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STUDIES ON MANGANESE BINDING BY SELECTIVE SOLUBILIZATION OF PHOTOSYSTEM-II POLYPEPTIDES

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Deoxycholate was used to solubilize the 16 and 24 kDa polypeptides from spinach thylakoids, resulting in the loss of oxygen evolution. Manganese was retained in the membrane. When the deoxycholate-extracted membranes were subjected to a mild heat treatment, the water-soluble 33 kDa protein was selectively released. Less than one manganese per reaction center was lost on heating but this loss was not correlated to the solubilization of protein. Most of the manganese bound to the membrane remained EPR-undetectable and could be released by 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) or hydroxylamine treatments. This indicates that the manganese involved in oxygen evolution remains in its native binding site despite the loss of the 33 kDa protein. These results contradict the hypothesis that the 33 kDa protein is responsible for manganese binding at the photosynthetic oxygen-evolving site.

Introduction

The mechanisms of photosynthetic oxygen evolution has been the subject of great interest during the past few years, with particular emphasis on possible proteins involved in the binding of manganese. Several treatments, involving high pH, Tris, salt or detergents, result in the loss of proteins from the photosynthetic membrane and inhibition of oxygen evolution [1–5]. In a few cases, oxygen evolution has been partially restored by the readdition of extracted proteins to the depleted membranes. The requirement for oxygen evolution of a 24 kDa protein and possibly a 16 kDa component (both hydrophilic) has been inferred from such studies [3,4]. However, neither of these proteins bind manganese.

Tris, at high concentrations a potent inhibitor of oxygen evolution, removes a high proportion of the bound manganese, and at the same time releases a water-soluble 33 kDa protein together with the above-mentioned 24 and 16 kDa proteins from PS-II preparations [6,7]. The apparent correlation between the release of manganese and the 33 kDa component has been taken as evidence for an association of manganese with the protein in vivo [6,7]. Mutant studies have also indicated the involvement in manganese binding of a protein of similar mass [8]. This appears to be an intrinsic protein dissimilar to the soluble one [9].

While manganese is retained, deoxycholate inhibits photosynthetic oxygen evolution [10]. The inhibition results from the solubilization of the 16 and 24 kDa PS-II polypeptides [11]. The extracted thylakoid membrane, when subjected to a mild heat treatment, is depleted of most of the 33 kDa protein while only small amounts of manganese are lost. In similarity with oxygen-evolving membranes [12,13], these membrane fragments loose manganese when treated with Tris or hydroxyl-

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Abbreviations: Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PS II, Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

amine. These results may necessitate a reevaluation of the earlier proposed role of the 33 kDa component.

Methods

Chloroplast preparation. Spinach leaves were homogenized in a Waring blender in 400 mM sucrose/50 mM Tricine-NaOH (pH 7.5)/10 mM NaCl. The homogenate was strained through four layers of 20 mesh nylon cloth. After centrifugation at 3000 × *g* for 5 min, the pellet was suspended in 50 mM Hepes-NaOH (pH 7.5)/10 mM NaCl (Hepes buffer), and centrifuged at 5000 × *g* for 3 min. The last step was repeated once, and the broken chloroplasts were finally suspended in Hepes buffer to a chlorophyll concentration of 4–7 mg/ml, as determined by the method of Arnon [14]. The uncoupled PS-II activity was about 300 μmol O_2/mg Chl per h. The manganese content was 1 Mn/90 Chl.

Deoxycholate treatment. Class-II chloroplasts, 5 mg chl/ml, in Hepes buffer were incubated with 2% deoxycholate on ice for 15 min and centrifuged at 40000 × *g* for 1 h. To remove excess detergent, the pellet was suspended in Hepes buffer and centrifuged at 40000 × *g* for 30 min. The membrane fraction was suspended in Hepes buffer to a final chlorophyll concentration of 4–5 mg/ml.

Activity measurements. Oxygen evolution was measured in saturating white light at 25 °C with a Hansatech oxygen electrod in a medium containing 50 mM sodium phosphate (pH 6.5), 10 mM NaCl and 10 mM NH_4Cl with 1 mM phenyl-*p*-benzoquinone as electron acceptor.

Manganese analyses. Manganese was determined by atomic absorption or by EPR measurements at room temperature on acidified samples using the method of additions.

EPR measurements. Room temperature or liquid nitrogen temperature EPR spectra were recorded with a Varian E-3 spectrometer, and at lower temperatures with a Varian E-9 instrument [15].

SDS polyacrylamide gel electrophoresis. The samples were solubilized for 3 min at 80°C in 1% (w/v) SDS/5% (v/v) mercaptoethanol/1 mM EDTA, 10% (w/v) sucrose/10 mM Tris-HCl (pH 8.0). Electrophoresis was performed in the buffer system of Laemmli [16], using 10–20% gradient gels (2,6% crosslinking). The gels were stained with Coomassie Brilliant Blue R-250. Densitometric quantitation of proteins was made at 560 nm with a Gilford 240 spectrophotometer equipped with a gel scanning attachment.

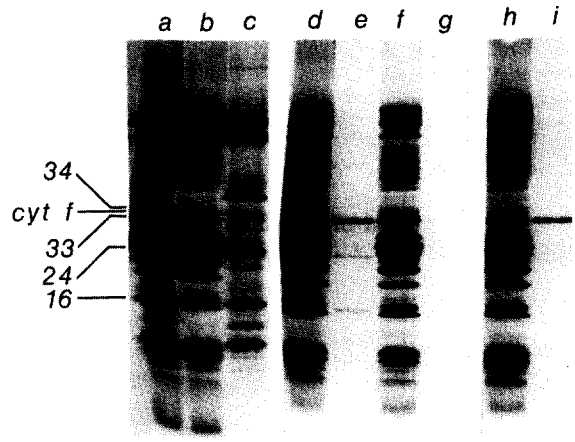

Fig. 1. Polypeptide analysis by electrophoresis in the presence of SDS. (a), class II chloroplasts; (b), deoxycholate-extracted chloroplasts; (c), supernatant from deoxycholate extraction; (d, e), pellet and supernatant after incubation of deoxycholate-extracted chloroplasts with 0.8 M Tris (pH 8.4); (f, g), pellet and supernatant after incubation of deoxycholate-extracted chloroplasts with 20 mM NH_2OH; (h, i), pellet and supernatant after heat treatment (55 °C, 3 min) of deoxycholate-extracted chloroplasts.

Results

The addition of deoxycholate to class-II chloroplasts (more than 0.6 mg deoxycholate/mg Chl) resulted in the inactivation of oxygen evolution and the concomitant solubilization of proteins (Fig. 1a–c). Only the solubilization of the 16 and 24 kDa polypeptides was correlated with the loss of oxygen evolution [11]. More than 90% of the water-soluble 33 kDa protein and most of the manganese remained bound to the membranes (Fig. 1b, Table I). The membrane-associated manganese was not EPR-detectable at room temperature or at 77 K.

The deoxycholate-extracted chloroplasts were subjected to treatments known to release the 33 kDa protein and/or manganese from PS-II preparations, such as incubation with Tris or hydroxylamine. Both treatments solubilized manganese

TABLE I

SOLUBILIZATION OF MANGANESE AND 33 kDa PROTEIN FROM DEOXYCHOLATE-EXTRACTED CHLOROPLASTS

Heat treatment and incubation with hydroxylamine were performed in 50 mm Hepes-NaOH (pH 7.5)/10 mM NaCl.

Method	Extracted (Mn/400 Chl)	Bound (Mn/400 Chl)	Extracted protein (%)
0.8 M Tris (pH 8.4)	3.2	1.1	82
20 mM NH_2OH	1.4	3.2	0
Heat treatment 55 °C, 3 min	0.9	3.2	72
Tris after heat treatment	2.3	1.1	26
NH_2OH after heat treatment	1.0	2.1	13

(Table I). The 33 kDa protein was released by Tris, but not by hydroxylamine (Fig. 1d–g, Table 1). Incubation at pH 9.3 [1] also resulted in the loss of manganese and protein (not shown).

Mild-heat treatment (up to 55 °C), which is known to inhibit oxygen evolution specifically, solubilized the 33 kDa protein from deoxycholate-extracted chloroplasts (Fig. 1h and i, Table I). However, most of the manganese remained bound (Table I), with less than 1 Mn/400 Chl released. The loss of manganese and protein were not obviously correlated. Fig. 2 shows that a small amount of manganese was more easily removed than the 33 kDa protein when samples were heated to temperatures between 35 and 55 °C. The released manganese was EPR-visible at room temperature with a spectrum indistinguishable from that of the manganese(II)hexaquo ion, and thus not protein-bound. About one-third of the bound manganese was detectable by EPR at liquid nitrogen temperatures as a six-line signal typical of monomeric protein-bound manganese.

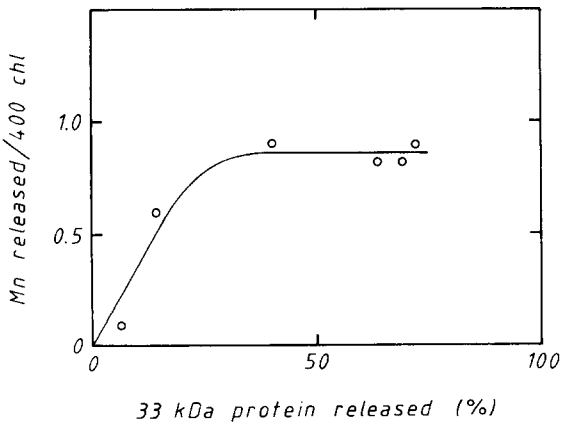

Fig. 2. Correlation between extraction of manganese and protein by heat treatment. Samples of deoxycholate-extracted chloroplasts were treated for various lengths of time at temperatures between 35 and 55 °C to remove increasing amounts of the 33 kDa protein.

The released protein was dialyzed against a weak ammonium bicarbonate buffer and freeze-dried. This protein did not contain manganese, and manganese (as $MnCl_2$) added to a solution of the protein could all be detected as manganese(II)hexaquo ion using EPR at room temperature.

After the mild heating, the membrane fraction was recovered by centrifugation and then subjected to Tris or hydroxylamine treatments (Table I). Both procedures released manganese to about the same extent as with unheated membranes.

Oxidizing conditions in the presence of $K_3Fe(CN)_6$ and diaminodurene [17] during the heat treatment did not significantly alter the solubilization pattern.

Discussion

The correlation between the inhibition of oxygen evolution and the solubilization of the 16 and 24 kDa polypeptides by deoxycholate [11] indicates the involvement of at least one of these on the donor side of PS II. Our results thus confirm those obtained after salt extraction of inside-out thylakoid vesicles [3]. The fact that a detergent causes the release of these proteins suggests that hydrophobic interactions may play a role in the binding of both proteins to the photosynthetic membrane. Electrostatic forces are probably also important for the binding [3]. The co-release of manganese and the 16, 24 and 33 kDa proteins by Tris suggested the involvement of one of these in manganese binding [2]. The deoxycholate extractions or experiments involving salt-washing of PS II particles [7,18] clearly demonstrate that neither the 16 kDa nor the 24 kDa polypeptide is involved in manganese binding. Some lack of correlation

between the release of a 34 kDa protein and manganese was noted in Ref. 5 but arguments for the involvement of the 33 kDa protein in manganese binding have been repeated recently [6,7], even though the protein did not contain manganese after release.

Abramowicz et al. [17] recently observed a very wide, complex EPR signal in chloroplasts and in an isolate containing the 33 kDa protein. They ascribed this signal to manganese bound to this protein. In oxygen-evolving, dark-adapted chloroplasts we have observed EPR signals with magnetic properties apparently identical to those observed by Abramowicz et al. The signals are unaffected by hydroxylamine or ascorbate (cf. [17]) and are strikingly dependent on the orientation of the sample in the magnetic field (cf. [19]). Thus, it is unlikely that they are associated with photosynthetic manganese. Protein impurities and a highly variable manganese to protein ratio in their preparation also complicates the interpretation of their results.

Our results from deoxycholate-extracted chloroplasts question the hypothesis that the 33 kDa protein is responsible for manganese binding. By the use of hydroxylamine or heat treatment, it is possible to selectively extract either manganese or protein into the surrounding medium (Table I). Deoxycholate has made the inside of the thylakoids accessible, probably by disrupting the vesicular structure. The extracted protein does not bind manganese, which agrees well with the results obtained from PS-II particles by incubation at a high pH or with Tris [6,7]. About one-third of the membrane-associated manganese is EPR-visible at low temperature after heat treatment. Thus, this fraction has been lost from its native binding site, as the manganese is not EPR-detectable in oxygen-evolving membranes or in deoxycholate-extracted chloroplasts before heat treatment. However, the fact that manganese is solubilized by Tris and hydroxylamine, which both are known to affect specifically the oxygen-evolving system (reviewed in Refs. 12 and 13) suggests that most of the manganese of the water-splitting site remains bound after heat treatment. Therefore, it seems clear that the 33 kDa protein is not involved in the binding of a major fraction of the photosynthetic manganese.

Studies on algal mutants have suggested that an intrinsic 34 kDa polypeptide is involved in manganese binding [8]. This polypeptide has been confused with the water-soluble, Tris- or heat-removable 33 kDa protein (discussed in Ref. 9). In fact, a 34 kDa polypeptide distinct from the 33 kDa protein can be seen in our gels (Fig. 1). This polypeptide remains bound to the membrane after deoxycholate extraction, heat treatment and incubation with Tris or hydroxylamine. The band from this polypeptide should not be confused with that from cytochrome *f*, which is solubilized by deoxycholate (Fig. 1a–c) and can be detected by optical methods in the extract.

Summarizing, the function of the water-soluble 33 kDa protein is still unclear. It is an extrinsic protein [9,20], located on the inside of the thylakoid membrane [20] and suggested to be a component of the oxygen-evolving site. Since it is solubilized at elevated temperatures, hydrogen bonding is expected to contribute to its association to the photosynthetic membrane. Our experiments show that it is not involved in the binding of a major fraction of the manganese in PS II. Most important, the Tris- and hydroxylamine-sensitive manganese which is considered to be uniquely associated with the oxygen-evolving system (reviewed in Refs. 12 and 13) remains in membranes depleted of the 33 kDa protein.

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