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EDTA-INDUCED RELEASE OF MANGANESE AND PROTEINS FROM INSIDE-OUT THYLAKOID VESICLES AND THE INHIBITION OF OXYGEN EVOLUTION

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Summary: Washing of inside-out, but not right-way-round, pea chloroplast thylakoid vesicles with 2 mM EDTA inhibits O_2 evolution. Artificial electron donor/acceptor studies indicate that the site of inhibition is on the oxidising side of photosystem two (PS2), a conclusion reinforced by chlorophyll fluorescence measurements. Evidence is presented that the EDTA inhibition of O_2 evolution is linked partly to the removal of one Mn atom per PS2 reaction centre and partly to the removal of extrinsic membrane proteins having apparent molecular weights between 58 and 70 kdaltons.

It has been clearly demonstrated by several workers that the treatment of isolated chloroplast thylakoid membranes and sub-chloroplast particles with high levels of Tris(hydroxymethyl)aminomethane (Tris) buffer at alkaline pH (1-3) and also other treatments (4,5) not only inhibit O_2 evolution but induce the release of polypeptides having apparent molecular weights of about 34, 24 and 15 kdaltons. These findings have led to the speculation that one or all of these polypeptides are involved in some way in the O_2 evolving process. The validity of this conclusion is, however, open to question because it has recently been shown (6,16)that the O_2 evolving capacity of well washed Tris treated inside-out thylakoid vesicles can be partially restored by the "dark reactivation" procedure of Yamashita et al. (7) which involves addition of reduced dichlorophenol indophenol (DCIP) or hydroquinone (HQ) to the inhibited membranes. The use of washed inside-out vesicles ensures that any proteins released from the O_2 evolving system by Tris treatment or any other components, such as manganese

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Abbreviations: PS2, Photosystem 2; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1-5 diphenyl carbazide; DCIP, 2,6 dichlorophenol indophenol; EDTA, ethylenediaminetetra-acetic acid (disodium salt); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

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(8,9), are not available for reincorporation during the reactivation process. Therefore these results indicate that O_2 evolution may not require the Tris removable proteins as has been suggested.

In this communication we report new observations regarding the relationship between inhibition of O_2 evolution and the release of membrane proteins. The approach adopted involves the use of inside-out thylakoid vesicles which expose the O_2 evolving components to the bulk medium rather than using normally orientated thylakoid membranes which have their O_2 evolving components on the inner lumenal surface (10). The inverted membranes have been subjected to EDTA washing to remove firmly bound manganese and surface proteins, and the removal of these components related with changes in O_2 evolving capacity.

METHODS

Chloroplast thylakoids were isolated from peas (Feltham First) as previously described (11), except the intact chloroplast fraction was subjected to osmotic shock. The membranes were then washed twice in an EDTA solution (0.1 M sucrose, 2 mM EDTA, 2 mM Tris-base brought to pH 8.0 with HCl) for 5 and then 20 minutes to remove extrinsic proteins from the outer surface and loosely associated manganese. Before subjecting the washed intact thylakoids to Yeda Press treatment they were incubated for 30 minutes at 0°C in a high salt medium (0.15 M NaCl, 50 mM Na_2HPO_4 brought to pH 7.5 with NaH_2PO_4) in order to induce membrane stacking. The details of the Yeda Press treatment and subsequent phase separation of inside-out (B3) and right-way-round (T3) vesicles have been given by Andersson and Akerlund (12). For EDTA treatment the two types of vesicles were subjected to 30 minutes incubation with the above EDTA containing solution. Control samples were subjected to the same washing procedures but uisng a low salt phosphate buffer (0.1 M sucrose, 5 mM NaCl, 10 mM Na_2HPO_4, pH 7.4 with NaH_2PO_4).

Manganese determinations were made on samples suspended in low salt phosphate buffer (made with deionised-distilled water) made to 1% HNO_3, using the flameless attachment (HGA 400) or a Perkin-Elmer Model 2280 atomic absorption spectrophotometer. Rates of O_2 evolution were measured at 18°C with a Rank O_2 electrode supplied with saturating white light and using membranes suspended in the low salt phosphate buffer in the presence of 0.5 mM p-benzoquinone as the electron acceptor. Rates of DCIP reduction were determined optically using a Perkin-Elmer 557 spectrophotometer with 600 nm measuring light and actinic light (100 J $m^{-2}s^{-1}$) transmitted by a Balzer 667 nm interference filter. Chlorophyll fluorescence was measured as previously described (13). EDTA removed polypeptides were subjected to polyacrylamide gel electrophoresis (PAGE) using a 7-17% linear gradient containing 0.1% sodium dodecyl sulphate (SDS). Before electrophoresis, the supernatants were passed through 0.22 μM Millipore filters until no chlorophyll could be detected in the extract. The proteins were then concentrated using a 10 kdalton cut-off filter and subjected to 20 minutes pre-digestion at room temperature in a medium containing: 20% glycerol, 4% SDS, 5% mercaptoethanol, 0.002% bromophenol blue and 0.125 M Tris-Cl at pH 6.8. Gels were run at 15 mA overnight at room temperature and were stained with 0.04% (w/v) coomassie blue in a 25% (v/v) isopropanol, 10% (v/v) acetic acid solution. Following destaining with 10% acetic acid, the gels were scanned using a Gilford apparatus linked to a Beckman U.V./Vis spectrophotometer.

Chlorophyll was determined by the method of Arnon (14).

RESULTS

Table 1 shows the effect of thirty minutes incubation with 2 mM EDTA on the Mn levels and the O_2 evolution rates of inside-out and right-way-round thylakoid membrane vesicles. It can be seen that almost 80% loss of O_2 evolving capacity occurred with the inverted membranes whereas normally orientated vesicles were barely affected. Moreover, the EDTA wash removed about 1 Mn per 200 chlorophyll *b* molecules from the inside-out vesicles but had less effect on the Mn content of right-way-round membranes. The inhibition of electron flow could be restored by introducing the photosystem two (PS2) artificial electron donor, diphenylcarbazide (DPC) as detected by an increase in the rate of DCIP reduction (see Fig. 1). This result suggests that the EDTA induced inhibition is likely to be on the oxidising side of PS2 at some position between the site of water oxidation and reduction of the photo-oxidised reaction centre chlorophyll $P680^+$. This suggestion was reinforced by chlorophyll fluorescence measurements. In Fig. 2 it can be seen that in the presence of DCMU, the fluorescence rose to the same maximum level (as indicated by dithionite addition) with both EDTA treated and control inside-out vesicles except the rate of the rise was slower in the inhibited sample. This result indicates that the action of EDTA is not on the acceptor side but supports the notion that inhibition is occurring in the electron transport chain between H_2O and $P680^+$.

TABLE 1 EFFECT OF EDTA-WASHING ON PHOTOSYNTHETIC OXYGEN EVOLUTION AND MANGANESE CONTENT OF INSIDE-OUT AND RIGHT-SIDE-OUT VESICLES.

Sample	Treatment	O_2 Evolution ($\mu eq.\ mg\ chl.^{-1}.\ hr^{-1}$)	Mn/200 chl *b*
Inside-out vesicles	Control washed	177 ± 33	5.36 ± 0.46
Inside-out vesicles	EDTA-washed	41 ± 7	3.91 ± 0.40
Right-side-out vesicles	Control washed	160 ± 22	5.63 ± 0.34
Right-side-out vesicles	EDTA-washed	152 ± 7	5.22 ± 0.55

Results ± SD (7 samples). Chl. a/Chl. b ratios: inside-out vesicles, 2.0-2.2; right-side-out vesicles, 2.5-3.0.

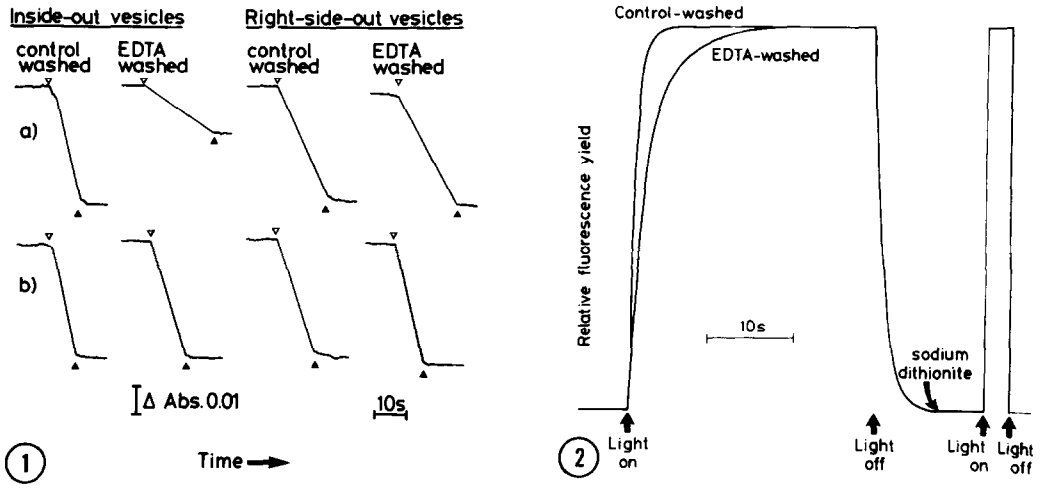

Fig. 1 DCIP reduction by (a) electrons derived from water, (b) as for (a) but supplemented with 5 mM DPC. Reaction medium 10 µg chl. cm^{-3}, 0.1 M sucrose, 5 mM NaCl, 10 mM Na_2HPO_4 at pH 7.4 with NaH_2PO_4, 30 µM DCIP.

Fig. 2 Chlorophyll fluorescence induction curves of control washed and EDTA washed inside-out vesicles in presence of 10 µM DCMU using low light conditions (1 J m^{-2} s^{-1}). No electron donors or acceptors were added. The $t_{\frac{1}{2}}$ for rise is 0.9s for control and 1.9s for EDTA washed membranes.

EDTA effect could be due to the loss of a crucial amount of Mn as indicated by the reduction of 1 Mn/200 chl *b* as shown in Table 1. As Fig. 3 shows, addition of Mn to the EDTA treated inside-out vesicles did bring about a stimulation of the inhibited rate with the maximum effect at about 0.5 mM Mn. This stimulation seemed to be specific to Mn and was not, for example, observed

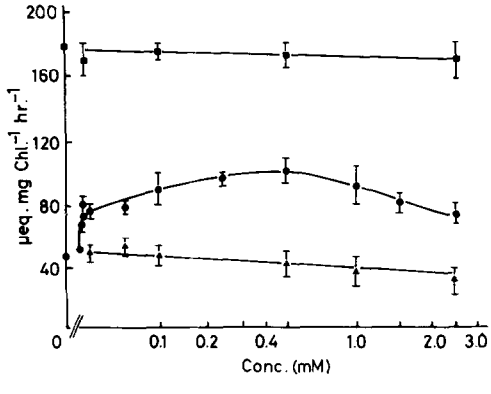

Fig. 3 Effect of $MnCl_2$ (■●) and $MgCl_2$ (▲) on O_2 evolution rates (with 0.5 mM p-benzoquinone as the electron acceptor) from control washed (■) and EDTA washed (■▲) inside-out vesicles. The concentration of salts is expressed on a logarithmic scale.

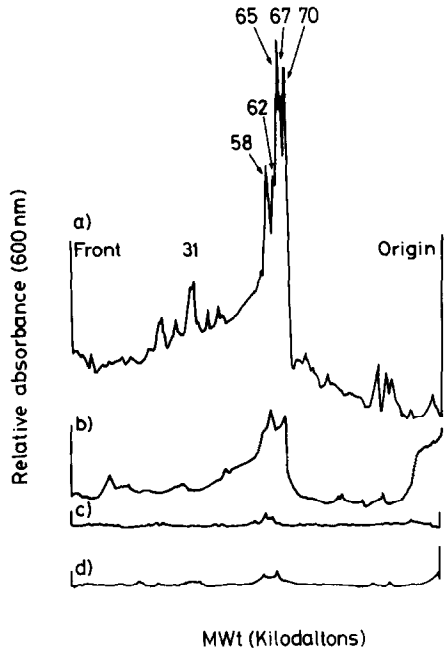

Fig. 4 Scan of coomassie blue stained SDS-polyacrylamide gel using 600 nm showing various polypeptides released from inside-out (a,c) and right-way-round (b,d) vesicles by EDTA (a,b) and control (c,d) washes.

with comparable levels of Mg (see Fig. 3). However, at best the stimulated rate did not reach that of the control untreated vesicles.

The possibility that EDTA washing not only removed Mn, but also proteins, was investigated. Fig. 4 shows the density scan of a coomassie blue stained gel obtained by subjecting the supernatant of the EDTA washed inside-out and right-way-round vesicles to PAGE. After centrifugation the supernatant was passed five times through 0.22 μm Millipore exclusion filters and then concentrated using a 10 kdalton cut-off filter. The supernatants of the control washed untreated vesicles were also subjected to the same procedures. Identical amounts of chlorophyll were used in all experiments so that the traces are comparable in terms of amounts of material subjected to the washing procedures. Using PAGE, the supernatant of the EDTA treated inside-out vesicles yielded a series of bands corresponding to apparent molecular weights ranging from 58 to 70 kdaltons (see Fig. 4). A minor band appeared at 31 kdaltons. Weaker bands in the same molecular weight regions were also obtained from the

TABLE 2 STIMULATION OF O_2 EVOLUTION FROM EDTA-WASHED INVERTED THYLAKOIDS BY RECONSTITUTION WITH A CRUDE PROTEIN EXTRACT

Sample	O_2 Evolution $\mu eq.mg\ chl^{-1}.hr^{-1}$	%
Washed-control	174 ± 6	100
EDTA-washed	40 ± 8	23
EDTA + protein extract	55 ± 11	32
EDTA + 10% glycerol	45 ± 10	26
EDTA + protein extract + 10% glycerol	104 ± 10	60
EDTA + BSA + 10% glycerol	60 ± 7	35

Rates ± SD (4 samples), BSA was added to give 50 µg protein cm^{-3}. Membranes were incubated in the dark for 20 minutes with the various additives and then recovered by centrifugation before measuring O_2 evolution.

EDTA washing of right-way-round membranes but these may be attributable to the contamination of this fraction by inside-out vesicles (10).

To test whether the removable polypeptides were involved in the EDTA inhibition of O_2 evolution, reconstitution experiments were conducted. A concentrated supernatant was added back to EDTA inhibited inside-out vesicles and no significant stimulation was observed until 10% glycerol was included in the incubation medium (see Table 2). In the presence of this agent, which enhances hydrophobic interactions (15), 60% of the control rate was obtained. A comparable stimulation was not observed with glycerol alone or with glycerol plus bovine serum albumin added as an arbitrary protein.

CONCLUSION

These preliminary investigations indicate that EDTA inhibition of O_2 evolution from inside-out thylakoid vesicles involves loss of several extrinsic membrane proteins and one manganese atom per PS2 reaction centre (assuming that there are approximately 200 chlorophyll *b* molecules per P680). Inhibition of O_2 evolution and the concomitant loss of components only occurred with inside-out vesicles where EDTA had ready access to the inner thylakoid membrane surface. Partial restoration of the O_2 evolving capacity was possible by adding back either low levels of Mn or proteins removed by the EDTA treatment. Further analyses of these effects will require separation and characterization of the

various EDTA released proteins in order to identify which components are active. Also, the synergistic action of Mn and the active proteins will need to be investigated to clarify any inter-relationship which may exist.

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REFERENCES

1. Akerlund, H-E. and Jansson, C. (1981) FEBS Lett. 124, 229-232.
2. Akerlund, H-E. Jansson, Ch. and Andersson, B. (1981) Proc. 5th Int. Cong. Photosynthesis (ed. G. Akoyunoglou) pp. 77-84. Pub. Balaban Int. Sci. Services, Philadelphia.
3. Yamamoto, Y., Doi, M. Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265-268.
4. Akerlund, H-E., Jansson, Ch. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1-10.
5. Kuwabara, T. and Murata, N. (1982) Plant and Cell Physiol. 23, 533-539.
6. Mansfield, R.W. and Barber, J. (1982) FEBS Lett. 140, 165-168.
7. Yamashita, T., Tsuji, J. and Tomita, G. (1971) Plant and Cell Physiol. 12, 117-126.
8. Cheniae, G.M. (1970) Ann. Rev. Plant Physiol. 21, 467-498.
9. Radmer, R. and Cheniae, G. (1977) in: Primary Processes of Photosynthesis (Barber, J. ed.) Topics in Photosynthesis Vol. 2, pp. 303-348, Elsevier Biomedical, Amsterdam, New York.
10. Andersson, B. (1978) Thesis, University of Lund, Sweden.
11. Nakatani, H.Y. and Barber, J. (1977) Biochim. Biophys. Acta 461, 510-512.
12. Andersson, B. and Akerlund, H-E. (1978) Biochim. Biophys. Acta 503, 462-472.
13. Barber, J., Chow, W.S., Scoufflaire, C. and Lannoye, R. (1980) Biochim. Biophys. Acta 591, 92-103.
14. Arnon, D.I. (1949) Plant Physiol 24, 1-15.
15. Stewart, A.C. and Bendall, D.S. (1981) Biochem. J. 194, 877-887.
16. Henry, L.E., Moller, B.L., Andersson, B. and Akerlund H-E. (1982) Carlsberg Res. Commun. 47, 187-198.