Binding and Functional Properties of Four Extrinsic Proteins of Photosystem II from a Red Alga, *Cyanidium caldarium*, As Studied by Release−Reconstitution Experiments

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ABSTRACT: Photosystem II (PSII) from a red alga, *Cyanidium caldarium*, contains four extrinsic proteins of 33, 20, and 12 kDa and cytochrome (cyt) c_{550} [Enami, I., et al., (1995) *Biochim. Biophys. Acta 1232*, 208−216]. The binding and functional properties of these four proteins in the red algal PSII were studied by release−reconstitution experiments. Of the four components, the 33 kDa protein binds to PSII completely by itself, and the 20 kDa protein binds to a level 61% of that in native PSII in the absence of other proteins. In contrast, cyt c_{550} and the 12 kDa protein cannot bind to PSII efficiently by themselves; their effective binding requires the other three extrinsic proteins. In particular, a strong interaction was observed between cyt c_{550} and the 12 kDa protein, and a weaker interaction was observed between cyt c_{550} and the 20 kDa protein. While binding of the 33 kDa protein alone or cyt c_{550} and the 12 kDa protein in the presence of the 33 and/or the 20 kDa protein generally enhanced oxygen evolution, binding of the 20 kDa protein did not. Oxygen evolution was strongly dependent on Ca^{2+} and Cl^- in the absence of cyt c_{550} and the 12 kDa protein, suggesting that these two proteins have functions similar to those of the 23 and 17 kDa proteins in higher plant PSII. From these results, we propose that the unique 20 kDa extrinsic protein found only in the red algal PSII functions in maintaining the proper binding of cyt c_{550} and the 12 kDa protein but is not involved directly in oxygen evolution. The binding and functional properties of these four proteins were compared with those of the three extrinsic proteins found in cyanobacterial and higher plant PSII in an evolutionary point of view.

Photosystem II (PSII)[1] complex contains a number of intrinsic membrane protein components commonly found from prokaryotic cyanobacteria to eukaryotic higher plants (*1*, *2*). There is, however, a remarkable difference in the extrinsic proteins associated with and functioning in the oxygen-evolving PSII complex between cyanobacteria and higher plants: In green algal and higher plant PSII, three proteins of 33, 23, and 17 kDa are present as extrinsic proteins functioning in maintaining the stability and activity of the oxygen-evolving complex (*3*). Of these three proteins, only the 33 kDa protein is found in cyanobacterial PSII but the other two proteins are absent. In contrast, cyanobacterial PSII contains two different extrinsic proteins, cyt c_{550} and a 12 kDa protein (*4*, *5*). The binding and functional features of these two extrinsic proteins resemble to some extent those of the 23 and 17 kDa proteins in higher plant PSII, but they are apparently different from the 23 and 17 kDa proteins in both the primary sequences and exact roles in oxygen evolution (*3*, *6*−*8*). These suggest the occurrence of a replacement of cyt c_{550} and the 12 kDa protein in cyanobacterial PSII by the 23 and 17 kDa proteins in green algal and higher plant PSII during evolution of the oxygen-evolving complex. The difference found in extrinsic proteins between cyanobacterial and higher plant PSII, therefore, provides a clue to elucidating the evolutional process of the oxygen-evolving complex from prokaryotes to eukaryotes.

In an attempt to address this question, we purified a PSII complex from an acidophilic and thermophilic red alga, *Cyanidium caldarium*, and found that the PSII from this red alga contains cyanobacterial-type extrinsic proteins, e.g., cyt c_{550} and the 12 kDa protein, in addition to the 33 kDa protein (*9*). This suggests that the red algal PSII is closely related to cyanobacterial PSII and that the replacement of the two extrinsic proteins by the 23 kDa and 17 kDa proteins occurred beyond the development of red algae. The PSII from *C. caldarium*, however, contained an additional extrinsic protein of 20 kDa which is present in neither cyanobacterial nor higher plant PSII (*9*). To clarify the role of this 20 kDa protein and also to compare the binding and functional properties of the red algal cyt c_{550} and the 12 kDa protein with those of the cyanobacterial proteins, we performed release−reconstitution experiments with the purified *C. caldarium* PSII and its extrinsic proteins. The results obtained suggest that the 20 kDa protein does not function directly in oxygen evolution but is required for maximum

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[1] Abbreviations: cyt, cytochrome; chl, chlorophyll; D1 and D2, reaction center proteins of photosystem II; DM, *N*-dodecyl β-D-maltoside; HTG, *N*-heptyl β-D-thioglucoside; PEG, poly(ethylene glycol); PSII, photosystem II; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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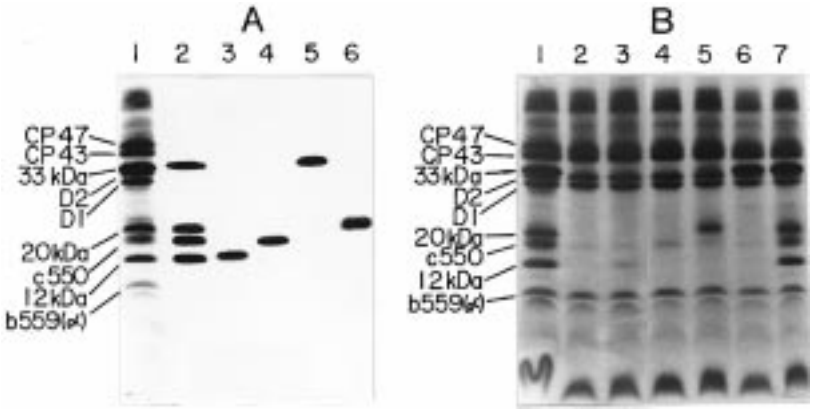

FIGURE 1: (A) Purification of the four extrinsic proteins from PSII of the red alga, *C. caldarium*. Lane 1, control PSII; lane 2, four extrinsic protein extracted from the red algal PSII by 1 M $CaCl_2$; lane 3, purified 12 kDa protein; lane 4, purified cyt c_{550}; lane 5, purified 33 kDa protein; and lane 6, purified 20 kDa protein. (B) Reconstitution of $CaCl_2$-treated PSII with each or all of the four extrinsic proteins. Lane 1, Control PSII; lane 2, 1 M $CaCl_2$-treated PSII; lanes 3−6, $CaCl_2$-treated PSII reconstituted with the 12 kDa protein (lane 3), cyt c_{550} (lane 4), the 20 kDa protein (lane 5) and the 33 kDa protein (lane 6); and lane 7, $CaCl_2$-treated PSII reconstituted with all the four extrinsic proteins.

binding of cyt c_{550} and the 12 kDa protein which are required for optimum activity of oxygen evolution. This is probably correlated with the fact that cyt c_{550} in the red alga was no longer able to bind to PSII in the absence of the other three extrinsic proteins, as opposite to the homologous cyt in cyanobacteria which can bind to PSII effectively independent of the other extrinsic proteins (*5*, *10*). The binding and functional properties found for the four extrinsic proteins of the red algal PSII were compared with those of cyanobacterial and higher plant PSII in an evolutionary point of view.

MATERIALS AND METHODS

Cells of the acidophilic as well as thermophilic red alga, *C. caldarium*, were grown in a medium of pH 3.0 at 40 °C as described previously (*9*, *11*). The harvested cells were broken by glass beads, and thylakoid membranes were prepared according to (*9*). Crude PSII was obtained by solubilizing the thylakoid membranes with HTG, and pure PSII was obtained by treating the crude PSII with DM followed by purification with a DEAE-Sepharose CL-6B column (Pharmacia Biotech Co.) with the procedure described previously (*9*). The pure PSII eluted from the column was concentrated by centrifugation in the presence of 10% PEG 6000, and the final pellet was suspended and stored in 25% glycerol, 50 mM MES (pH 6.5) (Buffer A).

To release the extrinsic proteins, the purified PSII particles were treated with 1 M $CaCl_2$ at 0.5 mg of chl/mL for 30 min at 0 °C, and the extrinsic proteins were separated from PSII particles by addition of PEG 6000 to a final concentration of 10% and centrifugation at 100000*g* for 30 min. The extrinsic proteins in the supernatant were dialyzed against 30 mM Tris/HCl (pH 9.0), and then purified with a DEAE-Toyopearl 650M column (Tosoh Inc.) equilibrated with 30 mM Tris/HCl (pH 9.0). The extrinsic proteins were eluted from the column by a stepwise addition of NaCl, with an elution order of the 12 kDa protein, the 33 kDa protein, cyt c_{550}, and the 20 kDa protein. Each purified protein was dialyzed against 50 mM Mes (pH 6.5) and then concentrated by centrifugation in a membrane filter (Centriprep 10, Amicon Co.). The concentrations of each purified extrinsic protein were determined by the method of Bradford (*12*), with bovine serum albumin as the standard.

For reconstitution experiments, the pure PSII was treated with 1 M $CaCl_2$ as above, washed twice with buffer A in the presence of 10% PEG 6000, and finally resuspended in buffer A. This extrinsic protein-depleted PSII was incubated with each purified protein either separately or in various combinations in buffer A for 15 min at 0 °C in the dark at a chl concentration of 0.1 mg/mL (equivalent to 2.2 μM PSII reaction center, assuming each PSII reaction center contains 50 molecules of chls). The amount of each protein added to the incubation mixture was 6.6 μM, which is approximately 3 times that of the reaction center. Following incubation, an aliquot of 50% PEG 6000 was added to a final concentration of 10%, and then the PSII particles were collected by centrifugation at 100000*g* for 30 min, washed once with and resuspended in buffer A.

SDS−PAGE was carried out with a gradient gel of 16−22% acrylamide containing 7.5 M urea (*13*). Samples were solubilized with 2% lithium dodecyl sulfate and 70 mM dithiothreitol in buffer A for 30 min. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250, dried, and scanned with a densitometer (Canoscan 600, Canon Co.). The peak area corresponding to each band was calculated with the NIH image software and used for estimation of the quantity of the corresponding proteins. Oxygen evolution was measured with a Clark-type oxygen electrode under saturating light in buffer A at 25 °C, with 0.4 mM phenyl-*p*-benzoquinone as the electron acceptor. Chl *a* concentration was determined according to (*14*).

RESULTS

Reconstitution with Each of the Extrinsic Proteins Separately. Treatment with 1 M $CaCl_2$ of the purified PSII particles from *C. caldarium* released four proteins, three of which are the 33 and 12 kDa proteins and cyt c_{550} similar to those found in cyanobacterial PSII, whereas the fourth one is a 20 kDa protein which is a novel extrinsic protein found only in the red algal PSII (Figure 1A) (*9*). These proteins can also be released by washing with urea/NaCl (*9*), but the release is sometimes incomplete. NaCl wash released none of the four proteins (*9*). It should be noted that some bands migrating slower than the CP47 bands remained in the purified red algal PSII; the identities of these bands are not

Table 1: Oxygen Evolution of PSII from the Red Alga, *C. caldarium*, Treated with 1 M $CaCl_2$ and Subsequently Reconstituted with Each of the Four Extrinsic Proteins Separately[a]

	oxygen evolution (μmol of O_2/mg of chl/h)			
	− ions	(%)	+50 mM $CaCl_2$	(%)
untreated PSII	2365	(100)	2486	(100)
$CaCl_2$-treated PSII	0	(0)	248	(10)
+ 12 kDa protein (21%)	0	(0)	265	(11)
+ cyt c_{550} (4%)	0	(0)	242	(10)
+ 20 kDa protein (61%)	0	(0)	260	(10)
+ 33 kDa protein (100%)	47	(2)	570	(23)
+ 33 kDa (100%) + 20 kDa (100%) + cyt c_{550} (93%) + 12 kDa (83%)	1324	(56)	1492	(60)

[a] Numbers in the parentheses following each protein represent the percent binding of that protein.

clear at present. Presumably, they are aggregates of some PSII intrinsic components. The presence of these bands, however, is not likely to interfere with the results of our following release−reconstitution experiments, since $CaCl_2$ wash specifically released the four extrinsic proteins leaving the slowly migrating bands unaffected, and rebinding of the extrinsic proteins resulted in an effective restoration of oxygen evolution.

The four extrinsic proteins released by the $CaCl_2$ wash were separated and purified by the DEAE-Toyoperal column to homogeneity, as judged from SDS−PAGE shown in Figure 1A. Using these purified proteins, we first carried out reconstitution experiments of $CaCl_2$-treated PSII with each of the four extrinsic proteins separately. The results obtained were shown in Figure 1B. The relative amounts of each protein rebound were estimated by scanning the dried gel with a densitometer and calculating the peak areas of each band, which were then normalized against the total area of the D1 and D2 protein bands. The abundance of each extrinsic protein in the native PSII was taken as 100%. The results (Table 1) showed that the 33 kDa protein rebound to the $CaCl_2$-treated PSII to a level equal to that in the native PSII, indicating that, similar to that in cyanobacterial and higher plant PSII, the 33 kDa protein alone is able to bind to PSII efficiently in the red algal PSII. When the 20 kDa protein was reconstituted alone, it rebound to a level 61% of that in the native PSII. In contrast, when the 12 kDa protein or cyt c_{550} was reconstituted alone, the level of rebinding was very low: the 12 kDa protein rebound was 21% whereas the level of cyt c_{550} rebound was only 4% of that in the native PSII. When all the four proteins were reconstituted together with the $CaCl_2$-treated PSII, they all rebound to PSII efficiently, i.e., the levels of rebinding of all the four proteins were equal to or close to those of the corresponding proteins in native PSII. These results suggested that, while the 20 kDa protein alone is able to bind to the red algal PSII to a significant extent, the 12 kDa protein and cyt c_{550} cannot bind to PSII efficiently by themselves. This is different from cyanobacterial PSII where cyt c_{550} alone can effectively bind to PSII in the absence of any other extrinsic proteins (*5*, *10*).

Table 1 shows the restoration of oxygen evolution of the $CaCl_2$-washed PSII upon reconstitution with each or all of the four extrinsic proteins. The native PSII of *C. caldarium* showed a high activity of 2365 μmol of O_2/mg of chl/h in the absence of $CaCl_2$ in the assay medium, and this activity did not increase much upon supplement of $CaCl_2$. This is similar to what was seen in higher plant PSII, where no $CaCl_2$ is required for normal oxygen evolution as far as the three extrinsic proteins are present. Upon $CaCl_2$ wash, the activity decreased to 0 in the absence of $CaCl_2$ and addition of 50 mM $CaCl_2$ slightly increased the activity to a level 10% of that in native PSII. Reconstitution with either the 12 kDa protein or cyt c_{550} separately did not give any restoration of the activity, consistent with the significantly low level of binding of these two proteins when reconstituted by themselves separately. Reconstitution with the 20 kDa protein also did not restore any oxygen evolution, despite the significant rebinding of the protein when reconstituted by itself. This may suggest that the 20 kDa protein itself does not function directly in the red algal oxygen evolution. Reconstitution with the 33 kDa protein alone yielded a partial restoration of oxygen evolution to a level 23% of that in native PSII in the presence of 50 mM $CaCl_2$. Reconstitution with all the four proteins increased the activity to 56% and 60%, respectively, in the absence or presence of $CaCl_2$, of that in the native PSII. These results suggest that, although the 33 kDa protein alone can bind to PSII efficiently, it is itself not enough to support the activity of oxygen evolution and that the other three proteins function properly only when reconstituted in combinations with part or all of the other proteins.

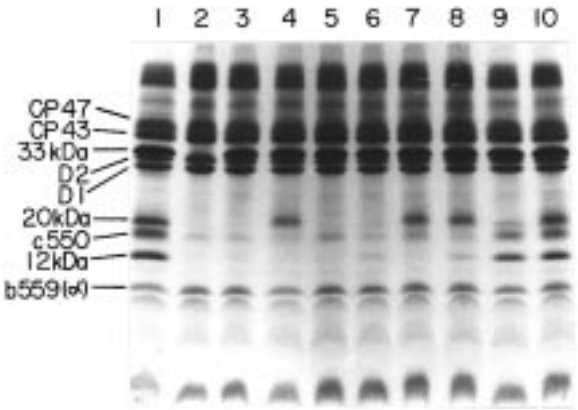

FIGURE 2: Reconstitution of the red algal PSII with the 33 kDa protein in various combinations with the other three extrinsic proteins. Lane 1, control PSII; lane 2, 1 M $CaCl_2$-treated PSII; lanes 3−10, $CaCl_2$-treated PSII reconstituted with the 33 kDa protein alone (lane 3), the 33 kDa protein *plus* the 20 kDa protein (lane 4), the 33 kDa protein *plus* cyt c_{550} (lane 5), the 33 kDa protein *plus* the 12 kDa protein (lane 6), the 33 kDa protein *plus* the 20 kDa protein and cyt c_{550} (lane 7), the 33 kDa protein *plus* the 20 and 12 kDa proteins (lane 8), the 33 kDa protein *plus* cyt c_{550} and 12 kDa protein (lane 9), and all four extrinsic proteins (lane 10).

Reconstitution with Various Combinations of the Four Extrinsic Proteins. Since the 33 kDa protein is able to bind to PSII by itself, we first carried out the reconstitution experiments with each of the other three proteins in combination with the 33 kDa protein. The results obtained were shown in Figure 2, and the relative amounts of each proteins rebound were summarized in Table 2. The amount of the 12 kDa protein rebound in the presence of the 33 kDa protein was 21%, which is the same as that in the absence of the 33 kDa protein. In the presence of both the 33 and 20 kDa proteins, the amount of the 12 kDa protein rebound increased slightly to 27% of that in native PSII, whereas in

Table 2: Relative Binding of Each Extrinsic Protein Reconstituted with the $CaCl_2$-Treated Red Algal PSII in Combination with the 33 kDa Protein

	amount of the 12 kDa protein rebound (%)
12 kDa protein only	21
12 kDa protein	
+ 33 kDa protein	21
+ 33 kDa + 20 kDa	27
+ 33 kDa + cyt c_{550}	72
+ 33 kDa + 20 kDa + cyt c_{550}	83
	amount of cyt c_{550} rebound (%)
cyt c_{550} only	4
cyt c_{550}	
+ 33 kDa protein	13
+ 33 kDa + 20 kDa	29
+ 33 kDa + 12 kDa	64
+ 33 kDa + 20 kDa + 12 kDa	93
	amount of the 20 kDa protein rebound (%)
20 kDa protein only	61
20 kDa protein	
+ 33 kDa protein	68
+ 33 kDa + cyt c_{550}	83
+ 33 kDa + 12 kDa	88
+ 33 kDa + cyt c_{550} + 12 kDa	100

the presence of the 33 kDa protein and cyt c_{550}, it increased to 72%. When all four proteins were reconstituted together, the amount of the 12 kDa protein rebound reached 83% of that in native PSII. These results suggest that, while the 33 kDa protein had no effect and the 20 kDa protein had only a small effect on rebinding of the 12 kDa protein to the purified *C. caldarium* PSII, cyt c_{550}, when supplemented together with the 33 kDa protein, had a significant effect on rebinding of the 12 kDa protein. This then implies that cyt c_{550} has a strong interaction with the 12 kDa protein in the red algal PSII. A similar situation was also seen in the rebinding of cyt c_{550}: While co-reconstitution with the 33 kDa protein gave a binding of 13%, and co-reconstitution with the 33 kDa protein plus the 20 kDa protein gave a slightly larger binding of 29% of cyt c_{550}, co-reconstitution with the 33 kDa protein plus the 12 kDa protein significantly increased the binding to a level 64% of that in native PSII. This reinforces the above implication that cyt c_{550} has a strong interaction with the 12 kDa protein and also suggests weak interaction of cyt c_{550} with the 20 kDa protein. The full rebinding of cyt c_{550}, however, required the presence of all three of the other proteins.

With respect to the 20 kDa protein, co-reconstitution with the 33 kDa protein virtually did not affect its binding and co-reconstitution with the combination of 33 kDa protein plus cyt c_{550} or 33 kDa protein plus 12 kDa protein slightly increased its rebinding.

Because the 20 kDa protein alone was able to rebind to PSII to a significant level, we subsequently performed reconstitution experiments with each of the proteins in combination with the 20 kDa protein. The results obtained, together with the results of reconstitution with combinations of cyt c_{550} and the 12 kDa protein, are shown in Figure 3 and Table 3. When the 12 kDa protein was reconstituted in combination with the 20 kDa protein, the amount rebound was essentially the same as that when it was reconstituted

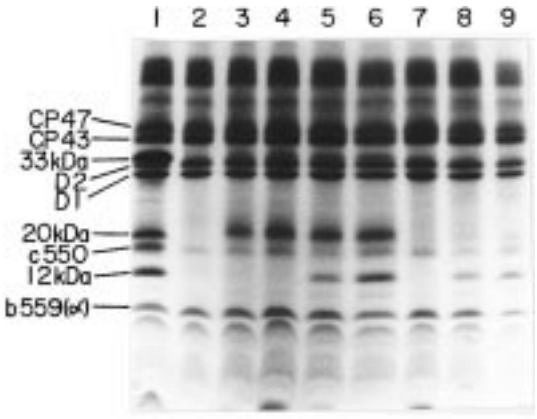

FIGURE 3: Reconstitution of the red algal PSII with various combinations of the extrinsic proteins in the absence of the 33 kDa protein. Lane 1, control PSII; lane 2, 1 M $CaCl_2$-treated PSII; lanes 3−9, $CaCl_2$-treated PSII reconstituted with the 20 kDa protein alone (lane 3), the 20 kDa protein *plus* cyt c_{550} (lane 4), the 20 kDa protein *plus* the 12 kDa protein (lane 5), the 20 kDa protein *plus* cyt c_{550} and the 12 kDa protein (lane 6), cyt c_{550} alone (lane 7), the 12 kDa protein alone (lane 8), and cyt c_{550} plus the 12 kDa protein (lane 9).

Table 3: Relative Binding of Each Extrinsic Protein Reconstituted with the $CaCl_2$-Treated Red Algal PSII in the Absence of the 33 kDa Protein

	amount of the 12 kDa protein rebound (%)
12 kDa protein	
+ 20 kDa protein	21
+ cyt c_{550}	24
+ 20 kDa + cyt c_{550}	57
	amount of cyt c_{550} rebound (%)
cyt c_{550}	
+ 20 kDa protein	16
+ 12 kDa	7
+ 20 kDa + 12 kDa	31
	amount of the 20 kDa protein rebound (%)
20 kDa protein	
+ cyt c_{550}	72
+ 12 kDa	67
+ cyt c_{550} + 12 kDa	83

alone, suggesting that the 20 kDa protein does not have a direct interaction with the 12 kDa protein. Co-reconstitution of the 12 kDa protein with cyt c_{550} in the absence of the 33 and 20 kDa proteins also did not enhance rebinding of the 12 kDa protein significantly. This is apparently due to the fact that cyt c_{550} itself cannot bind to PSII efficiently in the absence of the 33 and 20 kDa proteins. The amount of the 12 kDa protein rebound to PSII, however, increased to 57% of that in native PSII when reconstituted with the 20 kDa protein and cyt c_{550} together, and this amount increased further to 83% when all the other three extrinsic proteins were present. This suggests that the binding of the 12 kDa protein to PSII is facilitated by the co-binding of the 20 kDa protein and cyt c_{550}; this may be attributed mainly to an interaction of the 12 kDa protein with cyt c_{550} as mentioned above, as co-reconstitution with the 20 kDa protein alone did not give rise to a significant increase in binding of the 12 kDa protein. The full binding of the 12 kDa protein, however, requires all of the other three extrinsic proteins.

The binding of cyt c_{550} in the presence of the 12 kDa protein alone was not much different from that in the absence

Table 4: Oxygen Evolution of the $CaCl_2$-Treated PSII Reconstituted with the Extrinsic Protein in Various Combinations with the 33 kDa Protein[a]

	oxygen evolution (μmol of O_2/mg of chl/h)		
	− ions (%)	+100 mM NaCl (%)	+50 mM $CaCl_2$ (%)
untreated PSII	2207 (100)	2321 (100)	2321 (100)
$CaCl_2$-treated PSII	0 (0)	70 (3)	209 (9)
+ 33 kDa protein (100%)	0 (0)	116 (5)	533 (23)
+ 33 kDa + 20 kDa (100%) (68%)	0 (0)	298 (13)	596 (26)
+ 33 kDa + cyt c_{550} (100%) (13%)	0 (0)	182 (8)	605 (26)
+ 33 kDa + 12 kDa (100%) (21%)	88 (4)	187 (8)	562 (24)
+ 33 kDa + 20 kDa + cyt c_{550} (100%) (83%) (29%)	20 (1)	369 (16)	738 (32)
+ 33 kDa + 20 kDa + 12 kDa (100%) (88%) (27%)	146 (7)	383 (17)	765 (33)
+ 33 kDa + cyt c_{550} + 12 kDa (100%) (64%) (72%)	757 (34)	998 (43)	1101 (47)
+ 33 kDa + 20 kDa + cyt c_{550} + 12 kDa (100%) (100%) (93%) (83%)	1305 (59)	1341 (58)	1446 (62)

[a] Numbers in the parentheses below each protein represent the percent binding of that protein.

of the 12 kDa protein, reflecting the fact that the 12 kDa protein itself is not able to bind to PSII efficiently without the other two proteins of 20 and 33 kDa. The amount of cyt c_{550} rebound, however, increased slightly from 4% to 16% when reconstituted together with the 20 kDa protein; this is comparable with or slightly higher than the level of rebinding when reconstituted with the 33 kDa protein, consistent with the result that cyt c_{550} has a weak interaction with the 20 kDa protein (Table 2). The level of rebinding of cyt c_{550} further increased to 31% when reconstituted together with both the 20 and 12 kDa proteins. These data show that cyt c_{550} and the 12 kDa protein are able to bind to PSII partially in the presence of the 20 kDa protein but in the absence of the 33 kDa protein. The full rebinding of both cyt c_{550} and the 12 kDa protein, however, required all of the other three extrinsic proteins.

The effects of other proteins on rebinding of the 20 kDa protein was also investigated by various combinations of the proteins during reconstitution. Co-reconstitution of the 20 kDa protein with either the 12 kDa protein or cyt c_{550} slightly increased the amount of the 20 kDa protein rebound, and the presence of both cyt c_{550} and the 12 kDa protein further increased the amount of the 20 kDa protein rebound. In the presence of all the other three proteins, the 20 kDa protein rebound to a maximum level equal to that in the native PSII. These results imply that although the 20 kDa protein itself can bind to PSII to a significant level, it has interactions with all the other three extrinsic proteins and these interactions facilitated the full binding of the protein.

Restoration of Oxygen Evolution by Reconstitution with Various Combinations of the Extrinsic Proteins. Table 4 shows restoration of the oxygen-evolving activity upon reconstitution with the extrinsic proteins in various combinations measured either in the absence or presence of 100 mM NaCl or 50 mM $CaCl_2$. In the absence of the 33 kDa protein, no significant restoration of the activity was observed upon reconstitution with various combinations of each of the other three proteins, except PSII reconstituted with the 20 and 12 kDa proteins and cyt c_{550} together, of which the oxygen-evolving activity in the presence of 50 mM $CaCl_2$ increased slightly from 225 to 392 μmol of O_2/mg of chl/h. Thus, only the activity restoration upon reconstitution of various proteins in combinations with the 33 kDa protein was shown in Table 4.

As has been shown above, reconstitution of the $CaCl_2$-washed PSII with the 33 kDa protein alone increased the oxygen evolution slightly. Upon reconstitution with combinations of the 33 kDa protein with either cyt c_{550} or the 12 kDa protein, no further restoration of the oxygen evolution was observed. This is probably due to the significantly low level of binding of cyt c_{550} and the 12 kDa protein when co-reconstituted separately with only the 33 kDa protein. Co-reconstitution of the 33 and 20 kDa proteins also did not give rise to a remarkable restoration of oxygen evolution, although the 20 kDa protein binds to a significant level in the presence of the 33 kDa protein. This is similar to the situation observed when the 20 kDa protein is reconstituted alone and suggests that the 20 kDa protein itself does not have a significant functional role in the red algal oxygen evolution. Reconstitution with combinations of the 33 kDa protein plus 20 kDa protein and cyt c_{550}, or 33 kDa protein plus the 20 and 12 kDa proteins, resulted in a slight restoration of oxygen evolution. In contrast, reconstitution with the 33 kDa protein plus cyt c_{550} and the 12 kDa protein resulted in significant increases of oxygen evolution from 0, 70, and 209 μmol of O_2/mg of chl/h, respectively, to 757, 998, and 1101 μmol of O_2/mg of chl/h, in the absence of both Cl^- and Ca^{2+}, the presence of 100 mM NaCl or the presence of 50 mM $CaCl_2$. These increases in oxygen evolution were correlated with the significant binding of cyt c_{550} and the 12 kDa protein when reconstituted together with the 33 kDa protein, as shown in the previous section (Table

Table 5: Effects of Ca^{2+} and Cl^- Ions on Oxygen Evolution of the $CaCl_2$-Treated PSII Reconstituted with Various Combinations of the Extrinsic Proteins[a]

	oxygen evolution (μmol of O_2/mg of chl/h)				
	− ions	+5 mM $CaCl_2$	+50 mM $CaCl_2$	+100 mM NaCl	+50 mM $Ca(NO_3)_2$
untreated PSII	2670	2830	2825	2818	1883
$CaCl_2$-treated PSII	0	226	230	75	83
+ 33 kDa + 20 kDa + cyt c_{550} (100%) (83%) (29%)	21	604	610	320	220
+ 33 kDa + 20 kDa + 12 kDa (100%) (88%) (27%)	42	702	710	370	231
+ 33 kDa + cyt c_{550} + 12 kDa (100%) (64%) (72%)	756	1097	1194	1020	703
+ 33 kDa + 20 kDa + cyt c_{550} + 12 kDa (100%) (100%) (93%) (83%)	1449	1573	1683	1640	1075

[a] Numbers in the parentheses below each protein represent the percent binding of that protein.

2). In particular, the PSII reconstituted with the combination of the 33 kDa protein plus cyt c_{550} and the 12 kDa protein showed a significantly high activity of oxygen evolution even in the absence of both Ca^{2+} and Cl^-. When all four extrinsic proteins were reconstituted together, a further increase in the oxygen-evolving activity was achieved, and the requirement for Ca^{2+} and Cl^- disappeared completely.

Table 5 shows the effects of Ca^{2+} and Cl^- ions on oxygen evolution of the *C. caldarium* PSII reconstituted with various combinations of the extrinsic proteins. First of all, no significant effects of Ca^{2+} and Cl^- on oxygen evolution were observed in the native or $CaCl_2$-washed PSII reconstituted with all four extrinsic proteins. This is similar to that in higher plant PSII but somewhat different from that of the cyanobacterial PSII, where lack of Ca^{2+} in the assay medium resulted in a decrease of oxygen evolution even in the presence of all three extrinsic proteins (*5*). Partial reconstitution with cyt c_{550} (29%) or the 12 kDa protein (27%) in the presence of both the 33 and 20 kDa proteins resulted in virtually no restoration of oxygen evolution in the absence of both Ca^{2+} and Cl^-, but the activity recovered to a significant extent upon supplement of both Ca^{2+} and Cl^-. The concentration of $CaCl_2$ required for saturation is 5 mM, similar to that observed in higher plant PSII. When both cyt c_{550} (64%) and the 12 kDa protein (72%) were partially reconstituted together in the presence of the 33 kDa protein, the PSII showed a significant oxygen-evolving activity in the absence of both Ca^{2+} and Cl^- and the effect of Ca^{2+} completely disappeared; only Cl^- slightly enhanced the activity. When cyt c_{550} and the 12 kDa protein completely rebound to PSII in the presence of the 33 and 20 kDa proteins, the enhancement of Cl^- on oxygen evolution also disappeared completely. Rebinding of cyt c_{550} and the 12 kDa protein also protected PSII against inhibition caused by substitution of Cl^- with NO_3^- (Table 5). These results suggest that cyt c_{550} and the 12 kDa protein function in maintaining requirement for Ca^{2+} and Cl^- of the red algal PSII, resembling to some extent the functions of the 23 and 17 kDa proteins in higher plant PSII.

DISCUSSION

Binding and Functional Features of Four Extrinsic Proteins in the Red Algal PSII. The present results demonstrated that, among the four extrinsic proteins of the red algal PSII from *C. caldarium*, the 33 kDa protein can bind to PSII effectively by itself, and the 20 kDa protein binds to PSII to a level 61% of that in native PSII when reconstituted by itself. The other two proteins, cyt c_{550} and the 12 kDa protein, however, cannot bind to PSII by themselves unless reconstituted in combinations with the other proteins. The reconstitution experiments also suggested that cyt c_{550} and the 12 kDa protein have a specific, strong interaction whereas cyt c_{550} and the 20 kDa protein have a rather weak interaction. On the basis of these results, we propose a model for the association of the four extrinsic proteins with the red algal PSII (Figure 4). In this model, it is shown that the 33 and 20 kDa proteins have direct contact with the PSII complex whereas cyt c_{550} and the 12 kDa protein are associated with PSII through their contacts with both the 33 and 20 kDa proteins.

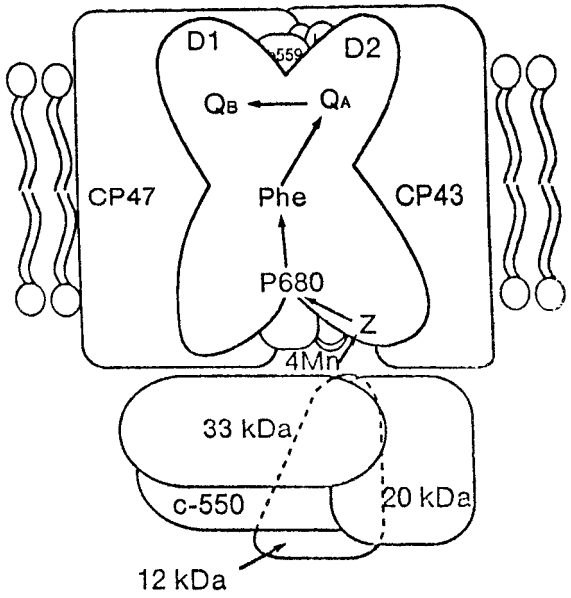

FIGURE 4: Model of red algal PSII showing association of the four extrinsic proteins at lumenal side of the thylakoid membrane. On the basis of the results obtained in the present study, it is shown that the 33 and 20 kDa proteins have direct contact with PSII intrinsic components, whereas cyt c_{550} and the 12 kDa protein associate with PSII indirectly and only through their interactions with the 33 and 20 kDa proteins. For further details, see Discussion.

Reconstitution with most of the individual proteins resulted in no restoration of oxygen evolution, except reconstitution

with the 33 kDa protein which resulted in a slight restoration of oxygen evolution. Reconstitution with any combinations of the three proteins without the 33 kDa protein also resulted in no significant restoration of the activity, of which only reconstitution with the combination of the 20 kDa protein, cyt c_{550}, and the 12 kDa protein slightly enhanced the activity, although their rebinding reached to levels of 83%, 31%, and 57%, respectively. Furthermore, the 33 kDa protein alone or in combinations with any one or two other proteins is not sufficient for the maximum activity; this was achieved only by rebinding of all the four components. These results suggest that, although the Mn can still bind to PSII in the absence of all the four extrinsic proteins, its proper functioning requires all of these components.

A lack of restoration of oxygen evolution upon reconstitution with cyt c_{550} or the 12 kDa protein alone is consistent with the fact that neither cyt c_{550} nor the 12 kDa protein can bind to PSII effectively by themselves. The lack of restoration of oxygen evolution upon reconstitution with the 20 kDa protein, however, somewhat contradicts the fact that the 20 kDa protein itself can bind to PSII to a level 60% of that in native PSII. One possible explanation for this is that the 20 kDa protein is not involved directly in the red algal oxygen evolution. Instead, it functions mainly in maintaining the binding of cyt c_{550} and the 12 kDa protein to the red algal PSII. In fact, in the absence of the 20 kDa protein, cyt c_{550} and the 12 kDa protein cannot bind to PSII to a maximum level needed for the maximum activity of oxygen evolution. In particular, a slight enhancement in binding of cyt c_{550} to the red algal PSII was observed upon co-reconstitution with the 20 kDa protein, suggesting a weak interaction of the cyt with the 20 kDa protein.

Comparison of Extrinsic Proteins of the Red Algal PSII with Those of the Cyanobacterial and Higher Plant PSII. Red algae are the eukaryotic algae most closely related to the prokaryotic algae cyanobacteria from an evolutional point of view. Consistent with this, PSII from the red alga *C. caldarium* contains cyanobacterial-type extrinsic proteins, e.g., cyt c_{550} and the 12 kDa protein instead of the 23 and 17 kDa proteins that are found in green algal and higher plant PSII. The functional properties of the red algal cyt c_{550} and 12 kDa protein, however, are more similar to those of the 23 and 17 kDa proteins as absence of cyt c_{550} and the 12 kDa protein created a large requirement for Ca^{2+} and Cl^- of oxygen evolution which disappears upon binding of the two components. This is different from what was seen in cyanobacterial PSII where absence or presence of cyt c_{550} and the 12 kDa protein does not have a direct correlation with the requirement for Ca^{2+} and Cl^- of oxygen evolution (*5*).

All the four extrinsic proteins could be effectively dissociated from the red algal PSII by $CaCl_2$ or urea/NaCl wash but not by NaCl treatment (*9*). This is similar to the three extrinsic proteins found in cyanobacterial PSII but apparently different from those in higher plant PSII where 1 M NaCl wash effectively released the 17 and 23 kDa proteins. The binding property of the red algal 12 kDa protein, however, is similar to both of the cyanobacterial 12 kDa protein and the higher plant 17 kDa protein in that none of them can bind to PSII independently (*5, 15*). On the other hand, the binding property of cyt c_{550} in the red algal PSII is different from both cyt c_{550} in cyanobacteria and the 23 kDa protein in higher plant PSII: The binding of cyt c_{550} in the red algal PSII requires all of the other three extrinsic proteins, whereas the cyanobacterial cyt c_{550} can bind to PSII by itself (*5, 10*), and the 23 kDa protein in higher plant PSII can bind in the presence of only the 33 kDa protein (*15*). It is interesting to note here that the red algal PSII contains a 20 kDa protein which is found in neither cyanobacterial nor higher plant PSII. The 20 kDa protein seems to function not directly in oxygen evolution but in maintaining the binding of cyt c_{550} and the 12 kDa protein. One possible hypothesis is that, during the evolution of the oxygen-evolving complex from the prokaryotic cyanobacteria to eukaryotic red algae, cyt c_{550} was changed so that it can no longer bind to PSII by itself; concomitant with this, the 20 kDa protein was newly developed in order to keep binding of the cyt to PSII. During evolution from the red algae to higher plants, however, cyt c_{550} was replaced by the 23 kDa protein which does not require the 20 kDa protein for binding; this then resulted in loss of the protein.

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