The role of lipids and 17-kDa protein in enhancing the recovery of O_2 evolution in cholate-treated thylakoid membranes

Kozo Akabori, Akiko Imaoka and Yoshinori Toyoshima*

Faculty of Integrated Arts and Sciences, Hiroshima University, 1-1-89 Higashisenda-machi, Naka-ku, Hiroshima 730, Japan

Received 30 April 1984; revised version received 28 May 1984

The recovery of O_2 evolution of cholate-treated thylakoids induced by the simultaneous addition of the 17- and 23-kDa proteins was enhanced by the further addition of thylakoid total lipids up to about 75% of the non-depleted original broken thylakoids at the optimal concentration of the lipids, through reactivation of electron transfer between the sites of DPC donation and DCIP acceptance. Novel findings clarifing the cause of the discrepancies, with regard to the requirement for the 17-kDa protein on the O_2 evolution of thylakoid membranes, were also provided, i.e., when the assay of O_2 evolution for the reconstituted systems was carried out at a low level of NaCl (e.g., 0.1 mM), besides the 23-kDa protein, the 17-kDa protein was also required for O_2 evolution, however, at a higher level of NaCl (>5 mM) the 17-kDa protein was not. The results suggest that the 17-kDa protein takes the place of Cl^- or acts as a constituent protecting Cl^- in the Cl^- activating sites against fluctuation of the Cl^- level in the inner compartment of thylakoid membranes.

Photosystem II *O_2 evolution* *Reconstitution* *Thylakoid lipid* *17-kDa protein* *Cl^-*

1. INTRODUCTION

Over the past two years several studies to identify the constituents of the water-splitting enzyme complex in thylakoid membranes by means of the disintegration and reconstitution of O_2 evolution were reported [1–4]. From these and other studies, either all or a part of the 3 proteins (15–18, 23–24, 32–34 kDa; referred to as 17-, 23- and 34-kDa proteins, respectively) have been suggested to be associated with the oxygen-evolving enzyme complex [1,3–12].

Previously we reported the recovery of O_2 evolution up to about 40% of the original tylakoids by simultaneous reinsertion of the 17- and 23-kDa proteins in the cholate-depleted thylakoids, which throughout lost O_2 evolution activity, using an improved reconstitution method. We also showed enhancement up to about 70% by further addition of a particular unknown component involved in a filtrate of the supernatant obtained after cholate extraction of broken thylakoids, through an ultrafiltration membrane (Amicon Ultrafiltration Membrane Corns CF-25) [4].

However, there are some discrepancies regarding the requirements for the 17- and 23-kDa proteins. The inhibition of O_2 evolution in inside-out thylakoids, caused by washing at different NaCl concentrations, was reported to be directly proportional to the amount of 23-kDa protein released but not 17-kDa protein [1]. Others reported that the extraction of the 23-kDa protein completely inhibits O_2 evolution [8]. Authors in [13] showed that O_2 evolution activity is linearly correlated to

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol; PBQ, phenyl-*p*-benzoquinone; chl, chlorophyll; PS II, Photosystem II

* To whom correspondence should be adressed

Published by Elsevier Science Publishers B.V.

the amount of 23-kDa protein remaining in PS II particles after treatment with various concentrations of NaCl but the particles from which the 23-kDa protein was completely depleted still retained activity at about 40% of the original level [11], and the activity was recovered almost to the original level with 10 mM Ca^{2+}, in a similar manner to the rebinding of the 23-kDa protein [13]. They also claimed that the 17-kDa protein did not restore O_2 evolution in the presence or absence of the 23-kDa protein [11]. Recently, it was reported that O_2 evolution activity was restored, at about 30% of the original level, in the 1 M $CaCl_2$ washed PS II particles which totally lost the 17-, 23- and 34-kDa proteins concomitant with rebinding of the 34-kDa protein alone [14].

First, we report here that some lipid components involved in the supernatant obtained after cholate extraction of thylakoid membranes are responsible for enhancing the O_2 evolving activity in our reconstituted membrane system with the 17- and 23-kDa proteins.

Second, we provide novel findings to clarify the cause of the discrepancies with regard to the requirement for the 17-kDa protein, in connection with the effect of Cl^- on O_2 evolution.

2. MATERIALS AND METHODS

Broken thylakoid membranes, named UP-10, were prepared from spinach class 2 chloroplasts as in [3]. The 17- and 23-kDa proteins were isolated and purified as in [4]. All protein samples were dialyzed against buffer solution A (0.2 M sucrose, 20 mM Mops, 20 mM sodium cholate, pH 7.0, at 4°C) for 24 h, changing the buffer solution more than 4 times. Complete removal of Cl^- from each sample solution was confirmed by a test with silver nitrate.

Isolation of thylakoid total lipids was done as follows. Methanol and chloroform were added to the supernatant obtained after cholate extraction of UP-10 [4] ($H_2O/CH_3OH/CHCl_3$, 1:1:1, v/v/v), and the chloroform layer collected using a separatory funnel. The solvent was evaporated to dryness under reduced pressure. The resultant materials were dissolved in *n*-hexane/2-propanol (20:1, v/v) and applied to a Sepharose CL-6B column as in [15]. The thylakoid lipid fractions which are completely free from proteins and pigments (carotenoids, chl *a* and chl *b*), were collected and dried prior to use in reconstitution experiments.

Depletion and reconstitution of UP-10 were taken as follows. UP-10 was suspended in buffer solution B (0.2 M sucrose, 3 mM $MgCl_2$, 20 mM Tricine, 50 mM sodium cholate, 1 M NaCl, pH 8.4, at 4°C) at a concentration of 2 mg chl/ml and stirred on ice for 15 min, followed by centrifugation at 30 000 × *g*. The pellet was resuspended in the same solution to repeat the extraction. The final pellet, centrifuged at 30 000 × *g*, was well dispersed by buffer solution A (pellet/buffer, 1:10, v/v), followed by centrifugation at 30 000 × *g*, and suspended and homogenized in the same solution ([chl] ~4 mg/ml). To this suspension, either the purified 17- and 23-kDa proteins, the lipids, their combinations, or some other components, were added together with glycerol at a final concentration of 25 vol% and with buffer solution C (0.2 M sucrose, 20 mM Mops, pH 7.0, at 4°C) containing sodium cholate at a final concentration of 15 mM. After incubation for 2 h at 4°C under gentle shaking, the mixed dispersion was diluted about 50 times with buffer solution C to give a final [chl] of 8.3 μg/ml, and termed RUP-10. Control RUP-10 (CRUP-10) was also prepared by the same method, without the addition of either protein or lipids to the reconstitution medium.

The measurement of O_2 evolution was carried out with UP-10, RUP-10 and CRUP-10 samples at various Cl^- concentrations using a Teflon-covered oxygen electrode (Bionics Instruments) at 25 ± 0.1°C. PBQ was used as an electron acceptor at a concentration of 0.3 mM. Continuous illumination of saturating light (0.13 W/cm^2) between 600 and 800 nm was provided by a 750 W tungsten lamp through a pair of colored glass filters (Toshiba, R-60 and IRA-25S) and a 10 cm water layer. The assay for the protoinduced electron transfer reaction from DPC to DCIP and from H_2O to DCIP was carried out spectrophotometrically (using a stopped-flow–rapid-scan instrument; Union Giken RA-1300) by following DCIP reduction at 605 nm upon illumination with 600–800 nm light at a very low intensity (0.15 mW/cm^2).

3. RESULTS AND DISCUSSION

It was recently shown that simultaneous reinsertion of the 17- and 23-kDa proteins into the

cholate/salt-depleted thylakoid membranes only restored the rate of O_2 evolution up to 30–40% of the original thylakoids, and further addition of certain unknown low-M_r components contained in the cholate/salt extract increased it up to 70% [4]. To determine which component in the cholate/salt extract is responsible for enhancing the O_2 evolving activity of the RUP-10, several components contained in a filtrate of the supernate after cholate/salt extraction of UP-10, through an ultrafiltration membrane (Amicon, ultrafiltration membrane corns CF-25), were separated by SDS–PAGE, acetone treatment, column chromatography on Sepharose CL-6B and thin-layer chromatography on silica. Besides the 17- and 23-kDa proteins, the dominant components contained in the filtrate were found to be a protein with an apparent molecular mass of 13 kDa, pigments (carotenoids, chl *a* and chl *b*) and thylakoid lipids, and their effects on O_2 evolution were examined. The recovery of O_2 evolution induced by the 17- and 23-kDa proteins was enhanced by the addition of thylakoid lipids, but the addition of 13 kDa protein or carotenoids or their combinations did not show any observable effect on recovery.

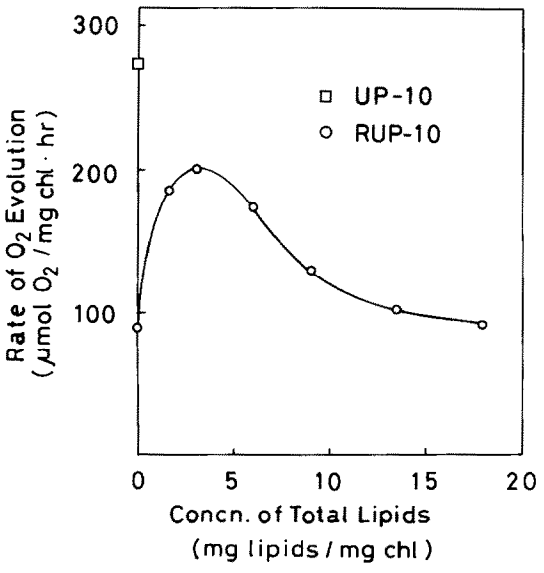

Fig.1. Effect of the amount of thylakoid total lipids on O_2 evolving activity of RUP-10. The amounts of the 17- and 23-kDa proteins are fixed at a 17-kDa protein/23-kDa protein/chl ratio of 0.45 : 0.60 : 1.0 (w/w/w). The assay was carried out in buffer solution C containing 20 mM NaCl.

The effect of thylakoid lipids on the recovery of O_2 evolution in RUP-10 is shown in fig.1. The recovery of O_2 evolution induced by simultaneous addition of the 17- and 23-kDa proteins without the total lipids was at a level of about 30% of original UP-10. Further addition of the total lipids increased the recovery of O_2 evolution of RUP-10 to about 75% of that of UP-10 at a concentration of 3 mg lipids/mg chl. Beyond this concentration the rates declined gradually with increasing lipid concentration. The effect of thylakoid lipids on an O_2 evolving PS II preparation has been reported, but the role of the lipids was not elucidated [16].

Table 1 shows the rates of photoinduced DCIP reduction with and without DPC and of O_2 evolution by UP-10, CRUP-10 and RUP-10 prepared under different conditions. With DPC as supplemental electron donor the rate of DCIP reduction by CRUP-10 was 26% of the rate in UP-10 with intact oxygen evolution. This rate was increased to 66% when reconstitution was carried out with total lipids. A similar stimulation was not observed in the rate of electron transfer from H_2O to DCIP and of O_2 evolution, i.e., the total lipids alone showed no significant effect on O_2 evolution. These results suggest that the total lipids mainly contribute to restoration of the electron transfer between the sites of DPC donation and DCIP acceptance. The addition of the total lipids to the 17- and 23-kDa proteins led to an increase in the rate of O_2 evolution from 32 to 73%. This result, together with those mentioned above, seems to indicate that without the total lipids, restoration of O_2 evolution by the 17- and 23-kDa proteins is only possible in PS II units in which electron transfer activity from DPC to DCIP still remains after the cholate/salt treatment. This conclusion is consistent with the results in [17] on $P680^+$ decay kinetics of systems similar to ours.

As the requirement of the total lipids for restoration of O_2 evolution in the RUP-10 was suggested, all the reconstitution experiments hereafter were carried out with total lipids at optimal concentration (3 mg lipids/mg chl) to determine the role of the 17-kDa protein in O_2 evolution.

Fig.2 shows the effect of NaCl concentration in the assay medium on the O_2 evolution of UP-10 and RUP-10 prepared under different conditions.

Table 1

Effect of the cholate-extracted components from UP-10 on electron transfer rates from DPC to DCIP and from H_2O to DCIP, and on oxygen evolution rates of CRUP-10 and RUP-10 prepared under different conditions

Sample	DCIP reduction (μequiv.·mg chl^{-1}·h^{-1})		Oxygen evolution (μmol O_2·mg chl^{-1}·h^{-1})
	With DPC	Without DPC	
UP-10	55	54	273
RUP-10 (17-, 23-kDa proteins and lipids)	47	38	202
RUP-10 (17- and 23-kDa proteins)	–	–	89
RUP-10 (lipids)	36	0	0
CRUP-10	14	0	0

All assays were carried out in buffer solution C containing 20 mM NaCl. Measurements of DCIP reduction were done at concentrations of 0.05 mM DCIP and 0.025 mg chl/ml with and without 0.25 mM DPC under illumination with 600–800 nm light at 0.15 mW/cm^2 and those of O_2 evolution at 0.3 mM PBQ and 8.3 μg chl/ml under illumination with saturating light of 600–800 nm (0.13 W/cm^2): RUP-10 (17-, 23-kDa proteins and lipids), 17-kDa protein/23-kDa protein/lipid/chl ratio of 0.45:0.60:3.0:1.0 (w/w/w/w); RUP-10 (17- and 23-kDa proteins), 17-kDa protein/23-kDa protein/chl ratio of 0.45:0.60:1.0 (w/w/w); RUP-10 (lipids), lipid/chl ratio of 3.0:1.0 (w/w)

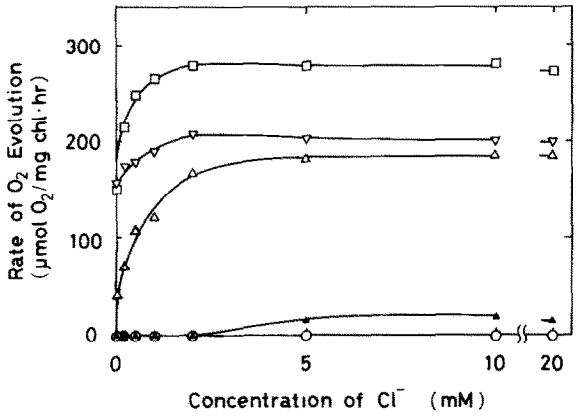

Fig.2. Effect of NaCl concentration on the O_2 evolving activity of UP-10 and RUP-10 prepared under various conditions: (□) UP-10 (the sample was washed with buffer solution C twice before assay); (▽) RUP-10 (17-, 23-kDa proteins and lipids), 17-kDa protein/23-kDa protein/lipid/chl ratio of 0.45:0.60:3.0:1.0 (w/w/w/w); (△) RUP-10 (23-kDa protein and lipids), 23-kDa protein/lipid/chl ratio of 0.60:3.0:1.0 (w/w/w); (▲) RUP-10 (17-kDa protein and lipids), 17-kDa protein/lipid/chl ratio of 0.45:3.0:1.0 (w/w/w); (○) RUP-10 (lipids), lipid/chl ratio of 3.0:1.0 (w/w). The assay was carried out in buffer solution C containing various amounts of NaCl.

At [NaCl] between 10 and 20 mM, both the RUP-10 prepared with the 23-kDa protein and total lipids (△) and RUP-10 with the 17- and 23-kDa proteins and total lipids (▽) exhibit large O_2 evolving activity (68 and 74%, respectively). However, the O_2 evolving activity of the former decreases with decreasing NaCl concentration in the assay buffer and falls to 15% when NaCl is not added to the assay buffer, while the activity of the latter remains almost constant independent of [NaCl] even in the range of less than 0.1 mM. The essentially similar, but more significant effect of the 17-kDa protein on O_2 evolution activity of the reconstituted system at low Cl^- level was obtained with PS II particles prepared using Triton X-100. The reconstituted PS II preparations from 1 M $CaCl_2$ washed PS II by the readdition of the 23- and 34-kDa proteins and of the 17-, 23- and 34-kDa proteins showed almost identical activity, e.g., 160 μmol O_2/mg Chl·h at 20 mM NaCl. At 0.5 mM NaCl, however, the activity of the former fell to 25 μmol O_2/mg Chl·h, while that of the latter remained almost at the same level, 140 μmol O_2/mg Chl·h. Furthermore, the reconstituted PS II with 34 kDa protein alone exhibited no significant O_2 evolution activity at 0.5 mM NaCl. This behavior may be the cause of the discrepancies between the requirement of the 17-kDa protein for O_2 evolution. As shown in our previous report, the assay of O_2 evolution of our reconstituted UP-10 was carried out in the absence of Cl^-, where besides the 23-kDa protein, the 17-kDa protein was

also required for O_2 evolution, while the assay in the other groups was done in the presence of 10–20 mM NaCl, where the 17-kDa protein was not required. It should be mentioned here that judging from SDS–PAGE, two extractions with 50 mM cholate and 1 M NaCl totally removed the 17- and 23-kDa proteins from UP-10, while the 34-kDa protein was retained to a considerable extent in the resulting pellet with which the reconstitution was carried out. This may explain why the RUP-10 prepared from the cholate and NaCl-treated UP-10 did not require the 34-kDa protein for recovery of O_2 evolution.

It has been shown tht photosynthetic O_2 evolution requires Cl^- as a cofactor [18–20], but understanding of its role is still speculative. The present results seem to suggest that the 17-kDa protein takes the place of Cl^-. However, it should be noted that if the 17-kDa protein has specific affinity to Cl^-, the purified 17-kDa protein used here might combine a trace amount of Cl^- even after complete dialysis against buffer solution A, although Cl^- was not detected in the 17-kDa protein solution by the test with silver nitrate. If this is the case, it may be possible that Cl^- bound to the 17-kDa protein is responsible for O_2 evolution and the 17-kDa protein acts, in vivo, as a constituent protecting Cl^- in the Cl^--dependent active sites of the water-splitting enzyme complex against fluctuation of the Cl^- level in the inner compartment of thylakoid membranes.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (58470136) and by special coordination funds from the Science and Technology Agency of Japan for promoting science and technology.

REFERENCES

[1] Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10.
[2] Sayre, R.T. and Cheniae, G.M. (1982) Plant Physiol. 69, 1084–1095.
[3] Toyoshima, Y. and Fukutaka, E. (1982) FEBS Lett. 150, 223–227.
[4] Fukutaka, E., Imaoka, A., Akabori, K. and Toyoshima, Y. (1983) FEBS Lett. 158, 217–221.
[5] Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268.
[6] Metz, J.G., Wong, J. and Bishop, N.I. (1980) FEBS Lett. 144, 61–66.
[7] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[8] Sandusky, P.O., DeRoo, C.L.S., Hicks, D.B., Yocum, C.F., Ghanotakis, D.F. and Babcock, G.T. (1983) in: The Oxygen Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp. 189–199, Academic Press, New York.
[9] Åkerlund, H.-E. (1983) in: The Oxygen Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp. 201–208, Academic Press, New York.
[10] Yamamoto, Y. and Nishimura, M. (1983) in: The Oxygen Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp. 229–238, Academic Press, New York.
[11] Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93.
[12] Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260.
[13] Miyano, M. and Murata, N. (1984) FEBS Lett. 168, 118–120.
[14] Ono, T. and Inoue, Y. (1984) FEBS Lett. 166, 381–384.
[15] Omata, T. and Murata, N. (1983) Plant Cell Physiol. 24, 1093–1100.
[16] Gounaris, K., Whitford, D. and Baber, J. (1983) FEBS Lett. 163, 230–234.
[17] Eckert, H.-J., Toyoshima, Y., Akabori, K. and Dismukes, G.C. (1984) submitted.
[18] Warburg, O. and Lüttgens, W. (1944) Naturwissenschaften 30, 301.
[19] Izawa, S., Heath, R.L. and Hind, G. (1969) Biochim. Biophys. Acta 180, 388–398.
[20] Critchley, C., Baianu, I.C., Govindjee and Gutowsky, H.S. (1982) Biochim. Biophys. Acta 682, 436–445.