Binding and Functional Properties of Two New Extrinsic Components, Cytochrome *c*-550 and a 12-kDa Protein, in Cyanobacterial Photosystem II†

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ABSTRACT: Cytochrome *c*-550, a low-potential *c*-type cytochrome, and a 12-kDa protein were recently shown to be associated extrinsically and stoichiometrically with purified photosystem II (PSII) complex of the thermophilic cyanobacterium *Synechococcus vulcanus* [Shen, J.-R., Ikeuchi, M., & Inoue, Y. (1992) *FEBS Lett. 301*, 145–149]. The binding and functional properties of these two extrinsic components in PSII were studied by means of release-reconstitution and thermoluminescence techniques. The following results were obtained: (i) cyt *c*-550 rebound appreciably to cyanobacterial PSII in the absence of the 33- and 12-kDa extrinsic proteins, but the presence of these two proteins facilitated the rebinding, affording a full level of binding equal to that in native PSII. (ii) The 12-kDa protein did not rebind to PSII at all unless the 33-kDa protein or cyt *c*-550 was present. It rebound only partially in the presence of either of these two proteins, but it rebound maximally when reconstituted together with both of them. (iii) Reconstitution with cyt *c*-550 or the 12-kDa protein alone in the absence of the 33-kDa protein did not restore the O_2-evolving activity of $CaCl_2$-washed PSII. Reconstitution with cyt *c*-550 in combination with the 33-kDa protein appreciably enhanced the activity, but the activity restoration was much more marked and reached a level close to that of the original activity when all three extrinsic proteins were included. (vi) Analysis of the light intensity dependence of O_2 evolution and thermoluminescence glow curves revealed that cyt *c*-550 regulates the efficiency of S-state transition between S_1 and S_2 or S_2 and S_3 or both, whereas the 12-kDa protein modulates a dark step(s) in O_2 evolution. It was inferred from these results that the two new cyanobacterial extrinsic components, cyt *c*-550 and the 12-kDa protein, are closely dependent on each other and also on the extrinsic 33-kDa protein with respect to their binding and functional properties. They bind to the donor side of cyanobacterial PSII and play important roles in regulating the O_2-evolving activity.

Cyanobacterial photosynthetic oxygen evolution takes place in a PSII[1] complex similar to those in higher plants. Both the PSII complexes from cyanobacteria and higher plants consist of the D1 and D2 reaction center proteins, the 47- and 43-kDa Chl proteins that serve as proximal antennae, the 33-kDa extrinsic protein that stabilizes the Mn cluster, two subunits of cyt *b*-559, and several other low molecular weight polypeptides whose functions are not well established.

In higher plants, two more extrinsic proteins of 23- and 17-kDa other than the 33-kDa protein are associated with native PSII. The functions of these two extrinsic proteins are closely related with the unique requirement of Ca^{2+} and Cl^- for O_2 evolution by higher plant PSII: the 23-kDa protein mitigates the demand for Ca^{2+} while the 17-kDa protein does for Cl^- [for a review, see Hansson and Wydrzynski (1990)]. These two proteins, however, have not been found in cyanobacterial PSII complexes. Experiments employing either release-reconstitution techniques or immunodetection methods have failed to detect any homologous polypeptides in cyanobacterial PSII (Koike & Inoue, 1985; Stewart et al., 1985a). Furthermore, the features of Ca^{2+} and/or Cl^- requirement for O_2 evolution by cyanobacterial PSII differ in many respects from those in higher plant PSII. Early release-reconstitution experiments with cyanobacterial PSII have suggested that the extrinsic 33-kDa protein alone is enough to maintain O_2 evolution by cyanobacterial PSII, although the restored activity was not very high as was that observed in their original PSII particles (Koike & Inoue, 1985).

In addition to the extrinsic 33-kDa protein, however, Stewart et al. (1985a,b) have reported the association of another extrinsic protein of 9 kDa with their PSII particles from thermophilic *Phormidium*. This protein had no sequence homology with any of the 23- or 17-kDa extrinsic protein found in higher plant PSII (Wallace et al., 1989). Importantly, however, release of the 9-kDa protein from PSII decreased the O_2-evolving activity, and the decrease could be reversed by rebinding of the protein (Stewart et al., 1985a,b; Rolfe & Bendall, 1989). Recently, we found an extrinsic 12-kDa protein in a highly purified PSII complex from another thermophilic cyanobacterium, *Synechococcus vulcanus*, and proved that its partial amino acid sequence is homologous to that of the 9-kDa protein of *Phormidium* (Shen et al., 1992). We also showed that our purified PSII complex contains a stoichiometric amount of cyt *c*-550, a low-potential *c*-type cyt, in addition to the 12-kDa protein. This cyt shows no homology with any of the 23- or 17-kDa proteins, but it exhibits features typical of an extrinsic component of PSII: it is easily released from cyanobacterial PSII concomitant with the 33- and 12-kDa proteins upon washing with 1 M Tris or $CaCl_2$ (Shen et al., 1992). It thus appears that the cyanobacterial O_2-evolving PSII has two more extrinsic proteins in addition to the 33-kDa protein.

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[1] Abbreviations: cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetate; Fecy, potassium ferricyanide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TL, thermoluminescence.

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In this study, we investigated the binding and functional properties of cyt *c*-550 and the 12-kDa protein by releasing them from PSII by $CaCl_2$ wash followed by reconstitution with each of the two proteins in stepwise or in various combinations. It was revealed that cyt *c*-550 can rebind to PSII in spite of the absence of the 33- or the 12-kDa protein, whereas rebinding of the extrinsic 12-kDa protein definitely requires co-rebinding of both the 33-kDa protein and cyt *c*-550. In good correlation with these, the maximum restoration of O_2-evolving activity close to the original level was obtained only when both cyt *c*-550 and the 12-kDa protein were rebound in combination with the 33-kDa extrinsic protein. Possible roles of cyt *c*-550 and the 12-kDa protein in S-state transitions were also studied. Part of the results has been reported preliminarily (Shen & Inoue, 1993).

MATERIALS AND METHODS

Crude PSII particles were obtained from thylakoids of thermophilic *Synechococcus vulcanus* by solubilization with lauryldimethylamine *N*-oxide according to Koike et al. (1987). Purified PSII complexess were prepared from the crude PSII particles as described by Shen et al. (1992) with slight modifications. The crude PSII particles were solubilized with 2.0% (w/v) *n*-dodecyl β-D-maltoside in 40 mM Mes (pH 6.0), 20 mM NaCl, and 25% glycerol at 0 °C for 5 min under dim light and loaded onto a Mono-Q column equilibrated with 40 mM Mes–NaOH (pH 6.0), 0.05% *n*-dodecyl β-D-maltoside at 18 °C. Phicobiliproteins and other contaminating components were removed by washing the loaded column with 200 mM NaCl containing 40 mM Mes–NaOH (pH 6.0) and 0.05% *n*-dodecyl β-D-maltoside. Then, the PSII complexes were eluted with the same medium containing 330 mM NaCl. The concentration of NaCl required to elute phicobiliproteins and other contaminating components varied slightly depending on the column condition and batch of the samples. After dilution with 20 mM Mes (pH 6.0) containing 20 mM NaCl and reconcentration by ultrafiltration, the purified PSII complexes were supplemented with 20 mM $MgCl_2$ and collected by centrifugation at 560000*g* for 2 h. The pelleted PSII complexes were suspended in 40 mM Mes–NaOH (pH 6.0), 50 mM NaCl, and 25% glycerol (medium A) and stored at –80 °C until use. The presence of 50 mM NaCl in the suspending medium was necessary to suppress proteolytic digestion of the extrinsic proteins.

For releasing the extrinsic proteins, the purified PSII complexes were incubated with 1 M $CaCl_2$ for 30 min at 0 °C in darkness at a Chl concentration of 0.5 mg/mL. The released extrinsic proteins were separated from the PSII complexes by centrifugation and dialyzed against 30 mM Tris-HCl (pH 9.0) containing 50 mM NaCl. Then, the three extrinsic proteins were separated from each other by chromatography with a Mono-Q column equilibrated in 30 mM Tris-HCl (pH 9.0) using a linear gradient of 0–0.1 M $MgSO_4$. The three proteins were eluted from the column in an order of 33-kDa protein, 12-kDa protein, and cyt *c*-550. The separated proteins were dialyzed against 20 mM Mes–NaOH (pH 6.0), 10 mM NaCl and stored at –80 °C.

$CaCl_2$-washed PSII complexes were reconstituted with each of the three extrinsic proteins either alone or in combination by incubating a mixture of the proteins and PSII in medium A at 0 °C for 15 min under room light at a Chl concentration of 0.1 mg/mL. Proteins were supplemented at protein to Chl ratios of 1.0, 0.4, and 0.6 (w/w) for the 33- and 12-kDa proteins and cyt *c*-550, respectively. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Otherwise indicated, 10 mM $CaCl_2$ was included in the reconstitution medium. Immediately after reconstitution, O_2 evolution was assayed at 35 °C under illumination with saturating light (10.4 mE m^{-2} s^{-1}) from a tungsten projector lamp in the same medium supplemented with 20 mM NaCl and 10 mM $CaCl_2$. In measuring O_2 evolution by the PSII depleted of all the three proteins, 100 mM NaCl was included in the assay medium to eliminate the effects of Cl^- in the absence of extrinsic proteins (Miayo & Murata, 1987). In determining the light intensity dependence of O_2 evolution, light intensity was varied by combinations of several neutral density filters. In determining the pH dependence of O_2 evolution, the same buffer with different pHs was used (40 mM citrate–NaOH was exceptionally used for pH 4.5). For electrophoresis and thermoluminescence (TL) measurements, the PSII samples were pelleted by centrifugation and resuspended in medium A. Samples were treated with 2% (w/v) lithium dodecyl sulfate, 60 mM dithiothreitol, and 60 mM Tris-HCl (pH 8.5) at 0 °C and subjected to SDS–PAGE as described by Ikeuchi and Inoue (1988) with a 16%–22% gradient gel containing 7.5 M urea. The Coomassie blue-stained gel was scanned at 560 nm, and the peak area of each extrinsic protein was normalized on the basis of the sum of the peak areas of the 43-kDa protein and the large subunit of cyt *b*-559.

TL glow curves were recorded as described by Ono and Inoue (1986). Samples at 0.1 mg of Chl/mL were supplemented with 20 mM $CaCl_2$ and dark-adapted at 0 °C for 1 h, and a 100-μL aliquot of the sample was illuminated with a single turnover flash prior to the measurement.

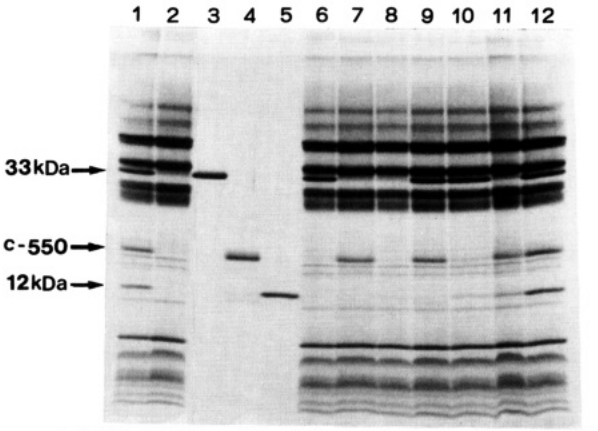

FIGURE 1: SDS–PAGE profiles of the purified cyanobacterial PSII complex after one wash with $CaCl_2$ and subsequent reconstitution with the three extrinsic proteins each separately or in combination: lane 1, untreated PSII; lane 2, $CaCl_2$-washed PSII; lanes 3, 4, and 5, chromatographically purified 33-kDa protein, cyt *c*-550, and 12-kDa protein, respectively; lanes 6, 7, and 8, $CaCl_2$-washed PSII reconstituted with 33-kDa protein, cyt *c*-550, and 12-kDa protein, respectively; lanes 9, 10, and 11, $CaCl_2$-washed PSII reconstituted with 33-kDa protein *plus* cyt *c*-550, 33-kDa protein *plus* 12-kDa protein, and cyt *c*-550 *plus* 12-kDa protein, respectively; lane 12, $CaCl_2$-washed PSII reconstituted with all three extrinsic proteins.

RESULTS

Rebinding of cyt c-550 and the 12-kDa Protein to $CaCl_2$-Washed PSII. One wash with 1 M $CaCl_2$ of purified cyanobacterial PSII removed all three extrinsic proteins, of 33- and 12-kDa and cyt *c*-550 [Figure 1, lane 2, see also Shen et al. (1992)]. Lanes 3–5 prove that these three extrinsic proteins were successfully purified to homogeneity without or with minimized proteolysis during purification procedures. When these three extrinsic proteins were subjected to recon-

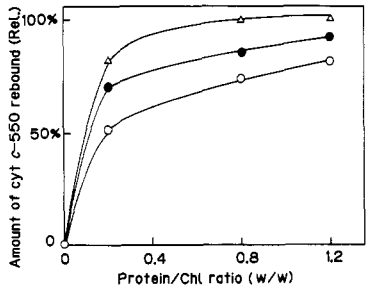

FIGURE 2: Effects of 33- and 12-kDa proteins on rebinding of cyt c-550 to $CaCl_2$-washed PSII. $CaCl_2$-washed PSII was incubated with cyt c-550 alone or in combination with the other two extrinsic proteins for 15 min at 0 °C and then pelleted by centrifugation at 560000*g* for 2 h. Symbols: (○) reconstituted with cyt c-550 alone; (●) reconstituted with cyt c-550 *plus* 33-kDa protein; (△) reconstituted with cyt c-550 *plus* 33- and 12-kDa proteins.

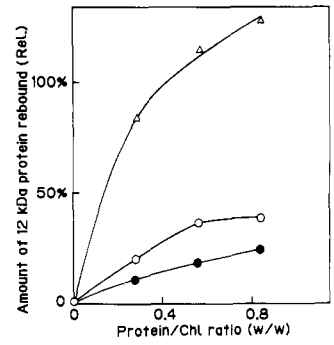

FIGURE 3: Effects of cyt c-550 and 33-kDa protein on rebinding of the 12-kDa protein. Conditions for reconstitution were the same as in Figure 2. Symbols: (○) reconstituted with 12-kDa protein *plus* cyt c-550; (●) reconstituted with 12-kDa protein *plus* 33-kDa protein; (△) reconstituted with 12-kDa protein *plus* 33-kDa protein and cyt c-550.

stitution, variously different rebinding features were observed. Both the extrinsic 33-kDa protein and cyt c-550 each supplemented alone could rebind to the $CaCl_2$-washed PSII (lanes 6 and 7). The rebinding of cyt c-550 did not change much when added together with the 33-kDa protein (lane 9, see also below). In contrast, the 12-kDa protein supplemented alone did not rebind at all to the washed PSII. Its rebinding was slightly stimulated by the presence of either the 33-kDa protein or cyt c-550 in the reconstitution mixture (lanes 10 and 11). However, when supplemented together with both the 33-kDa protein and cyt c-550, the 12-kDa protein exhibited its full rebinding (lane 12).

The features of rebinding of cyt c-550 and the 12-kDa protein were further studied by varying the concentrations of the proteins in the reconstitution mixture. The amounts of proteins rebound were expressed in a percentage as compared with their abundance in untreated PSII. As Figure 2 shows, cyt c-550 supplemented alone could rebind to 80% of the abundance in untreated PSII. The co-presence of the 33-kDa protein slightly enhanced the rebinding, but the maximum rebinding could be achieved only when both the 33- and 12-kDa proteins were simultaneously added. In the presence of these two proteins, the amount of cyt c-550 rebound reached a saturation level equivalent to the original level in untreated PSII and did not increase any more on further increasing the concentration of cyt c-550 in the reconstitution mixture, suggesting a stoichiometric association of cyt c-550 with PSII. These results indicate that cyt c-550 is able to bind to PSII essentially independent of the 33- and 12-kDa proteins, but its rebinding is facilitated by the presence of these two proteins.

The full binding of cyt c-550 in the presence of the 33- and 12-kDa proteins was achieved at protein to Chl ratios above 0.45 (w/w). Assuming the number of Chl molecules per a unit of cyanobacterial PSII to be 45 (Rogner et al., 1990), and the molecular weight of cyt c-550 to be 17-kDa (Shen et al., 1992), the minimum ratio for full binding corresponds to 1.07 mol of cyt c-550 per reaction center. This indicates that approximately one cyt c-550 apoprotein is associated with a unit of reaction center, in agreement with the previous estimation based on Coomassie-stained SDS–PAGE profile (Shen et al., 1992).

Figure 3 shows the rebinding features of the extrinsic 12-kDa protein in the presence or absence of cyt c-550 and the 33-kDa protein. As already shown by Figure 1, the 12-kDa protein supplemented alone did not rebind to PSII. However, when supplemented in combination with either the 33-kDa protein or cyt c-550, the 12-kDa protein showed a partial rebinding. The amount of the 12-kDa protein rebound increased with increasing the protein to Chl ratio in the reconstitution mixture containing either the 33-kDa protein or cyt c-550, but the amount rebound did not exceed 25 or 40% in the presence of 33-kDa protein or cyt c-550, respectively. When the 12-kDa protein was supplemented together with both the 33-kDa protein and cyt c-550, the rebinding was significantly enhanced and reached a level of 100% of its abundance in untreated PSII at a rather low protein to Chl ratio. These results clearly indicate that the binding site of the 12-kDa protein is provided through a concerted contribution by both the 33-kDa protein and cyt c-550. Upon raising the protein to Chl ratio, the amount of 12-kDa protein rebound further increased to exceed 100%. This excess rebinding may have resulted from its nonspecific interaction with PSII in the presence of both the 33-kDa protein and cyt c-550, as reported for the rebinding of the 17-kDa extrinsic protein in higher plant PSII (Miyao & Murata, 1983, 1987).

The 100% rebinding of the 12-kDa protein in the presence of both the 33-kDa protein and cyt c-550 was achieved at a protein to Chl ratio of about 0.40 (w/w). By a calculation similar to that described for cyt c-550, this corresponds to 1.35 mol of protein per reaction center, assuming the molecular weight of the protein as 12-kDa (Shen et al., 1992). This value is slightly larger than 1 mol of protein per reaction center, as we reported previously from estimation on the basis of gel electrophoresis (Shen et al., 1992).

Restoration of O_2 Evolution by Rebinding of the Extrinsic Proteins. Table I shows the O_2 evolution by $CaCl_2$-washed PSII after reconstitution with the extrinsic proteins each alone or in combination. When PBQ or PBQ *plus* Fecy was used as an electron acceptor(s), our purified PSII complex showed a high activity comparable to the activity of crude PSII (Koike & Inoue, 1985), indicating that it retained functionally intact reaction centers. The activity was much lower when Fecy alone was used as acceptor, indicative of intactness of the Q_B site. Upon $CaCl_2$-wash, O_2 evolution was severely inhibited to less than 10% of the original activity, and reconstitution with cyt c-550 alone or the 12-kDa protein alone gave no restoration. In contrast, reconstitution with the 33-kDa protein alone partially restored the activity to 41–47% with PBQ or PBQ *plus* Fecy as acceptor. This restoration extent did not change when the 12-kDa protein was additionally supplemented to the reconstitution mixture, probably because the 12-kDa protein could not bind significantly to PSII in the absence of cyt c-550 as indicated in Figure 3. When cyt c-550 was reconstituted in combination with the 33-kDa protein but without the 12-kDa protein, the activity was only slightly increased as compared with the level after reconstitution with

Table I: Restoration of O_2 Evolution upon Reconstitution of $CaCl_2$-Washed Cyanobacterial PSII with the Three Extrinsic Proteins Each Separately or in Combination

samples/additions	O_2 evolution[a] (μmol of O_2/(mg of Chl·h)) with these electron acceptors		
	PBQ/Fecy	PBQ	Fecy
control	2514 (100)[b]	1900 (100)	1195 (100)
$CaCl_2$-washed,			
no additions	242 (10)	154 (8)	217 (18)
c_{550}	284 (11)	250 (13)	246 (21)
12 kDa	254 (10)	165 (9)	250 (21)
33 kDa	1180 (47)	773 (41)	892 (75)
33 kDa + 12 kDa	1121 (45)	796 (42)	923 (77)
33 kDa + c_{550}	1423 (57)	896 (47)	1084 (91)
33 kDa + 12 kDa + c_{550}	1980 (79)	1329 (70)	1371 (115)

[a] Assay medium contained 40 mM Mes (pH 6.5), 25% glycerol, 20 mM NaCl, and 10 mM $CaCl_2$, except for $CaCl_2$-washed samples in which 100 mM NaCl was used instead of 20 mM. The concentrations of PBQ and Fecy used were 0.6 and 2 mM, respectively. [b] Percent activity relative to that of control PSII.

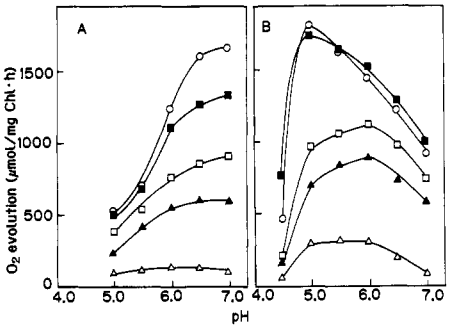

FIGURE 4: pH-dependence of O_2 evolution with PBQ (panel A) or Fecy (panel B) as the electron acceptor. Symbols: (△) $CaCl_2$-washed PSII; (▲) $CaCl_2$-washed PSII reconstituted with 33-kDa protein; (□) $CaCl_2$-washed PSII reconstituted with 33-kDa protein *plus* cyt *c*-550; (■) $CaCl_2$-washed PSII reconstituted with 33-kDa protein *plus* 12-kDa protein and cyt *c*-550; (○) control PSII.

the 33-kDa protein alone. However, when the three proteins were reconstituted altogether, the activity was markedly restored to 70% and 79% of the activity of original PSII with PBQ and PBQ *plus* Fecy as acceptors, respectively. These results clearly indicate the presence of cooperativity between the three proteins in restoring O_2 evolution in cyanobacterial PSII.

When O_2-evolving activities were measured with Fecy alone as acceptor, the restoration features were somewhat different from those obtained with PBQ or PBQ *plus* Fecy: reconstitution with all the three extrinsic proteins gave a higher activity exceeding the control level (Table I). This turned out to be due to the change in pH dependence of O_2-evolving activity during the release and reconstitution procedures. With PBQ as acceptor, our control PSII exhibited the maximum activity at around pH 7.0 (Figure 4A), and this optimum pH did not change much after $CaCl_2$ wash or subsequent reconstitution with any of the three extrinsic proteins either separately or in combination, so that the slight but significant stimulation of O_2 evolution induced by the additional reconstitution of cyt *c*-550 to the 33-kDa protein-reconstituted PSII could be similarly detected at all the pH ranges tested. With Fecy alone as the acceptor, however, our control PSII exhibited a sharp optimum at pH 5.0 (Figure 4B), in agreement with the previous observation with spinach PSII membranes (Ikeuchi & Inoue, 1986) which has been explained as due to the presence of negative surface charges on PSII. This profile with a sharp, low optimum pH was converted by $CaCl_2$ wash to a profile

Table II: Effects of Divalent Cations on O_2 Evolution in Native or Reconstituted Cyanobacterial PSII

samples/additions	O_2 evolution[a] (μmol of O_2/(mg of Chl·h)) in the presence of		
	EDTA[b] (2 mM)	$MgCl_2$[b] (10 mM)	$CaCl_2$[b] (10 mM)
control	1377 (65)[c]	1874 (88)	2135 (100)
$CaCl_2$-washed			
no additions	0 (0)	115 (47)	242 (100)
33 kDa	230 (25)	734 (79)	923 (100)
33 kDa + c_{550}	430 (32)	871 (66)	1323 (100)
33 kDa + c_{550} + 12 kDa	719 (42)	1384 (81)	1700 (100)

[a] Electron acceptor was 0.6 mM PBQ + 2 mM Fecy. [b] Salts (10 mM $MgCl_2$ or $CaCl_2$) or EDTA (2 mM) was included in both the reconstitution and assay media. [c] Percent activity relative to that in the presence of $CaCl_2$.

having a significantly broadened optimum at pH 5.0–6.0. The broadened profile did not change much upon reconstitution with the 33-kDa protein alone or with the 33-kDa protein *plus* cyt *c*-550, presumably because the high concentration of $CaCl_2$ had already eliminated some of the negative surface charges and enabled an easier access of Fecy to PSII at a broader pH range. This profile was altered again to show a downshifted optimum at pH 5.0, when all three proteins were reconstituted. The resulting pH dependence remained slightly broader than the original one in spite of the same optimum at pH 5.0, showing a higher activity at pH 6.5. This accounts for the excess activity restoration after the reconstitution (Table I). Although the reason for this downshift in optimal pH is not very clear, it is clearly due to the rebinding of the 12-kDa protein among others.

Table II shows the effects of $CaCl_2$ on O_2 evolution by our purified cyanobacterial PSII before and after depletion of extrinsic proteins by $CaCl_2$ wash. The untreated control PSII can evolve O_2 in the absence of Ca^{2+} (in the presence of EDTA), but addition of $CaCl_2$ appreciably enhanced the activity, indicating that the cyanobacterial PSII requires Ca^{2+} for maximum O_2 evolution even in the presence of the three extrinsic proteins. This enhancement, however, is not specific for Ca^{2+}: $MgCl_2$ could also appreciably stimulate the O_2 evolution, although the stimulation was not as pronounced as that by $CaCl_2$. Since similar stimulation could be observed with PBQ alone as electron acceptor (not shown), this $MgCl_2$ effect cannot be due to elimination of negative surface charges on the acceptor side but is a sort of divalent cation effect on the donor side. These observations are compatible with those reported for PSII from other cyanobacteria (Stewart et al., 1985b; Rolfe & Bendall, 1989; Pauly et al., 1992) but differ significantly from the Ca^{2+} effect observed with higher plant PSII. Upon $CaCl_2$ wash of cyanobacterial PSII, no O_2 evolution could be observed in the presence of EDTA even if the assay mixture was supplemented with a high concentration of Cl^- (100 mM NaCl). Addition of $MgCl_2$ again partially restored the activity, and the restoration extent increased further upon addition of $CaCl_2$. These results are compatible with the observations with cyanobacterial cells genetically depleted of the 33-kDa protein (Philbrick et al., 1991) but differ from those with higher plant PSII in which Cl^- alone effectively restores the O_2 evolution by PSII depleted of all the three extrinsic proteins (Miyao & Murata, 1984). Upon reconstitution with the 33-kDa protein, the activity was partially restored even in the presence of EDTA in both the reconstitution and assay media, and the restoration extent was increased by further reconstitution with cyt *c*-550 or cyt *c*-550 *plus* the 12-kDa protein. These results clearly indicate

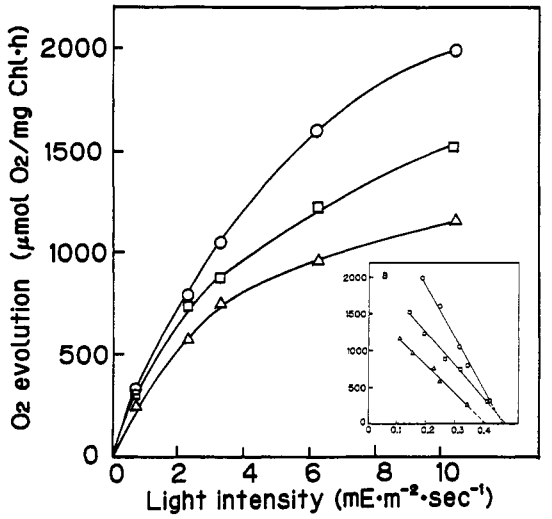

FIGURE 5: Light intensity dependence of O_2 evolution by $CaCl_2$-washed PSII reconstituted with three extrinsic proteins in different combinations. Activity was assayed under continuous light with PBQ *plus* Fecy as acceptor. Symbols: (△) Reconstituted with 33-kDa protein alone; (□) reconstituted with 33-kDa protein *plus* cyt *c*-550; (○) reconstituted with 33-kDa protein *plus* cyt *c*-550 and 12-kDa protein. Inset: O_2 evolution (micromoles of O_2 per milligram of Chl per hour) replotted against O_2 evolution/light intensity (μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}/E m^{-2} s^{-1}).

that in cyanobacterial PSII Ca^{2+} alone cannot substitute for cyt *c*-550 and the 12-kDa protein, while in higher plant PSII it substitutes for the 23- and 17-kDa extrinsic proteins.

Effects of cyt c-550 and the 12-kDa Protein on S-State Transitions. Figure 5 shows the light intensity dependence of O_2 evolution by variously reconstituted PSII. Under high light intensities, the extents of activity restoration of the three samples reconstituted with the 33-kDa protein alone, the 33-kDa protein *plus* cyt *c*-550, and all three extrinsic proteins were significantly different. Under low light intensities, however, the difference was much smaller between the former two samples, and practically no difference existed between the latter two samples. By replotting the data of Figure 5, we obtain a series of straight lines as depicted in the inset of Figure 5. By employing the analysis method documented in Lumry and Rieske (1959), Rieske et al. (1959), and Satoh et al. (1972), it was deduced that the absence of cyt *c*-550 affected the relative quantum yield for O_2 evolution but the 12-kDa protein did not. These results suggest that cyt *c*-550 modulates a light step(s) in O_2 evolution, whereas the 12-kDa protein modulates a dark step(s). The possibility that the effect of cyt *c*-550 on the relative quantum yield is due to impairment at the acceptor side of PSII can be ruled out, since Fecy *plus* PBQ was employed in O_2 assays, where Fecy accepts electrons from Q_A^- and thereby compensates for the impairment (if any) between Q_A and Q_B. On the other hand, similar results have been reported for PSII of *Phormidium* about the function of a 9-kDa protein homologous to our 12-kDa protein (Rolfe & Bendall, 1989).

Figure 6 shows the effects of the three extrinsic proteins on the properties of TL emission from purified cyanobacterial PSII core complex. After excitation with one flash, our control PSII showed a small glow peak at around 30 °C (panel A, curve a), the so-called TL B-band originating from charge recombination between S_2 (or S_3) and Q_B^- (Vass & Inoue, 1992). Upon addition of DCMU, this glow peak downshifted to 5 °C (broken glow curve in panel A), indicative of conversion of B-band to the Q-band that originates from $S_2Q_A^-$ charge recombination (Vass & Inoue, 1992). After excitation with two flashes, the emission intensity of the 30 °C band was significantly increased and remained almost unchanged after three or more flashes (not shown). The low intensity of the 30 °C band after one flash but markedly high intensity after two flashes implies that a large part of the reaction centers in our purified PSII preparation are in the state of $S_1Q_B^-$ after dark adaptation (1 h). This state will be converted by the first flash to the S_2Q_B state that does not thermoluminescence, and then by the second flash to the S_3Q_B state that thermoluminesces more strongly than $S_2Q_B^-$ by a factor of 1.8 (Vass & Inoue, 1992). The reason for unchanged emission intensity after three or more flashes is not clear because of our limited knowledge and experience about TL properties of cyanobacterial purified PSII core complex, but it is seemingly due to an increased miss factor and the absence of free plastoquinone in our purified PSII complex (note that the experiments were done in the absence of artificial acceptors).

Removal of extrinsic proteins by $CaCl_2$ wash affected the TL properties in two ways: (i) peak temperature of the Q-band was upshifted from 5 °C to 27 °C (broken glow curves in panels A and B), while that of the B-band (30 °C band) remained unchanged, and (ii) emission intensity of the B-band after the first and second flashes was reduced approximately to half. The former effect (i) is consistent with the previous observation with $CaCl_2$-washed higher plant PSII (Vass et al., 1987) or cyanobacterial cells genetically depleted of the 33-kDa protein (Burnap et al., 1992) and implicates that the properties of the S_2-state were modified to require a higher temperature for recombination of the $S_2Q_A^-$ charge pair (but not for $S_2Q_B^-$ charge pair, since Q_B was also modified as reported for higher plant PSII (Vass et al., 1987)) due to the absence of the 33-kDa protein. The latter effect (ii) implies that the absence of extrinsic proteins lowers the efficiency of PSII photoreaction to create charge pairs.

Of these two effects brought about by removal of all three extrinsic proteins, the former effect (i) was reversed by reconstitution with the 33-kDa protein alone as shown by the downshifted glow peak of the Q-band (broken glow curve in panel C) in agreement with the conversion of modified abnormal S_2 to normal S_2 by reconstitution with the 33-kDa protein as reported for higher plant PSII (Vass & Inoue, 1992). Reconstitution with the 33-kDa protein alone did not reverse the latter effect (ii), the low efficiency of PSII photoreaction, but led to generation of a shoulder on the glow curve at around 0 °C after the second and third flashes (solid glow curves in panel C). From its emission temperature, this shoulder is attributed to the A-band that arises from recombination of $S_3Q_A^-$ charge pair (Vass & Inoue, 1992). Notably, this shoulder disappeared upon reconstitution with both the 33-kDa protein *plus* cyt *c*-550 (panel D). Since the glow peak at 30 °C arises from charge recombination of S_2 or S_3 with Q_B^- the present results are interpreted as indicating that in samples reconstituted with the 33-kDa protein alone the electron transfer from Q_A to Q_B was somehow impaired, but the impairment could be reversed by additional reconstitution of cyt *c*-550. One may wonder why the A-band could not be detected in the absence of all the three extrinsic proteins (panel B). This is probably because in the absence of the three extrinsic proteins, the peak temperature of the TL emission arising from charge recombination between modified abnormal S_2 (or S_3) with Q_A^- was markedly upshifted to be superimposed on the B-band at 30 °C that arises from $S_2Q_B^-$ (or $S_3Q_B^-$) charge recombination, as documented previously (Vass et al., 1987; Burnap et al., 1992).

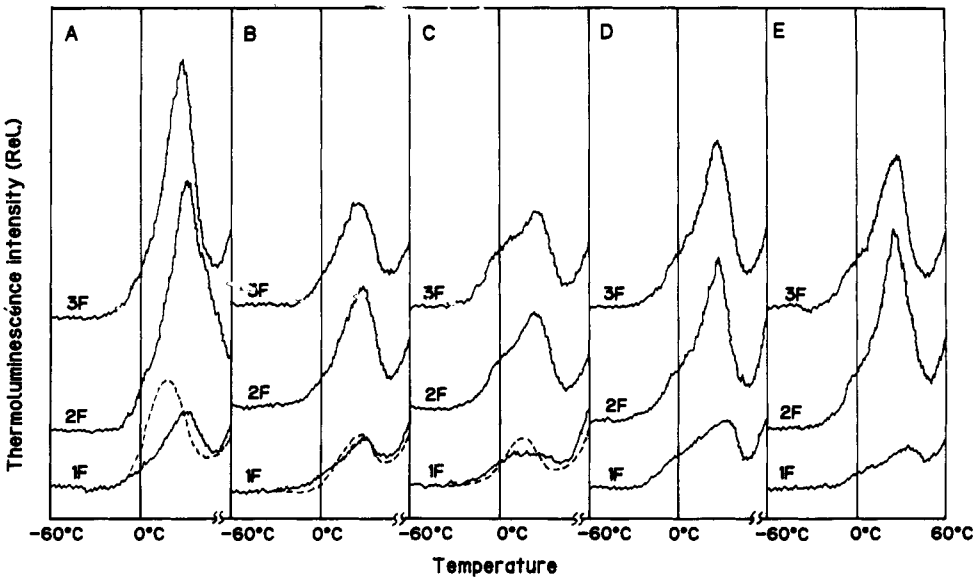

FIGURE 6: Thermoluminescence glow curves of purified cyanobacterial PSII core complex after $CaCl_2$ wash and reconstitution with three extrinsic proteins in different combinations. 1F to 3F stand for the number of excitation flashes given at 0 °C. (A) Control untreated PSII; (B) $CaCl_2$-washed PSII; (C) $CaCl_2$-washed PSII reconstituted with 33-kDa protein; (D) $CaCl_2$-washed PSII reconstituted with 33-kDa protein *plus* cyt *c*-550; (E) $CaCl_2$-washed PSII reconstituted with 33-kDa protein *plus* cyt *c*-550 and 12-kDa protein. Broken glow curves in pane A, B, and C were recorded in the presence of 10 μm DCMU.

When reconstituted with the 33-kDa protein *plus* cyt *c*-550, the TL intensity after two or three flashes was markedly increased (panel D), although further reconstitution with the 12-kDa protein did not increase the TL intensity any more (panel E). This indicates that the low efficiency of photoreaction in the 33-kDa protein alone-reconstituted PSII was restored to a higher efficiency by the additional reconstitution with cyt *c*-550. One might argue that the increased TL intensity could be due to the conversion of the 0 °C shoulder component to the 30 °C component, but a careful estimation of the total area under the TL glow peaks unambiguously indicated that additional reconstitution with cyt *c*-550 substantially increased the total TL intensity. Presumably, the S-state turnover was partially impaired either in S_1–S_2 or S_2–S_3 transition or both in the absence of cyt *c*-550, but the impairment was restored by reconstitution with cyt *c*-550. In contrast to cyt *c*-550, the 12-kDa protein did not influence these reactions. These results are compatible with those deduced from the analysis of light intensity dependence of O_2 evolution (Figure 5) that cyt *c*-550 regulates a light step(s) while the 12-kDa protein regulates a dark step(s) in O_2 evolution.

DISCUSSION

The present study demonstrated that the binding affinity for PSII core complex of the two newly found cyanobacterial extrinsic components, cyt *c*-550 and the 12-kDa protein, depends variously on the presence or absence of the well-characterized extrinsic 33-kDa protein. Of these three extrinsic members, the 33-kDa protein rebinds to PSII independent of the other two. Cyt *c*-550 also rebinds almost (ca. 80%) independent of the other two, although its rebinding is slightly enhanced (by ca. 10%) in the presence of the 33-kDa protein, and some more (by another ca. 10%) in the presence of both the 33- and 12-kDa proteins. In contrast, the 12-kDa protein does not rebind at all in the absence of the other two, but rebinds only slightly (by ca. 25%) in the presence of the 33-kDa protein, appreciably (by ca. 40%) in the presence of cyt *c*-550, and rebinds remarkably efficiently in the presence of the other two proteins, exceeding the original amount presented in untreated PSII.

By taking into consideration the established fact that the 33-kDa protein binds to the donor side of PSII, we can discuss the binding sites of cyt *c*-550 and the 12-kDa protein as follows. Since cyt *c*-550 is an extrinsic component that can bind to PSII almost independent of the other two proteins, we may assume its binding site on either the acceptor or the donor side of PSII.

Assumption 1: cyt *c*-550 binds to the acceptor side of PSII. This assumption is in line with the historical view that cyt *c*-550 is a soluble protein of cytoplasmic origin (Krogmann & Smith, 1990; Kienzl & Peschek, 1983). This model explains the enhancing effect of cyt *c*-550 on the binding of the 12-kDa protein (by ca. 40%), if we assume that the binding site of the 12-kDa protein is located on cyt *c*-550. However, the enhanced binding of cyt *c*-550 (ca. by 10%) and the enhanced binding of the 12-kDa protein (ca. by 25%) upon co-reconstitution with the 33-kDa protein cannot be explained, unless we assume that binding of the 33-kDa protein at the donor side of PSII elicits a membrane-spanning conformational regulation at the acceptor side. To make the matter worse, this model does not explain the remarkable enhancement of binding of the 12-kDa protein when both the 33-kDa protein and cyt *c*-550 are co-reconstituted. Since this model assumes the binding site of the 12-kDa protein as located on cyt *c*-550, the only possible way to explain this remarkable enhancement is to presume that binding of the 33-kDa protein influences the binding site of the 12-kDa protein at the surface of cyt *c*-550 across the PSII core complex (and the cyt *c*-550 molecule as well). This model thus forces us to attribute the clearest phenomenon, the cooperative effect of cyt *c*-550 and the 33-kDa protein in facilitating the binding of the 12-kDa protein, as due to an indirect conformational regulation across not only the membrane but also the cyt *c*-550 molecule. On the basis of these considerations, we abandon this model.

Assumption 2: cyt *c*-550 binds to the donor side of PSII. This model might appear difficult to accept in view of the past understanding about this protein. However, it easily explains the enhanced binding of cyt *c*-550 (ca. 10%) by the 33-kDa

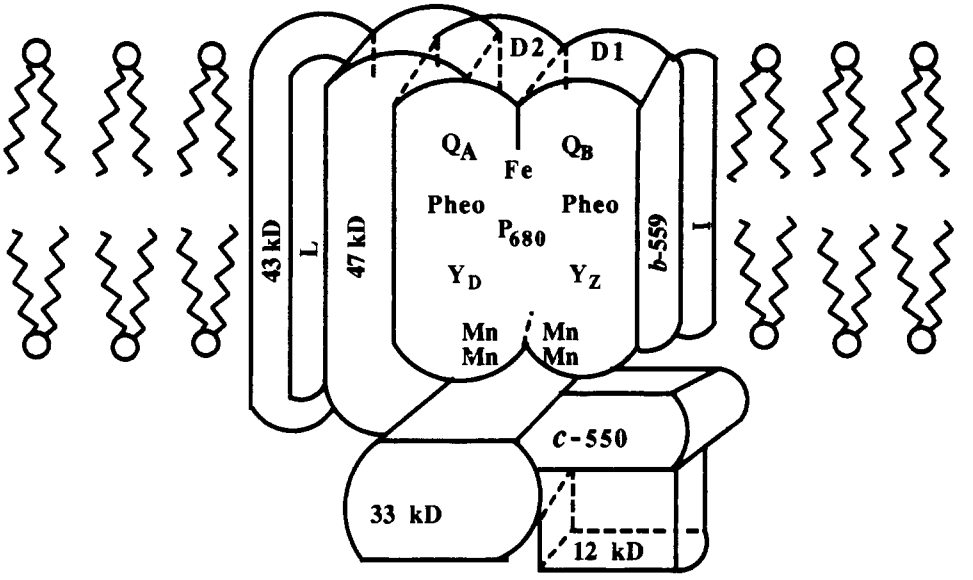

FIGURE 7: Schematic presentation of the probable association of three extrinsic proteins in cyanobacterial PSII. Both cyt c-550 and the 12-kDa protein were assumed to be localized on the lumen side of thylakoid as well as the 33-kDa protein (for details see text). Abbreviations: P_{680}, reaction center of PSII; Y_Z and Y_D, the secondary and auxiliary donors of PSII, tyrosine residues on D1 and D2 polypeptides, respectively; Q_A and Q_B, the primary and secondary quinone acceptors of PSII, respectively. I and L subunits are the low-molecular-mass proteins tightly associated with the cyanobacterial PSII reaction center (Ikeuchi et al., 1989). Other small proteins identified in the PSII core complex were omitted for simplicity.

protein, by simply assuming that cyt c-550 has affinity for the 33-kDa protein. It also easily explains the enhanced binding of the 12-kDa protein upon co-reconstitution with either cyt c-550 alone or the 33-kDa protein alone if we simply assume that the 12-kDa protein has affinity for both of the two extrinsic proteins, slight affinity (ca. 25%) with the latter, and appreciable affinity (ca. 40%) with the former. Moreover, this model explains reasonably the cooperativity between the 33-kDa protein and cyt c-550 in remarkably enhancing the binding of the 12-kDa protein: in the presence of the two extrinsic proteins both being bound at the donor side and having affinity for the 12-kDa protein, the binding of the 12-kDa protein will be much stabilized to achieve full binding. Importantly, we do not have to assume any (membrane-spanning) conformational regulation in this model in order to explain the cooperative effects among the three extrinsic members.

On the basis of these considerations, we conclude that both the 12-kDa protein and cyt c-550 bind to the donor side of PSII as well as the 33-kDa protein does, as schematized in Figure 7. It may be of note in this connection that this model is compatible with the fact that the gene for the *Phormidium* 9-kDa protein (homologous to the 12-kDa protein in this study) has a leader sequence that will be needed for its transport across the thylakoid membrane (Wallace et al., 1989), although corresponding sequence data for cyt c-550 are not available at present.

As to the relationships between binding features and functional properties, a good correlation can be pointed out for the 12-kDa protein. The 12-kDa protein alone cannot bind at all to PSII in the absence of the other two proteins, corresponding to the observation that no enhancement of O_2 evolution occurred when $CaCl_2$-washed PSII was reconstituted with this protein alone. Inversely, the activity enhancement by the 12-kDa protein occurs only in the presence of both the 33-kDa protein and cyt c-550, corresponding to the fact that the 12-kDa protein efficiently rebound to PSII only in the presence of both the other two proteins. As to cyt c-550, on the other hand, such correlation does not work: cyt c-550 reconstituted alone did not enhance O_2 evolution in spite of its significant binding. This implies that cyt c-550 functions in O_2 evolution only when the 33-kDa protein is present.

These binding and functional features resemble those of the 23- and 17-kDa proteins in higher plant PSII. In higher plant PSII, the 23-kDa protein can partially rebind to PSII in the absence of the 33-kDa protein, but its function manifests only in the presence of the 33-kDa protein (Murata et al., 1983; Andersson et al., 1984). Rebinding of the 17-kDa protein requires the presence of both the 33- and 23-kDa proteins, and consequently, it functions only in the presence of these two extrinsic proteins (Miyao & Murata, 1983, 1984). It thus appears as if the three cyanobacterial extrinsic proteins share their roles in a manner similar to the manner of those in higher plant PSII. However, there lies a profund difference between the two systems, particularly regarding the action of Ca^{2+}: (i) Cyanobacterial PSII requires Ca^{2+} for its maximum rate of O_2 evolution even in the presence of all the three extrinsic proteins, whereas plant PSII manifests its demand for Ca^{2+} only after removal of the 23- and 17-kDa proteins or modulation of their binding. (ii) The Ca^{2+} effect in cyanobacterial PSII is not so specific as that in plant PSII: $MgCl_2$ can support a significant part of O_2 evolution in place of $CaCl_2$ (Stewart et al., 1985b; Rolfe & Bendall, 1989; Pauly et al., 1992; this study). It is thus inferred that the enhancement of O_2 evolution by Ca^{2+} in cyanobacterial PSII is rather a sort of nonspecific effect of divalent cations, facilitating the maintenance of the optimal conformation of the extrinsic or intrinsic PSII proteins. This contrasts to that in higher plant PSII where O_2 evolution is stimulated specifically by Ca^{2+} but not by Mg^{2+}. (iii) There is an additional difference in the binding affinity of these extrinsic proteins. The 23- and 17-kDa proteins of higher plants can be released by a NaCl wash, whereas cyt c-550 and the 12-kDa protein of cyanobacteria are not (Shen et al., 1992). We also note that the 23- and 17-kDa proteins of higher plants never bind to PSII core complex unless the core complex retains some of the light-harvesting chlorophyll proteins (Ikeuchi & Inoue, 1986; Enami et al., 1989), whereas in cyanobacterial PSII, cyt c-550 and the 12-kDa protein are able to functionally bind to a purified

core PSII consisting of only the major membrane-spanning proteins and several low-molecular-mass subunits.

According to our analysis of the light intensity dependence of O_2 evolution by reconstituted PSII, the function of the 12-kDa protein seems to be related to a dark step(s) of O_2 evolution, in agreement with the previous results by Rolfe and Bendall (1989) about the homologous 9-kDa protein in *Phormidium* PSII. Our results also suggest that cyt *c*-550 supports the high efficiency of S-state transitions either in the S_1–S_2 or S_2–S_3 step or both. In addition, our TL analysis suggests that cyt *c*-550 facilitates the electron transfer from Q_A to Q_B. (Note that this effect manifests only in the absence of artificial electron acceptor, Fecy.) We consider, however, that this will be due to some secondary effect induced by this extrinsic cyt, since this cyt seems most likely to bind to the donor side of PSII as discussed above and therefore will have no direct interaction with the acceptor side of PSII. We note that similar indirect effects at the acceptor side of PSII have been reported in higher plant PSII upon removing and rebinding of the 23- and 17-kDa proteins at the donor side of PSII (Rashid & Carpentier, 1990).

The structural model of the cyanobacterial extrinsic protein system presented in this study contradicts, among others, the so far reported location and function of cyt *c*-550 (Krogmann & Smith, 1990; Kienzl & Peschek, 1983). An approach to clarify or reconcile these discrepancies will be a careful investigation of localization of cyt *c*-550 in vivo. Such trials are now underway.

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