Evolution, 37(1), 1983, pp. 30–37

KINETIC AND ELECTROPHORETIC DIFFERENTIATION OF LACTATE DEHYDROGENASES OF TELEOST SPECIES-PAIRS FROM THE ATLANTIC AND PACIFIC COASTS OF PANAMA

JOHN E. GRAVES[1], RICHARD H. ROSENBLATT, AND GEORGE N. SOMERO
Marine Biology Research Divison (A-002), Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093

Received October 6, 1981. Revised April 8, 1982

The important role of molecular adaptations in evolution has been demonstrated in studies of enzymic and respiratory proteins of organisms living in different habitats. Investigations of homologous proteins of warm- and cold-adapted ectotherms, for example, have elucidated many of the critical molecular properties that are involved in adaptation to different environments (Somero, 1978). However, even though substantial information is available on adaptive differences among homologous proteins of widely different species, e.g., those belonging to different families and genera, there remains much to be learned of the role of fine-scale adaptations, as might be important in populations of a single species that inhabit slightly different environments.

Substantial amounts of protein variation, both between populations of a single species and between closely related species, have been demonstrated with electrophoretic techniques (reviewed in Powell, 1975), and the existence of allelic variants which cannot be distinguished by standard electrophoretic methods has also been reported in comparisons of populations and species (Bernstein et al., 1973; Johnson, 1975; Siebenaller and Somero, 1978). The role of these electrophoretically detectable and cryptic allelic variants in molecular evolution has become an important focus of contemporary evolutionary study (Lewontin, 1974). Is the observed allelic variation of functional significance in many cases? Or is this variation generally lacking in selective importance? A closely related question, and one serving as a major focus of the present study, is what degree of environmental variation is required to select for functionally different proteins?

One approach to addressing these questions experimentally is to study a given class of enzyme in several populations of closely related organisms that are separated by a common faunal barrier into habitats that differ in an environmental factor such as temperature. Comparisons of protein homologues of these populations may provide especially good insight into the role of fine-scale molecular adaptation. However, in order for such a comparison to be meaningful, several conditions must be met. First, one must know in advance, from broadly comparative studies of the protein in question, what values are to be expected for the functional trait(s) under investigation. Only with this strong empirical base can the observed allelic variations be interpreted. Second, the populations studied must have existed in the different habitats for long enough periods of time for the accumulation of new protein variants. Third, functional analysis of the allelic variants must be performed under *in vitro* conditions which simulate, as closely as possible, the *in vivo* conditions to which the proteins are normally exposed.

An excellent opportunity for an investigation which meets these criteria is provided by the Panama land bridge, which arose approximately 3.1 million years ago (Keigwin, 1978), isolating a presumably very similar fauna of tropical marine shore fishes in significantly different thermal environments. During the dry season (December to May), strong offshore winds

[1] Current address: Southwest Fisheries Center, NMFS, P.O. Box 271, La Jolla, California 92038.

cause the upwelling of cold, nutrient-rich waters in the Pacific. Water temperatures in the Gulf of Panama have been recorded to drop by as much as 15 C in 48 hours (Hildebrand, 1939). The upwelling events are unpredictable, and rapid cooling is often followed by a gradual rise in surface temperatures. Not all areas of the eastern tropical Pacific experience intense upwellings, and there is much annual and geographical variability in both the duration and intensity of the upwelling events (Schaeffer et al., 1958). The Atlantic waters do not undergo these changes, and have a mean temperature 2–3 C higher than the Pacific waters, as well as a much smaller temperature range. The differences in thermal regime on the two coasts of Panama have favored selection for altered thermal tolerances between Atlantic and Pacific species. Graham (1971) found that the Pacific members of species pairs (congeners having close relatives on either side of the Isthmus) have lower lethal temperatures than their Atlantic congeners, when acclimated to the same temperature.

In this study we have examined four species pairs to determine if adaptation at the enzyme level has also been favored by the different thermal regimes of the Atlantic and Pacific coastal waters. A considerable amount of electrophoretically detectable protein variation has been found between congeneric fishes from these two habitats (Vawter et al., 1980), suggesting the possibility of functional differentiation between the proteins of the species pairs. That the slight temperature differences between these two habitats may be sufficient to lead to adaptive differences in enzyme function is suggested by the fact that, of the few studies which have demonstrated functional allelic differences between closely related populations and species, most have involved kinetic differences with respect to temperature (Koehn, 1969; Merritt, 1972; Vigue and Johnson, 1973; Day et al., 1974; Place and Powers, 1979; Graves and Somero, 1982). For example, comparisons of allelic variants of lactate dehydrogenases in congeners of the barracudas (genus *Sphyraena*) showed that temperature differences of only 5–8 C are sufficient to select for temperature-adaptive differences in this enzyme (Graves and Somero, 1982).

The present study examines variants of skeletal muscle type (M_4 or A_4) lactate dehydrogenase (LDH. E.C. 1.1.1.27; lactate : NAD oxidoreductase) due to the wealth of kinetic information available for interspecific homologues of this protein and its critical role in locomotory function of fishes (Somero and Childress, 1980). Fully purified M_4-LDHs from four species pairs are characterized in terms of apparent Michaelis constants (K_m) of pyruvate and catalytic rate constants (k_{cat}), using physiologically realistic temperature and pH values. In two of the four species pairs, K_m and k_{cat} values displayed temperature-adaptive patterns between congeners comparable to those found in other studies of the enzyme. In the two other cases, the LDHs of the congeners were kinetically indistinguishable. There was no correlation between kinetic differences and electrophoretic differentiation.

Materials and Methods

The four species pairs chosen for this study ([Atlantic and Pacific species of each pair, respectively] damselfishes: *Abudefduf taurus* and *A. concolor*; groupers: *Epinephelus cruentatus* and *E. panamensis*; blennies: *Ophioblennius atlanticus* and *O. steindachneri*; and wrasses: *Thalassoma bifasciatum* and *T. lucasanum*) are common on both coasts of Panama. Members of a pair are ecologically and morphologically extremely similar (Meek and Hildebrand, 1927), and they occupy the same types of habitat, occur over the same depth ranges, and have similar diets. The major physical difference between the environments of the pairs is temperature.

Specimens were collected on trolled lures, with pole spears, and with quinaldine and hand nets on the two coasts of Panama. Collections were made during March 1979 and March 1980 (Panama Atlantic water temperature of 26–28 C; Panama Pacific water temperature of 15–19

C) and during September 1977 (Panama Atlantic water temperature of 27–30 C; Panama Pacific water temperature of 27–30 C). The fish were immediately frozen and shipped to the laboratory at Scripps on dry ice, where they were stored until use at −35 C.

Purification of the M_4-isozyme of LDH followed the procedures of Graves and Somero (1982), with the exception that the fractions containing the highest LDH activity from the affinity chromatography step were not concentrated by ultrafiltration, but were pooled and stored at 5 C. Loss of enzymic activity of the pooled peak fractions over the three days required to perform the kinetic studies was less than 5%. Enzyme purity was determined in each case by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970). M_4-LDH was purified from two individuals of each *Abudefduf* and *Epinephelus* species. Because of their small sizes, several individuals of each *Ophioblennius* and *Thalassoma* species were pooled for purification after they were electrophoretically phenotyped. Kinetic differences were not detected between Pacific fishes collected in March (cold temperatures) and September (warm temperatures).

Two parameters of enzyme function were determined from the fully purified enzyme preparations. The apparent (=graphically determined) Michaelis constant (K_m), which for this reaction provides an approximation of enzyme-substrate affinity, was determined at 10 C, 20 C, and 30 C. The catalytic constant (k_{cat}), which is a measure of maximal catalytic efficiency (the maximal reaction velocity that a molecule of the enzyme can generate) was determined at 20 C. Both K_m and k_{cat} values were determined using a weighted linear regression analysis of Lineweaver-Burk plots (Wilkinson, 1961). Eight concentrations of pyruvate (0.2 to 1.0 mM) were used for each K_m and k_{cat} determination.

Horizontal starch gel electrophoresis of the purified M_4-LDHs was performed after the kinetic studies to determine within species variability of M_4-LDH and to determine whether a correlation existed between kinetic differentiation and electrophoretic variation. 12.5% (weight : volume) starch gels (Electrostarch lot #307) were run in tris-citrate buffer, pH 8.0, and Poulik buffer, pH 8.7, for 6 h at 60 mA (Selander et al., 1971). At least 10 individuals of each species were electrophoretically surveyed, and no within species variability of M_4-LDH phenotype was noted in any of the eight species.

Results and Discussion

Functional allelic differences were discovered between the M_4-LDHs of two of the species pairs. The kinetic data (Table 1) show that differences in both the apparent K_m of pyruvate and k_{cat} exist between the Pacific and Atlantic species of *Thalassoma* and *Ophioblennius*. In both pairs the enzyme of the Pacific species appears to be "cold-adapted" relative to the enzyme of the Atlantic congener. Thus, in agreement with studies of widely different vertebrates (fishes, reptiles and mammals) having extremes of body temperature between −2 C (Antarctic fishes) and 47 C (desert reptile), the LDHs of these two Pacific species have inherently higher K_m of pyruvate values at any given temperature than the LDHs of the more warm-adapted Atlantic congeners. These temperature-related differences in K_m favor a marked stabilization of K_m at physiological temperatures, as shown by the summary of existing data given in Figure 1. This high degree of conservation of K_m in different species at their appropriate body temperatures has been interpreted as an adaptation insuring that enzymes retain a reserve capacity for increasing rate of catalysis as substrate concentrations rise during metabolic activations, e.g., during bursts of locomotion. Thus, by stabilizing K_m values in a range that is close to, or somewhat greater than, physiological substrate concentrations, an enzyme is able to attain a significant fraction of its potential velocity (V_{max}) under normal physiological conditions and also increase its activity as substrate levels rise (Fersht, 1977; Graves and Somero, 1982). It bears noting that

TABLE 1. *Kinetic parameters of M_4-LDHs purified from closely related species separated by the Isthmus of Panama.* K_m *of pyruvate values were determined from Lineweaver-Burk plots of reaction velocity versus pyruvate concentration (eight different [pyruvate] were used in each case), using the weighted linear regression method of Wilkinson (1961). The* k_{cat} *values are based on theoretical maximum reaction velocities, which also were computed by the weighted linear regression method of Wilkinson (1961).*

Species	K_m of pyruvate (mM) ± 95% CI			k_{cat} (sec^{-1}) ± 95% CI
	10 C	20 C	30 C	20 C
Abudefduf taurus (Atlantic)	0.121 ± 0.007	0.172 ± 0.022	0.309 ± 0.030	357 ± 10
Abudefduf concolor (Pacific)	0.123 ± 0.009	0.154 ± 0.018	0.298 ± 0.024	332 ± 8
Epinephelus cruentatus (Atlantic)	0.073 ± 0.007	0.121 ± 0.006	0.212 ± 0.023	243 ± 3
Epinephelus panamensis (Pacific)	0.086 ± 0.012	0.125 ± 0.013	0.208 ± 0.011	294 ± 6
Ophioblennius atlanticus (Atlantic)	0.121 ± 0.007	0.188 ± 0.018	0.292 ± 0.013	173 ± 5
Ophioblennius steindachneri (Pacific)	0.189 ± 0.017	0.237 ± 0.022	0.396 ± 0.033	280 ± 9
Thalassoma bifasciatum (Atlantic)	0.072 ± 0.008	0.115 ± 0.012	0.248 ± 0.027	125 ± 2
Thalassoma lucasanum (Pacific)	0.106 ± 0.010	0.142 ± 0.012	0.298 ± 0.027	370 ± 8

this strong conservation of K_m of pyruvate is seen only when the *in vitro* assay medium has the appropriate pH for the temperature of measurement (Yancey and Somero, 1978; Somero, 1981). Thus, the detection of functionally significant differences between enzyme variants may be contingent on the use of physiologically realistic assay conditions, especially pH and temperature (cf. Place and Powers, 1979).

The differences in k_{cat} between the M_4-LDHs of the Atlantic and Pacific congeners of *Thalassoma* and *Ophioblennius* also are temperature compensatory. For both pairs of fishes, the Pacific species possesses the LDH variant with the higher catalytic rate potential. These k_{cat} data agree with findings made using widely different vertebrate species, which show that k_{cat} values for a variety of different enzymic reactions including the LDH reaction vary inversely with adaptation temperature (Low et al., 1973; Borgmann and Moon, 1975; Low and Somero, 1976; Johnston and Walesby, 1977; Somero, 1978; Somero and Siebenaller, 1979; Graves and Somero, 1982). Thus, the differences between the M_4-LDHs of the congeners of *Thalassoma* and *Ophioblennius* suggest that very small differences in body temperature are sufficient to favor selection for temperature adaptive differences in enzyme kinetic properties, a finding consistent with the comparisons of barracuda congeners having 5–8 C differences in average body temperature (Graves and Somero, 1982).

The lack of kinetic differences between the M_4-LDH homologues of the *Abudefduf* and *Epinephelus* species pairs cannot be explained in adaptive terms, and may simply reflect the lack of appropriate mutations (i.e., mutations of the types which we propose to have occurred in the other two species pairs) subsequent to the splitting of the original populations of these species.

The K_m of pyruvate values of the LDHs of the four species pairs all fall within the range of K_m values expected for vertebrate M_4-LDHs at normal body temperatures, 0.15 to 0.30 mM pyruvate (Fig. 1). How-

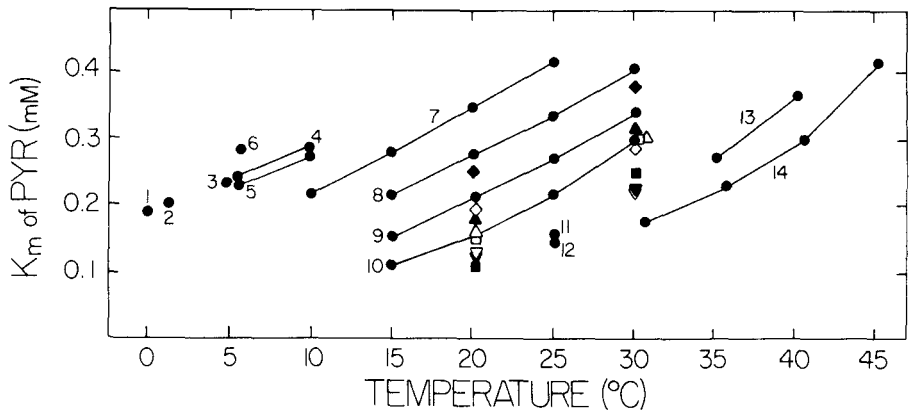

FIG. 1. Apparent K_m of pyruvate values for purified M_4-LDHs of selected vertebrate species having widely different body temperatures. Assays were performed using the physiologically realistic, temperature-dependent pH regime described by Yancey and Somero (1978). Assays were conducted at normal body temperatures of each species plus, in some cases, at temperatures outside the physiological range (see below). The species studied and their temperature ranges are 1. *Pagothenia borchgrevinki* (Antarctic nototheniid fish; −1.86 C), 2. *Halosauropsis macrochir* (deep-sea halosaur fish; 2–3 C), 3. *Coryphaenoides acrolepis* (Pacific rattail fish; 2–3 C), 4. *Sebastolobus alascanus* (shortspine thornyhead fish; 3–10 C), 5. *Hippoglossus stenolepis* (Pacific halibut fish; 4–12 C), 6. *Antimora rostrata* (deepsea flatnose codling fish; 2–6 C), 7. *Thunnus thynnus* (bluefin tuna fish; 5–30 C), 8. *Sphyraena argentea* (California barracuda fish; 14–22 C), 9. *Sphyraena lucasana* (Cortez barracuda fish; 16–28 C), 10. *Sphyraena ensis* (Panama barracuda fish; 22–30 C), 11. *Hypostomus plecostomus* (Amazon armoured catfish; 20–30 C), 12. *Potamotrygon* sp. (South American freshwater stingray fish; 25–30 C), 13. *Oryctolagus cuniculus* (New Zealand white rabbit; 37–39 C), and 14. *Dipsosaurus dorsalis* (desert iguana reptile; preferred temperatures of 36–42 C, and upper distribution limit at 47 C; Norris [1953]). K_m data are from Yancey and Somero (1978) (species 1, 5, 7, 11–13), Graves and Somero (1982) (species 8–10), Somero et al. (1982) (species 2–4, 6) and Donahue and Somero (unpubl.) (14). Symbols for this study: *Abudefduf taurus* (▲), *A. concolor* (△), *Epinephelus cruentatus* (▼), *E. panamensis* (▽), *Ophioblennius atlanticus* (◇), *O. steindachneri* (◆), *Thalassoma bifasciatum* (■), and *T. lucasanum* (□).

ever, within the 0.15 to 0.30 mM range, there is variation among species that appears related to taxonomic affinity. For example, both *Abudefduf* LDHs have K_m values that are statistically indistinguishable, but which are significantly lower at all temperatures than the K_m values of the *Epinephelus* species' LDHs, which also are statistically indistinguishable at all temperatures. The values for the K_m of the *Ophioblennius* species' LDHs at physiological temperatures are near 0.27 mM pyruvate, a value close to that found for the K_m of pyruvate for LDHs of four barracuda species (0.26 mM) at these species' normal physiological temperatures (Graves and Somero, 1982). For the *Thalassoma* species, however, the K_m of pyruvate is conserved near 0.21 mM. The significance of what appear to be slightly different "set points" for the K_m of pyruvate in different genera of fishes remains to be established.

Of the two species pairs that displayed functional differences in their M_4-LDHs, the *Thalassoma* species had alleles which were electrophoretically distinguishable, whereas the *Ophioblennius* species were electrophoretically indistinguishable (Fig. 2). In addition, of the two pairs which did not demonstrate functional allelic differences at the *M_4-Ldh* locus, one pair (*Epinephelus*) exhibited electrophoretic differences and the other (*Abudefduf*) did not. Thus, in the four species pairs examined, there was no correlation between electrophoretic and functional differentiation.

The lack of correlation between electrophoretic and functional differentiations of these species may be a consequence of the small number of species studied, but this

lack of correlation does reflect on the possible outcomes of protein variation. Certain sections of protein amino acid sequences are well-conserved between homologous proteins and between proteins with similar functions, but which are coded by different gene loci (analogous proteins; see Dayhoff, 1972). X-ray diffraction studies have demonstrated several of these well-conserved sequences in active sites and in areas which are important for determining conformational folding (Buehner et al., 1973; Taylor et al., 1973; Rossmann et al., 1974). As suggested by King and Jukes (1969), non-conservative amino acid substitutions in these regions are likely to produce dramatic changes in protein function, while amino acid substitutions in other, less-conserved sequences may produce no detectable change in enzyme function. An example of the latter type has been reported for LDH (Eventoff et al., 1977). The number of histidyl residues (a residue which is approximately half-protonated at physiological pH) on the exterior of the enzyme varies greatly, both between species and between isozyme forms. However, the internal and active site histidyl residues are highly conserved. Thus, depending on the location of an amino acid substitution involving a charge change, functional differences may or may not result. The demonstration of electrophoretically cryptic kinetic differentiation in LDHs (this study; Siebenaller and Somero, 1978) and the occurrence of electrophoretic differentiation in kinetically indistinguishable LDHs (this study) may reflect variations in charged amino acid residues, e.g., histidyls, at both conserved and non-conserved sites. Furthermore, kinetic differences may be due to amino acid substitutions causing no net charge alteration. The kinetic differences between the heart- and muscle types of LDHs of vertebrates are due largely to an alanyl to glutaminyl substitution in the cofactor binding domain, a substitution which conserves net charge (Eventoff et al., 1977).

One objective of this study was to determine the fraction of functionally significant allelic variation between two closely related species at a single gene locus. Two of the four species pairs displayed significant kinetic differences in their M_4-LDHs and, in both cases, the Pacific species was more "cold-adapted." These data are of course insufficient to allow predictions about the abundance of functional allelic variation in other instances, as the amount of kinetic variation detected between proteins of other species may be dependent on the protein investigated, the magnitude and constancy of the environmental differences between the species, and the length and nature of the barriers to gene flow between groups. However, the results of this study, and others in which closely related species from different environments have been investigated, clearly demonstrate that substantial functional allelic variation exists within and between natural populations.

FIG. 2. Electrophoretic mobilities of M_4-LDHs of closely related fish species separated by the Isthmus of Panama. From left to right: *Abudefduf taurus, A. concolor, Epinephelus cruentatus, E. panamensis, Ophioblennius atlanticus, O. steindachneri, Thalassoma bifasciatum*, and *T. lucasanum*.

The use of enzyme kinetics to investigate functional allelic variation has advantages over other approaches which have been used, e.g., intensity of gel staining and thermal stability. Of key importance is that kinetic measurements, when obtained in physiologically appropriate media, reflect enzyme performance under typical cellular conditions. Thus, kinetic differences detected *in vitro* could reflect true differences in metabolic flux and power output during times of thermal stress to the organism.

It is not known how large an environmental, e.g., thermal, difference needs to be before it becomes selective for molec-

ular fine-tuning. Previous work with congeneric barracudas demonstrated that mean environmental (body) temperature differences of only 5–8 C were sufficient to promote molecular adaptation. In this study a mean environmental temperature difference of 2–3 C and, possibly of more importance, a much cooler temperature range in the Pacific, appears to have provided a selective force favoring fine-scale molecular adaptation. It is also important to note that these adaptations have occurred even though the Pacific fishes do not experience upwelling (cooling) events over their entire ranges. Thus, in the absence of gene flow, very small changes in the physical environment may be sufficient to promote enzymic adaptations.

Summary

Many populations of marine shore fishes were separated by the uplift of the Isthmus of Panama approximately three million years ago. Since the time of separation, the populations have been exposed to different thermal environments. Due to strong, but geographically and temporally variable, upwelling events, the Pacific fishes experience a more variable temperature regime and a cooler mean temperature than their Atlantic congeners. Kinetic analysis of muscle-type lactate dehydrogenases, purified from white skeletal muscle of four species pairs, revealed significant functional differences in both apparent Michaelis constants of pyruvate and catalytic rate constants between members of two of the four pairs. In both cases the Pacific species were more cold-adapted than their Atlantic congeners, demonstrating that in the absence of gene flow, small environmental differences can lead to molecular adaptation. Horizontal starch gel electrophoresis of the purified enzymes revealed distinct allelic forms between members of two of the four pairs. However, the electrophoretic differences were not correlated with the functional differences. This lack of correlation is consistent with the hypothesis that the nature and location of an amino acid substitution determines whether the allele is selectively significant or neutral.

Acknowledgments

This work benefitted from discussions with Drs. G. S. Greaney and P. J. Walsh. The studies were supported by the Smithsonian Tropical Research Institute, the Foundation for Ocean Research, National Science Foundation grant PCM80-01949 (GNS) and U.S. Public Health Service grant 1 R01 GM2203 (RHR).

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Corresponding Editor: G. B. Johnson

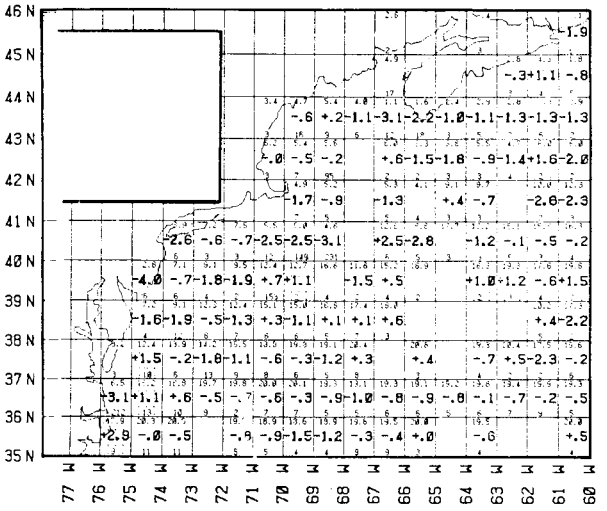

Figure 34. January 1981.

M. C. INGHAM
Atlantic Environmental Group, NMFS/NOAA
RR7, South Ferry Road
Narragansett, Rhode Island 02882, USA
D. R. MCLAIN
Pacific Environmental Group, NMFS/NOAA
c/o Fleet Numerical Oceanography Center
Monterey, California 93940, USA