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IMMUNOLOGICAL STUDIES ON THE ORGANIZATION OF PROTEINS IN PHOTOSYNTHETIC OXYGEN EVOLUTION

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Studies on inside-out thylakoid vesicles and several Photosystem-II particles have suggested the involvement of three proteins of 33, 23 and 16 kDa in photosynthetic oxygen evolution. In this study, monospecific antibodies were raised against the purified 33, 23 and 16 kDa proteins. The antibodies were used to investigate the organization and function of these proteins in the oxygen-evolving complex. Quantification of the 33, 23 and 16 kDa proteins by rocket immunoelectrophoresis revealed one copy of each polypeptide per some 200 chlorophylls in unfractionated thylakoids. Isolated inside-out thylakoids, derived from the grana partitions, showed 6–8 times more of the 33, 23 and 16 kDa proteins, on a chlorophyll basis, compared to the stroma lamellae vesicles. Agglutination studies revealed that the proteins are exposed on the lumenal side of the thylakoid membrane. An obligatory role for the 23 kDa protein in the photosynthetic water oxidation is suggested from the close correlation obtained between the inhibition of oxygen evolution and the release of this protein as caused by washing the inside-out thylakoids with increasing concentrations of NaCl. For the 16 kDa protein no such correlation was obtained. Rebinding experiments, using both salt-washed and alkaline Tris-washed inside-out thylakoids revealed that the 33 kDa protein was required for the binding of the 23 kDa protein, which in turn enhanced the binding of the 16 kDa protein. It is concluded that the three proteins are closely organized as a complex at the inner thylakoid surface in association with membrane spanning proteins of Photosystem II.

Introduction

Recent studies on the composition of the photosynthetic oxygen-evolving complex have suggested the involvement of three extrinsic polypeptides with apparent molecular weights of 33 000, 23–24 000 and 16–18 000. These three polypeptides can be released from inside-out thylakoid vesicles and other oxygen-evolving Photosystem-II preparations by washing with alkaline Tris or with NaCl at high concentrations, treatments that also inhibit oxygen evolution [1–4]. The 23 kDa protein was shown by Åkerlund et al. [5] to restore partly oxygen evolution in salt-washed inside-out vesicles. This restorative effect on oxygen evolution by the 23 kDa protein has later been demonstrated also with other Photosystem II preparations [6–9]. Furthermore, recent studies on mutants support these findings [10]. Restoration with the 16 kDa protein has so far only been reported for thylakoid particles isolated by a cholate-procedure [9]. No marked restoration with the 33 kDa protein has yet been demonstrated. In mutant studies [11,12] the disappearance of a 34 kDa protein correlated with a decrease in oxygen-evolving capacity and manganese content, although the cor-

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Abbreviations: IgG, immunoglobulin-G, Chl, chlorophyll.

respondence of this protein to the extrinsic 33 kDa protein discussed above has recently been questioned [13]. The isolation of a 34 kDa protein-containing manganese was recently reported [14]. Thus, a protein in the 33–34 kDa region is a likely candidate for the postulated mangano-protein in photosynthetic oxygen evolution. Since no prosthetic groups have so far been detected in the isolated 23 and 16 kDa proteins [7,15–18], their precise roles in oxygen evolution remain to be established.

To date the analyses of the 33, 23 and 16 kDa proteins have been confined to SDS-polyacrylamide gel electrophoresis, which has certain limitations. Due to the complexity of the thylakoid polypeptide composition interpretations concerning one individual polypeptide can be seriously hampered by staining properties, limits of detection and the problem of comigration. The later problem particularly concerns the 33 and 23 kDa polypeptides of oxygen evolution. The 33 kDa region also contains cytochrome *f* [19] and two polypeptides of the Photosystem II core complex [20], one of which is the herbicide-binding protein [21]. The broad apopolypeptides of LHC II [22] cover the 23 kDa region which also contains polypeptides of LHC I [23] and the cytochrome *b/f* complex [19].

To overcome these problems, we have used in the present study immunological methods for specific and sensitive analyses of the organization and function of the oxygen-evolution complex. Quantification by rocket immunoelectrophoresis showed one copy of each polypeptide per some 200 chlorophylls. The three proteins are arranged in a complex at the inner thylakoid surface, where the 33 kDa protein is required for the binding of the 23 kDa protein. The 23 kDa protein in turn, enhanced the binding of the 16 kDa protein. Correlation between the inhibition of oxygen evolution and release of the proteins after salt-washing of inside-out vesicles indicates an obligatory role for the 23 kDa protein but not for the 16 kDa protein.

Materials and Methods

Isolation of proteins

The 33, 23, and 16 kDa proteins were isolated as in Ref. 18. Briefly, isolated thylakoids were treated with 90% acetone to remove lipids. The water-soluble proteins were extracted, and fractionated on a DEAE-Sephacell^R column using 10 mM sodium phosphate (pH 6.4)/50 mM NaCl. The 33 kDa protein was further purified by affinity chromatography using Blue Sepharose^R, while the 23 and 16 kDa proteins were purified through repetitive runs on a CM-Sepharose column, using a linear gradient of 0–400 mM NaCl, containing 10 mM sodium phosphate (pH 6.4).

Immunological methods

Each of the three isolated proteins (200–300 μg) was mixed with one volume of Freund's complete adjuvant (Difco) and injected behind the scapulae of the rabbits. After 5 weeks, the rabbits were booster-injected in the same manner except that incomplete adjuvant was used. Antisera were collected 2, 3 and 4 weeks after the booster injection. IgG was prepared from the sera by affinity chromatography on a Protein A-Sepharose CL-4B column. After elution of the bulk protein with 50 mM K_2HPO_4/0.5 M KCl, the IgG was eluted with 0.1 M glycine (pH 3.0), dialyzed against 10 mM sodium phosphate (pH 7.4), and concentrated by ultrafiltration. Control IgG was prepared from serum obtained from non-immunized rabbits. The IgG concentration was calculated from the absorbance at 280 nm, using $A_{280} = 1.27$ for 1 mg IgG/ml (Hansson, U.-B., personal communication). The specificity of the antisera was determined by 'Western' blotting [24]. Thylakoid polypeptides were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane by electrophoresis. The blot was cut into strips; one strip was stained with Amido Black and the others incubated sequentially with antiserum (0.5–5 μl serum/10 ml medium) and peroxidase-conjugated swine anti-rabbit IgG. The peroxidase-containing bands were developed by incubating the strips with H_2O_2 and *o*-dianisidine. Rocket immunoelectrophoresis [25] was run in 0.8% agarose containing 20 mM barbital buffer (pH 8.6)/0.6% Zwittergent TM-314 (Calbiochem)/0.4% Triton X-100. 20 mM barbital buffer was used as electrode buffer. In quantification of the 16 kDa protein, the pH of the barbital buffer was lowered to 7.0, since the protein has a

high isoelectric point [18], so that cathodic rockets were obtained. The electrophoresis was run at 200 V at 5 °C for 18 h. After electrophoresis, the plates were washed, dried and finally stained with Coomassie brilliant blue. The amount of each antigen was determined from the rocket area. Before electrophoresis, thylakoid membranes were solubilized in a 3% detergent mixture of Triton X-100 and Zwittergent TM-314 (2:3). The purified proteins were treated in the same way and used as standards.

Agglutination of thylakoid vesicles was investigated by mixing 10 μl of IgG (1–4 mg/ml) and 10 μl of vesicle suspension (100 μg Chl/ml) at 20 °C. The aggregation was followed using a phase contrast microscope and was completed within 5 min.

Preparation and treatments of inside-out thylakoid vesicles

Inside-out and right-side out thylakoid vesicles were prepared by phase partition after Yeda press treatment in the presence of 5 mM $MgCl_2$ as previously described [5,26]. In some experiments, inside-out vesicles with a particularly high Photosystem-II enrichment and Photosystem-I-rich stroma lamellae were prepared as in Ref. [27]. Inside-out vesicles were washed in NaCl solutions of increasing concentrations (10–300 mM, pH 7.4) or in 100 mM Tris (pH 9.0), and finally suspended in 500 mM sucrose/5 mM sodium phosphate (pH 7.4)/2.5 mM NaCl.

Rebinding of the purified proteins to the inner thylakoid surface was studied by adding 20 μg of purified protein to washed inside-out vesicles (100 μg chlorophyll) suspended in 5 ml 70 mM sucrose/30 mM sodium phosphate (pH 6.5)/3 mM NaCl. After incubation for 30 min on ice the vesicles were sedimented at 100000 × *g* for 30 min. In the case of rebinding more than one type of protein, each addition was followed by the incubation and centrifugation steps to allow for a sequential rebinding.

Oxygen evolution with phenyl-*p*-benzoquinone as Photosystem-II acceptor was followed in 1 ml of a medium composed of 70 mM sucrose, 30 mM sodium phosphate (pH 6.5), 3 mM NaCl and thylakoids corresponding to 20 μg chlorophyll. Chlorophyll was determined according to Arnon [28] and protein according to Bearden [29].

Results

In order to obtain monospecific antibodies, the individual 33, 23 and 16 kDa proteins were purified by ion-exchange and affinity chromatography [17,18]. The purity and properties of the isolated proteins have been given elsewhere [17,18]. When

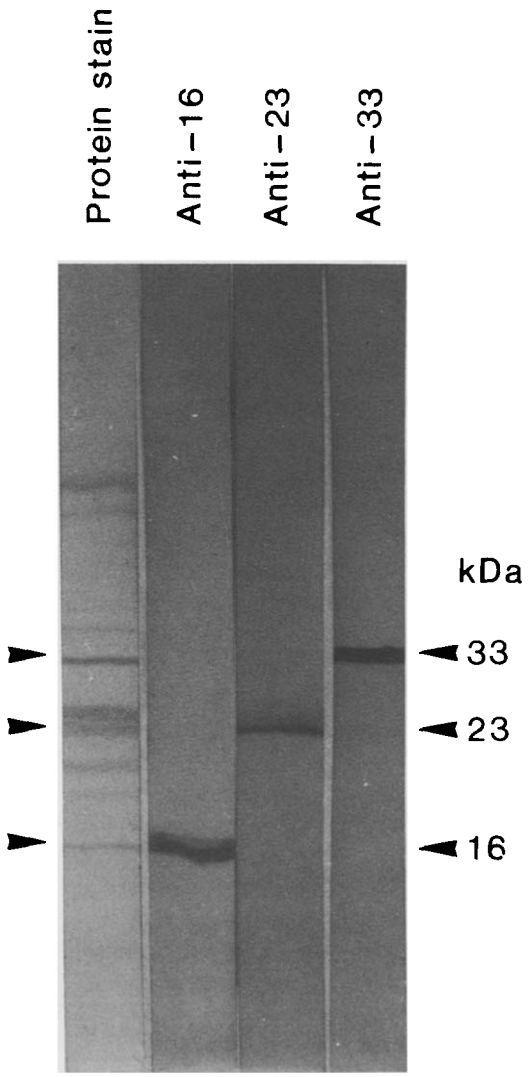

Fig. 1. The specificity of the antisera used for immunoelectrophoresis as demonstrated by 'Western' blotting. Thylakoid polypeptides were separated by SDS-polyacrylamide gel electrophoresis and then blotted on to a nitrocellulose membrane. The concentrations of the antisera against the 16, 23 and 33 kDa proteins were 5, 0.5 and 5 μl/10 ml medium, respectively. Thylakoid polypeptides reacting with IgG in the sera were specifically stained.

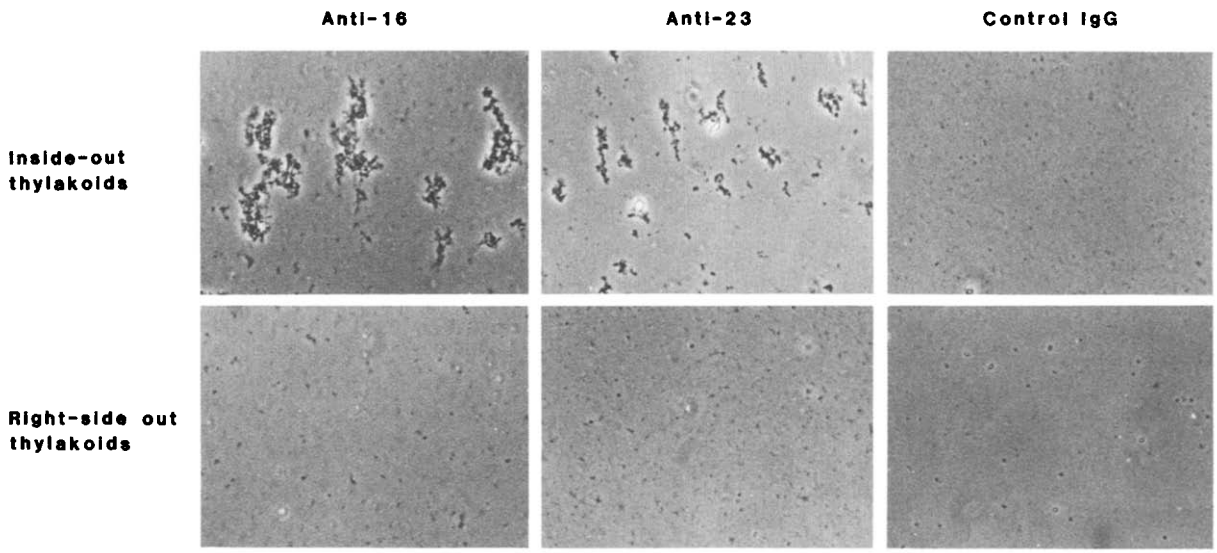

Fig. 2. Immunoprecipitation of inside-out and right-side out thylakoid vesicles. IgG prepared from antisera against the 23 and 16 kDa proteins, and from non-immune serum was used. Final concentrations in 20 μl were 2 mg IgG/ml and 50 μg chlorophyll/ml.

injected into rabbits, each protein gave rise to a monospecific antiserum. The monospecificity of the antisera was shown by 'Western'-blotting using dissociated intact thylakoids (Fig. 1). Each antiserum gave rise to one band at the expected apparent molecular weight. Also, in rocket immunoelectrophoresis with thylakoids each antiserum gave one main precipitation arc only.

In order to establish the location of the 23 and 16 kDa proteins in the thylakoid membrane, agglutination of inside-out and right-side out vesicles were compared. For that purpose, the IgG fraction of each antiserum was used to minimize unspecific agglutination caused by the bulk serum proteins. As can be seen in Fig. 2, the IgG against the 23 and 16 kDa proteins caused agglutination of inside-out thylakoids, clearly distinguishable from the background aggregation seen with the control IgG. In contrast, the agglutination of right-side out vesicles was weak with both anti-IgG and control IgG. This shows that the 23 and 16 kDa proteins are exposed on the inner thylakoid surface which is in accordance with the release-pattern upon salt-washing [15]. The same agglutination results have previously been observed using antibodies to the 33 kDa protein (Andersson, B., Klein-Hitpass, L., Jansson, C. and Berzborn, R., unpublished data). None of the antisera tested showed any inhibitory effect on oxygen evolution with untreated inside-out thylakoids. Thus the antibodies did not interact with any active sites of the proteins.

The amounts of the 23 and 16 kDa proteins were determined by rocket immunoelectrophoresis in the membranes of unfractionated thylakoids, inside-out thylakoids (derived from the appressed regions [30]) and stroma lamellae vesicles. This technique, which allows the quantification of a small amount of a certain protein species in a complex protein mixture, has previously been used in studies on thylakoid proteins such as CF_1, ferredoxin, reductase and plastocyanin [31–33]. Our results show that unfractionated thylakoids contained one 23 kDa protein per some 170 chlorophylls. The value for the 16 kDa protein was one per 205 chlorophylls. Previously, the 33 kDa protein was shown to be present in one copy per 200 chlorophylls (Andersson, B., Klein-Hitpass, L., Jansson, C. and Berzborn, R. unpublished data). Fractionation of the thylakoids revealed that both the 23 and 16 kDa proteins were highly enriched in the appressed regions, as judged by their content in highly purified inside-out vesicles as compared to stroma lamellae vesicles. On a chlorophyll basis, there was 6–8 times more of these proteins in appressed regions as compared to non-ap-

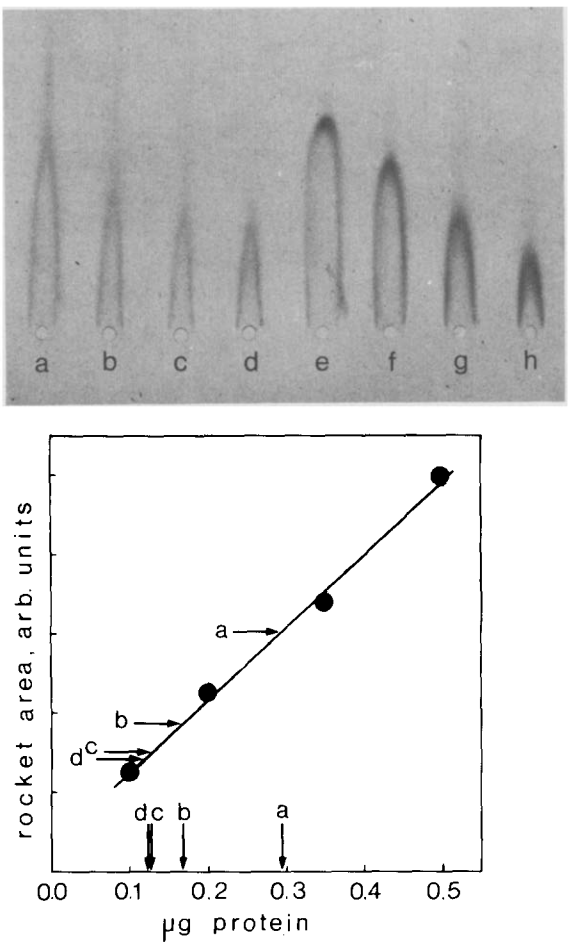

Fig. 3. Rocket immunoelectrophoresis, using the antiserum against the 23 kDa protein. Samples were (a) untreated inside-out thylakoids corresponding to 1.8 µg chlorophyll, (b–d) inside-out thylakoids washed with 100, 125 and 150 mM NaCl, respectively, (e–h) 0.50, 0.35, 0.20 and 0.10 µg of the purified 23 kDa protein, respectively. The diagram shows the corresponding calibration curve and the quantification of the thylakoid samples.

pressed regions.

Washing inside-out vesicles with increasing concentrations of NaCl (10–300 mM) resulted in a increasing deactivation of oxygen evolution (Fig. 4). Using rocket immunoelectrophoresis the amounts of the 33, 23 and 16 kDa proteins remaining on the membrane after washing were quantified. Fig. 3 shows an example of the rockets obtained for the 23 kDa protein remaining in inside-out vesicles after washing with increasing concentrations of salt. The area of these rockets was determined and is depicted in the diagram of Fig. 3. The same procedure was used for the 16 and 33 kDa proteins. Based upon such immunoelectrophoresis data, we have correlated the release of these proteins with the inhibition of oxygen evolution (Fig. 4). Strikingly, the release of the 23 kDa protein followed very closely the degree of inhibition. In contrast, the release of the 16 kDa protein markedly exceeded the inhibition; at 30% inhibition as much as 75% of the protein was released. Usually not more than 80% of the 23 and 16 kDa proteins could be released, probably due to the approx. 20% contamination by right-side out vesicles [34]. Virtually no 33 kDa protein was released by the salt washings. Moreover, salt-washing did not release any manganese [17]. This is in contrast to alkaline Tris treatment which released all three proteins (Table I) and which is known to release manganese also.

In order to investigate the molecular organization of the 33, 23 and 16 kDa proteins on the inner thylakoid surface rebinding of the three proteins to either salt or Tris-washed inside-out vesicles was performed (Fig. 5). Usually a 5–10 times excess of each protein (based on the stoichiometry in untreated material) was added to depleted inside-out vesicles under low ionic strength. Upon readdition of the 23 kDa protein to salt-washed inside-out vesicles, it was rebound to 93% of the original

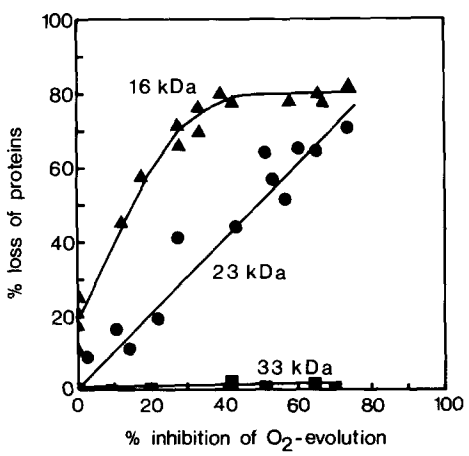

Fig. 4. Correlation between the release of the 33, 23 and 16 kDa proteins and the inhibition of oxygen-evolution after washing inside-out thylakoids with increasing concentrations of NaCl.

TABLE I

PROTEIN REBINDING TO SALT OR TRIS-WASHED INSIDE-OUT PARTICLES

The inside-out thylakoid vesicles were washed in 250 mM NaCl/10 mM sodium phosphate buffer (pH 7.4) or in 100 mM Tris-HCl, (pH 9.0). For rebinding, each of the three proteins (20 μg) was added individually to washed vesicles (100 μ Chl), suspended in 5 ml 70 mM sucrose/30 mM sodium phosphate buffer (pH 6.5)/3 mM NaCl. After incubation for 30 min, the vesicles were sedimented before being analysed. When more than one protein was added, the vesicles were sedimented and resuspended before the second protein was added, to achieve a sequential rebinding. The amounts of protein were determined by rocket immunoelectrophoresis. The level of each protein (33, 23, and 16 kDa) in untreated vesicles was set to 100%.

Materials	Additions	Levels of protein (%)		
		33 kDa	23 kDa	16 kDa
Untreated vesicles		100	100	100
Salt-washed vesicles		98	34	16
	+23		93	
	+16			54
	+23+16			89
Tris-washed vesicles		34	17	11
	+33	91		
	+23		23	
	+33+23		47	
	+16			24
	+33+16			19

level. Readdition of the 16 kDa protein resulted only in a partial rebinding. However, if the 16 kDa protein was added after the 23 kDa protein was rebound, the binding of the 16 kDa protein was markedly enhanced reaching nearly 90% of the original level. This indicates that the 23 kDa protein is involved in the binding of the 16 kDa protein. When the 33, 23 and 16 kDa proteins were added individually to the Tris-washed vesicles, only the 33 kDa protein showed any significant rebinding. A partial rebinding of the 23 kDa protein to the Tris-washed vesicles was obtained after the 33 kDa protein had been rebound. In contrast, the presence of the 33 kDa protein did not enhance the rebinding of the 16 kDa protein. Thus the 33 kDa protein seems necessary for the binding of the 23 kDa protein but not directly for the 16 kDa protein.

Discussion

Quantification of the 33, 23 and 16 kDa proteins by rocket immunoelectrophoresis showed that there is approximately one copy of each polypeptide per 200 chlorophylls in intact spinach thylakoids. The stoichiometry between these proteins and the Photosystem-II reaction centre depends on which chlorophyll : P-680 or chlorophyll : Q ratio applies to spinach thylakoids. Using repetitive short flashes, a chlorophyll : Q ratio of 550 was obtained [35]. Using another spectrophotometric method, Melis and Harvey reported a chlorophyll : Q ratio of 250–300 [36]. The first ratio results in 2–3 molecules of each polypeptide per Photosystem-II reaction centre, while the latter ratio gives a value of 1–2. Moreover, the 23 and 16 kDa proteins were highly enriched in subfractions originating from the appressed thylakoid region, demonstrating their association with Photosystem II. Although quite depleted in stroma lamellae vesicles, reproducible amounts of the proteins could always be detected. This may suggest that Photosystem II in non-appressed thylakoids; possible Photosystem II β [37], also contains the 33, 23 and 16 kDa proteins.

The agglutination studies show that these proteins are exposed on the inner side of the thylakoid membrane. Furthermore, the conditions needed to release the proteins [2,5], and their solubility in water without the use of detergents [17,18] suggest that they are extrinsic proteins located at the inner thylakoid surface. The fact that these proteins show pronounced enrichment in the appressed thylakoid regions is quite interesting. Previously, such an extreme lateral heterogeneity between the appressed and non-appressed thylakoids has only been demonstrated for proteins exposed at the outer membrane surface [38]. For those, a lateral displacement may be explained by the influence of attractive and repulsive forces in the tight appressions [39,40]. The 33, 23 and 16 kDa proteins probably belong to the Photosystem II membrane spanning complex able to sense the outside environment [41]. This is supported by a preliminary immunoprecipitation study [42]. This showed that when added to a partly solubilized Photosystem II preparation [43], antibodies against the 33 and 23 kDa proteins did not only precipitate the antigenic

proteins but also membrane proteins of 24, 22 and 10 kDa. Additionally, attractive and repulsive forces on the lumenal side of the thylakoid [44] could also contribute to the lateral heterogeneity of the 33, 23 and 16 kDa proteins.

The rebinding studies showed that the 33 kDa protein could rebind independently of the 23 and 16 kDa proteins suggesting that it is bound to some intrinsic membrane protein. The 23 kDa protein appears to bind to the 33 kDa protein. Probably, the 23 kDa protein also has a binding site on an intrinsic membrane protein, since it can be retained on the membrane when the 33 kDa protein is released by mild Tris treatment (Andersson, B., Klein-Hitpass, L., Jansson, C. and Berzborn, R., unpublished data) or urea treatment [45]. Finally, the 16 kDa protein appears to be bound to the 23 kDa protein. A similar rebinding pattern for these three proteins has recently been demonstrated from studies on Photosystem-II particles [8,45]. Thus, the three proteins seem to be arranged in one complex at the inner thylakoid surface and in probable association with intrinsic Photosystem II proteins. Both the 23 and 16 kDa proteins are likely to be bound by electrostatic forces, since their release and rebinding is dependent on the ionic environment. In contrast, the 33 kDa protein was not released by high salt (Fig. 5) indicating that other than electrostatic forces may be involved in its binding to the membrane. A hydrophobic binding is not likely, since the protein is released by increasing the temperature to 53°C (Andreasson, L.E., personal communication) and hydrophobic interactions are strengthened at higher temperatures [46]. Rather, hydrogen bonding may be involved in the attachment of the 33 kDa protein, in accordance with its release at high pH or increasing temperature.

The strict correlation between the release of the 23 kDa protein and the inactivation of the oxygen evolving activity (Fig. 4) after salt-washing suggests an obligatory role for this protein in photosynthetic water oxidation. Furthermore, if the protein is an oligomer, as may be suggested from its stoichiometry in relation to the Photosystem-II reaction centre, it is also released in that form. An obligatory role for the 23 kDa protein has also been supported by recent kinetic studies [47,48].

Very recent results [49] show that the 23 kDa protein increases the affinity of the water oxidation site for chloride. This indicates that the obligatory role for this protein in oxygen evolution may apply only under low chloride conditions. This may explain discrepancies in the assignment of the 23 kDa protein as obligatory (Fig. 4, [47,48]) or supplementary [4,8] for oxygen evolution.

The release pattern of the 16 kDa protein (Fig. 4) argues against an obligatory role in oxygen evolution. However, considering that the protein may be a dimer, a release curve close to that observed would be obtained if the subunits were released independently and only one was necessary for the activity.

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