The Subunit Structure of Mammalian Fructose Diphosphate Aldolase*

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ABSTRACT: (1) Five-membered hybrid sets are produced by reversible dissociation and reassociation of binary mixtures of aldolases A, B, and C and are found in extracts of tissues from a number of species. (2) The hybrids of the A–C set have been isolated in pure form from rabbit brain and from artificial mixtures formed by reversible dissociation of aldolases A and C. (3) The molecular weights of the hybrids are similar to those of the parental aldolases. (4) Reversible dissociation of each hybrid produces all five members of the A–C hybrid set. Reversible dissociation of aldolases A or C, on the other hand, produces only the parent aldolases A or C, respectively. (5) A and C subunits have been resolved from each of the hybrids of the A–C set by electrophoresis under dissociating conditions. (6) Dissociation and reassociation of [^{3}H]leucyl aldolase A (rat) and unlabeled aldolase C (rabbit) produce a typical five-membered set. Each of the members has been isolated; the relative specific radioactivity was approximately 1, 0.75, 0.5, 0.25, and 0 for aldolase A, hybrids 1–3, and aldolase C, respectively. This experimental result indicates the combinative unit is one-quarter of the native molecule. (7) Electron micrographs of aldolases A, B, and C suggest these enzymes contain four subunits.

The data obtained in the present study indicate fructose 1,6-diphosphate aldolases A, B, and C are composed of four subunits.

It is generally believed that large proteins are composed of subunits. The architecture of these polymeric molecules is currently of great interest, not only because it is a fundamental aspect of protein structure, but also because it is the basis for one form of enzymatic multiplicity (multiple combinations of different subunits) and, further, because the allosteric regulation of enzymatic activity may involve structural modulations at the level of subunit interaction (Monod *et al.*, 1965). Current models of protein structure assume a symmetrical association of subunits leading to even-numbered polymers (for example, combinations of monomers with identical "binding sets" result in 2, 4, or higher even-numbered oligomers) (Monod *et al.*, 1965; Hanson, 1966). The available evidence suggests most small polymeric proteins are composed of two or four subunits (Klotz, 1967). In the past, a number of proteins have been considered to be three-subunit molecules (for example, glyceraldehyde 3-phosphate dehydrogenase and myosin), but further investigations (Harris and Perham, 1965; Harrington and Karr, 1965; Weeds and Hartley, 1967) have usually substantiated an even-numbered stoichiometry. Fructose 1,6-diphosphate (FDP)[1] aldolase has been the sole "well-documented" example of a three-subunit protein. Most of the currently available methods for estimation of the number of polypeptide chains per molecule have been employed in various studies of this protein, and until recently, the experimental data have strongly supported the three-subunit model.

Drechsler *et al.* (1959) first reported the presence of three carboxy-terminal tyrosine residues per mole (assuming 150,000g of protein = 1 mole). Subsequently, a number of laboratories have found similar stoichiometry for aldolases A and B (Kowalsky and Boyer, 1960; Rutter *et al.*, 1961; Winstead and Wold, 1964; Spolter *et al.*, 1965). Several recent analyses suggest three N-terminal proline residues per mole (Edelstein and Schachman, 1966; Sine and Hass, 1967; T. V. Rajkumar and W. J. Rutter, unpublished observation). The earliest experimental results suggested only one substrate binding site per molecule (Grazi *et al.*, 1962; Westhead *et al.*, 1963), but more recent investigations suggest three binding sites for fructose diphosphate (Ginsburg and Mehler, 1966) and arabinotol diphosphate (Castellino and Barker, 1966). Similarly, Horecker and associates now report three moles of dihydroxyacetone phosphate bound in the presence of sodium borohydride (Kobashi *et al.*, 1966).

Deal *et al.* (1963), Stellwagen and Schachman (1962), Schachman (1963), and Schachman and Edelstein (1966) found that native aldolase A (mol wt 142,000–

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[1] Abbreviation used: FDP, fructose 1,6-diphosphate.

150,000) is reversibly dissociated in acid, detergents, urea, or guanidine into a form with apparently one-third the molecular weight of the original molecule. The results of experiments in which the enzyme was dissociated under basic conditions at first suggested the presence of even smaller units (approximately one-sixth the mass of the native molecule) (Hass and Lewis, 1963), but subsequent experiments indicated that partial degradation of the enzyme had occurred under these conditions, and when similar experiments were performed in which degradation was minimal, dissociation of the native molecule to approximately one-third of the original molecular weight was observed (Sine and Hass, 1967). By their nature, most of the described experiments yield minimal values for the number of subunits in the molecule. Nevertheless, the variety of studies and the general congruity of the results have been persuasive in favor of the three-subunit model of aldolase structure.

In contrast to the above, the results of recent studies have suggested that aldolase is composed of four instead of three subunits. We have reported that five-membered hybrid sets are formed by reversible dissociation from binary mixtures of the three homologous aldolases (A-B, A-C, and B-C) (Rajkumar *et al.*, 1966; Penhoet *et al.*, 1966; Rutter *et al.*, 1967). Such five-membered sets are also detected in extracts of various tissues obtained from a variety of organisms (Penhoet *et al.*, 1966; Christen *et al.*, 1966; Foxwell *et al.*, 1966; Rutter *et al.*, 1967; Herskovits *et al.*, 1967; H. G. Lebherz and W. J. Rutter, unpublished). These results are difficult to reconcile with the three-subunit molecule but would be predicted if the enzyme contained four subunits. On the basis of sedimentation studies, Kawahara and Tanford (1966) have proposed that the molecular weight of native aldolase is 160,000 (compare with 142,000–150,000 obtained in the other studies mentioned above) and dissociates into four subunits of approximately 40,000 mol wt in 6 M guanidine hydrochloride (Kawahara and Tanford, 1966).

We have attempted to resolve the present structural dilemma by methods which do not inherently yield minimal stoichiometry. The present paper reports a qualitative and quantitative analysis of the composition of members of the A–C hybrid set. The results of these experiments are incompatible with various postulated three-subunit models and directly support the four-subunit model. In addition, electron micrographs of aldolases A, B, and C suggest that these enzymes are composed of four subunits.

Methods

Aldolase Assay and Preparation of Aldolases A and C. Aldolase activity was assayed by methods previously described (Blostein and Rutter, 1963). One unit of activity is defined as the cleavage of 1 μmole of FDP/min under assay conditions; the specific activity is then in units per milligram of protein. Protein determinations were performed according to the method of Lowry *et al.* (1951) using aldolase A as a standard (the concentration of aldolase A was determined spectrophotometrically assuming 0.91 absorbance unit/mg of protein (Baranowski and Niederland, 1949)). The method for resolution of aldolases by zone electrophoresis on cellulose acetate and the detection of aldolase activity on the polyacetate strips have been described previously (Penhoet *et al.*, 1966).

Aldolase A was isolated from rabbit muscle by a method similar to that employed for the isolation of [^{3}H]leucyl aldolase A (see below); the procedure will be described more fully elsewhere (E. Penhoet, M. Kochman, and W. J. Rutter, in preparation). Aldolase C and the hybrids of the A–C set were isolated from rabbit brain according to a method previously outlined (Penhoet *et al.*, 1966; Rutter *et al.*, 1967).

The Preparation of [^{3}H]Leucyl Aldolase A. A total of 1 mc of [^{3}H]leucine (specific radioactivity 23 c/mmole) was injected into each of five 50-g rats over a period of 72 hr (0.2 mc was injected into the sublingual veins at zero time, and after 48 hr; 0.4- and 0.2-mc doses were injected intraperitoneally at 60 and 72 hr). After 80 hr, the animals were sacrificed, decapitated, and eviscerated (thus removing aldolases B and C); the remaining carcasses (100 g) were homogenized in 200 ml of 10^{-3} M Tris-chloride–10^{-3} M EDTA (pH 7.5) in a blender for 2 min. The resulting homogenate was centrifuged at 105,000g for 90 min. The precipitate was discarded and the clear supernatant was placed on a 2 × 20 cm cellulose phosphate column previously equilibrated with 0.01 M Tris-chloride–0.001 M EDTA (pH 7.5). The column was washed with 0.05 M Tris-chloride–0.005 M EDTA (pH 7.5) until the absorbance at 280 mμ of the effluent was less than 0.05, and then eluted with 0.05 M Tris-chloride–0.005 M EDTA–0.0025 M FDP (pH 7.5). The fractions containing significant quantities of aldolase activity were pooled (175 ml, 0.78 mg/ml) and concentrated to approximately 4 mg/ml by ultrafiltration (Amicon Inc., Cambridge, Mass.). The solution was then dialyzed against 0.01 M Tris-chloride–0.001 M EDTA (pH 7.5). The labeled aldolase preparation (sp act. 11) was further purified by passage through a DEAE Sephadex A-50 column equilibrated with 0.005 M Tris-chloride–0.001 M EDTA (pH 7.5). The aldolase activity was not absorbed in the column, and the breakthrough effluent exhibited a specific FDP-cleavage activity of 14. The sample was crystallized by dialysis against 50% saturated ammonium sulfate solution (with no change in specific activity). There was no evidence of heterogeneity in the analysis of a sedimentation equilibrium study of the labeled aldolase; furthermore, the enzyme gave a single band of catalytic activity, radioactivity, and protein on electrophoresis on cellulose acetate (pH 8.6). The final product had a specific radioactivity of 124,000 dpm/mg when solubilized in hydroxide of Hyamine (Rohm and Haas) and counted in Kinard solution in a Packard scintillation spectrometer (Kinard, 1957). The counting efficiencies were determined by employing the external standard and by using tritiated toluene as an internal standard.

Preparation of [^{3}H]Aldolase A–C Hybrids. [^{3}H]Leucyl

aldolase A (10 mg) and pure aldolase C (10 mg, sp act. 4) in 20 ml of 0.01 M Tris-chloride–0.001 M EDTA (pH 7.5) were acidified to pH 2.0 with 1 N H_3PO_4 at 0° (approximately 2 ml were required). The mixture was allowed to stand at 0° for 30 min and then diluted into 400 ml of 0.01 M Tris-chloride–0.001 M EDTA (pH 7.5). The pH of this mixture (6.5) was immediately adjusted to 7.5 by addition of 2 ml of 1 M Tris (unneutralized). The recovery of the aldolase activity was maximal (76%) after 40 min, and the preparation was concentrated to approximately 10 ml by ultrafiltration (approximately 16 mg of protein was recovered). Zone electrophoresis on cellulose acetate indicated that the above treatment had produced the members of the A–C hybrid set. The concentrated sample was then equilibrated with 0.01 M Tris-chloride–0.001 M EDTA (pH 7.5) by passage over a Sephadex G-25 column and then placed on an 0.8 × 42 cm DEAE Sephadex column equilibrated with 0.005 M Tris-chloride–0.001 M EDTA (pH 7.5). The A–C hybrids were resolved by elution with a linear gradient of 0–0.35 M NaCl in 0.01 M Tris-chloride–0.001 M EDTA–0.005 M β-mercaptoethanol–0.0005 M FDP (pH 7.5). The catalytic activity, specific radioactivity, and the absorbance at 280 mμ were determined on each 2-ml sample. These parameters were redetermined on selected samples after dialysis against 0.01 M Tris-chloride–0.001 M EDTA (pH 7.5).

Starch Gel Electrophoresis under Dissociating Conditions (Acid). Prior to electrophoresis, the aldolase sample (approximately 1 mg/ml in 0.1 M sodium glycylglycinate–0.01 M EDTA (pH 7.5) containing 0.07 ml of β-mercaptoethanol/100 ml of solution) was adjusted to pH 2.0 by the addition of cold 1 M H_3PO_4.

A 12% starch gel (hydrolyzed starch procured from Connaught Medical Research Laboratories, Toronto, Canada) was prepared in the same solution (pH 2.0) used for dissociation of the enzyme. The best resolution was obtained when the H_3PO_4 and β-mercaptoethanol were added just before the preparation of the gel. About 0.1 ml of sample was subjected to horizontal electrophoresis according to Smithies (1955). The buffer solutions in the electrode vessels were identical with those described above. A voltage gradient of 3–4 volts/cm was applied and electrophoresis was performed for 38 hr. The protein was stained using 1% Amido Black 10B in methanol–water–acetic acid (50:50:10).

Electron Microscopy. The enzyme was diluted to 50 μg/ml in 0.05 M potassium phosphate buffer (pH 7.0). Specimens were prepared on very thin carbon films and negatively stained with a 2% solution of sodium tungstosilicate. The preparations were examined in a Philips EM 200 electron microscope and electron micrographs were recorded on Ilford N 60 plates using 2-sec exposures at a magnification of 55,000×.

Results

Characteristics of Aldolase A–C Hybrid Set. Five-membered aldolase sets have been detected under a variety of conditions of pH, electrophoretic supporting media, chromatography, etc. Figure 1 illustrates the resolution of the aldolase A–C set under both acidic and basic conditions on cellulose acetate and in polyacrylamide gels. Attempts to resolve additional bands of activity under different conditions of zone electrophoresis have been unsuccessful.

Members of the set have been isolated from rabbit brain by a combination of cellulose phosphate and DEAE Sephadex chromatography. Zone electrophoresis of the peaks indicates resolution of each of the members of the A–C set and gives no indication of heterogeneity. In collaboration with Dr. David C. Teller using high-speed equilibrium techniques (Yphantis, 1964), the number-average and weight-average molecular weights of aldolases A, C, and the middle hybrid have been determined. The results are presented in Table I. The data indicate that the molecular weights of the members of the hybrid set are similar.

TABLE I: Molecular Weights of Adolases A, C, and Hybrid 2.[a]

Aldolase	A	Hybrid 2	C
$M_{(n)} \times 10^{-3}$	150 ± 9	156 ± 7	156 ± 4
$M_{(w)} \times 10^{-3}$	151 ± 6	157 ± 11	155 ± 1

[a] High-speed equilibrium analysis was carried out essentially according to Yphantis (1964). The protein (0.75 mg/ml) was dissolved in 0.1 M Tris-chloride (pH 7.5). The solution was centrifuged at 15,000 rpm, 5°, in a Spinco ultracentrifuge equipped with Rayleigh optics. Photographs of the fringe pattern were taken after 12 hr and analyzed with a modified Nikon optical comparator (Teller, 1967).

Production of Complete A–C Hybrid Sets by Dissociation and Reassociation of Isolated A–C Hybrids. After elution by the substrate, FDP, from cellulose phosphate columns, aldolase C and the intermediate members of the A–C hybrid set can be resolved by DEAE Sephadex chromatography. DEAE Sephadex has proved to be superior to DEAE-cellulose (previously used; Penhoet *et al.*, 1966); there is less trailing and overlap of the separated protein peaks, presumably because there is less nonspecific binding. Zone electrophoresis and subsequent detection of activity of each of the resolved peaks gave a single band of aldolase activity corresponding to hybrids 1–3 and aldolase C. The specific activities of the isolated hybrids were 9.8, 9.2, and 8.2 for 1, 2, and 3, respectively.

As shown in Figure 2, dissociation and reassociation of aldolase A or C gives a single activity band with an electrophoretic mobility corresponding to that of the parent aldolase molecule. On the other hand, dissociation and reassociation of the hybrids 1, 2, and 3 results in the production of complete A–C hybrid sets. The terminal band corresponding to aldolases A and C

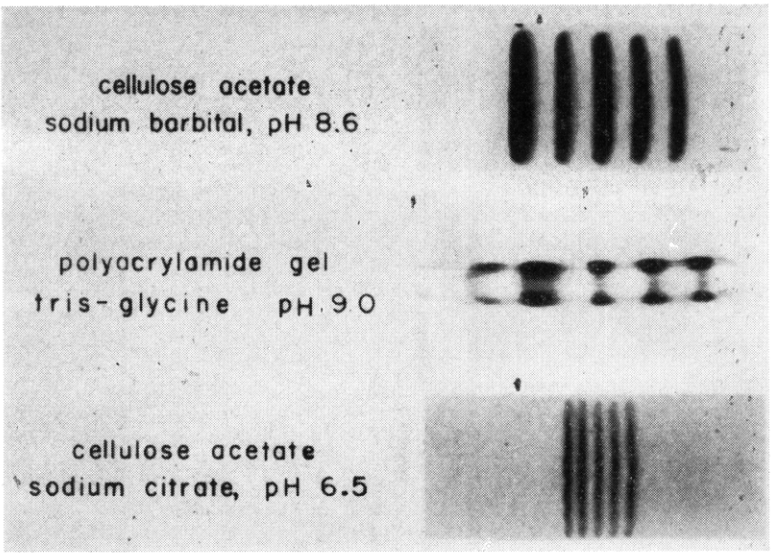

FIGURE 1: Resolution of the aldolase A–C set under various conditions. Supernatants (100,000*g*) from rabbit brain homogenates were prepared in the appropriate electrophoresis buffers. Activity stains were performed as previously described (Penhoet *et al.*, 1966). In all cases the anode is at the right of the figure. Top: Cellulose acetate electrophoresis was performed at 0.06 M sodium barbital–0.01 M β-mercaptoethanol (pH 8.6) at 250 V for 90 min in Gelman electrophoresis chambers as described previously (Penhoet *et al.*, 1966). Center: Polyacrylamide disc gel electrophoresis was performed according to Ornstein (1964) and Davis (1964) with a 3.5% running gel. Neutralized thioglycolate (1 μmole) was added to the sample to reduce any persulfate remaining after polymerization of the gel. Bottom: Cellulose acetate electrophoresis was performed as noted above except that 0.5 M sodium citrate (pH 6.5) was substituted for barbital. Under these conditions some members of the hybrid appear more anioic than on electrophoresis at pH 8.6 in barbital. This may be a reflection of citrate binding.

in the five-membered sets derived from hybrids 1 and 3, respectively, are not easily seen in the photographic reproductions but were clearly visible on the original strips. As might be expected from members of a hybrid series, the proportion of the parental subunits varies among the members of the hybrid set. Thus, form 1 produces a hybrid set skewed toward aldolase A; form 3 yields a hybrid set skewed toward aldolase C; the middle hybrid forms a symmetrically disposed hybrid set.

The results of these experiments are incompatible with various three-chain models of the enzyme. A binary mixture of trimers composed of three identical subunits in the simplest case would produce four-membered hybrid sets ($\alpha\alpha\alpha$, $\alpha\alpha\gamma$, $\alpha\gamma\gamma$, and $\gamma\gamma\gamma$; if α = A subunits and γ = C subunits). Binary combinations of the widely proposed three-subunit molecule containing two different subunits (Chan *et al.*, 1967) would predict a six-membered hybrid set ($\alpha\alpha\alpha'$, $\alpha\alpha\gamma'$, $\alpha\gamma\gamma'$, $\alpha\gamma\alpha'$, $\gamma\gamma\alpha'$, and $\gamma\gamma\gamma'$, assuming the primed subunit is present only once in the molecule). It could be argued that one of the six hybrids is either not formed or has not been resolved by the various procedures employed (Chan *et al.*, 1967). This possibility would predict that in the five-membered sets isolated by us in the present experiments, at least two of the intermediate hybrid forms would be composed of only three of the four possible subunit types. Therefore, it would not be possible to produce all members of the hybrid set on reversible dissociation of these members.

Three subunit molecules containing two or three different subunits with no combinative restrictions would yield hybrid sets with many more members (Rutter *et al.*, 1967).[2] There is no evidence supporting these structures, and they have never been seriously considered.

In contrast to the above, binary mixtures of tetrameric molecules would be expected to produce five-membered sets (α_4, $\alpha_3\gamma$, $\alpha_2\gamma_2$, $\alpha\gamma_3$, and γ_4). Reversible dissociation of each of the intermediate forms $\alpha_3\gamma$, $\alpha_2\gamma_2$, and $\alpha\gamma_3$ should produce complete hybrid sets.

[2] This condition would result in the formation of 20-membered hybrid sets composed of four groups, one group (four members) containing α subunits ($\alpha\alpha\alpha$, $\alpha'\alpha'\alpha'$, $\alpha\alpha\alpha'$, and $\alpha\alpha'\alpha'$), another group (four members) similarly containing all γ subunits, another group (six members) containing two α and one γ subunit, and a final group (six members) containing one α and two γ subunits. If combination is random, the relative proportions of the four groups are 0.2, 0.2, 0.3, and 0.3, respectively. There is no rational means of segregation of the 20-membered sets into five groups with the proportion of α and γ subunits being equivalent to that found for the A–C set (*viz.*, α_4, $\alpha_3\gamma$, $\alpha_2\gamma_2$, $\alpha\gamma_3$, and γ_4), and in the ratio of approximately 1:4:6:4:1 when the combinants in the hybrid set are derived from equal concentrations of α and γ subunits. For these reasons, we have discounted the three-subunit model with two different types of subunits.

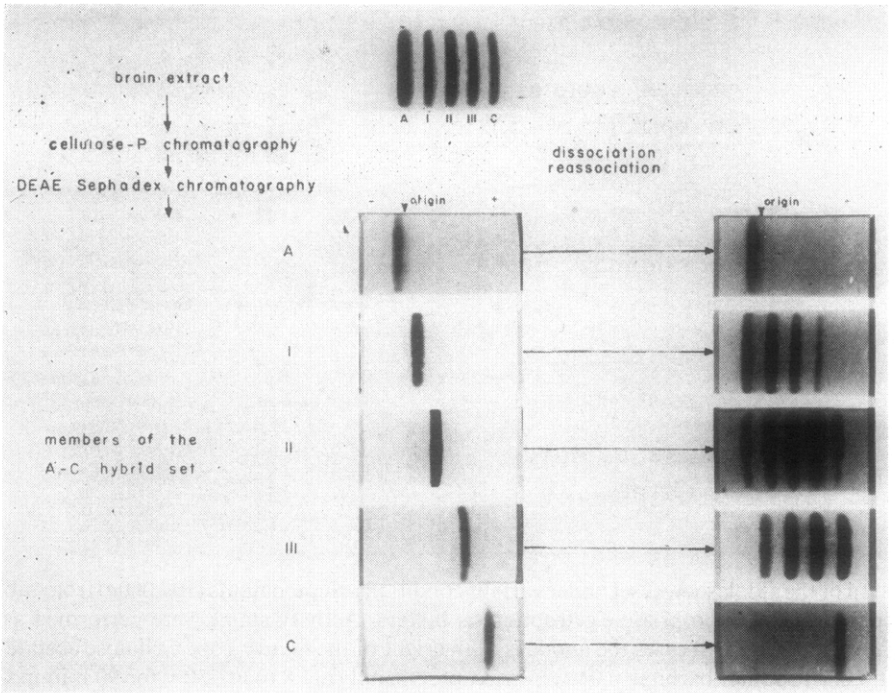

FIGURE 2: Reversible dissociation of the members of the A–C hybrid set. Members of the A–C hybrid set were isolated and subjected to dissociation and reassociation by methods previously described (Penhoet *et al.*, 1966; Rutter *et al.*, 1967). At least 75% recovery of activity was obtained in each case. Solutions were then subjected to cellulose acetate zone electrophoresis in sodium barbital buffer at pH 8.6, and aldolase activity was detected as described (Penhoet *et al.*, 1966).

If the reversible dissociation is random, then sets of the following distribution should result: hybrid 1 ($\alpha_3\gamma$) = 0.32(α_4), 0.42($\alpha_3\gamma$), 0.21($\alpha_2\gamma_2$), 0.05($\alpha\gamma_3$), and 0.004(γ_4); hybrid 2 ($\alpha_2\gamma_2$) = 0.06, 0.25, 0.38, 0.25, and 0.06; hybrid 3 ($\alpha\gamma_3$) = 0.004, 0.05, 0.21, 0.42, and 0.32. Although the ratios of the components in the hybrid sets resulting from reversible dissociation of the hybrids 1–3 have not been accurately quantitated, they are in qualitative agreement with the predicted distribution pattern.

Resolution of Aldolase A and C Subunits from Members of the A–C Hybrid Set. If the A–C hybrid set is subjected to electrophoresis on starch gel under non-dissociating conditions (Veronal buffer, pH 8.6), typical resolution of the five members of the A–C set is obtained; *i.e.*, there is a regularly ordered pattern of mobilities from aldolase A to aldolase C. As shown in Figure 3, a radically different pattern is obtained on zone electrophoresis under acidic dissociating conditions (see Methods). The subunits of aldolases A and C migrated as distinct single bands. In the first hybrid, the aldolase A subunits predominated; in the second hybrid, approximately equal amounts of aldolase A and C subunits were present; and in the third hybrid, the aldolase C subunits were present in higher concentration. Similar but less satisfactory resolution of A and C subunits were obtained by zone electrophoresis on cellulose acetate using a similar buffer system. In this instance, it was shown that on reneutralization catalytic activity was present in the area of the separated aldolase A and C subunits.

Definition of the "Combinative Unit" in the Formation of Hybrids from Aldolases A and C. The proportion of aldolases A and C in the intermediate forms of the A–C hybrid set was defined by reversible dissociation of crystalline [^{3}H]leucyl aldolase A (rat) and aldolase C (rabbit brain) (see Methods), and subsequent determination of the specific radioactivity in each of the members of the hybrid set. In order to obtain high specific radioactivity, it was advantageous to obtain [^{3}H]leucyl aldolase A from young rat tissues. Appropriate experiments demonstrated that the molecular weight and other molecular properties of rat aldolase A closely resembled those of the rabbit aldolase A. Moreover, hybrid sets between rat aldolase A and rabbit aldolase C are formed in excellent yield (greater than 75% recovery of initial activity). The electrophoretic and chromatographic properties of the heterologous A–C hybrid set are indistinguishable from those of the homologous A–C set.

To effect reversible dissociation, a mixture of equal parts of [^{3}H]leucyl aldolase A and aldolase C was acidified and subsequently reneutralized. On zone electrophoresis, an aliquot of the mixture produced a pattern similar to that obtained by reversible dissociation of the middle hybrid in Figure 2; all the members

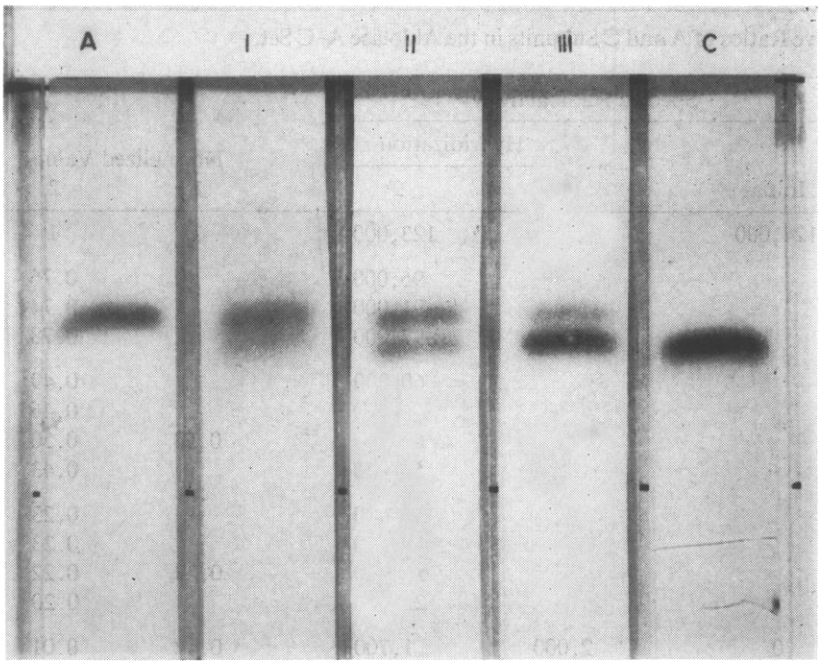

FIGURE 3: Starch gel electrophoresis of the aldolase A–C set under dissociation conditions. Electrophoresis of aldolase A, hybrids 1–3, and aldolase C were performed at pH 2 as described in Methods. The protein was stained using 1% Amido Black 10B in methanol–water–acetic acid (50:50:10). The protein migrated toward the cathode (upper part of the figure).

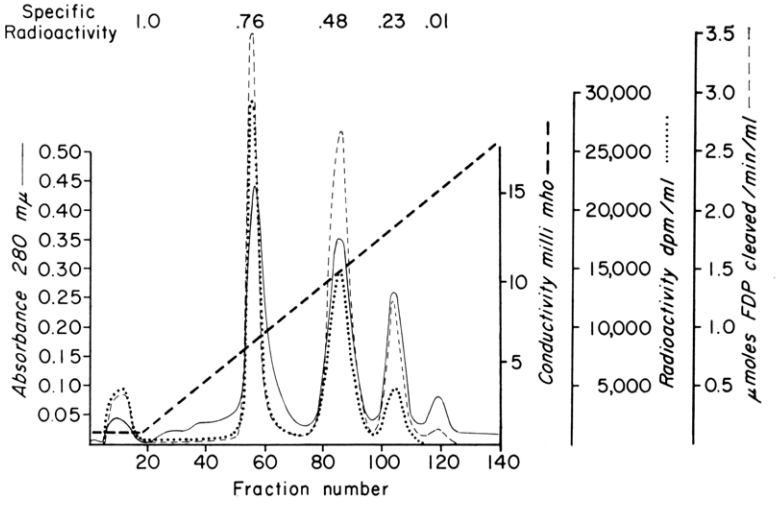

FIGURE 4: Chromatographic resolution of the labeled A–unlabeled C hybrid set. The radioactive hybrids were prepared as described in Methods. After concentration and dialysis, the sample was placed on a DEAE Sephadex A-50 column. The fractions were eluted with a linear gradient of NaCl from 0 to 0.35 M in 0.01 M Tris-chloride–0.001 M EDTA–0.0005 M FDP–0.005 M β-mercaptoethanol (pH 7.5). Fractions (2 ml) were collected and analyzed for absorbance at 280 mμ, FDP-cleavage activity, and radioactivity.

of the A–C set were formed by this treatment. As shown in Figure 4, the members of the hybrid set were resolved by DEAE Sephadex chromatography. The column fractions were analyzed for absorbance at 280 mμ, radioactivity, and FDP-cleavage activity. The relative specific radioactivity of the peak fraction are indicated in the figure (aldolase A = 124,000 dpm/mg = 1). There was excellent relative correspondence between the three parameters in all fractions of each peak except on the trailing edge of peak 2,

TABLE II: Combinative Ratios of A and C Subunits in the Aldolase A–C Set.

Combinants	Specific Radioactivity			Normalized Values		Theoretical Value (4 subunits)
	Initial	After Hybridization		1	2	
		1[a]	2[b]			
A	124,000	124,000	123,000	1	1	1
1			95,000		0.76	
			91,000		0.74	0.75
			97,000		0.78	
2			60,000		0.49	
			57,000		0.46	
		62,000	62,000	0.50	0.50	0.50
			53,000		0.43	
3			29,000		0.23	
			29,000		0.23	
		34,000	27,000	0.27	0.22	0.25
			25,000		0.20	
C	0	2,000	1,700	0.02	0.01	0

[a] Pooled fractions from each peak. [b] Individual fractions from each peak.

the first hybrid. After dialysis, which removed some contaminating material absorbing at 280 mμ, the values of these fractions agreed with those of other fractions in the same peak. The specific activities of typical fractions obtained from two separate experiments are summarized in Table II. In the first experiment, the fractions of each peak were combined, concentrated, and the specific activity was determined directly on the concentrate. In the second, fractions were selected from the leading and trailing edges of the peak. The relative specific activity of members of the A–C set in all the experiments are very close to 1, 0.75, 0.5, 0.25, and 0 for aldolase A, hybrids 1, 2, 3, and aldolase C, respectively. From these data it is apparent that the "combining unit" of aldolases A and C in the hybrid sets is one-quarter of the molecule. The constancy of the specific radioactivity in all regions of the peaks as well as the inability to resolve additional compounds by electrophoresis or chromatography suggest that the hybrid fractions are homogeneous. From these and other data, we infer that the combining unit is equivalent to the basic subunit of both aldolases A and C (one-quarter of the native molecule).

Visualization of Aldolase Subunits by Electron Microscopy. Aldolases A, B, and C have been examined by electron microscopy employing the negative staining procedure which has been employed in the determination of the structure of a number of proteins (for example, pyruvate carboxylase (Valentine *et al.*, 1966) and glutamine synthetase (Shapiro *et al.*, 1967)).

Although the subunit structure of a molecule as small as aldolase is not perfectly resolved by negative staining, this is the only technique presently available that is capable of showing directly the number and arrangement of the subunits. In the best preparations, two appearances of the molecule could be recognized in all the samples studied (aldolases A, B, and C and various hybrids). These had either square (or diamond shaped) profiles with a definite indication of four subunits or, less frequently, a triangular profile (Figure 5; see also Rutter *et al.*, 1967). This dual appearance is characteristic of a molecule consisting of four approximately spherical subunits arranged at the vertices of a tetrahedron. In such a tetrahedral arrangement the center-to-center spacing of the subunits is given by the diagonal distance between centers when the units appear as a square. This measures 40 A. A spherical subunit of mol wt 40,000 would have a diameter between 40 and 45 A. The tetrahedral architecture implies at least two types of bonds between the units. The dissociation of the molecule would then be expected to proceed in two steps: first, the formation of dimers; second, individual subunits. The original studies of Deal *et al.* (1963) suggested the presence of an intermediate in the dissociation process. Whether this is a dimer or an unfolded tetramer has not been defined. The electron micrographs appear inconsistent with any symmetrical (hence self-assembling) arrangement of either three or six similar subunits.

Discussion

Confirmation of the Four-Subunit Model. We believe the data obtained in the present experimental investigations establish the four-subunit model for fructose diphosphate aldolase by eliminating all reasonable

alternatives. Only the four-subunit model can account for (1) the five-membered hybrid sets produced by reversible dissociation and reassociation of binary mixtures of aldolases A, B, and C and found in extracts of tissues from a large number of different species; (2) the production of all members of the hybrid set by reversible dissociation and reassociation of each hybrid; (3) the qualitative resolution of A and C subunits present in the hybrids of the A–C hybrid set; (4) a combinative unit of one-quarter of the native molecule in the formation of hybrids (in this case, the combinative unit equals the basic subunit); and (5) the appearance of the molecules in electron micrographs.

We conclude that the basic composition of members of the aldolase A–C set is α_4, $\alpha_3\gamma$, $\alpha_2\gamma_2$, $\alpha\gamma_3$, and γ_4. We infer that other hybrid sets formed from binary mixtures are similar (the AB set: α_4, $\alpha_3\beta$, $\alpha_2\beta_2$, $\alpha\beta_3$, and β_4; the BC set: β_4, $\beta_3\gamma$, $\beta_2\gamma_2$, $\beta\gamma_3$, and γ_4).

The Contradictory Data. The conclusion that aldolase contains four subunits necessitates consideration of the substantial body of evidence which has supported the three-subunit hypothesis.

A. THE QUANTITATIVE DETERMINATION OF C-TERMINAL AND N-TERMINAL AMINO ACIDS. It is well known that these determinations of terminal amino acids provide minimal stoichiometry. We know of no study maximizing the yields of either N- or C-terminal amino acids obtained from aldolase. The early stoichiometry (Udenfriend and Velick, 1951) for N-terminal prolines (two per mole) obtained by the formation of the *N*-pipsyl derivatives were admittedly minimal; the values more recently obtained by other methods may also be low (Fraenkel-Conrat *et al.*, 1955; Drechsler *et al.*, 1959). The possibility of blocked N-terminal amino acids in aldolase has not been eliminated; in fact, Stegink (1967) has found approximately 0.3 mole of bound acetyl group/mole of aldolase A. Hass (1964) found 1.3 proline phenylthiohydantoins/150,000 g on initial reaction with the enzyme, and an additional 2.59 by repeating the procedure on the same sample (total 3.98). Further repetition yielded no additional amino acids. (These data were not interpreted by Hass as an indication of four subunits.)

The method most frequently employed for the determination of carboxy-terminal amino acids involves measurement of the amino acids released by carboxypeptidase. The obtained values may be limited by: (a) incomplete release of the terminal amino acids, perhaps by conformational alterations of the protein after partial release of C-terminal residues; (b) incomplete determination of C-terminal residues released (some of the C-terminal residues could, for example, be coprecipitated with the protein by the procedures (trichloroacetic or perchloric acid precipitation) employed; and (c) variable degradation of one of the chains might yield smaller quantities of several amino acids different from tyrosine, the acknowledged C terminus.

B. THE NUMBER OF SUBSTRATE BINDING AND CATALYTICALLY ACTIVE SITES. It is possible but unlikely that a tetrameric molecule with identical or closely related subunits has three active sites. It is more likely that some of the substrate binding sites in the isolated molecules have different kinetic constants and remain undetected in previous studies.

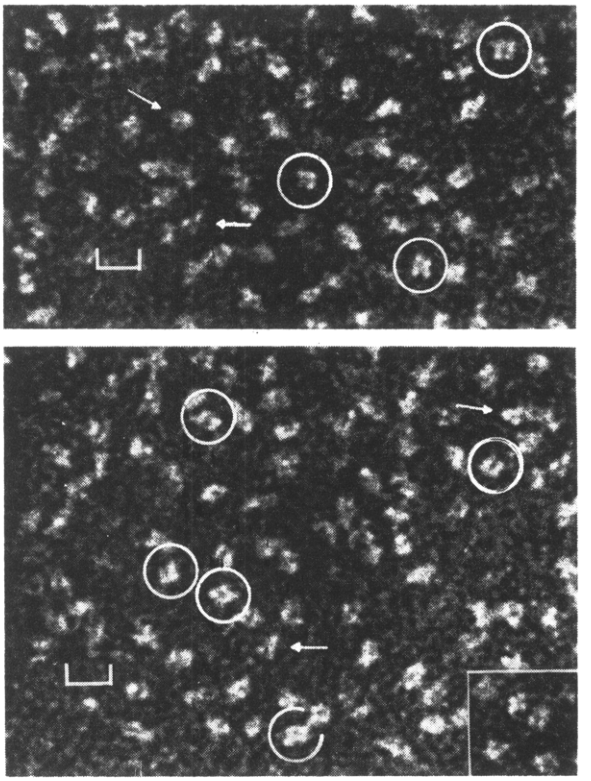

FIGURE 5: Electron micrograph of aldolase molecules. Characteristic appearances are a square of four subunits (circles) and a triangular outline (arrows) suggesting a tetrahedral molecule with four subunits. The scale indicates 100 A; magnification 500,000×.

The determination of the stoichiometry of dihydroxyacetone phosphate binding by borohydride reduction may be subject to various limitations as suggested by variation in the values reported with this method (Grazi *et al.*, 1962; Lai *et al.*, 1965; Kobashi *et al.*, 1966). The recent observations (B. M. Woodfin, unpublished results) that aldolase binds FDP or a moiety derived from FDP with a concomitant reduction in the specific activity of the enzyme may provide one explanation for underestimation of the number of active sites observed in previous studies.

C. DETERMINATION OF THE MOLECULAR WEIGHT OF THE NATIVE MOLECULE AND THE DISSOCIATED SUBUNITS. The values of the molecular weight of the native aldolase molecule and subunits obtained by physical methods have varied widely. During recent years, for example, the reported molecular weight of native aldolase molecule has varied from 142,000 (Stellwagen and Schachman, 1962) to 160,000 (Kawahara and Tanford, 1966). Similarly, the values for dissociated molecules have ranged between 37,000 and 51,000. Within these ranges are several investigations which suggest the molecular weight of the dissociated form is one-

third the native molecule (*ca.* 150,000 mol wt) (Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Schachman, 1963; Schachman and Edelstein, 1966; Sine and Hass, 1967), and the study of Kawahara and Tanford (1966) which yields a value of one-fourth the native molecule (mol wt 160,000). The reason for the discrepant results is not yet clear, but the conditions employed for determination of the molecular weights of native and dissociated molecules are different. Different means of evaluation of solvent effects have been employed in the various studies.

The experiments presented here require that the molecular weight of the subunit be one-fourth that of the native molecule but, of course, do not define the molecular weight of either. In view of the considerable variance in molecular weight values obtained by the ultracentrifugal procedures, it is desirable to define the molecular weight of the native tetramer by other methods. The determination of the molecular weight of the aldolase molecules by appropriate X-ray crystallographic techniques is in progress under the direction of P. E. Wilcox and L. H. Jensen at the University of Washington, and F. S. Mathews at Washington University.

Does the Aldolase Tetramer Contain More Than One Kind of Subunit? The present experiments distinguish aldolase A and C subunits but do not define whether all subunits of aldolase A or C are identical. The prediction of the numbers of subunits in the various hybrid sets has been based on the assumption that the tetrameric molecule is composed of identical subunits, but the arguments presented only require that pairs of subunits in combination have indistinguishable electrophoretic and chromatographic properties.

The available experimental evidence suggests the presence of two distinct peptide chains in aldolase A preparations. The initial studies involving carboxypeptidase degradation of aldolase A (Kowalsky and Boyer, 1960) first suggested this possibility. The results of Winstead and Wold (1964), who measured the kinetics of release of amino acids from aldolase A by carboxypeptidase, further supported the concept that aldolase contained two different subunit types. A similar interpretation could be placed on the data obtained by carboxypeptidase treatment of aldolase B (Rutter *et al.*, 1961). Furthermore, polyacrylamide gel electrophoresis under dissociating conditions of aldolase (Edelstein and Schachman, 1966) or carboxymethyl aldolase resolves two peaks (Chan *et al.*, 1967). Chan and Morse (1967) have recently reported that the separated chains have a different amino acid sequence as indicated by the release of amino acids by carboxypeptidase and by the distinct appearance of their respective peptide maps. The most straightforward accommodation of the above data would be the assumption that aldolase A contains two very similar but distinct subunit types. From a consideration of symmetry and by analogy with the structure of hemoglobin, the structures of $\alpha_2\alpha_2'$ might be proposed. However, there are other interpretations of the data which would allow an oligomeric aldolase structure. (1) Two different alleles or duplicate genes for aldolase A might cause the formation of two closely related aldolase A subunits (polypeptide chains) in the system studied. In this case, various combinations of the closely related aldolase A subunits would be expected. Molecular heterogeneity of aldolase A has not been detected, but still remains possible if the two subunits are closely related. (2) A single aldolase polypeptide chain might be formed and subsequently modified either within the cell or during the isolation procedure to produce two different components. It is well known that some of the procedures employed for analyses can lead to structural modifications, and it is apparent that the chemical properties of aldolase are quite distinctive. In view of the above, it would be premature to draw a specific conclusion regarding the kinds of subunits present in the aldolase tetramer. Because of the more facile prediction of five-membered hybrid sets from tetrameric molecules composed of identical subunits, however, we do not believe a structure of aldolase involving two different subunit types will be inviolate. At least some of the FDP aldolases will probably be oligomers composed of a single subunit type. But, what is the general structure, and why?

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