IMMUNOCHEMICAL HOMOLOGIES AMONG SUBUNITS OF TROUT LACTATE DEHYDROGENASE ISOZYMES*

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Abstract.—Lactate dehydrogenase is a tetrameric enzyme, generally composed of one or two kinds of subunits each encoded in a separate gene. Most vertebrates synthesize five major isozymic forms of lactate dehydrogenase, but the salmonid fish, particularly trout, synthesize many more. The numerous lactate dehydrogenase isozymes of trout can be selectively removed from tissue homogenates by suitably prepared antisera to specific lactate dehydrogenase subunits of fish. Electrophoretic resolution of such antisera-treated homogenates then permits an identification of each isozyme and a determination of its subunit composition and probable genetic basis. Such data indicate that trout are tetraploid organisms and have duplicate and slightly different loci for two and perhaps three of the lactate dehydrogenase loci found in other fish. The evolutionary relationships among the lactate dehydrogenase loci can also be assessed by these immunochemical data.

Introduction.—Many investigators have demonstrated that mammals contain five major isozymes of lactate dehydrogenase formed by the random combination of two different subunits into tetramers.¹⁻³ Each of these subunits represents a distinct gene product. An apparent contrast to this simple picture is provided by the isozyme patterns of many fish.⁴ The salmonoid fish, particularly the trout, show exceedingly complex isozyme patterns involving more than 20 isozymes in homozygous individuals.^{5, 6} This paper describes methods whereby this great molecular complexity can be resolved and the subunit composition of each isozyme identified through the use of immunochemical techniques.

Of primary importance is the fact that antibodies made against the **A** or **B** subunits of mammals, birds, or fish are highly specific for that subunit but do cross-react with the homologous subunits from other organisms.⁷ The degree of cross-reaction reflects the degree of identity between the subunits synthesized by the different species.⁸⁻¹² Each antiserum contains a complicated population of antibody molecules, and immunochemical investigations exploit their precipitating, inactivating, or complement fixation properties. In our investigation we have exploited the selective precipitating activity of the antibodies as applied to homogenates containing many different isozymes.

Materials and Methods.—Two species of fish were used, brook trout (*Salvelinus fontinalis*) and rainbow trout (*Salmo gairdneri*). Tissues were dissected from freshly killed specimens, homogenized, and centrifuged at 100,000 *g* for 30 min. The supernatants were used as the crude isozyme preparations for electrophoretic resolution and assay or for reaction with antisera.

The general procedures for starch gel electrophoresis and for staining for lactate dehydrogenase have been fully described in other papers.^{4, 6}

Antisera were prepared in rabbits against lactate dehydrogenase isozymes A₄ and B₄

extracted and purified from tissues of the sea trout *Cynoscion regalis*.¹³ Antisera of high titers were obtained that reacted strongly with the provoking antigen and with homologous isozymes from other species of fish. Some cross-reaction of the anti-B sera and A subunits of the brook trout was demonstrated. This cross-reaction cannot be due to contaminating A subunits in the B₄ antigenic preparation used for immunizing the rabbits because contamination would certainly have led to an antibody-antigen reaction with the A₄ isozyme of the sea trout. And no such cross-reaction with heterologous isozymes of the sea trout was observed for either the anti-A or anti-B sera. Such antisera made against the isozymes of a fish provide an extremely specific means of identifying homologous fish isozymes and are, moreover, more sensitive detectors of differences among fish than are antisera made against proteins from more distantly related organisms such as birds.¹²

The antisera were added in various concentrations to homogenates containing the isozymes from different tissues of the brook trout and the rainbow trout. The antisera were allowed to react overnight in the cold. The antiserum-homogenate preparation was then centrifuged (100,000 *g* for 30 min) and the supernatant applied to starch gels for electrophoresis. This combination of selective precipitation by antibodies followed by electrophoretic resolution of the unprecipitated isozymes provides a very sensitive identification of each isozyme and permits an assessment of its subunit composition.¹³ The selective removal from tissue homogenates of individual isozymes with somewhat different immunochemical specificities can be controlled by adjusting the titer of the appropriate antiserum. Moreover, only fractions of an international unit of enzyme are required to carry out these reactions since the stain for lactate dehydrogenase involves the accumulated product of a prolonged enzyme reaction rather than a direct measurement of the enzyme molecules themselves.

Results.—The multiplicity of isozymes in the tissue patterns of the brook trout and the rainbow trout may be seen in Figure 1. One complexity in these patterns occurs as a result of overlapping positions for isozymes of different subunit composition. Note, for example, in the brook trout zymogram that the A'₄ tetramer and the B'₄ tetramer occupy the same electrophoretic position; similarly in the rainbow trout zymogram, the slowest-migrating isozyme of the d group (D₄) and the fastest migrating isozyme of the b group (B'₄) occupy the same position. Thus, the complete removal of one or the other of these tetramers would still leave the position occupied by a lactate dehydrogenase isozyme. Such double occupancy of an electrophoretic position can be detected by successive antibody treatments that remove first one and then the other of the two isozymes at that electrophoretic position.

Note in the rainbow trout zymogram that the intestinal extract isozymes show distinctly greater mobility towards the anode than do the isozymes found in the heart or liver. These have been designated D isozymes and are composed of subunits that are electrophoretically but not immunochemically distinct from B subunits. The zymogram of the brook trout shows that this particular fish is heterozygous at the B' locus, whereas the rainbow trout is homozygous at all loci. The multiplicity of isozymes evident in these fish has been noted previously by other investigators, and they have offered various suggestions concerning the isozyme subunit composition.^{5, 6, 12, 14, 15}

Figure 2 shows the results of treating the homogenates from the various tissues with antisera. First, in interpreting the results of the antisera treatment, it is important to realize that any tetramer containing either an A or B subunit reacts with the corresponding antiserum. Notice that the channel containing the

FIG. 1.—LDH isozyme patterns in brook trout (*Salvelinus fontinalis*) and rainbow trout (*Salmo gairdneri*). In the brook trout LDH zymogram, homotetramers are indicated at the side of the photograph in line with the corresponding isozyme. For the rainbow trout zymogram, groups of LDH isozymes are represented by the small letters: *a* for A and A' subunit containing isozymes, *b* for those with B and B' subunits, and *d* for those with D and D' subunits.

homogenate from heart tissues shows, in the control, nine isozymes in the brook trout and five isozymes in the rainbow trout. In both of these fish, all of the isozymes in heart muscle are removed by treatment with the antisera made against B subunits and are not affected at all by treatment with the anti-A sera. Previous reports have suggested that the repertory of isozymes in the heart tissue involves both A and B subunits.⁵ The results of the immunochemical tests shown here indicate that this is not true. Clearly, all of these isozymes of heart muscle are homologous to the B subunits of higher organisms.

Several investigations have indicated that the trout are derived from tetraploid ancestors.^{6, 12, 15} Thus, each gene should be duplicated so that in the genome of these fish there should be two A loci for lactate dehydrogenase, two B loci, and so forth. This appears to be true although the isozymes coded for by these duplicated genes are now somewhat different from one another. Apparently the genes have diverged slightly during the course of evolution. This can be readily seen in Figures 1 and 2 where duplicate sets of subunits at the A and B loci of the brook and rainbow trout and at the D locus of the rainbow trout would account for the multiplicity of isozymes at these several positions. The E₄ isozyme, however, shows only one electrophoretically distinct form in these trout.

The tetramers containing A or B subunits are clearly identified in Figure 2 by the corresponding antiserum, which removes them. In contrast to results reported for the lactate dehydrogenases of other vertebrates, we find some cross-

FIG. 2.—Immunochemical specificity of trout LDH isozymes. The subunit composition of the homotetramers is indicated at the side of the photograph. The white circles identify rabbit LDH in the antiserum. Abbreviations used in this legend are as follows: BM, brook trout muscle extract; BH, brook trout heart extract; RI, rainbow trout intestine extract; RH, rainbow trout heart extract; AA, anti-A serum; AB, anti-B serum; CS, control serum. The tissue extracts and serum were mixed in ratios listed below and treated as described in the text. Channel 1, BM:AA = 1:4; channel 2, BM:AB = 1:10; channel 3, BM:CS = 1:10; channel 4, BM:AA = 1:1; channel 5, BM:AB = 1:3; channel 6, BM:CS = 1:3; channel 7, BH:AA = 1:2; channel 8, BH:AB = 1:2; channel 9, BH:CS = 1:2; channel 10, RH:AA = 1:1; channel 11, RH:AB = 1:1; channel 12, RH:CS = 1:1; channel 13, RH:AA = 1:2; channel 14, RH:AB = 1:2; channel 15, RH:CS = 1:2. The virtual absence of isozymes in channel 1 demonstrates that all of the isozymes apparent in the adjacent control channels are composed of A type subunits; similarly, for channels 8, 11, and 14 B type subunits must have composed all isozymes.

reaction between antisera to B subunits and the trout A subunits. Note in Figure 2, channel 2, that high concentrations of anti-B serum do, in fact, greatly reduce the concentration of A tetramers. Moreover, the effect is differential: the tetramers with the greatest electrophoretic mobility (A') are slightly more sensitive to the anti-B serum, whereas those with the least mobility (A) are relatively more sensitive to the anti-A serum (channel 4). Of course, the anti-A serum eliminates both A and A' subunits (channel 1) at much lower antibody concentrations than is required for the anti-B serum to have any effect on A' subunits. Thus, these A subunits of the trout are very similar, though not identical, and moreover are immunochemically related to the B subunits. No such cross-reactivity between A and B subunits in other fish has so far been observed.

Aside from the A and B subunits, these fish also synthesize isozymes containing D and E subunits.⁶ The D subunits apparent in the intestine of the rainbow trout are completely inactivated by anti-B serum but are not touched by the anti-A serum. Thus, the D subunits are either epigenetic modifications of B subunits or are encoded in genes that have diverged so slightly as to be immunochemically indistinguishable. The E subunits (Fig. 1) were also inactivated by the anti-B serum at high concentrations but were not affected by the anti-A serum.^{16, 17}

Thus, the E subunits are distinct from the B subunits but are more closely

related to them than to the A's. Our immunochemical tests clearly distinguish between the two kinds of A subunits, but so far no immunochemical distinction is apparent between the two B's. Nevertheless, both the A's and the B's are regulated differentially as shown by their differential tissue distribution. Note, for example, that in the liver (Fig. 1) only the B gene is activated and the B' is not. Similarly, muscle tissue contains all five of the A-A' tetramers; eye tissue in the brook trout contains only the A tetramer. This was observed by incubating brook trout eye tissue extracts with increasing quantities of anti-B serum. It was found that the B' subunits could be removed preferentially revealing the presence of the A₄ isozyme at the same location. Thus, in these different tissues, molecular mechanisms for identifying and separately regulating the different A and B genes must exist.

From these results it is possible to develop a scheme showing the evolutionary relationships among these different genes for the synthesis of lactate dehydrogen-

FIG. 3.—Suggested evolutionary relationships of LDH genes in salmonoid fish.

ase (Fig. 3). Presumably a single ancestral gene encoded a polypeptide with lactate dehydrogenase activity. This gene then duplicated to form the **A** and **B** genes which in the salmonids still show some relationship as indicated by our immunochemical data. Later the **A** and **B** genes, at the time of tetraploidization of the salmonids, were duplicated. They then diverged slightly so as to produce two different kinds of **A** and **B** genes. From the **B** line must have evolved the **D** gene, if, in fact, it is genetically distinct, and also the **E** gene, for which there is good evidence of genetic distinctiveness.^{16, 17}

Several reports have recently been concerned with the rate of evolution of genes.¹⁸ These reports all deal with homologous proteins in different organisms. In the salmonids, however, an exceedingly opportune arrangement exists: namely, duplicated genes evolving in the same organisms—the two **A**'s, two **B**'s, and so on. Thus, a careful chemical analysis of the different subunits of lactate dehydrogenase that are encoded by these multiple genes should provide critical evidence concerning the intrinsic rates of evolution of proteins under nearly identical conditions—that is, in the same organism. Such data should prove more valuable than data for homologous proteins extracted from different organisms.

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