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# A SHUTTLE SYSTEM FOR THE TRANSFER OF REDUCING EQUIVALENTS IN MOUSE SPERM MITOCHONDRIA

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SUMMARY

Studies have been carried out on an *in vitro* reconstituted system composed of mouse lactate dehydrogenase isozyme X or $C_4$, branched chain amino acid aminotransferase , NAD , alpha-hydroxy isocaproate, glutamate and mouse sperm mitochondria. This system demonstrated capacity for the oxidation of extramitochondrial NADH . It is proposed that a branched chain alpha-hydroxyacid / amino acid shuttle for the transfer of reducing equivalents from cytosol to mitochondria may be functional in mouse spermatozoa.

Spermatozoa from many species can oxidize a variety of substrates like fructose, glucose, lactate, etc. (1). Aerobic utilization of these substrates produces an increase of cytoplasmic NADH which can not be directly oxidized in the respiratory chain because of the impermeability of the inner membrane of mitochondria to nicotinamide adenine dinucleotides. The reducing equivalents produced in cytosol during aerobic utilization of glucose or other substrates are transferred to mitochondria by the reduced product of a reaction catalyzed by a cytoplasmic NAD-linked dehydrogenase . So far, two of these shuttle systems have been demonstrated in different organisms and tissues . One utilizes the redox couple alpha-glycerophosphate / dihydroxyacetone phosphate and the other is the so-called malate / aspartate shuttle (2).

Keyhani and Storey (3) have presented evidence indicating that the malate / aspartate shuttle may be the principal system

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commuting hydrogens from cytoplasm to mitochondria in rabbit spermatozoa.

Previous studies in our laboratory had demonstrated a very peculiar substrate specificity (4) and subcellular localization (5) of the lactate dehydrogenase isozyme X or $C_4$ present in mouse sperm cells. Other observations demonstrated a high activity of branched chain amino acid aminotransferase in mouse testis (6).This enzyme showed the same cellular distribution as lactate dehydrogenase isozyme $C_4$, e.g., it is located in cytosol and in the matrix of mitochondria (6).

On the basis of these findings,we have proposed the possible existence of an additional shuttle system in mouse spermatozoa. Alpha-ketoacids derived from transamination of branched chain aminoacids can be readily converted to alpha-hydroxyacids by the "soluble" isozyme $C_4$ , while oxidizing cytoplasmic NADH . The alpha-hydroxyacids traverse the mitochondrial membrane to be oxidized by isozyme $C_4$ in the inner compartment of the organelle, transferring hydrogen to NAD of the mitochondrial pool.The alpha ketoacids thus formed could be transaminated to their respective amino acids, which may return to the cytoplasm.

We have performed experiments on an *in vitro* reconstituted system which indicate the effective functioning of such a shuttle.

## MATERIAL AND METHODS

*Lactate dehydrogenase* (EC 1.1.1.27) was purified from adult mouse testes with the method of Goldberg (6).

*Branched chain amino acid aminotransferase* (EC 2.6.1.6) was purified from mouse kidney and testis following the procedure described by Aki et al. (8). Studies in our laboratory (6) have indicated that the enzymes from mouse kidney and testis present very similar properties and that they must be the same . Units of this enzyme are defined according to Aki et al. (8).

After purification , the enzymes were dialyzed overnight against 0.05 M Tris-HCl pH 7.4 . The preparations did not show glutamate dehydrogenase activity.

NAD, NADH, ATP, rotenone, 2,4-dinitrophenol, alpha-hydroxy-isocaproate and imidazole were purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.).

*Mitochondria*. "Heavy" mitochondrial fraction from adult mouse testis , containing a homogeneous population of the organelles present in the mid-piece of spermatozoa , was obtained as described by Machado de Domenech et al. (9).

*Incubation*. The system was reconstituted by adapting the conditions designed by Bremer and Davis (10) for the malate / aspartate shuttle. The preincubation mixture contained glutamate 3.3mM, alpha-hydroxy-isocaproate 25mM, NAD 1.7mM , lactate dehy-

drogenase isozyme $C_4$ 5 IU , branched chain amino acid aminotransferase 3 U, imidazole-HCl buffer pH 7.4 25mM and KCl 0.075M in a final volume of 3 ml. This mixture was left at room temperature (20°-23°C) for 20 min . Concentrations of alpha-hydroxy-isocaproate and glutamate correspond to those assuring maximal activity for isozyme $C_4$ and the aminotransferase (4, 6).

After preincubation , mitochondria were added in a proportion of 10 mg of protein per ml . Other additions are indicated in Table 1 . Blanks contained the same mixture, except that KCl 0.15M was replaced for the mitochondrial suspension . Tubes were then incubated for 20 min in a water bath at 37°C . Samples were taken at the same time as from the mitochondrial incubations.

Formation and/or disappearance of NADH was measured after inactivation in a boiling water bath during 90 sec . Tubes were immediately cooled on ice and centrifuged . Supernatants were used for NADH determinations.

NADH assays. They were performed according to the method proposed by Klingenberg (11).

## RESULTS AND DISCUSSION

In the preincubated system containing the "cytosolic" half of the shuttle , with the enzymes lactate dehydrogenase isozyme $C_4$ and branched chain amino acid aminotransferase , a certain ratio NADH/NAD is in equilibrium with alpha-hydroxy-isocaproate, alpha-ketoisocaproate,leucine,glutamate and alpha-ketoglutarate.

As presented in Table 1,which shows results of one representative experiment, more than 50% of the total NADH formed during preincubation became oxidized when mitochondria were added . In controls containing only the imidazole-HCl buffer , KCl and 0.115mM NADH plus the preparation of mitochondria , there was absolutely no oxidation of the reduced dinucleotide.

When rotenone , an inhibitor of the respiratory chain , was added to the reconstituted system , the oxidation of NADH was strikingly reduced . Addition of ATP to rotenone-inhibited mitochondria produced a partial reversion of that effect, suggesting that the energy state is an important factor in the transfer of reducing equivalents through the mitochondria.

On the other side , uncoupling of mitochondria with 2,4-dinitrophenol increased markedly the oxidation of extramitochondrial NADH.

Addition of 1mM arsenite, which inhibits alpha-ketoglutarate dehydrogenase, did not modify oxidation of external NADH by mitochondria . This indicates that oxidation of alpha-ketoglutarate is not a factor affecting the changes of NADH observed in the system.

TABLE 1. Changes of external NADH in an in vitro reconstituted shuttle with mouse sperm mitochondria

During preincubation (see text), 0.488 µmoles of NADH were formed. Indicated additions were made immediately before mouse sperm mitochondria were added to the mixture. Incubation was continued for 20 min at 37°C.
Results are given in µmoles of NADH oxidized and in percentages of the NADH formed prior addition of mitochondria, taking 0.488 µmoles as 100 %.

| Additions | NADH oxidized | |
|---|---|---|
| | µmoles | % |
| None | 0.283. | 58.0 |
| Rotenone (15µM) | 0.037 | 7.6 |
| Rotenone (15µM) + ATP (3.3mM) | 0.137 | 28.0 |
| Dinitrophenol (0.33mM) | 0.449 | 92.0 |
| Arsenite (1mM) | 0.280 | 59.0 |

Results presented indicate a significant transference of reducing equivalents into mitochondria utilizing the branched chain alpha-hydroxyacid / alpha-ketoacid redox couple.

The operation of this shuttle would imply uptake of alpha-hydroxyisocaproate and glutamate and expulsion of leucine and alpha-ketoglutarate by the mitochondria. The effect of ATP on rotenone-inhibited mitochondria suggests the possibility that active transport may be involved in the process. For the alpha-hydroxyacids, there is evidence indicating that monocarboxylates can be transported by the carrier system for pyruvate described in liver and heart mitochondria (12). If a similar system exists in spermatozoa, the monocarboxylates could be actively carried through the internal membrane. However, the presence of a carrier does not seem to be essential, since monocarboxylates traverse freely in and out of the inner space of the mitochondria (13).

Theoretically, it is possible that this shuttle may work in both directions. However, metabolic requirements of spermatozoa would impose the transfer of hydrogen preferentially from cytosol to mitochondria. There is no evidence of significant gluconeo-

genic activity in spermatozoa (1) , a metabolic function which would require reversal of the shuttle.

Although utilization of alpha-ketoacids of carbon chain longer than pyruvate is a common feature for lactate dehydrogenase isozyme $C_4$ from many species (14) , not all of them possess significant activity with the alpha-ketoacids related to branched chain amino acids . Isozyme $C_4$ from certain species, like rabbit for example , utilizes very poorly those alpha-ketoacids . In rabbit spermatozoa , as Keyhani and Storey (3) have pointed out , the malate / aspartate shuttle may be the main commuter system for hydrogens . In addition , a recent report by Storey and Kayne (15) indicated that lactate dehydrogenase isozyme $C_4$ could function in a pyruvate / lactate shuttle.

In sperm cells of the mouse , it appears that the additional "branched chain alpha-hydroxyacid / amino acid" shuttle may contribute to the reoxidation of cytosolic NADH. Further studies are necessary to stablish its real functioning *in vivo* and the relative contribution of the different systems to the total hydrogen transfer.

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