EVOLUTION OF LACTATE DEHYDROGENASE GENES

Roger S. HOLMES
Department of Biochemistry, La Trobe University, Bundoora, 3083, Victoria, Australia

Received 29 August 1972

1. Introduction

The enzyme lactate dehydrogenase (LDH) exists as a complex system in vertebrate organisms and is encoded in two major structural genes (A and B), each resulting in a different subunit of LDH [1–3]. These polypeptides usually assemble randomly to form five tetrameric isozymes (A_4, A_3B, A_2B_2, AB_3, and B_4), although in certain fish, restriction in subunit assembly does occur and the expected five isozymes are not formed [4–7]. A third locus (C) is present in mammals and birds and functions only in primary spermatocytes producing a distinct LDH-C_4 isozyme [8–10]. An additional structural gene (E) has been established in teleosts (bony fish) and is expressed in retinal and nervous tissue resulting in the synthesis of LDH-E_4 as well as other isozymes containing A, B, and E polypeptide subunits [7, 11]. Studies on LDH from gadoid fish (eg. haddock, cod) have shown the presence of an LDH isozyme specific to liver tissue. Genetic and evolutionary variation of the LDH isozymes in these fish indicate that this isozyme is encoded at a separate locus (F) [12]. LDH from salmonid fish is determined by five gene loci [13, 14] resulting in the synthesis of more than fifteen isozymes in homozygous individuals. Cytological and biochemical studies have verified the existence of duplicated A (A and A′) and B (B and B′) loci in addition to the E locus in these fish [13–18].

Immunochemical procedures have been found to be very useful in studies related to biochemical evolution and phylogeny [19–21]. Antibodies to specific enzymes or isozymes may be used in the investigation of the structural similarities of gene products at different stages of evolution [20–22] or in establishing subunit homologies among isozyme systems [7, 17, 18, 23]. Immunochemical cross reactivity indicates similarities between the structure of the antigenic determinants which implies some degree of sequence resemblance. Recent studies on animal lysozymes have shown a correlation between the degree of cross reaction and their amino acid sequence and have determined that these enzymes fail to cross react if they differ by more than 30–40% in their amino acid sequence [24]. The selective precipitation activities of antibodies prepared against teleost homotetrameric isozymes of LDH (A_4 and B_4) with LDH isozymes from teleost and mammalian sources have been used in this study to describe the common evolutionary origin of this multiple enzyme system.

2. Methods

Antisera were prepared in rabbits against LDH isozymes A_4 and B_4 purified from muscle extracts of the sea trout (*Cynoscion regalis*) [23]. Antisera of high titres were obtained which cross reacted strongly with the antigen and with homologous isozymes from other species of fish but not with the heterologous isozyme [18, 23]. The antibodies were partially purified by ammonium sulphate precipitation (33% satn.), washed in 33% ammonium sulphate solution, and subsequently stored as a precipitate at 4°. This procedure removes from the antibody preparations the bulk of rabbit serum proteins including rabbit LDH isozymes. Prior to use in immunochemical experiments, the antibody preparations were dialysed against 30 mM Tris-HCl buffer, pH 7.4. The immunochemical cross reactivity of these antibodies with fish and mam-

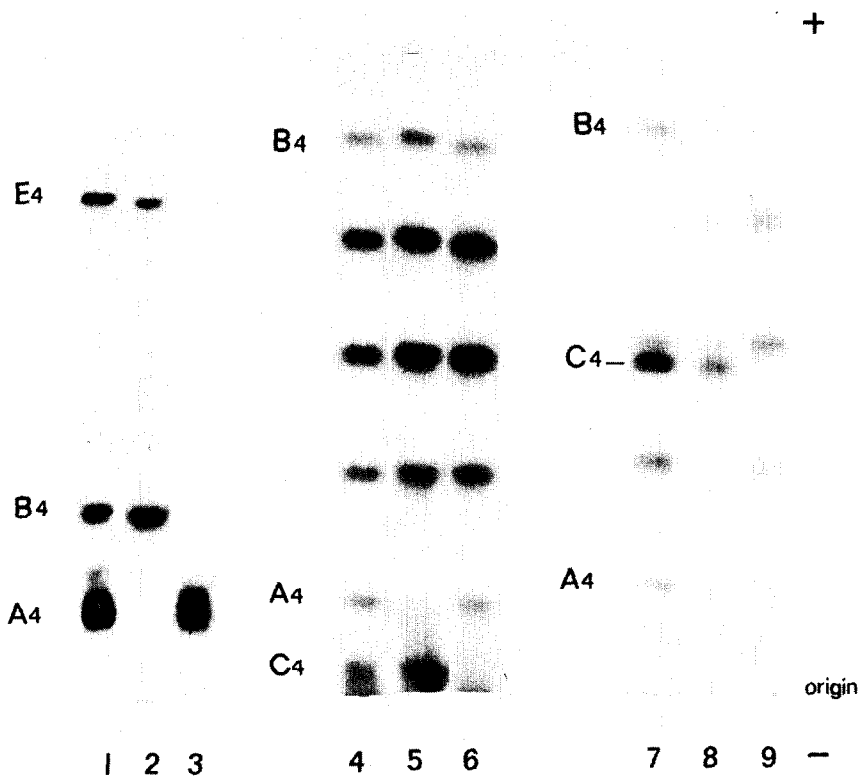

Fig. 1. Immunochemical precipitation of vertebrate LDH isozymes. The subunit composition of the homotetramers is indicated at the side of the photograph. Following pretreatment of tissue homogenates with antibodies, the samples were electrophoresed on 11% starch gels with Tris-citrate buffers [23]. Abbrevations in the legend are as follows: FRM, fish (flounder, *Ammotretis rostratus*) retina (30%)/muscle (5%) extract; PT, possum (*Trichosurus vulpecula*) testis extract (30%); MMT, marsupial mouse (*Sminthopsis crassicaudata*) testis extract (30%); CA, control rabbit antibodies; AB, anti-B antibodies; AA, anit-A antibodies. The tissue extracts and antibodies were mixed in ratios listed below and treated as described in the text. (1) FRM: CA = 9:1; (2) FRM: AA = 9:1; (3) FRM: AB = 9:1; (4) PT: CA = 1:9; (5) PT: AA = 1:9; (6) PT: AB = 1:9; (7) MMT: CA = 1:6; (8) MMT: AA = 1:6; (9) MMT: AB = 1:6.

malian isozymes have been investigated by a procedure combining selective precipitation by antibodies followed by the electrophoretic resolution of the unprecipitated isozyme [18, 23]. This method provides a sensitive measure of the immunochemical specificities of individual isozymes present in tissue homogenates and enables isozymes of differing specificities to be distinguished by adjusting the titre of the antibody added [18, 23]. The antibodies were added in various concentrations to mammalian tissue homogenates containing LDH's A_4, B_4, and C_4 and to fish tissue homogenates containing LDH's A_4, B_4, and E_4. The mixtures were allowed to react overnight in the cold and were then subjected to centrifugation (48,500 *g* × 30 min) and the supernatant applied to starch gels for electrophoresis and subsequent histochemical staining [18]. Using suitable controls with normal rabbit antibodies, it may be concluded that those isozymes, which have been removed from a zymogram following pretreatment with antibodies, are immunochemically related to the original antigen used in provoking anitbody production.

3. Results and discussion

The results on the immunochemical specificities of fish LDH isozymes show that the B and E subunits are

immunochemically related since the anti-B antibodies precipitated LDH-B_4 and E_4 isozymes but not LDH-A_4 (fig. 1, slot 3). Conversely, the anti-A antibodies cross reacted with LDH-A_4 and did not precipitate the other isozymes (fig. 1, slot 2). These results confirm previous studies [7, 18, 23], and indicate that a degree of sequence resemblance exists between the LDH-B_4 and E_4 isozymes. Further evidence for their homology is exemplified by the studies of Whitt [7] who has reported striking similarities in their kinetic and physical properties. A recent detailed investigation into the phylogenetic distribution of the LDH-E gene in fish has shown that it is absent in primitive fish but present in most teleosts and expressed specifically in retinal and neural tissue [25].

Fig. 1 also demonstrates cross reaction of antibodies prepared against fish LDH-A_4 and B_4 with mammalian LDH isozymes. An increased ratio of antibody to antigen (approx. 100 times) is required to precipitate mammalian LDH's in comparison to those from fish. Protein structure studies for homologous proteins of different species have shown that the number of amino acid replacements is inversely proportional to the phylogenetic relatedness of the species being investigated [20, 26]. Proteins from closely related species showed few or no differences while those from distantly related organisms are more dissimilar. As a result, it is expected that homologous mammalian LDH isozymes should exhibit decreased immunochemical reactivity with antibodies prepared against teleost LDH's. The results show that anti-A antibodies precipitate LDH-A_4 from testis extracts of two marsupial species, possum (*Trichosurus vulpecula*) and marsupial mouse (*Sminthopsis crassicaudata*) (slots 5 and 8), whereas the anit-B antibodies cross react with LDH-C_4 and do not show any activity with LDH's A_4 and B_4 (slots 6 and 9). These observations indicate some degree of sequence homology between LDH-A_4 isozymes from teleost fish as well as between LDH-B_4 from fish and LDH-C_4 from marsupials.

Evolution at the molecular level may be regarded as a process in which the DNA content of cells increases and the nucleotide sequences in molecules of DNA changes so as to increase and adapt the total genetic information. Gene duplication has emerged as playing an important role in evolution [27] and has been proposed as the mechanism for the creation of multiple genes for proteins of similar amino acid sequence which perform the same function [15, 18, 27] or different functions [26], as well as in the creation of larger genes by duplication and fusion (eg. ferredoxin and immunoglobulins) [26]. Gene duplication is considered to have been achieved by unequal crossover of chromosomes or by chromosomal duplication [15]. Evidence is available which indicates that both of these processes have played some role in evolution. The four polypeptides of hemoglobin (alpha, beta, gamma, and delta) and myoglobin are highly homologous in their amino acid sequences and Ingram [27] has suggested that their genes were all derived from a common ancestor. By comparing the extent of homology of the various chains, he has reconstructed the most probable order of duplications during evolution. The delta and beta chains are most closely related and are proposed as being products of the most recent duplication event. If duplication was achieved by unequal crossover, then genes should be closely linked which is in fact the case [28]. Evidence for chromosomal duplication arose from enzyme and karyotypic studies on trout which indicated that these fish are derived from tetraploid ancestors [13–18]. If polyploidization had occurred and the resultant duplicated loci undergone some degree of divergence, there should be two loci present for each original locus in the genome. This has been

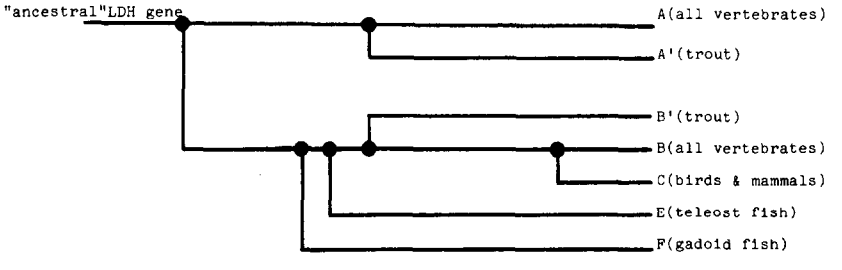

Fig. 2. Proposed evolutionary relationships of LDH genes in vertebrates. • Represents a gene duplication event.

found for at least a number of multiple protein systems: LDH [13–18]; malate dehydrogenase [29]; 6-phosphogluconate dehydrogenase [30]; creatine kinase [31]; glycerophosphate dehydrogenase [32]; and hemoglobin [33].

LDH genes most probably arose from a single ancestral locus by gene duplication events during vertebrate evolution in a similar fashion to that proposed by Ingram for hemoglobin (fig. 2). Although the homology of the LDH-A and B polypeptides is as yet unknown in terms of their amino acid sequence, other information supports the concept of the LDH A and B loci evolving from a single ancestral gene: (a) the LDH A and B polypeptides readily copolymerize thus indicating similarities in subunit–subunit binding sites [4]; (b) the amino acid sequence of the dodecapeptide at the active site is identical for the A and B polypeptides [34]; and (c) LDH-A_4 and B_4 are immunochemically distinct in most vertebrates [7, 21, 23, 35] but to some degree of identity in certain fish. Anti-B antibodies prepared against sea trout LDH-B_4 cross react with LDH-B_4 and to some extent with LDH-A_4 in trout [18] while both anti-A and anti-B antibodies cross react with LDH-B_4 from shark tissues [25]. The E and F loci for LDH presumably arose from duplications of the B locus during the course of teleost evolution. The similarity in immunochemical, kinetic, and physical properties between LDH's B_4, E_4, and F_4 from teleosts provide good evidence for their homology and common evolutionary origin.

An indication of the homologous nature of LDH-C_4 and B_4 is provided by their similarities in physical, chemical, and kinetic properties [10, 37, 38] as well as the immunochemical cross reaction of anti-B antibodies with LDH-C_4 from marsupials (fig. 1). Other immunochemical studies have shown that anti-A antibodies prepared against beef LDH-A_4 do not cross react with LDH-C_4 or LDH-B_4 from marsupials but do precipitate isozymes containing A subunits [39], and that mammalian anti-C antibodies do not cross react with either LDH-A_4 or LDH-B_4 isozymes from mammals [40]. Apparently, the antigenic sites of the B and C polypeptides have diverged to a sufficient extent in mammals to be immunochemically distinct with the C subunit retaining some degree of homology with the ancestral B locus because of its reactivity with fish anti-B antibodies (fig. 1). The proposal that the C gene arose from a duplication of the B gene (fig. 2) is also supported by recent genetic evidence which has shown the B and C loci in pigeons to be tightly linked [41]. Unequal crossover between sister chromatids of the chromosome containing the B locus or between two homologous chromosomes during meiosis at a stage of avian evolution would have resulted in duplicated B loci being tightly linked on one chromosome. Subsequent evolution and divergence of these loci would differentiate them in terms of their amino acid sequence and transcription during cellular development.

In summary, it is most probable that the mechanism of establishing multiple loci for LDH in vertebrate organisms is similar to that reported for the hemoglobin system although duplicate gene loci for LDH-A (A and A′) and B (B and B′) subunits in salmonid fish appear to be a result of tetraploidization during the evolution of these species. The A locus of LDH is considered to be more representative of the ancestral LDH gene because of its greater activity and wider distribution in vertebrate tissues. Immunochemical and other studies indicate some degree of homology between LDH's A and B, B and C, B and E, and B and F polypeptides which provides evidence for a common evolutionary origin of this enzyme system.

Acknowledgements

This work was supported by a grant (D70/17375) of the Australian Research Grants Committee. I thank Dr. D.W. Cooper for supplying the marsupials and Terri Jenkins for her technical assistance.

References

[1] C.L. Markert, in: Hereditary, Developmental and Immunochemical Aspects of Kidney Disease, ed. J. Metcoff (Northwestern University Press, 1962).
[2] C. Shaw and E. Barto, Proc. Natl. Acad. Sci. U.S. 50 (1963) 211.
[3] R.D. Cahn, N.O. Kaplan, L. Levine and E. Zwilling, Science 136 (1962) 962.
[4] C.L. Markert, Science 140 (1963) 1329.
[5] C.L. Markert and I. Faulhaber, J. Exp. Zool. 159 (1965) 319.
[6] C.L. Markert, Ann. N.Y. Acad. Sci. 151 (1968) 14.
[7] G.S. Whitt, J. Exp. Zool. 175 (1970) 1.
[8] A. Blanco and W.H. Zinkham, Science 139 (1963) 601.

[9] E. Goldberg, Science 139 (1963) 602.
[10] W.H. Zinkham, Ann. N.Y. Acad. Sci. 151 (1968) 598.
[11] G.S. Whitt, W.F. Childers and T.E. Wheat, Biochem. Genet. 5 (1971) 257.
[12] G.F. Sensabaugh and N.O. Kaplan, J. Biol. Chem. 248 (1972) 585.
[13] W.J. Morrison and J.E. Wright, J. Exp. Zool. 163 (1966) 259.
[14] W.J. Morrison, Trans. Amer. Fish. Soc. 99 (1970) 193.
[15] S. Ohno, U. Wolf and N.B. Atkin, Hereditas 59 (1968) 169.
[16] E.J. Massaro and C.L. Markert, J. Exp. Zool. 168 (1968) 223.
[17] G.S. Bailey and A.C. Wilson, J. Biol. Chem. 243 (1968) 5843.
[18] R.S. Holmes and C.L. Markert, Proc. Natl. Acad. Sci. U.S. 64 (1969) 205.
[19] C.A. Leone, Taxonomic Biochemistry and Serology (Ronald Pr., 1964).
[20] V. Bryson and H.J. Vogel, Evolving Genes and Proteins (Academic Press, N.Y., 1965).
[21] A.C. Wilson, N.O. Kaplan, L. Levine, A. Pesce, M. Reichlin and W.S. Allison, Federation Proc. 23 (1964) 1258.
[22] R. Arnon and H. Neurath, Proc. Natl. Acad. Sci. U.S. 64 (1969) 1323.
[23] C.L. Markert and R.S. Holmes, J. Exp. Zool. 171 (1969) 85.
[24] E.M. Prager and A.C. Wilson, J. Biol. Chem. 246 (1971) 5978.
[25] J.J. Horowitz and G.S. Whitt, J. Exp. Zool. 180 (1972) 13.
[26] Structure, Function, and Evolution of Proteins, Brookhaven Symp. Biol. (1968) 21.
[27] V.M. Ingram, Nature 189 (1961) 704.
[28] R. Ceppellino, in: Biochemistry of Human Genetics, eds. G. Wolstenholme and C. O'Connor (J. and A. Churchill, London, 1959).
[29] G.S. Baily, G.T. Cocks and A.C. Wilson, Biochem. Biophys. Res. Commun. 34 (1969) 605.
[30] K. Bender and S. Phno, Biochem. Genet. 2 (1968) 101.
[31] H.M. Eppenberger, A. Scholl and H. Ursprung, FEBS Letters 14 (1971) 317.
[32] W. Engel, J. Schimdtke and U. Wolf, Experentia 27 (1971) 1489.
[33] H. Tsuyuki and A.P. Ronald, Comp. Biochem. Physiol. 39B (1971) 503.
[34] K. Mella, E. Folsche, H. Torff and G. Pfleiderer, Z. Physiol. Chem. 349 (1968) 891.
[35] C.L. Markert and E. Appella, Ann. N.Y. Acad. Sci. 103 (1963) 915.
[36] G.S. Whitt, Science 166 (1969) 1156.
[37] L.J. Ballellino and A. Blanco, J. Exp. Zool. 174 (1970) 173.
[38] E. Goldberg, J. Biol. Chem. 247 (1972) 2044.
[39] R.S. Holmes, D.W. Cooper and J.L. van de Berg, J. Exp. Zool., in press.
[40] E. Goldberg, Proc. Natl. Acad. Sci. U.S. 68 (1971) 349.
[41] W.H. Zinkham, H. Inensee and J.H. Renwick, Science 164 (1969) 185.