Immunochromical Specificity of Lactate Dehydrogenase-X

ERWIN GOLDberg

Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201

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ABSTRACT Sperm-producing testes of mammals and some birds contain a unique form of lactate dehydrogenase (EC 1.1.1.27) which is a tetramer composed of C subunits, rather than the A and B subunits of lactate dehydrogenase isozymes 1-5. Immunochemical evidence is presented which confirms the hypothesis that the C subunit is encoded in a separate gene from the A and B polypeptides. The evolutionary relationships of these genes are discussed.

Studies by many investigators have clearly established that the five major types of lactate dehydrogenase (LDH; EC 1.1.1.27) in mammals are formed by the random combination of two different subunits into tetramers. Each subunit (A and B) is the product of a distinct gene locus (1). There is now substantial evidence that the isozyme LDH-X of the sperm cell is also a tetramer and that it is composed of a third polypeptide type (C), which is encoded in a separate gene (2-5). Apparently this gene is active only during the primary spermatocyte stage of the spermatogenic process and is inactive in all other cells of the organism (6, 7).

Immunological procedures have proved to be potent tools for establishing subunit homologies (8) and for examining the evolution of lactate dehydrogenases (9). The selective inhibiting and precipitating activities of antibodies have been used in the present investigation to demonstrate that the C subunit of LDH-X is indeed distinct from the A and B polypeptide, thus confirming its independent genetic control, and further, to show that this isozyme from several vertebrate species has a common evolutionary origin.

METHODS

LDH-X was purified from mouse testes by a combination of ammonium sulfate fractionation and DEAE-Sephadex chromatography. The most active fractions from the column were combined and concentrated to contain approximately 760 international units of LDH-X activity per ml. This unit is defined as that amount of enzyme which will oxidize 1 μmol of NADH/min at 25°C. While this material contained only the X isozyme of LDH, other contaminating proteins with different electrophoretic mobility were present. Therefore, as a final step in this purification a 2-ml sample, containing approximately 20 mg of protein, was subjected to electrophoresis on a preparative polyacrylamide slab-gel in the Ortec pulsed constant power system. The LDH-X band was identified by nitroblue tetrazolium staining as previously described (10) sliced from the gel, and homogenized in 0.05 M phosphate buffer at pH 7.4 that contained 0.85% NaCl. The gel slurry was shown to be homogeneous by disc gel electrophoresis with the protein band, stained by amido black, and the LDH-X band having coincident electrophoretic mobility. 71% of the enzyme activity applied to the gel was recovered.

Approximately 4500 units of LDH-X was injected into three albino female rabbits. Each rabbit received three injections, of 1-ml each, into a footpad and the dorsal thoracic muscles. 2 weeks later, each doe was given a single injection containing 600 units of LDH-X. As Weintraub and Raymond (11) have shown, the acrylamide serves as an adjuvant in this procedure. Blood was withdrawn from the heart at weekly intervals, commencing on the 15th day, for a period of 2 months. In subsequent work, crystalline LDH-X in Freund's adjuvant was used as the provoking antigen for antisem production. Antibody specificity and titer was established by the Ouchterlony diffusion method applied as recommended by Stollar and Levine (12), by the immunodiffusion experiments method of Wasserman and Levine (13), and by measuring inhibition and precipitation of enzyme activity. From the latter measurements, equivalence points for the reaction between antibody and antiserum were calculated (14). Finally the procedure combining selective precipitation by antibodies followed by electrophoretic resolution of the unpurified isozyme was as described by Markert and Holmes (14). This method provides a sensitive measure of antibody specificity in a mixture of isozymes. Suitable controls with normal rabbit serum were performed.

RESULTS

Immunodiffusion experiments were performed in an agar gel containing a center well for antiserum and four test wells. A single, intense precipitin line was formed against purified LDH-X. This merged with the precipitin line with crude testis extract in the adjacent well. The fact that no spur was formed indicates a "reaction of identity" (12). However, a second, faint precipitin line was observed with the crude extract. Only the intense precipitin lines stained for LDH when the nitroblue tetrazolium reaction mixture was added to the plates. Lack of staining of the faint band reflected the presence of antibodies to impurities in the immunizing antigen. No precipitin reaction could be detected with homogeneous preparations of mouse LDH-1 and LDH-5 in the other wells on the agar plate. Double diffusion analyses of antisera produced by rabbits injected with crystalline LDH-X indicated a high probability of immunochemical homogeneity, since a single precipitin line of identity was obtained from LDH-X and from crude extracts of testis. In no case was a reaction observed with homogeneous preparations of mouse LDH-1 and LDH-5 at several concentrations of antiserum and of enzyme.

Abbreviation: LDH, lactate dehydrogenase.
Similarly, there is no cross reaction by anti-LDH-X serum with LDH-1 and LDH-5 in enzyme inhibition measurements. These experiments measured both the immediate enzyme-inhibiting capacity of the antiserum and the residual activity after maximal precipitation of enzyme by antibodies. Increasing quantities of antiserum to LDH-X were mixed with a fixed amount of homologous or heterologous enzyme activity (2.5 units of enzyme activity in the reaction mixture) for 15 min and the residual enzyme activity was determined spectrophotometrically. Then the antiserum-enzymo mixtures were incubated at 25°C for 1 hr and at 4°C overnight, centrifuged at 100,000 × g for 30 min, and the supernatants were tested for LDH activity. From these data it appears that the antiserum used contain primarily enzyme-inactivating antibodies. The equivalence points (Fig. 1) are virtually the same before and after removal of the enzyme-antibody precipitate. There are apparently no protective antibodies produced (14) since no enzyme activity remains after maximum treatment with antiserum. No cross reaction with A or B subunits could be detected even at a 100-fold increase in antiserum concentration. The equivalence points calculated (Fig. 1) are about 30-times higher than those obtained with antiserum produced from animals receiving LDH-X in acrylamide. Antiserum to the pure, crystalline LDH-X was used in all subsequent work.

Antiserum was mixed with extracts of heart tissue and of testes and then these preparations were subjected to electrophoresis on polyacrylamide slab gels or disc gels. The data obtained are a striking demonstration of the specificity of the antiserum for LDH-X (Fig. 2), which is selectively removed from the testis homogenate while the other bands of LDH are unaffected. That no cross reaction of antiserum with A or B subunits occurs is even more evident from the heart extract pattern. Mouse heart muscle contains the usual five somatic-tissue LDH isozymes and, typically, most of the enzyme activity is associated with the more anodal forms (i.e., LDH-1, 2, and 3). There is neither a qualitative nor quantitative change in the isozyme pattern in the presence of antiserum to LDH-X (Fig. 2). Furthermore, these data strongly support the conclusion that the A₄ homotetramer, which has an electrophoretic mobility coincident with LDH-X, is absent from mature testes and, more importantly, that B-C subunit combinations do not occur in vivo (15, 16). When the BC₄ and B₄C₄ tetramers, produced by in vitro dissociation-recombination of subunits (5) according to the procedure of Markert (17), were treated with antiserum, they were also selectively removed.

This procedure of antiserum treatment and electrophoresis is extremely useful in demonstrating homologies among LDH loci (8, 14, 18). The antiserum was tested in this manner against preparations of human spermatozoa and of testes from rat, rabbit, hamster, guinea pig (Fig. 3), and pigeon (Fig. 4). LDH-X from rats and humans migrates on polyacrylamide disc gels between LDH-3 and 4, and LDH from the rabbit migrates between LDH-4 and 5. Clearly, the antiserum specifically and selectively removes LDH-X. In hamster tissues, as in the mouse, LDH-X and LDH-5 possess the same net charge and show coincident electrophoretic mobility. There is obviously only the C₄ homotetramer occupying this zone on the gel after electrophoresis of hamster-testis extract.

Guinea pig testes contain three X-bands rather than two as previously reported (19, 20). Two of these bands are readily discernible on the basis of electrophoretic mobility and migrate between LDH-3 and 4. The third is cathodal to, and barely separated from, LDH-4 on disc gels, and thus could be easily overlooked except when the gel containing it is compared with an antiserum-treated preparation (Fig. 3). While the existence of a third gene locus coding for the C polypeptide accounts for a single "band X" in testes, it is more difficult to explain the occurrence of multiple forms of LDH-X. Zinkham et al. (6) observed that the C locus was polymorphic in some pigeons so that both C and C' alleles directed the synthesis of five forms of LDH-X. Both the number of LDH bands and their relative mobilities would seem to support an argument against the formation of

![Fig. 1. Inactivation of LDH-X by homologous antisera against LDH-X; ○-○, inhibition of LDH-X by antisera; ●-●, precipitation of LDH-X by antisera; ▲-▲, attempted inactivation of nonhomologous LDH (LDH-1 and LDH-5).](image)

![Fig. 2. Immunochemical specificity of mouse LDH isozymes. Abbreviations used in this legend: AS, rabbit antiserum to LDH-X; CS, rabbit control serum; T, testes extract; H, heart extract. The tissue extracts and serum were mixed in ratios listed below and treated as described in the text. Channel 1, T:AS = 1:1; channel 2, T:AS = 1:2; channel 3 and 5, T control; LDH-X designated by closed circle; channel 4, AS; channel 6, T:CS = 1:1; channel 7, T:CS = 1:2; channel 8, CS; channel 9, H control; channel 10, H:AS = 1:4.](image)
Fig. 3. Disc gels showing the effect of antiserum to LDH-X on isozymes of various mammalian testes extracts. The tissue extracts and antiserum were mixed 1:1 in each case and treated as described in the text. Closed circles designate LDH-X, open circles mark the LDH isozymes in rabbit serum. Gels numbered 2, 5, 7, 9, and 11 show the isozyme pattern after antiserum treatment of the appropriate extract. Gel 3 is a diaphragm extract. All of the extracts were adjusted to contain the same total LDH activity prior to electrophoresis. 1, 2, human; 3-5, hamster; 6, 7, guinea pig; 8, 9, rabbit; 10, 11, rat.

C–C' as well as B–C and A–C heterotetramers in the guinea pig testes. As an alternative, in animals with more than one "X band," there may be an epigenetic modification of the C subunit which alters the net charge of the tetramer, perhaps by combination with a small molecule, analogous, for example, to the association of alkaline phosphatase isozymes with varying amounts of sialic acid (21).

The pigeon used in these experiments was apparently heterozygous at the C locus (6) since five X bands were removed by antiserum (Fig. 4). Under these conditions, A and B subunit-containing tetramers were not affected. Confirmation of this result was obtained by incubating a gel in a reaction mixture in which 0.5 M α-hydroxyvalerate was substituted for lactate so that only the five forms of LDH-X showed enzymatic activity (6).

The antibody–antigen reactions were quantitatively measured by micro-complement fixation analyses (13). Fig. 5 shows that mouse LDH-X reacted with antiserum at a concentration of 1:7500. The degrees of cross-reaction with homogeneous preparations of LDH-1 and LDH-5 were expressed quantitatively as the index of dissimilarity or immunological distance (ID); that is, the relative concentration of antiserum required to produce a complement fixation curve whose peak was as high as that given by the homologous LDH (22). Values of 75 were obtained with both LDH-1 and LDH-5. Thus, there would appear to be little structural similarity between mouse LDH-X as compared to either LDH-1 or 5.

DISCUSSION
The evidence presented here clearly establishes that LDH-X is immunologically distinct from LDH-1 and LDH-5, and confirms the concept that the C subunit is encoded in a separate gene in mammals as well as in pigeons. These results rule out the suggestion by Stambaugh and Buckley (23), based on an assumed octameric structure for LDH, that the C subunit is in fact a hybrid subunit involving the same

Fig. 4. Pigeon LDH isozyme patterns. Abbreviations used are the same as in Fig. 2. Channel 1, AS; channel 2, T alone; channel 3, T:AS = 1:2; channel 4, T:AS = 1:4; channel 5, H alone; channel 6, H:AS = 1:2; channel 7, H:AS = 1:4; channel 8, H:AS = 1:8. There are five bands of LDH-X in the testis preparation, all of which appear to be equally affected by AS.
Fig. 5. Micro-complement fixation. Antiserum at a dilution of 1:7500 was tested against a crystalline preparation of LDH-X, and homogeneous preparations of mouse LDH-1 and LDH-5. A curve coincident with that of LDH-X was obtained with crude testis extract, thus confirming the purity of the antigen and specificity of antiserum.

genes as the A and B polypeptides. This would require that the antiserum to LDH-X cross-react with isozymes containing B subunits.

The data obtained allow the conclusion that the C subunits in various mammals and in the pigeon are homologous polypeptides which are probably derived from a common ancestral gene. Presumably, the different genes for LDH synthesis evolved from a single ancestral gene which encoded a polypeptide with LDH activity. This gene duplicated to form the A and B genes, which then diverged to code for products that were immunochemically and kinetically distinct (14). The C gene must also have evolved as a consequence of a duplication event. Whether this involved the common evolutionary progenitor of the A and B genes, or one of these genes at a later time, is uncertain. The micro-complement fixation data reveal that LDH-X is as unrelated to LDH-1 as it is to LDH-5. Nevertheless, all of these enzymes must have some structural features in common since they catalyze the same reaction and their subunits can combine, at least in vitro. At the present time it seems reasonable to propose that the primary duplication event(s) produced three genes (A, B, and C) and that the C locus came under control of a regulator (gene?) so that its expression was limited to the primary spermatocyte either by activation restricted to this cell type or repression in all other cells. The finding that LDH-X is indeed a unique protein of the sperm cell may be of considerable practical significance by providing a new approach to contraception in the male. Experiments are in progress to determine whether interference with LDH-X synthesis or activity will affect spermatogenesis or the fertilizing capacity of spermatozoa.

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