A PRELIMINARY IMMUNOCHEMICAL STUDY OF *E.coli* ASPARTOKINASE I-HOMOSERINE DEHYDROGENASE I

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Received May 15, 1973

SUMMARY : The preparation of immunoadsorbents against aspartokinase I-homoserine dehydrogenase I from *E.coli* is described. In the presence of aspartate, considerably less enzyme is bound by the fixed antibodies. The fixed protein can be displaced by a protein extracted from a nonsense mutant.

Physical chemical studies of aspartokinase I-homoserine dehydrogenase I from *Escherichia coli* K 12 have shown that two enzyme activities are carried by a single protein (1, 2, 3), a tetramer with identical subunits (4). This protein exists in two main conformations in equilibrium, an active form R having a low affinity for the regulatory ligand L-threonine and an inactive form T, having a high affinity for the same ligand (5). This protein was selected to investigate evolutionary protein divergence within the genus *Enterobacteriaceae*, using immunological methods as a tool to detect differences that might exist among aspartokinases I-homoserine dehydrogenases I produced by different species. During the elaboration of the methodology, it was found that the protein reacted differently with its specific antibodies according to whether or not aspartate was present.

MATERIALS AND METHODS

Enzymes and measurements of activity

Aspartokinase I-homoserine dehydrogenase I was prepared from *E.coli* K 12, strain Tir ^{8}, as previously described (3, 5). Homoserine dehydrogenase was assayed in the direction homoserine → aspartate semialdehyde, following $NADP^{+}$ reduction (6). Aspartokinase activity was measured either by the formation of aspartohydroxamate (7) or by a coupled assay using pyruvate kinase and lactate dehydrogenase (8).

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Crude extracts of *E.coli*, strain Gif 108, a mutant containing a tetramer formed of shortened polypeptide chains with aspartokinase activity, but devoid of homoserine dehydrogenase activity (9, 10), were also used.

Buffers

Buffer A. - 2×10^{-2}M potassium phosphate, pH 7.2, containing 2×10^{-3}M Mg Titriplex and 0.15 M KCl.

Buffer AT.- Buffer A + 2×10^{-3}M L-threonine.

Immunizations

Two series of sera were used. The first sera were those used in a previous publication (11). One of these sera, originating from a single rabbit, was used for the preparation of the immunoadsorbent A. The second series of sera was obtained as follows : rabbits were immunized by intradermic injections in several depots in the back ; four immunizations were carried out at one week interval. One booster intravenous injection was performed 10 days after the last of the four immunizations. The intradermic injections were performed in complete Freund's adjuvant (0.25 ml) mixed with a solution composed of 0.1 ml of buffer A containing 80 µg of pure enzyme and 0.25 ml of saline solution containing 50 µg of methylated bovine serum albumin. The total volume (0.60 ml) was injected. The booster injection was performed with 1.25 ml of saline solution containing 250 µg of enzyme and 50 µg of methylated bovine serum albumin. The rabbits were bled from the ear eleven days after the booster injection. The γ-globulins were obtained by ammonium sulfate fractionation and were used for the preparation of the immunoadsorbent B.

Activation of Sepharose and preparation of the immunoadsorbent columns

Sepharose 4B (Pharmacia, Uppsala) was activated by cyanogen bromide by the method described by Cuatrecasas and Anfinsen (12) : 300 mg of CNBr were used per ml of packed Sepharose ; the coupling was performed in 0.1 M sodium citrate buffer, pH 6.5, using 9 mg of protein per ml of packed activated Sepharose. Immunoadsorbent A was prepared with crude serum, while immunoadsorbent B was prepared by coupling the purified immunoglobulin fraction described above. All sera and fractions used as source of antibodies were dialyzed against saline prior to coupling. Both columns (A : 0.9 cm diameter by 10 cm height ; B : 0.9 cm diameter by 7 cm height) were packed and equilibrated with the appropriate buffers.

Chromatography of the enzyme on the immunoadsorbent columns

Pure enzyme was loaded on the column ; buffer was then passed at a flow rate of 0.1 ml/min for column A and of 0.8 ml/min for column B. Eluates were tested for enzyme activities and sensitivity to 2 x 10^{-3}M L-threonine.

The capacities of the immunoadsorbents were determined in the following manner : successive loads of enzyme were added to the columns ; after each load, a volume of A or AT buffer equal to twice the column bed volume was used to wash the immunoadsorbent. When enzyme appeared in the eluate, this meant that the column was saturated, which defined its capacity. All operations were performed at room temperature (18 - 20°).

After each experiment, the columns were washed with 6 M urea (containing 0.001 N hydrochloric acid) ; this procedure removes all the enzyme bound to the antibodies. The columns were then washed with the appropriate buffer. The capacities of the columns were not affected by this treatment and remained constant for several months.

R E S U L T S

Fixation of aspartokinase I-homoserine dehydrogenase I to the antibody column

When pure enzyme is loaded on column B in buffer AT, and washed with the same buffer, 1.4 mg of enzyme is bound. In buffer A, the same capacity is found.

However, if 10^{-2}M L-aspartate is added to buffer A and used for loading and washing, the capacity falls to 0.56 mg. The enzyme in the eluate displays a normal sensitivity towards L-threonine.

This lowered capacity obeys a narrow stereospecificity : in the presence of 10^{-2}M L-glutamate or D-aspartate, the normal capacity of 1.4 mg is found.

Displacement of antibody-bound aspartokinase I-homoserine dehydrogenase I by an aspartokinase fragment extracted from a mutant

Experiments were carried out to determine whether antibody-bound aspartokinase I-homoserine dehydrogenase I could be displaced by the aspartokinase from Gif 108, which is devoid of homoserine dehydrogenase activity. The immunoadsorbent (column A) was saturated with the pure enzyme from the wild-type (90 µg) and it was carefully checked that no activity was eluted by washing with buffer A. Then 0.87 units of aspartokinase I contained in 2 ml of crude

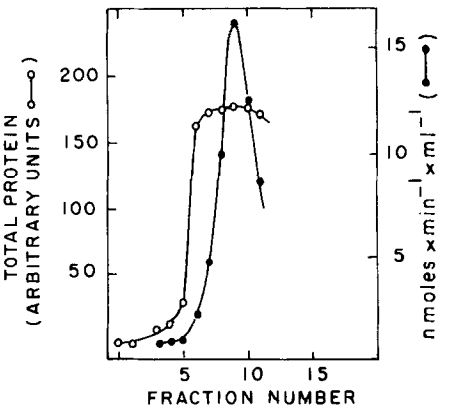

Fig. 1. - Exchange of bound wild-type aspartokinase I-homoserine dehydrogenase I with aspartokinase I from the nonsense mutant Gif 101 (crude extract).

●—● homoserine dehydrogenase activity

0—0 absorbance at 280 nm.

extract from Gif 108 were loaded onto the column which was then washed slowly (flow rate of 0.1 ml/min) with buffer A. Figure 1 shows the appearance of protein followed by homoserine dehydrogenase activity in the eluate fractions. Since the enzyme from the mutant Gif 108 had no homoserine dehydrogenase activity, exchange must have taken place. The properties of the eluted enzyme were unusual : when tested for threonine sensitivity, it was discovered that the homoserine dehydrogenase activity was enhanced rather than inhibited, as is the case with the enzyme from the wild-type. This enhancement was always greater than 100 %.

D I S C U S S I O N

It is evident that the antibodies against aspartokinase I-homoserine dehydrogenase I are able to distinguish between two conformations of the enzyme. The form which predominates in the presence of threonine, called T form in our previous publications (5, 13), must have some antigenic determinants exposed to react with the corresponding sites on the antibodies, whereas they must be less available in the presence of aspartate, that is in the R form. However, the transition from the T to the R form can be achieved not only with aspartate, but also with K^+ ions ; the concentration of K^+ in buffer A is such that the protein is already in the R form (as defined by its spectroscopic properties), before aspartate is added. Therefore, we can define the following equilibria :

$$R_2 \underset{\text{aspartate}}{\rightleftharpoons} R_1 \overset{\text{threonine}}{\underset{\text{KCl}}{\rightleftharpoons}} T$$

In this model, R_1 and R_2 have identical or similar spectroscopic (5) or hydrogen exchange (14) properties, since KCl and aspartate bring about the same variations of these properties. On the other hand, the conclusion that R_1 and R_2 differ by their reactivity to their specific antibodies is inescapable.

Similar differences in reactivity with antibodies have been found for rat liver tryptophan oxygenase apo- and holo-enzyme suggesting that not all forms of an enzyme need react equally with the same antisera (15).

The exchange reaction observed when crude extracts of aspartokinase from Gif 108, devoid of homoserine dehydrogenase, could be due either to the displacement of the wild-type enzyme or to the formation of hybrid molecules containing protomers from both mutant and wild-type enzymes. Whereas the enhancement by threonine would rather favour the hypothesis that the molecules eluted are different from those of the wild-type aspartokinase I-homoserine dehydrogenase I, the possibility remains that the wild-type enzyme is eluted in a stable, less active conformation, and that the enhancement by threonine reflects a reactivation to the normal conformation.

ACKNOWLEDGEMENTS : This work has been supported by the Délégation Générale à la Recherche Scientifique et Technique, the Commissariat à l'Energie Atomique, the National Institutes of Health and the Fondation pour la Recherche Médicale Française.

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