GENE DUPLICATION IN FISHES: MALATE DEHYDROGENASES OF SALMON AND TROUT*

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ABSTRACT - Three forms of supernatant malate dehydrogenase occur in most salmon and trout. Dissociation and recombination experiments on the purified forms show that these three isoenzymes arise from the association of two types of subunits (A and B) into dimers. The A and B subunits are shown to be under the control of separate genes. The detection of duplicate genes for supernatant malate dehydrogenase provides additional support for the hypothesis that salmonid fishes are tetraploid.

Biochemical and genetic studies have established that in fishes of the family Salmonidae, the gene coding for the enzyme H_4 lactate dehydrogenase (LDH) has undergone duplication (1-5). Several other proteins exhibit an unusually large number of electrophoretic forms in these fishes (6-9), which suggests that duplication of the whole genome has occurred and that salmonids are therefore tetraploid.

The tetraploidy hypothesis was first proposed to account for the finding that salmonid fishes contain about twice as much DNA per nucleus and about twice as many chromosomes as other fishes in the same order, Clupeiformes (10, 11). Before this hypothesis can be considered established, the subunit composition and genetic control of proteins other than H_4 LDH need to be investigated in salmonids. In this communication we show that the multiple forms of supernatant malate dehydrogenase in representative salmonids can be accounted for by the tetraploidy hypothesis.

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METHODS

Animals - Tissues from freshly killed king salmon (Oncorhynchus tshawytscha) and rainbow trout (Salmo gairdnerii) were obtained through the cooperation of Dr. Alex Calhoun at the Nimbus Hatchery, California Department of Fish and Game, and Dr. John Halver at the Western Fish Nutrition Laboratory and Spring Creek Hatchery, Cook, Washington.

Assays - LDH, MDH, and protein were determined as previously described (3). Succinate-cytochrome c reductase was assayed by a previously described method (12) except that DPNH was omitted from the assay mixture.

Starch Gel Electrophoresis - Electrophoresis was routinely performed at pH 6.0 as previously described (3), at a gradient of 14 volts/cm for 19 hours.

Subcellular Fractionation - Mitochondria were prepared from fresh salmon skeletal muscle. Epaxial muscle (165 g) was diced, rinsed in cold 0.15 M KCl and blended for 10 seconds in 3 volumes (w/v) cold Tris KCl buffer (0.01 M Tris-Cl pH 7.8, 0.10 M KCl). Cell debris was sedimented at 600 x g for 10 minutes. The pellet was washed 3 times with 100 volumes Tris-KCl buffer. Mitochondria were ruptured by suspending the pellet in 2 volumes of a buffer containing 10 mM Tris-Cl, pH 7.0, 1 mM EDTA, and 1 mM 2-mercaptoethanol, followed by homogenization in a glass/glass TenBroek homogenizer, and one freeze-thaw cycle. The thawed suspension was centrifuged at 10,000 x g for 10 minutes. The supernatant, containing soluble mitochondrial enzymes, was made 40 percent saturated in ammonium sulfate and stored at 6°C.

The supernatant fractions of heart, liver, spleen, skeletal muscle, and red blood cells were isolated at 5°C by gentle grinding in glass/Teflon homogenizers in 4 volumes of 0.25 M sucrose, 0.01 M Tris-Cl, pH 7.0, followed by centrifugation at 15,000 x g for 20 minutes and discarding of the pellet. Red blood cell supernatant was also obtained by diluting washed cells in 4 volumes of buffered 0.25 M sucrose, sonicating for 3 seconds at maximum intensity on a Bronwill BIOSONIC II, and centrifuging at 15,000 x g for 20 minutes.

Subunit Recombination - The fastest and slowest anodal isoenzymes of supernatant MDH were purified to specific activities of 520 and 560 units/mg, respectively, by a procedure to be reported in detail elsewhere. The purified isoenzymes were placed in separate dialysis bags (90 units MDH, 1/4 ml) and a 1:1 mixture of the two was placed in a third bag (90 units each, 180 units total, 1/2 ml). The isoenzymes were dissociated into subunits and recombined by the following modification of a published procedure (14). Dialysis was carried out against 200 ml 7.6 M guanidine HCl, 0.01 M Tris-Cl pH 7.0, 0.1 M 2-mercaptoethanol for three hours at 4°C. The bags were removed, rinsed, and dialyzed against 200 ml 0.01 M Tris-Cl pH 7.0, 0.1 M 2-mercaptoethanol for 1/2 hour at 4°C, then 3 hours at 35°C. The samples were assayed for recovery of MDH activity (25-35 percent) and analyzed by electrophoresis at pH 6.0.

RESULTS AND DISCUSSION

Subcellular Fractionation - Mitochondria were isolated from the skeletal muscle of a single salmon, using succinate-cytochrome c reductase as a mitochondrial marker. Activity for this system was low for the untreated suspension of mitochondria, but was elevated ten-fold after rupture of the suspension, indicating that the initial preparation consisted largely of intact mitochondria.

Table I. Subcellular Fractionation of Salmon Muscle Tissue

Cell Fraction	Succinate-Cytochrome c Reductase (Units/ml)	LDH (Units/ml)	MDH (Units/ml)
Supernatant	0	250	48
Mitochondrial	91 [a]	0.2 [b]	70 [b]

[a] Measured by assaying 0.01 ml of a suspension of 1 g mitochondrial pellet in 2 ml buffer (see Methods). Following incubation of the enzyme in the assay mixture for 5 minutes, the reaction is started by adding 0.1 ml of 2 μM horse cytochrome c. Without incubation, the assay rate is about 90 percent diminished, indicating that the mitochondrial preparation is essentially intact. One unit is defined as that amount of enzyme which reduces 1 μmole of cytochrome c per minute, assuming ϵ cyt c (red.-oxid.) = 2.1×10^4 cm^{-1} M^{-1}.

[b] Measured by assaying the extract obtained by rupturing the mitochondria, centrifuging, and discarding the pellet.

The very low levels of LDH in this fraction served as a measure of the absence of contamination by cytoplasmic enzymes (Table I). Rupture of the preparation resulted in the release of an active, although labile, MDH component, which was used as the source of mitochondrial MDH.

The supernatant fraction of various tissues was obtained by extraction under conditions where mitochondrial rupture was minimized. Each of the tissues examined yielded high levels of LDH and MDH activity in the supernatant fraction (Table I) which were subjected to electrophoretic analysis.

Electrophoresis - The MDH's contained in the mitochondrial and supernatant fraction of salmon tissues are electrophoretically distinct (Figure 1). Mitochondrial MDH migrates slowly, as a diffuse band, toward the anode at pH 6.

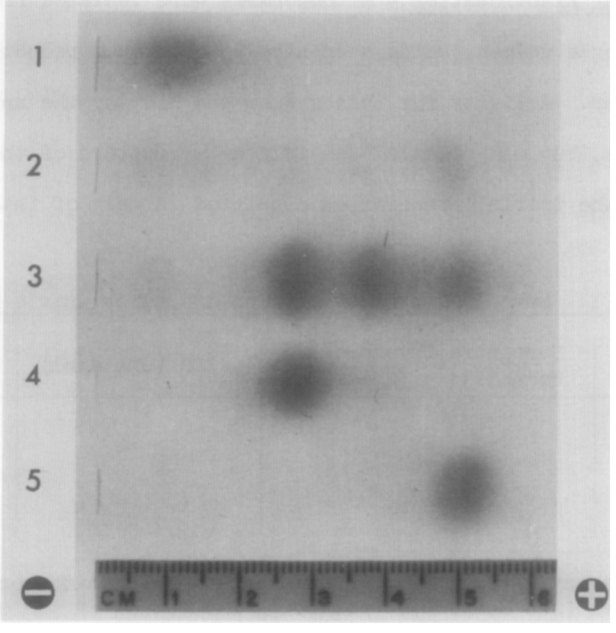

Figure 1. Electrophoresis of king salmon MDH's 1 - Mitochondrial extract. 2 - Crude muscle extract. 3 - Crude heart extract. 4 - Slowest anodal form of supernatant MDH, partially purified. 5 - Fastest anodal form of supernatant MDH, partially purified. MDH was visualized by a specific tetrazolium staining method (14).

In a few experiments this enzyme appeared to resolve into a small number of sub-bands; its isoenzymic properties were not further investigated.

Supernatant MDH, on the other hand, is composed of three sharp, equally spaced, anodal bands (Figure 1). The relative proportions of the three supernatant bands varies markedly from tissue to tissue. For example, in skeletal muscle the most anodal band predominates; in brain, eye, and liver the least anodal band predominates; in heart and red blood cells all three supernatant isoenzymes occur in approximately equal amounts.

Subunit Recombination - The fastest and slowest supernatant MDH's were purified to high specific activity and separated from each other by ion exchange chromatography (Figure 1). When the two forms were dissociated in guanidine HCl and reassociated together, all three supernatant forms were regenerated (Figure 2) with electrophoretic mobilities identical to the untreated

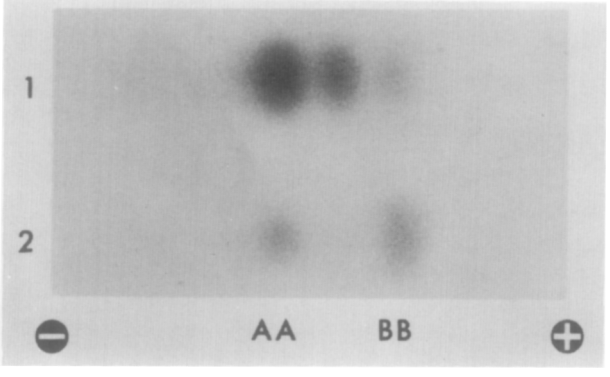

Figure 2. Electrophoresis of highly purified supernatant MDH's subjected to molecular hybridization tests (see Methods). 1 - Mixture of fastest and slowest supernatant forms dissociated and reactivated together. All three supernatant bands were reformed with mobilities identical to untreated isoenzymes. 2 - Fastest and slowest supernatant forms dissociated and reactivated separately, then subjected to electrophoresis together. As measured by spectrophotometric assay, recovery of the fastest form was 25 percent and of the slowest form 35 percent.

isoenzymes. When the two forms were dissociated and reassociated separately, only the two parental forms were regenerated. Therefore the three forms do not appear to be conformers (15) or artifacts of the analytical procedure. The simplest interpretation of these results is that there are two kinds of subunits (A and B) which associate to produce three dimeric forms (AA, AB, BB) of supernatant MDH in salmon.

Genetic Control of the Supernatant MDH's - Electrophoretic surveys of wild and hatchery populations of salmon and trout revealed the presence of some individuals with six, rather than three supernatant MDH's (Figure 3). Three of the six bands are identical in mobility with AA, AB, and BB. The three additional bands can be accounted for by the assumption that the gene for the B subunit is present in two allelic forms, B and B'. The association of three gene products - A, B, and B' - would produce the six dimers AA, AB', AB, BB, BB', B'B'. The electrophoretic spacing of the observed six bands fits this hypothesis.

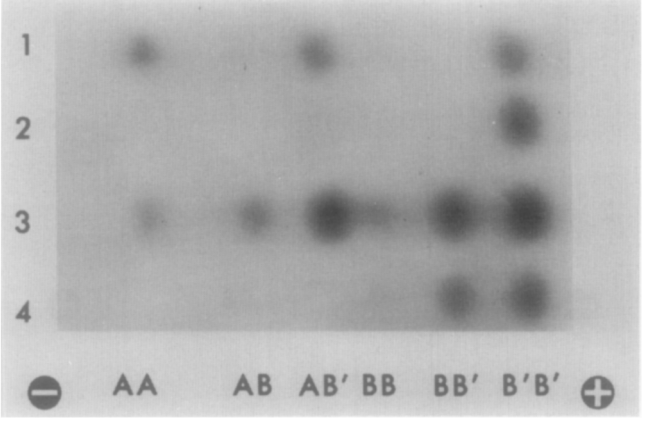

Figure 3. Electrophoresis of supernatant MDH's of rainbow trout, showing two types of B subunits. 1 - Heart extract of individual presumed to be homozygous for one allele at the B locus. 2 - Muscle extract of same individual 3 - Heart extract of individual presumed to be heterozygous at the B locus. 4 - Muscle extract of the heterozygous trout.

This genetic interpretation is strengthened by the finding that the frequency of occurrence of B alleles, of which three are known, varies widely among populations and among species. For example, most rainbow trout are B'B' homozygotes, whereas most salmon tested are BB homozygotes.* Furthermore, the frequency of occurrence of B variants appears to be unrelated to the age of the individuals tested (13). It is thus unlikely that specific or nonspecific somatic alterations of a single polypeptide are responsible for the observed isoenzyme patterns. The simplest interpretation of the above data is that two genes, A and B, dictate the formation of salmonid supernatant MDH.

CONCLUSION

We have presented biochemical evidence that salmonid fishes, unlike higher vertebrates (16,17), contain duplicate genes for supernatant MDH. Three forms of this dimeric enzyme, therefore, exist in these fishes. Enolase, another enzyme which is dimeric in higher vertebrates, also occurs in three forms in salmonids (6). M_4 LDH, a tetrameric enzyme, exists as a series of 5 isoenzymes in salmonids (2-5). Four independent genetic loci therefore appear to exist in duplicated form in salmonids - viz the loci for H_4 LDH, M_4 LDH, enolase, and supernatant MDH. It is unlikely that this represents four isolated and independent instances of regional chromosomal duplication. These multiple cases of gene duplication afford substantial confirmation of the hypothesis that salmonids are tetraploid.

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*Brown trout and brook trout show added electrophoretic complexities possibly indicating extensive polymorphism at both loci A and B.

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