Investigation of the substrate specificity of thylakoid protein kinase using synthetic peptides

I.R. White, P.J. O'Donnell, J.N. Keen, J.B.C. Findlay and P.A. Millner

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, UK

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Synthetic peptide analogues of the N-terminal region of the light harvesting chlorophyll *a/b* binding polypeptide of photosystem II (LHC II) were used to probe the effect of charged groups on the protein kinase activity of pea (*Pisum sativum*) thylakoid membranes. The effectiveness of the synthetic peptides as substrates for protein kinase activity or as inhibitors of LHC II phosphorylation was correlated with their net positive charge, which ranged between +2 and +5. The effects of the synthetic peptides on phosphorylation of other, non-LHC II, thyakoid polypeptides are also discussed.

Protein kinase; Synthetic peptide; LHC II; Substrate specificity

1. INTRODUCTION

The dynamic regulation of light harvesting in higher plant chloroplasts is brought about by the reversible phosphorylation of light harvesting polypeptides associated with PS II [1–3]. Other polypeptides associated with PS II [4,5], including the reaction centre polypeptides themselves, also undergo reversible phosphorylation. However, the kinetics of phosphorylation are somewhat slower than for LHC II polypeptides and the consequences of phosphorylation are less clear [6,7]. The kinases responsible for these phosphorylation events appear to respond to the redox state of the plastoquinone pool [1,8,9], or some factor that is isopotential with this pool, i.e. a component of the cytochrome b_6f complex [10–12]. Currently, the molecular identity of the thylakoid protein kinases responsible for the phosphorylation of LHC II and the other PS II associated polypeptides is uncertain, although based on the differential inhibition of phosphorylation of LHC II and other PS II associated polypeptides by a number of different agents, two protein kinases have been proposed [9,12–14]. Candidate kinases of 23 kDa and 38 kDa [15,16] and also of 64 kDa [17] have been isolated although only the latter protein kinase was able to phosphorylate purified LHC II polypeptides [18]. Previously, a number of laboratories have demonstrated the value of using synthetic peptides to characterise the substrate specificity of protein kinases [19–22,30], and in particular the putative LHC II kinase [21,22]. In the present work we have investigated the relationship between net charge carried by synthetic peptides and their ability to act as substrates for thylakoid protein kinase activity or as inhibitors of thylakoid membrane polypeptide phosphorylation.

Correspondence address: P.A. Millner, Department of Biochemistry, University of Leeds, Leeds LS2 9JT, UK

Abbreviations: LHC II, light harvesting chlorophyll *a/b* binding protein of photosystem II; PS II, photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

2. MATERIALS AND METHODS

Peptides were synthesized using Fmoc-based chemistry [23] on a MilliGen 9050 solid-phase peptide synthesizer. The peptides were purified using reverse-phase hplc on a Pharmacia Pep-RPC/C_{18} column and their sequences verified by automated solid phase protein sequencing. The peptides were quantified by Lowry protein assay [24].

Thylakoid membranes were isolated from 14-day-old *Pisum sativum* (var. Feltham First) seedlings as previously described [25] and resuspended in Buffer A comprising 330 mM sorbitol, 25 mM tricine-NaOH, pH 8.0, and 1 mM $MgCl_2$.

Peptide and protein phosphorylation was assayed in a total volume of 50 μl, containing Buffer A plus 10 mM NaF, 200 μM unlabelled ATP, 3.7 kBq·nmol^{-1} of [γ^{32}P]ATP, various concentrations of synthetic peptide (see figure legends) and thylakoids equivalent to 200 μg chl·ml^{-1}. Protein kinase activity was initiated by the addition of freshly prepared $Na_2S_2O_4$ solution to a final concentration of 5 mM, or by illuminating the sample with white light. The reaction was terminated after 10 min by addition of an equal volume of ice-cold phosphoric acid saturated with EDTA [26]. With light-induced kinase activity, control incubations were also carried out in the presence of 5 μM DCMU.

Electrophoresis of 20 μl aliqots of the reaction mixture, containing ^{32}P-labelled thylakoid polypeptides and synthetic peptides, was carried out after the addition of 20 μl of sample buffer comprising 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 125 mM Tris base and 20% (w/v) sucrose and heating the samples for 3 min at 95°C. Following electrophoresis of the samples on a linear 10% to 30% (w/v) SDS-polyacrylamide gel using the Laemmli buffer system [27], the wet gels were wrapped in clingfilm and ^{32}P-labelled material visualised by autoradiography. In other cases, the gels were first stained with Coomassie blue R250 in 50% (v/v) methanol, 7% (v/v) acetic acid and dried prior to autoradiography. Incorporation of $^{32}PO_4$ into thylakoid proteins was quantified by densitometry of

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Table I

Sequences of synthetic peptides

Peptide	Sequence	Charge[a]
(A)	M-R-*D*-S-A-T-T-K-K-V-A-C	+2
(N)	M-R-*Nle*-S-A-T-T-K-K-V-A-C	+3
(S)	M-R-K-S-A-T-T-K-K-V-A-C	+4
(B)	M-R-*K*-K-S-A-T-T-K-K-V-A-C	+5

All of the peptides carry a C-terminal cysteine residue in addition to the sequence deduced by Cashmore [29]. Residues in italics represent substitutions or additions to the native sequence represented by peptide (S)

[a] Net charge at pH 7; Nle = norleucine

autoradiograms using an LKB-Ultrascan XL laser-densitometer. The exposure time, typically 2–3 h, was chosen to ensure that all bands on the autoradiograph lay on the linear portion of an exposure versus film density calibration curve.

Peptide phosphorylation was assayed as described in [28]. After termination of the phosphorylation reaction, thylakoid polypeptides were sedimented at 12 000 × *g* for 3 min and 25 μl of the supernatant spotted onto Whatman P81 paper strips. After drying and successive 5 min washes in 1 M phosphoric acid plus 10% (w/v) trichloroacetic acid (4 washes) and absolute ethanol (1 wash), the strips were dried again and incorporation of $^{32}PO_4$ into peptides estimated by scintillation counting of the strips in 2 ml of Optiphase Safe scintillation cocktail.

3. RESULTS

The synthetic peptides used are shown in Table I. Peptide S corresponds to the N-terminal amino acid sequence of pea LHC II deduced by Cashmore [29] and carries a net charge of +4 at neutral pH. Peptides N and A were successively less basic analogues of peptide S while peptide B is more basic. All of the peptides were synthesized with a C-terminal cysteine residue additional to the native sequence, in order to facilitate the future preparation of affinity matrices for protein kinase isolation. The effect of the synthetic peptides on phosphorylation of LHC II and other thylakoid polypeptides is shown in Fig. 1. The ability of the synthetic peptides to inhibit the phosphorylation of the LHC II polypeptides (Fig. 1) was clearly correlated with the net peptide charge. The most basic peptide, peptide B, was the most effective inhibitor. Under the same conditions the ability of the peptide to act as kinase substrates was also correlated with the net peptide charge. The most basic peptide, B, appeared to be the most heavily phosphorylated whilst peptide S was labelled to an intermediate level. The least basic peptide, A, was not phosphorylated. If the polyacrylamide gels were dried prior to autoradiography then phosphorylation of other thylakoid associated polypeptides of 58 kDa and 17–20 kDa was also seen to be inhibited by the synthetic peptides, with the most basic peptides being the most effective inhibitors (not shown). In Fig. 2 the effects of the various peptides on the relative level of LHC II phosphorylation are presented. At peptide concentrations of 100 μM and

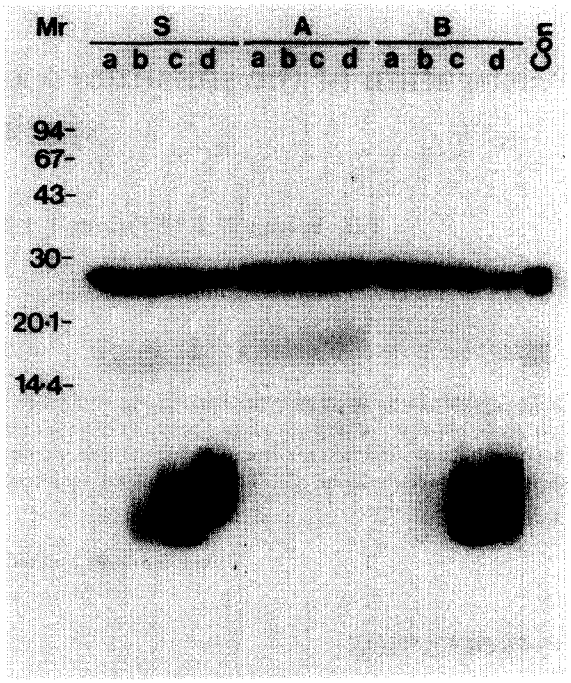

Fig. 1. Phosphorylation of thylakoid polypeptides in the presence of synthetic peptides S, A and B. The autoradiograph of the wet SDS-polyacrylamide gel was exposed for 2 h at −70°C. Peptide concentrations were: (a) 10 μM; 100 μM; (c) 1 mM; and (d) 5 mM. Con = control.

above, the inhibitory potency of the peptides was clearly correlated with net positive charge and with their concentration. Peptide A, which carries an acidic substitution as compared with the native sequence, was barely effective at the higher concentrations used. At

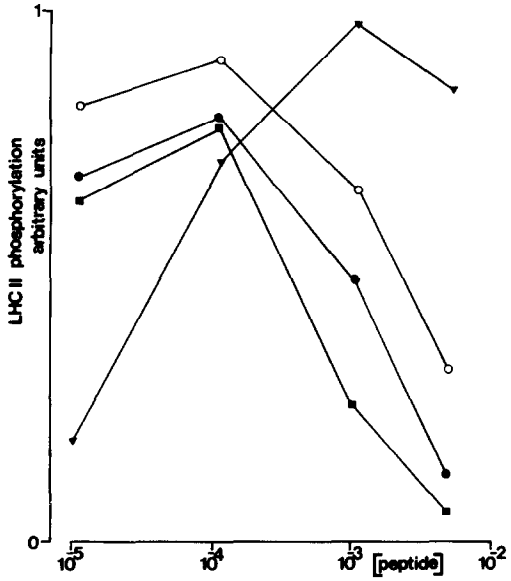

Fig. 2. Quantification of LHC II phosphorylation in the presence of synthetic peptides by densitometry. Autoradiographs were scanned using a LKB Ultroscan XL laser densitometer. Values for the integrated density are arbitrary. (▼) + peptide A; (○) + peptide N; (●) + peptide S; (■) + peptide B.

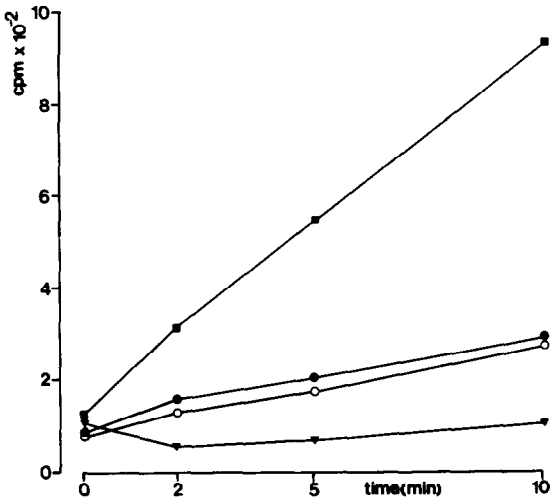

Fig. 3. Time course of synthetic peptide phosphorylation by thylakoid membranes. Incorporation of $^{32}PO_4$ into 1 mM synthetic peptides was determined using Whatman P81 paper as described in section 2. (▼) peptide A; (○) peptide N; (●) peptide S; (■) peptide B.

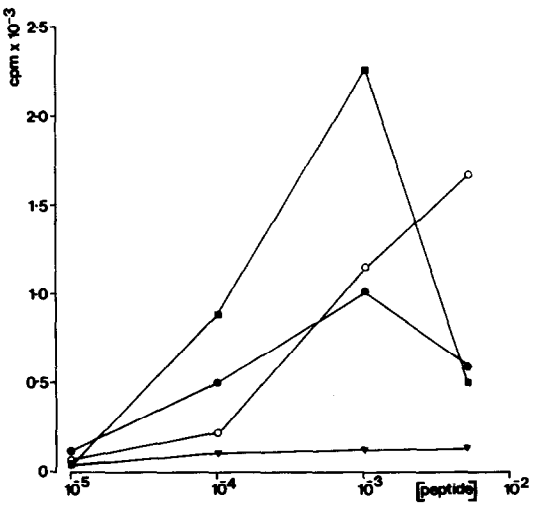

Fig. 4. Concentration dependence of synthetic peptide phosphorylation by thylakoid membranes. Incorporation of $^{32}PO_4$ into 10 μM to 5 mM synthetic peptides was determined as described in section 2 after incubation with pea thylakoids for 10 min. (▼) peptide A; (○) peptide N; (●) peptide S; (■) peptide B.

peptide concentrations of 10 μM, all of the peptides appeared to reduce LHC II phosphorylation to a slightly greater extent than 100 μM peptide. Peptide A frequently had the greatest inhibitory effect at this concentration, although in most cases the inhibition was substantially less than shown in Fig. 2. In Fig. 3 the time course for phosphorylation of 1 mM synthetic peptides is presented. This concentration of synthetic peptide was chosen since the greatest differences in LHC II inhibition by the various peptides were observed at this concentration. Additionally, on the basis of previous data with synthetic peptides [21,22] it was expected that this concentration of peptide would be saturating. All of the peptides examined showed a linear rate of phosphorylation over 10 min. The concentration dependance of peptide phosphorylation is shown in Fig. 4. The degree of phosphorylation of the various peptides, at concentrations up to 1 mM, was directly correlated with their ability to inhibit LHC II phosphorylation, i.e. peptide B was the best substrate and peptide A was barely phosphorylated. Peptide S and N were of intermediate activity. At 5 mM peptide, the phosphorylation of peptides S and B decreased.

4. DISCUSSION

In the present communication we have clearly shown that the inhibitory effect of the synthetic peptides on LHC II phosphorylation was correlated with their ability to act as substrates for thylakoid protein kinase activity, i.e. they appeared to be acting as competitive inhibitors. In addition, increasing the degree of positive charge (peptide B) N-terminal to the phosphorylated Thr residue on the native peptide (peptide S) produced a more effective substrate. Decreasing the positive charge of this N-terminal region by the substitution of lys_3 with a norleucine residue decreased the substrate effectiveness of the peptide N slightly as compared to peptide S, whilst the substitution of Lys_{-3} with an acidic residue (peptide A) almost completely abolished its ability to act as substrate. In these respects the kinase responsible for LHC II phosphorylation appeared to show a requirement for blocks of basic amino acids located N-terminal to the phosphorylation site as was shown for protein kinase C [19] and cyclic AMP-dependent protein kinase [20] using synthetic peptide substrates. The presence of basic residues C-terminal to the phosphorylation site of LHC II also suggests similarities between the substrate site specificity of protein kinase C and the LHC II kinase. Further analyses of LHC II kinase activity, using peptides with residues that have been altered C-terminal to the phosphorylated Thr residue, would allow additional scrutiny of this possibility. In contrast to LHC II kinase, the other major protein kinase studied in animal cells, i.e. casein kinase, shows specificity for peptides with blocks of acidic residues surrounding the phosphorylation site [30]. The observation that low concentrations of peptide A inhibited LHC II phosphorylation (Fig. 2) but was barely phosphorylated even at higher concentrations is difficult to explain. Possibly this peptide, in which and Asp residue has been substituted for Lys_{-3}, can bind to the kinase but is unable to be phosphorylate due to the presence of the acidic residue. This hypothesis could be tested by binding studies with the various peptides.

In recent work, Michel and Bennett [22] used synthetic peptides corresponding to the N-terminal sequence of pea LHC II [29], to study the effects of alterations to the hydroxyamino acids at the

phosphorylation site, and of the separation between the N-terminal basic block of amino acids and the phosphorylated residue, normally Thr-6. Changes of this sort had a relatively minor effect on apparent K_m for the peptides, as compared with the K_m for the native sequence, and increased its value by less than two-fold. In the same study [22] peptides corresponding to the N-terminal region of tomato LHC II [31] were used to study the effect of peptide length on the phosphorylation reaction. A peptide truncated by three residues at the N-terminal end, as compared with the native sequence, was a very poor substrate. In contrast, one extended by four residues was a somewhat better substrate than the native peptide. Since the former peptide bore two less basic residues and the latter (extended) peptide an additional N-terminal basic residue, the data presented in that study can be explained in terms of the relationship between net positive charge carried by the peptides and their effectiveness as substrates revealed in the present report. This conclusion is reinforced by preliminary work (not shown) using an extended peptide corresponding to the first 21 residues of the pea LHC II sequence. The latter peptide appeared to be similarly effective as a substrate to the 12 residue peptide (Table I, peptide S) used in the work here.

Thus, in the present report we have shown the feasibility of preparing synthetic peptides which are substrates for the LHC II kinase and also which bind with greater affinity than the native substrate. Previous methodologies for isolation of putative LHC II kinase [17,18] have utilised the 'pseudo affinity' purification step involving histone-agarose. In future work, the peptides characterised here will be utilised as a rational basic for the construction of crosslinker-peptide constructs and affinity matrices for the identification and purification of the authentic LHC II kinase.

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