Branched-chain amino acid aminotransferase in mouse testicular tissue

E. E. Montamat, J. Moreno and A. Blanco

Cátedra de Química Biológica, Facultad de Ciencias Médicas, Universidad de Córdoba, 5000 Córdoba, Argentina

Summary. Branched-chain amino acid aminotransferase (L-leucine:2-oxoglutarate aminotransferase, EC 2.6.1.6) activity was determined in several tissues of the mouse. Testis homogenates presented a specific activity very close to that of heart extracts which were the most active. Enzyme activity was detectable in testes from 5-day-old mice and increased steadily during development to reach a maximum at the 20th day of life. The transaminase was present in the cytosol of testicular homogenates and also associated, probably in the matrix, with a special type of mitochondria present in spermatozoa and gametogenic cells. The enzyme from testis is active against the three branched-chain amino acids and catalyses the reaction in both directions. Highest activity and lowest \( K_m \) were obtained with L-leucine. Activity with L-valine was the lowest. The enzyme from the mitochondrial fraction showed identical properties to that from the soluble phase. The possible participation of this aminotransferase in a shuttle system transferring reducing equivalents from cytoplasm to mitochondria is postulated.

Introduction

A previous report from this laboratory has presented evidence showing the wide substrate specificity of the lactate dehydrogenase isoenzyme peculiar to mature testicular tissue and spermatozoa (LDH isoenzyme X) (Blanco, Burgos, Gerez de Burgos & Montamat, 1976). This isoenzyme catalyses the interconversion 2-oxoacid = 2-hydroxyacid, linked to NAD and the natural metabolites produced by transamination of amino acids are among the wide variety of substrates utilized. For example, the LDH isoenzyme X (LDH-X) from mouse testis exhibits relatively high activity against 2-oxo-4-methylpentanoate, 2-oxo-3-methylpentanoate and 2-oxo-3-methylbutanoate, which are derived from the branched-chain amino acids leucine, isoleucine and valine respectively. LDH-X has been shown to have a rather peculiar subcellular distribution: it is localized in the soluble phase of testicular homogenates and in the mitochondria of spermatozoa and gametogenic cells (Machado de Domenech, Domenech, Aoki & Blanco, 1972; Blanco et al., 1976).

On the basis of these observations, Blanco et al. (1976) proposed that a possible functional role for LDH-X was to integrate a shuttle system which transferred hydrogen from the cytoplasm to the mitochondria. If such a commuter system does exist in gametogenic and sperm cells, then another enzyme, the transaminase of branched-chain amino acids, would also be necessary, and an aminotransferase should be present in cells of the sperm line and should be intracellularly distributed in the same sites as LDH-X.

This paper presents a study of the activity, subcellular distribution and kinetic properties of branched-chain amino acid aminotransferase (L-leucine: 2-oxoglutarate aminotransferase, EC 2.6.1.6) in mouse testis during development.

Materials and Methods

Adult male albino Swiss mice were killed by decapitation. Testes, kidney, liver, heart, brain and skeletal muscle were removed and rinsed briefly with ice-cold 9·0 \% \,(w/v) \,NaCl. Total homogenates of these tissues were prepared by suspending one part of tissue in 4 parts (1:5 w/v) of distilled water and grinding with an all-glass Potter-Elvejhem homogenizer.
**Cellular fractionation**

*Testis.* About 4 g decapsulated testes were homogenized in 12 ml (1:4 w/v) 0:32 m-sucrose solution, by using a Potter–Elvejhem grinder provided with a Teflon pestle. Fractionation was performed with the method described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) for liver. This technique produces two mitochondrial fractions, designated 'heavy' and 'light'. Pellets of these particular fractions were washed twice with 0:32 m-sucrose.

*Kidney.* Tissue was homogenized in 9 parts (1:10 w/v) of 0:25 m-sucrose in 0:0025 m-tris–HCl, pH 7-4. Fractions were obtained by the procedure of Ernster, Siekevitz & Palade (1962).

*Rabbit spermatozoa.* Spermatozoa were obtained with an artificial vagina. Isolation and treatment of spermatozoa were performed as indicated by Keyhani & Storey (1973). After gentle homogenization and centrifugation at 25 000 g, the supernatant was saved for enzyme analyses.

**Mitochondrial subfractionation.** The 'heavy' mitochondrial fraction from testis homogenates was submitted to centrifugation in a discontinuous density gradient after swelling and shrinking, as indicated by Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967). This technique provides three fractions: (a) 'heavy', comprising intact inner membrane and matrix; (b) 'light', rich in outer membranes, and (c) 'soluble', containing material from the intermembrane space and matrix from disrupted mitochondria. After separation, the 'heavy' and 'light' subfractions were washed with 0:32 m-sucrose. Final pellets of these subfractions were homogenized in 0.1 ml 0.15 m-NaCl with 1% Triton X-100 per g of original tissue. The suspensions were centrifuged at 18 000g for 30 min and the supernatants were used for enzyme assays. To the soluble phase Triton X-100 was added up to a 1% final concentration.

**Enzyme assays**

*Branched-chain amino acid aminotransferase.* Activity was determined with the method of Wada & Snell (1962) by using the technique proposed by Ichihara & Koyama (1966) with the following minor modifications. For the forward reaction, the reagent mixture contained, in a 3 ml final volume, 0-4 µmol pyridoxal-5'-phosphate, 36 µmol 2-mercaptoethanol, 105 µmol sodium pyrophosphate buffer, pH 8-6, the substrates L-leucine, L-isoleucine or L-valine and 2-oxoglutarate, at the concentrations indicated in the 'Results'. The enzyme preparation was added after appropriate dilution. For the reverse reaction, the assay mixture contained the same reagents as for the forward reaction, except for the amino acids and 2-oxoglutarate, which were replaced by L-glutamate and 2-oxoisocaproate.

The mixture was incubated at 37°C for 10 min. Enzymic activity was expressed in units which corresponded to µmol 2-oxoacid (2-oxo-4-methylpentanoate, 2-oxo-3-methylpentanoate and 2-oxo-3-methylbutanoate for the forward reaction, 2-oxoglutarate for the reverse) formed during 10 min.

*Lactate dehydrogenase isoenzyme X* was determined with the method utilized by Machado de Domenech et al. (1972) except that 0.1 mm 2-oxo-4-methylpentanoate was used as substrate instead of pyruvate. One enzyme unit (U) is the amount producing the conversion of 1 µmol NADH per min.

**Marker enzymes.** Malate dehydrogenase, monoamine oxidase and succinate dehydrogenase were determined in the mitochondrial subfractions as indicated by Montamat & Blanco (1976).

**Total protein**

This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

**Electron microscopy**

The nature of the pellets from testicular preparations was checked by electron microscopy as indicated by Machado de Domenech et al. (1972). Fractions of mouse testis gave the same images as those described previously for rat testes. The 'heavy' mitochondrial fraction exhibited a homogeneous population of mitochondria with a dense matrix and dilated cristae, identical to those present in the middle piece of spermatozoa (Machado de Domenech et al., 1972).
Aminotransferase activity in mouse testicular tissue

Chemicals

Pyridoxal-5'-phosphate, 2-oxo-4-methylpentanoate (sodium salt), 2-oxo-3-methylpentanoate, 2-oxo-3-methylbutanoate, 2-mercaptoethanol and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). 2-oxoglutarate (free acid) and oxaloacetate (free acid) were obtained from Koch-Light Laboratories (Colnbrook, Bucks, England). L-Leucine, L-isoleucine, L-valine, L-glutamic acid and tetrasmium pyrophosphate were purchased from Carlo Erba (Milano, Italy). Acid substrates were neutralized with NaOH solution before utilization. Other reagents used were of the highest purity commercially available.

Results

Branched-chain amino acid aminotransferase activity in mouse tissues

Activity in total homogenates was determined by using final concentrations of 40 mM-L-leucine and 12 mM-2-oxoglutarate. We did not examine the existence of multiple forms of this enzyme in mouse tissues. It is possible, as Ichihara, Yamasaki, Masui & Sato (1975) have demonstrated in the rat, that testis, kidney and heart possess only one molecular form (isoenzyme 1).

The tissues showing highest activity were kidney (17-9 U/g wet tissue or 347 mU/mg protein), heart (15-8 U or 448 mU) and testis (11-2 U or 426 mU). The lowest activity was recorded for liver homogenates (0-53 U/g wet tissue or 7-4 mU/mg protein). Brain (8-8 U or 403 mU) and skeletal muscle (1-2 U or 73 mU) presented intermediate values.

Enzyme activity was already present in testes from 5-day-old mice. Specific activity increased progressively during development, to reach a maximum at the 20th day of life. This level attained was maintained without further significant variations up to 9 months of age (Table 1).

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>60</th>
<th>90</th>
<th>270</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity (U/mg protein)</td>
<td>0-19</td>
<td>0-24</td>
<td>0-33</td>
<td>0-43</td>
<td>0-38</td>
<td>0-40</td>
<td>0-37</td>
<td>0-40</td>
</tr>
<tr>
<td>±0-03</td>
<td>±0-13</td>
<td>±0-05</td>
<td>±0-11</td>
<td>±0-07</td>
<td>±0-08</td>
<td>±0-10</td>
<td>±0-14</td>
<td></td>
</tr>
</tbody>
</table>

Values at 5, 10, 15, 20 and 25 days of age represent means from three pools of 10 testes each. Values at 60, 90 and 270 days are means for individual testes from 5 animals.

Subcellular distribution of enzymic activity in testis

Transaminase activity was assayed in the fractions of adult testes with 40 mM-L-leucine and 12 mM-2-oxoglutarate.

About 80% of the total branched-chain amino acid aminotransferase activity was recovered in the soluble phase and in the 'heavy' mitochondrial fraction which contained the organelles typical of spermatozoa (Table 2). The 'light' fraction, in which mitochondria of the common type were predominant, contained 12% of the total activity. The microsomal fraction showed very poor activity.

Determination of the activity of LDH-X in the same preparations (Table 2) confirmed previous results (Blanco et al., 1976). Although the proportions of LDH-X present in 'heavy' mitochondria and cytosol were reversed in relation to those of the transaminase, these two fractions were the main sites of localization for both enzymes.

The activity of marker enzymes was determined in the fractions, and the results indicated that the soluble fraction was not contaminated with mitochondrial material, for example, succinate dehydrogenase activity was negligible in the soluble phase. Moreover, thorough washing of mitochondrial pellets reduced the possibility of contamination of this particulate fraction with cytoplasmic 'soluble' enzymes.


Table 2. Distribution of branched-chain amino acid aminotransferase and lactate dehydrogenase isoenzyme X (LDH-X) activity (Units, see text) in subcellular fractions of mouse testis

<table>
<thead>
<tr>
<th></th>
<th>Aminotransferase total activity</th>
<th>LDH-X total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>First supernatant</td>
<td>34-74 ± 3-2 (100)</td>
<td>93-3 ± 11 (100)</td>
</tr>
<tr>
<td>'Heavy' mitochondria</td>
<td>21-60 ± 1-8 (60)</td>
<td>18-4 ± 1-8 (19-7)</td>
</tr>
<tr>
<td>'Light' mitochondria</td>
<td>4-40 ± 0-7 (12)</td>
<td>0-5 ± 0-18 (0-53)</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0-5 ± 0-2 (1-43)</td>
<td>1-3 ± 0-3 (1-39)</td>
</tr>
<tr>
<td>Soluble phase</td>
<td>6-5 ± 0-4 (19)</td>
<td>60 ± 7-7 (64)</td>
</tr>
</tbody>
</table>

Values are mean ± s.d., with % in parentheses, from 4 fractionations. Total activity of the first supernatant after separation of nuclei and debris is taken as 100%.

In rabbit spermatozoa, about 18% of the total aminotransferase activity was contained in the 'soluble' phase. Succinate dehydrogenase determination indicated that there was no contamination with mitochondrial material.

Distribution of branched-chain amino acid aminotransferase within the testicular 'heavy' mitochondrial fraction

Transaminase activity was determined in the subfractions obtained from the 'heavy' mitochondrial fractions with 40 mM-L-leucine and 12 mM-2-oxoglutarate as substrates. The results are shown in Table 3. Most (94%) of the total activity was recovered in the 'heavy' subfraction and in the soluble phase.

Table 3. Distribution of branched-chain amino acid aminotransferase and lactate dehydrogenase isoenzyme X activity (Units, see text) in 'heavy' mitochondria from mouse testis

<table>
<thead>
<tr>
<th></th>
<th>Aminotransferase</th>
<th>LDH-X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mitochondria</td>
<td>21-6 ± 1-3 (100)</td>
<td>17-2 ± 4-6 (100)</td>
</tr>
<tr>
<td>'Heavy' subfraction</td>
<td>15-3 ± 0-89 (70)</td>
<td>2-0 ± 0-25 (12)</td>
</tr>
<tr>
<td>'Light' subfraction</td>
<td>1-2 ± 0-2 (5-5)</td>
<td>—</td>
</tr>
<tr>
<td>Soluble phase</td>
<td>5-3 ± 1-5 (24)</td>
<td>13-0 ± 2-09 (75)</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. (with % in parentheses) for six preparations.

Determinations of LDH-X activity confirmed previous findings (Montamat & Blanco, 1976) and showed that it was distributed in the same two fractions as the transaminase, although the relative proportions were reversed. As postulated elsewhere (Montamat & Blanco, 1976), the higher proportion of LDH-X in the soluble phase is possibly explained by the diffusibility of the isoenzyme.

These results can be interpreted as an indication that the aminotransferase and LDH isoenzyme X are both located in the mitochondrial matrix.

The results (not shown) of determinations of marker enzymes in the subfractions were identical to those obtained previously (Montamat & Blanco, 1976): the 'heavy' and 'soluble' subfractions contained matrix material.
Catalytic properties of branched-chain amino acid aminotransferase from mouse testis

Studies were carried out on 'heavy' mitochondrial extracts and on the soluble or cytosolic phase from testis homogenates. Only results obtained with the mitochondrial extracts are presented: those for the soluble phase enzyme were practically identical.

**Forward reaction.** The initial reaction velocity with L-leucine, L-isoleucine and L-valine was determined with final amino acid concentrations of 0-4, 0-6, 0-8, 1-6 and 4-0 mM; the concentration of the other substrate, 2-oxoglutarate, was 12 mM. The highest activity was recorded with L-leucine, the lowest with L-valine. The curve for 2-oxoglutarate was obtained by using 0-45, 0-581, 0-83, 0-96, 1-6 and 5-0 mM concentrations of the 2-oxoacid; L-leucine was maintained at 40 mM. In all cases, plots of initial velocity against substrate concentration gave hyperbolic curves.

**Reverse reaction.** Enzymic activity against L-glutamate was determined by using 0-4, 0-8, 1-2, 1-6 and 4-0 mM final concentrations of the amino acid, while maintaining the concentration of 2-oxo-4-methylpentanoate at 12 mM. The curve for 2-oxo-4-methylpentanoate was obtained with 0-4, 0-8, 1-2, 1-6 and 4-0 mM concentrations of the 2-oxoacid and with 40 mM L-glutamate. For both substrates, plots gave Michaelian curves.

These results showed that the aminotransferase works effectively in both directions, and were used to estimate $K_m$ and $V$ values (Table 4) by means of double reciprocal plots. The $K_m$ for L-leucine was the lowest; those for L-isoleucine and L-valine were more than four times higher.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V$ (mU/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0-83</td>
<td>119</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>2-70</td>
<td>71-5</td>
</tr>
<tr>
<td>L-Valine</td>
<td>2-63</td>
<td>31</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>1-58</td>
<td>349</td>
</tr>
<tr>
<td>Reverse reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxo-4-methylpentanoate</td>
<td>2-4</td>
<td>245</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>3</td>
<td>80-2</td>
</tr>
</tbody>
</table>

* As the values were determined with crude preparations, $V$ indicates maximal specific activity.

The enzyme from kidney mitochondria gave an apparent $K_m$ for L-leucine of 0-6 mM, while the value for the enzyme of the soluble phase of kidney was 0-49 mM. The substrate specificity of enzymes from kidney was very similar to that shown by the aminotransferase from testis.

**Discussion**

The relative activities of branched-chain amino acid aminotransferase in mouse heart, liver, kidney and brain crude homogenates presented here are very similar to those reported by Ichihara & Koyama (1966) for the rat: heart, brain and kidney showed a relatively high activity, while that of liver was very poor. We also found a high activity, however expressed, in testis homogenates as first reported by Awapara & Seale (1952) for rat testicular tissue. The age at which the enzyme activity increased markedly in the mouse testicular tissue (10–20 days) is the period when spermatogenesis is initiated in the mouse (Goldberg & Hawtrey, 1967). Unlike LDH-X, however, which is specific to sperm cells, and appears in mouse testis about the 14th day of life, when primary spermatocytes first appear (Goldberg & Hawtrey, 1967; Battellino & Blanco, 1970), branched-chain amino acid aminotransferase does not show the cellular specificity of LDH-X.
While LDH-X is contained exclusively in spermatozoa and cells of the gametogenic stem line starting with spermatocytes (Zinkham, Blanco & Clowry, 1964), the branched-chain amino acid aminotransferase is widely distributed in many different tissues and cells (Ogawa, Yokojima & Ichihara, 1970). The LDH-X found in the cytosol of total testis homogenates can only be derived from spermatozoa and spermatogenic cells, but it is possible that the 'soluble' transaminase is contributed by other cellular elements present in testis.

However, it appears logical to assume that at least part of the branched-chain amino acid aminotransferase found in the soluble phase of testicular homogenate arises from the cytosol of spermatogenic and sperm cells. In all tissues where the distribution of the enzyme has been studied, the aminotransferase has been found in mitochondria and in the soluble phase (Ichihara & Koyama, 1966; Aki, Ogawa, Shirai & Ichihara, 1967; Aki, Ogawa & Ichihara, 1968). Cells of the sperm line would be a very rare exception if the transaminase were present only in mitochondria and not in the cytoplasm. However, in mature testes with active spermatogenesis, such as the ones studied here, there is a marked predominance of spermatogenic and sperm cells over other types of elements and the relative contribution of these elements to the total homogenate must be low. Further indirect evidence of the presence of the aminotransferase in the soluble phase of spermatozoa was obtained from the study of rabbit spermatozoa which could be obtained in adequate numbers.

The subcellular distribution of the branched-chain amino acid aminotransferase in testis appeared to be very similar to that of the isoenzyme X of lactate dehydrogenase: both enzymes are located in the cytosol and in a peculiar type of mitochondrion found in spermatozoa and spermatogenic cells. Within these mitochondria, the enzyme appeared to be located in the matrix. The common type of mitochondria which, in our preparation, were mainly contained in the 'light' fraction, showed lower transaminase activity than the sperm-specific organelles. Storey & Kayne (1977) have obtained evidence that rabbit spermatozoa contain an intramitochondrial LDH, probably LDH-X, which represents 2–6% of the total LDH activity of the sperm cell.

The branched-chain amino acid aminotransferase from mouse testis exhibited catalytic properties similar to those of the enzyme from kidney and resembled very closely the isoenzyme I described by Ichihara et al. (1975) in most tissues of the rat. There were no significant differences in substrate specificity and Michaelis constants between the enzymes from mitochondrial and soluble fractions from mouse testicular homogenates.

Although indirect, the findings presented here lend support to the hypothesis advanced in a previous report (Blanco et al., 1976) of the existence of a shuttle system for the transfer of reducing equivalents from cytosol to mitochondria. The requirements for the existence of an additional commutator system are met in sperm cells of the mouse and the system might be used for oxidation of cytoplasmic NADH formed during aerobic glycolysis or fructolysis, or oxidation of exogenous lactate. Further studies are necessary to establish the mechanism of this proposed system in spermatozoa.

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References


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