Cytosolic and mitochondrial isoenzymes of branched-chain amino acid aminotransferase during development of the rat

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The isoenzymic forms of branched-chain amino acid aminotransferase in mitochondria of rat tissues were compared with the better-known cytosolic forms in order to find any regular pattern of expression of these isoenzymes during development. Mitochondria of all tissues examined except brain contained only a type-I isoenzyme differing from the cytosolic type-I isoenzyme in heat stability and activation by mercaptoethanol. Foetal and adult brain mitochondria contained isoenzymes type III as well as type I. The large excess of type-I isoenzyme in foetal liver was localized in mitochondria, apparently of haematopoietic cells. The activity of this isoenzyme declined precipitously (by 80%) from day 19 of gestation at the same period and rate as does the volume fraction of haematopoietic cells that are then leaving the liver. Cortisol treatment accelerated the loss of these cells, and proportionally accelerated loss of the mitochondrial isoenzyme I. A developmental succession of type-I isoenzyme by the unique type II of liver parenchymal cell cytosols could not be demonstrated, since small, about equal, amounts of types I and II were always present in cytosols of foetal and adult liver. Developmental succession of isoenzymes within tissues was limited to cytosols and was demonstrated by the presence of cytosolic isoenzyme III in foetal and newborn skeletal muscle and kidney, organs which contain only isoenzyme I in the adult.

Isoenzymes have been used with some success in monitoring cell differentiation, when a succession of 'foetal' and 'adult' types could be identified, even though a general distinction between such isoenzyme classes is not yet clearly defined. The orderly expression of the cytosolic isoenzymes of branched-chain amino acid aminotransferase (EC 2.6.1.42, isoenzymes I and III; EC 2.6.1.6, leucine-specific isoenzyme II) appears to be irregular, since at about the time of birth type I is succeeded by type II in rat liver (Ichihara & Takahashi, 1968), but type III by type I in rat kidney (Roth *et al.*, 1977). Generally it is the foetal form of an isoenzyme that reappears in neoplasms (Knox, 1976). It is the cytosolic type III that appears in hepatomas (Ogawa *et al.*, 1970; Ogawa & Ichihara, 1972) and in virally transformed liver and kidney cells (Roth *et al.*, 1977).

A substantial proportion of the total activity of branched-chain amino acid aminotransferase of cells is present in mitochondria, especially in foetal liver (Cappuccino *et al.*, 1978), and the nature of the isoenzymes in mitochondria might clarify the developmental succession. From the few studies of these mitochondrial isoenzyme types it is clear that

they can be classified by the same DEAE-cellulose-chromatographic procedures as for the better-known cytosolic isoenzymes, and that the cytosolic and mitochondrial types are not always identical in the same cell (Aki *et al.*, 1967; Ogawa *et al.*, 1970). The types and amounts of isoenzymes of branched-chain amino acid aminotransferase were therefore determined in parallel in the cytosols and mitochondria of selected organs of the developing and adult rat.

Experimental

Materials

Animals. Inbred Kx (formerly New England Deaconess Hospital) rats were fed on Purina chow and water *ad libitum* and killed at 90–100 days of age by cervical dislocation. Foetal ages were estimated by the method of Knox & Lister-Rosenoer (1978). When indicated, foetal rats were injected with cortisol acetate, either 0.125 mg/foetus, 36 h before use, intraperitoneally through the uterine wall as described by Greengard *et al.* (1972), or 2.5 mg/100 g body wt. into pregnant rats. Lac-

tating-mammary-gland weights were corrected for milk content by measurement of lactose (Slater, 1957) on the basis of 30 mg of lactose/ml of milk.

Chemicals. [$1\text{-}^{14}\text{C}$]Leucine and [$1\text{-}^{14}\text{C}$]valine were obtained from New England Nuclear Corp., Boston, MA, U.S.A. L-Amino acids and pyridoxal 5'-phosphate were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (Cellex D) was purchased from Bio-Rad, Richmond, CA, U.S.A., and $(\text{NH}_4)_2\text{SO}_4$ (special Enzyme Grade) from Schwarz-Mann, Orangeburg, NY, U.S.A. β -Mercaptoethanol was obtained from Eastman Kodak, Rochester, NY, U.S.A. Hydroxide of Hyamine 10-X was obtained from Packard Instrument Co., Downers Grove, IL, U.S.A.

Methods

Enzyme assays. The activity of branched-chain amino acid aminotransferase was measured by using L-[$1\text{-}^{14}\text{C}$]leucine and L-[$1\text{-}^{14}\text{C}$]valine by $^{14}\text{CO}_2$ release from the product on oxidative decarboxylation with H_2O_2 by the method described by Cappuccino *et al.* (1978).

Cell fractionation. The tissues (except brain and skeletal muscle) were homogenized in 4 vol. (w/v) of 0.25 M-sucrose solution with a glass-Teflon homogenizer (0–4°C). Homogenates and intact mitochondria to be assayed were kept on ice with 0.2% Triton X-100 for 30 min before use. Mitochondria were prepared as follows. Nuclei and cell debris were removed from homogenates by centrifugation at 700g for 5 min and washed twice. The supernatant and washings from the nuclear fraction were re-centrifuged at 15000g for 30 min to pellet the mitochondria. The once-washed mitochondrial fraction was suspended in 1 mM-potassium phosphate (pH 7.8) containing 10 μM -pyridoxal phosphate. Mitochondrial-matrix fractions were prepared by sonication of the mitochondrial fractions (approx. 10 mg of mitochondrial protein/ml) for 2 min with a Fisher Ultrasonic Probe (model BP-2) and centrifugation at 100000g for 60 min. The mitochondrial enzyme was quantitatively solubilized by this treatment, except for that of placenta, which lost two-thirds of its mitochondrial activity at this step.

Subcellular fractionation of the brain was performed by the method of Dienel *et al.* (1977), monitored with the same marker enzymes. The crude mitochondria, and the myelin, synaptosome and purified mitochondrial particles after re-fractionation in a sucrose gradient, were left for 30 min at 0°C in the presence of 0.5% Triton X-100 before assay of marker enzymes (glucose 6-phosphate dehydrogenase for cytosol, glutamate dehydrogenase for mitochondria and glutamate decarboxylase for synaptosomes). Another part of the fractions from brain for assay of branched-chain amino acid

aminotransferase was sonicated and centrifuged as described above for mitochondria.

Mitochondria from skeletal muscle were prepared by the method of Ernster & Nordenbrand (1967).

All cytosol fractions were prepared from the postmitochondrial supernatants by centrifugation at 100000g for 60 min.

Isoenzyme separations with DEAE-cellulose. Cytosol fractions were dialysed for 3 h against 1 mM-potassium phosphate buffer, pH 7.8, containing 10 μM -pyridoxal phosphate. The very low activities in foetal liver cytosol, and in adult liver cytosol and mitochondrial matrix, were concentrated with $(\text{NH}_4)_2\text{SO}_4$ (75% saturation) and dialysed in the same way. Samples of the dialysed cytosols and the undialysed mitochondrial extracts containing less than 10 mg of protein in less than 3 ml were applied to small columns of DEAE-cellulose (0.8 cm $\times$ 1.7 cm) that had been washed and equilibrated with 1 mM-potassium phosphate, pH 7.8. Limitation of the sample volumes to be applied avoided tailing of isoenzymes in later fractions. Columns were eluted as described by Roth *et al.* (1977) with four successive concentrations of potassium phosphate buffer (pH 7.8) without added β -mercaptoethanol. β -Mercaptoethanol was avoided, since its use in the chromatography decreased the recovery of activity well below the 75% or more achieved in its absence. After the wash with 5.25 ml of 5 mM buffer, the fraction eluted with 8.75 ml of 65 mM buffer was taken as isoenzyme I, and, after a further wash with 7.0 ml of 100 mM buffer, the fraction eluted with 8.75 ml of 300 mM buffer combined isoenzyme II, when present, with isoenzyme III. Isoenzyme II reacts only with leucine, and it was quantified by the excess of activity with leucine over that with valine as substrate.

The properties of the isoenzymes separated from adult kidney, brain, skeletal muscle and mammary gland were studied after the eluates of each were concentrated with $(\text{NH}_4)_2\text{SO}_4$ and dialysed as described above. Protein contents were adjusted to 3 mg/ml before heat treatments. Protein was determined by the method of Lowry *et al.* (1951).

Results

The total branched-chain amino acid aminotransferase activity in adult rat tissues varied 100-fold, highest in lactating mammary gland and lowest in liver (Table 1). The mitochondrial portions of these totals ranged from 74% in kidney and placenta to 25% in brain, as previously reported (Cappuccino *et al.*, 1978). In agreement with previous work based on isoenzyme separation by DEAE-cellulose chromatography (Ichihara, 1975), the cytosolic fractions of lactating mammary gland,

kidney and skeletal muscle contained only isoenzyme I, and those of ovary, brain and placenta contained both isoenzymes I and III (with type III equal to or greater than type I). The cytosol of liver contained low amounts of isoenzymes I and II in nearly equal proportions.

Separations by DEAE-cellulose columns were also used to identify the isoenzymes of the mitochondrial-matrix fractions of the adult tissues (Table 1). Only isoenzyme I was detected in mitochondria of any of these tissues except brain. The mitochondrial fraction of brain contained both isoenzymes I and III, with type III predominating to the same degree as it did in brain cytosol. Since the crude mitochondrial fraction of brain as isolated is mixed with synaptosomes and myelin vesicles encapsulating some cytosol (Dienel *et al.*, 1977; Kapatos & Kaufman, 1981), it was possible that at least some of the isoenzyme III found in the crude

brain mitochondrial fraction originated from the cytosolic fraction. However, re-fractionation of the brain particles in a sucrose gradient (Dienel *et al.*, 1977) separated from the mitochondria most of the glucose 6-phosphate dehydrogenase and glutamate decarboxylase used as markers of cytosol and synaptosomes, respectively, and left with the mitochondrial fraction most of its glutamate dehydrogenase marker and the branched-chain amino acid aminotransferase. The purified mitochondria contained both isoenzymes I and III in the same proportion as in the crude mitochondria (Table 1). Isoenzyme III without type I was found in traces in the myelin and synaptosome particles.

Changes in the isoenzymes present during development were sought in the placenta (Table 1) and in foetal liver and other tissues (Table 2). Placentae from days 16 to 21 of gestation maintained constant activities of the cell fractions, with

Table 1. *Branched-chain amino acid aminotransferase activities and isoenzyme patterns in various tissues of the adult rat*

Activities are means $\pm$ s.d., and isoenzyme percentages are the means for the numbers of measurements given in parentheses. Zero percentages represent actual zero, or negligible elutions of less than 3%. Kidney, muscle, brain and liver were from 100-day-old male rats. Placentae were from 17–19 days of gestation. Mammary glands were from day 20 of lactation, and the activities are per g of milk-free weight. All isoenzyme recoveries were 75% or greater.

Tissue	Activity before DEAE-cellulose treatment ($\mu\text{mol}/\text{min}$ per g of tissue)	Proportions of isoenzymes (% after DEAE-cellulose)		
		I	II	III
Mammary gland				
Homogenate	7.76 ± 1.09 (3)			
Cytosol	4.49 ± 0.34 (3)	100	0	0 (3)
Mitochondrial matrix	2.87 ± 0.23 (3)	100	0	0 (3)
Kidney				
Homogenate	1.98 ± 0.35 (3)			
Cytosol	0.44 ± 0.10 (3)	100	0	0 (3)
Mitochondrial matrix	1.47 ± 0.40 (3)	100	0	0 (3)
Skeletal muscle				
Homogenate	1.41 ± 0.16 (3)			
Cytosol	0.62 ± 0.04 (3)	100	0	0 (3)
Mitochondrial matrix	0.76 ± 0.01 (3)	100	0	0 (3)
Ovary				
Homogenate	1.32 ± 0.05 (4)			
Cytosol	0.85 ± 0.03 (4)	51	0	49 (3)
Mitochondrial matrix	0.39 ± 0.10 (3)	100	0	0 (3)
Brain				
Homogenate	1.18 ± 0.19 (4)			
Cytosol	0.88 ± 0.07 (4)	10	0	90 (4)
Mitochondrial matrix				
Crude	0.27 ± 0.03 (4)	20	0	80 (4)
Purified	0.17 (1)	15	0	85 (1)
Placenta				
Homogenate	0.75 ± 0.11 (7)			
Cytosol	0.17 ± 0.07 (7)	11	0	89 (3)
Mitochondrial matrix	0.19 ± 0.02 (3)*	100	0	0 (3)
Liver				
Homogenate	0.07 ± 0.01 (4)			
Cytosol	0.02 ± 0.005 (4)	39	61	0 (2)
Mitochondrial matrix	0.04 ± 0.007 (4)	100	0	0 (3)

* After sonication the activity recovered from the matrix fraction of placenta was only one-third of that in mitochondria.

the predominant isoenzyme III occurring only in the cytosol. The total aminotransferase activity of foetal liver was considerably higher than that of adult liver, owing solely to the larger amount of isoenzyme I in mitochondria. Isoenzyme II was restricted to the cytosol in low amounts that were unchanged in 17–21-day-foetal livers and in adult livers (Tables 1 and 2, Fig. 1). Analysis of the enzyme in foetal livers of accurately known ages (Knox & Lister-Rosenoer, 1978) showed that a precipitous fall in the mitochondrial activity of isoenzyme I (homogenate minus cytosolic activities) occurred from day 19 of gestation (Fig. 1). The time and extent of this fall closely paralleled the decrease in the volume fraction of haematopoietic cells in foetal rat livers that was previously measured in this laboratory (Greengard *et al.*, 1972). Also in parallel with the volume fraction of hematopoietic cells, the activity of isoenzyme I in foetal liver mitochondria was decreased prematurely by treatment of the foetuses *in vivo* with cortisol.

The unchanging amounts of cytosolic isoenzyme II in foetal and adult livers, together with only small amounts of isoenzyme I that were ascribable to the

parenchymal cells, eliminated the possibility of any developmental replacement of isoenzyme I by type II in the liver cells examined. Additional tissues from 17–19-day foetuses and 1–5-day newborns were examined for evidence of other developmental successions. In addition to isoenzyme I, isoenzyme III was found in decreasing proportions in 19-day-foetal brain, the eviscerated foetal carcass, and foetal kidney, but none in foetal heart. No other sources of isoenzymes II or III were found. In newborn-rat tissues isoenzyme I predominated and was the only isoenzyme found in heart and lung, but there were substantial proportions of isoenzyme III limited to the cytosols in skeletal muscle and kidney. The proportions of isoenzyme III present in the last two tissues decreased smoothly from days 1 to 5 after birth (Table 2).

Certain properties (activation by mercapto-ethanol, heat stability and K_m for leucine, valine and α -oxoglutarate) of the ubiquitous mitochondrial type I isoenzyme and other isoenzymes I and III from selected tissues were compared after separation and concentration. There were no differences between the cytosolic and mitochondrial isoenzymes III of

Table 2. *Activities and isoenzymes of branched-chain amino acid aminotransferase in foetal and newborn rat tissues*
Results are presented as in Table 1. Those from foetal livers represent means of measurements on four pools of tissue at days 17–19 of gestation, and the others are each from single pools of tissue from rats 1 to 5 days of age.

Age and organ	Activity of fractions ($\mu\text{mol/min per g of tissue}$)	Proportions of isoenzymes (% of total eluted)		
		I	II	III
Foetal liver				
Homogenate	1.19			
Cytosol	0.02	46	54	0
Mitochondrial matrix	0.64	100	0	0
1-Day-newborn heart				
Cytosol	0.14	100	0	0
Mitochondrial matrix	0.42	97	0	0
1-Day-newborn lung				
Cytosol	0.09	100	0	0
Mitochondrial matrix	0.49	97	0	0
1-Day-newborn kidney				
Cytosol	0.07	82	0	18
Mitochondrial matrix	0.43	97	0	0
4-Day kidney				
Cytosol	0.06	91	0	9
Mitochondrial matrix	0.78	100	0	0
1-Day-newborn skeletal muscle				
Cytosol	0.11	48	0	52
Mitochondrial matrix	0.06	97	0	0
4-Day skeletal muscle				
Cytosol	0.20	69	0	31
Mitochondrial matrix	0.20	100	0	0
5-Day skeletal muscle				
Cytosol	0.23	83	0	17
Mitochondrial matrix	0.20	100	0	0

brain, and none between the same isoenzyme-I fractions of these adult tissues (Table 3). Addition of β -mercaptoethanol to the assay did not activate enzyme not yet treated with DEAE-cellulose columns, and its presence decreased recovery of activity from the columns by at least 60%. In the

Fig. 1. Perinatal changes of branched-chain amino acid aminotransferase activities (left ordinate) in comparison with simultaneous changes in the volume fractions of haematopoietic cells (right ordinate) in rat liver

The low, unchanging activity of the cytosols (●) consisted of approximately equal amounts of isoenzymes I and II. The activity of the homogenates (○) was mainly that of isoenzyme I in mitochondria (Tables 1 and 2). It decreased sharply before birth and decreased prematurely after cortisol treatment of foetuses (□) or of the dam (■). The values for 5-day-olds are means from earlier data (Cappuccino *et al.*, 1978). Triangles (▲) give the haematopoietic-cell volume fractions that were measured previously in this laboratory (Greengard *et al.*, 1972) over the same age period. The open triangle (arrow) is an example of the premature decrease of this cell volume fraction caused by the same cortisol treatment used here.

optimal concentration of 50mM in the assay it activated all the isoenzyme fractions that had been separated by the DEAE-cellulose column. The degree of activation was about 5-fold for isoenzyme I of mitochondria from three tissues, but only 2-fold or less for the cytosolic isoenzymes I and III of these tissues and brain (Table 3). Heating at 50°C for 5 min in the presence of 5 mM- α -oxoglutarate did not affect the activity of any isoenzymes, but in the absence of α -oxoglutarate the mitochondrial isoenzyme I was less stable to heat than the cytosolic isoenzymes I and III when all were heated at constant protein concentrations (Table 3). The same relative heat stabilities were observed in assays with or without activation by β -mercaptoethanol.

The K_m values for valine were regularly 3 times those for leucine but were not substantially different for the isoenzymes from different tissues. There were no substantial differences between the isoenzymes in their affinities for α -oxoglutarate in reactions with leucine (results not shown) or valine (Table 3). A single difference in the isoenzyme proteins could account for the properties observed, since the greater dependence on mercaptoethanol activation and greater heat lability of the mitochondrial isoenzyme I could both be caused by a more reactive thiol group than that of cytosolic isoenzyme 1.

Discussion

The developmental change in the isoenzymes of liver was believed to involve the replacement at birth of the isoenzyme I by isoenzyme II (Ichihara & Takahashi, 1968) in both cytosol and mitochondria of liver. However, we found small, almost unchanging, amounts of isoenzyme II present only in the cytosols of both foetal and adult liver. Iso-

Table 3. Properties of separated branched-chain amino acid aminotransferase isoenzymes
Isoenzymes were separated with DEAE-cellulose columns, concentrated with (NH₄)₂SO₄ and dialysed. All assays except the controls for mercaptoethanol activation contained 50mM- β -mercaptoethanol. Thermostability was tested at 50°C for 5 min in the absence of α -oxoglutarate with 3 mg of protein/ml. K_m values for valine and leucine were determined in the presence of 3 mM- α -oxoglutarate; K_m for α -oxoglutarate is that in 6 mM-valine.

Isoenzyme type	Cell fraction	Tissue source	Activity (% of control)		K_m (mM)		
			Mercaptoethanol activation	Heat at 50°C for 5 min	Valine	Leucine	α -Oxoglutarate
I	Cytosol	Kidney	241	74	1.23	0.47	1.24
		Skeletal muscle	132	84	1.27	0.44	1.21
		Mammary gland	128	79	1.04	0.30	1.21
I	Mitochondria	Kidney	456	36	1.17	0.40	1.48
		Skeletal muscle	617	40	1.30	0.40	1.50
		Mammary gland	517	30	1.15	0.62	2.02
III	Cytosol	Brain	129	73	1.74	0.62	0.99
III	Mitochondria	Brain	140	78	2.00	0.56	1.05

enzyme II is inducible by various hormones and dietary treatments (Shirai & Ichihara, 1971), which is one characteristic of some adult-type isoenzymes. It has not as yet been shown to replace an earlier form in the same cells. Isoenzyme II may first appear earlier than the 17–19-day gestational stage that we studied, possibly in succession to a type-III isoenzyme, since foetal liver is already a relatively well-differentiated tissue at this stage (Knox, 1976).

The relatively high activity of the mitochondrial isoenzyme I in foetal liver is apparently concentrated in non-parenchymal cells. It disappears at parturition in parallel with the decreasing volume of haematopoietic cells in the liver at this time and also in parallel with the accelerated loss of haematopoietic cells that follows cortisol treatment. The change from isoenzyme I to II during development of liver is therefore most probably due to the change in cell composition of liver and not to a developmental succession of isoenzyme types I and II within the liver parenchymal cells. Neither is therefore a candidate for the earliest, foetal, form.

Isoenzyme III is characteristic of some undifferentiated cells. Both isoenzymes I and III are present in the cytosol of kidney in the newborn, but only type I is found in adult kidney (Roth *et al.*, 1977), findings that we have confirmed. The changes from types I and II in liver to types I and III in hepatomas (Ogawa *et al.*, 1970; Ogawa & Ichihara, 1972), and from type I to III in virally transformed kidney cells (Roth *et al.*, 1977), represent an expected chemical de-differentiation of neoplastic cells to ones with a more embryonic enzyme pattern. The presence of isoenzyme III limited to the cytosols of placenta and ovary, which contain relatively undifferentiated cells, is consistent with type III being the isoenzyme of undifferentiated cells, even though it also persists uniquely in the highly differentiated brain.

A foetal-type isoenzyme should also be more widely distributed among undifferentiated tissues than among adult tissues. Examination of the isoenzymes in foetal and newborn tissues showed a more widespread distribution of isoenzyme III at this age than in adult rats. We found it in kidney cytosol in the newborn and an even larger proportion in the cytosols of foetal and newborn skeletal muscle, though both these tissues in the adult contain only isoenzyme I. In these tissues only isoenzyme I was present in the mitochondria. The expected decreases in the proportions of isoenzyme III during development of these tissues were observed already during the first 5 days after birth. Therefore isoenzyme III has characteristics of an enzyme of undifferentiated tissues in that it is distributed more widely among foetal than in adult tissues, and it reappears after neoplastic transformation of cells not normally containing it.

Examination of the isoenzymes of mitochondria did not help to describe any developmental changes, since a particular type-I isoenzyme was found in all the mitochondria examined and as the only form there except in brain. The presence of isoenzyme I along with type III in the mitochondria of brain was confirmed in mitochondria separated from myelin and synaptosome particles. Only isoenzyme I was in the mitochondrial fractions of placenta and ovaries and of newborn kidney and muscle, whose cytosols contained isoenzyme III. Yoshida hepatoma also contained only isoenzyme I in its mitochondria in association with its cytosolic types I and III (Ogawa *et al.*, 1970).

There now appear to be four more or less distinct proteins of branched-chain amino acid aminotransferase, with only three isoenzyme types distinguished by DEAE-cellulose columns. Besides the newly studied mitochondrial type I, these are the leucine-specific type II of liver cytosol (Aki *et al.*, 1968) and the cytosolic types I and III that are immunologically distinct (Aki *et al.*, 1969). Cytosolic type III of hepatoma was identical with that of brain (Ogawa *et al.*, 1970), and no difference was seen here between brain cytosolic and mitochondrial type-III isoenzymes. The mitochondrial type-I isoenzyme differed from the cytosolic type I in two properties suggesting that the thiol groups of the former are more reactive. Hog heart isoenzymes I of cytosol and mitochondria differed in much the same way (Aki *et al.*, 1967).

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