Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue

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Abstract The site of phosphorylation of phenylalanine ammonia-lyase (PAL) has been identified as a threonine residue. A Ca^{2+}-stimulated protein kinase of approximately 55 kDa has been partially purified from elicited cells. The kinase can phosphorylate a synthetic peptide derived from PAL and a recombinant poplar PAL. PAL phosphorylation was associated with a decrease in V_{max} in agreement with the suggestion that protein phosphorylation is involved in marking PAL subunits for turnover. The phosphorylation site in French bean PAL is most likely Thr^{545} in the sequence VAKRTLTT (539–546). Conservation of the phosphorylation site in PAL from diverse species suggests that phosphorylation of PAL may be a ubiquitous regulatory mechanism in higher plants.

Key words: Phenylalanine ammonia-lyase; Protein kinase; Phosphorylation; Higher plant

1. Introduction

Reversible phosphorylation of target proteins by protein kinases and phosphatases is a universal mechanism in all eukaryotes but only relatively recently has the extent of the involvement of such mechanisms in regulatory processes begun to be appreciated in plant systems [1,2]. A number of protein kinases and protein phosphatases have now been characterised at the gene and protein levels in plants although the target proteins for only relatively few have been identified. The work described here concerns the phosphorylation of a key enzyme in phenolic metabolism and therefore the possible involvement of phosphorylation in the overall regulation of the phenylpropanoid pathway.

Secondary metabolism is highly regulated in plants. Such regulation is complex and can involve transcriptional activation, mRNA processing and stability, enzyme activation and inactivation and regulation of turnover. Phenolic metabolism and its regulation shows many of these features [3,4] and in particular, the first step, catalysed by phenylalanine ammonia-lyase (PAL), is subject to a number of control mechanisms. For example, PAL activity is induced by light, wounding, plant growth regulators, and biotic and abiotic elicitor molecules. In all these cases the initial appearance of the enzyme activity is dependent upon transcriptional activation. In French bean there are at least four gene products that are modulated by stress and during xylogenesis [5–7]. These all give rise to inducible polypeptides of M_r 77 000. In addition, a constitutive form of PAL of M_r 83 000 has been identified [8] but the exact relationship between this isoform and the inducible gene products is not yet known.

In French bean, following a relevant stimulus, PAL mRNA is expressed transiently [7,9]. Following translation, the enzyme is subject to protein inactivation, and down-regulation of transcription occurs, both of which are possibly regulated by the level of phenylpropanoids [10,11]. PAL turnover involves the degradation pathway through M_r 70 000, 53 000 and 4 000 polypeptide intermediates, whether or not the 83 000 or 77 000 forms are the initial target [12]. However, there appears to be an additional involvement of phosphorylation, which has been demonstrated in vivo but not in vitro [13,14]. The phosphorylation site has been mapped to an M_r 18 000 peptide whose N-terminus coincides with a residue 248 amino acids from the C-terminus [14]. The present work describes the identification of the likely target sequence and describes preliminary characterisation of the protein kinase thought to be responsible for the phosphorylation.

2. Materials and methods

2.1. Materials

Cell suspension cultures of French bean were prepared and elicitor preparations from the fungus *Colletotrichum lindemuthianum* were made as described previously [15]. γ-[^{32}P]ATP (3000 Ci/mmol) and γ-[^{33}P]ATP (3000 Ci/mmol) were obtained from Amersham International. Kemptide and malantide were obtained from Sigma. Two synthetic PAL peptides (peptide A, SRVAKRTLTTA and peptide B, LISARKTNEA) were made by the peptide synthesising service, Durham University, Durham, UK.

2.2. Phosphorylation of peptides

Extracts of French bean suspension cultured cells for routine kinase assays were prepared as described [13] except that Tris-HCl was replaced with MOPS/NaOH pH 7.0. Protein kinase assays were carried out using a modification of the method of Roskoski [16]. Peptides were incubated with 50 mM MOPS pH 7.0, 10 mM $MgCl_2$, 0.25 mg/ml BSA, 1 μM Microcystin-LR, 100 μM γ-[^{32}P] or γ-[^{33}P]ATP (0.05 μCi) and extract, in a total volume of 50 μl. Following incubation, portions (40 μl) were taken and spotted on to Whatman P81 phosphocellulose strips (2×2 cm) and immersed in 75 mM phosphoric acid to terminate the reaction. The strips were washed for 10 min, then washed with two changes of phosphoric acid for 10 min each, dried and then subjected to liquid scintillation counting.

Amino acid sequence for the peptides was determined in an ABI Model 477 Sequencer. The phosphorylated peptides were covalently coupled through their C-termini to Sequelon membranes (Sequelon-AA Reagent Kit, Millipore Corporation), then sequenced and fractions were collected at each cycle for liquid scintillation counting to locate the position of the phosphorylated amino acid.

2.3. Partial purification of PAL kinase free of endogenous PAL

Cultured bean cells were harvested by vacuum filtration, frozen in

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Abbreviations: PAL, phenylalanine ammonia-lyase; PKA, protein kinase A; CDPK, calmodulin-like domain protein kinase

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liquid nitrogen and extracted with an equal volume of 50 mM MOPS, 1 mM DTT, 2 mm EDTA and sodium ascorbate (4 mg/ml), pH 7.0. The extract was centrifuged at 20 000×*g* for 20 min and a 40–70% ammonium sulphate fraction prepared which was redissolved in buffer containing 50 mM MOPS, 1 mM DTT and 2 mM EDTA, pH 8.0 (buffer A). The redissolved pellet was desalted using a PD-10 column and the eluate applied to a DEAE cellulose column equilibrated with buffer A. The protein kinase was eluted with a 0–500 mM NaCl gradient in buffer A. Active fractions were pooled and concentrated, and then applied to a Sephacryl S-300 column and eluted with buffer A. Active fractions were pooled and then applied to a Cibacron blue-agarose column equilibrated in buffer A and eluted with a 0–1 M NaCl gradient in buffer A. The kinase was purified free of endogenous bean PAL by ammonium sulphate precipitation and DEAE cellulose chromatography. In some purification procedures Sephadex G100 replaced Sephacryl S-300 chromatography.

2.4. Phosphorylation of recombinant PAL

Recombinant poplar PAL was produced as previously described [17]. PAL kinase was prepared from elicited French bean cell cultures. Phosphorylation reactions with recombinant PAL were carried out as for peptides except that the total incubation volume was reduced to 20 μl, 1 μCi of γ[^{32}P]ATP and 20 μM ATP were added and 5 μl of enzyme, purified to the Cibacron blue-agarose stage. A peptide competition experiment was conducted using the partially purified enzyme with recombinant PAL in the presence of varying amounts of peptide. In both cases radiolabelled PAL was separated by SDS-PAGE on an 8% acrylamide gel and subjected to autoradiography. For studies on the effect of phosphorylation on PAL kinetics, the kinase was purified to the DEAE cellulose stage which effectively removed endogenous bean PAL, and non-radiolabelled ATP (1 mM) was substituted for radiolabelled ATP in the incubation medium. PAL activity was determined spectrophotometrically by following the formation of cinnamic acid by measuring the absorbance at 290 nm in the presence of L-phenylalanine in 100 mM Tris-HCl buffer pH 8.5 in a total volume of 1 ml and at 25°C [5].

2.5. In-gel assay of the kinase using recombinant PAL as substrate

Control and elicited cells were harvested and homogenised in 50 mM HEPES KOH (pH 7.6), 2 mM DTT, 2 mM EDTA, 20 mM β-glycerophosphate, 20% glycerol (v/v), 1 mM sodium vanadate, 1 mM NaF and 50 μg/μl protease inhibitors (Complete, Boehringer Mannheim). The homogenate was centrifuged at 14 000×*g* for 20 min and the supernatant was subjected to ammonium sulphate precipitation. A 40–70% ammonium sulphate fraction was prepared and desalted against the same buffer on a Sephadex G-25 column. A kinase preparation partially purified to the blue agarose stage was suspended in the same buffer. These fractions were analysed by an in-gel assay [18] by separation by SDS-PAGE, on an 8% polyacrylamide gel, polymerised in the presence of 100 μg/ml recombinant PAL. The same samples were also separated on an identical gel without recombinant PAL as a control to check for autophosphorylation. Following electrophoresis, the gels were washed in 25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 1 mM sodium vanadate, 5 mM NaF, 0.5 mg/ml BSA, 0.1% Triton X-100 while shaking at room temperature for 3×30 min. The gels were then equilibrated with 25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 1 mM sodium vanadate, 5 mM NaF at 4°C overnight and were then incubated for 60 min at room temperature with 25 mM Tris-HCl pH 7.5, 2 mM EDTA, 12 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM DTT and 1 mM sodium vanadate. The buffer was replaced with an identical solution containing 50 μCi γ[^{32}P]ATP and incubated for 60 min at room temperature with shaking. The gels were then fixed and washed in 5% trichloroacetic acid containing 1% sodium pyrophosphate with five changes for at least 6 h at room temperature. The gels were then dried and subjected to autoradiography.

Bean PAL 491-500	(peptide B)§	L I S A R K T N E A
Bean PAL 537-547	(peptide A)§	S R V A K R T L **T** T A
Poplar PAL 540-550		S Q V S K R V L **T** T G
PAL peptide A consensus†		X X V X K [K/R] X L **T** X
Malantide		R T K R S G **S** V Y E P L K I
Kemptide		L R R A S L G

Fig. 1. Comparison of the predicted phosphorylated peptides and a conserved sequence in PAL with the PKA substrates malantide and kemptide. The phosphorylated residue is shown in bold type. †Some 18 PAL sequences from 15 different plant species were matched in the various databases. §Numbering based on the sequence of PAL-2 from *Phaseolus vulgaris* [6].

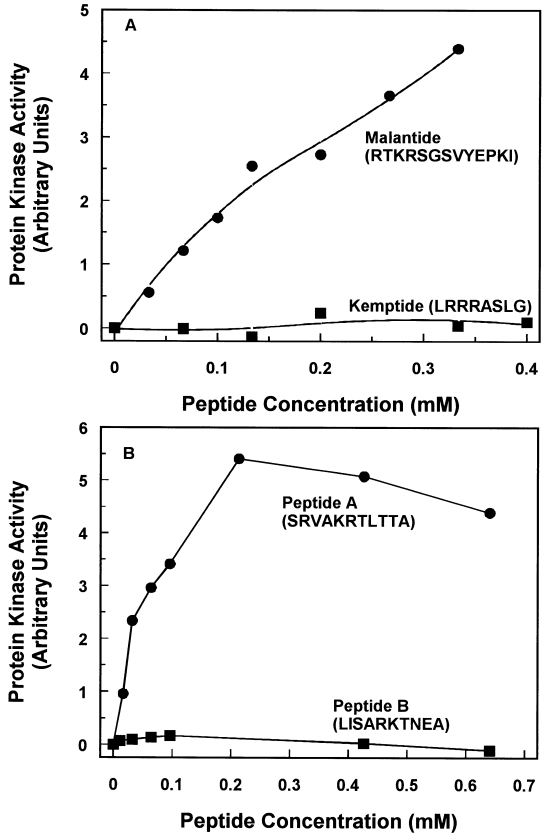

Fig. 2. Effect of peptide concentration on rate of phosphorylation of peptides catalysed by protein kinase. A: Protein kinase activity determined with a range of malantide and kemptide concentrations, only malantide was phosphorylated. B: Protein kinase activity with PAL peptides A and B, only peptide A was phosphorylated to a significant extent.

3. Results

3.1. Phosphorylation of peptides

Analysis of the single phosphorylated peptide derived from PAL localised the phosphorylation site between residues 465 and 662 [14]. Total acid hydrolysis of this peptide in 5.7 M HCl, followed by thin layer chromatography [19] with phosphoserine, phosphothreonine and phosphotyrosine standards, showed that only threonine was phosphorylated (data not shown). This information allowed a search of sequence databases for conserved threonine flanked by conserved amino acid residues in PAL and led to the identification of two possible sites for phosphorylation within this region, and two peptides (A and B) corresponding to these sites were synthesised (Fig. 1). Since they had some features of protein kinase A (PKA) target sites, two commercially available peptides known to be PKA substrates, malantide and kemptide,

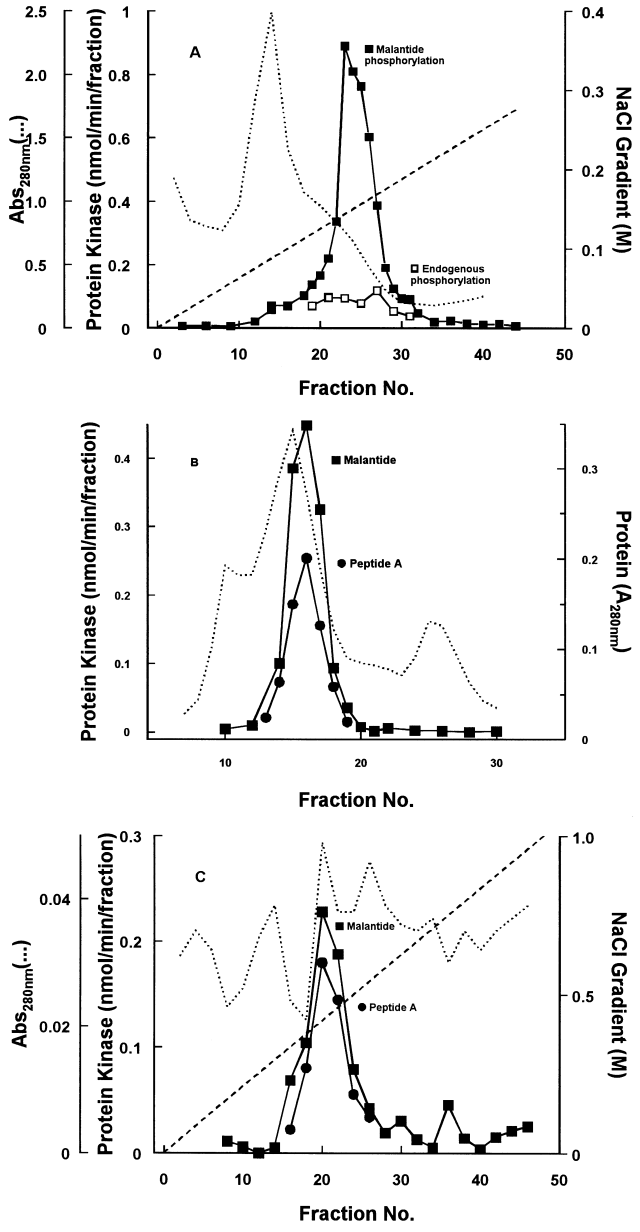

Fig. 3. Purification of protein kinase free of endogenous PAL. Protein kinase was subjected to ammonium sulphate precipitation, (A) DEAE cellulose ion exchange chromatography, (B) gel filtration on Sephacryl S300, and (C) dye ligand chromatography on Cibacron blue-agarose. This procedure effectively removed all endogenous PAL at the DEAE cellulose chromatography step.

were initially used for characterisation of the specificity of the protein kinase.

Extracts of French bean suspension cultured cells subjected to elicitation, previously shown to phosphorylate endogenous proteins, could also phosphorylate malantide but not kemptide (Fig. 2A). More importantly, of the two peptides modelled on PAL sequences, only peptide A was phosphorylated (Fig. 2B). The kinase extracted from cells at various time points after addition of elicitor showed some increase in extractable activity (40%) between 15 and 60 min (data not shown) and consequently extracts from elicited cells were used for all subsequent experiments unless otherwise stated. The activity of the kinase in the desalted ammonium sulphate fraction was not potentiated by the addition of cyclic AMP, tautomycin or microcystin but showed some inhibition (23%) by EGTA (100 μM) and some activation (50%) in the presence of Ca^{2+} (200 μM) and EGTA (100 μM) suggesting the presence of a calmodulin-like domain protein kinase (CDPK) [20]. The kinase responsible for the phosphorylation of PAL, routinely assayed using 100 μM malantide as substrate and with microcystin added to assay media, was partially purified (40-fold; 5–10% recovery) by fractionation by ammonium sulphate precipitation, DEAE cellulose chromatography, Sephadex G100 or Sephacryl S-300 chromatography, and affinity chromatography on Cibacron blue-agarose (Fig. 3) and was used to phosphorylate peptide A. The authenticity of the peptide and its phosphorylated derivative was checked by reversed phase HPLC followed by sequence analysis (Fig. 4). This analysis showed that the second threonine in the sequence, corresponding to Thr^{545} in PAL-2 [6] from French bean, was the target amino acid. The protein kinase had a K_m of about 50 μM for peptide A and 500 μM for malantide, although the V_{max} was similar, suggesting that the former is a preferred substrate. The partially purified enzyme became more sensitive to Ca^{2+} on purification with a 10-fold increase in activity in the presence of Ca^{2+} (200 μM) and EGTA (100 μM) compared to a control with EGTA only.

3.2. Phosphorylation of recombinant PAL

Recombinant poplar PAL [17] was used in a heterologous system to study the phosphorylation of PAL and the effect of phosphorylation on PAL kinetics. Protein kinase was purified to the Cibacron blue-agarose chromatography stage and used to phosphorylate PAL. Fig. 5A shows an autoradiograph of an SDS-PAGE of a typical phosphorylation experiment. A strongly labelled 55 kDa phosphorylation product is observed in both control and PAL incubation mixtures while phosphorylation of a 77 kDa protein corresponding to PAL is only observed where the recombinant PAL is added. The major phosphorylation product is most probably the result of autophosphorylation of the kinase. Competition between PAL and the PAL peptide for phosphorylation by the kinase was shown in an experiment where increasing concentrations of the peptide progressively inhibited the phosphorylation of

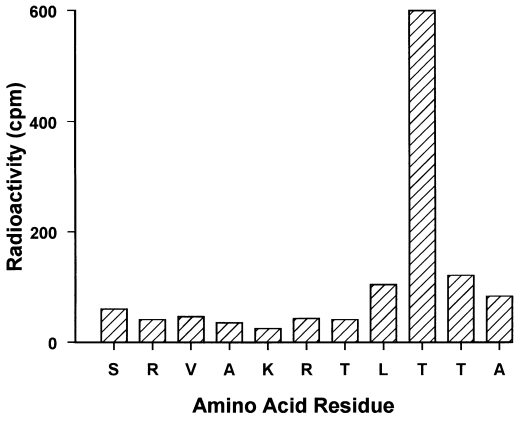

Fig. 4. Identification of the phosphorylation site in peptide A by Edman degradation. Peptide A was phosphorylated using [^{33}P]ATP as substrate, the [^{33}P]phosphopeptide separated by HPLC, covalently bound to a Sequelon membrane and sequenced in order to establish that only the second threonine (Thr^9 in the phosphopeptide) was phosphorylated.

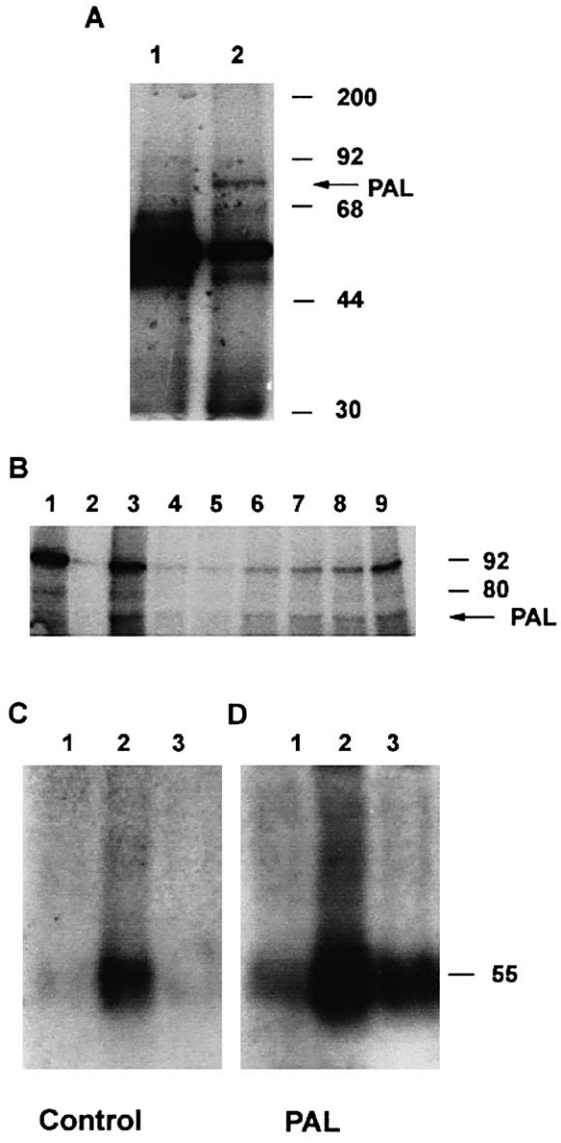

Fig. 5. A–D: SDS-PAGE analysis of phosphorylation of recombinant poplar PAL. Recombinant PAL was incubated in the presence of protein kinase with [^{32}P]ATP for 30 min. Proteins were separated by SDS-PAGE on an 8% acrylamide gel and subjected to autoradiography. A: Track 1, kinase preparation showing phosphorylation of a protein of 55 kDa; track 2, kinase preparation plus recombinant PAL showing phosphorylation of a protein at 55 kDa and of PAL at 77 kDa. B: Phosphorylation of recombinant PAL in the presence of peptide A. Track 1, autophosphorylation control; track 2, recombinant PAL only; tracks 3–9, recombinant PAL and kinase; track 3, no peptide A added to reaction mixture; tracks 4–9, reaction carried out in the presence of: 4, 4 mM; 5, 2 mM, 6, 1 mM, 7, 0.4 mM; 8, 0.2 mM; 9, 0.1 mM peptide A. Note the progressive inhibition of the phosphorylation of the 77 kDa PAL band (arrowed) by the peptide in the presence of increasing peptide concentrations. C and D: In-gel assay of kinase preparations. C: Control incubation, without recombinant PAL in the gel. Track 1, a desalted, 40–70% ammonium sulphate fraction from unelicited cells; track 2, a desalted, 40–70% ammonium sulphate fraction from cells extracted 1 h after elicitation; track 3, kinase preparation purified to the Cibacron blue-agarose stage, after extraction from elicited cells. D: The same preparations, incubated with recombinant PAL polymerised into the gel.

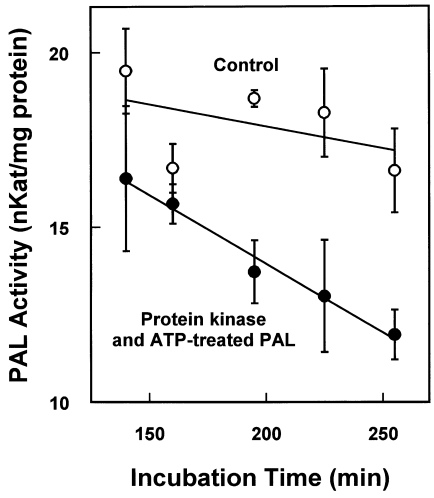

Fig. 6. The effect of phosphorylation on the stability of recombinant PAL. Recombinant PAL was incubated in the presence of protein kinase with 1 mM ATP-Mg (●) or without ATP-Mg (○) for times up to 4.5 h. PAL activity was subsequently determined spectrophotometrically. The results are plotted as mean values ± S.D., for three separate experiments.

PAL (Fig. 5B). Similar competition for the phosphorylation of two other, unidentified, proteins of 92 and 80 kDa, which were present in the kinase preparation, was also found (Fig. 5B). The phosphorylation of PAL, together with further evidence that the M_r of the protein kinase is 55 kDa, was achieved by in-gel assays (Fig. 5C,D). Fig. 5C shows three different kinase preparations incubated in-gel in the absence of substrate. Significant autophosphorylation of a protein of 55 kDa was only seen with the ammonium sulphate precipitate from elicited cells. Fig. 5D shows the result when the same preparations were treated in exactly the same way and exposed for the same time as in Fig. 5C but with recombinant PAL in the gel. There is clearly an increased phosphorylation in the presence of PAL, particularly with extracts from elicited cells and with the purified kinase, confirming that PAL is indeed phosphorylated by a 55 kDa protein kinase.

The kinetic parameters for recombinant PAL were also determined, with or without phosphorylation by protein kinase purified to the DEAE cellulose chromatography stage. After 1 h only a minor change in K_m from 0.052 ± 0.014 to 0.044 ± 0.009 mM (mean ± S.D., $n = 3$) for control and kinase-treated PAL, respectively, was observed. There was also a small decrease in V_{max}, from 11.3 ± 1.4 to 9.55 ± 0.50 nKat/mg protein (mean ± S.D., $n = 3$) when the PAL was treated with kinase. However, the phosphorylated PAL appeared to be less stable when incubated for a further period of up to 4 h, with the V_{max} decreasing 3-fold faster when the PAL was treated with kinase plus ATP (Fig. 6). It was ascertained that ATP, in the absence of the kinase, but with other conditions identical, had no effect on the enzyme stability over this period.

4. Discussion

The results obtained in the present study confirm our previous finding that PAL is phosphorylated in vivo and extend our knowledge of this phosphorylation in several important ways. We have established that the phosphorylated residue is

a threonine residue and we have used that information plus our previous data on the localisation of the phosphorylation site to predict the possible target site. Based on an assay involving the phosphorylation of a synthetic PAL-derived peptide we have isolated and partially purified a protein kinase from elicited bean cells and have used the purified kinase to show that a recombinant poplar PAL may also be phosphorylated in vitro.

One of the substrates phosphorylated by the kinase is malantide, which is a peptide based on the phosphorylation site in the β-subunit of phosphorylase kinase and is a potent substrate for PKA in mammalian tissues [21]. Protein kinase and protein phosphatase inhibitors have been used to investigate the modulation of the induction of PAL activity in elicitor-treated and control cells [14,22]. The specificity of these gave some indication for the direct or indirect phosphorylation of PAL by a protein kinase which may mark it for turnover and thus reduce the activity of PAL once it has exerted an effect on phenylpropanoid synthesis. The adenylate cyclase modulators, forskolin, cholera and pertussis toxins, also regulate PAL activity but the effects are complex [13,14,22] with both activation and inhibition of the appearance of PAL activity observed, depending on the conditions used. The production of elevated levels of cAMP in cells by the administration of relatively high concentrations of forskolin can be shown to be followed by increased PAL phosphorylation [13]. However, it has been found subsequently that the effect of cAMP is likely to be indirect and that the activation of the kinase is probably dependent upon Ca^{2+} influx into elicited cells.

The identification of a threonine phosphorylation site (VXKK/RXL$\underline{T}^{545}$) in French bean PAL, which is conserved in all PAL where the sequence is known, reveals it to be similar to PKA target sites and to the phosphorylation site of some plant protein kinases. These typically have basic residues, usually Arg or Lys, at P−3 or P−4 relative to a Ser or Thr residue [1]. However, the bean PAL kinase lacks some of the features of a PKA in that it does not phosphorylate kemptide, has a different molecular weight and is not activated by cAMP. There is rather tenuous evidence for the existence of PKA in plants and amongst the plethora of gene sequences for plant protein kinases now available, none has been unequivocally designated as a PKA.

It is clear that PAL is not the only target for phosphorylation by the protein kinase found in the present study and work is in progress to identify other possible targets. The target phosphorylation site in PAL has some similarities to that of a maize sucrose synthase kinase, probably a CDPK, where sequencing analysis revealed that Ser^{15} of sucrose synthase in the sequence RVLSRLH$\underline{S}$VR is phosphorylated [23]. Similarly, a spinach CDPK has been found which phosphorylates nitrate reductase at Ser^{534}, with the major specificity requirements being a basic residue at P−3 and a hydrophobic residue at P−5 [24,25]. Three related CDPKs from soybean, each exhibiting different sensitivity to Ca^{2+}, have a similar phosphorylation motif, Basic-X-X-Ser(Thr), to the kinase described in the present study, although flanking hydrophobic residues at P−5, P+1 and P+4 may also be important in determining specificity. All three are activated at micromolar Ca^{2+} in the presence of peptide substrates and are all capable of autophosphorylation particularly in the presence of Ca^{2+} [26]. Multiple forms of protein kinases which phosphorylate nitrate reductase have been isolated from spinach, one of which is Ca^{2+}-independent [27]. HMG-CoA reductase kinase, a member of the SNF-1-related protein kinase family [28] which also phosphorylates nitrate reductase and sucrose phosphate synthase, has a substrate recognition motif with hydrophobic residues at P−5 and P+4 and a basic residue at P−4 relative to the phosphorylated serine [29]. The SNF-1-related kinases are however not Ca^{2+}-dependent and are therefore unlikely to be involved in the phosphorylation of PAL, particularly since PAL lacks the hydrophobic residues at these particular positions flanking the phosphorylation site. On the basis of the evidence in the literature and data in the present study, we propose that the kinase responsible for the phosphorylation of PAL is a CDPK.

The activity of the protein kinase responsible for the phosphorylation of PAL is clearly increased following elicitation. Rapid events following elicitor action include an increase in O_2 consumption, accumulation of active oxygen species, alkalinisation of the cell wall and depletion of ATP [30] and an increase in the level of cAMP been shown in a number of systems [31–34]. This increased level of cAMP may also be related to opening of ion channels leading to the influx of Ca^{2+} and H^+ and efflux of K^+ [34]. These metabolic changes and ion fluxes are probably involved in regulation of a number of protein kinases, including the enzyme under investigation in the present study, which coincide with profound changes in the oxidative state of French bean cells following elicitor treatment. Some of these signals may be related to the eventual down-regulation of stress metabolism during a recovery phase of metabolism, one event of which may be phosphorylation-mediated PAL turnover. To our knowledge this is the first demonstration of a protein kinase involved in the phosphorylation of a key enzyme of secondary metabolism in plants and that this covalent modification results in modulation of enzyme activity. However, the level of modulation is not great and the phosphorylation may have other functions. There is some evidence for the association of PAL with membranes following elicitation. We have previously shown that nearly 20% of the newly radiolabelled PAL subunits can be recovered by immunoprecipitation from membranes [8]. It is possible that phosphorylation or dephosphorylation may be involved in the targeting of PAL to membranes. It is known that over-expression of PAL leads to an increase in microsome-associated PAL possibly allowing metabolic channeling in phenylpropanoid metabolism [35]. In this context, it has been shown that phosphorylation of sucrose synthase by exogenous PKA leads to dissociation of the synthase from microsomal membranes [36].

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