J. comp. Physiol. 125, 129–134 (1978)

Journal of Comparative Physiology · B

Temperature Dependence of Intracellular pH: Its Role in the Conservation of Pyruvate Apparent K_m Values of Vertebrate Lactate Dehydrogenases

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Accepted December 28, 1977

Summary. When apparent Michaelis constants (K_m's) for pyruvate of M_4-lactate dehydrogenases from differently thermally adapted vertebrates are measured at the species' normal cell temperatures, a marked degree of conservation in K_m is observed, but only when the pH of the assay medium is varied in the manner in which intracellular pH varies with temperature in most animals (Fig. 2). K_m measurements performed at a constant pH do not yield this high degree of interspecific conservation in K_m (Figs. 2 and 3).

The temperature dependence of intracellular pH preserves the charge states of imidazoles of protein histidines during temperature transitions. Thus under intracellular conditions the ionization state of the active site histidine of LDH will be independent of temperature, reducing the temperature dependence of pyruvate binding. This effect appears important in the contexts of short-term temperature variation experienced by an individual ectotherm and of long-term, evolutionary temperature changes important in speciation processes.

These findings emphasize the importance of utilizing biologically realistic pH values in enzyme studies if major adaptive trends are to be observed.

Introduction

Hydrogen ion activity is an important factor in many enzymatic reactions. Substrate binding and catalysis, for example, often involve ionizing groups whose charge states are sensitive to the hydrogen ion activity in the local environment (Laidler and Bunting, 1973). Thus, to avoid complicating effects on kinetics, most in vitro studies of enzymes have been conducted in buffers at a constant pH. However, it is well established that pH values of extra- and intracellular fluids of most animals are dependent on body temperature and, in fact, approximately parallel the pH of water neutrality, decreasing by 0.015–0.02 pH units per °C. Furthermore, pH values are similar among animals adapted to the same temperature (Rahn et al., 1975; Heisler et al., 1976; Malan et al., 1976). The effect of this variable pH on ionizing groups may be important not only in the function of enzymes in eurythermal ectotherms but also in evolutionary adaptations of enzymes to different temperatures. Significant adaptive processes may be missed in temperature studies of enzymes if buffer pH is held constant.

It has been suggested that the temperature dependence of intracellular pH may indeed play a role in the function of at least one enzyme, the M_4 (skeletal muscle) isozyme of lactate dehydrogenase (LDH, EC 1.1.1.27; lactate: NAD^+ oxidoreductase). Apparent Michaelis constants (K_m's)[1] for pyruvate have been found to be quite sensitive to pH, decreasing as pH is lowered (Hochachka and Lewis, 1971; DeBurgos et al., 1973; Wilson, 1977a). Thus, in the cell of an ectotherm, the increase in pyruvate K_m with increasing temperature (a characteristic of M_4-LDH's) may be substantially reduced by the simultaneously decreasing pH (Wilson, 1977a).

This pH dependence of pyruvate binding will be biologically significant only if the magnitude of the K_m of pyruvate is important for the function of M_4-LDH's. It has indeed been argued that the substrate binding abilities of enzymes are highly critical for optimal catalytic and regulatory functions, and that optimal enzyme performance is attained when apparent K_m values fall in the range of intracellular substrate concentrations (Atkinson, 1976). Thus substrate binding abilities should be tightly controlled by natural selection, and should be adapted to cope with environmental and physiological factors which

[1] K_m refers to apparent K_m throughout the text

0340-7616/78/0125/0129/$01.20

alter K_m values. There is a simple test of these arguments: in many cases, an enzyme and its homologues from other species should have similar K_m values since concentrations of substrates are generally similar in homologous tissues from different organisms (Sacks et al., 1954; Vesell and Pool, 1966; Freed, 1971). Such conservation in K_m values should be detectable in vitro only when major physiological factors affecting binding are adjusted in the assay medium to values appropriate for each enzyme. Support for this line of reasoning is found with several enzymes and their homologues which fit these predictions when one of these major physiological factors, adaptation temperature, is so adjusted (Baldwin, 1971; Kobayashi et al., 1974; Wedler and Hoffmann, 1974; Low and Somero, 1976).

Nevertheless, even when assayed at respective adaptation temperatures, M_4-LDH's do not show consistent evidence of conservation in pyruvate K_m values when data from various studies in the literature are compared. However, most of these studies used a constant pH buffer, usually at pH 7.4. Since intracellular pH varies with adaptation temperature within and among organisms, and is probably less than 7.4 in most species (Rahn et al., 1975), the present study was conducted to test for conservation in pyruvate K_m values under biologically realistic conditions of pH as well as temperature. Results indicate that pyruvate K_m values have indeed been conserved among vertebrate M_4-LDH's, and that the normal change in intracellular pH which occurs with changing temperature may stabilize pyruvate binding abilities during short-term and evolutionary adaptation to temperature.

Materials and Methods

Experimental Animals

The following animals were used in this study: Teleost fishes – *Trematomus* (= *Pagothenia*) *borchgrevinki* (Nototheniidae); *Sebastolobus alascanus* (Scorpaenidae); *Hippoglossus stenolepis*, pacific halibut (Pleuronectidae); *Thunnus thynnus*, bluefin tuna (Scombridae); *Hypostomus plecostomus*, Amazon armored catfish (Loricariidae); *Gillichthys mirabilis*, longjaw mudsucker (Gobiidae); Elasmobranch fishes – *Potamotrygon* sp. (South American freshwater stingray) (Potamotrygonidae); Mammal – rabbit.

Purification of M_4-Lactate Dehydrogenases

M_4-LDH's from halibut and bluefin tuna were generously provided by the laboratory of Dr. N.O. Kaplan. Rabbit enzyme was purchased from Sigma Chemical Company, St. Louis, Mo. M_4-LDH's from the remaining organisms were purified on an oxamate-Sepharose affinity column, prepared in collaboration with Joseph Siebenaller of this institution according to the following procedure modified from Spielmann et al. (1973): 5 g of aminohexyl Sepharose 4B were swelled in 200 ml of 0.5 M NaCl, then washed on a sintered glass filter with 1 l each of 0.5 M NaCl and distilled water, yielding 20 ml of gel. 5 g of potassium oxalate in 20 ml water were added to 3 g of fresh 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide dissolved in 10 ml of water. The pH was not adjusted. After 4 min this solution was added to the gel in a flask and gently swirled at room temperature for 28 h. The gel was then washed with 2 l of water, then with 1 l of 0.5 M KCl in 0.05 M potassium phosphate buffer, pH 6.8 (column buffer). The trinitrobenzene-sulfonate test (Cuatrecasas, 1970) indicated complete substitution. Each muscle LDH purification followed the procedure of O'Carra et al. (1974) for placental LDH, but using several grams of white skeletal muscle, a 5 ml bed column at 5 °C, and eluting the M_4 isozyme with 2.5 mM NAD^+ and 0.25 M KCl in the column buffer. For species possessing only the M_4 isozyme in white muscle, LDH was eluted with 0.5 M KCl. Enzyme preparations were examined by starch gel electrophoresis, using an activity stain, to ascertain that only the M_4 isozyme was present in the final fraction. Isozyme separation was complete in all cases, and SDS-acrylamide gel analysis, using a general protein stain, with some of the preparations revealed only a single protein species. All LDH's were stored as ammonium sulfate precipitates and were highly diluted or dialyzed before assaying.

Determination of Enzyme Velocities and Apparent Pyruvate K_m's

Activities of the enzymes were assayed in a Varian-Techtron 635 spectrophotometer by monitoring the decrease in absorbance at 340 nm with time. Temperature of the spectrophotometer cell was controlled to ±0.2 °C with a circulating water bath. Assays were conducted in the following buffer systems: 1) *constant pH* – 66.7 mM potassium phosphate buffer, adjusted to different pH values by varying the proportions of K_2HPO_4 and KH_2PO_4, and 2) *temperature-dependent pH* – 80 mM imidazole-HCl buffer, which has a temperature dependence of −0.02 pH units/°C (Perrin and Dempsey, 1974), similar to that of biological fluids (Rahn et al., 1975); the pH was adjusted to 6.98 at 20 °C and followed the temperature curve shown in Figure 2A, an approximation of known intracellular pH values in vertebrate muscle. Assay solutions also contained 0.15 mM reduced nicotinamide adenine dinucleotide (NADH) and various concentrations of sodium pyruvate. At each temperature activity was measured at seven to ten pyruvate concentrations between 0.08 and 4 mM. Apparent K_m values and 95% confidence limits were calculated using a weighted linear regression method according to the technique of Wilkinson (1961).

Reagents

All reagents were purchased from Sigma Chemical Company, St. Louis, Mo.

Results

Using M_4-LDH's from a number of teleost fishes, a freshwater elasmobranch and a mammal, pyruvate K_m values were determined as a function of temperature under two different pH regimes.

Constant pH

K_m values were determined at a constant pH of 7.4 and compared over the respective body temperature

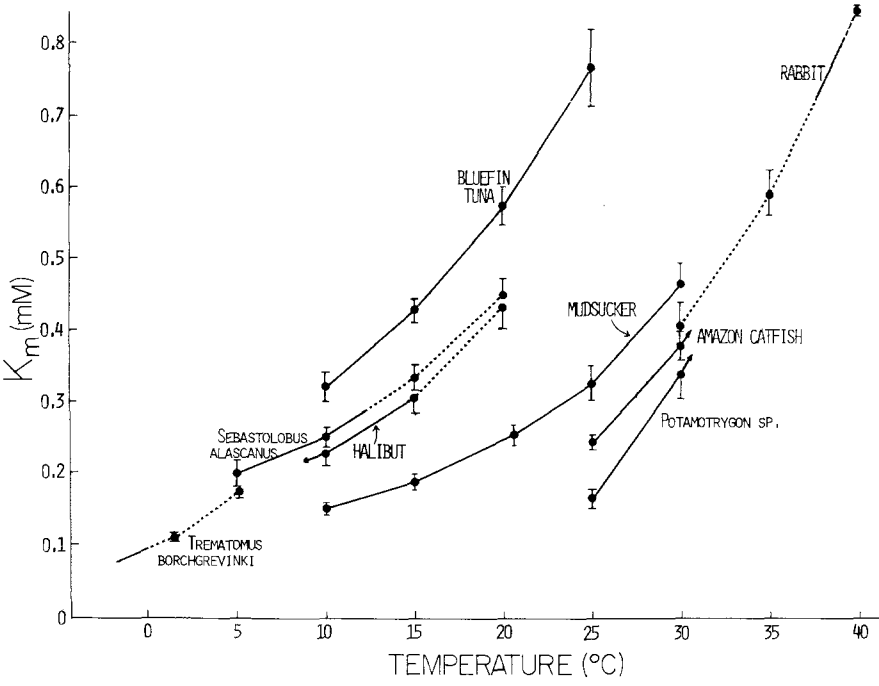

Fig. 1. Effect of temperature on apparent Michaelis constants (K_m) for pyruvate of M_4-LDH's of several vertebrate species. Assays were conducted in phosphate buffer, pH 7.4 at all temperatures. Solid lines represent approximate temperature ranges of these species (the plot for *Trematomus* is extrapolated below 1.5 °C). 95% confidence limits are shown

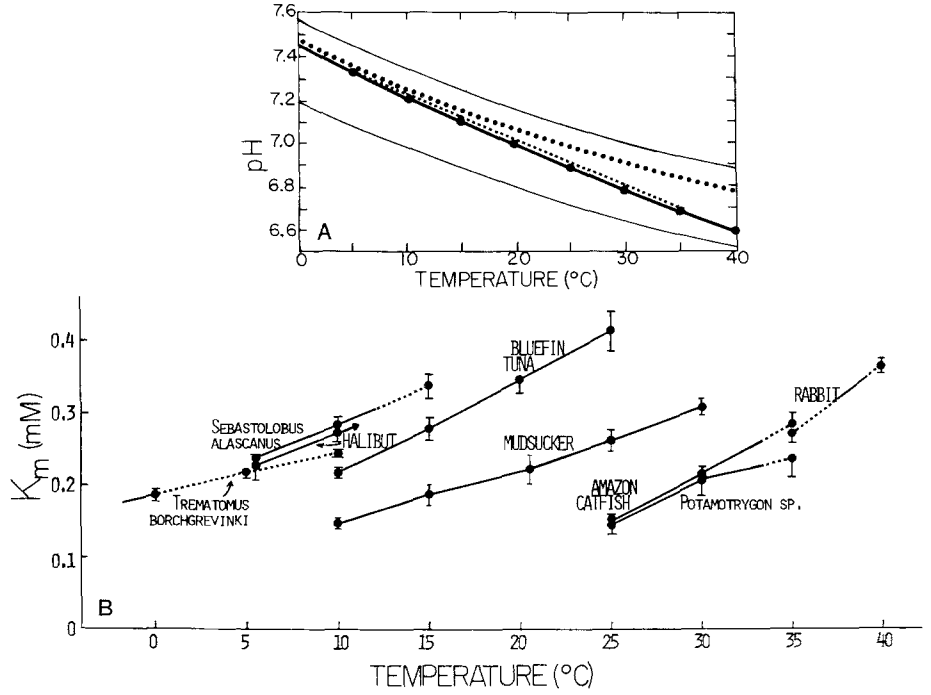

Fig. 2. **A** Effect of temperature on the pH of imidazole-HCl buffer (●——●) (determined by the authors); water neutrality(● ● ● ●) (Rahn et al., 1975); and intracellular fluid in turtle skeletal muscle (------) (from Malan et al., 1976). The upper and lower lines represent the range of known intracellular pH values from numerous animals and tissues (Rahn et al., 1975). **B** Effect of temperature on apparent Michaelis constants (K_m) for pyruvate of M_4-LDH's of several vertebrate species. Assays were conducted in imidazole-HCl buffer with temperature-dependent pH values shown in **A**. Solid lines represent approximate temperature ranges of the species (the plot for *Trematomus* is extrapolated below 0 °C). 95% confidence limits are shown

ranges of the different species (Fig. 1). Two trends are apparent. First, K_m values for each enzyme increase rapidly with temperature, spanning a wide range in the case of eurythermal species such as the bluefin tuna and mudsucker. Second, among different LDH's K_m values vary more than ten-fold (from approximately 0.07 to 0.80 mM) and tend to be higher for enzymes from species adapted to higher temperatures.

Temperature-Dependent pH

K_m values determined in imidazole-HCl buffer are plotted in Figure 2B (Fig. 2A shows the pH at each assay temperature). The reduction in pH with increasing temperature significantly alters the trends seen in Figure 1. First, the K_m of each LDH is much less temperature-sensitive in the imidazole system. For example, the K_m of the mudsucker LDH increases 200% between 10 and 30 °C in the constant pH system, but only 100% in the imidazole system. Second, K_m values at normal body temperatures of the different species fall within a much narrower range, approximately 0.15 to 0.35 mM, in the variable pH imidazole-HCl buffer system. That is, a marked interspecific conservation of K_m is observed when biologically realistic pH values are employed.

For all of the enzymes examined, the maximal velocity (V_{max}) of the reaction was independent of pH over the pH range used in the assays. Thus the pH dependence of the apparent K_m of pyruvate appears to derive entirely from pH effects on the binding constant for pyruvate (see Discussion).

Effects of Buffer Type and Absolute pH on K_m Values

Phosphate buffer at pH 7.4 was chosen for the constant pH experiments because this is the most common buffer system used in previous studies of LDH's. In comparison to the standard phosphate buffer system, the pH of the imidazole-HCl buffer used in this study ranged from about 7.4 to 6.6 between 0 and 40 °C, and the K_m values observed in the imidazole system were higher, at a given pH, than values obtained with the phosphate system (Fig. 3). In order to be certain that the different patterns of pyruvate K_m values seen in the two buffer systems are due to the different temperature dependencies of buffer pH and not to the absolute pH or the buffer type, several of the enzymes were assayed in phosphate buffer at pH 6.98 and at pH values adjusted to match those of the imidazole buffer at each temperature.

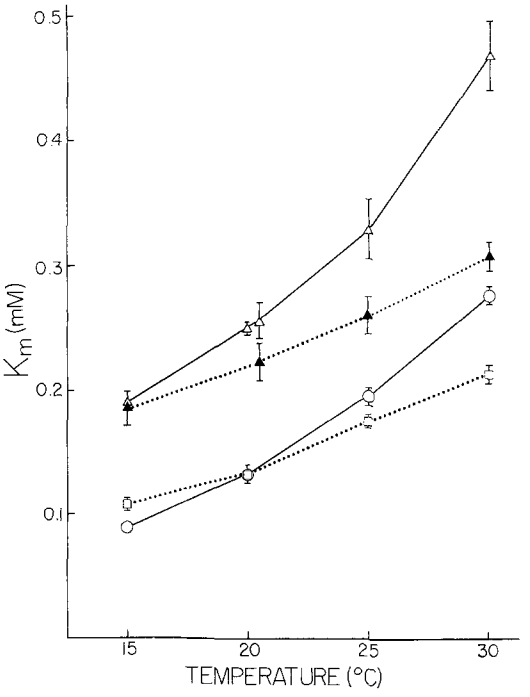

Fig. 3. Comparison of the temperature dependence of pyruvate K_m values for mudsucker M_4-LDH determined in different buffers and at different pH values. Constant pH: 66.7 mM potassium phosphate buffer, pH 7.4 (△——△); pH 6.8 (○——○). Temperature-dependent pH: phosphate buffer (□ · · · □); 80 mM imidazole-HCl buffer (▲ · · · ▲). For both buffers the pH values were: 7.09 (15 °C), 6.98 (20 °C), 6.88 (25 °C) and 6.78 (30 °C). 95% confidence intervals are shown

The K_m values for mudsucker LDH under these two sets of conditions are shown in Figure 3. Identical effects were found with the other LDH's. It is apparent that, although K_m values are considerably higher in imidazole buffer, the temperature dependence of K_m is due strictly to the change in buffer pH with temperature, not to buffer type.

Discussion

The data presented here demonstrate that, if intracellular pH values do vary with evolutionary adaptation temperature as they do when the body temperature of an individual ectotherm is varied, then pyruvate K_m values have been conserved among vertebrate M_4-LDH's in two ways: 1) within each organism, such that changes in pyruvate K_m with temperature are relatively small (Fig. 2B; see also Hochachka and Lewis, 1971; DeBurgos et al., 1973; Wilson, 1977a); and 2) throughout much of vertebrate evolution, such that the pyruvate K_m values of all M_4 homologues are very similar when measured at different species' cell pH and temperature values (Fig. 2B). Thus pyru-

vate K_m values are probably critical for optimal catalytic and regulatory capabilities of M_4-LDH's (the importance of pyruvate binding ability in LDH function is discussed in detail in the following paper, Yancey and Somero, 1978).

The results also indicate that intracellular pH plays an important role in this conservation of pyruvate K_m values. The pH of intracellular fluids during temperature changes closely follows not only the pH of water neutrality, but also the pK values of histidine imidazole groups (Roberts et al., 1969; Rahn et al., 1975) and of some sulfhydryl groups (Wilson, 1977b). Since histidine imidazoles are the principal ionizing groups of proteins in the physiological pH range, the overall charge states of proteins will remain approximately constant during temperature transitions. This may be an important mechanism in preventing temperature-induced disruptions in protein structure and function (Malan et al., 1976). In particular, intracellular pH may be important in the function of histidines (and possibly cysteines) involved in binding and catalysis in many enzymes. The active site of LDH, for example, has a histidine residue which must be protonated for pyruvate binding to be possible. Wilson (1977a) has interpreted the temperature dependence of pyruvate K_m to be the result of two enthalpy changes: the ionization enthalpy of this histidine imidazole (ΔH_i^0) and the enthalpy of pyruvate binding to the protonated LDH-NADH complex (ΔH_{so}). In a constant pH environment, the histidine will lose ability to bind pyruvate with increasing temperature because of its sizeable ionization enthalpy (which gives histidines their characteristic temperature-dependent pK values). However, if pH varies inversely with temperature in the cell of an animal, the contribution of ΔH_i^0 to the temperature dependence of pyruvate binding will be essentially zero (Wilson, 1977a, b). Thus the temperature dependence of intracellular pH will not only stabilize the pyruvate affinity of the M_4-LDH of an ectotherm which experiences fluctuating temperatures, but may also minimize binding site modifications via amino acid substitutions which are required for evolutionary adaptation to a new thermal regime. Modifications in the ΔH_{so} contribution to pyruvate binding enthalpies may also occur during temperature adaptation, of course, but these changes may be but a concomitant to adjustments in the free energy of pyruvate binding. The latter have been suggested to play an important role in the temperature adaptation of vertebrate LDH's (Borgmann and Moon, 1975).

The results presented in this paper demonstrate that two important factors must be carefully chosen in enzyme studies: 1) the buffer type, since kinetic values may not be comparable between different buffers (Fig. 3); and 2) experimental pH, particularly if experimental and/or adaptation temperature is a variable under study. With M_4-LDH's the use of a constant pH buffer system could lead to false conclusions about the temperature sensitivity and selective importance of pyruvate K_m values (Fig. 1). Although this study uses pH values close to that of water neutrality to mimic the intracellular environments, it should be realized that techniques for measuring intracellular pH are not highly accurate (Waddell and Bates, 1969) and cannot reveal the "true" pH of local microenvironments within a cell. Nevertheless, available values for intracellular pH are probably good estimates of the actual pH of the cytosol in which the M_4-LDH's function. In any case, the relative change in pH with temperature is probably similar in most intracellular environments, an effect which can easily be taken into account in enzyme assay systems.

These studies were supported by National Science Foundation grant PCM-09498.

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