CHEMICAL MODIFICATION OF THE TRYPTOPHAN RESIDUES OF THE L-ASPARAGINASE OF E. coli WITH N-BROMOSUCCINIMIDE

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Recently, intensive investigations of the structure of the active center of L-asparaginase have been performed. The role of the serine, histidine, and tyrosine residues and of −SH and of −S−S− groups in the catalytic activity of this enzyme has been studied [1, 2]. We previously put forward a hypothesis on the importance of the tryptophan residue in L-asparaginase [2]. In the present paper we give the results of a study of the role of the tryptophan residues by the chemical modification of the enzyme with N-bromosuccinimide (N-BS). The action of this reagent on L-asparaginase both in acid (pH 4.0) and in alkaline (pH 8.6) media leads to a considerable fall in the catalytic activity of the enzyme (Fig. 1). In an acid medium, N-BS in a concentration of $5 \cdot 10^{-7}$ M inhibits the asparaginase activity by 70%, and in a concentration of 10^{-6} M by 100%. In an alkaline medium, complete inactivation of the enzyme is observed only at a concentration of 10^{-4} M. In view of this, the further investigation of the influence of N-BS on L-asparaginase was performed in an acid medium.

The dependence of the L-asparaginase activity on the action of N-BS in various molar ratios to the tryptophan residues of the enzyme molecule is shown in Table 1. It has been established that 5.25 moles of N-BS per mole of tryptophan is sufficient for the complete inactivation of the L-asparaginase.

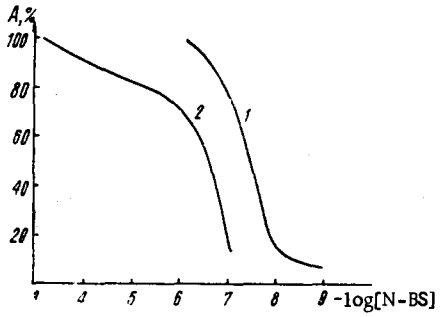

Fig. 1. Dependence of the enzymatic activity of L-asparaginase on the molar concentration of N-bromosuccinimide.

TABLE 1. Action of N-BS on L-Asparaginase at Various Molar Ratios to the Tryptophan Residues

$M_{N\text{-}BS}$: $M_{tryptophan}$	Inhibition, %		
	enzyme + N-BS	enzyme + substrate + N-BS	enzyme + inhibitor + N-BS
5,25 : 1	100	12	—
5,00 : 1	96	5	4
3,00 : 1	60	0	0
1,00 : 1	40	0	0

In order to avoid the oxidation of the tryosine [3, 4], for the cleavage of a peptide bond [5, 6] by an excess of N-BS this reagent was used to modify L-asparaginase in molar ratios with respect to the tryptophan not exceeding 5.25 : 1.

The homogeneity of the enzyme that we had modified was confirmed by gel filtration through Sephadex G-150.

The UV spectra of the native and modified L-asparaginases showed that the absorption maximum at 280 mμ characteristic for the protein and due to the tryptophan and tyrosine residues became weaker when N-BS was added to the enzyme, and a new absorption zone appeared at 250-260 mμ (Fig. 2) which, according to the literature [7, 8], is characteristic for oxidized indole rings of tryptophan.

Using the method of Patchornic and Witkon [7], from the maximum reduction in λ_{max} at 280 mμ we calculated that under the conditions of our experiments N-BS oxidizes one (1.01) tryptophan residue per subunit (mol. wt. = 33,000) or four residues per molecule of L-asparaginase, i.e., all the tryptophan residues in the enzyme molecule. Under

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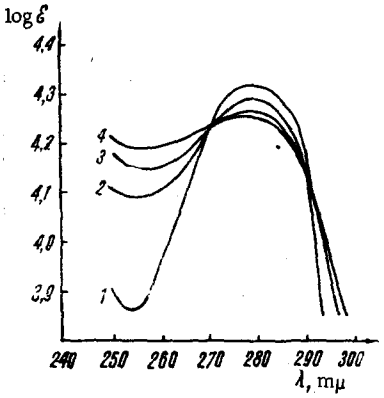

Fig. 2. UV spectrum of native (1) and N-bromosuccinimide-modified (2, 3, 4) L-asparaginases.

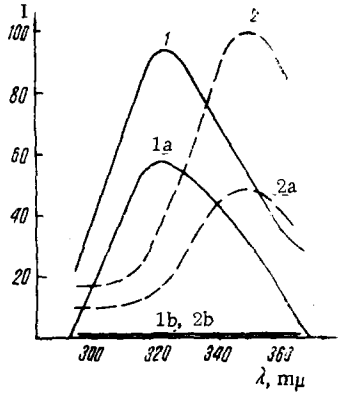

Fig. 3. Influence of N-BS on the fluorescence of L-asparaginase (1, 1a) and of L-tryptophan (2, 2a). 1, 2) Native enzyme and L-tryptophan; 1a, 2a) $M_{N\text{-}BS} : M_{tryptophan} = 5 : 1$; 1b, 2b) $M_{N\text{-}BS} : M_{tryptophan} = 15 : 1$.

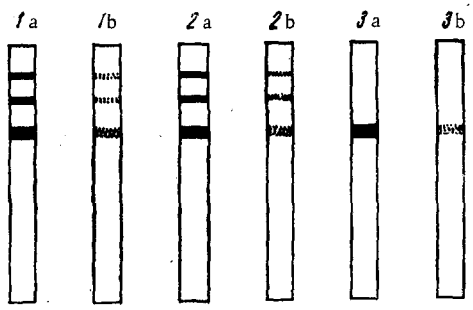

Fig. 4. Electrophoresis of the native and modified L-asparaginases: 1) native enzyme, 2) enzyme modified with substrate protection, 3) modified enzyme; a) protein, b) enzyme activity.

these conditions the catalytic activity of the L-asparaginase was completely suppressed.

In view of the large excess of tyrosine residues with respect to tryptophan residues (11 : 1) in the molecule of L-asparaginase, fluorescence spectra of this enzyme were taken with excitation at 295 mμ, which is characteristic only for the excitation of tryptophan. The luminescence maximum of L-asparaginase, unlike that due to tryptophan residues at 345-350 mμ, is observed at 320 mμ (Fig. 3), which is obviously explained by the hydrophobic environment of the tryptophan [9, 10].

The addition of N-BS to a solution of the enzyme lowers the intensity of fluorescence while the maximum at 320 mμ is retained. From the latter circumstance it is assumed that this action does not cause conformational changes in the enzyme molecule connected with a change in the position of the tryptophan residues.

Calculated to 1 mole of tryptophan in the L-asparaginase molecule, 1 mole of N-BS lowers the absorption maximum by 40%, and 2.5 moles cause the complete quenching of the fluorescence of this protein (Fig. 3a).

The decrease in the intensity of the fluorescence of L-asparaginase is apparently connected just with the modification of the tryptophan residues, as is also confirmed by the fluorescence spectra of a solution of the amino acid L-tryptophan, the nature of which coincides with that of the spectra of L-asparaginase. This phenomenon is obviously also connected with changes in the interaction of the modified tryptophan with other surrounding amino acids. It is extremely likely that such an amino acid is the histidine residue [10] which, according to our results [2, 11], is present in the active center of the L-asparaginase, is located close to the tryptophan, and has direct interaction with it.

Electrophoresis in acrylamide gel showed that in the modified L-asparaginase, as compared with the native protein, two small protein fractions had disappeared, but the position of the main zone was preserved (Fig. 4). In a determination of enzyme activity, this zone was either not stained or was stained very feebly, confirming the inhibition of the activity of the L-asparaginase on treatment with N-BS.

In an electrophoretogram of L-asparaginase modified with substrate protection, the number and pattern of the electrophoretic distribution of the fractions and also the activities of the zones did not differ from those of the native enzyme.

The addition of the substrate, L-asparagine, to a solution of L-asparaginase directly before the addition of N-BS sharply reduced the inhibitory effect of the latter (see Table 1). In an amount suppressing asparaginase activity by 96% after preincubation for 10 min, the inactivation of the enzyme with substrate protection under the same conditions was only 5%. A similar effect is also observed in the protection of L-asparaginase by a competing inhibitor, S-benzyl-N-benzyloxycarbonyl-L-cysteine. In the presence of this inhibitor in a concentration of $3 \cdot 10^{-3}$ M, inhibiting the enzyme

activity by 95%, the action of N-BS on L-asparaginase was greatly reduced, and after the separation of the S-benzyl-N-benzyloxycarbonyl-L-cysteine and the excess of N-BS on Sephadex G-25, the activity of the enzyme was 96% restored.

The pronounced suppression of asparaginase activity under the influence of N-BS, which is considerably diminished on substrate or inhibitor protection of the enzyme, shows a definite role of the tryptophan residues in the catalytic action of L-asparaginase, indicating their position directly in the active center or close to catalytically important amino-acid residues and in direct interaction with them.

EXPERIMENTAL METHOD

The work was carried out with highly purified L-asparaginase from E. coli B 675 with a specific activity of ~250 IU/mg of protein, obtained by a modification of Roberts' method [12].

The reagent N-bromosuccinimide was recrystallized from benzene (mp 173°C), and solutions of it were prepared immediately before each experiment. The preincubation of the enzyme with the modifier was performed in the appropriate buffer at room temperature for 10 min. To check the substrate or inhibitor protection, a 300-fold molar excess of L-asparaginase (Reanal) or S-benzyl-N-benzyloxycarbonyl-L-cysteine was added.

The L-asparaginase activity was determined by direct Nesslerization [13]. The excess of N-BS was separated by gel filtration on a column of Sephadex G-25. The homogeneity of the modified L-asparaginase was checked by gel filtration on a column of Sephadex G-150.

The UV spectra of L-asparaginase were taken on a Specord UV-VIS spectrophotometer. The fluorescence spectra were taken on a home-made instrument consisting of two SPM-1 monochromators. The fluorescence was excited by a DDS-250 lamp. The spectral slit width of the exciting light was 6 mμ and that of the fluorescent light 3 mμ. The intensity of fluorescence was measured by the synchrodetection principle, using a FÉU-39 photomultiplier. Disk electrophoresis of the L-asparaginase was performed by Davis's method [14], and the activity of the zones was determined by a method described previously [15].

SUMMARY

1. The role of the tryptophan residues in the L-asparaginase molecule has been studied by the method of chemical modification with N-bromosuccinimide, and it has been established that in an acid medium this reagent modifies all four tryptophan residues present in the molecule, completely suppressing the activity of the enzyme.

The substrate – L-asparagine – and a competing inhibitor – S-benzyl-N-benzyloxycarbonyl-L-cysteine – protect the L-asparaginase from the action of N-bromosuccinimide, which shows the role of the tryptophan in the catalytic center of the L-asparaginase.

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