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STUDIES ON ASPARTASE

III. ALTERATION OF ENZYMATIC PROPERTIES UPON TRYPSIN-MEDIATED ACTIVATION

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Summary

Highly purified aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) from *Escherichia coli*, already of full activity, is further activated 3.3-fold by limited treatment with trypsin. The activation requires a few minutes to attain maximum level, and hereafter the activity gradually decreases to complete inactivation. Prior or intermediate addition of soybean trypsin inhibitor results in an immediate cessation of any further change in the enzyme activity. Upon trypsin-mediated activation no appreciable change is detected in the molecular weight of the enzyme subunits as judged from sodium dodecyl sulfate polyacrylamide gel electrophoresis, nor in the pH vs. activity profile in the presence of added metal ions. However, $S_{0.5}$ and Hill coefficient for L-aspartate considerably increase upon activation. As the trypsin-mediated activation proceeds, a marked absorbance difference spectrum of the trypsin-treated aspartase vs. untreated aspartase appears with negative absorbance maxima at 278 and 285 nm. When the trypsin-activated enzyme is denatured in 4 M guanidine · HCl, followed by removal of the denaturant by dilution, the enzyme activity is readily restored to as much as 1.5 times that of the native enzyme, indicating that the trypsin-activated enzyme is rather a stable molecule.

Introduction

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and NH_4^+. The enzyme purified from

A preliminary account of this work has been published [1].

Abbreviations used: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, tris(hydroxymethyl)methylaminopropanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid; SDS, sodium dodecyl sulfate.

Escherichia coli W cells has a molecular weight of 193 000 and is composed of four subunits of seemingly identical molecular weight [2]. One or two sulfhydryl groups per subunit have been found to be essential for enzymic activity [3]. In an effort to elucidate the nature and the role of the sulfhydryl groups in the enzymatic reaction, limited proteolysis of the enzyme protein was attempted whereupon it was observed that the enzyme was markedly activated by trypsin treatment without an appreciable alteration of the molecular weight. In this communication the catalytic and the regulatory properties of the trypsin-activated enzyme are described.

Materials and Methods

Materials. Bovine pancreatic trypsin (twice crystallized) was obtained from Worthington and treated with diphenyl carbamyl chloride [4]. Soybean trypsin inhibitor, Tris, 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)methylaminopropanesulfonic acid (TAPS) and cyclohexylaminopropanesulfonic acid (CAPS) were from Sigma. Guanidine · HCl (ultrapure) was from Mann and Sepharose 6B from Pharmacia. All other chemicals were of analytical grade.

Enzyme preparation. Aspartase was purified from *E. coli* W cells as described previously [2]. The enzyme preparations used in this investigation were homogeneous as judged by ultracentrifugation and polyacrylamide gel disc electrophoresis.

Enzyme assay. The activity of aspartase was routinely determined spectrophotometrically by measuring the formation of fumarate following the increase in absorbance at 240 nm at 39°C, with a Hitachi 124 recording spectrophotometer equipped with a constant-temperature cell housing. The standard assay mixture contained, in a total volume of 1.0 ml, 0.1 M sodium L-aspartate (pH 7.0), 2 mM $MgCl_2$, 0.1 M Tris · HCl buffer, pH 7.0 *, and the enzyme.

Other determinations. Polyacrylamide gel disc electrophoresis was carried out according to the method of Davis [5]. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Laemmli [6] by using 10% acrylamide gels. Protein concentration was determined by the method of Lowry et al. [7]. Difference absorbance spectra were measured with a Hitachi 323 automatic recording spectrophotometer equipped with a constant-temperature cell housing.

Results

Effects of trypsin treatment on enzyme activity and its molecular size

When aspartase was incubated with trypsin at pH 7.4 and 30°C, a marked increase in catalytic activity was observed. The activity reached 3.3 times that of the untreated enzyme in 7 min, and hereafter it gradually decreased reaching almost complete inactivation after 4 h (Fig. 1). Inactivation of trypsin by heat

* Although the maximum activity was observed at alkaline pH, the effect of trypsin on the enzyme activity was more noticeable at neutral pH than at alkaline pH. Therefore, the enzyme activity was assayed at pH 7.0.

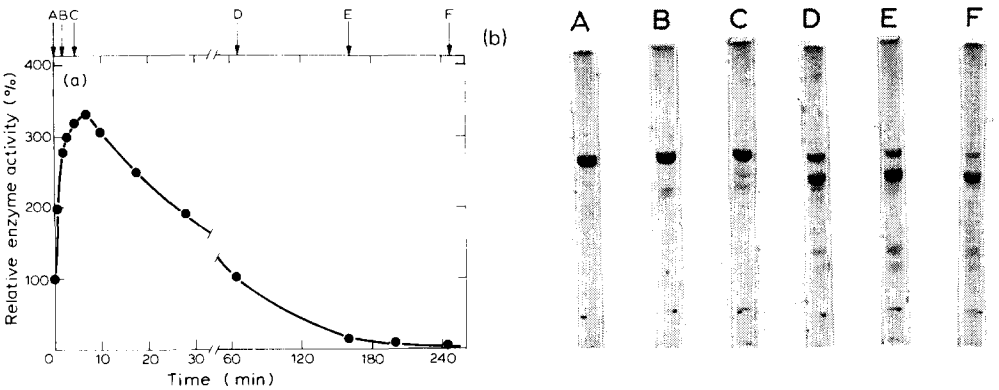

Fig. 1. Alteration of enzyme activity and molecular size of aspartase by trypsin treatment. The reaction mixture contained 0.1 M Tris · HCl buffer, pH 7.4, 180 μg of aspartase and 10 μg of trypsin in a total volume of 1.0 ml. The reaction was carried out at 30°C. (a) At designated time intervals aliquots were removed and analyzed for enzyme activity. (b) Simultaneously, other aliquots for SDS polyacrylamide gel electrophoresis were transferred into test tubes containing an equal volume of a solution consisting of 0.125 M Tris · HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% Bromo-Phenol blue. The sample proteins for electrophoresis were 18 μg (A,B,C and D) or 27 μg (E and F). Electrophoresis was carried out at a constant current of 3 mA per gel (0.6 × 10 cm) for 7 h. Direction of migration was from top to bottom. The gels were stained with Coomassie Brilliant Blue.

treatment prior to the addition to aspartase or addition of soybean trypsin inhibitor completely prevented the trypsin-mediated alteration of the aspartase activity. Simultaneous inspection of the change in the molecular weight of subunits revealed that the maximally active enzyme had the same mobility as that of the native enzyme (M_r = 48 500) on electrophoresis in the presence of SDS. As the enzyme activity decreased, faster-moving components appeared and after prolonged exposure to trypsin (for 4 h) only a faint band was seen corresponding to the native subunit. Furthermore, in order to examine whether or not the dissociation of subunits occurred concomitantly with activation, Sepharose 6B column chromatography was carried out. The activated enzyme was eluted at the same elution volume as that of the native enzyme (data not shown). These results indicate that the activated enzyme remained as a tetramer and the molecular weight of its subunits did not change to an appreciable extent.

pH vs. activity profile of trypsin-activated enzyme

Frustose-1,6-bisphosphatase (EC 3.1.3.11) from rabbit liver has been known to undergo marked activation upon limited proteolysis with subtilisin, as Traniello et al. reported [8]. In this case, the shift of the optimum pH for activity was found to be responsible for the activation. In order to examine this possibility, pH vs. activity profiles were compared before and after trypsin activation. The results are shown in Fig. 2. In the presence of added Mg^{2+}, a shift of the optimum pH for the activity was not detected and the optimum pH was about 8.8 in both cases. In the absence of added Mg^{2+}, however, a slight shift of the optimum pH was observed. Furthermore, in the case of the native enzyme, the enzyme activity in the absence of added Mg^{2+} was slightly higher than that in the presence of Mg^{2+} in the pH range lower than pH 7.5 (Fig. 2A). However, in

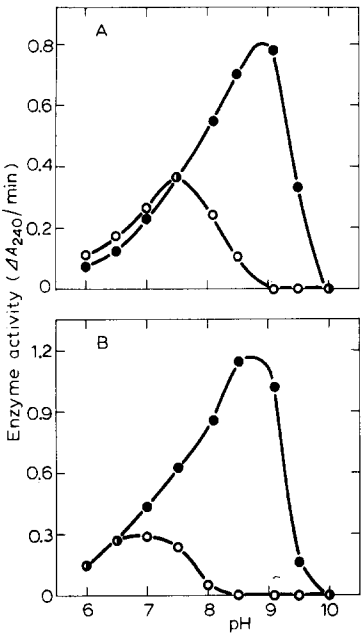

Fig. 2. pH vs. activity profile of trypsin-activated enzyme. Aspartase (130 μg) was incubated with 1 μg of trypsin in 0.1 M Tris · HCl buffer, pH 7.4 at 30° C in a total volume of 0.8 ml. After 26 min trypsin inhibitor (5 μg) was added and the test tube was transferred into an ice bath. This preparation was designated as the trypsin-activated enzyme. Initial velocity of the enzyme reaction was determined at 30° C using a reaction mixture which contained 0.1 M L-aspartate (pH was adjusted to indicated values with KOH), the designated buffer (0.1 M), the enzyme (1.6 μg) and 2 mM $MgCl_2$ (●——●) or in its absence (○——○) in a total volume of 1.0 ml. Buffers were: MES/KOH (pH 6.0—7.0), HEPES/KOH (pH 7.0—8.0), TAPS/KOH (pH 8.0—9.0), CAPS/KOH (pH 9.5—10.0). A, native enzyme; B, trypsin-activated enzyme.

the pH range higher than pH 7.5, the ratio of the activity with and without Mg^{2+} markedly increased as the pH increased. In contrast, in the case of the trypsin-activated enzyme, the ratio of the activity with and without Mg^{2+} was unity in the pH range lower than pH 6.5 and it increased in the pH range higher than pH 6.5 (Fig. 2B).

Kinetic properties of trypsin-activated enzyme

As previously reported from this laboratory, aspartase exhibits, in its substrate saturation profile, a negative cooperativity at pH 7.0 and, conversely, a positive cooperativity at pH 8.5 [2,3,9]. The substrate saturation profiles of the enzyme before and after the trypsin treatment are shown in Fig. 3. At pH 7.0 the enzyme activity of the trypsin-activated enzyme was higher than that of the native enzyme throughout the substrate concentrations tested. In contrast, at pH 8.5 the enzyme activity of the trypsin-activated enzyme was lower than that of the native enzyme at substrate concentrations lower than 25 mM, while it was higher than the native enzyme activity at substrate concentrations above 25 mM. The differences in the substrate saturation profiles are not due to the presence of trypsin and trypsin inhibitor in the enzyme solution, since prior addition of trypsin inhibitor to aspartase before trypsin treatment did not affect the substrate saturation profile of the native enzyme. The insets of Fig. 3

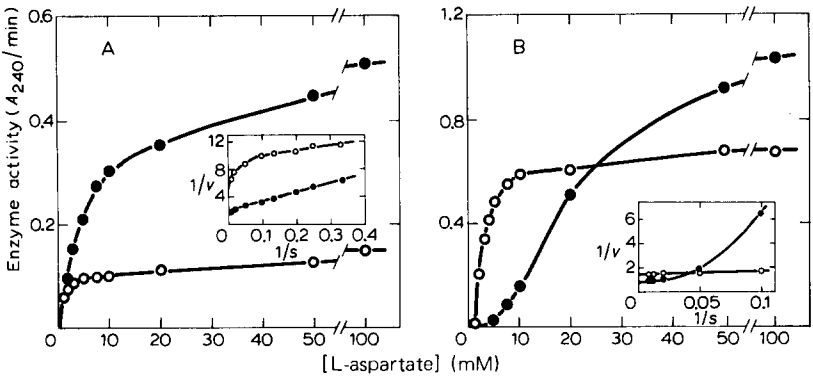

Fig. 3. Substrate saturation curves of trypsin-activated enzyme. Aspartase (150 μg) was incubated with 4 μg of trypsin in 0.1 M Tris · HCl buffer, pH 7.4 at 30°C in a total volume of 0.8 ml. After 3 min trypsin inhibitor (20 μg) was added and the test tube was transferred into an ice bath. This preparation was designated as the trypsin-activated enzyme. Initial velocity of the enzyme reaction was determined at various concentrations of Na L-aspartate in the presence of 0.1 M Tris · HCl buffer, pH 7.0 (A) or pH 8.5 (B), and 2 mM $MgCl_2$. ○———○, native enzyme (1.9 μg of protein/ml); ●———●, trypsin-activated enzyme (1.9 μg of protein/ml). The insets show double reciprocal plots of the data.

are double reciprocal plots made according to the method of Lineweaver and Burk [10]. At pH 7.0 the value of the half-saturation concentration ($S_{0.5}$ *) for L-aspartate of the trypsin-activated enzyme was 12 mM. This value is virtually the same as that of the native enzyme (10 mM). The V value increased about 3-fold upon trypsin activation. At pH 8.5 the $S_{0.5}$ value of the trypsin-activated enzyme was 21 mM, 7 times as high as that of the native enzyme (3.1 mM). The V value increased about 1.6-fold upon trypsin activation.

The data in Fig. 3 were replotted to obtain Hill coefficients. The results are shown in Fig. 4. At pH 7.0 the Hill coefficient (n value **) of the native

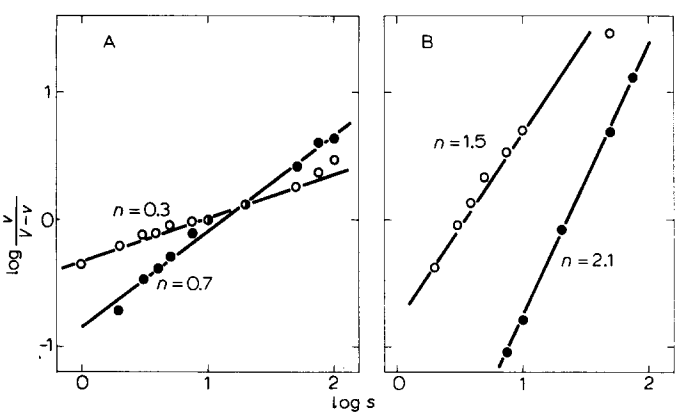

Fig. 4. Hill plots of trypsin-activated enzyme reaction. Data in Fig. 3 were replotted by using the V values obtained by extrapolation of the curves in the double reciprocal plots. (A), pH 7.0; (B), pH 8.5. ○———○, native enzyme; ●———●, trypsin-activated enzyme.

* Since the double reciprocal plot was not linear, the V value was estimated by extrapolation of the curve.

** As the Hill plot profile of aspartase is complex [9], n values were obtained for a limited range of L-aspartate concentrations.

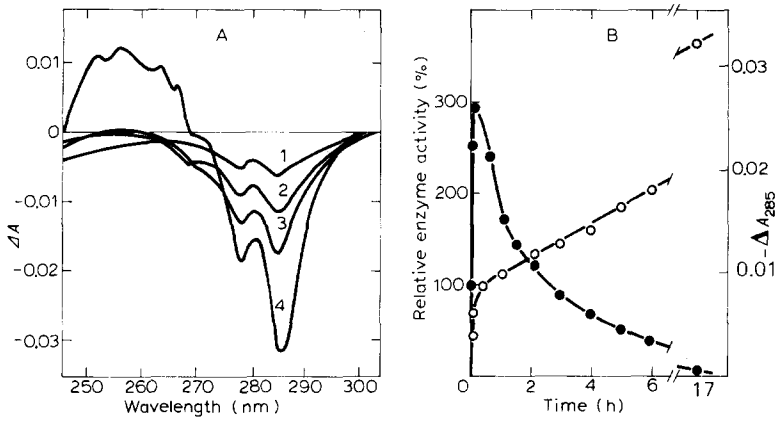

Fig. 5. Difference absorbance spectra of trypsin-treated aspartase vs. untreated enzyme. A; In both the sample- and the reference compartments of the spectrophotometer, two cuvettes (5 mm light path) were placed in tandem, one containing 2.6 mg of aspartase in 1.1 ml of 90 mM Tris · HCl buffer, pH 7.4 and the other 1.1 ml of deionized water. Trypsin (5 μg) was added to the cuvette which contained aspartase (sample) or to that which contained water (reference). The spectra were recorded at 25°C after 10 min (curve 1), 131 min (curve 2), 6 h (curve 3) and 17 h (curve 4). B; Difference absorption at 285 nm determined at indicated time intervals was plotted (○———○). The enzyme activity was simultaneously determined using small aliquots (●———●).

enzyme was 0.3, and it increased to 0.7 upon trypsin activation. At pH 8.5 the n value increased from 1.5 (native enzyme) to 2.1. Thus, the subunit interaction of the enzyme tends to increase upon trypsin activation.

Spectral change during trypsin treatment

In Fig. 5A the difference absorbance spectra of the trypsin-treated aspartase vs. untreated aspartase are shown. As the trypsin-mediated modification of the enzyme proceeded, marked absorbance peaks appeared in the ultraviolet region in a time-dependent fashion. The difference spectra had negative peaks at 278 nm and 285 nm. Fig. 5B shows the time course of the difference absorbance at 285 nm and that of the enzyme activity. The time course consisted of two phases: first, a rapid increase in the absorption was observed concomitant with the activation and second, a slower increase in the absorbance was observed as the inactivation proceeded. A marked positive absorbance in the range between 246 nm and 269 nm in curve 4 seems to result from denaturation of the enzyme polypeptides.

Reversible denaturation

As will be reported separately (Tokushige, M., Eguchi, G. and Hirata, F. (1976) Biochim. Biophys. Acta, submitted), aspartase denatured in 4 M guanidine · HCl regains its activity and quaternary structure upon dilution. In order to examine the stability of the trypsin-activated enzyme preparation, the activated enzyme was first denatured in 4 M guanidine · HCl and then 30 min later the solution was diluted 21-fold with a buffer mixture containing 50 mM potassium phosphate buffer, pH 6.8, 0.1 M KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA. The enzyme activity was assayed at designated time intervals using

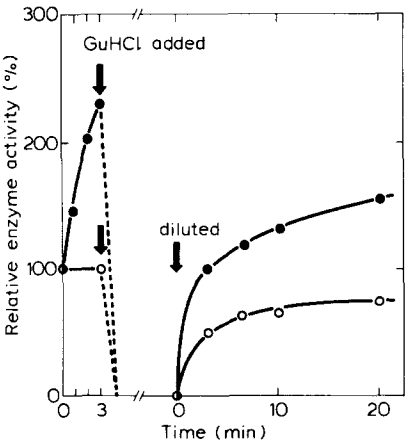

Fig. 6. Reactivation of denatured aspartase in guanidine · HCl. Aspartase (160 μg) was incubated with 1 μg of trypsin in 0.2 ml of 60 mM Tris · HCl buffer, pH 7.4 at 30° C. After 3 min, 10 μg of trypsin inhibitor was added. From this enzyme preparation (●———●) or the native enzyme (160 μg in 0.2 ml, ○———○) 50 μl portions were transferred into test tubes containing 50 μl of 8 M guanidine · HCl (at the arrow). After a 30-min incubation at 30° C, the reaction mixture was diluted 21-fold at 30° C with 2 ml of 50 mM potassium phosphate buffer, pH 6.8 containing 0.1 M KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA (at the arrow). The restored activity was determined at designated time intervals using small aliquots.

small aliquots of the renatured enzyme solution. The untreated (native) enzyme was also examined for reversible denaturation under the same conditions. The results are shown in Fig. 6. After 20 min, almost 70% of the original activity, i.e. 150% of the activity of the native enzyme (before addition of trypsin), was restored. These results suggest that the trypsin-activated enzyme species is a fairly stable molecule.

Discussion

Limited proteolysis has been widely employed as a useful technique for investigation of the structure-function relationship in various enzymes. As revealed in the present investigation, however, aspartase purified from *E. coli* cells as an active enzyme is unexpectedly activated several-fold by limited treatment with trypsin. The degree of peptide cleavage directly associated with the activation seems to be extremely small as judged from the fact that no appreciable change in the molecular weight of the enzyme subunits is detected by SDS-polyacrylamide gel electrophoresis. Upon activation, pH vs. activity profile of the enzyme is essentially unchanged. However, the ultraviolet absorbance of the enzyme protein decreases, as revealed by difference spectroscopy. Furthermore, $S_{0.5}$ and Hill coefficient for the substrate are significantly altered, indicating that subunit interaction of the enzyme is modified to an appreciable extent, although neither dissociation nor association of subunits is involved in the activation, as judged by gel permeation chromatography. At pH 7.0 the activity increases throughout the substrate concentration tested, while at pH 8.5 a significant lowering of the activity and a marked activation are observed

at the substrate concentrations lower than 25 mM and higher than 25 mM, respectively. Thus, the catalytic and the regulatory properties of the enzyme were modified by trypsin treatment to a considerable degree.

As will be reported in a separate paper (Tokushige, M., Eguchi, G. and Hirata, F. (1976) Biochim. Biophys. Acta, submitted), aspartase is reversibly denatured. After denaturation of the trypsin-activated enzyme in guanidine · HCl, dilution results in reactivation to as much as 1.5 times the native enzyme activity. This is a strong indication that the trypsin-activated enzyme is a stable molecular species distinct from the native enzyme.

Activation of enzymes is sometimes brought about by non-covalent protein-protein interactions. In the present case, however, this possibility seems quite unlikely, since (1) prior addition of trypsin inhibitor prevents the activation, (2) when the ratio of the concentration of trypsin to that of aspartase decreases, the extent of the maximal activation is not affected (Figs. 1 and 5), and (3) the trypsin-activated enzyme denatured in 4 M guanidine · HCl is readily reactivated upon dilution.

The activating effect of trypsin appears to be rather specific. When chymotrypsin is used in lieu of trypsin, no activation but inactivation is observed.

Several enzymes have been reported to be activated by limited proteolysis. Among them, acetyl-CoA carboxylase (EC 6.4.1.2) from rat liver [11], phosphorylase kinase (EC 2.7.1.38) from rabbit skeletal muscle [12] and tyrosine hydroxylase (EC 1.14.16.2) from rat brain [13] were reported to be activated by trypsin. In the former two enzymes, trypsin-catalyzed activation is assumed to be mediated by a conformational change following peptide modification, which was otherwise induced by citrate binding (acetyl-CoA carboxylase) or phosphorylation of the enzyme protein (phosphorylase kinase). In contrast, replacement of the ligand-mediated activity control does not seem to be involved in the present case.

Although we have no experimental evidence at present, it is a plausible conjecture that enzyme-catalyzed covalent activation of aspartase actually occurs in *E. coli* cells. It is noteworthy that Pacaud and Richaud have recently purified a trypsin-like enzyme from *E. coli* cells [14]. The molecular mechanism of the trypsin-mediated activation of aspartase including the site of peptide cleavage is under investigation.

Acknowledgement

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