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Use of synthetic peptides to study the substrate specificity of a thylakoid protein kinase

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Seven synthetic peptide analogs of the phosphorylation site of the light-harvesting chlorophyll *a/b* complex II (LHC II) were used to examine the substrate specificity of thylakoid-bound LHC II kinase in higher plants. The peptides were phosphorylated by spinach and pea thylakoid membranes in which LHC II kinase had been activated by illumination. Phosphorylation was under redox control, as shown by the inhibitory ability of 20 μM diuron. Apparent K_m(peptide) values ranged from 26 μM for a highly basic peptide with multiple sites for phosphorylation to 1.2 mM for a peptide lacking basic residues on the N-terminal side of the phosphorylation site. Both serine and threonine residues were phosphorylated but the relative rates depended on peptide sequence. A comparison of our data with published LHC II sequences indicates that almost all known LHC II molecules have phosphorylatable sequences.

Chloroplast; Protein phosphorylation; Peptide phosphorylation

1. INTRODUCTION

LHC II is a family of abundant pigment-protein complexes in the photosynthetic membrane of green plant chloroplasts. Its primary function is to act as an antenna for PS II, but here is evidence that it may also do so for PS I, with the distribution of the complexes between the two photosystems apparantly being controlled by reversible phosphorylation of LHC II itself [1–3]. The LHC II kinase is thylakoid-bound and regulated by the redox state of the electron-transport chain. Both plastoquinone [2,4,5] and the cytochrome *bf* complex [6–9] have been implicated in this control. The phosphorylation site of one LHC II of pea leaves has been identified by a combination of protein sequencing and cDNA cloning [10,11]. The phosphorylated threonine is located very close to the N-terminus of the protein, in a segment that is exposed at the stromal (or outer) surface of the membrane. This segment is the most variable part of LHC II, both between *cab* genes of the same plant and between genera [12,13]. Although the segment is always very basic, the number and location of potentially phosphorylatable hydroxyamino acids are variable, and as we do not know enough about the substrate specificity of the LHC II kinase, it is not clear as to which *cab* genes encode phosphorylatable proteins and which do not. We have already established the feasibility of studying the substrate specificity of the LHC II kinase with synthetic peptide substrates [6,14]. We now report the results of a study with seven different peptides: four are analogs of the phosphory-

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Abbreviations: Chl, chlorophyll; diuron, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; LHC, light-harvesting chlorophyll *a/b* complex; PMSF, phenylmethylsulfonyl fluoride; PS, photosystem

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lation site of pea, while three are analogs of the putative phosphorylation site of a tomato LHC II.

2. MATERIALS AND METHODS

Chloroplasts were isolated from 6–8 weeks-old spinach (*Spinacia oleracea* L. cultivar hybrid 424) as described [15]. The chloroplasts were broken by suspension in buffer containing 10 mM Tricine-NaOH (pH 7.8), 10 mM EDTA, 10 mM NaCl, 0.2 mM PMSF to a concentration of 0.2 mg Chl/ml. The membranes were sedimented and washed twice with a buffer containing 10 mM Tricine-NaOH (pH 7.8), 0.2 mM PMSF. Sedimentation was performed at 4°C and 15 000 × g, for 5 min. Finally, the membranes were suspended in the washing buffer to a concentration of 1 mg Chl/ml. The same procedure was used for preparation of thylakoid membranes from 14-day-old pea (*Pisum sativum* L. var. Alaska).

Peptides were synthesized on a solid support either manually or in a Bioresearch model 9600 synthesizer, using t-Boc chemistry [16]. The completed peptides were deblocked and cleaved from the resin with HF/anisole and washed with ether. The crude peptide was extracted from the resin with a total of 15 ml of 2 M acetic acid and lyophilized. Normally, 40 mg crude peptide was dissolved in 4 ml of 0.5 M Tric-HCl (pH 8.0), 6 M guanidine HCl and the cysteinyl residues were blocked by addition of dithiothreitol (54 mg), then iodoacetamide (145 mg), and finally dithiothreitol (27 mg). After each addition, the solution was incubated for 1 h at 37°C under nitrogen and in darknes. After desalting over Sephadex G-15 in 1% ammonium bicarbonate the peptide-containing fractions were lyophilized. Each peptide was purified by HPLC using a reverse-phase column (C_{18}, Vydac, 22 × 250 mm, 10 μm pore size) and a water/sodium phosphate/hexanesulfonate/acetonitrile buffer system [17]. Peptide-containing fractions from the column were lyophilized and desalted over Sephadex G-15 in 0.1 M acetic acid. A single peak was obtained when the purity of each peptide was checked by analytical HPLC, including cation exchange as described [18]. The identity of each peak was confirmed by sequence analysis on an Applied Biosystems gas-phase sequencer. Peptides were quantified by amino acid compositional analysis.

Time-dependent phosphorylation of the peptides was carried out in a volume of 100 μl of 150 μM peptide, 0.1 mg Chl/ml thylakoid membranes, 20 mM NaF, 1mM $MgCl_2$, 50 mM Tricine (pH 7.8), 200 μM ATP and 200–300 μCi/μmol [γ-^{32}P]ATP in an illuminated water bath (23°C, unfiltered tungsten lamp, 200 $\mu E \cdot m^{-2} \cdot s^{-1}$). At appropriate times 15-μl aliquots were diluted into 45 μl of 20 mM NaF, 20 mM EDTA to stop the reaction. After centriguation (at 12 000 × g for 40 s), 50 μl supernatant were directly injected onto 400 μl anion-exchange column (AG1 × 8, acetate form, Bio-Rad) in 30% acetic acid to remove phosphate and unreacted ATP [19]. After 10–20 min the peptide was eluted from the resin by brief centrifugation directly into a scintillation vial. Quantitative recovery of the peptide was ensured by washing the resin with an additional 600 μl of 30% acetic acid. Reverse-phase HPLC with a Radiomatic Flow-One detector revealed a single peak of radioactive peptide in each case. The extent of peptide phosphorylation was estimated by Cerenkov counting. To measure K_m and V_{max} different concentrations of peptide were assayed as above but in a volume of 20 μl. Kinetic data were ploted on double-reciprocal plots and fitted to the Michaelis-Menten equation by the method of least squares.

3. RESULTS

Table 1 lists the seven synthetic peptides used as LHC II kinase substrates in this study. Peptides 1–4 are analogs of a pea LHC II phosphorylation site [11] and were synthesized to vary the site and chemical nature of the hydroxyamino acids. Peptides 5–7 are analogs of a putative LHC II phosphorylation site from tomato [20] and were chosen to explore the importance of peptide length and composition. The peptides were all synthesized with an N-terminal methionine, because it is generally assumed that the pre-LHC II is cleaved immediately in front of the methionine to yield mature LHC II and a transit peptide.

Fig.1 shows the time course of phosphorylation of 150 μM peptides 1 and 5 by spinach thylakoids. Incorporation was linear for at least 40 min, with peptide 5 being consistently more heavily labelled than peptide 1. Background activities, obtained by incubating membranes under identical conditions without peptide, were typically less than 1 nmol phosphate incorporated per mg chlorophyll and did not increase with time during 50 min incubations. Thyulakoid preparations usually contain trace contamination by chloroplast envelopes, chloroplast stroma and chromatin, all of which contain protein kinase activity. To establish that the peptides were being phosphorylated by the redox-controlled protein kinase characteristic of

Table 1

Kinetic parameters for phosphorylation of synthetic peptides.

		K_m(peptide)[a]	V_{max}[b]
Pea analogs			
(1)	MRKSATTKKAVC[c]	69 ± 11	113 ± 10
(2)	MRKSASSKKAVC	115 ± 27	38 ± 3
(3)	MRKAAATKKAVC	112 ± 16	51 ± 2
(4)	MRKAATAKKAVC	118 ± 19	49 ± 8
Tomato analogs			
(5)	GRVTMRKTATKAKPASSC	26 ± 6	130 ± 4
(6)	MRKTATKAKPASSC	102 ± 29	67 ± 16
(7)	TATKAKPASSC	1200 ± 500	31 ± 10

[a] Mean ± SD of at least three different experiments (μM)
[b] Exposed as nmol/h mg Chl
[c] Range of peptide concentration 20–500 μM (peptides 1–6), and 0.2–2 mM (peptide 7)

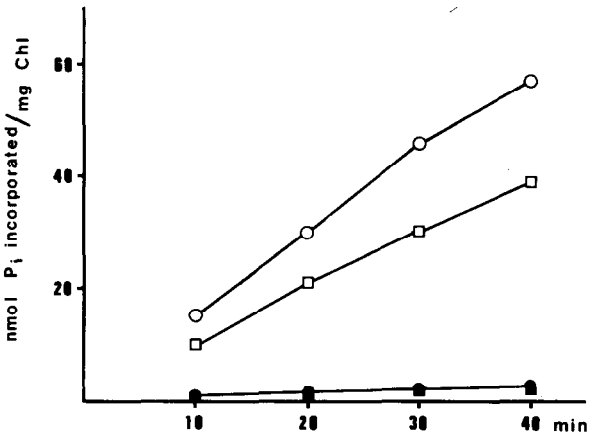

Fig.1. Inhibition of peptide phosphorylation by diuron. Peptide 1 (□,■) and peptide 5 (○,●) were phosphorylated at 150 μM with spinach thylakoid membranes in the light in the absence (□,○) and presence (■,●) of 20 μM diuron. At the indicated times aliquots were taken and radioactivity incorporated into peptide was measured. See table 1 for peptide sequences.

thylakoids, we followed incorporation in the presence of 20 μM diuron, which is a potent inhibitor of the redox activation of LHC II kinase [2]. Fig.1 shows that diuron inhibited phosphorylation of both peptides by 95%, eliminating the possibility that an extraneous kinase was responsible for phosphorylation of these peptides.

Fig.2 shows the phosphorylation of all seven peptides by spinach thylakoids in a typical experiment, when peptides were present at 150 μM. Peptide 1, which most closely resembles the actual pea LHC II sequence [11], was phosphorylated about 3-times more rapidly than peptides 2–4. Peptide 1 contains three hydroxyamino acids: Ser-4, Thr-6 and Thr-7. Sequencing of the phosphorylated peptide indicated that Thr-6 was the major phosphorylation site (fig.3). There may have been a small amount of incorporation into Thr-7 but Ser-4 was not significantly phosphorylated. Peptides 3 and 4 were phosphorylated on the only available residues (Thr-7 and Thr-6, respectively) but peptide 2 was phosphorylated mainly on Ser-4 and detectably on Ser-6; it is not clear whether Ser-7 was also phosphorylated.

Peptide 6 corresponds to the presumed N-terminus of a tomato LHC II [20]. Peptide 5 differs from peptide 6 in containing what are assumed to be the last four residues of the transit peptide. In contrast, peptide 7 is a truncated peptide which has lost the first three residues of the presumed N-terminus of mature LHC II. These changes in sequence have a significant effect on the ability of the peptides to act as kinase substrates. Peptide 5 is about twice as effective as peptide 6 as a substrate. In contrast, peptide 7 is only phosphorylated at higher concentrations. Sequencing of phospho-

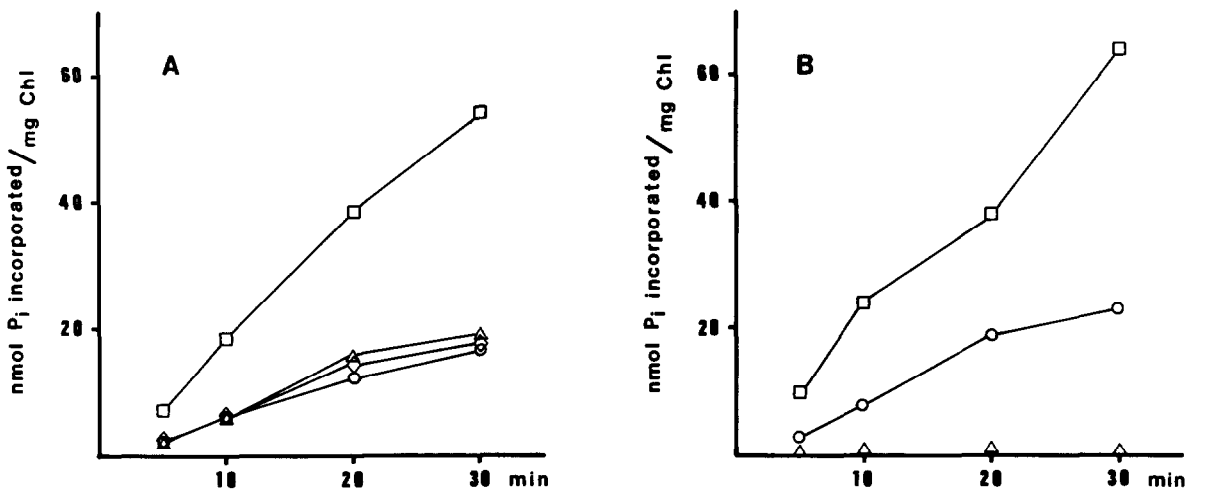

Fig.2. Time course of peptide phosphorylation. (A) Peptides 1 (□), 2 (△), 3 (○) and 4 (◇) were phosphorylated at 150 μM with spinach thylakoid membranes in light. At the indicated times aliquots were taken and radioactivity incorporated into peptide was measured (B) As above, but peptides 5 (□), 6 (○) and 7 (△). See table 1 for peptide sequences.

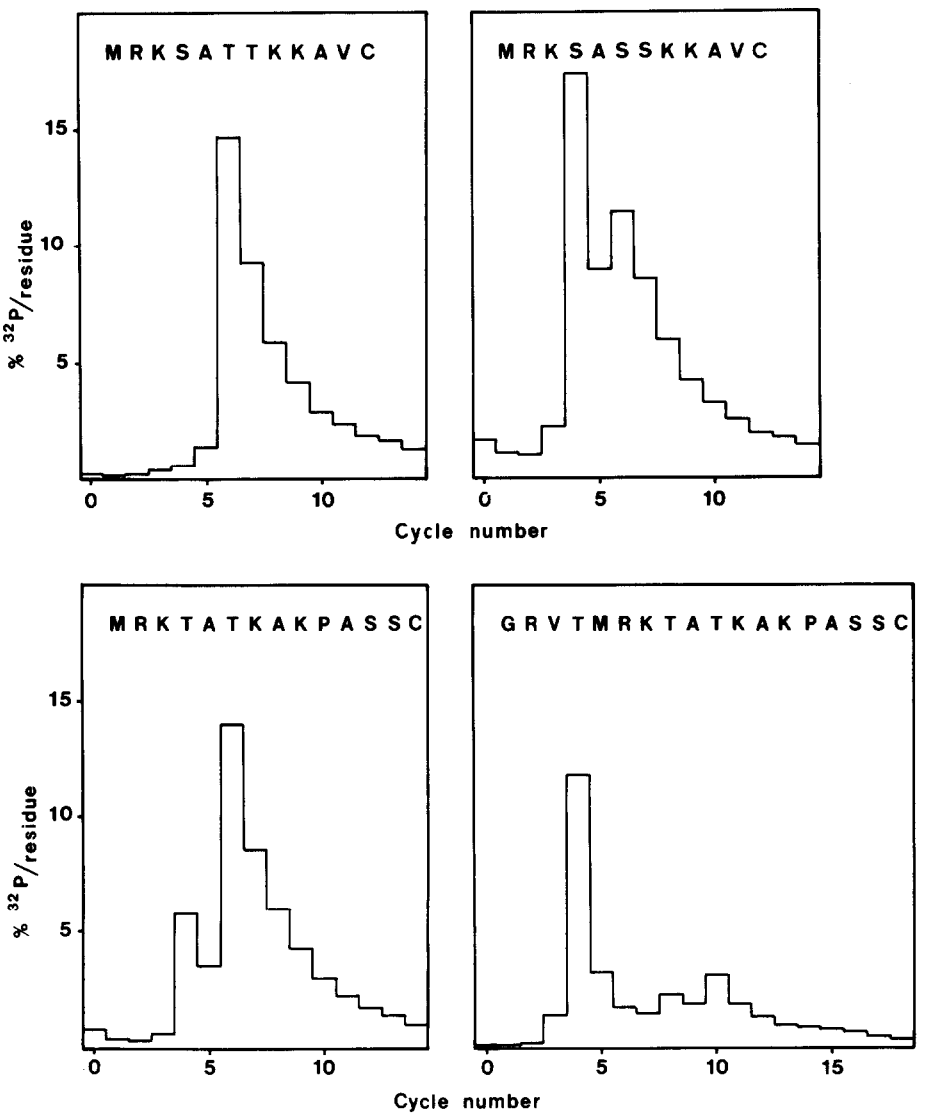

Fig.3. Identification of phosphorylated residues. Peptides 1, 2, 5 and 6 were labelled for 30 min at 150 μM in the light with spinach thylakoids. Membranes as well as unreacted ATP and phosphate were removed and the phosphorylated residues were identified by stepwise degradation on a Beckman 890 C sequencer. See table 1 for peptide sequences.

peptides 5 and 6 established the sites of phosphorylation (fig.2). Peptide 6 was phosphorylated principally on Thr-6 and also on Thr-4. Peptide 6 was also phosphorylated on the same residues but the principal site of phosphorylation was Thr-(-1), i.e. the first residue before the assumed processing site of pre-LHC II.

To estimate K_m and V_{max} values for the phosphorylation of the seven peptides, we determined the rate of phosphorylation of each peptide at different peptide concentrations (see table 1). Spinach thylakoids were used as source of kinase. Table 1 lists the apparent K_m and V_{max} values calculated from the rates of phosphate incorporation over the first 20 min. We use the term 'apparent' for two reasons (i) the ATP concentration was 200 μM rather than saturating, and (ii) the results apply to the phosphorylation of each peptide as a whole and not to the phosphorylation of individual residues where multiple hydroxyamino acid exist. The use

of 200 μM ATP is unlikely to distort the values of K_m(peptide) greatly, because the K_m(ATP) for spinach LHC II kinase is reported to be only 35 μM [21]. Data are reported for whole peptides because of the impracticability of sequencing each peptide at each data point. The V_{max} values are expressed relative to peptide 1. Only peptide 5 has a larger V_{max} and a smaller K_m than peptide 1. As expected, peptide 7 differed markedly from the others, exhibiting a much higher K_m and a slightly lower V_{max}. The other peptides showed apparent K_m values in the range 26–118 μM and V_{max} values in the range 38–130 nmol/mg per h.

4. DISCUSSION

The thylakoid-bound LHC II kinase of spinach chloroplasts phosphorylates the seven peptides used in this study. Virtually identical results were obtained with thylakoid membranes of pea (not shown). Among the phosphorylated peptides were peptide 1 (MRKSATTKKAVC), an analog of the pea LHC II phosphorylation site identified by Mullet [11], and peptide 6 (MRKTATKAKPASSC), an analog of a putative tomato LHC II phosphorylation site deduced by Pichersky et al. [20]. In both peptides, Thr-6 was phosphorylated in preference to other hydroxyamino acids. This result is consistent with the data of Mullet [11] who found that a pea LHC II was phosphorylated on threonine near the N-terminus but could not determine whether phosphorylation occurred on Thr-6 or Thr-7 or both. However, the pea phosphorylation site is atypical of higher plant LHC II. Most of the more than 20 LHC II sequences which have been inferred from DNA sequences do not contain threonine or serine residues at position 6 or 7. With only one known exception (a tomato LHC II [20]), they all contain a serine or threonine at position 4. Our data indicate that this site is phosphorylatable with either serine (peptide 2) or threonine (peptide 6) but position 4 may be completely ignored if site 6 is available for phosphorylation (peptide 1). We conclude that the vast majority of known LHC II genes would appear to encode phosphorylatable proteins.

Our results reveal a major difference between the LHC II kinase and the cAMP-dependent kinase of animal cells. Peptides containing the sequence RRXSZ (where X and Z are small, non-polar residues) are readily phosphorylated by cAMP-dependent enzyme [19,22,23]. When X is removed from RRXSZ or an additional residue is inserted between the basic residues and the hydroxyamino acid [22], the K_m(peptide) of the kinase increases by about 2 orders of magnitude. If K_m(peptide) is regarded as a measure of the affinity between substrate and enzyme, this affinity is markedly reduced for the cAMP-dependent kinase when the location of the phosphorylatable hydroxyamino acid is moved by even one residue. In contrast, variations in the location of the phosphorylatable hydroxyamino acid do not appear to alter the affinity between the LHC II kinase and its substrate (table 1), even though such changes may lead to a significant alteration in the rate and preferred site of phosphorylation. Thus, there is little difference in K_m(peptide) for phosphorylation of Ser-4 in peptide 2, Thr-6 in peptides 1, 4 and 6 and Thr-7 in peptide 3.

Two other serine/threonine protein kinases have been extensively studied in animal cells: protein kinase C and casein kinase (review [24]). Whereas the cAMP-dependent protein kinase recognizes basic residues on the N-terminal side of the hydroxyamino acid, protein kinase C recognizes sites where the hydroxyamino acid is flanked on both sides by basic residues [22,25]. In contrast, casein kinase recognizes peptides with acidic residues flanking the hydroxyamino acid [26]. The conservation of basic residues on both sides of the phosphorylation site of LHC II [12,13] would suggest a similarity with protein kinase C, which also resembles the LHC II kinase in being membrane-bound and being regulated by lipoidal components of the membrane [24]. Two explanations can be put forward for the poor phosphorylation of peptide 7 (TATKAKPASSC): (i) the peptide is too short to bind avidly to the kinase, or (ii) the hydroxyamino acids cannot be phosphorylated because they are not flanked on both sides by basic residues. We are addressing this issue through the synthesis of additional peptides.

Peptide 5 includes four residues of the transit peptide of pre-LHC II and is phosphorylated avidly on Thr-(−1). This might be explained by the fact that Thr-(−1) is flanked on both sides by basic residues. Whether pre-LHC II molecules are phosphorylated on this residue in vivo is unknown, but this possibillity should be considered, given the

evidence that processing of pre-LHC II can occur on thylakoids [27]. What effect such phosphorylation might have on the cleavage of the precursor remains to be determined.

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