Pressure-Adaptive Differences in Lactate Dehydrogenases of Congeneric Fishes Living at Different Depths

Abstract. *The muscle-type (M_4) lactate dehydrogenases of* Sebastolobus altivelis, *a deep-water scorpaenid, and* S. alascanus, *a shallower species, are electrophoretically indistinguishable, yet differ in pressure sensitivities. The lactate dehydrogenase of* S. altivelis *exhibits lower pressure sensitivities of substrate and coenzyme binding and catalytic rate. Such apparently pressure-adaptive kinetic properties may be important for establishing species depth zonation patterns in the ocean.*

Despite a lack of physical barriers, marine species often display characteristic depth distribution patterns throughout their geographical ranges (*1*). The factors responsible for establishing these vertical zonation patterns have been widely discussed, but they remain poorly understood. Various workers have placed different emphases on the potential roles of biological interactions, food supply, and environmental factors (such as temperature, hydrostatic pressure, current regimes, and sediment types) in establishing species depth distributions. Indeed, different parameters or combinations of parameters may set the upper and lower distributional bounds of any species. Although correlations between these parameters and species distributions have been observed, experimental verification of proposed causal relationships is apt to be very difficult, especially when such factors must be studied in situ. The factors most amenable to laboratory testing are hydrostatic pressure and temperature, variables that have profound effects on the physiological and biochemical—for example, enzymatic—processes of organisms (*2*, *3*) and that can be readily manipulated in the laboratory.

These considerations have led us to initiate studies designed to elucidate the significance of pressure-related adaptations in enzyme systems of marine organisms adapted to different depths. Although there is evidence that enzymatic adaptations to pressure are important in deep-living species (*3*), most of this evidence derives from studies of phylogenetically distant species whose habitats may differ in a number of important factors, for example, in both temperature and pressure. The confounding effects of phylogenetic distance and influences of other habitat conditions on enzymatic properties render the delineation of strictly pressure-related adaptations difficult. Thus, to isolate the influences of hydrostatic pressure on enzyme evolution one would ideally wish to compare species which (i) are phylogenetically close, (ii) have similar life histories, (iii) encounter similar thermal regimes, but (iv) exhibit different depth distributions. Studies of species fitting these criteria may yield insights not only into the nature of enzymatic pressure adaptations, but, also, evidence on the minimal pressure differences that appear to necessitate adaptive modifications in the functional and structural properties of enzymes.

The two congeneric scorpaenid fishes which were examined in our study fit the above criteria very closely. *Sebastolobus altivelis* and *S. alascanus* are morphologically similar and have similar life histories (*4*). Based on an electrophoretic survey, the genetic similarity (*5*) of these two species is high (*6*). These species occur together in the eastern North Pacific but have different bathymetric distributions (Fig. 1). *Sebastolobus altivelis* commonly occurs between 550 and 1300 m; *S. alascanus* is common between 180 and 440 m. The temperature difference between these two depth regimes is slight (< 5°C) (*7*).

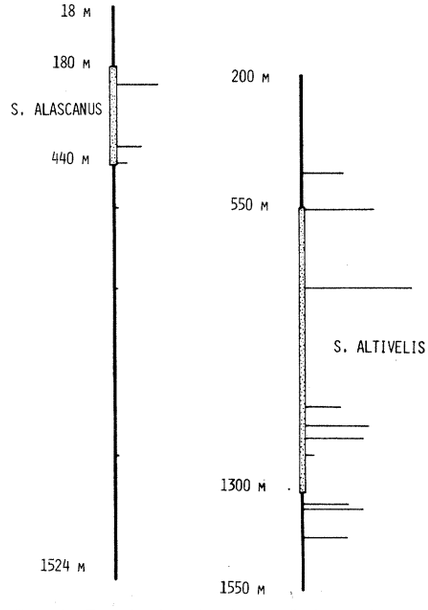

Fig. 1. Depth distributions of the *Sebastolobus* species. The widened region on the vertical bars indicates depths at which the species is most common, and the extending bars represent the depths at which any specimens have been taken (*4*). The horizontal bars indicate the numbers of specimens taken for an electrophoretic survey of populations (*6*). The longest horizontal bar represents 73 specimens; the shortest bar, one specimen. A depth change of 10 m represents a hydrostatic pressure change of approximately 1 atm.

In spite of the small differences in hydrostatic pressure between the two habitats, the skeletal muscle type (M_4) lactate dehydrogenases (LDH) (E.C. 1.1.1.27) of the two species differ in apparently adaptive ways in their pressure sensitivities, even though the two LDH's are electrophoretically indistinguishable. Enzymatic adaptations may thus be important in establishing and maintaining even fine differences in depth distribution patterns. Furthermore, electrophoretic identity may belie adaptively important functional differences between enzyme homologs.

Pressure effects on enzymatic catalysis may derive from two primary sources: (i) pressure-induced changes in enzyme structure and (ii) the volume changes that accompany the individual steps in the enzymatic reaction sequence. If a step occurs with an increase in system (enzyme plus solvent) volume, pressure will inhibit that step. If a decrease in volume occurs, pressure will enhance the rate of that step. Only if no volume change occurs will the step be pressure-insensitive. Among the steps in an enzymatic reaction sequence that are likely to be pressure-sensitive are ligand (substrate, cofactor, and modulator) binding and catalytic activation (*8*, *9*). Since the binding and catalytic functions of enzymes have been shown to be critically important sites of adaptations to temperature (*10*, *11*), conservation of these properties may also be expected to be an important component of adapatation to hydrostatic pressure.

The effects of hydrostatic pressure on substrate and coenzyme binding of the M_4 LDH's (Figs. 2 and 3) of the two species were examined with dialyzed enzyme purified by oxamate-Sepharose affinity chromatography (*11*). For pyruvate binding (Table 1) (*6*), the apparent Michaelis constants (K_m's) of the two LDH's are identical under all of the assay conditions employed at 1 atm, but at 68 atm, a pressure typical of the depth range of the deeper-living *S. altivelis*, the apparent K_m of the LDH of *S. alascanus* significantly increased, differing from the 1-atm value and from the value for the *S. altivelis* LDH at this higher pressure (*t*-test, $P < .01$) (Fig. 3). The apparent K_m of the LDH from *S. altivelis* was unaffected by pressures up to 476 atm, the highest pressure in our study (Fig. 3).

Coenzyme (reduced nicotinamide adenine dinucleotide, NADH) binding abili-

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Table 1. The effect of temperature on the apparent K_m (mM) for pyruvate of the *Sebastolobus* LDH's at 1 atm. The assay mixture contained 80 mM tris-HCl buffer, pH 7.5 at the assay temperature, 0.15 mM NADH, 100 mM KCl, and varying concentrations of pyruvate (0.10 to 4.0 mM). The K_m values, based on seven to nine pyruvate concentrations, were calculated by a weighted linear regression (*17*). Pyruvate K_m values at 1 atm were also identical in these two species, at these temperatures, when 66.7 mM potassium phosphate buffer (pH 7.4) and 80 mM imidazole-HCl buffer (pH varying with temperature) were used (*6*).

Temperature (°C)	Apparent K_m of pyruvate			
	Sebastolobus altivelis		*Sebastolobus alascanus*	
	K_m	C.I*	K_m	C.I*
5	0.395	0.422 to 0.368	0.399	0.425 to 0.372
10	0.398	0.411 to 0.355	0.341	0.376 to 0.306
15	0.531	0.598 to 0.464	0.460	0.482 to 0.437

*Ninety-five percent confidence interval.

ties of the two LDH's are also identical at 1 atm (Fig. 3). At increasing pressures, the apparent K_m values for NADH rise for both species, but the increase is much less for the LDH of *S. altivelis* (Fig. 2). Thus, for both substrate and cofactor binding, the LDH of the deeper-occurring species, *S. altivelis*, is less pressure-sensitive than is the LDH of *S. alascanus*. As is discussed in the context of temperature adaptation (*10, 11*), conservation of a proper apparent K_m value is a critical requirement in enzyme evolution in different environments because of the importance of this characteristic for both the regulatory and catalytic functions of enzymes (*12*). Offsetting the effects of pressure perturbation on these binding events would appear to be an important evolutionary event in the histories of marine species that are successful in colonizing deeper regions of the water column.

The structural bases of the different pressure sensitivities in ligand binding obviously cannot be deduced from these kinetic data. However, the variation in apparent K_m values with pressure (Figs. 2 and 3) does not display the log-linear relation expected if pressure sensitivity were due to a pressure-insensitive volume change on ligand binding (*8, 9*). Thus, the initial increase in pressure at 68 atm leads to an increase in the apparent K_m, but further pressure increases are not as effective in raising the K_m. We suggest that the initial pressure rise at 68 atm causes a change in enzyme structure which, in turn, leads to altered K_m's.

Pressure effects on the M_4 LDH reaction velocities of *S. altivelis* and *S. alascanus* in the pyruvate reductase direction also differed in apparently adaptive ways. Using both purified and crude homogenate enzyme preparations we monitored reaction velocities at 68, 204, and 340 atm (*13*). Variation in reaction velocity with pressure gave a log-linear relation, in contrast to the data from K_m as a function of pressure. From 3 to 16 ($\bar{x}$ = 9.5) determinations were made on 11 specimens of each species. The volume changes calculated from the log-linear velocity changes with pressure (*14*) differed significantly between the two species (one-way analysis of variance, $P << .001$). The volume change of the *S. altivelis* LDH reaction (8.1 cm^3 mole^{-1}) was smaller than that of the shallow-living *S. alascanus* LDH reaction (12.8 cm^3 mole^{-1}). The LDH reaction of *S. altivelis* is thus less pressure-sensitive than that of the homologous enzyme from *S. alascanus*. All individuals of a species gave comparable results, and the pressure effects on purified and crude homogenate LDH's were the same.

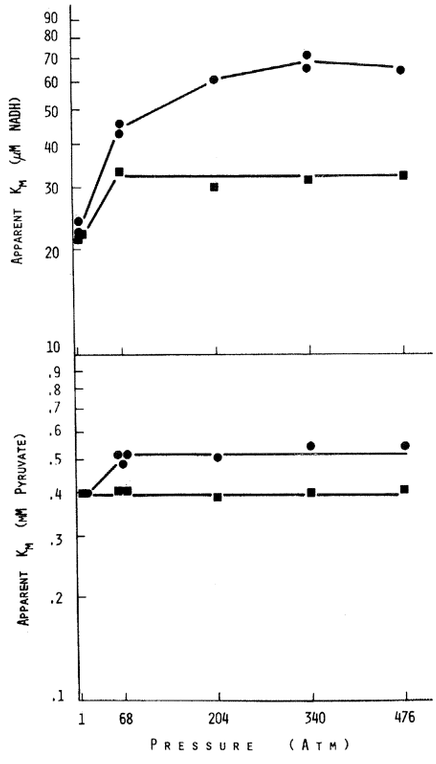

Fig. 2 (top). Effects of hydrostatic pressure on the apparent K_m for NADH of the *Sebastolobus* LDH's. The assay mixture contained 80 mM tris-HCl, pH 7.5, at the assay temperature of 5°C, 5 mM sodium pyruvate, and 100 mM KCl. Seven to nine NADH concentrations (200 to 10 μM) were used. The K_m's were calculated with a weighted linear regression (*17*). ■, *S. altivelis*; ●, *S. alascanus*. Fig. 3 (bottom). Effects of hydrostatic pressure on the apparent K_m for pyruvate of the *Sebastolobus* LDH's. The assay mixture contained 80 mM tris-HCl, pH 7.5, at the assay temperature of 5°C, 0.15 mM NADH, and 100 mM KCl. Tris was used because of the relative pressure-insensitivity of the tris pK (*18*). Apparent pyruvate K_m's were determined as described in Table 1. See (*13*) for a description of the high-pressure optical cell used. ■, *S. altivelis*. ●, *S. alascanus*.

Although the LDH's of the two *Sebastolobus* species differed in their responses to hydrostatic pressure, the enzymes could not be distinguished under any of the electrophoretic conditions used, including gel sieving (up to 17 percent, weight to volume, on starch gels, and up to 15 percent on acrylamide gels) (*6, 15*). Gel sieving techniques have, in other instances, led to successful resolution of genetic variation that is cryptic under standard electrophoretic conditions (*16*). It appears that the kinetic differences between the two *Sebastolobus* LDH's are based on relatively minor differences in amino acid composition.

Our findings have several implications for questions of biochemical adaptation and species zonation in the marine environment.

1) It appears that relatively small differences in hydrostatic pressure, of the order of 100 atm and less, are adequate to favor adaptive modifications in the functional properties of enzymes. These modifications, namely preservation of stable ligand-binding abilities and catalytic rates, are comparable to the temperature-adaptive changes noted among enzymes from differently thermally adapted species (*9, 10*). Thus hydrostatic pressure may play a major and heretofore unappreciated role in biochemical adaptation to the marine environment.

2) The foregoing conclusion suggests that depth zonation patterns may be critically linked to adaptations of enzymes to hydrostatic pressure. Successful colonization of deeper regions by a population may be attendant on the acquisition of pressure-adaptive changes in enzymatic properties.

Such biochemical adaptations could be a significant factor in speciation events in the marine environment. The regular variation of hydrostatic pressure with depth contrasts with the relative uniformity of other physical environmental parameters, such as temperature and salinity. Different pressure regimes

may provide sufficiently different forces of natural selection to promote the genetic divergence necessary for speciation. Finally, the discovery that kinetically different enzyme homologs are indistinguishable when conventional electrophoretic techniques are used emphasizes again the importance of electrophoretically silent mutations in the protein divergence of species.

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L_{III}-Edge Anomalous X-ray Scattering by Cesium Measured with Synchrotron Radiation

Abstract. *Diffraction of monochromatized synchrotron radiation by crystals of cesium hydrogen tartrate has been used to measure the magnitude and phase of x-ray scattering for cesium near the* L_{III} *absorption edge. In this wavelength region the scattering amplitude of cesium is reduced by as much as 25 electrons per atom, compared to scattering of copper* Kα *x-rays. This change, which varies as a function of wavelength, affects the diffraction intensities in a manner similar to isomorphous substitution, and it is large enough to have promise for phase determination in the study of macromolecular structures. This experiment also demonstrates that accurate diffractometer measurements are possible with synchrotron radiation produced by an electron storage ring.*

We report here x-ray diffraction experiments carried out with an automatic diffractometer and a focused monochromatic x-ray beam of synchrotron radiation from the SPEAR storage ring at the Stanford Synchrotron Radiation Laboratory (*1*). Diffraction measurements with crystals of cesium hydrogen (+)-tartrate have shown that this equipment can give reproducible results at wavelengths chosen from the continuous spectrum of the synchrotron radiation. We have derived from these data the anomalous scattering terms for cesium near the L_{III} absorption edge and find that the scattering power of cesium is reduced by as much as 25 electrons. To our knowledge this is the largest such effect yet observed in an x-ray diffraction experiment. This reduction in scattering power, which is approximately equivalent to removing a rubidium atom from the structure, could be used as a substitute for or a complement to isomorphous replacement in solving the phase problem for macromolecular structures. This technique has the advantage that the crystal structure is exactly the same as that studied at another wavelength, thus avoiding the imperfect isomorphism that generally occurs when atoms are added to or replaced in the crystal.

An atom scatters x-rays with an amplitude and phase that can be represented by the complex number $f = f_0 + f' + if''$, where f_0 is the value appropriate for very short wavelengths (*2*). The anomalous scattering terms f' and f'', which are functions of the wavelength, describe the in-phase and out-of-phase components of the change due to finite binding energies of the electrons in the atom. While f_0 decreases as the scattering angle increases, f' and f'' are nearly independent of angle.

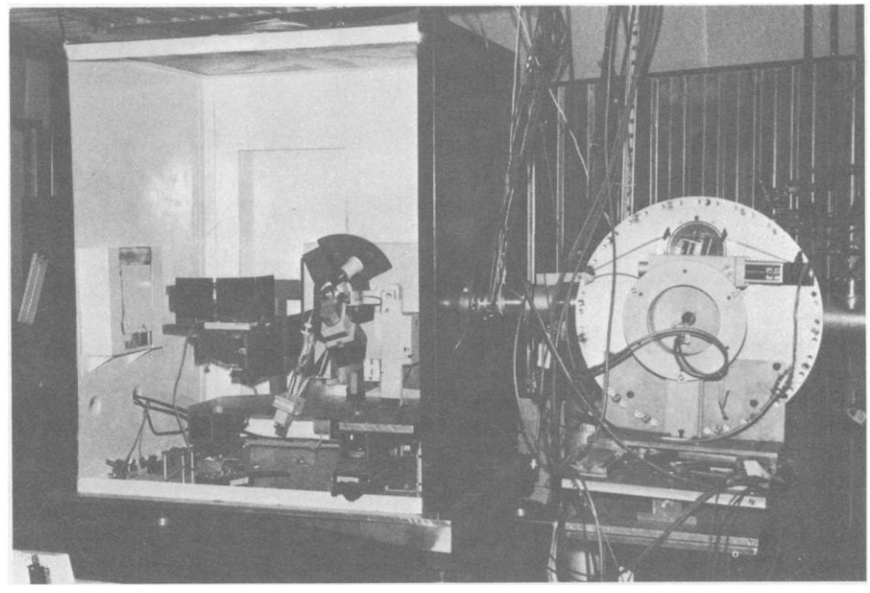

Fig. 1. View of the experimental apparatus. The tank housing the two-crystal monochromator is seen on the right. The monochromatized x-ray beam enters the hutch (which has one side removed to show the inside) and passes into the entrance collimator of the diffractometer. The diffractometer is supported by the computer-controlled carriage, seen in the lower part of the hutch. The 2θ arm holding the detector can be seen below the median plane, and the film carousel (which is removed when the detector is being used) is to the left.

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