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Subunit Interactions of Vertebrate Lactate Dehydrogenase: II. Molecular Hybridizations

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척추동물 젖산탈수소효소 하부단위체의 상호작용 Ⅱ. 분자 교잡

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적 요

척추동물 6종의 젖산탈수소효소의 초자내 종간 및 종내 분자교잡을 실시한 결과 드렁허리 H_4 동위효소와 가물치 M_4 동위효소의 교잡 및 가물치 M_4동위효소와 메기 동위효소의 교잡에서 H_2M_2 동위효소라고 생각되는 교잡형 동위효소를 얻었다.

하등 척추동물 젖산탈수소효소의 하부단위체 M과 H의 상호간 조합에 관한 한 3차구조의 하부단위체는 동일한 구조를 가진 하부단위체와 이량체를 형성하고 이러한 동질이량체가 사량체를 형성한다고 사료된다.

INTRODUCTION

On the basis of a variety of observations (Appella and Markert, 1961; Cahn *et al.*, 1962) it is generally accepted that lactate dehydrogenase (EC. 1.1.1.27; LDH) is composed of four subunits and that two types of subunit are present in tissues of most species. These two monomers, the subunit M and subunit H, combine each other to produce five distinct tetramers.

The electrophoretic patterns of LDH isozymes from numerous vertebrates reveal the tissue specificity (Fine *et al.*, 1963). The M_4 isozyme is the principal form in skeletal muscle and the H_4 isozyme predominates in heart (Dawson *et al.*, 1964), although there is some evidence which renders the terms muscle-prominent or heart-prominent inadequate (Peter *et al.*, 1971).

This simple model, however, cannot be applied straight-forwardly to the LDHs of teleost fishes and amphibians. In the tissues of many teleosts, the *in vivo* assembly of two subunits appears to be restricted such that only two isozymes (M_4 and H_4) or three (M_4, M_2H_2 and H_4) are formed as determined by electrophoresis (Markert and Faulhaber, 1965). Furthermore, isozyme patterns in teleosts also suggest a third locus responsible for subunit E (Whitt, 1969). This third subunit is highly tissue-specific in its function and produces with the subunit M and/or H several heterotetramers (Whitt, 1970a).

The absence of the two heterotetramers, M_3H and MH_3, is not due to a temporal or spatial isolation of subunit synthesis but due to the intrinsic properties of the M and H polypeptides. Those epigenetic control mechanism upon the subunit assembly is primarily concerned with the structure of hydrophilic surface of each subunit.

The elucidation of structure and function of a protein needs large observations on the phylogeny of the protein and *vice versa* (Nelson, 1970). The LDH isozymes separated by various electrophoretic techniques reveal species-specificity (Shows *et al.*, 1969; Scholl and Holzberg, 1972), suggesting that there are exquisite intracellular mechanisms controlling the subunit assemblies.

In the present work with vertebrate LDH isozymes, the authors have suggested possibilities that, once the tertiary polypeptides are discharged from their polysomes, the like subunits interact with the like subunits and that the tetramer isozyme is a dimer of homodimer.

MATERIALS AND METHODS

Specimens

The fresh-water fishes *Fluta alba* and *Ophicephalus argus* and the chicken were obtained from commercial dealer in Seoul. The squamate *Rhabdophis tigrinus* were collected in the Yong Moon Mt. area, the frog *Hyla arborea japonica* at the southern vicinity of Seoul and the fresh-water fish *Parasilurus asotus* at the Choon Chun Reservoir.

Purification of LDH isozymes

Four hundred and fifty grams of *R. tigrinus* skeletal muscle was homogenated with 1350 ml cold distilled water in a homogenizer. The first and second precipitations by ammonium sulfate were made according to the method of de Burgos *et al.* (1973). The resulting precipitate was collected by centrifugation and dialyzed against 0.01 M potassium phosphate buffer, pH 7.0.

The dialysate was heated at 60°C for 3 minutes and allowed to stand at 4°C for 2 hrs. The solution was made clear by centrifugation and the still red supernatant was reduced to its half volume by the treatment of the polyethylene glycol (M.W. 6,000) on the surface of dialysis sack (Sigma) within 40 minutes at 10°C.

The resulting solution was layered on the Sephadex G-100 column (4.9×34 cm) and eluted with 0.05 M potassium phosphate buffer.

The enzyme solution was added to a DEAE-cellulose column (2.5×25 cm) and the fractions with the M_2H_2 LDH isozyme activity were pooled, dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, and stored at −20°C.

Three isozymes of *F. alba* H_4, *F. alba* M_4 and *O. argus* M_4 were purified by the method of Park *et al.* (1979).

Electrophoresis on cellulose acetate strips

The cellulose acetate strip (Millipore) was cut in dimension of 2.5×5 cm, prebuffered with the barbital buffer (pH 8.6, ionic strength 0.075) and placed between two cells bathed in sufficient amount of ice. The proteins were migrated for 1 hr at a constant 100 volts per 7.5 cm. LDH isozymes were stained with mixture of nicotinamide adenine dinucleotide, lactate, phenazine methosulfate and nitro blue tetrazolium (Park and Cho, 1972) and allowed to be fixed in diluted acetic acid solution.

Heat denaturation

For studies of temperature inactivation, 0.5 ml of an extract was placed in each of six glass test tubes (1.3×11 cm) and then simultaneously placed in a 60°C water bath. After a given time, each test tube was cooled rapidly to 4°C and the precipitate was removed by centrifugation. The clear supernatant was subjected to the electrophoresis.

When larger amount of enzyme solution were heat-denatured, 80 ml of the solution in 250 ml Erlenmeyer flask was placed in a 60°C water bath and constantly swirled for a given time.

LDH activity and protein assays

LDH activity was assayed colorimetrically by determining the formation of NAD in the mixture of 48 mM potassium phosphate buffer, 0.6 mM pyruvate and 0.18 mM NADH (Bergmeyer and Bernt, 1974) at 340 nanometers with Spectronic 20 (Bausch & Lomb). Protein concentration was measured according to the method of Lowry *et al.* (1951) with the bovine serum albumin as a standard protein.

In vitro molecular hybridization

The LDH isozymes to be hybridized were mixed together and the sodium chloride was added to the enzyme solution to both final concentration of 1.0 M (Markert, 1963) and 0.1 M (Whitt, 1970a). When needed the equal enzyme activity in the mixture was established. The mixture was frozen at −20°C for 12 hrs and thawed at 4°C. After centrifugation, the supernatant was electrophoresed. The tank buffer was replaced by fresh one after every run. During the studies with the crude extract, the solution was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0.

RESULTS

Electrophoresis

Upon electrophoresis at pH 8.6, tissue extracts of *F. alba* showed characteristic tissue-specificity; the skeletal muscle and heart tissues possess two homotetramers of M_4 and H_4 isozymes with their relative activities reversed and the brain tissue revealed the M_2H_2 isozyme equally spaced from both homotetramers and one LDH isozyme considered to have at least one product of gene E at the anodal side of the M_4 isozyme (Fig. 1). The zymograms of the liver and kidney tissues were similar to that of the heart tissue.

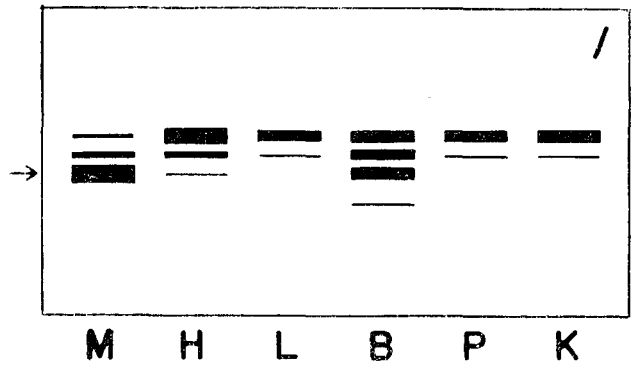

Fig. 1. Tissue specificity of LDH isozymes from *F. alba* tissues. An LDH isozyme containing E subunit is shown at the anodal side of the M_4 isozyme of brain tissue. M, skeletal muscle; H, heart; L, liver; B, brain; P, pancreas; K, kidney. Upper side is the cathode and arrow indicates the origin.

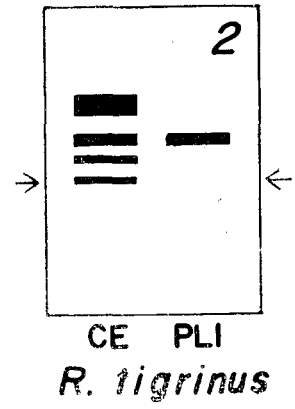

Fig. 2. Final purification of the M_2H_2 isozyme of *R. tigrinus* skeletal muscle. CE, crude extract; PLI, purified LDH isozyme.

Heat inactivation

The inactivation rate of a protein by heat is not dependent upon the protein concentration. The heat inactivation of *F. alba* H_4 isozyme is more sensitive than that of the M_4 isozyme (Fig. 3). LDH isozymes in *O. argus* skeletal muscle after concentration by the ammonium sulfate precipitation were heated, leaving the results that M_3H isozyme is thoroughly inactivated by nearly one-minute heating at 60°C and that while the M_4 isozyme activity is diminished by time consumption, those of the three isozymes containing more than one subunit H is not changed at least in ten minutes (Fig. 4). The M_3H isozymes in brain and heart tissues are also labile to heat inactivation just as that of skeletal muscle. The squamate *R. tigrinus* has the M_4 isozyme which is more heat sensitive than the other three isozymes (Fig. 5).

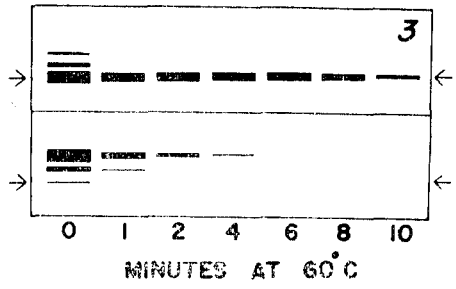

Fig. 3. Comparison of heat stability of the isozymes M_4 and H_4 from *F. alba* skeletal muscle(upper) and kidney (lower). The H_4 isozyme is more heat sensitive than the M_4 isozyme.

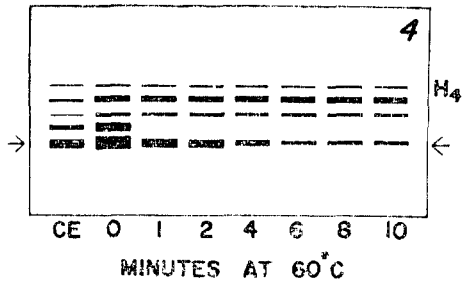

Fig. 4. Heat stability of LDH isozymes from *O. argus* skeletal muscle. CE means the crude extract solution. The concentrated solution of CE by ammonium sulfate precipitation and subsequent dialysis was subjected to heat inactivation. The M_3H isozyme is dramatically heat inactivated.

In vitro molecular hybridizations

The subunits of the M_4 and H_4 isozymes in *F. alba* were dissociated from their parental isozymes by treatment of sodium chloride and by subsequent freezing at −20°C and

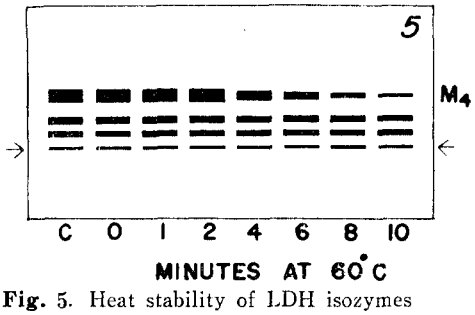

Fig. 5. Heat stability of LDH isozymes from *R. tigrinus* skeletal muscle. C means the crude extract. The M_4 isozyme is more labile to heat inactivation.

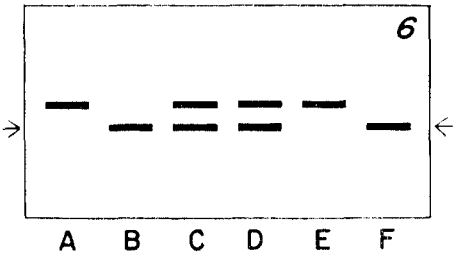

Fig. 6. Hybridization between *F. alba* M_4 isozyme and *F. alba* H_4 isozyme. A mixture in a desired concentration of sodium chloride was freezed at −20°C for 12 hrs and then thawed at 4°C. A means the purified *F. alba* H_4 isozyme; B, the purified *F. alba* M_4 isozyme; C, hybridization between M_4 and H_4 isozymes in 0.1 M sodium chloride; D, hybridization between M_4 and H_4 isozymes in 1.0 M sodium chloride; E and F, respectively H_4 and M_4 isozyme after freezing-thawing in 1.0 M sodium chloride.

reassociated to yield only the parental isozymes (Fig. 6). The activity of the parental isozymes in 1.0 M sodium chloride solution was not affected by freezing at −20°C. The hybridization between LDH isozymes in crude extracts of skeletal muscle and kidney tissues also failed to produce the expected M_2H_2 isozyme.

In the mixture of *F. alba* H_4 isozyme and *O. argus* M_4 isozyme, a hybrid isozyme of equal distance from the parental isozymes was made when these parental isozymes were led to hybridize in sodium chloride in the concentration of 0.1 M (Fig. 7). The effect of the high concentration of the salt on the activity of *O. argus* M_4 isozyme was observed. The binomial distribution of isozymes was not obtained after various hybridizations between *O. argus* crude extracts of skeletal muscle and liver tissues (Fig. 8).

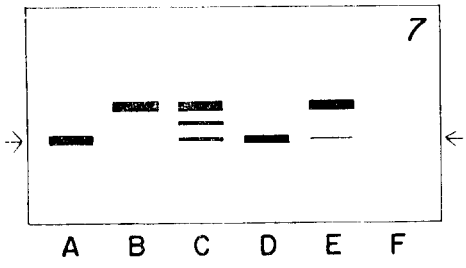

Fig. 7. Hybridization between *F. alba* H_4 isozyme and *O. argus* M_4 isozyme. A means *O. argus* M_4 isozyme; B, *F. alba* H_4 isozyme; C, hybridization between *O. argus* M_4 isozyme and *F. alba* H_4 isozyme in 0.1 M sodium chloride; D, *O. argus* M_4 isozyme in 0.1 M sodium chloride; E, hybridization between *O. argus* M_4 isozyme and *F. alba* H_4 isozyme in 1.0 M sodium chloride; F, *O. arugs* M_4 isozyme in 1.0 M sodium chloride.

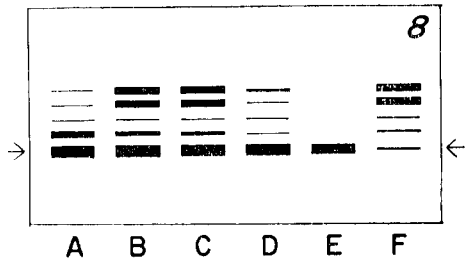

Fig. 8. Hybridization between isozymes in the crude extract mixture of *O. argus* skeletal muscle and *O. argus* liver. A means the isozymes of skeletal muscle; B, the mixture of A and F; C, hybridization between isozymes of B in 0.1 M sodium chloride; D, hybridization between isozymes of B in 0.3 M sodium chloride; E, hybridization between isozymes of B in 0.5 M sodium chloride; F, the isozymes of liver.

The rate of electrophoretic migration of *R. tigrinus* M_2H_2 isozyme is almost between the rates of the M_4 isozyme and the H_4 isozyme of *F. alba*. No hybrid isozymes were made between the *R. tigrinus* M_2H_2 isozyme and *F. alba* two homotetramers (Fig. 9). The M_2H_2 isozyme in *R. tigrinus* was strongly inactivated even at the sodium chloride concentration of 0.1 M.

The hybridization between the *R. tigrinus* M_2H_2 isozyme and *O. argus* M_4 isozyme was led to the failure because of the instability of both parental isozymes to the low concentration of sodium chloride (Fig. 10).

Each of the skeletal muscle tissues of the frog *H. arborea japonica* and the chicken shows one LDH isozyme with the electrophoretic migration rate nearly the same as that of *F. alba* H_4 isozyme. No subunit exchange in 1.0 M sodium chloride solution was made in the hybridizations between the *F. alba* M_4 isozyme and chicken isozyme and between the

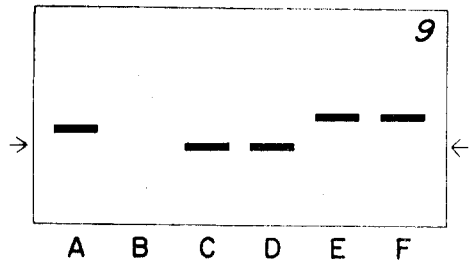

Fig. 9. Hybridization between *F. alba* isozymes and *R. tigrinus* M_2H_2 isozyme. A means the *R. tigrinus* M_2H_2 isozyme; B, *R. tigrinus* M_2H_2 isozyme in 0.1 M sodium chloride; C, hybridization between *R. tigrinus* M_2H_2 isozyme and *F. alba* M_4 isozyme in 0.1 M sodium chloride; D, *F. alba* M_4 isozyme; E, hybridization between *R. tigrinus* M_2H_2 isozyme and *F. alba* H_4 isozyme in 0.1 M sodium chloride; F, *F. alba* H_4 isozyme.

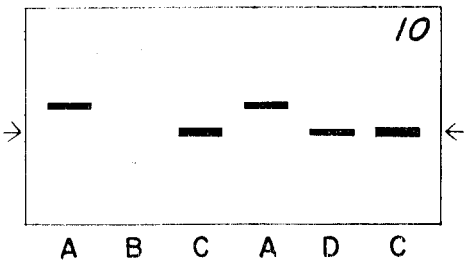

Fig. 10. Hybridization between *O. argus* M_4 isozyme and *R. tigrinus* M_2H_2 isozyme. A means *R. tigrinus* M_2H_2 isozyme; B, hybridization between *R. tigrinus* M_2H_2 isozyme and *O. argus* M_4 isozyme in 1.0 M sodium chloride; C, *O. argus* M_4 isozyme; D, hybridization between *R. tigrinus* M_2H_2 isozyme and *O. argus* M_4 isozyme in 0.1 M sodium chloride.

F. alba M_4 isozyme and the frog isozyme (Fig. 11). The factor of sodium chloride concentration of 0.1 M did not make any hybridizations.

Another fresh-water fish *P. asotus* has one LDH isozyme with almost similar rate of

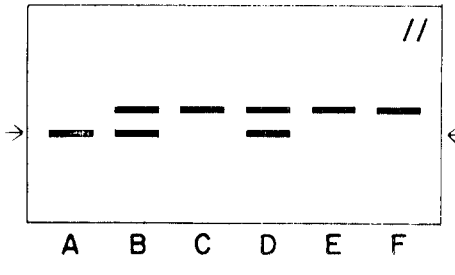

Fig. 11. Hybridization between *F. alba* M_4 isozyme and chicken and between *F. alba* M_4 isozyme and *H. arborea japonica* isozyme. A means *F. alba* M_4 isozyme; B, hybridization between *F. alba* M_4 isozyme and chicken isozyme in 1.0 M sodium chloride; C, chicken isozyme; D, hybridization between *F. alba* M_4 isozyme and *H. arborea japonica* isozyme in 1.0 M sodium chloride; E, *H. arborea japonica* isozyme; F, *H. arborea japonica* isozyme in 1.0 M sodium chloride.

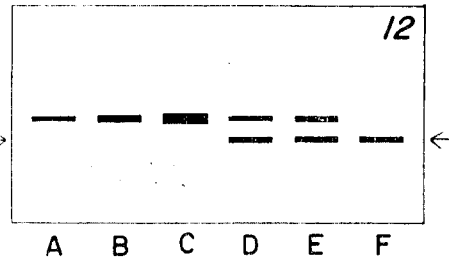

Fig. 12. Hybridization between *F. alba* M_4 isozyme and *P. asotus* isozyme. A means *P. asotus* isozyme in 1.0 M sodium chloride; B, *P. asotus* isozyme in 0.1 M sodium chloride; C, *P. asotus* isozyme; D, hybridization between *F. alba* M_4 isozyme and *P. asotus* isozyme in 1.0 M sodium chloride; E, hybridization between *F. alba* M_4 isozyme and *P. asotus* isozyme in 0.1 M sodium chloride; F, *F. alba* M_4 isozyme.

electrophoretic migration to that of the *F. alba* H_4 isozyme. When the *P. asotus* isozyme was forced to make hybridization with *F. alba* M_4 isozyme, the subunit exchange was not observed (Fig. 12).

The subunits of *O. argus* M_4 isozyme were again hybridized with those of the *P. asotus* isozyme when the mixture was in the sodium chloride solution of 0.1 M (Fig. 13). No hybrid tetramers were made in the hybridizations between *O. argus* M_4 isozyme and chicken isozyme and between *O. argus* M_4 isozyme and *H. arborea japonica* isozyme (Fig. 14). The M_4 isozyme in the crude extract of *O. argus* skeletal muscle still reserves its activity in the sodium chloride solution of 2.0 M. At this concentration, however, the other four isozymes lose their activities to zero.

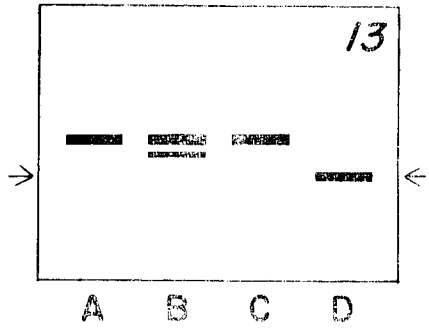

Fig. 13. Hybridization between *O. argus* M_4 isozyme and *P. asotus* isozyme. A means *P. asotus* isozyme; B, hybridization between *O. argus* M_4 isozyme and *P. asotus* isozyme in 0.1 M sodium chloride; C, hybridization between *O. argus* M_4 isozyme and *P. asotus* isozyme in 1.0 M sodium chloride; D, *O. argus* M_4 isozyme.

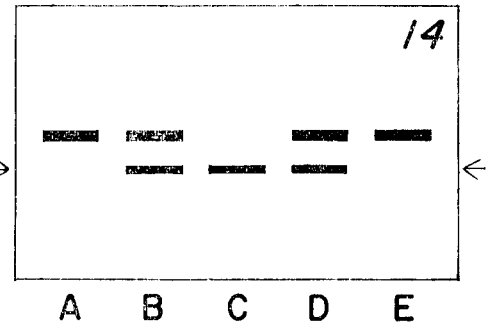

Fig. 14. Hybridization between *O. argus* M_4 isozyme and chicken isozyme and between *O. argus* M_4 isozyme and *H. arborea japonica* isozyme. A means the chicken isozyme; B, hybridization between *O. argus* M_4 isozyme and chicken isozyme in 0.1 M sodium chloride; C, the *O. argus* M_4 isozyme; D, hybridization between *O. argus* M_4 isozyme and *H. arborea japonica* isozyme in 0.1 M sodium chloride; E, the *H. arborea japonica* isozyme.

DISCUSSION

In this work, four kinds of electrophoretic patterns of vertebrate LDH isozymes were considered; one isozyme in *P. asotus*, *H. arborea japonica* or chicken, three isozymes in *F. alba*, four isozymes in *R. tigrinus* and five isozymes in *O. argus*. The results from polyacrylamide gel electrophoresis of the LDH isozymes from these six species carried out by the method of Tombs and Akroyd (1967) were similar to the results from the

cellulose acetate electrophoresis.

Of the four groups, the first could be considered to be two isozymes of M_4 and H_4 having same net charge at their subunit surface or to be one isozyme resulted from the absence of gene action of presumably H gene. The latter is more acceptable on the basis of several observations (Pesce *et al.*, 1964). The second takes large parts in teleost taxon and the absence of the M_2H_2 isozyme is frequently found. Third is shown in the skeletal muscle, liver and heart of the squamates which demonstrate the absence of the M_3H isozyme (Park *et al.*, 1976). The fourth is the most old type in the history of LDH isozyme studies.

Molecular weight of teleost LDH isozymes

D-Lactate dehydrogenase was found in horseshoe crab (Selander and Yang, 1970) and in octopus spermatozoa (Mann *et al.*, 1974). Although there has been a change of substrate specificity from D-lactate to L-lactate in the LDH evolution, the molecular weight of the enzymes in teleost is reported to be approximately 140,000 (Massaro, 1973) as is that of beef and chicken (Pesce *et al.*, 1964). Supposing that a trace of the molecular weight 65,000 of horseshoe crab D-LDH revealed by Long and Kaplan (1968) could exist in Pisces taxon, the three-isozyme pattern demonstrated by electrophoresis of several tissues in *F. alba* might be interpreted such that the subunit assemblies of these three isozymes are M_2, MH and H_2.

This consideration, however, cannot be accepted on the basis of the fact that, when the two purified LDH isozymes of *F. alba* and the M_2H_2 isozyme of *R. tigrinus* were forced to be cofractionated on a Sephadex G-100 column with sufficient height (2×100 cm) by the method of Determann (1969), they were found in similar fraction. This result, the electron micrograph of LDH isozyme (Dudman and Zerner, 1973) and other several clues later discussed might suggest that the subunit combinations of LDHs in *F. alba* and, by extension, in most teleost fishes seem to be $(M_2)_2$, $(M_2)(H_2)$ and $(H_2)_2$.

In vitro molecular hybridizations of LDH isozymes

Since hybridization could occur in the crude extract (Salthe *et al.*, 1965), the degree of purity of the parental enzyme apparently is not critical. The temporal or spatial compartmentalization is not required as far as assembly of subunits of cytoplasmic enzymes is concerned (Teipel and Koshland, 1971a). The *in vitro* molecular hybridization of *F. alba* $M_4\times$*F. alba* H_4 did not produce the M_2H_2 isozyme as well as the other two hybrid isozymes in which the former was expected to be assembled because of the *in vivo* occurrence of the isozyme in brain tissue. The failure of the hybrid isozymes may verify the suggestion of Lindy (1974) that the reassociation of subunits M is more rapid and extensive than subunits H in rat liver.

The amino acid sequence of LDH active site from mammals to fishes are reported to be almost identical (Taylor *et al.*, 1973). Homologies between isozymes of fishes and

those of higher vertebrates are apparent to be true by physical, catalytic and immunological criteria (Bailey and Wilson, 1968). It could be said, however, that the configuration of polypeptide at the subunit combining site of subunit M is different from that of subunit H on the basis of above result of intraspecific molecular hybridization.

Each of the interspecific molecular hybridization of *F. alba* $H_4 \times$ *O. argus* M_4 and that of *O. argus* $M_4 \times$ *P. asotus* isozyme showed a hybrid isozyme which is considered, by the criterion of electrophoretic mobility, to have two subunits from one parental isozyme and two from the other parental one. Electrophoresis of the hybrid isozyme through polyacrylamide gel also showed the mean mobility.

It is evident that environmental factors have a decided influence on the *in vitro* refolding of denatured polypeptide chains. The concentration of sodium chloride is believed to influence differentially on the formation of tertiary structure of protein. *In vitro* hybridization experiments employing conditions previously used by several investigator (Markert, 1963), i.e., 1.0 M sodium chloride, did not induce satisfactory hybridization between M_4 and H_4 isozymes of teleost fishes. The M_4 or H_4 isozyme of *F. alba* was not affected at 1.0 M sodium chloride concentration whereas the M_4 isozyme of *O. argus*, which has the same enzyme activity as the isozymes of *F. alba*, was seriously affected at that concentration.

It is highly accepted that the subunit interactions are essential for LDH activity (Chan and Mosbach, 1976). In the hybridization between the *O. argus* M_4 isozyme and the *P. asotus* isozyme, the subunit M of *O. argus* was strongly denatured to produce a polypeptide which lost its native tertiary structure. This severe denaturation is exemplified by the *R. tigrinus* M_2H_2 isozyme bathed in 0.1 M sodium chloride solution. Teipel and Koshland (1971a) reported their results that the per cent regain of biological activity varied dramatically from enzyme to enzyme when they were denatured and renatured under identical conditions and that the recovery of porcine heart LDH activity was relatively low to other enzymes. And the LDH was reported to be classified as a protein regaining only limited native structure after renaturation (Teipel and Koshland, 1971b). For the pig or rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, sodium chloride inhibits the catalytic activity even at low ionic strengths (Harrigan and Trentham 1973). The dissociation constant of the yeast glyceraldehyde-3-phosphate dehydrogenase is appreciably less than that of the rabbit muscle enzyme (Mockrin *et al.*, 1975).

Conservatism of subunit M

Some amino acid sites in a protein molecule vary considerably from species to species, while other sites are very conservative (Fitch and Markowitz, 1970). The total amounts of the highly variable sites in subunit M seems to be relatively less than those in subunit H. In other words, the subunit M of vertebrate LDH has been relatively conservative in its evolution.

The consevatism of subunit M could be revealed by several evidences. The genes M and H were diverged from a common ancestral gene and the third gene, gene E, was derived from the gene H (Whitt, 1970a). The subunit M locus was expressed almost exclusively in the white skeletal muscle of medaka, *Oryzias latipes*, while the subunit H locus in nearly all tissues (Philipp and Whitt, 1977). The subunits H in two *Rana* populations are far more variable than the subunits M (Salthe, 1969). Throughout the subgenus *Luxillus* (Cypriniformes) the subunits H have two electrophoretic mobilities while the subunits M are virtually indistinguishable (Rainboth and Whitt, 1974). This conservatism of subunit M may, in part, account for the observation that no hybrid isozymes were made in the intraspecific hybridization.

Heat instability of heterotetramers

The M_4 and H_4 isozymes differ not only in their electrophoretic and immunochemical properties but also in their heat stabilities. In general, the H_4 isozyme is more stable than the M_4 isozyme (Whitt, 1970a). The *F. alba* H_4 isozyme, however, is more sensitive to heat inactivation.

The temperature required for 50% inactivation of lower vertebrate LDH isozyme in 20 minutes is near 60°C. By contrasts, the birds and higher reptiles have LDH isozymes whose inactivation temperature is in the neighborhood of 80°C (Wilson *et al.*, 1964). The *R. tigrinus* LDH isozymes did not lose its activity after 10 minutes heating at 60°C.

It is interesting that the M_3H isozyme of *O. argus* skeletal muscle appears to dramatically lose its quaternary structure after relatively mild heat inactivation. The subunit combination of the 3M+H is likely to be more fragile when the intracellular buffer systems are damaged. This fragility of the M_3H isozyme is also shown in the *Barbus conchonius* (Cyprinidae) (Shaklee *et al.*, 1973). The marked instability of the M_3H isozyme is also dependent upon the pH of enzyme medium. The asymmetric heteropolymers of *Alosa pseudoharengus* (Clupeidae) LDHs, the M_3H and MH_3 isozymes, are strongly inactivated under more basic pH condition (Shaklee, 1975). It could be likely suggested that intersubunit interaction is considerably weaker in heterotetramers than it is in homotetramers.

In squamate tissue, *in vivo* absence of the M_3H isozymes is frequently found. Although the squamate skeletal muscle and liver tissues are pools of sufficient amounts of both the subunits M and H, there is no assembly of the M_3H isozyme (Markert, 1968; Park *et al.*, 1976). Electrophoretic analysis of amphibian tissue extracts has shown that most do not fit the five isozyme hypothesis and usually lack the heterotetramers (Moyer *et al.*, 1968).

Being a dimer of homodimer

The possibility that each of the three isozymes of *F. alba* is a dimer of homodimer could be substantiated by following considerations: (1) dimeric structure is the most com-

mon form which constitutes 52% of enzymes so far studied by sufficiently precise electrophoretic techniques (Harris, 1975); (2) *in vitro* molecular hybridization between the *F. alba* H_4 isozyme and the *O. argus* M_4 isozyme or between the *O. argus* M_4 isozyme and the *P. asotus* isozyme produced only one hybrid isozyme and (3) *in vivo* absence of the asymmetric heterotetramers is highly accepted by electrophoretic and immunochemical criteria (Shaklee, 1975; Park *et al.*, 1976) and the M_3H isozymes in teleost fishes are fragile when the buffer systems are damaged.

The subunit E in teleost are synthesized almost exclusively in eye and brain (Whitt *et al.*, 1971). The presence of a subunit E permits the assembly of subunits M and H into a heterotetrameric isozyme detected by electrophoresis even though the subunits M and H by themselves could not form heterotetrameric isozymes detectable by electrophoresis (Whitt, 1970a). This observation led him to make a hypothesis that the subunit E would exert its effect by changing the conformation of any subunit it binds to and thus the subunit E would act by removing conformational differences between the subunits M and H that normally prevent their association (Whitt, 1970b).

The two heterotetrameric isozymes, the M_3H and MH_3 isozymes, are not assembled in eye and brain. It might then be assumed that the tetramer is a dimer of homodimer as far as only the assemblies of subunit M and subunit H are concerned.

SUMMARY

In vitro intra- and interspecific molecular hybridizations of lactate dehydrogenase isozymes from six species were performed by freezing and thawing the isozymes in sodium chloride, resulting that each of the interspecific hybridization of *Fluta alba* $H_4 \times$ *Ophicephalus argus* M_4 and that of *O. argus* $M_4 \times$ *Parasilurus asotus* isozyme showed one hybrid isozyme considered to be an M_2H_2 isozyme.

The results fit with the suggestion that the isozyme of lower vertebrates is a dimer of homodimer as far as the assemblies of subunit M and subunit H are concerned.

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