*MULTIPLE FORMS OF FRUCTOSE DIPHOSPHATE ALDOLASE IN MAMMALIAN TISSUES**

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Many of the molecular and catalytic properties of aldolase A, the classical muscle enzyme, and aldolase B, liver aldolase, have been defined.[1] Although these enzymes have generally similar molecular properties, they have different primary structures and can also be readily distinguished by their catalytic properties.[2] In this paper, we report the existence of a third fructose diphosphate aldolase termed "aldolase C" which is distinct from aldolases A and B. These three parent forms of aldolase appear to be structurally homologous since hybrids of these enzymes can be formed by dissociation and reassociation of mixtures of the parent aldolases. Five forms, including three hybrids, are detectable by zone electrophoresis and by chromatography for each two-membered set of parent aldolases. Hybrid forms of the aldolase A-B and aldolase A-C sets are found in extracts of tissues which are known to contain aldolases A and B or A and C, respectively. The fructose diphosphate aldolase activity of all tissues examined can be related to the presence of the parent FDP aldolases A, B, C, or their hybrids. The number of hybrids found (three) in each two-membered aldolase set is difficult to reconcile with a three-chain model of the enzyme, but it is easily harmonized with a molecule composed of four subunits. In spite of considerable chemical and physical evidence supporting the three-chain model, we have been led to consider the latter possibility seriously.

Materials and Methods.—Enzymes and reagents: Rabbit muscle aldolase A (Boehringer) had a specific activity ranging from 10 to 12 units/mg protein. Aldolase B, prepared by the method of Rajkumar, Woodfin, and Rutter,[3] had a specific activity of 1.5 units/mg protein. Glyceraldehyde-3-phosphate dehydrogenase was purchased from Boehringer. NaFDP, NAD, and NADH were procured from Sigma Chemical Co. Nitroblue tetrazolium and phenazine methosulfate were obtained from Aldrich Chemical Co.

Assays: Aldolase activity was assayed as described elsewhere.[4] A unit of enzymatic activity is defined as the cleavage of 1 μmole of substrate (FDP or F1P) per min.

Immunochemical analysis: Preparation of antibodies against aldolases A and B and the methods of immunochemical analyses have been described elsewhere.[4, 5]

Preparation of tissue extracts: The tissues were excised, minced, and homogenized in the cold for 15 sec in presence of 2 vol (w/v) of 0.01 *M* Tris Cl and 0.001 *M* EDTA, pH 7.5. The homogenate was centrifuged at 100,000 *g* at 2° for 60 min.

Zone electrophoresis: Zone electrophoresis was performed in 0.06 *M* barbital, 0.01 *M* β-mercaptoethanol, pH 8.6, on $6^3/_4''$ cellulose acetate strips (Gelman) at 250 v for 90 min with the origin equidistant from the electrodes. The strips were stained for aldolase activity by a modification of a procedure developed by Crawford.[6]

A 0.5% Noble agar solution in 0.01 *M* $NaAsO_4$, pH 7.5, containing 0.01 *M* NaFDP (Calbiochem), 0.001 *M* NAD, 0.12 mg/ml glyceraldehyde-3-phosphate dehydrogenase, 0.024 mg/ml phenazine methosulfate, and 0.4 mg/ml nitroblue tetrazolium chloride was poured into shallow dishes (4 ml per Petri dish) at 42° and allowed to solidify at 4°. After electrophoresis, the cellulose acetate strips were placed on this agar and incubated at 37° for 10–20 min to allow color development.

In vitro hybridization: The indicated proportions of the aldolases were mixed in 0.1 *M* glycylglycine and 0.01 *M* EDTA, pH 7.5, to obtain a final concentration of 1.0 mg/ml. Cold 1 *M* H_3PO_4

(0.2–0.4 ml) was added to the mixture at 2° to bring the pH to 2.0. After about 30 min, the enzyme was diluted to approximately 0.1 mg/ml in a medium containing 1 mg/ml albumin in 0.1 *M* glycylglycine and 0.01 *M* EDTA, pH 7.5, at 0°. The pH of the solution was adjusted to 7.5 by careful addition of cold 1 *N* NaOH or 1 *M* Tris base (0.2–0.3 ml). The solution then was concentrated to approximately 1 mg/ml by ultrafiltration or vacuum dialysis.

Results.—Immunochemical evidence for a third aldolase: The activity of aldolases A and B can be distinguished in crude extracts by substrate specificity (FDP/F1P activity ratio = $\sim$ 50 and 1 for aldolases A and B, respectively), or by inhibition with specific antibodies. (Antibodies prepared against aldolase A precipitate and specifically inhibit aldolase A activity, but are indifferent to aldolase B; and, conversely, antibodies prepared against aldolase B precipitate and inhibit aldolase B activity, but have no effect on aldolase A activity.) Using these criteria, Blostein[4] and Weber[5] in this laboratory defined the distribution of aldolases A and B in various tissues. The results are summarized in Table 1. In all tissues examined, except brain, the aldolase activity behaved as aldolase A, aldolase B, or a mixture of the two. The aldolase activity in muscle, heart, and spleen exhibited an FDP/-F1P activity ratio approaching 50, the value for pure aldolase A, and was also quantitatively inhibited by anti-A. On the other hand, the aldolase activity in liver and kidney extracts exhibited an FDP/F1P activity ratio of approximately 1 and 2, respectively. The degree of inhibition by anti-A and anti-B corresponded to that predicted from the FDP/F1P activity ratios of pure aldolases A and B. In contrast to the above, the FDP/F1P activity ratio of brain tissue was 26, a value which would predict more than 95 per cent inhibition by anti-A. Only partial (80%) inhibition by excess anti-A was observed, and no further inhibition was detected on addition of excess anti-B. The residual aldolase activity after treatment with excess anti-A and anti-B exhibited an FDP/F1P activity ratio of 5 in this experiment (in other experiments, the ratios have been somewhat higher). The activity refractory to anti-A and anti-B could be associated with a new enzyme or could be the result of a "bound form" of aldolases A and B not readily accessible to the antibody. Experiments to be presented indicate the former explanation is valid.

Resolution of FDP aldolases by zone electrophoresis: The difference in mobilities of aldolases A and B in free-boundary electrophoresis[2] suggested the possibility of resolution of these proteins by zone electrophoresis. In the experiments to be described, electrophoresis was carried out on cellulose acetate strips. Aldolase activity was detected by means of an activity stain. In this system, aldolase is

TABLE 1

ALDOLASE CONTENT OF ADULT RABBIT TISSUES

	FDP/F1P activity ratio	Inhibition FDP activity by excess anti-A (%)	Residual FDP/F1P Activity ratio in excess anti-A	Inhibition FDP activity by excess anti-B (%)	Residual FDP/F1P activity ratio in excess anti-A and B
Muscle	55	96	—	0	—
Heart	44	100	—	0	—
Spleen	49	100	—	0	—
Brain	26	80	5.1	0	5.1
Kidney	2.7	70	0.81	30	—
Intestine	1.4	24	1.1	—	—
Liver	1.2	7	1.1	94	—

Assay procedures and details of the immunochemical studies are discussed in *Materials and Methods*.

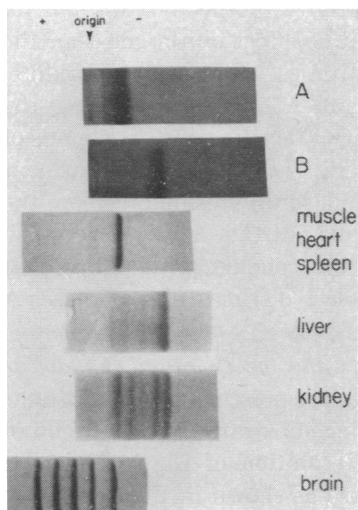

FIG. 1.—Patterns of aldolase activity of adult rat tissues after zone electrophoresis. Aldolase, 0.01–0.03 units (1–3 μg protein), was applied on the cellulose acetate strips. Electrophoresis and staining for aldolase activity was carried out as described in *Materials and Methods*.

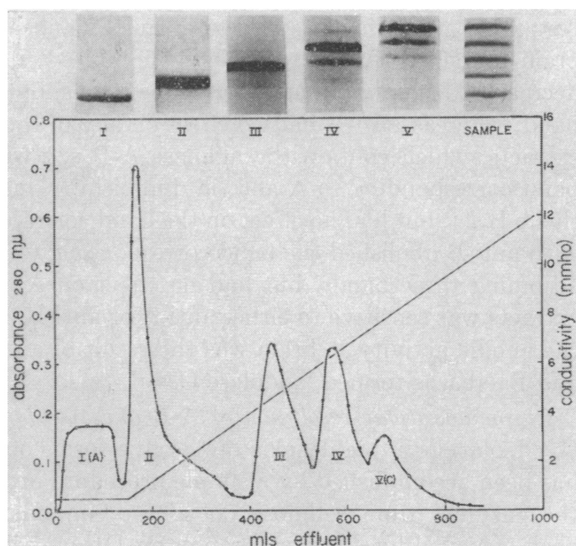

FIG. 2.—DEAE-cellulose chromatography of A-C aldolase set isolated from rabbit brain. The aldolase activity was purified by ammonium sulfate fractionation and cellulose phosphate chromatography. The sample placed on the DEAE column had a specific FDP activity of 10 μM/min/mg protein. The fractions were eluted with a linear gradient from 0 to 0.4 M NaCl in 0.01 M Tris Cl, 0.001 M EDTA, and 0.0005 M FDP, pH 7.5. Protein was determined by absorbance at 280 mμ. Pooled fractions were analyzed by zone electrophoresis as described in *Materials and Methods*. The patterns of aldolase activities are shown.

coupled with triosephosphate dehydrogenase so that cleavage of FDP results in the formation of NADH which in turn reduces nitroblue tetrazolium via the intermediation of phenazine methosulfate (see *Materials and Methods* for details). This assay is relatively more sensitive to aldolase A than to aldolase B, because the former has a turnover number (with FDP as substrate) approximately ten times that of aldolase B. Relatively low levels of aldolase A contamination in mixtures can thus be detected, but not vice versa. As shown in Figure 1, three patterns of aldolase activity were detected after zone electrophoresis of extracts from various tissues. The aldolase activity in skeletal muscle, heart, and spleen migrated as a single band with the same mobility as crystalline aldolase A. In contrast, five bands of activity were discernible in tissues containing mixtures of A and B. These bands are termed the A-B set. The most anodic and cathodic bands had a mobility coinciding with pure A and B, respectively. Moreover, added aldolases A and B reinforced the most divergent anodic and cathodic bands, respectively. Five bands were readily apparent in kidney extracts. Even in liver extracts, where more than 98 per cent of aldolase protein is aldolase B,[5, 7] the four other bands of the A-B set were discernible. Five bands of aldolase activity were also observed in brain extracts: one band with a mobility corresponding to that of aldolase A, the other four bands migrating toward the anode (in contrast to the A-B set). The possibility that the extreme anodic band was a result of a new aldolase activity was

tested by measuring the effects of anti-A and anti-B on the aldolase activity of brain extracts. Anti-A completely inhibited the activity of the A band and, in decreasing fashion, the adjacent three bands, but had no effect on the most anodic band, whereas anti-B had no effect on any of the activities. Treatment of kidney extracts, which contain the aldolase A-B set, with anti-A selectively removed the band corresponding to A and, in diminishing quantity, the adjacent three bands of the A-B set, but had no effect on the band corresponding to aldolase B. Treatment with anti-B abolished the band corresponding to B and, in diminishing fashion, the adjoining three bands, but had no effect on A. Thus, all the activity in kidney extracts was sensitive to either anti-A or anti-B. It was concluded, therefore, that the anodic activity in brain was the result of an aldolase distinct from aldolases A and B; it was termed "aldolase C."

Chromatographic resolution of A-B and A-C hybrids and the isolation of aldolase C: Isolation in high yields of the five forms of aldolase present in brain extracts has been accomplished by a simple procedure involving substrate (FDP) elution of the enzymes from cellulose phosphate columns. Resolution of the hybrids has been achieved by chromatography on DEAE cellulose as shown in Figure 2. Aldolase C (the terminal peak) catalyzes the cleavage of approximately 7 μmoles FDP/min/mg protein under standard assay conditions, and exhibits an FDP/F1P activity ratio of approximately 10. The molecular and catalytic properties of aldolase C and the hybrids of the A-C set will be reported in another communication.

Members of the A-B hybrid set have been partially resolved by chromatography on cellulose phosphate. Electrophoretic analysis shows that the first and last peaks contain only aldolases A and B, respectively. The three central peaks are enriched for the corresponding band in the zymogram, but are not completely free of neighboring hybrid species. The FDP/F1P activity ratios of the A-B hybrids fall in an ordered series between those of the parent molecules (aldolase B = 1, aldolase A = 50). The specific activities of the bands also fall in an ordered series between 1.5 and 15, the activities of aldolases B and A, respectively.

Formation of hybrids of aldolases A, B, and C: The possibility that the intermediate three bands in the five-membered sets detected in tissue extracts were hybrids was confirmed by experiments in which bands of similar mobility were produced from appropriate mixtures of aldolases A, B, and C after dissociation and reassociation *in vitro.*[8] Under the conditions employed, 100, 25, and 50 per cent recovery of aldolase activity was obtained by reversible dissociation of aldolases A, B, and a mixture of A and B. Figure 3 shows the patterns produced after dissociation and reassociation of aldolases A, B, and C singly, and in various combinations. Three intermediate bands were observed in each of the A-B, A-C, and B-C sets. The inclusion of native enzyme into the reassociated hybrid mixtures always reinforced the outer bands. Reversible dissociation of mixtures of aldolases A, B, and C resulted in the formation of a complex pattern containing at least 11 discernible bands. In more recent experiments giving similar results, more than 50 per cent recovery of activity has been obtained in reversible dissociation of aldolase B, and correspondingly higher recoveries with mixtures of A and B.

Production of members of the A-C set from a single hybrid form: The middle hybrid of the A-C set was crystallized by dialysis against ammonium sulfate solu-

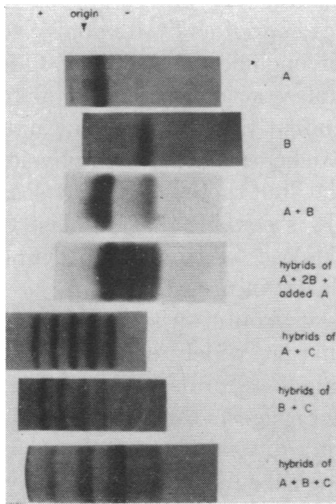

FIG. 3.—Electrophoretic patterns of the aldolase A, B, and C hybrid sets. Solutions of the hybrid sets were obtained as described in *Materials and Methods*. In the case of A-B hybrids, pure A was added to the hybrid mixture. The parent enzymes here were obtained from the rabbit. Electrophoresis and activity stain were carried out as described in *Materials and Methods*.

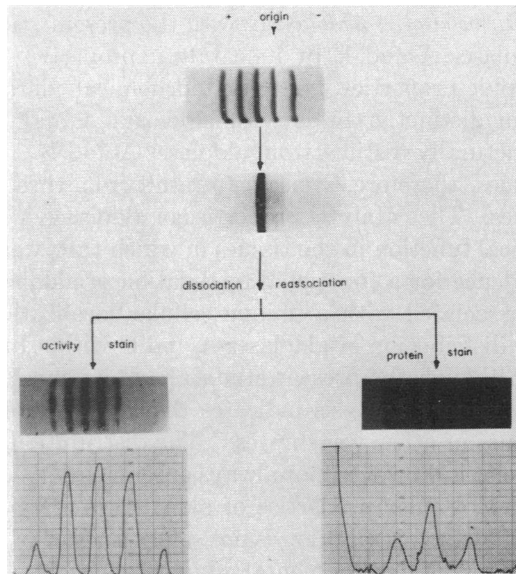

FIG. 4.—Formation of the A-C hybrid set by reversible dissociation of the middle A-C hybrid is depicted. *Top*, pattern obtained from the original brain extract; *middle*, the middle hybrid isolated from the brain by chromatography on DEAE cellulose. The middle hybrid was then dissociated into subunits and recombined as described in *Materials and Methods;* one set of electropherogram (*bottom left*) was obtained according to the procedure outlined in Fig. 1 and stained for aldolase activity as described in *Materials and Methods*. Another was run for 30 min longer (120 min total) at 250 v and stained for protein (*bottom right*) using Coomassie brilliant blue, R-250. The darkest protein band on the left side of the strip is due to the albumin used in the reassociation medium.

tions. The molecular weight is similar to that of aldolases A and B as determined by sedimentation equilibrium.[9] Its electrophoretic mobility corresponded to that of the middle band in the crude extracts. The enzyme was reversibly dissociated according to the procedure detailed in the *Materials and Methods* section. Appropriate aliquots were subjected to electrophoresis and stained for activity and protein. As shown in Figure 4, all members of the A-C set were formed after reversible dissociation of the middle hybrid. The densitometric tracing of the activity stain showed the relative intensity of the peaks to be 1:4.7:5.8:4.2:1.3 (reading from C to A). For analysis with a protein stain, larger quantities of protein were employed and a longer time was required for electrophoretic resolution of the most anodic aldolase band from the albumin present in the system. Although five protein bands corresponding to the bands of aldolase activity were discernible, the densitometric tracing indicated that only the middle three peaks were above background noise; the area under these peaks was approximately 1:2:1.

From the above experimental results, it is concluded that the intermediate three bands seen in the five-membered sets in tissue extracts are hybrids composed of subunits of the parent aldolases.

Discussion.—*Aldolase C:* In the present study, aldolase C is differentiated from aldolases A and B by its catalytic properties, electrophoretic mobility, chromatographic properties, and immunochemical characteristics as well as by its ability to form distinct hybrids with aldolases A and B. We conclude that aldolase C is structurally distinct from aldolases A and B. The aldolase activity in mammalian tissues, therefore, appears to result from three independently regulated structural genes. The catalytic properties of aldolases A and B can be correlated with physiological function in the tissues in which they were found,[5,7] but there is as yet no clear evidence for a functional modulation of aldolase C. It is possible that aldolase C is associated with a unique cellular localization of aldolase. Attempts to define the distribution of aldolases A and C within brain cells are in progress.

Although the present studies have involved the tissues from rats and rabbits, a preliminary analysis indicates that three aldolase forms are widely distributed in tissues of other vertebrates. The distribution of the aldolase forms in the various species is, however, not always organ-specific. This fact suggests that the catalytic and molecular properties of aldolases A, B, and C may differ in various species, or that the distribution is not solely a result of physiological suitability but rather, perhaps, of developmental advantage.

Hybrid forms: The existence of hybrids in tissues containing more than one parental form of aldolase indicates the synthesis of the subunits of both aldolase molecules in the same cell. Thus, it excludes the possibility of synthetic exclusivity of aldolases A, B, and C in specific cells. This was a strong possibility, at least for aldolases A and B, since the distribution of these enzymes appeared to be segregated in an all-or-none fashion.[5,7] As shown by the data in Figure 4, the distribution of hybrids formed by reversible dissociation of two parental aldolases approximates that expected in a random process. The favored explanation of a zymogram pattern which is divergent from a binomial distribution is synthetic compartmentalization. Thus, the patterns obtained for liver and kidney are most easily explained in terms of cell heterogeneity in those organs: in liver, presumably the parenchymal cell synthesizes predominately aldolase B, and another cell type synthesizes the subunits of both aldolase A and aldolase B in similar proportions. The relatively weak central band observed in extracts of kidney tissue could be the result of two populations of cells, one synthesizing primarily aldolase A, and the other, aldolase B.

Number of hybrid forms and molecular structure: The demonstration of three hybrid forms for each pair of parental aldolases (Fig. 3) indicates a homologous structural relationship among the parental aldolases. Considerable catalytic and structural evidence suggesting a homologous relationship between aldolases A and B has already been presented.[10]

The number of hybrids produced in a given set must be related to the subunit structure of the parental aldolase molecules. Most of the accumulated evidence for aldolase A is consistent with a three-subunit molecule. Three C-terminal tyrosines,[11,12] three N-terminal prolines,[13,14] three substrate binding sites,[15,16] and three subunits of molecular weight approximately 50,000 in a molecule of molecular weight 150,000[17,18] have been reported for aldolase A. In contrast to the above, Kawahara and Tanford[19] have claimed that four subunits of molecular weight approximately 40,000 are present in the aldolase molecule.

The stoichiometry of hybrid formation is relevant to the problem of the subunit composition of the aldolase molecule. The symmetry of a three-subunit system results in the formation of even-numbered sets. In contrast, recombination of the homologous proteins containing four identical subunits results in a five-membered set containing three hybrid species. Therefore, an unequivocal demonstration of a five-membered set (three hybrids) becomes a strong argument for a structure containing four identical subunits. Hence, the validity of the demonstration of five-membered sets containing three hybrids must be carefully evaluated. The present experiments, of course, have analytical limitations; combinative restrictions as well as compensatory electrophoretic and chromatographic properties could limit the number of enzymatic forms detected. For a number of reasons, however, we believe the data may be a reflection of molecular reality: (1) Similar results have been obtained by electrophoresis in polyacrylamide gels or cellulose acetate using different buffer systems. There is little effect of added salts on the resolution of the five bands (three hybrid forms). (2) Aldolases A, B, and C exhibit similar electrophoretic mobilities when present in crude or highly purified systems. (3) There is no detectable change in the electrophoretic pattern observed by incubating extracts prior to analysis. (4) The various members of the A-C set are resolved chromatographically with no evidence of enzymatic heterogeneity in the purified fractions. (5) Five members of the aldolase A-C set are found on reversible dissociation of a crystalline preparation of a single hybrid. (6) The same number of hybrids is found in each of the three two-membered sets studied, even though members of each of the sets vary widely in the degree of resolution by the electrophoretic and chromatographic means employed. It appears unlikely that in all these experiments the estimation of the number of hybrids formed by mixtures of the parental aldolases would be in error. However, the chemical and physical measurements. which have led to the three-subunit model for the aldolase molecule cannot be easily ignored; each determination has its own uncertainties, but the congruity of the results is persuasive. We believe that the evidence on balance is sufficiently contradictory to force a reconsideration of this fundamental structural characteristic of the FDP aldolase molecules. A direct test of combinative proportions is currently under way in our laboratory. We hope that the resolution of this structural dilemma will lead to an understanding of a molecular idiosyncrasy which is as interesting as it is momentarily obfuscating.

Summary.—(1) A new fructose diphosphate aldolase termed "aldolase C" has been isolated from rabbit brain. Aldolase C can be distinguished from aldolase A and B by its catalytic properties, electrophoretic and chromatographic properties, and its immunochemical characteristics. Antibodies prepared against aldolase A and B do not cross-react with aldolase C.

(2) Five-membered aldolase sets including three hybrid forms are produced *in vitro* by reversible dissociation of a mixture of any two of the parental aldolases (A, B, or C). All five members of the A-C set can be produced on reversible dissociation of a single crystalline hybrid of the A-C set. Such hybrid forms are found *in vivo* in tissues which contain more than one parental aldolase type.

(3) The number of hybrid forms (three) can be easily reconciled with an enzyme composed of four similar subunits, but not with three-chain models of the enzyme.

The analysis of aldolase content of tissues reported in Table 1 was performed by Drs. R. Blostein and Carl Weber. The technical assistance of Mr. David A. Walsh is gratefully acknowledged.

Abbreviations employed in this paper: NaFDP, fructose-1,6-diphosphate sodium salt; NaF1P, fructose-1-phosphate sodium salt; NADH, reduced nicotinamide adenine dinucleotide; NAD, oxidized nicotinamide adenine dinucleotide; EDTA, ethylene diamine tetraacetic acid; Tris Cl, tris-(hydroxymethyl)-amino methane hydrochloride.

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