The 33 kDa Protein Can Be Removed without Affecting the Association of the 23 and 17 kDa Proteins with the Luminal Side of PS II of Spinach†

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ABSTRACT: An earlier study shows that a 30 min incubation of spinach PS II submembrane fragments at pH 6.3 in the presence of 10 μM $HgCl_2$ induces a 40% depletion of the 33 kDa protein without the apparent release of the 17 and 23 kDa proteins [Bernier, M., and Carpentier, R. (1995) *FEBS Lett. 360*, 251−254]. Here we report that the photosystem II 33 kDa extrinsic protein is fully removed by $HgCl_2$ added at micromolar and higher concentrations (0.25, 20, and 50 μM), with the 17 and 23 kDa extrinsic proteins and other intrinsic proteins remaining bound to the reaction center. The data presented here put in doubt the "regulatory cap" model of PS II, which follows the OEC−33 kDa−23 kDa−17 kDa binding order, as these results directly demonstrate that the 33 kDa protein can be removed without affecting the binding of the 23 and 17 kDa proteins to the intrinsic subunits of PS II. This suggests that each extrinsic protein may possess its own binding site on PS II. A possible mechanism for $HgCl_2$ upon the release of the 33 kDa protein is discussed.

Photosystem II (PS II)[1] is a membrane-bound chlorophyll−protein complex that utilizes the energy of visible light to catalyze the reduction of plastoquinone to plastohydroquinone and the oxidation of water to molecular oxygen and protons (*1*, *2*). The structure of PS II has been explored by high-resolution electron microscopy and X-ray crystallography (*3*−*9*). Seven major intrinsic polypeptides, CP47, CP43, D1, D2, α and β subunits of cytochrome b_{559}, and the 4 kDa *psb*I gene product, form the membrane-associated core of PS II (*10*, *11*). In higher plants and green algae, three extrinsic proteins with apparent molecular masses of 33, 23, and 17 kDa (*psb*O, *psb*P, and *psb*Q nuclear-encoded gene products, respectively) bind to the lumenal side of plant PS II and modulate the properties of the OEC (*10*−*12*). The key roles observed for the 33 kDa protein are to stabilize the tetranuclear manganese cluster under physiological salt concentrations and to accelerate O_2 evolution activity, and therefore, the 33 kDa protein is termed the "manganese stabilizing protein" (MSP). The 23 and 17 kDa proteins appear to play a role in regulating calcium and chloride concentrations within PS II (*10*, *11*, *13*). In red algae and cyanobacteria, the 23 and 17 kDa proteins were generally thought to be absent and apparently substituted with a 12 kDa protein (*psb*U) and cytochrome c_{550} (*psb*V) (*14*, *15*). However, a recent proteomic analysis showed that both the PsbP and PsbQ proteins are present in the cyanobacterium *Synechocystis* 6803 and could have a similar function in cyanobacteria and in higher plants (*16*).

Although many reports have appeared concerning the functional and biochemical aspects of these three extrinsic proteins, relatively little is known of their binding properties or spatial arrangement within PS II. On the basis of removal and reconstitution experiments, researchers have suggested several schematic presentations of the probable association of the extrinsic proteins at the lumenal side of the thylakoid membrane (*11*, *17*, *18*). Moreover, on the basis of the analysis of protease cleavage sites, it has been suggested that the structure of the 33 kDa protein is different in different organisms and can be divided into three major groups: a higher plant type, a cyanobacterial type (red algae and cyanobacteria), and an intermediate type (green algae and euglena) (*19*). However, it is not clear whether these structural differences translate to differences in the binding properties of the 33 kDa proteins among different organisms. In green algae, the 23 and 17 kDa proteins still bind to the thylakoid membrane in a mutant of *Chlamydomonas reinhardtill* lacking the ability to synthesize the 33 kDa protein (*20*), hence indicating that the binding of the 23 and 17 kDa proteins to PS II does not require the presence of the 33 kDa protein. Moreover, in release reconstitution experiments, Suzuki et al. observed that three extrinsic proteins independently re-bound to their original binding sites within PS II of *C. reinhardtii* (*21*), suggesting that each of the three extrinsic proteins has its own binding site independent of the others in the green algae PS II. In higher plants, however, the most popular model is still the "regulatory cap" model, which states that the 23 kDa protein cannot directly bind to PS II and that the 23 kDa protein associates with PS II only through its interaction with the 33 kDa protein, with the 17 kDa protein, in turn, functionally associated with PS II by

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[1] Abbreviations: BBY, PS II-enriched thylakoid membrane; Chl, chlorophyll; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MSP, manganese-stabilizing protein; OEC, oxygen-evolving complex; pI, isoelectric point; PS II, photosystem II.

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its interaction with both the bound 33 and 23 kDa proteins (*22*−*24*). As the 33 kDa protein is much more tightly associated with the intrinsic PS II proteins than the 23 and 17 kDa proteins (*10*, *25*, *26*), the full release of the 33 kDa protein from BBY samples by alkaline Tris, $CaCl_2$, or urea-NaCl washing of BBY samples (*27*−*29*), or through high hydrostatic pressure treatments (*30*), results in either concomitant or subsequent depletion of the 17 and 23 kDa proteins. It should be pointed out, however, that an earlier study shows that a 30 min incubation of spinach PS II submembrane fragments at pH 6.3 in the presence of 10 μM $HgCl_2$ induces a 40% depletion of the 33 kDa protein without the apparent release of the 17 and 23 kDa proteins (*31*). This study hints at a possibility that the binding of the 23 and 17 kDa proteins to PS II in higher plants could also be independent of the 33 kDa protein just as in green algae (*21*). Obviously, additional experiments must be performed to either support or disprove this possibility. Here we report results that show that the 17 and 23 kDa proteins still bind to the thylakoid membranes following $HgCl_2$ treatments of PS II membrane samples that provide the near-complete release of the 33 kDa protein.

MATERIALS AND METHODS

Materials. $HgCl_2$ (ACS reagent) was used as received from Sigma. SDS−PAGE standards (low range) were used as received from Bio-Rad. All other chemicals were used as received from Fisher Scientific or Sigma.

Preparation and Characterization of BBY Samples. PS II-enriched "BBY" membranes were prepared from spinach on the basis of the procedure developed by Berthold et al. (*32*, *33*) with some modifications provided by Campbell et al. (*34*). All steps were performed in a cold room (4 °C) under dim green light or in darkness. Isolated PS II membranes were resuspended in SMNCE buffer, which contains 400 mM sucrose, 20 mM MES-NaOH (pH 6.0), 15 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 1 mM EDTA, and were analyzed by SDS−urea−PAGE using a gel containing 6.0 M urea as described previously (*35*). The polyacrylamide concentrations of the stacking and separation gels were 4 and 15%, respectively. The gels were stained with Coomassie blue R-250. Using a luminescent image analyzer (Fujifilm, LAS-3000) and image reader, individual protein bands were quantified. Lanes were normalized relative to one another using the 47 kDa protein band (whose intensity is a function of Chl concentration only) as a standard to account for small variations in protein loading. For the graphs showing polypeptide compositions, error bars represent the standard deviation from the average of at least three trials.

$HgCl_2$ Treatments of BBY Samples. BBY samples were washed twice with a buffer containing 400 mM sucrose, 1 mM EDTA, and 20 mM MES-NaOH (pH 6.0) and then suspended to a concentration of 0.05 mg of Chl/mL in buffers (without Cl^- or with the addition of both 15 mM Cl^- and 3 mM Ca^{2+}) at various pHs (HEPES at pH 7 or 8 or CAPS at pH 9), and with $HgCl_2$ at the desired concentrations. After incubation in room temperature for ~30 min in the dark, all BBY samples were centrifuged at 30000*g* for 30 min. The final pellets were washed once in a buffer containing 400 mM sucrose, 50 mM MES-NaOH, and 200 mM NaCl (pH 6.0) and then analyzed by SDS−urea−PAGE. The supernatants were centrifuged to remove the remaining membrane fragments, concentrated against a sucrose gradient, dialyzed against the buffer as described above at 4 °C, and analyzed by SDS−urea−PAGE. The Chl concentration and Chl *a*/Chl *b* ratios were determined by the method of Arnon (*36*).

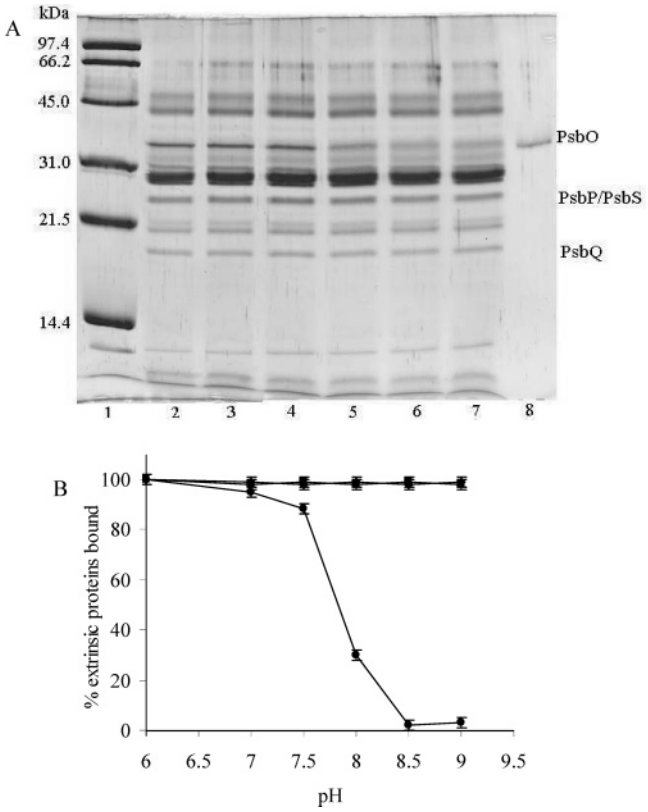

FIGURE 1: Effects of 0.25 μM $HgCl_2$ on the release of the extrinsic proteins from PS II membranes without addition of Cl^- in buffer at different pHs (7.0−9.0). (A) SDS−urea−PAGE: lane 1, protein standards; lane 2, control (BBY); lane 3, BBY incubated at pH 7.0; lane 4, BBY at pH 7.5; lane 5, BBY at pH 8.0; lane 6, BBY at pH 8.5; lane 7, BBY at pH 9.0; and lane 8, supernatant of $HgCl_2$-treated BBY at pH 8.5. (B) Quantitative analysis of effects of $HgCl_2$ on individual proteins: (◆) PsbQ, (■) PsbP, and (●) PsbO. All experimental conditions are given in Materials and Methods.

RESULTS

Polypeptide Composition of BBY Samples. The polypeptide composition of extracted BBY samples prepared as described in Materials and Methods is shown in Figure 1A as analyzed by SDS−urea−PAGE. The gel pattern of the control sample (Figure 1A, lane 2) shows several major polypeptides, including D1, D2, CP47, CP43, three extrinsic proteins, etc.

Effects of $HgCl_2$ at Different Concentrations on the Release of the 33 kDa Protein from PS II in Various pH Buffers in the Absence of Cl^-. Bernier et al. reported that the incubation of PS II samples in the presence of 10 μM $HgCl_2$ induced a 40% depletion of the 33 kDa protein, and this depletion was strongly reduced either in the presence of 5 mM Cl^- or by treatment with a higher concentration of $HgCl_2$ (>10 μM) at pH 6.3 (*31*). In our experiment, 95% of the 33 kDa protein remained bound in a 30 min incubation with 0.25 μM $HgCl_2$ at pH 7.0 buffer in the absence of Cl^- (Figure 1A, lane 3). As the pH of the buffer was increased, the 33 kDa protein was gradually released; 12% of the 33 kDa protein was released from the PS II membrane at pH 7.5 (Figure 1A,

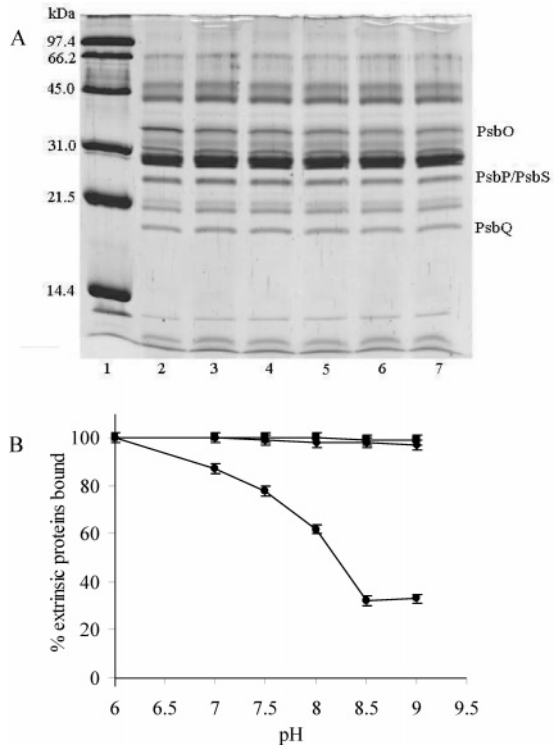

FIGURE 2: Effects of 20 μM $HgCl_2$ on the release of the extrinsic proteins from PS II membranes without addition of Cl^- in buffer at different pHs (7.0−9.0). (A) SDS−urea−PAGE: lane 1, protein standards; lane 2, control (BBY); lane 3, BBY incubated at pH 7.0; lane 4, BBY at pH 7.5; lane 5, BBY at pH 8.0; lane 6, BBY at pH 8.5; and lane 7, BBY at pH 9.0. (B) Quantitative analysis of effects of $HgCl_2$ on individual proteins: (◆) PsbQ, (■) PsbP, and (●) PsbO. Error bars represent the standard deviation from the average of at least three trials.

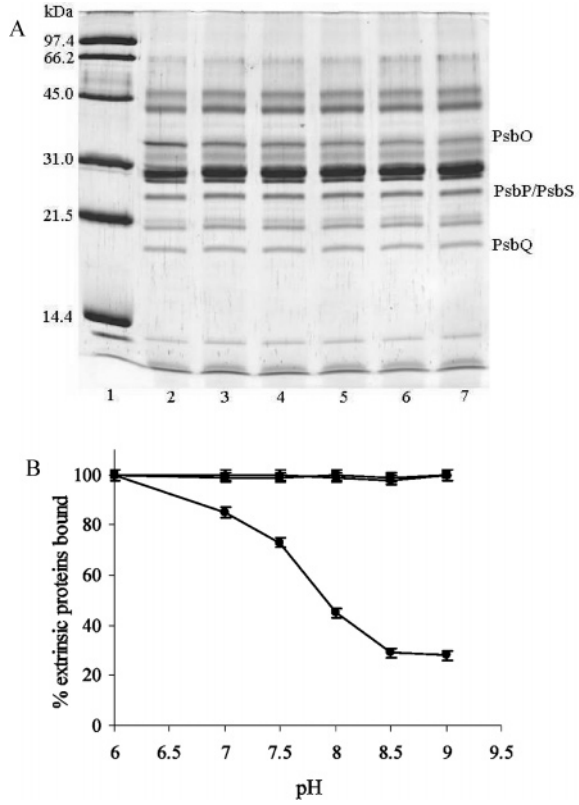

FIGURE 3: Effects of 0.25 μM $HgCl_2$ on the release of the extrinsic proteins from PS II membranes with addition of 15 mM Cl^- in buffer at different pHs (7.0−9.0). (A) SDS−urea−PAGE: lane 1, protein standards; lane 2, control (BBY); lane 3, BBY incubated at pH 7.0; lane 4, BBY at pH 7.5; lane 5, BBY at pH 8.0; lane 6, BBY at pH 8.5; and lane 7, BBY at pH 9.0. (B) Quantitative analysis of effects of $HgCl_2$ on individual proteins: (◆) PsbQ, (■) PsbP, and (●) PsbO.

lane 4, and Figure 1B) and 70% at pH 8.0 (Figure 1A, lane 5, and Figure 1B). Both the 23 and 17 kDa proteins were still bound to the PS II intrinsic proteins even after nearly all of the 33 kDa protein (>97%) had been released during the 30 min incubation with 0.25 μM $HgCl_2$ at pH 8.5 in the absence of chloride (Figure 1A, lane 6, and Figure 1B). This suggests that pH may be one of many important factors which control the release of the 33 kDa protein from PS II during its incubation with $HgCl_2$ (0.25 μM) in the absence of chloride.

Interestingly, the depletion of the 33 kDa protein was obviously inhibited in BBY samples incubated with a higher concentration of $HgCl_2$ (20 μM) for ~30 min in various pH buffers in the absence of chloride (Figure 2A). A maximal removal of the 33 kDa protein was observed, reaching ~70% of the control level after a 30 min incubation at pH 8.5 in the absence of chloride (Figure 2A, lane 6, and Figure 2B), while both the 17 and 23 kDa proteins still remained unaffected (Figure 2B). This result is consistent with a previous report that depletion of the 33 kDa protein from the PS II membrane was strongly attenuated by treatment with a higher concentration of $HgCl_2$ (>10 μM) (*31*). This shows that the effects of $HgCl_2$ on the removal of the 33 kDa protein from PS II are related to its concentration as well as to the pH of the incubation buffer in the absence of Cl^-.

Effects of $HgCl_2$ at Different Concentrations on the Release of the 33 kDa Protein from PS II in Various pH Buffers in the Presence of 15 mM Cl^-. Chloride is an essential inorganic cofactor for the normal function of the OEC (*37*). However, the effects of Cl^- on the depletion of the 33 kDa protein from PS II during the incubation with $HgCl_2$ are poorly understood. In our experiment, we observed a 2-fold effect of additional Cl^- (15 mM) on the release of the 33 kDa protein from PS II membranes during incubation with different concentrations of $HgCl_2$ in various pH buffers. Figure 3A shows that the release of the 33 kDa protein was partly suppressed by the addition of 15 mM Cl^- during the incubation with a lower concentration of $HgCl_2$ (0.25 μM) in various pH buffers (Figure 3B) with a maximal removal of 70% at pH 8.5 (Figure 3A, lane 6, and Figure 3B), consistent with a previous report (*31*). However, quantitative analysis showed that the 33 kDa protein was almost completely released (>97%) after incubation at a higher concentration of $HgCl_2$ (20 μM) with the addition of 15 mM Cl^- at pH 8.5 (Figure 4A, lane 6, and Figure 4B), in conflict with this report (*31*). This indicates that the optimal condition for depleting the 33 kDa protein from PS II varies with the concentration of both $HgCl_2$ and chloride, and the suppression effects of Cl^- are weakened with an increase in the concentration of $HgCl_2$.

Effects of $HgCl_2$ at Different Concentrations on the PS II Samples with the Addition of both 15 mM Cl^- and 3 mM Ca^{2+} at pH 8.5. Calcium is another essential inorganic cofactor for the normal function of the OEC (*1*, *2*). The effects of calcium on the release of the 33 kDa protein from PS II membranes during incubation with $HgCl_2$ have not been

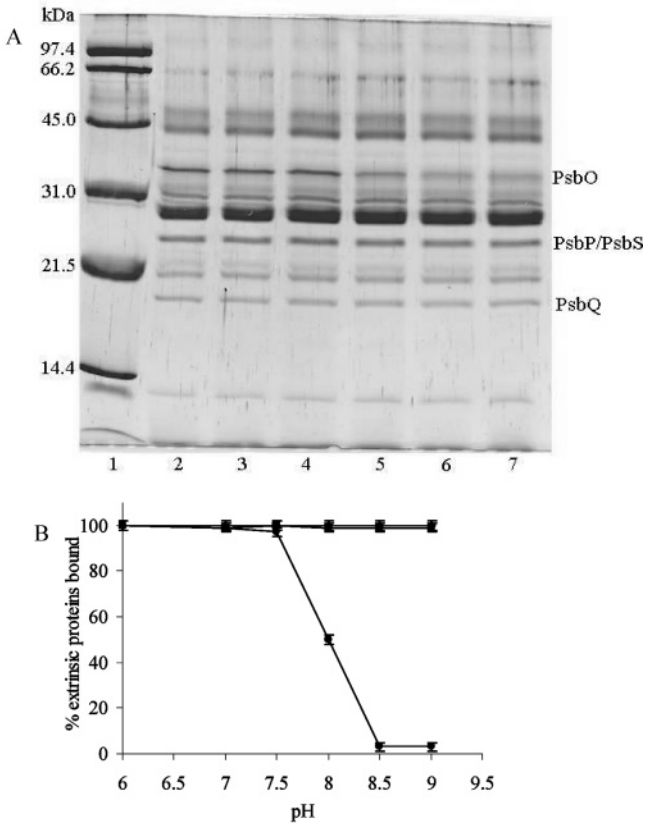

FIGURE 4: Effects of 20 μM $HgCl_2$ on the release of the extrinsic proteins from PS II membranes with addition of 15 mM Cl^- in buffer at different pHs (7.0−9.0). (A) SDS−urea−PAGE: lane 1, protein standards; lane 2, control (BBY); lane 3, BBY incubated at pH 7.0; lane 4, BBY at pH 7.5; lane 5, BBY at pH 8.0; lane 6, BBY at pH 8.5; and lane 7, BBY at pH 9.0. (B) Quantitative analysis of effects of $HgCl_2$ on individual proteins: (◆) PsbQ, (■) PsbP, and (●) PsbO.

reported. In this experiment, BBY samples were incubated with $HgCl_2$ in the presence of 15 mM Cl^- and 3 mM Ca^{2+} at pH 8.5. Surprisingly, nearly all of the three extrinsic proteins were released from PS II samples during a 5 min incubation period regardless of whether BBYs were incubated in 0.25 μM (Figure 5A, panel a, and Figure 5B, panel a) or 20 μM $HgCl_2$ (Figure 5A, panel b, and Figure 5B, panel b). When BBY samples were incubated with 50 μM $HgCl_2$ for 5 min in the same buffer, however, only the 33 kDa protein was released from PS II (Figure 5A, panel c, and Figure 5B, panel c). Again, these data demonstrate that the effects of $HgCl_2$ on PS II vary with experimental conditions.

DISCUSSION

Several three-dimensional X-ray crystal structures of cyanobacterial PS II core complexes have revealed the mutual orientations of membrane-spanning α-helices, and the ligation of various cofactors (*3*−*6*). Neither the PsbP protein nor the PsbQ protein is present in the current crystallographic models of the PS II structure, and it is even unknown if they contribute to PS II water oxidation activity in cyanobacteria (*16*). Thus, the role and location of the three extrinsic proteins on the lumenal side of PS II in higher plants remain to be resolved. Electron cryomicroscopy is a complementary technique suitable for obtaining an intermediate-resolution structure when ordered three-dimensional crystals are not available (*7*−*9*). In particular, the information about the structure and organization of the three extrinsic proteins of higher plants has been derived from single-particle analysis of the PS II supercomplex isolated from spinach (*38*). The structural model derived for three extrinsic proteins on the lumenal side of PS II from one of the recent EM studies is consistent with the earlier studies which showed that the binding of the PsbP protein to PS II requires the presence of the PsbO protein and that the PsbP protein is required for the binding of the PsbQ protein (*22*, *38*). Interestingly, our data presented above demonstrate that the 33 kDa extrinsic protein can be removed by $HgCl_2$ at a micromolar concentration (0.25 μM) as well as by higher concentrations (20 and 50 μM), with the 17 and 23 kDa extrinsic proteins and the intrinsic proteins of PS II left intact. The results given above also demonstrate that the optimal condition for the release of the 33 kDa protein from PS II varies with the treatment conditions, which seems to partly explain why the effects of $HgCl_2$ on PS II appear to be so diverse (*31*, *39*−*49*).

In principle, the binding of the 33 kDa protein to the intrinsic PS II proteins could be through either ionic or hydrophobic interactions, as this protein can be removed by either 1 M $CaCl_2$ or 2.6 M urea (*22*, *50*). As is known, Hg is a potential environmental contaminant. Unfortunately, the mechanism by which plants respond to toxic Hg is still poorly understood except for its general high affinity of sulfhydryl groups and/or disulfide bonds. According to the analysis of the complete amino acid sequence of the 33 kDa protein of spinach, it contains 64 charged amino acid residues (*22*). It has been suggested that the negative charges of carboxylic residues and the C-terminus of the 33 kDa protein do not participate in its binding to PS II, whereas the positive charges of lysyl and arginyl residues are important for its binding (*51*). The 23 and 17 kDa proteins are not removed by $HgCl_2$ during incubation in the absence of chloride and calcium (Figure 1), though both of them also contain many charged amino acid residues (*22*, *52*), suggesting that the release of the 33 kDa protein from PS II by $HgCl_2$ may have nothing to do with the interaction between charged amino acid residues of the 33 kDa protein and $HgCl_2$.

The 33 kDa protein is quite acidic (pI = 5.2); the 23 kDa protein is only slightly acidic (pI = 6.5), and the 17 kDa protein is alkaline (pI = 9.2) (*11*). After incubation in high-pH buffer, the "acidic" 33 kDa protein should therefore have more negative charges on the surface, which could change the whole distribution of electric charges and the conformation of the 33 kDa protein. Our experiment shows that high pH favors the release of the 33 kDa protein, although this protein cannot be released even if incubated at pH 9.8 without $HgCl_2$ (not shown).

It is worth noting that the 17 kDa protein has no cysteine residues, the mature 23 kDa protein only one, and the 33 kDa protein two conserved cysteines (C28 and C51) that form an intrachain disulfide linkage (*52*). The 33 kDa protein is quite flexible, and the secondary structure of the 33 kDa protein undergoes conformational changes upon binding to PS II (*53*−*55*). It has been suggested that the disulfide bond of the 33 kDa protein is located in the interior of the molecule but may not be completely buried or is located in a part where local fluctuation occurs frequently (*56*). Moreover, the sequences around C28 and C51 are -Gln-Cys-Pro- and -Phe-Cys-Leu-, respectively (*52*). It is clear that prolines can cause

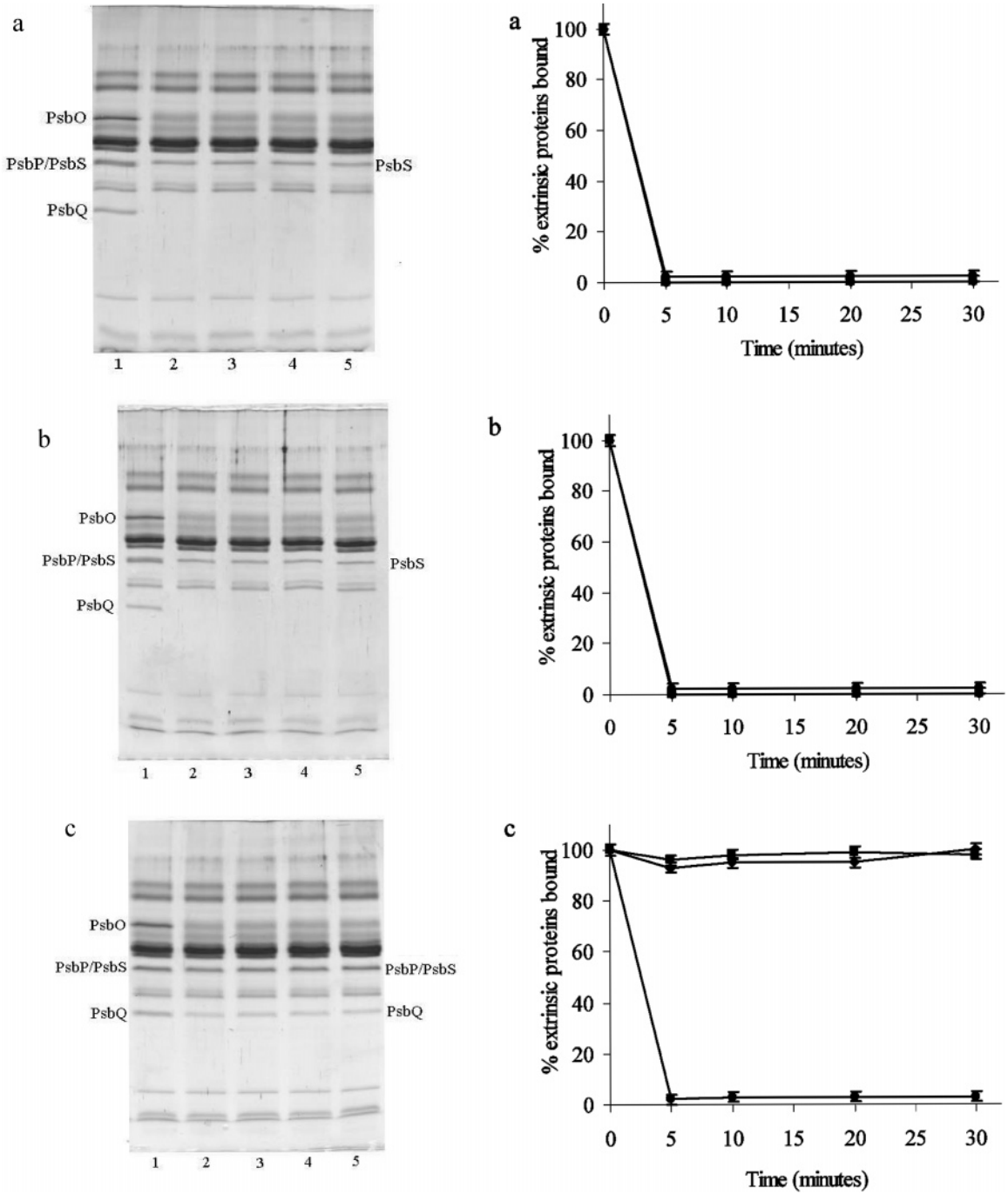

FIGURE 5: (A) SDS−urea−PAGE analysis of the effects of varied concentrations $HgCl_2$ on the release of the extrinsic proteins from PS II membranes with addition of 15 mM Cl^- and 3 mM Ca^{2+} at pH 8.5. The concentration of $HgCl_2$ was 0.25 (panel a), 20 (panel b), or 50 μM (panel c): lane 1, control (BBY); lane 2, BBY incubated at pH 8.5 for 5 min; lane 3, BBY incubated for 10 min; lane 4, BBY incubated for 20 min; and lane 5, BBY incubated for 30 min. (B) Quantitative analysis of effects of $HgCl_2$ on individual proteins. The concentration of $HgCl_2$ was 0.25 (panel a), 20 (panel b), or 50 μM (panel c): (◆) PsbQ, (■) PsbP, and (●) PsbO.

special bends in the protein backbone and mark the edges of specific domains. In view of the large radius of Hg and the possible location of the disulfide bond in the 33 kDa protein (*55*), once the disulfide linkage of the 33 kDa protein is attacked and replaced with Hg to form an -S-Hg-S- motif at high pH, the adjacent special bend structure made by proline in the protein backbone or the hydrogen bond within the β-turn could be changed and damaged, which could further induce the conformational change of the 33 kDa protein, finally resulting in the release of the 33 kDa protein from PS II as its conformation is no longer adapted for binding to PS II.

The effects of $HgCl_2$ on the 33 kDa protein are very complicated, and the accelerated release of three extrinsic proteins from PS II with the addition of an appropriate amount of Cl^- and Ca^{2+} cannot be explained immediately. It could be related to the change in the dielectric constant after the addition of Cl^- and Ca^{2+} or to the synergism effects among different ions in the buffer, which needs further study. Additionally, the gel electrophoresis approach used here has limitations. For example, the observed high level of retention of the 33 kDa protein at higher $HgCl_2$ concentrations could be explained by aggregation effects, with the Hg-induced aggregate remaining bound to the PS II membranes during

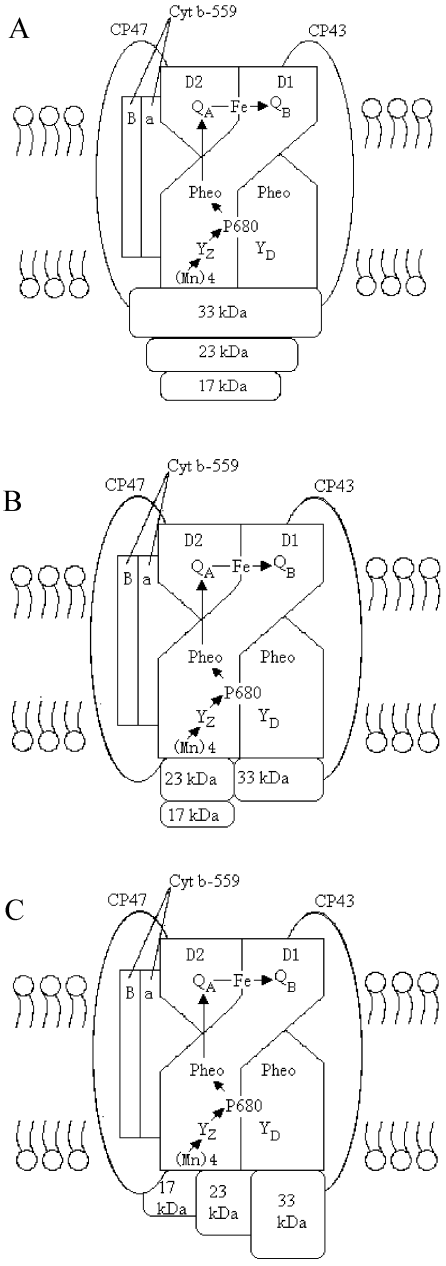

FIGURE 6: (A) Regulatory cap model of higher-plant PS II showing the association of the three extrinsic proteins at the lumenal side of the thylakoid membrane. (B) Model of higher-plant PS II showing the 23 kDa extrinsic protein directly binds to PS II. (C) Model of higher-plant PS II showing each of the three extrinsic proteins possesses its own binding sites on some intrinsic components of PS II.

the wash steps. More work is definitely needed to address the complicated observations described here.

Of the three extrinsic proteins, the 33 kDa protein appears to play a central role in the stabilization of the manganese cluster, and it is essential for efficient and stable oxygen evolution (*10*−*12*). As described in the introductory section, relatively little is known about the binding properties of the three extrinsic proteins or the architecture within plant PS II. At present, there are mainly three models showing how the three extrinsic proteins could be attached to the lumenal site of the OEC. The first is the above-mentioned regulatory cap model (*17*, *22*−*24*, *38*, *57*−*59*) (Figure 6A). Second, biochemical and genetic studies have suggested that the 23 kDa protein, along with the 33 kDa protein, also binds directly to intrinsic proteins such as D1 and CP43 (*11*, *18*) (Figure 6B). The third model states that the three extrinsic proteins each possess different binding sites on some intrinsic components of PS II (*60*, *61*) (Figure 6C). Our results create some doubt about the regulatory cap model. Since the 33 kDa protein can be removed while the 23 and 17 kDa proteins remain bound to PS II, the binding sites of the 23 and/or 17 kDa protein appear to be directly located on the intrinsic subunits of PS II (Figure 6C). In green algae, this has been recently proven by release reconstitution experiments (*21*). Whether this motif is used in higher plants requires further study. For example, it is possible that the interaction between PS II intrinsic proteins and the 23 kDa protein creates a binding site for the 17 kDa protein (*11*, *18*) (Figure 6B).

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