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Wound-induced phenylalanine ammonia-lyase in potato (*Solanum tuberosum*) tuber discs

Significance of glycosylation and immunolocalization of enzyme subunits

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1. Excised discs of potato (*Solanum tuberosum*) tuber were incubated with [^{3}H]mannose or [^{3}H]fucose and extracts were prepared and incubated with an antibody to phenylalanine ammonia-lyase. Analysis of the resulting immunoprecipitated proteins by SDS/PAGE showed [^{3}H]mannose- and [^{3}H]fucose-labelled bands with M_r values corresponding to those of phenylalanine ammonia-lyase subunits. 2. When potato discs were incubated with [^{3}H]sugars in the presence of tunicamycin, an inhibitor of *N*-linked protein glycosylation, incorporation of radioactivity from [^{3}H]mannose into the immunoprecipitated enzyme subunits was virtually eliminated, whereas that from [^{3}H]fucose was only marginally inhibited. 3. Tunicamycin reduced the level of extractable phenylalanine ammonia-lyase activity induced in excised potato tuber discs. Kinetic analysis revealed that the $V_{max.}$ value of the enzyme in crude extracts from tunicamycin-treated tissue was reduced, whereas the apparent K_m values were unaffected. 4. Immunoprecipitation of the enzyme labelled *in vivo* with [^{35}S]methionine showed that tunicamycin did not inhibit the synthesis of the enzyme protein *per se*, nor did it increase the degradation of the enzyme protein. 5. Immunoprecipitation of the enzyme labelled *in vitro* with [^{14}C]nitromethane showed that tunicamycin did not affect the introduction of the dehydroalanine residue into the active site. 6. These results are consistent with the following hypothesis: tunicamycin inhibits the *N*-linked glycosylation of phenylalanine ammonia-lyase which, in turn, results in imperfect folding of the enzyme protein. The orientation of the active site is changed in such a way that the affinity of the enzyme for its substrate is unaffected, whereas the catalytic activity of the enzyme is reduced. 7. Both optical- and electron-microscopic immunolocalization studies with antibody to phenylalanine ammonia-lyase showed increased deposition of silver granules in cells in sections of potato discs in which induction of the enzyme was allowed to occur compared with cells from newly wounded tissue. The enzyme was located in the cytoplasm, and was possibly membrane-associated.

INTRODUCTION

Phenylalanine ammonia-lyase (EC 4.3.1.5.) catalyses the elimination of ammonia and the *pro-S* hydrogen atom from L-phenylalanine to form *trans*-cinnamic acid. This reaction is the first step in the biosynthesis of phenolic compounds in plants, and the enzyme has been widely studied in plant tissues, especially with regard to its induction by various environmental factors such as light, wounding excision and infection [1,2]. Early studies on the enzyme protein focused on its purification, kinetics and the unusual dehydroalanine residue at the active site [3–7]. Characterization of the structure of the protein has proved difficult: the enzyme is not easy to purify from plants, and multiple molecular forms are generated both *in vivo* and *in vitro* [8–10]. Among plant tissues, the potato (*Solanum tuberosum*) tuber disc is a particularly convenient experimental system in which to study the regulation of phenylalanine ammonia-lyase in response to wounding. Enzyme activity is present in very low amounts in the intact potato tuber. Upon excision, there is rapid increase in solute uptake and respiration, together with increased RNA and protein synthesis [11]. A number of enzyme activities are induced, one of which is phenylalanine ammonia-lyase [12].

Tunicamycin is a nucleoside antibiotic that inhibits the dolichol phosphate-mediated *N*-linked glycosylation of proteins [13]. It has been widely used to study the effects of glycosylation on protein structure and functions, and there are several reports concerning its use in plant glycoprotein studies [14–16]. There are two reports which suggest that a plant phenylalanine ammonia-lyase [from maize (*Zea mays*)] may be covalently glycosylated [17,18], and it has been debated whether the removal of an *O*-linked glycosyl chain from a serine residue may be the mechanism for the post-translational production of the active-site dehydroalanine [6,18]. In the present paper we report that potato phenylalanine ammonia-lyase is glycosylated and examine the effects of tunicamycin upon the enzyme in terms of its glycosylation, activity, synthesis and the post-translational introduction of the active-site dehydroalanine residue. In addition, we present immunocytochemical results that demonstrate the induction and subcellular location of phenylalanine ammonia-lyase protein in excised potato tuber discs, and a possible pool of inactive enzyme in uninduced tissue.

MATERIALS AND METHODS

Materials

Tunicamycin (isomer composition: A, 9%; B, 32%, C, 46%; D, 12%) and Protein A–Sepharose were from Sigma. L-[2,3,4,5,6-^{3}H]Phenylalanine (sp. radioactivity 100 Ci·mmol^{-1}), D-[2,6-^{3}H]-mannose (54 Ci·mmol^{-1}), [^{14}C]nitromethane (9.5 mCi·mmol^{-1}), Rainbow molecular-mass markers for gel electrophoresis, Amplify autoradiography enhancer and β-max X-ray film

Abbreviations used: GMA, glycol methacrylate-low acid; LRG, London Resin Gold.

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were from Amersham. Tran^{35}S-label ([^{35}S]methionine, 1067 Ci·mmol^{-1}) was from ICN Biomedicals, and X-Omat AR X-ray film was from Kodak. L-[6-^{3}H]Fucose (86.3 Ci·mmol^{-1}) was from New England Nuclear. Polyclonal antibody and purified anti-(phenylalanine ammonia-lyase)IgG were raised against a preparation of phenylalanine ammonia-lyase from *Phaseolus vulgaris* (French bean) containing predominantly the 77000-M_r subunit [9]. LRG was from the London Resin Co., Woking, Surrey, U.K.; glycol methacrylate-low acid (GMA) was from Polysciences, Warrington, PA, U.S.A., and Ortho tissue adhesive was from Ortho Diagnostics, High Wycombe, Bucks., U.K. Goat anti-rabbit IgG conjugated to colloidal gold and Intense II were from Janssen, ICN Biomedical, High Wycombe, Bucks., U.K. All other chemicals were of the highest quality commercially available.

Methods

Plant material and enzyme induction, extraction and assay. Potato tubers were bought locally. Discs, 2.5 g per batch, 10 mm (diameter) × 1 mm (thick) were cut, washed extensively in cold tap water, rinsed in deionized water and blotted dry. They were then incubated in closed Petri dishes in 5.0 ml of buffer containing 10 mM-Hepes, adjusted to pH 7.2 with NaOH, and $CaSO_4$ (30 mg·ml^{-1}), under cool-white and natural-daylight fluorescent light at 25 °C. Radioactive precursors and tunicamycin (5 mg·ml^{-1} in dimethylformamide) were added at the start of the incubation period. Controls received an equivalent volume of dimethylformamide (see the Figure legends for details). Enzyme was extracted by grinding the discs in a pestle and mortar in 5 ml of ice-cold sodium borate buffer (100 mM-boric acid adjusted to pH 8.7 with NaOH at 25 °C) containing 1 mM-dithiothreitol and 0.5 g of insoluble polyvinylpyrrolidone. The resulting homogenate was centrifuged for 10 min at 15000 *g*, then the supernatant was passed through a column (1.5 diameter × 5.0 cm long) of Sephadex G-25M (Pharmacia PD10). The desalted solution was used for the spectrophotometric assay of phenylalanine ammonia-lyase at 37 °C in sodium borate buffer, with 15 mM-phenylalanine as substrate [19]. The absorbance at 290 nm of 1 μmol of *trans*-cinnamic acid in a volume of 1 ml was assumed to be 9.0 [3].

Immunoprecipitation of radioactively labelled phenylalanine ammonia-lyase. Enzyme extract (2.5 ml) in borate buffer was passed through a column (Pharmacia, PD10) of Sephadex G-25M that had been equilibrated with 100 mM-Tris/HCl buffer, pH 7.8, and 1 mM-dithiothreitol. A 1 ml portion of this preparation was incubated end-over-end for 16 h at 4 °C with 25 μl of anti-(phenylalanine ammonia-lyase) serum. Protein A–Sepharose [4 mg; which had been incubated overnight in wash buffer: 50 mM-Tris/HCl, pH 7.4, containing 1% Triton X-100, 0.75 M-NaCl and 3% bovine serum albumin (fraction V; Sigma)] was added to each sample and the incubation was continued for a further 2 h at 4 °C. The immunoprecipitate–protein A–Sepharose complex was then centrifuged and washed four times with wash buffer minus bovine serum albumin, and then once with 50 mM-Tris/HCl buffer, pH 7.4. Immunoprecipitated protein was analysed by SDS/PAGE and autoradiography.

SDS/PAGE. This was carried out in 10% (w/v) slab gels as described in [20], except that coloured M_r standards were used and the gels were not stained. The gels were dried and those containing ^{35}S- or ^{14}C-labelled samples were exposed to β-max X-ray film at −70 °C. Gels containing ^{3}H-labelled samples were treated with Amplify autoradiography enhancer before drying and then exposed to Kodak X-Omat AR X-ray film at −70 °C. For comparative purposes certain bands on the X-ray films were scanned with a Joyce–Loebl densitometer, and the results are presented in the legends to the relevant Figures.

Active-site labelling of phenylalanine ammonia-lyase with [^{14}C]nitromethane. Aliquots (1 ml) of enzyme extract in 100 mM-Tris/HCl buffer (pH 7.8)/1 mM-dithiothreitol were incubated with [^{14}C]nitromethane (6.25 μCi; added as a solution in 50 μl of ethanol) at room temperature for 4 h and then at 4 °C overnight. Radiolabelled phenylalanine ammonia-lyase was then immunoprecipitated and analysed by SDS/PAGE and autoradiography as described above.

[^{3}H]Phenylalanine incorporation into protein. Trichloroacetic acid (1 ml; 20%, w/v) was added to 1 ml of undesalted enzyme extract from potato discs incubated with [^{3}H]phenylalanine (3 μCi) and stored overnight at 1 °C. The precipitate was centrifuged, washed twice with 10% (w/v) trichloroacetic acid, resuspended in 0.25 ml of 0.1 M-NaOH and mixed with 4.75 ml of Picofluor 30 (Packard) for liquid-scintillation counting.

Protein concentration determinations. These were carried out by the method of Bradford [21], with bovine serum albumin (fraction V; Sigma) as standard.

Preparation of tissue for immunomicroscopy. Potato tuber material was fixed in freshly prepared 1% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.05 M-sodium phosphate buffer, pH 6.8, at 4 °C for 2 h. After an overnight incubation in 0.05 M-sodium phosphate buffer, pH 6.8, the tissues were dehydrated through increasing concentrations of ethanol and placed in several changes of hydrophilic resin [3 parts LRG, 2 parts GMA and 0.1% (v/v) benzoin ethyl ether]. The tissue was finally embedded in gelatin capsules or flat-bottomed polypropylene capsules and polymerized for 24 h at room temperature by illumination with u.v. light at 360 nm.

Immunostaining–optical microscopy. Clean dry microscope slides were prepared by dipping them into a solution [3% (v/v) in pure water] of Ortho tissue adhesive; the excess solution was allowed to drain and the slides dried overnight at 40 °C. Sections (1 μm thick) of tuber tissue were cut on a glass knife and transferred to drops of water on a prepared slide. The water was evaporated slowly using a warm hotplate and sections were maintained overnight at 30 °C. Immunogold labelling was carried out by first incubating sections at 37 °C in 20 μl of 3% (v/v) ovalbumin in 50 mM-Tris buffer, pH 7.4, containing 0.15 M-NaCl (TBS) for 30 min. Excess solution was then drained from the sections and replaced with 20 μl of purified anti-(phenylalanine ammonia-lyase) IgG [1:4000 dilution (v/v) in TBS; initial concn. 2 mg/ml] for 60 min at 37 °C. They were then washed thoroughly and repeatedly for 5 min in TBS, pH 7.4, and the area around the sections was carefully dried. Sections were immersed in 20 μl of goat anti-rabbit IgG conjugated to colloidal gold (5 nm particle size; 1:200 working solution in TBS, pH 7.4) for 30 min at 37 °C. Sections were then washed as described above, followed by a final rinse in distilled water.

Silver enhancement, where used, was then carried out by using a modification of the method of Danscher [22]. This method employed incubation of slides with a mixture of 2 ml of 1 M-sodium citrate buffer, pH 3.5, 3 ml of 30% (w/v) gum arabic (Sigma), 1.5 ml of 100 mM-silver lactate (freshly prepared) and 1.5 ml of 50 mM-hydroxyquinoline (freshly prepared) in total darkness. The exact incubation time was determined by periodic monitoring of the enhancement reaction under the optical microscope. Slides were rinsed in distilled water, subjected to a fixation in 25% (w/v) sodium thiosulphate for 2 min and then

washed for 5 min in tap water before counterstaining with 0.05% (w/v) Malachite Green.

Immunostaining–electron microscopy. Ultra-thin sections of tissue were collected on Formvar (2% in amyl acetate)-coated nickel grids and placed in 10 μl aliquots of 1% (w/w) ovalbumin in 0.2 M-Tris/HCl (pH 7.4)/450 mM-NaCl (TBS) for 20 min at 37 °C. The grids were then transferred without washing to 10 μl aliquots of rabbit anti-(phenylalanine ammonia-lyase) IgG (1:8000 in TBS containing 0.1% Tween 20 for 60 min at 37 °C). Grids were then washed thoroughly with TBS and then incubated with goat anti-rabbit IgG colloidal gold at 1:2000 dilution in TBS/ovalbumin for 30 min at 37 °C. The grids were then washed thoroughly with TBS, followed by distilled water and dried. Silver enhancement of the colloidal gold was carried out using IntenSE II for 2–4 min at room temperature. After a thorough washing with distilled water, the grids were counterstained with aq. 2% (w/v) uranyl acetate and lead citrate.

RESULTS

To determine whether potato phenylalanine ammonia-lyase is glycosylated, excised tuber discs were incubated with [^{3}H]mannose or [^{3}H]fucose, protein extracts were prepared, and incorporation of radioactivity into the enzyme assessed by autoradiography of immunoprecipitated subunits after SDS/PAGE. The results, presented in Fig. 1, show the incorporation of radioactivity from [^{3}H]mannose (lane A) and [^{3}H]fucose (lane C) into proteins precipitated by anti-(phenylalanine ammonia-lyase) serum. The main bands labelled with [^{3}H]mannose are seen at M_r values of 83000, 77000, 63000, 48000, 44000 and 41000. The 77000-M_r band corresponds to the native subunit size of the *Phaseolus* enzyme [9], whereas the band at M_r 83000 corresponds to the reported [23] subunit size of the potato enzyme. However, the difference in M_r between these two subunits is probably not due to mannosylation, since both are mannosylated to some extent. The lower-M_r bands may represent phenylalanine ammonia-lyase degradation products [9]. With [^{3}H]fucose labelling most of radioactivity is associated with the 83000-M_r subunit rather than with the 77000 M_r subunit. In similar experiments where tunicamycin was included in the incubation medium, the incorporation of radioactivity from [^{3}H]mannose into phenylalanine ammonia-lyase subunits is virtually eliminated (Fig. 1, lane B). Although the incorporation of radioactivity from [^{3}H]fucose was only slightly affected by tunicamycin treatment (Fig. 1, lane D), the M_r of the major fucosylated band of interest was shifted down from 83000 to 77000. This could be due to degradation *in vivo* or *in vitro* of the M_r-83000 subunit when it is not mannosylated.

Having established that tunicamycin inhibits the incorporation of mannose into the enzyme, and therefore probably the formation of an *N*-linked glycosyl side chain, we decided to investigate

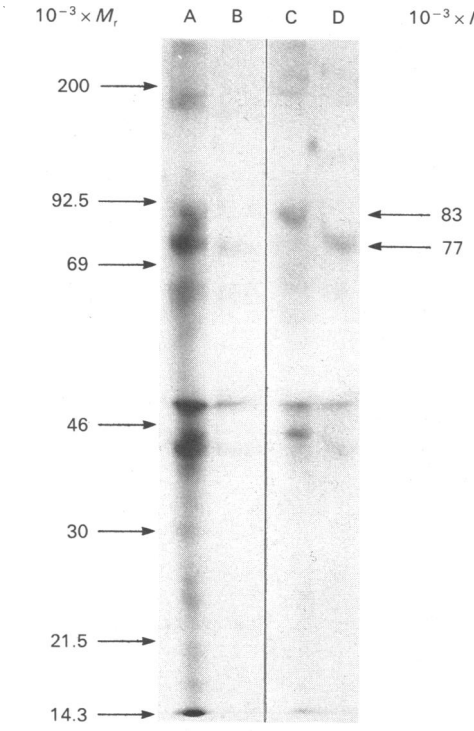

Fig. 1. Incorporation of [^{3}H]mannose and [^{3}H]fucose into immunoprecipitated phenylalanine ammonia-lyase subunits: inhibition of [^{3}H]mannose incorporation by tunicamycin

Potato discs were incubated for 16 h with 50 μCi of [^{3}H]mannose (lanes A and B) or [^{3}H]fucose (lanes C and D) with (lanes B and D) or without (lanes A and C) tunicamycin (10 μg·ml^{-1}). Immunoprecipitates of phenylalanine ammonia-lyase subunits were prepared and analysed as described in the Experimental section. The relative intensities of the 77000-M_r bands labelled with [^{3}H]mannose are: control/tunicamycin, 16.7:1. The relative intensities of the bands labelled with [^{3}H]fucose at M_r 83000 and 77000 respectively are: control/tunicamycin, 1.3:1.

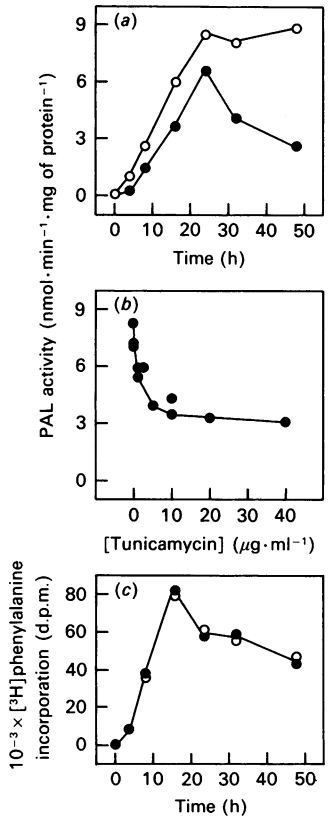

Fig. 2. Inhibition of the induction of extractable phenylalanine ammonia-lyase in potato discs by tunicamycin

(*a*) Time course for the induction of phenylalanine ammonia-lyase in potato (var. Cara) discs incubated with (●) or without (○) tunicamycin (10 μg·ml^{-1}). (*b*) Dose–response curve for the inhibition of extractable phenylalanine ammonia-lyase by tunicamycin. (*c*) Time course for the incorporation of [^{3}H]phenylalanine into the protein of potato discs incubated with (●) or without (○) tunicamycin (10 μg·ml^{-1}).

the effect of tunicamycin on the induction of extractable enzyme activity. This was found to vary within and between experiments. However, with a tunicamycin concentration of 10 $\mu g \cdot ml^{-1}$, inhibition was generally in the range from 40 to 60%. Representative data are presented in Fig. 2. Fig. 2(*a*) shows a time course for the induction of the enzyme in the presence or absence of tunicamycin (10 $\mu g \cdot ml^{-1}$). Fig. 2(*b*) shows a dose–response curve for extractable enzyme activity versus tunicamycin concentration at 16 h after excision. Half-maximal inhibition occurred at approx. 3.0 μg of tunicamycin $\cdot ml^{-1}$, and maximal inhibition (approx. 60%) was reached between about 20 and 40 $\mu g \cdot ml^{-1}$. Concentrations of tunicamycin between 5 and 10 $\mu g \cdot ml^{-1}$ are known to result in inhibition of protein glycosylation in other plant tissues [15].

Since tunicamycin can be separated into a number of isomers and homologues [24], some of which can inhibit protein synthesis at high concentrations in addition to protein glycosylation [25], it was necessary to establish that the tunicamycin preparation used in the present study resulted in a loss of phenylalanine ammonia-lyase activity by inhibiting its glycosylation rather than by a general effect on protein synthesis. Thus the effect of tunicamycin on [^{3}H]phenylalanine incorporation into total protein was investigated. Tunicamycin at a concentration of 10 $\mu g \cdot ml^{-1}$ had no effect on [^{3}H]phenylalanine incorporation into protein (Fig. 2*c*), nor had it an effect on the overall pattern of protein synthesis as assessed by labelling *in vivo* with [^{35}S]methionine followed by analysis of labelled proteins by SDS/PAGE and autoradiography (Fig. 3).

The kinetic constants of the enzyme from control and tunicamycin-treated potato discs were determined, and the results are shown in Fig. 4. Crude enzyme extracts from potato discs incubated for 16 h with or without 10 μg of tunicamycin $\cdot ml^{-1}$ were assayed at substrate concentrations from 10 μM to 10 mM. The apparent K_m values for the enzyme from both control and tunicamycin-treated discs were the same, i.e. 400 μM and 14.0 μM.

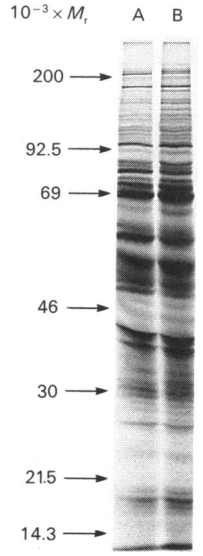

Fig. 3. Effect of tunicamycin on the overall pattern of protein synthesis in potato discs

Potato (var. Estima) discs were incubated for 16 h with 50 μCi of Tran^{35}S-label ([^{35}S]methionine). Protein was extracted as described in the Experimental section and analysed by SDS/PAGE and autoradiography. Lane A, control; lane B, tunicamycin (10 $\mu g \cdot ml^{-1}$). (Note: var. Estima showed similar properties to var. Cara with respect to inhibition of phenylalanine ammonia-lyase activity by tunicamycin.)

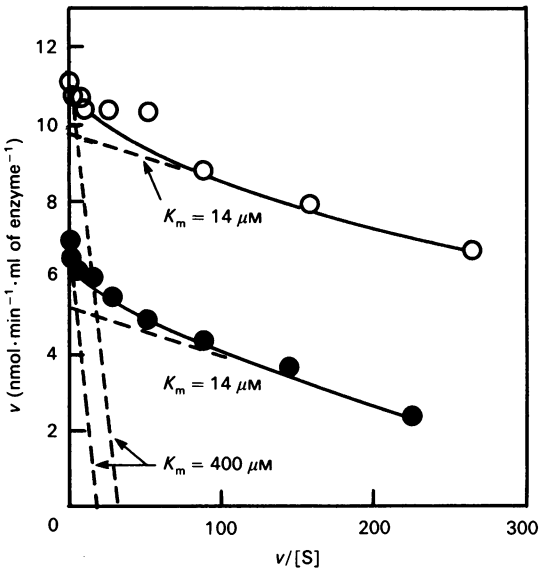

Fig. 4. Kinetics of phenylalanine ammonia-lyase activity in crude extracts extracted from potato discs incubated with or without tunicamycin

Enzyme extracts were prepared from potato discs incubated with or without tunicamycin for 16 h. Velocity measurements were made at various substrate concentrations and the results are shown by using an Eadie–Hofstee plot.

By contrast, the $V_{max.}$ value for the enzyme from the tunicamycin-treated discs was lowered (6.9 nmol $\cdot min^{-1} \cdot$ mg of protein^{-1}) compared with that from the control discs (11.1 nmol $\cdot min^{-1} \cdot$ mg of protein^{-1}). The complex kinetic behaviour observed in [5] was again apparent in the present study.

To investigate whether the reduction of extractable phenylalanine ammonia-lyase activity by tunicamycin (Fig. 2) was due to an effect on the synthesis of the enzyme protein as well as on its glycosylation, potato discs were incubated with [^{35}S]methionine with or without tunicamycin and enzyme protein was immunoprecipitated and analysed by SDS/PAGE and autoradiography. The results, shown in Fig. 5, show a major immunoprecipitated band with an M_r of 77000, with an equal intensity regardless of whether extraction was from control (lane A) or tunicamycin-treated (lane B) potato discs. This indicates that tunicamycin does not inhibit the synthesis of the potato enzyme protein *per se*. The lack of an obviously interpretable shift of M_r to a lower value for the 77000 M_r subunit from the tunicamycin-treated tissue (Fig. 5, lane B) shows that the mannose-containing constituent of the subunit does not contribute substantially to its M_r.

The active site of phenylalanine ammonia-lyase contains an unusual dehydroalanine residue that is involved in the catalytic mechanism of the enzyme [26]. This residue is susceptible to modification by a number of nucleophilic reagents, including nitromethane [1,7]. Nitromethane reacts only with dehydroalanine in proteins, therefore labelling with [^{14}C]nitromethane, combined with immunoprecipitation, provides an excellent probe for the dehydroalanine residue of phenylalanine ammonia-lyase.

Protein extracts from potato discs incubated with or without tunicamycin were incubated with [^{14}C]nitromethane and then radiolabelled phenylalanine ammonia-lyase was immunoprecipitated and analysed by SDS/PAGE and autoradiography. The results (Fig. 6) show a single band with an M_r of 77000 in both the control extract and the extract from tunicamycin-treated discs. The intensities of both bands are equal, which indicates that tunicamycin, and the consequent inhibition of

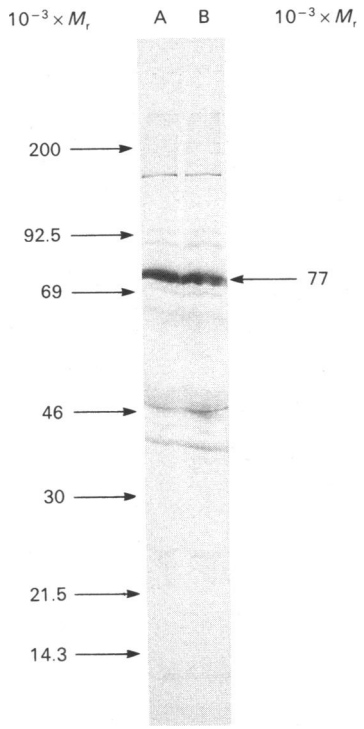

Fig. 5. Effect of tunicamycin on the synthesis of the polypeptide chain of phenylalanine ammonia-lyase

Potato (var. Estima) discs were incubated for 16 h with 50 μCi of Tran^{35}S-label ([^{35}S]methionine) plus or minus tunicamycin (10 $\mu g \cdot ml^{-1}$). Immunoprecipitates of the enzyme were prepared and analysed as described in the Experimental section. Lane A, control; lane B, tunicamycin (10 $\mu g \cdot ml^{-1}$). The relative intensities of the 77000-M_r bands labelled with [^{35}S]methionine are: control/tunicamycin, 1.04:1.00.

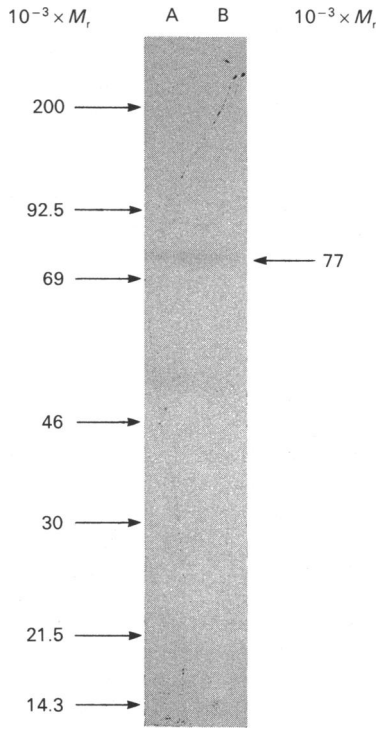

Fig. 6. Effect of tunicamycin on the introduction of the dehydroalanine residue into the active site of phenylalanine ammonia-lyase

Enzyme extracts were prepared from potato (var Estima) discs incubated with or without tunicamycin (10 $\mu g \cdot ml^{-1}$) for 16 h. These extracts were incubated with [^{14}C]nitromethane and then radiolabelled enzyme immunoprecipitated and analysed by SDS/PAGE and autoradiography as described in the Experimental section. Lane A, control; lane B, tunicamycin (10 $\mu g \cdot ml^{-1}$). The relative intensities of the 77000-M_r bands labelled with [^{14}C]nitromethane are: control/tunicamycin, 0.99:1.00.

glycosylation, do not affect the introduction of the dehydroalanine residue into the active site of phenylalanine ammonia-lyase.

In order to study the effect of wounding on the subcellular distribution of phenylalanine ammonia-lyase subunits by immunogold localization, a number of control incubations were investigated. No binding of gold particles was observed when tissue was incubated with pre-immune serum and second antibody–gold complexes or with second antibody alone. In uninduced potato tissue, use of an anti-(potato lectin) serum showed a different distribution to the highly purified anti-(*Phaseolus* phenylalanine ammonia-lyase) IgG, being found associated with the inner face of the cell wall rather than distributed throughout the cytoplasm (results not shown). A similar difference in the distribution of the binding of these two antibody preparations was also seen in *Phaseolus* tissue (also not shown). Together, these results largely rule out non-specific binding of serum components.

The distribution of phenylalanine ammonia-lyase subunits was compared in both uninduced and induced tissue by immunogold localization and observed under the optical microscope with silver enhancement (Figs. 7*a*–7*d*). Some binding to uninduced tissue is observed, possibly representing a pool of relatively inactive phenylalanine ammonia-lyase or a level of enzyme activity below that detected with current assay methods and is found evenly distributed throughout the cytoplasm. Upon induction there is a considerable increase in the density of labelling throughout cells at the wound site by 24 h after excision. When gold complexes were revealed under the electron microscope (Figs. 8*a*–8*d*), they were found associated with cytoplasmic structures, and the level of induction was revealed to be much more striking. A considerable accumulation of subunits occurs in accordance with the increased activity observed. Since tuber tissue is relatively devoid of membranous structures, association of phenylalanine ammonia-lyase subunits with, for example, endoplasmic reticulum, is not ruled out. Where membrane is observed, particle clusters can be seen in the vicinity.

DISCUSSION

Mannose is a major constituent of both the high-mannose and modified asparagine-linked (*N*-linked) oligosaccharide side chains of eukaryotic glycoproteins [27], and tunicamycin is an inhibitor of *N*-linked glycosylation. The results presented here indicate that phenylalanine ammonia-lyase is glycosylated and that the mannose residue(s) occur on an *N*-linked oligosaccharide side chain. The different effect of tunicamycin on mannose and fucose incorporation suggests that the enzyme is glycosylated at more than one site, with fucose possibly as part of an *O*-linked side chain. Sequence analyses of phenylalanine ammonia-lyase genes from bean (*Phaseolus vulgaris*) and parsley (*Petroselinum crispum*) show potential glycosylation sites [28,29].

The issues concerning the subunit size of phenylalanine ammonia-lyase from plant sources and the specificity of anti-

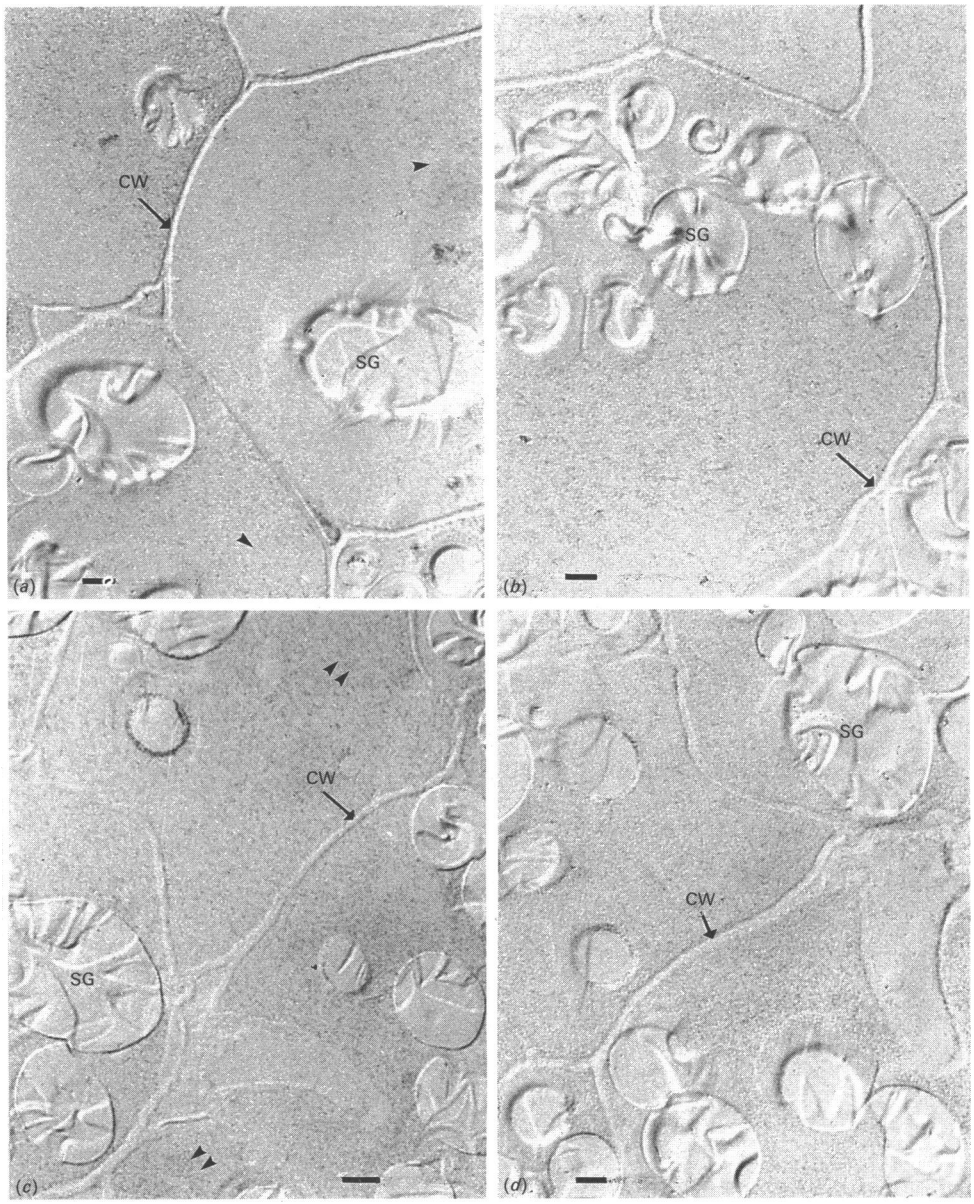

Fig. 7. Immunolocalization of potato phenylalanine ammonia-lyase subunits detected by optical microscopy

(*a,b*) Newly excised tissue; (*c,d*) induced tissue; abbreviations: CW, cell wall; SG, starch grain. Small arrows indicate examples of immunogold deposits; the double arrows indicate the heavier deposition associated with wounding. Magnification ×375; the scale bar indicates 10 μm.

bodies raised against the enzyme have been difficult to resolve [8,9]. In the present study, subunits of 83000 and 77000 M_r were immunoprecipitated. Although the 77000-M_r subunit predominates in terms of newly synthesized protein, the 83000-M_r subunit becomes glycosylated to a proportionally greater extent. However, there appears to be no simple relationship between glycosylation and M_r of the two subunits.

It is not known at present whether the M_r-83000 subunit represents a precursor of the M_r-77000 form or vice versa, or which form predominates *in vivo* or has the highest specific catalytic activity. The antiserum used in the present study was raised against a preparation of the enzyme containing predominantly the M_r-77000 subunit from *Phaseolus*, has been extensively characterized [9], and preferentially precipitates a protein with an M_r of 77000 from crude *Phaseolus* extracts from cells subjected to induction by pathogenic stress.

Confirmation that the [^{3}H]mannose-labelled M_r-77000 band seen in Fig. 1 is, in fact, a phenylalanine ammonia-lyase subunit, is supported by the experiments which show major M_r-77000 bands, labelled with [^{35}S]methionine or [^{14}C]nitromethane, immunoprecipitated from potato extracts by antiserum raised against the enzyme from *Phaseolus* (Figs. 5 and 6). The M_r of the potato enzyme subunit has been reported to be 83000, and so the relative proportions of the two subunits with M_r values of 83000 and 77000 seen in the present study may be a true representation of the situation *in vivo* or could be a function of the preferential immunoprecipitation of the 77000-M_r subunit by the antibody to the 77000-M_r *Phaseolus* subunit. Alternatively the 83000-M_r subunit may be rapidly degraded to the 77000 M_r subunit *in vitro*.

The enzyme from *Phaseolus* has multiple molecular forms with differing pI values [9]. However, the native subunit M_r of all these

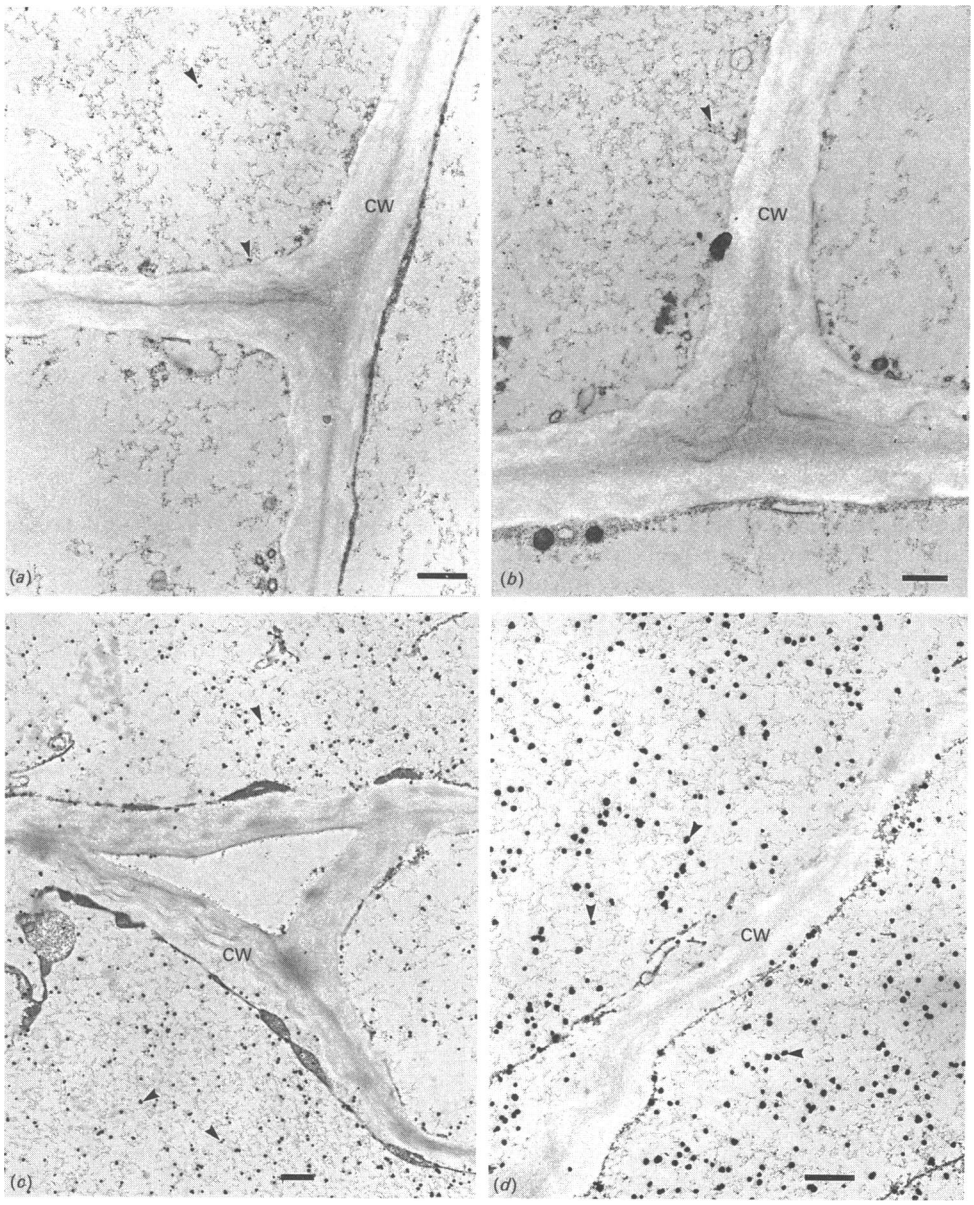

Fig. 8. Immunolocalization of phenylalanine ammonia-lyase subunits in tuber cells: electron microscopy

(*a*,*b*) Newly excised tissue; (*c*,*d*) induced tissue. Abbreviation: CW, cell wall. Arrows indicate examples of gold particle deposition. Magnifications: (*a*) ×6000; (*b*) ×6000; (*c*) 4050; (*d*) ×6000. The scale bar indicates 1 μm.

forms appears to be 77000, with degradation products with M_r values of 70000, 53000 and 46000. It is noteworthy that a subunit of M_r 83000 is occasionally seen that might correspond to the M_r-83000 subunit observed in the present study (Fig. 1), whereas the M_r-77000 is the form induced by stress.

The decrease in extractable enzyme activity resulting from decreased glycosylation could be for one of a number of reasons: the peptide chain of the non-glycosylated enzyme may not fold correctly after passing through the endoplasmic reticulum, which in turn could affect the physicochemical stability of the enzyme, make the enzyme more susceptible to proteolysis [13] or adversely affect the orientation of the active-site amino acid residues. The post-translational introduction of the active-site dehydroalanine could also be affected or, alternatively, when the enzyme is not glycosylated the polypeptide chain may not be synthesized: a form of feedback control in the interests of cellular economy [14].

The present study shows that the enzyme protein is present in equal amounts and is therefore probably synthesized and degraded at similar rates in control and tunicamycin-treated tissues. Tunicamycin also has no effect on the introduction of dehydroalanine into the active site. The present results are therefore more consistent with decreased glycosylation resulting in a conformational change or imperfect folding of the enzyme protein. This, in turn, leads to a decrease in catalytic activity, owing to a change in the orientation of the active-site residues.

Multiple molecular forms of phenylalanine ammonia-lyase with differing pI values occur in both *Phaseolus vulgaris* and in potato cells grown in suspension culture [8,9]. Differential glycosylation of the enzyme in plants might help to explain this phenomenon and perhaps also the complex kinetic data reported in [5].

The subcellular distribution of phenylalanine ammonia-lyase

in plant tissues has been investigated, and although it is thought to be largely cytosolic, there is evidence for association of the enzyme with some membranous organelles [2]. The present study employed an antiserum initially raised against *Phaseolus* phenylalanine ammonia-lyase. This antiserum is cross-reactive with potato phenylalanine ammonia-lyase subunits from the results described above, from inhibition, immunoprecipitation and Western-blotting studies (G. P. Bolwell, D. J. Millar, A. K. Allen & A. R. Slabas, unpublished work), which have confirmed the inducibility, and from the work of Vayda & Schaeffer [30], who have also employed the same antiserum. A purified IgG fraction from this antiserum was then used to immunolocalize phenylalanine ammonia-lyase subunits in newly excised and in induced tuber tissue. A number of control incubations indicate that the interaction with the tissue was specific. Both optical and electron microscopy detected immunogold labelling in newly excised tissue and which was considerably increased in wounded tissue. These results clearly confirm the inducibility of phenylalanine ammonia-lyase protein in response to wounding. Electron-microscopic studies demonstrate a largely cytoplasmic localization for the subunits, and since glycosylated proteins undergo processing through the endomembrane system, a loose association with the endoplasmic reticulum may be indicated. However, owing to the paucity of membranous structures in this tissue, this association may not be readily observed. Furthermore, wounded plant cells are characterized by breakdown of membranous structures [31].

A number of studies [32] have begun to address the molecular basis of increased transcription of phenylalanine ammonia-lyase genes in response to wounding. The present study indicates that several post-transcriptional and post-translational processes may be essential before the arrival of active phenylalanine ammonia-lyase subunits at their designated subcellular sites.

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