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Epigenetic Control of Lactate Dehydrogenase Subunit Assembly

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Fish lactate dehydrogenase differs from the mammalian enzyme by virtue of tissue-specific non-random tetramerization of its two subunits, which can be random *in vitro*. The primary structures of the subunits are therefore not the sole determinants of the quaternary structure of the completed molecule.

IT is well known that the synthesis of a protein is accomplished by transcription of the structural gene followed by translation of its message, and it is generally agreed that the amino-acid sequence thus assembled, and perhaps modified, is the final determinant of the tertiary and quaternary structure as well[1]. Furthermore, the reversible denaturation of ribonuclease[1] and X-ray crystallography of haemoglobin[2] have indicated that there is a single fairly rigid tertiary and quaternary structure for every protein which is not affected by chemical changes including those as great as species haemoglobin differences. The evidence presented here demonstrates that, at least in the case of fish lactate dehydrogenase, the primary structure of the protein is not the sole determinant of its quaternary structure as evidenced by subunit association. Tissue-specific mechanisms beyond the level of the structural gene apparently play a role in determining the final protein product.

Lactate dehydrogenase (LDH) is a tetrameric protein, present in most mammalian tissues as five different molecular forms, called isozymes, distinguishable by charge and hence separable by electrophoresis (Fig. 1*a*)[3] and ion-exchange chromatography[4]; the five forms are determined by the random association of two different subunits, A and B, encoded by non-allelic genes, into A4, A3B, A2B2, AB3 and B4 in the binomial proportions 1 : 4 : 6 : 4 : 1 (refs. 5–7). Differential tissue distributions of these five forms are explained by excess of one subunit over the other, so that preferential synthetic rates of the subunits by the cell are sufficient explanation for the tissue distributions generally seen in mammals.

In more than 50% of the fish studied in this laboratory, however, there are only one, two or three LDH isozymes (Fig. 1*b*). Other species have five isozymes, but the distributions are non-random with the central isozymes reduced in proportion (Fig. 1*c*), and still other fish have different isozyme numbers in different tissues (Fig. 1*d*). These fish lactate dehydrogenases were purified[4], and their subunit compositions were elucidated by the following experiments.

Subunit Structure of LDH

Dissociation–recombination[8,9] of LDH from whole three isozyme fish yielded five molecules in approximately random distributions (Fig. 2*a*). The same result was obtained from dissociation–recombination of the purified[5] anodal and cathodal isozymes from three isozyme fish (Fig. 2*b*), and from dissociation–recombination of the purified central isozyme; furthermore, the five products of these two experiments were reproducibly superimposable (Fig. 2*b*), indicating that they were the same. Five products would be expected from binomial theory[10] if two subunits associate randomly into tetramers. Thus these experiments indicated that the subunit structure of three isozyme fish LDH is A4, A2B2, B4 (Fig. 2). Confirmation of these compositions was obtained by immunochemistry. Monospecific antibody was induced in rabbits[11] to pure LDH isozymes from a related species, *Merluccius bilinearis*; antibody to A4 inactivated the cathodal and central isozymes of three isozyme fish, presumably A4 and A2B2 (Fig. 2*c*); similarly, antibody to B4 inactivated the anodal and central isozymes, and antibody to A2B2 inactivated all three.

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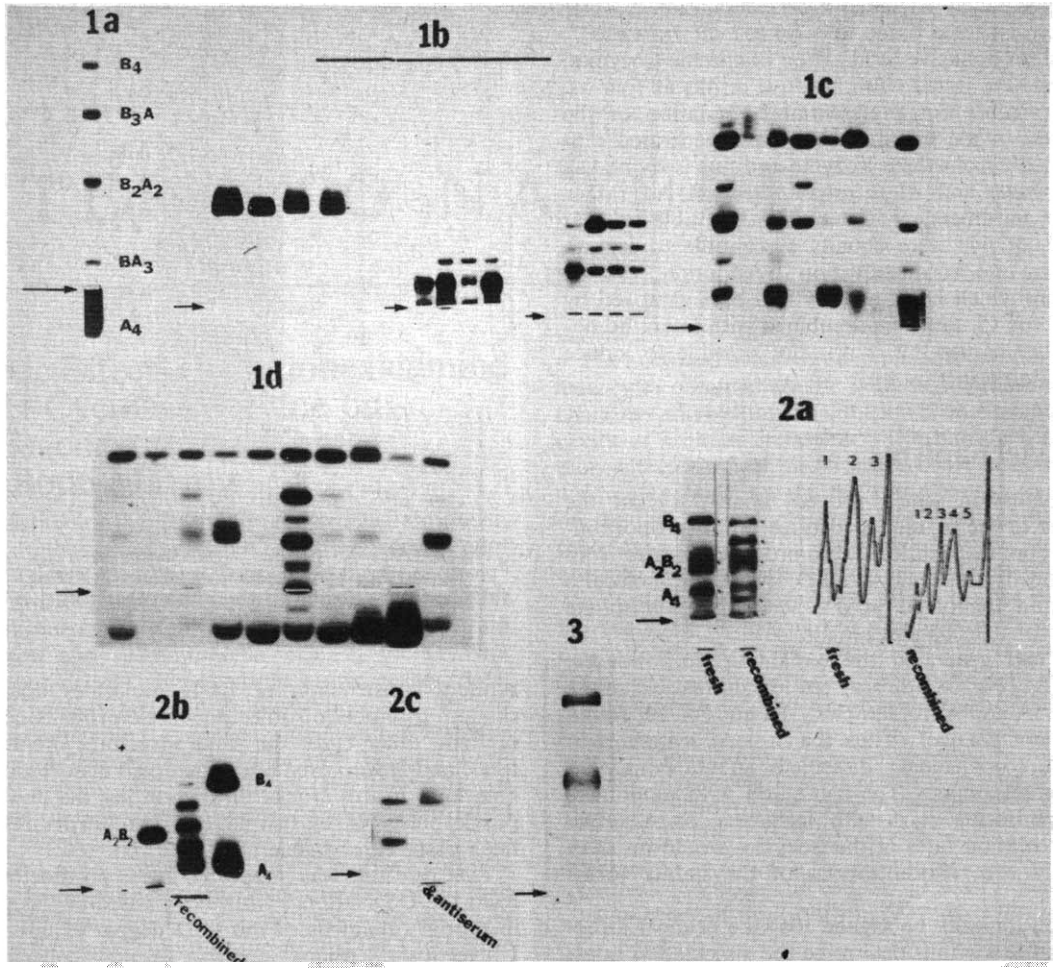

Fig. 1 *a*, LDH of the dog, *Canis familiaris*, showing five molecular forms (isozymes) and their subunit composition. Starch gel electrophoresis, *p*H 8.6. Arrows indicate sample origin. *b*, Gel at left: LDH isozymes of smelt, *Osmerus mordax*. Sample slots contain extracts of, left to right: gonad, muscle, kidney, heart. Centre gel: LDH of porgy, *Stenotomus versicolor*: left to right: kidney, eye, brain, air sac. Gel at right: sergeant major, *Abudefduf marginatus*: heart, muscle, eye, brain. Starch, *p*H 8.6. *c*, Alewife, *Alosa pseudoharengus*: liver, gut, brain, spleen, eye, heart, gills, kidney. Starch, *p*H 8.6. *d*, Silver hake, *Merluccius bilinearis*: gills, brain, eye, kidney, gonad, heart, stomach, gut, liver, muscle. Preparation of samples for electrophoresis was by homogenization in three volumes of electrophoresis buffer at 4° C, followed by centrifugation at 10,000*g* for 20 min.

Fig. 2 *a*, Dissociation–recombination of LDH from the three isozyme mackerel, *Scomber scombrus*, to yield five isozymes. Left, acrylamide gels, *p*H 9.5; right, densitometry. *b*, Left, pure mackerel A2B2: right, pure A4 and B4; centre: five products of dissociation–recombination of A4 × B4 and of A2B2 × itself. Starch, *p*H 8.6. *c*, Left, sergeant major LDH; right, sergeant major LDH treated with antiserum to LDH A4 of a related species. Starch, *p*H 8.6. For dissociation, pure LDH samples were reduced with 8 M urea, alkylated with 0.01 M iodoacetamide, at 20° C, under nitrogen, for 30 min. Urea gel electrophoresis is described in ref. 3, and with a more detailed technique in ref. 12.

Fig. 3 Dissociation of pure fluke (*Paralichthys dentatus*) LDH into two subunits. Urea-acrylamide, *p*H 4.5.

Similar experiments could be performed with five isozyme fish LDH; A3B, A2B2 and AB3, reduced in fresh tissue, were generated *in vitro* by dissociation–recombination of whole LDH or of A4 with B4 (ref. 12), and the subunit structures were confirmed with antibody. By similar experiments, two isozyme fish LDH was shown to be A4 and B4; one isozyme fish LDH was shown to be A2B2 because it dissociated into two subunits when reduced and alkylated (Fig. 3) and because dissociation–recombination with known homopolymers yielded twelve to fifteen products[12].

These fish LDH patterns pose a theoretical dilemma: if the gene, as encoded in primary protein structure, were the sole determinant of tertiary and quaternary structure, then subunit assembly should be identical in fresh tissue *in vivo* and *in vitro*, and identical in different tissues. In this case subunit assembly is restrictive in fresh tissue, random *in vitro*, and different in different tissues.

The simple explanation, that two isozyme LDH is monomeric-A and B, whereas three isozyme LDH is dimeric-AA, AB and BB, would not hold because a tetrameric structure was demonstrated by the dissociation–recombination experiments described, and also by molecular weight studies: both dextran gel chromatography[13] and ultracentrifugation[14] gave identical molecular weights for all fish and mammalian LDH studied. These were: fish, *Paralichthys dentatus* (one isozyme); *Stenotomus versicolor* (two isozymes); *Abudefduf marginatus* and *Scomber scombrus* (three isozymes); *Alosa pseudoharengus* (five isozymes); mammals, *Bos bovis* and *Equus caballus*. The explanation that the sites of subunit synthesis are isolated was excluded by cloning cultures[15,16] of two isozyme fish tissues; each clone produced the same LDH pattern as whole fresh tissue (Fig. 4). Furthermore, electrophoresis of single cells from two isozyme and three isozyme fish[17] revealed two and three LDH isozymes respectively. Subcellular isolation

of subunit synthesis was excluded by fractionation of two isozyme and three isozyme tissue into subcellular fractions[18], and this revealed no exclusive localization of specific isozymes. Other hypotheses were tested, with negative results as follows. There was no evidence for preferential degradation of the missing structures: when five molecules were generated randomly and purified[4], from three isozyme and five isozyme fish (*Abudefduf marginatus* and *Alosa pseudoharengus*), A4, rather than the missing tetramers, proved most susceptible to heat inactivation and all five were equally susceptible to trypsin and chymotrypsin and to inactivation by dilution. Tissue homogenates from which LDH activity had been removed by heating to 60° C for 15 min were combined with intact homogenates from other tissues but it was not possible to induce tissue-specific patterns in this way.

Epigenetic Mechanisms

The only explanation which proved tenable is that the dissociation rates of the missing structures are disproportionately high. Random distributions were produced *in vitro* from three isozyme and five isozyme fish LDH, and the molecules were purified[4] and incubated in 0.1 M sodium phosphate (*p*H 7), at 4° C with 1/1,000 merthiolate. After 2–4 weeks, the A3B molecules had generated the remaining isozymes A4, A2B2, AB3 and B4 (Fig. 5). Similar but less dramatic changes occurred in the B3A and A2B2 samples. B4 and A4, separated or together, did not change. Thus the missing structures in fresh fish LDH complements dissociate spontaneously in favour of stabler tetramers. The fish starts with monomers whereas the experiments start with tetramers, so a subtle difference in dissociation rates of the isozymes would result in disproportionately high concentrations of the stabler forms *in vivo*.

Although it can logically be argued that the primary structures of the A and B subunits determine the dissociation rates, this fails to explain the fact that the degree and mode of restrictive association differs in different tissues of the same organism. Because different tissues have different concentrations of various ions, protein and coenzyme factors known to affect dissociation rates in LDH[8,9], it seems likely that there would be tissue-specific dissociation rates producing tissue-specific patterns. The conclusion remains, however, that beyond the level of synthesis of the two subunits, epigenetic mechanisms determine the mode of subunit association and the number of molecular forms of LDH.

Regulation of structure and synthesis of proteins by cells is known to be achieved by induction, repression, hormonal stimulation, translational control[19,20]; all these are mechanisms which operate at the level of the genetic message, but non-

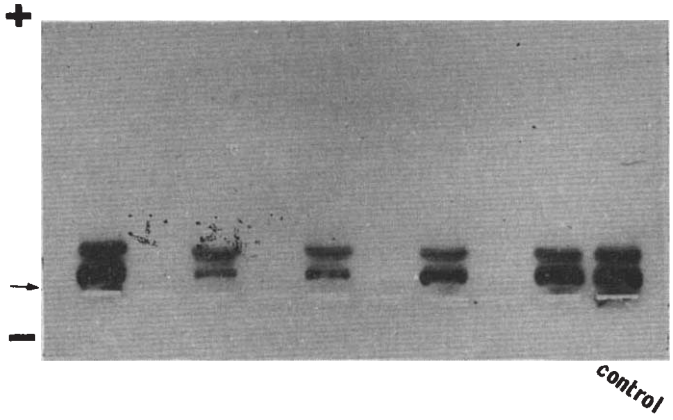

Fig. 4 LDH from clones of porgy (*Stenotomus versicolor*) kidney. Each clone has the same pattern as the whole fresh tissue (last slot on right). Starch, *p*H 8.6.

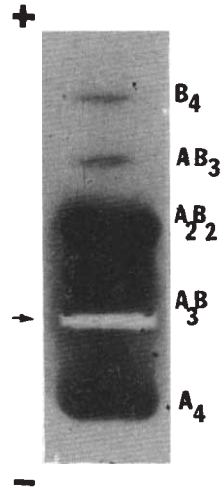

Fig. 5 Alewife (*Alosa pseudoharengus*) A_3B, generated *in vitro*, purified, and stored at 4° C for 3 weeks. Spontaneous dissociation equilibria favour the other forms A4, A_2B_2, B4. Starch, *p*H 8.6.

random construction of LDH molecules does not. Non-random assembly of other polymeric proteins occurs[21,22], but in these other cases the same structures are found in fresh tissue and *in vitro* even after attempted dissociation and recombination; in this crucial distinction lies the uniqueness of the puzzle presented by fish LDH, where structures absent from fresh tissue can be generated *in vitro*.

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