Biochimica et Biophysica Acta, 616 (1980) 319—328

BBA 69154

STUDIES ON ASPARTASE

VI. TRYPSIN-MEDIATED ACTIVATION RELEASING CARBOXY-TERMINAL PEPTIDES

NOBORU YUMOTO [a], MASANOBU TOKUSHIGE [a] and RIKIMARU HAYASHI [b]

[a] *Department of Chemistry, Faculty of Science, and* [b] *Research Institute for Food Science, Kyoto University, Kyoto (Japan)*

(Received May 29th, 1980)

Key words: Aspartase; Limited proteolysis; Trypsin activation; Carboxy-terminal peptide; Activation; (E. coli)

Summary

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) of *Escherichia coli*, already of full activity, is 3—5-fold activated by a limited proteolysis with trypsin (Mizuta, K. and Tokushige, M. (1976) Biochim. Biophys. Acta 452, 253—261). Structural bases for the activation were investigated.

The NH_2-termini of the native enzyme and of the trypsin-activated enzyme were found to be equally serine, as analyzed by the dansylation method. However, the COOH-terminal glutamate of the native enzyme was altered to arginine upon activation, as revealed by treatments with carboxypeptidases Y, A and B. The released peptides were obtained by molecular sieve membrane filtration following trypsin activation of the enzyme. The peptides were separated by high voltage paper electrophoresis, and the amino acid composition and the terminal residues were determined. The results showed that one or a few related peptides consisting of 7—17 residues were released from the COOH-terminal upon activation.

The circular dichroism spectrum of the enzyme suggested that the helical content of the activated enzyme was about 5% less than that of the native enzyme, an indication that the trypsin-activated enzyme has a somewhat looser conformation than the native enzyme. Determination of the fluorescence decay time of the enzyme protein indicated that the tryptophan residue became more exposed to outer environment than that of the native enzyme upon trypsin-activation.

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 *Escherichia coli* W cells has a molecular weight of 193 000 and is composed of four subunits of identical molecular weight [1]. One or two sulfhydryl groups per enzyme subunit have been found to be essential for enzymatic activity [2]. As previously reported [3,4], the enzyme is markedly activated by trypsin (EC 3.4.21.4) treatment without an appreciable alteration of the molecular weight. Not only the V value but also the $S_{0.5}$ value and Hill coefficient for L-aspartate considerably increase upon activation. As the trypsin-mediated activation proceeds, a marked difference absorbance spectrum of the trypsin-treated aspartase vs. untreated aspartase appears with negative absorbance maxima at 278 and 285 nm, indicating that some aromatic amino acid residue(s) of the enzyme protein becomes exposed to hydrophilic environment upon trypsin-activation. When the trypsin-activated enzyme is denatured in 4 M guanidine-hydrochloride, 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 a stable molecule. The results obtained in previous reports indicate that the trypsin-activated aspartase is a more efficient catalyst than the native enzyme. It is of interest to note how such a highly active enzyme species is produced in spite of the minor degree of peptide cleavage. In this communication, chemical and conformational features of the trypsin-mediated activation are described.

Materials and Methods

Materials. Insoluble trypsin on polyacrylamide and dansyl (5-dimethyl-amino-naphthalene-1-sulfonyl) chloride were obtained from Sigma. Carboxy-peptidase A and B (EC 3.4.17.1 and 3.4.17.2), chymotrypsinogen A, hen egg and bovine serum albumin, and phosphorylase *b* (EC 2.4.1.1) were from Boehringer Mannheim. All other chemicals were of analytical grade.

Enzyme preparations. Aspartase was purified from *E. coli* W cells as described previously [1]. The enzyme preparations used in this investigation were homogeneous as judged by ultracentrifugation and polyacrylamide gel electrophoresis. Carboxypeptidase Y was prepared from baker's yeast (Oriental Yeast Co.) as described previously [5]. The preparation was extensively purified by repeated chromatography (three times) on DEAE-Sephadex A-50. Finally, the enzyme was lyophilized after exhaustive dialysis against distilled water.

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 30°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 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.

Preparation of trypsin-activated aspartase. Aspartase (15 mg) was incubated with 200 mg insoluble trypsin in 0.1 M Tris-HCl buffer, pH 7.4 at 30°C in a total volume of 30 ml with constant stirring. After 30 min, the reaction was

terminated by removing the insoluble trypsin by filtration. The filtrate was concentrated with a membrane filter (Amicon PM 10) and this preparation was designated as the trypsin-activated enzyme. The activity of the trypsin-activated enzyme was 2.4-times that of the untreated enzyme. The filtrate of Amicon PM 10 membrane was used for the analysis of released peptides.

Electrophoresis. Polyacrylamide gel disc electrophoresis was carried out according to the method of Davis [6]. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out using the methods of Laemmli [7] or Weber and Osborn [8], using 7.5% gel containing 0.1% SDS. Slab gel electrophoresis was carried out in an ATTO apparatus.

NH_2-terminal analysis. Identification of the NH_2-terminal amino acid in aspartase was made by reaction of the enzyme protein with dansyl chloride followed by acid hydrolysis and polyamide TLC according to the procedure of Gray [9].

COOH-terminal analysis. Aspartase (0.18—0.5 mg) was digested either with carboxypeptidase Y [10] or with carboxypeptidase B (1.8—5.0 μg). Reactions were terminated at different time intervals by lowering the pH to 1.5—2.0 and the precipitate was removed by centrifugation. The supernatant was dried and redissolved in 0.2 M sodium citrate buffer, pH 2.2, and amino acid analysis was performed.

High voltage paper electrophoresis. The solution containing released peptides was lyophilized and redissolved in 50 μl pyridine acetate buffer, pH 3.7 (pyridine/acetic acid/water, 1 : 10 : 289) [11] and spotted onto sheets of Toyo 51A chromatography paper (20 × 40 cm). High voltage paper electrophoresis was then performed in pyridine acetate buffer, pH 3.7, for 150 min at 1000 V.

Amino acid analysis. Amino acid analysis was performed in the 1—10-nmol range using a narrow bore column (0.3 cm diameter) as described by Liao et al. [12].

Circular dichroism (CD) spectroscopy. CD spectra were measured in a JASCO automatic recording spectropolarimeter model J-20 with a CD attachment in N_2 stream using 0.1- or 1.0-mm light path cells. Mean residue weight was assumed to be 128 based on the amino acid composition [1]. The unit of the molecular ellipticity, $[\theta]$ is degree · cm^2 per dmol. The CD spectra of the enzyme were measured at a protein concentration of 1—5 mg/ml. All recordings were made at 25°C. The $[\theta]$ values were calibrated with androsterone.

Fluorescence decay measurements. Fluorescence decay was measured by the single photon counting method (half-width of the light pulse ≃2 ns) at 20°C using an Ortec, 9200 ns fluorescence spectrophotometer with a Hitachi multichannel analyzer, model 505. The decays were deconvoluted by the multicomponent decay function,

$$G(t) = \sum_{k=1}^{n} a_k \exp(-t/\tau_k)$$

with the excitation light (290 nm), using the least-squares fitting method [13].

Other determinations. Aspartase concentration was determined based on an $E^{1\%}_{1cm}$ value of 5.9 at 280 nm [14]. Other protein concentrations were determined by the method of Lowry et al. [15] using bovine serum albumin as the standard. All spectrophotometric determinations were carried out in a Hitachi model 101 spectrophotometer.

Results

Change in the molecular weight upon trypsin-activation

As previously reported [3,4], SDS-electrophoresis on rod gel revealed that the subunit molecular weight of the trypsin-activated aspartase did not change to an appreciable extent. The effect of trypsin-activation on the molecular weight was reexamined by electrophoresis on slab gel. Fig. 1 shows the results of electrophoresis on 7.5% gel according to the method of Laemmli [7]. The activated enzyme migrated slightly ahead of the native enzyme. A mixture of the two enzyme preparations gave a broad band. Additional experiments with the method of Weber and Osborn [8], which is expected to be more reliable for estimation of the molecular weight, exhibited similar results. These results indicate that the change in the molecular weight of aspartase is quite small and oligopeptides, having a molecular weight of more or less 1000, seem to be released. Judged from a narrow and sharp appearance of the band on gel electrophoresis, the trypsin-activated aspartase does not contain an appreciable amount of the native enzyme. In other words, the trypsin treatment causes 100% conversion of the native enzyme into the activated enzyme species under the experimental conditions.

Determination of the terminal amino acid residues

When the NH_2-termini of the native and trypsin-activated enzyme were analyzed by the dansylation method, only *N*-dansyl serine was detected for both

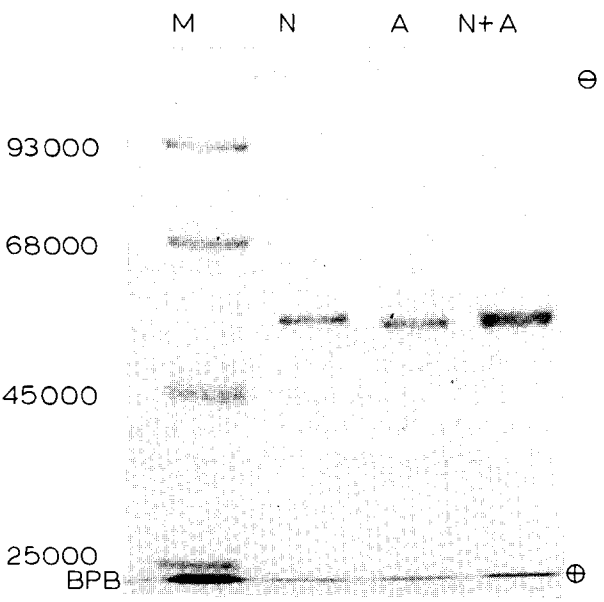


Fig. 1. SDS-polyacrylamide gel electrophoresis on slab gel. N, native enzyme (10 μg); A, trypsin-activated enzyme (10 μg); M, molecular weight markers; 93 000, rabbit muscle phosphorylase *b*; 68 000, bovine serum albumin; 45 000, hen egg albumin; 25 000, pig pancreatic chymotrypsinogen A; BPB, Bromophenol blue. Electrophoresis was carried out according to the method of Laemmli [7]. Proteins were stained with Coomassie brilliant blue.

TABLE I

COOH-TERMINAL ANALYSIS USING CARBOXYPEPTIDASE Y

The native enzyme (250 μg) was treated with carboxypeptidase Y (2.5 μg) at pH 5.0 and 30°C. For the trypsin-activated enzyme, carboxypeptidase Y was added at pH 5.5 and 4°C. When the trypsin-activated enzyme was treated at 30°C, the reaction was too rapid to follow.

Amino acids	Amino acids released (mol/enzyme subunit)				
	Native			Trypsin-activated	
	2 h	5 h	20 h	10 min	20 min
Lysine	0	0	0	0.19	0.26
Arginine	0	0	0	1.03	1.20
Threonine	0.48	0.83	0.88	0.57	0.82
Serine	0.28	0.50	0.86	0	0
Glutamic acid	0.60	1.02	2.08	0	0
Alanine	0	0.52	0.92	0	0
Valine	0	0.40	1.16	0.61	0.95
Tyrosine	0	0	0.72	0.97	1.03

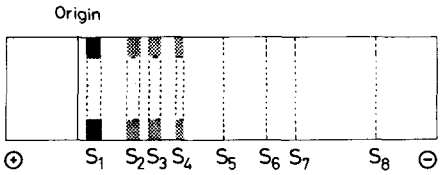
enzyme preparations. A preliminary sequence analysis also revealed that both enzyme preparations have serine at the NH_2-termini and the same sequence in the NH_2-terminal region. It seems, therefore, unlikely that the limited proteolysis for activation occurs at the NH_2-terminal end.

When the native enzyme was treated with carboxypeptidase Y at pH 5.0, 1.02 mol glutamate and 0.83 mol threonine per enzyme subunit were released after 5 h (Table I). In contrast, when the native enzyme was treated with carboxypeptidase Y at pH 5.5, the amount of the amino acids released was only slight even after a prolonged treatment. When carboxypeptidase A was employed in lieu of carboxypeptidase Y, no amino acid was released. It is well known that carboxypeptidase Y releases COOH-terminal glutamate at pH 5.0 but not at pH 5.5 [10], whereas carboxypeptidase A hardly releases any [16]. In view of these results, the COOH-terminal amino acid of the native enzyme was identified to be glutamate. In contrast, the trypsin-activated enzyme released 1.03 mol arginine and 0.97 mol tyrosine per enzyme subunits after a 10 min-treatment with carboxypeptidase Y (Table I). When carboxypeptidase B was employed in lieu of carboxypeptidase Y, arginine was rapidly released. From these results, the COOH-terminal amino acid of the trypsin-activated enzyme was identified to be arginine.

When the native enzyme was treated with carboxypeptidase Y for as long as 20 h, several amino acids other than arginine were released at the amount of 1 or 2 mol per enzyme subunit.

Isolation and analysis of released peptides upon trypsin-activation

Proteolytic products having a molecular weight smaller than 10 000 were separated from the trypsin-activated enzyme preparation by filtration with Amicon PM 10 membrane (see Materials and Methods). The peptides obtained were then separated by high voltage paper electrophoresis. Approx. 8 bands were detected and the most slowly migrated peptide (S_1) exhibited the strongest fluorescence (Fig. 2). The next three peptides (S_2—S_4) were also



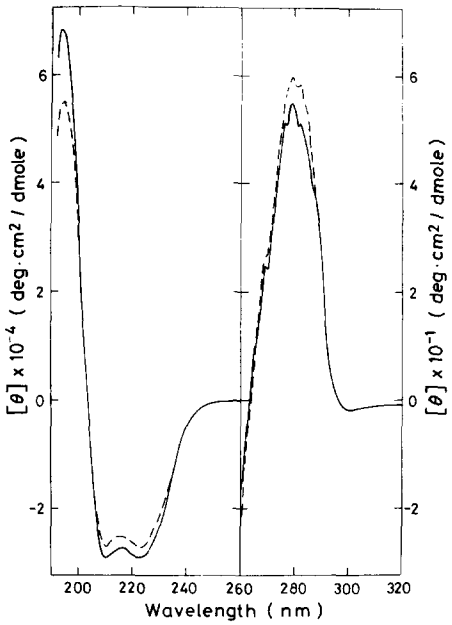


Fig. 2. Separation of released peptides by high voltage paper electrophoresis. Peptides were detected using fluorescamine at both margins of paper and then each peptide was extracted with 5% acetic acid from paper strips (indicated by half-tone).

Fig. 3. CD spectra. The solid line represents the CD spectrum of the native enzyme (1.46 mg/ml for 190—260 nm and 2.68 mg/ml for 260-320 nm) and the dashed line represents that of the trypsin-activated enzyme (1.65 mg/ml for 190—260 nm and 3.02 mg/ml for 260—320 nm). For shorter wavelength range, 0.1-mm light path cells and for longer wavelength range, 1.0-mm cells were used.

TABLE II

AMINO ACID COMPOSITION AND TERMINAL AMINO ACID RESIDUES OF RELEASED PEPTIDES

The possible number of residues is in parentheses.

Amino acids	S_1	S_2	S_3	S_3
Lysine	0.1	0.7 (1)	0.8 (1)	0.8 (1)
Histidine	0	0.2	0.4	0.4
Arginine	0	0.7 (1)	2.0 (2)	1.7 (2)
Aspartic acid	1.1 (1)	1.6 (2)	2.0 (2)	0.5 (1)
Threonine	0.9 (1)	1.1 (1)	1.0 (1)	0.4
Serine	0.8 (1)	1.1 (1)	0.4	1.0 (1)
Glutamic acid	3.1 (3)	2.6 (3)	3.3 (3)	0.5 (1)
Proline	0	0	0.8 (1)	0
Glycine	0.3	0.4	0	1.0 (1)
Alanine	0.3	0.9 (1)	1.3 (1)	0.6 (1)
Valine	0.2	0.7 (1)	3.2 (3)	1.6 (2)
Methionine	0	0	0	0
Isoleucine	0.1	0.4	0.7 (1)	0.4
Leucine	0.2	0.7 (1)	3.0 (3)	0.4
Tyrosine	0.7 (1)	0.9 (1)	0.8 (1)	0.3
Phenylalanine	0	0	0	0
Half-cystine	0	0	0	0
Tryptophan	0	0	0	0
Total	(7)	(13)	(17)	(10)
Yield (%)	10.2	4.4	2.4	3.9
NH_2-terminus *	Tyr	—	—	—
COOH-terminus **	Glu	Glu	Lys ***	Lys

* Identified by the dansylation method.

** Identified with carboxypeptidase Y.

*** Not conclusive due to inavailability of an adequate amount.

reasonably strong. The amino acid composition and terminal amino acids of the major four peptides are shown in Table II. S_1 was a heptapeptide having tyrosine and glutamate at the NH_2- and COOH-termini, respectively. The peptides S_2—S_4 contained 13, 17 and 10 residues, respectively.

CD spectroscopy

The CD spectra of the trypsin-activated and native enzyme preparations are shown in Fig. 3. The native enzyme exhibited a marked positive Cotton effect centered at 194 nm with a $[\theta]$ value of 68 200 and negative troughs at 210 ($[\theta] = -29\,300$) and 222 nm ($[\theta] = -29\,300$). From these values the helical content of the native enzyme was estimated to be at least 70% taking that of poly-L-glutamate as a standard (100%). The $[\theta]$ values of the trypsin-activated enzyme at 194, 210, and 222 nm were 55 130, —27 300, and —27 000, respectively. The helical content of the trypsin-activated enzyme appears to be at least 5% less than that of the native enzyme. Although both enzyme preparations exhibited a weak Cotton effect in the longer wavelength range (260—320 nm), the difference in the two preparations cannot be regarded as significant.

Determination of fluorescence decay time

Aspartase of *E. coli* contains only one tryptophan residue per enzyme subunit [1]. When the native enzyme was excited at 290 nm, a non-exponential

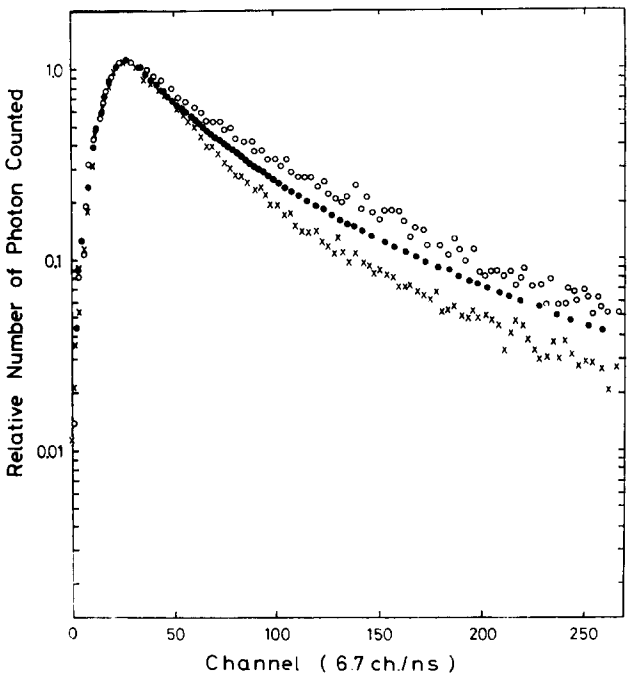


Fig. 4. Fluorescence decay measurements. Protein concentration was adjusted to 1 mg/ml using KCl-potassium phosphate buffer (50 mM potassium phosphate, pH 6.8/0.1 M KCl/5 mM 2-mercaptoethanol/1 mM EDTA). Excitation, 290 nm; fluorescence emission, 340 nm. ○, native aspartase; ●, trypsin-activated enzyme; X, denatured enzyme in 4 M guanidine-hydrochloride. The data for the trypsin-activated enzyme were treated by the least-squares method to avoid complexity.

TABLE III

FLUORESCENCE DECAY OF ASPARTASE

The experimental procedures were described in Materials and Methods.

Samples	Excitation life time (τ, ns) and fraction (a)						
	τ_1	a_1	τ_2	a_2	τ_3	a_3	a_3/a_2
Native enzyme	1.02	0.40	3.10	0.39	7.70	0.21	0.54
Native enzyme denatured in 4 M guanidine-hydrochloride	1.29	0.49	3.30	0.45	9.40	0.06	0.13
Trypsin-activated enzyme	1.02	0.49	3.40	0.36	9.60	0.15	0.42

decay curve was observed and the exponential decay was well simulated with a function, $G(t)$, of three terms (approx. 1, 3 and 8 ns). The native enzyme has a considerable fraction of the longest component ($a_3/a_2 = 0.54$). When the enzyme was excited after denaturation in 4 M guanidine-hydrochloride, the shortest component increased and the decay curve was essentially the same as that of aqueous tryptophan (data not shown). When the trypsin-activated enzyme was excited at the same wavelength, a decay curve having an intermediate feature was obtained. These results shown in Fig. 4 and Table III, indicate that the tryptophan residue in the trypsin-activated enzyme is more exposed than that of the native enzyme, in spite of its higher enzyme activity. ity.

Discussion

Although several examples are known of activation of already active enzymes by limited proteolysis, molecular mechanisms for the phenomena are not necessarily as simple as follows.

Tyrosine hydroxylase (EC 1.14.16.2) of rat brain is 2-fold activated by treatment with trypsin [17]. Concomitant with the activation, the sedimentation coefficient of the enzyme (9.2 S) is decreased to 3.7 S, indicating that the tetrameric enzyme having a molecular weight of 200 000 is dissociated into monomers having a molecular weight of 50 000. In contrast, the trypsin-activated aspartase retains the tetrameric structure, as judged from disc electrophoresis and Sepharose 6B column chromatography [3,4].

Phosphorylase kinase (EC 2.7.1.38) of rabbit muscle is several-fold activated by treatment with trypsin [18]. This activation appears to be a modification of a specific serine residue at the regulatory site, which is otherwise enzymatically phosphorylated [19]. The activation of acetyl-CoA carboxylase (EC 6.4.1.2) of rat liver by trypsin [20] is also a similar example, in that the trypsin-activation is associated with a modification of the regulatory site for citrate activation [21]. No evidence is so far available which suggests that the trypsin-activation of aspartase is associated with the regulatory site modification.

When fructose-1,6-bisphosphatase (EC 3.1.3.11) is activated by a limited

proteolysis, the optimum pH for the activity is altered to a marked extent and an apparent activation is attained by this pH shift [22]. In the case of aspartase, such a pH shift can not be regarded as the cause for the activation.

In the case of phenylalanine hydroxylase (EC 1.14.16.1), the protease-mediated activation involves a release of the specific peptide region, which is inhibitory for the activity [23].

Thus, a variety of features are known for protease-activation. However, the mechanism for aspartase activation can so far be explained by none of them.

Available experimental evidence suggests that the trypsin-mediated activation of aspartase proceeds as follows: trypsin cleaves the polypeptide chain in the COOH-terminal region of the native enzyme and releases oligopeptides S_1—S_4. In view of the fact that all preparations of the trypsin-activated enzyme were essentially homogeneous, the larger peptides such as S_2 and/or S_3 appear to be released first, followed by secondary cleavage to yield S_1 and/or S_4. Consequently, the COOH-terminal glutamate of the native enzyme is altered to arginine upon activation.

As for the conformational alteration of the enzyme molecule upon trypsin-activation, the following observations support the view that the activated enzyme species has somewhat looser conformation than that of the native enzyme: (1) tryptophan residue became more exposed to outer environment; (2) the helical content of the enzyme protein was decreased.

The non-exponential features of the fluorescence decay curves of the enzyme tryptophan are of interest. Fleming et al. [24] reported that aqueous tryptophan and two related peptides give non-exponential fluorescence decay which can be analyzed in terms of two exponentials. The results were interpreted in terms of two kinds of trapped conformers in the excited state that interconvert no quicker than the time scale of the fluorescence [24]. Furthermore, the three fractions obtained in the present study appear to be quite similar to those of pig heart lactate dehydrogenase (EC 1.1.1.27) as reported by Torikata et al. [25]. They studied the decay of tryptophan emission from lactate dehydrogenase following pulsed excitation. The pulsed and steady-state fluorescence was discussed in terms of a model with three life time classes of tryptophan, viz. 1, 4 and 8 ns.

In the light of the above information, the trypsin-mediated activation of aspartase is regarded as a rare example for production of a highly efficient biocatalyst endowed with a strong cooperativity, in spite of a minor degree of limited proteolysis. In addition, it is of interest that the trypsin-mediated activation is associated with the peptide cleavage at the COOH-terminal.

The physiological significance of the above phenomenon remains to be elucidated.

Acknowledgements

The authors wish to express their sincere gratitude to Professor H. Katsuki of this Department for continuous encouragement and valuable discussions during this investigation. We are also indebted to Professors Y. Nishijima and M. Yamamoto, Department of Polymer Chemistry, for measurements of fluorescence decay time, to Professors S. Takahashi and T. Ooi, Institute for Chemical

Research, for CD measurements, and to Professor T. Murachi, Faculty of Medicine, Kyoto University, for help with the sequence analysis. Thanks are also due to Dr. K. Mizuta for her cooperation in the initial part of this work and Miss I. Kameda for devoted assistance. This investigation was supported in part by Grants-in-Aid for Fundamental Research in Chemistry from the Japan Society for the Promotion of Science, from the Ministry of Education, Science, and Culture of Japan, No. 558060, 521312 and 438020, and from the Naito Foundation.

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