REDUCTIVE ALKYLATION OF *E. coli* L-ASPARAGINASE

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Alkylasparaginases have been obtained with the far-reaching modification of 1-10 lysine residues per subunit of the enzyme. It has been established that alkylation does not affect the stability of the enzyme, and the change in the pH optimum of the catalytic action is due to the chemical nature of the alkyl substituent.

The biological properties of an antileukotic drug — the enzyme L-asparaginase — are determined mainly by its asparaginamide hydrolase activity, and therefore an all-sided study of the dependence of the catalytic function on the physicochemical state of the protein molecule of the enzyme is important. A convenient instrument for these purposes is chemical modification and, in particular, the reductive alkylation of the amino groups of the protein. It is to be expected that the reactivity and the spatial and electronic structures of carboxylic agents will affect not only the degree of modification and catalytic activity but also the isoelectric point, the pH optimum of the catalytic action, the thermal stability, the intraprotein hydrophobic interactions, etc.

The reductive alkylation of L-asparaginase was carried out without the isolation of the intermediate Schiff's bases. Under identical conditions of the reaction with different agents, different degrees of modification were obtained, which are obviously determined by the reactivity, volume, and electronic structures of the aldehydes:

Modification of L-asparaginase	Number of modifications per subunit*	Specific activity, IU/mg of protein	IP of the main zone
Adenyl-N_9-ethyl-	undet.	129	5.43
6-Mercaptopurinyl-N_9-ethyl	2	128	5.48
p-Diethylaminobenzyl-	1	127	5.44
Uracilyl-N_1-ethyl	3	122	5.46
Hydroxyethyl-	3	118	5.42
5-Nitrofurfuryl-	undet.	116	4.70
γ-Picolyl-	10	93	5.43
Native enzyme	0	184	5.40

The specific catalytic activity of alkylasparaginases depends on the degree of modification. In particular, the use of pyridine-4-aldehyde led to the formation of 10 γ-picolylaminolysine groups per subunit, which was accompanied by the loss of 50% of the catalytic activity. We observed the same level of inactivation as the result of the trinitrophenylation of eight Lys residues per subunit. As is well known, the pK values of monoalkylamino groups are only 0.1-0.7 pH units higher than the pK values of the primary amino groups [1] of Lys and, consequently, reductive alkylation only slightly raises the isoelectric point of L-asparaginase. An exception proved to be 5-nitrofurfurylasparaginase, the isoelectric point

*According to the results of amino acid analysis.

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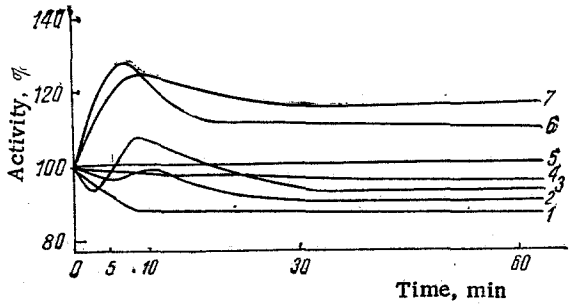

Fig. 1. Stability of solutions of alkylasparaginases at 37°C for concentrated solutions of 1 mg of protein/ml of 0.05 M K-Na phosphate buffer, pH 7.2, containing 0.1 M NaCl; 1) γ-picolylaminoasparaginase; 2) 6-mercaptopurinyl-N_9-ethylaminoasparaginase; 3) uracilyl-N_1-ethylaminoasparaginase; 4) hydroxyethylaminoasparaginase; 5) native *E. coli* L-asparaginase; 6) p-diethylaminobenzylaminoasparaginase; 7) adenyl-N_9-ethylaminoasperaginase.

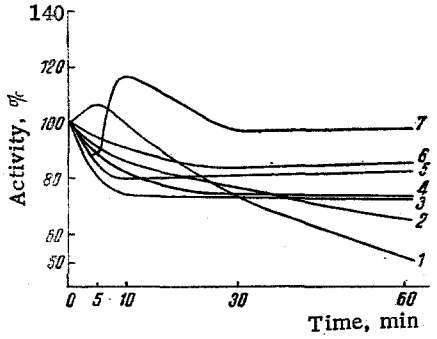

Fig. 2. Stability of solution of alkylasparaginases at 55°C, and of concentrated solutions of 1 mg of protein/ml of 0.05 M K-Na phosphate buffer, pH 7.2, containing 0.1 M NaCl; 1) γ-picolylaminoasparaginase; 2) native *E. coli* L-asparaginase, 3) hydroxyethylaminoasparaginase; 4) 6-mercaptopurinyl-N_9-ethylaminoasparaginase; 5) adenyl-N_9-ethylaminoasparaginase; 6) uracilyl-N_9-ethylaminoasparaginase; 7) p-diethylaminobenzylaminoasparaginase.

of which was greatly lowered as a consequence of the electronegative effect of the nitro group, and on electrofocusing all the bands of the modified protein were located in a narrow group at pH 4.6-4.8, resembling the single diffusion band in the pI 5.0-5.1 region of TNP* asparaginase (degree of modification: 2-3 Lys residues per subunit).

Characteristic for the modified L-asparaginases is an increased stability on storage and an increased thermal stability in comparison with the native enzyme [2, 4].

The introduction of heterylalkyl groupings exerts some stabilizing influence on the enzyme (Figs. 1 and 2). The results of heat denaturation of the alkylasparaginases indicates that the additional residues in the protein derivatives can take part in protein hydrophobic interactions and intensify them thereby stabilizing the enzyme, this effect being inversely proportional to the degree of modification [5].

For some samples, we observed a flash of catalytic activity after 5-15 minutes' heating (Figs. 1 and 2). It is likely that initially, under the influence of the temperature, a catalytically more suitable form of the enzyme molecule is formed which creates a so-called apparent temperature optimum [6]. This form can be fixed as a consequence of the appearance of new and additional hydrophobic interactions as obviously takes place in the case of p-diethylaminobenzylasparaginase.

The native *E. coli* enzyme L-asparaginase is catalytically active at pH 5-10.5, i.e., over a wide range of ionization constants of the protein (Fig. 3) and has a maximum at pH 8.5. A chemically modified protein usually consists of a set of molecules of different degrees of modification for which the mean degree of modification is determined analytically. If the optimum ionization states of the individual molecules are sufficiently close, we observe some shift in the pH dependence of the catalytic activity. However, the formation of 6-mercaptopurinyl-N_9-ethylaminoasparaginase leads to a marked change in the optimum

*TNP) Trinitrophenyl group.

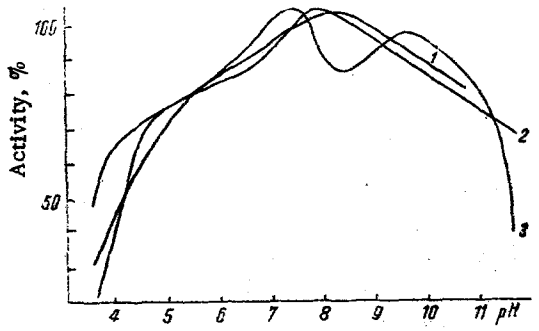

Fig. 3. Dependence of the activity of L-asparaginase on the pH of the medium (0.04 M universal buffer mixture, phosphage—acetate—borate—NaOH—0.1 M NaCl): 1) native *E. coli* L-asparaginase, 2) uracilyl-N_1-ethylaminoasparaginase, 3) 6-mercaptopurinyl-N_9-ethylaminoasparaginase.

ionization state of the enzyme which is shown in the form of a second activity maximum at pH 10.

Amino acid analysis* showed that only in the case of the reaction of L-asparaginase with uracil-N_1-acetaldehyde is it possible to assume the modification of the N-terminal Leu residue, since in this preparation a decrease in the amount of Leu by 5.6 residues per tetramer was observed:

Modification of L-asparaginase	Lys	Leu	Ile	Val
Native enzyme	22.8 (24)†	23.1 (24)†	8.2 (11)†	23.4 (29)†
p-Diethylaminobenzyl-	22.3	22.7	6.7	17.2
6-Mercaptopurinyl-N_9-ethyl	21.4	24.1	6.9	19.6
Uracilyl-N_1-ethyl-	21.2	17.5	5.8	17.5
Hydroxyethyl-	20.3	22.9	7.1	18.7
γ-Picolyl-	13.1	22.4	8.2	22.4
Trinitrophenyl-	1.7	22.7	8.5	22.8

In all cases of reductive alkylation, some lowering of the Val and Ile contents was observed when an amino acid analysis was performed in a manner identical with that of the native protein. This is an indirect indication of an increase in the compactness of the hydrophobic sections of the protein as a consequence of reductive alkylation, leading to the necessity for more severe conditions for complete hydrolysis. This observation is in good agreement with the facts given above on the thermal stability of the preparation.

EXPERIMENTAL

We used L-asparaginase from the Riga medical preparations factory of the Institute of Organic Synthesis of the Academy of Sciences of the Latvian SSR with a specific activity of 180 IU/mg of protein.

Reductive alkylation was carried out by a modified method [5]: with stirring, the appropriate aldehyde was added to a final concentration of 1% to a solution of L-asparaginase with a concentration of 15 mg of protein/ml in 0.1 M Na borate buffer, pH 9.0. The mixture was stirred at room temperature for 15 min, and then an equal volume of aqueous $NaBH_4$ solu-

*Determined after hydrolysis under standard conditions for 24 h.
†Calculated from the results of analysis after hydrolysis of the sample for 72 h.

tion with a concentration of 2 mg/ml was added; the mixture was stirred for another 30 min, and an additional portion of reducing agent was added. After 20 min, the mixture was left for dialysis against 3 × 100 volumes of double-distilled water. The sample was dialyzed for 36 h and was then concentrated to its initial volume by strewing the dialysis bag with dry polyethyleneglycol having a molecular weight of 40,000.

The hydrolase activity of the L-asparaginase and its alkylated derivative was determined by direct Nesslerization using a method that we have proposed previously [7].

Concentrations were determined by the Lowry method [8] or spectrophotometrically on a Specord UV-VIS instrument (GDR) using an absorption coefficient for asparaginase of $D^{0,1\%}_{278\,nm} = 0.71 \pm 0.02$ [9]. Amino acid compositions were determined by the generally adopted method [10] in the accelerated variant on a 6020 A amino acid analyzer (Czechoslovakia).

To determine thermal stability, samples of the alkylasparaginases were incubated at a concentration of 1 mg of protein/ml in 0.05 M K-Na phosphate buffer, pH 7.2 prepared in 0.1 M NaCl at 37 and 55°C. Samples for the determination of catalytic activity were taken after 0, 5, 10, 30, and 60 minutes' incubation.

The pH dependence of the hydrolase activity was determined in 0.04 M universal buffer mixture, phosphate–acetate–borate–NaOH–0.1 M NaCl, the activity of the enzyme being determined every 0.5 pH unit in the pH range from 3.0 to 11.5.

The isoelectric points (pI) of the samples of asparaginase were determined from the position of the main electrofocusing zone in a thin layer (140 × 100 × 1 mm) of 5% polyacrylamide gel containing 2% of ampholine pH 4-6 LKB (Sweden) according to a published method [11] with a field strength of 20 V/cm for 48 h.

The initial aldehydes were provided by M. Yu. Lidak. The amino acid analyses were performed by V. M. Grishchenko.

SUMMARY

Depending on the chemical nature of the alkylating agent, reductive alkylation does not change or increase the thermal stability of L-asparaginase, but it does affect the pH optimum and catalytic activity of the enzyme. This modification has practically no effect on the isoelectric point of the protein.

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