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IDENTIFICATION OF POLYPEPTIDES ASSOCIATED WITH THE 23 AND 33 kDa PROTEINS OF PHOTOSYNTHETIC OXYGEN EVOLUTION

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An immunological approach was used for nearest-neighbor analyses for the 23 and 33 kDa proteins of the oxygen-evolving complex. Functional Photosystem II particles with a simple polypeptide composition were partly solubilized with detergent and incubated with monospecific antibodies against either the 23 or the 33 kDa protein. SDS-polyacrylamide gel electrophoresis revealed that the immunoprecipitates, apart from the antigenic proteins, also contained polypeptides at 24, 22 and 10 kDa. In contrast, polypeptides of the light-harvesting and Photosystem II core complexes showed very poor coprecipitation with the 23 and 33 kDa proteins. The 24, 22 and 10 kDa polypeptides were not precipitated by the antibodies if the 23 and 33 kDa proteins had been removed from the particles prior to solubilization. These observations demonstrate a close association between the 24, 22 and 10 kDa polypeptides and the 23 and 33 kDa proteins of the oxygen-evolving complex. None of these precipitated polypeptides contained any manganese. It is suggested that the 24, 22 and 10 kDa polypeptides are subunits of the oxygen-evolving complex and involved in the binding of the extrinsic 23 and 33 kDa proteins to the inner thylakoid surface.

Introduction

The identification and characterization of proteins in the thylakoid membrane is of fundamental importance for understanding the complex energy conservation process of photosynthesis. For Photosystem II, including photosynthetic oxygen evolution, the polypeptide composition has been unknown, but progress has been made in recent years. The isolation of functional Photosystem II preparations with a simple polypeptide composition has limited the number of possible polypeptides in Photosystem II to approx. 10–15 [1–9]. Out of these polypeptides, several have been identified and at least partly characterized. Prominent bands on SDS-polyacrylamide gel electrophoresis are 2–4 bands in the 26–27 kDa region belonging to the light-harvesting complex of Photosystem II [10]. The two polypeptides of 47 and 43 kDa belong to the chlorophyll *a* complex of Photosystem II [11], in which the former polypeptide has been shown to be the actual reaction centre apopolypeptide [12]. The Photosystem II preparations are sensitive to DCMU and bind atrazine [13], and should therefore contain the 32 kDa herbicide-binding protein harbouring the second quinone acceptor B [14]. Cytochrome *b*-559 is also present and usually identified as a 9–10 kDa polypeptide [15]. These 47, 43, 32 and 10 kDa polypeptides and an uncharacterized 34 kDa polypeptide can be isolated as one complex [16]. This Photosystem II core complex is able to perform DCIP reduction in the presence of diphenylcarba-

Abbreviations: Chl, chlorophyll; IgG, immunoglobulin-G; LHC-II, light-harvesting complex II; SDS, sodium dodecyl sulphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCIP, 2,6-dichlorophenolindophenol.

zide [17]. An intrinsic 34 kDa polypeptide has been identified in Photosystem II particles from maize [7] and has been suggested to be the polypeptide whose absence in certain mutants correlates with decreased manganese contents and loss of oxygen evolution [18,19]. The Photosystem II preparations also contain varying amounts of cytochrome *f* [3,6,8,9]. Moreover, there are several polypeptides of unknown properties and function.

In addition to the polypeptides mentioned above, which all appear intrinsic, the Photosystem II particles contain three extrinsic proteins of 33, 23 and 16 kDa, which have been ascribed a role in photosynthetic oxygen evolution [20]. The 33 kDa protein has been suggested to be associated with manganese [21], while the 23 kDa protein seems to play a mediating role for the chloride [22,23] and/or calcium [24,25] necessary for oxygen evolution. The 33, 23 and 16 kDa proteins are bound to the inner thylakoid surface at low ionic strength and neutral pH but can all be released by alkaline Tris [20], and in the case of the 23 and 16 kDa polypeptides, also by high concentrations of sodium chloride [26].

How all these polypeptides are arranged in the thylakoid membrane to provide the functional machinery of Photosystem II is far from understood. In this study, we have investigated which polypeptides are specifically associated with the extrinsic 23 and 33 kDa proteins of oxygen evolution. This has been done by immunoprecipitation of partly solubilized Photosystem II particles using monospecific antibodies against each of the two proteins. When immunoprecipitation was performed at low ionic strength and neutral pH, the antibodies not only precipitated the antigenic proteins themselves, but also proteins of 24, 22 and 10 kDa. It is suggested that these three polypeptides, due to their close association with the extrinsic 23 and 33 kDa proteins, are subunits in the oxygen-evolving complex.

Materials and Methods

Thylakoid membranes from spinach [27] were fractionated by the detergent method of Berthold et al. [1] yielding Photosystem II particles. These particles were suspended and stored in 5 mM sodium phosphate (pH 6.5)/2.5 mM NaCl/500 mM sucrose. For one aliquot of the preparation, the 23 and 33 kDa proteins were removed by incubation in 800 mM Tris-HCl (pH 8.4) for 15 min on ice in room light, followed by sedimentation at $100\,000 \times g$ for 45 min. This treatment was repeated once. The particles were washed in 5 mM sodium phosphate (pH 6.5)/2.5 mM NaCl/500 mM sucrose and finally stored in this medium. Rocket immunoelectrophoresis showed that this Tris treatment quantitatively removed the 23 and 33 kDa proteins.

The normal and the Tris-washed Photosystem II particles (200 μg/ml in 10 mM sodium phosphate (pH 6.5)) were further solubilized using a detergent mixture (40% Triton X-100/60% Zwittergent TM-314) at a ratio of 3, 4, 5 and 10 mg detergent/mg chlorophyll, to obtain samples solubilized to different degrees. After 30 min incubation on ice, unsolubilized material was pelleted at $150\,000 \times g$ for 45 min. As judged by SDS-polyacrylamide gel electrophoresis, the pellet and the supernatant contained the same polypeptides, demonstrating that no preferential solubilization of certain polypeptides occurred during this treatment (not shown).

Antibodies against each of the 23 and 33 kDa proteins were obtained from rabbits using standard immunological procedures [28]. An IgG fraction was prepared from each serum by affinity chromatography on a protein A-Sepharose CL-4B column and dialysed against 10 mM sodium phosphate (pH 6.5). Furthermore, nonspecific antibodies normally occurring in preimmune rabbit sera, and mainly crossreacting with the galactolipids [29] were removed by adsorbing the IgG fractions with the Tris-washed Photosystem II particles, devoid of the antigenic 23 and 33 kDa proteins, and solubilized at the various detergent-to-chlorophyll ratios. To these solubilized Tris-washed Photosystem II particles, IgG was added (30% of the volume). The mixture was incubated for 4 h on ice, and centrifuged at $10\,000 \times g$ for 10 min to remove unspecific precipitates. When these adsorbed IgG fractions were reincubated with detergent-solubilized, Tris-washed particles, no precipitate was formed. Monospecificity of the original antisera was shown by 'Western' blotting [28].

Immunoprecipitation was performed by mixing the final IgG fraction, obtained as the supernatant

after the 10000 × *g* centrifugation, with a double volume of normal Photosystem II particles, solubilized at the same detergent-to-chlorophyll ratio as the Tris-washed particles. After incubation for 2 h on ice, the precipitate was collected by centrifugation at 10000 × *g* and washed once in 10 mM sodium phosphate (pH 6.5).

For SDS-polyacrylamide gel electrophoresis, Photosystem II particles and the various precipitates were suspended in solubilizing buffer [30], sonicated in a water-bath-type sonicator for 1 min, and kept at 65°C for 2 min to solubilize the samples. Reducing substances, such as mercaptoethanol, were omitted to avoid dissociation of the IgG into the light and heavy chains. Electrophoresis was run in the buffer system of Laemmli [30] using 12–22.5% gradient gels. The gels were stained with Coomassie brilliant blue R-250 and the polypeptide bands quantified with an LKB 2202 laser gel scanner connected to a computing integrator.

Metal analyses of the precipitates were performed with particle-induced X-ray emission [31]. Prior to these analyses, the samples were solubilized in 1% Triton X-100/10 mM sodium phosphate (pH 6.5).

Chlorophyll *a* and *b* were determined according to Arnon [32]. Cytochrome *b*-559 was prepared mainly according to Metz et al. [15].

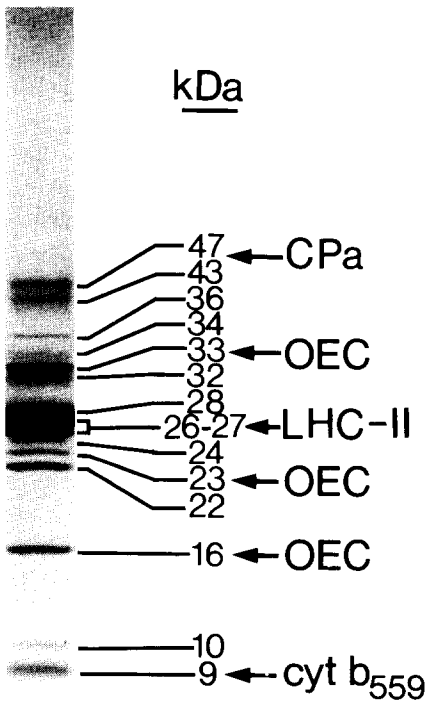


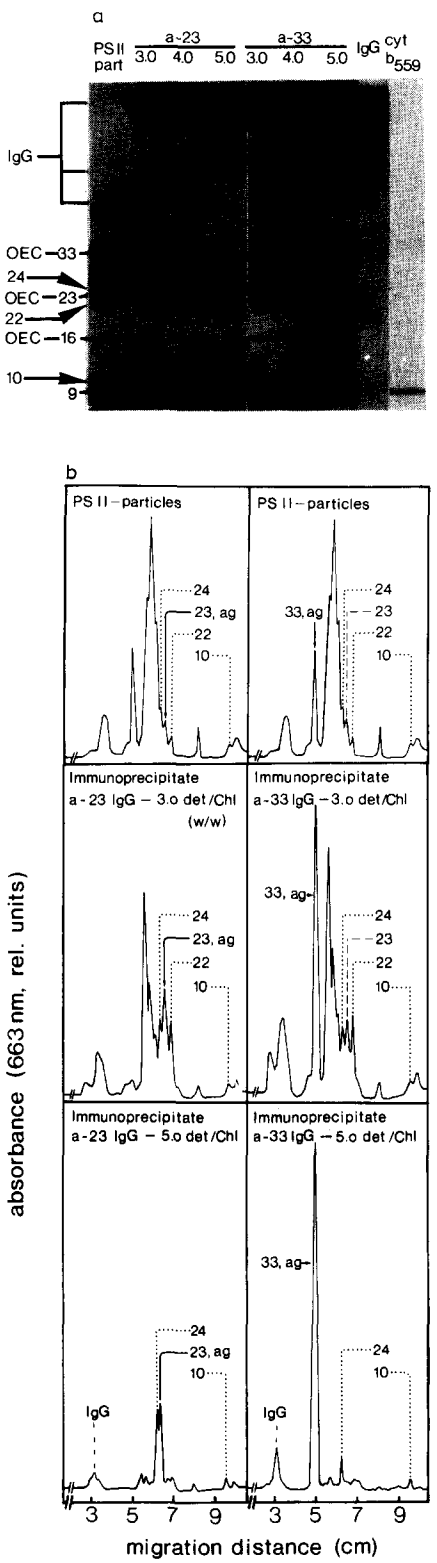
Fig. 1. Polypeptide composition of the Photosystem II particles used as starting material for the immunoprecipitations. CPa, Photosystem II reaction centre complex; OEC, oxygen-evolving complex.

Results

As revealed by SDS-polyacrylamide gel electrophoresis, the Photosystem II particles showed a simple polypeptide composition (Fig. 1), in agreement with several previous studies [1–8]. Present among the 14 polypeptides detected by Coomassie blue staining were the extrinsic 23 and 33 kDa proteins of the oxygen-evolving complex. To identify further the polypeptides associated with oxygen evolution, we attempted to find out which polypeptides of the Photosystem II particles (Fig. 1) are specifically associated with the 23 and 33 kDa proteins. To this end, we analyzed the composition of the immunoprecipitates obtained when partly solubilized Photosystem II particles were incubated with monospecific antibodies against either the 23 or the 33 kDa protein. The solubilization preceeding immunoprecipitation was designed to segregate discrete protein complexes and, at the same time, allow a sequential dissociation of polypeptides from the individual complexes. This was achieved by using a mixture of the mild detergents Zwittergent TM-314 and Triton X-100 at low concentrations (3,4,5 and 10 detergent/chlorophyll (w/w)). Furthermore, the solubilization was performed at neutral pH and low ionic strength to maintain the 23 and 33 kDa proteins attached to their binding sites. In these experiments, 20–60% of the antigenic proteins were precipitated (see Table II).

Immunoprecipitation with IgG against the 23 kDa protein

When monospecific antibodies, directed against the 23 kDa protein, were added to the solubilized Photosystem II particles, not only the antigenic 23 kDa protein itself was precipitated but also a number of other polypeptides (Fig. 2). The polypeptide composition of the immunoprecipitates



was markedly different from the starting material, demonstrating that the various Photosystem II polypeptides showed different degrees of coprecipitation with the 23 kDa protein.

To allow for comparison between different polypeptides, their coprecipitation was quantified from gel scans (Fig. 2b). The ratio between each polypeptide and the 23 kDa protein was calculated for the precipitates and finally divided by the corresponding value in the Photosystem II particles, used as starting material. These ratios, expressed as percentage coprecipitation, are given in Table I. When the antibodies were added to Photosystem II particles, solubilized at a detergent-to-chlorophyll ratio of 3, a 22 kDa polypeptide showed a coprecipitation as high as 71%. The values for two other polypeptides at 24 and 10 kDa were also quite prominent, 41 and 43% respectively. In sharp contrast, the 26–27 kDa apopolypeptides of the light-harvesting complex and the 43 and 47 kDa apopolypeptides of the Photosystem II reaction centre showed a coprecipitation of less than 8%. The quantification of the 43 and 47 kDa polypeptides was corrected for the comigrating IgG component at 50 kDa (Fig. 2). Another protein which also showed a low coprecipitation was the 33 kDa protein. Polypeptides at 34, 28, 16 and 9 kDa showed intermediate values ranging from 15 to 25%. When a detergent-to-chlorophyll ratio of 4 was used for solubilization, the precipitation pattern observed at the lower ratio was further accentuated. The coprecipitations of the apopolypeptides of the light-harvesting and Photosystem II reaction centre complexes were now below 5% and also the 34, 28, 16 and 9 kDa polypeptides were now quite depleted in relation to the 23 kDa protein. The 22, 24 and 10 kDa polypeptides still showed a quite high coprecipitation. However, the coprecipitation for the 22 kDa polypeptide had dropped below

Fig. 2. (a) Polypeptide patterns of immunoprecipitates obtained from solubilized Photosystem II particles incubated with antibodies against either the 23 kDa protein (a-23) or the 33 kDa protein (a-33), (slot 2–7). Slots 1, 8 and 9 show the original Photosystem II particles, IgG and purified cytochrome *b*-559, respectively. (b) Densitometric traces of gel patterns presented in (a). Each polypeptide was quantified by determination of the area under the corresponding peak. ag, the antigenic protein; det, detergent.

TABLE I

DEGREE OF COPRECIPITATION OF PHOTOSYSTEM II (PS II) POLYPEPTIDES WITH THE ANTIGENIC 23 AND 33 kDa PROTEINS

The percentage coprecipitation was calculated by the following formula: $((x/ag)_{\text{precipitate}}/(x/ag)_{\text{PS II particles}})\times 100$. x, amount of a particular PS II polypeptide; ag, amount of antigenic protein. For each degree of solubilization, the average of three experiments are given. a-23, a-33, see legend to Table II.

PSII polypeptides (kDa)	Degree of coprecipitation (%)					
	Detergent/ Chl (w/w): a-23 IgG			a-33 IgG		
	3.0	4.0	5.0	3.0	4.0	5.0
22	71	23	8	85	47	13
24	41	24	30	51	35	38
10	43	44	19	41	20	20
23	100	100	100	49	39	8
28	25	8	3	31	10	1
34	16	1	2	32	13	8
9	16	14	5	20	11	8
16	15	8	4	24	8	4
47	8	4	4	16	9	3
43	7	5	4	18	9	3
33	7	2	1	100	100	100
26–27	7	1	2	9	5	3

that of the 24 and 10 kDa polypeptides. After solubilization at a detergent-to-chlorophyll ratio of 5, only the 24 and 10 kDa polypeptides still showed a high coprecipitation with the 23 kDa protein. Virtually the same pattern was obtained when the experiment was carried out at a detergent-to-chlorophyll ratio of 10 (not shown).

The various immunoprecipitates were also analysed with respect to their thylakoid protein-to-chlorophyll ratio and manganese contents (Table II). Compared to the starting material, the protein-to-chlorophyll ratio was markedly increased in the precipitates. In the precipitate obtained after solubilization at 10 mg detergent/mg chlorophyll, the ratio was some 10-times higher than in the starting material, after correcting for the IgG polypeptides. The colour of the pellets was only faintly green. This is consistent with the poor coprecipitation of the chlorophyll-carrying light-harvesting and Photosystem II reaction centre complexes. Manganese was found only in the precipitate obtained at a detergent-to-chlorophyll ratio of 3. It was of interest to find out whether the 10 kDa polypeptide, showing a high coprecipitation, was cytochrome *b*-559. This was tested by running a purified aliquot of the cytochrome next to the Photosystem II particles on SDS-polyacrylamide gel electrophoresis (Fig. 2a). The purified cytochrome comigrated with a 9 kDa polypeptide and not with the 10 kDa polypeptide.

TABLE II

CHLOROPHYLL, MANGANESE AND ANTIGEN CONTENTS OF IMMUNOPRECIPITATES FROM SOLUBILIZED PHOTOSYSTEM II (PS II) PARTICLES

Thylakoid protein was determined from Coomassie-stained SDS-polyacrylamide gel electrophoresis omitting the IgG-bands. a-23 and a-33 denotes IgG against the 23 and 33 kDa proteins, respectively. Values in parentheses denote detergent/chlorophyll ratios (w/w). d.l., detection limit.

Materials	Protein/Chl (w/w)	Mn/protein ((w/w)($\times 10^6$))	Amount antigen precipitated (%)
PS II particles	5	110	
Precipitates:			
a-23 (3.0)	17	14	48
a-23 (4.0)	18	–	58
a-23 (5.0)	34	d.l.	53
a-23 (10.0)	50	d.l.	53
a-33 (3.0)	11	34	22
a-33 (4.0)	15	24	30
a-33 (5.0)	65	d.l.	32
a-33 (10.0)	67	d.l.	25

It should be mentioned that in order to obtain the coprecipitation patterns shown in Fig. 2, Tables I and II, some pretreatment of the IgG fractions was necessary. Although immunoblotting showed that the antisera were monospecific, unspecific precipitation occurred. The main reason was probably that the rabbits already before immunization have antibodies crossreacting with the galactolipids of the thylakoid membrane [29]. When mild solubilizations are made, lipids remain on the proteins, in particular the hydrophobic ones [33,34]. This problem was overcome by adsorbing the IgG preparations with solubilized Tris-washed Photosystem II particles, devoid of the antigenic 23 and 33 kDa proteins. In this way, IgG fractions were obtained which did not react with any other thylakoid components than the 23 and 33 kDa proteins, respectively. This was demonstrated by reincubating the adsorbed IgG fractions with the Tris-washed, detergent-solubilized Photosystem II particles devoid of the 23 and 33 kDa proteins. Upon this reincubation, no precipitates were formed.

Immunoprecipitation with IgG against the 33 kDa protein

When antibodies against the 33 kDa protein, pretreated as above, were used for immunoprecipitation, the results resembled in many respects those obtained with antibodies against the 23 kDa protein (Fig. 2, Tables I and II). The polypeptides at 22, 24, and 10 kDa showed a high coprecipitation with the antigenic 33 kDa protein. This was also the case for the 23 kDa protein, while the light-harvesting and the reaction centre apopolypeptides showed a low coprecipitation. The polypeptides at 34, 28, 16 and 9 kDa showed intermediate values also in this experiment. At a detergent-to-chlorophyll ratio of 5, both the 22 and 23 kDa polypeptides were no longer coprecipitating, leaving mainly the 23 and 10 kDa polypeptides in the precipitate together with the 33 kDa protein. Manganese, in low amounts, was detected only in the precipitates obtained at the detergent-to-chlorophyll ratios of 3 and 4 (Table II).

Discussion

By immunoprecipitation, the specificity of an antibody is used to remove the antigenic protein selectively from a complex mixture such as a solubilized biomembrane. If the antigenic protein is not free but bound to other protiens, the antibody may precipitate not only the antigenic protein but also proteins attached to it. Thus, immunoprecipitation when performed under controlled conditions can be used for nearest-neighbor analyses. In the present study, solubilized Photosystem II particles were incubated with antibodies against the 23 and the 33 kDa protein, respectively, and three polypeptides of 24, 10 and 22 kDa were found to coprecipitate. This result suggests a close association between these three polypeptides and the 23 and 33 kDa proteins of the oxygen-evolving complex. We exclude the possibility that the present coprecipitations are caused by lack of monospecificity since: (i) immunoblotting showed that each of the two original antisera reacted only with one thylakoid polypeptide [28]; and (ii) no precipitation occurred when the adsorbed IgG fractions used for immunoprecipitation were incubated with detergent-solubilized Photosystem II particles devoid of the antigenic 23 and 33 kDa proteins.

It should be emphasized that evaluation of the coprecipitation requires that the amount of each polypeptide is quantified and related to the amount of the antigenic protein. Simple visual inspection of the polypeptide pattern of a precipitate is not sufficient, since even a poor coprecipitation of a polypeptide that is prominent in the starting material may give rise to a significant band. Thus, in a previous preliminary study [35], a high coprecipitation was implied also for the 27, 43 and 47 kDa polypeptides, but as shown in Table I, quantification reveals a quite low coprecipitation of these polypeptides.

It remains to be seen whether the structural association of the 24, 10 and 22 kDa polypeptides with the 23 and 33 kDa proteins can be extended to also include a functional role in oxygen evolution. However, so far, no oxygen evolution or any other Photosystem II activity has been detected after the further solubilization of the Photosystem II particles. None or only small amounts of manganese were found in the precipitates, suggesting that either the manganese had been released by the detergents or that none of the dominating polypeptides in the precipitates was associated with manganese.

At present, very little data is at hand concerning the 24, 10 and 22 kDa polypeptides. The 24 and 22 kDa polypeptides are likely to be intrinsic, since they are not released by treatments known to release intrinsic membrane proteins. The possibility that the 10 kDa polypeptide represents cytochrome *b*-559 was not supported by coelectrophoresis of the pure cytochrome with the Photosystem II particles. Recent studies of the Photosystem II particles have shown that the 10 kDa polypeptide can be released by alkaline Tris [42] in the same way as has previously been described for the 33, 23 and 16 kDa proteins. This implies that also the 10 kDa protein is an extrinsic membrane protein. The 22 kDa protein differs from the 24 and 10 kDa polypeptides in showing an extremely high coprecipitation at low detergent concentrations but virtually none at higher concentrations. This implies that the 22 kDa polypeptide is closely associated with the 23 and 33 kDa proteins by hydrophobic forces that are very sensitive to the presence of detergents. On the other hand, the 24 and 10 kDa polypeptides exhibit a significant coprecipitation even at high detergent concentrations. Probably, these two polypeptides are bound to the 23 and 33 kDa proteins by electrostatic forces and/or hydrogen bonding, thereby being less affected by the detergents.

Several of the polypeptides in the Photosystem II particles are arranged either in the light-harvesting chlorophyll *a/b* complex or in the Photosystem II core complex. It is of interest to note that no subunit of these two complexes showed a high coprecipitation with either the 23 or the 33 kDa protein. This is consistent with a recent fractionation study by Westhoff et al. [36]. Gradient centrifugation following dodecylmaltoside solubilization of Photosystem II particles separated the extrinsic proteins of oxygen evolution from both the Photosystem II core complex and the light-harvesting complex. Moreover, a polypeptide of approx. 22 kDa was found in a fraction next to the 23 and 33 kDa proteins. These observations suggest that proteins involved in oxygen evolution are arranged in a distinct complex separated from the other two Photosystem II complexes. The suggestion that an individual Photosystem II reaction centre may be able to interact with several oxygen-evolving complexes [37] is consistent with such an arrangement. Still, most kinetic data [38] suggest a need for a functional close association between a separate oxygen-evolving complex and a Photosystem II core complex. Recent studies by Ono and Inoue [39,40] have suggested a proximity between manganese, the 33 kDa protein and the 47 kDa reaction centre polypeptide. This may seem to be a contradiction to our present data which show poor coprecipitation of the 47 kDa polypeptide with the 33 kDa protein. It must be stressed, however, that although our immunoprecipitations do give evidence for a close association of the 24, 10 and 22 kDa polypeptides with the oxygen-evolving complex, our data do not exclude the association of additional polypeptides. A low coprecipitation could simply mean that a certain polypeptide is bound to one of the antigenic proteins with forces that are very sensitive to detergents even in the low concentrations used here, or its presence may shield the antigenic sites, thereby preventing the antibodies from reacting. In fact, the latter seems to be the case for the immunoprecipitation using the antibody against the 23 kDa protein, which does not cause much coprecipitation of the 33 kDa protein, although previous studies [28,41] have suggested a close interaction between these two proteins.

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