Molecular and Catalytic Properties of Aldolase C*

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ABSTRACT: The catalytic, physical, and immunochemical properties of aldolase C are presented and compared with the corresponding properties of aldolases A and B. The three enzymes have different fructose 1,6-diphosphate:fructose 1-phosphate ratios (A = 50, B = 1, and C = 10), Michaelis constants for fructose 1-phosphate (A = 10^{-2} M, B = 3×10^{-4} M, C = 4×10^{-3} M), and specific fructose 1,6-diphosphate cleavage activites (A = 2900, B = 250, and C = 1000) and are inhibited to different extents by treatment with carboxypeptidase. They have similar Michaelis constants for fructose 1,6-diphosphate ($1–3 \times 10^{-6}$ M), similar catalytic properties after carboxypeptidase treatment, and nearly identical pH profiles for the fructose 1,6-diphosphate and fructose 1-phosphate cleavage reactions. The enzymes have different amino acid compositions and different primary structures as demonstrated by peptide mapping. In addition, they are immunochemically distinct with no cross-reactions observed among the three proteins. The data obtained suggest that aldolase A, B, and C are three distinct proteins which are structurally closely related and functionally distinct but homologous.

Although multiple forms of many enzymes have been detected in recent years (*e.g.*, see *N. Y. Acad. Sci. 151*, 1968) the structural bases of the various forms have been rarely defined and the physiological roles (if any) have been even more elusive.

The studies elucidating the molecular basis for the multiple forms of LDH[1] are well known. The typical five-membered LDH activity profile is caused by the random interaction of two distinct subunit types to form tetrameric molecules: two unique parental homomeric species HHHH and MMMM, and three heteromeric species HHHM, HHMM, and HMMM. The parental molecules derived from skeletal muscle and heart tissue exhibit different kinetic properties (especially susceptibility to substrate inhibition), and a distinct physiological role based on this characteristic has been proposed (Cahn *et al.*, 1962). The validity of this proposal has however been challenged on the basis that nonphysiological concentrations of substrate are required for inhibition, there is a large excess of LDH activity over known glycolytic rates in tissues, and the inhibitions observed are quite dependent upon the assay conditions employed (Vesell, 1968).

Two parental types of fructose diphosphate aldolase are also well known: the classical enzyme derived from skeletal muscle tissues (aldolase A) and the enzyme isolated from liver (aldolase B) (Rutter, 1960, 1964). In this instance, the physiological roles of the enzymes seem more readily apparent. A difference in substrate specificity (FDP:F-1-P activity ratio) corresponds to the demonstrated function of liver aldolases in the aldol cleavage of fructose 1-phosphate during the metabolism of fructose by this tissue. In addition, other catalytic differences between the molecules suggest aldolase B is tailored for a role in gluconeogenesis while aldolase A is more effective in a degradative glycolytic capacity (Leuthardt *et al.*, 1953; Hers and Kusaka, 1953; Rutter *et al.*, 1963).

Recently, we have detected a third aldolase (termed aldolase C) in brain and other tissues (Penhoet *et al.*, 1966). Five-membered hybrid sets are produced *in vitro* by reversible dissociation of binary mixtures of the parental aldolases. Such sets are also detected in extracts of tissues containing two of the aldolase types, indicating the simultaneous synthesis of both types of subunits in the same cells. Other experiments have demonstrated conclusively that the parental and hybrid species are tetramers (Penhoet *et al.*, 1967; Kawahara and Tanford, 1966) instead of trimers as had been proposed earlier (Chan *et al.*, 1967).

During the course of our recent investigations, methods for the isolation of the A–C hybrid set from brain tissue as well as new methods for the purification of aldolases A and B have been developed (Penhoet *et al.*, 1969). This paper presents many of the molecular and catalytic properties of aldolase C. In addition, several basic characteristics of aldolases A and B have been redetermined for comparative purposes. Although structurally and functionally similar, aldolases A, B, and C are nevertheless distinct molecules; they are immunochemically distinct, and have unique amino acid sequences and significantly different catalytic properties.

Materials

Fructose 1-phosphate and fructose diphosphate were obtained from the Sigma Chemical Co. Both preparations were greater than 99% pure. The diacetal barium salt of glyceraldehyde 3-phosphate (Sigma Chemical Co.) was deionized on Dowex 50-X4. The resulting solution was converted into the free aldehyde by heating for 3 min at 100°. Dihydroxyacetone phosphate, dimethyl ketal, and dimonocyclohexylamine salt (Sigma Chemical Co.) were converted into dihydroxyacetone

* From the Departments of Biochemistry and Genetics, University of Washington, Seattle, Washington 98105. *Received June 24, 1969.* The experimental work described in this paper was supported in part by the U. S. Public Health Service Grant No. HD-02126.

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[1] Abbreviations used are: LDH, lactate dehydrogenase; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; TPCK, (tosylamido-2-phenyl)ethyl chloromethyl ketone; FDP, fructose 1,6-diphosphate; F-1-P, fructose 1-phosphate.

phosphate by the method of Ballou and Fischer (1956). Glycerol phosphate dehydrogenase–triosephosphate isomerase mixture was obtained from the Boehringer-Mannheim Corp. DFP-treated carboxypeptidase A was procured from the Worthington Biochemical Corp. TPCK-treated bovine trypsin was obtained from the Gallard-Schlesinger Corp. All other compounds were reagent grade materials used without further purification. Aldolases were purified as described in the previous paper (Penhoet *et al.*, 1969).

Methods

Aldolase activity was assayed at 25° according to the method of Blostein and Rutter (1963) except that 0.1 M Tris-Cl was substituted for glycylglycine as the buffer in the system. In the kinetic experiments a Cary Model 16 spectrophotometer was employed for determination of critical initial rates. A unit of activity is defined as the cleavage of 1 μmole of substrate/min. Specific activity is defined as the unit of aldolase activity per milligram of protein. Protein determinations were performed spectrophotometrically at 280 mμ assuming an absorption of 0.91/mg of protein per ml in a 1-cm cell for aldolase A (Baranowski and Neiderland, 1949), 0.89 for aldolase B, and 0.88 for aldolase C. The value for aldolase B was determined by dry weight analysis by Dr. T. V. Rajkumar in this laboratory. The value for aldolase C was obtained by differential refractometry using aldolase A as a standard and by correlation with the yield of the amino acid analysis.

FDP and F-1-P concentrations were determined enzymatically using the above assay conditions, but with added aldolase (approximately 0.1 unit). DHAP and GA3P concentrations were determined enzymatically with an excess of DPNH and glycerol phosphate dehydrogenase, or DPN and glyceraldehyde 3-phosphate dehydrogenase, respectively.

The aldolase-catalyzed synthesis of FDP was determined by incubation of the enzymes with varying levels of triose phosphates in 0.1 M Tris-Cl (pH 7.5) in a final volume of 0.5 ml. The reactions were terminated at appropriate intervals by the addition of 1.75 ml of 30% HCl to the reaction mixture. FDP concentrations were determined by the method of Roe (1934) using FDP as a standard.

Molecular weight determinations were performed according to Yphantis (1964) in a Spinco Model E ultracentrifuge equipped with Rayleigh optics. Aldolase solutions (0.75 mg/ml in 0.1 M Tris-Cl, pH 7.5) were run at 5° in 3-mm cells at 16,000 rpm. Photographs were taken after equilibrium was established (usually 12 hr) and read with a modified Nikon optical comparator (Teller, 1967). The data obtained were analyzed with the aid of a computer utilizing the program derived by Dr. David Teller and associates (Teller *et al.*, 1969).

Amino acid compositions were determined on duplicate samples hydrolyzed in 6 N HCl for 18, 30, 48, and 96 hr. The hydrolysates were analyzed according to Moore and Stein (1963) on a Beckman 120C analyzer in the laboratory of Dr. H. Neurath.

Carboxyl-terminal residues were determined by assaying the amino acids released from the enzymes on digestion with a 1:20 molar ratio of carboxypeptidase A to aldolase in 0.01 M Tris-Cl–1% LiCl (pH 7.5). Digestions were terminated by adding 0.05 ml of 1 N HCl/ml of reaction mixture. The acidified reaction mixture was diluted with an equal volume of pH 2.2 sodium citrate buffer and analyzed on a Beckman 120C analyzer. Control autodigestions of carboxypeptidase A were performed for each series of incubations.

Peptide maps were prepared by chromatography and electrophoresis of trypsin-digested aldolases in a manner identical with that recently reported for triosephosphate dehydrogenases isolated from skeletal muscle and brain tissues (Kochman and Rutter, 1968).

Antisera were produced by injecting purified aldolase solutions emulsified with an equal volume of Freund's adjuvant into adult male white rock chickens; 5 mg was injected intraperitoneally on day 1; 5 mg was injected intramuscularly on days 3 and 5. On days 7, 9, and 11, each animal was injected intravenously in the wing vein or breast vein with 5 mg of aldolase dissolved in 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5) without adjuvant. This injection schedule was followed by a rest period of from 4 to 6 weeks, at the end of which a second series of injections was begun. After five intravenous injections of 5 mg each on alternate days, the animal was allowed to rest for a period of 5–7 days before being bled by cardiac puncture.

Antisera were tested by the immunodiffusion test of Ouchterlony (1953) in 0.8% noble agar–4% NaCl (pH 7.5). Aldolase solutions were equilibrated with 0.01 M Tris-Cl–0.001 M EDTA–4% NaCl (pH 7.5) before analysis. For the tests of inhibition of aldolase activity, the samples were dissolved in 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5), mixed with equal volumes of the appropriate antisera, and allowed to stand at 4° for 1 hr prior to assay. The low level of FDP cleavage activity found in the serum without added aldolase was subtracted from all values obtained.

Results

Catalytic Properties of Aldolases A, B, and C. The basic catalytic properties of aldolase C as well as those of aldolases A and B are presented in Table I. Although the kinetic parameters of the latter two enzymes have been reported in a number of earlier studies (Rutter *et al.*, 1963), they were redetermined in this study so that meaningful comparisons of the three enzymes, assayed under identical conditions, could be made. The data obtained for aldolase C, plotted according to the method of Lineweaver and Burk, are presented in Figure 1.

In agreement with previous data, aldolase A cleaves FDP at a rapid rate compared to F-1-P (FDP:F-1-P ratio $\simeq$ 50). Liver aldolase B, on the other hand, cleaves FDP and F-1-P at very nearly equal rates. The FDP:F-1-P activity ratio of aldolase C is intermediate between those of A and B. The Michaelis constants of aldolases A, B, and C with FDP as a substrate are all very similar, falling in the range 1–4 $\times$ 10^{-6} M. The value reported here for aldolase A is considerably lower than that obtained in most previous studies but is in agreement with values recently obtained by Rose and O'Connell (1969). Mehler (1963) as well as Rose and O'Connell (1969) have shown that anions such as Cl^- are competitive inhibitors; thus, the apparent K_m is dependent upon the anion concentration in the buffer medium. The Michaelis constants of aldolases A, B, and C with F-1-P as substrate are more varied from 10^{-2} M for aldolase A with aldolase C having an intermediate value to 3 $\times$ 10^{-4} M for aldolase B.

The pH–cleavage activity relationships of aldolases A, B, and C are presented in Figure 2. The FDP cleavage activities of

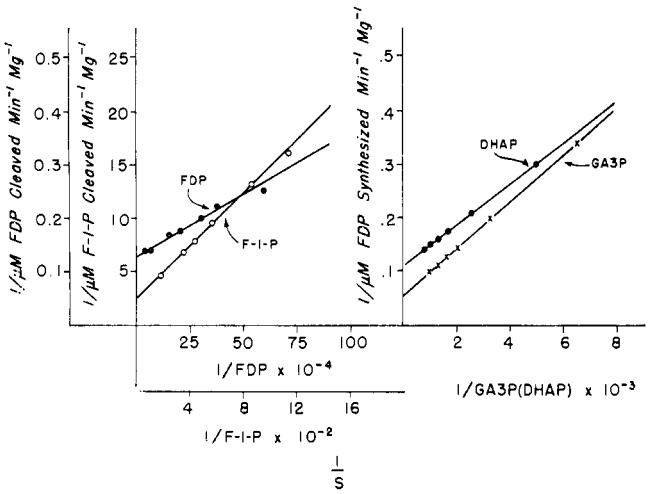

FIGURE 1: Lineweaver–Burk plots: aldolase C with FDP, F-1-P, DHAP, and GA3P. Aldolase C activity was assayed with varying levels of FDP or F-1-P using the assay described in Methods. The cleavage reactions were monitored with a Cary Model 16 spectrophotometer and were carried out at 25°. The 3-ml reaction mixtures contained 0.12 mmole of Tris-Cl (pH 7.5), 0.63 μmole of DPNH, 0.04 mg of glyceraldehyde dehydrogenase–tricosephosphate isomerase stock, 2 mg of bovine serum albumin, and 2 μg of aldolase C (FDP) or 4 μg of aldolase C (F-1-P). The fructose diphosphate synthesis reactions were carried out at 25° in a final volume of 0.5 ml containing 0.04 mmole of Tris-Cl (pH 7.5) and 0.85 μmole of GA3P (in the experiment varying the DHAP) or 1 μmole of DHAP (in the experiment varing the GA3P). FDP was measured by the method of Roe (1934).

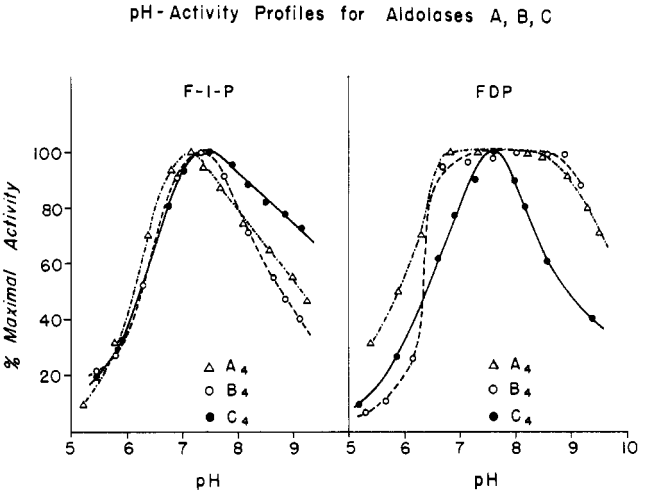

FIGURE 2: The effect of pH on the FDP and F-1-P cleavage activities of aldolases A, B, and C. FDP and F-1-P cleavage activity was determined as described in Methods except that 0.1 M Tris-malonate buffer was used in place of Tris-Cl. Reaction mixtures were adjusted to constant ionic strength by the addition of appropriate amounts of saturated NaCl. The pH was determined immediately following the completion of each assay using a radiometer pH meter. These values were obtained at substrate concentrations of 0.0025 M (FDP) and 0.1 M (F-1-P).

aldolases A and C are essentially independent of pH in the region 7–9. Aldolase B, on the other hand, has a sharp optimum peak occurring at pH 7.5–8.0. Mehler (1963) has suggested that changes in ionization of the substrate (pK_a = 6.8) may be responsible for the acid side of the curve of aldolase A. If this is indeed the case, the same factor might be responsible for the acid portion of the B and C curves. The FDP and F-1-P activity profiles are different for each of the enzymes, pointing out the fact that the FDP:F-1-P ratio determined for any one of them is dependent upon the pH of the activity measurements. The ratios presented in Table I were determined at pH 7.5, the optimum for the F-1-P cleavage reactions.

Treatment of aldolase A with small amounts of carboxypeptidase A results in alteration of its catalytic properties. Concommitant with the loss of carboxyl-terminal tyrosine residues, the enzyme exhibits a 20-fold fall in the rate of FDP cleavage (Drechsler *et al.*, 1959) and a 500-fold decrease in the rate of exchange detritiation of dihydroxyacetone phosphate (Richards and Rutter, 1961). Similar treatment of aldolase B results in only a twofold decrease in FDP cleavage activity (Rutter *et al.*, 1963). The effect of carboxypeptidase treatment on the FDP cleavage activity of aldolase C as well as aldolases A and B is presented in Figure 3. The loss of FDP cleavage activity is rapid for all three of the enzymes although they are inhibited to different extents in order of their original specific activities (A > C > B). The catalytic properties of the carboxypeptidase-treated enzymes (Table II) are remarkably similar in

TABLE 1: Properties of (Class I) Homologous FDP Aldolase Variants (Rabbit).

	A, Muscle	B, Liver	C, Brain
V_{max} (FDP cleavage)[a]	2,900	250	1,000
V_{max} (FDP synthesis)[a]	10,000	2,600	4,900
K_m (M) FDP	3×10^{-6}	1×10^{-9}	3×10^{-6}
F-1-P	1×10^{-2}	3×10^{-4}	4×10^{-3}
DHAP	2×10^{-3}	4×10^{-4}	3×10^{-4}
GA3P	1×10^{-3}	3×10^{-4}	8×10^{-4}
FDP:F-1-P activity ratio	50	1	10

[a] Moles of FDP cleaved (synthesized) per mole of enzyme per minute at 25°.

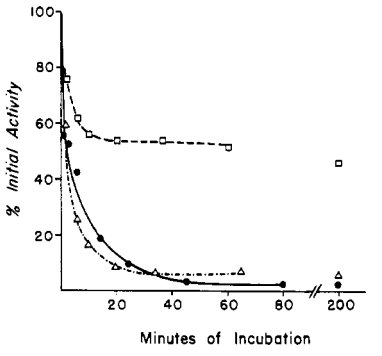

FIGURE 3: The effect of carboxypeptidase A treatment on the FDP cleavage activities of aldolases A, B, and C. Aldolases A, B, and C were dissolved in 0.1 M Tris-Cl–1% LiCl (pH 7.5) and were incubated at 37° with a 1:300 molar ratio of carboxypeptidase A. Aliquots were removed from the reaction mixture at the indicated times and were assayed by the spectrophotometric assay. (●) Aldolase A, (□) aldolase B, and (△) aldolase C.

TABLE II: The Effect of Carboxypeptidase A on the Catalytic Activity of Aldolases A, B, and C.

Aldolase	Initial Sp Act. FDP	Initial Sp Act. F-1-P[a]	Initial FDP:F-1-P	Final Sp Act. FDP	Final Sp Act. F-1-P	Final FDP:F-1-P
A	19	0.40	47	0.51	0.26	2.0
B	0.98	0.99	1.0	0.45	0.53	1.02
C	6.6	0.53	12.5	0.46	0.24	1.9

[a] F-1-P = 10^{-2} M.

spite of the considerable differences noted between the native enzymes.

Physical Properties. MOLECULAR WEIGHT DETERMINATIONS. The molecular weights of aldolases A, B, C, and A_2C_2 were determined by sedimentation equilibrium. The weight and number average molecular weights of these enzymes, expressed as a function of protein concentration in the cell at equilibrium, are presented in Figure 4. The values obtained for all four proteins are very similar (probably within the error of the methods). The constancy of apparent molecular weight as a function of protein concentration in all of these experiments indicates that there is very little molecular weight heterogeneity in any of the aldolase preparations and that the dissociation constant for the reaction tetramer $\rightleftharpoons$ monomer of all of the aldolases studied including the heteromeric ones must be very small.

AMINO ACID COMPOSITION. The amino acid compositions of aldolases B and C, determined the present study, are presented in Table III together with values obtained for aldolases A and B in the earlier studies of Woodfin (1963). Although the compositions of the three proteins are broadly similar, there are significant differences, some of which are quite pronounced (*e.g.*, aldolase C has 44 fewer lysines than does aldolase A and 28 fewer than does aldolase B).

PEPTIDE MAPS. The differences in amino acid composition between the three proteins are reflected in unique polypeptide sequences which can be visualized by peptide mapping. Peptide "maps" of chymotryptic digests of aldolases A and B have been published previously (Rutter *et al.*, 1963). Maps of tryptic digests of aldolases A and C and the intermediate members of the A–C hybrid set are presented in Figure 5. As expected from a homologous but nonidentical pair of proteins, aldolases A and C have many peptides which appear identical by the procedures employed and many others which are clearly different. The number of peptides detected (A = 38 and C = 29) approached the values predicted on the basis of the number of lysine and arginine residues present in each of the protein subunits (A = 48 and C = 37, assuming that aldolases A and C are each tetramers composed of identical or nearly identical subunits). The hybrid nature of the interme-

TABLE III: Amino Acid Composition of Aldolases A, B, and C.

Amino Acid	Moles of Amino Acid/Mole of Enzyme: Aldolase A[a]	Aldolase B[a]	Aldolase B	Aldolase C
Alanine	154	144	160	177
Arginine	67	65	60	67
Aspartic acid	112	140	136	131
Half-cystine	28	31	31	22
Glutamic acid	160	172	171	166
Glycine	116	116	120	127
Histidine	45	41	35	29
Isoleucine	83	76	77	84
Leucine	140	134	136	135
Lysine	121	111	105	77
Methionine	12	20	23	12
Phenylalanine	29	43	42	32
Proline	78	61	56	84
Serine	75	64	73	80
Threonine	80	77	91	68
Tryptophan[b]	12	14	(14)	14
Tyrosine	44	38	41	50
Valine	90	94	99	115
Sum	1446	1441	1470	1470

[a] From Woodfin (1963). The values were taken from one of the two aldolase forms (B_1) isolated from liver by Woodfin.
[b] Determined by the method of Edelhoch (1948) (aldolase C).

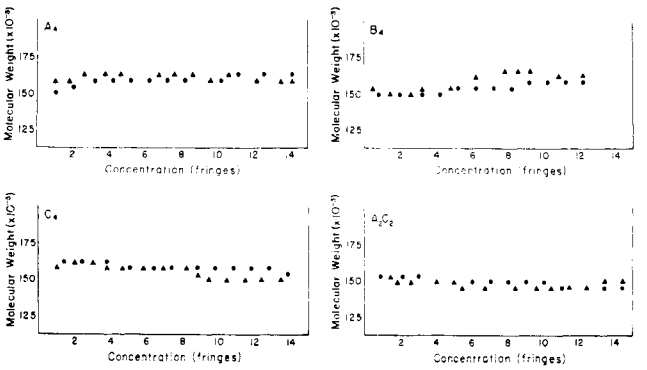

FIGURE 4: Molecular weight *vs.* concentration at equilibrium: aldolases A, B, C, and A_2C_2. Proteins dissolved in 0.1 M Tris-Cl (pH 7.5) were centrifuged at 15,000 rpm at 5°. The data were analyzed as described in Methods. The data for aldolase B were taken from an experiment by Dr. David Teller. (▲) Number-average molecular weights and (O) weight-average molecular weights.

TABLE IV: Carboxypeptidase A Digestion of Aldolases A and C. Number of Residues per Mole of Enzyme (160,000 Molecular Weight).

	Aldolase C, Expt 1, Sp Act. 2			Aldolase C, Expt 2, Sp Act. 5.5, 20 min	Aldolase C, Expt 3, Sp Act. 7.2, 20 min	Aldolase A, Sp Act. 17, 12 hr
	Time of Digestion					
	45 min	200 min	12 hr			
Tyrosine	Trace	0.75	1.3	2.4	3.1	3.48
Alanine	4.5	7.8	10.8	2.4		4.33
Serine	2.6	3.7	5.3	1.4		4.37
Leucine	0.9	2.0	4.9	0.4		2.46
Phenylalanine	Trace	1.4	3.5	Trace		2.2
Isoleucine	Trace	0.3	0.9	0		2.3
Valine	0	Trace	1.6	0		
Methionine	0	Trace	1.2	0		
Glycine	0	Trace	Trace	0		Trace
Threonine	0	0	Trace	0		
Aspartic acid	0	0	Trace	0		
Histidine				0		2.37

diate members of the A–C set, clearly demonstrated in earlier studies (Penhoet *et al.*, 1966, 1967; Rutter *et al.*, 1963), is graphically portrayed in the peptide maps of Figure 5. All of the peptides of A and C are present in the hybrid molecules and the intensities of the spots unique to either aldolase A or C are proportional to the number of A and C subunits present in a given heteromer.

CARBOXYL-TERMINAL RESIDUES OF ALDOLASE C. As noted above, treatment of aldolase A with carboxypeptidase A results in the removal of carboxyl-terminal tyrosine residues

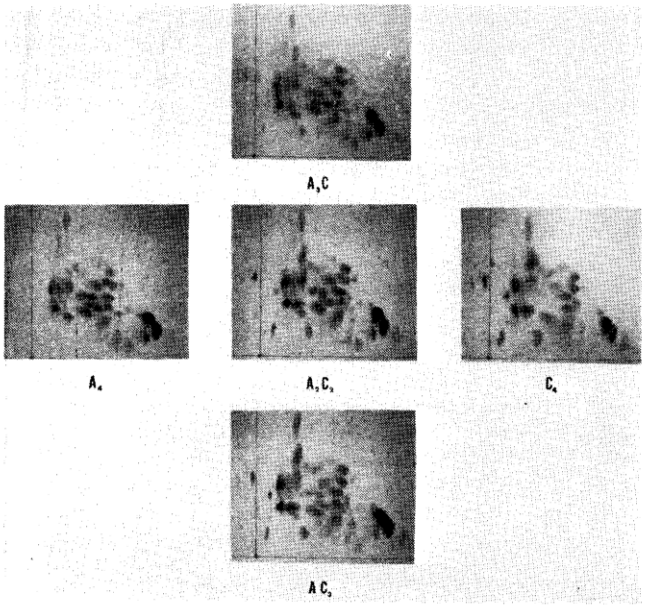

FIGURE 5: Peptide maps of aldolases A, C, and the A–C hybrids. Aldolases purified as previously described (Penhoet *et al.*, 1969) were carboxymethylated, digested with trypsin, and subjected to paper electrophoresis and chromatography as described by Kochman and Rutter (1968).

(Dreschler *et al.*, 1959; Kowalsky and Boyer, 1960; Winstead and Wold, 1964). Similar results were obtained with aldolase B (T. V. Rajkumar and W. J. Rutter, 1967, unpublished observations). Since the catalytic properties of all three aldolases were modified in a similar manner by carboxypeptidase treatment, it was considered likely that tyrosine also occupied a carboxyl-terminal position in aldolase C. Because aldolase C is difficult to purify in large amounts, a preparation of high purity but low specific activity was used for an analysis of the amino acids released during carboxypeptidase digestion. As shown in Table IV, little tyrosine was released during the digestion period and the results of this experiment alone would not suggest tyrosine as the carboxyl terminal. Because of the strong suggestion from the catalytic experiments cited above, however, the digestions of aldolase C were repeated on higher specific activity preparations which were unfortunately available only in smaller amounts. In these experiments, a much greater total and relative amount of tyrosine was released according to the specific activities of the preparations. It is likely that the variations in specific activity noted in the several aldolase C preparations are due to limited proteolysis during the isolation procedures. A similar phenomenon may cause variations in the specific activities of aldolase A preparations (Penhoet, 1968; Rose and O'Connell, 1969).

The data of Table IV strongly suggest that the carboxyl-terminal residue of aldolase C is tyrosine, and a tentative assignment of the carboxyl-terminal amino acid sequence of aldolase C is --------Ser-Ala-Tyr. This may be compared with the sequence --------(His,Ser)-Ala-Tyr previously determined for aldolase A (Chan *et al.*, 1967; Rutter *et al.*, 1963).

IMMUNOCHEMICAL PROPERTIES. Antisera were prepared against purified solutions of aldolases A, B, and C as described in Methods. The immunological properties of the aldolases were examined by double diffusion analysis as shown in Figure 6. Antiserum prepared against rabbit muscle aldolase A forms precipitin bands with aldolase A purified from either muscle or brain tissue, but not with aldolase B or aldolase C. The precipitin bands formed by reaction of aldolase A from brain

muscle with anti-A serum met each other in a reaction of identity. This suggests that the molecule and brain aldolases are the same proteins. Antiserum produced against purified liver aldolase B reacted only with aldolase B and with neither aldolase A nor aldolase C. In a similar manner, antialdolase C reacted only with aldolase C and did not cross-react with either aldolase A or aldolase B.

In addition to forming specific precipitates in the double-diffusion system, antisera produced against aldolases A, B, and C each inhibited almost completely the FDP cleavage activity of their specific antigens, while having no effect on the activity of either of the other homomeric aldolases. These inhibitions occurred rapidly upon mixing the antibody and antigen and took place in solutions of relatively low ionic strength where precipitation of the antibody–antigen complexes was minimal. Thus, the inhibition of activity is due to the formation of the antibody–antigen complex rather than the removal by precipitation of the enzyme from the solution.

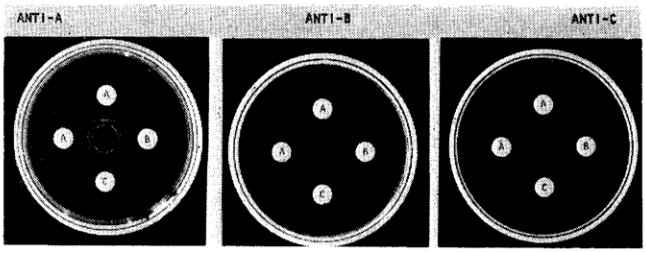

FIGURE 6: Double diffusion analysis: aldolases A, B, and C. Antibodies prepared against purified aldolases A, B, and C as described in Methods were placed in the center wells. Solutions of aldolases A from brain (left), A from muscle (top), B from liver, and C from brain were adjusted to a concentration of 1 mg/ml in 0.01 M Tris-Cl–0.001 M EDTA–4% NaCl (pH 7.5) and were placed in the outer wells cut in the 4% NaCl–0.8% noble agar. The precipitin bands formed after several days incubation at 4°.

Discussion

Aldolases A, B, and C are tetrameric molecules which can be differentiated by their catalytic properties, electrophoretic mobilities, chromatographic properties, amino acid compositions, peptide maps, and immunochemical characteristics as well as by their ability to interact with one another in a specific manner to form heteromeric tetramers (Penhoet *et al.*, 1966, 1967).

In spite of the many differences demonstrated between the proteins, they appear homologous in the sense that they catalyze the same basic reactions and are structurally closely related. All three have the same number of subunits and have similar molecular weights. In addition, they must have very similar over-all conformations and similar if not identical subunit interaction sites since heteromers form in a random fashion with no apparent preferential interactions (Penhoet *et al.*, 1966, 1967; Rutter *et al.*, 1963). The amino- and carboxyl-terminal sequences determined for aldolases A, B, and C provide indications of homology at the level of the primary structure. Primary sequence homology has also been noted for the active site sequences of aldolases A and B as determined by Lai *et al.* (1965) and Morse and Horecker (1965). The mechanisms by which the three enzymes catalyze the reversible cleavage of FDP and F-1-P are undoubtedly very similar. They have similar pH profiles, are all inhibited by carboxypeptidase treatment, and all have nearly identical catalytic properties after carboxypeptidase treatment. The work of Grazi *et al.* (1962) and Morse *et al.* (1965) has shown that the reaction of both aldolases A and B involves the formation of a Schiff's base intermediate between the ϵ-amino group of a lysine on the protein and the carbonyl moiety of DHAP. Although similar experiments have not been performed with aldolase C, it has been shown that the activity of the enzyme is inhibited by $NaBH_4$ in the presence of FDP (M. Kochman and W. J. Rutter, unpublished data). Presumably the same basic reaction mechanism is involved for the three enzymes and a Schiff's base intermediate will undoubtedly be delineated for aldolase C.

The apparent immunochemical uniqueness of aldolases A, B, and C was unexpected in view of the rather extensive structural homologies demonstrated. This suggests that the homologous regions must somehow be shielded from the antibody-forming systems of the animal in which the antibodies were prepared. The active-site sequences determined do have a high proportion of hydrophobic residues suggesting that they may be located in the interior of the molecule.

Alternatively, those sites such as the active sites and subunit interaction sites which have been maintained in the evolution of aldolases A, B, and C within a given species are likely to have been maintained in the phylogenetic evolution of the proteins. If such sites are identical in the aldolases of the rabbit and the chicken (the animal used to prepare the antisera), it is likely that the chicken antibody forming system will not form antibodies against these regions of the molecule. Many of the evolutionary variations in the primary structure of these three proteins may have taken place on the portions of the molecule which are exposed to the outer environment as it is folded in solution. The studies of Perutz *et al.* (1955) demonstrating the three-dimensional structure of hemoglobin molecules and the amino acid sequences of hemoglobins from several species provide a clear precedent for this interpretation. The phylogenetically invariant regions of the hemoglobin molecule seem to be located on the inside of helical portions and in internal cleft while the outside of the hemoglobin molecule had tolerated many amino acid replacements during the course of evolution.

Amino acid analysis, peptide maps, and limited sequence analysis have demonstrated that aldolases A, B, and C have different primary structures, and they must, therefore, be the products of three different cistrons. The activities of these three cistrons are apparently strictly and independently regulated in the various tissues of higher organisms, since previous studies have shown that there are characteristic and specific distributions of these enzymes which differ markedly from tissue to tissue (Penhoet *et al.*, 1966, 1967; Rutter *et al.*, 1963; Lebherz and Rutter, 1969). Aldolase A is the most widely distributed of the three proteins, being present in all rat or rabbit tissues examined. Aldolase B, on the other hand, is apparently confined to liver, kidney, and some sections of the intestine, while aldolase C is located primarily in nervous tissues of the rat or rabbit and is more widely distributed in the tissues of the fowl (Lebherz and Rutter, 1969). The strict control of these cistrons and the resultant tissue specificity indicate that there are probably distinct physiological roles for all three of the enzymes. The role of aldolase B in the liver seems quite apparent from the catalytic properties of the isolated enzyme

(Peanasky and Lardy, 1958; Rutter *et al.*, 1963; Woodfin, 1963).

The low K_m for F-1-P of the liver enzyme (see Table I) emphasizes the catalytic effectiveness of aldolase B for this substrate. At likely physiological concentrations of F-1-P (10^{-4} M), aldolase A would have very little F-1-P cleavage activity and aldolase B would catalyze the cleavage of F-1-P at a rate 60–70 times greater than would aldolase A. On the other hand, unique roles for aldolase A and C are not apparent from the catalytic properties of the isolated enzymes. A similar situation exists with lactate dehydrogenase M and H and with hexokinases I and II where the properties of each of these isolated enzymes do not unequivocally suggest a specific metabolic role. It is interesting to note that a number of these enzymes have similar distributions. Hexokinase I, LDH $^{-}$H, and aldolase C are frequently found together in tissues of constant metabolic activity (brain and heart, for example). In a similar manner, hexokinase type II, LDH M, and aldolase A are found to predominate in tissues such as skeletal muscle which are subject to wide variations in metabolic activity.

It is possible that failure to discern the metabolic relevance of these pairs of enzymes and particularly the present failure to detect a functional difference between aldolases A and C may stem from the level of analyses employed. Of course there may be an undiscovered difference in substrate specificity, but in addition, intracellular localization and compartmentalization or specific enzyme–enzyme interactions may depend on unique structural characteristics of the enzymes. Besides a more extensive analysis of the catalytic properties of these enzymes, what is needed is an investigation of the interaction of aldolases with other cellular components at higher levels of organization.

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