ACTA CHEMICA SCANDINAVICA 17 (1963) S 226 – S 232

# Enzymic Homology

## Structural and Catalytic Differentiation of Fructose Diphosphate Aldolase*,**

WILLIAM J. RUTTER, BEULAH M. WOODFIN and RHODA E. BLOSTEIN

*Department of Chemistry and Chemical Engineering, Biochemistry Division, University of Illinois, Urbana, Illinois, USA*

The catalytic and structural properties of aldolase isolated from muscle (aldolase A) and two aldolases isolated from liver (aldolases $B_1$ and $B_2$) have been summarized. The immunochemical properties as well as analysis of fingerprint patterns of peptides have indicated that aldolases $B_1$ and $B_2$ have a closely similar structure but that aldolase A has a different primary structure. It is thus presumed that the synthesis of aldolase A is at least partially defined by separate regions of the genome than that of aldolases $B_1$ and $B_2$. In spite of the specific differences reported, the general molecular structure and certain properties of the catalytically active site of aldolases A, $B_1$ and $B_2$ are reminiscent of each other. It is proposed that these two species of aldolase thus are related phylogenetically.

The biological significance of these enzyme homologs may be in the regulation of metabolic processes in specific cells.

Metabolic pathways may be defined as a sequence of enzyme-catalyzed reactions. It has been frequently assumed that a single enzymatic species is associated with a particular reaction. The credence of this concept has been strengthened by the demonstration that certain metabolic pathways can be blocked by a genetic mutation affecting the structure of a single enzymatic entity. The availability in recent years of more discriminating procedures for resolution of protein mixtures has resulted in the demonstration of heterogeneity in enzymatic populations catalyzing a single reaction. These multiple molecular forms associated with one enzyme activity have been termed "isozymes" by Markert and Møller[1]. Isozymes need not have an independent genetic origin,

* This investigation was supported in part by a grant from the *National Institutes of Health, United States Public Health Service.*

** Part of the data are taken from a dissertation submitted by Beulah M. Woodfin to the Graduate College of the University of Illinois in partial fulfilment of the requirements for the degree of Doctor of Philosophy, 1963.

since multiple forms may also represent 1) heterogeneity in a genetic product, 2) multiple, stable conformations of the same structure, or 3) the modification of a given gene product by other enzymatic systems or by the chemical environment.

The existence and biological significance of structurally unique enzymes catalyzing the reversible aldol cleavage of fructose-1,6-diphosphate (FDP)* is considered here. It has been previously demonstrated[2–4] that the aldolase found in muscle differs from the enzyme found in liver tissue, both catalytically (as evidenced by differences in the relative specific activity toward the substrates FDP and F1P) and structurally (as evidenced by the different immunochemical properties of the molecules against anti-rabbit muscle aldolase). Two proteins exhibiting aldolase activity have now been isolated from rabbit liver in crystalline form. The catalytic and structural properties of liver aldolases (hereafter called aldolases $B_1$ and $B_2$) are very similar, but they are significantly different from the primary structure and catalytic activity of muscle aldolase (hereafter called aldolase A).

## MATERIALS AND METHODS

*Enzymes.* The isolation and assay of the enzymes are described in detail elsewhere[5]. The enzymatic assays were performed using the coupled $\alpha$-glycerophosphate dehydrogenase system. One unit of enzymatic activity is defined as the amount of enzyme facilitating the aldol cleavage of 1 $\mu$mole of substrate/min. The specific activity is defined in terms of units of activity/mg of protein. Protein was estimated by the absorption at 280 m$\mu$ (O.D. of .91 = 1 mg protein/ml).

*Structural studies.* Immunochemical procedures are detailed elsewhere[4]. Antibodies to crystalline rabbit muscle aldolase (anti-aldolase A) and to crystalline rabbit liver aldolase 2 (anti-aldolase $B_2$) were prepared in chickens. Studies of the molecular properties have included: electrophoretic and ultracentrifugal analysis, molecular weight determinations, amino acid analysis, two-dimensional chromatographic-electrophoretic (fingerprint) analysis, and amino acid terminal group estimations; detailed procedures are described elsewhere[5].

## RESULTS

*Catalytic activity.* The comparative catalytic properties of aldolases A, $B_1$ and $B_2$ are presented in Table 1. The activities of aldolases $B_1$ and $B_2$ are similar but not identical. The basic difference in catalytic properties of aldolases A and B, originally suggested by studies of rabbit muscle and bovine liver aldolases[2], is clearly indicated by the data obtained on enzymes from the same species. The catalytic activity of aldolase A is an order of magnitude greater than aldolases B with respect to FDP cleavage. In contrast, aldolases B exhibit a greater activity toward F1P than aldolase A. Aldolases B are also relatively more effective in catalyzing the synthesis of FDP from triose phosphates under the experimental conditions employed. Aldolases B have lower $K_m$ values for FDP and F1P and the triose phosphates.

The pH optima of the aldolases A and B are different. The acidic portions of the pH profile are similar but the activity of aldolase A is unaltered from pH 6.5 to 9.0, while the activity of aldolases B declines above pH 8.0 giving a maximum at approximately pH 7.5. A difference of at least one dissociable group at the

* The following abbreviations are employed in this paper: FDP, fructose-1,6-diphosphate; FIP, fructose-1-phosphate.

*Table 1.* Catalytic properties of aldolases.

| Aldolase | Substrate | $K_m$(M) | $V_{max}$* | pH optimum | % activity remaining after carboxypeptidase treatment |
|---|---|---|---|---|---|
| A | FDP | $6.1 \times 10^{-5}$ | 5 300 | 7–9 | 5 |
| | F1P | $1.2 \times 10^{-2}$ | 105 | 7.0 | 50 |
| | DHAP | $2.1 \times 10^{-3}$ | 9 450 | – | – |
| | GA3P | $1.1 \times 10^{-3}$ | 10 400 | – | – |
| $B_1$ | FDP | $1.2 \times 10^{-6}$ | 230 | 7.7 | 48 |
| | F1P | $8.7 \times 10^{-4}$ | 460 | 7.6 | 50 |
| | DHAP | $4.2 \times 10^{-4}$ | 2 500 | – | – |
| | GA3P | $2.8 \times 10^{-4}$ | 2 600 | – | – |
| $B_2$ | FDP | $2.3 \times 10^{-6}$ | 460 | 7.8 | 49 |
| | F1P | $8.3 \times 10^{-4}$ | 460 | 7.7 | 48 |
| | DHAP | $3.7 \times 10^{-4}$ | 2 800 | – | – |
| | GA3P | $3.0 \times 10^{-4}$ | 3 000 | – | – |

* Moles of hexose cleaved or synthesized per min per mole of enzyme at 30°C.

active site of the two enzymes is necessary to explain the difference observed in the basic regions of the profile.

It has been shown that carboxypeptidase treatment of muscle aldolase lowers the FDP cleavage activity to 5 % and that of aldolases B to approximately 50 % of the activity of the native enzyme[5,11]. Three, one to two, and no tyrosines are released from aldolases A, $B_1$ and $B_2$, respectively, by this treatment[5]. The substrate specificity and specific catalytic activity of carboxypeptidase-degraded aldolases A and B are similar.

*Molecular properties.* The general molecular properties of the aldolases are presented in Table 2. A single symmetrical boundary was observed in both

*Table 2.* Molecular properties of aldolases. Conditions of sedimentation constant determinations: for the native aldolases, 8–10 mg/ml enzyme in 0.015 M sodium phosphate, pH 7.4 at 20°C; for acidified aldolases, 1.4 mg/ml enzyme in 0.05 M citrate, pH 2.2 – 2.6, at 5°C.

| Aldolase | Molecular weight | $S_{20,w}$ sec $\times 10^{-13}$ | $D_{20,w}$ cm$^2$ sec$^{-1}$ $\times 10^{-7}$ | $\frac{f}{f_0}$ | $V_{20}$ ml g$^{-1}$ | $\mu$(pH 7.5) cm$^2$ v$^{-1}$ sec$^{-1}$ $\times 10^{-5}$ | $S_{20,w}$ (pH < 3.0) sec $\times 10^{-13}$ |
|---|---|---|---|---|---|---|---|
| A | 147 000 | 7.35 | 4.70 | 1.27 | .742 | –1.50 | 2.13 |
| $B_1$ | 152 000 | 7.49 | 4.55 | 1.32 | .735 | –0.83 | 2.54* |
| $B_2$ | 154 000 | 7.56 | 4.35 | 1.35 | .730 | –0.91 | 2.08* |

* Rapidly sedimenting material (10 S – 22 S) also appeared in acidified solutions of aldolases B.

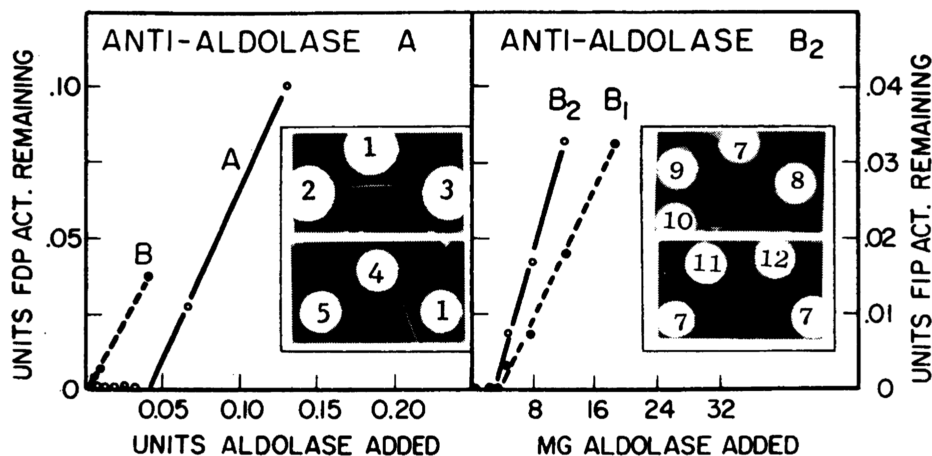


*Fig. 1.* Immunochemical analysis of aldolases. Increasing amounts of the aldolases A, $B_1$, $B_2$ or B (crude liver extract), as indicated, were incubated with .025 ml anti-aldolase A or .05 ml anti-aldolase $B_2$ in a final volume of 1.0 ml or 0.5 ml, respectively, centrifuged and assayed. For the double diffusion analysis, immune sera were placed in center wells and aldolases (mg/ml A, $B_1$ or $B_2$) in numbered wells as follows: (1) .10, A; (2) 1.0, $B_2$; (3) 1.7, $B_2$; (4) .34, $B_1$; (5) .68, $B_1$; (7) .06, $B_2$; (8) .14 units/ml liver extract; (9) .5, A; (10) 1.0, A; (11) .25 A; (12) .06, $B_1$.

electrophoretic and sedimentation analysis suggesting homogeneity of the preparations. The electrophoretic mobility and sedimentation constants of the three enzymes are in the same general range, but there are significant differences, especially between aldolases A and B. The molecular weight of aldolase A, as determined from hydrodynamic data, is significantly lower than that of aldolases B.

Recently it has been shown that aldolase A may be dissociated in acid to subunits having about one-third the molecular weight of the original molecule[7,8]. These subunits reassociate on reneutralization to form a product with the catalytic and molecular properties of the native molecule. Under appropriate conditions, essentially complete recovery of catalytic activity has been achieved. In acid solution, aldolases $B_1$ and $B_2$ both yield materials having low sedimentation constants like the sub-units derived from aldolase A and, in addition, aggregates of high sedimentation constant. Aldolase A exhibits similar behavior at higher protein concentrations, or at somewhat higher pH[7]. On reneutralization, recovery of only 30—50 % of the initial activity of aldolases B has been achieved. The general behavior in acid, the demonstration of three C-terminal tyrosines in aldolase A by Drechsler *et al.*[9], together with the finding of three N-terminal residues for aldolases A, $B_1$ and $B_2$ in the present work are in agreement with the postulate that these aldolases are each composed of three subunits.

An immunochemical analysis of the aldolases was performed using anti-aldolase A and anti-aldolase $B_2$. The results of double diffusion on agar plates as well as quantitative precipitin tests are presented in Fig. 1. Anti-aldolase A quantitatively precipitates aldolase A. Moreover, there is no detectable catalytic activity in the

*Table 3.* Total amino acid analysis of aldolases.

| Aldolase | Ala | Arg | Asp | Cys | Glu | Gly | His | Ileu | Leu | Lys | Met | Phe | Pro | Ser | Thr | Try | Tyr | Val |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 143 | 62 | 104 | 29 | 147 | 108 | 42 | 77 | 129 | 112 | 12 | 27 | 72 | 69 | 74 | 11 | 41 | 83 |
| $B_1$ | 139 | 63 | 136 | (30) | 167 | 113 | 39 | 73 | 130 | 108 | 19 | 42 | 59 | 62 | 75 | 14 | 37 | 91 |
| $B_2$ | 131 | 63 | 141 | (19) | 159 | 107 | 34 | 71 | 137 | 112 | 17 | 47 | 64 | 66 | 74 | 12 | 37 | 88 |

antigen-antibody complex[4]. In contrast, this antibody cross-reacts only very slightly with aldolases $B_1$ and $B_2$. Anti-aldolase $B_2$ quantitatively precipitates aldolases $B_1$ and $B_2$. There is no detectable cross-reaction of anti-aldolase $B_2$ with aldolase A. A slight cross-reaction between anti-aldolase A and aldolases B suggests the presence of (a) common immunological determinant(s) in the two proteins. The method of isolation precludes contamination of aldolases $B_1$ and $B_2$ with traces of native aldolase A, but it is, of course, possible that traces of aldolase A have been degraded in such a way that they migrate in the various chromatographic procedures and co-crystallize with aldolases B. These results indicate a structural distinction between aldolases A and B and suggest a close structural relationship between aldolases $B_1$ and $B_2$. These conclusions are confirmed and amplified by studies of the specific composition of the proteins[5]. The relative amino acid contents of the three proteins are broadly similar. The compositions of aldolases $B_1$ and $B_2$ appear almost identical (Table 3), but the composition of aldolase A is significantly different. A fingerprint analysis of the peptides produced from aldolases A and B is presented in Fig. 2. Separate analyses of increasing concentrations of partially-degraded enzymes allow the resolution of 74, 67, and 78 peptides for aldolases A, $B_1$ and $B_2$, respectively. Aldolases $B_1$ and $B_2$ give very similar patterns, but the distribution of the peptides on the fingerprint of aldolase A is distinctly different.

## DISCUSSION

From the experimental evidence presented, it is concluded that aldolases A and B are distinct entities. The immunochemical analyses indicate that few immunological determinants are shared by these enzymes. The marked contrast in the fingerprint patterns obtained from these enzymes together with the demonstration that there are probably three polypeptide subunits in the molecules, indicate that there are qualitative differences in the amino acid sequence of these enzymes. On the other hand, aldolases $B_1$ and $B_2$ resemble each other so strongly in overall structure that the differences reported may be a result of modification of a single protein. The possibility of a hybrid of similar polypeptide chains or even of independent genetic derivation is not excluded.

The difference in primary structure of aldolases A and B implies that the synthesis of at least some portions is controlled by separate regions on the genome. Since the proportion of the two types varies in different tissues and changes markedly in the liver during embryological development[3], it is presumed that the synthesis of these two types of aldolases is controlled separately.

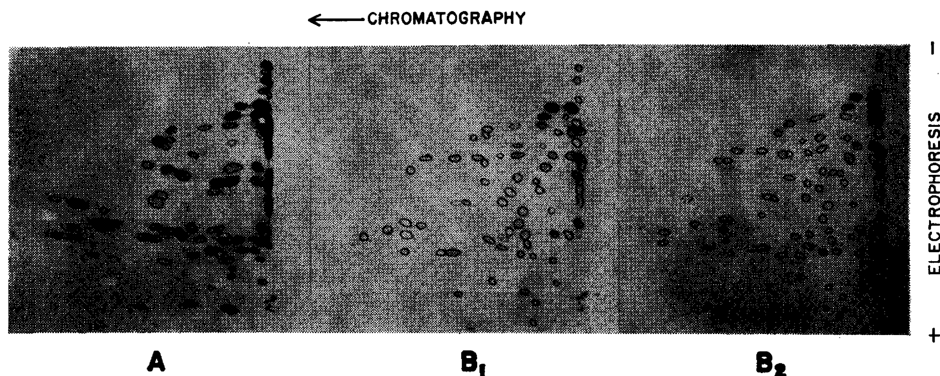


*Fig. 2.* Qualitative analysis of peptides produced by partial degradation of aldolases A, $B_1$ and $B_2$. 20 mg of each protein were subjected to performic acid oxidation at −10°C. The lyophilized proteins were dissolved in 3.0 ml of 0.1 M ammonium carbonate, pH 8.2, and incubated for 2.5 h with 0.2 mg trypsin at 37°C, followed by incubation with 0.2 mg α-chymotrypsin for 2.5 h at 37°C. Hydrolysate corresponding to 3 mg protein was placed at the origin (×). The chromatograms were run in butanol: acetic acid: water, 4 : 1 : 5 (upper phase) for 16 h, descending. This was followed by electrophoresis for 90 min in pyridine: acetic acid: water, 1 : 10 : 289, pH 3.2. Peptide maps were developed with 0.2 % ninhydrin in 95 % ethanol, and outlined before photographic recording.

Hers[10] has pointed out that certain cases of fructose intolerance in humans may be a result of the absence of liver type aldolase (aldolase B) and its partial replacement by muscle type aldolase (aldolase A) in the liver of the patients.

In spite of the conclusion of the independent genetic origin of these enzymes, it is clear from the data available that the molecular features of all three enzymes are similar. The molecular size, the reversible dissociation of the molecule in acid, the probable number of polypeptide chains, the general amino acid composition, as well as the noticeable resemblance in the fingerprint patterns, all indicate that the general molecular architecture, and to some degree the specific structure of the molecules must be related. The specific alteration in the catalytic properties of aldolases A and B by removal of C-terminal residues with carboxypeptidase reflects a basic similarity in the active sites of these enzymes. Previous comparative studies of bovine liver aldolase and rabbit muscle aldolase present evidence for a basic catalytic similarity[11].

These facts thus suggest that even though these molecules are genetically distinct, they may be phylogenetically related. A similar conclusion has been drawn by Ingram for fetal, adult and the various hemoglobin variants[12]. In this instance, hybridization of the different polypeptide chains in the complex hemoglobin molecule has been clearly demonstrated. The interesting and informative studies of Markert and Appella[13] and Cahn *et al.*[14] on lactic dehydrogenase (LDH) in mammalian tissue are relevant. Of the five forms of this enzyme present in mammalian tissues, two are apparently distinct molecules. It has been proposed[14] that the other three, which exhibit intermediate properties, are hybrid combinations of the two different polypeptide chains. The fingerprint analysis

of aldolases A and B tend to rule out the possibility that these enzymes are simply hybrid variants, since in this instance the number and distribution of peptides in the analysis would be similar. The possibility that aldolases $B_1$ and $B_2$ are hybrid variants must be resolved by a more discerning analysis of these proteins.

It is proposed that the term "enzyme homologs" be employed for the fraction of isozymes which have distinct primary structure and therefore are presumably of independent genetic origin but nevertheless have strong similarities in structure and thus may be phylogenetically related. "Enzyme analogs" may then refer to enzymes of independent genetic origin and with apparently independent phylogenetic origin. An example of the latter case is found in *Euglena gracilis* where two structurally and catalytically unrelated forms of aldolases have been discovered[15]. Multiple forms of a single genetic product can appropriately be termed "enzyme derivatives", or simply enzyme variants. On this basis, aldolases A and B and probably the LDH isozymes would be termed enzyme homologs.

Whereas it is not yet possible to define the biological significance of all isozymes, the properties of the enzyme homologs may be related to specific control of metabolic processes. The catalytic properties of aldolase A are tailored primarily to the aldol cleavage of FDP in glycolysis and those of aldolases B to the broadened substrate specificity required for the metabolism of F1P and also for the synthesis of FDP from triose phosphates in the process of glyconeogenesis. A major catalytic difference between LDH variants is the sensitivity of the active site to substrate inhibition. The desirability of this feature in skeletal muscle and the lack of desirability of it in heart muscle has been argued by Cahn *et al*[14].

A more comprehensive analysis of enzymatic populations facilitating the same reaction may elucidate hitherto unappreciated aspects of the control of metabolic processes and provide the basis for definition at the molecular level of one aspect of cellular differentiation.

## REFERENCES


1. Markert, C. L. and Møller, F. *Proc. Natl. Acad. Sci, U. S.* **45** (1959) 753.
2. Rutter, W. J. *The Enzymes*, Academic Press, New York and London, 1961, Vol. V, p. 341.
3. Rutter, W. J., Blostein, R. E., Woodfin, B. M. and Weber, C. S. In *Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Liver,* Indianapolis, Indiana, 1962, Pergamon Press. *In press.*
4. Blostein, R. E. and Rutter, W. J. *J. Biol. Chem.* (1963). *In press.*
5. Woodfin, B. M. and Rutter, W. J. *J. Biol. Chem. In preparation.*
6. Richards, O. C. and Rutter, W. J. *J. Biol. Chem.* **236** (1961) 3177.
7. Deal, W. C., Jr., Rutter, W. J. and Van Holde, K. E. *Biochemistry* **2** (1963) 246.
8. Stellwagen, E. and Schachman, H. K. *Biochemistry.* **1** (1962) 1056.
9. Drechsler, E. R., Boyer, P. D. and Kowalsky, A. G. *J. Biol. Chem.* **234** (1959) 2627.
10. Hers, H. G. *Chemisch Weekblad* **57** (1961) 437.
11. Rutter, W. J., Richards, O. C. and Woodfin, B. M. *J. Biol. Chem.* **236** (1961) 3193.
12. Ingram, V. M. In *The Molecular Control of Cellular Activity,* McGraw-Hill Company, New York, 1962, p. 181.
13. Markert, C. L. and Appella, E. *Ann. N. Y. Acad. Sci.* **94** (1961) 678.
14. Cahn, R. D., Kaplan, N. O., Levine, L. and Zwilling, E. *Science* **136** (1962) 962.
15. Rutter, W. J. and Groves, W. E. In *International Conference on Taxonomic Biochemistry, Physiology and Serology,* University of Kansas, Lawrence, Kansas, September, 1962.


Received March 30, 1963.