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AMINO ACID RESIDUES IN ASPARAGINASE (*ESCHERICHIA COLI* HAP) ASSOCIATED WITH ITS ENZYMIC ACTIVITY

YOSHIHISA NISHIMURA, HIROSHI MAKINO, OSAMU TAKENAKA AND YUJI INADA
Laboratory of Biological Chemistry, Tokyo Institute of Technology, Meguroku, Tokyo (Japan)

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SUMMARY

The relationship of the amino groups and tyrosine, arginine, tryptophan, serine and cysteine residues in asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) isolated from *Escherichia coli* HAP with the enzymic activity was investigated by measuring the reactivity of each residue or group with various chemicals, together with the change of enzymic activity. The following facts were revealed: The total 90 amino groups in the asparaginase molecule were classified into three types; (1) approximately 32 residues reactive with monochlorotrifluoro-*p*-quinone (CFQ), (2) approximately 45 residues becoming reactive with CFQ after alkali denaturation and (3) the remaining 13 residues nonreactive with CFQ. 4 ± 0.5 amino groups reactive with CFQ in the native enzyme were closely associated with the enzymic action. About 15 out of 47 tyrosine residues in the enzyme molecule were modified with tetranitromethane and the activity of the modified molecule decreased to 8% of the original level. About 12 out of the total 32 arginine residues and 2 or 3 out of the total 5 tryptophan residues per molecule were modified with glyoxal and H_2O_2–dioxane, respectively, and the activities of the modified enzymes decreased to 25% and 40% of the original. These activity drops may be attributed to a destruction of the tertiary structure of the enzyme. Serine residue reactive with diisopropyl-fluorophosphate (DFP) and cysteine were not present in the asparaginase molecule. The subunit structure of the asparaginase molecule was discussed in relation to these data.

INTRODUCTION

The finding that L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1), an enzyme catalyzing the hydrolysis of L-asparagine to L-aspartic acid and ammonia, acts as antitumor agent[1–3] in some kinds of mammals led to intensive clinical investigations for remission in human leukemia[4,5] and to studies on the physicochemical properties of the asparaginase molecule. HILL *et al.*[6] obtained an asparaginase preparation in a highly purified state, and recently, TANAKA *et al.*[7] succeeded in preparing

Abbreviations: CFQ, monochlorotrifluoro-*p*-quinone; DFP, diisopropyl fluorophosphate.

crystalline asparaginase from *Escherichia coli* HAP in a large scale, and they determined the amino acid composition and the molecular weight of the enzyme to be 141000. KIRSCHBAUM *et al.*[8] found that the molecular weight of asparaginase from *E. coli* B was 255000 and the molecular weight decreased to 132000, 68000 and 19000–24000 by treatments with 2 M NaCl, 1% sodium dodecyl sulfate and 8 M urea, respectively.

ALTENBERN AND HOUSEWRIGHT[9] reported that asparaginase isolated from *Brucella abortes* 19 appeared to be a sulfhydryl type enzyme. WHELAN AND WRISTON[10] found one cysteine residue per minimum molecular weight of 22170 for the enzyme (*E. coli* B) reduced by dithiothreitol. ARENS *et al.*[11] found no cysteine residue in the enzyme molecule and showed that the enzymic activity was not inhibited by *p*-chloromercuribenzoate nor by *N*-ethylmaleimide.

The identification of amino acid residues associated with the active site of enzyme is clearly of supreme importance for studying the mechanism of enzymic action. However, there has been little information concerning the amino acid residues in the asparaginase molecule involved in the enzymic activity.

Extensive studies on the states of amino acid residues in enzymes in relation to the enzymic activities have been performed in our laboratory and others by spectrophotometrical methods[12,13] and by the modification of amino acid residues with chemicals such as glyoxal for the arginine residue[14], tetranitromethane for the tyrosine residue[15], H_2O_2–dioxane for the tryptophan residue[16], ethyl morpholinyl carbodiimide for the carboxyl group[17] and monochlorotrifluoro-*p*-quinone (CFQ) for the amino group[18]. The present paper deals with the identification of amino acid residues associated with the enzymic activity of asparaginase and their states in the asparaginase molecule by means of the chemical modification with various compounds.

EXPERIMENTAL

Material

Crystalline L-asparaginase obtained from *E. coli* HAP was donated from the Kyowa Hakko Kogyo Co. The specific activity of this enzyme was 220 I.U./mg protein. The molar extinction coefficient of the enzyme was determined in the present study to be $8.83 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 278 nm, assuming its molecular weight to be 141000 (ref. 7). *N*-Ethylmaleimide, *p*-chloromercuribenzoic acid, glyoxal and dioxane were purchased from Tokyo Kasei Kogyo Co., and monochlorotrifluoro-*p*-quinone (CFQ) from Seikagaku Kogyo Co. Tetranitromethane and the Ellman reagent of 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from the Wako Junyaku Co. and the Nakarai Chemical Co., respectively. Dioxane was redistilled before use.

Procedure

Modifications of amino group and tyrosine, tryptophan and arginine residues were carried out with CFQ, tetranitromethane, H_2O_2–dioxane and glyoxal as modifying reagents, respectively. The method of modification of amino groups with CFQ was described in detail by NAKAYA *et al.*[18]. To 4.5 ml of 1.1 μM asparaginase in 0.2 M phosphate buffer, pH 8.5, was added 0.5 ml of CFQ at various concentrations, and the reaction mixture was left standing for 2.5 h at room temperature. The degree of modification of amino groups in the enzyme molecule was estimated spectrophoto-

metrically by measuring the difference in absorbance at 353 nm between the sample solution and the control without the enzyme. The difference in molar extinction coefficient of one amino group was 21600 $M^{-1} \cdot cm^{-1}$. The same experiment as above was carried out for the denatured asparaginase incubated in alkaline solution, pH 12.9, for 4 h.

The reactivity of tyrosine residues with tetranitromethane was measured spectrophotometrically by the modified method of RIORDAN *et al.*[15]; 4.5 ml of 11.1 μM asparaginase in 0.1 M Tris, pH 8.0, were mixed with 0.5 ml of ethanolic solution of tetranitromethane, and the reaction mixture was incubated for 2 h at room temperature. The number of the modified tyrosine residues in the enzyme molecule was calculated by assuming the difference in the molar extinction coefficient of modified tyrosine ethyl ester with tetranitromethane at pH 4.0–9.0 to be $1.26 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 427 nm. The number of tyrosine residues modified with tetranitromethane was also determined with a Hitachi amino acid analyzer model KLM-3.

Arginine residues were modified with glyoxal according to the method of NAKAYA *et al.*[14]; to a mixture of 150 μM asparaginase (1.0 ml) and 3.0 ml of 0.5 M bicarbonate buffer, pH 8.6, was added 1.0 ml of glyoxal at various concentrations. The reaction mixture was kept standing for 3 h at room temperature, and then an appropriate amount of 10% trichloroacetic acid was added to the reaction mixture. Modified protein thus obtained as precipitate was subjected to amino acid analysis.

Tryptophan residues were oxidized with H_2O_2 in 10% dioxane[16], and the degree of oxidation of the residues was determined spectrophotometrically by measuring the increase of absorbance at 320 nm due to the oxidation of tryptophan chromophore. The difference in the molar extinction coefficient between intact tryptophan and its oxidized form was calculated to be $1.48 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 320 nm. The reaction system was a mixture of 2.0 ml of 37.5 μM asparaginase, 2.5 ml of 0.02 M phosphate buffer, pH 8.0, and 0.5 ml of H_2O_2 in aqueous 10% dioxane.

The enzymic activity of asparaginase was measured in relation to the modification of amino group and tyrosine, arginine and tryptophan residues in the enzyme molecule. The activity was determined by measuring the amount of ammonia released from asparagine with the Nessler reagent. To 2.0 ml of 15 mM asparagine in 0.02 M phosphate buffer, pH 8.0, was added 0.5 ml of 0.005 μM enzyme solution. After incubation for 30 min at 37°, the reaction mixture was diluted ten times with distilled water and 0.5 ml of the Nessler reagent was added, and then the absorbance at 425 nm was measured. Three different control solutions were required for an accurate determination of ammonia with the Nessler reagent; the first with distilled water (0.5 ml) instead of the asparaginase solution, the second with 0.02 M phosphate buffer (2.0 ml) instead of the asparagine solution and the last with 2 mM NH_4Cl solution in 0.02 M phosphate buffer (2.0 ml) instead of the asparagine solution.
The first one was the reference for the measurement of the absorbance of a sample solution at 425 nm, and the latter two were employed to examine possible disturbance of color development by chemicals and ions in the reaction mixture.

The question whether asparaginase is a "serine enzyme" or a "sulfhydryl enzyme" was examined with DFP or with *N*-ethylmaleimide, *p*-chloromercuribenzoic acid and Ellman reagent. A mixture of 10 μM asparaginase (10 ml) and 10 mM DFP (0.1 ml) was incubated for 2.5 h at pH 8.0, and its enzymic activity was measured as described above. The reactions of the sulfhdryl group in the enzyme with *p*-chloro-

mercuribenzoate[19], *N*-ethylmaleimide[20] and ELLMAN's[21] reagent were followed at pH 8.0 at room temperature. The systems were as follows: 1.0 μM asparaginase (1.0 ml), 0.1 mM *p*-chloromercuribenzoate (1.0 ml) and 0.1 M phosphate buffer, pH 8.0, (1.0 ml) for *p*-chloromercuribenzoate; 10 μM asparaginase (1.0 ml), 10 mM *N*-ethylmaleimide (1.0 ml) and 0.1 M phosphate buffer (1.0 ml) for *N*-ethylmaleimide; 300 μM asparaginase (0.5 ml), 2 mM Ellman reagent (0.5 ml) and 0.1 M phosphate buffer (1.0 ml) for the Ellman reagent. Spectral measurements were made with a Cary recording spectrophotometer model 14M-50 using 1.0-cm cells. Throughout this paper, the number in moles of amino acid residues modified with a reagent per mole of asparaginase is represented by n.

RESULTS

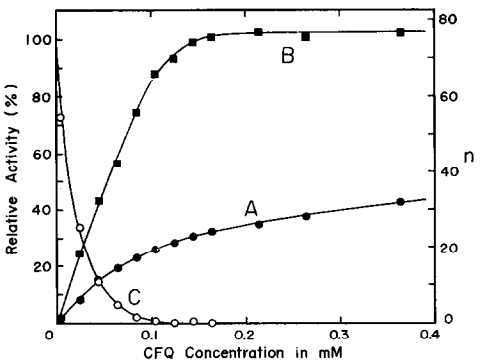
Serine and cysteine residues

The enzymic activity of asparaginase was not inhibited by DFP, which is known to react specifically with the hydroxyl group of the serine residue involved in the catalytic site of proteolytic enzymes[22,23]. The molar contents of cysteine residues in the asparaginase molecule were determined to be 0.03 for native asparaginase and 0.04 for the denatured one treated with 7 M urea for 24 h by measuring the degree of the modification of cysteine residue with Ellman reagent. These values were obtained by assuming the molecular weight of the enzyme to be 141000 and by assuming the molar extinction coefficient of sulfhydryl group reacted with Ellman reagent to be $1.1 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 412 nm. *p*-Chloromercuribenzoate, *N*-ethylmaleimide and Ellman reagents all did not inhibit the enzymic activity. These results indicate that cysteine or serine residue reactive with DFP is not present in the asparaginase molecule.

Amino groups

The molar content of lysine residues in the asparaginase molecule is 90 according to the amino acid analysis by TANAKA *et al.*[7]. The reaction curves (the degree of reaction is plotted against the reagent concentration) obtained for native and alkali-denatured preparations of asparaginase are reproduced in Curves A and B in Fig. 1, respectively. The number of amino groups in the enzyme molecule reactive with CFQ increases with increasing CFQ concentration, and reaches a constant level of $n = 32$ for native asparaginase. For the denatured asparaginase, the number reaches a level of $n = 77$. 45 amino groups have been made reactive with CFQ, presumably by the breaking of the tertiary structure of asparaginase by contact with alkali. This result indicates that approximately 32 out of the total 90 amino groups per molecule of the enzyme are free to react with CFQ and, therefore, are present at the surface of the molecule, and about 45 residues becoming reactive after denaturation and the remaining 13 nonreactive groups are embedded in the interior of the protein molecule or bound with other amino acid residues in the enzyme molecule.

The enzymic activity of asparaginase decreases with increasing CFQ concentration, as is clear from Curve C in Fig. 1, and is completely lost above 0.1 mM of CFQ. An abrupt decrease of the enzymic activity at such a low concentration of CFQ implies that some amino groups reactive with CFQ are associated with the enzymic activity of asparaginase. The relation between the enzymic activity and the degree



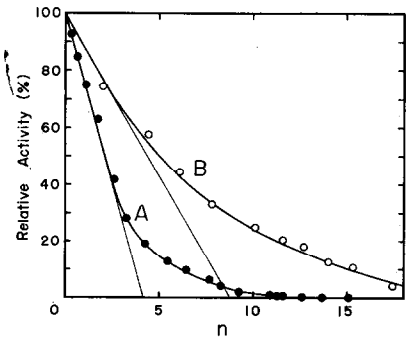


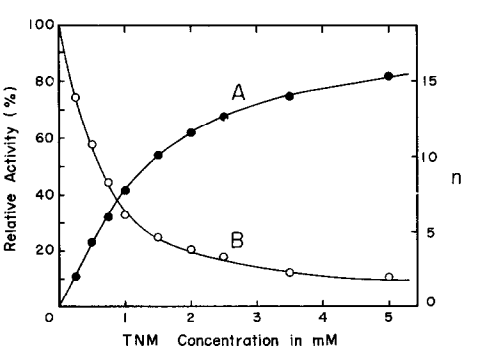
Fig. 1. Reaction curves obtained for amino groups of asparaginase at pH 8.5. Curves A and B, native and denatured asparaginase (1 μM) treated in alkaline solution of pH 12.9 for 4 h, respectively. Curve C shows the enzymic activity plotted against CFQ concentration.

Fig. 2. The relation between the enzymic activity of asparaginase and the mole of amino acid residues modified with a reagent per molecule of the enzyme. Curve A, amino group of asparaginase (5 μM); Curve B, tyrosine residue of the enzyme (50 μM). The straight line extrapolations intersect abscissa at 4.1 $\pm$ 0.5 for amino group and at 9 $\pm$ 1 for tyrosine residue.

of modification of amino groups was studied in more detail at fairly low concentrations of CFQ. The activity of asparaginase was plotted against the number of the modified amino groups (Curve A in Fig. 2). The extrapolation of the initial linear stage of the modification with CFQ intersects the abscissa at 4.1 $\pm$ 0.5 amino groups per mole of asparaginase. This indicates that about 4 amino groups in the enzyme molecule are closely related to the enzymic action of this enzyme.

Tyrosine residues

Curve A in Fig. 3 shows the reaction curve obtained by plotting the moles of modified tyrosine residues in the asparaginase molecule against tetranitromethane concentration. Curve B in the same figure represents the course of the asparaginase



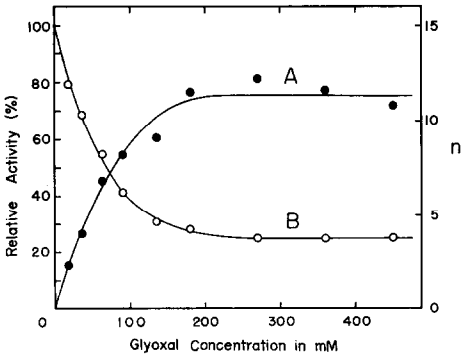


Fig. 3. The reaction curve obtained for tyrosine residues in asparaginase (50 μM) at pH 8.0 (Curve A). Curve B shows the enzymic activity plotted against TNM concentration.

Fig. 4. The reaction curve obtained for arginine residues in asparaginase (30 μM) at pH 8.6 (Curve A). Curve B shows the enzymic activity plotted against glyoxal concentration.

activity which drops upon treatment with tetranitromethane. The degree of modification of the tyrosine residues increases with increasing tetranitromethane concentration, and above 3.5 mM of tetranitromethane, the number of the modified residues tends to approach a constant level of $n = 15$. The enzymic activity decreases sharply with increasing tetranitromethane concentration and approaches a level of 8% of the original level. These results indicate that about 15 of the total 47 tyrosine residues in the asparaginase molecule are reactive with tetranitromethane, and the remaining 32 residues are nonreactive with tetranitromethane in the native state of asparaginase and that some tyrosine residues reactive with tetranitromethane of low concentrations may be associated with the enzymic activity. The number of tyrosine residues involved in the enzymic activity was roughly estimated to be 9 ± 1 from Curve B in Fig. 2. The extrapolation of the linear portion of Curve B intersects the abscissa at $n = 9 \pm 1$.

Arginine residues

E. coli HAP asparaginase contains 32 arginine residues per molecule (mol. wt. 141000). Curve A in Fig. 4 is the reaction curve obtained for arginine residues with glyoxal as a modifying reagent. The number of modified arginine residues increases with increasing glyoxal concentration and reaches a constant level of $n = 12$ above 300 mM of glyoxal. This indicates that approximately 12 out of 32 arginine residues in the asparaginase molecule are reactive with glyoxal.

The enzymic activity of asparaginase drops to 25% of the original at low concentrations of glyoxal, but no further drop of activity is observed above 0.2 M of glyoxal (Curve B in Fig. 4).

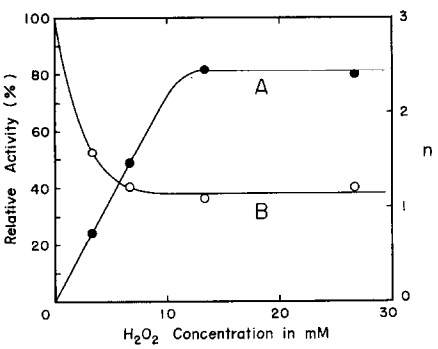


Fig. 5. The reaction curve obtained for tryptophan residues in asparaginase (13.3 μM) at pH 8.2 (Curve A). Curve B shows the enzymic activity plotted against H_2O_2 concentration.

Tryptophan residues

The reaction curve obtained for tryptophan residues in asparaginase is reproduced in Curve A in Fig. 5. The curve rises steeply up to 10 mM H_2O_2 and above this concentration, shows a constant level of $n = 2.5$, indicating that 2 or 3 out of the total 5 tryptophan residues in the enzyme molecule are oxidized, and the remaining 3 or 2 residues are not oxidized with H_2O_2. The enzymic activity does not decrease below 40% with increasing H_2O_2 concentration. This implies that tryptophan residues oxidizable with H_2O_2 have little association with the enzymic action.

TABLE I

ENZYMIC ACTIVITY OF ASPARAGINASE TREATED WITH MODIFYING REAGENTS

The activity is expressed in % of the control without reagents. n_t stands for the total number of each amino acid residue in the asparaginase molecule, assuming its molecular weight to be 141 000.

Residue	*Reagent*	*Concentration*			*Number of residues modified*	*Activity (%)*
		Enzyme (μM)	*Reagent (mM)*	*Ratio*		
Serine ($n_t = 67$)	DFP	10	0.1	10	—	100
Cysteine	Ellman	40	0.2	5	0	99
Arginine ($n_t = 32$)	Glyoxal	30	300	1000	12	25
Tryptophan ($n_t = 5$)	H_2O_2	13.3	13	1000	2–3	40
Tyrosine ($n_t = 47$)	Tetranitromethane	50	6	120	15	8
Amino group ($n_t = 90$)	CFQ	5	0.2	40	8	1

DISCUSSION

The relationships between the enzymic activity and the modification with various chemicals of amino acid residues in asparaginase from *E. coli* HAP are summarized in Table I, where n_t stands for the total number of each amino acid residue per asparaginase molecule. The total number of each amino acid residue was calculated from the minimum number of residues obtained by amino acid analysis[7], assuming its molecular weight to be 141000. This molecular weight is in agreement with the molecular weight, 139000, of *E. coli* B asparaginase determined by the method of WHELAN AND WRISTON[10] and also with the molecular weight, 132000, of *E. coli* B asparaginase determined by the method of KIRSCHBAUM *et al.*[8] in the presence of 2 M NaCl. The molar amino acid composition of asparaginase listed in Table I is quite similar to that obtained by WHELAN AND WRISTON[10], ARENS *et al.*[11] or HILL *et al.*[6] except for tryptophan.

As is clear from Table I, serine and cysteine residues reactive with DFP and Ellman reagent, respectively, were not present in the asparaginase molecule. 12 out of the total 32 arginine residues and 2 or 3 out of the total 5 tryptophan residues per molecule were modified with glyoxal and H_2O_2–dioxane, respectively, and the enzymic activities decreased to 25% and 40% of the original level on the modifications. The decrease of activity may be attributed to some destruction of the tertiary structure of the enzyme since the inactivation was incomplete.

The modification of 15 out of 47 tyrosine residues and 8 out of 90 amino groups by tetranitromethane and CFQ, respectively, at fairly low concentrations gave rise to almost complete inactivation of the enzymic activity. The numbers of tyrosine residues and amino groups per molecule of asparaginase associated with the enzymic activity were estimated from Curves B and A in Fig. 2 to be 9 ± 1 and 4 ± 0.5, respectively. A question arises whether these residues or groups directly participate in the enzymic activity or the modification of these residues induce the dissociation

of the enzyme molecule into inactive subunits. The subunit structure of asparaginase was extensively studied with ultracentrifugal technique by KIRSCHBAUM *et al.*[8] and with Sephadex chromatography by ARENS *et al.*[11]. KIRSCHBAUM *et al.*[8] found that the asparaginase molecule is dissociated either by dilution or by adding salts, dodecylsulfate and urea, and a component with a sedimentation constant of $s^\circ_{20,w} = 8.6$ S is transformed into a component with $s^\circ_{20,w} = 5.6$ S or $s^\circ_{20,w} = 4$ S. ARENS *et al.*[11] demonstrated that a component showing a sedimentation constant of 5.6 S apparently has the highest activity. In our laboratory, asparaginase with three modified amino groups showing an enzymic activity of 26% of the control without CFQ was subjected to ultracentrifugal analysis. Although the analysis is not yet complete enough for publication, it can safely be said that the ultracentrifugal pattern thus obtained shows a major symmetrical peak with a sedimentation coefficient of 8.6 S and a minor peak with a larger Svedberg unit. The content of the minor component was roughly 7% of the major component. These results dismiss the latter possibility mentioned above, and they indicate that the modification of 4 amino groups in the asparaginase molecule causes complete inactivation without dissociation of the molecule into subunits and that these groups play an important role in enzymic action of asparaginase. The authors would like to propose the hypothesis that the asparaginase molecule of the molecular weight of 141000 consists of 4 subunits (mol. wt. 35000), having one amino group in each subunit involved in the enzymic activity. This seems to be supported by the following experimental results of ARENS *et al.*[11]. The molecular weight of a single chain of the asparaginase molecule was calculated to be 35000 by the quantitative determination of the N-terminal amino acid.

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