Biochimica et Biophysica Acta 808 (1985) 323–327
Elsevier

BBA 41784

Characterisation of the Photosystem II polypeptides of *Anacystis nidulans* by trypsin digestion, Tris washing and lead incubation

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(Received January 22nd, 1985)

Key words: Photosystem II; Oxygen evolution; Pb^{2+} effect; (*A. nidulans*)

Mild trypsin digestion of *Anacystis nidulans* thylakoids results in the removal of peptides of 30 and 36 kDa over a similar time scale to the loss of oxygen evolution. A conformational change occurs in the pigment system over a longer time of incubation. Tris washing of thylakoids also removes the 36 kDa peptide together with peptides of 27, 15 and 12 kDa. No manganese is lost during trypsin digestion, about 25% by Tris washing and 58% by incubation with lead. No peptides are lost during lead incubation. Half the pool of Ca^{2+} is removed by Tris washing, or by washing with NaCl or Hepes. It is proposed that the 36, 27 and 15 kDa peptides are analogous to the 33/34, 24/25 and 16/18 kDa peptides of chloroplasts, and that the 12 kDa peptide is related to that of 36 kDa. It is also suggested that the 36 kDa is required for O_2 evolution and also linked to Ca^{2+} binding, but that none of these peptides is an Mn-binding protein. The role of Ca^{2+} is discussed.

Introduction

There is considerable current interest in the individual polypeptide functions with Photosystem II (PS II). The majority of published data concerns inside-out chloroplast thylakoids (e.g., Refs. 1 and 2) or PS II particles prepared from chloroplasts (e.g., Refs. 3–6). Three peptides have been implicated in the oxygen-evolving steps, namely peptides 32/33 kDa, 25 kDa and 16/18 kDa [1,2]. However, recent data suggest that the 32/33 kDa peptide is more closely involved in reactivation of $CaCl_2$-washed [4] or salt-washed PS II particles [5], and this peptide is also involved in the binding of Ca^{2+} to the thylakoid membrane. Franzen and Andreasson [8] have concluded that the 32/33 kDa peptide is not involved in the binding of Mn to the thylakoid as it can be selectively removed from chloroplasts by mild deoxycholate treatment which leaves the Mn pool unaltered. However, they conclude from ESR data that this peptide is required for rapid electron transfer to Z [9]. They also believe that the peptides 16/18 and 24/25 kDa are concerned with interconversion of the S-state cycle. In contrast, Miyao and Murata [7] found that the 16/18 kDa peptide was inessential to O_2 evolution and Ca^{2+} could be substituted for the 24/25 kDa peptide. Ca^{2+} has been implicated in the mechanism of oxygen evolution in chloroplasts [10] and more especially in cyanobacteria [11–13]. We have shown that Ca^{2+} is essential for preparation of active PS II particles from *Anacystis nidulans*, and, in the absence of Ca^{2+}, peptides of 30, 33 and 36 kDa are lost from the particles. We present data here on the effects of mild trypsin treatment and Tris-washing of thylakoids from *A.*

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Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PS II, Photosystem II; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea.

nidulans and compare these results with those gained from higher plant preparations.

Methods

Anacystis nidulans (Collection of Algae; UTEX 625; University of Texas, Austin, TX, U.S.A.) was a gift from Dr. N.G. Carr. The organism was maintained and grown, cell-free membrane preparations extracted and O_2-evolving PS II particles prepared as described previously [14].

Trypsin digestion was carried out by suspending thylakoids to a chlorophyll concentration of 0.2 mg Chl *a* per cm^3 in 0.4 M Sorbitol/10 mM $CaCl_2$/50 mM Hepes (pH 7.5). Trypsin was added to a final concentration of 10 μg per cm^3 and the thylakoids stirred gently at room temperature in the dark. Aliquots of 1 cm^3 were removed at specified times, and proteolysis halted by immediate addition of 200 $\mu g \cdot cm^{-3}$ soya bean trypsin inhibitor.

Tris washing of thylakoids and PS II particles was carried out by suspending the preparation to 0.2–0.3 mg Chl *a* per cm^3 in 0.8 M Tris (pH 8.0) for 20 min at 4°C followed by sedimentation of thylakoids and resuspension in preparation buffer. Electron-transport measurements were made by measuring O_2 evolution, using a Rank O_2 electrode under saturating white light with potassium ferricyanide as electron acceptor or by the reduction of DCIP (20 μM) at 590 nm in a Perkin Elmer 550 spectrophotometer. When O_2 evolution was inhibited, diphenylcarbazide (0.1 mM) was used as electron donor. Manganese was estimated using a Perkin Elmer atomic absorption spectrometer.

Polyacrylamide gel electrophoresis was performed using the method of Weber and Osborn [15]. Densitometric quantitation of proteins was made at 560 nm using a Perkin Elmer 5 spectrophotometer equipped with a gel scanning attachment.

Fluorescence was measured using a modular fluorimeter manufactured by Applied Photophysics Ltd., as previously described [16].

Chemicals were of Analar grade wherever possible, otherwise of the highest grade commercially available.

Results and Discussion

Figs. 1 and 2 summarise the results of a mild trypsin digestion of *A. nidulans* thylakoids. Fig. 1 shows that within 10 min incubation, 70% of electron-transport activity has been lost from water to an acceptor, either ferricyanide or DCIP. Between 10 and 20 min after incubation commenced, the thylakoids showed a three-fold increase in fluorescence over the whole of the emission spectrum, and between 0 and 20 min about 20% of electron-transport activity from the electron donor diphenylcarbazide to DCIP was lost. Fig. 2 shows scans of polyacrylamide gel electrophoresis of thylakoid samples taken periodically during the incubation period. After 5 min two polypeptides of 30 and 36 kDa were missing, correlating with the marked drop in light-induced oxygen evolution. Over 60 min incubation there was a reduction in a peptide of 27 kDa. No manganese was lost from the membranes throughout the experiment. This suggests that the peptides 30 and 36 kDa are required for electron-transport between water and Z, but do not bind manganese. A further alteration in the thylakoids must occur up to 20 min

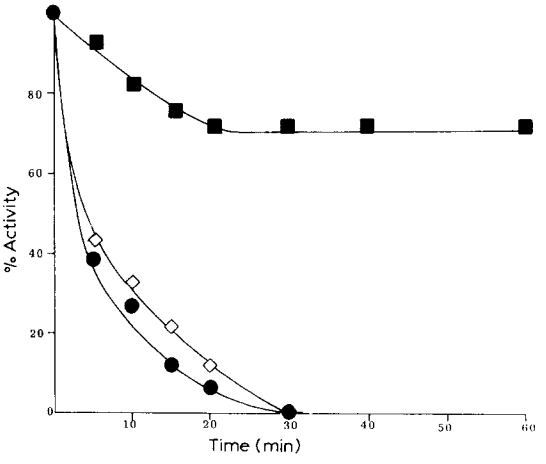

Fig. 1. Effect of mild trypsin treatment upon photosynthetic electron transport rates of thylakoid membranes, prepared from *Anacystis nidulans*. Thylakoid membranes (0.2 mg Chl *a* per cm^3) were treated with 10 $\mu g \cdot cm^{-3}$ trypsin. Proteolysis was halted by addition of 200 $\mu g \cdot cm^{-3}$ soya bean trypsin inhibitor. Electron transport rates were measured as described in methods. ●———●, electron transport $H_2O \rightarrow$ potassium ferricyanide; ◇———◇, electron transport $H_2O \rightarrow$ DCIP; ■———■, electron transport diphenylcarbazide → DCIP.

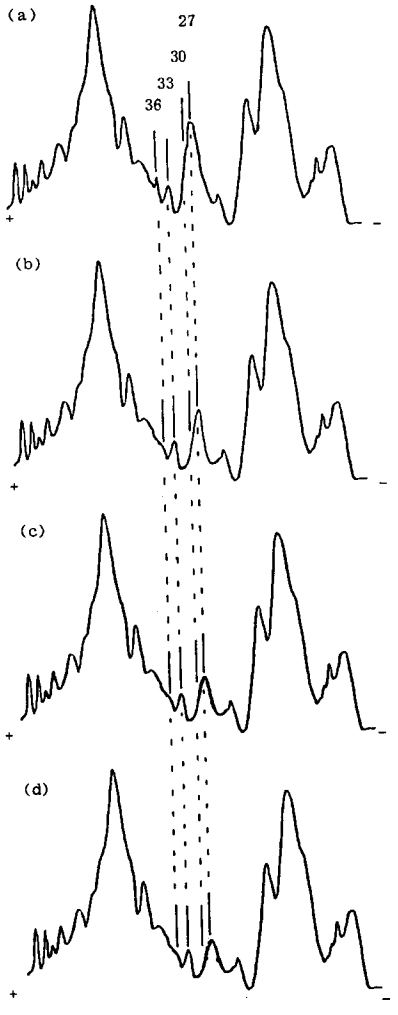

Fig. 2. Densitometer scans of the polypeptide profiles of thylakoid membranes after mild trypsin treatment. (a) Control; (b) 5 min trypsin incubation; (c) 30 min trypsin incubation; (d) 60 min trypsin incubation.

into the incubation to change the environment of the pigments, resulting in increased fluorescence. This, however, does not occur within the timescale of the inhibition of oxygen evolution, contradicting the suggestion [17] that conformational changes in phycobilisomes or phycobilisome detachment may be linked, in cyanobacteria, with oxygen evolution. In any case, PS II particles active in oxygen evolution can be prepared lacking phycobilisomes [13,14]. The increase in fluorescence may be the result of decreased efficiency of energy transfer to PS II reaction centers, causing the decrease in electron transport between diphenylcarbazide and DCIP.

Fig. 3 shows scans of polyacrylamide gel electrophoresis of Tris washed thylakoids together with controls. Four polypeptides have been washed out, namely 36, 27, 15 and 12 kDa. Only 25% of the Mn pool is removed by this treatment, but 50% of the bound Ca^{2+} is lost (Table I). Light-induced oxygen evolution is totally inhibited and electron transport between diphenylcarbazide and DCIP is reduced slightly. Table I also demonstrates that washing with NaCl or Hepes also removes Ca^{2+} from the thylakoids, and, in the former case about 10% of the Mn pool. In both cases there was inhibition of light-induced oxygen evolution. Some slight inhibition is, however, observed at electron transport between diphenylcarbazide and DCIP. Glycerol appears to protect the membranes against the effects of Hepes, which is one of many buffers which have well-documented chelation properties [18]. All electron transport rates were inhibited 100% by 20 μM DCMU.

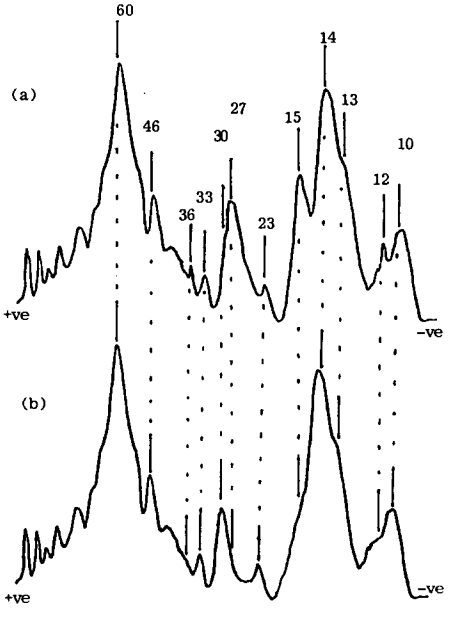

Fig. 3. Densitometer scans of the polypeptide profiles of thylakoid membranes after Tris-washing. Thylakoid membranes (0.2–0.3 mg Chl *a* per cm^3) were incubated in 0.8 M Tris (pH 8.0) for 20 min at 4°C. Electron-transport rates were measured as described in methods.

TABLE I

THE EFFECTS OF TRIS, NaCl OR HEPES WASHING ON LIGHT-INDUCED OXYGEN EVOLUTION Mn^{2+} AND Ca^{2+} OF *ANACYSTIS NIDULANS* THYLAKOIDS

O_2 evolution measured as described in Methods using 2 mM potassium ferricyanide as electron acceptor in a buffer, containing 25% (v/v) glycerol/50 mM Hepes (pH 7.5).

Treatment	O_2 evolution (μmol/mg Chl per h)	Diphenylcarbazide (0.1 mM)/DCIP (20 M) (μmol DCIP/mg Chl per h)	Mn^{2+} (μg/mg Chl)	Ca^{2+} (μg/mg Chl)
Control	230	5.0	1.14	6.0
Tris washed	0	4.8	0.74	2.95
NaCl-washed	25	5.0	0.89	3.14
Hepes washed	0	4.3	1.03	3.13

Conditions which remove up to 60% Mn from *A. nidulans* thylakoids are shown in Table II. Incubation with Pb^{2+} (0.5 mm) reduced the Mn pool to 42% of the control, and Ca^{2+} (0.5 mM) provided no significant protection against this loss. Light-induced oxygen evolution was completely inhibited, but electron transport between diphenylcarbazide and DCIP was stimulated. No proteins were lost from the thylakoids during incubation with Pb^{2+}.

It seems that these *A. nidulans* thylakoids are broadly comparable with the inside-out chloroplast thylakoids in their response to trypsin digestion and Tris washing [19]. Trypsin digestion of inside out chloroplast thylakoids releases the 33/34 kDa peptide, and peptides of 30 and 36 kDa in *A. nidulans*. Tris washing releases proteins of 33/34 and 23 kDa from inside out chloroplast thylakoids and 36, 27, 15 and 12 kDa in those of *A. nidulans*. Loss of light-induced oxygen evolution can be correlated with the loss of the 30 and 36 kDa peptides in *A. nidulans* from the trypsin digestion experiment. These peptides are also implicated in Ca^{2+} binding to the membrane as they were lost in the preparation of PS II particles lacking Ca^{2+} [13]. Only the 36 kDa peptide, not that of 30 kDa, was removed by Tris washing, suggesting that this is analogous to that of the 33/34 kDa peptide of chloroplasts. From our experiments this peptide is clearly not an Mn-binding protein as no Mn was lost during the tryptic digestion. This finding is in agreement with Ono and Inoue [4,20], Miyao and Murata [21] and Franzen and Andreasson [8]. The 33/34 kDa peptide is also implicated in Ca^{2+}-binding to the reaction centre of chloroplasts according to Ono and Inoue [4]. This washing of *A. nidulans* thylakoids also removed peptides of 27, 15 and 12 kDa. The first two are probably analogous to 24/25 and 16/18 kDa peptides of chloroplasts, as they are enriched in PS II particles [13], and the 12 kDa is probably related to the 36 kDa peptide. A lead-tolerant *A. nidulans* shows a marked increase in the 12 kDa peptide and concomitant reduction in that of 36 kDa [22]. We have, as yet, not identified a role for the peptides of 27 and 15 kDa, as conditions that remove these peptides also halve the pool of bound Ca^{2+} (Table I). Washing the thylakoids with Hepes, which does not remove any peptides, inactivates light-induced oxygen evolution presumably also by removing the Ca^{2+}. We therefore have some reservations about experiments in which a role has been ascribed to the 24/25 and 16/18 kDa peptides which have

TABLE II

THE EFFECTS OF INCUBATION WITH Pb ON LIGHT-INDUCED OXYGEN EVOLUTION AND Mn CONTENT OF *ANACYSTIS NIDULANS* THYLAKOIDS

Incubation for 30 min at 4°C in a buffer containing 25% (v/v) glycerol/50 mM Hepes (pH 7.5). O_2 evolution: using 2 mM potassium ferricyanide as electron acceptor as in Table I.

Treatment	O_2 evolution (μmol/min per mg Chl)	Mn^{2+} (μg/mg Chl)
Control	209	4.35
Incubation with 0.5 mM Pb^{2+}	0	1.82
Incubation with 0.5 mM Pb^{2+} + 0.5 mM Ca^{2+}	0	2.20

involved extraction procedures in which Ca^{2+} will also have been lost. It is noteworthy that Miyao and Murata have found that Ca^{2+} reconstituted activity to PS II particles from which the 24/25 kDa peptide had been removed [7]. Also, a recent paper by Ghanotakis et al. [23] suggests that Ca^{2+} is essential to reconstitution of oxygen evolution even when the 16/18 and 24/25 kDa proteins are replaced.

Incubation with Pb^{2+} removes over 50% of the Mn pool from *A. nidulans* thylakoids, but with no loss of any peptides. Exchange of Pb^{2+} and Mn is not 1 : 1, so it seems that the Pb^{2+} binds to proteins around the Mn-binding site, causing the Mn-binding conformation to be lost. We suggest that this Mn is not coordinated to any single peptide of those discussed, but located at a position in the membrane such that the correct conformation of the active site requires the 36 kDa peptide and Ca^{2+}. It may be that under certain extraction conditions this Mn can be bound on to this peptide [24], but this is not generally the case.

In common with other workers (e.g., Ref. 18) we find that electron transport between diphenylcarbazide and DCIP is not inhibited by any of the treatments. However, it is worth remembering that a second site of action for Ca^{2+} must exist, as PS II particles prepared without Ca^{2+}, and incubated with Pb^{2+}, show a stimulation of electron transport between diphenylcarbazide and DCIP [13].

Acknowledgements

We thank the Science and Engineering Research Council for financial support.

References

1 Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10
2 Fukutaka, E., Imaoka, A., Akabori, K. and Toyoshima, Y. (1983) FEBS Lett. 158, 217–221
3 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539
4 Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286
5 Nakatani, H.Y. (1984) Biochem. Biophys. Res. Commun. 120, 299–304
6 Wensink, J., Dekker, J.P. and Van Gorkom, H.J. (1984) Biochim. Biophys. Acta 765, 147–155
7 Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120
8 Franzen, L.-G. and Andreasson, L.-E. (1984) Biochim. Biophys. Acta 765, 166–170
9 Andreasson, L.-E., Franzen, L.-G., Hansson, O. and Vånngard, T. (1984) Abstracts of the 8th International Congress on Biophysics, Bristol, U.K., No. 205, p. 149
10 Yerkes, C.T. and Babcock, G.T. (1981) Biochim. Biophys. Acta 64, 19–29
11 Brand, J.J. (1979) FEBS Lett. 103, 114–117
12 Yu, C. and Brand, J.J. (1980) Biochim. Biophys. Acta 59, 483–487
13 England, R.R. and Evans, E.H. (1983) Biochem. J. 210, 473–476
14 England, R.R. and Evans, E.H. (1981) FEBS Lett. 134, 175–177
15 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4416
16 Pullin, C.A., Brown, R.G. and Evans, E.H. (1979) FEBS Lett. 101, 110–112
17 Stewart, A.C. and Larkum, A.W.D. (1983) Biochem. J. 210, 583–589
18 Kapoor, R.C., Jailwal, J.K. and Kishan, J. (1978) J. Inorg. Nucl. Chem. 40, 155–158
19 Åkerlund, H.-E., Jansson, Ch. and Andersson, B. (1981) in Photosynthesis III Structure and Molecular Organisation of the Photosynthetic Apparatus (Akoyunoglou, G., ed.), pp. 77–83, Balaban International Science Services, Philadelphia, PA
20 Ono, T.-A. and Inoue, Y. (1983) FEBS Lett. 164, 255–260
21 Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354
22 England, R.R. and Evans, E.H. Plant Sci. Lett., in the press
23 Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173
24 Abramowicz, D.A. and Dismukes, G.C. (1984) Biochim. Biophys. Acta 765, 309–317