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The chymotrypsin-catalysed activation of bovine liver glutamate dehydrogenase

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1. Ox liver glutamate dehydrogenase is activated by bovine pancreatic α-chymotrypsin, but the extent of activation is dependent on the age of the dehydrogenase preparation. 2. The degree of activation is constant and the pseudo-first-order rate constant of activation is directly proportional to the concentration of proteinase used. 3. Commercial preparations of α-chymotrypsin differ in their ability to produce a secondary inactivation phase, and this was shown to be due to low tryptic contamination. The 'superactive' form of glutamate dehydrogenase has an increased sensitivity to tryptic inactivation as compared with the native enzyme. 4. Analysis of the activation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed that the subunit molecular weight of 'superactive' glutamate dehydrogenase differs by less than 5% from that of the native subunit.

Limited proteolysis of native enzymes can provide a valuable tool for the analysis of conformational transitions, ligand interaction and for the assignment of functional properties to specific domains in the structure (Anfinsen & Scheraga, 1975; Citri, 1973). In particular, limited proteolysis can occasionally generate a product that retains partial functional identity with the parent structure, permitting the resolution of different catalytic properties of the enzyme. Several enzymes undergo a limited proteolytic digestion that causes an increase in activity, often at the expense of some other property. These artificial activation phenomena, produced *in vitro* and distinguishable from natural events such as zymogen activation, include the activation of pyruvate oxidase (Russell *et al.*, 1977*a*,*b*), adenylate cyclase (Anderson *et al*, 1978; Marshak & Neer, 1980), aspartase (EC 4.3.1.1; Mizuta & Tokushige, 1975) and, of particular relevance to the present paper, glutamate dehydrogenase [L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3] (Beynon & Kay, 1976).

The kinetic (enzymic) and structural properties of glutamate dehydrogenase have been the subjects of extensive investigation that has included the limited proteolysis of the native enzyme. Hucho *et al.* (1975) observed that limited tryptic digestion at the *N*-terminus of the native polypeptide caused a 10% decrease in molecular weight of the monomer and produced a form of the enzyme that exhibited different structural and allosteric properties. However, the enzyme was fully inactivated upon extended digestion, making resolution of the properties of proteolysed glutamate dehydrogenase difficult.

The effect of trypsin was confirmed by Beynon & Kay (1976, 1978), who demonstrated the effects of several proteinases on this enzyme. Of particular interest was the observation that two proteinases, α-chymotrypsin and a proteinase from *Armillaria mellea* (honey fungus), were able to activate glutamate dehydrogenase. Treatment of the enzyme with either of these proteinases generated a transient intermediate that was severalfold more active than the starting material but which had lost the ability to respond to the allosteric activator, ADP. Those authors proposed that a 'superactive' form of the enzyme was generated by limited chymotryptic proteolysis, but that it was inactivated by a subsequent proteolytic step.

During further examination of this phenomenon, we have noted that the activation process failed to give consistent results with different commercial preparations of chymotrypsin or glutamate dehydrogenase. These findings were of interest, since it has been shown that some batches of glutamate dehydrogenase have suffered limited proteolysis (McCarthy *et al.*, 1980, 1981).

Abbreviations used: Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Arg-OMe, N^{α}-tosyl-L-arginine methyl ester; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester; SDS, sodium dodecyl sulphate.

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The present paper discusses the activation of glutamate dehydrogenase, with particular reference to the problems that we have encountered.

Materials and methods

Materials

Preparations of ox liver glutamate dehydrogenase were obtained from Boehringer Corp. (London) Ltd. as a solution in 50% (v/v) glycerol. α-Chymotrypsin (EC 3.4.21.1) was purchased from Sigma (London) Chemical Co. or Miles Research Laboratories, Stoke Poges, Slough, U.K. Performic acid-oxidized B chain of insulin was obtained from Boehringer. Trypsin (EC 3.4.21.4), azocasein, Tos-Lys-CH_2Cl, Tos-Arg-OMe, Bz-Tyr-OEt and CNBr were purchased from Sigma. Lima-bean trypsin inhibitor and $NADP^+$ were purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. Sepharose 6B was obtained from Pharmacia, Hounslow, Middx., U.K. Carrier-free [^{125}I]iodine (IMS 30) was purchased from Amersham International, Amersham, Bucks., U.K.

All other reagents were of AnalaR grade and double-distilled water was used throughout.

Assay of glutamate dehydrogenase

The oxidation of L-glutamate by glutamate dehydrogenase was measured as described previously (Beynon & Kay, 1976) with 33 mM-L-glutamate and 0.5 mM-$NADP^+$ in 0.1 M-sodium pyrophosphate buffer, pH 8.5.

Assays of proteolytic enzymes

Chymotrypsin and trypsin were assayed with the ester substrates Bz-Tyr-OEt and Tos-Arg-OMe respectively (Hummel, 1959). Absorption coefficients ($A_{280}^{1\%}$) of 15.4 and 20.0 were used to calculate the concentrations of trypsin and chymotrypsin respectively.

For the measurement of general endopeptidase activity, two substrates were employed: (*a*) azocasein (Beynon & Kay, 1978) and (*b*) ^{125}I-labelled B chain of insulin. The latter substrate was prepared and used by the methods of Kenny (1977), but the oxidized B chain of insulin was substituted for the reduced carboxymethylated peptide in the original methods. Results are expressed as percentage digestion of the substrate to yield peptides soluble in 12.5% (w/v) trichloroacetic acid.

Proteolytic digestions of glutamate dehydrogenase

All incubations of glutamate dehydrogenase with proteinases were conducted at 30°C in a total volume of 1.0 ml of 0.1 M-sodium phosphate buffer (pH 7.5). All digestions were initiated by the addition of proteinases from stock solutions prepared in 1 mM-HCl, and at various times samples were removed and assayed immediately for glutamate dehydrogenase activity. The addition of the proteinase had a negligible effect on the pH or volume of the digestion mixture.

Preparation of samples for gel electrophoresis

Samples for gel electrophoresis were prepared by the procedures described by Beynon & Kay (1976). It proved necessary to remove the chymotrypsin from digestion mixtures before denaturation of the samples (by heating in a solution of sodium dodecyl sulphate) because extensive proteolysis otherwise occurred during this phase (R. J. Beynon, unpublished work). Samples from the digestions were removed and treated with two successive quantities of lima-bean trypsin inhibitor, covalently attached to agarose and prepared as described by Otsuka & Price (1974). The capacity of the adsorbent was determined to be 1.7 mg of chymotrypsin/ml of settled gel.

SDS/polyacrylamide-gel electrophoresis

The separation of the digestion products was performed in 17.5% (w/v) polyacrylamide gels in the presence of 0.1% (w/v) SDS, by the method of Studier (1973).

Inhibition of contaminant trypsin by Tos-Lys-CH_2Cl

Stock solutions of chymotrypsin (in 1 mM-HCl) were treated with an excess of Tos-Lys-CH_2Cl at room temperature until tryptic activity was completely inhibited. Typical incubations consisted of 2 mg of chymotrypsin and 0.5 mg of inhibitor in a total volume of 250 μl. Trypsin activity was determined with Tos-Arg-OMe as substrate (Hummel, 1959).

Analysis of data

The time course of changes in glutamate dehydrogenase (GDH) activity were analysed as two sequential first-order reactions specifying the process GDH → GDH* → inactive products (GDH* specifies the activated proteolysed derivative). The activity at time t (A_t) during this process is given by:

$$A_t = A_0 e^{-k_{+1}t} + F \cdot \frac{k_{+1}}{k_{+2} - k_{+1}} \cdot A_0 (e^{-k_{+1}t} - e^{-k_{+2}t}) \quad (1)$$

where k_{+1} is the first-order rate constant for GDH → GDH*, k_{+2} is the rate constant for GDH* → inactive products and A_0 in the activity at zero time. The factor, F, represents the activation factor of glutamate dehydrogenase during the activation reaction. In practice, all data have been plotted as a percentage activation ($A_0 = 100$).

Eqn. (1) above is linear with respect to F but non-linear in k_{+1} and k_{+2}, meaning that simple linear regression techniques should not be used to find the best-fit estimates of these parameters. Thus in order

to fit eqn. (1) to experimental data a non-linear curve-fitting method was employed which provided estimates of the parameters k_{+1}, k_{+2} and F that minimized the error sum (unweighted sum of squares of differences between the experimental data and the theoretical function).

The non-linear curve fitting process was performed by using Patternsearch, a direct search method for parameter optimization. A computer program based on the algorithm described by Colquhoun (1971) was written in floating-point BASIC for the Apple II microcomputer (Beynon, 1982). Parameters were optimized to an accuracy of better than 0.1%, requiring between 300 and 400 evaluations of the error sum, and in all cases fitting was conducted by using several different initial guesses to decrease the possibility of the program locating a subminimum that represented a poor fit to the data.

Where appropriate, the Figures show the data plotted with the theoretical curve drawn according to eqn. (1) by using the parameter values obtained from Patternsearch. The software for the graph-plotting package was written by the authors for the Apple II microcomputer linked to a Houston Instruments DMP 3 A4 digital plotter.

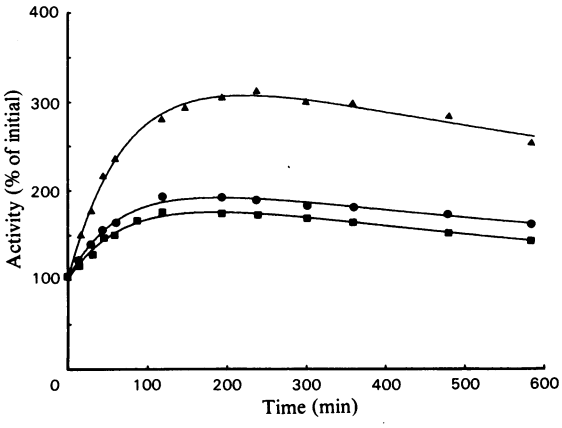

Fig. 1. *Effect of chymotrypsin on batches of glutamate dehydrogenase of different ages*

Glutamate dehydrogenase (200μg) was incubated with 200μg of chymotrypsin (obtained from Sigma) at 30°C in 0.1M-sodium phosphate buffer, pH 7.5 (1.0ml). At suitable times samples were removed and assayed for glutamate dehydrogenase activity. Reference dates of the different batches were: ▲, 1981; ●, 1979; ■, 1975.

Results and discussion

When chymotrypsin was incubated with three different batches of commercial glutamate dehydrogenase, the subsequent change in the activity of the dehydrogenase was different in the three cases (Fig. 1). The oldest of the preparations showed limited activation (1.5–2-fold), lower than observed by Beynon & Kay (1976), whereas the most recent preparation was more responsive and was activated to a transient plateau approx. 3-fold greater than the activity of the starting material. This result was similar to that obtained previously (Beynon & Kay, 1976), although the inactivation phase was slower. Beynon & Kay (1976) suggested that the activation of glutamate dehydrogenase by α-chymotrypsin was due to limited proteolysis at a sensitive site on the substrate polypeptide chain. The transient nature of the activation process was proposed to be due to a second, inactivating, proteolytic step that resulted in complete digestion of the substrate to limit peptides. It would appear that the older batches of glutamate dehydrogenase have a lower response to proteolytic digestion.

McCarthy *et al.* (1980, 1981) have shown that commercial preparations of ox liver glutamate dehydrogenase have undergone limited proteolysis. To assess whether our preparations were contaminated with proteinase(s), we attempted to measure proteolytic activity by using azocasein or ^{125}I-labelled B chain of insulin, both substrates for the determination of general endopeptidase activity. No proteolytic activity was detectable in any preparation with azocasein, but significant activity could be measured by using the more sensitive radiochemical assay. The older batches of glutamate dehydrogenase exhibited the greatest endogenous proteinase activity (1975 batch, 4μg of substrate digested/h per mg of GDH protein; 1979 batch, 9.0μg/h per mg of GDH protein), whereas the most recent batch possessed very little activity (<0.5μg/h per mg of GDH protein). The proteolytic activity was greatest in those preparations of glutamate dehydrogenase that were least responsive to chymotryptic digestion, implying that digestion of stock solutions of glutamate dehydrogenase at 4°C was decreasing the response. Interestingly, the specific activities of all three preparations were similar [nkat/mg; mean ± S.D. (*n*): 1975 batch, 12.30 + 0.28(5); 1979 batch, 11.21 ± 0.28(5); 1981 batch, 9.80 ± 0.17(5)], although we have measured a change in the K_a for, and extent of stimulation by, the allosteric activator ADP (G. A. Place, unpublished work). This suggests that the allosteric properties of the older glutamate dehydrogenase preparations have been altered during storage. In this respect it is noteworthy that previous work of the proteolysis of glutamate dehydrogenase by trypsin or chymotrypsin suggests that the loss of stimulation by ADP is one of the first events (Hucho *et al.*, 1975; Beynon & Kay, 1976). The 1981 preparation of glutamate dehydrogenase was the most responsive to

chymotrypsin and contained very low activity of endogenous proteinase, and was therefore used for all subsequent studies.

The time course of the activation by different amounts of α-chymotrypsin (obtained from Sigma) was analysed by non-linear curve fitting of a sequential reaction to time-course data. The estimates of the rate constants k_{+1} and k_{+2}, and the factor for activation of glutamate dehydrogenase, F, are summarized in Table 1. The rate constant for the first, activation, phase was related linearly to the concentration of chymotrypsin (correlation coefficient = 0.9955, $P < 0.002$ by linear regression), but the rate constants for inactivation (k_{+2}) were too low for meaningful analysis. Finally, the glutamate dehydrogenase was activated 3-fold irrespective of the proteinase concentration. These results suggest that chymotryptic digestion of glutamate dehydrogenase generates a 'superactive' form that is quite stable to further digestion, although the term 'superactive' may be an oversimplification (Aitchison & Engel, 1980).

This superactive form of the enzyme was considerably more stable than was reported originally (Beynon & Kay, 1976). However, when glutamate dehydrogenase was incubated with α-chymotrypsin from Miles (as used by Beynon & Kay, 1976), the activity of glutamate dehydrogenase attained a transient maximum, followed by a relatively rapid decline in activity, in keeping with the original findings (Fig. 2). This suggested that the two preparations of α-chymotrypsin were able to produce different effects on glutamate dehydrogenase, although both preparations of the proteinase were obtained from the same source. However, the preparation from Miles, when assayed for tryptic activity with Tos-Arg-OMe, was contaminated with trypsin to the extent of 5 μg of trypsin/mg of protein.

Table 1. *Effect of chymotrypsin on the activation parameters for glutamate dehydrogenase*

Glutamate dehydrogenase (200 μg) was incubated at 30°C in 1.0 ml of 0.1 M-sodium phosphate buffer, pH 7.5, in the presence of various amounts of α-chymotrypsin (Sigma). At suitable times, samples were removed and assayed for dehydrogenase activity. The time courses were analysed by using non-linear curve fitting to obtain values of the two rate constants, k_{+1} and k_{+2}, and F, the fractional activation constant.

Chymotrypsin (μg/ml)	k_{+1} (min^{-1})	k_{+2} (min^{-1})	F
42	0.0034	<0.00001	2.95
84	0.0075	<0.00001	3.04
210	0.0146	<0.00001	3.00
420	0.0289	0.0005	3.27

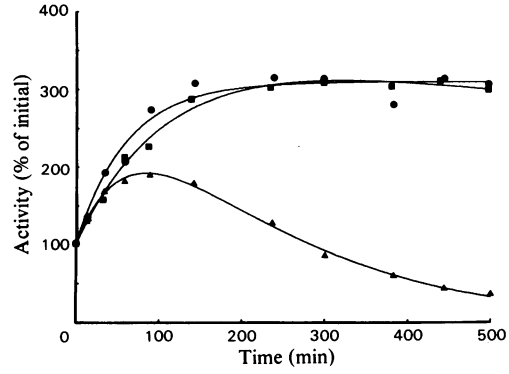

Fig. 2. *Effect of trypsin-inhibited α-chymotrypsin preparations on glutamate dehydrogenase*

Glutamate dehydrogenase (200 μg) was incubated at 30°C in 1.0 ml of 0.1 M-sodium phosphate buffer, pH 7.5, with: 200 μg of Sigma α-chymotrypsin, pretreated with Tos-Lys-CH_2Cl (●); 200 μg of Miles α-chymotrypsin, pretreated with Tos-Lys-CH_2Cl (■); or 200 μg of untreated Miles α-chymotrypsin (▲). At suitable intervals, samples were removed and assayed for dehydrogenase activity.

In contrast, the α-chymotrypsin from Sigma contained no trypsin detectable by this assay, suggesting that the rapid inactivation phase obtained with the preparation from Miles could have been due to contaminating trypsin. To test this, samples of Miles or Sigma α-chymotrypsin were pretreated with Tos-Lys-CH_2Cl, a potent irreversible inhibitor of trypsin that has no effect on chymotrypsin (Shaw *et al.*, 1965). The concentrations of inhibitor were sufficient to inactivate all of the contaminating trypsin as measured with Tos-Arg-OMe, but were without effect on the glutamate dehydrogenase in subsequent incubations.

After inactivation of contaminating trypsin and incubation with Miles chymotrypsin, the activity of glutamate dehydrogenase increased to a stable plateau value 3-fold higher than the starting activity (Fig. 2). The inactivation phase was abolished and the activated form of the enzyme appeared to be stable for extended periods, being approx. 1000 times less sensitive to further chymotryptic digestion. Treatment of the Sigma preparation of α-chymotrypsin with Tos-Lys-CH_2Cl gave no change in the activation process, and the plateau obtained was identical with that produced for untreated α-chymotrypsin. It would appear that contaminating trypsin was responsible for the inactivation of the superactive form of glutamate dehydrogenase.

The amount of trypsin that was present in incubations with Miles α-chymotrypsin was approx. 1 μg/ml. However, it has been shown previously that

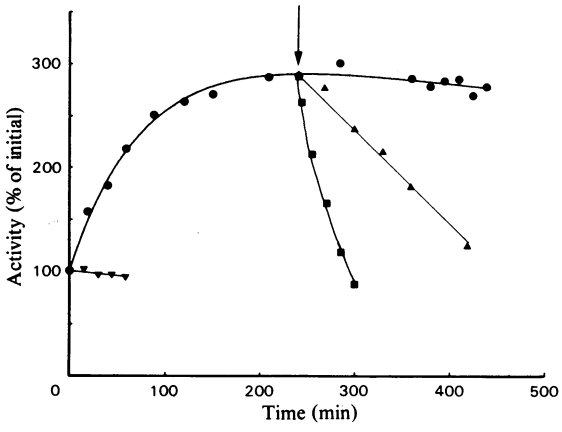

Fig. 3. *Effect of trypsin on native and chymotrypsin-activated glutamate dehydrogenase*

Glutamate dehydrogenase (200μg) was incubated with 200μg of α-chymotrypsin (Sigma) in 1.0ml of 0.1M-sodium phosphate buffer, pH7.5, at 30°C (●). At the point indicated by the arrow, samples were removed from the incubation mixture and incubated under the same conditions in the presence of trypsin (▲, 1μg/ml; ■, 5μg/ml). Additionally, native glutamate dehydrogenase (200μg) was incubated under the same conditions with 5μg of trypsin (▼). At the times indicated samples were removed and assayed for dehydrogenase activity.

native glutamate dehydrogenase is relatively resistant to trypsin (Hucho *et al.*, 1975; Beynon & Kay, 1976, 1978), and this concentration of contaminating proteinase could not have been expected to effect such a rapid inactivation. One possible explanation for this anomaly was that the superactive glutamate dehydrogenase exhibited an enhanced susceptibility to tryptic inactivation as compared with the native enzyme. To test this, glutamate dehydrogenase was activated with 200μg of trypsin-free Sigma α-chymotrypsin/ml until a plateau value for the activity of the substrate was attained. At this point, small amounts (final concentrations 1.1 or 5.2μg/ml) of trypsin were added (Fig. 3). With the higher trypsin concentration, glutamate dehydrogenase activity decayed rapidly, losing 70% of the activity in less than 1h. This contrasts markedly with the effect of trypsin on the starting material, where the same concentration of trypsin caused only a 6% inactivation in the same period. In the absence of trypsin the superactive enzyme retained its activity for at least a further 4h, suggesting that the transience of the activation observed previously (Beynon & Kay, 1976) was due to contamination of chymotrypsin by low amounts of trypsin sufficient to cause a rapid inactivation of the superactive form of glutamate dehydrogenase but unable to affect the native enzyme. The differences in susceptibility of the superactive glutamate dehydrogenase to the different proteinases may be due to conformational changes on the surface of the enzyme that alter the accessibility of critical peptide bonds.

To establish that trypsin had no effect on the activation process, glutamate dehydrogenase was incubated with trypsin-free α-chymotrypsin to which known amounts of trypsin were added. The time-course data were analysed by non-linear curve fitting, and the best-fit values for k_{+1}, k_{+2} and F are summarized in Table 2. The pseudo-first-order rate constant for the inactivation phase (k_{+2}) is related linearly to the concentration of trypsin (correlation coefficient 0.995, $P < 0.0003$ by linear regression), whereas the rate constants for activation (k_{+1}) and the fractional activation constant (F) are similar and appear to be independent of the trypsin concentration.

The extent of proteolysis of glutamate dehydrogenase during activation by chymotrypsin was investigated by SDS/polyacrylamide-gel electrophoresis. During an activation reaction samples were removed and treated with insolubilized lima-bean trypsin inhibitor to inhibit and physically remove all of the chymotrypsin from the incubation mixture (as measured by the hydrolysis of Bz-Tyr-OEt). The chymotrypsin-free samples were then subjected to electrophoresis, and the resulting gel pattern is shown in Fig. 4. The activation of glutamate dehydrogenase was accompanied by a slight shift in mobility of the subunit, suggesting a decrease in molecular weight of less than 5%. Some additional products of lower molecular weight were also visible, possibly owing to alternative proteolytic reactions, for example by small quantities of contaminating trypsin. It would appear that the hydrolysis of a peptide bond close to a terminal of the enzyme was

Table 2. *Effect of trypsin on the activation/inactivation parameters for glutamate dehydrogenase*

Glutamate dehydrogenase (200μg) was incubated at 30°C in 1.0ml of 0.1M-sodium phosphate buffer, pH7.5, in the presence of a constant amount of 200μg of α-chymotrypsin (Sigma) and various amounts of trypsin. At suitable times, samples were removed and assayed for dehydrogenase activity. The time courses were analysed by using non-linear curve fitting to obtain values of the two rate constants, k_{+1} and k_{+2}, and F, the fractional activation constant.

Trypsin (μg/ml)	k_{+1} (min^{-1})	k_{+2} (min^{-1})	F
0	0.0173	0.00004	3.73
0.26	0.0192	0.00140	3.64
0.51	0.0183	0.00258	3.58
1.03	0.0210	0.00645	3.20

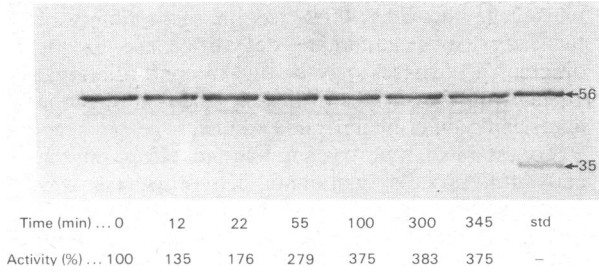

Fig. 4. *Extent of digestion of glutamate dehydrogenase by α-chymotrypsin*

Glutamate dehydrogense (800μg) was incubated in 4.0ml of 0.1M-sodium phosphate buffer, pH 7.5, at 30°C with 800μg of chymotrypsin (Sigma). At the indicated times, samples (0.5ml) were removed and treated with two successive quantities (0.15ml) of insolubilized lima-bean trypsin inhibitor. After final centrifugation, samples of the supernatant (0.15ml) were freeze-dried before analysis by SDS/polyacrylamide-gel electrophoresis. Marker proteins (std) were glutamate dehydrogenase (56kDa) and malate dehydrogenase (35kDa).

responsible for a 3-fold increase in specific activity of glutamate dehydrogenase. Some evidence of further proteolysis could be seen, but the extent of digestion was clearly limited.

Proteolytic activation of native enzymes has been observed by several workers. Mizuta & Tokushige (1975) have shown that aspartase from *Escherichia coli* can be activated by trypsin, the activation being due to a severalfold increase in $V_{max.}$ for the reaction, without appreciable change in the molecular weight of the subunit. The activation of pyruvate oxidase from *E. coli* by chymotrypsin was accompanied by a 7% decrease in subunit molecular weight; in this case activation was associated with an insensitivity to binding of amphiphilic molecules that normally activated the unproteolysed enzyme (Russell *et al.*, 1977*a*,*b*). Activation of rabbit liver fructose 1,6-bisphosphatase by subtilisin was associated with a change in the allosteric properties of the enzyme (Traniello *et al.*, 1972; Pontremoli *et al.*, 1973). Thus there is no common mechanism for activation of native enzymes by limited proteolysis. However, we have noted that the superactive form of glutamate dehydrogenase exhibits altered allosteric properties, including insensitivity to the allosteric activator ADP (G. A. Place, unpublished work), suggesting that activation is associated with a release from a conformational restraint normally elicited upon binding of ADP. Indeed, the extent of the conformational changes produced in the enzyme is confirmed by the marked increase in susceptibility to trypsin that is shown by superactive glutamate dehydrogenase.

It is doubtful whether the activation phenomenon has any physiological significance. However, the possibility of producing a modified form of glutamate dehydrogenase that exhibits partial function but has altered allosteric properties may provide a valuable tool for the elucidation of the mechanism of this complex enzyme. Limited proteolysis by chymotrypsin may aid in the elucidation of the functional areas of glutamate dehydrogenase and should provide a fruitful area for further work.

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