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

I. A 56 KILODALTON MANGANESE-CONTAINING PROTEIN, A PROBABLE COMPONENT OF THE COUPLING FACTOR ENZYME

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The binding of endogenous manganese (Mn) to proteins released from spinach grana-thylakoid membranes by 2% cholate detergent or by osmotic shock is investigated. A mixture of 15–20 proteins is released by cholate and has been separated by isoelectric focusing in a sucrose gradient or by chromatofocusing. Mn coelutes with several proteins, but is lost upon dialysis. A dramatic redistribution of this Mn occurs in proteins released by osmotic shock in the presence of hydrophobic and hydrophilic oxidants. Maintaining an oxidizing solution potential during extraction apparently precludes reduction of the higher oxidation states of Mn to the labile Mn(II) state by reducing agents released from the membranes during lysing. This allows proteins to be separated which bind non-labile Mn ions. Under these extraction conditions, a protein is isolated which has an apparent molecular weight (M_r) of 65000 or 56000 on SDS-polyacrylamide gel electrophoresis depending on the sample buffer system used. The nondissociated protein occurs as a monomer of 58 kDa (90%) and an apparent dimer of 112 kDa (10%) by gel filtration. This protein binds little Mn if extracted by cholate and separated by isoelectric focusing. However, extraction by osmotic shock in the presence of oxidants and separation by chromatofocusing results in the retention of 1.9 ± 0.3 Mn ions per monomer. This protein is identical to that reported by Spector and Winget (Spector, M., and Winget, G.D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 957–959). Contrary to their result, this protein does not reconstitute O_2 evolution when added to depleted membranes. Rabbit antibody to this purified protein inhibits O_2 evolution by 20% when incubated with intact grana-thylakoid membranes or 10–20% with partially inverted, French-pressed thylakoids. This inhibition is completely removed by 10^{-3} M NH_3Cl as an uncoupler of photophosphorylation. These results support a role in Phosphorylation and a location on the outer surface of the thylakoids. This antibody also selectively binds purified coupling factor, CF_1, the multisubunit phosphorylation enzyme which is located on the outer thylakoid surface and which is known to bind two Mn ions tightly (Hochman, Y. and Carmeli, C. (1981) Biochemistry 20, 6293–6297). Thus the β-subunit of CF_1, which has a molecular weight of 56 kDa, can be identified as the locus of Mn binding in CF_1 and as the Mn protein isolated by Spector and Winget. This protein plays no role on O_2 evolution.

Introduction

Several proteins released from photosynthetic membranes have been reported to play a role in oxygen evolution (reviewed in Refs. 1 and 2). Of

Abbreviations: CF, coupling factor; PPBQ, phenol-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Hepes (4-(2-hydroxyethyl)-1-piperazineethanosulfonic acid; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

special interest has been the search for the manganese binding complex thought to represent the active site for water oxidation. EPR evidence on intact membranes suggests that this complex is comprised of a binuclear manganese center in the S_2 Kok oxidation state, which is two equivalents above the lowest state [3]. Four manganese ions have been found to be necessary for O_2 evolution, from which it has been suggested that two such binuclear units may interact to yield a tetranuclear Mn complex [4]. Isolation of some Mn-binding components from chloroplast membranes is now reviewed.

Foyer and Hall [5] have isolated a light-harvesting chlorophyll protein complex with M_r values of 29000, 26000 and 23000. This complex was found to contain Mn and superoxide dismutase activity. Okada and Asada [6] have recently isolated a 13 kDa protein containing 0.2–1 atom Mn from PS-II particles of the alga *Plectonema boryanum*. Although they claim that this protein is the water dehydrogenase, its location on the outer surface of the thylakoid membrane is inconsistent with data from spinach thylakoids. Spector and Winget [7,8] reported that sodium cholate detergent extracts a polypeptide from spinach thylakoid membranes having an M_r of 65000 on SDS-polyacrylamide gel electrophoresis which contains 1.6–2.6 atoms Mn and one cytochrome *b*. They claim this protein is capable of reconstituting more than 85% of the original O_2 evolution rate to depleted membranes. Although significant effort has been concentrated on repeating these results in many laboratories, no one has succeeded in reproducing them. Nakatani and Barber [9] extracted a polypeptide under identical conditions with and M_r of 231000 (58 kDa subunits on SDS-polyacrylamide gel electrophoresis) which contains heme but no Mn. This protein was reported to have an isoelectric point (p*I*) of 6.0 and to catalyse the release of O_2 upon reconstitution to membranes, a result thought to represent a catalase activity in which H_2O_2 is disproportionated. This activity may not be associated with chloroplasts but could arise from incomplete separation of peroxisomes. Sayre and Chenaie [11] have observed a 65 kDa protein extracted under similar conditions which has no Mn and does not reconstitute oxygen evolution to depleted membranes. We reported in preliminary accounts conditions which permit the extraction of this 65 kDa protein in a state which retains bound Mn [12,13]. In this account we present these results in detail and give evidence which indicates that this protein is the β-subunit of the phosphorylation enzyme fragment CF_1.

Methods and Materials

Thylakoid membranes were isolated from local farm spinach within 24 h of cutting. Leaves were deveined and finely chopped prior to homogenizing in grinding medium (50 mM K_2HPO_4/50 mM KH_2PO_4/2 mM EDTA/350 mM NaCl, at pH 7.4). 450 g of cold, dry spinach were homogenized in 800 ml of grinding medium for 4 s, using a Waring blender. All operations were performed at 0–4 °C. The homogenate was filtered through eight layers of cheesecloth and spun at $15000 \times g$ for 2 min (after achieving $15000 \times g$). The pellet was gently suspended with a paint brush in suspension medium (200 mM sucrose/50 mM tricine/3 mM KCl/3 mM $MgCl_2$, at pH 8.0). This first step should be completed in less than 20 min, to limit the time the membranes are exposed to the grinding medium. This suspension is spun at $600 \times g$ for 30 s and filtered through Whatman No. 4 filter paper to remove large debris. The supernatant is then spun at $26000 \times g$ for 10 min and the pellet suspended in suspension medium. Washing once more yielded Photosystem-II enriched, grana-thylakoid membranes which support O_2 evolution at good rates with added acceptors (typically 400–600 μmoles O_2/mg Chl per h; Chl $a/b = 2.3$). The washed thylakoid membranes were stored at −20 °C in the dark in 50% glycerol at approx. 2 mg Chl/ml, until needed. Chlorophyll concentration was determined by extraction in 80% acetone with the procedure of Arnon [14].

Thylakoid membranes were extracted with 2% cholate at pH 8.0 while incubated in light, as described by Winget et al. [15]. The solubilized, straw-colored protein mixture was separated from the depleted membranes by centrifugation at $150000 \times g$ for 60 min. Proteins were collected by precipitation in 1.2 M $(NH_4)_2SO_4$ or by ultrafiltration using an Amicon TCF-2 thin-channel system with a PM-10 membrane and dialyzed (two

volumes, at dilution 100-fold each) before addition to the columns.

Thylakoid membranes stored in glycerol were washed and concentration by centrifugation at $26\,000 \times g$ for 10 min after dilution with suspension media. For extractions by osmotic shock, the pellet was suspended in three times the original volume with a low-salt buffer, containing 2 mM Hepes/2 mM EDTA, at pH = 8.0, to a Chl concentration of 0.5 mg/ml. Oxidizing buffers also included 0.4 mM $K_3Fe(CN)_6$ or 1.2 mM $K_3Fe(CN)_6$ and 0.4 mM diaminodurene. The diaminodurene is oxidized quantitatively to diiminodurene by two equivalents of $K_3Fe(CN)_6$, as was determined spectrophotometrically. Solutions containing diaminodurene were freshly prepared. The redox potential of this buffer prior to the addition of membranes was +400 mV vs. normal hydrogen electrode. The sample was incubated in room light on ice for 20 min and pelleted at $150\,000 \times g$ for 2 h. The supernatant (wash 1) was set aside on ice. The lysed pellet was then resuspended in the same volume of oxidizing buffer with a paint brush and a Vortex-Genie K-550-G mixer and again incubated on ice for 20 min before pelleting at $215\,000 \times g$ for 2 h. This is referred to as the 'second wash'. The membranes were then washed a third time, and the remaining pellet after ultracentrifugation is referred to as the 'lysed' or 'depleted' membranes. Each supernatant (washes 1, 2 and 3) was analyzed for protein and Mn content before combining and concentrating for use. Osmotic shock with or without oxidants released the same pattern of proteins observable by SDS-polyacrylamide gel electrophoresis.

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 in suspension media and temperature equilibrated to 20 °C. The depleted membranes were then added and incubated with mixing in either light or dark for 3 min.

PS-II activity was measured spectrophotometrically at 424 nm using H_2O or 0.5 mM 1,5-diphenylcarbazide as donor and 0.4 mM $K_3Fe(CN)_6$ as acceptor. Electron transport was inhibited with 5 μM DCMU. The 1,5-diphenylcarbazide was used from a 100 × stock solution in 50% ethanol/acetic acid.

Isoelectric focusing was initially performed on Ampholine PAG plates (LKB Chemical) in a range of pH 3–10. This method was modified to preparative scale in a 30 ml linear sucrose gradient of 0–35%, as described by Kint [16]. For the pH range of 3–10, the dense electrolyte was 63 mM H_3PO_4 and the light electrolyte was 20 mM triethylene. For the pH range of 5–8, the dense electrolyte was 63 mM NaOH and the light electrolyte was 50 mM H_3PO_4. The sucrose medium contained ampholines at 2.5% (w/v). Electrical contact was made through a polyacrylamide plug to prevent gases formed at the electrodes from disturbing the focusing. Measurements were conducted at 0–4 °C in a cold room and with the aid of a water jacket.

Isoelectric focusing on ion-exchange columns, also known as chromatofocusing, was performed in a Pharmacia K9/30 chromatographic column as described by Sluyterman and coworkers [17,18]. For the pH range of 5–8, the ion -exchanger was polybuffer exchanger 94 and the start buffer was 25 mM tris-acetic acid at pH 8.3. The running buffer used was 3% polybuffer 96 and 7% polybuffer 74 (v/v) adjusted to pH 5.0 with CH_3COOH. After equilibration of the column with start buffer, the concentrated protein sample was applied. Elution was performed at 4 °C at a rate of 30–40 cm/h with running buffer and 2 ml fractions collected.

Fractions were examined by SDS-polyacrylamide gel electrophoresis in 12.4% gels according to the method of Laemmli [19]. Samples were run with two sample buffer systems. Method I utilized a buffer with a composition of 2.5 ml 0.5 M Tris-HCl (pH 6.8)/2.0 ml 10% SDS/2.0 ml glycerol/100 μl 1% Bromophenol blue/3.2 ml H_2O. Method II involved a modified buffer system containing 15 ml glycerol/15 g SDS.18.75 ml 0.5 M Tris-HCl (pH 6.8)/2 ml 1% Bromophenol blue/7.5 ml β-mercaptoethanol/6.75 ml H_2O. Although the samples displayed no difference in migration either with or without heating or in sample buffer I or II the migration of the molecular weight standards does vary, as described in the text (see Fig. 3 and Results). Gel were stained with Coomassie R250 or the Bio-Rad silver staining kit. Fractions were assayed for Mn ($\lambda = 279.5$ nm) and Fe ($\lambda = 248.3$ nm) by flameless atomic ab-

sorption (AA) spectroscopy using a Perkin-Elmer 305B spectrophotometer and a Perkin-Elmer HGA-2000 graphite furnace (sensitivity, 0.001 ppm). Standards used for AA calibration contained metal ion, salt, buffer and protein (bovine serum albumin or lysozyme at a concentration of 0.1 mg/ml).

O_2 evolution was measured using a YSI model 53 oxygen monitor and a Clark-type electrode at 20 °C after a 3 min dark adaptation. The isolated thylakoid membranes have a half-life of 15 min at this temperature. Measurements at pH 7.5 and 8.0 were not significantly different. The oxygen evolution rates continue at close to the initial value during the measurement. Electron acceptors, 2 mM $K_3Fe(CN)_6$ and 1 mM PPBQ, are added to the system to support maximal electron transport. Illumination was provided with heat-filtered light at saturating intensity from a GE ELH 300 W (quartz dichromic lamp) filtered by a 0.04 M $CuSO_4$ solution (16 cm) and a 580 nm cutoff red filter. This afforded a maximum intensity of 1 W/cm^2 when focused at the electrode chamber, as monitored by a Scientech 362 power/energy meter.

Gel filtration was performed on a G100 Sephadex column with a bed volume of 115 ml at a flow rate of 0.3 ml/min. Column standardization utilized Blue dextran 2000 (2000 kDa), β-galactoside (115 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and myoglobin (17.8 kDa).

Ultraviolet-VIS absorbance was measured on an HP 8450 A spectrophotometer. Protein concentration was determined by either a Coomassie G250 Brilliant Blue assay [20] with the change in absorbance at 600–470 nm or by the absorbance at 280 nm compared to a standard curve for bovine serum albumin. Antibody was prepared according to Garvey et al. [19]. The isolation of gamma globulin (IgG) from serum involved repeated precipitation with ammonium sulfate.

Cholic acid (Sigma Chemical) was twice recrystallized from 70% aqueous ethanol after treatment with activated charcoal. Special enzyme grade sucrose and ultrapure ammonium sulfate were obtained from Schwarz-Mann. Electrophoresis purity Tris and glycine were obtained from Bio-Rad Laboratories. Carrier ampholytes 'Ampholines' were obtained from LKB Chemical. Polybuffer and polybuffer exchanger were obtained from Pharmacia Fine Chemicals. All other chemicals were reagent grade.

Results

The isolated grana-thylakoid membranes typically evolve oxygen at rates of 400–600 μmoles O_2/mg Chl per h. These consistently good values are the result of modifications made in the thylakoid extraction procedures and the use of fresh, locally-available, farm spinach. The Chl a/b ratio of 2.3 indicates significant enrichment in PS-II over chloroplasts (Chl a/b, 3.0) arising from removal of stromal lamellae. The ratio of total Chl to P-680 was estimated by measurement of the O_2 yield with single turnover laser flashes [22]. This gave a ratio of 330 ± 40 Chl per P-680.

Since freeze-thaw cycling can seriously damage biological membranes, our samples were stored at −20 °C in 50% glycerol in the dark. It was found that the samples did not freeze and they retained 90–100% of their original O_2 activity until used (up to 12 months).

Fig. 1 displays the SDS-polyacrylamide gel electrophoresis of the cholate-solubilized proteins (lane 3) and the proteins released by osmotic shock in the oxidizing buffer (lanes 1 and 2). Both methods release proteins of 59, 56, 37, 34, 24, 20–21 and 18

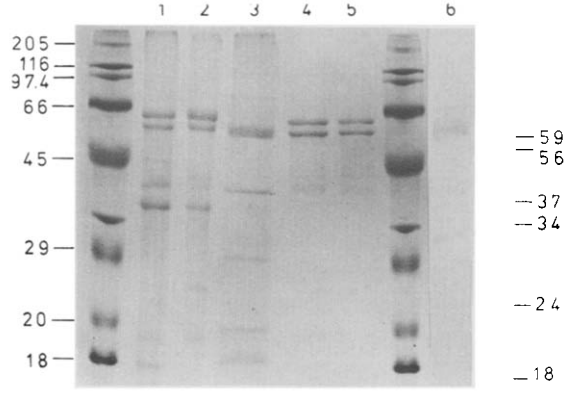

Fig. 1. SDS-gel of proteins released from spinach grana thylakoid membranes. Lane 1, proteins released by osmotic rupture of thylakoids, first wash; lane 2, second wash; lane 3, cholate-solubilized proteins released from thylakoids; lane 4, CF_1; lane 5, protein extracted by antibody to the 56 kM_r protein (bound to CNBr-activated Sepharose-4B) from a protein mixture including CF_1; lane 6, chromatofocusing fraction, pI = 6, of osmotically released proteins.

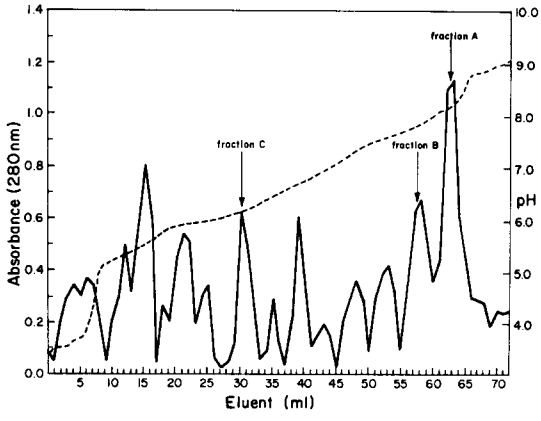

Fig. 2. Isoelectric focusing (pH 8.3–5.2) of proteins released from spinach grana thylakoid membranes by 50 mM cholate extraction. pH (- - - - - -); absorbance (———).

kDa, while cholate releases additional proteins at 54 and 29 kDa. Weaker bands are seen upon silver staining at 80, 45 and 39 kDa. The cholate extraction releases more total protein by a factor of 3–4.

The separation of the cholate extract by isoelectric focusing between pH 8.3 and 5.2 is shown in Fig. 2. 1 ml fractions obtained from this column were analyzed for Mn content and by SDS-polyacrylamide gel electrophoresis. Fig. 1, lane 6, represents the pI = 6.0–6.2 fraction which occurs as a single protein of M_r 56000 when standards are

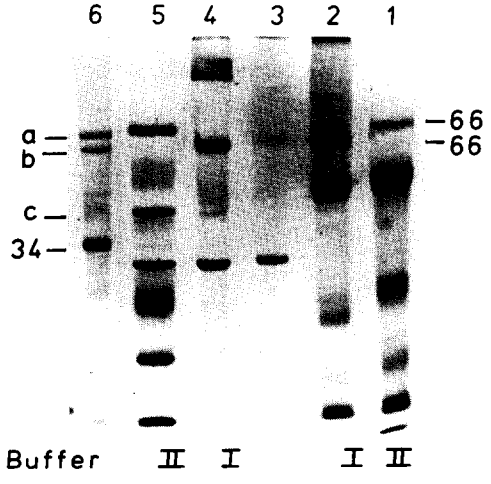

Fig. 3. SDS-gel comparing the mobility of standards in buffer I and II. Lanes 1 and 2, Sigma marker SDS-6 in buffer II and I, respectively; Lane 4, Sigma marker SDS-6H in buffer, I; lane 5, Sigma marker SDS-7 in buffer, II; lane 6, proteins released by osmotic rupture of thylakoids membranes.

made in sample buffer II. This same fraction will give a molecular weight of 65000 relative to standards made in same buffer I. This difference in the M_r of this protein is due to the change in migration of the closest molecular weight standard, bovine serum albumin with M_r = 66000 in different sample buffer systems. This is displayed in Fig. 3. Lanes 1 and 2 (Fig. 3) compare the migration of Sigma SDS-6 standards in sample buffer systems II and I, respectively. Note that the M_r value for the β band (56 kDa) would be calculated as 56000 in buffer system II, and 65000 in buffer system I. This same difference in migration is seen for bovine serum albumin in lanes 4 and 5. Lane 4 displays Sigma marker SDS-6H in buffer system I (β = 65 kDa) and lane 5 displays Sigma marker SDS-7 in buffer system II (β = 56 kDa). All of these Sigma molecular weight kits use bovine serum albumin as the protein of M_r = 66000. Lane 6 displays the proteins released by osmotic shock, with the main bands at 59, 56, 37 and 34 kDa. From this point on, all M_r values will be given for migration computed with standard in buffer system II, the Laemmli buffer system. Thus, the 56/65 kDa protein will be referred to as the 56 kDa protein.

From the cholate solubilization data (Fig. 2), this pI = 6.0–6.2 fraction contains 0.02 ppm Mn which is less than 0.02 atoms Mn per molecule of protein. All the fractions shown in Fig. 2 contain approximately the same Mn concentration (less than 0.03 ppm). Most of the Mn from the original sample is located near the cathode. This suggests

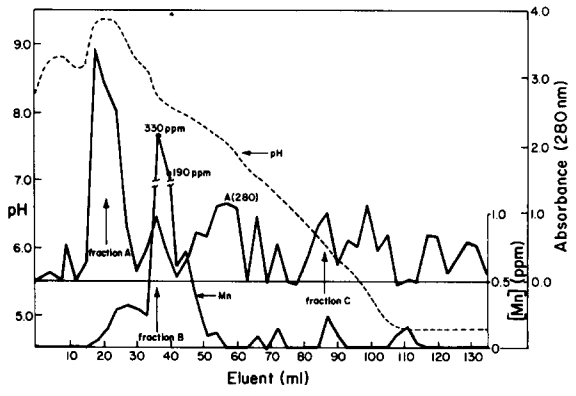

Fig. 4. Chromatofocusing (pH 8.3–5.2) of proteins released from spinach grana thylakoid membranes by 50 mM cholate extraction.

TABLE I

EFFECT OF CHEMICAL OXIDANTS ON THE YIELD OF NON-DIALYZABLE Mn BOUND TO TOTAL SOLUBILIZED PROTEINS

Cholate in suspension medium released by osmotic shock in:	μmoles Mn/g protein	
	wash 1	wash 2
2% Cholate in suspension medium released by osmotic shock in:	6.6	–
2 mM Hepes [a]	4	63
2 mM Hepes [a] + 0.4 mM $K_3Fe(CN)_6$	10	117
2 mM Hepes [a] + 1.2 mM $K_3Fe(CN)_6$ + 0.4 mM DAD	20	164

[a] Contains 2 mM EDTA, pH = 8.0.

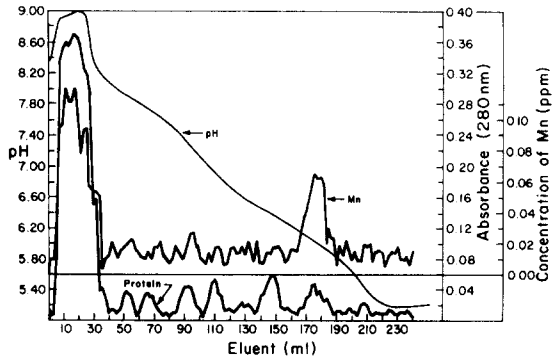

Fig. 5. Chromatofocusing (pH 8.3–5.2) of proteins released from spinach grana thylakoid membranes by osmotic rupture in oxidizing buffer (2 mM Hepes, 2 mM EDTA, 1.2 mM $K_3FE(CN)_6$, 0.4 mM diaminodurene).

that cholate, in combination with the high ionic strength and ohmic heating involved in isoelectric focusing, causes a separation of Mn from proteins.

Chromatofocusing of cholate-extracted membranes was investigated as a milder alternative to isoelectric focusing (Fig. 4). The overwhelming majority of Mn comigrates with protein at pI = 8.4–8.0, fraction B, Fig. 4. When examined by SDS-polyacrylamide gel electrophoresis, this fraction contains bands at 59, 56 and 37 kDa corresponding to subunits of CF_1, as well as unresolved components. Fraction C at a pI = 5.9–6.1 contains essentially a single protein of 56 kDa. The amount of Mn in this fraction varies from 0.3 to 0.5 Mn per molecule of protein and is a factor of 10–20 greater than observed with isoelectric focusing. This Mn was not removed by dialysis against 5 mM Mes buffer at pH = 6.0.

If most of the CF_1 is removed prior to protein extraction by repeated EDTA washings in suspension medium, then the amount of 59, 56 and 37 kDa proteins obtained is greatly reduced. Accordingly, the amount of protein and Mn purified in chromatofocusing fractions B and C (Fig. 2 and 4) is greatly reduced.

Although the chromatofocusing technique increases the amount of Mn retained in the 56 kDa protein, we were concerned that some Mn might still be released by cholate or by the material it extracts from the membranes. Therefore, extraction of thylakoid membranes by osmotic shock was used to release weakly bound proteins which might be, hopefully, less denatured (Fig. 1; lanes 1 and 2). In addition, chemical oxidants were included in the buffer to test if maintaining a high solution redox potential would affect the binding of Mn to released proteins. The results are summarized in Table I. The amount of nondialyzable Mn bound to the total protein fraction increases by a factor of 3–5. Separation of these proteins by chromatofocusing is shown in Fig. 5. A dramatic redistribution of Mn amongst the protein fractions occurs. Now only a single protein fraction in the

TABLE II

SUMMARY OF PROPERTIES OF 56 kDa protein

Isolation-purification procedure	Isoelectric point	Mn content/protein	Fe	λ_{max} (ϵ_{max} $M^{-1} \cdot cm^{-1}$)	EPR
cholate – isoelectric focusing	6.0–6.2	0.02 ± 0.05	–	–	–
cholate – chromatofocusing	5.9–6.1	0.4 ± 0.3	–	–	–
oxidizing buffer – chromatofocusing	6.0–6.2	1.9 ± 0.3	0.7 ± 0.2	280 nm (42000) 420 nm (15000)	silent

TABLE III

EFFECT OF ANTIBODY TO THE 56 kDa PROTEIN (BOUND TO CNBr-SEPHAROSE) ON THE RATE OF O_2 EVOLUTION IN THYLAKOID MEMBRANES

Inhibitor (5 μM DCMU)	Antibody	Uncoupler (1 mM NH_4Cl)	μmoles O_2 / mg Chl per h	Relative rate	Percentage inhibition
−	−	−	350	1.00	
−	+	−	280	0.80	20
−	−	+	540	1.54	
−	+	+	550	1.57	0
+	−	−	0	0	
+	+	−	0	0	0

pH range 8.3–5.2 is observed to bind a significant amount of Mn. This fraction has an isoelectric point of 6.0–6.2 and occurs predominantly as a single peptide of $M_r = 56000$ on SDS gels (Fig. 1, lane 6). It contains 1.9 ± 0.3 Mn per molecule of protein (average of five isolations).

Gel filtration of the $pI = 6.0–6.2$ fraction on Sephadex G-100 resulted in the separation of two proteins with M_r of 58000 (90%) and 112000 (10%). The value of 58 kDa agrees well with the value of 56 kDa obtained by SDS gels (Fig. 1, lane 6).

This 56 kDa protein also contains 0.8 ± 0.2 Fe/protein which are not removed by dialysis. This Fe could be due to the binding of $K_3Fe(CN)_6$ during the isolation procedure. The protein has an absorptive spectrum (with peaks) at 280 nm ($\epsilon = 42000\ cm^{-1} \cdot M^{-1}$) and at 420 nm ($\epsilon = 15000\ cm^{-1} \cdot M^{-1}$). Similar peaks are observed in the spectrum of ferricyanide at $\lambda = 260$ nm ($\epsilon = 1400\ M^{-1} \cdot cm^{-1}$), 305 nm ($\epsilon = 1800\ M^{-1} \cdot cm^{-1}$) and 420 nm ($\epsilon = 1150\ M^{-1} \cdot cm^{-1}$). The absorptivity coefficient at 420 nm is larger by a factor of 13 for the protein suggesting that an additional chromophore probably contributed. Binding of ferricyanide to nitrogen donor ligands of proteins is well established.

The purified 56 kDa protein containing 2 Mn ions is EPR-silent from 4.2 to 300 K. Our previous report [13] of an EPR signal displaying Mn hyperfine structure from this protein was due to contamination by another closely migrating Mn protein of $pI = 5.2–5.0$ and M_r of 34000. A discussion of the 34 kDa protein is included in the following paper. A summary of the chemical and spectroscopic properties of the 56 kDa protein is given in Table II.

Rabbit antibody was raised against the purified 56 kDa protein. Antibody bound to Sepharose-4B reproducibly inhibits 20% of O_2 evolution when

TABLE IV

EFFECT OF PROTEIN EXTRACTION ON Mn CONTENT AND PS II ACTIVITY IN SPINACH GRANA THYLAKOID MEMBRANES

DPC, 1,5-diphenylcarbazide; PPI,

Membrane sample [a]	Mn/400 Chl	Mn/PS II	(DPC → $Fe(CN)_6^{-3}$) relative PS II activity	(H_2O → $Fe(CN)_6^{-3}$ + PPBQ) relative O_2 rate
Grana-thylakoids	7–8	5.5–6.4	1.0	1.0
Cholate extracted	1–2	0.8–1.7	0.0	0.0
Osmotically shocked (oxidizing)	1.8–2.7	1.4–2.2	0.3	< 0.1
CF_1 extracted [b]	5–6	4–4.8	1.0	1.0

[a] Conditions for sample preparation are given in the text.

[b] CF_1 was removed by washing in 2 mM EDTA in suspension buffer.

incubated with stacked grana-thylakoids and 10–20% with French-pressed grana-thylakoids, which contain a mixed population of inverted and right-side-out membranes. This effect is cancelled by 1 mM NH_4Cl, an uncoupler of photophosphorylation (Table III). The inhibition is absent in heat-denatured, inactive antibody. These results indicate that this protein is located on the outer surface of the thylakoids and has a role in phosphorylation. Also, this antibody to the 56000 M_r protein binds CF_1 from a mixture of proteins containing CF_1 (fig. 1, lanes 4 and 5). CF_1 is that part of the ATPase in chloroplasts which is exposed to the outer surface of the membrane.

A summary of the effect of cholate and osmotic shock on Mn content and membrane activity is given in Table IV. Note that the oxidizing buffer and cholate extractions remove a similar amount of Mn and O_2 evolving capacity.

Discussion

The results of Tables I and II and Figs. 2, 4 and 5 establish that Mn associated with thylakoid membranes is rendered labile during the extraction of weakly associated proteins. Detergents such as cholate release more total protein than does osmotic shock, and both methods yield labile Mn. Further loss in Mn bound to the solubilized proteins occurs when isoelectric focusing is used for purification instead of the milder conditions of chromatofocusing. The choice of isolation and purification strategy seems to be crucial in affecting the amount of Mn bound to these proteins.

The most dramatic parameter which we find influences the binding of Mn to these proteins is the solution redox potential. Of the six major polypeptides released by osmotic shock and resolved by chromatofocusing between pH 8.3 and 5.2, only one of these at 56 kDa is found to bind stoichiometric quantities of Mn when isolated in oxidizing buffers. The released Mn is rendered non-labile when proteins are extracted under oxidizing conditions and a major redistribution of Mn amongst the released proteins accompanies this. Inclusion of the non-ionic redox mediator diiminodurene ($E_0 = +260$ mV, vs. normal hydrogen electrode) further increases the quantity of Mn associated with extracted proteins (Table I), presumably because of a faster equilibration rate than for the negatively charged ferricyanide ion. The origin of the increased binding of Mn to this protein may involve a decrease in the loss of natively bound Mn to reducing agents released into solution during the protein extraction.

The origin of the Mn associated with the 56 kDa protein appears to come from the fraction of 2–3 Mn/400 Chl which is separated by successive washings of isolated grana-thylakoids (7–8 Mn/400 Chl) with 2 mM EDTA in sucrose buffer (Table IV). This washing removes CF_1 and some Mn, but retains highly active O_2-evolving membranes (300–600 μmol O_2/mg Chl per h; 4–5 Mn/PS II). The resulting membranes are also depleted of the 56 kDa protein. If CF_1 is not completely extracted prior to cholate extraction or osmotic shock, then the 56 kDa protein is released along with additional Mn and protein. Loss of this addition Mn correlates with the loss of O_2 evolution activity (Table IV) as is well-known [23,24]. This additional Mn can bind to the intact CF_1 fragment released by cholate as shown in Fig. 4, fraction B. CF_1 is known to bind as many as five Mn(II) ions from solution, two of these bind strongly [25].

The identity of the 56 kDa protein correlates well with the β-subunit of CF_1. There is an equivalence in the M_r [10]; the β subunit exists as a β_2 dimer, as does the 56 kDa protein when observed by gel filtration; removal of CF_1 from the membrane correlates with loss of the 56 kDa protein; antibody to the 56 kDa protein localizes it to the outer thylakoid surface and to a function linked to photophosphorylation, both of which are true of CF_1; and this antibody selectively removes CF_1 from a mixture of proteins in solution, thus establishing immunological equivalence. Our results indicate that the β-subunit is the site for binding of the two strongly bound Mn ions associated with CF_1 [25]. The absence of EPR from the 56 kDa protein implies that the Mn ions are either interacting antiferromagnetically to yield a nonmagnetic ground state that is EPR-silent or, alternatively, may exist as isolated Mn(III) ions which are invariably EPR-silent owing to large zero-field splittings.

These results establish that the report by Spector and Winget [7] concerning a role for the 56

kDa (65 kDa) protein in O_2 evolution is probably incorrect. Also, the difference in M_r values calculated for this protein could explain why Nakatani and Barber [9] isolated a 58 kDa protein with the same method used by Spector and Winget [7]. However, we do observe a correlation between O_2 evolution and a 34 kDa manganoprotein which is discussed in the following article.

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It has recently been reported that isolation of CF_1 by the standard acetone extraction or EDTA extraction methods and purification by ion exchange chromatography leads to copurification of a major fraction containing the isolated CF_1-β subunit, free of other subunits (Finel, M., Rubenstein, M. and Pick, U. (1984) FEBS Lett. 166, 85). The present findings are in accord with these results showing that significant release of CF_1-β occurs.

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