PHOTOSYSTEM II-ASSOCIATED PROTEIN KINASE PHOSPHORYLATES A NOVEL 6.3 kDa PROTEIN WHICH SUBSEQUENTLY DISSOCIATES FROM PHOTOSYSTEM II CORE COMPLEXES.

Helen L. Race*, Jens Forsberg*, Geoffrey Hind† and John F. Allen*
*Plant Cell Biology, Lund University, S-220 07 Lund, Sweden;
†Biology Dept., Brookhaven National Laboratory, Upton, NY 11973, USA.

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1. Introduction

The enzymes responsible for reversible phosphorylation of components of photosynthetic complexes are presumed to be associated with the thylakoid membrane. Evidence supporting the presence of multiple protein kinases in this system is mounting. Phosphorylation appears to be redox-regulated with activity favoured by reducing conditions. Mild neutral detergents release the bulk of thylakoid kinases (1, 2, 3) but the unsolubilised membranes consistently retain enzyme activity. This protein kinase activity copurifies with a photosystem II (PS II) core complex and catalyses phosphorylation of intrinsic protein components of PS II and light harvesting complex II (LHC II) (4). Characterisation of a constitutively active, photosystem II-associated protein kinase (PS II-PK) revealed it to be a distinct and novel protein with an apparent molecular mass of 58 kDa, as determined from its electrophoretic migration (4). The enzyme activity is rapidly ($t_{0.5} \approx 40$ s) and irreversibly photoinactivated by strong red light illumination (4, 5). Additional detergent and chromatographic treatment yields a preparation enriched in two proteins, PS II-PK and a 37 kDa protein identified as FNR by Western blotting (5).

Here we report the presence of an additional, novel protein associated with PS II core complexes containing PS II-PK. The 6.3 kDa phosphoprotein was visualised only after electrophoretic separation of proteins on polyacrylamide gels designed to resolve low molecular weight proteins (6). Centrifugation of PS II cores after phosphorylation reveals that the 6.3 kDa phosphoprotein dissociates from the complex. *In vivo* this phosphoprotein could provide a mechanism for signalling photosynthetic activity within the thylakoid membrane to the stroma and perhaps ultimately to the cytosol.

2. Procedures

PS II core complexes containing PS II-PK activity and LHC II proteins were prepared as described in (4). *In vitro* phosphorylation of PS II proteins and LHC II (7) was monitored at the times indicated in the figure legends. Phosphorylated proteins were separated on 13.5% polyacrylamide/ 4 M urea gels with the buffer system of Laemmli (8). Resolution of low molecular weight proteins was achieved using 16.5 % T/ 2.6 % C polyacrylamide gels according to (6). Incorporated radioactivity was visualised by exposure to a PhosphorImager plate and quantified using ImageQuant software.

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Proteins were electrophoretically transferred to nitrocellulose and probed with antibodies directed towards the PS II-W protein (kind gift of Dr W. Schröder). Immunoreactions were visualised with an ECL kit. To assess the stability of phosphoamino acids proteins were transferred to PVDF membrane and washed with 50 mM Hepes pH 8.0 or 0.5 M NaOH or 0.5 M HCl for 1 h at 65°C (9). After drying in air incorporated radioactivity was visualised as described above.

3. Results and Discussion

3.1 *Endogenous protein phosphorylation catalysed by PS II-PK.*
The phosphorylation of endogenous proteins catalysed by PS II-PK is shown in Figure 1. Within 3 minutes phosphate is incorporated into the CP43 antenna protein. After 15 minutes the D1, D2 and PS II-H proteins are also labeled as is the 27 kDa component of LHC II which is believed to be more tightly associated with PS II than the 25 kDa polypeptide (Figure 1, lanes 1 and 2). Incubation of purified LHC II proteins with [γ-^{32}P] ATP does not result in phosphorylation (Figure 1, lanes 3 and 4) but when this preparation is mixed with PS II core complexes radiolabeling of the 25 kDa component is detected after 3 minutes and increases after longer incubation (Figure 1, lanes 5 and 6).

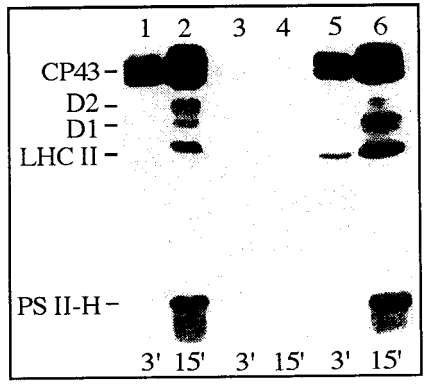

Figure 1. Phosphorylation of endogenous PS II proteins and LHC II catalysed by PS II-PK. Lanes 1 & 2, PS II core complexes; lanes 3 & 4, LHC II; lanes 5 & 6, PS II core complexes + LHC II. Incubation times (min) are indicated on the figure.

Analysis of phosphorylation of low molecular weight proteins revealed two phosphoproteins with apparent molecular weights of 7.2 kDa and 6.3 kDa in addition to the PS II-H protein which migrates at 9.6 kDa in the gel system used (Figure 2A, lane 1). We examined the supernatant remaining after centrifugation of phosphorylated PS II core complexes and identified a 6.3 kDa phosphoprotein in this soluble phase (Figure 2A, lane 2) which stains poorly with Coomassie blue (data not shown). Western blotting with antibodies directed towards the 6.1 kDa product of the *psb*W gene demonstrated that the 6.3 kDa protein is not a post-translationally modified form of PS II-W.

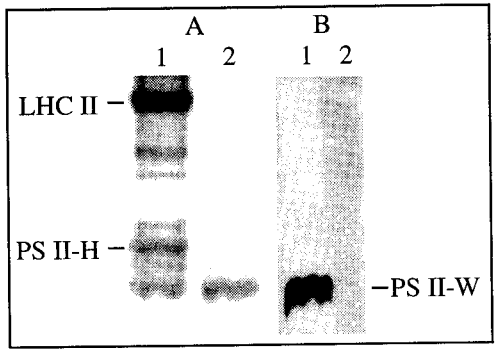

Figure 2. Separation of low molecular weight protein components of PS II core complexes. A: autoradiograph of proteins remaining associated with (lane 1) or dissociated from (lane 2) PS II core complexes after phosphorylation. B: Western blot of identical samples probed with antibodies directed towards PS II-W protein.

3.2 *Kinetics of phosphorylation of 6.3 kDa protein.*

A time course of incorporation of phosphate into PS II core proteins, precipitated by centrifugation after phosphorylation, and the 6.3 kDa protein is shown in Figure 3. The phosphorylated 6.3 kDa protein can be detected in both the PS II complexes and the supernatants from these samples when phosphorylation is terminated after 3 or 5 minutes (Figure 3A and B, lanes 2 and 3). After longer incubation times the 6.3 kDa phosphoprotein is predominantly in the soluble phase after centrifugation (Figure 3A and B, lanes 4 and 5). A comparison of the amount of phosphate incorporated into the PS II-H and 6.3 kDa proteins is shown in Figure 4. Phosphorylation of the two proteins appears to proceed at a similar rate.

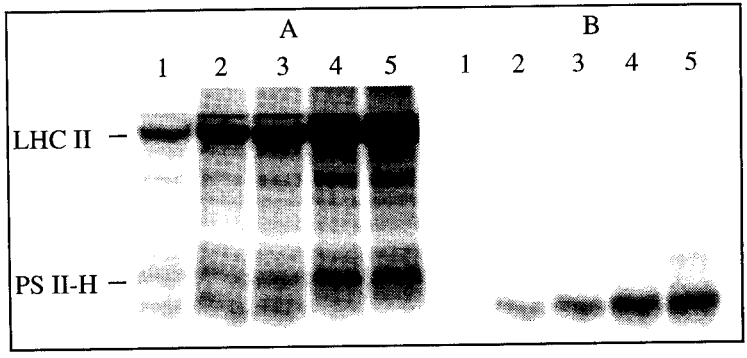

Figure 3. Time course of incorporation of ^{32}P into PS II core complex proteins. Autoradiographs show phosphoproteins remaining associated with (A) or dissociated from (B) PS II complexes after 0, 3, 5, 10 and 15 min (lanes 1-5 respectively) incubation with [γ-^{32}P] ATP.

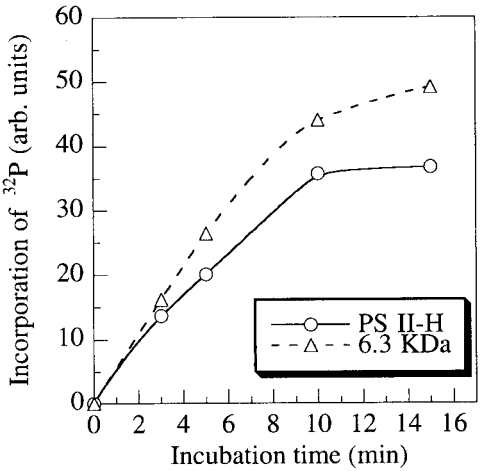

Figure 4. Quantification of ^{32}P associated with PS II-H and soluble 6.3 kDa phosphoproteins from Figure 3A and B respectively.

3.3 *Acid/ alkaline stability of phosphoamino acids with the 6.3 kDa phosphoprotein.*

A simple approach to determine the type of phosphoamino acid modified is to subject phosphoproteins, previously separated and immobilised on PVDF membrane, to washes with strongly acidic or alkaline solutions (9). The significant loss of radiolabel after alkaline washing suggests that phosphate may be associated with serine or threonine residues (Figure 5, lane 3). The residual phosphate remaining after this treatment might indicate additional phosphotyrosine residues but could also originate from phosphothreonine residues in an environment where they are protected them from the effects of the stringent wash. The presence of phosphohistidine residues is ruled out by the stability of phosphate groups under acidic conditions (Figure 5, lane 4).

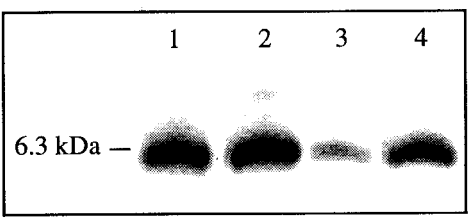

Figure 5. Acid/ alkaline stability of ^{32}P incorporated into the 6.3 kDa protein after immobilisation on PVDF membrane. Lane 1, unwashed; lane 2, 50 mM Hepes pH 8.0; lane 3, 0.5 M NaOH; lane 4, 0.5 M HCl.

Preliminary sequencing studies suggest that this protein, in common with other thylakoid proteins, is post-translationally modified at the N terminus. It is tempting to speculate that the gradual release of the 6.3 kDa phosphoprotein from the PS II complex points to a two step process with dissociation requiring modification of two residues. The high level of phosphate incorporated into this protein relative to that measured for the PS II-H protein, previously believed to be the second most heavily phosphorylated protein in the thylakoid membrane, provides support for the idea of multiple phosphorylation sites within the smaller protein.

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