17. Studies on the phosphorylation and dephosphorylation reactions of thylakoid membrane proteins using synthetic peptides

LÜLING CHENG and JOHN F. ALLEN

Plant Cell Biology, Lund University, Box 7007, S-220 07 Lund, Sweden

Introduction

Reversible protein phosphorylation of chloroplast thylakoid membranes regulates the distribution of excitation energy between the two photosystems during linear electron flow (Allen 1992). The protein kinase responsible for phosphorylation of a number of thylakoid proteins is membrane bound, and is regulated by the redox state of a component of the photosynthetic electron transport chain, probably by a quinone–semiquinone couple associated with the cytochrome b_6f complex (Silverstein *et al.* 1993*a*). The dephosphorylation reactions of all thylakoid membrane phosphoproteins are catalysed by a phosphoprotein phosphatase which is not under redox control (Bennett 1980; Silverstein *et al.* 1993*b*). However, the actual number of the protein kinases and phosphatases responsible for all thylakoid protein phosphorylation–dephosphorylation reactions is not clear, and the regulatory mechanism of these enzymes, the structure–function relationships and their substrate specificity are still unknown. Previous studies on chloroplast thylakoid protein kinase and phosphatase using synthetic peptides have shown that peptides could act as substrates of the LHCII kinase (Michel and Bennett 1989) and phosphoprotein phosphatase (Sun *et al.* 1993). Here we describe effects on the kinetics and substrate specificity of both thylakoid protein kinase and phosphatase using synthetic peptides corresponding to an N-terminal fragment of pea LHCII in both its phosphorylated and dephosphorylated forms. We found that both the thylakoid protein kinase and the phosphatase exhibit broad substrate specificity. We suggest that amino acid residues in the N-terminal domain of LHCII take part in protein–protein interactions between the LHCII, PSII core proteins and the protein kinase.

Materials and methods

The synthetic peptide corresponding to the N-terminal fragment of pea LHCII (RKSATTKKVASSGSP) was synthesized by a solid phase, t-Boc strategy as described by Barany and Merrifield (1980). For the synthetic phosphopeptide (RKSAT(PO4)TKKVASSGSP), in place of phosphorylated threonine, a Boc-Thr[OPO(OPh)$_2$] group was incorporated and a deprotection step carried out as described by Grehn *et al.* (1987).

Pea (*Pisum sativum L.*) thylakoid membranes were isolated from pea chloroplasts by the method described by Harrison and Allen (1991) and the thylakoid suspension at approximately 2 mg chlorophyll ml^{-1} was stored on ice in darkness for 60 min prior to radiolabelling. Phosphorylation of thylakoid membrane proteins and of synthetic peptide (RKSATTKKVASSGSP) was carried out by incubation of washed thylakoid membranes equivalent to 200 μg chlorophyll ml^{-1} with concentrations of peptide ranging from 0 to 4 mM in a reaction medium as described by Allen and Findlay (1986). The phosphorylation reaction was started by illuminating the samples with a desk-lamp giving a light intensity ~130 μmol m^{-2} s^{-1}. The reaction was terminated by addition of 8 volumes of acetone (precooled to −20 °C). The time-course of dephosphorylation was obtained by incubation of the labelled thylakoid membranes in darkness with either 167 μM synthetic phosphopeptide (RKSAT(PO4)-TKKVASSGSP), 10 mM NaF or 15 μM antimycin A. Time-courses were started by switching off the light, and samples (100 μl) were withdrawn at appropriate time intervals and immediately precipitated by mixing with 0.8 ml acetone (precooled at −20 °C). The protein pellets were solubilized in SDS–sample buffer and separated on 12–25 per cent SDS-PAGE gel. Gels were stained (Coomassie Blue or silver nitrate), dried, and subsequently analysed by phosphorimaging using a Fuji Bio-Imaging analyzer BAS 2000.

Results and discussion

Effects of a synthetic peptide on protein phosphorylation

The synthetic peptide analogue of the N-terminal fragment of pea LHCII (RKSATTKKVASSGSP) became phosphorylated by the action of a thylakoid protein kinase and had a marked effect on the phosphorylation of thylakoid membrane proteins. Figure 17.1 shows a typical pattern of SDS-PAGE analysis and a phosphorimage of ^{32}P-labelled pea thylakoid membrane proteins in the presence of various concentrations of the peptide. Phosphoproteins can be seen at 56, 45 (CP43), 40, 32 (D2), 31 (D1), 20, 18, 16, and 12 kDa, in addition to the heavily labelled light harvesting complex II (LHCII), 9 kDa and synthetic peptide (1.5 kDa). Different

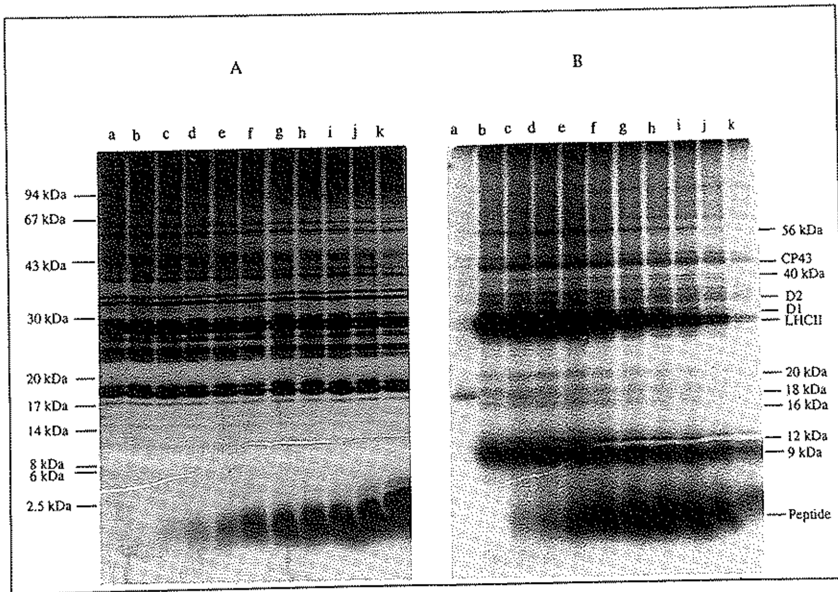

Fig. 17.1. SDS-PAGE silver stained (A) and phosphorimage (B) of phosphorylation of pea thylakoid membrane proteins and synthetic peptide. Samples were phosphorylated by illumination for 5 min. Dark incubation served as a control and is shown in track a. Synthetic peptide concentrations were: 0, 0, 0.084, 0.17, 0.33, 0.67, 1.0, 1.34, 2.0, 2.67, and 4.0 mM corresponding to tracks a–k respectively. The sample illuminated for 5 min in the absence of peptide served as 100% ^{32}P incorporation (track b). Tracks were loaded with protein equivalent to 2 μg chlorophyll.

concentrations of the peptide had different effects on phosphorylation of LHCII, photosystem II (PSII) core proteins and other minor proteins of thylakoid membranes. By increasing the peptide concentration, most thylakoid proteins underwent two changes: at low peptide concentrations the phosphorylation of LHCII, PSII core proteins, and some minor thylakoid proteins increased to a certain extent, whereas phosphorylation of LHCII increased to a greater extent. Maximum stimulation was obtained at 0.33 mM peptide concentration (Fig. 17.1B lane e and Fig. 17.2), where the extent of phosphorylation varied from 120 to 180 per cent of the control value. Thereafter, phosphorylation of all the thylakoid proteins decreased with increasing peptide concentration, with LHCII being the most affected. Figure 17.2 shows the quantification of ^{32}P labelling of pea thylakoid membrane proteins in the presence of various concentration of the peptide. The stimulation of phosphorylation at low peptide concentration may result from activation of the protein kinase whereas the inhibitory effect of the peptide may be explained by a competition between the peptide and the other substrates of the kinase.

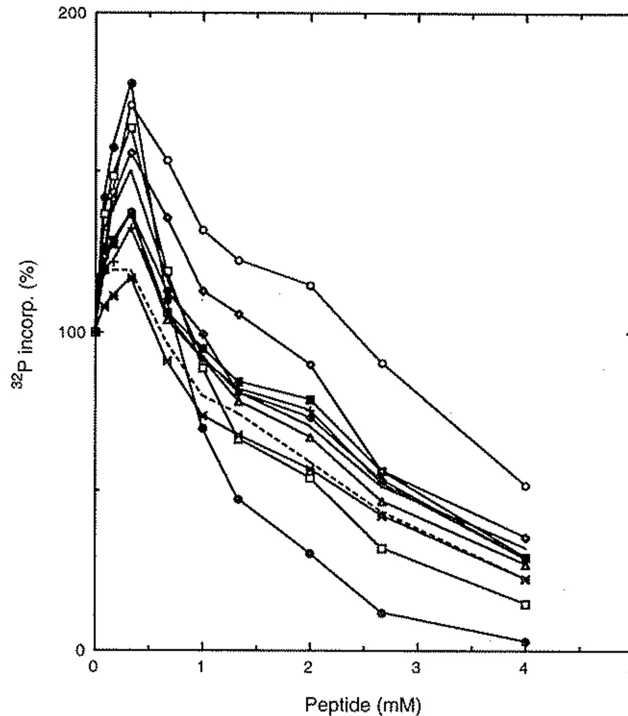

Fig. 17.2. Quantification of the ^{32}P labelling in specific bands of the phosphorimage shown in Fig. 17.1. LHCII, *filled circles*; 9 kDa, *open circles*; CP43, *filled squares*; 56 kDa, *open squares*; 12 kDa, *open rhombus*; 20 kDa, *open triangles*; 18 kDa, *dotted line*; 16 kDa, *solid line*; 40 kDa, *filled rhombus*; D1, *plus*; D2, *asterisks*.

Light-state transitions are a mechanism by which photosynthetic organisms regulate the distribution of light excitation between the two photosystems (Allen *et al.* 1981; Allen 1992). It has been proposed that the transient existence of a 'supercomplex' of LHCII with PSII and the cytochrome b_6/f complex, interconnected by confined plastoquinone molecules, is a control mechanism for linear electron flow from PSII (Lavergne and Joliot 1991), and that a specific diffusion of cytochrome b/f complex from the grana to the stromal domain during the state 2 transition activates cyclic electron flow in photosystem I. Based on the structure of LHCII (Kühlbrandt and Wang 1991; Kühlbrandt *et al.* 1994), it might be expected that most of the subunit–subunit interactions of the LHCII monomer will be mediated by residues in the N-terminal domain. Alterations near the N-terminus may affect trimer formation or association of LHCII with the PSII core proteins. The N-terminal domain of LHCII and other thylakoid proteins may thus play an important part in protein–protein interactions within the thylakoid membrane. The LHCII

associated with the membrane-bound protein kinase by the residues in the N-terminal domain, together with the cytochrome *b/f* complex or some other proteins, may form a supercomplex. The peptide analogue of the N-terminal segment of LHCII would be recognized by the kinase and occupy the binding site between LHCII and the kinase, changing their structural interactions. As soon as the LHCII binding site becomes saturated by the peptide, the peptide may compete for the kinase with thylakoid protein, producing an inhibitory effect (Fig. 17.2). The synthetic peptide analogue of the N-terminal segment of LHCII was originally expected to act as a substrate for LHCII kinase and to inhibit competitively the phosphorylation only of the LHCII, but it was found that the effects of the peptide on phosphorylation of different thylakoid proteins are very similar (Fig. 17.2). This observation indicates that all of the recognition features required by the protein kinase could be present in most thylakoid proteins including LHCII, PSII core proteins as well as the 15-residue synthetic peptide. We thus suggest that thylakoid protein kinase exhibits broad specificity and phosphorylates multiple thylakoid membrane proteins.

Effects of a synthetic phosphopeptide and phosphatase inhibitors on phosphoprotein dephosphorylation

Figure 17.3. shows a phosphorimage of the time-course of dephosphorylation reactions of pea thylakoid phosphoproteins separated by SDS-PAGE. Purified pea thylakoid membranes became phosphorylated under illumination. When phosphorylated thylakoid membranes were then incubated in darkness, the phosphoproteins became dephosphorylated with very different kinetics. The relative quantities of ^{32}P in specific bands of the phosphorimage are shown in Fig. 17.4. The dephosphorylation half-time (the time when 50 per cent of the initial ^{32}P label remained) of each phosphoprotein is shown in Table 17.1. The most rapidly dephosphorylated protein was phospho-LHCII, with a half-time of 7 min. The most slowly dephosphorylated proteins were the 18 kDa and 9 kDa phosphoproteins, with about 60–70 per cent ^{32}P remaining in these bands even after 180 min.

Fluoride inhibits the dephosphorylation of LHCII and 9 kDa proteins (Bennett 1980), but whether this inhibitor has a similar effect on other phosphorylated components in thylakoid membranes is not clear. Antimycin A is a cytochrome b_6f complex inhibitor, interacting with the quinone reductase site of the complex, and has been found to activate LHCII phosphorylation (Gal *et al.* 1988). To investigate further the substrate specificity of thylakoid protein phosphatase, and to examine the effect of inhibitors on the phosphatase, a synthetic phosphopeptide (RKSAT(PO4)TKKVASSGSP) analogue of the N-terminal

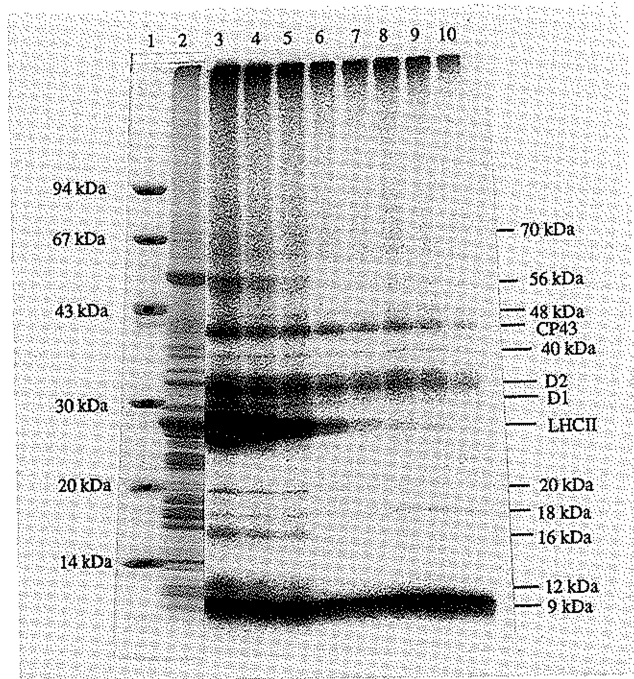

Fig. 17.3. SDS-PAGE of the time-course of dephosphorylation of pea thylakoid membrane phosphoproteins. Track 1 shows the molecular weight markers. Track 2 shows the Coomassie blue stained gel for the 0 time incubation in darkness. Stained gels for all the samples are identical. Tracks 3–10 are phosphorimages showing the extent of ^{32}P labelling at different incubation time in darkness. Track 3 (0 min), track 4 (10 min), track 5 (20 min), track 6 (40 min), track 7 (60 min), track 8 (90 min), track 9 (120 min), track 10 (180 min).

phosphorylation site of LHCII was used in conjunction with sodium fluoride and antimycin A. Figure 17.5 shows a phosphorimage of the time-course of dephosphorylation of pea thylakoid membrane phosphoproteins in the presence of peptide, NaF, and antimycin A. The synthetic phosphopeptide analogue of the N-terminal segment of LHCII acts as a competitive inhibitor not only of phospho-LHCII, but of all the phosphoproteins. NaF at 10 mM partially inhibits most phosphoprotein dephosphorylations. Antimycin A at 15 μM causes slight inhibition of the dephosphorylation of some phosphoproteins, notably of CP 43. The half-times of dephosphorylation reactions of thylakoid phosphoproteins in presence of the phosphopeptide, NaF, and antimycin A are listed in Table 17.1.

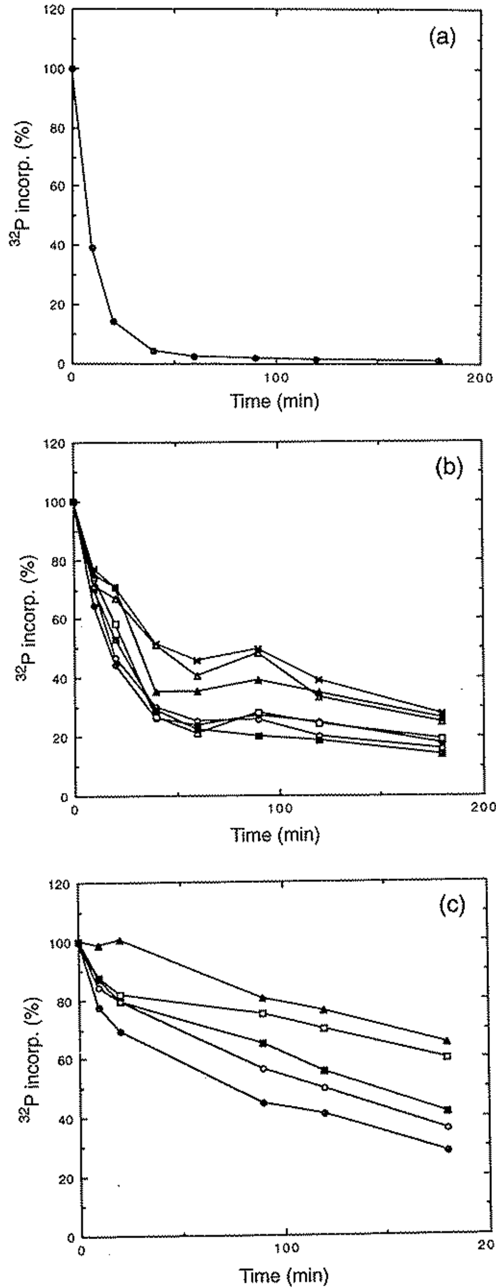

Fig. 17.4. Quantification of the ^{32}P Labelling in specific bands of the phosphorimage shown in Fig. 17.3. The sample incubated at zero time served as 100% ^{32}P incorporation. (a), LHCII, *filled circle*. (b), 56 kDa, *filled circles*; 12 kDa, *open circles*; D1, *filled squares*; 16 kDa, *open squares*, 20 kDa, *filled triangles*; CP43, *open triangles*; D2, *asterisks*. 70 kDa, *filled circles*; 48 kDa, *open circles*; 40 kDa, *filled squares*, 18 kDa, *open squares*; 9 kDa, *filled triangles*.

Table 17.1 The half-times of the dephosphorylation of thylakoid phosphoproteins in the absence and presence of synthetic phosphopeptide, NaF, and antimycin A.

Phosphoproteins	No addition (min)	Synthetic phospho- (min)	NaF (min)	Antimycin A (min)
LHCII	7	86	98	7
56 *kDa*	17	125	109	18
12 *kDa*	19	>180	141	27
D1	23	125	116	25
16	26	129	156	33
20 kDa	33	180	>180	73
CP43	43	98	37	98
D2	47	131	102	59
70 kDa	80	147	103	96
48 kDa	122	159	–	141
40 kDa	147	180	147	147
18 kDa	>180	>180	>180	>180
9 kDa	>180	>180	>180	>180

^{32}P-labelled thylakoid proteins were incubated in darkness in absence and presence of synthetic phosphopeptide, NaF and antimycin A for a range of times between 10 min and 180 min. The samples were fractionated by SDS-PAGE and subsequent analysis by phosphorimaging using a Fuji Bio-Imaging analyzer BAS 2000 (see Figs 17.1 and 17.3). The half-times were calculated when 50% of the initial ^{32}P label remained.

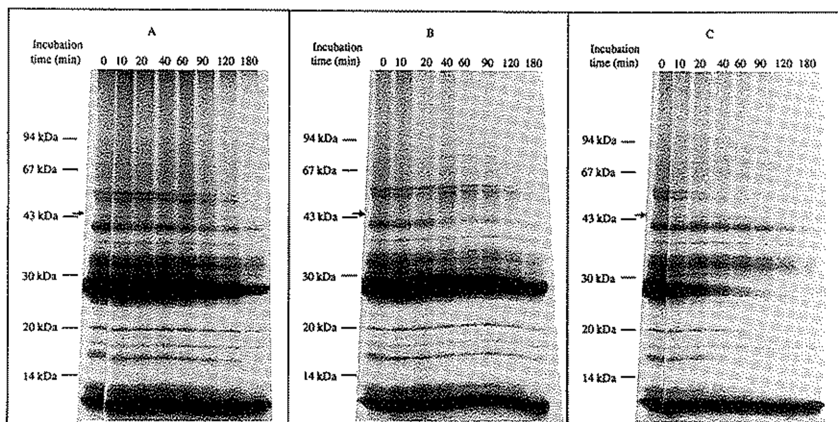

Fig. 17.5. Phosphorimage of the time-course of dephosphorylation of pea thylakoid membrane proteins in presence of (A) synthetic phosphopeptide, (B) NaF, and (C) antimycin A.

Conclusions

We demonstrated that the peptide (RKSATTKKVASSGSP) affects the phosphorylation of different thylakoid proteins. These results provide direct evidence for the existence of a thylakoid protein kinase which exhibits broad substrate specificity and phosphorylates a large number of thylakoid proteins including LHCII, PSII core proteins and some minor thylakoid proteins. Activation of thylakoid protein kinase by the synthetic peptide indicates that the protein–protein interactions between the kinase and thylakoid proteins maybe mediated by residues in the N-terminal domain. We also suggest that structural features required for recognition of the phosphoprotein phosphatase are common to different thylakoid phosphoproteins as well as to the phosphopeptide itself: at least one thylakoid phosphoprotein phosphatase exhibits a broad substrate specificity. The synthetic peptide analogue of an N-terminal segment of phosphorylated thylakoid protein is of particular interest for characterization of structure, function and substrate specificity of the protein kinase. It will be important to know if the synthetic peptides with the N-terminus of the 9 kDa protein act as substrates for the thylakoid protein kinase. The possible mechanism of regulation of both thylakoid protein kinase and phosphatase are currently under further investigation.

Acknowledgements

We thank Drs Dalibor Stys and Michael D. Spangfort for discussions, Henry Franén and Ivo Bláha for peptide synthesis. This work was supported by grants from the Swedish Natural Science Research Council and the Per-Eric Ulla Schyberg Foundation.

References

Allen, J. F. (1992). Protein phosphorylation in regulation of photosynthesis. *Biochimica et Biophysica Acta* **1098**, 275–335.

Allen, J. F. and Findlay, J. B. C. (1986). Amino acid composition of the 9 kDa phosphoprotein of pea thylakoids. *Biochemical and Biophysical Research Communications* **138**, 146–52.

Allen, J. F., Bennett, J., Steinback, K. E. and Amtzen, C. J. (1981). Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. *Nature* **291**, 25–9.

Barany, G. and Merrifield, R. B. (1980). Special methods in peptide synthesis. In *The peptides*, (ed. E. Gross and J. Meienhofer), **vol 2**, pp. 1–284, Academic Press, New York.

Bennett, J. (1980). Chloroplast phosphoproteins. Evidence for a thylakoid-bound phosphoprotein phosphatase. *European Journal of Biochemistry* **104**, 85–9.

Gal, A., Schuster, G., Frid, D., Canaani, O., Schwieger, H. G. and Ohad, I. (1988). Role of the cytochrome b_6f complex in the redox-controlled activity of *Acetabularia* thylakoid protein kinase. *Journal of Biological Chemistry* **263**, 7785–91.

Grehn, L., Fransson, B. and Ragnarsson, U. (1987). Synthesis of substrates of cyclic AMP-dependent protein kinase and use of their protected precursors for the convenient preparation of phosphoseiine peptides. *Journal of the Chemical Society Perkin Transactions I*, 529–35.

Harrison, M. A. and Allen, J. F. (1991). Light-dependent phosphorylation of photosystem II polypeptides maintains electron transport at high light intensity: separation from effects of phosphorylation of LHCII. *Biochimica et Biophysica Acta* **1058**, 289–96.

Kühlbrandt, W. and Wang, D. (1991). Three-dimensional structure of plant light-harvesting complex determined by electron crystallography. *Nature* **350**, 130–4.

Kühlbrandt, W., Wang, D. and Fujiyoshi, Y. (1994). Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367**, 614–21.

Lavergne, J. and Joliot, P. (1991). Restructed diffusion in photosynthetic membranes. *Trends in Biochemical Sciences* **16**, 129–34.

Michel, H. and Bennett, J. (1989) Use of synthetic peptides to study the substrate specificity of a thylakoid protein kinase. *FEBS Letters* **254**, 165–70.

Silverstein, T., Cheng, L. and Allen, J. F. (1993*a*). Redox titration of multiple protein phosphorylations in pea chloroplast thylakoids. *Biochimica et Biophysica Acta* **1183**, 215–20.

Silverstein, T., Cheng, L. and Allen, J. F. (1993*b*). Chloroplast thylakoid protein phosphatase reactions are redox-independent and kinetically heterogeneous. *FEBS Letters* **334**, 101–5.

Sun, G., Sarath, G. and Markwell, J. (1993). Phosphopeptides as substrates for thylakoid protein phosphatase activity. *Archives of Biochemistry and Biophysics* **304**, 490–5.