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ENOLASE ISOENZYMES

III. CHROMATOGRAPHIC AND IMMUNOLOGICAL CHARACTERISTICS OF RAT BRAIN ENOLASE

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Summary

1. The chromatography of rat brain enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) reveals three distinct components. One of these appears to be isoenzyme 1 ($\alpha\alpha$) but isoenzymes 2 ($\alpha\beta$) and 3 ($\beta\beta$) are absent.

2. The most acidic form of brain enolase was partially purified and an antiserum raised against it in the chicken.

3. A combination of chromatographic and immunological studies showed that a third type of subunit (γ) is present in the brain giving rise to two further isoenzymes ($\alpha\gamma$ and $\gamma\gamma$).

4. Developmental studies on the brain enzyme show an increase in total enolase activity from foetal life to maturity and a concurrent rise in the proportion of brain specific dimers.

5. It is therefore concluded that there are three genetic loci α, β and γ, coding for the enolase isoenzymes of rat tissues.

Introduction

We have previously reported [1] chromatographic and immunological evidence for the existence of a brain specific isoenzyme of enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) in the rat.

On QAE-Sephadex ion exchange chromatography the enolase activity of this tissue eluted as a complex profile with a substantial portion of the activity bound more strongly to the exchanger than are the isoenzymes 1 ($\alpha\alpha$), 2 ($\alpha\beta$) and 3 ($\beta\beta$) [1,2]. This activity, apparently unique to the brain failed to cross-react with antisera specific for muscle and liver enolases. In the present paper we describe further the characterization of brain enolase and give an account of the developmental changes in the total activity of the enzyme and in the relative proportions of its isoenzymes.

Experimental

Animals. Wistar rats of the Sheffield University animal house colony maintained and fed as previously described [2] were used throughout.

Enzyme assays. Enolase activity was assayed as reported earlier [3] by coupling with pyruvate kinase and lactate dehydrogenase. Activities are expressed in enzyme units (1 unit = amount of enzyme converting 1 μmol of substrate in 1 min at 30°C, except for the analytical column chromatography eluates which were assayed at 37°C). Specific activities were expressed as units/mg protein; protein was determined by the method of Lowry et al. [4].

Analytical column chromatography. Homogenates of rat whole brain (1 : 5 w/v) made in 15 mM sodium phosphate buffer, pH 7.9, containing 4 mM $MgSO_4$, 0.1 mM EDTA and 0.1 M NaCl were centrifuged at 125 000 × *g* for 40 min at 5°C and the supernatant fluids dialysed against 20 vols. of buffer for 2 h. Samples (5 ml) were applied to DEAE-Sephadex (A-50) columns (2.5 × 10 cm) previously equilibrated with the above buffer, 160 ml of which was also used for the initial stage of elution and followed by a linear gradient in which the NaCl concentration was raised to 0.6 M. The ionic concentration of the eluate was monitored by conductivity measurements.

Immunological procedures. The production of the anti-muscle and anti-liver enolase sera used here has been described previously [3]. Antiserum to the partially purified acidic brain enolase (see Results section) was raised in chickens by the following schedule. 1 mg doses of the protein preparation which had been concentrated by ultrafiltration to 0.6 ml were emulsified with equal volumes of Freund's adjuvant and injected subcutaneously into the wing at 0, 23, 39 and 50 days. For the first and last of these injections Freund's complete adjuvant was used otherwise the incomplete form was employed.

Serum was separated from blood taken by venepuncture and assayed for antibody activity against the purified brain enolase fraction. The immunological titration method was as described previously [3] except that the mixture of enzyme and antiserum was centrifuged at 20 000 × *g*. Antisera neutralizing 0.4 and 0.6 units of acidic brain enolase per ml serum were obtained at 49 and 57 day respectively.

Results

DEAE-Sephadex chromatography of adult brain enolase

Fig. 1 shows a better resolution of rat brain enolases on DEAE-Sephadex than we have previously been able to achieve [1]. The immunological properties of the three peaks were investigated. The activity of the first peak to be eluted is susceptible entirely to antiserum raised against liver enolase but that of the third and most acidic peak is unaffected. The second peak is inhibited 50% by the anti-liver enolase serum while none of the three peaks is significantly affected by anti-muscle enolase serum. Insofar as the immunological properties indicate the nature of the subunits, peak 1 would appear to be isoenzyme 1, the $\alpha\alpha$ dimer, peak 3 an isoenzyme with subunits distinct from α and β, whereas peak 2 has intermediate properties immunologically and chromatographically.

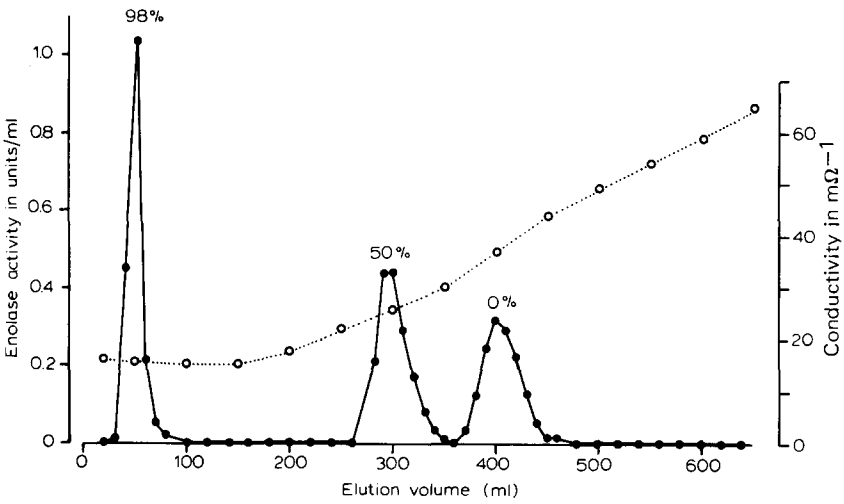

Fig. 1. Separation of enolase isoenzymes of rat brain by DEAE-Sephadex chromatography. The column (2.5 × 10 cm) was eluted with 160 ml 15 mM sodium phosphate buffer, pH 7.9 containing 4 mM $MgSO_4$, 0.1 mM EDTA and 0.1 M NaCl, followed by a linear gradient in which the NaCl concentration was raised to 0.6 M. Enolase activity •; conductivity of eluate ○. The values shown above each peak indicate the percentage of the activity susceptible to anti-liver enolase serum.

Purification of "acidic brain enolase"

The third or most acidic fraction eluting from DEAE-Sephadex was purified as follows. All steps were carried out at 5°C. Batches of 200 g rat brain were homogenized in 4 vols. 15 mM sodium phosphate buffer, pH 6.9, containing 5 mM $MgSO_4$ and 0.1 mM EDTA and the homogenate centrifuged at 130 000 × *g* for 80 min. The supernatant fluid was applied to a CM-Sephadex column (5 × 40 cm) previously equilibrated with the homogenization buffer which was also used for the elution. Fractions (10 ml) were collected and assayed for enolase activity and protein content. A single broad peak of activity was eluted without retardation. Fractions with specific activities over 4 units/mg were pooled, adjusted to pH 7.9 with 2 M NaOH and dialysed overnight against 4 vols. 15 mM sodium phosphate buffer pH 7.9 containing 4 mM $MgSO_4$ and 0.1 mM EDTA. The dialysate was run on to a DEAE-Sephadex column (4.5 × 20 cm) equilibrated with that buffer, 250 ml of which was also used for the initial elution followed by a linear gradient, 1500 ml, of sodium chloride (0.1—0.7 M) in the same buffer. Three peaks of enolase activity were obtained and the elution profile was similar to that seen in Fig. 1. Those fractions of the third peak having a specific activity greater than 2.5 units/mg were pooled and dialysed against 4 vols. of saturated ammonium sulphate. The resulting suspension was centrifuged for 20 min at 14 000 × *g* and resuspended in a minimum volume of the sodium phosphate buffer, pH 7.9, without sodium chloride. Substantial but reversible inactivation of enolase occurred at this stage, between 0 and 20% of the pre-dialysis activity remaining. The inactive preparation (20 ml) was applied to a Sephadex G-150 column (5 × 100 ml) equilibrated and eluted with the sodium phosphate buffer, pH 7.9. All the enolase activity lost in the ammonium sulphate step was recovered as a single peak. Fractions with a specific activity greater than 5.0

TABLE I

SUMMARY OF TYPICAL PREPARATION OF RAT BRAIN ENOLASE 'ACIDIC FRACTION'

For the DEAE-Sephadex separations results for the first, second and third elution peaks are given. Specific activities are given only for the third elution peak which is the acidic fraction being sought.

Purification step		Volume (ml)	Total enolase activity (units)	Total protein (mg)	Specific activity (units/mg)	% yield
Crude homogenate supernatant		800	8500	4500	1.9	100
CM-Sephadex eluate		1000	7870	2130	3.7	93
Dialysate		905	5140	2130	2.4	61
DEAE-Sephadex I eluate	1.	1400	2210	—	—	26
	2.	150	230	—	—	3
	3.	800	1125	415	2.7	13
$(NH_4)_2SO_4$ precipitate		20	nil	—	—	nil
Sephadex G-150 eluate		250	1400	235	6.0	16
DEAE-Sephadex II eluate	1.	—	nil	—	—	nil
	2.	60	500	—	—	5.9
	3.	50	500	24	21	5.9

units/mg were pooled and applied to a second DEAE-Sephadex column (2.6 × 15 cm) which was equilibrated and eluted as before except that 100 ml buffer was used for the initial elution and 500 ml for the gradient (0.1—0.7 M sodium chloride).

In this elution the first and second peaks of activity are very much reduced and the third peak, designated acidic brain enolase, accounts for 50% of the activity remaining and 6% of the initial homogenate activity. The results of the purification procedure are summarised in Table I.

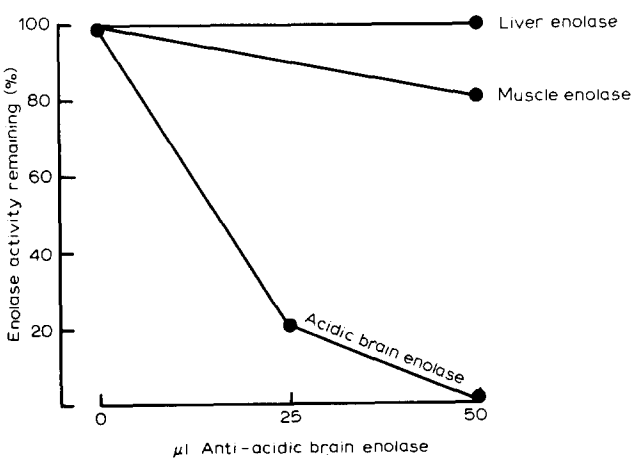

Fig. 2. Titration of the enolase activity of rat liver, muscle and 'acidic brain fraction' with anti-acidic brain enolase serum. The 'acidic brain fraction' used here and against which the serum had been raised in chickens, is the third peak of activity eluting in the DEAE-Sephadex chromatography of crude brain extract further purified as indicated in Table I. For each type of enolase the initial activity was 0.01 units. The enzyme sample, 10 μl, was mixed with the volume of antiserum indicated and made up to a final volume of 65 μl with control serum. After standing for 15 min the mixture was centrifuged at 20 000 × *g* for 15 min and the activity remaining in the supernatant fluid determined.

Immunological characterization of brain enolase

An antiserum was raised against the partially purified acidic brain enolase preparation, as described in the methods section, and its immunological properties are seen in Fig. 2. The antiserum completely blocks the activity of the brain fraction against which it was prepared whereas the liver enzyme (isoenzyme 1) is unaffected and the muscle enzyme (isoenzyme 3) is inhibited to a small but significant extent. Thus the anti-acidic brain enolase serum completely inhibits that portion of the brain activity which was unaffected by the anti-liver and anti-muscle enolase sera. This is consistent with our previous suggestion [1] that there exists a further type of subunit, designated γ, and that the third or most acidic fraction of brain enolase is entirely composed of dimers of this immunologically distinct subunit. The second or intermediate DEAE-Sephadex peak is shown by Table II to be partially susceptible to both anti-liver enolase serum and anti-acidic brain enolase serum and this indicates that this fraction contains α and γ subunits. The fact that the whole of the crude brain enolase activity (Table II) can be inhibited by a combination of the individual antisera raised against liver and acidic brain enolase means that this activity can be accounted for entirely in terms of α and γ subunits which would appear to be present in approximately equal amounts.

We have previously reported [1] no effect of anti-muscle enolase serum on total brain enolase activity, yet as shown in Fig. 2 there is some cross-reaction between the anti-acidic brain enolase serum and crude muscle enolase. The effect of anti-muscle enolase was re-investigated (Fig. 3) and it was found that massive amounts of the antiserum do in fact inhibit the acidic brain fraction, but 70% inhibition is achieved only with thirty times the amount of antiserum required when muscle enolase is titrated.

Fig. 4 shows developmental changes in the total enolase activity of brain from foetal life to maturity and also the corresponding values for susceptibility

TABLE II

THE EFFECT OF EXCESS ANTI-LIVER ENOLASE SERUM AND ANTI-ACIDIC BRAIN ENOLASE SERUM ON TOTAL BRAIN ENOLASE AND AN INTERMEDIATE CHROMATOGRAPHIC FRACTION

A: brain intermediate fraction (the 2nd peak of activity eluting in the DEAE-chromatography of brain enolase as shown in Fig. 1.) and B: total enolase activity of high-speed supernatant of rat whole brain homogenate. Immunochemical tests were carried out as described in Fig. 2, in each case sufficient antiserum was used to give maximal inhibition. The initial activities were 0.012 units and 0.014 units respectively for A and B.

	Percentage of enloase activity remaining	
	A Intermediate fraction of brain enolase	B Total brain enolase
Control serum	100	100
Anti-liver enolase serum	66	49
Anti-acidic brain enolase serum	40	42
Anti-liver enolase serum + anti-acidic brain enolase serum	12	4

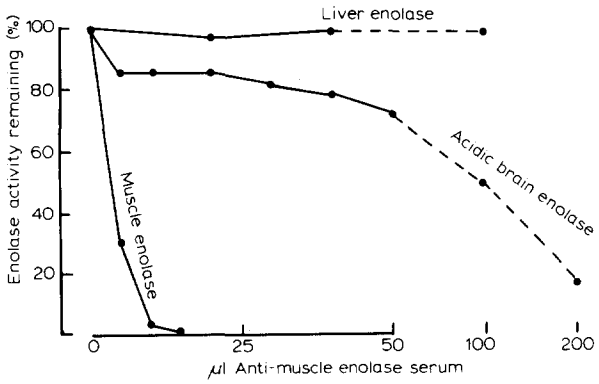

Fig. 3. Immunological titration of liver enolase, 'acidic brain enolase' and muscle enolase of rat with anti-muscle enolase serum. The experiments were carried out as indicated in Fig. 2. In each case the initial activity was 0.01 units of enolase.

to anti-liver enolase serum. The total activity increases from 15 units/g in the late foetal and early neonatal periods to reach the adult value 45 units/g at 80 days. The period of greatest change is between 10 and 30 days. In the foetus inhibition by anti-liver enolase serum is virtually complete indicating that only the α containing isoenzymes are present. The effect of the antiserum decreases steadily until at 50 days 30% of the activity is resistant.

The developmental changes in brain enolase were further investigated chromatographically as shown in Fig. 5. It is seen that changes do occur but only within the three-peak elution pattern already described in Fig. 1. The main feature of development is that the third peak, the brain specific isoenzyme, is absent in the foetus, just detectable at 10 days and increases progressively until

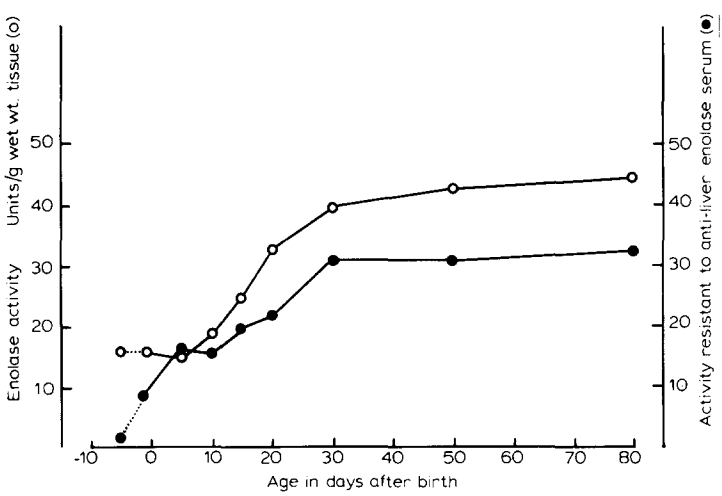

Fig. 4. Changes in the total activity and immunological properties of brain enolase during development. Unpurified high speed supernatants were used for the determination of total enolase (o); the percentage of the enolase resistant to anti-liver enolase serum is shown for each age (●). All points except that for five days before birth represent the means from at least four animals and in no case did the standard error of the mean exceed 6% of the value given. The early foetal values are for the pooled heads of a whole litter of six.

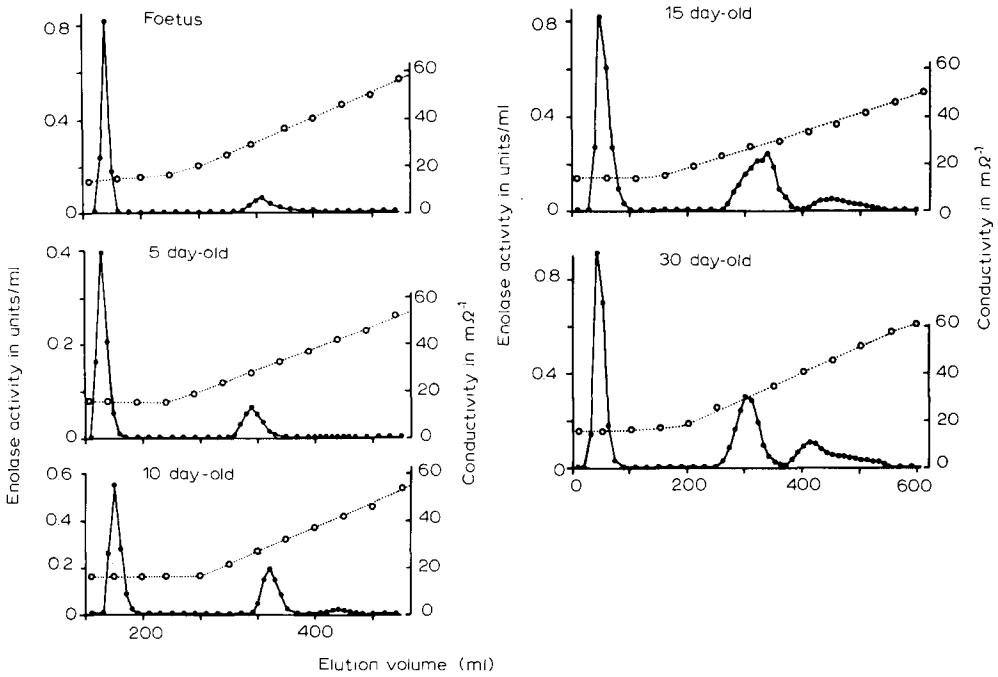

Fig. 5. DEAE-Sephadex chromatography of rat brain enolase during development. These separations were carried out as indicated in Fig. 1. Enolase activity •; conductivity of eluate ○.

adult levels are reached. All chromatographic peaks were tested with the antisera against liver and muscle enzymes and immunological properties were found to be similar to those of the corresponding peaks separated from adult brain. Testing with anti-muscle enolase serum failed to show the presence of β subunits at any stage of development. Calculation of the relative proportions of the three isoenzymes from the elution peaks gives the following ratios for $\alpha\alpha$: $\alpha\gamma$: $\gamma\gamma$; foetus, 79 : 21 : 0; 5 days, 69 : 28 : 3; 10 days, 59 : 34 : 7; 15 days, 52 : 38 : 10; 30 days, 45 : 34 : 22; adult, 37 : 34 : 30.

Discussion

We have previously shown that in order to explain the multiple forms of enolase found in rat brain it is necessary to postulate a third genetic locus (γ) in addition to the α and γ loci which appear to account adequately for the enolase isoenzymes of other tissues. In this paper the product of this putative gene has been isolated and an antiserum prepared against it. Titrations with this antibody combined with improved chromatographic separation of the brain enolases have shown that the brain specific isoenzymes are $\alpha\gamma$ and $\gamma\gamma$ dimers. We have found no evidence for the existence of any dimers containing β subunits in the brain.

Recently three groups, Kamel and Schwarzfischer [5], Pearce et al. [6], and Chen and Giblett [7] have reported electrophoretic studies on human enolase and all indicate heterogeneity of the brain enzyme. The most complete of these

studies [6] suggests, in agreement with our own conclusions for the rat [1,2], that enolase isoenzymes of human tissues may be accounted for by three independent genetic loci.

The developmental studies show that in foetal brain isoenzyme 1 predominates, to be partially replaced by increasing proportions of dimers containing γ subunits, a developmental change entirely analogous to that found with muscle and heart in which tissues β subunits replace α subunits as development proceeds [2].

Whereas no immunological cross-reaction between α and β subunits has been found, a definite cross-reaction (see Figs. 2 and 3) occurs between β and γ subunits. This suggests that the β and γ loci must be related more closely one to another than either is to the α gene. It is also interesting to note that the β and γ subunits seem never to be expressed together in the same tissue and that furthermore, in those tissues where they do occur, muscle and heart for the β subunit and brain for the γ subunit, the developmental patterns are similar.

Recently it has been suggested that the two proteins associated with brain specific antigen 14.3.2 [8] possess enolase activity and it seems that these enolases must be $\alpha\gamma$ and $\gamma\gamma$ forms described in this paper. The presence of the γ subunit in both of these dimers would explain their apparent antigenic identity.

In conclusion strong chromatographic electrophoretic and immunological evidence has now been presented for the existence of $\alpha\alpha$, $\alpha\beta$, $\beta\beta$, $\alpha\gamma$ and $\gamma\gamma$ enolase dimers in rat tissues. As yet the remaining $\beta\gamma$ combinant has not been found. It is noteworthy that the molecular heterogeneity of enolase is comparable with that of aldolase [9] in that each of these glycolytic enzymes is encoded on three independent structural genes, one of which is active only in the brain.

Acknowledgments

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