Preparation of Isolated Multiple Forms of Rabbit Brain Aldolase

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1. A method is described for partial purification of the total aldolase of rabbit brain by ammonium sulphate precipitation followed by fractionation of the aldolase into five electrophoretically distinct forms. 2. The method allows a laboratory-scale preparation of the five aldolases. 3. With the exception of band 3 the fructose 1,6-diphosphate/fructose 1-phosphate activity ratios of the isolated multiple forms show a continuous increase from isoenzyme 1 to isoenzyme 5; this evidence supports the four-sub-unit model.

Recent work has shown that aldolase (ketose 1-phosphate aldehyde-lyase, EC 4.1.2.7; fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13) in mammalian brain is composed of five multiple forms (Foxwell, Cran & Baron, 1966; Christen, Rensing, Schmidt & Leuthardt, 1966; Penhoet, Rajkumar & Rutter, 1966). It has been suggested that these five forms are derived by hybridization from the classical aldolase A (of muscle), and from a new aldolase, named aldolase C. Herskovits, Masters, Wasserman & Kaplan (1967) have found aldolase C in brain, heart and embryonic and infant leg muscle of the chicken.

The observations are of particular interest if it is assumed that the aldolase C protein is composed of three sub-units, analogous to the postulated structure for aldolase A (Horecker, 1965; Sine & Hass, 1967), as the five multiple forms in brain cannot then be formed from these parent molecules. However, work by Kawahara & Tanford (1966), Penhoet, Kochman, Valentine & Rutter (1967) and Sia & Horecker (1968) has tended to question the widely held belief that aldolase A is composed of three sub-units, and indications are that a four-sub-unit model may be correct. If this is the case, and aldolase C is similar, then hybridization between aldolases A and C would give rise to the five multiple forms, as for lactate dehydrogenase. The present paper describes a method for the separation of the individual forms of aldolase in reasonable quantity.

EXPERIMENTAL

Animals. New Zealand White male rabbits weighing 2.8–4.3 kg. were used.

Chemicals. All chemicals (purest grades) were purchased

from British Drug Houses Ltd., Poole, Dorset, except for the barium salt of fructose 1-phosphate [Sigma (London) Chemical Co. Ltd., London, S.W. 6], Pevikon C870 (Shandon Scientific Co. Ltd., London, N.W. 10), hydrolysed starch (Connaught Medical Supplies, Toronto, Ont., Canada) and barbitone-acetate buffer for electrophoresis (Oxoid Ltd., London, E.C. 4). Saran-wrap was obtained from Dow Chemical Co. (Midland, Mich., U.S.A.), u.v. aldolase test kit from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) and dialysis tubing from Solmedia Ltd. (London, E. 17).

Buffer solutions. Buffer A consisted of 1 mM-tris-HCl, pH 7.6, containing 1 mM-iodoacetamide and 0.2 mM-EDTA. Buffer B was 1 M-tris-HCl, pH 7.6, containing 1 mM-iodoacetamide and 0.2 mM-EDTA.

Block electrophoresis. Pevikon C870 was washed with tap water, twice with distilled water and at least twice with 0.05 M-barbitone-acetate buffer, pH 8.6. Each time the suspension was allowed to settle out, and then the supernatant solution was decanted off. After the final wash in 0.05 M-barbitone-acetate buffer, pH 8.6, the Pevikon slurry was poured into a Perspex tray (25.3 cm. × 50.5 cm. × 0.5 cm.), covered with Saran-wrap, blotted dry and levelled off. The surface of the block was covered with Saran-wrap and then it was equilibrated for 45 min. at 4° by using a 0.1 M-barbitone-acetate buffer, pH 8.6, bridge solution with absorbent lint wicks and platinum electrodes at 3 mA/cm. width of block (constant current) with a voltage of about 360 V obtained by using an APT power pack (Electronic Industries Ltd., Byfleet, Surrey).

Preparation of haemoglobin solution. The method of Lehmann & Ager (1966) for the preparation of oxyhaemoglobin solution (about 10 g./100 ml.) was used.

Preparation of homogenate. Eight rabbits were killed by injecting about 20 ml. of air into the marginal ear vein. The brains were rapidly removed, pooled, rinsed with cold buffer A, blotted dry and weighed (total weight 70–80 g.). The pooled brains were homogenized in the cold in an equal quantity (w/v) of cold buffer A for 30 sec. in an MSE homogenizer. The homogenate was centrifuged at 78410 g (30000 rev./min. with rotor no. 30) for 1 hr. at 4° in a Spinco model L ultracentrifuge. The supernatant (S1) was decanted off and the precipitate discarded. Saturated (NH₄)₂SO₄ solution, pH 7.0, was added to the supernatant

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until 45% saturation was achieved. After 30 min. at 4° the precipitate was removed by centrifugation and discarded and the $(\text{NH}_4)_2\text{SO}_4$ saturation of the supernatant was increased to 60%. The 45–60% saturation precipitate after 30 min. at 4° was collected as before, taken up in buffer A (2 × 2 ml.: pooled) and dialysed against the same buffer (11.) overnight. The non-diffusible material was centrifuged at 104000g (40000 rev./min. with rotor no. 40) for 15 min. at 4° and gave a clear supernatant (S2).

This supernatant (S2) was mixed with 1 ml. of 10% (w/v) haemoglobin solution and, by using a 10 ml. syringe and a no. 20 needle, was applied to a slit (0.1 cm. × 23 cm. × 0.5 cm.) cut in the Pevikon block about 7 cm. from the cathode. Blotting paper was placed about 2 cm. on either side of the slit to absorb the displaced buffer. The slit was filled in with surrounding Pevikon and then the block was covered with Saran-wrap. The block was developed at 3 mA/cm. constant current at about 360 v for 26 hr. at 4°. The haemoglobin was used as a marker, and experience showed that when the haemoglobin was approximately half way along the total length of the block the five multiple forms of aldolase were sufficiently separated to allow extraction of the individual bands.

Location of aldolase on Pevikon block. Location and staining of aldolase on the Pevikon block was by a modification of the Pietruszko & Baron (1967) method. Three strips of Whatman no. 1 chromatography paper (51 cm. × 5 cm.) soaked in 2% (w/v) fructose 1,6-diphosphate in buffer A were placed on the Pevikon block lengthwise. The block and papers were covered with Saran-wrap and incubated at about 20° for 15 min. To be certain of locating the enzymes it was found necessary to place weights on the filter-paper strips so that intimate contact between the Pevikon and the paper was achieved.

The multiple forms of aldolase, located as given above, were then separated by cutting out the relevant sections of the block and washing with buffer A (2 × 50 ml.) on a sintered-glass funnel, which was sucked dry after the second washing. Buffer B (10 ml.) was then added to each of the five solutions and the proteins were precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 60% saturation. The solutions were kept at 4° for 30 min. and then centrifuged at 40210g (20000 rev./min. with rotor no. 21) for 30 min. at 4°.

The supernatants were discarded, and the precipitates were taken up in a total of 5 ml. of buffer A and dialysed against the same buffer (11.) overnight at 4°. After dialysis the solutions containing the non-diffusible material were centrifuged at 105400g (40000 rev./min. with rotor no. 40) for 15 min. at 4° to give clear solutions of the enzyme fractions. The enzyme solutions were stored at 4°.

Starch-gel electrophoresis. Vertical starch-gel electrophoresis of enzyme samples (approx. 50 $\mu\text{l.}$) was carried out at pH 7.0 and at pH 8.6 by the Smithies (1959) procedure.

The phosphate-citrate gel, pH 7.0, described by Pietruszko & Baron (1967), was developed at 3 mA/cm. constant current and about 120 v. For electrophoresis with barbitone-acetate buffer, pH 8.6, the starch was gelled in a solution of 0.05 M-barbitone-acetate buffer and the bridge solution was 0.1 M-barbitone-acetate buffer of the same pH. The gel was developed at 8 mA/cm. and about 100 v.

Location of aldolase on starch gels. The method used for demonstration of aldolase on starch gels was essentially that of Pietruszko & Baron (1967) except that fructose

1,6-diphosphate was dissolved in buffer A and the incubation time at 37° was 60 min.

Enzyme assay. Aldolase activity was determined with the substrates fructose 1,6-diphosphate and fructose 1-phosphate. Details of the fructose 1,6-diphosphate aldolase u.v. assay method are given by Pietruszko & Baron (1967). The fructose 1-phosphate determination was carried out by essentially the same method. The barium salt of fructose 1-phosphate (90.5%) was suspended in 1 ml. of water, and 0.38 ml. of ice-cold N-HCl was added. A clear solution was obtained, 0.19 ml. of M- Na_2SO_4 was added and the pH of the solution adjusted to pH 7.0 with N-NaOH. The volume was made up to 4.4 ml. and the solution centrifuged. To part of this solution (2.67 ml.) 1 ml. of 0.28 M-collidine-HCl buffer, pH 7.4, containing 1.5 mM-iodoacetate was added, and the volume made up to 5 ml. with distilled water. Solutions 2 and 3 from the Boehringer aldolase u.v. test kit were added in the proportions indicated and the solution was equilibrated at 28° for 5 min. The same procedure for fructose 1-phosphate enzyme activity was then used as for the fructose 1,6-diphosphate enzyme assay. Solutions from which fructose 1-phosphate and fructose 1,6-diphosphate were absent were used as blanks. Each enzyme assay was done in duplicate.

Fig. 1. Electrophoretic separation of multiple forms of rabbit brain aldolase after block electrophoresis on Pevikon in 0.1 M-barbitone-acetate buffer, pH 8.6. The block was developed at 3 mA/cm. (constant current) and 360 v for 26 hr. To locate the enzymes, three strips (5 cm. wide) of Whatman no. 1 paper soaked in 2% (w/v) fructose 1,6-diphosphate in buffer A were placed centrally and on the left and the right side of the Pevikon. The block was incubated at 20° for about 15 min.

It was desirable to carry out the fructose 1-phosphate determination as rapidly as possible because of instability of reagents.

Protein determinations. These were carried out by measuring extinction at 260 and 280 nm. in a Hilger Uvispek and protein content was read off with a nomogram derived from the equation of Warburg & Christian (1942).

RESULTS AND DISCUSSION

The electrophoretic separation given by the Pevikon block is shown in Fig. 1.

The protein concentration and the fructose 1-phosphate and fructose 1,6-diphosphate activities of the crude homogenate supernatants (S1 and S2) and the five multiple forms of brain aldolase were measured; the measurements were repeated again the next day (Table 1).

Starch gels were run at pH 7.0 and pH 8.6. The

best separation was obtained at pH 7.0 (Fig. 2). The multiple forms 1-5 migrated as electrophoretically homogeneous bands, which corresponded to those seen in the crude brain homogenate.

The five multiple forms of aldolase were first observed after electrophoresis of crude brain homogenates on starch gel. However, this technique did not allow a preparative separation of the multiple forms, and for this purpose the technique of block electrophoresis was found to be very satisfactory. It was possible to put at least 25 times more crude brain homogenate on the Pevikon block than on a starch gel; and, after separation of the five forms, removal of the enzymes from the Pevikon was simply achieved by washing the Pevikon with buffer, whereas complete removal of starch from the enzyme solutions was a much more difficult procedure.

Table 1. *Protein concentrations and enzyme activities of the crude brain homogenate (S1), the ammonium sulphate concentrate (S2) and the multiple forms 1-5 of rabbit brain aldolase separated by Pevikon-block electrophoresis*

The aldolase activity was assayed with fructose 1,6-diphosphate (Fru-1,6-P₂) and with fructose 1-phosphate (Fru-1-P) as substrate. When calculating ratios the measured fructose 1,6-diphosphate activity is halved to compensate for the formation of two active trimers in this enzyme action.

Sample	Day	Protein (mg./ml.)	Volume (ml.)	Activity ($\Delta E/\text{min.}/0.01 \text{ ml.}$)		Mean Fru-1,6-P ₂ / Fru-1-P activity ratio	Mean specific activity			
				Fru-1,6-P ₂	Fru-1-P		Fru-1,6-P ₂	Fru-1-P		
S ₁	3	50	60	{ 0.66 0.66 0.70 0.60 }	{ 0.0064 0.0065 0.0064 0.0064 }	51	1.3	0.01		
	4					52	1.3	0.01		
	3			20	6.4	{ 2.75 2.80 2.45 2.32 }	{ 0.0340 0.0360 0.0310 0.0310 }	39	13.8	0.17
	4							38	11.9	0.15
Multiple forms										
1	3	2.8	5.8			{ 0.358 0.361 0.318 0.330 }	{ 0.0084 0.0088 0.0070 0.0070 }	21	12.8	0.30
4					23	11.5	0.25			
2	3			1.3	5.4	{ 0.213 0.219 0.145 0.138 }	{ 0.0042 0.0040 0.0037 0.0035 }	26	17.3	0.31
4							19.5	10.9	0.27	
3	3	1.2	5.4			{ 0.147 0.151 0.089 0.092 }	{ 0.0033 0.0029 0.0031 0.0033 }	24	12.4	0.25
4							14	7.6	0.26	
4	3			2.3	5.3	{ 0.175 0.173 0.170 0.188 }	{ 0.0024 0.0021 0.0022 0.0023 }	38	7.5	0.10
4							39	7.7	0.10	
5	3	0.8	5.2			{ 0.053 0.051 0.061 0.057 }	{ 0.0005 0.0007 0.0007 0.0004 }	43	6.5	0.07
4							54	7.3	0.07

Fig. 2. Electrophoretic separation of individual multiple forms of rabbit brain aldolase on starch gel at pH 7.0 with a citrate-phosphate buffer containing 6mm-citric acid and 48mm- Na_2HPO_4 . The gel was developed at 3mA/cm. (constant current) and 120v for 16-18hr. Whatman no. 1 paper soaked in fructose 1,6-diphosphate was overlaid on the gel, and incubation was for 60min. at 37°.

The hypothesis of the three-chain model for the structure of aldolase, supported by physical and chemical evidence (Penhoet *et al.* 1966), could not adequately explain the finding of the five multiple aldolase forms in brain. If, however, we consider a three-chain model and two parent forms x_3 and y_3 with hybrids x_2y and xy_2 , it can be seen that only four forms are possible. If, however, we consider a four-chain structure with two parent forms x_4 and y_4 and the hybrids x_3y , x_2y_2 and xy_3 , we have the possibility of the five multiple forms as found in rabbit brain. Penhoet *et al.* (1966) and Rensing, Schmidt, Christen & Leuthardt (1967) have shown that this is in fact possible by their hybridization experiments with pure aldolase A and C obtained from rabbit and rabbit and ox respectively.

The fructose 1,6-diphosphate/fructose 1-phosphate activity ratios given in Table 1 on day 3 indicate a gradual increase in ratio from aldolases 1 to 5 with the exception of band 3. These results are

similar to those obtained by Rensing *et al.* (1967), and are a correction of our earlier results (Foxwell *et al.* 1966). The fructose 1,6-diphosphate/fructose 1-phosphate activity ratio of aldolase 5 on day 3 was 48:1, which is very similar to the ratio found for the classical aldolase A, which is 50:1. The fructose 1,6-diphosphate/fructose 1-phosphate activity ratio for aldolase C (band 1) was found to be about 20:1.

The enzyme-activity assays repeated on the next day showed that the fructose 1,6-diphosphate/fructose 1-phosphate activity ratios of aldolases 1, 5 and the hybrid 4 did not change appreciably, whereas those of the hybrids 2 and 3 did. The fall in the fructose 1,6-diphosphate activity in hybrid 2 (C_3A) and hybrid 3 may possibly reflect the unstable character of the A aldolase hybrid when the C form predominates, especially as the hybrid aldolase 4 (A_3C), which is predominantly A, did not show the fall in activity.

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