

Effects of synthetic peptides on thylakoid phosphoprotein phosphatase reactions

Lüling Cheng, Dalibor Stys and John F. Allen

Cheng, L., Stys, D. and Allen, J. F. 1995. Effects of synthetic peptides on thylakoid phosphoprotein phosphatase reactions. – *Physiol. Plant.* 93: 173–178.

A synthetic peptide analogue of the phosphorylation site of LHC II, when phosphorylated by thylakoid membranes, served as a substrate for the thylakoid phosphoprotein phosphatase. The phosphopeptide became dephosphorylated at a low rate, comparable to that of the 9 kDa phosphoprotein. Phospho-LHC II itself became dephosphorylated much more rapidly, at a rate unaffected by endogenous phosphorylation of the peptide. Endogenous phosphorylation of the peptide was also without effect on other thylakoid protein phosphorylation and dephosphorylation reactions. In contrast, dephosphorylation of many thylakoid phosphoproteins was inhibited by addition of a pure, chemically-synthesised phosphopeptide analogue of phospho-LHC II. This result suggests that at least one thylakoid phosphoprotein phosphatase exhibits a broad substrate specificity. The results indicate that any one of a number of amino acid sequences can give a phosphoprotein configuration that is recognised by a single phosphatase.

Key words – Chloroplast thylakoids, LHC II, molecular recognition, phosphoprotein phosphatase, protein kinase, synthetic phosphopeptide.

L. Cheng, D. Stys and J. F. Allen (corresponding author), Plant Cell Biology, Lund Univ., Box 7007, S-220 07 Lund, Sweden.

This paper is part of the contributions to a Plant Cell Biology Workshop on Thylakoid Protein Phosphorylation, held at Lund University, Sweden, 27–29 March, 1994.

Introduction

Reversible protein phosphorylation regulates protein function in many aspects of prokaryotic and eukaryotic metabolism, gene expression and response to environmental change (Edelman et al. 1987). Protein phosphorylation is catalysed by protein kinases and dephosphorylation by protein phosphatases. In plants, a number of protein phosphorylation systems have been found (Ranjiva and Boudet 1987). One of these is the chloroplast thylakoid system that regulates photosynthesis (Bennett 1991, Allen 1992). Phosphorylation of thylakoid proteins is stimulated by light (Bennett 1979) and when phosphorylated material is then placed in the dark, it becomes dephosphorylated (Bennett 1980). The phosphatase that catalyses dephosphorylation of thylakoid phosphoproteins is membrane bound (Bennett 1980) and redox-inde-

pendent (Silverstein et al. 1993). The enzyme was found to be inhibited by classical phosphatase inhibitors fluoride (Bennett 1980) and molybdate (Owens and Ohad 1982) but not by okadaic acid (Mackintosh et al. 1991, Sun and Markwell 1992), an inhibitor of specific mammalian phosphatases (Cohen et al. 1990).

A central question is how protein kinases and phosphatases recognise their diverse protein substrates. Since structural information is available for only a handful of protein substrates (Johnson and Barford 1993), synthetic peptides have played an important role in assessing the specificity and recognition features of protein kinases and phosphatases (Kemp and Pearson 1990, Kennelly and Krebs 1991). Synthetic phosphopeptides have been used to distinguish different protein phosphatases (Donella-Deana et al. 1990, 1991) and as model substrates to investigate their specificity (Agostinis et al. 1987, 1990),

Received 27 June, 1994; revised 26 August, 1994

providing clues to sequence requirements for protein phosphatase recognition. For the thylakoid phosphoprotein phosphatase, synthetic phosphopeptides mimicking the N-terminal phosphorylation site of the LHC II have been shown to act as substrates of a phosphoprotein phosphatase. Dephosphorylation of phosphopeptides by thylakoid membranes was similar to dephosphorylation of endogenous LHC II in its pH-dependence profile, in its sensitivity to inhibitors, and in its divalent cation requirement (Sun et al. 1993). To test further the regulatory properties of thylakoid phosphoprotein phosphatase and the structural requirements of its substrates, we used both unphosphorylated and phosphorylated synthetic peptides. Our results demonstrate that dephosphorylation of a phosphopeptide that has been phosphorylated by a thylakoid-bound protein kinase proceeds at a much lower rate than that of phospho-LHC II. We also show that the presence of endogenously phosphorylated peptide has no effect on the dephosphorylation of LHC II or other thylakoid phosphoproteins. However, the corresponding chemically-synthesised phosphopeptide exhibits an inhibitory effect on all phosphoprotein phosphatase reactions. We suggest that any one of a number of amino acid sequences can give a phosphoprotein configuration that is sufficient for recognition of the phosphoprotein by a single phosphatase. However, the configuration of other domains in the native protein, remote from the phosphorylation site, may also be essential for efficient catalysis. The possible regulatory role of molecular recognition between thylakoid phosphoprotein phosphatase and its substrates is discussed.

Abbreviations – Chl, chlorophyll; CP43, PS II (psbC) polypeptide; D1 and D2, 31 (psbA) and 32 (psbD) kDa PS II reaction centre polypeptides; DCC, dicyclohexylcarbodiimide; HF, hydrogen fluoride; HOBT, 1-hydroxybenzotriazole; LHC II, light harvesting complex II; 9 kDa, PS II (psbH) polypeptide; PQ, plastoquinone; PS I and II, photosystem I and II; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Materials and methods

Plant material

Pea seedlings (*Pisum sativum* L. cv. Sockerärt de grace) were grown at 20°C with a 12 h light period. Leaves were harvested during the light period 14–16 days after sowing.

Preparation of the synthetic peptide

A 15 amino-acid synthetic peptide corresponding to an N-terminal fragment of pea LHC II (RKSATTKK-VASSGSP) and its phosphorylated form (RKSAT[PO₄]TKKVASSGSP) were synthesised by a solid phase, t-Boc strategy according to Barany and Merrifield (1980) and Grehn et al. (1987). The phosphopeptide was synthesized using the standard Boc-protocol on an ABI 430A solid phase peptide synthesizer. In place of phosphorylated threonine in the synthetic peptide, a Boc-

Thr[OPO(OPh)₂] was incorporated. Boc-Thr[OPO(OPh)₂] was obtained by phosphorylating Boc-Thr-OBn with diphenylphosphochloridate according to Perich (1991) and then hydrogenated using 5% Pd/C in ethyl-acetate:acetic acid (25:1) for 2 h. The phosphothreonine was coupled twice for 3 h using DCC and HOBT. The peptide was cleaved from the resin with HF:Anisole (9:1) for one hour at –5°C. After washing the resin with ether and extracting the peptide with 30% acetic acid, the peptide was lyophilized. The product was then hydrogenated in the solution containing 200 mg peptides, 185 mg platinum oxide (PtO₂) in H₂ at twice atmospheric pressure in 12 ml 80% acetic acid for 4 days. The product was purified on a 250 × 20 mm Kromasil 5 µm, C8 column with 1% TFA-acetonitrile gradient. The purity of the phosphopeptide was >90% as revealed by HPLC and mass spectroscopy (not shown). The correct mass according to mass spectroscopy was 1585.5 Da (theoretical mass: 1584.7 Da). High specific activity [γ -³²P]ATP was obtained from Amersham. Other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

In vitro phosphorylation and dephosphorylation

Thylakoid membranes were isolated from pea chloroplasts by the method described in Harrison and Allen (1991) and then stored on ice in darkness for 60 min prior to radiolabelling. Phosphorylation of thylakoid membrane proteins and of the synthetic peptide was carried out by incubation of purified thylakoids at 0.2 mg Chl ml⁻¹ together with 334 µM synthetic peptide in a medium containing 0.1 M sorbitol, 50 mM HEPES (pH 7.6) and MgCl₂, NaCl, NH₄Cl, all at 5 mM and [γ -³²P]ATP (0.2 mM) at a molar activity of 3.7 TBq mol⁻¹ at 22°C (Allen and Findlay 1986) and by illuminating the samples for 10 min with a desk-lamp giving a light intensity ~ 130 µmol m⁻² s⁻¹. Dephosphorylation was obtained by incubation of the ³²P-labelled samples in darkness from 10 to 180 min with or without chemically-synthesised phosphopeptide (final concentration 167 µM). Dephosphorylation time-courses were started by switching off the light, and samples (100 µl) were withdrawn at intervals and immediately precipitated by mixing with 0.8 ml acetone (pre-cooled at –20°C). The sample at zero time served as the control (100% phosphorylation). The acetone suspension was stored on ice for at least 30 min before centrifugation at 13 000 g for 2.5 min. The protein pellets were prepared for gel electrophoresis as described below.

Polyacrylamide-gel electrophoresis

Samples were solubilized in 62.5 mM Tris-HCl (pH 6.8) buffer, containing 12% glycerol, 5% 2-mercaptoethanol, 4.5% SDS, and 0.01% bromophenolblue by vortexing and incubation at room temperature for 2 h until the pellets were dissolved. The samples were heated to 70°C for 5 min, and centrifuged at 13 000 g for 5 min before loading on the slab gel. SDS-PAGE was performed on

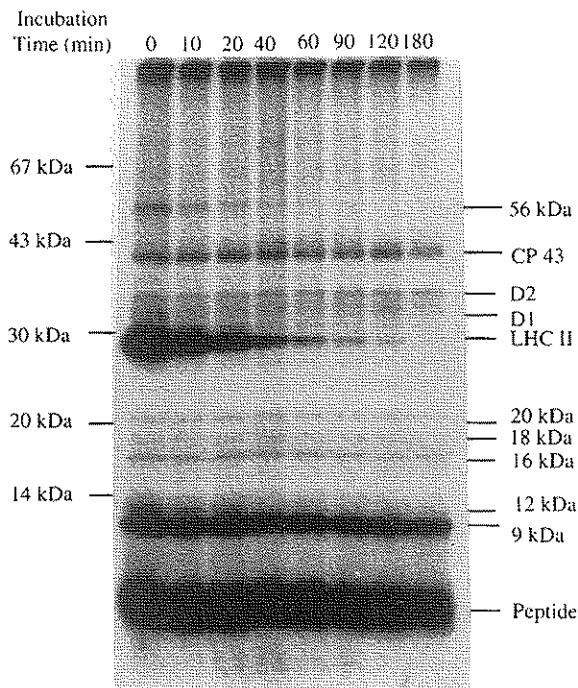


Fig. 1. SDS-PAGE phosphorimage of the time-course of dephosphorylation of both endogenously phosphorylated peptide and pea thylakoid phosphoproteins. Purified pea thylakoid membranes were incubated with the unphosphorylated peptide in a reaction medium with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, illuminated for 10 min. The ^{32}P labelled samples were subsequently incubated in darkness for 0, 10, 20, 40, 60, 90, 120 and 180 min. Stained gels for all the samples are identical (not shown). Tracks were loaded with protein equivalent to 2 μg chlorophyll.

10–25% gradient gels with the buffer system of Laemmli (1970). Silver staining of the gels was performed according to manufacturer's instructions for Bio-Rad 'silver stain plus'. Gels were dried and subsequently analysed by phosphorimaging. The quantity of radioisotope present in the specific bands was measured using a Fuji Bio-Imaging analyzer BAS 2000.

Chlorophyll determination

Chlorophyll determination in 80% acetone was performed by the method of Arnon (1949).

Results and discussion

The potentially phosphorylated residues in pea LHC II have been identified (Mullet 1983) and shown to be preferentially Thr-5 (Thr-6 if the N-terminal Met is counted as the first amino acid) (Bennett et al. 1987). From the pea LHC II sequences deduced by Cashmore (1984), a synthetic peptide (RKSATTKKVASSGSP) analogue of an N-terminal segment of LHC II and its phosphorylated form (RKSAT[PO_4]TKKVASSGSP) were synthesised. The unphosphorylated synthetic peptide was phosphorylated by a thylakoid protein kinase simulta-

neously with native thylakoid proteins when the peptide was incubated together with thylakoid membranes illuminated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Dephosphorylation of this phosphopeptide (here termed 'endogenously phosphorylated peptide') and of the other thylakoid phosphoproteins started when the light was switched off. Figure 1 is a phosphorimage of an SDS-PAGE gel showing a typical time-course of dephosphorylation of endogenously phosphorylated peptide and thylakoid phosphoproteins. The relative quantities of the ^{32}P -labelling in specific bands of the phosphorimage are shown in Fig. 2. As can be seen in Figs 1 and 2, despite its identical sequence with a fragment of LHC II, the endogenously phosphorylated peptide was dephosphorylated slowly, at a rate comparable to that of dephosphorylation of the 9 kDa phosphoprotein. Dephosphorylation of phospho-LHC II was much more rapid, with a half-time of 7 min, in agreement with results obtained in the absence of peptide (Cheng et al. 1994). The phosphopeptide served as a weak substrate for the phosphatase, indicating that the recognition features required by the phosphatase were present in the phosphopeptide. However, since dephosphorylation of the endogenously phosphorylated peptide was slow compared to the dephosphorylation of its parent protein LHC II, some structural feature of the phosphopeptide must be unfavourable for the phosphatase activity. Slower dephosphorylation of the phosphopeptide may also be caused either by low stability of the structure of the phosphopeptide or by a higher probability that membrane proteins interact with each other than with a

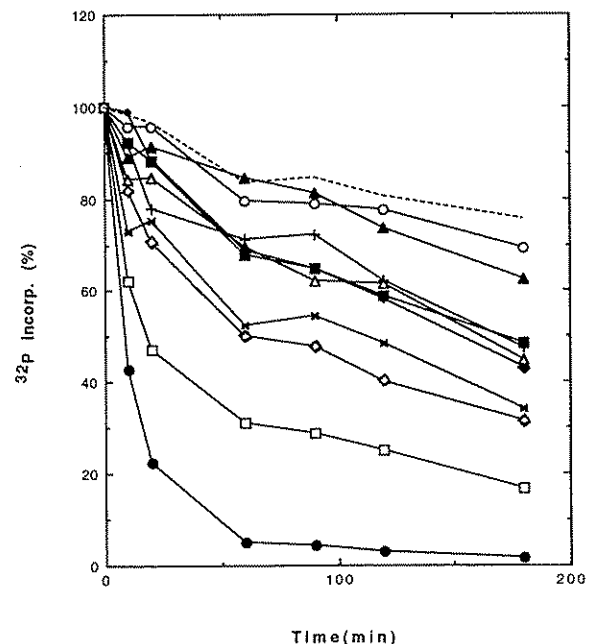


Fig. 2. Quantification of ^{32}P -labelling in specific bands of the phosphorimage shown in Fig. 1. The sample incubated at zero time served as 100% ^{32}P incorporation. Peptide (---), LHC II (●), 9 kDa (○), CP43 (■), 56 kDa (□), 12 kDa (◇), 20 kDa (△), 18 kDa (▲), 16 kDa (◆), D1 (+), D2 (x).

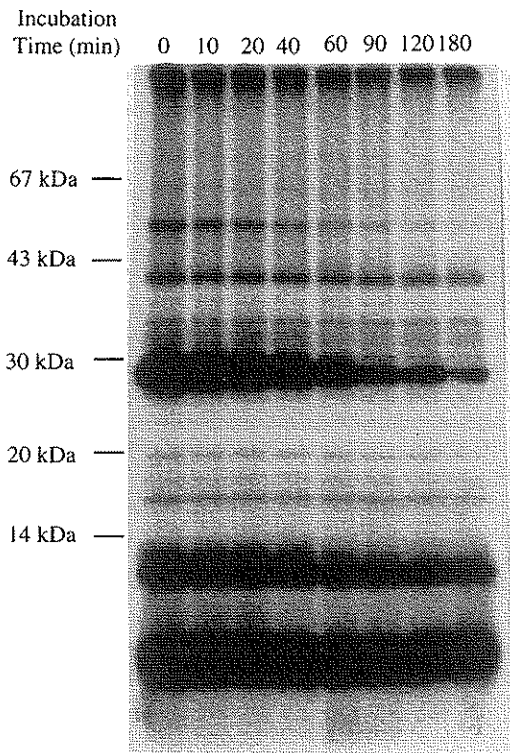


Fig. 3. SDS-PAGE phosphorimage of the time-course of dephosphorylation of both endogenously phosphorylated peptide and pea thylakoid phosphoproteins in presence of exogenous phosphopeptide. The ^{32}P labelled samples were incubated with $167 \mu\text{M}$ of exogenous phosphopeptide in darkness for 0, 10, 20, 40, 60, 90, 120 and 180 min. Stained gels for all the samples are identical (not shown). Tracks were loaded with protein equivalent to $2 \mu\text{g}$ chlorophyll.

protein in solution. Competition of a membrane protein with a soluble protein for the same binding site is incompletely understood. Nevertheless, other factors may be responsible for less efficient catalysis of the endogenously phosphorylated peptide by the thylakoid phosphoprotein phosphatase. These factors could include absence of other domains in the native protein, remote from the phosphorylated site, or a low stability of the proper configuration on the phosphopeptide.

The presence of endogenously phosphorylated peptide did not interfere with the dephosphorylation of phospho-LHC II. Similar results were observed on the dephosphorylation of most other thylakoid phosphoproteins (Fig. 2). The results show that the thylakoid phosphoprotein phosphatase is activated not only by its native substrates, but also by an exogenous product of the kinase reaction. On the other hand, although the total concentration of the phosphopeptide is higher than those of the phosphoproteins (Fig. 1, Lane time 0), it does not compete significantly with them for the phosphatase. The difference between the phosphoproteins and the phosphopeptide in recognition by the phosphoprotein phosphatase

may be responsible for their different rates of dephosphorylation.

Figure 3 shows a phosphorimage of SDS-PAGE fractionation of the time-course of dephosphorylation of both endogenously phosphorylated peptide and thylakoid phosphoproteins in presence of the chemically-synthesised phosphopeptide (RKSAT[PO_4]TKKVASSGSP). The chemically-synthesised phosphopeptide acts as a competitive inhibitor not only of phospho-LHC II, but also of all the thylakoid phosphoproteins (Cheng et al. 1994). By addition of the same phosphopeptide in the system with the endogenously phosphorylated peptide present, the dephosphorylation of most thylakoid phosphoproteins was inhibited (Fig. 4). The inhibitory effects are stronger on the rapidly dephosphorylated phosphoproteins such as LHC II and 56 kDa phosphoprotein compared to those slowly dephosphorylated phosphoproteins such as 9 kDa and endogenously phosphorylated peptide, the dephosphorylation of which were hardly detectable. However, the inhibitory effects of the chemically-synthesised phosphopeptide on the dephosphorylation of thylakoid phosphoproteins in the presence of endogenously phosphorylated peptide are slightly less compared with the dephosphorylation of thylakoid phosphoproteins in the absence of endogenously phosphorylated peptide. We have measured the concentration of the phosphorylated peptide in the solution of the endogenous peptide by HPLC. The chromatographic peak of the phosphopeptide was not detectable although the phosphorylated and unphosphorylated peptides were clearly

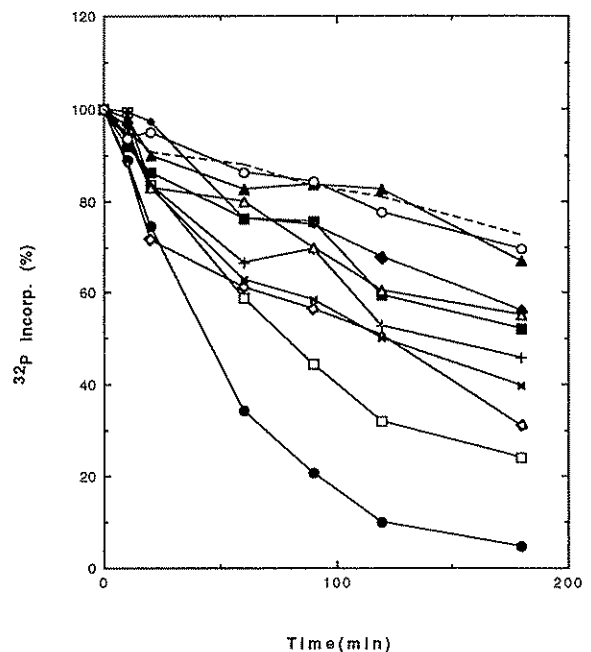


Fig. 4. Quantification of the ^{32}P labelling in specific bands of phosphorimage shown in Fig. 3. The sample incubated at zero time served as 100% ^{32}P incorporation. Peptide (---), LHC II (●), 9 kDa (○), CP43 (■), 56 kDa (□), 12 kDa (◇), 20 kDa (△), 18 kDa (▲), 16 kDa (◆), D1 (+), D2 (x).

resolvable on the control assay (data not shown). The concentration of the phosphopeptide in the solution containing endogenously phosphorylated peptide is therefore negligible compared to that of the unphosphorylated peptide. This again supports the conclusion that the phosphopeptide is a weak substrate compared to the membrane phosphoproteins and is able to inhibit the phosphatase only at high concentration. We cannot exclude very limited feedback control of the phosphatase by the concentration of the substrate but our experiments make such control less probable.

In summary, our results show that the presence of the endogenously phosphorylated peptide served only as a weak substrate of the phosphatase, with a dephosphorylation rate similar to that of the 9 kDa phosphoprotein, which has a sequence at the phosphorylation site unrelated to that of LHC II (Michel and Bennett 1987) and the phosphopeptide. When the chemically-synthesised phosphopeptide was present at a concentration much higher than that of the endogenously phosphorylated peptide, it acted as a competitive inhibitor of the phosphatase, not only for dephosphorylation of LHC II but also for dephosphorylation of other thylakoid phosphoproteins. The results are in agreement with our previous observation and support the conclusion that at least one phosphoprotein phosphatase in thylakoid membranes exhibits a broad substrate specificity. The results indicate that any one of a number of amino acid sequences can give a phosphoprotein configuration that is sufficient for recognition of the phosphoprotein by a single phosphatase. However, the configuration of other domains remote from the phosphorylated site of the substrate are likely to be essential for efficient catalysis. The availability of phosphopeptides creates the possibility of using an artificial structure for specific detection and assay the thylakoid phosphoprotein phosphatase. Future experiments involving structural and enzymological characterisation of synthetic phosphopeptide analogues of the phosphorylation site of other thylakoid phosphoproteins as substrates for the thylakoid phosphoprotein phosphatase should clarify the general structural features required for substrate recognition.

Acknowledgments – We thank Dr Ivo Bláha for the unphosphorylated synthetic peptide and Dr Henry Franzén for the synthetic phosphopeptide. We also thank the Swedish Natural Science Research Council (NFR) and Per-Eric and Ulla Schyberg Foundation for research grants and NFR and Federation of European Biochemical Societies for fellowships to D.S.

References

- Agostinis, P., Goris, J., Waelkens, E., Pinna, L. A., Marchiori, F. & Merlevede, W. 1987. Dephosphorylation of phosphoproteins and synthetic phosphopeptides. – *J. Biol. Chem.* 262: 1060–1064.
- , Goris, J., Pinna, L. A., Marchiori, F., Perich, J. W., Meyer, H. E. & Merlevede, W. 1990. Synthetic peptides as model substrates for the study of specificity of the polycation-stimulated protein phosphatases. – *Eur. J. Biochem.* 189: 235–241.
- Allen, J. F. 1992. Protein phosphorylation in regulation of photosynthesis. – *Biochim. Biophys. Acta* 1098: 275–335.
- & Findlay, J. B. C. 1986. Amino acid composition of the 9 kDa phosphoprotein of pea thylakoids. – *Biochem. Biophys. Res. Commun.* 138: 146–152.
- Arnon, D. I. 1949. Copper enzymes in chloroplasts. Polyphenol-oxidases in *Beta vulgaris*. – *Plant Physiol.* 24: 1–15.
- Barany, G. & Merrifield, R. B. 1980. Special methods in peptide synthesis. – *In* The Peptides (E. Gross and J. Meienhofer, eds), Vol. 2, pp. 1–284. Academic Press, New York, NY. ISBN 0-12-304202-X.
- Bennett, J. 1979. Chloroplast phosphoproteins. The protein kinase of thylakoid membranes is light-dependent. – *FEBS Lett.* 103: 342–344.
- 1980. Chloroplast phosphoproteins. Evidence for a thylakoid-bound phosphoprotein phosphatase. – *Eur. J. Biochem.* 104: 85–89.
- 1991. Protein phosphorylation in green plant chloroplast. – *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42: 281–331.
- , Shaw, E. K. & Bakr, S. 1987. Phosphorylation of thylakoid proteins and synthetic peptide analogs. – *FEBS Lett.* 210: 22–26.
- Cashmore, A. R. 1984. Structure and expression of a pea nuclear gene encoding a chlorophyll *a/b*-binding polypeptide. – *Proc. Natl. Acad. Sci. USA* 81: 2960–2964.
- Cheng, L., Spangfort, M. D. & Allen, J. F. 1994. Substrate specificity and kinetics of thylakoid phosphoprotein phosphatase reactions. – *Biochim. Biophys. Acta* (In press).
- Cohen, P., Holmes, C. F. B. & Tsukitani, Y. 1990. Okadaic acid: a new probe for the study of cellular regulation. – *Trends Biochem. Sci.* 15: 98–102.
- Donella-Deana, A., Mac Gowan, C. H., Cohen, P., Marchiori, F., Meyer, H. E. & Pinna, L. A. 1990. An investigation of the substrate specificity of protein phosphatase 2C using synthetic peptide substrates; comparison with protein phosphatase 2A. – *Biochim. Biophys. Acta* 1051: 199–202.
- , Meyer, H. E. & Pinna, L. A. 1991. The use of phosphopeptides to distinguish between protein phosphatase and acid/alkaline phosphatase activities: opposite specificity toward phosphoserine/threonine substrates. – *Biochim. Biophys. Acta* 1094: 130–133.
- Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. 1987. Protein serine/threonine kinases. – *Annu. Rev. Biochem.* 56: 567–613.
- Grehn, L., Fransson, B. & Ragnarsson, U. 1987. Synthesis of substrates of cyclic AMP-dependent protein kinase and use of their protected precursors for the convenient preparation of phosphoserine peptides. – *J. Chem. Soc. Perkin Trans. I.* 529–535.
- Harrison, M. A. & Allen, J. F. 1991. Light-dependent phosphorylation of photosystem II polypeptides maintains electron transport at high light intensity: separation from effects of phosphorylation of LHC-II. – *Biochim. Biophys. Acta* 1058: 289–296.
- Johnson, L. N. & Barford, D. 1993. The effects of phosphorylation on the structure and function of proteins. – *Annu. Rev. Biophys. Biomol. Struct.* 22: 199–232.
- Kemp, B. E. & Pearson, R. B. 1990. Protein kinase recognition sequence motifs. – *Trends Biochem. Sci.* 15: 342–346.
- Kennelly, P. J. & Krebs, E. G. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. – *J. Biol. Chem.* 266: 15555–15558.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. – *Nature* 227: 680–685.
- Mackintosh, C., Coggins, J. & Cohen, P. 1991. Plant protein phosphatases. – *Biochem. J.* 273: 733–738.
- Michel, H. & Bennett, J. 1987. Identification of the phosphorylation site of an 8.3 kDa protein from photosystem II of spinach. – *FEBS Lett.* 212: 103–108.
- Mullet, J. E. 1983. The amino acid sequence of the polypeptide

- segment which regulates membrane adhesion (grana stacking) in chloroplasts. – J. Biol. Chem. 258: 9941–9948.
- Owens, G. & Ohad, I. 1982. Phosphorylation of *Chlamydomonas reinhardtii* chloroplast membrane proteins in vivo and in vitro. – J. Biol. Chem. 93: 712–718.
- Perich, J. W., Alewood, P. F. & Johns, R. B. 1991. Synthesis of casein-related peptides and phosphopeptides. VII* The efficient synthesis of ser(P)-containing peptides by the use of Boc-ser(PO₃R₂)-OH derivatives. – Aust. J. Chem. 44: 233–252.
- Ranjeva, R. & Boudet, A. M. 1987. Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. – Annu. Rev. Plant Physiol. 38: 73–93.
- Silverstein, T., Cheng, L. & Allen, J. F. 1993. Chloroplast thylakoid protein phosphatase reactions are redox-independent and kinetically heterogeneous. – FEBS Lett. 334: 101–105.
- Sun, G. & Markwell, J. 1992. Lack of types 1 and 2A protein serine (P)/threonine (P) phosphatase activities in chloroplasts. – Plant Physiol. 100: 620–624.
- , Sarath, G. & Markwell, J. 1993. Phosphopeptides as substrates for thylakoid protein phosphatase activity. – Arch. Biochem. Biophys. 304: 490–495.