SOME ASPECTS OF THE STRUCTURE OF THE ACTIVE CENTER OF THE ENZYME L-ASPARAGINASE

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Recently, the enzyme L-asparaginase (L-asparagine amidohydrolase 3.5.1.1) has attracted the attention of many workers as an antileukemic agent [1, 2] and immunodepressant [3]; however, the structure of this enzyme has so far been little studied. There is information in the literature only on some L-asparaginase inhibitors of different origins [4-6], without their systematic study, although inhibitory analysis provides the possibility of determining the nature and topochemistry of the functional groupings of the active center of the enzyme.

The aim of the present work was to determine by the method of inhibitory analysis the functional groupings playing an important part in the behavior of the enzymatic reaction of the L-asparaginase of E. coli B675. The amino-acid composition of the L-asparaginase of E. coli B675 is close to that of the L-asparaginase of E. coli B [12], but differs strongly from that of the L-asparaginase of guinea-pig serum [13]. The amino-acid composition of the L-asparaginase of E. coli B675 is as follows:

Amino acid	Number of amino acid residues per mol. wt. of 25,000	Amino acid	Number of amino acid residues per mol. wt. of 25,000
Tryptophan	2	Alanine	20.8
Lysine	14.2	Valine	20.2
Histidine	2.2	Methionine	4.0
Arginine	6.0	Isoleucine	8.5
Aspartic acid	33	Leucine	16.0
Threonine	21.2	Tyrosine	8.2
Serine	10.8	Phenylalanine	6.1
Glutamic acid	13.6	Cystine	0.5
Proline	8.4	Ammonia	30
Glycine	19.1		

The molecule of the enzyme L-asparaginase of E. coli B675 has an enormous number of L-aspartic acid residues and, obviously (judging from the amount of ammonia) asparagine residues. The amounts of threonine, alanine, and valine are also fairly large, while the amounts of tryptophan and histidine are small.

The chemical modification of the protein permits an idea of the nature of the most important functional groups in the molecule, and therefore we have tested as inhibitors reagents specifically blocking the individual functional groups of the enzyme. Figures for the inhibition of the activity of the L-asparaginase of E. coli B675 are given below.

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Compound	Concentration, M	Inhibition, %
Iodoacetic acid	$1 \cdot 10^{-2}$	15
Iodoacetamide	$1 \cdot 10^{-3}$	10
o-Iodoazobenzoate	$1 \cdot 10^{-3}$	8
p-Chloromercuribenzoate	$1 \cdot 10^{-3}$	0
Methylmercuribenzoate	$1 \cdot 10^{-3}$	0
N-Ethylmaleimide	$1 \cdot 10^{-2}$	0
Mercaptoethanol	$1 \cdot 10^{-3}$	10
Thioglycolic acid	$1 \cdot 10^{-3}$	0
Acetic anhydride	$1 \cdot 10^{-3}$	0
Succinic anhydride	$1 \cdot 10^{-3}$	0
Diisopropyl phosphorofluoridate	$1 \cdot 10^{-3}$	13
Diazotized sulfanilic acid	$1 \cdot 10^{-3}$	100
1-Fluoro-2,4-dinitrobenzene	$1 \cdot 10^{-2}$	50
Methylene Blue (with photooxidation)		70
Methylene Blue		10
Bromosuccinimide	$1 \cdot 10^{-3}$	96
	$1 \cdot 10^{-4}$	25
Carbodiimide (solution)	$1 \cdot 10^{-3}$	0

Consequently, such specific blocking agents of sulfhydryl groups as N-ethylmaleimide and mercaptide-forming reagents – p-chloromercuribenzoate and methylmercury nitrate – are not inhibitors of the enzymatic activity of the L-asparaginase of E. coli B675. Reagents alkylating sulfhydryl groups (iodoacetic acid, iodoacetamide) or oxidizing them (cystine, o-iodoazobenzoate) are less specific, since they react partially with methionine and histidine [14]. These reagents give a slight decrease in enzymatic activity.

Since after the blocking of sulfhydryl groups by different more-or-less specific reagents the enzymatic activity is retained, sulfhydryl groups play no fundamental role in the course of the enzymatic reaction.

It is interesting to observe that the L-asparaginase of guinea-pig serum is inhibited by p-chloromercurisulfonate (to the extent of 56%) but is not inhibited by N-ethylmaleimide [6]. Disulfide groups obviously do not form part of the active center of the enzyme, since mercaptoethanol and thioglycolic acid do not suppress the activity of the L-asparaginase of E. coli B675.

The results obtained in the experiments with acetic anhydride (it does not inhibit the L-asparaginase activity) make the participation of amino groups in the action of the enzyme unlikely. Even after partial deamination the enzyme retains its activity [15]. The hydroxy groups of serine and threonine, which are bound by diisopropyl phosphorofluoridate are probably of no importance for the catalytic action of the enzyme, either.

Iodine, 1-fluoro-2,4-dinitrobenzene, diazotized sulfanilic acid, and also photooxidation in the presence of Methylene Blue lead to the inactivation of the enzyme. Consequently, the phenol and imidazole groupings of tyrosine and histidine, respectively, are present in the catalytic section of the enzyme L-asparaginase from E. coli B675.

EXPERIMENTAL

Isolation and Purification of the L-Asparaginase. The experiments were performed with highly purified E. coli B675 L-asparaginase obtained by a method proposed by us previously [7] and purified by Roberts' method [8]. The specific activity of the enzyme was ~250 IU/mg of protein.

Amino-Acid Composition. The amino-acid analysis was performed as described by Moore and Stein [9]. Samples of dry lyophilized L-asparaginase (10-15 mg) were hydrolyzed with 6 N hydrochloric acid for 22, 48, and 72 h. The tryptophan was determined after alkaline hydrolysis by Spies' method [10]. The amino-acid composition was determined on a "Bio-Cal" BC-200 automatic amino-acid analyzer.

Investigation of Inhibitory Properties. The samples contained 0.1 ml of enzyme solution (~ 10 IU of E. coli B675 L-asparaginase) stabilized with 0.1% of albumin in 0.03 M tris-HCl buffer, pH 8.6, and 0.6 ml of the substrate – a 0.04 M solution of L-asparagine in the same buffer; the solutions of the inhibitors and the buffer solution were made up to a final volume of 1.8 ml.

Preincubation of the enzyme with the inhibitor for 30 min (with the exception of 1-fluoro-2,4-dinitrobenzene, for which it was 3 h); incubation of the reaction mixture with the substrate at 37°C for 15 min.

The enzymatic reaction was stopped by the addition of TCA at 0°C to a final concentration of 5%. The activity of the enzyme was determined by the direct Nesslerization of the reaction product – ammonia [11].

SUMMARY

Some functional groups in the active center of the enzyme L-asparaginase have been studied by the method of inhibitory analysis.

It has been established that the phenol groups of the tyrosine residues and the imidazole groups of the histidine residues are important for the occurrence of the enzymatic reaction.

The blocking of sulfhydryl, disulfide, and amino groups, and also of the hydroxy groups of serine and threonine, does not suppress the enzymatic activity.

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