NATURE OF FUNCTIONAL GROUPS OF THE ACTIVE CENTER OF ANTITUMOR GLUTAMIN(ASPARAGIN)ASE

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KEY WORDS: Woodward's reagent; 2,3-butandione; glutamin(asparagin)ase.

The antitumor glutamin(asparagin)ase from Pseudomonas aurantiaca VKM-548 is being investigated in the writer's laboratory. Methods of isolation of the enzyme in a homogeneous state have been developed, and its physicochemical and catalytic properties, amino-acid composition, and quaternary structure have been determined [1-4]. Attempts are now being made to investigate the identity of the amino-acid residues essential for activity of the enzyme [5].

At physiological pH values the substrates of the enzyme L-glutamine and L-asparagine are in an ionized state in solution in the form of zwitterions, i.e., compounds with both a positive and a negative charge. This suggests that anionic and cationic binding sites exist on the substrate-binding region of the active center of the enzyme. It was therefore necessary in the first place to study the role of residues of arginine and dicarboxylic amino acids in the action of the enzyme. α-Dicarbonyl compounds such as phenylglyoxal [13] and 2,3-butandione [11] are highly specific reagents which can be used for chemical modification of arginine residues in proteins. To modify carboxyl groups in proteins, N-ethyl-5-phenyl-isoxazolium-3^1-sulfonic acid, or Woodward's reagent K (WK) is widely used, for it can selectively modify carboxyl groups in proteins at close to neutral pH values [6, 9, 12].

The aim of this investigation was a preliminary evaluation of the inhibitory action of 2,3-butandione and of WK reagent on activity of glutamin(asparagin)ase (GA-ase).

EXPERIMENTAL METHODS

GA-ase was isolated from the biomass of Ps. aurantiaca VKM-548 by the method developed by the writers previously, with a few modifications [3]. The degree of purity of the enzyme preparation, as shown by the results of polyacrylamide gel disk-electrophoresis in the presence of sodium dodecylsulfate, was not below 84%. The enzyme was kept in a lyophilized state.

Activity of the enzyme was measured with the aid of glutamate dehydrogenase at 25°C [7]. Incubation samples 1 ml in volume contained: 0.05 M Tris-HCl (pH 7.4) or 0.025 M borate buffer (in the experiments with butandione; pH 8.6), 0.25 mM NADH, 5 mM sodium α-ketoglutarate, 0.5 mg glutamate dehydrogenase, 1 mM L-glutamine or L-asparagine, and 0.005-0.010 unit GA-ase. The decrease in optical density at 340 nm with time was recorded.

To study the role of arginine residues, modification of the enzyme by 2,3-butandione (Aldrich, USA) was used [11]. The GA-ase was incubated with a freshly prepared solution of 0.1 M butandione at 25°C in 0.05 M borate buffer (pH 8.4) in a tube wrapped in aluminum foil. After 2.5 h aliquots were taken for determination of asparaginase and glutaminase activities in medium of the above-mentioned composition. In the control samples, 0.05 M borate buffer was added instead of butandione. The modification reaction was begun by addition of butandione.

Modification of the carboxyl groups was carried out with WK reagent (Aldrich) [6, 9]. A freshly prepared solution of the reagent in 0.001 N HCl was used. The enzyme was incubated with 4.4-19.7 mM WK at 25°C in 0.05 M MES (pH 6.1). The reaction was started by addition of WK. At certain time intervals aliquots were taken to measure glutaminase and asparaginase

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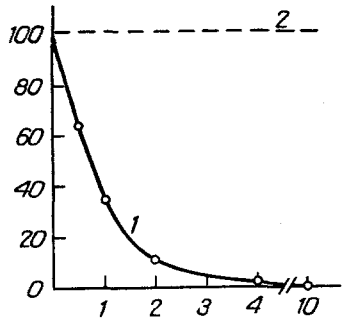

Fig. 1

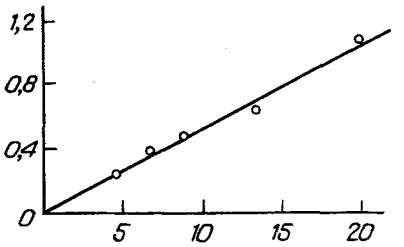

Fig. 2

Fig. 1. Inactivation of GA-ase by the action of 19.7 mM WK (1) and activity of the enzyme in the absence of inhibitor (2). Here and in Fig. 3: abscissa, time (in min); ordinate, activity (in %). Conditions of incubation: 25°C, 0.05 M MES (pH 6.1), substrate L-glutamine.

Fig. 2. Dependence of initial velocity of enzyme inactivation on WK concentration. Abscissa, concentration of inhibitor (in mM); ordinate, velocity of inactivation (in min^{-1}). Conditions of incubation: 25°C, 0.05 M MES (pH 6.1).

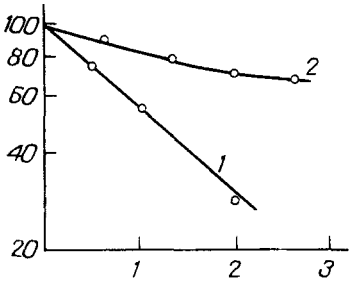

Fig. 3. Effect of L-glutamate on inactivation of enzyme by the action of 13.1 mM WK. Conditions of incubation: 25°C, 0.05 M MES (pH 6.1), in absence (1) and in presence of 5 mM L-glutamate (2).

activities. To study the effect of reaction products and their analogs on the degree of inhibition of the enzyme by WK, the GA-ase was incubated in the presence of these compounds for 2 min at 25°C.

Measurements were made on a Specord M-40 spectrophotometer (East Germany), with thermostatically controlled cuvettes.

EXPERIMENTAL RESULTS

In the experiments of series I the effect of 2,3-butandione on GA-ase activity was studied. To prevent side reactions of photo-oxidation, catalyzed by this reagent in light, modification was undertaken in darkness, under conditions analogous to those used to modify arginine residues in the active centers of various other enzymes [8, 10, 11]. Incubation of the enzyme with 0.1 M 2,3-butandione for 2.5 h at pH 8.4 was found not to lead to a reduction in glutaminase and asparaginase activities, measured at saturating concentrations of

TABLE 1. Effect of Products of Enzyme Reactions and Their Analogs on Inactivation of Enzyme by WK Reagent

Compound	Concn., mM	Residual acivity, %
L-aspartate	5	90
L-glutamate	5	66
Glycine	5	60
γ-Aminobutyrate	5	47
Acetate	10	35
No additives	—	28

Legend. Conditions of incubation: 25°C, 0.05 M MES (pH 6.1), 2 min, 13.1 mM WK.

the substrates (1 mM). However, the possibility could not be ruled out that although the modification did not affect the maximal velocity of the enzyme reaction, it did cause a sharp decrease in affinity of the enzyme for the substrate and an increase in the Michaelis constant (K_m) if the arginine residue was located close to groups participating in direct contact with the substrate. To test this hypothesis activity was measured with a nonsaturating concentration of L-asparagine (0.02 mM). No difference was found in activity of the native and butandione-treated enzyme. The degree of lowering of activity in both specimens of enzyme, during a change in substrate concentration from 1.0 to 0.02 mM was 19%, in agreement with the value of K_m, lying between 10^{-6} and 10^{-5} M [2]. The results serve to eliminate any role of the arginine residue in binding and catalytic conversion of the substrates.

The problem of participation of carboxyl groups in the manifestation of GA-ase activity was studied with the aid of WK. It was found that incubation with this reagent causes virtually complete inactivation of the enzyme (Fig. 1). Plotting the data between semilogarithmic coordinates showed that in the initial period inactivation proceeded in the manner of a pseudo-first-order reaction. The deviation from a pseudo-first-order kinetics observed during more prolonged incubation may have been the result of hydrolysis of the WK itself. The velocity of inactivation depended on concentration of inhibitor (Fig. 2). The velocity constant of the second-order inactivation reaction was 52 $M^{-1} \cdot min^{-1}$.

The results of investigation of inactivation of GA-ase by WK in the presence of competitive inhibitors (L-glutamate and L-aspartate), and also of their analogs, are given in Table 1. All the substances used were found to protect the enzyme to a greater or lesser degree. The two most likely causes of the protective effect observed may be: lowering of the WK concentration in the reaction medium due to its interaction with substances used for protection [12, 14], and direct interaction of competitive inhibitors with the active center of the enzyme. If the lowering of the degree of inactivation were due to interaction between WK and protective substances, if the concentrations of L-aspartate and L-glutamate were the same, an identical protective effect ought evidently to be observed. If, however, it were due to binding of these ligands in an active center, with the concentration of L-aspartate used, which has affinity for the enzyme an order of magnitude higher than L-glutamate [2], L-aspartate ought to protect the enzyme better than L-glutamate, and much better than acetate which, because of the absence of an α amino group, does not in general bind with the active site [1].

It will be clear from Table 1 that L-aspartate gives a stronger protective effect than L-glutamate. It is essential to note that 10 mM acetate protects the enzyme only to a very slight degree. The results are evidence that the protective effect of amino acids is largely determined by their direct interaction with the enzyme. To confirm this hypothesis, the effect of L-glutamate on the initial velocity of inactivation (Fig. 3) was studied. Since the initial WK concentration was independent of the presence of L-glutamate in the medium, the observed reduction of the initial velocity of inactivation can be attributed only to competition between L-glutamate and WK for binding with the enzyme.

Investigation of the effect of pH on enzyme inactivation during incubation for 10 min with 5 mM WK in 0.05 M phosphate buffer, showed that with a decrease in pH from 7.0 to 6.1 the degree of inactivation increases, and this is characteristic of modification of carboxyl

groups. Under these circumstances changes in glutaminase and asparaginase activities of the enzyme were found to be similar in character. Inactivation of GA-ase by WK is also accompanied by spectral changes characteristic of interaction of the reagent with the accessory peak, with a maximum in the 340 nm region and with reduction of the ratio A_{280}/A_{250} [6, 9].

Investigation of the effect of two chemical agents on GA-ase thus revealed that 2,3-butandione, which modifies arginine residues in proteins, does not affect activity of the enzyme. The enzyme is completely inactivated in the presence of WK. Data obtained by a study of the effect of concentration of the modifying agent, pH of the medium, competitive inhibitors, and certain other substances, on the degree of inactivation suggest that carboxyl groups, essential for substrate binding, are present in the active center of glutamin-(asparagin)ase.

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MECHANISM OF ACTION OF KETAMINE DERIVATIVE ANESTHETICS ON PHOSPHOLIPID MEMBRANES

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Cell membranes have for a comparatively long time been considered to be the site of action of anesthetics. As regards ketamine derivatives, which are powerful anesthetics of the latest generation, no systematic studies of the mechanism of their action on membranes have yet been undertaken, and the available data on this matter are few in number and contradictory in nature. Some workers consider that ketamines "liquefy" the lipid phase of membranes [8], whereas others consider that they make it more "rigid" [10]. Finally, information has been obtained to the effect that ketamine, which has a positive charge at neutral pH values, modifies the surface potential of the ganglioside films [7].

In the investigation described below the effect of the ketamine derivatives Calypsol and Ketalar on viscosity and surface charge was studied on model phospholipid membranes (liposomes).

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