



The Use of Advanced Mass Spectrometry to Dissect the Life-Cycle of Photosystem II

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Photosystem II (PSII) is a photosynthetic membrane-protein complex that undergoes an intricate, tightly regulated cycle of assembly, damage, and repair. The available crystal structures of cyanobacterial PSII are an essential foundation for understanding PSII function, but nonetheless provide a snapshot only of the active complex. To study aspects of the entire PSII life-cycle, mass spectrometry (MS) has emerged as a powerful tool that can be used in conjunction with biochemical techniques. In this article, we present the MS-based approaches that are used to study PSII composition, dynamics, and structure, and review the information about the PSII life-cycle that has been gained by these methods. This information includes the composition of PSII subcomplexes, discovery of accessory PSII proteins, identification of post-translational modifications and quantification of their changes under various conditions, determination of the binding site of proteins not observed in PSII crystal structures, conformational changes that underlie PSII functions, and identification of water and oxygen channels within PSII. We conclude with an outlook for the opportunity of future MS contributions to PSII research.

Keywords: Photosystem II, Photosystem II life-cycle, mass spectrometry, post-translational modification, chemical cross-linking, protein footprinting

OPEN ACCESS

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Specialty section:

This article was submitted to
Plant Cell Biology,
a section of the journal
Frontiers in Plant Science

Received: 05 February 2016

Accepted: 22 April 2016

Published: 10 May 2016

Citation:

Weisz DA, Gross ML and Pakrasi HB
(2016) The Use of Advanced Mass
Spectrometry to Dissect the
Life-Cycle of Photosystem II.
Front. Plant Sci. 7:617.
doi: 10.3389/fpls.2016.00617

INTRODUCTION

Since the late 1990s, mass spectrometry (MS) has become a central tool for the study of proteins and their role in biology. The advent of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) permits the ionization of peptides and proteins and their introduction into the gas phase, enabling their analysis by MS. The typical “bottom-up” workflow that emerged in the wake of these breakthroughs involves: (1) enzymatic digestion (often by trypsin) of a protein to produce peptides of small enough size (typically 1–3 kDa) to be ionized

Abbreviations: *A.thaliana*, *Arabidopsis thaliana*; BS³, (bis(sulfosuccinimidyl)suberate); *C. reinhardtii*, *Chlamydomonas reinhardtii*; DSP, dithiobis(succinimidyl propionate); DTSSP, 3,3'-dithiobis(sulfosuccinimidyl propionate); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ESI, electrospray ionization; FAB, fast atom bombardment; FTICR, Fourier transform ion cyclotron resonance; GEE, glycine ethyl ester; LC, liquid chromatography; LHCII, Light-harvesting complex II; LMM, low-molecular-mass; LTQ, linear ion trap quadrupole; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; *N. tabacum*, *Nicotiana tabacum*; NHS, N-hydroxysuccinimide; OCP, orange carotenoid protein; PBS, phycobilisome; PSII, Photosystem II; PC, plastocyanin; PTM, post-translational modification; QqQ, triple-quadrupole; SCP, small CAB-like protein; *Synechococcus* 7002, *Synechococcus* sp. PCC 7002; *Synechocystis* 6803, *Synechocystis* sp. PCC 6803; *T. elongatus*, *Thermosynechococcus elongatus*; TOF, time-of-flight; WOC, water-oxidizing complex.

and fragmented efficiently in a mass spectrometer; (2) liquid chromatographic (LC) separation of the peptides; and (3) online (or offline) injection of the separated peptides into a mass spectrometer. The “top-down” approach is an attractive alternative that eliminates the protein digestion step, but the subsequent steps are generally more difficult for intact proteins than peptides, and this approach is currently best-suited for small, soluble proteins. After injection of the peptides, a typical tandem MS analysis consists of: (1) ionization of the peptide sample by ESI or MALDI and introduction into the gas phase; (2) measurement of the mass-to-charge (m/z) ratio of the intact peptide (also referred to as “MS¹” analysis); and (3) fragmentation of the precursor ion and measurement of its “product-ion” spectrum (“MS/MS” or “MS²” analysis), which provides information about the peptide’s amino acid sequence. When genomic information is available to predict the sequence of all proteins in the organism, computer analysis of the peptide masses and product-ion spectra can determine the highest-scoring match for each peptide from the protein database. This highest-scoring match is taken as the identity of the peptide, assuming data quality meets certain statistical criteria. A given protein is then determined to have been present in the sample if the quality and number of its peptide hits meet an additional set of statistical criteria. The ability to identify many proteins in a sample at once by MS has become the cornerstone of the field of proteomics.

Protein identification is only the most basic application of MS-based proteomics, and it has traditionally been described as the first “pillar” of the field. The second pillar is characterization of the many proteoforms that exist for each protein, arising, e.g., from splice variants and post-translational modifications (PTMs). These two pillars address questions about the *composition* of a protein sample. The third pillar is quantification—either absolute or relative—of proteins using isotopic labeling or label-free approaches. This pillar is typically used to address questions about the *dynamics* of a system—how composition of proteins or proteoforms changes over time, space, or under different environmental conditions or perturbations. A proposed fourth pillar focuses on the emerging area of structural proteomics that uses MS-based techniques to address questions about the three-dimensional *structure* of proteins and protein complexes in a cell.

These four pillars of proteomics have each become indispensable tools for gleaning information about photosynthesis (Battchikova et al., 2015; Bricker et al., 2015; Heinz et al., 2016) and, in particular for this review, the life-cycle of PSII. A search for publications containing both “Photosystem II” and “mass spectrometry” in the article title, abstract, and/or keywords was performed on the Scopus database. The results, displayed in **Figure 1**, show that prior to the advent of ESI and MALDI in the late 1980s, publications were nearly zero per year. Starting in the early 1990s and continuing through 2015, publications have risen steadily, with around 20–30 publications per year in the last several years. The rise can be attributed to method and instrument development, and to increasing accessibility of MS instrumentation to biology researchers. An overview of how MS-based tools are typically applied to PSII

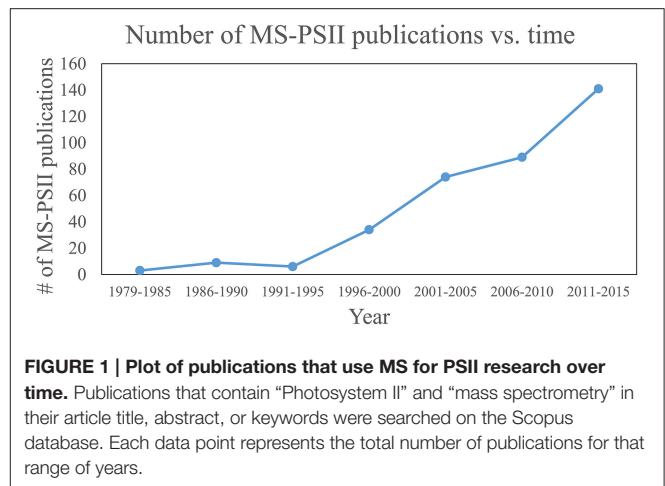


FIGURE 1 | Plot of publications that use MS for PSII research over time. Publications that contain “Photosystem II” and “mass spectrometry” in their article title, abstract, or keywords were searched on the Scopus database. Each data point represents the total number of publications for that range of years.

life-cycle research is given in **Table 1**. This review focuses on MS of proteins. However, it should be noted that another widely used application of MS in PSII research is the analysis of the isotopic composition of evolved oxygen by membrane-inlet mass spectrometry. This technique has yielded significant insight into the mechanistic aspects of water oxidation by PSII (reviewed in Shevela and Messinger, 2013).

In the sections that follow, we consider questions of PSII composition, dynamics, and structure separately. For each area, a brief overview of the relevant MS-based tools is given, followed by examples of several PSII life-cycle research areas that have benefitted from these techniques. In the final section, the outlook for future contributions of MS techniques to PSII life-cycle research is discussed.

COMPOSITION OF PSII COMPLEXES

MS-Based Methods to Study the Composition of PSII Complexes PSII Subunits with Soluble Domains

The bottom-up MS workflow is highly effective at identifying soluble proteins or proteins with soluble domains. It is, therefore, the main MS strategy that has been used to detect the core PSII proteins D1, D2, CP43, and CP47, which are transmembrane proteins but have multiple soluble domains, the extrinsic (soluble) PSII proteins, or unknown PSII-bound proteins. Bottom-up MS analysis can be preceded by either in-gel or in-solution digestion of the protein, each with advantages. Gel electrophoresis serves as a one- or two-dimensional fractionation step, simplifying the mixture to be analyzed by MS. Using this approach to remove interferences can improve instrument sensitivity toward proteins in the band of interest. Native PAGE, either alone or followed by denaturing SDS-PAGE (2D-BN-PAGE), is a common choice for resolving multiple protein complexes in a thylakoid membrane or purified PSII preparation; unknown bands can be excised and analyzed by MS to identify components of specific complexes (Granvogl et al., 2008; Pagliano et al., 2014; Gao et al., 2015). However, targeted band excision can miss potentially important proteins that migrated at

TABLE 1 | Overview of the role of MS in PSII life-cycle research.

Kind of information	Information desired	MS-based technique
Composition	PSII subunits present in a complex	Bottom-up MS (intact or top-down MS for LMM subunits)
	Accessory proteins that associate with PSII	Bottom-up MS
	PTMs	Bottom-up MS
Dynamics	Protein and PTM changes between samples	Label-free or isotopic-label-based relative quantification
	PSII subunit lifetime	Rate of unlabeled protein disappearance after isotopic label exposure
	Relative position of subcomplexes in PSII life-cycle	Relative isotopic label incorporation after pulse
Structure	Binding site of proteins not found in PSII crystal structures	Cross-linking, footprinting
	Conformational changes	Footprinting, quantify changes in modification extent
	Water and oxygen channel detection	Footprinting

positions not selected for in-gel digestion. In theory, native PAGE can remove unbound proteins from complexes, simplifying MS analysis; however, disruption of certain relevant protein-protein interactions in complexes cannot ever be fully excluded. Alternatively, in-solution digestion allows a more comprehensive analysis of the protein components in a sample, but without the sample simplification or complex-specific resolution provided by prior SDS-PAGE or native PAGE.

MS instrumentation, as well as membrane-protein sample preparation (Whitelegge, 2013; Battchikova et al., 2015; Heinz et al., 2016) and bioinformatics capabilities, has improved over the last two decades to facilitate PSII life-cycle research (Table 2 summarizes the kinds of experiments that have been performed and the main MS instrumentation and features that enable them). Early mass spectrometers that were applied to PSII research, especially triple-quadrupole (QqQ) and MALDI-time-of-flight (MALDI-TOF) instruments, had relatively low sensitivity, resolving power, and mass accuracy (on the order of 100-several hundred ppm; Michel et al., 1988; Sharma et al., 1997a,b,c; Frankel et al., 1999). Scarcity of genomic sequence data combined with low instrument sensitivity, mass accuracy, and fragmentation efficiency meant that sample analysis was mainly restricted to highly purified PSII complexes or individual subunits, with poor capability for novel protein identification. The mid-2000s saw the appearance of higher-performing instruments, especially the hybrid quadrupole-TOF (Q-TOF) and increasing availability of genomic sequence data for commonly studied photosynthetic organisms. These enabled routine bottom-up identification of the main subunits of PSII complexes (those with soluble domains) from more complex starting mixtures and identification of novel PSII-associated proteins (Kashino et al., 2002; Heinemeyer et al., 2004; Komenda et al., 2005). The fragmentation efficiency of the Q-TOF, however, still limited sequence coverage of proteins. The development and distribution of Fourier transform instruments (ion cyclotron resonance and orbitraps) sometimes interfaced with ion traps provided improved fragmentation efficiency and enabled analysis of highly complex mixtures with higher sequence coverage than ever before. These instruments allow proteome-wide experiments, enable routine confident PTM site identification, and have opened the door for bottom-up MS experiments on photosynthetic systems not before feasible (see Table 2 and sections below).

The Low-Molecular-Mass (LMM) Subunits

Fully assembled PSII contains around 13 low-molecular-mass (LMM) proteins (<10 kDa) whose transmembrane domains account for around 40–85% of the sequence. Identification of these very hydrophobic proteins by bottom-up LC-MS/MS is challenging, with typically four or fewer LMM proteins detected (Granvogl et al., 2008; Haniewicz et al., 2013; Pagliano et al., 2014). Difficulties are associated with the proteins' hydrophobicity and lack of soluble domains, which lead to sample losses during preparation, poor tryptic digestion due to infrequent arginines and lysines, slow elution during chromatography, and poor ionization efficiency due to lack of abundant proton-accepting residues. Fractionation by gel electrophoresis carries the additional challenge of extracting the protein from the gel, made more difficult because tryptic digestion sites are infrequent (Granvogl et al., 2008).

To circumvent these difficulties, intact-mass measurement (no MS/MS fragmentation of the protein) and more recently top-down MS strategies have been employed, both of which avoid protein digestion and are able to identify nearly all the LMM subunits in a purified complex (summarized in Table 3). Intact-mass measurement of the LMM subunits was demonstrated by both ESI and MALDI methods, using QqQ and MALDI-TOF instruments (see references cited in Table 3). Both methods achieve roughly 50–200 ppm mass accuracy; especially without fragmentation data, this would typically not be enough for confident identification of an unknown protein. However, because there are only approximately 13 LMM subunits, predicted masses, which are available from genomic sequences in many organisms, are distinctive, and because the starting sample is typically a purified PSII complex, these intact-mass measurements are routinely accepted as confident identifications.

MS/MS fragmentation of intact LMM subunits, however, can be induced using both ESI and MALDI, although ESI has been more successful (see Table 3 and references cited therein). Whitelegge and co-workers (Thangaraj et al., 2010) identified 11 LMM proteins in purified PSII from *G. sulphuraria* with a linear ion trap quadrupole-Fourier transform ion cyclotron resonance (LTQ-FTICR) instrument after offline LC and confirmed several modifications. They employed both collisional-activated dissociation (CAD) and electron-capture dissociation (ECD) to fragment the proteins, but CAD gave better results for all

TABLE 2 | MS instruments and instrument features for PSII life-cycle research applications.

Biological application	Mass accuracy (MS ¹)	Mass accuracy (MS ²)	Sensitivity/Good sequence coverage	QqQ	TOF	Q-TOF	LTQ-Orbitrap	Q-Exactive ^a	LTQ-FT-ICR
ID proteins-purified PSII complex/simple mixture/gel band	Med	Low	Low	+	+	++	++	++	++
ID LMM subunits-purified PSII complex-intact/top-down	Med	Low	Low	+	+	++	++	++	++
ID proteins-membranes/complex mixture/unknown protein search	High	Med	Med	-	-	+	++	++	++
ID modifications-targeted search	High	Med	Med	+	+	+	++	++	++
ID modifications-non-targeted search (PTMs, footprinting)	High	Med	High	-	-	-	+	++	+
Quantification of proteins/modifications ^b -targeted search	High	Med	Med	+	+	+	++	++	++
Quantification of proteins/modifications ^b -non-targeted search	High	Med	High	-	- ^c	-	+	++	+
Cross-linking	High	Med-High	High	-	-	-	+	++	+

High: high priority; Med: medium priority; Low: low priority. "-", "undesirable instrument choice"; "+", "acceptable instrument choice"; "++", "desirable instrument choice.

^aThe Q-Exactive is the most sensitive instrument listed. For experiments where it is given an equal rating as other instruments, high sensitivity was not deemed absolutely critical to the experiment. However, if a Q-Exactive is readily accessible, it is generally the preferred choice of the instruments listed. Other high-performing instruments have been released recently and are expected to be highly useful for PSII research as well.

^bRatings are assuming precursor-ion-based quantification, as has been used in the large majority of studies focused on the PSII life-cycle. Product-ion-based quantification is relevant for studies that use iTRAQ and some forms of spectral counting.

^cAs an exception, rough quantification of relative LMM subunit stoichiometry between samples has been performed by intact-mass measurement on a MALDI-TOF (Sugiura et al., 2010a).

LMM proteins. Eichacker and co-workers (Granvogel et al., 2008) demonstrated top-down analysis on a Q-TOF with sequence coverage ranging from 14 to 82%. This method has been used in several other recent studies (Plösch et al., 2009; Boehm et al., 2011, 2012). Notably, Eichacker and co-workers (Granvogel et al., 2008) developed a protocol to perform in-gel extraction of intact LMM proteins prior to top-down analysis (capable of extracting all but the PsbZ protein from the gel matrix). This technique can be used to analyze individual BN-PAGE bands and, thus, identify the LMM components specific to individual types of PSII complexes in heterogeneous mixtures such as a thylakoid membrane proteome or affinity-tagged PSII complexes.

PSII Life-Cycle Application: Composition of Subcomplexes

Many subcomplexes form during the PSII life-cycle, and MS has played a critical role, in combination with gel electrophoresis, immunoblotting, crystallography, electron microscopy and other biochemical techniques, in identifying their components (Heinz et al., 2016). A schematic of the life-cycle is shown in **Figure 2**

(for reviews of the life-cycle and the subcomplexes that form, see Baena-González and Aro, 2002; Aro et al., 2005; Nixon et al., 2010; Shi et al., 2012; Komenda et al., 2012b; Nickelsen and Rengstl, 2013; Järvi et al., 2015; Heinz et al., 2016). A summary of the main subcomplexes whose composition has been studied by MS is found in **Table 4** (for completeness, several other subcomplexes are also included). MS analysis generally allows more rapid, comprehensive, and definitive profiling of PSII subunits than other methods, and is especially useful for the LMM subunits that tend to stain poorly on gels. However, owing to the high sensitivity of MS and because relative quantification by MS is not straightforward, it can be difficult to distinguish a trace component of a complex from one that is stoichiometric. Immunoblotting, therefore, complements MS for characterizing composition of subcomplexes.

At the start of *de novo* PSII assembly, each of the four core subunits D1, D2, CP47, and CP43, forms a pre-complex with specific LMM components. Using a $\Delta D1$ mutant in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) and top-down ESI-MS on a Q-TOF, Nixon and co-workers (Boehm et al., 2011) showed that the CP47 pre-complex contains the LMM subunits

TABLE 3 | Identification of LMM subunits by MS.

MS technique	Ionization method	Number of LMM subunits identified	Instrument	Mass accuracy (MS ¹)	References
Bottom-up	ESI, MALDI	0–4	Variety	~1–100 ppm (peptides)	Many, e.g., Kereiche et al., 2008; Plöschner et al., 2011; Haniewicz et al., 2013; Pagliano et al., 2014
Intact	ESI	9–11	QqQ	~50–200 ppm	Sharma et al., 1997a; Gómez et al., 2002; Laganowsky et al., 2009; Thangaraj et al., 2010
	MALDI	9–13	MALDI-TOF	~50–200 ppm	Sugiura et al., 2010a; Pagliano et al., 2011; Nowaczyk et al., 2012; Nakamori et al., 2014; Pagliano et al., 2014
Top-down	ESI	8–13	Q-TOF, LTQ-FTICR	3–30 ppm (Q-TOF), <1–5 ppm (LTQ-FTICR)	Granvogl et al., 2008; Plöschner et al., 2009; Thangaraj et al., 2010; Boehm et al., 2011, 2012
	MALDI	5	MALDI-TOF/TOF	~50–200 ppm	Pagliano et al., 2014

PsbH, PsbL, and PsbT, whereas the CP43 pre-complex contains the LMM subunits PsbK and Psb30. In this study, it was not possible by MS alone to demonstrate fully stoichiometric binding, just co-purification, of those LMM subunits to CP47 and CP43. However, these results are consistent with the PSII crystal structures and other non-MS-based results (Boehm et al., 2011 and references cited therein). Previous evidence implies PsbZ could also associate with the CP43 pre-complex (Iwai et al., 2007; Guskov et al., 2009; Takasaka et al., 2010), but it was not detected by MS in this study. As determined by affinity purification and immunoblotting, the D1 pre-complex contains PsbI and possibly Ycf48 (Dobáková et al., 2007). It was suggested that a Ycf39-ScpB-ScpE complex may also associate as early as this stage to insert chlorophyll into D1 (Knoppová et al., 2014). The D2 pre-complex contains PsbE and PsbF (Müller and Eichacker, 1999; Komenda et al., 2008).

The D1 and D2 pre-complexes merge to form the reaction center (RC) complex, the earliest subcomplex capable of charge separation (Baena-González and Aro, 2002; Dobáková et al., 2007). The RC complex, initially isolated from spinach and wheat by detergent solubilization of thylakoid membranes, was characterized by gel electrophoresis and immunoblotting to contain D1, D2, PsbE, PsbF, and PsbI (Nanba and Satoh, 1987; Ikeuchi and Inoue, 1988). Intact-mass and bottom-up MS studies later confirmed this composition (Sharma et al., 1997a,b,c). Several biochemical studies detected the 10-kDa PsbW subunit, which is found in green algae and higher plants but not in cyanobacteria, as an additional component (Irrgang et al., 1995; Lorković et al., 1995; Shi and Schröder, 1997). Subsequently, more specific studies (including an MS-based one, Granvogl et al., 2008) showed that PsbW associates later, to dimers during formation of PSII-Light-harvesting complex II (LHCII) supercomplexes (see below; Shi et al., 2000; Thidholm et al., 2002; Rokka et al., 2005; Granvogl et al., 2008). Despite attaching to PSII at a late stage of assembly, PsbW may bind tightly to the D1/D2 surface and, thus, remain partially attached to the RC complex during solubilization, while other peripheral PSII subunits are removed, explaining the controversy (Rokka et al., 2005). This case highlights that subcomplexes obtained from detergent solubilization, a technique used especially in early PSII subcomplex studies, do not necessarily represent

subcomplexes that form *in vivo*. An alternative major method for isolating PSII subcomplexes is purifying them from mutant strains that are “blocked” at a particular stage of assembly. Such complexes are indeed formed *in vivo*, but it is possible that the altered relative quantity of PSII subunits in the thylakoid membrane arising from the mutation may lead to artefactual binding of certain subunits to some subcomplexes (Thidholm et al., 2002). In cyanobacteria, two slightly different forms of the RC complex were observed, labeled RCII* and RCIIa, which differ slightly in accessory protein content (see **Table 3** and the section below). MS was critical in RCII* component characterization, and was indirectly used for RCIIa characterization as well by gel and immunoblot comparison (Knoppová et al., 2014).

The next complex formed during PSII assembly is the RC47 intermediate, also called the CP43-less core monomer in plants, formed by attachment of the CP47 pre-complex to the RC complex. In 1998, Barber and co-workers (Zheleva et al., 1998) showed by MS that the monomeric RC47 complex from spinach contains the D1, D2, CP47, PsbE, PsbF, PsbI, PsbT_c, and PsbW proteins, and the dimeric form contains, in addition, PsbK and PsbL. From the later studies on PsbW cited above, PsbW presence may arise from a tight binding to the D1/D2 surface, not *in vivo* presence in the RC47 complex during assembly. Based on Nixon and co-workers’ study (Boehm et al., 2011) on the CP47 pre-complex in *Synechocystis* 6803, it would be expected that RC47 also contains PsbH. Indeed, a more recent MS-based study of the RC47 complex from *Synechocystis* 6803 identified all the proteins found by Barber and co-workers (Zheleva et al., 1998) in their monomeric RC47 complex (except PsbW which is not found in cyanobacteria), plus PsbH, PsbM, PsbX, PsbY, and Psb28 (Boehm et al., 2012).

Attachment of the CP43 pre-complex to RC47 forms the inactive PSII monomer (Nickelsen and Rengstl, 2013). Active monomeric PSII is formed upon D1 processing (Liu et al., 2013a), dissociation of Psb27 (Liu et al., 2013a), assembly of the water-oxidizing manganese-calcium cluster and photoactivation (Dasgupta et al., 2008), and binding of PsbO, PsbU, and PsbV (cyanobacteria) or PsbO, PsbP, and PsbQ (algae and higher plants; Bricker et al., 2012). Active monomers dimerize and can attach to the phycobilisome antenna complex (cyanobacteria)

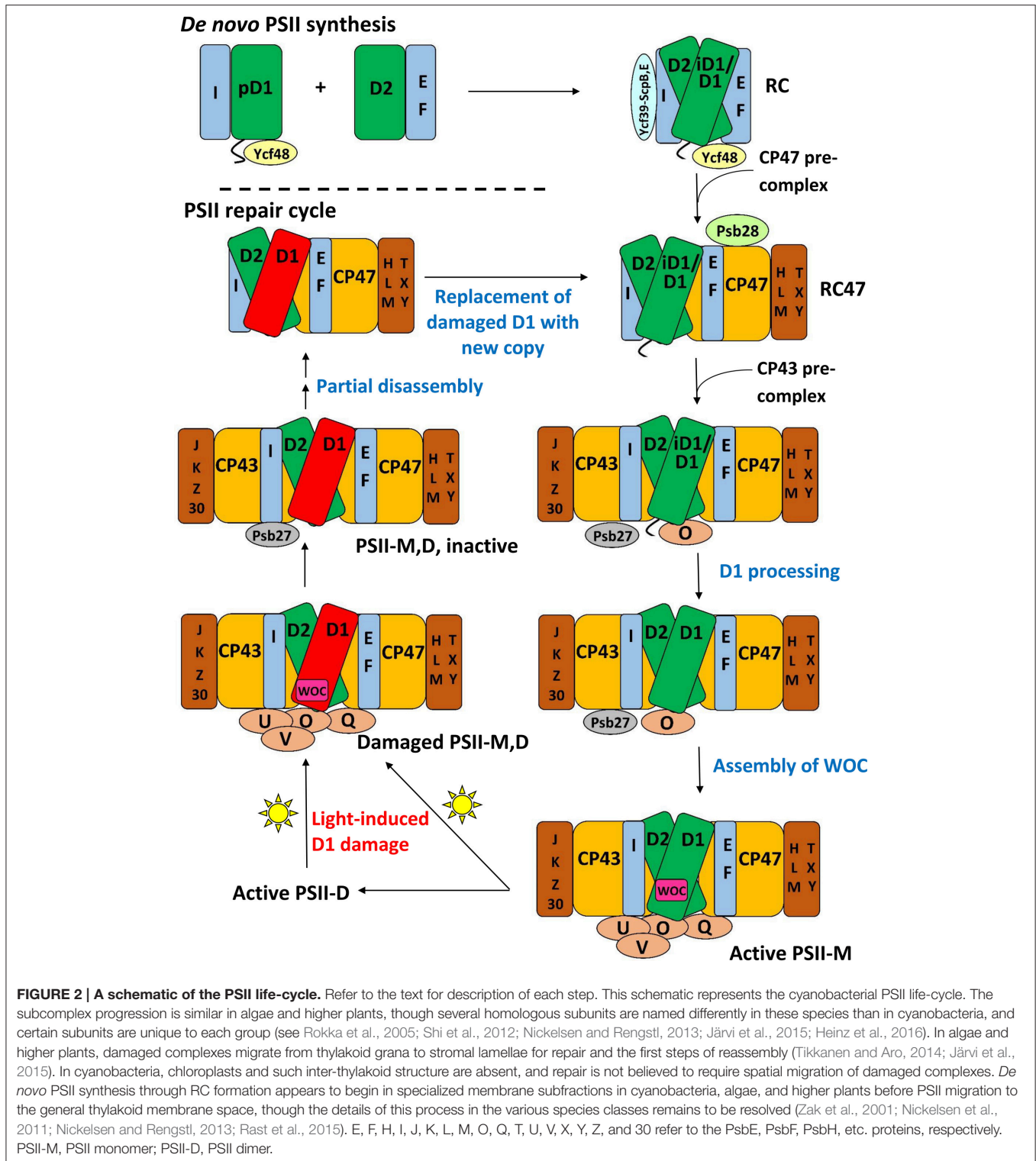


FIGURE 2 | A schematic of the PSII life-cycle. Refer to the text for description of each step. This schematic represents the cyanobacterial PSII life-cycle. The subcomplex progression is similar in algae and higher plants, though several homologous subunits are named differently in these species than in cyanobacteria, and certain subunits are unique to each group (see Rokka et al., 2005; Shi et al., 2012; Nickelsen and Rengstl, 2013; Järvi et al., 2015; Heinz et al., 2016). In algae and higher plants, damaged complexes migrate from thylakoid grana to stromal lamellae for repair and the first steps of reassembly (Tikkanen and Aro, 2014; Järvi et al., 2015). In cyanobacteria, chloroplasts and such inter-thylakoid structure are absent, and repair is not believed to require spatial migration of damaged complexes. *De novo* PSII synthesis through RC formation appears to begin in specialized membrane subfractions in cyanobacteria, algae, and higher plants before PSII migration to the general thylakoid membrane space, though the details of this process in the various species classes remains to be resolved (Zak et al., 2001; Nickelsen et al., 2011; Nickelsen and Rengstl, 2013; Rast et al., 2015). E, F, H, I, J, K, L, M, O, Q, T, U, V, X, Y, Z, and 30 refer to the PsbE, PsbF, PsbH, etc. proteins, respectively. PSII-M, PSII monomer; PSII-D, PSII dimer.

(Mullineaux, 2008) or various oligomeric states of LHCII complexes (algae and higher plants) (Kouřil et al., 2012).

Although, crystal structures of active PSII dimers from cyanobacteria are available, several MS studies of fully-assembled cyanobacterial PSII have provided independent confirmation of

the subunits present in purified complexes under more native conditions (Sugiura et al., 2010a; Nowaczyk et al., 2012). Using native conditions has even helped discover a component (PsbQ) that was lost during crystallization (Kashino et al., 2002; Roose et al., 2007). The majority of PSII from algae and higher plants

is found in several PSII dimer-LHCII supercomplexes (for a review see Kouřil et al., 2012). MS studies (in concert with other techniques) have identified their subunit compositions, even in the absence of crystal structures of the complexes from these organisms. Eichacker and co-workers (Granvogl et al., 2008) showed that the four PSII-LHCII supercomplexes in *Nicotiana tabacum* contain identical PSII core and LMM subunits (of the eight LMM subunits identified), and that only PSII-LHCII supercomplexes contain the PsbW protein. These results support previous studies that suggest that PsbW may facilitate linkage of LHCII trimers to PSII (Shi et al., 2000; Thidholm et al., 2002; Rokka et al., 2005). Using both bottom-up and top-down MS techniques, Pagliano et al. (2014) found that the various supercomplexes in pea contain identical core and LMM subunits, but that the C₂S₂M₂ supercomplex contains the PsbQ, PsbR, PsbP, Lhcb3, and Lhcb6 proteins whereas the C₂S₂ supercomplex does not. In light of the stabilizing effect of the PsbQ and PsbP proteins on oxygen evolution, this finding raises interesting questions about the role of the C₂S₂ supercomplex. Another recent study used MS to characterize PSII-LHCII supercomplexes in *N. tabacum* and found a few differences in subunit composition; in particular, the C₂S₂ supercomplex contained Lhcb1 isoform CB25, while the C₂S₂M₂ supercomplex did not (Haniewicz et al., 2015).

Several studies indicate that PSII-PSI-antenna megacomplexes can form in both cyanobacteria and higher plants. Using *in vivo* cross-linking, Blankenship and co-workers (Liu et al., 2013b) captured a PSII-PSI-phycoobilisome megacomplex in *Synechocystis* 6803. The authors used MS to demonstrate presence of subunits from each complex in the preparation (Tables 3, 5), and identified cross-links revealing specific inter-complex subunit interactions. Aro and co-workers (Tikkanen et al., 2008b, 2010) showed that LHCII can transfer excitation energy to PSI in grana margins of higher plants as a means of balancing energy flux under varying light conditions. In support of this hypothesis, two PSII-PSI-LHCII megacomplexes from *Arabidopsis thaliana* were observed by a novel large-pore BN-PAGE system (Järvi et al., 2011), and more recently, a PSII-PSI-LHCII megacomplex was identified by MS from the macroalga *Ulva* sp. under drought stress conditions (Gao et al., 2015).

PSII Life-Cycle Application: Identification of Accessory Proteins

Many accessory proteins bind transiently to PSII subcomplexes during the PSII life-cycle, serving key regulatory roles, but are not present in the crystal structure owing to their absence in fully assembled PSII. For reviews of the accessory proteins of PSII (see Shi et al., 2012; Komenda et al., 2012b; Nickelsen and Rengstl, 2013; Mabbitt et al., 2014; Järvi et al., 2015; Heinz et al., 2016). Bottom-up MS has played a key role in identifying some of the known ones, and others likely remain to be identified. Identifying a previously unknown PSII-associated protein in this manner, however, is not straightforward because the mass spectrometers used for bottom-up analysis are so sensitive that dozens of contaminant proteins are often

detected even in “purified” complexes. Low signal intensity of a peptide compared to those of known PSII peptides does not necessarily indicate a contaminant at low abundance because different peptides have different intrinsic ionization efficiencies, and many accessory proteins bind sub-stoichiometrically to PSII. Certain contaminant proteins such as NDH-1 complex subunits (Nowaczyk et al., 2012), ATP synthase subunits (Komenda et al., 2005), phycobilisome subunits (Kufryk et al., 2008), certain ribosomal proteins (Liu et al., 2011b), and several carbon dioxide-concentrating mechanism proteins (Kufryk et al., 2008; Liu et al., 2011b) are frequently observed. Careful examination of the full list and consideration of the experimental conditions are needed to distinguish plausible PSII-interaction candidates from contaminant proteins (Kashino et al., 2002). Although, different MS search software packages use different algorithms for scoring protein hits, a strict statistical confidence threshold should be employed and reported. Overall, although a simple bottom-up experiment is a powerful tool to suggest new candidate proteins that associate with PSII, subsequent targeted experiments on each one are needed to confirm the interaction.

This strategy has proven successful many times for identifying new PSII interaction partners. An early example (Kashino et al., 2002) analyzed SDS-PAGE bands by MALDI-TOF MS from a highly purified PSII preparation and identified several novel proteins, Sll1638 (PsbQ), Sll1252, and Sll1398 (Psb32), that appeared to be plausible PSII interaction partners. Follow-up biochemical studies targeting these proteins confirmed their role in the PSII life-cycle and elucidated functional aspects of each (Inoue-Kashino et al., 2011; Wegener et al., 2011; Bricker et al., 2012). A later proteomic study of purified PSII complexes revealed that the Slr0144-Slr0152 proteins, all part of one operon, associate with PSII, leading to further characterization of their role in PSII assembly (Wegener et al., 2008). In other cases, specific subcomplexes were isolated before MS analysis and identification of accessory proteins. For example, analysis of a gel band from $\Delta ctpA$ -HT3-PSII revealed that the Psb27 protein binds specifically to a PSII subcomplex that accumulates before D1 processing (Roose and Pakrasi, 2004), initiating the studies that ultimately elucidated its role in PSII assembly (Nowaczyk et al., 2006; Roose and Pakrasi, 2008; Grasse et al., 2011; Liu et al., 2011a,b; Komenda et al., 2012a). MS analysis showed that the Ycf39, ScpB (HliC), and ScpE (HliD) proteins bind specifically to the RCII* form of the reaction center complex, but not the related RCIIa form (Knoppová et al., 2014). The specific binding of the accessory proteins Psb28 (Dobáková et al., 2009; Boehm et al., 2012) and Psb28-2 (Boehm et al., 2012) to the RC47 complex, and of Ycf48 to RCII* and RCIIa (Knoppová et al., 2014), was initially discovered by immunoblotting, but the proteins' presence was confirmed by MS, strengthening the finding.

PSII Life-Cycle Application: Identification of PTMs

Identification of Processing Events to Form Mature PSII Proteins

The D1 protein is synthesized as a precursor protein (pD1) with a C-terminal extension that gets cleaved during PSII

TABLE 4 | Composition of complexes in the PSII life-cycle by MS and other methods.

Sub-complex	Composition by MS	Species ^a	References	Composition by other methods	Species ^a	References
D1 pre-complex	ND	-	-	pD1, I, Ycf48 ^c	Syn. 6803	Dobáková et al., 2007
D2 pre-complex	ND	-	-	D2, E, F	Syn. 6803	Komenda et al., 2008
CP47 pre-complex	CP47, H, L, T	Syn. 6803	Boehm et al., 2011	CP47, H	Syn. 6803	Komenda et al., 2005
CP43 pre-complex	CP43, K, Psb30	Syn. 6803	Boehm et al., 2011	CP43, K, Z, Psb30 ^c	<i>C. reinhardtii</i> , <i>T. elongatus</i>	Sugimoto and Takahashi, 2003; Iwai et al., 2007
RC	D1, D2, E, F, I	Pea	Sharma et al., 1997a,b	D1, D2, E, F, I, W ^d	Spinach	Nanba and Satoh, 1987; Ikeuchi and Inoue, 1988; Irgang et al., 1995
RCII*	D1/ID1, D2, E, F, I, Ycf48, Ycf39, ScpB, ScpE	Syn. 6803	Knoppová et al., 2014	D1/ID1, D2, E, F, I, Ycf48, Ycf39, ScpE	Syn. 6803	Komenda et al., 2004; Dobáková et al., 2007; Komenda et al., 2008; Knoppová et al., 2014
RCIIa	ND	-	-	D1/ID1, D2, E, F, I, Ycf48	Syn. 6803	Dobáková et al., 2007; Komenda et al., 2008; Knoppová et al., 2014
RC47 monomer	D1, D2, CP47, E, F, I, L, M, T, X, Y, Psb28, Psb28-2	Syn. 6803	Boehm et al., 2012	D1, D2, CP47, E, F, H, Psb28, Psb28-2	Syn. 6803	Komenda et al., 2004; Dobáková et al., 2009; Boehm et al., 2012
RC47 monomer	D1, D2, CP47, E, F, I, T _c , W	Spinach	Zheleva et al., 1998	D1, D2, CP47, E, F, H, I, M, R, T _c	Spinach	Rokka et al., 2005
RC47 dimer	D1, D2, CP47, E, F, I, K, L, T _c , W	Spinach	Zheleva et al., 1998	ND	-	-
PSII monomer, inactive	Psb27	Syn. 6803, <i>T. elongatus</i>	Grasse et al., 2011; Liu et al., 2013a	RC47 components + CP43, Psb27	Syn. 6803, <i>T. elongatus</i>	Roose and Pakrasi, 2008; Grasse et al., 2011; Liu et al., 2013a
PSII monomer/dimer, active	D1, D2, CP47, CP43, E, F, H, I, J, K, L, M, O, Q, U, V, T, X, Y, Z, Psb30	Syn. 6803, <i>T. elongatus</i>	Kashino et al., 2002; Nowaczyk et al., 2012	Crystal structure shows all components as by MS except PsbQ	Syn. 6803, <i>T. elongatus</i>	Roose et al., 2007; Umena et al., 2011
PSII monomer/dimer, active	D1, D2, CP47, CP43, E, F, H, I, K, L, M, O, R, T _c , X	<i>N. tabacum</i>	Granvogl et al., 2008; Haniewicz et al., 2015	D1, D2, CP47, CP43, O, P, S; R, W (pea)	<i>A. thaliana</i>	Caffarri et al., 2009; Pagliano et al., 2011
PSII-LHCII supercomplexes ^b	D1, D2, CP47, CP43, E, F, H, I, K, L, M, O, R, T _c , W, X; Lhcb1-4, 6	<i>N. tabacum</i>	Granvogl et al., 2008; Haniewicz et al., 2015	D1, D2, CP47, CP43, O, P, Q, S; Lhcb1-6; W (pea)	<i>A. thaliana</i>	Thidholm et al., 2002; Caffarri et al., 2009; Kouřil et al., 2012
PSII-PSI-PBS megacomplex	D1, D2, CP47, CP43, O, U, V; PsaA, B, C, D, E, F, L, Siro172, Ycf4, PC; ApcA, B, C, D, E, F, CpcA, B, D, G1, G2	Syn. 6803	Liu et al., 2013b	ND	-	-
PSII-PSI-LHCII megacomplex	D1, D2, CP47, CP43, E; PsaA,B,L; LhcbM1, LhcbM10, Lhca2	<i>Ulva</i> sp.	Gao et al., 2015	D1, D2, CP47, CP43; PsaB,D,F,G, K,L; unspecified LHCII subunits	<i>A. thaliana</i>	Järvi et al., 2011

ND, not determined.

^aWhen two species are listed in the same subcomplex entry, the protein components are the union of those found in the individual studies.

^bCharacterization of specific PSI-LHCII supercomplexes.

^cUncertain; evidence is suggestive.

^dSubsequent studies indicate PsbW presence in this complex may be an artifact of solubilization conditions (discussed in the text).

TABLE 5 | Summary of MS-based PSII cross-linking studies^a.

Species	Cross-linked subunit 1	Cross-linked subunit 2	Cross-linker	Method notes	References
<i>Syn. 6803</i>	Psb27	CP43	EDC, DTSSP	<ul style="list-style-type: none"> • Cross-linked species enriched on gel • In-gel digestion; trypsin or chymotrypsin • LTQ-Orbitrap XL • MassMatrix search software 	Liu et al., 2011a
<i>Syn. 6803</i>	PsbQ	CP47, PsbO	EDC, DTSSP	<ul style="list-style-type: none"> • No cross-link enrichment • In-solution digestion; trypsin • LTQ-Orbitrap XL • MassMatrix search software 	Liu et al., 2014b
<i>Syn. 6803</i>	D2, CP43, CP47	ApcE	DSP	<ul style="list-style-type: none"> • No pre-MS cross-link enrichment • In-solution digestion; trypsin + LysC • LTQ-Orbitrap XL • $\geq +3$ charge states selected for MS² to maximize cross-link selection • MassMatrix search software 	Liu et al., 2013b
<i>T. elongatus</i>	Psb27	CP43	BS ³ d0/d12	<ul style="list-style-type: none"> • Isotope-encoded cross-linker • No cross-link enrichment • In-solution digestions; trypsin • Orbitrap Elite Velos Pro • StavroX search software 	Cormann et al., 2016
<i>C. reinhardtii</i>	PsbP	PsbQ	EDC	<ul style="list-style-type: none"> • Wash step isolates extrinsic proteins after cross-linking • Cross-linked species enriched on gel • In-gel digestion; trypsin or Asp-N • Ultraflex MALDI-TOF • MS¹ only; trypsin and Asp-N samples independently indicate the same cross-linked residues 	Nagao et al., 2010
Spinach	PsbP	PsbQ	BS ³	<ul style="list-style-type: none"> • Wash step isolates extrinsic proteins after cross-linking • Cross-linked species enriched on gel • In-gel digestion; trypsin \pm LysC • LTQ-FTICR • MassMatrix search software 	Mummadiseti et al., 2014
Spinach	PsbP	PsbE	EDC	<ul style="list-style-type: none"> • Biotin-tagged PsbP isolates the free protein + its cross-linked partners • Cross-linked species enriched on gel • In-gel digestion; trypsin • LTQ-Orbitrap XL • MassMatrix search software 	Ido et al., 2012
Spinach	PsbP	PsbR, CP26	EDC	<ul style="list-style-type: none"> • Biotin-tagged PsbP isolates the free protein + its cross-linked partners • Cross-linked species enriched on gel • In-gel digestion; trypsin • LTQ-Orbitrap XL • MassMatrix search software 	Ido et al., 2014

^aOnly inter-protein cross-links that reveal interactions not detectable in the available PSII crystal structures are shown here.

assembly (Takahashi et al., 1988). An early study using peptide sequencing showed that in spinach, cleavage occurs after Ala-344, removing nine C-terminal residues (Takahashi et al., 1988). Several years later, it was found that, in *Synechocystis* 6803, cleavage also occurs after Ala-344, removing 16 C-terminal residues (Nixon et al., 1992). In this study, peptide sequencing as well as fast atom bombardment (FAB)-MS (a predecessor for ESI and MALDI) were used to pinpoint this cleavage site. Ala-344 serves as a ligand for a Mn ion in the water oxidation cluster (Umena et al., 2011) so that without cleavage, PSII remains incapable of oxygen evolution (Roose and Pakrasi, 2004). The extension, thus, protects early assembly intermediates from harmful premature water oxidation activity. Interestingly, although D1 in higher plants is cleaved in a single step, cyanobacterial D1 is cleaved in two steps, and an intermediate D1 (iD1) is formed transiently (Inagaki et al., 2001). Although, the iD1 cleavage site remained unknown for two decades, in 2007, MS and biochemical evidence demonstrated that the CtpA protease cleaves after Ala-352 to form iD1, which is then cleaved again after Ala-344 to form mature D1 (Komenda et al., 2007). The significance of the two-step cleavage remains unknown, although iD1 may serve as a signal for transferring an early PSII assembly intermediate from the cytoplasmic to the thylakoid membrane (Komenda et al., 2007).

The CP43 protein also appears to be cleaved before, or during an early stage of, PSII assembly. Tandem MS analysis identified a CP43 peptide in spinach starting with a modified form of Thr-15 (Michel et al., 1988). Based on the genomic sequence, the preceding residue is a leucine, so this peptide would not be a predicted trypsin cleavage product. It was also found that the N-terminus of CP43 is blocked from analysis by Edman degradation, likely owing to N-terminal modification. Taken together, these results show that the first 14 residues of CP43 are cleaved, leaving Thr-15 as the mature protein's N-terminus (Michel et al., 1988). Subsequent studies identified the corresponding CP43 peptide in *A. thaliana* (Vener et al., 2001) and *Synechocystis* 6803 (Wegener et al., 2008), suggesting that this cleavage is conserved. Crystal structures of cyanobacterial PSII were not able to resolve the most N-terminal portion of CP43, so those structures do not address this question of CP43 cleavage (Loll et al., 2005; Umena et al., 2011).

Cyanobacterial Psb27, PsbQ, and PsbP have unusually hydrophobic properties for soluble lumen-localized proteins and contain a lipoprotein signal motif and conserved cysteine in their N-terminal regions (Thornton et al., 2004; Nowaczyk et al., 2006; Fagerlund and Eaton-Rye, 2011). This led to the suggestion that they are N-terminally lipid-modified and, thus, anchored to the luminal surface of the thylakoid membrane. Using lipase treatment and MALDI-TOF MS, Rögner and co-workers (Nowaczyk et al., 2006) showed that Psb27 from *Thermosynechococcus elongatus* does indeed contain such a modification. Also using MALDI-TOF MS, Wada and co-workers (Ujihara et al., 2008) confirmed this finding with Psb27 from *Synechocystis* 6803 and also found that *Synechocystis* 6803 PsbQ, recombinantly expressed in *E. coli*, is also N-terminally lipid modified. Notably, this group developed a method to extract

lipid-modified peptides from a gel matrix after in-gel digestion, enabling downstream MS analysis (Ujihara et al., 2008). During PSII assembly, it is important that Psb27 binds to the luminal surface before the other extrinsic proteins (Liu et al., 2013a), and the lipid anchor may facilitate this sequence by keeping Psb27 in close proximity at all times. A similar role for the lipid anchor of PsbQ was proposed recently (Liu et al., 2015). A lipid modification on PsbP has not yet been demonstrated although strong suggestive evidence indicates its presence (Fagerlund and Eaton-Rye, 2011).

Identification of Phosphorylation Sites

In the early 1980s, phosphorylation of the four PSII subunits that later came to be known as D1, D2, CP43, and PsbH, was observed. These studies were conducted *in vivo* and *in vitro* using ^{32}P labeling of whole cells and thylakoid membranes from *Chlamydomonas reinhardtii* and pea, with detection of phosphoproteins by autoradiography (Owens and Ohad, 1982, 1983; Steinback et al., 1982). Immunoblotting with antibodies that recognize phosphorylated residues was introduced later and became another popular detection method (Rintamäki et al., 1997). Neither of these methods, however, reveal the modified residue. This information was first obtained by gas-phase sequencing using Edman degradation, which demonstrated that the PsbH phosphorylation site is Thr-2, its N-terminus, in spinach (Michel and Bennett, 1987) and *C. reinhardtii* (Dedner et al., 1988). Since then, MS analysis has replaced Edman degradation and become the dominant method for phosphorylation-site determination, as it is higher-throughput, more definitive, more sensitive, and not limited by N-terminal blockage (e.g., acetylation). The main sites identified are presented below (for reviews, see Vener, 2007; Pesaresi et al., 2011; Puthiyaveetil and Kirchhoff, 2013).

Tandem MS demonstrated phosphorylation of D1-Thr-2, D2-Thr-2, and CP43-Thr-15, the mature proteins' N-termini, in spinach (Michel et al., 1988), *A. thaliana* (Vener et al., 2001), and *C. reinhardtii* (Turkina et al., 2006). Phosphorylation of CP43 was also observed at Thr-20, Thr-22, and Thr-346 in spinach (Rinalducci et al., 2006), and at Thr-346 and Ser-468 in *A. thaliana* (Sugiyama et al., 2008; Reiland et al., 2009). MS analysis showed that PsbH is phosphorylated at its N-terminus in *A. thaliana*, supporting the Edman degradation data from spinach and *C. reinhardtii*, and additionally demonstrated phosphorylation of Thr-4 (Vener et al., 2001). Intact-mass MS evidence also indicates double PsbH phosphorylation in spinach and pea (Gómez et al., 1998, 2002). More recently, phosphorylation of the extrinsic proteins PsbP, PsbQ, and PsbR was observed in phosphoproteomic studies of *A. thaliana* (Sugiyama et al., 2008; Lohrig et al., 2009; Reiland et al., 2009). Although, not discussed here, phosphorylation of LHCII is well-documented, and it regulates state transitions in green algae and higher plants (for reviews see Lemelle and Rochaix, 2010; Minagawa, 2011; Schönberg and Baginsky, 2012; Tikkanen and Aro, 2014; Tikhonov, 2015).

Phosphorylation of PSII subunits is not absolutely required for PSII repair (Bonardi et al., 2005) but assists in transferring damaged PSII complexes from the stacked thylakoid grana to

stromal lamellae, where repair occurs. Phosphorylation appears to induce architectural changes in the stacked grana and increase membrane fluidity in such a way as to promote mobility of damaged PSII centers to the stromal lamellae for repair (Tikkanen et al., 2008a; Fristedt et al., 2009, 2010; Herbstová et al., 2012; Järvi et al., 2015). For many of the PSII phosphorylation sites, light intensity and/or other environmental conditions affect the phosphorylation extent, with implications for the functional significance of these modifications. MS analysis has played a critical role in these quantitative studies, and methodology for such measurements is discussed in the dynamics section below. For reviews that discuss the role of PSII phosphorylation (see Pesaresi et al., 2011; Mulo et al., 2012; Schönberg and Baginsky, 2012; Järvi et al., 2015).

PSII phosphorylation may not be needed in cyanobacteria owing to the lack of spatial organization of thylakoids (Mulo et al., 2012). However, a recent global proteomics study of the cyanobacterium *Synechococcus* sp. PCC 7002 (hereafter *Synechococcus* 7002) found that a portion of D1 copies are phosphorylated at their N-terminus, Thr-2 (Yang et al., 2014), as in higher plants. This finding opens the possibility for a role of phosphorylation in PSII turnover in cyanobacteria.

Identification of Oxidative and Other Modifications

Light is necessary for PSII function, but even low light intensities can lead to PSII damage, particularly of the D1 protein. Damage triggers partial PSII disassembly, D1 degradation, insertion of a new D1 copy, and PSII re-assembly (Nickelsen and Rengstl, 2013). When the rate of damage exceeds that of repair, photosynthesis is inhibited, referred to as photoinhibition. Photodamage can be initiated in several ways, but a common result of each mechanism is production of highly oxidizing species (e.g., singlet O₂, other reactive oxygen species (ROS), or radical PSII cofactors). These species rapidly oxidize PSII subunits, ultimately rendering the complex non-functional. For reviews of the photoinhibition process (see Barber and Andersson, 1992; Adir et al., 2003; Pospíšil, 2009; Allahverdiyeva and Aro, 2012; Tyystjärvi, 2013).

Although oxidative damage of PSII was long believed to be responsible for photoinhibition (Telfer et al., 1994), MS studies provided the first concrete evidence for specific oxidative modifications of PSII. Bottom-up MS analysis of the D1 and D2 subunits from pea PSII found up to three +16 oxidative modifications (each representing incorporation of an oxygen atom) on certain peptides (Sharma et al., 1997c). Interestingly, not all peptides were oxidized, but the oxidized ones were all located near the predicted D1 and D2 redox cofactor sites, supporting the idea that radical redox cofactors themselves, or ROS produced by reaction with them, cause oxidative damage to PSII. More recently, Bricker and co-workers (Frankel et al., 2012, 2013b) used tandem MS to identify oxidized residues on spinach D1, D2, and CP43 that are located near the Q_A, Phe_{D1}, and manganese cluster sites, all reasonable sources of oxidizing species. Additionally, tryptophan oxidation products in spinach were identified on CP43-Trp-365 and D1-Trp-317, which are located near the manganese cluster (17 and 14 Å, respectively, in the crystal structure from *T. elongatus*; Anderson et al., 2002;

Dreaden et al., 2011; Kasson et al., 2012). By monitoring the digested peptides' absorption at 350 nm, the authors found that these tryptophan oxidations are correlated with increased light intensity and decreased oxygen evolution. Other modifications to PSII subunits were also detected by MS (Gómez et al., 2002, 2003; Anderson et al., 2004; Rexroth et al., 2007; Sugiura et al., 2013). Notably, a recent global proteomics study of *Synechococcus* 7002 identified many new PSII PTMs (Yang et al., 2014), but the functional significance of these modifications remains to be determined.

DYNAMICS: QUANTITATIVE OR SEMI-QUANTITATIVE CHANGES IN PSII PROTEINS AND PTMS

MS-Based Methods to Study PSII Dynamics

Most MS-based quantification experiments seek the relative, not absolute quantity of a protein or PTM in one sample compared to another. We focus here on relative quantification methods because nearly all the work on PSII dynamics fell into that category.

Gel-Based Quantification

Perhaps the most basic MS-based semi-quantitative method is in-gel digestion at the same band in two different sample lanes, prompted by a significant staining-intensity difference between the two bands. This approach was used frequently when analyzing different purified PSII complexes (Liu et al., 2011b; Knoppová et al., 2014), yielding information about accessory proteins that bind specifically to certain subcomplexes. A proper loading control (typically equal chlorophyll) must be used to ensure a meaningful comparison. Multiple proteins are typically identified by MS in both bands, however, so it may not be immediately apparent which protein is the main component (Liu et al., 2011b). Confirmation may be necessary by western blotting or one of the more quantitative MS-based techniques described below.

The accuracy of gel-based quantification can be improved by introducing a second electrophoretic separation dimension before in-gel digestion and LC-MS/MS. Semi-quantitative two-dimensional denaturing gel electrophoresis (2DE) (distinct from 2D BN-PAGE described above), a popular technique especially in early proteomics studies, usually first separates proteins by size and then on the basis of pI (Rabilloud et al., 2010). The difference in staining intensity indicates the relative content of that protein in each sample. Because two proteins migrate less often together in two dimensions than in one, separation and quantification accuracy are improved. 2DE is useful for large-scale studies such as whole-cell or whole-organelle proteome profiling that require higher-resolution separation than a 1D gel provides. However, in recent years, 2DE has declined in popularity owing to its numerous drawbacks (reviewed in Rabilloud et al., 2010) and the improvements in other more versatile quantitative MS methods. Such large-scale proteomics studies have detected expression-level changes in several PSII proteins in response to a variety of

stress conditions (e.g., Ingle et al., 2007; Aryal et al., 2011; Li et al., 2011; Guerreiro et al., 2014). However, insights into the PSII life-cycle have mainly emerged from more focused studies on purified PSII complexes.

Label-Free Quantification

Some MS-based relative quantification methods use a so-called label-free approach, but the better approach, when feasible, is to introduce a stable isotope into the sample. For label-free quantification, the samples to be compared are analyzed by LC-MS/MS separately. A variety of software tools can then be used to obtain an extracted ion chromatogram (EIC) of any peptide. The EIC displays the total intensity (peak area) of that peptide. Comparing the intensities of the same peptide from two different samples indicates the relative content of that peptide in those samples. Although the concept is simple, accurate label-free quantification depends on a number of factors: equal sample loading (on a relevant basis, e.g., chlorophyll concentration), reproducible LC runs, lack of ion suppression, and appropriate normalization during data analysis. For quantification of proteins, data from component peptides must be merged in a statistically sound way (Bantscheff et al., 2012; Nahnsen et al., 2013). Thorough mass spectral sampling of possible precursors—not as crucial in non-quantitative experiments—is necessary for accurate peak definition, but that typically diverts instrument time from obtaining product-ion spectra that give information for peptide identification and sequence coverage (Bantscheff et al., 2012). Various strategies have been designed to address this challenge (e.g., data-independent acquisition approaches such as MS^E (Silva et al., 2006; Grossmann et al., 2010) and “all-ion fragmentation” (Geiger et al., 2010) especially when combined with Ultra-Performance LC (UPLC) (Bantscheff et al., 2012). Label-free quantification by spectral counting, which involves comparing the total number of product-ion (MS/MS) spectra obtained for a given peptide or protein, is a common approach (Lundgren et al., 2010), although that has been used in fewer PSII-related studies (Fristedt and Vener, 2011; Stöckel et al., 2011). Label-free quantification of intact proteins is more direct than comparing peptides, but best applied for small proteins. Intact-mass spectra (MALDI and ESI) of the LMM PSII proteins indeed have been used in a number of instances for label-free quantification between states (Laganowsky et al., 2009; Sugiura et al., 2010a).

Isotope Label-Based Quantification

The alternative to label-free quantification is introduction of a stable isotope label into one of the two samples being compared (certain methods also allow greater multiplexing, see below). In contrast to the label-free approach, the labeled and unlabeled samples (often called “heavy” and “light”) are mixed and analyzed in a single LC-MS/MS run. The mass spectra of the light and heavy peptide show two peaks shifted slightly in mass. Comparison of their peak areas, just as in label-free quantification, indicates the relative amount of that peptide in each sample (Bantscheff et al., 2012). Although, comparing peak areas from a single LC-MS/MS run eliminates

the concerns of label-free LC reproducibility and ion suppression, labeling introduces additional sample preparation steps and often involves costly reagents.

Isotopic labeling (with ²H, ¹³C, ¹⁵N, or ¹⁸O) of all proteins can be accomplished during cell growth (metabolic labeling), or by labeling a subset of proteins or peptides at various stages after cell lysis (chemical or enzymatic labeling). In the SILAC method (“stable isotope labeling by amino acids in cell culture”; reviewed in Chen et al., 2015), addition of labeled arginine or lysine to the growth medium results in incorporation of only the labeled form of that amino acid into all proteins. Hippler and co-workers (Naumann et al., 2007) used a SILAC-based method to measure changes in expression of PSII subunits and other proteins in *C. reinhardtii* under iron deficiency, and Jacobs and co-workers (Aryal et al., 2011) used this method to measure light-dark diurnal cycles in *Cyanothece* sp. ATCC 51142. A more common approach in PSII life-cycle research, however, has been ¹⁵N metabolic labeling (see “Measuring the temporal dynamics of life-cycle events using isotopic labeling” below), in which the growth medium is modified so that the only nitrogen source is a labeled salt such as potassium nitrate or ammonium chloride (Gouw et al., 2010).

Isotopic labeling at the peptide or protein level during downstream processing after cell lysis is an alternative to metabolic labeling. Tandem mass tags (TMT) (Thompson et al., 2003), isotope tags for relative and absolute quantification (iTRAQ) (Ross et al., 2004), enzymatic ¹⁸O labeling, and isotope-coded affinity tags (ICAT) can be used in proteomics experiments in photosynthetic organisms (Thelen and Peck, 2007). TMT and iTRAQ are related approaches that have become popular recently (Bantscheff et al., 2012). Both modify peptides with one of several possible isobaric tags that produce reporter ions during MS/MS fragmentation. Each sample is labeled with a different tag, but owing to the tags’ isobaric nature, identical peptides from each sample are observed together chromatographically and as a single peak in a low-resolving power mass spectrum. Each tag, however, contains a unique reporter ion that appears as a distinct peak in the product-ion (MS/MS) spectra, and the ratio of these ions reveals the relative amounts of that peptide in each sample. The iTRAQ reagent modifies primary amines, and TMT tags are available that modify primary amines, thiols, or carbonyl groups. Advantages of these labeling approaches include the ability to multiplex up to 8 or 10 samples, greater than with metabolic and other chemical labeling methods, and the isobaric nature of the same peptide across all samples reduces both LC separation demands and MS data complexity (Bantscheff et al., 2012). Although, many proteomics studies on photosynthetic organisms have used these chemical labeling methods, most have not focused on PSII life-cycle issues (Thelen and Peck, 2007; Battchikova et al., 2015). Two relevant examples include the detection of elevated PsbO cysteine oxidation under DCMU and dark conditions (Guo et al., 2014), and intriguing evidence that PSII thermotolerance in *Synechocystis* 6803 may arise in part from antenna trimming and an increased rate of electron transfer to the cytochrome *b₆/f* complex (Rowland et al., 2010).

PSII Life-Cycle Application: Measuring Changes in Phosphorylation Levels

As mentioned in the composition section above, phosphorylation of PSII subunits affects membrane fluidity and inter-thylakoid dynamics, thus playing a role in facilitating PSII turnover in green algae and higher plants (see the reviews cited in that section for in-depth treatment of this topic). Many of the studies that have contributed to our current understanding of this process used MS quantification techniques to compare phosphorylation levels between samples and under different environmental conditions.

When using peak-area-based label-free quantification to determine the change in a modified peptide between samples, it is crucial that the peak area of the unmodified peptide be taken into account as well, to distinguish a true change in modification *extent* from simply an increased level of protein expression in one of the states. This method is demonstrated in a study of phosphorylation and nitration in *A. thaliana* grown under low and high light regimes (Galetskiy et al., 2011b). The authors first normalized each modified-peptide peak area in each sample to that of its unmodified counterpart and then compared the modified peptides' normalized peak area to each other. This method can reveal *fold-changes in modification extent* between the two states, but not the absolute percentage of that peptide that contains the modification (the "*modification stoichiometry*").

To find the modification stoichiometry, it is necessary to know in addition the relative "flyability" (ionization efficiency) of the modified and unmodified peptides. Vierstra and co-workers (Vener et al., 2001) showed that the relative flyabilities of six synthetic phosphopeptides and their non-phosphorylated counterparts are nearly identical. Suggesting this as a general phenomenon for phosphorylated peptides, they estimated the modification stoichiometry for the phosphorylated peptides of D1, D2, CP43, PsbH, and an LHCII protein. In 2010, Vener and co-workers (Fristedt et al., 2010) calculated the actual relative flyability ratio for these PSII peptide pairs, and reported reliable modification stoichiometry for these proteins for the first time under the various conditions in their study. Interestingly, the flyability ratios were indeed close to 1 for each pair (ranging from 0.89 to 1.23), supporting the earlier suggestion that this may be the case for most phosphorylated/non-phosphorylated peptide pairs (Vener et al., 2001). Other studies have since used those flyability ratios to determine changes in the modification stoichiometry of those same phosphorylation sites under other growth conditions (Fristedt and Vener, 2011; Romanowska et al., 2012; Samol et al., 2012). Knowledge of modification stoichiometry under different conditions is quite valuable; it enabled, for example, a greater level of confidence and detail in the model proposed for how phosphorylation affects thylakoid membrane stacking than would have been possible with fold-change data alone (Fristedt et al., 2010).

Chemical isotopic labeling of peptides has also been applied fruitfully to the study of PSII phosphorylation. Immobilized metal-ion affinity chromatography (IMAC) is a standard protocol for enrichment of phosphopeptides, taking advantage of the interaction between phosphoryl groups and a Fe^{3+} -agarose matrix (Andersson and Porath, 1986). Given that free carboxyl groups can also interact with the resin, it has become common

to convert free carboxylates to methyl esters after digestion and prior to IMAC, to avoid this interaction (Ficarro et al., 2002). Vener and co-workers (Vainonen et al., 2005) modified this approach by using deuterated methanol (CD_3) as the esterification reagent for one sample, and unlabeled methanol for a second sample to quantify by "isotope encoding." After mixing the samples and analyzing by LC-MS/MS, the relative amount of each phosphorylated peptide in the two samples is quantified by comparison of their mass spectral peak areas. It should be noted that this approach does not reveal the modification stoichiometry of any phosphorylation site; rather the techniques described above still need to be performed to gain that information. Instead, as with other isotope-labeling strategies, it enables more confident and straightforward comparisons of the level of any given peptide between samples. This labeling method was used to study phosphorylation of PSII under a variety of conditions and genetic backgrounds (Vainonen et al., 2005; Lemeille et al., 2010; Fristedt and Vener, 2011; Samol et al., 2012).

PSII Life-Cycle Application: Measuring Changes in Oxidation Levels

As discussed above, oxidation of PSII subunits is a well-documented phenomenon, and occurs, at least partially, from oxidizing species generated during the electron transfer reactions of PSII, especially under stress. However, relatively few studies have quantified changes in PSII subunit oxidation under different controlled conditions. Adamska and co-workers (Galetskiy et al., 2011a) used label-free quantification to compare oxidation and nitration (also associated with oxidative stress) levels of thylakoid membrane protein complexes from *A. thaliana* grown under low and high light. They found significantly more modified sites in PSII than in the PSI, cytochrome b_6/f , and ATP synthase complexes. Interestingly, the modified D1, D2, and PsbO sites increased around 2-5-fold, whereas CP47, CP43, PsbE, and PsbR oxidation levels remained roughly constant. D1 and D2 bind most of the redox-active cofactors of PSII, so the increased oxidation especially of these two proteins is not surprising. Similarly, by measuring the increase in 350 nm absorption, Barry and co-workers (Dreaden et al., 2011; Kasson et al., 2012) found that two tryptophan oxidation products increase after exposure to high light, with a corresponding decrease in oxygen evolution activity. Adamska and co-workers (Galetskiy et al., 2011b) found that nitration levels in assembled PSII complexes decrease after exposure to high light, but increase in PSII subcomplexes. This may imply that once nitrated, PSII complexes are damaged and targeted for disassembly and repair.

PSII Life-Cycle Application: Measuring the Temporal Dynamics of Life-Cycle Events using Isotopic Labeling

Measurement of PSII subunit lifetimes has focused mainly on D1, using immunodetection following addition of a protein-synthesis inhibitor or by radioisotope pulse-chase labeling with detection by autoradiography or phosphorimaging (Aro et al., 1993; Mullet and Christopher, 1994; Ohnishi and Murata, 2006). Recently, several studies used ^{15}N labeling pulses and quantified the disappearance of unlabeled PSII subunits using MS. This method

enables simultaneous detection of a larger number of PSII subunits and eliminates any concern of overlapping signal from proteins with similar electrophoretic mobility (Yao et al., 2012b). From surveying nine PSII subunits from *Synechocystis* 6803, Vermaas and co-workers (Yao et al., 2012a) found that protein half-lives range from 1.5 to 33 h in a PSI-less mutant grown under low light ($4 \mu\text{mol m}^{-2}\text{s}^{-1}$ photon flux). In WT *Synechocystis* 6803 grown under $75 \mu\text{mol m}^{-2}\text{s}^{-1}$ photon flux, half-lives of D1, D2, CP47, and CP43 ranged from <1 to 11 h (Yao et al., 2012b). In both studies, D1 exhibited the shortest half-life. These studies highlight the wide range in PSII subunit lifetime and the tight regulation of protein synthesis and PSII assembly that must occur to ensure constant proper stoichiometric availability of all subunits. Interestingly, the chlorophyll half-life was several times longer than that of the core chlorophyll-binding proteins, but the half-life was reduced in the absence of the small CAB-like proteins (SCPs), implying that SCPs play a role in chlorophyll recycling during PSII turnover (Yao et al., 2012a).

Rögner, Nowaczyk, and co-workers demonstrated an elegant application of ^{15}N labeling by purifying several subcomplexes in the PSII life-cycle after a pulse with ^{15}N (from $^{15}\text{NH}_4\text{Cl}$). Comparing extents of incorporation of ^{15}N in different subcomplexes (e.g., monitoring D1 and D2 peptides) reveals the subcomplexes' position in the PSII life-cycle. Using this method, the authors demonstrated that in *T. elongatus*, Psb27 binds to a monomeric subcomplex early in the PSII assembly process (Nowaczyk et al., 2006), and that Psb27 binds again during disassembly to inactive dimers (Grasse et al., 2011). This information fits well with the current understanding of Psb27 as a gatekeeper preventing manganese cluster assembly in immature complexes (Liu et al., 2013a; Mabbitt et al., 2014).

Cyanobacteria contain multiple versions of the *psbA* gene, and the resulting versions of the D1 protein have some different properties and are expressed preferentially under different environmental conditions (for reviews see Mulo et al., 2009; Sugiura and Boussac, 2014). For example, the *psbA1* gene product in *T. elongatus* is dominant under standard growth conditions, but expression of the *psbA3* gene product, which differs from the PsbA1 copy by ~21 residues, increases under high light conditions (Clarke et al., 1993; Kós et al., 2008; Mulo et al., 2009). Characterization of PSII from mutants that express only specific versions of the gene has shown differences in electron-transfer properties, with the implication that PsbA3 assists in photoprotection of PSII under light stress conditions (Sander et al., 2010; Sugiura et al., 2010b). D1-copy expression was mainly monitored on the transcript level (Golden et al., 1986; Komenda et al., 2000; Kós et al., 2008; Sugiura et al., 2010b). However, using ^{15}N labeling and MS-based quantification, Rögner and co-workers showed that PsbA3 incorporation on the protein level could be monitored unambiguously in *T. elongatus* under high light conditions (Sander et al., 2010) and in the ΔpsbJ mutant (Nowaczyk et al., 2012). Those studies used ^{15}N -labeled PSII from a strain that only expresses the PsbA3 copy as a standard for 100% incorporation; relative peak area of the unlabeled PsbA3 peptides compared to this standard is a measure of the incorporation. Such definitive monitoring should allow further detailed studies of *psbA* gene incorporation dynamics.

Progress has also recently been made on the role of the PsbA4 D1 copy; an iTRAQ labeling study found elevated expression of PsbA4 in *Cyanothece* sp. PCC 7822 in the dark (Welkie et al., 2014), providing complementary evidence to that of Pakrasi and co-workers (Wegener et al., 2015) who found that PsbA4 incorporation into PSII renders the complex non-functional. PsbA4 replaces PsbA1 at night in cyanobacterial species that fix nitrogen during this time, protecting against even the trace levels of oxygen evolution that could occur and damage the nitrogenase enzyme (Wegener et al., 2015).

STRUCTURE: DETERMINING PROTEIN-PROTEIN INTERACTIONS IN PSII COMPLEXES

MS-Based Methods to Study PSII Structure

X-ray crystallography remains the benchmark for determining the structure of protein complexes, but besides fully-assembled active PSII, many complexes that form during the PSII life-cycle are too transient and low in abundance to be easily amenable to crystallography. Valuable information about protein-protein interactions within PSII was obtained from immunogold labeling (Tsiotis et al., 1996; Promnares et al., 2006) and yeast two-hybrid assays (Schottkowski et al., 2009; Komenda et al., 2012a; Rengstl et al., 2013), but the former is primarily suitable for large PSII complexes (Dobáková et al., 2009), and the latter is time-consuming and low-throughput. Both provide relatively low-resolution structural information. Recently, advanced structural proteomics techniques bypass the limitations of the above techniques and offer higher-resolution structural data (although still lower than X-ray crystallography). Either chemical cross-linking or protein footprinting followed by MS detection of these modifications are enabled by MS instruments with high sensitivity, resolving power, and <1–5 ppm mass accuracy on orbitrap- and FTICR-based instruments (Table 2). These methods allow not only identification of the binding partners of a specific protein but also a low-resolution mapping of the binding site.

Chemical Cross-Linking

Briefly, the chemical cross-linking technique (reviewed in Sinz, 2014) uses a small molecule with two functional groups on either end that can react with protein residues, separated by a spacer arm (typically less than 14 Å). Many types of cross-linkers are available (Paramelle et al., 2013). The ones most commonly used in PSII research (Bricker et al., 2015) can react with either the primary amine of a lysine and protein N-terminus (and under certain conditions, to a lesser extent with the hydroxyl group of a serine, threonine, or tyrosine, Mädler et al., 2009), or with the carboxylate of aspartate and glutamate side chains and protein C-termini. After both sides of the cross-linker react with neighboring proteins, digestion, LC-MS/MS, and specialized data analysis can identify cross-linked peptides. Inter-protein cross-linked peptides provide structural information about the complex because the two linked residues are constrained to the spacer arm-length distance from each other.

Cross-linking has been used for decades to study protein-protein interactions (Clegg and Hayes, 1974; Wetz and Habermehl, 1979; Waliczek et al., 1989; Back et al., 2003; Sinz, 2014), but its power was limited until modern MS instrumentation and the proteomics platform enabled high-throughput analysis and confident identification of linked peptides (Rappsilber, 2011). Identification of cross-linked peptides by MS is more challenging than for a typical protein digest, especially for large complexes, because the candidate peptide database increases roughly with the square of the number of peptides. As a result, false positives based on the mass spectrum are common even with high mass accuracy instruments, making high-quality product-ion spectra critical for a confident assignment. Despite powerful and constantly improving cross-link search algorithms (Rinner et al., 2008; Xu and Freitas, 2009; Petrotchenko and Borchers, 2010; Götze et al., 2012, 2015; Yang et al., 2012; Hoopmann et al., 2015), manual verification of the product-ion spectra of hits is highly recommended. Successful cross-linking requires high sequence coverage and high mass accuracy as is now practical with orbitrap- and FTICR-based instruments (**Table 2**).

Because cross-linked peptides give typically low-intensity signals compared to those of unlinked peptides, they are often not selected for fragmentation by the instrument's traditional "highest-abundance ion" selection criteria. Several strategies have been developed to improve cross-link selection and/or reduce false positives. They include various methods to enrich for cross-linked peptides before LC-MS/MS (Chu et al., 2006; Kang et al., 2009; Fritzsche et al., 2012; Leitner et al., 2012); use of isotope-coded linkers whose "fingerprint" increases confidence in an identification and can enable real-time guided selection of cross-links for fragmentation (Müller et al., 2001; Pearson et al., 2002; Seebacher et al., 2006; Petrotchenko et al., 2014); and MS-cleavable linkers that simplify data analysis by cleaving a cross-linked peptide into its component peptides before fragmentation (Kao et al., 2011; Petrotchenko et al., 2011; Weisbrod et al., 2013; Buncherd et al., 2014).

Protein Footprinting

Protein footprinting is another MS-based structural technique that has been used to study PSII. Its principle is that a protein residue's solvent accessibility determines its susceptibility to modification by a reagent in the solution; residues buried in a protein-protein interface are less susceptible to modification than surface-exposed residues. These modifications are then detected by MS. Instruments with high sensitivity, resulting in high sequence coverage, are critical so that footprinting experiments yield maximal information (**Table 2**). A common approach is hydroxyl radical footprinting using the well-established technique of synchrotron radiolysis of water to generate the radicals (Takamoto and Chance, 2006; Wang and Chance, 2011). Fast photochemical oxidation of proteins (FPOP) is a more recent hydroxyl radical footprinting technique that uses a laser pulse to generate the radicals and can probe protein dynamics that occur on a faster timescale, down to microseconds (Gau et al., 2011). Hydroxyl radical footprinting can modify 14 of the 20 amino acid side chains (Wang and Chance, 2011). Another technique,

glycine ethyl ester (GEE) labeling, adapts a long-standing method for modifying and cross-linking carboxylate groups in proteins (Hoare and Koshland, 1967; Swaisgood and Nataka, 1973) for protein footprinting (Wen et al., 2009; Gau et al., 2011). It is easier to implement than hydroxyl radical footprinting, and data interpretation is simpler, but it can only probe changes on aspartate, glutamate, and protein C-termini.

PSII Life-Cycle Application: Cross-Linking and Footprinting to Determine Interactions among PSII Subunits

Early cross-linking studies on PSII provided information about subunit connectivity before PSII crystal structures were available. Many studies focused on the luminal extrinsic proteins (Enami et al., 1987; Bricker et al., 1988; Odom and Bricker, 1992; Han et al., 1994), which are more easily accessible to soluble cross-linkers, but interactions involving the transmembrane subunits can also be detected (Tomo et al., 1993; Seidler, 1996; Harrer et al., 1998). In the absence of the MS-based platforms currently available, gel electrophoresis and immunoblotting identify cross-linked products and their likely component proteins. Those methods are still helpful today as confirmation and when cross-linked peptides are not detected by MS (Hansson et al., 2007; Nagao et al., 2010; Liu et al., 2011a, 2014b), but MS provides much greater confidence in the identification and pinpoints the exact cross-linked residues. Notably, Satoh and co-workers (Enami et al., 1998) used FAB-MS to identify intramolecular cross-linked peptides in PsbO, and deduced the linked residues even without MS/MS capability.

Since these early studies, crystal structures have elucidated the connectivity between the components of active cyanobacterial PSII. As a result, more recent cross-linking studies have focused on accessory proteins that bind only to subcomplexes and/or that are not found in the crystal structures, though work has continued on the luminal extrinsic PSII subunits from algae and higher plants, PsbP and PsbQ, which differ significantly from their cyanobacterial counterparts (Bricker et al., 2012; results are summarized in **Table 5**). Cross-linking-MS has also been recently applied to study interactions within the phycobilisome (Tal et al., 2014) and between the phycobilisome and the photoprotective orange carotenoid protein (OCP) (Zhang et al., 2014; Liu et al., 2016), reviewed in Bricker et al. (2015).

With complementary use of the cross-linkers EDC and DTSSP, Pakrasi and co-workers (Liu et al., 2011a) demonstrated that the accessory protein Psb27 binds on the luminal surface of CP43. Because this interaction is transient and occurs in only a small fraction of PSII centers in the cell at a given time, the authors purified PSII complexes from the $\Delta ctpA$ mutant strain of *Synechocystis* 6803 that accumulates such complexes (Liu et al., 2011b), maximizing chances of capturing and observing Psb27 inter-protein cross-links. The two cross-linked species detected were used to map Psb27 onto the PSII crystal structure, showing how Psb27 accomplishes its role as a gatekeeper, protecting partially assembled PSII complexes from gaining premature harmful water oxidation activity (Roose and Pakrasi, 2008). Recently, Nowaczyk and co-workers (Cormann et al., 2016)

identified a different cross-link between Psb27 and CP43 in *T. elongatus* using an isotope-encoded version of the BS³ cross-linker. Despite the different cyanobacterial species used in the two studies, and the different Psb27 residues that were cross-linked, both cross-links localize Psb27 to the same domain on CP43 (Liu et al., 2011a; Cormann et al., 2016).

Cyanobacterial PsbQ is a component of active PSII (Roose et al., 2007), but is not found in any of the crystal structures, presumably because it is destabilized under crystallization conditions. Pakrasi and co-workers (Liu et al., 2014b) again used EDC and DTSSP in parallel and detected a PsbQ-CP47 and two PsbQ-PsbO cross-links by MS. A PsbQ-PsbQ cross-link that appears to arise from two different copies of the protein was also detected. Taken together, these results position PsbQ along the luminal PSII dimer interface, consistent with evidence that PsbQ stabilizes the PSII dimer (Liu et al., 2014b). In this study, in-solution digestion was used instead of in-gel digestion to avoid losses of large cross-linked peptides that are difficult to extract from the gel matrix.

Several recent studies have probed the binding sites of the higher plant luminal extrinsic proteins PsbP and PsbQ, which help optimize Ca²⁺ and Cl⁻ binding properties at the oxygen-evolving center (Bricker et al., 2012). Ifuku and co-workers (Ido et al., 2012, 2014) identified cross-links in spinach PSII between PsbP and PsbE, PsbR, and CP26 by MS and provided MS-based evidence for PsbP-CP43, PsbQ-CP43 and PsbQ-CP26 cross-links. The suggestive evidence arose from MS identification of CP43 or CP26 in individual cross-linked gel bands after affinity pull-downs using biotin-tagged PsbP or PsbQ (Ido et al., 2014). Their binding model for PsbP is different than that proposed by Bricker and co-workers (Mummadiseti et al., 2014), who identified nine intra-protein cross-links between the N-terminal and C-terminal regions of spinach PsbP that constrain significantly its binding conformation. The authors also identified a PsbP-PsbQ cross-link, consistent with that observed in *C. reinhardtii* by Enami and co-workers (Nagao et al., 2010).

The PsbQ-CP43 interaction in spinach PSII suggested by Ifuku and co-workers (Ido et al., 2014) contrasts with the PsbQ-CP47 cross-link identified in *Synechocystis* 6803 by Pakrasi and co-workers (Liu et al., 2014b) and their evidence for a PsbQ-PsbQ interaction at the PSII dimer interface. Significant sequence differences between cyanobacterial and plant PsbQ may explain this discrepancy. Bricker and co-workers (Mummadiseti et al., 2014) also found cross-linking evidence for a PsbQ-PsbQ interaction in spinach that may require a position at the dimer interface, consistent with the Pakrasi group's results in *Synechocystis* 6803. However, they suggest that that interaction could in theory arise from an inter-PSII-dimer interaction, and, thus, the results could alternatively be consistent with the Ifuku group's positioning of spinach PsbQ near CP43. Interestingly, the recently published crystal structure of PSII from the eukaryotic red alga *Cyanidium caldarium* indeed shows PsbQ' binding to the luminal surface of CP43 (Ago et al., 2016). PsbQ' shares relatively low sequence homology to green algal or higher plant PsbQ; and though PsbQ' can functionally replace PsbQ at least partially in *C. reinhardtii*, it cannot bind to spinach PSII (Ohta et al., 2003). Therefore, the red algal PsbQ'-CP43 interaction supports Ifuku

and co-workers' (Ido et al., 2014) similar conclusion in spinach, but at the same time it does not necessarily contradict the alternate PsbQ-CP47 interaction observed by the other groups in spinach and *Synechocystis* 6803. The recent characterization of an active PSII complex from *Synechocystis* 6803 with multiple copies of the PsbQ protein (Liu et al., 2015) hints at one possible reconciliation of these findings, if such a complex is present in other species as well. Despite some discrepancies, these results begin to elucidate the binding orientation of the higher plant luminal extrinsic proteins, suggesting a mechanism for stabilization of PSII-LHCII supercomplexes (Ido et al., 2014), and paving the road for further structural studies.

Although the advanced techniques for improving cross-link identification described in the methods section above have largely not yet been applied to PSII studies (with the exception of the recent use of isotope-encoded BS³ by Nowaczyk and co-workers, Cormann et al., 2016), several other creative approaches have been used. Enami and co-workers (Nagao et al., 2010) improved identification confidence by detecting the same cross-linked residues in peptides from two separate digestion experiments, one with trypsin and one with Asp-N. Pakrasi and co-workers (Liu et al., 2011a) provided strong evidence, using the thiol-cleavable cross-linker DTSSP and 2D gel electrophoresis, that Psb27 and CP43 cross-link to each other, allowing targeted data analysis and providing higher confidence in the subsequent MS cross-link identification. Ifuku and co-workers (Ido et al., 2012, 2014) used a biotin-tagged PsbP or PsbQ to purify only those cross-linked proteins. Although this method is not as efficient as purifying only cross-linked peptides by means of a tagged linker, because following digestion many non-linked peptides from the tagged protein will be present, it does simplify sample complexity and focuses on cross-links containing a particular protein of interest. Notably, Blankenship and co-workers (Liu et al., 2013b) demonstrated that *in-vivo* cross-linking of thylakoid membrane complexes is possible and can capture interactions between protein complexes that are otherwise difficult to preserve after cell lysis. Using the membrane-permeable cross-linker DSP, they captured a PSII-PSI-phycoobilisome megacomplex and identified five cross-links between PSII subunits and the PBS, and five between PSI subunits and the PBS, providing the first molecular-level description of the interface of these complexes.

Like cross-linking, protein footprinting is a technique that has long been used in PSII structural studies but that has become significantly more powerful in combination with modern MS. Early studies using N-hydroxysuccinimidobiotin (NHS-biotin) and other modification reagents investigated the binding site of higher plant PsbO to PSII. In the absence of MS detection, specific modification sites could either not be identified (Bricker et al., 1988) or were localized to particular protein domains by N-terminal sequencing of peptides (Frankel and Bricker, 1992). With the rise of protein MS in the mid-1990s, MALDI-TOF and FAB-MS were used to identify modified peptides; lack of MS/MS capability, however, produced lower-confidence peptide identification than is achievable today, and meant that specific modified residues could only be pinpointed in favorable cases (Frankel and Bricker, 1995; Miura et al., 1997; Frankel

et al., 1999). Nonetheless, these pioneering footprinting studies demonstrated, e.g., that PsbO interacts with Loop E of CP47 (Frankel and Bricker, 1992), and that charged residues on the surface of PsbO are involved in its interaction with PSII (Miura et al., 1997; Frankel et al., 1999).

Recently, hydroxyl radical footprinting using synchrotron radiolysis of water was used to study the binding surfaces of spinach PsbP and PsbQ to PSII, with detection of modified residues by MS (Mummadiseti et al., 2014). The results reveal buried regions on the surface of these proteins that complement the authors' cross-linking data and suggest these proteins' binding interfaces to other PSII subunits. The data also confirm and elaborate on the binding region identified by this group in a previous study using NHS-biotin as footprinting reagent (Meades et al., 2005).

Although the above footprinting studies detected whether or not a residue was modified in a given state, it is also possible to analyze footprinting data quantitatively to detect a conformational change in a complex in two different states. The label-free approaches described above can be used to monitor the relative change in modification, normalized to the unmodified peptide, in different PSII complexes. The utility of this approach was demonstrated in a study of the role of Psb27 in PSII assembly (Liu et al., 2013a) using GEE labeling. The authors monitored the relative changes in aspartate and glutamate modification of three PSII complexes representing different stages of PSII assembly, not only extending previous information about the Psb27 binding site (Liu et al., 2011a; Komenda et al., 2012a), but also demonstrating a conformational change upon D1 processing that prompts Psb27 dissociation and permits assembly of the oxygen evolving complex (Liu et al., 2013a). Blankenship and co-workers have also used quantitative GEE labeling to detect a light-dependent conformational change in the OCP protein that appears to underlie its photoprotective function (Liu et al., 2014a). The recent implementation of isotopically-labeled GEE (iGEE) footprinting (Zhang et al., 2016) will streamline, and increase confidence in, quantitative comparisons of modification extent between states.

Hydroxyl radical footprinting has also been used to identify putative water and oxygen channels in PSII (Frankel et al., 2013a), a topic that has been explored previously through computational studies (Murray and Barber, 2007; Ho and Styring, 2008; Gabdulkhakov et al., 2009; Vassiliev et al., 2012). This study provides general experimental support for the existence of such channels, confirms specific channel identifications from computational work (Ho and Styring, 2008; Vassiliev et al., 2012), and proposes a previously unidentified putative oxygen/ROS exit channel (see Bricker et al., 2015 for a discussion of the MS-based and computational results).

FUTURE DIRECTIONS

MS technology and associated sample preparation techniques are evolving rapidly. Increasing sensitivity and speed of instruments for bottom-up proteomics allows better coverage of transmembrane PSII proteins; for example, coverage of the core D1, D2, CP47, and CP43 proteins is routinely ~50–85% on a Thermo Q-Exactive Plus instrument, whereas ~20–40%

coverage was reported on LTQ-Orbitrap, LTQ-FTICR, and MALDI-TOF instruments (Aro et al., 2005; Frankel et al., 2012; Liu et al., 2013a,b). This increased coverage will mean that more PTMs and cross-linked peptides can be identified, and a larger portion of the PSII complex can be mapped by footprinting. PTM analysis, especially using quantitative techniques to compare complexes exposed to different conditions, may help elucidate signals (largely unknown in cyanobacteria) that trigger D1 degradation. The increasing availability of high-sensitivity instruments that can achieve high sequence coverage is enabling detailed quantitative and non-quantitative global proteomic studies. The new challenge is to reduce the large amounts of information becoming available into specific testable hypotheses for targeted follow-up studies.

Improvements at all stages of the cross-linking workflow are occurring, from linker design to linked-peptide enrichment and software analysis. Specifically, isotope-labeled and MS-cleavable linkers are powerful tools that are just beginning to be applied to PSII research. *In-vivo* cross-linking is a promising approach to detect transient or unstable interactions that are difficult to capture after cell lysis. Cross-linking may enable binding site identification for at least some of the approximately 30 accessory proteins now known or believed to associate with PSII during its life-cycle (Nickelsen and Rengstl, 2013; Järvi et al., 2015). Detecting interactions between PSII subcomplexes and, e.g., D1 degradation proteases or proteins involved in chlorophyll loading would also be of prime interest.

Intact-mass measurements of the large core PSII proteins D1, D2, CP47, and CP43 were reported in several studies (Sharma et al., 1997b; Whitelegge et al., 1998; Huber et al., 2004; Thangaraj et al., 2010), with detection of the phosphorylated form of D1 as well in some cases (Whitelegge et al., 1998; Huber et al., 2004). However, their top-down analysis has not yet been achieved. Top-down technology is continuously developing, especially methods for increased product-ion sequence coverage (Frese et al., 2012; Shaw et al., 2013; Brunner et al., 2015) and analysis of larger integral membrane proteins and their PTMs (Ryan et al., 2010; Howery et al., 2012). Such analysis will make it easier to identify nearly-stoichiometric (and potentially important) PTM events from trace ones under different conditions, not an easy task using bottom-up MS. Native MS is capable of analyzing certain intact membrane protein complexes, although the technology is still developing, and no one approach works for all protein complexes (reviewed in Mehmood et al., 2015). Native MS analysis of PSII has not yet been demonstrated, but the technique could in theory serve as a complementary method to native gels to characterize the distribution of PSII subcomplexes under various conditions, and their components. This might be particularly useful to address the stoichiometry of accessory proteins and cofactors, and could add a new tool to address the long-standing question of chlorophyll loading in PSII.

CONCLUSION

The use of MS has been fueled by improvements in sample preparation methods for analysis of membrane proteins, increasing availability of MS instrumentation, and significant advances in instrument sensitivity, speed, and mass accuracy.

Techniques from each of the four pillars of proteomics will continue to be employed to study the PSII life cycle. These techniques have addressed a wide range of questions regarding the composition of PSII complexes, the time-dependent dynamic changes of individual subunits and complexes under different environmental conditions, and the tertiary and quaternary structure of PSII complexes.

Modern MS techniques provide a higher level of detail and confidence than previous methods; examples are identification of a protein's phosphorylation site instead of mere detection of a phosphorylated protein, and identification of specific cross-linked residues instead of only suggestive evidence that two particular proteins are cross-linked to each other. For other applications, the use of MS permits entirely new questions to be asked (e.g., what proteins are present on a proteome-wide scale for a purified PSII complex).

The new information has opened up new questions about function. For example, what are the physiological roles of the many new PTMs that have been identified? What purpose does an accessory protein serve by binding at this particular location on a PSII complex? In some cases, the sensitivity of MS is a potential pitfall: identification of a protein in a PSII sample does not necessarily mean it is a stoichiometric component, or that it associates specifically with the complex at all. Thus, information from MS should be a starting point for more targeted

genetic and biochemical studies, and MS is one component of an expanding toolbox for PSII life-cycle research. Rapidly developing MS technology promises continued contributions to this field, which has a wide range of fascinating questions about membrane protein complex composition, dynamics, and structure yet to be answered.

AUTHOR CONTRIBUTIONS

HP, MG conceived of the article. DW collected data from the literature and drafted the manuscript. HP, MG, and DW revised the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. Hao Zhang (Washington University) and Dr. Phil Jackson (University of Sheffield) for helpful discussions. This work was supported by the Photosynthetic Antenna Research Center, an Energy Frontier Research Center funded by the U.S. Department of Energy (DOE), Office of Basic Energy Sciences (Grant DE-SC 0001035 to MG and HP), by the Chemical Sciences, Geosciences, and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (grant no. DE-FG02-99ER20350 to HP), and by the NIH (Grant P41GM103422) to MG.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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