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MANGANESE PROTEINS ISOLATED FROM SPINACH THYLAKOID MEMBRANES AND THEIR ROLE IN O_2 EVOLUTION

II. A BINUCLEAR MANGANESE-CONTAINING 34 KILODALTON PROTEIN, A PROBABLE COMPONENT OF THE WATER DEHYDROGENASE ENZYME

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Extraction conditions have been found which result in the retention of manganese to the 33–34 kDa protein, first isolated as an apoprotein by Kuwabara and Murata (Kuwabara, T. and Murata, N. (1979) Biochim. Biophys Acta 581, 228–236). By maintaining an oxidizing-solution potential, with hydrophilic and lipophilic redox buffers during protein extraction of spinach grana-thylakoid membranes, the 33–34 kDa protein is observed to bind a maximum of 2 Mn/protein which are not released by extended dialysis versus buffer. This manganese is a part of the pool of 4 Mn/Photosystem II normally associated with the oxygen-evolving complex. The mechanism for retention of Mn to the protein during isolation appears to be by suppression of chemical reduction of natively bound, high-valent Mn to the labile Mn(II) oxidation state. This protein is also present in stoichiometric levels in highly active, O_2-evolving, detergent-extracted PS-II particles which contain 4–5 Mn/PS II. Conditions which result in the loss of Mn and O_2 evolution activity from functional membranes, such as incubation in 1.5 mM NH_2OH or in ascorbate plus dithionite, also release Mn from the protein. The protein exists as a monomer of 33 kDa by gel filtration and 34 kDa by gel electrophoresis, with an isoelectric point of 5.1 ± 0.1. The protein exhibits an EPR spectrum only below 12 K which extends over at least 2000 G centered at $g = 2$ consisting of non-uniformly separated hyperfine transitions with average splitting of 45–55 G. The magnitude of this splitting is nominally one-half the splitting observed in monomeric manganese complexes having O or N donor ligands. This is apparently due to electronic coupling of the two ^{55}Mn nuclei in a presumed binuclear site. Either a ferromagnetically coupled binuclear Mn_2(III,III) site or an antiferromagnetically coupled mixed-valence Mn_2(II,III) site are considered as possible oxidation states to account for the EPR spectrum. Qualitatively similar hyperfine structure splittings are observed in ferromagnetically coupled binuclear Mn complexes having even-spin ground states. The extreme temperature dependence suggests the population of low-lying excited spin states such as are present in weakly coupled dimers and higher clusters of Mn ions, or, possibly, from efficient spin relaxation such as occurs in the Mn(III) oxidation state. Either 1.5 mM NH_2OH or incubation with reducing agents abolishes the low temperature EPR signal and releases two Mn(II) ions to solution. This is consistent with the presence of Mn(III) in the isolated protein. The intrinsically unstable Mn_2(II,III) oxidation state observed in model compounds favors the assignment of the stable protein oxidation state to the Mn_2(III,III) formulation. This protein exhibits characteristics consistent with an identification with the long-sought Mn site for photosynthetic O_2 evolution. An EPR spectrum having qualitatively similar features is observable in dark-adapted

Abbreviations: CF_1, spinach coupling factor; Chl, chlorophyll; p*I*, isoelectric point (units-pH); PS II, Photosystem II; DCIP, 2,6-dichlorophenolindophenol.

intact, photosynthetic membranes (Dismukes, G.C., Abramowicz, D.A., Ferris, F.K., Mathur, P., Upadrashta, B. and Watnick, P. (1983) in The Oxygen-Evolving System of Plant Photosynthesis (Inoue, Y., ed.), pp. 145–158, Academic Press, Tokyo) and in detergent-extracted, O_2-evolving Photosystem-II particles (Abramowicz, D.A., Raab, T.K. and Dismukes, G.C. (1984) Proceedings of the Sixth International Congress on Photosynthesis (Sybesma, C., ed.), Vol. I, pp. 349–354, Martinus Nijhoff / Dr. W. Junk Publishers, The Hague, The Netherlands), thus establishing a direct link with the O_2 evolving complex.

Introduction

Many recent experiments have sought the enzyme responsible for the four electron oxidation of water to molecular oxygen in photosynthetic membranes. The requirement for manganese in this enzyme is well established (reviewed in Refs. 1–4) and much work has centered on the isolation of manganese-containing proteins as candidates for the water dehydrogenase. A minimum of four Mn ions is functionally associated with O_2 evolution activity in intact thylakoid membranes [5] and in subchloroplast particles enriched in Photosystem II (PS II) [6]. It is widely accepted that this Mn is associated with protein bound to the inner surface of the thylakoid membrane [7]. EPR studies on intact membranes have established that at least part of this manganese is organized as a binuclear [8], or possibly tetranuclear [9], cluster of electronically interacting manganese ions in the S_2 Kok oxidation state. Extraction of some of this manganese followed by reconstitution with other divalent metal ions in PS-II particles results in a partial reconstitution of PS-II electron transport [10]. This has been interpreted to mean that only two Mn ions are needed for catalytic activity while the other two are possibly structurally important, since divalent metal ions other than manganese can replace them. A quantitative correlation with O_2 evolution activity was not reported in this latter experiment and electron transport rates were only 10–20% of the rates observed in chloroplasts.

There has been no reported success in isolating a protein which binds stoichiometric levels of Mn and which is involved in O_2 evolution. This appears to be caused by the fragility of the oxygen-evolving complex when exposed to conditions necessary for the release of this membrane-associated complex, especially to the lability of Mn. In this article and in the preceding one [11] we report the conditions necessary to inhibit the lability of this manganese while still allowing protein isolation.

Despite this lack of success in isolating the manganese complex, a number of recent studies have suggested the involvement of at least three polypeptides as components of the oxygen-evolving complex. Åkerlund et al. [12,13] have isolated proteins of apparent molecular weight (M_r) 23 000 and 16 000, released by washing inverted thylakoid membranes in 1 M salt, a treatment which also inhibits O_2 evolution. Rebinding of one 23 kDa protein is observed to reconstitute 60% of the O_2 evolution activity, although optimal rates are well below intact membranes. Kuwabara and Murata [14,15] have extended these results with PS-II enriched detergent particles. The M_r and isolectric points of the polypeptides they release are 33 000 (pI = 5.1), 24 000 (pI = 6.5) and 18 000(pI = 9.2). They also observe that conditions which result in the selective release of the 33 kDa protein (0.8 M Tris or high pH) from PS-II particles prewashed with high salt, also cause release of manganese to solution [16]. Partial reconstitution of O_2 evolution to salt-washed particles by the 24 kDa and 18 kDa proteins occurs only if the 33 kDa protein and Mn are not released from the membrane. Thus, an indirect association between Mn and this protein or its receptor site on the membrane can be inferred. Yamamoto et al. [17,18] have also observed these three polypeptides through their preferrential release under similar conditions. Henry and Møller [19] have optimized the purification of PS-II enriched O_2-evolving, inside-out, thylakoid membranes, as originally reported by Albertsson [20]. These membranes display eight bands on SDS-polyacrylamide gel electrophoresis: four chlorophyll proteins (51, 43, 30 and 24 kDa) and four nonpigmented bands (33, 22, 19 and 18 kDa). No functional assignment was given. In all these reports no evidence for metal ion binding by any of these proteins is found.

Metz et al. [21] isolated a low-fluorescent mutant of the green algae *Scenedesmus obliquus* which lacks oxygen-evolving capability, has low manganese content, and lacks a polypeptide of an M_r of 34000, while retaining PS-II electron transport. This indirectly indicates the possible involvement of a 34 kDa protein in manganese binding and O_2 evolution.

Okada and Asada [22] have recently isolated a 13 kDa protein containing 0.2–1 atom manganese from PS-II particles from the blue-green alga *Plectonema boryanum*. Although they claim this protein is the water dehydrogenase, an antibody to this protein inhibits 50% of the PS-II activity (1,5-diphenylcarbazide → DCIP) even with water oxidation uncoupled. The antibody also localizes the protein-binding site to the outer surface of the thylakoid membrane, just the opposite side to that observed for the oxygen-evolving complex in spinach thylakoids. Although these may represent real differences between the organisms, further work is needed to clarify these seemingly inconsistent results.

In this paper we report isolation conditions which result in the binding of two manganese ions to the water-soluble 34 kDa protein isolated from spinach thylakoids. This manganese is associated with the fraction of Mn present in PS II and normally assigned a role in O_2 evolution. It thus appears that this protein is a good candidate for the long-sought manganese site of water oxidation.

Methods and Materials

Spinach grana-thylakoid membrane isolation procedures, chromatofocusing, oxygen evolution, flameless atomic absorption, chlorophyll determination, osmotic rupture of membranes, gel filtration, and SDS-polyacrylamide gel electrophoresis were performed as previously described [11]. Protein concentrations were evaluated by the methods of Lowry [23], Coomassie G 250 brilliant blue protein assay with the change in absorbance at 600–470 nm compared to a standard curve for bovine serum albumin, bovine γ globin and lysozyme [24], and by the absorbance at 280 nm compared to a standard curve for bovine serum albumin. It is important to realize that absolute measurements for protein concentrations will vary by a factor of 2 between the different methods, although the precision with any of the individual methods is good. This difference between the protein assays is inherent and due to varying dye/protein bindings. The Mn/protein values were calculated using the modified Bradford assay [24] with bovine-γ-globin as a standard.

Protein stoichiometry relative to PS II was estimated by the relative staining on SDS-gels by comparison to a 45 kDa Chl-containing band attributed to a PS II reaction center protein. A comparison between individual protein concentration and the total protein removed from the membranes was also used.

Incubation of PS-II enriched membranes and isolated protein in 1.5 mM NH_2OH was performed for 30 min in the dark on ice. This releases 60% of the bound Mn and destroys 80% of O_2 evolution activity. Incubation of isolated protein with reducing agents (1 mM ascorbate and 1 mM dithionite) was performed for 30 min in the dark on ice.

Reconstitution of purified protein fractions to depleted membranes was assayed for O_2 evolution and PS-II activity. A 5-fold excess of protein per PS II was added to membranes in suspension media, incubated with stirring for 3 min and temperature equilibrated to 20.0 °C.

PS-II membrane fragments were prepared from spinach thylakoids according to Yamamoto et al. [18]. The membrane fragments evolve oxygen at 550–700 μmoles O_2/mg Chl per h and contained 4–5 Mn/PS II.

EPR spectra were measured with a Varian E12 spectrometer at 9.5 GHz. Samples were cooled by liquid helium in an Oxford Instruments ESR-900 continuous flow cryostat. Experimental conditions are as described in the figure legends. Electron microscopy was performed on a Philips EM 400 transmission electron microscope.

Results

The spinach grana-thylakoid membranes isolated for these experiments displayed good rates of oxygen evolution (400–600 μmoles O_2/mg Chl per h), contained 7–8 Mn/400 Chl, and are enriched in PS II, as shown by an enrichment in Chl *b* (Chl $a/b = 2.3$ vs.3.0 in chloroplasts) [25]. Rates were

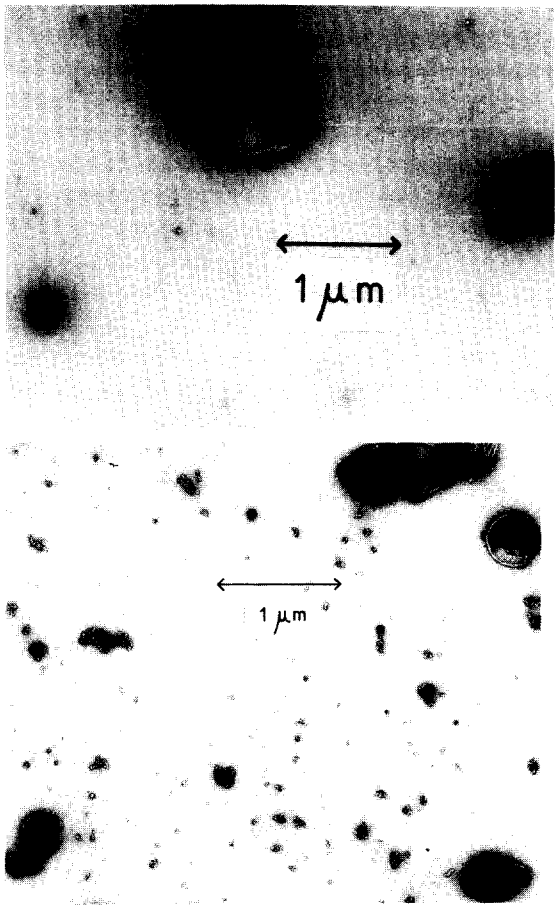

Fig. 1. Electron micrographs of grana thylakoid membranes from spinach in suspension buffer (above) and depleted membranes after osmotic rupture in hypotonic buffer (below). Magnification, ×11700.

increased by 25–50% upon addition of 1 mM NH_4Cl to aid in the uncoupling of photophosphorylation. Proteins were released by osmotic shock from stacked grana-thylakoid membranes in suspension buffer by washing in a hypotonic buffer [11]. Electron microscopy (Fig. 1) and equilibrium centrifugation demonstrated that the membranes were lysed by this procedure and reduced in size by a factor of ×2–3 to an average dimension of less than 0.3 μm. The osmotic shock removed 70% of the PS-II activity (1,5-diphenylcarbazide → $Fe(CN)_6^{-3}$) and more than 90% of O_2 evolution (Table IV, [11]).

Separation, by chromatofocusing in the pH range 8.3–5.2, of the protein mixture released from thylakoid membranes by osmotic shock is shown in the preceding article to resolve a single manganoprotein at an isoelectric point of $pI = 6.0 \pm 0.1$. A second manganoprotein can be isolated if the resolving range is extended down to pH 4.9. By degassing the buffers prior to use in the chromatofocusing separations, dissolved CO_2 is removed and the working pH range is extended to 4.9. The resulting separation is shown in Fig. 2. In addition to the manganese-containing protein at pI 6.0, another manganese-containing band is observed at $pI = 5.1 \pm 0.1$. An unresolved peak is observed for proteins above pH = 8.3 and for solubilized Mn which elute directly from the column without binding. This band also contains a significant fraction of unresolved proteins which do not equilibrate with the column.

If grana thylakoid membranes are washed two or three times with 2 mM EDTA in suspension medium in order to remove CF_1 prior to lysing in the hypotonic oxiding buffer, the $pI = 6.0$ protein is no longer observed. These membranes contain significantly less Mn (4–4.8 Mn/PS II) and retain high O_2 evolution rates of 300–600 μmoles $O_2 \cdot h^{-1} \cdot$ mg Chl^{-1}. The resulting protein separation is shown in Fig. 3. Now the only manganese-containing fraction separated between pH 8.3 and 4.9 is the band at $pI = 5.1$. This fraction migrates primarily as a single protein band of M_r 34000 (minor components at 36000 and 32000) on SDS-polyacrylamide gel electrophoresis. (Fig. 4, lane 5) and as a single peak at 33 kDa by gel filtration on Sephadex G-100 (not shown). No additional protein is released from membranes after three washes. This yields an initial stoichiometry of 1–2 34

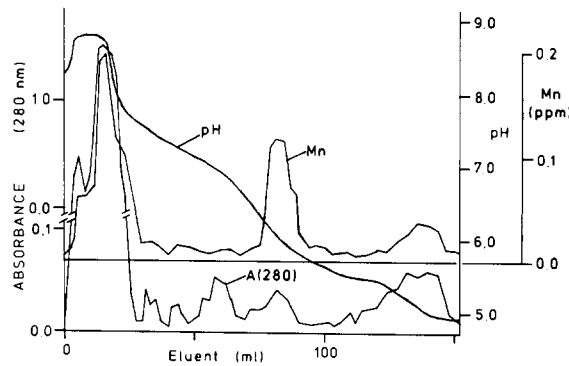

Fig. 2. Chromatofocusing between pH 8.3 and 4.9 of proteins released from spinach grana thylakoids by osmotic shock in hypotonic oxidizing buffer.

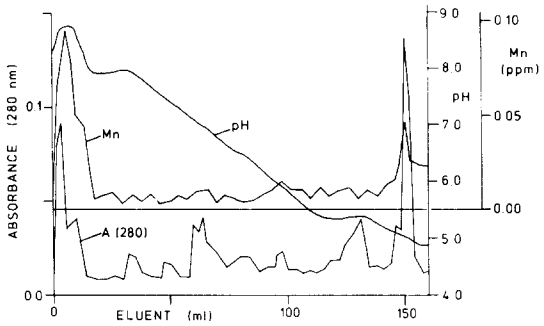

Fig. 3. Chromatofocusing between pH 8.3 and 4.9 of proteins released from spinach grana thylakoids by osmotic shock in hypotonic oxidizing buffer; CF_1 removed prior to protein extraction.

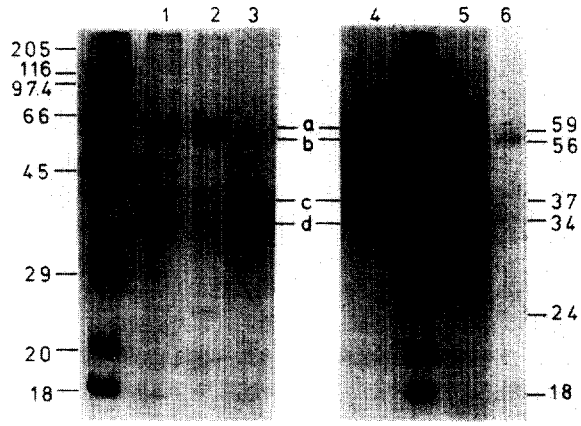

Fig. 4. 12.4% SDS-polyacrylamide gel electrophoresis of proteins released from spinach grana thylakoid membranes by osmotic shock in hypotonic buffer. All samples heated to 100 °C for 5 min. Lane 1, first washing; lane 2, second washing; lane 3, proteins released by osmotic shock of PS-II particles; lane 6, chromatofocusing fraction $pI = 6.0 \pm 0.1$; lane 5, chromatofocusing fraction $pI = 5.1 \pm 0.1$; lane 4, CF_1.

kDa/PS II using a ratio of 330 Chl/PS II for our enriched membranes. Manganese analysis by flameless atomic absorption spectrophotometry following dialysis obtains 1.8 ± 0.3 Mn/protein, an average of three determinations. This result agrees with the manganese content estimated by EPR of the depleted protein (see below). The iron content is less than 0.1 Fe/protein. Dialysis was performed at 4 °C against three volume changes at $\times 1000$ each of chromatofocusing buffer at pH = 8 [11]. A summary of some chemical and physical properties of this protein is given in Table I. Manganese analysis on the depleted membranes obtains approx. 2 Mn/PS II. This means that approx. 3 Mn/PS II are released by osmotic shock from membranes initially at 4–4.8 Mn/PS II.

We found, unlike Pharmacia [26], that the eluting polybuffer used for chromatofocusing interferes with both the Lowry and Coomassie G-250 brilliant blue standard protein assays. Dialysis of the proteins to remove polybuffer reduced this interference to 25% error. In these cases, two additional methods for protein estimation were used. An estimate of protein concentration was obtained by comparison of the absorbance at 280 nm to a standard solution of bovine serum albumin in polybuffer at the same absorbance using a molar absorptivity coefficient of 44000 $cm^{-1} \cdot M^{-1}$. For the 34 kDa protein, concentration was also determined by using the published extinction coefficient of 22000 $M^{-1} \cdot cm^{-1}$ at 276 nm for the Mn free protein [15].

Detergent solubilized PS-II enriched membranes, prepared as in Ref. 18, were examined to see if they too released the same 34 kDa protein upon osmotic shock in the hypotonic oxidizing buffer. The proteins released are shown on SDS gels in Fig. 4, lane 3. For comparison, lane 4 in

TABLE I

SUMMARY OF PROPERTIES OF THE 34 kDa PROTEIN PAGE

Molecular Weight	pI	Mn [a]	Fe	$\lambda_{max}(\varepsilon_{max})$	EPR
34 kDa SDS-gel 33 kDa gel filtration	5.0–5.2	1.8 ± 0.3	< 0.1	278 nm (25000 $M^{-1} \cdot cm^{-1}$) [b]	active below 12 K; $\geq$ 2000 G width; 45–55 G hyperfine splitting, $g \approx 2$

[a] Dissociates as free Mn(II) upon incubation in reducing agents such as 1.5 mM NH_2OH or 1 mM ascorbate plus 1 mM dithionite.
[b] Apoprotein.

this figure corresponds to subunits of the isolated CF_1 with bands labeled a, b and c at 59, 56 and 37 kDa [27]. As expected, these bands are not present in the proteins released from PS-II membranes by the osmotic shock. As noted earlier, the pI = 6.0 protein from thylakoid membranes (lane 6) corresponds to band b at 56 kDa [11]. Band d represents a 34 kDa protein present in the protein extract from both thylakoid membranes (lanes 1 and 2) and enriched PS-II membranes (lane 3). This is the same 34 kDa protein isolated at pI = 5.1 by chromatofocusing.

An EPR spectrum at 4.2 K of the 34 kDa protein at a concentration of 74 μM protein and containing 1.8 $\pm$ 0.3 Mn/protein was recorded in order to test for the oxidation state and interaction of the Mn ions. As shown in Fig. 5A, the spectrum exhibits an extensive hyperfine structure extending over more than 2000 G and centered at $g \approx 2$. The splittings are non-uniform and occur, on average, every 45–55 G. The spectrum is strongly dependent on temperature and disappears above 12 K reversibly (Fig. 6). There is no evidence for microwave power saturation at 4.2 K and 200 mW, indicating a rapidly relaxing spin system. This spectrum is contrasted in Fig. 5C to the spectrum of 90 μM Mn(II) in 50% glycerol/water at the same (saturating) power which shows the usual six-line hyperfine pattern for ^{55}Mn with peak separation of 110–130 G.

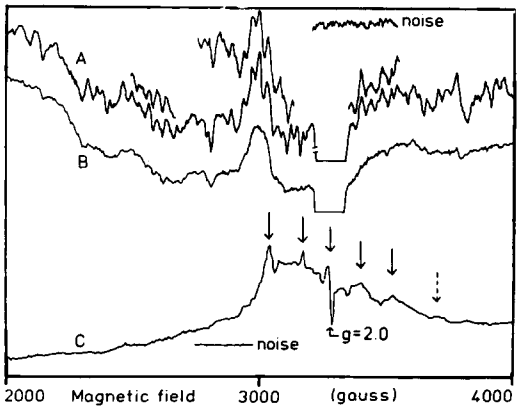

Fig. 5. (A) EPR of pI 5.1 chromatofocusing fraction at 75 μM protein. MA = 16 G, RG = 20000, P = 100 mW, T = 4.3 K. (B) same as A, after 1.5 mM NH_2OH incubation. (C) 90 μM Mn^{2+} standard, in 50% glycerol/buffer. RG = 2000. The large peak at 3300 G is a cavity impurity.

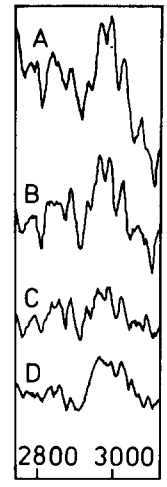

Fig. 6. Temperature dependence of hyperfine structure for 34 kDa protein (pI 5.1 fraction); MA = 16 G, RG = 20000, p = 100 mW, (A) 4.3 K; (B) 5.6 K; (C) 9 K; (D) 12 K.

The effect on the EPR spectrum of the 34 kDa protein from incubation in 1.5 mM NH_2OH was examined because of the well-known inactivation of O_2 evolution and release of Mn from photosynthetic membranes which this treatment induces. Fig. 5B shows that the low-temperature multiline signal observed at high microwave power is removed by NH_2OH incubation. In its place, a new EPR signal is observed at room temperature at low microwave power (non-saturating) as shown in Fig. 7B. This six-line spectrum is due to released Mn(II). For comparison, the spectrum of a Mn(II) standard is shown at the same spectrometer conditions in Fig. 7C. The 34 kDa protein when observed at room temperature under these conditions is EPR-silent (Fig. 7A). The amount of Mn(II) released is approx. 85% of the total manganese content of the protein, as measured by atomic absorption. Incubation of a detergent extracted PS-II particle in 1.5 mM NH_2OH under the same conditions releases 60–80% of the Mn and abolishes 80–90% of O_2 evolution (originally containing 4 Mn/PS-II and having an activity of 750 μmoles O_2/mg Chl per h). Incubation of the 34 kDa protein in 1 mM ascorbate and 1 mM dithionite also abolishes the low-temperature signal and releases Mn(II) that is detectable by EPR at room temperature (data not shown).

Attempts at reconstitution with the 34 kDa

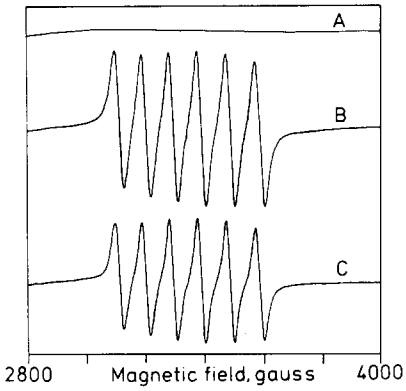

Fig. 7. (A) EPR of pI = 5.1 chromatofocusing fraction at 75 μM protein MA = 16 G, RG = 400, P = 10 mW, T = 295 K. (B) Same as A, after 1.5 mM NH_2OH incubation. (C) 90 μM Mn^{2+} standard with 1.5 mM NH_2OH, conditions same as for A.

protein alone were not successful. It was possible to restore up to 25% of the original O_2 evolution rate by adding back the crude protein mixture to the depleted membranes. This suggests that the other proteins removed may also play a role in electron transport.

Discussion

Electrophoresis

When spinach grana-thylakoid membranes are lysed upon transfer to a low osmotic medium, a number of proteins are released that can be observed on SDS-polyacrylamide gel electrophoresis [11]. The 59, 56 and 37 kDa bands correspond to subunits of Coupling Factor, CF_1 [27]. The 34 kDa band is undoubtedly the same water-soluble protein initially characterized by Kuwabara and Murata [14] which has been found to be released by chaotropic agents like Tris and urea [17,28] and by mild detergents like sodium cholate [11,29]. The results of this work establish that osmotic shock is an alternative method for release of the 34 kDa protein and evidently at a quantitative level judging from the stoichiometry with PS II. This method is presumably less disruptive to protein structure than the previous methods which rely upon detergent action or protein denaturing agents.

The coremoval of CF_1 and the 34 kDa protein by the same conditions suggests that their mode of binding to their respective sites on the membrane may be quite similar. Notable in this regard is the stabilizing influence of divalent ions in the binding of CF_1 to the membrane and the destabilizing effect of chelating agents like EDTA. Electrostatic repulsion between the negative membrane and negatively charged CF_1 is thought to be the cause of this lability. CF_1 is known to bind up to five divalent ions [30]. We presume that the highly acidic 34 kDa protein, owing to its isoelectric point of 5.1, would bear a large negative charge under ambient pH conditions if it were not for the binding of polyvalent metal ions such as manganese and, possibly, other metal ions.

Manganese binding

Manganese is released into the supernatant upon osmotic shock of membranes and becomes weakly bound to several proteins, especially if detergents such as sodium cholate are employed to facilitate release of proteins [11]. When chemical oxidants are present during lysing, two proteins are observed to bind a non-dialyzable stoichiometric amount of manganese. These appear at 56 kDa and 34 kDa on SDS-gels (Fig. 4, bands b and d). The mechanism for the stabilizing effect of oxidants on the Mn content appears to be the suppression of chemical reduction of natively-bound high-valent states of Mn to the labile Mn(II) state by reducing agents released from the membranes.

Mn(II) complexes with various inorganic and organic coordinating groups undergo rapid ligand substitution reactions and have stability constants which are 10^{12}–10^{18} smaller than the corresponding Mn(III) complexes [31]. In the case of $Mn(H_2O)_6^{2+}$, the substitution rate for water is 10^3 faster than for $Mn(H_2O)_6^{3+}$ [32]. In a protein where all six ligands must be replaced to achieve release this means a difference in rates of approx. 10^{18}. There is, therefore, good reason to expect the higher valent states of Mn to be less susceptible to displacement from their binding sites than is Mn(II).

The average initial manganese content of our EDTA-washed grana membranes which retain CF_1 is 5.5–6.4 Mn/PS II (7–8 Mn/400 Chl). This compares favorably with the range 5–8 Mn/400 Chl reported by Cheniae [3] for EDTA-washed, broken chloroplasts. Part of this manganese is bound to the Coupling Factor fragment CF_1 [30].

We observe that 1.6–2.1 Mn ions bind to the 56 kDa protein, now known to be the β-subunit of CF_1 [11]. Prior removal of CF_1 using successive EDTA washings reduces the Mn content of membranes to 4–4.8 Mn/PS II while retaining high O_2 evolution rates, in agreement with others [5]. Subsequent lysing in the presence of chemical oxidants of these active membranes releases additional protein bound Mn to solution, but does not release the 56 kDa protein, since it is now absent. Only the 34 kDa protein is then found to bind Mn amongst the proteins resolved between pH 8.3 and 4.9 by chromatofocusing.

The manganese content of the depleted membranes, obtained from grana membranes initially containing 5.5–6.4 Mn/PS II, after three extractions to remove all of the labile 34 kDa protein is 1.4–2.2 Mn/PS II. Thus, we infer that the loss of approx. 4 Mn/PS II occurs upon lysing of grana thylakoid membranes with a concomitant loss of more than 90% O_2 evolution activity.

Protein properties

The stoichiometry we find for the 34 kDa protein is 1–2 per PS II, which brackets the results observed for PS-II enriched membranes [28,33]. At present we cannot say whether the four manganese ions released from PS II are asssociated with two 34 kDa proteins or if only one copy of this protein exists per PS II, with the remaining two manganese ions arising from another, as yet, unidentified site.

This protein apparently exists as a monomer of 33–34 kDa(pI = 5.1), since even under the mild lysing conditions we use to separate it from the membrane, it exists as a single band on gel filtration. All previous accounts have not observed manganese binding to this protein. However, in all cases, the conditions used for the removal of the 34 kDa protein are well-known to release manganese from thylakoid membranes. Accordingly, these reports cannot exclude the possibility of intrinsic manganese binding to the 34 kDa protein. There is indirect evidence for the binding of manganese to a 34 kDa protein in the green alga *Scenesdesmus obliquus*. Metz et al. have developed several mutants of this alga, one of which is inactive in O_2 evolution, has low manganese content and has an alteration in a 34 kDa protein found in the wild phenotype such that it migrates at 36 kDa in the mutant [21]. Loss of manganese may induce this shift in M_r.

In our SDS-gels, although the 34 kDa protein predominates (more than 85%) in the pI = 5.1 fraction (Fig. 4, lane 5), weaker bands at 36 and 32 kDa are present. Similar behavior is observed by Kuwabara and Murata [14,33]. They observed a 36 kDa impurity and a higher mobility band thought to arise by reduction of disulfides in the 34 kDa protein in the presence of mercaptoethanol. We have not tested the influence of mercaptoethanol, but it is highly unlikely that a similar reduction could account for the band we see at 32 kDa, since we isolate this material under oxidizing conditions. The possibility of proteolytic cleavage was not investigated.

EPR

Evidence on the degree of interaction of the two manganese ions in the 34 kDa protein comes from the EPR results of Fig. 5. The spectrum of isolated protein in Fig. 5A is more complex than that observed from isolated Mn(II) ions in Fig. 5C. The extensive breadth of the apparent hyperfine features (more than or equal to 2000 G) centered around a g-value of 2, is typical of the fine structure transitions observed for ferromagnetically coupled manganese dimers having an even-spin ground state [34–36]. So also is the reduced separation of hyperfine peaks at 45–55 G compared to isolated Mn(II) ions at 95–130 G, Mn(III) at 80–100 G and Mn(IV) at 70–80 G for 0 or N donor ligands. Theoretical models predict and experiments show a halving of the hyperfine constant in symmetric dimers. Either a Mn_2(II,II) or Mn_2(III,III) oxidation state would be consistent with the data. Hence, there is good evidence for a close association of the Mn ions in this protein.

An alternative explanation of the hyperfine pattern may be that it arises from a mixed-valence Mn_2(II,III) state. Unfortunately, discrete model dimers in this oxidation state have not been characterized by EPR as of yet. Consequently, a comparison of spectral features can only be made based upon theoretical predictions for a (II,III) dimer. Since the theoretical spectra for the analogous Mn_2(III,IV) mixed valence dimers reliably reproduce the corresponding experimental spectra [37], it should be possible to correctly predict the

spectrum for a Mn_2(II,III) dimer. The spectrum of a (II,III) dimer is predicted to have 36 hyperfine transitions due to the interaction of the single unpaired electron ($S = \frac{1}{2}$ ground state presumed) with two ^{55}Mn nuclei. The hyperfine coupling constants for such a dimer are $A_1 = 7/3\ A'_1$ for Mn(II) and $A_2 = -4/3\ A'_2$ for Mn(III), where A' refers to the intrinsic coupling constant for a mononuclear Mn ion of the same oxidation state [37]. Taking $A'_1 = 100$ G and $A'_2 = 89$ G, which are typical values for Mn(II) and Mn(III) complexes with 0 and N donor ligands, respectively, the overall hyperfine field has a width of $5|A_1| + 5|A_2| = 1760$ G [8]. From this, we predict an average separation of 1760 G/36 = 49 G between hyperfine transitions, in agreement with the data of Fig. 5. Moreover, the transitions are expected to be separated non-uniformly owing to the higher-order corrections to the first-order hyperfine field necessary when large hyperfine fields such as this are present [8]. We can conclude that, at least at this qualitative level of comparison, the predictions of this model are also in agreememt with the observed spectrum. Further clarification of these two interpretations of the EPR spectrum should be possible when better data and well-characterized model dimers are available for comparison.

The results of the NH_2OH incubation provide compelling evidence that the manganese from O_2-evolving PS-II particles is derived from the bound 34 kDa protein, since the same treatment of the isolated 34 kDa protein releases an equivalent fraction of manganese (Figs. 5 and 7). The concurrent loss of O_2 evolution activity in the PS-II particles indicates a direct correlation between this bound Mn and the ability to oxidize water. The absence of an EPR spectrum for the isolated 34 kDa protein at room temperature would be consistent with either the presence of a coupled binuclear unit for the manganese ions, or the existence of EPR silent oxidation states of mononuclear manganese ions, such as Mn(III) and probably Mn(IV). The partial release of bound manganese to yield free Mn(II) ions upon reduction with ascorbate and dithionite is consistent with the presence of substitution inert oxidation states such as Mn(III) or higher states in the isolated protein. The mode of action of NH_2OH in the release of manganese from the 34 kDa protein is also presumed to occur by reduction of manganese followed by hydrolysis and release. The extreme temperature sensitivity and the complex hyperfine pattern observed in Figs. 5 and 6, suggests that the electron spin exchange interaction between the manganese ions in the protein may be weak, with magnitude comparable to the zero-field splitting of the individual manganese ions. Such weak coupling would yield a large number of closely spaced spin states, the population of which could account for the sharp temperature dependence.

An alternative reason for the strong temperature dependence is rapid spin relaxation. Mn(II) has a half-filled $3d^5$ electron configuration and zero spin-orbit coupling to first order. Consequently, it has a much slower spin relaxation rate than Mn(III) at low temperatures.

The EPR results taken as a whole are consistent with either a Mn_2(II,III) or Mn_2(III,III) formulation for the isolated protein. The inherent instability of binuclear Mn_2(II,III) model complexes to dissociation supports the latter assignment for the oxidation state of the isolated protein. This assignment would imply that the protein is isolated in an oxidation state equivalent to the S_1 Kok oxidation state of the intact oxygen-evolving complex [38], since comparison with the light-induced S_2 Kok oxidation state has been interpreted to be due to a Mn_2(III,IV) dimer unit based upon its characteristic EPR spectrum [8].

The EPR spectrum we observe for the isolated protein is qualitatively similar to the spectrum which can be observed in dark-adapted, intact photosynthetic membranes [34] and in detergent-extracted, O_2-evolving PS-II particles [39], thus establishing a direct link with the manganese involved in the oxidation of water to molecular oxygen.

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It has recently been reported that it is possible to dissociate the 33 kDa extrinsic protein (34 kDa extrinsic protein in the present work) using 1 M $CaCl_2$ washing of PS-II enriched thylakoid membranes. This treatment also releases polypeptides of 17000 and 23000 M_r(Ono, T.-A. and Inoue, Y. (1983) FEBS Lett. 164, 255–260). Furthermore, these authors report that no manganese is released from the membrane by this treatment, as detected by EPR spectroscopy of $Mn(H_2O)_6^{2+}$ in acidified membranes. These results suggest that either (1) the two manganese atoms found associated with the 34 kDa protein in the present work are also associated with the surface of the protein, and hence capable of facile and strong bonding to non-dissociating membrane components, or (2) that the present results are incorrect. We repeated the experiments described by these authors and find very different results (Dismukes, G.C., Abramowicz, D.A. and Rutherford, A.W., unpublished data). Our data show that for PS-II particles, prepared as mentioned elsewhere (Berthold, D.A., Babcock, G.T. and Yocum, C.F., (1981) FEBS Lett. 134, 231–234), at least 50% of the manganese released by 1–2 M HCl is lost by initial washing in 1M $CaCl_2$. Moreover, the acidified supernatant of the $CaCl_2$ washed membranes contains greater than 7-fold more manganese than the acid extractable manganese observed in the control membranes. This latter result indicates that there is a large fraction of manganese, other than that released by 1–2 M HCl, which requires harsher conditions for quantitative release than afforded by acidification.

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