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Substrate specificity and kinetics of thylakoid phosphoprotein phosphatase reactions

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Abstract

A synthetic 15-amino-acid phosphopeptide analogue of an N-terminal phosphorylated segment of LHC II was found to inhibit dephosphorylation not only of phospho-LHC II but of all other thylakoid phosphoproteins resolved by phosphorimaging. The results 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. Dephosphorylation reaction rates of all 13 thylakoid phosphoproteins were determined, and the dephosphorylation half-times were found to range from 7 min to more than 180 min. Most of the phosphoprotein dephosphorylation reactions were partially inhibited by NaF, and were insensitive to antimycin A and okadaic acid. Nevertheless, both antimycin A and NaF stimulated the phosphorylation of LHC II and the 9 kDa protein. Possible reasons for differences in sensitivity to these inhibitors are discussed.

Keywords: Synthetic phosphopeptide; Chloroplast thylakoid; LHC II; Phosphoprotein phosphatase; Protein kinase; Protein phosphatase inhibitor

1. Introduction

It is known that regulation of excitation energy distribution between the two photosystems in higher plant chloroplasts at limiting light intensity involves the phosphorylation of one or more thylakoid membrane polypeptides [1,2]. The protein kinase responsible for this phosphorylation is membrane bound, and is regulated by the redox state of a component of the photosynthetic electron transport chain [3–5]. The corresponding dephosphorylation is catalysed by one or more protein phosphatases which are not subject to redox control [6–8]. Previous studies on thylakoid phosphoprotein phosphatase reactions identify a pronounced kinetic heterogeneity among the phosphatase substrates [6,8]. Phosphatase activity is stimulated by Mg^{2+} and inhibited by the classical phosphatase inhibitors fluoride [6] and molybdate [9], but is insensitive to electron transport inhibitors. A thylakoid membrane-associated protein phosphatase was found by Sun et al. [10] to be readily removed by salt, the phosphatase activity being stimulated by reducing agents such as DTT and inhibited by chelating agents such as EDTA. Sun et al. also reported that the protein phosphatase activity in crude leaf extracts and in purified intact chloroplasts of wheat and pea was not inhibited by microcystin-LR or okadaic acid [11], suggesting that the phosphatase is not a type 1 or type 2A protein serine (P)/threonine (P) phosphatase.

Elich et al. [12] have suggested that the dephosphorylation of Photosystem (PS) II core proteins is light activated in vivo. However, light-driven net dephosphorylation of proteins was observed only in the presence of DCMU [12]. Under these conditions, light would be

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.

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expected to inactivate the protein kinase by oxidising the PQ pool. We suggest that the results of Elich et al. [12] can be more simply explained by PS I-driven inhibition of the protein kinase, which is active in darkness when the PQ pool is reduced by chlororespiration. Redox titrations of the protein phosphatase reactions of isolated pea chloroplast thylakoid membranes show that dephosphorylation reactions of all the thylakoid phosphoproteins are strictly redox-independent [8], which suggests a light-independent phosphatase both in vitro and in vivo.

The number of protein phosphatases present in the thylakoid membrane and their substrate specificities are unknown. Redox titration of thylakoid proteins phosphorylation has revealed two proteins whose phosphorylation undergoes 'reverse' redox dependence (becoming phosphorylated under oxidising conditions), suggesting that more than one protein kinase or phosphatase functions in the thylakoid membrane [13]. Several acid phosphatases [10,14,15] and alkaline phosphatase [16] isolated from thylakoids are able to dephosphorylate thylakoid phosphoproteins. Synthetic peptide analogues of the phosphorylation site of the LHC II have been used successfully to study the substrate specificity of the LHC II kinase [17–19]. Synthetic phosphopeptides mimicking the N-terminal phosphorylation site of the LHC II have been used to study thylakoid protein phosphatase activity [20]. Here a synthetic phosphopeptide analogue of the phosphorylation site of the LHC II is used to investigate the kinetics and substrate specificity of thylakoid protein phosphatase reactions. Our results demonstrate that the dephosphorylation reactions of thylakoid membranes involve different kinetic processes with different inhibitor sensitivities. Nevertheless, the general inhibitory effect of a single synthetic phosphopeptide suggests that at least one phosphoprotein phosphatase has a broad substrate specificity, and may be sufficient to account for all known thylakoid protein dephosphorylation reactions.

2. Materials and methods

2.1. Plant

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

2.2. Materials

The synthetic phosphopeptide corresponding to an N-terminal fragment of pea phospho-LHC II [21] (RKSAT(PO4)TKKVASSGSP) was synthesised by Dr. Henry Franzén in BM-unit in Lund University using a solid phase, *t*-butyloxycarbonyl (Boc) strategy as described below. The phosphopeptide was synthesised using the standard Boc-protocol on an ABI 430A solid phase peptide synthesiser. 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 Ref. [22] and then hydrogenated using 5% Pd/C in ethylacetate/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/Anisol (9:1) for 1 h at −5°C. After washing the resin with ether and extracting the peptide with 30% acetic acid, the peptide was lyophilised. The product was then hydrogenated in the solution containing 200 mg peptides, 185 mg platinum oxide (PtO_2) in H_2 at twice atmospheric pressure in 12 ml 80% acetic acid for 4 days as described in Refs. [23,24]. 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-^{32}P]ATP was obtained from Amersham. Other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

2.3. In vitro phosphorylation and dephosphorylation of thylakoid proteins

Thylakoid membranes were isolated from pea chloroplasts by the method described in Ref. [25] and then stored on ice in darkness for 60 min prior to radiolabelling. Phosphorylation was carried out by incubation of washed thylakoids at 0.2 mg Chl ml^{-1} in a medium containing 0.1 M sorbitol, 50 mM Hepes (pH 7.6) and $MgCl_2$, NaCl, NH_4Cl, all at 5 mM and [γ-^{32}P]ATP (0.2 mM) at a specific activity of 100 mCi $mmol^{-1}$ at 22°C [26]. The samples were illuminated for 10 min with a desk-lamp giving a light intensity ~ 130 $\mu E\ m^{-2}\ s^{-1}$. Dephosphorylation was obtained by incubation of the labelled thylakoid membrane in darkness with either synthetic peptide (final concentration 167 μM), NaF (final concentration 10 mM) or antimycin A (final concentration 15 μM). 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 (precooled at −20°C). The sample at zero time served as the control (100% phosphorylation).

The effect of antimycin A, NaF and okadaic acid on both thylakoid membrane phosphorylation and dephosphorylation was assayed by a method similar to that described above, but samples were pre-incubated with 15 μM antimycin A, 10 mM NaF or 1 μM okadaic acid

in darkness for 30 min. The phosphorylation reaction was started by addition of [γ-^{32}P]ATP (0.2 mM, at a specific activity of 100 mCi mmol^{-1}) and illumination was for only 5 min at the same light intensity as above. The subsequent dephosphorylation reaction was carried out by incubation of the sample in darkness.

The acetone suspension was stored on ice for at least 30 min before centrifugation at 13000 × g for 10 min. The protein pellets were prepared for gel electrophoresis as described below.

2.4. Polyacrylamide-gel electrophoresis

Samples were solubilised in 62.5 mM Tris-HCl sample buffer (pH 6.8), containing 10% glycerol, 5% 2-mercaptoethanol, 5.0% SDS, and 0.01% bromophenol blue. The samples were heated to 70°C for 20 min, and centrifuged at 13000 × g for 5 min before loading on the slab gel. SDS-PAGE was performed on 10–25% gradient gels with the buffer system of Ref. [27]. Gels were stained in 0.25% Coomassie brilliant blue R250 in 45% methanol and 7% acetic acid for 2 h, destained for 24 h, and dried. Measurement of the amount of radioisotope present in the specific bands was carried out by phosphorimaging and subsequent analysis using a Fuji Bio-Imaging analyzer BAS 2000.

2.5. Chlorophyll determination

Chlorophyll determination in 80% acetone was performed by the method of Arnon [28].

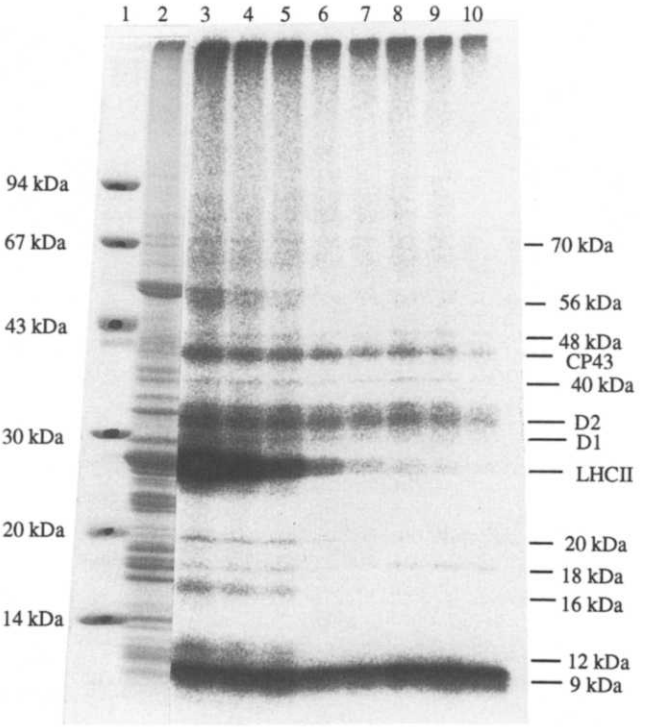


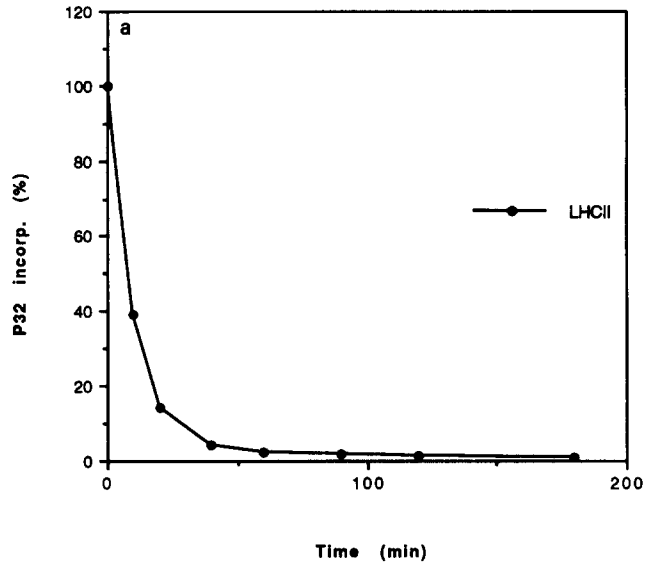
Fig. 1. SDS-PAGE of the time-course of dephosphorylation of pea thylakoid phosphoproteins. Purified pea thylakoid membranes were illuminated for 10 min and then incubated in darkness for 0, 10, 20, 40, 60, 90, 120 and 180 min. Track 1 shows the molecular mass markers (kDa). Track 2 shows the Coomassie 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 times 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).

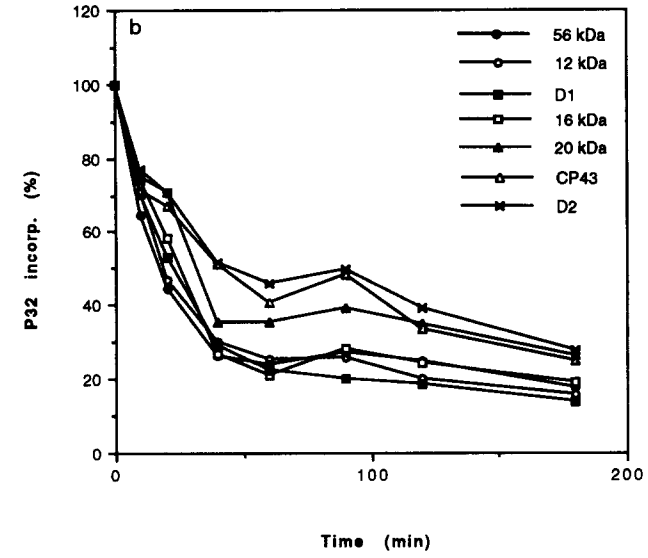
3. Results and discussion

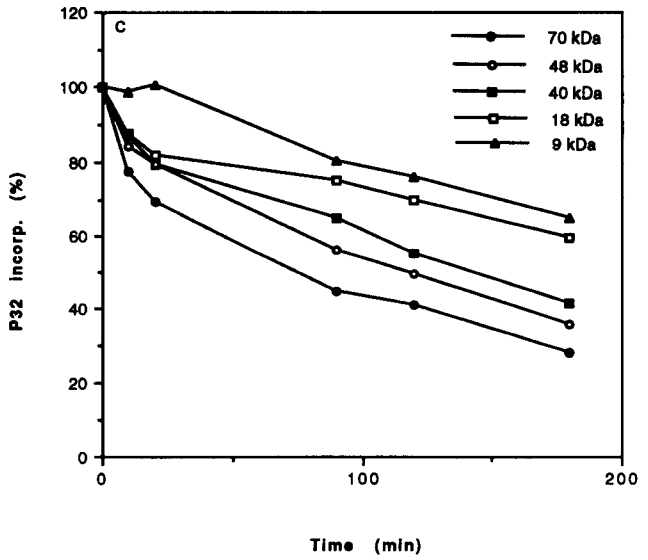
Fig. 1 shows a phosphorimage of the time-course of dephosphorylation reactions of pea thylakoid phosphoproteins separated by SDS-PAGE. At least 13 proteins in purified pea thylakoid membranes became phosphorylated under illumination. When phosphorylated thylakoid membranes were then incubated in darkness, the phosphoproteins became dephosphorylated with very different kinetics. The relative quantities of ^{32}P in specific bands of the phosphorimage are shown in Fig. 2. The dephosphorylation half-time (the time when 50% of the initial ^{32}P-label remained) of each phosphoprotein is shown in Table 1. The most rapidly dephosphorylated protein was phospho-LHC II, with a half-time of 7 min. The most slowly dephosphorylated proteins were the 18 kDa and 9 kDa phosphoproteins, with about 60% to 70% ^{32}P remaining in these bands even after 180 min. The apparent molecular masses of the thylakoid phosphoproteins were estimated according to the migration of molecular mass standards. The estimated molecular masses differ slightly from those in Ref. [8], which may be a result of differences in sample preparation: TCA precipitation was omitted in the present study.

It has been demonstrated that NaF inhibits the dephosphorylation of LHC II and 9 kDa proteins [6]. Antimycin A is a cytochrome b_6f complex inhibitor, interacting with quinone reductase site of the complex, and has been found to activate LHC II phosphorylation [29], whether this effect was due to the inhibition of the phosphatase is unknown. To investigate further the substrate specificity of thylakoid protein phosphatase and the effect of inhibitors on the phosphatase reactions, a synthetic phosphopeptide analogue of the N-terminal phosphorylation site of LHC II was used in conjunction with sodium fluoride and antimycin A.

It was found that synthetic peptide analogues of the phosphorylation site of pea LHC II are phosphorylated preferentially on Thr-5 rather than Ser-3 or Thr-6 [19]. In order to assess the substrate specificity of thylakoid phosphoprotein phosphatase(s), a 15-amino-acid synthetic phosphopeptide (RKSAT(PO4)TKKVASSGSP) analogue of the phosphorylation site of pea LHC II was used. This peptide was originally expected to act as a substrate for the phospho-LHC II phosphatase and







to inhibit competitively the dephosphorylation only of phospho-LHC II. However, the phosphopeptide was found to have an effect on all thylakoid phosphoprotein dephosphorylations. As can be seen from the phosphorimage of the time-course of dephosphorylation processes of pea thylakoid phosphoproteins in the presence of the phosphopeptide (Fig. 3A), the phosphopeptide competed for the phosphatase not only of phospho-LHC II, but also of all other thylakoid phosphoproteins. We suggest that the phosphopeptide may act as a substrate for a single phosphatase which catalyses dephosphorylation of all the phosphoproteins in the thylakoid membrane. The result suggests that the structural features necessary for recognition by the phosphoprotein phosphatase are present in all thylakoid phosphoproteins as well as in the phosphopeptide itself.

Fig. 3B and C shows phosphorimages of the time-course of dephosphorylation processes of pea thylakoid phosphoproteins in the presence of NaF and antimycin A. NaF at 10 mM partially inhibits most phosphoprotein dephosphorylations. However, the dephosphorylations of CP43 and the 40 kDa protein were insensitive to NaF (Table 1). A 48 kDa minor phosphoprotein was rapidly dephosphorylated even in the presence of NaF. 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 1. Since NaF is a non-specific protein phosphatase inhibitor, the observation that dephosphorylation of CP43 and the 40 kDa phosphoprotein is insensitive to NaF may be interpreted as evidence that different phosphatases act on these phosphoproteins. However, the common effects of the phosphopeptide suggest a single phosphatase, in which case the specific effects of NaF may reside in the specific interaction of enzyme and substrate.

Okadaic acid is an inhibitor of mammalian types 1 and type 2A, 2B phosphoprotein phosphatases [30]. It was found that okadaic acid has no marked inhibitory effect on LHCP dephosphorylation [11]. In agreement with this report, our results show the absence of any effect of okadaic acid at 1 μM final concentration on the phosphorylation and dephosphorylation of LHC II and the 9 kDa proteins as well as all the other phos-

Fig. 2. Measurement of ^{32}P-labelling in specific bands of the phosphorimage as shown in Fig. 1. The sample incubated at zero time served as 100% ^{32}P incorporation. (A) LHC II, 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. (C) 70 kDa, filled circles; 48 kDa, open circles; 40 kDa, filled squares, 18 kDa, open squares; 9 kDa, filled triangles.

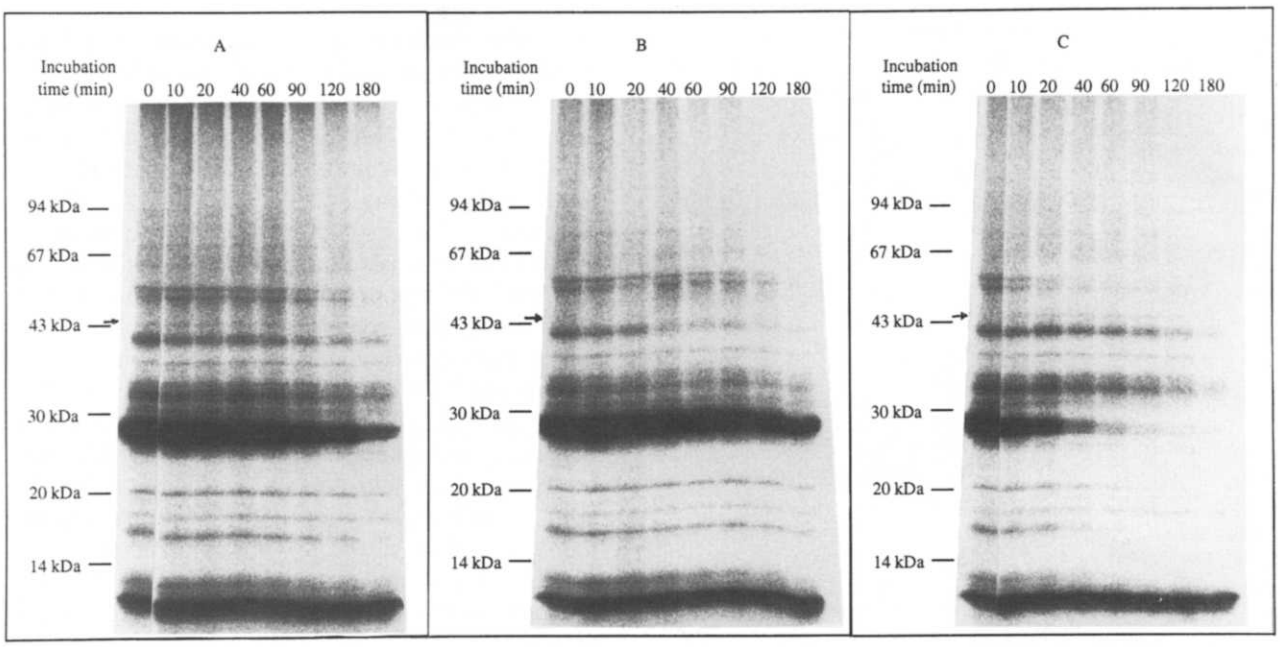


Fig. 3. Phosphorimage of the time-course of dephosphorylation of pea thylakoid phosphoproteins in presence of synthetic phosphopeptide (A), NaF (B) and antimycin A (C). Arrows indicates the 48 kDa protein which is missing in (B) in the presence of NaF.

phoproteins in thylakoid membrane (Fig. 4). The results indicate that the okadaic acid has no effect on thylakoid protein phosphorylation and dephosphorylation, contrary to the conclusion of Kinoshita et al. [31].

Fig. 5 shows the phosphorylation and dephosphorylation of thylakoid membranes in the presence and absence of antimycin A and NaF. With 5 min incubation under illumination in the presence of fluoride and antimycin A, the ^{32}P incorporated in the 9 kDa protein is respectively 170% and 190% of that of the control sample without addition of inhibitors, while ^{32}P incorporated in LHC II is respectively 280% and 160% of

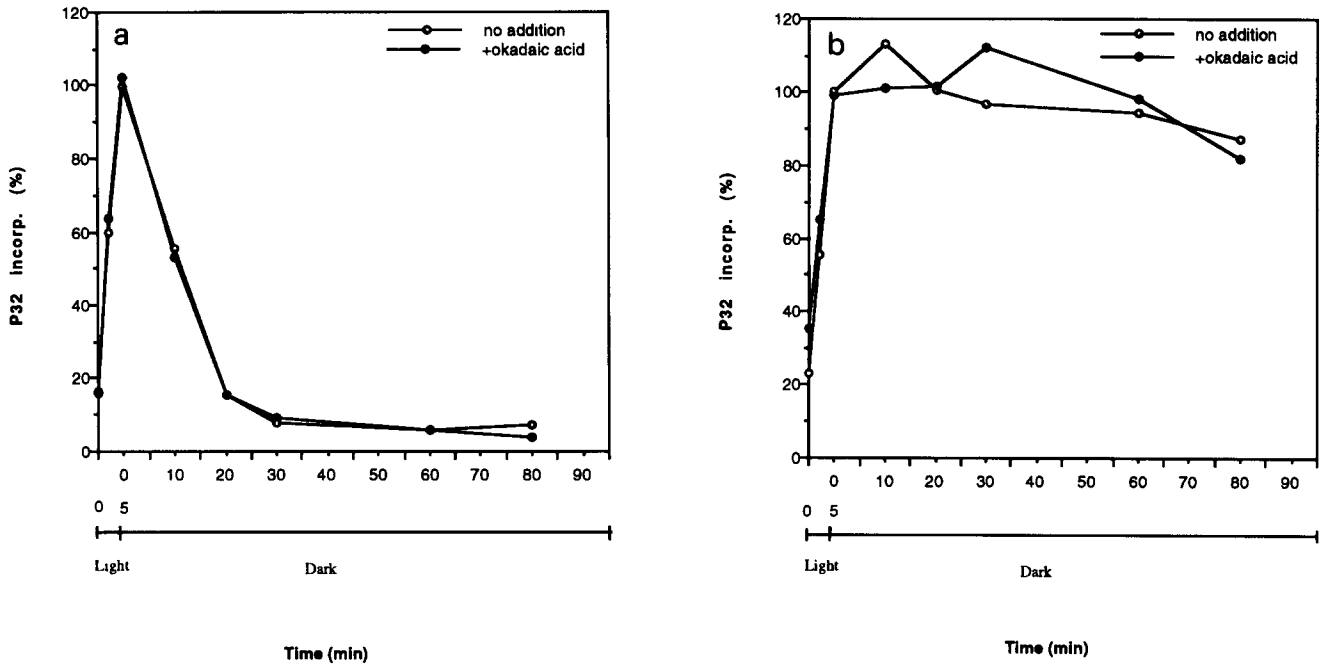


Fig. 4. The time-course of phosphorylation and dephosphorylation of the LHC II (A) and the 9 kDa protein (B) in thylakoid membranes in absence (open circles) and presence of okadaic acid (filled circles). Like LHC II and the 9 kDa protein, all the other proteins have the same kinetics in absence and presence of okadaic acid. The purified thylakoid membranes were illuminated for 5 min and then incubated in darkness for 10, 20, 30, 60 and 80 min. The sample illuminated for 5 min without okadaic acid served as 100% ^{32}P incorporation.

Table 1
The half-times of the dephosphorylation of thylakoid phosphoproteins in absence and presence of synthetic phosphopeptide, NaF and antimycin A

Phospho-proteins	No addition (min)	Synthetic phospho-peptide (min)	NaF (min)	Antimycin A (min)
LHC II	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. 1 and 3). The half-times were calculated when 50% of the initial ^{32}P-label remained.

that of the control sample. It is known from previous experiments that antimycin A has insignificant inhibitory effect on the dephosphorylation of LHC II and 9 kDa proteins. Dephosphorylation of the 9 kDa protein in the presence of NaF is very slow, with a half-time of longer than 98 min, so the observed increase in protein phosphorylation was not caused by inhibition of the phosphoprotein phosphatase. It seems that both antimycin A and NaF have an activation effect on the thylakoid protein kinase. The possible role of NaF and antimycin A in stimulating the phosphorylation of LHC II and the 9 kDa protein is under investigation.

The results presented here suggest that the differences in dephosphorylation kinetics of thylakoid phosphoproteins may result from substrate difference between reactions catalyzed by a single phosphoprotein phosphatase. While it is also possible that there is more than one phosphoprotein phosphatase present in thylakoid membranes, at least one of them apparently exhibits a broad substrate specificity and dephosphorylates multiple phosphoproteins. Design of specific phosphopeptide substrates is useful in assessing the substrate specificity of target phosphoprotein phosphatases. It will be interesting to know whether a synthetic phosphopeptide analogue of an N-terminal segment of the 9 kDa protein also competes with multiple native substrates for the phosphoprotein phosphatase. The possible regulatory mechanism of thylakoid phosphoprotein phosphatase(s) and protein kinase(s) are currently under further investigation.

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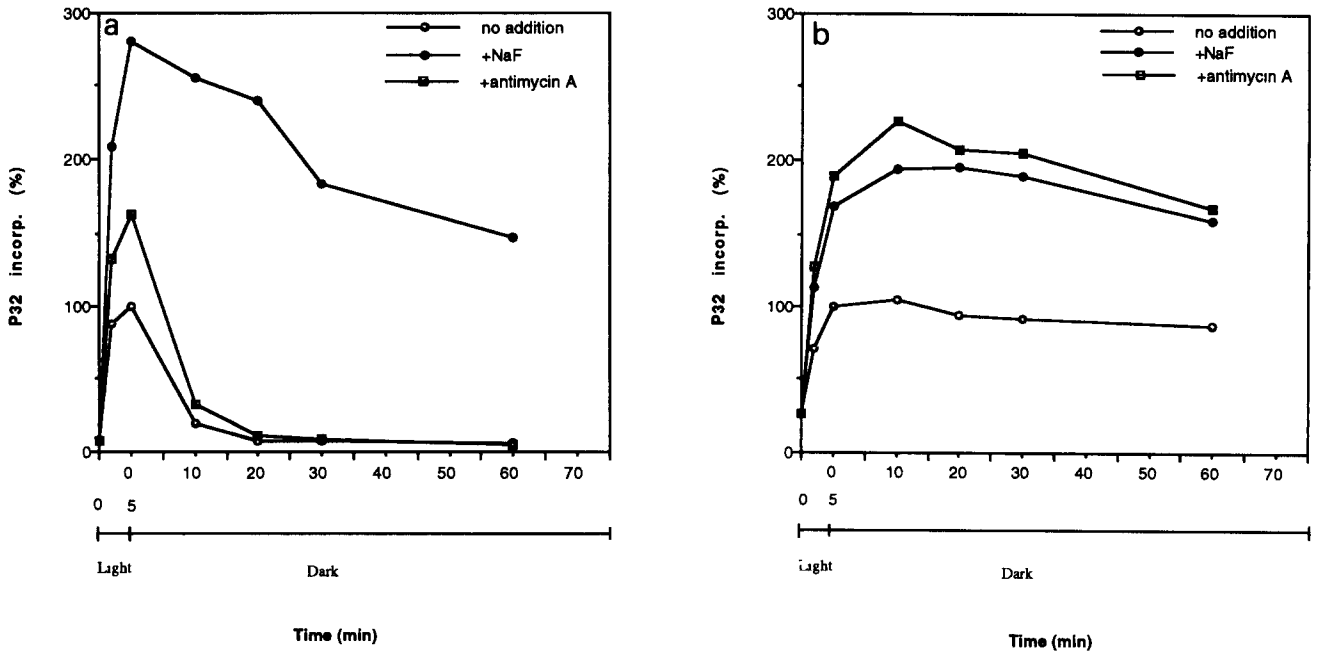

Fig. 5. The time-course of phosphorylation and dephosphorylation of the LHC II (A) and the 9 kDa protein (B) in thylakoid membrane in absence (open circles) and presence of NaF (filled circles) and Antimycin A (open squares). The purified thylakoid membranes were illuminated for 5 min and then incubated in darkness for 10, 20, 30, 40, and 60 min. The sample illuminated for 5 min without inhibitors served as 100% ^{32}P incorporation.

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