RELEASE OF POLYPEPTIDES FROM HIGHLY ACTIVE O_2-EVOLVING PHOTOSYSTEM-2 PREPARATION BY TRIS TREATMENT

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1. Introduction

Oxygen evolution in higher plants is thought to be mediated by an enzyme system containing membrane-bound manganese where positive charges might be accumulated by illuminating the chloroplasts and oxidation of water occurs subsequently [1–3]. The enzyme system is so labile that the isolation and characterization of the enzyme complex have not met much success. Several attempts were made to identify the polypeptide(s) involved in the O_2-evolution enzyme complex by SDS–PAGE, using plant and algal mutants with deletions in PS-2 [4–6]. These results showed a total or partial loss of polypeptides or a change in the electrophoretic mobility of 32–36 X 10^3 M_r polypeptide bands, associated with the mutation. An Mn-protein of 65 X 10^3 M_r isolated by cholate treatment of chloroplasts was also reported to be involved in the O_2-evolution enzyme [7].

Here, we isolated a highly active O_2-evolving PS-2 preparation from spinach chloroplasts using a low concentration of digitonin and Triton X-100, and examined the effect of Tris treatment on the preparation. The subsequent release of polypeptides from the PS-2 preparation was analysed by SDS–PAGE and the result was compared with that obtained by Tris treatment of unfractionated broken cloroplasts.

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DCIP, 2,6-dichloroindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PS-1(2), photosystem 1(2); CP 1, chlorophyll- protein complex 1; CF_1, chloroplast coupling factor; chl, chlorophyll; RuBP carboxylase, ribulose-1,5-bisphosphate carboxylase; M_r, relative molecular mass

2. Materials and methods

2.1. *Preparation of highly active O_2-evolving PS-2 subchloroplasts from spinach chloroplasts*

An O_2-evolving PS-2 preparation was obtained by treating spinach chloroplasts with a low concentration of digitonin and Triton X-100. Broken chloroplasts prepared as in [8] were incubated with 0.25% digitonin (digitonin:chl, 5:1,w/w) for 5 min with stirring at 4°C. After the digitonin treatment, the suspension was diluted 10-fold with a buffer medium containing 0.33 M sorbitol, 10 mM sodium pyrophosphate and 4 mM $MgCl_2$ (pH adjusted to 6.5 with HCl), and centrifuged at 10 000 X *g* for 30 min. The pellet suspended in the same buffer medium was then incubated with 0.2% Triton X-100 (Triton X-100:chl, 4:1,w/w) for 5 min. The suspension was diluted 10-fold with the buffer after the Triton treatment and centrifuged at 12 000 X *g* for 30 min. A PS-2 subchloroplast preparation was obtained as a dark-green pellet. The preparation procedure and characteristics of the preparation will be detailed in [9].

2.2. *Tris treatment*

The broken chloroplasts and the PS-2 preparation (0.1 mg chl/ml) were incubated with 0.8 M Tris–HCl (pH 8.4) for 10 min at 4°C. The suspension was centrifuged at 20 000 X *g* for 1 h and the supernatant was separated from the pellet. Before being applied to SDS–PAGE, the supernatant was concentrated using an Amicon ultrafiltration cell model 52.

2.3. *O_2 assay*

O_2 evolution and PS-1-dependent O_2 uptake were measured with a Rank oxygen electrode. The assay medium for O_2 evolution (2 ml) contained 0.5 mM

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p-benzoquinone and 5 mM NH_4Cl in the above buffer medium. O_2 uptake was assayed in the presence of 0.1 mM methylviologen, 50 μM DCIP, 0.5 mM sodium ascorbate, 0.5 mM sodium azide and 10 μM DCMU in the buffer. Broken chloroplasts and subchloroplast preparations were used at 10 μg chl/ml. Red actinic light was provided by a 650 W projector with a Toshiba R-62 filter. Temperature of the reaction mixture was adjusted to 20°C by circulating thermostatted water around the reaction vessel.

2.4. *SDS–polyacrylamide gel electrophoresis*

Samples were solubilized for 30 min in a solution containing 65 mM Tris–HCl (pH 9.8), 12% sucrose, 2% β-mercaptoethanol and 2% SDS at 1 mg chl/ml. Analysis of polypeptides using SDS–PAGE was done as in [10]. Electrophoresis was performed at a constant current (10 mA) for 5 h at 4°C in a slab-gel apparatus (140 × 140 mm, 1 mm thick) using a stacking gel (6% acrylamide) and a resolving gel (15–7.5% gradient acrylamide). The gel stained by Coomassie brilliant blue R 250 was analyzed by a Toyo digital densitometer DMU-33c.

3. Results

3.1. *Measurements of O_2 evolution and O_2 uptake in PS-2 preparation*

Fig.1 shows the time-courses of O_2 evolution and PS-1-dependent O_2 uptake in the broken chloroplasts and PS-2 preparation. The PS-2 preparation lost O_2-uptake activity completely, but still had a high O_2-evolution activity equivalent to 20% of that observed in NH_4Cl-uncoupled broken chloroplasts. The O_2-evolution activity in the PS-2 preparation was inhibited by DCMU or 0.8 M Tris treatment, suggesting its PS-2 nature.

3.2. *Assay of polypeptides released from membrane of broken chloroplasts and PS-2 preparation after Tris washing*

Fig.2 shows the densitometric patterns of SDS–PAGE of broken chloroplasts and the PS-2 preparation before and after the 0.8 M Tris treatment. The Tris treatment of broken chloroplasts released 52, 34 and 16 × 10^3 M_r polypeptide bands. In the PS-2 preparation, the amounts of CP 1 and large subunits of CF_1 which showed strong bands in the high M_r region of SDS- PAGE in chloroplasts [11,12] were

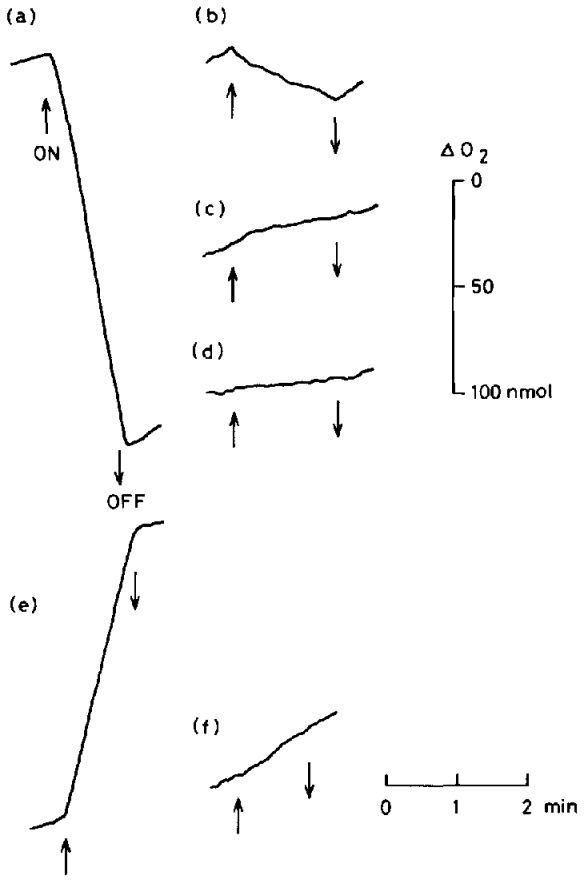

Fig.1. Time-courses of O_2 evolution and PS-1-dependent O_2 uptake in broken chloroplasts and PS-2 subchloroplast preparation. O_2 evolution in (a) broken chloroplasts, (b) PS-2 preparation, (c) PS-2 preparation with 10 μM DCMU and (d) Tris-washed PS-2 preparation. O_2 uptake in (e) broken chloroplasts and (f) PS-2 preparation.

reduced significantly. The Tris treatment of the PS-2 preparation removed 3 polypeptides from the membrane of 33, 24 and 18 × 10^3 M_r. A small band observed at 28 × 10^3 M_r in the supernatant of Tris-washed PS-2 preparation might be due to a slight contamination of the depleted PS-2 preparation that was not separated from the supernatant by the centrifugation at 20 000 × *g* for 1 h.

4. Discussion

The Tris treatment of chloroplasts releases manganese from the O_2-evolution enzyme system and

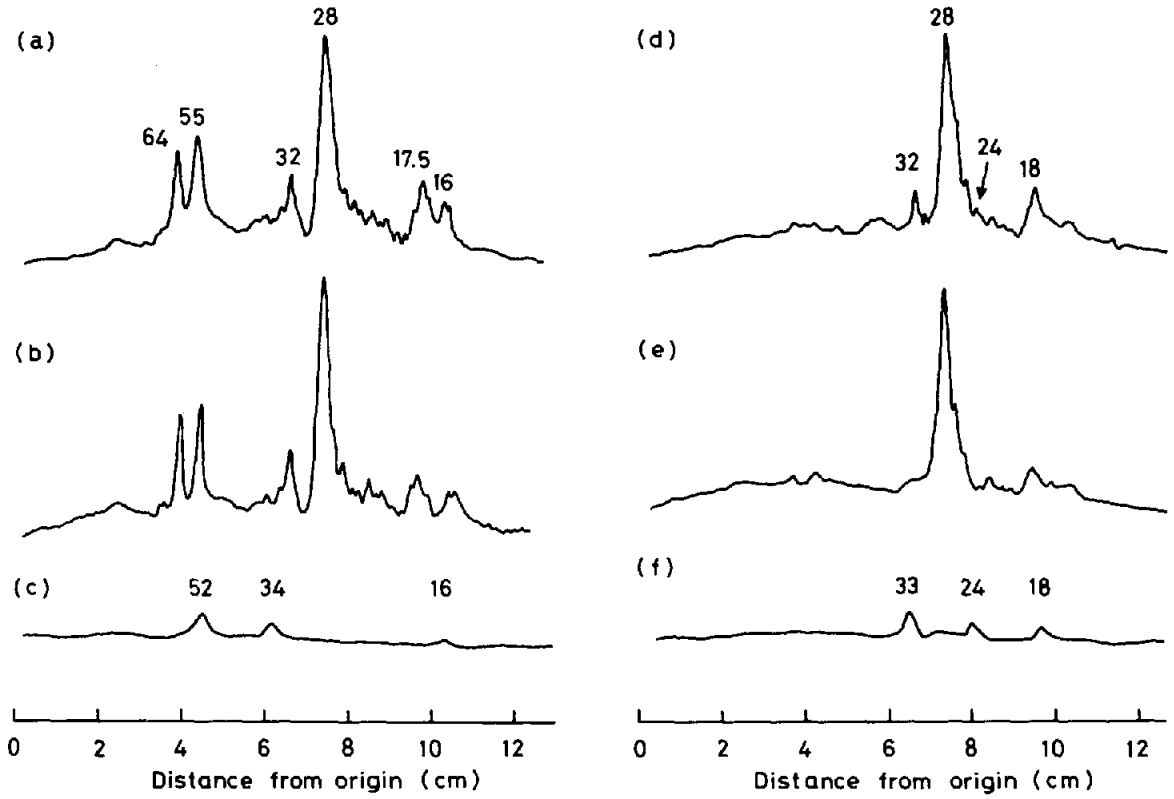

Fig.2. Densitometric traces of SDS–PAGE before and after Tris treatment of broken chloroplasts and PS-2 preparation: (a) broken chloroplasts; (b) Tris-washed broken chloroplasts; (c) supernatant of the Tris-washed broken chloroplasts; (d) PS-2 preparation; (e) Tris-washed PS-2 preparation; (f) supernatant of the Tris-washed PS-2 preparation.

induces subsequent loss of O_2-evolution capacity. The site of water splitting is believed to be exposed on the lumenal side of thylakoid [2]. EPR measurement also showed the manganese release to the lumenal side [13].

The Tris treatment of broken chloroplasts removed 52, 34 and 16 X 10^3 M_r polypeptides. Two of these bands (the 52 and 16 X 10^3 M_r bands) may be attributed to large and small subunits of RuBP carboxylase [14] still remaining on the outer surface of thylakoid membranes after the thylakoids were hypotonically disrupted and washed by low ionic buffer solution. The 34 X 10^3 M_r band remains unidentified, but probably corresponds to cytochrome *f* of 31–36 X 10^3 M_r in its monomeric form [15,16]. As far as the usual broken chloroplasts are used as a material for the Tris treatment, the proteins released by Tris on the inner side of the thylakoid may not be detected by SDS–PAGE. In [17] the inside-out thylakoid vesicles were treated with 0.8 M Tris, and 34 and 23 X 10^3 M_r polypeptides were shown, both being exposed to the lumenal side of thylakoid, were totally removed by the treatment [17].

Judging from the SDS–PAGE densitograms, the O_2-evolving PS-2 preparation does not have the PS-1 complex, CF_1 and other peripheral membrane proteins, thus the interference of these polypeptides on the assay of SDS–PAGE of PS-2 is reduced significantly. This makes structural analysis of the O_2-evolution enzyme complex easier. Our result shows that at least 3 polypeptides were removed by the Tris washing the PS-2 preparation, of 33, 24 and 18 X 10^3 M_r, two of which are consistent with [17]. Although the exact information on the structure of our PS-2 preparation is not available now, Tris seems to release a protein from the active water-splitting site of the PS-2 preparation to the outer aqueous phase. Possibly, the protein carries the binding sites from Mn comprising the labile Mn pool that is readily extracted by the Tris treatment [18,19].

The 33 X 10^3 M_r protein, one of the polypeptides

that was removed by the Tris washing of the PS-2 preparation, is probably the same as that reported [20] to be associated with PS-2. The 33×10^3 M_r polypeptide seems also to be similar, in its M_r-value, to that lost in the algal and mayze mutants with deletions in PS-2 [4,5]. A manganese protein isolated from *Zea mays* [21] has M_r 25×10^3, which is consistent with the 24×10^3 M_r protein released by the Tris washing of our PS-2 preparation. In the $50-70 \times 10^3$ M_r range of the PS-2 preparation, there were small bands due to contaminations of CP 1 and large subunits of CF_1, but we are not convinced of the presence of a 65×10^3 M_r polypeptide band reported in [7]. Further work is being undertaken to identify the 3 polypeptides released by the Tris washing of the O_2-evolving PS-2 preparation.

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