Evidence of Lactate Dehydrogenase-B Allozyme Effects in the Teleost, *Fundulus heteroclitus*

LEONARD DIMICHELE, KENNEDY T. PAYNTER, DENNIS A. POWERS

The evolutionary significance of protein polymorphisms has long been debated. Exponents of the balanced theory advocate that selection operates to maintain polymorphisms, whereas the neoclassical school argues that most genetic variation is neutral. Some studies have suggested that protein polymorphisms are not neutral, but their significance has been questioned because one cannot eliminate the possibility that linked loci were responsible for the observed differences. Evidence is presented that an enzymatic phenotype can affect carbon flow through a metabolic pathway. Glucose flux differences between lactate dehydrogenase-B phenotypes of *Fundulus heteroclitus* were reversed by substituting the *Ldh-B* gene product of one homozygous genotype with that of another.

THE DEBATE OVER THE EVOLUTIONARY significance of protein polymorphisms has continued for over 20 years without resolution (*1, 2*). Yet, many of the recent advances in evolutionary biology depend on the implicit assumption that the majority of this genetic variation is selectively neutral. Arguments for the neutral theory are largely derived from the theoretical considerations of population genetics (*2*). They are bolstered by the view, advanced by Newsholme and Start (*3*), that metabolism is controlled by a few key regulatory or rate-limiting enzymes in a pathway, whereas other enzymes such as lactate dehydrogenase (LDH) participate in reactions that are near equilibrium and therefore of little or no importance in flux generation or metabolic control (*3*). Kacser and Burns (*4*) suggested that the control of metabolic rate is more generally shared among enzymes, including the so-called "equilibrium" enzymes like LDH. Because the neutralist hypothesis implies that polymorphic enzymes (allozymes) are functionally equivalent, some investigators have sought to resolve the controversy by examining the biochemistry of allozymes, while others have concentrated on life history correlates (*5–9*). Although these exciting studies present convincing evidence that one or more polymorphic loci are correlated with physiological or other differences between genotypes, they cannot eliminate the possibility that some unknown locus is actually responsible for the differences observed. Thus, the debate remains an important evolutionary question. We addressed this question by examining the metabolism of mummichog, *Fundulus heteroclitus* (Teleostei, Cyprinodontidae), embryos in which the native LDH-B isozyme was directly replaced with heterologous enzymes.

In the mummichog, LDH is a multilocus system that produces three gene products (LDH-A_4, LDH-B_4, and LDH-C_4): the *Ldh-A* locus is expressed in white muscle, *Ldh-B* in a variety of tissues including oocytes, and *Ldh-C* in eye and some nerve tissue (*10*). The *Ldh-B* locus has two major alleles, *Ldh-B*^{a} and *Ldh-B*^{b}, that vary clinally over the geographical range of the species (*11*). The allelic isozymes (allozymes) encoded by this locus have been purified and kinetically characterized (*12–14*). Differences have been observed in product inhibition and reaction velocities at low substrate concentrations (*14*). Within a local population, *Ldh-B*^{a} homozygotes develop faster than *Ldh-B*^{b} homozygotes (*15*). This developmental variation has been correlated with differences in oxygen consumption (*16*), lactate and glucose utilization (*17*), and survival, that is, Darwinian fitness (*6*).

Recently, we have shown that mummichog eggs contain 40 to 50 mM lactate at the time of fertilization and that a major function of LDH-B in early development is oxidation of the lactate pool (*17*). Specific activity of LDH-B is similar between genotypes (1.2×10^{-3} IU per egg), but lactate concentrations found in newly fertilized eggs are correlated with LDH-B phenotype and are essentially equal to the inhibition constant of the purified allozymes of LDH-B: 51.8 ± 5.9 mM for LDH-B_4^a and 36.6 ± 3.5 mM for LDH-B_4^b (*14*). Thus, in vivo, the *Ldh-B*^{a} homozygote accumulates more lactate and uses it at a higher rate. The *Ldh-B*^{a} homozygote also uses glucose at a higher rate even though the glucose pool size is the same for both phenotypes. Since the LDH reaction and glycolysis are both functioning to produce pyruvate in mummichog embryos during the first 24 hours, it is difficult to explain how the two LDH-B allozymes could differentially affect glucose utilization.

L. DiMichele, Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843.
K. T. Paynter, Chesapeake Bay Institute, Johns Hopkins University, Shady Side, MD 20764.
D. A. Powers, Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950.

The ideal way to determine whether genetic variation at a particular locus directly affects a biochemical or metabolic function would be to study organisms that only vary at that locus. The construction of such strains can take years or decades and is not always feasible. An alternate experiment would be to change an organism's enzyme phenotype by exchanging its allozyme with that of a different genotype.

Klyachko *et al.* (*18*) successfully inserted exogenous LDH in loach blastomeres by microinjection into the yolk shortly after fertilization. These experiments showed that LDH was disproportionately accumulated in the blastomeres through binding of the enzyme to some cellular component and that the foreign protein competes with the native enzyme for binding sites. We hypothesized that it is possible to quantitatively replace an embryo's native LDH-B_4 (the only LDH isozyme present in eggs) with exogenous enzyme by injecting an excess of foreign protein and then, after an incubation time to allow the enzyme to distribute itself, wash out the yolk and excess LDH. We tested this hypothesis by attempting to create blastulas whose native LDH content was replaced by either the LDH-B_4 allozyme of a different genotype or one of two porcine LDH isozymes. Our results generally confirmed those of Klyachko *et al.* (*18*). The blastulas from injected eggs contain about 62% of the LDH originally present in oocytes; the same amount as in uninjected or saline injected blastulas (Table 1). Electrophoretic analysis showed that the LDH in the blastulas was of the type injected, whereas no detectable amounts of the native enzyme remained. Because it is possible to quantitatively replace an embryo's native LDH-B with foreign enzymes of different kinetic or structural properties, we used these preparations to test whether it is the physical LDH type present in the blastula that influences glucose utilization or some other factor.

Blastulas were prepared as above, but ^{14}C-6-glucose (specific activity, 0.2 μCi/μmol) was added to the washing buffer. Ten embryos were sealed in 1.2-ml tubes without additional buffer. A KOH trap was affixed to the tube cap to collect CO_2 given off by the eggs. These were incubated at 20°C for an additional 12 hours. The tubes were then acidified with trichloroacetic acid and equilibrated for 1 hour. The traps were then removed and counted. The data in Table 2 show that the actual LDH-B_4 homotetramer incorporated into the blastula affected glucose utilization rates. Genotypes injected with their own enzyme used glucose in a pattern similar to whole eggs; that is, the *Ldh-B*^{a} homozygote used glucose at a higher rate (*17*). However, when a genotype's allozyme was replaced by the alternate allozyme, glucose was used at a rate determined by the injected allozyme rather than by the genotype.

These results indicate that the genetic differences between LDH-B allozymes can alter glycolytic flux by approximately 30% (Table 2). We hypothesized that the substitution of other, genetically more divergent LDH isozymes, might have an even greater influence. Therefore, we replaced the native enzyme of *Ldh-B*^{a} homozygotes with two porcine LDH isozymes. Porcine heart type LDH is physiologically optimized to oxidize lactate to pyruvate. In this regard it is more like mummichog LDH-B_4^b than mummichog LDH-B_4^a (*12*), and its effect on the *Ldh-B*^{a} homozygote was in the same direction and of a greater magnitude than mummichog LDH-B_4^b (Table 3). Conversely, the physiological function of the porcine muscle type LDH is the reduction of pyruvate to lactate. Its effect on the *Ldh-B*^{a} homozygote was to increase glucose utilization. Thus, the results of the porcine LDH replacement studies are consistent with and reinforce the native allozyme replacement studies.

The mechanism by which LDH influences glucose metabolism in mummichog embryos is not clear. Since both the LDH reaction and glycolysis are functioning to produce pyruvate and the phenotype that is using glucose faster is also using lactate faster (*17*), it is unlikely that feedback inhibition, competition for nicotinamide adenine dinucleotide (NAD), or some similar mechanism is responsible for the observed differences. Srivastra and Bernhard and their colleagues (*19*) have shown that enzyme-enzyme interactions, including those between LDH and other glycolytic enzymes, have significant effects on the kinetics of

Table 1. Activity of LDH in isolated blastulas after microinjection of exogenous enzyme. Ripe adults were captured near Shady Side, Maryland, and genotyped for LDH-B. Pooled gametes were crossed to produce either *Ldh-B*^{a} or *Ldh-B*^{b} homozygotes with a common genetic background (*15, 16, 21*), except the controls, which were based on crosses of mixed genotype. The eggs were then microinjected with 1.2×10^{-2} IU of LDH (approximately 10× the native enzyme concentration) purified from either mummichog liver or from porcine heart or muscle (Sigma). The eggs were incubated at 20°C for 6 hours. The yolk sac was then pierced with two needles. Yolk sac contents were withdrawn from one needle and 10 to 15 egg volumes (40 to 60 μl) of a buffer (tris-HCl, pH 7.0; 40 mM lactate; 5 mM glucose) was injected through the other. This procedure effectively washed out the entire contents of the yolk sac while leaving it inflated. The buffer lactate and glucose concentrations mimicked whole egg concentrations (*15*). Thus, the experimental preparation consisted of a developing blastula resting on a nutrient-enriched buffer-filled sac. These preparations were viable for at least 24 hours and appeared to blastulate normally. However, they did not gastrulate. The experiments were conducted on four occasions to obtain three replicates of each treatment (a sufficient number of preparations for all eight treatments could not be prepared each time). The SAS general linear models procedure (*22*) was used to correct small differences in enzyme concentration between replications (significant at $P > 0.001$). There were no differences between treatment ($F7,13) = 1.46$; $P = 0.26$). LDH activity was determined by the decrease in absorbance of NADH at 25°C under saturating conditions of pyruvate and NADH (*14*). Specific activities of the enzymes injected were: 425 IU/mg protein for both *Fundulus* allozymes; 386 IU/mg protein for the porcine heart LDH; and 450 IU/mg protein for the porcine muscle LDH. At the activity levels accumulated in the blastulas, differences in apparent enzyme concentration due to the small activity differences would be below detection limits.

Injected enzyme	Phenotype injected	LDH activity* (IU × 10^{-3}/egg)
None		0.72 ± 0.03
Saline		0.81 ± 0.03
Porcine (heart)	LDH-B^aB^a	0.76 ± 0.03
Porcine (muscle)	LDH-B^aB^a	0.79 ± 0.03
LDH-B_4^a	LDH-B^aB^a	0.83 ± 0.03
LDH-B_4^b	LDH-B^aB^a	0.78 ± 0.03
LDH-B_4^a	LDH-B^bB^b	0.72 ± 0.03
LDH-B_4^b	LDH-B^bB^b	0.78 ± 0.03

*Data are the means (± SEM) of three pools of ten eggs each.

Table 2. Glucose utilization rates (μmol × 10^{-8} s^{-1} per egg) of injected eggs. Data are means (± SD) of three pools of ten eggs each. Saline-injected controls were not significantly different from phenotypes injected with their own allozyme; *Ldh-B*^{a} and *Ldh-B*^{b} homozygotes injected with the same allozyme were not different from each other; phenotypes injected with LDH-B_4^a were significantly different from those injected with LDH-B_4^b (ANOVA, $P < 0.005$).

Phenotype	Injected enzyme		
	Saline	LDH-B_4^a	LDH-B_4^b
LDH-B^aB^a	6.6 ± 0.5	7.1 ± 0.9	5.1 ± 0.4
LDH-B^bB^b	4.3 ± 0.8	6.3 ± 0.8	4.4 ± 0.5

Table 3. Glucose utilization rates (μmol × 10^{-8} s^{-1} per egg) of injected eggs. Data are means (± SD) of three pools of ten eggs each. All means are significantly different from each other (ANOVA, $P < 0.001$).

Original phenotype	Injected enzyme		
	Porcine (heart)	LDH-B_4^a	Porcine (muscle)
LDH-B^aB^a	3.2 ± 0.4	7.1 ± 0.9	14.7 ± 0.5

enzyme reactions and consequently metabolic flux. They suggested that an important aspect of enzyme evolution may be related to changes in protein structure that alter metabolite transfer within a pathway. Perhaps genetic variation in the LDH-B isozyme of the mummichog affects its association with other glycolytic enzymes and thereby affects the rate of glucose use.

Evidence from a variety of other animals and enzyme systems has also indicated that allozyme variation is associated with metabolic and organismal level differences that may affect fitness (*5–17, 20*). However, our experiments suggest that the organismal differences correlated with the *Ldh-B* polymorphism in the mummichog are causally linked. If this is not a unique observation, allozymic variation may be important in the evolution of species.

REFERENCES AND NOTES

1. R. C. Lewontin, *The Genetic Basis of Evolutionary Change* (Columbia Univ. Press, New York, 1974); H. Harris, *Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2079 (1976).
2. M. Nei and R. K. Koehn, *Evolution of Genes and Proteins* (Sinauer, Sunderland, MA, 1983).
3. E. A. Newsholme and C. Start, *Regulation in Metabolism* (Wiley, New York, 1973).
4. H. Kacser and J. A. Burns, *Genetics* **97**, 639 (1981).
5. D. A. Powers, L. DiMichele, A. R. Place, in *The Isozymes: Current Topics in Biological and Medical Research*, M. C. Ratassi, J. G. Scandalois, G. S. Whitt, Eds. (Liss, New York, 1983), vol. 10, pp. 147–170.
6. L. DiMichele, J. A. DiMichele, D. A. Powers, *Am. Zool.* **26**, 201 (1986).
7. R. K. Koehn, *Am. Sci.* **74**, 659 (1986).
8. E. Zouros and D. W. Foltz, in (*5*), vol. 13, pp. 1–59.
9. W. B. Watt and C. L. Boggs, in (*5*), vol. 15, pp. 27–47.
10. A. R. Place and D. A. Powers, *Biochem. Genet.* **16**, 577 (1978).
11. ———, *ibid.*, p. 593.
12. A. R. Place and D. A. Powers, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2354 (1979). Both allozymes were shown to have the same first-order rate constant (k_{cat}). However, they have different K_m's for both lactate and pyruvate and different inhibition constants for lactate. Thus at similar enzyme concentration, both allozymes have the same reaction velocity at saturating substrate concentration (V_{max}), but different reaction velocities at low substrate concentration.
13. A. R. Place and D. A. Powers, *J. Biol. Chem.* **76**, 1299 (1984).
14. ———, *ibid.*, p. 1304.
15. L. DiMichele and D. A. Powers, *Nature* **296**, 563 (1982).
16. ———, *Physiol. Zool.* **57**, 52 (1984).
17. K. T. Paynter, L. DiMichele, S. C. Hand, D. A. Powers, *J. Exp. Zool.* **257**, 24 (1991).
18. O. S. Klyachko, V. P. Korzh, S. I. Gorgolyuk, A. V. Timofeev, A. A. Neyfakh, *ibid.* **222**, 137 (1982).
19. D. K. Srivastava and S. A. Bernhard, *Biochemistry* **23**, 4538 (1984); *ibid.* **24**, 623 (1985) ———, R. Langridge, J. A. McClarin, *ibid.*, p. 629; D. K. Srivastava and S. A. Bernhard, *Science* **234**, 1081 (1986); *Annu. Rev. Biophys. Biochem.* **16**, 175 (1987); D. K. Srivastava *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6464 (1989).
20. R. K. Koehn, R. I. E. Newell, F. Immermann, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5385 (1980); D. R. Cavener and M. T. Clegg, *ibid.* **78**, 4444 (1981); T. J. Hilbish, L. E. Deaton, R. K. Koehn, *Nature* **298**, 688 (1982); R. S. Burton and M. W. Feldman, *Biochem. Genet.* **21**, 239 (1983); W. B. Watt, R. C. Cassin, M. S. Swan, *Genetics* **103**, 725 (1983); W. B. Watt, P. A. Carter, S. M. Blower, *Genetics* **109**, 157 (1985); W. E. Zammer and R. J. Hoffmann, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2737 (1989).
21. The animals selected were randomly drawn from the same population, which has mitochondrial DNA haplotypes found only in southern waters [I. L. Gonzalez and D. A. Powers, *Evolution* **44**, 27 (1990)]. The Ldh-B alleles exist in Hardy-Weinberg equilibrium and are not linked to any known polymorphic locus [D. A. Powers *et al.*, *Am. Zool.* **26**, 131 (1986)].
22. SAS Institute, *SAS/STAT User's Guide, Release 6.03 Edition* (SAS Institute, Cary, NC, 1988), pp. 549–640.

23. Supported by NSF grant DEB-82-07006 and the Texas Agricultural Experiment Station.

29 April 1991; accepted 22 May 1991