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RECONSTITUTION OF PHOTOSYNTHETIC WATER SPLITTING IN INSIDE-OUT THYLAKOID VESICLES AND IDENTIFICATION OF A PARTICIPATING POLYPEPTIDE *

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Inside-out spinach thylakoid vesicles have been used for inhibition and reconstitution of photosynthetic water splitting. Washing inside-out vesicles with a buffer containing 250 mM NaCl inhibited 75% of the Photosystem II reduction of phenyl-*p*-benzoquinone. In contrast, the same treatment of right-side-out vesicles gave quite a small inhibition. The site of inhibition was shown by fluorescence induction to be located at the water-splitting side of P-680. The salt-induced inhibition was accompanied by the release of two polypeptides (23 and 16 kDa) from the inner thylakoid surface. Readdition of the salt-washed supernatant or a crude chloroplast extract to the washed inside-out vesicles at low ionic strength gave a 2.7-fold stimulation of the water-splitting activity, thereby restoring about 60% of the activity lost by salt washing. This stimulation was abolished by proteolysis of the reconstituting fractions. Restoration of the water-splitting reaction was accompanied by rebinding of the 23 and 16 kDa polypeptides to the inside-out vesicles. After polypeptide purification by ion-exchange chromatography it was shown that the 23kDa polypeptide was responsible for the restorative effect. These observations provide strong evidence that the 23 kDa polypeptide, electrostatically bound to the inner thylakoid surface, is involved in the photosynthetic water-splitting reaction.

Introduction

Photosynthetic water splitting is a fundamental process shown by all green plants and algae, including the cyanobacteria. The mechanism of water splitting is still far from understood despite intense research. There is, however, a considerable amount of kinetic information available [1,2] and the basic scheme for charge accumulation proposed by Kok et al. [3] has become widely accepted.

In contrast, information about protein components involved in the water-splitting reaction is very limited. Some polypeptides have, however, been suggested from circumstantial evidence. Metz et al. [4] reported the lack of a 34 kDa polypeptide from a *Scenedesmus* mutant specifically deprived of water-splitting capacity and with a low manganese-to-chlorophyll ratio. Åkerlund and Jansson [5] used mild trypsinization to digest a 34 kDa polypeptide specifically in inside-out thylakoids, a treatment shown earlier to inhibit the water-splitting activity [6]. They also demonstrated [5] release of 34 and 23 kDa polypeptides specifically from inside-out thylakoid vesicles by washing with alkaline Tris, known to inhibit water oxidation [7]. Later, Yamamoto et al. [8] reported the release of 33, 24 and 18 kDa polypeptides from oxygen-evolving PS II detergent particles on Tris washing.

* A preliminary report on part of this work has been published [16]

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; PS, photosystem; Chl, chlorophyll; Me_2SO, dimethyl sulfoxide.

Reconstitution obtained with purified polypeptides should be a direct way to identify water-splitting components. Some early studies using this approach [9–11] do not seem to have been confirmed or extended. More recently, Spector and Winget [12] reported the isolation of a 65 kDa polypeptide able to reconstitute water-splitting activity in cholate-extracted thylakoid membranes incorporated into liposomes. This protein contained manganese and was claimed to be the site for Tris action in thylakoids. Later, Nakatani et al. [13] following the same preparation procedure obtained a similar protein. However, this protein contained iron instead of manganese.

For direct reconstitution experiments on the water-splitting system, the use of inside-out thylakoid vesicles [14] should be advantageous. These vesicles retain high rates of oxygen evolution [15] and expose the water-splitting enzyme system to the surrounding medium [6]. Therefore, it should be possible to reach and to affect the components of the water-splitting system without the use of detergents or sonication. Thus, it should be possible to release any weakly bound water-splitting components by mild treatments without gross damage of the thylakoid membrane. This makes the conditions for reconstitution favorable, since the subsequent readdition of the released components to the native inner thylakoid surface is possible. In a preliminary study by Åkerlund [16] this approach was shown to be promising.

In the present study, the water-splitting activity of inside-out thylakoids was inhibited by washing with 250 mM NaCl and reconstituted by readdition of the released proteins. Two main polypeptides, with apparent molecular weights of 23000 and 16000, were released by the salt treatment and could be rebound to the membrane at low ionic strength. The 23 kDa polypeptide was shown by fractionation studies to be the component responsible for reconstitution.

Materials and Methods

Poly(ethylene glycol) 4000 (Carbowax 3350) was obtained from Union Carbide, New York, NY, U.S.A., and dextran T 500, batch No. 2836, from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Preparation of inside-out thylakoid vesicles

Inside-out thylakoid vesicles were prepared according to a modification of an earlier procedure [14]. Spinach leaves were homogenized in a medium composed of 300 mM sucrose, 50 mM sodium phosphate buffer (pH 7.4) and 10 mM $MgCl_2$. The slurry was filtered through four layers of nylon mesh (25 μm) and centrifuged (1000 $\times$ g, 1 min). The chloroplasts were resuspended in the same medium, pelleted by centrifugation (1000 $\times$ g, 10 min) and hypotonically shocked in 5 mM $MgCl_2$. The broken chloroplasts obtained were washed three times by centrifugation (2000 $\times$ g, 5 min) in 300 mM sucrose, 10 mM Tricine buffer (pH 7.4) and 5 mM $MgCl_2$ and finally suspended in 100 mM sucrose, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 5 mM $MgCl_2$. The stacked thylakoids were disintegrated twice in a Yeda press at a nitrogen pressure of 10 MPa. To attain further disintegration, EDTA to a final concentration of 5 mM was added followed by two more press treatments. After a low-speed centrifugation (1000 $\times$ g, 10 min) to remove starch grains and unbroken lamellae, the inside-out thylakoid vesicles formed during the disintegration procedure [17] were separated from right-side-out material by partition in an aqueous dextran/poly(ethylene glycol) two-phase system [14]. 1 ml of the Yeda press homogenate was added to 24 g of a polymer mixture to yield the following composition: 5.7% (w/w) dextran T 500, 5.7% (w/w) poly(ethylene glycol) 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl, 20 mmol/kg sucrose and thylakoid material corresponding to 4 mg Chl. The phase system was thoroughly mixed and allowed to settle. The inside-out material, partitioning to the lower phase, was purified by repeating the partition procedure twice with pure upper phase. The purified inside-out vesicles were freed from polymers by dilution (two to four times) in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4) and 2.5 mM NaCl, followed by centrifugation (100000 $\times$ g, 30 min). The vesicles were resuspended in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4) and 2.5 mM NaCl to a concentration of 500 μg Chl/ml. Part of the material was made 5% with respect to Me_2SO and stored in liquid nitrogen. The remaining material

was diluted to 200 μg Chl/ml and used for salt treatment. Broken chloroplasts were used as right-side-out material.

Salt washing of inside-out thylakoids

The inside-out thylakoid vesicles (200 μg Chl/ml) were diluted ten times with a high-salt medium composed of 10 mM sodium phosphate buffer (pH 7.4), 250 mM NaCl and 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride and incubated on ice for 30 min. After centrifugation (100000 × *g*, 30 min) the membranes were suspended in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4), 2.5 mM NaCl and 5% Me_2SO to a concentration of 500 μg Chl/ml and stored in liquid nitrogen. The supernatant was set aside for further processing.

Preparations of reconstituting fractions

Two alternative fractions were prepared.

(a) The supernatant of the salt-washed inside-out vesicles was concentrated with respect to protein by ultrafiltration (Amicon PM 10 Diaflo^R membrane, cutoff 10 kDa) and desalted to less than 5 mM NaCl by repeated dilutions and filtrations. This fraction is referred to as the salt-wash supernatant.

(b) Broken chloroplasts isolated from 2.5 kg spinach were lipid depleted with 90% acetone (precooled to −20°C) for 20 min. This was repeated once and the second sediment was pelleted by centrifugation (10000 × *g*, 10 min) and allowed to dry. 10 g of dry powder were homogenized with 300 ml water and incubated at 4°C for 1 h with stirring. The suspension was centrifuged (10000 × *g*, 10 min) and the supernatant was collected. This supernatant is referred to as the crude chloroplast extract.

A small portion of the crude chloroplast extract was incubated on ice for 10 min with subtilisin (Sigma, protease type VIII). The subtilisin/protein ratio (w/w) was 0.2. The proteolytic activity was stopped by addition of 1 mM phenylmethylsulfonyl fluoride (freshly made in ethanol).

The crude chloroplast extract was fractionated on a DEAE-Sephacel^R (Pharmacia Fine Chemicals) column. 20 ml of sample were diluted to 60 ml with 10 mM sodium phosphate buffer (pH 6.2) and 50 mM NaCl and loaded on the column. The two fractions were eluted with 10 mM sodium phosphate buffer (pH 6.2) and 50 mM NaCl and the remaining fractions with a linear NaCl gradient, 50–1000 mM in the same buffer. The fractions were desalted and concentrated as described above.

Protein determination was made according to the method of Bearden [18], adopted to suit dual-wavelength instrumentation.

Addition of reconstituting fractions to salt-washed inside-out thylakoids

The various protein fractions were added to washed inside-out thylakoid vesicles under low ionic strength and analysed for restorative effect on oxygen evolution and fluorescence induction. The incubation medium was composed of 70 mM sucrose, 30 mM sodium phosphate buffer (pH 6.5), 3 mM NaCl and thylakoid material corresponding to 20 and 7.2 μg Chl/ml for measurements of oxygen evolution and fluorescence, respectively. For analyses of rebinding the thylakoid vesicles were freed from the incubation medium and suspended in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4), 2.5 mM NaCl and 5% Me_2SO to a concentration of 400 μg Chl/ml and stored in liquid nitrogen.

Measurements of water-splitting activity

The oxygen evolution with phenyl-*p*-benzoquinone as PS II electron acceptor [19] was followed using a Clark-type oxygen electrode at 20°C. Continuous illumination was provided by a slide projector giving a quantum flux density of 2000 μE/m^2 per s between 400 and 700 nm. The measurements were performed in the incubation medium (see above) supplemented with 0.2 mM phenyl-*p*-benzoquinone. The thylakoid material corresponded to 20 μg Chl and protein fractions were added as indicated. The final volume was adjusted to 1 ml with water.

Fluorescence measurements

Room-temperature fluorescence was measured with continuous excitation light provided by the actinic source at 90° to the photomultiplier of an Aminco DW-2 spectrophotometer. The excitation light was passed through a 452 nm interference filter (half bandwidth 76 nm). The incident quantum density was 133 μE/m^2 per s. The emitted

fluorescence was filtered through a 682 nm interference filter (half bandwidth 11 nm). The photomultiplier output was amplified and recorded with a Nicolet 527 signal averager. The assay was performed in the incubation medium (see above) with the thylakoid material corresponding to 7.2 μg Chl/ml. Where indicated NH_2OH (final concentration 6 mM) or reconstituting fractions were added. The final volume was adjusted to 3 ml with water. For each measurement a new sample was dark adapted for 20 min.

SDS-polyacrylamide gel electrophoresis

Prior to electrophoresis, membrane fractions were centrifuged (100000 × *g*, 30 min) and suspended in water to a concentration of 400 μg Chl/ml. Membrane and protein samples were solubilized for 3 min at 80°C in a medium of the following composition: 5% (v/v) mercaptoethanol, 1% (w/v) SDS, 10% (w/v) glycerol and 62 mM Tris-HCl buffer (pH 6.81). Electrophoresis was performed in the buffer system of Laemmli [20], using slab gels with an acrylamide gradient of 12–20% (2.7% cross-linking). Electrophoresis was run for 19 h at a constant current density of 4 mA/cm^2. The gels were stained with Coomassie brilliant blue R 250. Molecular weight standards were bovine serum albumin (68000), ovalbumin (45000), carbonic anhydrase (30000), soybean trypsin inhibitor (22000), myoglobin (17000), ribonuclease (14000) and cytochrome *c* (12000).

Results

High-salt treatment of biological membranes is frequently used for the release of electrostatically bound components, like many peripheral proteins. Table I shows the effect on PS II electron transport after washing thylakoid membranes of opposite sidedness with a buffer containing 250 mM NaCl. The activity was measured as oxygen evolution under relatively low ionic strength, using the PS II acceptor phenyl-*p*-benzoquinone after suspension of the thylakoid membranes in a low-salt buffer. After the high-salt washing as much as 75% of the PS II activity was lost in the inside-out thylakoid vesicles while only 15% of the original activity was lost in thylakoids of normal sidedness (Table I). Such inhibition and reconstitution have been obtained in numerous experiments although the absolute values have varied. The same inhibitory effects were obtained by washing with 250 mM LiCl or KCl (not shown). (The higher control rate for the everted vesicles compared to right-side-out thylakoids reflects their PS II enrichment [15,21].)

Since the salt should easily penetrate the membrane during the 30 min incubation, the pronounced difference in inhibition between inside-out

TABLE I

INHIBITION AND RECONSTITUTION OF PS II OXYGEN EVOLUTION IN INSIDE-OUT THYLAKOID VESICLES

PS II activity from water to phenyl-*p*-benzoquinone was measured as oxygen evolution with a Clark-type electrode. Inside-out and right-side-out thylakoids (400 μg Chl/ml) were washed with a high-salt buffer (pH 7.4) containing 250 mM NaCl and assayed for activity under low-salt conditions before and after addition of the reconstituting fractions. The amount of protein added during reconstitution corresponded to 1.5 and 2.0 μg protein/μg Chl for the salt-wash supernatant and crude chloroplast extract, respectively

Material	Oxygen evolution (μmol O_2/mg Chl per h)	Inhibition (%)	Stimulation (%)	Reconstitution (%)
Inside-out thylakoids				
Control	153			
Salt washed	40	74		
Salt washed + salt-wash supernatant	109		172	61
Salt washed + chloroplast extract	107		168	59
Control + chloroplast extract	170		11	
Right-side out thylakoids				
Control	128			
Salt washed	109	15		
Salt washed + chloroplast extract	86		0	0

and normal thylakoids should not be due to accessibility restrictions but may be explained as illustrated in Fig. 1. Under high-salt conditions components are detached from the membrane in both right-sided and inside-out material. In the inside-out vesicles, components released from the original inner thylakoid surface are lost to the supernatant during centrifugation, while for the right-sided material these components are retained in the lumenal space and able to reassociate with the membrane after resuspension in a low-salt medium. This reversibility also requires that the released components have not been denatured by the treatment. (The lack of total inhibition in the inside-out fraction on salt treatment may be due to the contamination of right-side-out vesicles [22].)

The considerations above suggest that the supernatant after salt washing of inside-out thylakoids would contain active components able to restore the PS II-mediated oxygen evolution. This was tested by readdition of the concentrated salt-wash supernatant to the washed inside-out vesicles under low ionic strength. As shown in Table I, this led to an about 2.7-fold stimulation of the oxygen evolution, thereby restoring more than 60% of the activity lost due to salt washing.

Processing of the reconstituting salt-wash supernatant in terms of desalting and concentration was found cumbersome and gave quite a low recovery and therefore an alternative way for obtaining the reconstituting components was tried. Large amounts of chloroplasts were isolated followed by lipid depletion with acetone. Since the components responsible for reconstitution apparently are water soluble, the acetone powder was extracted with water. Such a crude chloroplast extract was added back to the washed inside-out thylakoids. Table I shows that this led to a stimulation of the oxygen evolution comparable to that obtained for the salt-wash supernatant. It should be pointed out that a high stimulation was obtained only for salt-washed inside-out thylakoids. For right-sided thylakoids or unwashed inside-out thylakoids there was no or only a small stimulation.

These observations (Table I) suggest that specific components involved in the electron transport through PS II were reversibly detached from the inner thylakoid surface. By only measuring the oxygen evolution associated with reduction of a PS II acceptor, one cannot judge whether the inhibitory and stimulatory effects are on the water-splitting or reducing side of the reaction center P-680 [6,23]. Since the water splitting complex is exposed at the inner thylakoid surface [6], it may be suggested that the reconstituting components released from this surface are involved in the water-splitting reaction. In order to confirm this, fluorescence induction was studied for the various thylakoids. Inhibitors on the water-splitting side of P-680 are known to reduce the variable fluorescence and also to give a slower rise time [24]. In contrast, inhibitors on the reducing side, like in the case of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), result in a more rapid rise of the variable fluorescence [24]. As shown in Fig. 2, the fluorescence induction curve for inside-out thylakoid vesicles after salt treatment shows a decrease in amplitude but a more pronounced increase in the rise time compared to the untreated material. Accordingly, this suggests that the inhibition site was on the water-splitting side. This is further supported by the result obtained after the addition of NH_2OH, a PS II donor [25] which restored the rapid rise time of the variable fluorescence of the washed inside-out vesicles. Faster rise times were also obtained after addition of the salt-wash supernatant ($t_{1/2} = 0.30$ s) and the crude chloroplast extract ($t_{1/2} = 0.25$ s), demonstrating restorative effects. Taken together with the effects on the electron-transport rates, these fluorescence measurements give strong evidence that the inhibitory and reconstituting effects are indeed exerted on the water-splitting side of P-680.

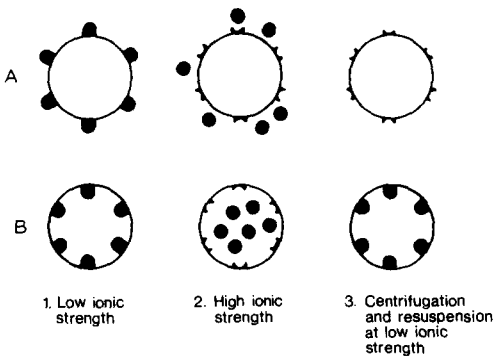

Fig. 1. Schematic representation of salt-dependent release and reassociation of components after washing thylakoid membranes of opposite sidedness. (A) Inside-out, (B) right-side-out.

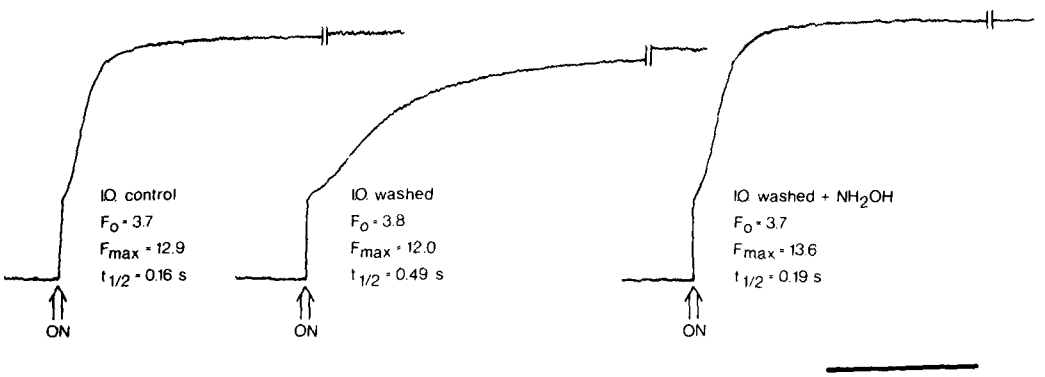

Fig. 2. Fluorescence induction curves for unwashed and salt washed inside-out (I.O.) thylakoids. Bar represents 1 s in the first part and 10 s in the second part of each trace.

In order to elucidate the nature of the reconstituting component(s), the crude chloroplast extract was treated with the proteolytic enzyme subtilisin before addition to the thylakoids. This treatment almost completely abolished the stimulating power of the extract, thereby strongly suggesting that the restoring components are proteins.

In an attempt to identify the protein components involved in this reversible inhibition of the oxygen evolution, SDS-polyacrylamide gel electrophoresis analyses were applied to the various fractions during the inhibition and reactivation experiments. Comparison of the polypeptide composition of control inside-out vesicles with that of the salt washed inside-out vesicles (Fig. 3a and b) shows that the salt released mainly two polypeptides with apparent molecular weights of 23000 and 16000 while other polypeptides were mainly unaffected. As expected, these two polypeptides were the major constituents of the salt-wash supernatant (Fig. 3e). This also contained a major band at 21 kDa which apparently was not released from the membrane upon washing and may therefore be a degradation product of the 23 kDa polypeptide. Salt treatment of right-sided thylakoids releases mainly subunits from the coupling fractor [16]. The polypeptide pattern of the crude chloroplast extract is shown in Fig. 3d. As expected from the way of preparation, it contained a lot of polypeptides, including the 23 and 16 kDa polypeptides. By readdition of this chloroplast extract under low ionic strength to the washed inside-out vesicles followed by centrifugation, the binding of polypeptides to the original inner thylakoid surface was studied. Fig. 3c shows that pronounced binding was seen only for the 23 and 16 kDa polypeptides. This points to quite a specific binding,

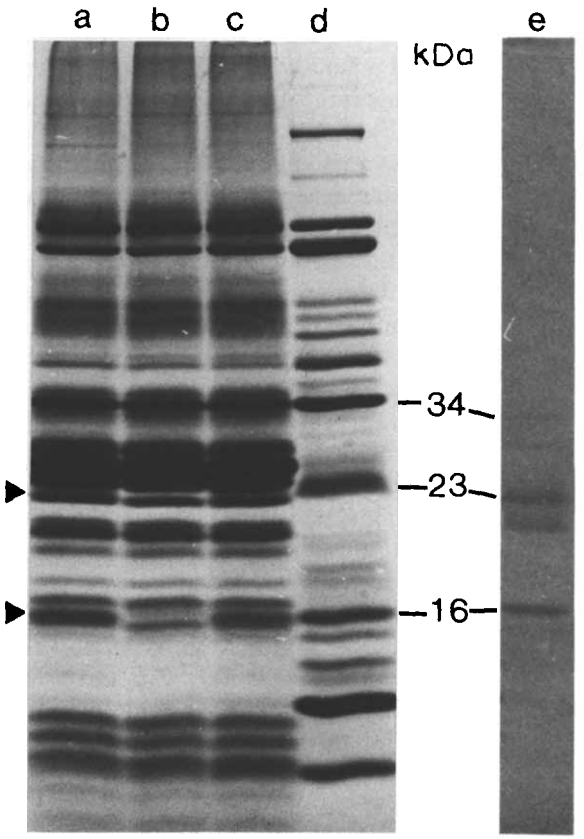

Fig. 3. Polypeptide pattern for inside-out thylakoid vesicles (a) Control, (b) salt washed, (c) salt washed after binding experiment with crude chloroplast extract, (d) crude chloroplast extract, (e) salt-wash supernatant. The arrows indicate the 23 and 16 kDa bands.

since the chloroplast extract contained numerous polypeptides, some in quite high amounts, which showed no or very little binding. Only two other polypeptides with apparent molecular weights of 62000 and 10000 showed some binding. Since they were not released from the membrane by the salt washing, their involvement in the inhibitory and restorative effects on the oxygen evolution is unlikely. Readdition of the concentrated salt-wash supernatant to washed inside-out vesicles under low ionic strength also led to rebinding of the 23 and 16 kDa polypeptides (not shown).

As shown in Table II, the stimulatory effect on oxygen evolution remained even after spinning the membranes down, following incubation with the protein fractions under low ionic strength. This result shows that the restoration of oxygen evolution was not due only to the presence of polypeptides but also to rebinding of the 23 and 16 kDa polypeptides to the original inner thylakoid surface. This gives further support to one or both of these polypeptides being responsible for the reconstitution of the oxygen evolution.

In order to purify the active polypeptide(s), ion-exchange chromatography on DEAE-Sephacel was performed. To obtain sufficient amounts of protein for this fractionation the crude chloroplast extract had to be used rather than the less complex salt-wash supernatant. The elution pattern in Fig. 4 shows several peaks from which fractions 1–9 were collected. For each fraction, tubes were pooled followed by concentration and desalting. Fig. 5 compares the stimulatory effect on oxygen evolution of two of these protein fractions to that of the unfractionated crude chloroplast extract. Only for fraction 2 was an improved stimulation obtained, with half-maximal stimulation at 50 μg protein/mg Chl compared to 300 μg/mg for the unfractionated material. Moreover, fraction 2 reached about the same maximal stimulation compared to the chloroplast extract. All other fractions gave a quite low stimulation, comparable to or less than that of fraction 1 (Fig. 5).

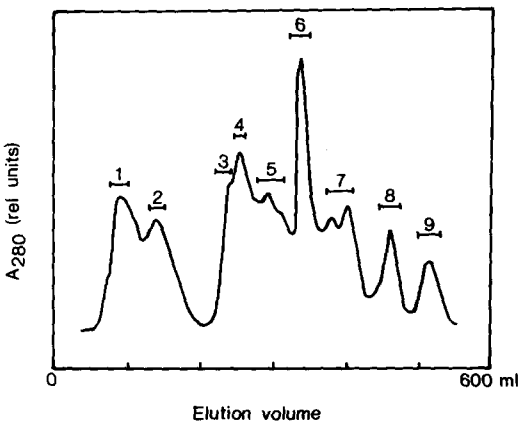

Fig. 4. Elution profile from ion-exchange chromatography on DEAE-Sephacel of the crude chloroplast extract. Fractions were collected as indicated.

TABLE II

RECONSTITUTION OF OXYGEN EVOLUTION BY REBINDING OF POLYPEPTIDES TO THE INNER THYLAKOID SURFACE

PS II activity from water to phenyl-*p*-benzoquinone was measured as oxygen evolution with a Clark-type electrode. Salt-washed inside-out thylakoid vesicles were incubated with the salt-wash supernatant or the crude chloroplast extract at low ionic strength. The amount of protein added during incubation corresponded to 1.5 and 2.0 μg protein/μg Chl for the salt-wash supernatant and crude chloroplast extract, respectively. The vesicles were spun down in order to remove unbound components and assayed for activity under low-salt conditions.

Material	Oxygen evolution (μmol O_2/mg Chl per h)	Stimulation (%)
Inside-out thylakoids		
Salt washed	50	
Salt washed after binding experiment with the salt-wash supernatant	121	142
Salt washed after binding experiment with the chloroplast extract	133	158

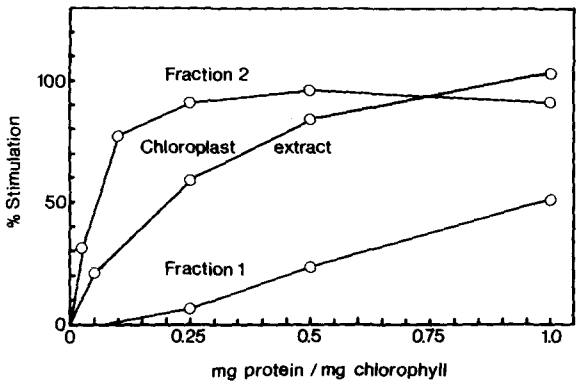

Fig. 5. Stimulation curves for oxygen evolution of salt-washed inside-out thylakoid vesicles obtained by addition of the crude chloroplast extract or fractions 1 or 2 from the chromatographic fractionation. Other fractions gave a stimulation comparable to or less than that of fraction 1.

Fig. 6 shows the polypeptides of the different fractions obtained after chromatography. The stimulating fraction 2 contained the 23 kDa polypeptide with very small amounts of other polypeptides. The 16 kDa polypeptide was the major band of fraction 1, which showed a rather poor stimulation. In the fractions that exhibited such a low stimulating capacity as in the case of fractions

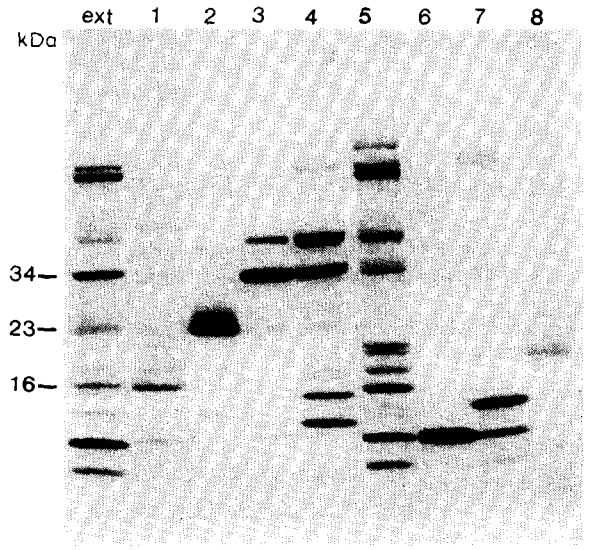

Fig. 6. Polypeptide pattern for the fractions obtained after ion-exchange chromatography of the crude chloroplast extract. 1–8, fraction numbers; ext, crude chloroplast extract.

1, 3 and 4, this could be attributed to the presence of small amounts of the 23 kDa polypeptide (Fig. 6). The amount of fraction 2 protein needed for half-maximal stimulation was 50 μg protein/mg Chl. Assuming a Chl/P-680 ratio of 300 for the inside-out thylakoids, taking their PS II enrichment into account, the number of 23 kDa polypeptides per reaction center was calculated to be 1.3. This stoichiometry is reasonable for a component involved in photosynthetic water splitting. These calculations makes the participation of any minor polypeptides of peak 2 (Fig. 6) unlikely, since their amount compared to P-680 would be far too low.

Discussion

By inhibition and restoration studies on inside-out thylakoid vesicles, we have demonstrated the involvement of a 23 kDa polypeptide in photosynthetic water splitting. That the reconstitution was specific and not due to some general effects of the assay conditions was concluded from the following observations. (a) Inhibition and restoration of the water-splitting activity was seen for inside-out thylakoids but not for right-side-out thylakoids. (b) The restorative capacity of the reconstituting fractions was abolished by proteolysis. (c) Reconstitution was accompanied by rebinding of specific polypeptides (23 and 16 kDa) to the inner thylakoid surface. (d) Only one polypeptide (23 kDa) out of several could stimulate.

The detailed properties of the stimulating 23 kDa polypeptide have not yet been established but the following characteristics are known. It is a water-soluble peripheral protein electrostatically bound to the inner thylakoid surface. This is suggested by its salt-dependent release from and reassociation with the inside-out vesicles without any need for detergents. Its weak binding to the ion-exchanger DEAE-Sephacel indicates that the protein is only slightly negative or even positively charged at neutral pH. The polypeptide is active after being kept frozen for several months. The polypeptide gives a fuzzy band in SDS-polyacrylamide gel electrophoresis, which may indicate the involvement of intrachain sulfhydryl groups that are hard to keep reduced throughout the electrophoretic run. Of special interest is whether

or not the 23 kDa polypeptide contains manganese. This metal is reversibly released by Tris washing [26] and has been ascribed a central role in the charge accumulation necessary for oxygen evolution [27]. The metal composition of the polypeptide is currently being investigated and preliminary analyses show no manganese associated with the polypeptide.

Apart from the 23 kDa polypeptide, a 16 kDa polypeptide was released from the inner thylakoid surface by high-salt washing and rebound under low-salt conditions. Moreover, this polypeptide is enriched in PS II fractions (not shown). However, when purified, this 16 kDa polypeptide could not contribute to any stimulation. Another polypeptide apparently lacking reconstituting effect was the 34 kDa polypeptide, highly purified in fraction 3 after chromatography (Fig. 6). Still, this polypeptide is completely released from inside-out thylakoids by Tris washing [5], a treatment known to cause inhibition of oxygen evolution [7]. Thus, circumstantial evidence supports the involvement of both the 16 and 34 kDa polypeptides in photosynthetic water splitting. This is also supported by recent results by other groups [4,8,28]. At present, the possibility cannot be excluded that these two polypeptides lost their activity during the isolation procedure. This may explain why total restoration of the lost water splitting was not obtained. Together with the 16 and 34 kDa polypeptides, the active 23 kDa polypeptide may be part of a multienzyme complex. Together with intrinsic polypeptides, these three water-soluble components may constitute a membrane-spanning complex in analogy with the mitochondrial cytochrome *c* oxidase [29].

There are a few early reports in the literature of components able to stimulate water-splitting activity. Fredricks and Jagendorf [9] reported a component, apparently a protein, which could stimulate the PS II activity in blue-green algae, but according to their own interpretation this component was not likely to be involved on the water-splitting side. Tel-Or and Avron [10] described a manganese-containing component from blue-green algae. This factor could reconstitute the PS II activity in hypotonically washed spheroplasts from *Phormidium luridum*. The site of reactivation was inferred to be on the water-splitting side. However, the heat stability and low molecular weight of this component make it unlikely as a polypeptide. Huzisige et al. [11] reported a factor derived from Tris-treated chloroplasts on sonication. This factor could stimulate the PS II activity in untreated but not in Tris-washed grana fractions. Although these works [9–11] were quite early, there have been no confirmational or extended studies presented. The different 65 kDa polypeptides more recently reported by Spector and Winget [12] and Nakatani et al. [13] could both restore water-splitting capacity in cholate-extracted thylakoid membranes, incorporated into liposomes. Cheniae and Sayre [30] could reconstitute the oxygen evolution in cholate-extracted thylakoids with components from the cholate supernatant. Since the 65 kDa polypeptides apparently are water soluble, the need for detergents and artificial membrane systems during isolation and reconstitution may reflect the internal location of the water-splitting components in the thylakoids. This explains why detergents did not have to be applied in the present study, where inside-out thylakoid vesicles were used. Whether there is any relation between our 23 kDa polypeptide and the proteins obtained by Nakatani et al. [13] and Spector and Winget [12] has yet to be established.

An obvious question is in what way our isolated 23 kDa polypeptide reconstitutes oxygen evolution. There are at least two possible alternatives. The first and perhaps the most straightforward explanation is that the protein is directly involved in the electron transport from water to P-680$^+$ as an electron carrier. The estimated 23 kDa polypeptide/P-680 ratio of 1.3 seems to support such a conclusion. The other possibility is that the isolated protein in some way regulates the activity of the water-splitting enzyme, possibly by influencing the deactivation of S-states. Further investigations have to be made to distinguish between these alternatives.

The weak binding of the protein to the membrane may suggest that it can be free in the intrathylakoid space under certain conditions, e.g., at high ionic strength. This is in accordance with a model proposed by Lavorel [31] to explain the damping of oscillation in oxygen yield under flashing light conditions. In this model it is suggested that at least a part of the water-splitting complex

is soluble in the intrathylakoid space. The recent results of Maison-Peteri et al. [32] indeed show that the damping of oscillation in oxygen yield measured on intact thylakoids was affected by the ionic composition of the medium: at pH 7.5 an increase in damping was observed on going from 100 mM KCl to 20 mM $MgCl_2$.

The present isolation of a protein able to reconstitute water splitting in inside-out thylakoids offers unique possibilities to study this process. Studies are in progress to investigate further the properties of the 23 kDa polypeptide and to ascribe to it a more specific function in the water-splitting complex.

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