Detection of the Homology among Proteins by Immunochemical Cross-Reactivity between Denatured Antigens. Application to the Threonine and Methionine Regulated Aspartokinases–Homoserine Dehydrogenases from *Escherichia coli* K 12†

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ABSTRACT: The two isofunctional enzymes aspartokinases–homoserine dehydrogenases I and II from *Escherichia coli* K 12 are compared using immunochemical techniques. The antibodies raised against one of these two proteins when in its native state can only recognize the homologous antigen, whether it is native or denatured. Contrarily, the antibodies raised against one of these two proteins when in its denatured state can recognize both the homologous and heterologous denatured antigens. The existence of this cross-reaction only between the two denatured aspartokinases–homoserine dehydrogenases suggests that these two enzymes have some similarity since such a reaction is not detected with several other denatured proteins. The regions involved in this similarity are buried inside the native proteins, and become exposed only upon denaturation. The same result, the existence of a cross-reaction between denatured species and none between the native ones, is obtained with proteolytic fragments derived from these two proteins and endowed with homoserine dehydrogenase activity. This resemblance between the two aspartokinases–homoserine dehydrogenases suggests that these proteins derive from a common ancestor. It is also proposed that such a cross-reaction between two denatured proteins is evidence for an homology between their amino acid sequences, and that the use of denatured proteins as both immunogens and antigens could be useful in detecting sequence homologies.

Evolutional relationships between proteins of either the same or different organisms are usually evidenced by the homology between the amino acid sequences of these proteins. Determining the primary sequences of several proteins in order to compare them is a long and difficult task, and an immunological approach might be a shortcut toward the detection of similarities among various proteins. This work shows this approach as applied to two isofunctional enzymes from *E. coli* K 12, the aspartokinases–homoserine dehydrogenases I (regulated by threonine) and II (regulated by methionine).

These two proteins indeed possess remarkable similarities (Truffa-Bachi et al., 1974; Dautry-Varsat & Cohen, 1977; Dautry-Varsat et al., 1977). They are both bifunctional enzymes which catalyze the same two reactions and, thus, have comparable sites for a variety of ligands; aspartate, ATP, potassium ions, NADPH, homoserine, aspartate semialdehyde. The subunits of each enzyme have almost the same molecular weight, 86 000 for AK I–HDH I[1] and 88 000 for AK II–HDH II, and in the native enzymes these subunits are folded in a similar manner, i.e., into two domains corresponding each to one of the enzymatic activities, kinase or dehydrogenase. Owing to the size of these two proteins, any other than functional resemblance would be difficult to establish without their sequences, none of which is known. Here we use immunochemistry as a tool to precisely determine the extent to which the functional similarity between these two enzymes has a counterpart in their structures. We find that these proteins do not have any significant immunochemical resemblance when native and do have one when denatured. The same immunochemical behavior is also observed with proteolytic derivatives of these enzymes, which are endowed with homoserine dehydrogenase activity (Véron et al., 1972; Dautry-Varsat & Cohen, 1977).

Materials and Methods

(a) *Proteins and Reagents.* AK I–HDH I and AK II–HDH II were prepared as previously reported (Truffa-Bachi & Cohen, 1970; Falcoz-Kelly & Cohen, 1970). AK I–HDH I comes from a strain (Tir 8) which has only about 0.1% of AK II–HDH II as compared with AK I–HDH I; AK II–HDH II was prepared from an *E. coli* strain (Gif 881 L) which has no AK I–HDH I activity. Each enzyme preparation was finally checked to ensure the absence of heterologous material. Both proteins showed a single band on polyacrylamide gels in the presence or absence of sodium dodecyl sulfate. The proteolytic fragments endowed with full dehydrogenase activity were prepared as previously described (Véron et al., 1972; Dautry-Varsat & Cohen, 1977). Gel electrophoresis in the presence of sodium dodecyl sulfate showed that these fragment preparations contained no more than a few percent of the corresponding entire proteins. β_2 subunit of tryptophan synthetase and tryptophanase were gifts from A. Riabaud, O. Riabaud, and M. Goldberg. HSK, γ-globulins, antinative AK I–HDH I, and serum antinative HSK were gifts from P. Truffa-Bachi. Bovine pancreatic ribonuclease A was obtained from Worthington. All other reagents were of AR grade.

(b) *Preparation of Denatured and Citraconylated Derivatives.* The various proteins were denatured in 6 M guanidine hydrochloride and were either alkylated with NEM or carboxymethylated with iodoacetic acid in the following conditions: either 1 to 3 mg/mL of protein, 20 mM NEM, 50 mM cacodylate buffer, pH 7, or 1 to 3 mg/mL of protein, 10 mM iodoacetic acid, 50 mM Tris buffer, pH 8.5. These conditions

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[1] Abbreviations used: AK I–HDH I, aspartokinase I–homoserine dehydrogenase I; AK II–HDH II, aspartokinase II–homoserine dehydrogenase II; HSK, homoserine kinase; NEM, *N*-ethylmaleimide; RNase A, bovine pancreatic ribonuclease A.

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TABLE I: Passive Hemagglutination Titers of Antisera Directed against Native AK I-HDH I, AK II-HDH II, and HSK.[a]

	antiserum against native		
	AK I-HDH I	AK II-HDH II	HSK
native AK I-HDH I	1/59 049	1/243	1/27
NEM-AK I-HDH I	1/6561	1/243	1/3
native AK II-HDH II	1/243	1/531 441	1/81
NEM-AK II-HDH II	1/729	1/19 683	1/3
native HSK	1/27	1/27	1/177 147
NEM-HSK		1/81	1/59 049

[a] In this table, NEM refers to NEM-modified derivatives.

lead to complete modification of all sulfhydryl groups, as verified by amino acid analysis. Either reaction was allowed to proceed for 2 h at room temperature. The modified proteins were dialyzed against 1 M phosphate buffer, pH 8, which caused them to precipitate. Citraconic anhydride was added to the precipitated proteins in phosphate buffer, three times at 30-min interval, up to a final anhydride concentration of 0.25 M, and citraconylation was allowed to take place for 2 h. The modified proteins were first dialyzed against 1 M ammonium bicarbonate, pH 9, then against various decreasing concentrations of the same salt, still at pH 9. After a final dialysis against 50 mM ammonium bicarbonate, pH 9, these modified proteins were soluble again and were lyophilized. In the case of the proteolytic fragments, citraconylation was not needed because the carboxymethylated derivatives are soluble enough to be used as such. Also RNase A was submitted only to the modification of its sulfhydryl groups after reduction by β-mercaptoethanol of its disulfide bonds, since this protein is soluble enough in this state. All these modified proteins were completely inactive, indicating that these denatured species were free from native enzymes.

(c) *Antisera.* Antibodies toward native AK I-HDH I, AK II-HDH II, and HSK were elicited in several rabbits as previously reported (Kaminsky et al., 1969). Antisera for AK I-HDH I and AK II-HDH II were pooled samples of six and three animals, respectively. Antibodies toward denatured and NEM-alkylated AK I-HDH I and AK II-HDH II were obtained in single rabbit each following multisite intradermal injections of the proteins emulsified in complete Freund's adjuvant. One inoculation of 0.5-1 mg of protein was given, and after 3 weeks two injections of 0.5-1 mg of protein were given weekly. Blood was collected about 10 days after the last inoculation. All the proteic species used for immunization were carefully checked for the absence of contaminant, the presence of which could have impaired the significance of immunochemical results.

(d) *Immunological Assays.* For immunochemical assays, aliquots of all modified proteins were taken and solubilized by adding 0.1 mL of water and 2 μL of 2 M sodium hydroxide; the volume was then adjusted to the final concentration with the appropriate buffer. Actual antigen concentrations were determined by amino acid analysis of the same aliquots.

(i) Passive hemagglutination was carried out according to Herbert (1967) using an antigen concentration of 20 μg/mL. Sheep erythrocytes were formalized, tanned, and then coated with the antigen dissolved in 0.15 M phosphate buffer, pH 6.4. Antisera dilutions were performed in 0.15 M phosphate buffer, pH 7.2. The results were scored after 2-6 h at room temperature. Inhibition experiments were performed by incubation of inhibitors with antiserum for 1 h at room temperature before adding the antigen-coated sheep erythrocytes. All samples were analyzed at least in duplicate.

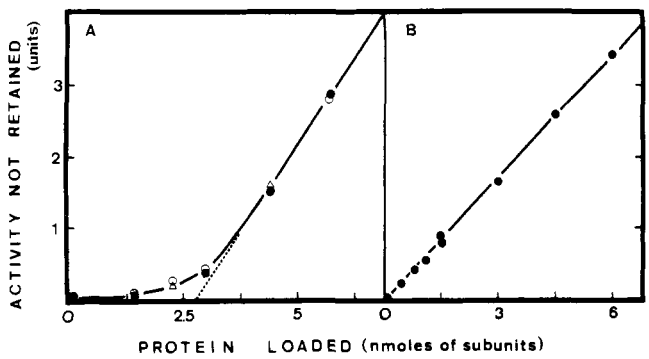

FIGURE 1: Binding of (A) AK I-HDH I and (B) AK II-HDH II to an immunoadsorbent gel prepared with γ-globulins directed against native AK I-HDH I. A: (●) AK I-HDH I, loaded at 1.3 mg/mL; (○) AK I-HDH I loaded at 13 μg/mL; (△) AK I-HDH I loaded at 1.3 mg/mL after trying to load AK II-HDH II at 1.4 mg/mL. B: (●) AK II-HDH II loaded at 1.4 mg/mL. The extent of binding of either protein to the gel is measured by the difference between the total activity loaded and the activity not retained by the gel. The extent of binding is expressed in nmol of subunits for either proteins, using their specific activities.

(ii) Complement fixation was performed in isotonic Veronal buffer containing 0.0005 M of $MgCl_2$, 0.00015 M of $CaCl_2$, and 0.1% bovine serum albumin, according to Osler et al. (1952). For each antigen concentration, two replicates were made and each experiment was run at least twice.

(iii) Immunoadsorbents. The activation of the sepharose 4B by cyanogen bromide, the coupling of the γ-globulin fraction, and the determination of the capacity of each immunoadsorbent were performed as previously described (Cowie et al., 1973). Eluates were tested for enzyme activities (Truffa-Bachi & Cohen, 1970; Falcoz-Kelly & Cohen, 1970).

Each experiment was independently performed by at least two methods, passive hemagglutination and complement fixation, the results of which were always consistent. In some of the experiments reported below, only one set of results will be given for concision of the text.

Results

I. *There Is No Immunological Similarity between Native AK I-HDH I and AK II-HDH II.* Several methods were used to search for an immunological resemblance between native AK I-HDH I and AK II-HDH II: passive hemagglutination, complement fixation, and binding to specific immunoadsorbent gel. In some of these experiments, HSK was also included since it has been reported that this enzyme shows some immunological similarity to AK I-HDH I, when both proteins are native (Truffa-Bachi et al., 1975). No cross-reaction is visible by either hemagglutination (Table I) or complement fixation between any pair of these three proteins, AK I-HDH I, AK II-HDH II, HSK. The homologous reactions showed more than 70% of complement fixation with antisera dilutions of 1/2000, while the efficiency of the heterologous reaction with a given specific antiserum was comparable to that obtained with the same antigen and serum from nonimmunized animals, both sera being used at the same dilution, 1/20. Also no inhibition by the heterologous proteins of the reaction between an antiserum and its homologous antigen can be seen, although the concentration of heterologous protein was higher than in direct hemagglutination tests (Table II).

A possible immunological resemblance between native AK I-HDH I and AK II-HDH II was also tested by the ability of the proteins to bind to a specific immunoadsorbent gel prepared with γ-globulins directed against native AK I-HDH I. The ability to bind to this gel was measured using enzyme con-

TABLE II: Inhibition of Passive Hemagglutination Reaction between AK I-HDH I or AK II-HDH II with the Homologous Antisera, by the Heterologous Protein.

	immunological system			
	native AK I-HDH I antinative AK I-HDH I		native AK II-HDH II antinative AK II-HDH II	
inhibitor	inhibitor concn (μM)	hemagglutination titer	inhibitor concn (μM)	hemagglutination titer
native AK I-HDH I	0	1/59 049	0.3	1/177 147
	0.5	1/729	0.6	1/177 147
			3.5	1/177 147
native AK II-HDH II	0.5	1/59 049	0	1/177 147
	1	1/59 049	0.1	1/6561
			0.5	1/729

centrations ranging from 10 μg/mL to above 1 mg/mL. Figure 1 shows that native AK I-HDH I is indeed able to bind this gel, whatever its concentration, until the gel is saturated, whereas native AK II-HDH II shows no detectable binding, even at a concentration of 1.4 mg/mL. These experiments would have allowed detection of an amount of bound AK II-HDH II of the order of 5% of that of bound AK I-HDH I. This lack of binding of native AK II-HDH II to a specific immunoadsorbent gel directed against native AK I-HDH I is at variance with another report (Truffa-Bachi et al., 1975), although we used the same preparation of γ-globulins as that utilized by Truffa-Bachi et al., and the same method and activity measurements to test the binding of AK II-HDH II to the gel. However, recent work on AK II-HDH II has indicated that precise measurements of this enzyme activity required special precautions (Dautry-Varsat & Garel, manuscript in preparation), which were taken here. Without these precautions, the amount of AK II-HDH II activity recovered in the flow-through eluate is quite underestimated, thus leading to the incorrect conclusion that part of the enzyme has been retained by the gel.

As far as a possible immunological resemblance between native AK I-HDH I and AK II-HDH II is concerned, the results of the three different sets of experiments presented here, as well as those of gel diffusion tests previously reported (Kaminsky et al., 1968), clearly show that no such resemblance can be detected. Also, no similarity between native HSK and AK I-HDH I (or AK II-HDH II) can be evidenced by either passive hemagglutination, its inhibition, or complement fixation tests.

II. *There Is An Immunological Similarity between Denatured AK I-HDH I and AK II-HDH II.* AK I-HDH I and AK II-HDH II, as well as other proteins were denatured in 6 M guanidine, and their sulfhydryl groups were fully alkylated with NEM. All these alkylated proteins, except RNase, are hardly soluble in aqueous solution, as expected for denatured proteins. The insoluble alkylated AK I-HDH I and AK II-HDH II were injected as such, as an emulsion in complete Freund's adjuvant. This procedure leads to a satisfactory immune response. In order to test the antisera thus obtained, the antigens had to be solubilized, which was achieved by citraconylation of their amino groups.

Two different solubilized antigens were prepared for each denatured protein, one in which the sulfhydryl groups were alkylated by NEM as used in immunization, and the other in which these groups were carboxymethylated. The antisera directed against native AK I-HDH I, AK II-HDH II, or HSK still recognize their homologous antigen when denatured and solubilized, albeit less efficiently that when it is native, and do not react with any heterologous antigen whether native or denatured (Table I). The antisera directed against denatured

TABLE III: Passive Hemagglutination Titers of Antisera Directed against Denatured AK I-HDH I and AK II-HDH II.[a]

	antiserum against denatured	
	AK I-HDH I	AK II-HDH II
NEM-AK I-HDH I	1/6561-1/19 683	1/6561
NEM-AK II-HDH II	1/729	1/19 683
NEM-HSK	1/729	1/729
NEM-tryptophanase	1/729	1/243
NEM-β_2 subunit of tryptophan synthetase	1/729	1/729
NEM-ribonuclease A	1/9	1/9
CM-AK I-HDH I	1/6561	1/729
CM-AK II-HDH II	1/729-1/2187	1/6561
CM-HSK	1/27	1/81
CM-β_2 subunit of tryptophan synthetase	1/81	1/27
CM-ribonuclease A	1/3	1/3

[a] In this table, NEM and CM refer respectively to NEM-modified or carboxymethylated derivatives.

AK I-HDH I or AK II-HDH II react efficiently with their homologous antigens, when solubilized and either alkylated or carboxymethylated (Table III and Figures 2 and 3). Also, the antiserum raised against denatured AK II-HDH II recognizes denatured AK I-HDH I when either alkylated or carboxymethylated and does not react with any of the other proteins (Table III and Figures 2 and 3). The antiserum raised against denatured AK I-HDH I seems to react with several of the denatured proteins, among which is AK II-HDH II, when alkylated with NEM (Table III and Figure 2). But in the case of carboxymethylated proteins, the same antiserum recognizes only AK II-HDH II as heterologous antigen (Table III and Figure 3). These results therefore show the existence of a definite immunological cross-reaction between denatured AK I-HDH I and AK II-HDH II, and no such reaction with the other proteins tested. This cross-reaction is independent of the nature of the group carried by the cysteine residues in the case of the antiserum directed against denatured AK II-HDH II, and can also be detected between the antiserum raised against denatured AK I-HDH I and denatured and carboxymethylated AK II-HDH II.

III. *The Proteolytic Fragments of AK I-HDH I and AK II-HDH II Show an Immunochemical Similarity When Denatured and None When Native.* From both AK I-HDH I and AK II-HDH II, one can obtain a proteolytic fragment endowed with full homoserine dehydrogenase activity of respective molecular weight 2 × 55 000 and 2 × 35 000 (Véron et al., 1972; Dautry-Varsat & Cohen, 1977). As for the entire proteins, the antisera raised against native AK I-HDH I or AK

TABLE IV: Passive Hemagglutination Titers of Antisera Directed against Native and Denatured AK I-HDH I and AK II-HDH II.

	antiserum against native		antiserum against denatured[a]	
	AK I-HDH I	AK II-HDH II	AK I-HDH I	AK II-HDH II
native fragment HDH I	1/59 049	1/81		
native fragment HDH II	1/27	1/177 147		
CM-fragment HDH I	1/19 683	1/243	1/6561	1/2187
CM-fragment HDH II	1/27	1/177 147	1/729-1/2187	1/6561

[a] Results for control proteins are given in Table III.

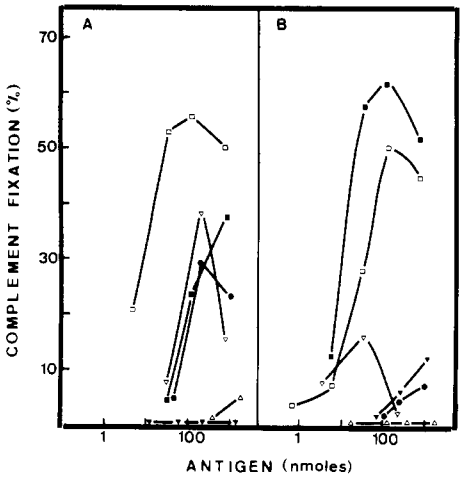

FIGURE 2: Complement fixation assays of the reaction of the antisera raised against denatured and alkylated with NEM (A) AK I-HDH I and (B) AK II-HDH II, with various proteins denatured, alkylated with NEM, and citraconylated. (□) AK I-HDH I; (■) AK II-HDH II; (△) HSK; (●) bovine pancreatic ribonuclease A; (▽) *E. coli* tryptophanase; (▼) β_2 subunit of *E. coli* tryptophan synthetase. The dilutions of the two antisera are 1/200.

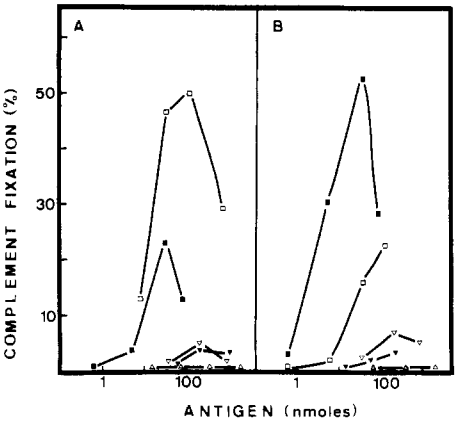

FIGURE 3: Complement fixation assays of the reaction of the antisera raised against denatured and alkylated with NEM (A) AK I-HDH I, and (B) AK II-HDH II, with various proteins denatured, carboxymethylated, and citraconylated. (□) AK I-HDH I; (■) AK II-HDH II; (△) HSK; (▽) *E. coli* tryptophanase; (▼) β_2 subunit of *E. coli* tryptophan synthetase. Bovine pancreatic ribonuclease A is not shown and gives no reaction with either antiserum. The dilutions of these two antisera are 1/200 in A and 1/100 in B.

II-HDH II recognize the homologous fragment when native, or when denatured, and do not recognize the heterologous fragment (Table IV). However, each denatured and carboxymethylated fragment is recognized by both the antisera raised against denatured AK I-HDH I and AK II-HDH II, i.e., against the homologous and heterologous entire protein (Table IV and Figure 4). We have checked that this recognition of a given denatured fragment by the heterologous antiserum is indeed due to the fragment and cannot arise from the possible contamination of this fragment by a few percent of the corresponding entire protein. These results show that the dehydrogenase regions contribute significantly to the immunochemical resemblance between denatured AK I-HDH I and AK II-HDH II.

Discussion

The two isofunctional enzymes AK I-HDH I and AK II-HDH II from *E. coli* K 12 show enough common functional and structural features, so that it has been proposed that these two proteins were evolved from a common ancestor (Falcoz-Kelly et al., 1969). The aim of this work is to characterize a possible structural similarity between these enzymes, using immunochemical methods, when either native or denatured.

I. *There Is No Immunological Cross-Reaction between Native AK I-HDH I and AK II-HDH II.* All the methods tested fail to reveal any immunological resemblance between native AK I-HDH I and AK II-HDH II. This lack of detectable cross-reaction is not due to the use of techniques such as hemagglutination and complement fixation, which both require rather low antigen concentrations, and could have masked a low affinity reaction. Tests of the inhibition of hemagglutination by a heterologous antigen were performed at a higher concentration of this antigen and were also negative. Also, the assays of binding AK II-HDH II to an immunoad-

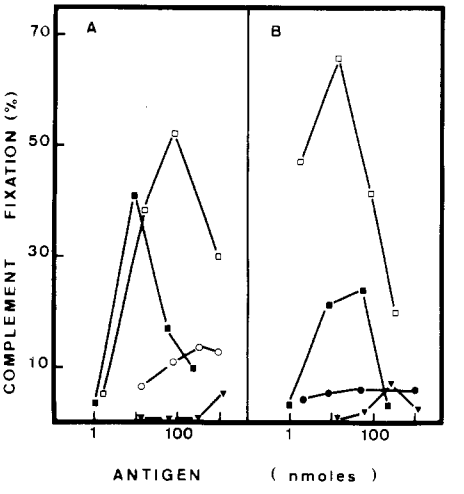

FIGURE 4: Complement fixation assays of the reaction of the antisera against denatured (A) AK I-HDH I and (B) AK II-HDH II with denatured (■) fragment HDH I and (□) fragment HDH II. Also shown are the results given by (▼) denatured β_2 subunit of tryptophan synthetase. Denatured bovine pancreatic ribonuclease A gives no reaction with either antiserum (*results not shown*). The dilutions of the antisera are 1/200 for the reaction with the fragment HDH I in A, 1/100 for that with the fragment HDH II in B, and 1/50 for all other reactions. Serum from nonimmunized rabbits (dilution 1/50) does not react with denatured (○) fragment HDH II and (●) fragment HDH I.

sorbent gel specifically directed against AK I-HDH I were carried out at an AK II-HDH II concentration as high as 1.4 mg/mL and were negative. Therefore no immunochemical similarity can be detected between AK I-HDH I and AK II-HDH II when native.

There have already been a few cases where proteins known to have homologous amino acid sequences did not show any resemblance when measured immunochemically in their native state (Arnon, 1974). These results are usually interpreted by assuming that the immunological properties of native proteins are due to the surface of these molecules, while most of the residues conserved in homologous proteins are those involved in structure and function and are rather buried in the inside. Unfolding homologous (or putatively homologous) proteins may render accessible the regions involved in the sequence homology, which are masked in the interior of the native molecules. This is indeed the approach used in the present work.

II. *There Is an Immunological Cross-Reaction between Denatured AK I-HDH I and AK II-HDH II.* Denatured AK I-HDH I and AK II-HDH II are hardly soluble in aqueous solvent. We have obtained antisera directed against these insoluble species, and tested the reactions of these antisera with various antigens which have been solubilized by citraconylation of their amino groups. This procedure probably masks some of the antigenic determinants present in the original proteins and thus may decrease the apparent immunochemical resemblance as compared with that which would be observed with nonmodified proteins.

The results given in Figures 2 and 3 and Table III clearly show that there is a definite cross-reaction between AK I-HDH I and AK II-HDH II when in their denatured state. The antiserum directed against denatured AK II-HDH II reacts only with denatured AK I-HDH I and AK II-HDH II, whether the protein sulfhydryl groups have been alkylated by NEM or carboxymethylated (Figures 2 and 3; Table III). Also, the antiserum directed against denatured AK I-HDH I reacts significantly with denatured and carboxymethylated AK II-HDH II (Figure 3 and Table III). However, the antiserum directed against AK I-HDH I, denatured and alkylated by NEM, can react with several other proteins, among which is AK II-HDH II, also denatured and alkylated by NEM (Figure 2 and Table III). This result suggests that this antiserum contains some antibodies directed against alkylated sulfhydryl groups and/or neighboring residues. But the specific reaction between this antiserum and denatured AK II-HDH II can be detected by changing the alkylating groups, which abolishes the interference of these antibodies.

The cross-reaction observed between denatured AK I-HDH I and AK II-HDH II is specific for the use of the denatured state of these proteins to produce antisera. Indeed, there is no cross-reaction between the antisera directed against the native proteins and the heterologous denatured protein, while there is still a measurable homologous reaction (Table I). This is a further argument to show that antibodies against native AK I-HDH I and AK II-HDH II do have strictly different specificities, and do recognize only the homologous antigen whether native or denatured. So, it is only the immunization with denatured proteins which has elicited the production of antibodies capable of recognizing both AK I-HDH I and AK II-HDH II in their denatured state, and responsible for the observed cross-reaction. The common antigenic determinants involved in this cross-reaction are thus completely masked in the native proteins.

The similar results obtained with the proteolytic fragments can be interpreted in the same way. Since they possess full dehydrogenase activity when native, these fragments should have a folded structure, where no common antigenic determinant is apparent. The common antigenic determinants appear only upon unfolding these fragments. The immunochemical resemblance between denatured AK I-HDH I and AK II-HDH II is confirmed by an independent set of experiments using their proteolytic derivatives; this resemblance is at least partly due to the homoserine dehydrogenase region of the polypeptide chains.

The significant recognition by an antiserum directed against one of the native enzymes, AK I-HDH I or AK II-HDH II, of an homologous denatured proteic species [entire protein, (Table I) or fragment (Table IV)] is quite surprising. This result might suggest that either denatured species are contaminated with native material, or the native enzymes used for immunization are contaminated with denatured material. The former possibility seems unlikely because all denatured proteins are completely devoid of the activity associated with their native state. The latter possibility seems also unlikely because it should have lead to the presence in these antisera of antibodies reacting with both the homologous and heterologous denatured proteins, which is not the case (Tables I and IV). In denatured species, either the survival of *"native"* antigenic determinants, or an antibody-induced local *"native"* structure, or both, could give rise to their observed reaction with antisera against the native enzymes.

III. *Significance of the Immunological Similarity between Denatured AK I-HDH I and AK II-HDH II.* Denatured proteins are assumed not to have any defined and stable conformation (Tanford, 1968). Some segments of the polypeptide chain may be in a preferred configuration, but only as the result of short range interactions due to the local amino acid sequence. The antigenic determinants present in a denatured protein should therefore strongly depend on the sequence and should correspond to a few residues, either to their sequence itself or to a particular configuration of this sequence, the one that it would take by itself. It is thus likely that an immunochemical resemblance between two denatured proteins reveals a similarity in the amino acid sequences of these proteins (Arnon & Maron, 1971; Arnheim et al., 1971). Also, it has been observed that the immunochemical properties of one of the denatured proteolytic fragment are the same as those of the pool of its cyanogen bromide peptides (Sibilli & Zakin, unpublished), which shows that only local structural elements are involved in the immunochemical behavior of denatured proteic species. It is impossible to correlate the extent of immunochemical resemblance between denatured AK I-HDH I and AK II-HDH II with the extent of homology of their amino acid sequences. That the cross-reaction between these two denatured proteins is specific for their amino acid sequences is supported by the fact that several other proteins do not show such a cross-reaction, when denatured. Finding such a similarity between the sequences of AK I-HDH I and AK II-HDH II strongly suggests that these two proteins derive from a common ancestor. Indeed we do not believe that the immunochemical resemblance observed could arise only from these two proteins having comparable sites for the same ligands. HSK is a kinase and can bind homoserine and ATP and does not show any similarity to AK-HDH I or II, when either native or denatured. Therefore we take our results as suggesting that AK I-HDH I and AK II-HDH II are evolutionally related and possess homologous amino acid sequences.

IV. *The Use of Denatured Proteins to Detect Immunochemically Sequence Homologies.* In all cases reported so far, the existence of a cross-reaction between two denatured pro-

teins and the antisera raised against them has been correlated with the existence of the homology between the amino acid sequences of these proteins (Arnon & Maron, 1971; Arnheim et al., 1971). The generalization of this correlation to cases where the sequences are not known appears reasonable considering both the properties of a denatured polypeptide chain and the specificity of the antigen–antibody reaction. Measuring cross-reactivity between denatured proteins could thus be a simple and rapid method to detect sequence homologies. In the present work, we show that such a cross-reaction between denatured proteins is not observed for arbitrarily taken proteins and, thus, has some specificity, and also that it is not related to similar functional features, like similar ligands or catalyzed reactions. These observations suggest that the method used here for AK I–HDH I and AK II–HDH II could be generalized and may be useful to characterize evolutional relationships, sequence redundancies in a protein, precursor to product relationships, gene fusion products, nonsense mutation products, etc. Further work is now in progress to relate the extent of cross-reaction between denatured proteins to the actual extent of homology between their amino acid sequences.

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