Mass Spectrometric Resolution of Reversible Protein Phosphorylation in Photosynthetic Membranes of Arabidopsis thaliana*

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The use of mass spectrometry to characterize the phosphorylome, *i.e.* the constituents of the proteome that become phosphorylated, was demonstrated using the reversible phosphorylation of chloroplast thylakoid proteins as an example. From the analysis of tryptic peptides released from the surface of *Arabidopsis* thylakoids, the principal phosphoproteins were identified by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. These studies revealed that the D1, D2, and CP43 proteins of the photosystem II core are phosphorylated at their N-terminal threonines (Thr), the peripheral PsbH protein is phosphorylated at Thr-2, and the mature light-harvesting polypeptides LCHII are phosphorylated at Thr-3. In addition, a doubly phosphorylated form of PsbH modified at both Thr-2 and Thr-4 was detected. By comparing the levels of phospho- and nonphosphopeptides, the *in vivo* phosphorylation states of these proteins were analyzed under different physiological conditions. None of these thylakoid proteins were completely phosphorylated in the steady state conditions of continuous light or completely dephosphorylated after a long dark adaptation. However, rapid reversible hyperphosphorylation of PsbH at Thr-4 in response to growth in light/dark transitions and a pronounced specific dephosphorylation of the D1, D2, and CP43 proteins during heat shock was detected. Collectively, our data indicate that changes in the phosphorylation of photosynthetic proteins are more rapid during heat stress than during normal light/dark transitions. These mass spectrometry methods offer a new approach to assess the stoichiometry of *in vivo* protein phosphorylation in complex samples.

The reversible phosphorylation of specific proteins participates in the regulation of virtually all aspects of cell physiology and development. The extent of its importance is illustrated by the hundreds of conventional protein kinases and phosphatases detected in various eukaryotic genomes (1–3). Whereas, serine, threonine, and tyrosine residues are the typical targets of these kinases, phosphorylation of at least six other amino acids is feasible, potentially expanding even further the dimensions of this post-translational modification (reviewed in Ref. 4). Despite the importance of this pool of phosphorylated proteins, our understanding of its depth and breadth remains sketchy. One barrier has been the lack of methods to define *en masse* the “phosphorylome,” *i.e.* the subset of proteins in the proteome that become modified *in vivo* by phosphorylation. Precise characterization of the phosphorylome will be essential to fully understand how proteins are activated or inhibited, encouraged to interact with other components in the cell, and selected for rapid degradation. Certainly, the dynamic and transient nature of many protein phosphorylation reactions underscores the difficulties of resolving the complete phosphorylome for a given organism. Nevertheless, the identification of even just the principal cellular phosphoproteins under distinct physiological conditions should bring significant biological insights.

The most common method for analysis of the phosphorylome involves the use of radioactive labeling either *in vivo* or *in vitro*. However, uneven uptake of the label in complex multicellular organisms, the large pools of endogenous free phosphate, and the presence of pre-existing bound phosphate often limit conclusions. Phosphoamino acid antibodies have been exploited recently but their use is restricted to tailor-made immunological applications and because these antibodies cannot detect the nonphosphorylated form they are unable to determine stoichiometry. More recently, mass spectrometry (MS) has been applied to analyses of protein phosphorylation (5–10). This highly sensitive technique offers the advantage of scanning complex mixtures for phosphoproteins that become modified *in vivo*. Moreover with appropriate considerations, we show here that MS can also be used to provide estimates of the phosphorylation state of specific proteins.

To demonstrate the utility of MS, we have applied this technique to the analysis of the major phosphoproteins in the chloroplast thylakoid, the membrane containing the photosynthetic light reactions of photosystem (PS) I and II, light-harvesting chlorophyll a/b complex (LHClI), cytochrome b6f complex, and the ATP synthase (11, 12). Multiprotein complexes within the thylakoids are responsible for light-driven oxidation of water with concomitant release of oxygen and the production of energy and reducing potentials. During these reactions, re-
versatile phosphorylation is thought to play critical roles in (i) the redistribution of excitation energy between PSI and II via modification of LHCII (11–13), and (ii) the maintenance of the PSI by controlling the turnover of its reaction center polypeptides (14–18). Studies in spinach and pea using $^{32}$P labeling and phosphoamino acid antibodies showed that a number of proteins are phosphorylated, including threonine residues at or near the N termini of LHCII (19) and PSII polypeptides, the D1 and D2 reaction center proteins, chlorophyll-binding protein CP43 (20), and peripheral polypeptide PsbH (21).

However, given the limitations of $^{32}$P labeling and immunohistological assays, it remains unclear how important chloroplast protein phosphorylation is to the normal function and regulation of the photosynthetic light reactions. In vitro studies using $^{32}$P labeling have suggested that a number of thylakoid proteins are extensively phosphorylated in the light (22) by a kinase controlled by the photosynthetic electron transport chain (12, 23, 24), and dephosphorylated in the dark by phosphatase(s) that are not light sensitive (12, 25). More recent studies using phosphothreonine antibodies questioned the magnitude of this phosphorylation by showing that some thylakoid phosphoproteins remain phosphorylated even in dark-adapted plants (26–28). Furthermore, the maximal phosphorylation of LHCII only occurs at low light and is drastically decreased at higher irradiations (26). Phosphorylation of spinach LHCII was also found to increase in darkness upon exposure of leaves to heat shock (27).

Here, we report the identification of the major phosphoproteins in the thylakoid membranes from *Arabidopsis thaliana* and map their phosphorylation sites using both matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) MS. Measurements of the phosphorylation level of each protein revealed that changes in the steady state phosphorylation during normal light/dark growth cycles may be less extensive and slower than previously thought and may be more significant and rapid during a response to stress (e.g., heat shock). The MS techniques described here should be applicable for many analyses involving complex mixtures of phosphoproteins.

**EXPERIMENTAL PROCEDURES**

**Preparation of Chloroplasts and Thylakoids—** *A. thaliana* ecotype Columbia-0 was grown at 21 °C either in soil or on 0.7% (w/v) agar containing one-half strength MS media (Life Technologies, Inc.). Plants were irrigated with 100 μmol m$^{-2}$ s$^{-1}$ of white light provided by fluorescent lights with a photoperiod of 16-h light/8-h dark. Chloroplasts and thylakoids were prepared from 3-week-old plants. Chloroplasts were extracted from the leaves and purified by Percoll gradient centrifugation according to Ref. 29. To isolate thylakoids, the chloroplasts were resuspended in 7 ml of 10 mM sodium phosphate (pH 7.5), 5 mM MgCl$_2$, 5 mM NaCl, and homogenized 10 times in a Potter grinder. The homogenate was diluted to 30 ml with the same buffer and thylakoids were collected by centrifugation for 5 min at 4000 × g.

For direct preparation of thylakoids, 5 g of leaves were homogenized with a Polytron (Brinkmann PT 10/35) in 25 ml of ice-cold extraction buffer, containing 300 mM sorbitol, 50 mM sodium phosphate (pH 7.5), 5 mM MgCl$_2$, 10 mM NaF. The suspension was filtered through four layers of Miracloth and centrifuged for 3 min at 1500 × g. The pellet was resuspended in 7 ml of lysis buffer (10 mM sodium phosphate (pH 7.5), 5 mM MgCl$_2$, 1 mM NaF) and homogenized 10 times in a Potter grinder. The suspension was diluted to 30 ml with the lysis buffer and centrifuged for 5 min at 4000 × g. The pellet was resuspended in 3 ml of the extraction buffer with the Potter grinder and layered on the top of a sucrose step gradient, containing (bottom to top): 10 ml of 1.8 M sucrose in 50 mM sodium phosphate (pH 7.5) and 10 mM NaF. After centrifugation in a swinging bucket rotor for 15 min at 4000 × g, the thylakoid fraction was collected from the 1.8 M sucrose interface. Thylakoids were diluted to 25 ml with extraction buffer and collected by a 5-min centrifugation at 4000 × g. The thylakoid pellet was resuspended in 1 ml of 25 mM NH$_4$HCO$_3$ (pH 8.0), 10 mM NaF, and pelleted again using a microcentrifuge.

**In Vitro Phosphorylation Reactions—** Chloroplasts (0.2 mg of chlorophyll) were gently resuspended in 1 ml of 20 mM Tricine (pH 8.0), 330 mM sorbitol, 6.6 mM MgCl$_2$, 1 mM Na$_2$HPO$_4$, and incubated at 22 °C. Phosphorylation was induced by a 10–20 min irradiation with 100 μmol m$^{-2}$ s$^{-1}$ of white light and terminated by addition of 10 μl of ice-cold 20 mM Tricine (pH 8.0), 5 mM NaF, 20 μM EDTA, 10 mM NaF. Thylakoids were prepared from chloroplasts as described above. When isolated thylakoids were used, they were resuspended in 20 mM Tricine (pH 8.0), 100 mM sorbitol, 5 mM MgCl$_2$, 1 mM ATP and irradiated as described above.

**Preparation of Thylakoid Peptides and Phosphopeptides—** Isolated thylakoids were washed twice with 25 mM NH$_4$HCO$_3$ (pH 8.0), 10 mM NaF by centrifugation and resuspension in the same buffer to a concentration of 1.3–1.5 mg of chlorophyll/ml. The suspension was incubated with sequencing-grade modified trypsin (Promega) (8 μg of trypsin/mg of chlorophyll) at 22 °C for 90 min. The digestion products were frozen, thawed, and clarified at 14,000 × g. The supernatant containing released thylakoid peptides was collected. Similar peptides were collected from spinach thylakoids as described (30).

Phosphopeptides were affinity enriched from the thylakoid peptide fraction by chromatography with immobilized Fe(III) or Ga(III) columns (31, 32). Typically, columns containing 50 μl of chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) beads were washed with 0.3 ml of water, 0.3 ml of 0.1% (v/v) acetic acid, charged with 0.3 ml of 0.1 mM FeCl$_3$ or GaCl$_3$, and washed with 0.5 ml of 0.1% (v/v) acetic acid. The thylakoid peptides (0.2–0.5 μg) were mixed with 20 μl of 0.1% (v/v) acetic acid and loaded onto the columns. After washing twice with 0.2 ml of 0.1% (v/v) acetic acid, bound phosphopeptides were eluted with 300 μl of either 20 mM sodium phosphate buffer (pH 7.0) (for Fe(III) (31)), or 20 μl of nonbuffered Na$_2$HPO$_4$ (for Ga(III) (32)).

**Synthetic Phosphopeptides—** Synthetic phosphopeptides included: APRT$^\ast$PGGR, CDGVTTKT$^\ast$TAGTPTD, CDGVTTKT$^\ast$FAQTD, LIPQQ$^\ast$INEAK, DRHD$^\ast$GLDS$^\ast$NKDE, DRHD$^\ast$GLDS$^\ast$NKDE, CDRHD$^\ast$GLDS$^\ast$NKDE, and GRPRRT$^\ast$FAE (where $^\ast$ and $^\ast$ indicate phosphothreonine and phosphoserine, respectively). To obtain the corresponding dephosphopeptides, 0.25 nmol of each phosphopeptide was dissolved in the phosphate buffer (25 mM NH$_4$HCO$_3$ (pH 8.0), 10 mM MgCl$_2$, 2 mM dithiothreitol) and incubated for 2 to 24 h at 37 °C with the addition of 1 to 5 units of alkaline phosphatase (New England Biolabs, Beverly, MA). The extent of dephosphorylation was monitored by MALDI-TOF MS.

**MALDI-TOF MS Analyses—** Samples were prepared by mixing 1–2 μl of each mixture with 1–2 μl of a c-acyclo-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile with 2% (v/v) trifluoroacetic acid. One μl of final mixture was spotted on the target. Linear and reflector mass spectra were recorded using a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker, Billerica, MA) operated in delayed extraction mode using an accelerating voltage of 19 kV. Spectra were calibrated externally. Post-source decay (PSD) spectra were recorded using Bruker's FAST procedure.

**LC-ESI MS Analyses—** Peptide mixtures were separated on 5 μm of C18 MetaChem 150 × 1.0-mm column at a flow rate 20 μl/min. A gradient of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) was distributed as follow: 0% B in first 3 min; 0–20% B in 3 to 20 min; 20–70% B in 20 to 105 min; 70–99% B in 105 to 115 min. The online detection with positive/negative-ion mode switching was performed using an API 365 triple quadrupole MS with a standard ionspray source (Applied Biosystems/MD Sciex, Foster City, CA). Each 5.2-s positive-ion scan in the m/z range from 320 to 1800 was followed by a 0.7-s pause for polarity switching and 1.5-s single ion-monitoring of negative 79 and another 0.7-s pause to return to positive-ion mode. In the positive-ion mode, ion source, orifice, and ring voltages were set at 5 kV, 12 V, and 140 V, respectively, to minimize fragmentation. However, in some cases a small amount of nonphosphorylated ion species was generated from the phosphopeptide by partial skimmer-induced fragmentation (see Fig. 3C, for example). In the negative-ion mode, the ion source, orifice and ring voltages were set at −4.5 kV, −200 V, and −300 V, respectively, to maximize the phosphoryl-79 signal.

For skimmer collision-induced dissociation (CID), the ion source, orifice, and ring voltages were set at 5 kV, 95 V, and 200 V, respectively, to maximize peptide fragmentation. Sequencing of high performance liquid chromatography-purified peptides was performed by tandem MSMS using conditions recommended by Applied Biosystems/MD Sciex (Foster City, CA).

**RESULTS**

Previous studies with spinach and pea chloroplast thylakoids showed that the primary sites for phosphorylation in...
volve polypeptide regions exposed to the outer surface of the membranes (30, 33). To enrich for these regions, thylakoid membranes were purified from chloroplasts isolated from Arabidopsis leaves and then “shaved” with trypsin to release surface-exposed peptides from the various constituent proteins (Fig. 1A). Although a variety of proteins were digested, several proteins and/or protein domains that we presumed were protected by the membrane remained intact. LC-ESI MS analyses of the released fraction revealed approximately a thousand major peptides liberated by this protease treatment. By using the characteristic decomposition products of phosphopeptides following the breakdown of phosphoryl-peptide linkages as a signature (5, 6, 8), numerous phosphopeptides were identified. We enriched for phosphopeptides by immobilized metal (Fe(III) and/or Ga(III)) affinity chromatography (IMAC) (31, 32). Because the binding specificity and elution properties of Fe(III) and Ga(III) IMAC differ, both were used to isolate a range of phosphopeptides (see Table I). Although nonphosphorylated peptides were also present in the eluted fractions, the partial purification of phosphopeptides by IMAC greatly simplified their identification and initial analysis.

During MALDI-TOF-MS, phosphopeptides lose phosphoric acid as $H_3PO_4$ (98 Da) and $HPO_3$ (80 Da) with the concomitant production of metastable ions (6). Whereas both the metastable and parent phosphopeptide ions arrive at the detector simultaneously and thus produce one coherent signal in the linear mode, they generate separate signals in the reflector mode, with the metastable ions arriving sooner than the parent ion. The appearance of this metastable ion is especially evident for peptides containing phosphoserine and phosphothreonine, which generate intense daughter signals upon losing $H_3PO_4$ (98 Da) (6). In our experimental settings, these metastable ions actually appeared as ions 86 $m/z$ rather than 98 $m/z$ smaller than the parent ions (Fig. 1D). This difference was a result of the metastable ion flying out of focus from the ion mirror, a phenomenon that also led to a broad ion signal that is characteristic of an ion lacking isotope resolution (Fig. 1B).

From the analysis of a series of synthetic phosphopeptides (see “Experimental Procedures”), we found that all generated this metastable ion peak in the reflector mode regardless of the amino acid sequence or position of the phosphoserine or phosphothreonine residue. In fact, doubly phosphorylated peptides produced two metastable ions in the reflector mode, 86 $m/z$ and 172 $m/z$ smaller than the parent ion. We could reliably detect these metastable ions with as little as femtomole amounts of these synthetic phosphopeptides, indicating that they could be detected with high sensitivity. Thus, we exploited the presence of metastable signal in the reflector mode at −86 $m/z$ and its unique shape as a reliable indicator for phosphopeptides.

Using this metastable ion signature, numerous phosphopeptides were detected by MALDI-TOF MS in the IMAC-enriched fractions of peptides released from thylakoids of light-adapted plants. As an example, Fig. 1 shows the MALDI-TOF MS detection of a single ion cluster at 1355.6 $m/z$ in the linear mode (Fig. 1C) that behaved as two ion clusters in the reflector mode, one for the parent ion at 1355.6 $m/z$ and another for the metastable ion at 1269.6 $m/z$, which was 86 $m/z$ smaller and devoid of isotope resolution (Fig. 1B). By this approach we detected eight phosphopeptides abundant in the IMAC-enriched fractions (Table I). In several cases, we also detected these phosphopeptides in the crude trypsin hydrolysates before IMAC enrichment. As shown in Fig. 1, D and E, the metastable 1269.6 $m/z$ ion for the 1355.6 parent ion was readily detected in the reflector mode but absent in the linear mode.

The thylakoid phosphopeptides identified by MALDI-TOF MS were sequenced using MALDI-TOF PSD, ESI MS/MS, and LC with online ESI-skimmer CID MS. MALDI-PSD MS of the 1355.6 $m/z$ phosphopeptide identified its sequence as Ac-RKTpVAKPK 1048.8 amu (Table I). This phosphopeptide identified its sequence as Ac-RKTpVAKPK 1048.8 amu, which was 86 $m/z$ smaller and devoid of isotope resolution (Fig. 1B). The molecular masses of these phosphopeptides were determined by MALDI-PSD MS (Table I). The sequence data base revealed that this sequence belonged to the chloroplast-encoded CP43 subunit of PSII, assuming that the first 14 amino acids of the initial translation product were removed and the resulting N-terminal threonine residue was acetylated. Because the phosphate moiety is readily lost from phosphopeptides during MALDI-TOF-MS, we used complementary ESI-CID MS sequencing to unambiguously identify the phosphorylation site(s). The most efficient method was the
ESI-skimmer CID MS when used online with LC separation of IMAC-enriched peptides. Fig. 2B shows the mass spectrum containing mostly y (C-terminal) and b (N-terminal) fragment ions of the N-terminal phosphopeptide from CP43. The fragmentation pattern was consistent with the N-terminal threonine being both N-acetylated and O-phosphorylated.

The LC with online ESI-skimmer CID MS also revealed the presence of an unexpected isomer of CP43 in which the amino acid at position 4 was aspartic acid not asparagine (Fig. 2C). This aspartate isomer was found in all thylakoid preparations isolated from plants under a variety of conditions and comprised ~15% of the total CP43 pool. Given that the Arabidopsis CP43 gene encodes asparagine at this position (34), it is likely that this isoform was created by a deamidation reaction, the nature of which is currently unknown.

By similar analysis, we determined the sequence of the seven other phosphopeptides and identified the corresponding proteins in the Arabidopsis sequence data bases (Table I). Besides CP43, the D1 and D2 proteins of the PSII reaction center were identified and found to also contain an N-terminal threonine that was both N-acetylated and O-phosphorylated. We identified two phosphopeptides that corresponded to the mature LHCIICII poly peptides phosphorylated at the Thr-3 (Table I). One represented the expected tryptic fragment (TVAKPK) whereas the other was two amino acids longer and N-acetylated (Ac-RKTVAKPK). The second form likely represented an incomplete digestion product caused by the phosphate at Thr-3 blocking trypsin cleavage after Lys-2. The PsbH protein of PSII was phosphorylated at Thr-2. Notably, we also found a doubly phosphorylated form of this peptide containing a second phosphate bound to Thr-4 (Fig. 2D and Table I). The doubly phosphorylated form of PsbH was also detected in spinach thylakoids, suggesting that its presence is widespread in higher plants.

To study the phosphorylation state of these principal Arabidopsis thylakoid proteins in different physiological conditions, we developed an ESI MS method to compare the levels of the phospho and nonphospho forms for each. First, we determined the LC elution positions of the eight phosphopeptides present in the complete tryptic peptide mixtures without IMAC enrichment. This LC separation did not completely resolve these complex mixtures but did make spectrometric identification of separate peptide ions in each fraction possible (Fig. 3A). Following LC, the peptides were detected online by ESI-MS and their masses were determined from full scan data in the positive-ion mode. Phosphopeptides were concurrently identified by switching to the negative-ion mode every 6 s; this mode led to peptide fragmentation and production of ions of ~79 m/z, which are diagnostic for PO<sub>4</sub> (5, 7). By simultaneous analysis in the positive-ion mode and single ~79 m/z ion monitoring in the negative-ion mode, the LC retention times of all thylakoid phosphopeptides described in Table I were determined (Fig. 3, A and B). The intensity of the ~79 m/z ion signal depended on the nature of a parent phosphopeptide and its propensity to decompose. For example, the ~79 m/z signals produced by fragmentation of N-terminal phosphothreonines of D1, D2, and CP43 peptides were more intense as compared with the internal phosphothreonine from the LHClCII and PsbH phosphopeptides (Fig. 3B). We could also detect this set of phosphopeptides in the negative-ion mode, using the characteristic fragment ions of ~97 m/z (H<sub>2</sub>PO<sub>4</sub>) and ~63 m/z (PO<sub>3</sub><sup>−</sup>). However, these signals appeared less specific for phosphate than ~79 m/z and gave higher noise during LC-ESI MS, thus reducing their reliability.

The intensity of ionic species detected by MS depends on the nature of a particular peptide and its ionization properties. Consequently, quantitative comparisons of different peptides is generally not allowed. However, because phosphorylation of a peptide adds just 80 m/z (HPO<sub>3</sub>) and does not change the ionization state of the peptide under acidic conditions, quantitation of a phosphopeptide relative to its parent peptide could be possible using LC-ESI MS in the positive-ion mode. In this approach, the total peptide mixture (without IMAC enrichment) is separated by LC and the phosphopeptides and peptides then are detected in the same run by ESI MS. Because both forms would be detected simultaneously in the same sample, the stoichiometry of phosphorylation could be measured for individual proteins. To test this approach, we subjected equimolar ratios of six different synthetic phosphopeptides and their corresponding dephospho forms (see “Experimental Procedures”) to LC-ESI MS. Under our LC conditions, the phosphopeptides eluted ~1 to 2 min earlier than the dephospho-
forms, which simplified detecting the ions derived from the dephosphorylated forms. In all cases, the sum of the peak intensities of each phosphopeptide ion species used to normalize the data for these polypeptides (D1, D2, CP43, and PsbH) was found to be completely phosphorylated or dephosphorylated in either preparation (Table II). The only exception was the doubly phosphorylated form of PsbH, which was absent in tissue harvested after dark adaptation. This result provided an initial indication that light is not strictly required for phosphorylation of these PSII proteins as had been previously proposed (reviewed in Refs. 12 and 13). However, for the LHCl polypeptides, these long-term light/dark treatments did significantly affect their phosphorylation state. Whereas a substantial portion of the LHCl pool was phosphorylated in the light-grown samples, the phosphorylated forms were undetectable after an extended dark adaptation (Table II).

Previous in vitro studies with spinach thylakoids, using either 32P labeling and/or phosphoamino acid antibodies, showed that heat appears to cause a rapid dephosphorylation of PSII core proteins D1, D2, and CP43, presumably by a heat-activated phosphatase (27). Here, we demonstrated a similar effect for Arabidopsis thylakoids by MS. Isolated thylakoid membranes were incubated in the light with or without a 15-min exposure to 40 °C and then the associated peptides were released by trypsinization. MS analysis of the released fraction revealed a significant dephosphorylation of the PSII reaction center proteins D1, D2, and CP43 after exposure to 40 °C. This was observed as a dramatic decrease in the phosphopeptide ion intensities as compared with those from their nonphosphorylated forms (Fig. 3, C and D, and Footnote 2). In contrast, the levels of LHCl and PsbH phosphopeptides were unaffected by this heat treatment. Collectively, these data imply that a heat-activated phosphatase is also present in Arabidopsis thylakoid membranes. Because only the PSII core proteins were dephosphorylated, we suggest that this phosphatase differs substrates bearing N-terminal N-acetyl-O-phosphothreonines.

In vitro 32P labeling of pea and spinach thylakoids showed that light rapidly stimulates the phosphorylation of both LHCl proteins and the components of PSII (12, 22). To measure the extent of this reaction by MS, we prepared intact chloroplasts or thylakoid membranes from dark-adapted Arabidopsis plants, added inorganic phosphate or ATP, respectively, and then initiated the kinase reaction by irradiating the preparations with white light for 10 to 20 min. Studies with spinach and pea thylakoids indicated that this kinase reaction is complete by this time (22, 23, 25), although more recent studies with Arabidopsis thylakoids suggested that additional time is required for completion (35). LC-ESI MS quantitation of the resulting phosphopeptides revealed that these short light treatments had little effect on the stoichiometry of phosphorylation for the D1, D2, CP43, LHCl, and PsbH proteins; light increased the amount in the phosphorylated form by only a few percent over that observed for the protein from dark-adapted plants without the in vitro light treatment. As a result, these well-characterized in vitro kinase reactions may reflect only a small fraction of that which occurs in vivo.
Thylakoids prepared from isolated chloroplasts

| Protein | Dark adapted | Continuous light | Night | Day |
|---------|--------------|-----------------|-------|-----|
| P-D1    | 18%          | 43%             | 31%   | 40% |
| P-D2    | 12%          | 37%             | 25%   | 35% |
| P-CP43  | 29%          | 43%             | 38%   | 43% |
| P-PsbH  | 44%          | 69%             | 40%   | 25% |
| PP-PsbH | 0%           | 16%             | 0%    | 43% |
| P-LHCII*/P-LHCII/LHCII | 0/0/1 | 0.8/0.3/1 | 0.14/0.02/1 | 0.41/0.07/1 |

Directly isolated thylakoids

| Protein | Dark (30 min) | 40 °C (15 min) |
|---------|---------------|----------------|
| P-D1    | 41%           | 19%            |
| P-D2    | 34%           | 24%            |
| P-CP43  | 49%           | 31%            |
| P-PsbH  | 38%           | 26%            |
| PP-PsbH | 0%            | 37%            |
| P-LHCII*/P-LHCII/LHCII | 0.40/0.06/1 | 0.38/0.06/1 |

Table II

Stoichiometry of in vivo phosphorylation for the major photosynthetic phosphoproteins in thylakoid membranes of Arabidopsis

The data are the average from two to three experiments in each condition and two LC-MS runs for every experiment.

![Image](327x201 to 536x541)

FIG. 4. Determination of the in vivo phosphorylation levels for Arabidopsis thylakoid proteins. Thylakoids were directly isolated in the presence of phosphatase inhibitors. A, SDS-polyacrylamide gel electrophoresis separation of the thylakoid membrane proteins before and after treatment with trypsin. B, MS spectra of the fractions containing the doubly phosphorylated form when the plants were transferred to darkness. This response was rapid, being complete within 30 min of transfer. A significant decrease in phosphorylation of D1, D2, and CP43 was evident upon heat shock, but was much less pronounced than that observed in vitro (see above). Collectively, the data indicate that none of the principal phosphoproteins in the Arabidopsis thylakoid membranes were completely phosphorylated at the time points examined. C, LC-MS profiles of the intensities of ions produced by AcRKTpVAKPK (P-LHCII*) and TpVAKPK (P-LHCII) from the same LC-MS run.

a Plants were either grown in continuous white light or continuous light followed by a 48-h incubation in darkness.

b For D1, D2, CP43, and PsbH, % of phosphorylation for each protein in particular condition is shown. Due to the alternative proteolysis of phospho-LHCII, the intensities of ions produced by AcRKTpVAKPK (P-LHCII*) and TpVAKPK (P-LHCII) were normalized against the intensities of TVAKPK (LHCII) from the same LC-MS run.

c Double phosphorylation form of PsbH.

By LC-ESI MS, we then attempted to quantitate the in vivo changes in phosphorylation state of the D1, D2, CP43, LHCII, and PsbH proteins when the Arabidopsis leaves were subjected to several environmental conditions. In an attempt to more effectively capture the phosphorylated forms, we developed a method to rapidly prepare thylakoid membranes directly from Arabidopsis leaves. It involved collecting a crude chloroplast preparation by centrifugation, osmotic shock to release the thylakoid membranes, and then enrichment of the membranes by sucrose step gradient centrifugation. All buffers contained 50 mM phosphate and 10 mM NaF to inhibit endogenous phosphatases. Previous studies showed that these levels of inhibitors used singly are sufficient to completely block most, if not all, protein phosphatases in thylakoid preparations from spinach and various other plant species (12, 13, 26, 30). The thylakoid protein patterns prepared by this method were identical to those prepared conventionally (Figs. 1A and 4A). Moreover, the LC-ESI MS analysis revealed similar levels of phosphorylation for the five phosphoproteins prepared from light-adapted samples (Table II). The only major change was an enrichment of the doubly phosphorylated form of PsbH and a concomitant reduction in the singly phosphorylated form using the new method.

For the environmental analysis, the Arabidopsis plants were grown in a 16-h light/8-h dark photoperiod and then harvested: 1) at the end of the 8-h dark period; 2) 4 h into the 16-h light period; 3) 4 h into the light period followed by a 30-min dark treatment; and 4) 4 h into the light period followed by a 15-min heat shock at 40 °C (Table II). After harvest, thylakoids were prepared and the shaved peptides were subjected directly to LC-ESI MS. As can be seen in Table II, the phosphorylation states of all five proteins were not radically altered by any of the treatments. A modest decrease in LHCII phosphorylation and slight decreases in D1, D2, and CP43 phosphorylation were evident in the dark period as compared with the light period. However, this change was not yet evident after a 30-min dark adaptation, suggesting that the kinetics of this dephosphorylation is slow. The only major light-dependent change was for PsbH, which revealed a near complete loss of the doubly phosphorylated form when the plants were transferred to darkness. Although the amount of peptides varied in the different LC-MS runs, the ratios of the phosphorylated over the non-phosphorylated form for specific peptides were highly reproducible among independent samples.
phosphorylated or nonphosphorylated under normal photoperiodic growth conditions. They also suggest that significant and rapid changes in the overall phosphorylation state of the photosynthetic complexes may occur in heat-stressed plants.

**DISCUSSION**

Attempts to assess the importance of phosphorylation in many cellular processes have been hampered by the dynamic and reversible nature of this process and by the lack of reliable methods to quantitate phosphorylation in intact cells. We show here that MS can be helpful in these studies by allowing the detection and mapping of phosphoproteins even in complex mixtures and by providing a semi-quantitative measure of the phosphorylation state for individual proteins. This approach improves upon current techniques using phosphoamino acid antibodies or $^{32}$P labeling because it can detect with high sensitivity both the phosphorylated and nonphosphorylated forms simultaneously and because it measures stoichiometry directly without the need for an exogenous tracer.

Using the regulatory phosphorylation system from *Arabidopsis* chloroplast thylakoids as an example, we detected a number of abundant phosphorylated proteins, easily mapped their phosphorylation sites, and estimated the effects of a range of environmental conditions on their phosphorylation state. In this case, the availability of the chloroplast and near-complete nuclear genomic sequences from *Arabidopsis* were instrumental for the unequivocal identification of the corresponding proteins following MS-MS sequencing of the peptides. Given the complexity of these thylakoid preparations, containing numerous proteins either peripheral or integral to the membrane, the success of MS analysis underscores its potential ability to dissect much more complex cell structures and organelles and may even be adapted to whole cell studies.

Several conditions helped simplify our characterization of the thylakoid system. First, the straightforward isolation of plant photosynthetic membranes allowed us to study protein phosphorylation in a well defined subcellular compartment rather than using the total cellular constituents. Second, the restriction of phosphorylation to surface exposed segments of the thylakoid proteins helped limit the number of peptides by focusing only on those accessible to trypsin. The remaining hydrophobic segments of membrane proteins were easily removed after proteolysis by centrifugation. This characteristic should also help simplify studies with other membrane protein phosphorylation cascades, including those involved in membrane receptor signaling. For more soluble systems, it is possible that complete trypsinization will generate many more peptides in any complex peptide mixture.

The recent use of phosphothreonine antibodies revealed previously unanticipated patterns of thylakoid protein phosphorylation in *Arabidopsis* thylakoid phosphopeptides in conjunction with the *Arabidopsis* genome sequence database allowed us easily to map for the first time the phosphorylation sites of central photosynthetic proteins in this plant species. These phosphoproteins included the chlorophyll-binding proteins LHCII and the D1, D2, CP43, and PsbH peptides of PSII. In all cases, the phosphate was bound to threonines at or near the N terminus. Coincidentally, the analysis of the corresponding peptides also allowed identification of the mature N-terminal residue of each of the five peptides and showed that three are both N-acetylated and O-phosphorylated (D1, D2, and CP43). The results obtained here with *Arabidopsis* are similar to those obtained earlier with spinach using conventional phosphate mapping, suggesting that this photosynthetic phosphorylation system is conserved among higher plants (19–21).

A novel finding of our study was the identification of a second phosphorylation site in PsbH protein at Thr-4. In contrast to phosphorylation at Thr-2, modification of Thr-4 is highly sensitive to the ambient light conditions, being rapidly dephosphorylated when plants are placed in darkness. In the green alga *Chlamydomonas reinhardtii*, PsbH is essential for assembling PSII in thylakoids (36); *psbH* mutants have a PSII-deficient phenotype and lack a functional PSII complex (36, 37). O'Connor et al. (37) concluded from studies showing that *C. reinhardtii* strains expressing a PsbH mutant lacking Thr-2 (Thr-2 to Ala) behave as wild-type, that phosphorylation of PsbH may not be important for its function. However, our finding of a second phosphorylation site in PsbH and the demonstration that modification of this site is light responsive *in vivo* reopens the role of phosphorylation in PsbH function.

Mapping of phosphopeptides using LC-ESI MS along with detection of both phosphorylated and nonphosphorylated forms obtained from the same physiological context shows that this approach can be used to estimate the *in vivo* phosphorylation state of many proteins simultaneously. When applied to the five thylakoid proteins characterized here, we demonstrated that both the phosphorylated and nonphosphorylated forms are present under various light/dark conditions *in planta*. Consequently, our data indicate that the current model of photosynthetic regulation in which thylakoid proteins are phosphorylated in light and dephosphorylated in darkness requires reconsideration at least in *Arabidopsis* (12, 22). Despite extensive dark adaptation, we found that only LHCII was completely dephosphorylated whereas a substantial percentage of D1, D2, CP43, and PsbH protein of PSII remained phosphorylated in *Arabidopsis* plants. Only the second phosphorylation site of PsbH followed the original model, being rapidly and completely dephosphorylated following the transition from light to dark. Whether these effects are universal to all plants remains to be demonstrated. Several studies suggest that thylakoid protein phosphorylation patterns can vary considerably among plant species (26, 28).

The recent use of phosphothreonine antibodies revealed previously unanticipated patterns of thylakoid protein phosphorylation *in vivo* (26–28). Irradiance-dependent phosphorylation of PSII and LHCII phosphopeptides was observed and connected to a regulatory mechanism involving *in vivo* changes in the thiol-disulfide redox state (26, 38). Moreover, significant phosphorylation of thylakoid proteins in the dark was found in plants after light and heat stress (27, 28). In a few plant
species, high-light stress led to sustained phosphorylation of D1 and D2 proteins in the dark, the extent of which correlated with a sustained xanthophyll cycle-dependent dissipation of energy (28). In spinach, high temperature stress induced fast and specific dephosphorylation of PSII reaction center proteins (27). Using MS, we confirm here the effects of high temperature stress in Arabidopsis and show that heat shock conditions induce specific dephosphorylation of PSII core phosphoproteins D1, D2, and CP43. For each of these proteins, dephosphorylation involves the N-terminal N-acetyl-O-phosphothreonyls, suggesting that the responsible heat-activated phosphatase prefers this type of substrate.

Collectively, our MS study shows that reversible regulatory protein phosphorylation in Arabidopsis photosynthetic membranes overall may be more rapid during stress than during the normal light-dark cycles. In this respect, longer and faster changes in the protein phosphorylation may be anticipated in response to high light stress, short light pulses, or limited light intensities when the influence of dominant wavelengths would be important. In contrast with all prior methods used to study thylakoid phosphorylation, our MS approach allowed objective quantitation of in vivo phosphorylation of multiple proteins obtained from plants exposed to different physiological conditions. The further application of MS should allow us to understand the role(s) of reversible phosphorylation in photosynthetic regulation and reveal additional components that are affected by this modification. Of particular interest will be a re-examination of the short-term effects of light previously shown by other methods to have rapid and substantial effects on thylakoid phosphorylation.

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