Subcellular localization of branched-chain amino acid aminotransferase and lactate dehydrogenase C₄ in rat and mouse spermatozoa

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Spermatozoa isolated from rat and mouse epididymes show a relatively high branched-chain amino acid aminotransferase (leucine aminotransferase, EC 2.6.1.6) activity. There is a significant reduction of leucine aminotransferase and of the isoenzyme C₄ of lactate dehydrogenase (EC 1.1.1.27) in the gametes during their epididymal transit. Studies of patterns of liberation of the leucine aminotransferase and of the lactate dehydrogenase C₄ from intact spermatozoa, treated with increasing concentrations of digitonin, indicate that both enzymes have the same dual subcellular location, i.e. in the cytosol and in the mitochondria.

INTRODUCTION

The isoenzyme C₄ of lactate dehydrogenase (LDH C₄; EC 1.1.1.27), originally designated LDH X, is a molecular form found exclusively in mature testis and spermatozoa of many mammalian and avian species [1–4].

The sperm-specific isoenzyme differs from the five molecular forms of lactate dehydrogenase common to somatic tissues (LDH 1 or B₄ to LDH 5 or A₄) in subcellular location and catalytic properties.

Studies in this laboratory [5–8], confirmed by other investigators [9–11], have shown that LDH C₄ has a peculiar subcellular distribution. At variance with the common isoenzymes, which are located only in the cytosol, it has a dual localization in the cytosol and in a special type of mitochondria, which appears in primary spermatocytes and is present in the subsequent elements of the spermatogenic line. In the middle piece of spermatozoa these organelles constitute the mitochondrial sheath surrounding the proximal segment of the flagellum [6]. Our observations showed that the enzyme is located in the mitochondrial matrix [8].

One of the catalytic properties distinguishing LDH C₄ from the common isoenzymes is its broader substrate specificity [12]. LDH C₄ from mouse and rat, for example, is able to catalyse the NAD⁺-linked interconversion 2-oxoacid ⇌ 2-hydroxyacid with a variety of substrates, including 5- and 6-carbon branched-chain compounds, like 4-methyl-2-oxopentanoate (α-ketoisocaproate), 3-methyl-2-oxopentanoate (α-keto-β-methylvalerate) and 3-methyl-2-oxobutanoate (α-ketoisovalerate) [7,12]. These 2-oxoacids are the products of transamination reactions with the amino acids leucine, isoleucine and valine, respectively. The enzyme responsible for those reactions, the branched-chain amino acid aminotransferase leucine aminotransferase (LAT; 2.6.1.6), is present at relatively high activity in mouse testes [13].

Since LDH C₄ and LAT share substrates and/or products, they could be integrated in the same metabolic pathways.

Studies carried out on mouse testes indicated that

LAT is located, like LDH C₄, in the cytosol and in the matrix of the mitochondria found in spermatozoa and spermatogenic cells [13].

It was proposed that, in mouse spermatozoa, there is a shuttle system for the transfer of reducing equivalents from cytosol to mitochondria, on the basis of knowledge of subcellular location and substrate specificity of LDH C₄. LDH C₄ and LAT would be the enzymes involved in this shuttle, which utilizes branched-chain 2-hydroxyacids/2-oxoacids as the redox couple [7]. The effective operation of the shuttle has been demonstrated *in vitro* in reconstituted systems [14–16].

Previous studies on subcellular location of LDH C₄ and LAT have been performed by the fractionation of total testis homogenates. We did not use spermatozoa as starting material, because it is practically impossible to obtain a satisfactory mitochondrial preparation from the gametes. Since LAT does not possess the cellular specificity of LDH C₄ and can be found in many different cellular species, some authors have expressed their doubts about results obtained with total testis preparations [17]. Instead we studied activity of LAT and LDH C₄ directly in mouse and rat spermatozoa and investigated the cellular location of both enzymes in intact gametes by using an indirect method. Results confirm our previous observations on testicular preparations.

EXPERIMENTAL

Animals

Adult Wistar rats and albino Swiss mice were used. Animals were killed by decapitation, and testes and epididymes were dissected immediately. Epididymes were sectioned into three segments. The proximal portion or 'caput' and the distal or 'cauda' were separated to collect spermatozoa. The medial section or 'corpus' was discarded.

Testis homogenates

After dissecting the capsule, testicular tissue was suspended in distilled water (1:5, w/v) by using an all-

Abbreviations used: LAT, leucine aminotransferase; LDH C₄, lactate dehydrogenase isoenzyme C₄.

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glass Potter–Elvehjem homogenizer. The suspensions were centrifuged at 20000 *g* and 4 °C for 3 min; supernatants were used for enzyme determinations.

Spermatozoa

The 'caput' and 'cauda' of epididymis were sectioned into small pieces and gently stirred in a buffer solution, pH 7.4, composed of 113 mM-KCl/12 mM-KH₂PO₄/3 mM-MgCl₂/0.4 mM-EDTA/20 mM-Tris [18]. The suspension was filtered through a fine-mesh silk cloth and centrifuged at 800 *g* for 10 min at room temperature. The sperm pellet was washed with the buffer solution and finally resuspended in 10 vol. of the same buffer. An aliquot of the suspension, diluted with 0.15 M-NaCl containing 0.2% formaldehyde, was used for spermatozoa count in a haemocytometer. Another aliquot was used to study enzyme liberation by digitonin treatment. The rest of the suspension was sonicated in a Branson Sonic Power Co. instrument, model B-12, by applying 4 × 15 s 70 W bursts, with a 10 s interval between pulses. Microscopic inspection showed that no intact spermatozoa were left in the preparation after this treatment. The suspension was centrifuged for 30 min at 20000 *g* and 4 °C. The supernatant was used for enzyme determinations.

Digitonin treatment

Aliquots of intact 'cauda' or 'caput' spermatozoa suspensions were incubated with the assay mixture used for each of the enzymes, with digitonin added up to the following final concentrations: 0.05, 0.1, 0.2, 0.3, 0.5, 0.8, 1.0, 1.5, 1.75 and 2.0 mg/mg of total protein in the preparation. Treatment was performed according to the technique described by Visser & Oppendoes [19]. No effect of digitonin upon the activity of analysed enzymes was observed at the concentrations used.

Enzyme assays

LAT activity was determined by the procedure of Montamat *et al.* [13]; LDH C₄ activity by the method of Coronel *et al.* [12]; malate dehydrogenase (EC 1.1.1.37) activity by the method of Ochoa [20]; glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity by the technique of Funayama *et al.* [21] and citrate synthase activity (EC 4.1.3.7) by the procedure of Srere [22]. All activities are expressed in international units (U).

Total protein determination

The method of Lowry *et al.* [23] was used.

RESULTS

Activity of LAT and LDH C₄ in testes and spermatozoa

Table 1 shows results of LAT LDH C₄ activity determinations in total testis and spermatozoa extracts. Activity for testis is expressed in units per milligram of protein (specific activity) and in units per gram of wet tissue, and for spermatozoa in specific activity and in units per 10⁹ cells. There is lower activity of both enzymes in 'cauda' spermatozoa compared with 'caput' spermatozoa. The difference is much more conspicuous for LAT than for LDH C₄.

Digitonin treatment

Fig. 1 shows curves obtained by plotting the percentage of enzyme liberated against the concentration of digitonin

Fig. 1. Liberation of enzymes from rat spermatozoa treated with digitonin

Enzyme activity in supernatants of 'whole' spermatozoa suspensions after exposure to increasing concentrations of digitonin. Activity is expressed as a percentage, taking as 100% the maximum activity liberated. (●), LDH C₄; (○), LAT; (△), malate dehydrogenase; (▲), glucose-6-phosphate dehydrogenase; (■), citrate synthase. Results are from one representative experiment out of eight performed under identical conditions.

that spermatozoa were exposed to. Activity determined in aliquots of the same spermatozoal suspensions submitted to sonication was taken as 100%, and, in all cases, was about the same as that liberated by 2 mg of digitonin/mg of protein.

Citrate synthase, located exclusively in the mitochondrial matrix, glucose-6-phosphate dehydrogenase, a cytosolic enzyme, and malate dehydrogenase, located in cytosol and in the mitochondrial matrix, were used as marker enzymes. Activity of citrate synthase in the medium was poor (< 20%) following incubation with up to 1 mg of digitonin/mg of protein. Leakage increased at 1.5 mg and reached 100% at 2 mg of digitonin. All the glucose-6-phosphate dehydrogenase diffused to the thesizing activity [25], most of the enzymes they contain gave a bimodal curve; about 75% of the total activity in spermatozoa was liberated at 0.2 mg of digitonin/mg protein, then there was a plateau up to 1 mg, at which concentration a steady increase of leakage started, with 100% activity reached at 2 mg.

LAT and LDH C₄ gave profiles very similar to that of malate dehydrogenase, with marked liberation at low digitonin concentrations, reaching 65–75% of the total activity with 0.2 mg and then plateauing up to 0.8 mg. From 1 mg on, enzyme liberation increases again to attain 100% at 2 mg of digitonin. Similar results were obtained with 'caput' or 'cauda' spermatozoa.

Table 1. Leucine aminotransferase (LAT) and lactate dehydrogenase isoenzyme C₄ (LDH C₄) activity in testis and spermatozoa

Values represent averages of duplicate determinations in five different samples. Numbers in parentheses are S.D. U/gwt: units per gram of wet tissue.

	Activity in spermatozoa					
	Activity in testis		'Caput'		'Cauda'	
	(U/mg of protein)	(U/gwt)	(U/mg of protein)	(U/10 ⁹ cells)	(U/mg of protein)	(U/10 ⁹ cells)
Rat						
LAT	0.005 (0.001)	0.19 (0.05)	0.03 (0.005)	1.57 (0.4)	0.007 (0.0007)	0.13 (0.05)
LDH C ₄	0.080 (0.009)	3.80 (0.12)	0.10 (0.01)	3.93 (0.50)	0.20 (0.04)	1.70 (0.40)
Mouse						
LAT	0.011 (0.0007)	0.52 (0.06)	0.016 (0.004)	1.10 (0.30)	0.006 (0.0008)	0.14 (0.07)
LDH C ₄	0.55 (0.10)	28.0 (2.5)	1.0 (0.75)	77.7 (9.0)	2.52 (0.42)	42.7 (11.4)

DISCUSSION

It is known that LDH C₄ appears in primary spermatocytes at the mid-pachytene stage [24] and is present in all the following cellular components of the gametogenic line up to spermatozoa. LAT, an enzyme related to LDH C₄ in that they have substrates and/or products in common, has been found in ejaculated rabbit spermatozoa [13]. Results presented here reveal its presence in mouse and rat epididymal spermatozoa.

Grootegoed *et al.* [17] studied cells of the seminiferous tubules of rat testes and reported that spermatocytes and spermatids do not possess LAT activity. They also suggested that branched-chain amino acid transamination would take place in Sertoli cells. The 2-oxoacids produced could then be offered to spermatogenic cells containing LDH C₄, which could use them as substrates.

It is difficult to reconcile this assumption with the demonstration of LAT activity in ejaculated and epididymal spermatozoa. Although we have not determined LAT activity directly in spermatocytes or spermatids, there are reasons to believe that the enzyme must be present in those cells.

The specific activity of LAT in mouse and rat 'caput' spermatozoa is higher than that in total testis, which is a tissue relatively rich in this enzyme [13]. This means that sperm cells are endowed with an important level of the branched-chain amino acid transaminating enzyme. Since spermatozoa possess very poor protein-synthesizing activity [25], most of the enzymes they contain must be received 'ready-made' from the precursor cells. On the other hand, studies of LAT ontogeny in mouse testes [13] showed an important increase of activity between the ages of 10 and 20 days, which is the time when spermatogenesis starts [26]. The enzyme increment is coincident with the appearance and proliferation of gametogenic cells, suggesting a relationship between LAT activity and proportion of spermatogenic cells in testis. These facts support the assumption that LAT is present in the precursor elements of the germinal stem line.

In the course of this investigation, it was observed that LDH C₄ and LAT activity per cell undergoes a significant reduction during the epididymal transit. The fall in C₄ isoenzyme activity in spermatozoa from 'caput' to 'cauda' in the rat has been previously reported [27]. The relative decrease of enzyme activity is strikingly larger

for LAT than for LDH C₄. We do not know the physiological significance of these enzymic changes, which adds to the complexity of biochemical modifications accompanying maturation of spermatozoa along the epididymal tract [28].

Patterns of liberation of several marker enzymes from spermatozoa treated with increasing concentrations of digitonin are in perfect agreement with those expected. The cytosolic glucose-6-phosphate dehydrogenase and the exclusively intramitochondrial citrate synthase and malate dehydrogenase gave the profiles expected from their respective subcellular distributions. LDH C₄ and LAT presented bimodal curves, almost identical to that of malate dehydrogenase, indicating that both enzymes have a dual location, in cytosol and mitochondria and probably in the mitochondrial matrix.

Our original recognition of the intramitochondrial location of LDH C₄ is supported by evidence from this and other laboratories [10,11,14-16,29]. Results presented afford additional proof of the dual location of the sperm-specific isoenzyme. For LAT, the subcellular distribution appears to be the same as that of LDH C₄. The bimodal location of LAT is not exclusive to spermatozoa. Studies using traditional cell-fractionation techniques [30] and fractional extraction by detergents [31] have revealed the presence of the enzyme in cytosol and mitochondrial matrix of different rat tissues.

These findings in intact spermatozoa, isolated from rat epididymis, confirm previous observations in mouse testis [7,13]. By being in the same cell, with identical intracellular distribution (cytosol and mitochondria), LDH C₄ and LAT meet the requirements to function in a shuttle system.

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina and by the Consejo de Investigaciones Científicas y Tecnológicas de la Provincia de Córdoba (CONICOR). N.T.V. and A.B. are Career Investigators of the CONICET.

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Received 25 April 1988/22 August 1988; accepted 24 August 1988