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EVIDENCE FOR THE EXISTENCE OF LACTATE DEHYDROGENASE X IN EMBRYONIC CHICK LIVER

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SUMMARY

Lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) from embryonic chick liver had different kinetic and thermal stability properties from lactate dehydrogenase from adult chick liver, and lactate dehydrogenase prepared from different sources known to contain varying percentages of the lactate dehydrogenases 1, 2, 3, 4 and 5 isozymes. The embryonic chick liver lactate dehydrogenase could not be separated from adult chick liver lactate dehydrogenase or lactate dehydrogenase 5 on the basis of its electrophoretic mobility on polyacrylamide disc gels. However, embryonic chick liver lactate dehydrogenase utilized α-ketoglutaric acid as a substrate, and 3-acetylpyridine NAD as a coenzyme more efficiently than any of the other lactate dehydrogenase isozymes. Also, embryonic chick liver lactate dehydrogenase was more resistant to heating at 65 °C than the other lactate dehydrogenase isozymes. Further experiments showed that the kinetic properties of embryonic chick liver lactate dehydrogenase were consistent with those demonstrated for lactate dehydrogenase X.

INTRODUCTION

The occurrence of five major types of lactate dehydrogenase isozymes (L-lactate:NAD oxidoreductase, EC 1.1.1.27) in most mammalian and avian tissues has been well documented[1–3]. These five isozymes are formed by the random combination of two different polypeptide subunits into tetramers[4–7]. The subunits have been designated as A and B by Markert[8]. Thus, lactate dehydrogenase 1 may be represented by BBBB, and lactate dehydrogenase 5 by AAAA. Lactate dehydrogenases 2, 3 and 4 are hybrids of lactate dehydrogenases 1 and 5, each one being a tetramer.

In addition to these molecular forms of lactate dehydrogenase, substantial evidence has been accumulated which shows that avian sperm and mammalian testes and sperm cells contain a sixth isozyme, lactate dehydrogenase X[9–12]. This isozyme is

composed of a third polypeptide which is also in the form of a tetramer, and is the product of a separate gene[9,11,13,14]. Various investigators have shown that lactate dehydrogenase X has distinct kinetic properties which can differentiate it from the other lactate dehydrogenase isozymes[10,12,15–19]. This is especially true in relation to the broad substrate and coenzyme specificity range, *plus* the thermal stability of lactate dehydrogenase X. Other experiments have established the fact that lactate dehydrogenase X is an immunochemically distinct lactate dehydrogenase isozyme[16,17]. Lactate dehydrogenase X from rat, human and rabbit tissues can also be easily separated electrophoretically as single bands on polyacrylamide disc gels from the other isozymes[16]. However, lactate dehydrogenase X and lactate dehydrogenase 5 from hamster and mouse tissues have the same net charge, and therefore cannot be separated on the basis of their electrophoretic mobilities[20]. However, recent evidence has shown that lactate dehydrogenase X from mouse testes has a different primary structure from mouse lactate dehydrogenase 1 and lactate dehydrogenase 5[21].

In the present report, data are presented to demonstrate that lactate dehydrogenase from embryonic chick liver has different kinetic and thermal stability properties from the lactate dehydrogenase from adult chick liver, and other lactate dehydrogenase preparations containing varying percentages of lactate dehydrogenases 1, 2, 3, 4 and 5. Further evidence is provided to show that the kinetic properties of embryonic chick liver lactate dehydrogenase are consistent with those properties demonstrated for lactage dehydrogenase X.

MATERIALS

White Leghorn fertilized chicken eggs were purchased from the Shamrock Poultry Farms, North Brunswick, N.J. The eggs were incubated at 38.5 °C in a Thelco incubator until needed. Chicken serum was obtained from the jugular vein of 1-year-old White Leghorn hens. These same hens were used to obtain the adult chicken liver samples. Mouse testes were obtained from Swiss mice.

L-Lactic acid, sodium pyruvate, α-hydroxyglutaric acid, α-ketoglutaric acid, 3-acetylpyridine NAD, NAD, and NADH were purchased from The Sigma Chemical Company. Partially purified lactate dehydrogenase 5 isozyme (from rabbit muscle), and lactate dehydrogenase 1 isozyme (from beef heart) were products of the Worthington Biochemical Corporation. The chemicals used to prepare the polyacrylamide gel electrophoresis were purchased from Eastman Organic Chemicals.

METHODS

Homogenates

Tissues were removed from embryonic chicks and placed in ice-cold water. The pooled tissues were blotted dry, washed, and then homogenized in a Potter–Elvehjem-type homogenizer in 0.1 M acetate buffer, pH 5.5 or 0.25 M sucrose (1:4, w/v). The homogenate was centrifuged at 105 000 × *g* for 1 h at 2 °C in a Spinco Model L centrifuge. The resultant supernatant was passed through two layers of cheesecloth, and then heated at 65 °C for 5 min (unless indicated), and immediately placed in ice. After centrifugation to remove proteins, this extract was used directly as the source of enzyme to measure keto acid reduction. To measure hydroxy acid oxidation, the

enzyme extract was first neutralized with 0.1 M sodium carbonate. All of the enzyme solutions were either used immediately or stored at −20 °C. Protein concentrations of the enzyme extracts were measured by the method of Lowry *et al.*[22].

Lactate dehydrogenase assays

Changes in 340 nm absorbance were measured at room temperature with a Beckman Model DU spectrophotometer equipped with a Guilford automatic sample changer. Reaction rates were obtained every 15 s for 2–3 min. Changes in absorbance were obtained from the initial linear portion of the curve. The enzyme solutions were diluted so that the rate of absorbance change was within the range of 0.025–0.075 unit per min.

The assay mixture for pyruvic acid reduction contained 150 μmoles of potassium phosphate (pH 7.4), 0.2 μmole of NADH, and enzyme, as indicated, in a volume of 2.9 ml. The enzyme reactions were then initiated with the addition of 0.1 ml of a solution containing 1.0 μmole of pyruvic acid (adjusted to pH 7.4). For α-ketoglutaric acid reduction, 100 μmoles of acetate buffer (pH 5.4) were employed. The reaction was initiated with 0.1 ml containing 1 μmole of α-ketoglutaric acid (pH 5.4). Blanks, in which the substrate was deleted, were assayed concurrently with all samples.

For hydroxy acid oxidation, the solutions contained 100 μmoles of Tris–HCl (pH 9.2), 0.2 μmole of NAD (Grade V), or 0.2 μmole of 3-acetylpyridine NAD, and enzyme in a volume of 2.9 ml. The reactions were initiated by the addition of 0.1 ml of a solution containing either 1.0 μmole of α-hydroxyglutarate or L-lactate (adjusted to pH 9.2). Blanks, without any substrate, were assayed concurrently with all the samples.

Lactate dehydrogenase activities are given in International Units (I.U.), defined as the amount of enzyme activity necessary to oxidize 1.0 μmole NADH or reduce 1.0 μmole NAD in 1 min at 25 °C.

Polyacrylamide gel electrophoresis

Gel electrophoresis was performed by the method of Davis[23], as modified by Dietz *et al.*[24]. 40 μl containing approximately $1 \cdot 10^{-4}$ I.U. of enzyme activity (with respect to NAD oxidation) were placed on the gels. The gels were 5.5% acrylamide made in 0.04 M Tris–HCl buffer (pH 8.9). The running buffer was 0.05 M sodium glycinate (pH 9.5). Bromophenol blue was employed as the tracking dye. The current flow was 4 mA per tube for approximately 30 min (the time for the tracking dye to reach the bottom of the gel column). The gels were then stained at pH 10.0 for lactate dehydrogenase activity, utilizing the *p*-nitro-blue tetrazolium reagent as described by Meizel and Markert[25]. The excess dye was removed by soaking overnight in 7% acetic acid.

RESULTS

Keto acid reduction

Lactate dehydrogenase from either embryonic or adult chicken liver was able to reduce pyruvic acid. However, when α-ketoglutaric acid was employed as the substrate in lieu of pyruvic acid, embryonic chick liver lactate dehydrogenase was readily measurable, while adult liver lactate dehydrogenase activity was barely detectable.

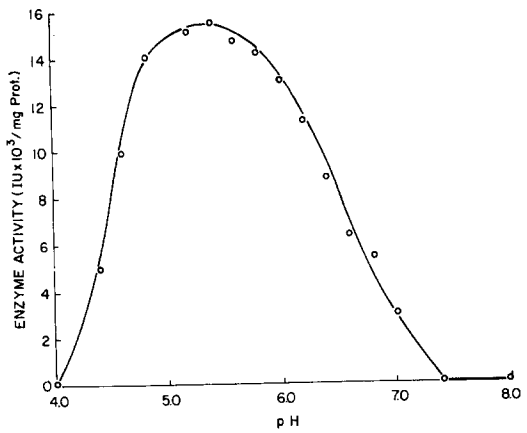

Fig. 1. pH profile curve for embryonic chick liver lactate dehydrogenase, utilizing α-ketoglutaric acid as a substrate. Acetate buffer was used for the pH 4.0–5.8 range, and phosphate buffer for the pH 6.0–8.0 range.

These results were consistent whether the liver extracts were 105 000 × *g* supernatants prepared from 0.25 M sucrose homogenates, or high speed supernatant solutions which had been prepared from pH 5.2 acetate homogenates, and then heated at 65 °C for 5 min.

Fig. 1 is a pH profile of embryonic chick liver lactate dehydrogenase activity utilizing α-ketoglutaric acid as a substrate. The pH maximum of the enzyme reaction was 5.4, the enzyme activity being undetectable at pH 7.4. This was in contrast to the reduction of pyruvic acid by adult or embryonic chick liver lactate dehydrogenase which had a pH maximum of pH 7.4 and was undetectable at pH 5.4.

TABLE I

SUBSTRATE AND COENZYME ACTIVITY RATIOS FOR LACTATE DEHYDROGENASE DERIVED FROM VARIOUS SOURCES

It was predetermined by gel electrophoresis that beef heart lactate dehydrogenase contained 75% lactate dehydrogenase 1 and 25% lactate dehydrogenase 2; rabbit muscle contained 85% lactate dehydrogenase 5, 15% lactate dehydrogenase 4, and 5% lactate dehydrogenase 3; fresh chicken serum contained 50% lactate dehydrogenase 5, 25% lactate dehydrogenase 4, 10% lactate dehydrogenase 3, 10% lactate dehydrogenase 2, and 5% lactate dehydrogenase 1; and mouse testes, heated at 65 °C for 10 min, contained 90% lactate dehydrogenase X, 5% lactate dehydrogenase 1, and 5% lactate dehydrogenase 2. NADH was the coenzyme employed with pyruvic acid as the substrate, while NAD was used with either α-ketoglutaric acid or lactic acid as the substrate. Lactic acid was used as the substrate when either 3-acetylpyridine NAD (3-AcPrNAD) or NAD was the coenzyme.

Source of lactate dehydrogenase	*Substrate activity ratio*		*Coenzyme activity ratio 3-AcPrNAD/NAD*
	Pyruvate/α-ketoglutarate	*Pyruvate/lactate*	
Embryonic chick liver	270:1	760:1	2.40:1
Mouse testes	120:1	690:1	1.80:1
Adult chicken liver	1150:1	510:1	0.45:1
Beef heart	1000:1	680:1	0.50:1
Rabbit muscle	1040:1	660:1	0.45:1
Chicken serum	900:1	640:1	0.70:1

Table I shows that embryonic chick liver lactate dehydrogenase was able to reduce pyruvic acid at pH 7.4 270 times more readily than α-ketoglutaric acid at pH 5.4. Mouse testes, consisting of 90% lactate dehydrogenase X, reduced pyruvic acid 120 times more readily than α-ketoglutaric acid. The mouse testes supernatant was preheated at 65 °C for 5 min, thus inactivating lactate dehydrogenases 1 and 2, as shown by gel electrophoresis. Lactate dehydrogenase extracts prepared from adult chicken liver, beef heart, rabbit muscle and chicken serum utilized pyruvic acid at pH 7.4 900–1200 times faster than α-ketoglutaric acid at pH 5.4. α-Hydroxyglutaric acid was identified as the product of α-ketoglutaric acid reduction by a previously described procedure[26]. This method entailed the use of paper chromatography with 3 different solvent systems. As a result of this study, the possibility of a contaminating glutamate dehydrogenase (EC 1.4.1.2) forming glutamic acid and NAD from α-ketoglutaric acid and NADH was eliminated.

The lactic acid/α-hydroxyglutaric acid oxidation ratio from the various lactate dehydrogenase samples could not be ascertained, since only embryonic chick liver and mouse testes oxidized the α-hydroxyglutaric acid. Both of these lactate dehydrogenase samples had a 100-fold preference for lactic acid in comparison with α-hydroxyglutaric acid.

Table I also shows that all of the lactate dehydrogenase isozymes had a 500–700 times preference for the reduction of pyruvic acid in comparison with the oxidation of lactic acid. However, with lactic acid as the substrate, lactate dehydrogenase from embryonic chick liver and mouse testes utilized 3-acetylpyridine NAD as a coenzyme about twice as readily as the natural coenzyme, NAD. All of the other lactate dehydrogenase enzymes, except the chicken serum preparation, had a 2-fold preference for NAD. Chicken serum lactate dehydrogenase had an approx. 1.4 times preference for NAD.

Heat stability of lactate dehydrogenase isozymes

Fig. 2 shows the heat stability of lactate dehydrogenase derived from embryonic and adult chick livers. Embryonic chick liver lactate dehydrogenase, with pyruvic acid as the substrate, was 45% active after heating at 65 °C for 5 min, while only 25% of the adult chick liver was detectable at those conditions. With α-ketoglutaric acid as the substrate, the embryonic liver lactate dehydrogenase was 65% active, while the adult liver retained 25% of its activity. Lactate dehydrogenase from beef heart and rabbit muscle was totally inhibited after heating at 65 °C for 5 min, while the mouse testes lactate dehydrogenase X retained 50% of its activity with either α-ketoglutaric acid or pyruvic acid as a substrate. Chicken serum was 40% active after heating at 65 °C for 5 min. Heating at 65 °C for 30 min resulted in the inhibition of 90% of the adult chick liver lactate dehydrogenase activity, and 45% of the embryonic and mouse testes lactate dehydrogenase activity. This was true whether pyruvic acid or α-ketoglutaric acid was used as the substrate.

Polyacrylamide gel electrophoresis

It was demonstrated by polyacrylamide gel electrophoresis that lactate dehydrogenases derived from embryonic and adult chick liver could not be separated from each other or chicken serum lactate dehydrogenase 5. The location of the embryonic chick liver lactate dehydrogenase band on the acrylamide gel remained

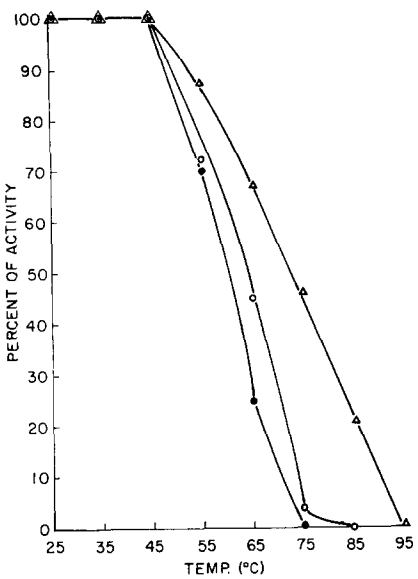

Fig. 2. Heat stability of lactate dehydrogenase from embryonic and adult chick liver, utilizing either pyruvic acid or α-ketoglutarate as a substrate. △—△, embryonic chick liver lactate dehydrogenase activity with α-ketoglutaric acid as the substrate; ○—○, embryonic chick liver lactate dehydrogenase activity with pyruvic acid as the substrate; ●—●, adult chick liver with pyruvic acid as the substrate. The enzyme assays are described in Methods.

constant whether 3-acetylpyridine NAD or NAD was used as the coenzyme in the staining mixture. This was also true whether lactic acid or α-hydroxyglutaric acid was employed as the substrate.

DISCUSSION

Previous experiments have demonstrated that lactate dehydrogenase X can utilize α-ketoglutaric acid as a substrate[12]. This fact has been used to differentiate lactate dehydrogenase X from the other lactate dehydrogenase isozymes. In mouse testes, where lactate dehydrogenase X cannot be separated from lactate dehydrogenase 5 on the basis of their electrophoretic mobilities, lactate dehydrogenase X was shown to utilize α-ketoglutaric acid at a pH maximum of 5.5[27]. Experiments detailed in this study show that lactate dehydrogenase activity from embryonic and adult chicken liver could not be differentiated from each other, or from chicken serum lactate dehydrogenase 5 on polyacrylamide gels. However, the embryonic chick liver lactate dehydrogenase resembled mouse testes lactate dehydrogenase X by the fact that they had similar pyruvate/α-ketoglutarate substrate activity ratios. Adult chicken liver lactate dehydrogenase had a similar pyruvate/α-ketoglutarate substrate activity ratio to lactate dehydrogenase 1 and lactate dehydrogenase 5.

Other investigators[21,27] have demonstrated that lactate dehydrogenase X has a greater resistance to heating at 65 °C than the other lactate dehydrogenase isozymes. This data has been exploited for the isolation and purification of lactate dehydrogenase X from various sources. Experiments in the present study showed that lactate

dehydrogenase 1 and lactate dehydrogenase 5 can be completely inactivated by heating at 65 °C for 5 min, while 75% of the adult chicken liver lactate dehydrogenase was inhibited at this temperature. Embryonic chick liver lactate dehydrogenase resembled mouse testes lactate dehydrogenase X in that only about 35% of the enzyme activity was inhibited after 5 min, and about 45% was inhibited after heating for 30 min. Lactate dehydrogenase activity from all sources was totally inactivated by heating at 90 °C for 5 min. The partial resistance of adult chicken liver to heating at 65 °C may be due to the presence of embryonic liver-type lactate dehydrogenase in the adult liver extracts. The initial inactivation of embryonic liver lactate dehydrogenase to 65 °C heat may have been a result of the presence of adult-type lactate dehydrogenase in the embryonic extracts. Chicken serum lactate dehydrogenase was only 60% inactivated by heating at 65 °C for 30 min. This may be due to the large amounts of serum albumin which is known to have a stabilizing effect on enzymes.

Gel electrophoresis experiments showed that it was possible to substitute α-hydroxyglutaric acid for lactic acid and/or 3-acetylpyridine NAD for NAD in the staining media for detecting embryonic chick liver lactate dehydrogenase activity. There was no shift in the location of the lactate dehydrogenase band on the gels when these replacements were made. These findings indicate that the enzymatic reduction of α-hydroxyglutaric acid and lactic acid are a result of the same enzyme.

Previous experiments[9] have shown that lactate dehydrogenase X reacts more readily with 3-acetylpyridine NAD than the natural coenzyme, NAD. The present study showed that embryonic chick liver had a 2-fold greater preference for the NAD derivative, while adult chicken liver lactate dehydrogenase, lactate dehydrogenase 1 and lactate dehydrogenase 5 had a 2-fold greater preference for NAD.

Other investigators[28–32] have studied lactate dehydrogenase isozymes in the developing chick liver without detecting an lactate dehydrogenase X type of activity. This probably was due to the fact that the embryonic chick liver lactate dehydrogenase cannot be differentiated from adult liver lactate dehydrogenase on the basis of their electrophoretic mobilities. Therefore, there appeared to be no reason to study in detail the comparative kinetic properties of embryonic and adult chicken liver lactate dehydrogenase. There is also the possibility that embryonic chick liver lactate dehydrogenase resembles lactate dehydrogenase X only in specific breeds of chicken.

In the present study, evidence is presented to show that lactate dehydrogenase derived from White Leghorn embryonic chick livers can substantially reduce α-ketoglutaric acid, has a preference for 3-acetylpyridine NAD in comparison to NAD, and has a greater resistance to heating at 65 °C than adult chicken liver lactate dehydrogenase, lactate dehydrogenase 1 and lactate dehydrogenase 5. These kinetic properties of embryonic chick liver lactate dehydrogenase have been shown to be consistent with those properties demonstrated for lactate dehydrogenase X from mouse testes. However, further experiments will be necessary to determine whether the embryonic chick liver lactate dehydrogenase is composed of a distinct polypeptide which is encoded in a separate gene, and has distinct immunological properties.

NOTE ADDED IN PROOF (Received December 28th, 1972)

In further experiments it was not possible to detect any lactate dehydrogenase X type of activity in rat embryonic liver and various rat hepatomas.

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