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Identification of the phosphorylation site of an 8.3 kDa protein from photosystem II of spinach

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The four principal phosphoproteins of PS II cores (8.3, 32, 34 and 44 kDa) give rise to distinct tryptic phosphopeptides which have been purified by affinity chromatography on Fe^{3+}-chelating Sepharose and reverse-phase HPLC. The tryptic phosphopeptide derived from the 8.3 kDa protein has the sequence NH_2-Ala-Thr-Gln-Thr-Val-Glu-Ser-Ser-Ser-Arg. It corresponds to the N-terminus of the chloroplast *psb*H gene product, except for the loss of the initiating *N*-formylmethionine. The peptide is phosphorylated on the first threonyl residue. Differences between the phosphorylation sites of the 8.3 kDa protein and LHC II are consistent with the hypothesis that thylakoids contain two distinct redox-controlled protein kinases differing in substrate specificity.

Phosphorylation site; 8.3 kDa protein; Photosystem II; Protein kinase; (Spinach)

1. INTRODUCTION

The photosynthetic membranes of green plants contain more than ten phosphoproteins. The two most conspicuous (25 and 27 kDa) are components of the light-harvesting Chl *a/b* complex (LHC II) [1]. Their phosphorylation is thought to control the distribution of excitation energy between the two photosystems (PS I and PS II) as part of a mechanism to maximize the efficiency of photon utilization [2]. Four other phosphoproteins (44, 34, 32 and about 10 kDa) are associated with PS II itself [3–5] but the significance of their phosphorylation is uncertain. Phosphorylation of the PS II proteins is inhibited by the herbicide diuron [6], suggesting that, like LHC II, they are substrates of the thylakoid-bound kinase that is activated by reduction of the PQ pool [7–9]. However, the fact that thiol reagents [10], the adenosine analogue FSBA [11], and the PQ antagonist DBMIB [12] all preferentially inhibit phosphorylation of LHC II compared with the 10 kDa protein, raises the possibility that there are two distinct redox-controlled kinases differing in substrate specificity.

A rigorous study of substrate specificity must involve determination of phosphorylation site sequences. The 27 kDa LHC II of pea is phosphorylated on a threonyl residue close to the N-terminus of the mature protein. From a combination of conventional protein sequencing [13] and sequencing of a genomic DNA clone [14], the phosphorylation site has been deduced to be: (Met)-Arg-Lys-Ser-Ala-Thr-Thr-Lys-Lys-Val-Ala-Ser-Ser-Gly-Ser. The methionine is either the first residue of the mature protein or the last residue of

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Abbreviations: DBMIB, 2,3-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FSBA, 5′-fluorosulfonylbenzyladenine; IDA, iminodiacetate; LHC, light-harvesting chlorophyll *a/b* complex; PQ, plastoquinone; PS, photosystem; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

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the transit peptide of the LHC II precursor. It is not clear whether one or both threonines are phosphorylated. For several years, this has been the only sequence information available on thylakoid phosphoproteins but now we report the sequence of the phosphorylation site of the '10 kDa phosphoprotein' of spinach thylakoids.

2. MATERIALS AND METHODS

Photosystem II particles were prepared as described [15] from ^{32}P-labelled chloroplasts which had been purified by centrifugation through dense sucrose [16]. PS II particles were also obtained from unlabelled chloroplasts which had been prepared without purification through dense sucrose. For isolation of phosphopeptides, PS II particles (15 mg Chl) were washed once with 1 M Tris (pH 9.25) to remove extrinsic proteins and with 20 mM NH_4HCO_3 and then resuspended in the same buffer (1 mg Chl/ml) for digestion with trypsin (TPCK-treated, 5 μg/ml, Sigma). After 12 h at room temperature the solubilized peptides were separated from the remaining complex by centrifugation (40000 $\times$ g, 30 min). Digestion of the solubilized peptides was completed after addition of an equal amount of trypsin (7 h, 37°C). Cysteinyl residues were acetylated by addition of first dithiothreitol (1.2 mg) then iodoacetamide (3.2 mg) and finally dithiothreitol (0.6 mg), each for 1 h at 37°C. After lyophilization, the phosphopeptides were purified by affinity chromatography through Fe^{3+}-IDA-Sepharose (Sigma) [17]. The sample was dissolved in 4 ml of 0.1 M acetic acid and applied to the column (1 $\times$ 5 cm), which had previously been saturated with Fe^{3+}. The column was then washed with 12 ml of 0.1 M acetic acid/NaOH (pH 5.0), 12 ml water, 12 ml ammonium acetate (1%, w/v) and finally ammonium acetate (1%, adjusted to pH 8.0 with NH_3) to elute the phosphopeptides. Fractions (1 ml) containing phosphopeptides were pooled and the affinity chromatography was repeated once. After lyophilization the phosphopeptides were dissolved in 50% acetic acid, 4 M guanidine-HCl and further purified by HPLC on a Beckman binary system equipped with a reverse-phase column (C18, Ultrasphere, 4.6 $\times$ 250 mm, 5 μm pore size). The two solvents for gradient elution were water and acetonitrile both containing 0.1% (w/v) TFA. Total peptides were detected at 210 nm and radioactivity was detected with a 2 ml flow cell (Radiomatic Instruments) in Cerenkov mode.

PS II proteins were analyzed by SDS-PAGE on a 15% acrylamide gel slab, followed by staining and autoradiography [18]. The 8.3 kDa protein was prepared by elution from a preparative gel [18]. Buffer salts and some of the SDS were removed by dialysis against water (48 h, 4°C). The remaining SDS was then removed by organic extraction [19]. The protein (obtained from a PS II preparation containing 1 mg Chl) was then digested with 50 μg trypsin. After acetylation of cysteinyl residues, the mixture was analyzed by HPLC.

Sequence analysis was performed on an Applied Biosystems gas-phase sequencer. For amino acid analysis [20] the peptide was hydrolyzed at 105°C for 22 h. The values for threonine and serine were corrected by 5 and 10%, respectively. For location of the phosphorylated amino acid, the isolated peptide was degraded stepwise in a Beckman 890C protein sequencer and the eluted fractions were measured for radioactivity by Cerenkov counting.

3. RESULTS

PS II core particles, prepared from ^{32}P-labelled chloroplasts, contained four proteins which were strongly labelled (fig.1): the 44 kDa Chl *a*-binding protein (CPa-2), two proteins of 34 and 32 kDa, which are probably identical with the reaction center proteins D1 and D2, and a 8.3 kDa protein. When the PS II particles were treated with trypsin and centrifuged, the phosphate-containing peptides and other protein fragments were released into the supernatant, whereas the protein fragments which bind the pigments remained in the pellet. Analysis of the released tryptic peptides by reverse-phase HPLC revealed four distinct phosphate-containing peptides (fig.2A). We determined which of the four phosphopeptides was derived from each of the major PS II phosphoproteins by isolation of individual proteins by preparative SDS-PAGE. After elution from the gel and digestion with trypsin, each protein yielded a single phosphopeptide on HPLC analysis. The most hydrophilic phosphopeptide, and the first to be eluted from the column, was derived from the 8.3 kDa protein (fig.2B). The other three peptides

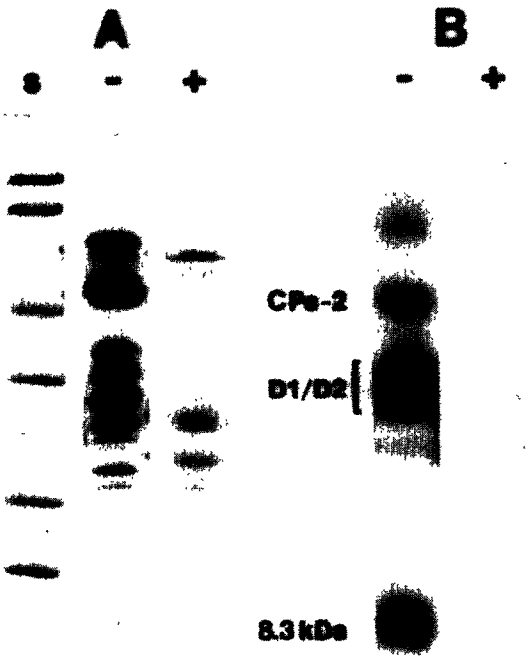

Fig.1. SDS-PAGE of PS II particles prepared from ^{32}P-labelled chloroplasts before (−) and after (+) treatment with trypsin (200 μg trypsin per mg chl). (A) Coomassie brilliant blue stained gel. (B) Autoradiograph of the same gel. s, standard proteins of 14.4, 21.5, 31, 45, 66.2 and 92.5 kDa.

were derived from D2, D1, and CPa-2, respectively. Each eluted protein was also subjected to brief acid hydrolysis and paper electrophoresis to determine the nature of the phosphorylated amino acid; each was labelled exclusively on threonine (not shown).

To sequence the phosphopeptide derived from the 8.3 kDa protein, it was necessary to remove the many non-phosphorylated peptides which had been released from PS II particles by trypsin. Two additional steps were introduced. Firstly, PS II particles were washed with alkaline Tris prior to trypsin treatment to remove extrinsic proteins. Secondly, the tryptic peptides were subjected to metal affinity chromatography with Fe^{3+} bound to chelating IDA-Sepharose [17]. Immobilized Fe^{3+} has great affinity for phosphate groups [17] and is a powerful tool for purification of phosphopeptides. The latter bind to the column at acid pH and

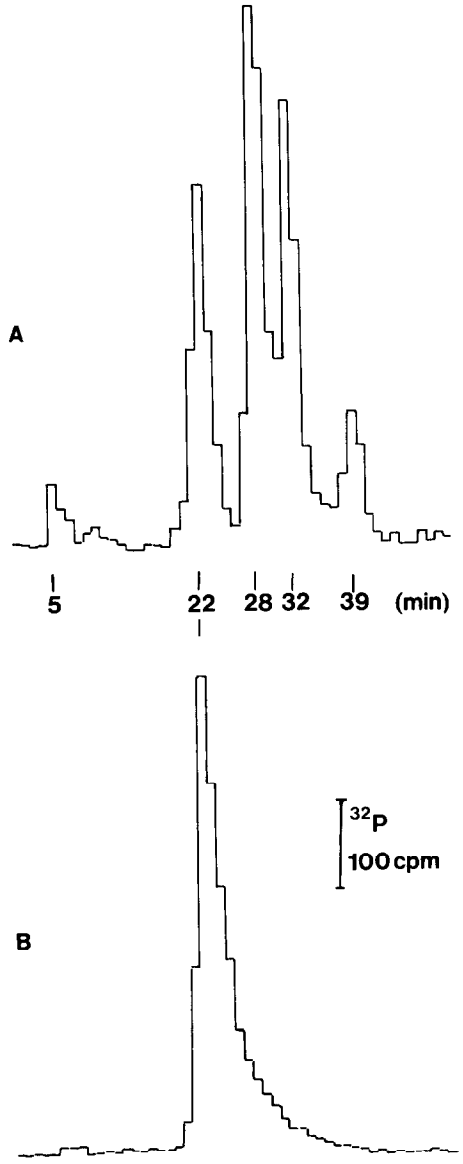

Fig.2. Analytical separation of tryptic peptides by reverse-phase HPLC. (A) Total peptide mixture from tryptic digestion of Tris-washed PS II particles. (B) Peptides after tryptic digestion of the isolated 8.3 kDa protein. Detection of ^{32}P-labelled peptides only. Gradient 0–60% of organic solvent, 1.5% per min, flow rate 1 ml/min.

are eluted at slightly alkaline pH. When total tryptic peptides released from PS II particles were subjected to two consecutive passages through the affinity column, the complexity of the peptide elu-

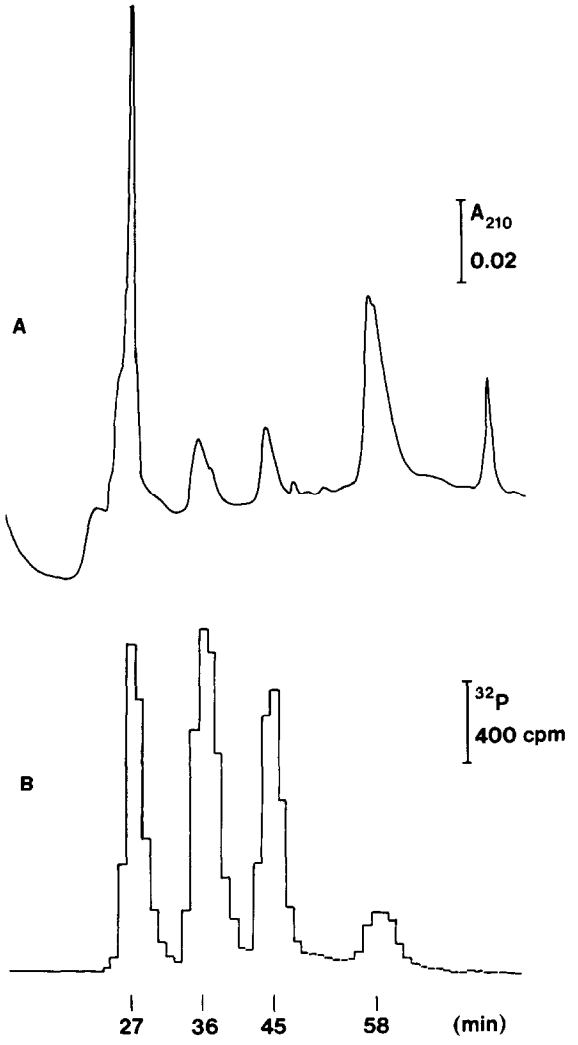

Fig.3. Preparative separation of tryptic phosphopeptides from Tris-washed PS II particles. Phosphopeptides, after elution from the metal affinity column, were purified by reverse-phase HPLC. Both A_{210} and ^{32}P were monitored. Gradient 0–60% organic solvent, 0.75% per min, 1 ml/min.

Table 1

Relative amino acid composition of the tryptic phosphopeptide of the 8.3 kDa protein

Ala	1.02 (1)
Arg	1.00 (1)
Asx	0.11 –
Glx	2.29 (2)
Gly	0.54 –
Ile	0.08 –
Leu	0.10 –
Val	0.99 (1)
Ser	3.02 (3)
Thr	1.66 (2)

All values were normalized to arginine (nearest integer in parentheses). Pro, Met, Tyr, Phe, His, Lys: not detected. Trp, Cys: not estimated

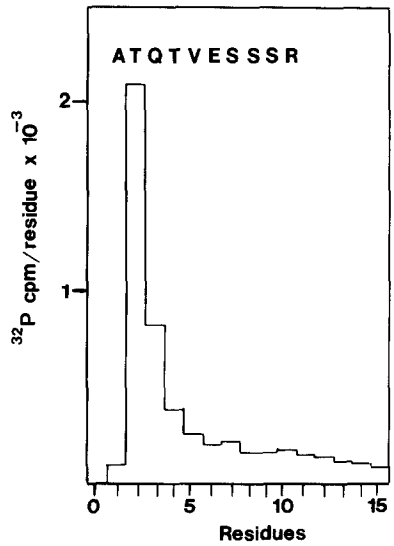

Fig.4. Identification of the phosphorylated amino acid by stepwise degradation of the isolated peptide in a Beckman 890C protein sequencer.

tion pattern seen subsequently at 210 nm after HPLC was reduced to just five peaks, four of which corresponded to the ^{32}P-labelled phosphopeptides (fig.3). Gas-phase sequencing and determination of amino acid composition (table 1) of the first ^{32}P-labelled peak in fig.3 revealed the following sequence: NH_2-Ala-Thr-Gln-Thr-Val-Glu-Ser-Ser-Ser-Arg. The substoichiometric recovery of threonine can be attributed to an incomplete conversion of phosphothreonine to threonine under the condition of acid hydrolysis (Michel, H.P., unpublished). Glycine which appears in the amino acid analysis was attributed to contamination. Stepwise degradation of the peptide using a Beckman spinning cup sequencer (fig.4) showed that the radioactivity was released with the threonine in the second position. The threonine in the fourth position was either not

phosphorylated or phosphorylated only to a minor extent.

4. DISCUSSION

Exhaustive tryptic digestion of the 8.3 kDa phosphoprotein of spinach PS II particles yields a single phosphopeptide. Its first 9 residues are identical to the N-terminal sequence reported [21]. The complete sequence of this protein from tobacco and wheat has been obtained from the corresponding gene (designated *psb*H) [22,23]. The N-terminal sequences are almost identical for the three plant species:

Spinach: Ala-Thr-Gln-Thr-Val-Glu-Ser- Ser-Ser-Arg

Tobacco: Met-Ala-Thr-Gln-Thr-Val-Glu-Asn-Ser-Ser-Arg...

Wheat: Met-Ala-Thr-Gln-Thr-Val-Glu-Asp-Ser-Ser-Lys...

The initiating *N*-formylmethionine appears to be lost in vivo, as found for several other chloroplast translation products [23]. The arginine that terminates our tryptic phosphopeptide has not been reported previously [21]; it is conserved in tobacco but in wheat it is replaced by lysine. The fact that the 8.3 kDa protein is phosphorylated close to the N-terminus establishes that the latter is exposed at the outer surface of the thylakoids, where ATP is available. Since the protein contains only one potential transmembrane segment [23], the C-terminus presumably protrudes into the lumen. Phosphorylation of this protein at the outer surface could affect events on the reducing side of PS II, including electron transport to PQ. Numerous changes in PS II behaviour have been attributed to phosphorylation (see, for example [24,25]). However, since PS II contains four phosphoproteins and is also closely associated with LHC II, it is not clear to what extent phosphorylation of the 8.3 kDa protein contributes to these effects. Another possible role of phosphorylation may be to control the rate of N-terminal degradation by aminopeptidases which are known to occur in chloroplasts [26]. In prokaryotes and eukaryotes, there appears to be a correlation between the stability of a protein and the chemical composition of its N-terminus [27]. Phosphorylation, by altering that chemistry, could regulate protein turnover.

LHC II and the 8.3 kDa protein appear to be phosphorylated by distinct redox controlled kinases [10–12]. The ability of these kinases to discriminate among possible substrates might depend on their ability to recognize different primary sequences. Knowledge of the primary structures of the phosphorylation sites of LHC II and the 8.3 kDa protein will enable us to synthesize oligopeptide substrates to test this hypothesis.

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