Mass spectrometry-based cross-linking study shows that the Psb28 protein binds to cytochrome b_{559} in Photosystem II

Daniel A. Weisz^{a,b}, Haijun Liu^{a,1}, Hao Zhang^{b,2}, Sundarapandian Thangapandian^c, Emad Tajkhorshid^c, Michael L. Gross^{b,3}, and Himadri B. Pakrasi^{a,3}

^aDepartment of Biology, Washington University, St. Louis, MO 63130; ^bDepartment of Chemistry, Washington University, St. Louis, MO 63130; and ^cDepartment of Biochemistry, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

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Photosystem II (PSII), a large pigment protein complex, undergoes rapid turnover under natural conditions. During assembly of PSII, oxidative damage to vulnerable assembly intermediate complexes must be prevented. Psb28, the only cytoplasmic extrinsic protein in PSII, protects the RC47 assembly intermediate of PSII and assists its efficient conversion into functional PSII. Its role is particularly important under stress conditions when PSII damage occurs frequently. Psb28 is not found, however, in any PSII crystal structure, and its structural location has remained unknown. In this study, we used chemical cross-linking combined with mass spectrometry to capture the transient interaction of Psb28 with PSII. We detected three cross-links between Psb28 and the α - and β -subunits of cytochrome b_{559} , an essential component of the PSII reaction-center complex. These distance restraints enable us to position Psb28 on the cytosolic surface of PSII directly above cytochrome b_{559} , in close proximity to the Q_B site. Protein-protein docking results also support Psb28 binding in this region. Determination of the Psb28 binding site and other biochemical evidence allow us to propose a mechanism by which Psb28 exerts its protective effect on the RC47 intermediate. This study also shows that isotope-encoded cross-linking with the “mass tags” selection criteria allows confident identification of more cross-linked peptides in PSII than has been previously reported. This approach thus holds promise to identify other transient protein-protein interactions in membrane protein complexes.

cyanobacteria | photosynthesis | membrane protein | Photosystem II | chemical cross-linking

Photosystem II (PSII) is a multisubunit pigment-protein complex embedded in the thylakoid membranes of cyanobacteria, algae, and plants. PSII uses light energy to oxidize water to molecular oxygen, simultaneously reducing plastoquinone. Active PSII consists of ~20 protein subunits and multiple light-harvesting and redox-active cofactors (1, 2).

As a result of demanding electron-transfer chemistry, PSII undergoes frequent oxidative damage, necessitating a complex cycle of repair and reassembly (reviewed in ref. 3). The assembly occurs stepwise via multiple transient intermediate complexes that are difficult to study because of their low abundance, relatively short lifetimes, and heterogeneity. Crystal structures of the active complex from thermophilic cyanobacteria are available (1, 4, 5), but they do not capture the transient interactions of the various accessory proteins that regulate the life cycle by binding exclusively to intermediate subcomplexes. Nevertheless, significant progress has been made in characterizing these intermediates through complementary use of genetic modification, biochemical analysis, and mass spectrometry (MS) (3, 6–9). Although crystal structures of assembly intermediate complexes are not available, the binding site of one accessory protein, Psb27, on the luminal surface of PSII has been determined by chemical cross-linking and MS (10, 11). This knowledge complements functional studies of Psb27 and provides mechanistic insight into how Psb27 protects PSII assembly intermediates from acquiring premature oxygen

evolution capability (12–15). The lack of comparable binding-site information for other PSII accessory proteins limits our understanding of their functions.

The Psb28 accessory protein was first identified in a proteomic analysis of PSII from *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) (16), and is likely the unidentified 12-kDa protein observed earlier in a cyanobacterial PSII preparation (17). Psb28 is found across a wide range of cyanobacteria and has homologs in higher plants, but relatively little is known about its binding site and function (18). Dobáková et al. (19) found that Psb28 binds mainly to the monomeric CP43-less PSII assembly intermediate, referred to as “RC47,” a finding confirmed subsequently (20–22). Although deletion of the *psb28* gene had little physiological effect under typical growth conditions (19, 20, 22), its absence impaired PSII recovery after photodamage under high-light conditions, especially at high temperature (20), and under intermittent high-light/dark conditions (22), suggesting a critical role of this protein during increased PSII turnover. Psb28 is the only known extrinsic cyanobacterial PSII protein found on the cytoplasmic surface of PSII (6, 8, 9, 19), and it may interact with CP47, based on the comigration of these two proteins during native gel electrophoresis (19).

To identify the Psb28 binding site, we used an isotope-encoded chemical cross-linker and MS to capture covalently the protein-protein interactions in PSII (23–25). We used on-the-fly precursor-ion selection that takes advantage of the isotopic “fingerprint” of cross-linked peptides to facilitate identification (26).

Significance

Photosystem II (PSII) is a multisubunit membrane protein complex that catalyzes light-driven oxidation of water, supplying energy to nearly all life on Earth. Because of the challenging chemistry it performs, PSII undergoes frequent damage, prompting an intricate cycle of disassembly, repair, and reassembly. Psb28, a protein that binds transiently to the RC47 intermediate complex, protects PSII from light-induced damage. The location of Psb28 in PSII has not been determined. In this study, we used chemical cross-linking, mass spectrometry, and protein docking to demonstrate that Psb28 binds to cytochrome b_{559} . Our results allow us to propose a mechanism by which Psb28 exerts its protective effect on the vulnerable RC47 complex.

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¹Present address: Benson Hill Biosystems, St. Louis, MO 63132.

²Present address: Amgen, Inc., Cambridge, MA 02142.

³To whom correspondence may be addressed. Email: pakrasi@wustl.edu or mgross@wustl.edu.

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Remarkably, Psb28 cross-linked to PsbE and PsbF, the α - and β -subunits, respectively, of cytochrome b_{559} (Cyt b_{559}), two of the five polypeptides that comprise the core “reaction center” of PSII (27, 28). Using these cross-links as distance restraints, and supported by protein–protein docking results, we generated a Psb28 binding model that shows Psb28 binding on the cytoplasmic surface of PSII directly above the heme of Cyt b_{559} and in close proximity to the Q_B site. On the basis of our results, we propose a mechanism for the protective effect of Psb28 on the RC47 assembly intermediate of PSII.

Results

Quantification of Psb28 Levels in PSII from Several Mutant Strains. Because Psb28 binds to a PSII assembly intermediate, we expected it to be present at a substoichiometric level in our purified PSII complexes, which would render cross-link identification challenging. To increase the chances of detecting Psb28 cross-linked peptides, we screened PSII complexes for elevated Psb28 content from the His47 strain (wild-type with a polyhistidine tag on the CP47 subunit) and several mutant strains that accumulate assembly intermediates (Fig. 1A). Only around one in seven PSII complexes purified from the His47 strain (wild-type with a polyhistidine tag on the CP47 subunit) contains Psb28 (Fig. 1B and C). This result is reasonable, given that Psb28 is found mainly in the RC47 intermediate, and RC47 accounts for around 10% of PSII complexes present in the wild-type cell (19, 21). PSII from the $\Delta psbO$ -His47 strain contains the most Psb28 and, therefore, was used in the subsequent experiments. The deletion of *psbO* prevents PSII dimerization (29), allowing the monomeric PSII fraction (ΔO -M) to be isolated by glycerol-gradient ultracentrifugation (*Materials and Methods*) and used in cross-linking. Pso binds on the luminal surface of PSII and stabilizes the manganese cluster, the site of water oxidation (2). The $\Delta psbO$ strain

still assembles active water-splitting PSII with the typical core components (Fig. 1D) and grows photoautotrophically, but the complexes are more susceptible to photodamage and require more frequent repair than wild-type cells (30). The elevated level of Psb28 implies an increased steady-state concentration of the RC47 complex (19–22), which is expected, as RC47 is a key intermediate in the PSII repair cycle (8).

We quantified the Psb28 content in ΔO -M, as well as the His47 PSII monomer and dimer, by calibrating with known quantities of recombinantly expressed and purified Psb28-His (Fig. 1B and C). Approximately one in three ΔO -M PSII complexes contained Psb28 compared with approximately one in seven His47-M and His47-D PSII complexes. Other studies found Psb28 mainly in the monomeric RC47 complex (19–22), whereas we detected approximately equal levels in monomeric and dimeric His47-PSII. A dimeric RC47 complex containing Psb28 was detected, however, in two previous studies (20, 21). This complex may form during disassembly, similar to the dimeric Psb27-containing species (31), implying a role for Psb28 during disassembly, as previously proposed (20, 32). The increased level of dimeric Psb28-containing complexes in our His47-PSII preparation compared with that in previous studies might have arisen from differences in the purification conditions.

Cross-Link Data Analysis. We subjected the ΔO -M sample to cross-linking with a 1:1 mixture of BS³ cross-linker labeled with 12 deuteriums and its unlabeled analog. This cross-linker can modify primary amines, and thus targets lysine residues and protein N-termini (33), and it yields an isotopic doublet in the mass spectra that facilitates identification of cross links. We evaluated the yield over a relative molar concentration of cross-linker:PSII between 50 and 300. We analyzed the cross-linked products by SDS/PAGE and immunoblotting, using anti-Psb28 antibodies (*SI Appendix, Fig. S1*), and found multiple Psb28-containing bands after cross-linking, but not in the nonlinked control sample, indicating successful cross-linking. Several faint bands do exist in the ~50–80-kDa region of the nonlinked sample, and increase in intensity after cross-linking. These bands apparently represent strong noncovalent interactions involving Psb28 that are retained during electrophoresis and that were captured and stabilized by cross-linking.

We chose the sample that was cross-linked with 300-molar excess linker:PSII for analysis by MS because it showed the highest number and intensity of Psb28-containing cross-linked bands. We identified 18 cross-links and 22 monolinked sites (for which only one side of the cross-linker reacted with a protein; *SI Appendix, Tables S1 and S2*). The isotopic doublet “fingerprint” of this cross-linker (26) enabled highly confident identification of cross-links, removing much of the ambiguity that often hinders cross-link analysis (24) (described further in *SI Appendix*).

Using the PSII crystal structure (4), we could measure the C α –C α distance between the linked residues for 13 of the linked peptides (*SI Appendix, Table S2*). Eleven of the 13 distances are within 30 Å, a typical benchmark (34–39), and the full distance distribution (*SI Appendix, Fig. S2*) matches the typical distributions found for this cross-linker (25, 37, 39–41). This critical control (described further in *SI Appendix*) demonstrates that the native structure of PSII was maintained in our experiments, and also verifies the accuracy of our data analysis.

Identification of Psb28 Cross-Links. We identified three inter-protein cross-links containing Psb28: Psb28-K8–PsbE-S2 (Fig. 2), Psb28-K8–PsbF-A2 (Fig. 3), and Psb28-A2–PsbF-A2 (*SI Appendix, Fig. S3*). PsbE-S2, PsbF-A2, and Psb28-A2 are susceptible to modification by BS³ because they are the N-terminal residues of the mature form of these proteins after in vivo cleavage of the N-terminal methionine residues (confirmed by intact-mass measurement; *SI Appendix, Fig. S4*). We identified a cross-link between PsbE-S2 and PsbF-A2 as well, indicative of the proximity of

Fig. 1. Screening of mutant strains for elevated Psb28 content, characterization of the $\Delta psbO$ PSII monomer, and quantification of Psb28 content in $\Delta psbO$ PSII. (A) Comparison of Psb28 content in PSII complexes from the His47 and several different mutant strains. (B) Immunoblot comparing Psb28 content in the $\Delta psbO$ PSII monomer, the His47 PSII monomer and dimer, and a dilution series of Psb28-His purified from *Escherichia coli*, respectively. (C) Calibration curve for quantification of Psb28 content in $\Delta psbO$ PSII and His47 PSII, using the *E. coli*-purified Psb28 as a standard. (D) Protein gel comparing PSII subunit composition of the His47 and $\Delta psbO$ PSII monomers.

Fig. 2. Mass spectrometric data showing a cross-link between Psb28-K8 and PseE-S2. The MS (precursor-ion) spectra for each cross-link is shown at the top of each figure; it displays the isotopic “fingerprint” of a real cross-link: a doublet of peaks of equal intensity, shifted from each other by 12 Da. The two lower spectra in each figure are the MS/MS (product-ion) spectra of the light and heavy forms of the cross-linked peptide, generated after fragmentation of that peptide by higher-energy collisional dissociation. The MS/MS spectra also display the “fingerprint” of a real cross-link: they are essentially identical, despite originating from different precursor ions, and cross-linker-containing peaks are shifted by +12 Da in the spectrum of the heavy form. The black notches in between residues in the peptide sequence indicate fragment ions that were observed in the MS/MS spectra of both the light and heavy forms (also labeled on the spectra themselves). For further details, see *SI Appendix*.

these two residues and their ability to each form a cross-link with Psb28-K8. The mass spectra for these cross-links, which were not detected in a control, nonlinked sample, display the doublet feature characteristic of the isotopic mixture of cross-linker used. Nearly all major fragments in the product-ion spectra match predicted peptide fragments, resulting in highly confident identification of these cross-links (Figs. 2 and 3, *SI Appendix*, Fig. S3, and *Discussion*). Furthermore, we also identified the monolinked peptide corresponding to each of these cross-linked residues (*SI Appendix*, Table S1), a valuable cross-check because for a given modifiable residue, a monolink is expected to form more readily than a cross-link. We selected the $\Delta psbO$ -His47 strain for our cross-linking-MS experiments because it accumulates more Psb28-containing PSII complexes than the other strains we screened.

However, after obtaining our results, we were able to detect the Psb28 cross-links in the MS-1 spectra of cross-linked His47 samples as well (*SI Appendix, Figs. S5–S7*), ruling out the possibility that the $\Delta psbO$ mutation led to a nonnatural interaction between Psb28 and PsbE and PsbF.

Psb28-PSII Docking. To examine possible binding modes of Psb28 to PSII by a method complementary to cross-linking, we performed protein docking with DOT 2.0 (for details, see *Materials and Methods*). The top 4,000 docked conformations clustered into four spatial groups. One of these clusters (“Cluster 1”), which contains 1,282 conformations, is on the cytosolic surface of PSII. Because Psb28 binds to the cytosolic surface of PSII (19), we restricted our analysis to the Cluster 1 conformations. Further examination of the top conformations showed that they are all localized near the surface above the *N*-termini of PsbE and PsbF (*SI Appendix*, Fig. S8 and Table S3), supporting the cross-linking results by showing that this area is favorable for Psb28 binding.

Discussion

Psb28 Dissociates from PSII Before Psb27 Attachment. In contrast to the other screened PSII mutants, His27-PSII contained almost no Psb28 (Fig. 14). The His27 strain is the only one in which the

Fig. 3. Mass spectrometric data showing a cross-link between Psb28-K8 and PsbF-A2. See Fig. 2 caption for interpretation of spectra.

The Psb28 Binding Site. We found cross-links between Psb28-K8 and the N-termini of PsbE and PsbF and between Psb28-A2 and the N terminus of PsbF. Protein–protein docking between Psb28 and the RC47 subunits of PSII provided independent support that the cytosolic surface in the region above PsbE and PsbF is a favorable site for Psb28 binding. The first 11 residues of PsbF are not resolved in the PSII crystal structure, and two of the three Psb28 cross-links are located at the PsbF N terminus. Therefore, we used the earliest resolved residue, PsbF-Y13, as a proxy for PsbF-A2 in positioning Psb28 above the cytosolic surface of PsbE and PsbF in a manner consistent with the cross-links (Fig. 4). We

The energetics of the top docked Psb28 conformations (*SI Appendix, Table S3*) show that electrostatic interactions dominate hydrophobic interactions as the major force stabilizing the binding of Psb28 to RC47. This is consistent with the fact that Psb28 can be removed from PSII membranes by washing with 1 M CaCl_2 , 0.1 M Na_2CO_3 , or 0.1 M NaOH (19). Previous work identified two Psb28 surface cavities that are mostly formed by highly conserved residues, and it was speculated that these cavities may be important for Psb28 binding (42). In our model, one of these cavities is present at the interface of Psb28 and RC47 (Cavity 4 in ref. 42).

The cross-linking study presented here shows that the Psb28 binding site is on the cytosolic surface above the Cyt b_{559} subunits PsbE and PsbF. Our model positions Psb28 ~ 9 – 12 Å directly above the heme of Cyt b_{559} (16 – 20 Å from the heme Fe) and 23 – 27 Å from the redox-active aromatic ring of the quinone Q_B (Fig. 4). With this information, we propose the following mechanism by which Psb28 might exert its protective effect on RC47.

Experimental evidence suggests RC47 is protected by an alteration in its acceptor-side electron-transfer dynamics; specifically, impaired electron transfer from Q_A to Q_B . This impaired

transfer is part of an established mechanism that protects partially assembled PSII complexes from damage by increasing the Q_A redox potential, minimizing a harmful redox back-reaction (43–47). Boehm et al. (21) purified the RC47 complex from a strain of *Synechocystis* 6803 that lacks the CP43 protein and, therefore, assembles PSII complexes only up to the RC47 stage. They found that the RC47 complex contains an intact electron-transfer chain from the primary donor P_{680} through reduction of Q_A , but that the final transfer step from Q_A to Q_B is blocked. An earlier study on intact cells from the CP43-deletion strain of *Synechocystis* 6803 also demonstrates poor electron transfer from Q_A to Q_B (48). Sharply decreased binding of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to the Q_B site suggests a structural alteration around the Q_B site (48, 49), and this could explain the impaired electron transfer. By binding in close proximity to the Q_B site, Psb28 may contribute to the structural perturbations in this area that protect RC47 by blocking electron transfer from Q_A to Q_B . Studying the electron transfer properties of RC47 purified from a strain containing and a strain lacking Psb28 could probe this possibility further.

A definitive recent study confirmed the importance of the protective Q_A redox switch under conditions when a functional Mn cluster is absent (47), such as occurs in the RC47 complex and other subcomplexes during PSII assembly. Because absence of PsbO destabilizes the Mn cluster, it seems reasonable that a greater proportion of PSII centers would require the protective effect of Psb28, explaining the increased levels of Psb28 in $\Delta psbO$ PSII. Cytosolic-side responses to a lumen-side stimulus, such as removal of an extrinsic protein, have long been known to occur in PSII. However, the underlying protein conformational changes that promote binding, and then release, of Psb28 are presently unknown. Future footprinting studies may be able to probe these changes, similar to the case of Psb27 (12).

We mention two additional functional considerations that arise from the structural location of Psb28 as possibilities requiring further investigation. PSII has evolved a complex toolkit of photoprotective methods, and it is quite possible that Psb28 is effective in multiple ways. First, the close proximity of Psb28 to Cyt b_{559} and its heme raises the question whether Psb28 binding affects its redox potential. Low-, intermediate-, and high-potential forms of Cyt b_{559} have been detected, each with different presumed photoprotective functions, but a detailed understanding of these forms and what causes their interconversion is missing (43, 50). With its lack of a functional Mn cluster, the RC47 complex is a good candidate for photoprotection via a Cyt b_{559} -mediated secondary electron-transfer pathway. Studying the redox properties of Cyt b_{559} after purification of the RC47 complex (21) from a strain containing and another lacking Psb28 may determine whether the interaction of Psb28 with Cyt b_{559} affects its function. Second, we note that Psb28 is the only known extrinsic cyanobacterial protein on the cytoplasmic surface of PSII (6, 8, 9, 19). Phycobilisomes, the large, light-harvesting antenna complexes that bind to this surface, supply mature PSII with excitation energy (13, 51). PSII assembly intermediates such as RC47, however, do not contain an active water-splitting complex, and accepting excitation energy in this state leads to photodamage (43). Our results position Psb28 just beside the cavity on PSII in which the phycobilisome apparently inserts (52) (*SI Appendix, Fig. S9*). Psb28 binding may block phycobilisome attachment, minimizing delivery of harmful excitation energy to RC47. Indeed, fluorescence emission spectra from the CP43-deletion strain of *Synechocystis* 6803 (which accumulates RC47) do not indicate coupled energy transfer from the phycobilisomes to assembled PSII subunits (53).

Combining evidence from previous research with the current results positions us to refine a model summarizing the steps in PSII assembly (Fig. 5). Psb28 binding occurs on formation of the RC47 complex, and Psb28 is positioned in Fig. 5 in a location consistent with our cross-linking results. Given the significant interface between Psb28 and CP47, PsbX, and PsbY, Psb28 would unlikely bind stably before the RC47 stage. Indeed, Psb28 has not

been detected in the preceding assembly intermediate, the RC complex (54, 55). Psb28 dissociation occurs in the next step, before binding of Psb27, as indicated by the lack of Psb28 in His27-PSII (Fig. 14).

Conclusion

We determined the structural location of Psb28, the only cytoplasmic extrinsic protein in PSII, in close proximity to the N-terminal domain of the Cyt b_{559} protein. With the structural information we have gained, we propose a mechanism by which Psb28 exerts its protective effect on the PSII subcomplex to which it binds, helping ensure optimal conversion of this subcomplex into functional PSII. This study also shows that the combination of isotope-encoded cross-linking with the “mass tags” selection criteria (26) can identify low-abundance cross-linked peptides originating from a large membrane protein complex. Such cross-links provide useful distance restraints for positioning subunits not present in crystal structures of protein complexes. This approach thus holds promise for identifying other transient protein–protein interactions, a burgeoning area of interest in biology.

Materials and Methods

PSII Purification. Histidine-tagged PSII complexes were purified as described previously (16), with minor modifications. After FPLC purification of $\Delta psbO$ -His47-PSII and His47-PSII, these complexes were purified further by glycerol gradient ultracentrifugation ($180,000 \times g$, 18 h, 4 °C), using a 5–30% (wt/vol) linear glycerol gradient. For further details, see *SI Appendix*.

Cross-Linking and Proteolytic Digestion. PSII samples were cross-linked using a 1:1 mixture of unlabeled BS^3 and BS^3 labeled with 12 deuteriums (Creative Molecules) for 50 min in the dark at room temperature, at a cross-linker:PSII molar ratio of 50:1, 100:1, and 300:1, with PSII sample containing 1 or 2 μg Chl *a*. After quenching, samples were precipitated, resuspended, and digested with lysyl endopeptidase (LysC), then trypsin. For details, see *SI Appendix*.

LC-MS/MS. Aliquots (5 μL) of the peptide samples were analyzed on a Q-Exactive Plus mass spectrometer (Thermo Fisher), and data in *SI Appendix, Fig. S3* were acquired on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher.) Cross-linked samples were analyzed with and without the “mass tags” feature enabled (26). For details, see *SI Appendix*.

Cross-Link Data Analysis. Protein identification was performed using PEAKS (ver. 7.0, Bioinformatics Solutions, Inc.). Cross-link analysis was performed using ICC-CLASS (26, 56) and Protein Prospector (57), with careful manual verification of hits. For search details, see *SI Appendix*.

Intact Protein LC-MS. His47-PSII samples were purified as described earlier, and a sample containing 1.2 μg Chl *a* was precipitated using the 2D Clean-up Kit (GE Healthcare), resuspended in 250 μL 90% formic acid, and spun down to remove insoluble material. The supernatant was filtered, and a portion containing $\sim 1.6 \mu g$ protein was injected immediately onto a PLRP-S column (Agilent, 2.1 $\times$ 150 mm, 300-Å pore size) controlled by an Agilent 1200 HPLC. Buffer A, Buffer B, and the LC gradient were described previously (58). Eluted proteins were analyzed online by a Maxis quadrupole-time-of-flight 4G mass spectrometer (Bruker Daltonics).

Protein–Protein Docking. We used the DOT 2.0 docking program (59) to predict the binding interface of PSII [Protein Data Bank (PDB) ID 4PJ0] (4) and Psb28 (PDB ID 2KVO) (42) with the 3D structures obtained from the PDB. Because Psb28 is known to bind to the RC47 subcomplex (19–22), the subunits that are not components of this assembly intermediate (CP43, PsbJ, PsbK, PsbO, PsbU, PsbV, Psb30, and PsbZ) (21) were removed from the structure before docking. For further details, see *SI Appendix*.

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