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The mode of binding of three extrinsic proteins of 33 kDa, 23 kDa and 18 kDa in the Photosystem II complex of spinach

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Binding of three extrinsic proteins of 33 kDa, 23 kDa and 18 kDa to the oxygen-evolving Photosystem II (PS II) complex was investigated under equilibrium conditions using spinach PS II membranes. Dissociation constants for the functional and stoichiometric binding of the extrinsic proteins were determined to be 12, 2.7 and 3.8 nM for the 33 kDa, 23 kDa and 18 kDa proteins, respectively. These values indicate the binding energy between the proteins and the PS II complex on the order of 10–11 kcal · mol^{-1}. Effect of removal of the extrinsic proteins and Mn atoms from the PS II complex on the dissociation constant and the number of binding sites for the proteins was also examined. The results indicate that the binding site for the 33 kDa protein is present in the PS II complex devoid of the functional Mn and that the Mn stabilizes the binding of the 33 kDa protein. The change in the binding energy of the 33 kDa protein afforded by the Mn was estimated to be about 1 kcal · mol^{-1}, indicating that the interaction between the 33 kDa protein and the functional Mn is, if any, very weak. It is also indicated that the 33 kDa protein bound to the PS II complex provides the high-affinity binding site for the 23 kDa protein in the complex and that the 23 kDa protein together with the 33 kDa protein does so for the 18 kDa protein. The measurement of the sedimentation coefficients of the extrinsic proteins and protein mixtures in an aqueous solution showed that the extrinsic proteins did not interact with each other when they were apart from the PS II complex. These observations suggest that the binding of the extrinsic proteins to the PS II complex alters the conformation of the extrinsic proteins themselves and/or that of the intrinsic part of the complex so as to create the binding sites for the other extrinsic proteins.

Introduction

Three extrinsic proteins of molecular mass 33, 23 and 18 kDa are involved in photosynthetic oxygen evolution of higher plants [1–31]. These proteins bind to the oxygen-evolving PS II complex (hereafter, designated PS II complex) in the inner surface of the closed thylakoid membrane [1]. By use of PS II membrane fragments in which the inner surface of thylakoid membrane is exposed to the outer aqueous phase [3,4], the extrinsic proteins can be readily manipulated and their function elucidated (for reviews, see Refs. 5, 6).

The PS II membranes contain one molecule each of the three extrinsic proteins, four Mn atoms and 220 Chl molecules per one PS II complex [7]. The three proteins can specifically be removed from the complex by treating the PS II membranes with concentrated salt or urea: treatment with 1.0 M NaCl removes the 23 kDa and 18 kDa proteins from the complex [8,9], and that with either alkaline Tris [3], 1.0 M $CaCl_2$ [10] or 2.6 M urea [11,12] removes the 33 kDa protein. The 23 kDa and 18 kDa proteins are likely to bind to the PS II complex by electrostatic interaction, since concentrated NaCl can remove them, and the 33 kDa protein by hydrophobic interaction since urea, but not NaCl, can remove it. The released proteins can be rebound to the PS II complex with saturation at the stoichiometric level when they are added to the depleted membranes at low salt conditions [6,9,13]. By manipulating this reconstitution system the function of each of the three extrinsic proteins has been clarified: the 33 kDa protein stabilizes two of the four Mn atoms which form a catalytic center of oxygen evolution [12–14] and accelerates the S-state transition from S_3 to S_0 in the Kok scheme [15], the 23 kDa protein acts to trap Ca^{2+} in the intrinsic part of the PS II complex [6,16,17] and the 18 kDa protein sustains the

Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; SDS, sodium dodecyl sulphate.

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oxygen evolution at low concentrations of Cl^-, an essential factor for the oxygen evolution [18,19].

Quantitative analysis of the release and rebinding of the extrinsic proteins has provided information as to the mode of binding of the extrinsic proteins. The 33 kDa protein binds to the PS II complex regardless of the presence or absence of the functional Mn [6] and enhances the binding of the 23 kDa protein to the complex [20,21], and the 23 kDa protein is required for the stoichiometric and functional binding of the 18 kDa protein [6,9,19]. These observations have suggested that the extrinsic proteins bind to the PS II complex in the order of the 33 kDa, 23 kDa and then 18 kDa protein. However, it is still obscure whether all the three proteins bind one after another to link in a row, or whether they can independently bind to the intrinsic part of the PS II complex. In addition, the strength of the protein binding to the PS II complex has not yet been determined.

In the present study, we analyzed the rebinding of the extrinsic proteins to the PS II complex in spinach PS II membranes under equilibrium conditions and determined the dissociation constant and number of binding sites for the extrinsic proteins, in order to investigate precisely the mode and affinity of the protein binding. The possible interaction between the extrinsic proteins in the complex was also examined.

Materials and Methods

Preparation of PS II membranes

PS II membranes were prepared from spinach chloroplasts with Triton X-100 according to Kuwabara and Murata [3] and stored at 77 K in the presence of 30% (v/v) ethylene glycol [9]. Before use, the membranes were thawed and washed three times with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) (designated hereafter as medium A) by centrifugation and resuspension, and finally suspended in the same medium and kept in darkness for 2 h.

PS II membranes depleted of the 23 kDa and 18 kDa proteins were prepared by treating the PS II membranes with 1.0 M NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) for 30 min in darkness [9]. PS II membranes depleted of all three extrinsic proteins were prepared by treating the PS II membranes with 2.6 M urea/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) for 30 min in darkness [12]. PS II membranes depleted of the three proteins and three of the four Mn atoms were prepared by treating the membranes with 1.0 M Tris-HCl (pH 9.3 at 4°C)/300 mM sucrose for 30 min under room light [3]. The treated membranes were collected by centrifugation at $35\,000 \times g$ for 20 min and washed once with 200 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) (designated hereafter as medium B) by resuspension and recentrifugation. The resultant pellets of the NaCl-treated and Tris-treated membranes were finally suspended in medium A and that of the (urea + NaCl)-treated membranes in medium B.

PS II membranes lacking only the 18 kDa protein were prepared by adding the purified 23 kDa protein to the NaCl-treated membranes at a protein-to-Chl ratio of 2:220 (mol/mol) and at 0.3 mg Chl · ml^{-1}. After being kept for 30 min, the membranes were collected by centrifugation at $35\,000 \times g$ for 10 min, washed twice with medium A by resuspension and recentrifugation and finally suspended in medium A.

Purification of extrinsic proteins

The 23 kDa and 18 kDa proteins were extracted from untreated PS II membranes with 1.0 M NaCl/25 mM Mes-NaOH (pH 6.5) and purified by high-performance liquid chromatography in a cation exchange mode (SP-650S, TOSOH) [22]. The 33 kDa protein was extracted from the NaCl-treated membranes with 1.0 M $CaCl_2$/25 mM Mes-NaOH (pH 6.5) and purified by high-performance liquid chromatography in an anion-exchange mode (DEAE-5PW, TOSOH) [22].

The purified protein preparations were concentrated, if necessary, by ultrafiltration with an Amicon YM 10 Diaflo membrane, dialyzed against 10 mM Mes-NaOH (pH 6.5) and kept frozen at −80°C. Before use, the protein preparations were thawed and passed through a Millipore filter (0.22 μm). The protein concentrations were determined using molar absorption coefficients of 16 at 276 nm, 22 at 277 nm, and 12 $mM^{-1} \cdot cm^{-1}$ at 277 nm for the 33 kDa, 23 kDa and 18 kDa proteins, respectively, which were calculated from the previously reported values [23] with correction for their molecular masses; molecular masses of 26.7 kDa for the 33 kDa protein [24], 20.2 kDa and 16.5 kDa for the 23 kDa and 18 kDa proteins [25] were used instead of those of 33 kDa, 24 kDa and 18 kDa estimated by SDS gel electrophoresis [23].

Determination of dissociation constant

The dissociation constants for the bindings between the extrinsic proteins and the PS II complex were determined by analyzing the rebinding of the proteins to PS II membranes as follows. PS II membranes were incubated with various amounts of the purified proteins in medium A for 30 min under room light, except for the (urea + NaCl)-treated membranes, which were kept in darkness. Suitable concentrations of the PS II membrane were designed depending on the binding affinity of the protein; the concentration corresponding to 3 μg Chl · ml^{-1} was employed for the dissociation constants lower than 10 nM, and that corresponding to 10 or 30 μg Chl · ml^{-1} for the dissociation constants greater than 10 nM. After the incubation the reconstituted membranes were collected by centrifugation at $220\,000 \times g$ for 2 h.

All the above procedures were performed at 0–4° C and all the handling of the (urea + NaCl)-treated membranes under dim green light.

Polypeptides of the reconstituted membranes were analyzed by SDS-urea polyacrylamide gel electrophoresis using the buffer system of Chua and Bennoun [26], except that the electrode buffer was 0.1% SDS/25 mM Tris-glycine (pH 8.3). The polyacrylamide concentrations of the stacking and separation gels were 5% and 12%, respectively. The urea concentrations of the gel were 6.0 M for determination of the 33 kDa protein and 5.0 M for determination of the 23 kDa and 18 kDa proteins. The gel was stained with Coomassie brilliant blue R-250 and the electrophoretic pattern was recorded at 560 nm with a TLC scanner (CS930, Shimadzu). The amount of each protein was determined according to the peak heights of the stained bands in the densitogram, with the purified proteins as a standard.

Determination of sedimentation coefficient

The sedimentation coefficients of the purified proteins or protein mixtures were determined by moving boundary sedimentation method using an analytical ultracentrifuge (282, Hitachi). The protein or protein mixture was dissolved in 10 mM Mes-NaOH (pH 6.5) containing a designated salt to give a final absorbance at 277 nm of 0.2–0.5 (about 10–30 μM protein) and placed in a sample chamber of a double sector cell with 10 mM Mes-NaOH (pH 6.5) with the salt in a reference chamber. The cell was set in a RA60HC rotor and span at 60000 rpm and 20° C. The sedimentation pattern of the protein(s) in the chamber was monitored with absorbance at 277 nm every 6 min for 2 h. The position at which the absorbance was half of the maximum plateau level of the protein solution was taken as the sedimentation boundary [27]. The logarithm of the distance of the sedimentation boundary from the rotation axis was plotted against centrifugation time, and the sedimentation coefficient was calculated from the slope of the line in the plot by dividing it by the square of angular velocity [28].

Other methods

Oxygen-evolution activity of PS II membranes was measured using a Clark-type oxygen electrode with 0.3 mM phenyl-*p*-benzoquinone as an artificial electron acceptor [3]. Chl concentration was determined according to Arnon [29].

Results

In order to determine the dissociation constant between the extrinsic proteins and the PS II complex, conditions under which the binding and release of the protein attain equilibrium were examined. Fig. 1 shows the effect of concentration of PS II membrane on the

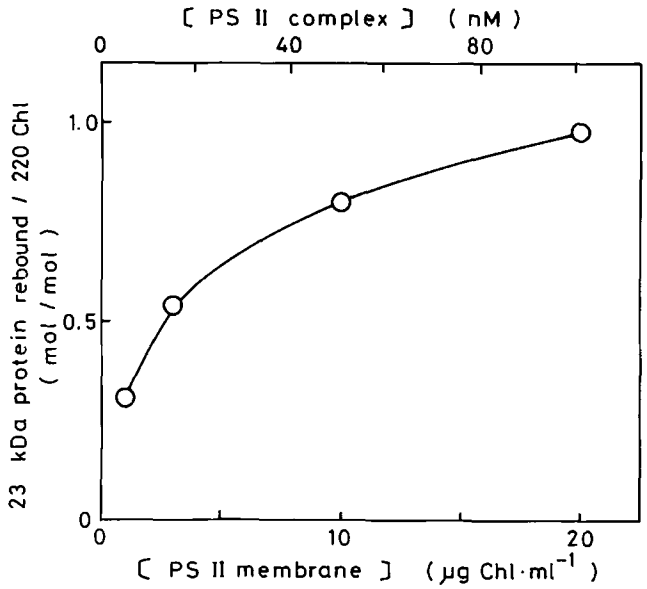

Fig. 1. Effect of concentration of PS II membrane on rebinding of the 23 kDa protein to the NaCl-treated PS II membranes depleted of the 23 kDa and 18 kDa proteins. The NaCl-treated PS II membranes were incubated for 30 min with the 23 kDa protein at a protein-to-Chl ratio of 1:220 (mol/mol) and at designated PS II membrane concentrations presented as Chl concentration in 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). Then, the PS II membranes were collected by centrifugation and the amount of the 23 kDa protein rebound to the membrane was determined by SDS polyacrylamide gel electrophoresis. Concentration of the PS II complex was calculated from the Chl concentration using the ratio of PS II complex to Chl of 1:220 (mol/mol) [7].

rebinding of the 23 kDa protein when the NaCl-treated PS II membranes previously depleted of the 23 kDa and 18 kDa proteins were incubated for 30 min with a stoichiometric amount of the protein, one per 220 Chl (mol/mol). As previously shown [9], the 23 kDa protein has a specific binding site on the PS II complex in the NaCl-treated PS II membranes. As shown in Fig. 1, almost all the added protein rebound to the membranes at PS II membrane concentration corresponding to 20 μg Chl · ml^{-1}, while the amount of the rebound protein decreased on lowering the PS II membrane concentration. At 1.0 μg Chl · ml^{-1} about 70% of the added protein remained unbound, though there were vacant binding sites for the protein in the membranes (Fig. 1). A prolonged incubation up to 2 h did not enhance the protein rebinding (data not shown). This suggests that at concentrations of PS II membrane corresponding to Chl concentrations lower than 20 μg · ml^{-1} the binding and release of the 23 kDa protein were equilibrated within 30 min.

Fig. 2A shows the binding profile of the 23 kDa protein when designated amounts of the protein were added to the NaCl-treated PS II membranes at 2.8 μg Chl · ml^{-1}. The amount of bound protein steeply increased to about 0.8 protein per 220 Chl (mol/mol)

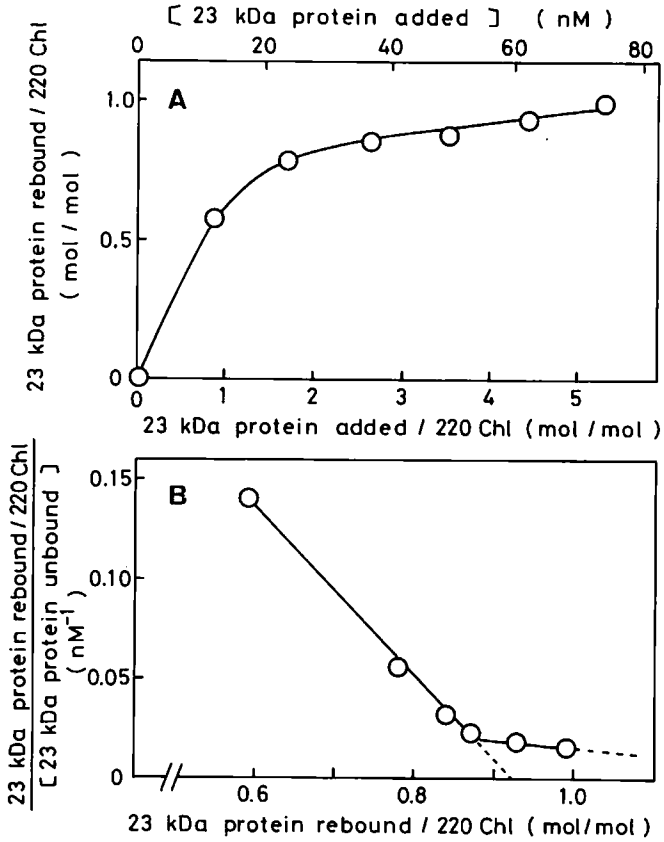

Fig. 2. Rebinding of the 23 kDa protein to the NaCl-treated PS II membranes depleted of the 23 kDa and 18 kDa proteins. Designated amounts of the 23 kDa protein were added to the NaCl-treated PS II membranes at 2.8 μg Chl·ml^{-1} (14 nM PS II complex) in 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). After being kept for 30 min, the PS II membranes were collected by centrifugation and amounts of the rebound protein were determined. (A) Plot of the amount of the 23 kDa protein rebound to the PS II membranes versus the amount of the 23 kDa protein added. (B) Scatchard plot of the data in Fig. 2A. Concentration of the 23 kDa protein remaining unbound was calculated by subtracting the amount of the rebound protein from that of the added protein.

and then gradually with the amount of added protein. The data were analyzed by the Scatchard plot in which the negative reciprocal of the slope and abscissa intercept of a line obtained give the dissociation constant and the number of binding sites, respectively. As shown in Fig. 2B, the plot could be fitted by two straight lines linked in series. The dissociation constant and the abscissa intercept of the line of steep slope were 2.4 nM and 0.9/220 Chl (mol/mol), respectively, and those of the other were 25 nM and 1.4/220 Chl (mol/mol), respectively. Thus, it is revealed that there are two types of binding site for the 23 kDa protein on the NaCl-treated PS II membranes; one binding site is of a low dissociation constant, that is, of high affinity, and amounts to about one per the PS II complex, the stoichiometric level, and the other is of low affinity and amounts to about a half per the complex. The latter binding appears not to be functional and specific, since it occurred after the tight and stoichiometric binding of the 23 kDa protein was completed.

Table I summarizes the dissociation constants and numbers of binding sites for the extrinsic proteins obtained by the analyses with the Scatchard plot as described above. The values for a binding of the lowest dissociation constant, that is, of the highest affinity in a combination of the extrinsic protein and the PS II membranes are listed.

The binding of the 33 kDa protein to the (urea + NaCl)-treated PS II membranes depleted of all the three extrinsic proteins gave a dissociation constant of 12 nM and number of binding sites of about 1/220 Chl, indicating a stoichiometric binding to the PS II complex as previously shown [6]. When the functional Mn was removed from the PS II complex by the Tris treatment, the number of binding sites was not substantially affected but the dissociation constant increased to 88 nM. This suggests that the binding site for the 33 kDa protein is present in the PS II complex devoid of the functional Mn and that the Mn only partially participates in the binding of the 33 kDa protein.

The binding of the 23 kDa protein to the NaCl-treated membranes depleted of the 23 kDa and 18 kDa proteins gave a low dissociation constant of about 3 nM and the number of binding sites almost equal to its stoichiometric level (Fig. 2, Table I), indicating a high-affinity and stoichiometric binding. When the 33 kDa protein was removed by the (urea + NaCl) or Tris treatment, the number of binding sites was reduced to about a half and the dissociation constant was much increased (Table I). This suggests that the 33 kDa protein is necessary for the high-affinity and stoichiometric binding of the 23 kDa protein to the PS II complex. One may suspect the possibility that unknown component(s) might participate in the binding of the 23 kDa protein and be partially removed upon the treatment with urea or Tris, resulting in the partial loss of the binding site. However, this is unlikely, since the stoichiometric binding of the 23 kDa protein was restored when the PS II complex was reconstituted with the purified 33 kDa protein (data not shown). It is likely that the low-affinity binding site of the 23 kDa protein in the absence of the 33 kDa protein differs from the high-affinity one in the presence of the 33 kDa protein.

The 18 kDa protein stoichiometrically rebound to the NaCl-treated membranes supplemented with the 23 kDa protein with a low dissociation constant of 4 nM (Table I). When the 23 kDa protein was removed from the PS II complex, the number of binding sites remained unaffected but the dissociation constant increased to 120 nM. This may suggest that the binding site for the 18 kDa protein was present on the PS II complex in the absence of the 23 kDa protein, although its affinity to the 18 kDa protein was much reduced. Whether this binding was functioning or not was examined by in-

TABLE I

Dissociation constant (K_d) and the number of binding sites (n/220 Chl) of the extrinsic proteins on the PS II membranes

The dissociation constant and number of binding sites were determined in 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). Data represent mean ± S.D. for two or three experiments. Binding energy was calculated from the dissociation constant according to the equation $\Delta G = -RT \ln(1/K_d)$.

Protein	Type of PS II membrane	Composition				K_d (nM)	n/220 Chl (mol/mol)	Binding energy (kcal·mol^{-1})
		18 kDa	23 kDa	33 kDa	Mn			
33 kDa	(Urea + NaCl)-treated	−	−	−	4	12 ± 4	0.95 ± 0.11	10.1 ± 0.2
	Tris-treated	−	−	−	1	88 ± 2	0.83 ± 0.03	8.9 ± 0
23 kDa	NaCl-treated	−	−	+	4	2.7 ± 0.1	0.92 ± 0.03	10.9 ± 0.1
	(Urea + NaCl)-treated	−	−	−	4	290 ± 80	0.46 ± 0.02	8.3 ± 0.2
	Tris-treated	−	−	−	1	100 ± 30	0.46 ± 0.08	8.9 ± 0.1
18 kDa	NaCl-treated and reconstituted with the 23 kDa protein	−	+	+	4	3.8 ± 0.3	0.86 ± 0.11	10.7 ± 0.1
	NaCl-treated	−	−	+	4	120 ± 30	0.84 ± 0.06	8.8 ± 0.1
	(Urea + NaCl)-treated	−	−	−	4	2500 [a]	2.4 [a]	7.1 [a]

[a] Values obtained in one experiment are shown. In another experiment the dissociation constant and number of binding site became infinite.

vestigating the effect of the 18 kDa protein on the Cl^- dependence of the oxygen-evolution activity. As shown in Fig. 3, the oxygen-evolution activity of the NaCl-treated PS II membranes depleted of the 23 kDa and 18 kDa proteins decreased steeply on lowering the Cl^- concentration below 10 mM. In the presence of the 18 kDa protein at a ratio of bound protein to Chl of 0.9 : 220 (mol/mol), no significant restoration of the oxygen evolution below 10 mM Cl^- was observed (Fig. 3). This indicates that the 18 kDa protein bound to the PS II membranes depleted of the 23 kDa protein cannot function. It is not clear at present whether the binding sites for the 18 kDa protein in the presence and absence of the 23 kDa protein are identical.

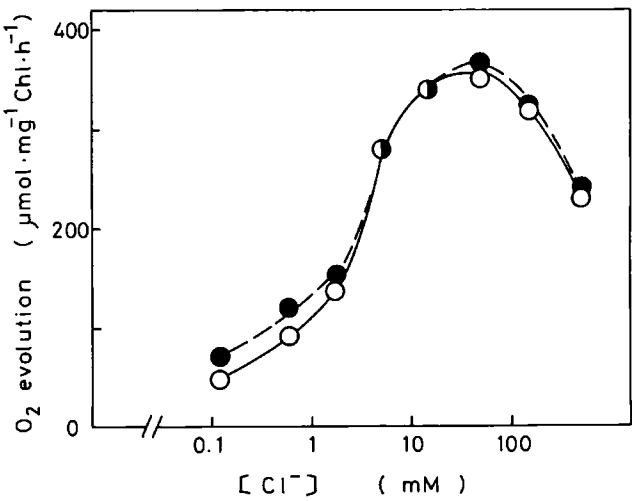

Fig. 3. Effect of the 18 kDa protein on Cl^- dependence of the oxygen-evolution activity of the NaCl-treated PS II membranes. The NaCl-treated PS II membranes were suspended at 6.0 μg Chl·ml^{-1} (30 nM PS II complex) in 1.0 mM $Ca(OH)_2$/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) containing a designated concentration of Cl^- with (●) or without (○) 1.11 mM of the 18 kDa protein. Chloride ion was added as NaCl. Under these conditions the amount of the 18 kDa protein bound to the NaCl-treated PS II membranes is estimated to be 0.9 : 220 Chl (mol/mol) from the dissociation constant of 120 nM (Table I). After incubation at 25 °C for 5 min, the oxygen-evolution activity was measured.

In the (urea + NaCl)-treated PS II membranes depleted of both the 23 kDa and 33 kDa proteins, the dissociation constant of the 18 kDa protein was very high and the number of binding sites reached far above its stoichiometric level. This indicates that this binding between the 18 kDa protein and the membranes is unspecific, and that both the 23 kDa and 33 kDa

TABLE II

Apparent sedimentation coefficient of the extrinsic proteins and the protein mixtures

The extrinsic protein or protein mixture was dissolved in 10 mM Mes-NaOH (pH 6.5) containing a designated salt. The apparent sedimentation coefficient was determined by the moving boundary method using an analytical ultracentrifuge (see Materials and Methods). The values for the protein mixtures were calculated assuming that samples consisted of a single sedimenting component.

Protein(s)	Sedimentation coefficient (S)		
Salt added:	None	10 mM NaCl	5 mM $CaCl_2$
33 kDa	2.6	2.5	2.5
23 kDa	2.2	2.2	2.1
18 kDa	1.7	1.8	1.8
33 kDa + 23 kDa (1 : 1, mol/mol)	2.4	–	–
23 kDa + 18 kDa (1 : 1, mol/mol)	2.1	2.0	–
33 kDa + 23 kDa + 18 kDa (1 : 1 : 1, mol/mol/mol)	2.2	2.3	2.1

proteins are necessary for the tight and stoichiometric binding of the 18 kDa protein.

In order to examine the possibility of direct binding between the extrinsic proteins, sedimentation analysis was applied to the proteins and protein mixtures in an aqueous solution. As shown in Table II, the extrinsic proteins gave sedimentation coefficients ranging from 1.7 to 2.6 S in reverse order of their molecular masses. These values are nearly equal to those of globular proteins of the same molecular sizes [30]. The presence of 10 mM NaCl or 5 mM $CaCl_2$ did not affect the sedimentation coefficients (Table II). If the extrinsic proteins could bind directly to each other in a solution, a resultant protein complex should sediment faster than its component proteins. When two or three proteins were mixed, no component of a higher sedimentation coefficient appeared. As shown in Table II, either mixture of the extrinsic proteins tested gave an apparent sedimentation coefficient close to an average of those of the respective proteins. This observation indicates that the proteins sedimented separately from each other, and therefore suggests that the extrinsic proteins do not interact with each other unless they are bound to the PS II complex.

Discussion

Previous studies on the rebinding of the extrinsic proteins to PS II membranes have provided information as to the mode of protein binding in the PS II complex [6,9,20,21]. These binding experiments were generally composed of three steps: incubation of PS II membranes with the extrinsic proteins, repetitive washing of the reconstituted membranes to remove weakly bound proteins, and then quantification of the rebound protein. Using this technique, only tightly bound protein which remained bound after washing could be analyzed and slight difference in the binding affinity could not be examined.

In the present study, we performed the rebinding experiment under equilibrium conditions using a very diluted suspension of PS II membranes, and the amount of the rebound protein was quantitated without washing. This technique enabled us to analyze not only tightly but also weakly bound proteins and to determine their dissociation constants (Fig. 2, Table I).

The results show that the 33 kDa protein could stoichiometrically bind to the PS II complex depleted of the three extrinsic proteins either in the presence or absence of the functional Mn in the complex. This is consistent with our previous observation in spinach PS II membranes [6] and those by other groups in wheat leaves grown under flash illumination [31] and in *Chlamydomonas* grown in an Mn-deficient medium [32]. The Mn in the complex, however, increased the binding affinity of the 33 kDa protein to the PS II complex. Inversely, it was previously shown that the 33 kDa protein stabilizes the binding of two of the four Mn atoms in the complex [12–14]. Therefore, it might be suggested that the 33 kDa protein directly interacts with the functional Mn in the PS II complex. As shown in Table I, the difference in the binding energy of the 33 kDa protein in the presence and absence of the functional Mn was estimated to be about 1 $kcal \cdot mol^{-1}$. This value is too small to be attributed to the ionic bond or coordinate bond between the 33 kDa protein and Mn atoms. Therefore, we conclude that the interaction between the 33 kDa protein and the functional Mn is, if any, very weak.

Previous studies showed that the 23 kDa protein could bind to the PS II complex containing the 33 kDa protein [20,21]. The present study confirms this and shows that the 33 kDa protein is required for the stoichiometric and tight binding of the 23 kDa protein to the PS II complex (Table I). The functional Mn in the complex, on the other hand, does not affect the binding of the 23 kDa protein.

The 18 kDa protein could stoichiometrically and tightly bind to the PS II complex containing both the 23 kDa and 33 kDa proteins as previously shown [6,9]. The protein could also stoichiometrically bind in the absence of the 23 kDa protein, but this binding was weak and not functional (Table I, Fig. 3). In the absence of the 33 kDa and 23 kDa proteins this weak and stoichiometric binding site disappeared. Thus, it is concluded that both the 23 kDa and 33 kDa proteins are necessary for the tight binding and functioning of the 18 kDa protein.

The previous and present studies suggest that the extrinsic proteins bind to the PS II complex in the order of the 33 kDa, 23 kDa and 18 kDa protein. This may suggest that the 23 kDa protein directly bind onto the 33 kDa protein in the PS II complex and the 18 kDa protein onto the 23 kDa protein. As shown in Table II, however, the extrinsic proteins cannot directly bind to each other when they are apart from the PS II complex. Therefore, it is likely that the binding site for the 23 kDa protein is formed in the PS II complex only when the 33 kDa protein binds to the complex, and similarly that for the 18 kDa protein is formed by the binding of the 23 kDa protein. There are two possible schemes considered: (1) the binding of the extrinsic protein alters the conformation of the intrinsic part of the complex to create the binding sites for the next extrinsic protein; and (2) the conformation of the extrinsic protein itself is changed in the bound form to provide the binding sites for the next protein on the protein itself. The localization of the binding sites for the extrinsic proteins requires further study.

In the intact thylakoid membrane the extrinsic proteins bind to the PS II complex within the lumen [1]. The volume of the lumen is calculated to be 0.001 μm^3,

on the assumption that the thylakoid is a vesicle of disk shape with a diameter of 300 nm and an internal thickness of 10 nm [33]. The concentration of the 33 kDa protein equivalent to its dissociation constant, 12 nM, corresponds to about 0.01 molecule of the protein in the lumenal space. This means that under the conditions of a half of the binding sites in this lumen being occupied by the 33 kDa protein and the other half open, only 0.01 molecule of the proteins remains unbound and dissolved in the lumen. At concentrations of the 23 kDa and 18 kDa proteins equivalent to their dissociation constants, about 3 nM, only 0.002 molecule each is dissolved in the lumenal space. This estimation suggests that the binding affinity of the extrinsic proteins is very high so that the proteins are bound to their own vacant binding site in the PS II complex at a very high probability when a molecule of the protein is introduced into the lumen.

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