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TESTICULAR LACTATE DEHYDROGENASE ISOZYME

NATURE OF MULTIPLE FORMS IN GUINEA PIG

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SUMMARY

1. Guinea pig's lactate dehydrogenase isozyme 1 (B_4) from heart, 5 (A_4) from skeletal muscle, and the two additional forms (X and X') from mature testis have been isolated and partially purified.

2. K_m, optimum substrate concentration, inhibition by high concentration of substrate and the effect of certain inhibitors and pre-heating, showed values for X' that were the average between those found for isozymes X and 5 (A_4).

3. Dissociation and recombination of polypeptides *in vitro* indicated that X is a homopolymer of chains (C) different from A and B, and that X' is a hybrid of A and C units.

4. Association of C and A polypeptides *in vivo* is possible in the precursor cells of the gametogenic line in which synthesis of the additional unit is started. In mature spermatozoa this hybridization does not occur, probably because the activity of genes controlling production of A and B chains is "turned off".

INTRODUCTION

Five molecular forms or isozymes of lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) are found in tissues from most vertebrates. These isozymes are the tetramers formed by random association of two polypeptide units (A, M or "muscle" type, and B, H or "heart" type)[1,2].

In the mature testis of many species, a sixth molecular form, designated lactate dehydrogenase-X, has been shown to occur[3,4]. This additional isozyme, found also in sperm[4,5], is a tetramer of polypeptide units (C) different from the A and B chains[6]. The three units are under the control of different genetic loci[7].

C polypeptides are able to recombine *in vitro* with A or B units to form active hybrids[6], but this recombination is not common *in vivo*. Many species present only one additional "X" isozyme. However, some species exhibit multiple "extra" fractions in the testis. The study of these multiple forms should afford further evidence as to the genetic and epigenetic control of isozymes.

The nature of these multiple forms has been clearly elucidated in pigeons with a set of five additional "X" bands[8]. In other animals, for example rat, guinea pig and bull[4], pig[9], and bat[10], two or more "X" fractions are present in the testis. It has not been demonstrated whether they represent the product of more than one extra polypeptide, or hybrids of C chains with the common polypeptide units.

This paper presents evidence supporting this last possibility.

METHODS

Guinea pigs were killed by decapitation and tissues removed and processed immediately. LDH-1 (B_4) was obtained from heart, LDH-5 (A_4), from skeletal muscle and LDH-X and X', from testis.

Sperm was collected by squeezing excised epididymes and subsequently washing them with 0.9% NaCl. On microscopic examination, this material was revealed to be composed almost exclusively of spermatozoa.

Preparation of homogenates and sperm lysates, electrophoresis on starch gel and purification of isozymes, were carried out as previously described[11].

Dissociation and recombination experiments

Dissociation and recombination of polypeptides from lactate dehydrogenase isozymes were performed according to the method of MARKERT[12]. Homogenates were subjected to two cycles of slow freezing and thawing in a medium containing 0.5 M NaCl, 0.1 M phosphate buffer, pH 7.0, and 4 mg per ml of NAD^+. Control mixtures were brought to the same salt concentration but were not frozen.

Lactate dehydrogenase assays

The method of WROBLEWSKI AND LA DUE[13] was applied for the direct reaction and that of MARKERT AND URSPRUNG[14] for the reverse. The absorbance change at 340 nm was read every minute for 6 min in a Beckman model DU spectrophotometer. Samples were diluted in order to obtain a $\Delta A_{340\ nm}$ of 0.030 to 0.040 per min in conditions assuring maximum activity. Assays were carried out at 20°.

Studies on the effects of oxalate, urea and pre-heating

These studies were carried out as previously reported for rabbit isozymes[11].

RESULTS

Electrophoretic patterns

Extracts from heart, diaphragm, skeletal muscle, tongue, esophagus, stomach, duodenum, large intestine, uterus, lung, liver, spleen, brain, kidney, eye, thyroid, adrenal gland, mature ovary and blood, showed characteristic patterns for lactate dehydrogenase isozymes. Enzymatic activity appeared distributed among five different fractions in proportions similar to those repeatedly described for the same tissues of other mammals. Sub-banding was commonly observed in the regions of isozymes 3, 4 and 5. Fig. 1 shows the lactate dehydrogenase patterns of several guinea pig tissues. In the system used, LDH-5 migrated toward the cathode.

Patterns from mature testis homogenates showed intense LDH-1 and -2, a faint

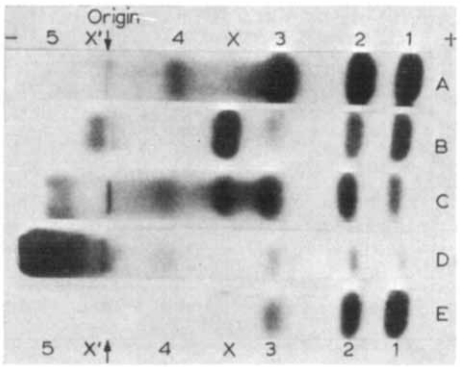

Fig. 1. Electrophoretic patterns of lactate dehydrogenases from guinea pig tissue homogenates. Numbers, X and X', indicate the position of corresponding isozymes. A. Heart (1:6, tissue to water, wt./vol). B. Mature testis (1:3). C. Mature epididymis (1:3). D. Skeletal muscle (1:7). E. Kidney (1:6). All homogenates were subjected to electrophoresis simultaneously in the same starch block. The gel was prepared with Tris–borate–EDTA (ref. 15).

LDH-3 and two prominent fractions with a mobility different from that of common isozymes or sub-bands observed in other tissues. One of these "extra" bands migrated between the areas of LDH-3 and -4 and will be designated LDH-X. The other was a cathodal band migrating between the origin and LDH-5. It will be named LDH-X'. The first (X) was more intensely stained. Isozymes 4 and 5 were not detected in fully developed testes. Testes from animals younger than a month did not exhibit the "extra" bands.

Mature epididymis extracts revealed only one additional fraction with the mobility of LDH-X (Fig. 1). Spermatozoa lysates had most of their activity localized

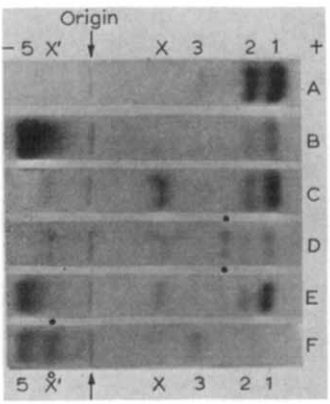

Fig. 2. Electrophoretic patterns of lactate dehydrogenases from guinea pig tissue homogenates. A. Heart (1:6). B. Skeletal muscle (1:7). C. Mature testis (1:3), control. D. Mature testis, same as C, after freezing and thawing. E. Mixture of testis and skeletal muscle homogenates (1:1), control. F. Mixture of testis and skeletal muscle, same as E, after freezing and thawing. All homogenates, including controls, contained 0.5 M NaCl, 0.1 M phosphate buffer (pH 7.0) and 4 mg/ml NAD^+, and were subjected to electrophoresis simultaneously in the same starch block. The gel was prepared with Tris–borate–EDTA (ref. 15). Numbers, X and X', indicate position of corresponding isozymes. ○ indicates new fractions formed after recombination.

in the region of LDH-X. The only other fraction demonstrated on these lysates was a very faint LDH-1.

Dissociation and recombination of polypeptide units

Dissociation and recombination of polypeptide units from lactate dehydrogenases contained in testicular extracts or in mixtures of testis and heart homogenates gave rise to a new fraction, never before observed in tissue extracts and which migrated between LDH-2 and -3 (Fig. 2). LDH-1, -2 and -X were markedly reduced, while LDH-X' did not change.

Studies carried out on mixtures of skeletal muscle and testis homogenates, produced a striking increase in the intensity of LDH-X' and LDH-3 and a reduction of LDH-1, -2, -X and -5 (Fig. 2).

These results did not change substantially when varying proportions of LDH-1 and LDH-X, or LDH-X and LDH-5 were mixed.

Recombination studies with mixtures of tissues containing the common isozymes produced a redistribution of activity among the five fractions, but no new bands with the mobility of those demonstrated when testis homogenates were included in the preparation.

In testicular extracts, or in mixtures of heart and testis homogenates, LDH-1 (B_4) and LDH-X are the predominant isozymes. If this last fraction were a tetramer

TABLE I

OPTIMUM SUBSTRATE CONCENTRATION AND K_m OF GUINEA PIG LACTATE DEHYDROGENASE ISOZYMES

For the reactions pyruvate → lactate and α-oxo-butyrate → α-OH-butyrate, final concentration of NADH was 0.115 mM and that of phosphate buffer (pH 7.4), 80 mM. For the reaction lactate → pyruvate, final concentration of NAD^+ was 0.9 mM, and that of Tris buffer, 10 mM (0.3 ml of 0.1 M Tris (pH 9.0) for 3.0 ml of reagent mixture). K_m is obtained from Lineweaver–Burk plots

Substrate	*LDH-1*	*LDH-5*	*LDH-X*	*LDH-X'*
	Optimum concentration (mM)			
Pyruvate	0.5	1.0	0.2	0.5
DL-Lactate	50.0	150.0	150.0	150.0
α-Oxo-butyrate	10.0	20.0	5.0	15.0
	K_m (mM)			
Pyruvate	0.051	0.13	0.039	0.071
DL-Lactate	4.3	25.0	5.1	14.0
α-Oxo-butyrate	1.0	2.2	0.8	1.5

of a different polypeptide (C_4), the random association of B and C in tetramers should produce three additional new forms. Instead, only one was detected, the mobility of which corresponded to that expected for a B_2C_2 hybrid.

In muscle-testis mixtures, where LDH-X and LDH-5 (A_4) are the predominant fractions, the only modification after recombination was the increase of activity in the area of LDH-X', indicating that this fraction has the same mobility as the A–C hybrid presumably formed. Again, only one of the three expected heteropolymers was found.

Effect of substrate concentration upon isozymes

Reaction pyruvate → lactate. LDH-X was the isozyme showing the highest affinity for pyruvate. K_m and optimum concentration were lower for LDH-X than those for LDH-1, -5 and -X′ (Table I).

Fig. 3 shows curves of rate of enzymatic activity against pyruvate concentration. At high concentrations of substrate, LDH-X exhibited the greatest inhibition. The inhibition for LDH-X′ was intermediate between the values for LDH-X and -5. At 10 mM pyruvate, the activity was 37% of the maximal for LDH-1, 68% for LDH-5, 21% for LDH-X and 43% for LDH-X′.

Reaction lactate → pyruvate. At low concentrations of lactate, the LDH-X activity was intermediate between the values for LDH-1 and -5. K_m for LDH-X is closer to that of LDH-1 (Table I). The optimum concentration and resistance to inhibition by high concentrations of lactate were identical for LDH-5, -X and -X′ (Fig. 4).

On studying the effect of substrate concentration on mixtures of purified isozymes, it was found that a 1:1 mixture of two different fractions gave a curve that

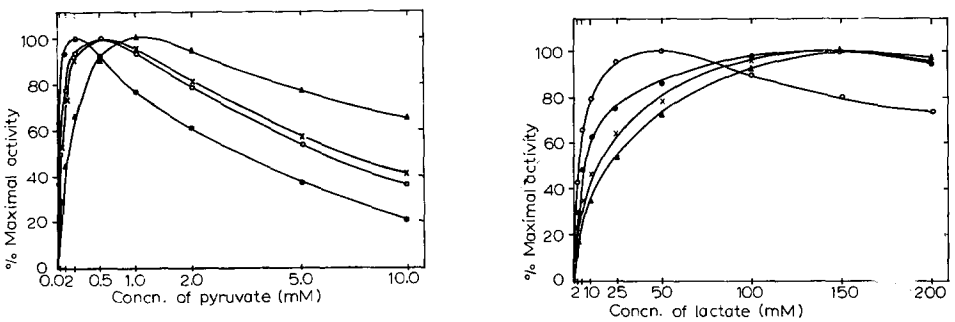

Fig. 3. Effect of pyruvate concentration upon activity of guinea pig LDH-1 (○), LDH-5 (▲), LDH-X (●) and LDH-X′ (×). Initial reaction velocity, expressed as a percentage of maximal activity, is plotted against concentration of pyruvate. Final concentration of NADH was 0.115 mM, that of phosphate buffer (pH 7.4), 80 mM, and the concentrations of pyruvate, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mM. Each point represents the mean value of six determinations carried out on different samples.

Fig. 4. Effect of lactate concentration upon activity of guinea pig LDH-1 (○), LDH-5 (▲), LDH-X (●) and LDH-X′ (×). Initial reaction velocity, expressed as a percentage of maximal activity, is plotted against concentration of lactate. Final concentration of NAD^+ was 0.9 mM, that of Tris, 10 mM and the concentrations of lactate, 2, 5, 10, 25, 50, 100, 150 and 200 mM. Each point represents the mean value of six determinations carried out on different samples.

was exactly the average of the curves corresponding to the original isozymes. For example, a 1:1 mixture of LDH-X and LDH-5 reproduced the curve obtained with LDH-X′, whereas a 1:3 mixture gave a curve almost identical to that of the predominant isozyme. Identical results were obtained when the mixtures were subjected to previous treatments producing dissociation and recombination of polypeptide units.

Activity with α-oxo-butyrate

As compared with testicular isozymes from other species, LDH-X from guinea pig showed good activity against α-oxo-butyrate. K_m values and optimum concen-

trations for the four isozymes are listed in Table I. The ratios of activity against α-oxo-butyrate to activity against pyruvate, both measured at their respective optimum concentration, were 0.84 for LDH-1, 0.88 for LDH-X, 0.72 for LDH-X′ and 0.54 for LDH-5 (values are averages of determinations on six different samples for each isozyme).

Effect of inhibitors

The effect of inhibitors upon the reaction pyruvate → lactate was studied. Results are the average values of determinations on six different preparations for each isozyme.

Oxalate. After incubation with 0.2 mM oxalate, the activity of LDH-1 was reduced to 35.5% of the original, that of LDH-X to 47%, that of LDH-X′ to 59.5% and that of LDH-5 to 73%.

Urea. LDH-1 was not inactivated by 2 M urea; the average remaining activity was 102%. LDH-X activity was 56% of the original, that of LDH-X′, 39% and in the case of LDH-5, 21%.

For both agents investigated, the effect on LDH-X′ was almost an exact average of the effects on LDH-X and LDH-5.

Effect of pre-heating

Fig. 5 shows curves depicting the remaining activity after heating. LDH-X

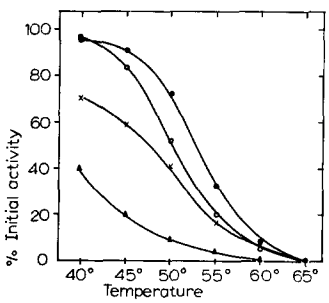

Fig. 5. Effect of pre-heating upon activity of guinea pig LDH-1 (○), LDH-5 (▲), LDH-X (●) and LDH-X′ (×). Activity, expressed as a percentage of the initial activity, is plotted against the heating temperature. The final concentration of NADH was 0.115 mM, that of phosphate buffer (pH 7.4), 80 mM and the concentrations of pyruvate, 0.5 mM for LDH-1 and LDH-X′, 1.0 mM for LDH-5 and 0.2 mM for LDH-X.

was the most stable isozyme, LDH-5 was very labile and LDH-X′ had a stability intermediate between the stabilities of LDH-X and -5.

DISCUSSION

In the mature testis of man, rabbit, dog, mouse and pigeon (type III), only one additional isozyme or LDH-X has been shown to occur[4,7]. Evidence accumulated indicates that the sixth isozyme is a tetramer of a polypeptide different from A and B, which has been designated C. Dissociation and recombination studies demonstrated

that hybrid tetramers between C and A or B units can be formed *in vitro* in most of those species[6].

Even when the three polypeptides co-exist in the same cell[4,5], the fact that they do not associate *in vivo* led MARKERT[16] to suggest that when the synthesis of C units starts, production of A and B polypeptides is "turned off" and there are no free chains available for association. Another explanation could be that in the cells where LDH-X appears, the corresponding chains are segregated to such a degree that they are unable to associate with other units. A report by CLAUSEN[17], together with results of studies in our laboratory, showed that LDH-X is predominantly located in the mitochondrial fraction of testicular homogenates.

A particular problem is posed by those species with more than one "X" band in the testis. The existence of multiple testicular forms has been well documented in the case of pigeons and their nature satisfactorily explained[7]. It was demonstrated that there may be allelism at the locus controlling the synthesis of C units, with the production of two different polypeptides (C and C'). Association in tetramers of those two chains give rise to a set of five additional forms.

The nature of two additional bands in some species has not been elucidated. The possible association of C and B units to form a second "extra" band in the testis was suggested for the type I pigeon[8]. From previous observations[11,18] *in vitro* it seemed that there was greater compatibility between C and B polypeptides to form hybrids than between C and A. The recent demonstration by ZINKHAM *et al.*[19] of a linkage of genetic loci for C and B would explain the existence of closer affinities between these units.

Our findings in guinea pig testis strongly indicate that one of the additional fractions, the one designated LDH-X, is a homopolymer of C chains. Its kinetic properties are very similar to those of the testicular isozyme from rabbit[11]. It showed a distinct sensitivity to increasing concentrations of substrate according to the direction in which the reaction is studied, a characteristic found in the "X" bands so far investigated[11,18]. It has the highest activity towards α-oxobutyrate and it is the predominant LDH isozyme found in sperm.

The properties exhibited by the X' fraction and the results of dissociation and recombination experiments, demonstrate that it is a heteropolymer of C and A units, and possibly an A_2C_2 combination.

Studies *in vitro* indicated that for either A–C or B–C associations, a dimer–dimer combination is favored in guinea pig. The reason why only the A–C hybrid is formed *in vivo* cannot be ascertained at present. It could indicate that A units are still being synthesized and are available for association when the production of C chains begins. From the patterns of whole testis homogenates one can assume that production of B units does predominate over that of A chains, but it is not known what happens at the particular cells where the C gene is activated. In any case, the possibility that some epigenetic factors are involved in the formation of isozymes instead of random association of sub-units produced at a regulated rate, cannot be excluded.

Epididymal and spermatozoal extracts possess LDH-X but no X' fraction. Obviously, the X band of epididymal homogenates comes from the sperm they contained.

It has been demonstrated that X and X' are related to cells of the gametogenic

line[4]. Probably they are initially synthesized at the stage of primary or secondary spermatocytes. Our observations suggest that at this point A and C chains are able to recombine but that at a later stage, in fully mature spermatozoa, only C polypeptides are synthesized, whilst production of other chains is stopped.

These findings emphasize the extraordinary cellular specificity and synchronization of mechanisms controlling the synthesis of lactate dehydrogenase sub-units. The appearance of the "hybrid" form X′ suggests the existence of epigenetic factors regulating the formation of certain isozymes.

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