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The effect of proteinases on phenylalanine ammonia-lyase from the yeast *Rhodotorula glutinis*

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Phenylalanine ammonia-lyase (EC 4.3.1.5) of the yeast *Rhodotorula glutinis* was rapidly inactivated by duodenal juice. It was susceptible to chymotrypsin and subtilisin and to a lesser extent trypsin. Initial proteolysis of the enzyme by chymotrypsin and trypsin resulted in cleavage of the monomeric subunit (75000 M_r) into a large (65000 M_r) and a small (10000 M_r) peptide. The small peptide was rapidly degraded. The 65000-M_r fragment was resistant to prolonged incubation with chymotrypsin, but was degraded by trypsin under the same conditions. Phenylalanine ammonia-lyase was cleaved into several polypeptides by subtilisin, the 65000-M_r peptide being totally absent. The *N*-terminal region of the enzyme was contained in the 65000-M_r fragment, as was the dehydroalanine moiety, the prosthetic group. Active-site-binding ligands protect the enzyme from inactivation by the three proteinases, and peptide-bond cleavage by trypsin and chymotrypsin. Several chemical modifications were performed on phenylalanine ammonia-lyase. Some decreased its antigenicity, and ethyl acetimidate decreased the rate of degradation of the 65000-M_r peptide by trypsin. The modification did not protect the enzyme from proteolytic inactivation of the enzymic activity. These observations are discussed in terms of the structure of phenylalanine ammonia-lyase and site of action of the proteinases.

Much information on the tertiary structure of proteins has been obtained by investigating the effects of proteinases on native enzymes. Neurath (1980) has proposed a model for the action of proteinases on native proteins, suggesting that functional domains of proteins have a high degree of tertiary structure, and regions most susceptible to proteinases are those linking domains known as 'hinge' regions. Three possible examples of this model are the limited proteolysis of IMP dehydrogenase (Gilbert *et al.*, 1979), isopropylmalate isomerase (Reichenbecker & Grass, 1978) and citrate synthase (Bloxham *et al.*, 1980). It is possible that cleavage occurs in the 'hinge' region, linking the structural domains of the enzymes. Another feature of proteolysis is the protection of enzymes by active-site-binding ligands. The apo forms of several pyridoxal-dependent enzymes, ornithine aminotransferase, cystathionase and δ-aminolaevulinate synthase, are less susceptible to serine proteinases than their holo forms (Bond, 1971). Ascorbic acid affords protection against proteolysis of dopamine β-hydroxylase (Wong *et al.*, 1981), and citrate synthase is protected from proteolysis by oxaloacetate (Bloxham *et al.*, 1980). It has been suggested that cofactor regulation of enzyme proteolysis is an important regulatory mechanism (Wong *et al.*, 1981).

Phenylalanine ammonia-lyase (EC 4.3.1.5), which occurs solely in plants and fungi, catalyses the deamination of L-phenylalanine to *trans*-cinnamic acid and ammonia. A dehydroalanine moiety acts as prosthetic group (Hodgins, 1971). Phenylalanine ammonia-lyase, besides playing an important role in phenylalanine metabolism, is of interest to clinicians as a possible treatment for phenylketonuria. At present the disease is treated by a rather unpalatable phenylalanine-free diet. The new treatment involves oral ingestion of phenylalanine ammonia-lyase, which on entering the small intestine converts free phenylalanine, produced by proteolysis of dietary proteins, to cinnamate, thereby decreasing intake of the amino acid (Hoskins *et al.*, 1980); it is hoped that phenylketonurics would then be able to consume a normal diet.

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Abbreviations used: SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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There are two main aims to the work described in the present paper: firstly, to use limited proteolysis to investigate the structure of the enzyme, and secondly, to ascertain the mechanism by which proteinase, particularly those in intestinal juice, inactivate phenylalanine ammonia-lyase, in order to assess the possibility of protecting the enzyme from proteolysis in the intestine. The results have shown phenylalanine ammonia-lyase to be rapidly inactivated by various proteinases. Chymotrypsin and trypsin release a 65000-M_r peptide that is resistant to further degradation by chymotrypsin. The tertiary structures of proteolytically inactivated and native enzyme are quite similar. Ligands that bind at the active site afford considerable protection from proteolysis. Several reagents that protect other proteins from proteolysis have no protective effect on phenylalanine ammonia-lyase. These results are discussed in terms of the tertiary structure of phenylalanine ammonia-lyase and the site of action of the proteinases.

Materials and methods

Chemicals

trans-Cinnamic acid was obtained from Aldrich Chemicals (Gillingham, Dorset, U.K.). Freund's complete adjuvant was obtained from Difco Laboratories (Detroit, MI, U.S.A.). NCS solubilizer was obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.). NE 260 scintillation fluid was obtained from Nuclear Enterprises Ltd. (Edinburgh, Scotland, U.K.). Blue Dextran 2000, Sepharose 4B, Sepharose 6B and CNBr-activated Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). NaB^3H_4 (sp. radioactivity 50–500mCi/mmol) and carrier-free [^{125}I]iodine (sp. radioactivity 100mCi/ml) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). α-Chymotrypsin (type II), subtilisin Carlsberg (Protease, type VIII) and *o*-tyrosine were obtained from Sigma (London) Chemical Co. All other chemicals were supplied by BDH, Poole, Dorset, U.K.

Assay for phenylalanine ammonia-lyase

The reaction mixture contained the following in a total volume of 3ml: Tris/HCl, pH8.5, 300μmol; L-phenylalanine, 39μmol; phenylalanine ammonia-lyase, 0.003–0.06 unit (one unit of enzyme activity is defined as the amount of enzyme required to transform 1μmol of substrate/min at 37°C). The reaction was initiated by the addition of enzyme to the reaction cuvette (10mm path length). The blank contained all components of the assay system except L-phenylalanine. The reaction was monitored continuously at 37°C in a Unicam SP.1800 spectrophotometer (Pye–Unicam), the production of *trans*-cinnamic acid being measured by its absorbance at 290nm. Cinnamate produced was calculated from a molar absorption coefficient of 1.07×10^4 litre·mol^{-1}·cm^{-1} at 290nm and pH8.5 (Emes & Vining, 1970).

Protein assay

Protein was determined by the automated method of Wade & Phillips (1971). Crystalline bovine serum albumin (fraction V) was used as standard.

Purification of phenylalanine ammonia-lyase

The enzyme was partially purified as described by Hoskins *et al.* (1980). Phenylalanine ammonia-lyase at this stage had a specific activity of 1.2units/mg. The enzyme was further purified by using affinity chromatography with phenylalanine as ligand (Jack, 1978). Partially purified enzyme (6ml of a 25mg/ml solution in 100mM-potassium phosphate buffer, pH6.1) was applied to a column (26mm × 70mm) containing phenylalanine–Sepharose 4B (35ml of settled gel) equilibrated with the same buffer. After washing through unbound protein with potassium phosphate buffer, protein non-specifically bound to the column was removed by washing with 50mM-$NaHCO_3$ buffer, pH9.6, containing 0.5M-NaCl. Finally, the enzyme was eluted from the column with 0.1M-Tris/HCl buffer, pH9.6. Fractions (5ml) were collected at a flow rate of 80ml/h. Purified phenylalanine ammonia-lyase had a specific activity of 4.75units/mg. A profile of the eluate from the affinity column is shown in Fig. 1.

Immunological methods

Antibodies to phenylalanine ammonia-lyase were raised in three 3kg New Zealand White male rabbits from the animal house of this institution. A solution of phenylalanine ammonia-lyase purified by affinity chromatography (3nM) was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected intramuscularly (2 × 0.25ml in the thigh) and subcutaneously (2 × 0.25ml in the neck). After 1 month a further 1mg of phenylalanine ammonia-lyase was injected as described above. After a further 2 weeks, 20ml of blood was bled from each animal. After 1h at 4°C the clot was removed by centrifugation.

Radioimmunoassay of phenylalanine ammonia-lyase was performed as follows. All components in the assay were diluted with 50mM-potassium phosphate buffer, pH7.4, containing 0.85% NaCl and 0.1% bovine serum albumin. Phenylalanine ammonia-lyase antiserum, 0.1ml of a 10^5-fold dilution, was mixed with 0.1ml of either a phenylalanine ammonia-lyase standard solution, 1μg–10ng/ml, or a dilution of the test sample. Finally, 0.1ml of ^{125}I-labelled phenylalanine ammonia-lyase (2×10^5 c.p.m./ml) (iodination performed as described by Fraker & Speck, 1978) was added and

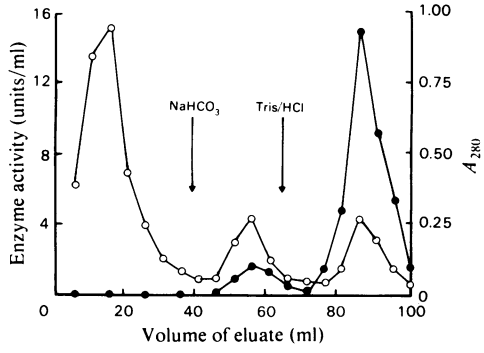

Fig. 1. *Elution of phenylalanine ammonia-lyase from the phenylalanine affinity column*

The elution of phenylalanine ammonia-lyase from the affinity column was performed as described in the Materials and methods section. After washing off unbound A_{280} (○) material, the column was washed in 50mM-$NaHCO_3$ buffer, pH9.6, containing 0.5M-NaCl, and enzyme (●) was eluted with 0.1M-Tris/HCl buffer, pH9.6, as indicated (↓).

tubes incubated overnight at 4°C. Donkey anti-(rabbit immunoglobulin G), 0.1ml of a 50-fold dilution of serum supplied by Wellcome Reagents Ltd. (Beckenham, Kent, U.K.) and 0.1ml of a 300-fold dilution of normal rabbit serum, were then added and the tubes incubated for 3h at room temperature. After adding 1ml of water, the tubes were centrifuged for 30min at 45000*g* at room temperature. The supernatants were aspirated and the tubes counted in a LKB 1270 Rackgamma counter. Blanks consisted of all components of the assay except phenylalanine ammonia-lyase antiserum. Approx. 15% of the ^{125}I-labelled phenylalanine ammonia-lyase bound to its antibody in the absence of unlabelled phenylalanine ammonia-lyase.

The antigenic character of different forms of phenylalanine ammonia-lyase was also judged by double-diffusion tests (Ouchterlony, 1949).

Analytical polyacrylamide-disc-gel electrophoresis

Non-denaturing gel electrophoresis was performed as described by Davis (1964), with a running-gel acrylamide concentration of 5% (w/v).

SDS/polyacrylamide-gel electrophoresis was based on the method of Laemmli (1970). Samples were prepared for electrophoresis by the addition of a 2% (w/v) SDS solution containing 10% (v/v) 2-mercaptoethanol and 50mM-$NaHCO_3$ to enzyme preparations [0.3–6nM; giving a 6:1 (w/w) ratio of SDS to protein], followed by heating for 5min at 100°C. Samples (10–100μl) were applied through the upper reservoir to 7.5% (w/v)- or 12%-acrylamide running gels and subsequently stained for protein with Coomassie Blue.

Determination of radioactivity in polyacrylamide gels

Destained polyacrylamide gels were placed in a Perspex tube containing slots 2mm apart. Slices 2mm thick were prepared by cutting the gel through the slots with a thin wire. The slices were placed in scintillation vials containing 0.5ml of an NCS solubilizer/water (10:1, v/v) mixture and heated overnight at 55°C. After cooling, 10ml of NE 260 scintillant was added and radioactivity counted in an Intertechnique SL 30 liquid-scintillation spectrometer.

N-Terminal sequence of phenylalanine ammonia-lyase

The method used was based on that described by Weiner *et al.* (1972). Native, chymotrypsin- and trypsin-inactivated phenylalanine ammonia-lyase (150μg/gel) were subjected to SDS/polyacrylamide-gel electrophoresis on 12 5%-acrylamide disc gels (110mm × 5mm) as described by Weber & Osborn (1969). One of the gels was stained with Coomassie Blue for 10min at 60°C, destained for 30min at 60°C, and used as a marker for the position of protein bands. Elution of protein from the gels, freeze-drying of samples and subsequent sequencing of amino acids were performed as described by Weiner *et al.* (1972). Approx. 2mg of phenylalanine ammonia-lyase, obtained from 33 gels, were used for four cycles of Edman degradation.

Amino acid analysis

Amino acid analysis was performed on a Technicon TSMI amino acid analyser by using a 0.2M-sodium citrate buffer system. Samples were deproteinized by adding an equal volume of 10% (w/v) sulphosalicylic acid.

^{3}H-labelling the dehydroalanine moiety of phenylalanine ammonia-lyase

A sample (5mg) of phenylalanine ammonia-lyase was made to react with NaB^3H_4 as described by Hodgins (1968).

Determination of the molecular weight of phenylalanine ammonia-lyase

The M_r of native and proteolytically inactivated phenylalanine ammonia-lyase was determined on a Sepharose 6B column (92mm × 26mm), by the method of Andrews (1964), with Blue Dextran 2000, bovine heart cytochrome *c*, bovine serum albumin, pig heart lactate dehydrogenase, bovine catalase, thyroglobulin and ferritin as standards. The column

was run at 30ml/h in 0.1 M-Tris/HCl buffer, pH 8.5, containing 0.1 M-NaCl.

Reaction of duodenal juice and various proteinases with phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase was added (to a final concentration of 3 nM) to duodenal juice at 37°C. At intervals, samples were removed, treated with phenylmethanesulphonyl fluoride (1 mM final concn.) for 15 min at 0°C, centrifuged for 5 min at 10000 *g* and the supernatant assayed for phenylalanine ammonia-lyase activity and amino acid composition.

To determine the effect of specific proteinases on phenylalanine ammonia-lyase activity, the enzyme (1 mg/ml) in 0.1 M-Tris/HCl buffer, pH 7.5, was incubated with various concentrations of proteinases at 37°C. At intervals, 30 μl were removed and assayed for phenylalanine ammonia-lyase activity; 100-fold dilution of the reaction mixture effectively stopped proteolysis of phenylalanine ammonia-lyase. To investigate the effect proteinases have on the structure of phenylalanine ammonia-lyase, the enzyme was incubated with proteinases as described above for duodenal juice. Samples (100 μl) were taken; 20 μl was assayed for phenylalanine ammonia-lyase activity and the remainder subjected to SDS/polyacrylamide-gel electrophoresis.

Covalent modifications of phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase was coupled to Dextran 40, 70 and 150 as described by Marshall & Rabinowitz (1976) in the presence of 2 mM-cinnamate, with 4 mg of phenylalanine ammonia-lyase per 200 mg of Dextran. The enzyme was attached to either poly(ethylene glycol) 6000 or monomethoxy poly(ethylene glycol) 5000 as described by Abuchowski *et al.* (1977). Modification of phenylalanine ammonia-lyase by Sepharose 4B was as follows. CNBr-activated Sepharose 4B (14 ml of swollen gel) after washing with 1 mM-HCl was resuspended in 20 ml of 0.1 M-borate buffer, pH 8.5, containing 0.5 M-NaCl. Phenylalanine ammonia-lyase (10 mg) was added and shaken for 16 h at 4°C. The gel was then washed with 0.1 M-Tris/HCl buffer, pH 8.5, containing 0.5 M-NaCl. No enzyme activity was observed in the washings. Finally, the gel was resuspended in 20 ml of 0.1 M-Tris/HCl buffer, pH 8.5. Phenylalanine ammonia-lyase attached to Sepharose 4B was assayed spectrophotometrically at 290 nm as follows. The assay medium (20 ml) was stirred at 37°C in a 50 ml beaker and recirculated at 10 ml/min through a 1 ml silica flow cuvette (10 mm path length). The reaction was initiated by the addition of 0.5 ml of gel, which was retained in the reaction vessel by a nylon filter on the outlet tube.

Modification by ethyl acetimidate or dimethyl suberimidate was performed as follows. Either ethyl acetimidate (0.2 ml of a freshly prepared 10 mg/ml solution in 0.5 M-borate buffer, pH 8.8) or dimethyl suberimidate (0.2 ml of a freshly prepared 20 mg/ml solution in 0.5 M-borate buffer, pH 8.5) was added to 1 ml of phenylalanine ammonia-lyase (7.5 nM in 0.1 M-borate buffer, pH 8.8), and incubated for 6 h at room temperature. The reaction was terminated by the addition of 1 ml of 1 M-Tris/HCl buffer, pH 7.5. The enzyme was then dialysed against 1000 vol. of 0.1 M-Tris/HCl buffer, pH 7.5.

For its reaction with acetic anhydride, phenylalanine ammonia-lyase (3 nM) was mixed with acetic anhydride (15 mM final concn.) in 0.1 M-borate buffer, pH 8.0, in the presence and absence of cinnamate. After 30 min the enzyme was dialysed against 1000 vol. of 0.1 M-Tris/HCl buffer, pH 7.5.

Results

Effect of proteinases on phenylalanine ammonia-lyase

Duodenal juice rapidly inactivated phenylalanine ammonia-lyase (there was a 50% decrease in activity after 3 min and no activity was observed after 30 min). This inactivation did not follow first-order kinetics. Instead, the rate of inactivation decreased with time. When the three major proteinases present in the juice (trypsin, chymotrypsin and carboxypeptidase A) were individually mixed with the enzyme, it was found that chymotrypsin inactivated phenylalanine ammonia-lyase approx. 30 times more rapidly than trypsin (Table 2), whereas the enzyme was totally resistant to carboxypeptidase A. Inactivation by trypsin and chymotrypsin followed first-order kinetics. Phenylalanine ammonia-lyase was stable when incubated with duodenal juice in which chymotrypsin and trypsin had been inactivated. The enzyme was able to decrease phenylalanine concentrations in juice that had been treated in this way (Table 1). Subtilisin was also shown to inactivate phenylalanine ammonia-lyase at approximately the same rate as chymotrypsin.

Subunit structure of phenylalanine ammonia-lyase after proteolysis

Phenylalanine ammonia-lyase consists of four polypeptides of identical M_r (Hodgins, 1971), which was determined as 75000 by SDS/polyacrylamide-gel electrophoresis. Purified enzyme appeared homogeneous by the presence of only one protein band on non-denaturing polyacrylamide-gel electrophoresis. When phenylalanine ammonia-lyase was subjected to SDS/polyacrylamide-gel electrophoresis, only one major polypeptide, over 90% of protein, with an M_r of 75000, was observed. On some occasions there was

Table 1. *Reaction of phenylalanine ammonia-lyase with duodenal juice*

Incubation of phenylalanine ammonia-lyase with duodenal juice, determination of enzyme activity and amino acid concentrations, were as described in the Materials and methods section. Amino acid concentrations are quoted as means ± S.E.M., with the number of determinations in parentheses.

Time (min)	Presence (+) or absence (−) of chymotrypsin and trypsin inhibitor	Phenylalanine ammonia-lyase activity (% of zero-time value)	Amino acid ...	Concentration (mM)			
				Phe	Ala	Gly	Glx
0	−	100		1.50 ± 0.23 (3)	1.08 ± 0.21 (3)	1.06 ± 0.11 (3)	0.56 ± 0.08 (3)
15	−	5		1.40 ± 0.16 (3)	1.38 ± 0.31 (3)	1.12 ± 0.06 (3)	0.6 ± 0.1 (3)
120	−	0		1.64 ± 0.25 (3)	1.90 ± 0.12 (3)	1.62 ± 0.14 (3)	0.86 ± 0.14 (3)
0	+	100		1.56 ± 0.31 (4)	1.04 ± 0.11 (4)	1.04 ± 0.16 (4)	0.62 ± 0.04 (4)
15	+	100		1.24 ± 0.2 (3)	1.26 ± 0.13 (3)	1.10 ± 0.1 (3)	0.65 ± 0.05 (3)
120	+	93		0.3 ± 0.006 (3)	1.68 ± 0.17 (3)	1.42 ± 0.21 (3)	0.9 ± 0.11 (3)

a minor protein band, less than 10% of the major protein band, with an M_r of 65000. Phenylalanine ammonia-lyase inactivated by either chymotrypsin or trypsin showed a similar pattern, in that the 75000 M_r polypeptide was converted into a 65000-M_r peptide and low-M_r (10000 and below) peptide that ran with the dye front (Fig. 2). These low-M_r peptides, when run on 12% gels, were shown to consist of a 10000-M_r peptide (approx. 60% of low-M_r peptides) together with other peptides of lower M_r. SDS/polyacrylamide-gel electrophoresis showed that phenylalanine ammonia-lyase inactivated by subtilisin was converted into many peptides; the 65000-M_r peptide was absent.

To investigate the stability of the 65000-M_r peptide, phenylalanine ammonia-lyase was incubated with either trypsin or chymotrypsin for 24h, under conditions that caused total inactivation within 20min. SDS/polyacrylamide-gel electrophoresis showed that the 65000-M_r peptide was converted into lower-M_r peptides in the presence of trypsin, but was resistant to cleavage by chymotrypsin. The low-M_r peptides released from phenylalanine ammonia-lyase by chymotrypsin were absent after this prolonged incubation with the proteinase.

Effect of ligands on proteolysis of phenylalanine ammonia-lyase

The effect of competitive inhibitors of phenylalanine ammonia-lyase on the rate of proteolysis was investigated. The results are presented in Table 2. It appears that each ligand tested was effective in protecting phenylalanine ammonia-lyase from inactivation by the three proteinases. In the presence of the ligands, inactivation still obeyed pseudo-first-order kinetics. The degree of protection depended on the concentration of ligand used.

To investigate the effect of proteinases on the structure of phenylalanine ammonia-lyase in the presence of the ligands, the formation of the 65000 M_r peptide was determined in the presence and absence of *o*-tyrosine when phenylalanine ammonia-lyase was incubated with either trypsin or chymotrypsin. The effect of trypsin (Fig. 3) was virtually identical with that of chymotrypsin: the ligands decreased the rate of proteolysis but did not alter the site of cleavage. It was not possible to perform the same experiment with subtilisin, as many peptides were released after protection from inactivation by the ligands.

Quaternary structure of inactivated phenylalanine ammonia-lyase

Column chromatography, with a Sepharose 6B column calibrated with M_r markers, of native, chymotrypsin-, trypsin- and subtilisin-inactivated phenylalanine ammonia-lyase showed that all forms

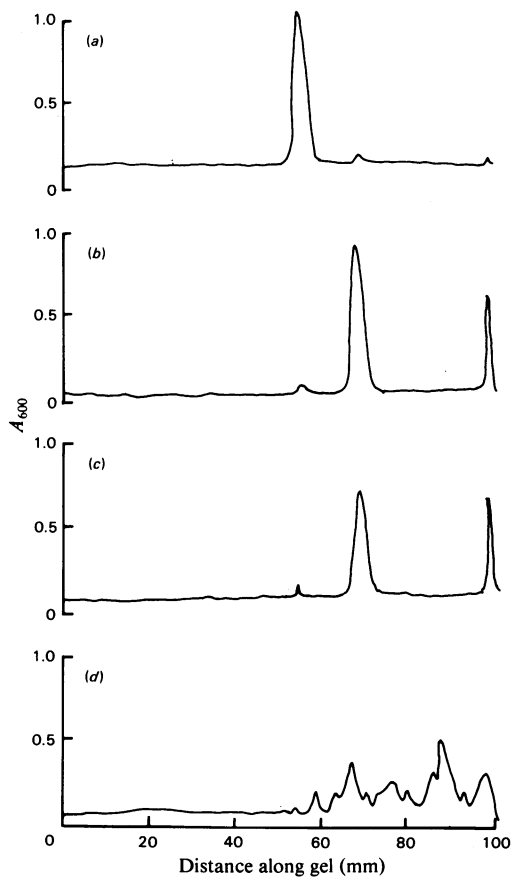

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of native (a), trypsin-inactivated (b), chymotrypsin-inactivated (c) and subtilisin-inactivated (d) phenylalanine ammonia-lyase.*

Phenylalanine ammonia-lyase was fully inactivated by the proteinases as described in the Materials and methods section. Approx. 50μg of protein were applied to the 7.5%-polyacrylamide gels.

of the enzyme had an M_r of 330000. This indicates that the quaternary structure of phenylalanine ammonia-lyase is maintained after proteolysis. SDS/polyacrylamide-gel electrophoresis of proteolytically modified enzyme revealed the same pattern of protein bands before and after column chromatography. Each of the inactivated forms of phenylalanine ammonia-lyase migrated to the same position as native enzyme on non-denaturing polyacrylamide-gel electrophoresis.

N-Terminal amino acid sequence of native and inactivated phenylalanine ammonia-lyase

The *N*-terminal amino acid sequence of native phenylalanine ammonia-lyase was:

Ala-Val-Leu-Leu-Asx

The *N*-terminal sequence of the 65000-M_r polypeptide released by chymotrypsin or trypsin treatment was the same as that of the native enzyme. Dansylation of chymotrypsin- or trypsin-treated enzyme before SDS/polyacrylamide-gel electrophoresis revealed a number of *N*-terminal amino acids. These results indicate that the 65000-M_r peptide is the *N*-terminal portion of the enzyme, and several peptide bonds are cleaved in the *C*-terminal region of the polypeptide.

Identification of the dehydroalanine moiety in phenylalanine ammonia-lyase

The prosthetic group, dehydroalanine, was ^{3}H-labelled as described in the Materials and methods section. Native labelled phenylalanine ammonia-lyase was incubated with either trypsin or chymotrypsin for the time required to inactivate active native phenylalanine ammonia-lyase. The labelled proteins (both native and proteolytically modified) were then subjected to SDS/polyacrylamide-gel electrophoresis and the gels analysed for ^{3}H. A typical result is presented in Fig. 4. The same result was obtained for both trypsin- and chymotrypsin-treated enzyme. It appears that the dehydroalanine moiety resides in the 65000-M_r peptide.

Immunoreactivity of phenylalanine ammonia-lyase inactivated by proteolysis

Valuable information on the tertiary structure of phenylalanine ammonia-lyase could be found by investigating the interaction of native and proteolytically modified forms of the enzyme with antisera. Ouchterlony double-diffusion tests showed no spur formation between native phenylalanine ammonia-lyase and enzyme treated with any of the three proteinases. Radioimmunoassay revealed that an equal quantity of native and proteolytically modified phenylalanine ammonia-lyase contained the same amount of antigenic material.

Modifications of phenylalanine ammonia-lyase

The results of various modifications of phenylalanine ammonia-lyase on enzyme activity, resistance to proteolysis and antigenicity of phenylalanine ammonia-lyase are presented in Table 3. Two types of modification were performed. One involved attachment of high-M_r molecules to phenylalanine ammonia-lyase so that proteinases should no longer attack the enzyme because of steric hindrance. The other type of modification involved reagents that react with lysine residues, such that peptide bonds involving modified amino acids are resistant to trypsin cleavage. Modifications by high-M_r compounds were shown to have occurred by the decrease in amino groups (monitored by the

Table 2. *Effect of ligands on proteolysis of phenylalanine ammonia-lyase*

Phenylalanine ammonia-lyase was incubated with the proteinases as described in the Materials and methods section. The final concentrations of chymotrypsin, trypsin and subtilisin were 85 nM, 2 μM and 85 nM. The final concentration of ligand was 2 mM.

Ligand	Proteinase ...	Second-order rate constants of inactivation ($M^{-1} \cdot min^{-1}$)		
		Chymotrypsin	Trypsin	Subtilisin
None		1.36×10^9	4.95×10^7	1.36×10^9
Amino-oxyacetic acid		2.63×10^8	1.23×10^7	2.55×10^8
Amino-oxyphenylpropionic acid		6.73×10^7	2.62×10^6	6.90×10^7
trans-Cinnamic acid		1.25×10^8	5.09×10^6	1.16×10^8
o-Tyrosine		6.27×10^7	2.37×10^6	6.68×10^7

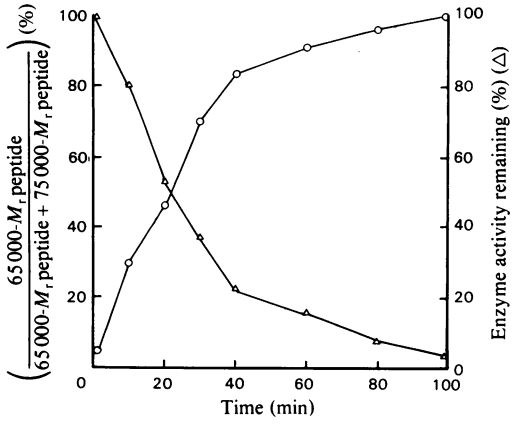

Fig. 3. *Effect of chymotrypsin on the structure and activity of phenylalanine ammonia-lyase*

Phenylalanine ammonia-lyase (1 mg/ml) was incubated with chymotrypsin (2 μg/ml final concn.) in 0.1 M-Tris/HCl buffer, pH 8.0, at 0°C. Assay of enzyme activity and determination of the 75000-M_r and 65000-M_r peptides was as described in the Materials and methods section. The ratios of the two peptides were determined from scans of the SDS/polyacrylamide gels. △, Enzyme activity; ○, proportion of the two peptides.

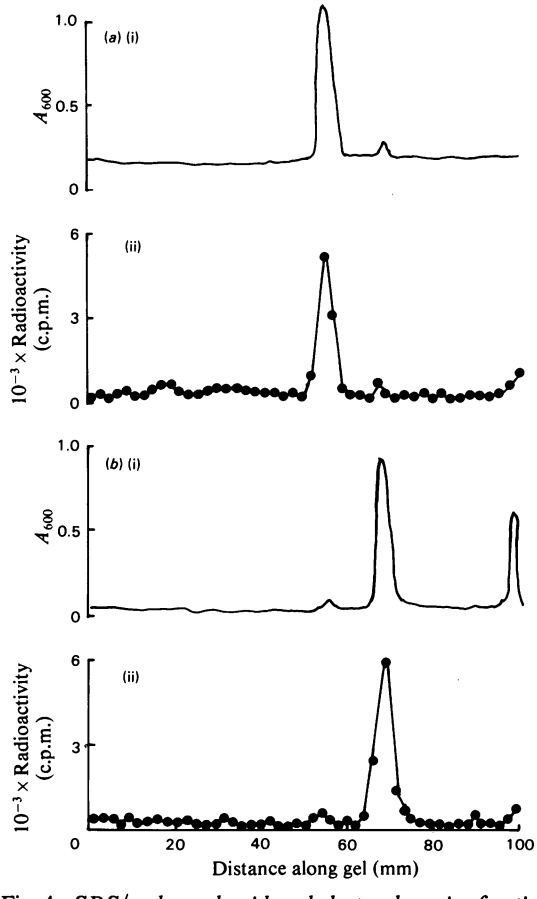

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of native and chymotrypsin-inactivated phenylalanine ammonia-lyase labelled with NaB^3H_4*

Electrophoresis of native phenylalanine ammonia-lyase (*a*) and chymotrypsin-inactivated phenylalanine ammonia-lyase (*b*) was performed on 7.5% gels with 50 μg of protein. Labelling with NaB^3H_4 and subsequent degradation of phenylalanine ammonia-lyase with chymotrypsin was as described in the Materials and methods section. (*a*) (i) and (*b*) (i), gel-scanning profiles; (*a*) (ii) and (*b*) (ii), radioactivity profiles.

method of Habeeb, 1966) and the large increase in M_r indicated by SDS/polyacrylamide-gel electrophoresis. The modifications rapidly inactivated phenylalanine ammonia-lyase unless cinnamate was added, but none protected the enzyme from proteinases. Guanidination of lysine residues with ethyl acetimidate did protect the 65000 M_r peptide from degradation by prolonged incubation with trypsin. Dextran- and poly(ethylene glycol)-modified phenylalanine ammonia-lyase was antigenically approx. 100 times less reactive than native phenylalanine ammonia-lyase (Table 3).

Table 3. *Effect of modifications on phenylalanine ammonia-lyase*

Phenylalanine ammonia-lyase was modified as described in the Materials and methods section. The effect of proteinases on modified phenylalanine ammonia-lyase was measured after removal of cinnamate present during the modification reaction.

Modification	Activity remaining after modification		Proteinase	Second-order rate constants of inactivation ($M^{-1} \cdot min^{-1}$)			Antigenicity (% of native phenylalanine ammonia-lyase antigenicity)
	No cinnamate	Cinnamate (2mM)		Chymotrypsin	Subtilisin	Trypsin	
None	100	100		1.16×10^9	1.36×10^9	4.33×10^7	100
Ethyl acetimidate	0	50		1.20×10^9	1.31×10^9	3.85×10^7	—
Dimethyl suberimidate	0	41		1.13×10^9	1.25×10^9	4.13×10^7	—
Soluble dextran	6	25		1.16×10^9	1.31×10^9	4.18×10^7	0.5
Sepharose 4B	28	72		1.09×10^9	1.25×10^9	9.63×10^7	—
Poly(ethylene glycol) 6000	0	30		1.09×10^9	1.16×10^9	3.47×10^7	0.6
Monomethoxy poly(ethylene glycol) 5000	0	42		1.02×10^9	1.29×10^9	4.08×10^7	1.8
Acetic anhyride	0	86		2.33×10^9	2.63×10^9	1.02×10^7	—

Discussion

Phenylalanine ammonia-lyase is more rapidly inactivated by chymotrypsin than by trypsin. It seems likely that most soluble enzymes are more susceptible to trypsin than to chymotrypsin, as peptide bonds, involving basic amino acids would be found on the surface of molecules, whereas those involving the more hydrophobic aromatic amino acids would be in the interior of the molecule and thus less accessible to proteinases. That phenylalanine ammonia-lyase is more susceptible to chymotrypsin suggests the presence of a hydrophobic region on the surface of the enzyme. This may be the region that binds the aromatic ring of phenylalanine.

Chymotrypsin and trypsin inactivate phenylalanine ammonia-lyase by cleaving peptide bond(s), which release a 65000-M_r peptide and several lower-M_r peptides. The small polypeptides are from the *C*-terminal region of the enzyme. As no polypeptide with an M_r between 75000 (subunit size) and 65000 was observed, cleavage of phenylalanine ammonia-lyase does not occur by sequential release of peptides from the *C*-terminal region. A more likely explanation is that initial cleavage converts the enzyme into a 65000-M_r peptide and a 10000-M_r peptide, the latter peptide then being further degraded. This is supported by the high proportion, in the initial period of incubation with chymotrypsin, of the 10000-M_r peptide, which disappears on prolonged incubation with the proteinase. From these data, two possible structures of phenylalanine ammonia-lyase present themselves. Firstly the enzyme may exist as two structural domains connected by a small 'hinge' region not containing a high degree of tertiary structure and so more susceptible to proteinases. The small domain is more susceptible to proteolysis, as is the postulated structure of isopropylmalate isomerase (Reichenbecker & Grass, 1978). Alternatively, phenylalanine ammonia-lyase may contain one large structural domain (65000-M_r peptide) and a region of less ordered structure. The initial cleavage of the enzyme occurs between the structural domain and the tail region, which is subsequently degraded.

Inactivation of phenylalanine ammonia-lyase is caused by cleavage of the enzyme into a 65000-M_r and a 10000-M_r peptide. This is indicated by inactivation closely paralleling the formation of the large peptide. If inactivation is caused by subsequent breakdown of the 10000-M_r peptide, a lag period would be observed between the formation of the two peptides and inactivation of the enzyme. In addition, the presence of a high proportion of 10000-M_r peptide after total inactivation indicates that initial cleavage causes inactivation. However, it should be pointed out that inactivation could be caused by a very small peptide (less than 10 amino

acids) being released from the 65000-M_r peptide, the 10000-M_r peptide or the native enzyme, since it would not be detected by SDS/polyacrylamide-gel electrophoresis.

Ligands that bind at the active site afford protection against proteolysis by each of the three proteinases tested. There are three possible mechanisms by which this protection could occur. Firstly, the ligands may stabilize the conformation of the active site after proteinases had cleaved the enzyme. This is unlikely, as *o*-tyrosine prevents actual cleavage of phenylalanine ammonia-lyase as well as protecting enzyme activity. Secondly, the ligands may form a binary complex with phenylalanine ammonia-lyase that undergoes a substantial conformational change compared with the native enzyme. This may result in decreased proteolytic susceptibility. Thirdly, proteinases may be acting at the active site, and ligands are therefore preventing proteolysis by steric hindrance. It is not possible to distinguish between the last two mechanisms.

The immunoreactivity, quaternary structure and mobility in polyacrylamide gels (non-SDS-containing) of native and proteolytically cleaved phenylalanine ammonia-lyase are very similar, indicating that the tertiary structure is highly conserved, even after phenylalanine ammonia-lyase cleavage into several polypeptides by subtilisin.

Numerous modifications of phenylalanine ammonia-lyase were shown to be ineffective in protecting the enzyme from proteolytic inactivation. Ethyl acetimidate did prevent further cleavage of the 65000-M_r peptide by trypsin, and high-M_r molecules decreased the antigencity of phenylalanine ammonia-lyase, suggesting that large areas of the protein can be made resistant to interaction with other proteins, either antibodies or proteinases, without decreasing proteolytic inactivation. This view is supported by the protection of activity by *o*-tyrosine to subtilisin attack, although a large number of peptides were released by the proteinase in the presence of the ligand. This suggests that inactivation by proteinases is caused by specific cleavage of the protein which is inhibited by active-site-binding ligands, and large regions of the polypeptide backbone can be broken without loss of catalytic activity. As the modifications were performed in the presence of cinnamate, the specific cleavage site is not modified by the reagents. If this is so, chemical modifications of phenylalanine ammonia-lyase are not likely to decrease its susceptibility to proteinases.

It is noteworthy that whereas phenylalanine ammonia-lyase is ineffective *in vitro* in decreasing phenylalanine concentrations in duodenal juice, *in vivo* it has proved effective in decreasing the normal rise in circulating phenylalanine concentrations after a high protein meal (Hoskins *et al.*, 1980), suggesting that phenylalanine ammonia-lyase is at least partially protected from proteolysis.

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