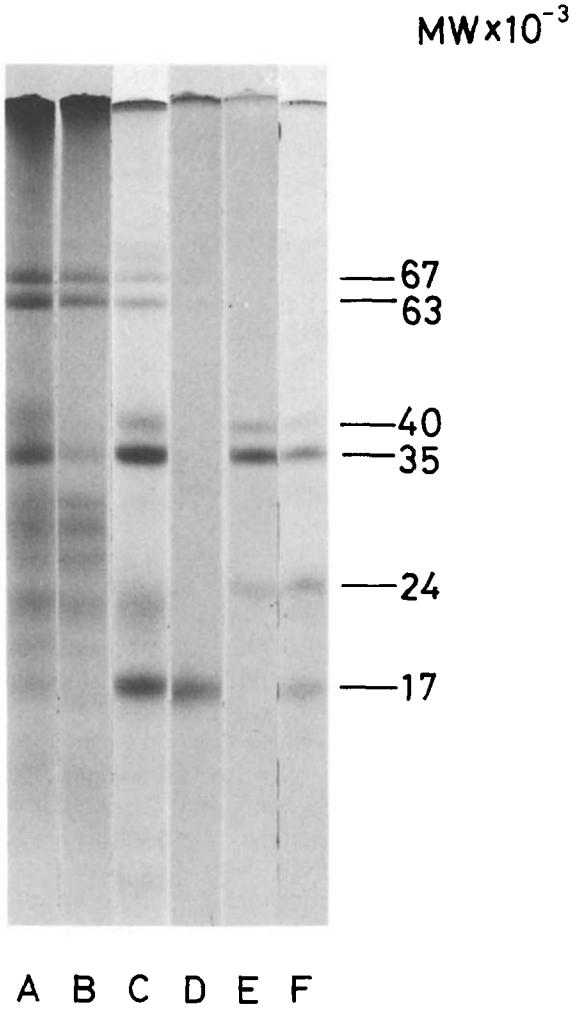

Fig. 2. SDS–polyacrylamide gel electrophoresis of the proteins obtained from UP-10 upon various treatments: (A) UP-10; (B,C) 144000 × *g* pellet and supernatant after cholate extraction of UP-10, respectively; (D,E) P-1 and P-4 fractions of DEAE-Sephacel column, respectively; (F) extracted proteins from inside-out thylakoid membranes by 0.8 M Tris washing. The M_r standards ($\times 10^{-3}$) were: bovine serum albumin (68); ovalbumin (43); α-RNA polymerase (39); α-chymotrypsinogen-A (25); soybean trypsin inhibitor (21.6); myoglobin (17.2); lysozyme (14.1); and ribonuclease A (13.7).

was provided by a 500 W xenon lamp focused on the sample chamber through an interference filter and a 10 cm water layer, while the assay for photo-induced electron transfer from DPC to DCIP was carried out spectrophotometrically by following DCIP reduction at 605 nm upon illumination with 680 nm light at 1.4 mW/cm^2.

3. RESULTS

Fig. 1 shows the elution profile of column chromatography monitored at 280 nm. Fig. 2 shows SDS–polyacrylamide gel electrophoresis of the proteins obtained from UP-10 upon different treatments. The proteins contained in the first peak fraction (P-1) of the DEAE-Sephacel column gave a single band on gel electrophoresis, indicating an app. M_r ~17000 (fig. 2, lane 4). About 1.5 mg of the purified, M_r 17000 protein was obtained from a UP-10 preparation containing 120 mg chl. The fourth peak fraction (P-4) contained mainly 3 kinds of proteins, 2 of which were identified as the M_r 23000 and 34000 proteins solubilized from the lumenal side of the thylakoid membrane in [10] with 0.8 M tris(hydroxymethyl)-aminoethane treatment (cf. lines 5,6 in fig. 2). Most remaining proteins solubilized from UP-10 by cholate were contained by some other peak fractions of the DEAE-Sephacel column.

Electron micrographs of negatively stained preparations of UP-10, RUP-10 formed in the presence of glycerol, and those of the cholate-extracted UP-10 are shown in fig. 3. Vesicular structures were observed in the UP-10 preparations, but not in the cholate-extracted UP-10. The sizes of the vesicles of UP-10 and RUP-10 are 0.1–1.0 μm.

Table 1 shows an example of photo-induced electron transfer from DPC to DCIP and shows the rates of oxygen evolution by UP-10 and RUP-10 preparations prepared under different conditions. Both the photo-induced electron transfer activity and oxygen evolution activity of UP-10 were ~70% of those of the original class 2 chloroplast prepara-

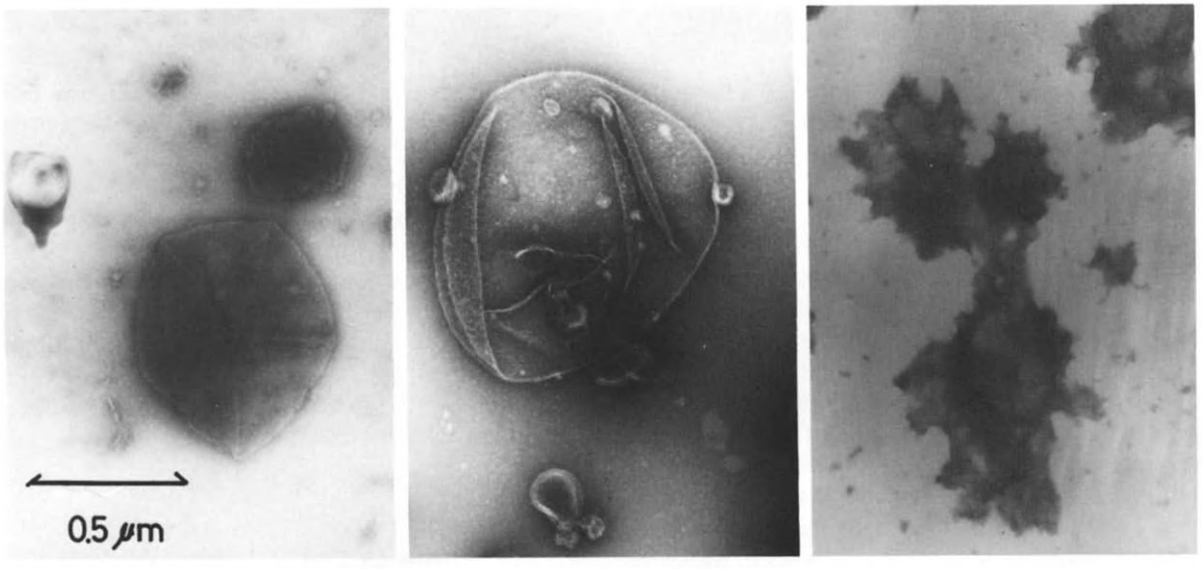

Fig. 3. Electron micrographs of negatively stained preparations of: (1) UP-10; (2) RUP-10; (3) the cholate-extracted UP-10.

Table 1

Effects of several cholate-extracted proteins from UP-10 and glycerol on electron transfer rates from DPC to DCIP and on oxygen evolution rates of RUP-10

Sample	Electron transfer from DPC to DCIP (μequiv.mg chl^{-1}.h^{-1})	Rate of oxygen evolution (μmol O_2.mg chl^{-1}.h^{-1})
UP-10	18.5	40.6
RUP-10 prepared without glycerol		
+ M_r 17000 protein	–	0
RUP-10 prepared with glycerol		
– additive	8.3	3.2
+ supernatant after cholate extraction of UP-10	–	8.5
+ M_r 17000 protein	8.9	11.8
+ P-2 fraction	–	1.5
+ P-4 fraction	–	4.2
+ M_r 17000 protein and P-2 fraction	–	3.0
+ M_r 17000 protein and P-4 fraction	–	19.3

All reaction mixtures contained 0.2 M sucrose, 3 mM $MgCl_2$, 20 mM tricine (pH 8.0). The mixture for the DPC to DCIP electron transfer contained 0.25 mM DPC, 0.05 mM DCIP and 0.025 mg chl/ml. The mixture for oxygen evolution contained 1 mM DCIP and 0.048 mg chl/ml. The experiments for the electron transfer and oxygen evolution were carried out under illumination by light of 680 nm at 1.4 mW/cm^2 and 2.1 mW/cm^2, respectively

tions. All RUP-10 preparations, including the samples prepared without any additives, exhibited reasonable rates of photosystem II activity measured as electron transfer from DPC to DCIP. However, significant photo-induced oxygen evolution was observed only in the samples prepared from the total cholate extracts or in the presence of the M_r 17000 protein. The samples prepared with the purified M_r 17000 protein, at a protein/chlorophyll ratio of 0.1 (w/w), usually represented 20–30% recovery of the oxygen evolution of the original UP-10 preparation, but in some cases, the recovery was as high as 60%. Although the solubilized proteins other than the M_r 17000 protein were unable to restore a significant oxygen evolution activity by themselves, the recovery of the oxygen evolution induced by the M_r 17000 protein seemed to be enhanced by the addition of a particular protein contained in the P-4 fraction of the DEAE-Sephacel column, presumably a M_r 34000 protein. However, recovery of the oxygen evolution induced by the M_r 17000 protein was inhibited by the addition of the polypeptides contained in the P-2 fraction of the DEAE-Sephacel column. Several proteins not derived from thylakoid membranes such as bovine serum albumin were tested but could not replace the M_r 17000 protein. The presence of glycerol in the incubation medium of the reconstitution process was one of the essential condition for restoring the oxygen evolution activity to RUP-10 (table 1). Photo-induced oxygen evolution was not observed with any sample prepared without glycerol in the incubation medium, even when the M_r 17000 protein had been added to the system.

4. DISCUSSION

The purified M_r 17000 protein exhibited almost the same M_r-value upon gel electrophoresis and on a Sephadex G-100 column, indicating that the native form of the protein is monomeric in aqueous solution. When photosystem II particles were treated at pH 9.5 [11], oxygen evolution was specifically inactivated, accompanied by the simultaneous release of 3 polypeptides having app. M_r

32000, 24000 and 15000. The last polypeptide might correspond to the M_r 17000 protein here. The purified M_r 17000 protein has no characteristic absorption between 300–800 nm and contains neither manganese nor iron, suggesting that this protein could not itself be the catalyst for water oxidation in the thylakoids. However, it may be a subunit of the catalyst or a component which is closely related to the electron transfer from water to the photo-oxidized photosystem II, because this protein was required for restoring the oxygen evolution activity in RUP-10, but not for the photo-induced electron transfer from DPC to DCIP. SDS–polyacrylamide gel electrophoresis clearly showed this protein to be distinct from both the M_r 23000 and 34000 proteins extracted from the lumenal side of the thylakoid membranes by Tris-treatment (fig. 2).

A possible cooperation of the M_r 17000 and 34000 proteins on oxygen evolution and the inhibition by the polypeptides present in the P-2 fraction remain open questions.

ACKNOWLEDGEMENTS

We would like to thank Miss Rieko Yogosawa for taking the electron micrographs. This work was supported in part by a Grant-in-Aid for Energy Research in Japan.

REFERENCES

[1] Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
[2] Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785.
[3] Wydrzynski, T. and Sauer, K. (1980) Biochim. Biophys. Acta 589, 56–70.
[4] Spector, M. and Winget, G.D. (1980) Proc. Natl. Acad. Sci. USA 77, 957–959.
[5] Winget, G.D. and Spector, M. (1981) in: Photosynthesis, vol. 2, pp. 453–464, Balaban International Science Services, Philadelphia PA.
[6] Nakatani, H.Y. and Barber, J. (1981) Photobiochem. Photobiophys. 2, 69–78.
[7] Newmark, P. (1981) Nature 293, 329.
[8] Sayre, R.T. and Cheniae, G.M. (1982) Plant Physiol. 69, 1084–1095.
[9] Toyoshima, Y. (1982) in: Research on Photosynthesis and Photoconversion of Solar Energy, pp. 155–158, The Ministry of Education, Science and Culture of Japan, Tokyo.
[10] Åkerlund, H. and Jansson, C. (1981) FEBS Lett. 124, 229–232.
[11] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.