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INACTIVATION OF GLUTAMIN-(ASPARAGIN)-ASE FROM *Pseudomonas aurantiaca* 548 BY AZASERINE AND 6-DIAZO-5-OXO-L-NORLEUCINE

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The bifunctional enzyme glutamin-(asparagin)-ase (GA-ase) was isolated comparatively recently in a pure form from microorganisms [1, 2, 5, 6, 9]. By now experimental and clinical evidence that GA-ase has an inhibitory effect on growth of cells from different types of human and animal tumors has been collected [4, 7, 8, 10]. Enhancement of the therapeutic effect of the enzyme has been observed when used in conjunction with other antitumor therapeutic preparations widely used in clinical oncology, for example, with chemical analogos of L-glutamine [8].

However, on direct contact with the enzyme, substrate analogs may induce its reversible or irreversible inhibition, and this usually leads to reduction or total loss of the catalytic activity of the enzyme.

The aim of this investigation was to make a preliminary assessment of the inhibitory action of glutamine analogs on GA-ase activity *in vitro*.

EXPERIMENTAL METHOD

Homogeneous GA-ase isolated from a biomass of *Pseudomonas aurantiaca* VKMV-548 by the method devised by the writers previously. Enzyme activity was measured the quantity of ammonia formed during hydrolysis of L-glutamine, by a continuous method, in the coupled glutamate dehydrogenase reaction at 25°C [3]. The reaction mixture (1 ml) contained 0.05M Tris-HCl (pH 7.4), 5 mM sodium α-ketoglutarate, 0.25 mM NADH, 0.5-10 mM L-glutamine, 0.5 mg of glutamate dehydrogenase, and 0.5 mg of bovine serum albumin.

To measure the degree of inactivation by the action of diazo coupling the enzyme (0.05-0.25 U/ml) was incubated with 0.05-8 mM of 6-diazo-5-oxo-L-norleucine (DON) or with 0.1-20 mM azaserine at 25°C in 0.1M Tris-HCl (pH 7.4) and aliquots were taken after known time intervals to measure enzyme activity in a medium of the above-mentioned composition. To investigate the effect of the reaction products and substrate analogs on the degree of inhibition of GA-ase by the action of DON the enzyme was incubated in the presence of the compounds indicated in Table 1 for 10 min at 25°C.

EXPERIMENTAL RESULTS

The effect of analogs of L-glutamine, most of them used in the treatment of cancer patients in conjunction with enzymes during combination treatment of malignant neoplasms, on activity of GA-ase from *Pseudomonas aurantiaca* 548 was investigated in the experiments of series I. These compounds were added directly to the system for measuring enzyme activity without preincubation with enzyme preparations. Table 2 shows that none of the substances tested had any inhibitory action, in a concentration of 0.5 mM, on enzyme activity in the presence of 0.5 mM L-glutamin

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TABLE 1. Effect of Enzymic Reaction Products and Substrate Analogs on Inactivation of GA-ase by 0.5 mM DON

Compound (0.5 mM)	Residual activity, %
L-aspartate	95
L-glutamate	57
L-γ-glutamyl-p-nitroanilide	84
L-methionine-DL-sulfoximine	57
L-methioninesulfone	54
N-acetyl-L-glutamin	47
L-carbobenzoxy-L-asparagin	47
Without additives	47

Legend. Conditions of determination: 0.1M Tris-HCl (pH 7.4), duration of preincubation with DON and stabilizer 10 min.

TABLE 2. Effect of Various Substrate Analogs on GA-ase Activity

Compound (0.5 mM)	Activity, %
6-Diazo-5-oxo-L-norleucine	97
O-Diazoacetyl-L-norleucine	100
L-methionine-DL-sulfoximine	95
L-methionine sulfone	95
Methotrexate (4-amino-10-methylpteroyl-glutamic acid)	102
Aminopterine (4-aminopteroylglutamic acid)	98
Folic acid (pteroylglutamic acid)	102

Legend. Activity determined with the aid of glutamate dehydrogenase. Conditions of determination: 30°C, 0.05M Tris-HCl (pH 7.4), substrate 0.5 mM L-glutamine.

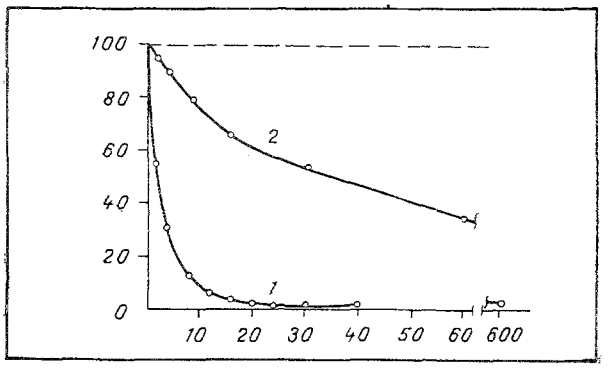

Fig. 1. Inactivation of GA-ase by 4 mM DON (1) and 20 mM azaserine (2). Abscissa, incubation time (in min); ordinate, enzyme activity (in % of control, taken as 100). Broken line) activity in absence of inhibitor. Conditions of incubation: 25°C, 0.1M Tris-HCl (pH 7.4).

Incidentally, of all the analogs tested only DON and azaserine contain a highly reactive functional group capable of interacting irreversibly with side chains of the amino-acid residues of the enzyme molecules. Consequently, when the effect of these compounds on enzyme activity was investigated, a different technique was used. The enzyme was preincubated with these analogs and aliquots were taken at definite time intervals, and later added to the activity measuring system. Ammonia formation as a result of the enzymic reaction took place as a linear function of time, and the velocity of this process characterized the residual activity of the enzyme. Keeping the enzyme with DON and azaserine in the absence of substrate was shown to lead to diminution of enzyme activity, and the degree of inhibition depended on the incubation time. Typical curves showing the decrease in enzyme activity with time under the influence of DON and azaserine are given in Fig. 1. In the presence of both DON and azaserine the velocity of hydrolysis fell gradually, whereas in the absence of the inhibitors enzyme activity remained unchanged within the time interval studied. Incidentally, the velocity of inhibition of enzyme activity by DON was much greater than the velocity of inactivation by azaserine. For instance, on incubation with DON, GA-ase activity fell by 50% after only 2.5 min, whereas during incubation with azaserine the same degree of inactivation was not reached until after 30 min. The results of investigation of the effect of the reaction product and certain substrate analogs on inactivation of GA-ase due to DON are given in Table 1. The enzyme was incubated against the background of 0.5 mM of the inhibitor and in the presence of the substances (0.5 mM) for 10 min. Of the substances investigated, L-aspartate had the strongest stabilizing action. Substrate analogs with a substituted α-amino group did not affect residual activity. This is evidence that the free α-amino group is essential for protection against inhibiton. L-γ-Glutamyl-p-nitroanilide possessed high protective activity, higher than that of L-glutamate, probably because its molecule contains a substitued amino group. Further investigations showed that L-aspartate acts as a competetive inhibitor of DON (K_i = 2.5×10^{-5} M).

The test of various therapeutic substances (substrate analogs) on GA-ase was thus investigated. It was shown that methotrexate and folic acid do not affect GA-ase activity. The enzyme is highly sensitive to the action of DON and azaserine. L-Isomers of glutamine, aspartate, and certain substrate analogs with a free α-amino group protect the enzyme against inactivation by the action of DON. In order that the use of this enzyme in conjunction with inhibitors for tumor chemotheraphy may be recommended, a thorough preliminary screening of suitable inhibitors and also selection of their doses and duration of treatment must first be undertaken.

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