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IMMUNOCHEMICAL COMPARISON OF A BIFUNCTIONAL ENZYME, ASPARTOKINASE II-HOMOSERINE DEHYDROGENASE II, AND ITS TWO PROTEOLYTIC FRAGMENTS

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SUMMARY. We report here a comparison between immunochemical properties of the bifunctional enzyme aspartokinase II-homoserine dehydrogenase II of *E.coli* K12 and of its two isolated proteolytic fragments. Both fragments, one inactive and one endowed with homoserine dehydrogenase activity, react with antibodies raised against the native enzyme. Some of the antibodies elicited against the dehydrogenase fragment can recognize regions of this fragment which are not exposed in the entire enzyme.

The immunochemical results are used to discuss a simple model in which this bifunctional enzyme is folded up in two domains. The organization of aspartokinase II-homoserine dehydrogenase II is compared to that of another bifunctional enzyme aspartokinase I-homoserine dehydrogenase I with which it shares some sequence homology.

INTRODUCTION

Large and complex proteins are often folded up in several domains. One way to approach experimentally the domains is to obtain fragments endowed with some of the properties of the proteins. Such fragments may be considered as the domains of the protein when isolated and some information about the organization and conformation of this protein may be obtained by comparing the fragments to the whole protein.

Aspartokinase II-homoserine dehydrogenase II (AK II-HDH II)[1] of *E.coli* K12 (1), a bifunctional enzyme, is a dimer composed of identical subunits (Mr = 2 x 88,000) (2). By limited proteolysis of AK II-HDH II two fragments can be obtained : one dimeric fragment endowed with full homoserine dehydrogenase activity (Mr = 2 x 35,000), which is an independent folding unit (3, 4), and another fragment, which although inactive has retained enough structure to also be a dimer (Mr = 2 x 24,000) (3).

[1] AK II-HDH II : aspartokinase II homoserine dehydrogenase II (EC. 2.7.2.4. and EC. 1.1.1.3.).

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The question arises how similar those two fragments are when isolated and when part of the whole protein.

In this paper we approach this problem by comparing the immunochemical properties of aspartokinase II homoserine dehydrogenase II and its two proteolytic fragments.

MATERIALS AND METHODS

Aspartokinase II-homoserine dehydrogenase II and its homoserine dehydrogenase (HDH II)[2] fragment were obtained as previously described (5, 3). The small inactive fragment (F II fragment)[3] after the usual purification (3) was filtered through a second G-100 superfine Sephadex column. All these preparations showed a single band upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 1). Homoserine dehydrogenase and aspartokinase activities were measured according to Patte et al. (6) and Wampler and Westhead (7) respectively, and antigen concentrations were measured using absorption at 280 nm. Rabbit antisera were obtained as previously described (8). The immunological assays used were passive hemagglutination (9, 8), complement fixation (10, 8) and a variation of a radioimmunoassay where antigens were detected by their enzymatic activity instead of radioactivity. For this assay immunoglobulins, from immunized rabbits and from non immunized rabbits as a control,

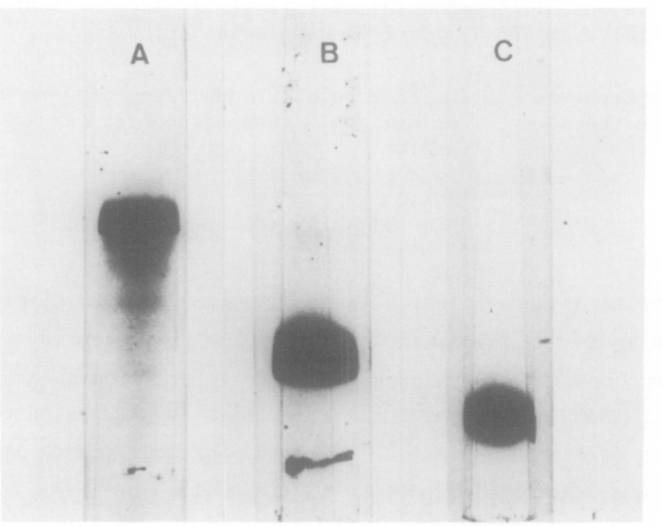

Figure 1. Gel electrophoresis in the presence of sodium dodecyl sulfate of (A) AK II-HDH II, (B) fragment HDH II and (C) fragment F II.

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[2] HDH II : proteolytic fragment of AK II-HDH II endowed with homoserine dehydrogenase activity.

[3] F II : Inactive proteolytic fragment of AK II-HDH II.

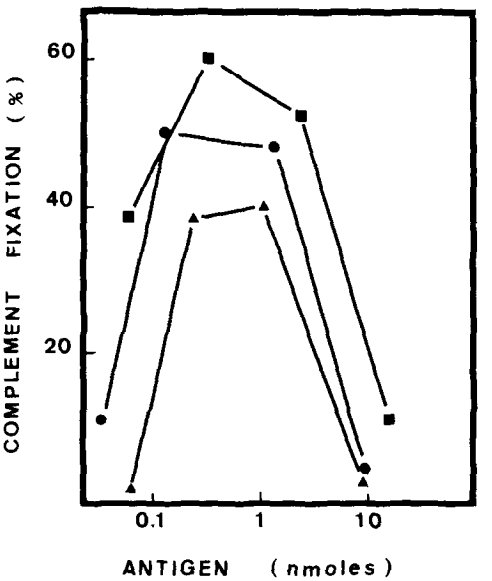

Figure 2. Complement fixation assays of the reaction of the antisera against native AK II-HDH II with (■) AK II-HDH II, (▲) fragment F II, and (●) fragment HDH II.

The antiserum dilution is 1/2000. When an antiserum dilution of 1/3000 is used 0.33 nanomoles of AK II-HDH II fix 55 % complement, 1.14 nanomoles of F II fix 11 % complement and 0.82 nanomoles of HDH II fix 21 % complement.

were coupled to Sepharose 4B-AH (Pharmacia, Sweden) according to Cambiasso et al. (11). In the assay free antigen was separated from antigen bound to immunoglobulins by centrifugation.

RESULTS AND DISCUSSION

Native proteins when injected to animals are known to elicit antibodies directed against antigenic determinants located on their surface (12). Thus immunochemical techniques give some information about that surface. This methodology is particularly interesting in the case of complex proteins folded in several domains, and for that reason it was used in the case of the complex protein AK II-HDH II.

Antibodies against native AK II-HDH II were elicited in rabbits and their average affinity constant, or avidity, was measured using the direct antigen-antibody assay described in Methods ; this avidity constant is 2×10^8 $1.mol^{-1}$.

The proteolytic fragments HDH II and F II react with these antibodies directed against native AK II-HDH II as measured by the three immunochemical assays used (Figure 2 and Tables I and II). Thus in the whole protein, the

TABLE I

Passive hemagglutination titers of antiserum directed against native AK II-HDH II

Antigen tested	Antiserum against AK II-HDH II
AK II-HDH II	1/531 441
F II	1/4 374
HDH II	1/39 366

TABLE II

Inhibition of the reaction of antibodies directed against AK II-HDH II with AK II-HDH II by the two proteolytic fragments HDH II and F II

Inhibitor	Inhibitor Concentration (10^{-6} M)	Inhibition (%)
F II	≥ 2	32
HDH II	≥ 2	46

AK II-HDH II was reacted with an immunoadsorbant directed against AK II-HDH II at 17 mg antibodies per ml of immunoadsorbant. 5 µl of a dilution 1 : 5 of this immunoadsorbant was added to AK II-HDH II at a final concentration of 0.2×10^{-6} M, which leads to 70% binding, in 100 µl final volume. The immunoadsorbant was incubated with increasing amounts of inhibitors for 1 hour and AK II-HDH II was then added. After incubation and agitation for 18 hours at room temperature, the tubes were centrifuged and aspartokinase activity was measured in the supernatant. Control experiments were carried with an immunoadsorbant made with immunoglobulins from non immunized rabbits.

regions from which the two proteolytic fragments arise are accessible to the immunogenic recognition system. The HDH II fragment is better recognized than the F II fragment by antibodies directed against native AK II-HDH II : this may simply reflect the size differences between these two fragments.

The reaction of the F II fragment with antibodies raised against AK II-HDH II, together with the fact that this fragment is dimeric, indi-

TABLE III

Percent of maximal complement fixation obtained with an antiserum directed against the proteolytic fragment HDH II

Antigen tested	Complement fixation (%)	Antigen (nanomoles)	Antiserum dilution
AK II-HDH II	42.5	16.2	1/500
F II	0	-	1/50
HDH II	68.5	0.82	1/500

cates that this fragment remains a compact and organized structure. Indeed this fragment also appears as a good approximation of a domain of AK II-HDH II when isolated.

The proteolytic fragment F II is not recognized by antibodies against the HDH II fragment (Table III). This result is in agreement with the fact that the two proteolytic fragments arise from different parts of the polypeptide chain of AK II-HDH II (3).

All these results support a simple model of AK II-HDH II which was proposed on the basis of limited proteolysis experiments (3). In this model, AK II-HDH II is folded in (at least) two domains, corresponding to the two proteolytic fragments. Each of these domains carries some of the antigenic determinants of the protein and thus some part of the accessible area of the protein.

The opportunity to isolate an active dehydrogenase fragment from AK II-HDH II allows to probe the whole protein by specifically looking at the dehydrogenase domain. This can be done using antibodies against the whole protein as seen above, or using antibodies directed against the fragment endowed with homoserine dehydrogenase activity. Such antibodies directed against the HDH II fragment were obtained : AK II-HDH II is recognized by these antibodies, but not as well as the HDH II fragment itself (Table III).

The reaction of AK II-HDH II with antibodies elicited against native HDH II fragment indicates that there are on the surface of the HDH II fragment some antigenic determinants which are not present on the surface of AK II-HDH II. The latter may correspond to regions of the homoserine

dehydrogenase domain that are involved in interactions with other domains of the protein and which are thus inaccessible to antibodies in the whole AK II-HDH II. The comparison of the immunochemical properties of a protein and its fragment maybe reveals in this particular case some interaction areas between a domain and the rest of the protein which may be important for the expression of one activity, the aspartokinase activity.

In the same organism, *E.coli* K12, another bifunctional enzyme aspartokinase I-homoserine dehydrogenase I (AK I-HDH I)[4] carrying the same two activities is present (13). AK I-HDH I and AK II-HDH II have some differences and similarities (14). It has been shown, using immunochemical techniques, that these two enzymes have some sequence homology (8). Chemical (15) and immunochemical studies (16) of AK I-HDH I show in this case that the homoserine dehydrogenase domain is located at the surface of the protein with the aspartokinase domain "buried" and inaccessible to antibodies. In the case of AK II-HDH II the model supported by the experimental results is quite different. Thus the complex enzymes AK I-HDH I and AK II-HDH II which have some sequence homology and which are thought to derive from a common ancestor (5, 8) are organized in a different manner.

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[4] AK I- HDH I : aspartokinase I-homoserine dehydrogenase I (EC. 2.7.2.4. and EC. 1.1.1.3.).

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