Planta (1986) 169:97–107

Planta

L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*: Modulation of the levels of active enzyme by *trans*-cinnamic acid

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Abstract. The extractable activity of L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) in cell suspension cultures of bean (*Phaseolus vulgaris*) is greatly induced following exposure to an elicitor preparation from the cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum*. Following exogenous application of *trans*-cinnamic acid (the product of the PAL reaction) to elicitor-induced cells, the activity of the enzyme rapidly declines. Loss of enzyme activity is accompanied by inhibition of the rate of synthesis of PAL subunits, as determined by [^{35}S]methionine pulse-labelling followed by specific immunoprecipitation; this is insufficient to account for the rapid loss of PAL enzyme activity. Pulse-chase and immune blotting experiments indicate that cinnamic acid does not affect the rate of degradation of enzyme subunits, but rather mediates inactivation of the enzyme. A non-dialysable factor from cinnamic-acid-treated bean cells stimulates removal of PAL activity from enzyme extracts in vitro; this effect is dependent on the presence of cinnamic acid. Such loss of enzyme activity in vitro is accompanied by an apparent loss or reduction of the dehydroalanine residue of the enzyme's active site, as detected by active-site-specific tritiation, although levels of immunoprecipitable enzyme subunits do not decrease. Furthermore, cinnamic-acid-mediated loss of enzyme activity in vivo is accompanied, in pulse-chase experiments, by a greater relative loss of ^{35}S-labelled enzyme subunits precipitated by an immobilised active-site affinity ligand than of subunits precipitated with anti-immunoglobulin G. It is therefore suggested that a possible mechanism for cinnamic-acid-mediated removal of PAL activity may involve modification of the dehydroalanine residue of the enzyme's active site.

Key words: Cell culture – *Phaseolus* (enzyme turnover) – Phenylalanine ammonia-lyase – Phenylpropanoid biosynthesis.

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Abbreviations: AOPP = L-α-aminoxy-β-phenylpropionic acid; CA = *trans*-cinnamic acid; PAGE = polyacrylamide gel electrophoresis; PAL = L-phenylalanine ammonia-lyase; SDS = sodium dodecyl sulphate

Introduction

L-Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the first reaction in the biosynthesis of a wide range of plant phenolic compounds from L-phenylalanine (Stafford 1974; Jones 1984). Transcriptional and translational regulation of PAL, particularly in response to external stimuli such as light and fungal elicitor macromolecules, has now been investigated using specific antibody and complementary DNA probes (Lawton et al. 1983a, b; Chappell and Hahlbrock 1984; Ebel et al. 1984; Kuhn et al. 1984; Bolwell et al. 1985a; Cramer et al. 1985a, b; Edwards et al. 1985). However, it is also clear that the level of PAL activity in elicitor-treated bean cell cultures may in part be modulated post-translationally (Lawton et al. 1980, 1983a). A number of studies have indicated that *trans*-cinnamic acid (CA), the product of the PAL reaction, may act as a modulator of PAL turnover; treatments which prevent the accumulation of endogenous CA in vivo may result in PAL superinduction (Amrhein and Gerhart 1979; Billet and

Smith 1980; Dixon et al. 1980; Gerrish et al. 1985; Lamb 1982), whereas conditions which inhibit CA metabolism correspondingly inhibit the appearance of PAL activity (Durst 1976; Shirsat and Nair 1981).

Exogenous application of CA to a number of plant tissues brings about prevention of PAL induction and-or rapid loss of PAL activity (Johnson et al. 1975; Dixon et al. 1980; Shields et al. 1982). By the use of density-labelling techniques it has been shown that the effect of CA on PAL activity in pea epicotyl sections is mediated by both inhibition of enzyme synthesis and stimulation of enzyme removal (Shields et al. 1982). Furthermore, in potato tuber tissues removal of PAL activity after attainment of its maximum level is inhibited by cycloheximide and cordycepin in a manner consistent with a requirement for a second, regulatory protein effecting PAL removal (Lamb 1977a; Lamb et al. 1981).

Treatment of suspension-cultured bean cells with elicitor macromolecules from the cell walls of the bean pathogen *Colletotrichum lindemuthianum* leads to rapid induction of enzymes involved in the accumulation of isoflavonoid phytoalexins (Dixon and Lamb 1979; Lawton et al. 1983a, b; Ryder et al. 1984; Cramer et al. 1985a, b; Robbins et al. 1985). This response is characterised by a rapid increase in PAL mRNA levels and translational activity leading to increased rates of enzyme synthesis (Lawton et al. 1983a, b; Cramer et al. 1985a; Edwards et al. 1985) and the differential induction of multiple tetrameric forms of PAL differing in isoelectric-point (pI) and k_m values (Bolwell et al. 1985a). The subsequent increase in PAL activity is, however, reversed by exogenous addition of CA at concentrations of around 1 mM (Dixon et al. 1980).

This loss of extractable PAL activity following exogenous addition of CA has now been investigated at the molecular level using specific anti-(PAL) immunoglobulin G (IgG) and immobilised affinity-ligand probes. A CA-mediated inhibition of the synthesis of M_r-77000 enzyme subunits has been demonstrated and it is further concluded that CA treatments lead to the appearance of an active factor which may bring about a specific and irreversible inactivation of PAL activity without an appreciable increase in the rate of removal of intact enzyme subunits.

Materials and methods

Chemicals. L-[^{35}S]Methionine (30 TBq·mmol^{-1}), [^{125}I]protein A (1.6 GBq·mg^{-1}) and NaB^{3}H$_4$ (185–740 GBq·mmol^{-1}) were purchased from Amersham International, Amersham, Bucks., UK. Protein A-Sepharose and Staphylococcal V8 protease were obtained from Sigma, Poole, Dorset UK. and nitrocellulose sheets were purchased from Anderman and Co., East Molesey, Surrey, UK. The preparation of anti-(PAL) IgG and L-aminooxy-*p*-hydroxyphenyl-propionic acid both linked to epoxy-activated Sepharose 6B, has been described previously (Bolwell et al. 1985a, b). Cinnamic acid (Sigma) was recrystallised three times from aqueous ethanol before use.

Growth, elicitation and treatment of cell cultures. Preparation of fungal elicitor and maintenance and growth of cell suspension cultures of dwarf French bean (*Phaseolus vulgaris* L. cv. Canadian Wonder) were as described previously (Dixon and Lamb 1979).

Cells were exposed to 20 µg glucose equivalents·ml^{-1} of *Collectotrichum* elicitor and subsequently sampled at various times by suction filtration and transfer to liquid nitrogen. Exogenous CA was added where described to a final concentration of 1 mM. For measurement of PAL synthesis in vivo, cells were transferred in 10-ml aliquots to presterilized plastic pots and 740 kBq L-[^{35}S]methionine was added 1 h before sampling. For pulse-chase experiments, 100 ml of cells were elicited and 3.7 GBq L-[^{35}S]methionine added after 2 h. Two hours later, unlabelled methionine was added to a final concentration of 100 µM and the cells divided into two 50-ml batches, CA being added to one of the batches to a final concentration of 1 mM. Samples (10 ml) were taken from each batch at subsequent time points.

Preparation and analysis of cell extracts. Cells were extracted in 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl buffer pH 8.0 (1 g cells·ml^{-1} buffer) and centrifuged at 1000 *g* for 15 min. The PAL activity was assayed spectrophotometrically, measuring the formation of CA at 290 nm as described by Lamb et al. (1979). Activities in extracts from CA-treated cells were determined after gel-filtration through Sephadex G 25. The levels of free and esterified CA were determined by high-performance liquid chromatography as described by Bolwell et al. (1985b). Immunoprecipitation of ^{35}S-labelled PAL subunits was carried out as described by Bolwell et al. (1985a). Affinity precipitation of radioactively labelled PAL subunits was accomplished by end-over-end incubation of 1 ml of cell extract with 50 mg L-aminooxy-*p*-hydroxyphenyl-propionic acid Sepharose overnight at 4° C. The gel was washed three times with 50 mM Tris-HCl pH 8.0 containing 1 M NaCl before preparation for gel electrophoresis. Both immunoprecipitated and affinity-precipitated PAL preparations were solubilised and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Bolwell et al. 1985a). Fluorography was carried out by the method of Bonner and Laskey (1975) and the relative level of radioactivity in the M_r-77000 PAL subunit was determined by densitometer scanning of gel tracks at 410 nm.

Measurement of total immunodetectable PAL subunits was carried out using a dot-blot immunoassay. Cell extracts (2.5 µl aliquots) were spotted onto nitrocellulose paper marked out in a grid with individual squares (0.4 mm^2). The grids (32 mm^2) were incubated in 100 mM Tris-HCl pH 9.0 containing 10% (v/v) ethanolamine and 0.25% (w/v) gelatine. They were washed briefly in water and incubated in coverslip boxes together with 4 ml of a 1:20 dilution of rabbit anti-(PAL) antibody (Bolwell et al. 1985b) in 50 mM Tris-HCl pH 7.4 containing 0.15 M NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.25% (w/v) gelatine and 0.5% Nonidet P-40 (TEGN) for 16 h at 37° C. The grids were washed briefly in water and then for 1 h in 250 ml TEGN buffer.

Protein-antibody complexes were detected by incubation with [^{125}I]protein A (18.5 kBq) in 4 ml TEGN buffer for 2 h at 37° C. Following extensive washing in TEGN the grids were dried and each individual dot was cut out for scintillation counting. The radioactivity was determined in eight replicates for each time point. The extent of non-specific binding was assesed by comparison of the binding of antibody to varying amounts of highly purified PAL with or without the presence of plant extracts containing very low PAL activity.

Treatment of cell extracts in vitro. These experiments were carried out using crude enzyme extracts prepared as described above, or using PAL preparations partially purified by ammonium-sulphate precipitation followed by gel filtration on Sephacryl S300 (Bolwell et al. 1985a).

For investigation of the effects of extracts from CA-treated bean cells on PAL activity in crude extracts from elicitor-treated cells or in partially purified enzyme preparations, aliquots of each extract were mixed and incubated at 37° C. The PAL activity was measured by continuous spectrophotometric assay over a 10-min period at various subsequent time points; all activities were normalised to the initial starting activity, correction being made for dilution of the mixed extracts.

For investigating the effect of extracts from CA-treated cells, the PAL prearations used were obtained from cells containing high PAL activity and high specific activity of L-[^{35}S]methionine-labelled proteins (in vivo labelled). Sufficient antibody (1:20 titre) was then added at subsequent time points to give a rapid immunoprecipitation of the PAL subunit. Antibody complexes were prepared and analysed by SDS-PAGE as described above.

Tritiation of the PAL active site. Preparations of PAL (900 µl) were mixed with 100 µl of 0.4 mM L-α-aminooxy-β-phenylpropionic acid (AOPP) in 50 mM Tris-HCl pH 8.0 and the mixture allowed to equilibrate on ice for 15 min. This treatment gave 100% inhibition of enzyme activity. Aliquots (5 µl) of 100 mM $NaBH_4$ in ice-cold 100 mM NaOH were then slowly added to the protected enzyme and to an equivalent batch of enzyme not pre-treated with AOPP, until the activity of the unprotected enzyme was fully inhibited. Both PAL samples were then extensively dialysed against 50 mM Tris-HCl, pH 8.0, before displacement of AOPP from the protected PAL with L-phenylalanine as described by Jones and Northcote (1984). The deprotected enzyme was then treated with 5-µl aliquots of NaB^3H_4 until the enzyme was fully inhibited. The unprotected enzyme, which was inactivated, was treated with an equivalent amount of NaB^3H_4, as a control. Samples were then analysed by immunoprecipitation and SDS-PAGE.

Peptide mapping. [^{35}S]Methionine-labelled PAL and ^{3}H-labelled PAL were immunoprecipitated as described above, subjected to digestion with Staphylococcus V8 protease by the method of Cleveland et al. (1977), and analysed by SDS-PAGE.

Results

Uptake of CA and effect on extractable PAL activity. Addition of CA to elicitor-treated cell cultures was followed by rapid uptake into the cells (Fig. 1). The CA could be recovered as both the free and bound (esterified) acid. High levels were maintained in CA-treated cells, approximate maximum intracellular concentrations being

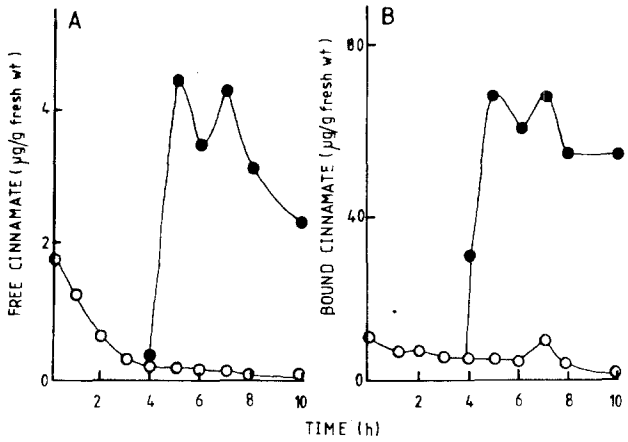

Fig. 1 A, B. Levels of endogenous free (**A**) and bound (**B**) cinnamic acid determined in extracts from suspension-cultured bean cells with (●—●) or without (○—○) exogenous addition of cinnamic acid (1 mM) 4 h after exposure of the cultures to *Colletotrichum* elicitor (20 µg glucose equivalents·ml^{-1} culture)

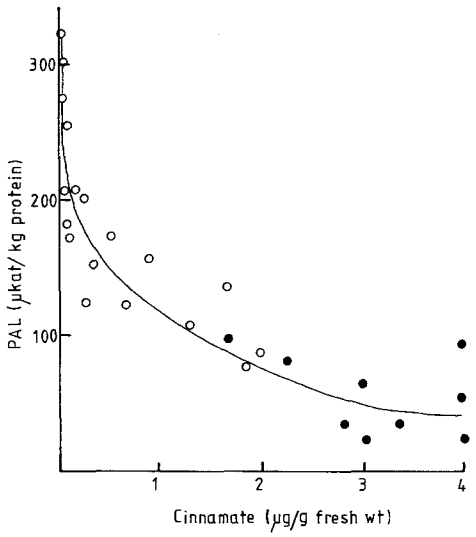

Fig. 2. The relation between phenylalanine ammonia-lyase extractable activities and free cinnamic-acid levels in suspension cultured bean cells. The data are collected from a number of experiments in which cells were harvested at various times after addition of *Colletotrichum* elicitor (20 µg glucose equivalents·ml^{-1} culture) (○—○) or elicitor plus cinnamic acid (1 mM) (●—●)

33 µM (free acid) and 400 µM (esterified). In contrast, rapid mobiliztion of free endogenous CA occurred in untreated, elicited cells as observed previously (Bolwell et al. 1985b). Direct comparison of the corresponding levels of endogenous CA with observed extractable PAL activities following removal of CA by gel filtration, without considerations of compartmentation, indicates simplistically an intimate reciprocal relationship between the two parameters (Fig. 2).

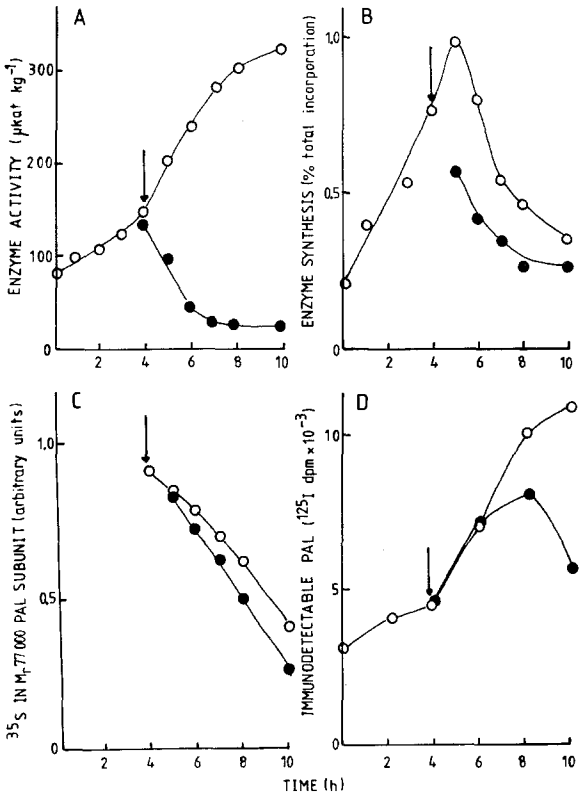

Fig. 3A–D. The effects of cinnamic acid on phenylalanine ammonia-lyase extractable activity, level, synthesis and turnover in elicitor-treated bean cell suspension cultures. Cells were treated with *Colletotrichum* elicitor (20 μg glucose equivalents · ml^{-1} culture) with (●——●) or without (○——○) addition of cinnamic acid (1 mM) 4 h later (*arrow*). **A** Extractable enzyme activity, **B** Rate of synthesis in vivo, measured as incorporation of [^{35}S]methionine into immunoprecipitatable M_r-77000 enzyme subunits. **C** Turnover of enzyme subunits, measured as loss of radioactivity in [^{35}S]-labelled immunoprecipitable M_r-77000 subunits in pulse-chase experiments; label was added 2 h before addition of unlabelled methionine with or without cinnamic acid . **D** Total immunodectable phenylalanine ammonia-lyase measured by dot-blot binding of anti-(phenylalanine ammonia-lyase) antibodies to immobilised antigens and detection with [^{125}I]protein A. The data in **B** are corrected for slight differences in the levels of intracellular [^{35}S]methionine incorporation into total newly synthesised proteins in the different experimental samples. Cinnamic acid did not appreciably decrease total incorporation of intracellular label into protein, but in some experiments did inhibit (up to 30%) the uptake of label into the cells

Addition of elicitor to the bean cell cultures led to rapid induction of PAL activity whereas addition of exogenous CA (or its analogue 4-fluorocinnamate, data not shown) during the period of enzyme increase led to a rapid loss in PAL activity (Fig. 3A). This activity was not regained after removal of CA by dialysis or gel filtration through Sephadex G 25, nor was it regained during extended incubations of the enzyme in the presence of substrate or by dialysis against saturated L-phenylalanine at 37° C, treatments known to overcome the more potent in-vitro inhibition of PAL by AOPP (Jones and Northcote 1984). This irreversible effect of CA in vivo is clearly different from the reversible effect of the acid observed in vitro, and could be the result of one or a combination of the following factors: (a) inhibition of transcription-translation, which could be sufficient to account for the decrease in extractable activity if PAL turnover were sufficiently rapid, (b) increased degradation of PAL subunits and (c) inactivation of the enzyme. Experiments were therefore designed to test these various possibilities.

Effects of CA on synthesis and turnover of PAL subunits in vivo. Rates of incorporation of [^{35}S]methionine into PAL subunits, rates of removal of labelled PAL subunits observed in pulse-chase experiments, levels of total immunodetectable PAL enzyme and extractable PAL activity were compared in elicitor-treated cell cultures, with or without addition of CA 4 h after addition of elicitor (Fig. 3).

In elicited cells, maximum PAL synthesis preceded maximum enzyme activity (Fig. 3B) as previously observed (Lawton et al. 1983a; Cramer et al. 1985a). However, addition of CA led to an appreciable reduction in PAL labelling at each subsequent time point when compared with untreated cells (Fig. 3B). In contrast, the level of total immunodetectable PAL protein (Fig. 3D) was not substantially reduced in CA-treated cells during the period of maximum enzyme activity decrease. Pulse-chase experiments (Fig. 3C) clearly demonstrated only slight effects of CA addition on removal of immunoprecipitable M_r-77000 PAL subunits, with a $t_1/_2$ of approximately 4 h in control cells being reduced to approximately 3.5 h in CA-treated cells. These values for $t_1/_2$ are similar to those calculated from the rates of labelling of active PAL in density-labelling experiments using ^2H from ^2H_2O (Lawton et al. 1980). Clearly, this loss in PAL subunits is insufficient to account for the loss in activity observed following inhibition of PAL synthesis and, in a subsequent experiment with a different batch of bean cells, no effects of CA additions on the $t_1/_2$ of the PAL subunit were observed (Fig. 5B).

Evidence for PAL inactivation in vivo. An affinity probe (L-aminooxy-*p*-hydroxyphenyl-propionic acid immobilised on epoxy-activated Sepharose 6B) for PAL has been developed (Bolwell et al. 1985c); partially purified PAL inactivated in vitro

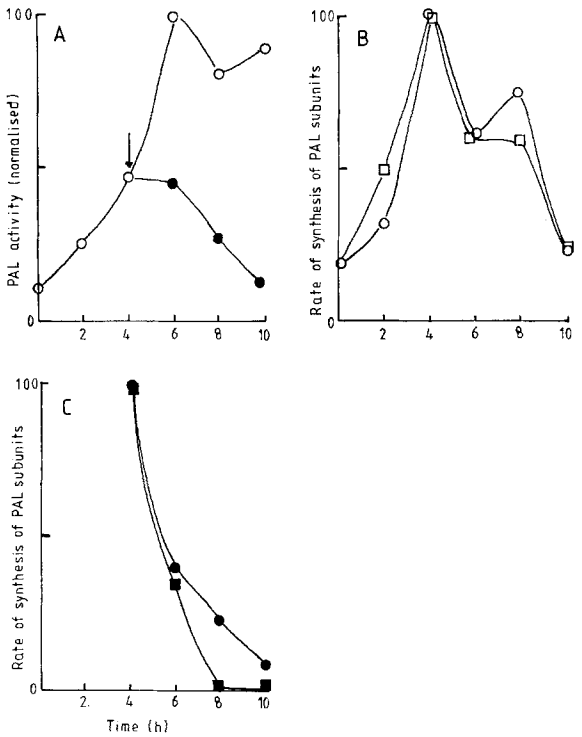

Fig. 4A–C. The effects of cinnamic acid on the synthesis of phenylalanine ammonia-lyase subunits in elicitor-treated bean cell suspension cultures. Cells were treated with *Colletotrichum* elicitor (20 µg glucose equivalents·ml^{-1} culture) with (*solid symbols*) or without (*open symbols*) addition of cinnamic acid 4 h later. **A** Extractable enzyme activity. **B** Rate of synthesis in vivo in cells treated with elicitor alone, measured as incorporation of [^{35}S]methionine into M_r-77000 subunits which were precipitated with anti-(phenylalanine ammonia-lyase) serum and protein A-Sepharose (o——o) or with Sepharose-bound AOPP (□——□). **C** Rate of synthesis in vivo in cells treated with elicitor followed by cinnamic acid; enzyme precipitated with antibody (●——●) or affinity matrix (■——■) as in **B** above. Data were corrected for incorporations as described in the legend to Fig. 3B

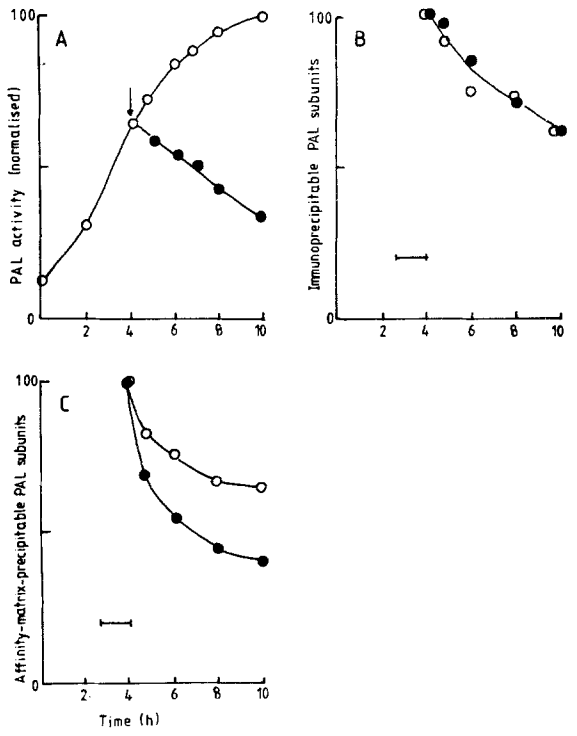

Fig. 5A–C. The effects of cinnamic acid on the turnover of newly synthesised phenylalanine ammonia-lyase subunits in elicitor-treated bean cell suspension cultures. Cells were treated with *Colletotrichum* elicitor (20 µg glucose equivalents·ml^{-1}) followed by [^{35}S]methionine (at 2 h) and unlabelled methionine with (●——●) or without (o——o) cinnamic acid at 4 h. **A** Extractable enzyme activity. **B** Loss of [^{35}S]-labelled M_r-77000 phenylalanine ammonia-lyase subunits immunoprecipitated by anti-(phenylalanine ammona-lyase) serum and protein A-Sepharose. **C** Loss of ^{35}S-labelled subunits precipitated by immobilised AOPP

by borohydride-reduction of the active site failed to bind to this probe.

Therefore, the in-vivo labelling and pulse-chase studies described above were repeated, comparing the relative amounts of [^{35}S]methionine-labelled PAL detected by immunoprecipitation or by the affinity probe. Fig. 4 shows the results of the in-vivo pulse-labelling experiment. In control elicitor-induced cultures the relative incorporation of label into immunoprecipitable M_r-77000 PAL subunits closely followed that measured after binding to the immobilised affinity probe (Fig. 4B), thus supporting the validity of the affinity-probe approach and indicating that PAL was either synthesised in an active form or was very rapidly activated following synthesis. In contrast, no binding to the affinity probe was detected in extracts from cells pulse-labelled at times from 4–6 h after CA treatment, even though some labelled enzyme subunits could be detected following immunoprecipitation (Fig. 4C). Similarly, in pulse-chase experiments (Fig. 5) the $t_1/_2$ of immunoprecipitable enzyme subunits was similar in untreated and CA-treated cells (Fig. 5B), whereas in extracts from CA-treated cells, less PAL subunit was precipitated by the affinity probe than was precipitated from untreated control cells at all times after CA addition (Fig. 5C). Taken together, these results strongly indicate an inactivation mechanism for the CA-mediated loss of PAL activity in vivo.

Effects of extracts from CA-treated bean cells on PAL removal in vitro. As the effects of CA on removal of PAL activity in vivo clearly differed from the reversible inhibitory effects of the compound on the enzyme activity in vitro, it is likely that CA modulates PAL via the intermediacy of some

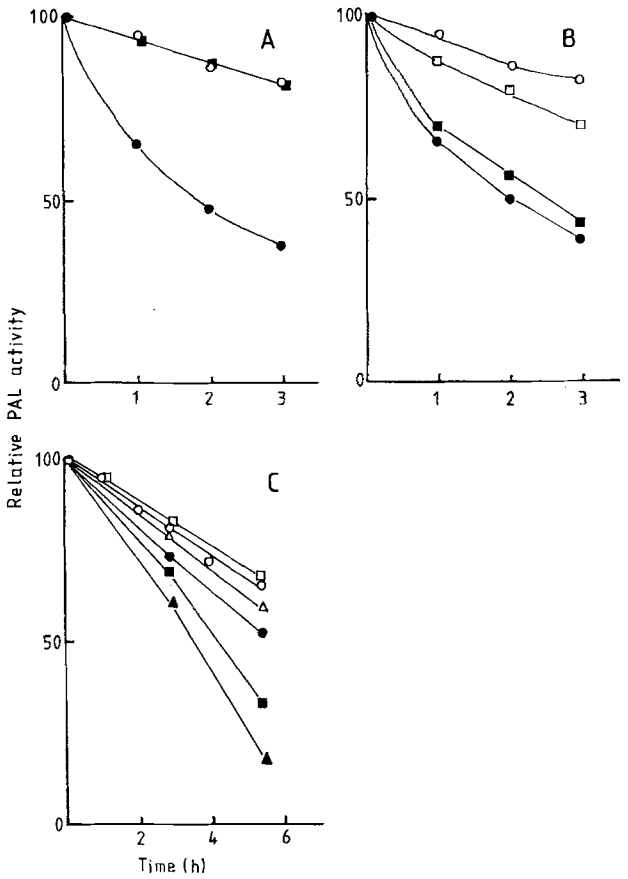

Fig. 6A–C. Loss of phenylalanine ammonia-lyase activity in vitro in mixing experiments with extracts from cinnamate-treated cells **A**. An extract from 6-h-elicited bean cells with high phenylalanine ammonia-lyase activity (300 μkat·kg^{-1} protein) was incubated at 37° C and assayed for enzyme activity every hour (o——o); parallel incubations were performed with a 1:1 (v/v) mixture of this extract and an equivalent extract prepared from unelicited cells 6 h after addition of cinnamic acid (1 mM) (●——●) or a mixture containing equal volumes of the phenylalanine ammonia-lyase extract and dialysed extract from cinnamate-treated cells (■——■). **B** Mixing experiments as in **A** above, where phenylalanine ammonia-lyase extracts were incubated at 37° C alone (or in the presence of cinnamic acid up to 1.0 mM) (o——o), with an equal volume of extract from cinnamate-treated cells (●——●) or with dialysed extracts from cinnamate-treated cells plus cinnamate at final concentrations of 50 μM (□——□) or 500 μM (■——■). **C** Mixing experiments in which phenylalanine ammonia-lyase extracts were incubated at 37° C alone (o——o), or with equal volumes of extracts from bean cells treated with cinnamic acid (1 mM) for 1 h (□——□), 2 h (△——△), 4 h (●——●), 5 h (■——■) and 6 h (▲——▲). All extracts from cinnamate-treated cells were dialysed and cinnamic acid added to a final concentration of 1 mM before addition to phenylalanine ammonia-lyase extracts

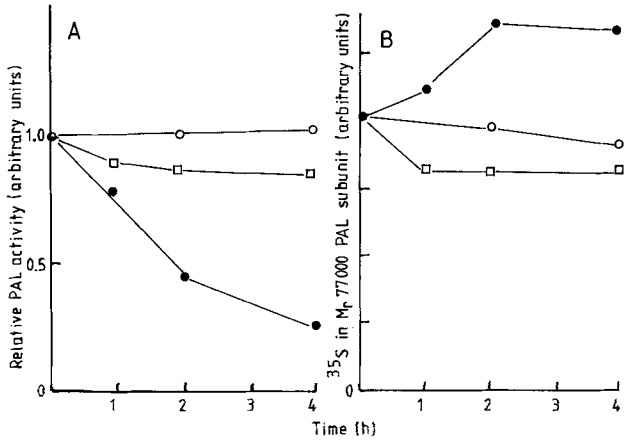

Fig. 7A, B. Loss of phenylalanine ammonia-lyase subunits and enzymic activity in vitro in response to extracts from cinnamic-acid-treated bean cells. A partially purified phenylalanine ammonia-lyase preparation from elicitor-induced bean cells labelled with [^{35}S]methionine was incubated alone at 4° C (o——o) or 37° C (□——□), or at 37° C in the presence of an equal volume of extract from cells exposed to cinnamic acid (1 mM) for 6 h (●——●). At 1-h intervals, measurements were made of **A** enzyme activity and **B** radioactivity in M_r-77000 enzyme subunits immunoprecipitated with anti-(phenylalanine ammonia-lyase) serum

other factor. Evidence for the existence of such a factor was therefore sought by examining, in mixing experiments, the effects of extracts from cinnamate-treated bean cells on the stability of PAL activity. On incubating crude enzyme extracts from elicitor-treated bean cells at 37° C, approx. 15% of the initial enzyme activity was lost after 3 h. When such extracts were mixed with similar extracts from bean cells which had been exposed to both elicitor and CA for 6 h, and which therefore contained negligible PAL activity, loss of initial PAL activity was much more rapid (Fig. 6A). However, no stimulation of activity loss was observed if the extracts from CA-treated cells were first dialysed (Fig. 6B). Interestingly, dialysed extracts regained the ability to stimulate PAL loss if CA was added back to the incubation mixtures, although CA itself was unable to stimulate PAL activity removal in the absence of the dialysed extracts (Fig. 6B).

By treating PAL-containing extracts with CA plus dialysed extracts taken from CA-treated bean cells at various times after addition to the cells of CA plus elicitor, it was shown that the non-dialysable activity responsible for PAL activity removal in vitro could be detected in extracts from treated cells approx. 2 h after addition of CA, and increased in activity until at least 6 h after addition (Fig. 6C).

Identical mixing experiments were performed using crude PAL preparations of high activity from [^{35}S]methionine-labelled elicitor-treated cells (Fig. 7). Immunoprecipitation of labelled PAL subunits during the period of loss of enzyme activity in response to addition of extracts from CA-treated cells indicated no loss of immunodetectable

Table 1. Tritiation of the phenylalanine ammonia-lyase active site with NaB^3H_4

Treatment[a]			Enzymic activity recovered (%)			Radioactivity incorporated mg^{-1} protein ($dpm \times 10^{-3}$)	% incorporated radioactivity in phenylalanine ammonia-lyase immunopreciptate
Protect with AOPP (stage 1)	$NaBH_4$/ dialysis (stage 2)	NaB^3H_4/ dialysis (stage 3)	Start	Stage 1+2	Stage 3		
+	+	+	100	69	13	250	16
−	+	+	100	0	0	64	2
+	−[b]	+	100	0	60	24	1
−	−[c]	+	100	75	15	116	4

[a] See *Material and methods*
[b] Sample was not dialysed after stage 1, and thus the active site was protected at time of addition of NaB^3H_4
[c] Control for recovery of enzyme activity after stages 1 and 2

enzyme. It is therefore clear that, as is the case in vivo, loss of PAL activity in in-vitro mixing experiments occurs by a process of inactivation rather than by stimulation of the degradation of enzyme subunits.

Effects of CA treatments on the PAL active site. The apparent irreversible inactivation of PAL observed in the above experiments, coupled with the loss of ability of the enzyme to bind to an active-site-specific immobilised affinity matrix during CA-mediated enzyme removal, indicates the possibility that the active site may become modified as an indirect result of intracellular CA accumulation. The active site of PAL contains the unusual amino acid dehydroalanine, and this may be reduced to alanine, with subsequent enzyme inactivation, following treatment with $NaBH_4$ (Jones and Northcote 1984). We therefore investigated whether the ability to tritiate PAL with NaB^3H_4 could be lost, either following exposure of the enzyme to extracts from CA-treated cells in vitro, or during CA-mediated enzyme removal in vivo.

The procedure for active-site-specific tritiation of PAL involves (a) protection of the active site with AOPP; (b) treatment of the preparation with unlabelled $NaBH_4$, to react any other available reducible groups, followed by removal of unreacted reagents and the blocking agent; and (c) reduction of the preparation with NaB^3H_4 followed by analysis of immunoprecipitates by SDS-PAGE.

The efficacy of this process was followed in a number of control incubations (Table 1) which demonstrated that by far the greatest radioactivity in immunoprecipitable PAL subunits was obtained under conditions for specific tritiation of the active site. Analysis of immunoprecipitates by SDS-PAGE (Fig. 8) indicated major tritiated bands at M_r 77000 and 46000, these corresponding to two

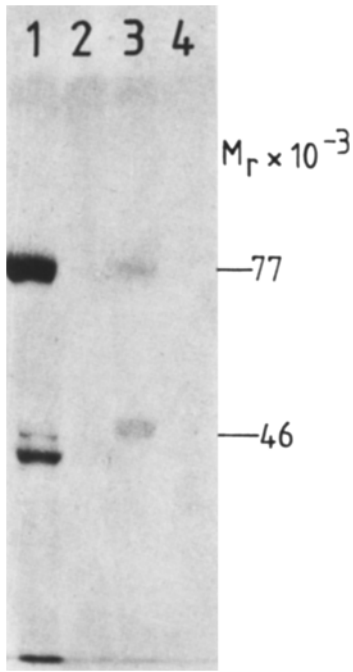

Fig. 8. Immunoprecipitates of phenylalanine ammonia-lyase subunits. *Track 1,* enzyme subunits labelled with [^{35}S]methionine in vivo. *Tracks 2–4,* enzyme partially purified by ammonium-sulphate fractionation and Sephacryl S-300 gel filtration and subjected to active-site tritiation with NaB^3H_4 as described in *Material and methods.* Sources of enzyme prior to tritiation were: *track 2,* bean cells incubated with cinnamic acid (1 mM) in vivo from 4–6 h after elicitation; *track 3,* cells exposed to elicitor alone for 6 h; *track 4,* cells exposed to elicitor for 6 h, with partially purified enzyme then incubated in vitro with an extract from cinnamate treated cells for 4 h at 37° C

of the major bands observed in immunoprecipitates of ^{35}S-labelled PAL (Bolwell et al. 1985c; see *Discussion*). The specificity of labelling was further determined by comparing peptide maps of proteolytically digested, immunoprecipitated PAL subunits labelled either with [^{35}S]methionine in vivo or with NaB^3H_4 in vitro (Fig. 9).

Table 2. Correlation between loss of phenylalanine ammonia-lyase activity and ability to reduce the active-site dehydroalanine residue in in-vitro mixing experiments

Mixed extracts[a]	Incubation temperature (°C)	% of initial level remaining 4 h after mixing			
		PAL activity		^{3}H in immunoprecipitated PAL	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
S-300 PAL + [crude + E]	4	100	100	100	100
S-300 PAL + [crude + E]	37	ND	75	ND	80
S-300 PAL + [crude + E + cinnamate]	37	20	16	22	27

[a] These refer to a partially purified phenylalanine ammonia-lyase preparation (S-300 PAL), a crude extract from 6-h-elicited cells ([crude + E]) and a crude extract from 6-h-elicited cells with cinnamate (1 mM) added concurrently with elicitor ([crude + E + cinnamate]). Extracts were mixed in a 1:1 (v/v) ratio. After 4 h, enzyme activity was determined, and enzyme specifically tritiated as described in *Material and methods*. ND = not determined

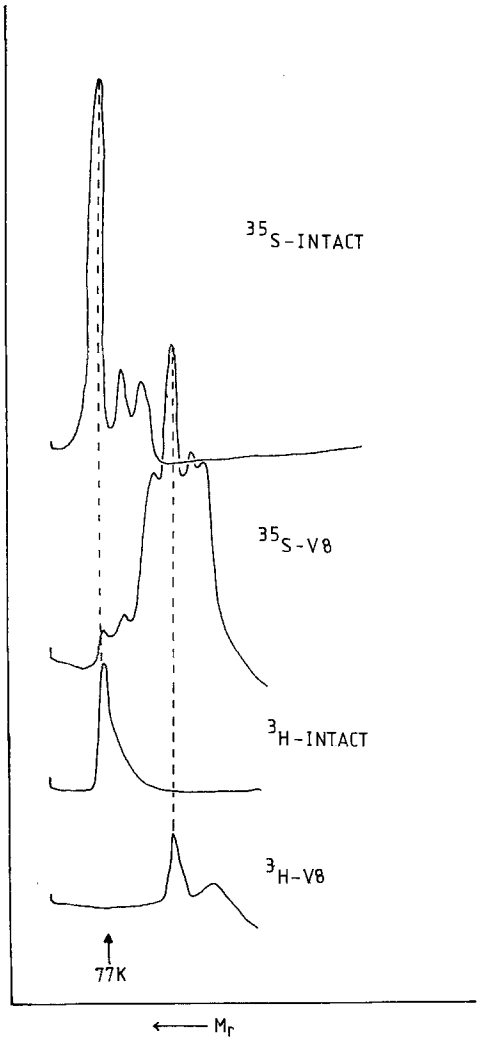

Fig. 9. Peptide maps of M_r-77000 phenylalanine ammonia-lyase subunits. Subunits from enzyme labelled with [^{35}S]methionine in vivo, or tritiated with NaB^3H_4 in vitro, were immunoprecipitated and separated by SDS-PAGE (^{35}S- and ^{3}H-intact). Immunoprecipitates were also digested with Staphylococcal V8 protease and analysed by SDS-PAGE (^{35}S- and ^{3}H-V8) according to the method of Cleveland et al. (1977). Traces were obtained by densitometric scanning of fluorographs

Whereas the intact subunits in both cases were predominantly of M_r 77000, digestion with V8 protease yielded at least six peptides detectable when the enzyme had been ^{35}S-labelled in vivo, whereas one major peptide, corresponding to the major ^{35}S-labelled peptide, was observed in digests of PAL labelled with ^{3}H in vitro. Some non-active-site-specific labelling may be indicated by the trailing peak of lower M_r in digests of ^{3}H-labelled enzyme.

Partially purified PAL preparations were incubated with extracts from CA-treated cells as described above. After 4 h incubation, enzyme activities were determined and aliquots of mixed extracts taken through the specific tritiation procedures, followed by immunoprecipitation and quantitation of labelled enzyme subunits by fluorography. The results (Table 2) indicate that loss of enzyme activity, although not correlated with loss of immunodetectable ^{35}S-labelled PAL subunits (Fig. 7) is associated with loss of a borohydride-reducible group(s) on the PAL molecule. In a separate experiment (data not shown) no labelling of PAL subunits was detected after incubation of the enzyme with extracts from CA-treated cells followed by treatment with NaB^3H_4.

Similar tritiation experiments were performed on PAL extracts from elicitor-treated bean cells and cells treated for 6 h with elicitor plus CA in vivo. Again, analysis of extracts from cells exposed to CA did not reveal the presence of ^{3}H-labelled PAL subunits following in-vitro tritiation and specific immunoprecipitation.

Discussion

Although elicitor-mediated induction of PAL in bean cell cultures appears to result initially from an increased rate of transcription (Edwards et al.

1985), there are many potential sites at which subsequent modulation of the appearance of active enzyme can occur. Earlier density-labelling studies, which demonstrated de-novo synthesis, rather than activation of pre-existing enzyme, as the mechanism for PAL induction in bean, have also indicated, in pea, a potential role for the product of the PAL reaction in bringing about a decrease in the zero-order rate constant for enzyme syntheis (k_s) and an increase in the first-order rate constant for the removal of active enzyme (k_d) (Shields et al. 1982). However, density-labelling analysis is essentially a kinetic rather than a molecular technique, and is limited by its reliance on the need to monitor enzyme activity. Such studies, therefore, do not distinguish the level at which enzyme synthesis is affected nor do they identify degradation or irreversible inactivation of enzyme subunits.

The effect of CA on elicitor-mediated induction of PAL in cell suspension cultures of bean has now been examined using an anti-(PAL) serum and an immobilised affinity probe (Bolwell et al. 1985a, c). The bean PAL subunit is inherently unstable in vitro; detailed characterisation by immunological, affinity and peptide-mapping techniques has shown that the native subunit of the tetrameric enzyme has an M_r of 77000, but that partial degradation products of M_r 70000, 53000 and 46000 are readily generated (Bolwell et al. 1985c). Furthermore, the tetramer can be resolved into four distinct forms by chromatofocusing; these differ in pI and K_m value, are differentially induced by fungal elicitor, but are not the result of partial degradation (Bolwell et al. 1985a). The intact, M_r77000 subunit, newly synthesised either in vivo or in vitro from mRNA, exists in a number of different charge forms, and enzyme turnover in vivo may involve degradation of the M_r-77000 subunits via the lower-M_r forms (Bolwell et al. 1985a, c).

In the present work the pulse-labelling experiments clearly show that exogenous addition of CA leads to a decrease in k_s for immunoprecipitable M_r-77000 PAL subunits, a decrease which, however, cannot account for all the losses in extractable PAL activity. The mechanism of CA repression of PAL synthesis is currently being investigated using the recently characterised cloned cDNA probes complementary to bean PAL mRNA (Edwards et al. 1985).

Effects of CA on PAL turnover have been examined in cells treated with a concentration of elicitor which induces PAL synthesis whilst maintaining the same $t_1/_2$ for active enzyme (approximately 4 h) as observed in unelicited control cultures (Lawton et al. 1980). At higher concentrations of elicitor the $t_1/_2$ of active enzyme is considerably extended (Lawton et al. 1980). From two sets of pulse-chase experiments it is clear that the increase in k_d observed following CA addition can not be accounted for by an increased rate of degradation of intact (M_r 77000) PAL subunits, this being confirmed by the demonstration of little loss of total immunodetectable PAL protein, as measured in dot-blot assays, over the period of maximum decrease of enzyme activity. The $t_1/_2$ of 3.5–4 h for the intact PAL subunit was the same in the presence or absence of CA, and was very similar to the value for the $t_1/_2$ of PAL activity determined from density-labelling studies (Lawton et al. 1980). The lack of effect of CA treatment on subunit k_d must therefore be associated with a process of enzyme inactivation which still maintains an intact immunodetectable PAL subunit.

Comparison of the relative amounts of M_r-77000 PAL subunit immunoprecipitated with those binding to the affinity matrix indicates that subunits are either newly synthesised in an active form or activated very rapidly after synthesis. However, in cells 4 h after exposure to 1 mM CA, no binding of newly synthesised M_r-77000 subunits to the affinity matrix was observed, although labelled enzyme was still immunodetectable. This difference may reflect the presence of an increasing proportion of newly synthesised enzyme that has become rapidly inactivated, and the timing of this effect indicates a lag period of approx. 1 to 2 h for its expression, as was observed for the enzyme activity in the same experiment. Such an effect on apparent enzyme inactivation can be more meaningfully observed in pulse-chase experiments, where no corrections are necessary for uptake of [^{35}S]methionine into PAL protein between different time points and where turnover, rather than synthesis, of enzyme subunits is being followed. In these experiments it is very clear that addition of CA has no effect on rates of removal of intact immunodetectable M_r-77000 PAL subunits whereas the $t_1/_2$ of subunits binding to the affinity matrix is appreciably reduced. In these cells, a notable lag period preceeding loss of enzyme activity was not observed. However, differences in the kinetics of CA-mediated induction of removal of enzyme activity are often observed between different bean cell batches, and this phenomenon may be a function of the levels of endogenous CA at the time of elicitation (Gerrish et al. 1985).

Studies using inhibitors of protein and mRNA synthesis have indicated that the CA-mediated removal of PAL activity in vivo, now shown to be

an inactivation process, may require protein synthesis (Lamb 1977a; Dixon et al. 1980; Lamb et al. 1981). The CA-mediated appearance of a factor involved in PAL inactivation has now been demonstrated by the effects of extracts from CA-treated cells on PAL activity in vitro, and the results indicate that the non-dialysable product increases in activity for at least 6 h after exposure of cells to CA. Addition of extracts containing this factor to ^{35}S-labelled PAL in vitro leads only to a loss in activity and not to loss of M_r-77000 subunits. In vitro, a requirement for CA for full expression of the action of this factor might allow for very rapid and flexible switching of enzyme inactivation in response to fluctuations in CA pool size, a phenomenon entirely consistent with a proposed role for pathway intermediates as 'sensors' of the flux through the phenylpropanoid pathway (Lamb 1979).

The mechanism of CA-mediated PAL inactivation has been investigated by titration of the active-site concentration of PAL using site-specific tritiation (Jones and Northcote 1984) of partially purified enzyme from cells treated with CA in vivo or of purified PAL treated with inactivation factor in vitro. These results strongly indicate that an irreversible modification of the enzyme active site occurs. The possibility of a protein-mediated dehydroalanine reduction is currently under investigation.

The effects of exogenous CA application on the loss of PAL activity appear to be specific effects operating via natural physiological mechanisms. Thus, the existence of an apparently complex and highly regulated mechanism for removal of PAL activity in response to CA, the effects of treatments which alter endogenous CA levels and affect PAL in a manner consistent with this role of CA, and a positive regulation by CA of later enzymes on branch pathways diverging from the central phenylpropanoid pathway (Lamb 1977b; Gerrish et al. 1985) have all been demonstrated. These conclusions contrast with a preliminary report of non-specific effects of CA on mRNA translation resulting in the loss of PAL activity (Walter and Hahlbrock 1984).

We thank Mr. C. Gerrish and Miss G. Manning for technical assistance, Dr. R. Sunley for providing substrate for the synthesis of the PAL affinity matrix, and Drs. D.K. Lawrence and I.G. Bridges for helpful discussions. This work was supported by grants from the Science and Engineering Research Council (R.A.D.) and Imperial Chemical Industries plc (C.J.L., R.A.D.).

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Received 13 February; accepted 16 March 1986