Interactions of temperature and pH on the regulatory properties of pyruvate kinase from organs of a marine mollusc

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Abstract: The effects of low temperature assay (5 °C) on the properties of the aerobic (low phosphate) vs. anoxic (high phosphate) forms of pyruvate kinase (PK) from foot muscle and gill of the whelk *Busycon canaliculatum* (L.) were assessed at two pH values, pH 7.00 and 7.25, and compared to control conditions of 20 °C and pH 7.00 (all assayed in imidazole buffer). When pH was held constant at 7.00, the decrease in assay temperature to 5 °C had large effects on the measured kinetic parameters of all PK forms, as compared to 20 °C and pH 7.00. However, when assay pH was allowed to rise, from 7.00 to 7.25, with the temperature decrease to 5 °C there were fewer alterations of kinetic parameters and quantitatively smaller changes to enzyme properties. It appears, then, that when pH rises with decreasing temperature following alphastat predictions, kinetic properties of PK are largely conserved. Low temperature, at either pH value, had several significant effects on PK properties. For example, low temperature raised the $S_{0.5}$ for phosphoenolpyruvate of PK-anoxic from gill by 3-6 fold and decreased the I_{50} Mg $\cdot$ ATP for PK-anoxic from foot by the same amount. Arrhenius plots of PK activity for the gill PK forms showed a distinct break at 10 °C; > 10 °C Q_{10} was 2.5 whereas < 10 °C Q_{10} was 8.4. Temperature-dependent changes in all cases affected enzyme properties in a manner that would restrict enzyme function at low temperature.

Key words: Busycon canaliculatum; pH effects on pyruvate kinase; Pyruvate kinase; Temperature effects on pyruvate kinase

INTRODUCTION

Blood pH of poikilothermic animals typically rises as body temperature is lowered due to either a ventilatory reduction in P_{CO_2} in air-breathing poikilotherms (Howell et al., 1970) or an elevation of plasma bicarbonate in aquatic poikilotherms (Randall & Cameron, 1973; Rahn & Baumgarder, 1972; Heisler et al., 1976). In most cases, the change in blood pH as a function of temperature parallels the effect of temperature upon the pH of neutral water such that the change is $\approx +0.017$ pH units per degree centigrade decrease in temperature (Wilson, 1977; Somero, 1981). A variety of studies have shown that intracellular pH values also frequently follow this pattern (Heisler et al., 1976; Malan et al., 1976; Reeves, 1972; Reeves & Malan, 1976).

Reeves (1972) found that adjustments of arterial pH in response to changing tempera-
ture minimized changes to the degree of dissociation of ionizable groups on proteins and
thus preserved the net charge on plasma proteins. He postulated that poikilotherms
regulate body fluids to maintain a constant net charge on proteins rather than a constant
net alkalinity. Within the physiological pH range the net charge on most proteins is
determined by the dissociation of histidine imidazole moieties (Hazel et al., 1978) and
the effect of temperature on the pK of these groups parallels the effect of temperature
on the dissociation constant of water. Thus, for a protein whose net charge is determined
largely by histidine imidazole residues, a constant charge state will be maintained as
temperature changes. The above postulation is known as the alphastat hypothesis
(Reeves, 1972).

The hypothesis suggests that the conservation of net charge on intracellular proteins
preserves their individual functional integrity as well as the integrated functioning of
metabolic pathways as body temperature changes (Malan et al., 1976). One would
expect, therefore, that enzyme kinetic constants (for substrates as well as effectors)
would be conserved as temperature changes provided that pH change follows alphastat
predictions. This has, indeed, been shown experimentally for some enzymes from
poikilothermic animals (Hazel et al., 1978; Yancey & Somero, 1978).

For many intertidal marine invertebrates in temperate zones, seasonal (or even acute)
decreases in temperature $<5 °C$ trigger metabolic arrest responses that reduce
metabolic rate to promote long term survival under conditions where food supplies are
typically scarce (Aarset, 1982; Murphy, 1983). For example, Q_{10} for oxygen con-
sumption was 22 between 4 and 0 °C for *Modiolus demissus* (Murphy, 1977). These same
animals also typically show a metabolic depression response to anoxia and, indeed, their
response to low temperature often includes actions (e.g., valve closure in bivalve
molluscs) that result in anoxia and its associated metabolic acidosis. We wondered,
then, about the interactions between low temperature and pH in the regulation of
enzymes from anoxia-tolerant marine molluscs. The present study examines the effects
of temperature change, with or without alphastat adjustment of assay pH, on the
properties of the aerobic (low phosphate) versus anoxic (high phosphate) enzyme forms
of pyruvate kinase (PK) from two organs of the marine whelk *Busycon canaliculatum*
(L.).

MATERIALS AND METHODS

CHEMICALS AND ANIMALS

Chemicals were obtained from Boehringer Mannheim GmbH, Sigma Chemical Co.,
or J. T. Baker Chemical Co. *B. canaliculatum* were purchased from the Marine Biologi-
cal Laboratory, Woods Hole, Massachusetts, and were kept until used in recirculating
seawater (1000 mOsmol) at 15–18 °C without feeding. Aerobic whelks were sampled
directly from the seawater tank. The shell was rapidly removed and tissues were

dissected out and immediately frozen in liquid nitrogen. All tissues were stored at $-80\,°C$ until used. To impose anoxia, whelks were placed in a large tub (10 l, two animals per tub) of seawater that had been previously deoxygenated by bubbling with a steady stream of nitrogen gas for 12–16 h. Whelks were exposed to anoxia for 16 h with the flow of nitrogen gas maintained throughout; time course studies have previously shown that PK in all whelk organs is completely converted to the anoxic, high phosphate form within this time. After anoxic exposure, tissues were rapidly dissected out and frozen as described above.

PREPARATION OF TISSUE EXTRACTS

Samples were prepared using a buffer that contained protein kinase and protein phosphatase inhibitors to prevent a change in the phosphorylation state of the aerobic or anoxic enzyme forms. Frozen tissues were rapidly weighed and homogenized 1:5 w/v in ice-cold 50 mM imidazole-HCl buffer, pH 7.00 containing 10 mM EDTA, 10 mM EGTA, 100 mM NaF, and 30 mM 2-mercaptoethanol with 0.1 mM PMSF (phenylmethylsulfonyl fluoride) added just prior to homogenization using a Polytron PT10 homogenizer (3×20 s). Homogenates were centrifuged for 20 min at 24 000 g in a Sorvall RC-5B refrigerated centrifuge at 5 °C. The supernatant was removed and then desalted by passage through a 5 ml column of Sephadex G-25 equilibrated in homogenization buffer and centrifuged for 1 min in an IEC desk-top centrifuge (Helmerhorst & Stokes, 1980). The filtrate was used for enzyme assay.

ENZYME ASSAY AND KINETIC STUDY

Enzyme activity was monitored at 340 nm using a Pye Unicam SP 1800 recording spectrophotometer with water-jacketed cell holder for temperature control. Cuvettes were pre-equilibrated in a water bath to the desired temperature (20 or 5 °C) and cuvette temperature was directly checked using a YSI telethermometer. Standard assay conditions for PK were 50 mM imidazole-HCl buffer, 2 mM ADP, 0.15 mM NADH, 50 mM KCl, 5 mM $MgCl_2$, 2 IU dialyzed lactate dehydrogenase and PEP at either 1 mM (aerobic enzyme) or 5 mM (anoxic enzyme) in a final volume of 1 ml. Assays at 20 °C were performed at pH 7.00. Assays at 5 °C were performed at either pH 7.00 or 7.25. Imidazole buffers for 5 °C assay were adjusted to pH 6.75 or 7.00 at 20 °C and then pH was allowed to change with cooling to 5 °C to pH 7.00 or 7.25, respectively.

$S_{0.5}$ PEP was determined at saturating ADP concentration and calculated from Hill plots. Activation constants (K_a) were determined using double reciprocal plots: $1/(V - V_0)$ vs. $1/[\text{activator}]$. I_{50} values (the inhibitor concentration producing 50% reduction in enzyme activity) were determined by the method of Job et al. (1978). For the determination of I_{50} Mg·ATP a 1:1 molar mixture of $MgCl_2$ and ATP was used as the source of ATP; Mg^{2+} added in this way was in addition to the 5 mM Mg^{2+} in the standard assay. For Arrhenius plots PEP substrate saturation plots were constructed at each assay temperature and the experimental V_{max} value was measured; for these

determinations, imidazole buffer pH was adjusted to pH 7.00 at 20 °C. All kinetic parameters are the $\bar{x} \pm$ SEM of determinations made on three separate enzyme preparations from different animals. Statistically significant differences were tested using the Student's t test.

<div align="center">RESULTS</div>

FOOT MUSCLE PK

The effects of temperature and pH change on the kinetic properties of the aerobic (low phosphate) and anoxic (high phosphate) forms of PK from whelk foot muscle are given in Table I. At 5 °C two pH values were tested: pH 7.00 (pH held the same as at 20 °C) and pH 7.25 (pH of the imidazole buffer allowed to rise with the decrease in temperature). When pH was held constant at 7.00 at 5 °C, the kinetic properties of PK-aerobic changed substantially: $S_{0.5}$ PEP rose 2.3-fold, the Hill coefficient decreased, I_{50} ATP increased and K_a FBP increased dramatically. When pH was allowed to rise to 7.25 with changing temperature, however, only the n_H and K_a FBP changed at 5 °C.

Changes in foot PK kinetic properties were similarly more pronounced for the anoxic enzyme form when pH was held constant at 7.00 at 5 °C (Table I). All the kinetic parameters measured at pH 7.00 and 5 °C were significantly different from those measured at pH 7.00 and 20 °C, including a 13-fold increase in I_{50} alanine and a 6-fold decrease in I_{50} ATP. When pH was 7.25 at 5 °C, however, I_{50} alanine and K_a FBP were not affected by the temperature change, I_{50} ATP decreased only 3-fold and $S_{0.5}$ PEP increased by 68%.

The effects of the change in assay pH alone, at constant 5 °C temperature, were relatively small for foot PK-aerobic; $S_{0.5}$ PEP changed by 2-fold and lesser changes occurred for I_{50} ATP and K_a FBP (Table I). For PK-anoxic, however, the change in enzyme kinetic constants with pH change at 5 °C was dramatic. At pH 7.00, compared to pH 7.25, $S_{0.5}$ PEP decreased 4-fold, I_{50} alanine increased by 18-fold, I_{50} ATP decreased 2-fold and K_a FBP decreased by 40%.

GILL PK

Temperature and pH change had different effects on the properties of PK from whelk gill (Table II). When pH was held constant at 7.00 with the temperature change, all of the kinetic properties of PK-aerobic were altered; particularly dramatic changes were an 8-fold decrease in I_{50} alanine and a change in ATP inhibition from no inhibitory action (at levels up to 30 mM) at 20 °C to strong inhibition (I_{50} 0.13 mM) at 5 °C. When pH was allowed to rise to 7.25 at 5 °C, however, the changes to the kinetics of gill PK-aerobic were less dramatic; I_{50} alanine decreased by only 5-fold, changes to $S_{0.5}$ PEP and K_a FBP were small, and ATP inhibited but only at high levels. There were, therefore, two large effects of pH change at 5 °C on gill PK-aerobic; at pH 7.00, compared to 7.25, K_a FBP increased by 5-fold whereas I_{50} ATP decreased by 100-fold.

TABLE I

Effect of pH change at low temperature on kinetic constants of aerobic and anoxic forms of pyruvate kinase from whelk foot muscle.

	PK-aerobic			PK-anoxic		
	20°C pH 7.00	5°C pH 7.00	pH 7.25	20°C pH 7.00	5°C pH 7.00	pH 7.25
$S_{0.5}$ PEP (mM)	0.092 ± 0.003	0.21 ± 0.015[a]	0.10 ± 0.009[c]	1.53 ± 0.06	0.67 ± 0.07[a]	2.57 ± 0.20[a,c]
n_H	0.94 ± 0.014	0.65 ± 0.068[b]	0.64 ± 0.030[b]	2.36 ± 0.07	0.44 ± 0.02[a]	0.60 ± 0.04[a,c]
I_{50} Ala (mM)	2.26 ± 1.63	2.54 ± 0.13	2.26 ± 0.16	0.067 ± 0.009	0.86 ± 0.077[a]	0.048 ± 0.007[c]
I_{50} Mg·ATP (mM)	22.0 ± 0.93	28.6 ± 0.80[a]	22.8 ± 1.63[d]	25.8 ± 0.83	4.27 ± 0.30[a]	8.26 ± 0.65[a,c]
K_a FBP*	0.046 ± 0.002	1.63 ± 0.095[a]	2.12 ± 0.18[a,d]	1.44 ± 0.027	0.94 ± 0.052[a]	1.60 ± 0.14[d]

Values are $\bar{x}$ ± SEM, $n = 3$ determinations on separate preparations of enzyme from different animals. Effector constants were determined at subsaturating levels of PEP, 0.2 mM PEP for the K_a FBP for both enzyme forms and the I_{50} values for aerobic enzyme and 1.50 mM PEP for I_{50} values for anoxic enzyme form. [a] Significantly different at either pH 7.00 or 7.25 at 5°C from corresponding value at pH 7.00 and 20°C, $P < 0.005$; [b] $P < 0.01$; [c] significantly different from corresponding value for enzyme at same (5°C) temperature but different pH value, $P < 0.005$; [d] $P < 0.05$. *K_a values are in mM for PK-aerobic at 5°C at both pH values and in µM for all others.

TABLE II

Effect of pH at low temperature on kinetic constants of aerobic and anoxic forms of pyruvate kinase from whelk gill.

Enzyme constants	Aerobic gill			Anoxic gill		
	pH 7.00 at 20°C	pH 7.00 at 5°C	pH 7.25 at 5°C	pH 7.00 at 20°C	pH 7.00 at 5°C	pH 7.25 at 5°C
PEP $S_{0.5}$ (mM)	0.83 ± 0.018	1.57 ± 0.081[a]	1.24 ± 0.040[a,d]	2.53 ± 0.10	7.45 ± 0.42[a]	15.46 ± 1.51[a,c]
n_H	1.14 ± 0.079	0.65 ± 0.023[a]	0.55 ± 0.021[a,d]	1.52 ± 0.081	0.59 ± 0.021[a]	1.04 ± 0.075[a,c]
I_{50} Ala (mM)	0.66 ± 0.030	0.085 ± 0.006[a]	0.13 ± 0.010[a,d]	0.062 ± 0.003	0.16 ± 0.02[a]	0.083 ± 0.004[a,d]
I_{50} MgATP (mM)	NE	0.13 ± 0.014	12.79 ± 1.16[a]	6.99 ± 0.83	7.90 ± 0.51	4.03 ± 0.33[c]
K_a FBP (µM)	1.07 ± 0.027	4.38 ± 0.35[a]	0.82 ± 0.035[a,c]	6.42 ± 0.61	9.83 ± 0.90[b]	4.34 ± 0.28[b,c]

Values are $\bar{x}$ ± SEM, $n = 3$ determinations on separate preparations of enzyme from different animals. Effector constants were determined at subsaturating levels of PEP, 0.2 mM PEP for K_a FBP for both enzyme forms and 1.5 mM PEP for I_{50} values for anoxic enzyme form. [a] Significantly different at either pH 7.00 or 7.25 at 5°C from corresponding value at pH 7.00 at 20°C, $P < 0.005$; [b] $P < 0.01$; [c] significantly different from corresponding values for enzyme at same temperature, $P < 0.005$; [d] $P < 0.025$. NE, no effect of MgATP on enzyme at levels up to 30 mM.

For the anoxic enzyme form from gill, the effect of temperature and pH change were most pronounced for $S_{0.5}$ PEP but most parameters were again altered from the values at pH 7.00 and 20 °C (Table II). When pH was held constant at 7.00, $S_{0.5}$ PEP rose 3-fold, I_{50} alanine rose 2.6-fold and K_a FBP increased 1.5-fold. When pH was allowed to rise to 7.25 at 5 °C, the effect of low temperature on PK-anoxic was even more pronounced, $S_{0.5}$ PEP increasing 6-fold over the value at 20 °C. However, I_{50} for alanine increased only slightly and I_{50} ATP and K_a FBP both decreased as compared to the values at 20 °C. For the anoxic enzyme form, then, the effects of pH change at a constant 5 °C were (at pH 7.00 compared to pH 7.25) a 2-fold decrease in $S_{0.5}$, and $\approx$ 2-fold increases in I_{50} and K_a values.
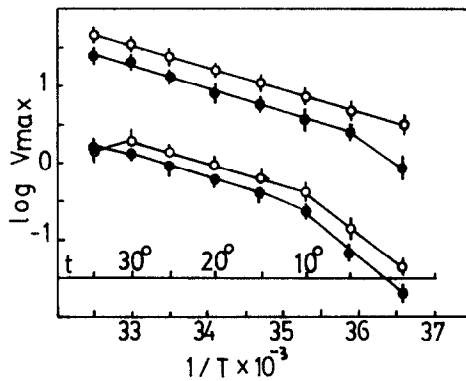

Fig. 1. Arrhenius plots of maximal activity of PK (U/g wet weight) assayed between 35 and 0 °C for muscle (upper curves) vs. gill (lower curves) enzymes. Open symbols are PK-aerobic; filled symbols are PK-anoxic. x axis shows $1/T$ (°K) with an inset of temperature (°C).

Arrhenius plots of the effect of temperature on the V_{max} of PK are shown in Fig. 1 for both aerobic and anoxic enzyme forms from whelk foot and gill. For PK-aerobic from foot, the relationship was a linear one over the entire temperature range, resulting in a Q_{10} of 2 and an activation energy for the enzyme of 12 436 cal/mol. PK-anoxic from foot paralleled the aerobic form over most of the temperature range but showed a sharp drop in activity between 5 and 0 °C. The relationship between temperature and activity for the gill enzyme was biphasic with two linear portions between 30 and 10 °C vs. 10 and 0 °C, respectively. Activation energies for the aerobic enzyme in the two portions were 13 993 vs. 32 345 cal/mol. Q_{10} values were $\approx$ 2.5 in the 30 to 10 °C interval but jumped to 8.4 between 10 and 0 °C.

DISCUSSION

We have previously documented the anoxia-induced phosphorylation of PK in several whelk organs and the resulting changes in enzyme kinetic parameters that produce less

active enzyme forms during anaerobiosis (Plaxton & Storey, 1984a,b, 1985; Whitwam & Storey, 1990; Michaelidis & Storey, 1990). PK from foot and gill of the whelk *B. canaliculatum* are two distinct isozymic forms (Plaxton & Storey, 1985) with different kinetic properties in both the aerobic (low phosphate) and anoxic (high phosphate) forms (Michaelidis & Storey, 1990). Each isozyme is subject to covalent modification via reversible protein phosphorylation and to allosteric regulation by L-alanine, Mg · ATP, and FBP. The kinetic constants of both aerobic and anoxic forms of each isozyme also respond differently to changes in assay pH (Michaelidis & Storey, 1990). In particular, deceasing pH (at 20 °C) increases inhibition of the enzyme by alanine and Mg · ATP; the natural decline in intracellular pH over the course of anaerobiosis would, therefore, further facilitate the inactivation of PK during anoxia.

pH AND TEMPERATURE INTERACTIONS

The present study examines the interactions of temperature and pH on the kinetic properties of PK. In general, the data show a greater conservation of kinetic constants between 20 and 5 °C when the assay pH at 5 °C was 7.25. In other words, when the pH of the imidazole assay buffer was allowed to rise with decreasing temperature following the effect of temperature on the pK for imidazole, then PK properties were more strongly conserved. Thus, in a variety of instances, kinetic constants at 5 °C and pH 7.25 showed no significant difference from the equivalent parameters measured at 20 °C. When the comparison was between 5 and 20 °C both at pH 7.00, many more significant changes in enzyme properties were noted (e.g., $S_{0.5}$ PEP and I_{50} ATP of foot PK-aerobic, among others). In other instances, kinetic constants at 5 °C were significantly different from the 20 °C values at both of the assay pH values but the magnitude of change at 5 °C was much smaller when the assay was at pH 7.25 (I_{50} values for alanine and ATP for foot PK-anoxic are examples here). Only in three instances was the reverse true with kinetic constants more strongly altered at low temperature at pH 7.25 compared to pH 7.00 (e.g., $S_{0.5}$ PEP and I_{50} ATP for gill PK-anoxic).

It appears, then, that alphastat predictions are upheld, in general, for the functioning of PK from whelk organs; kinetic parameters for both the aerobic and anoxic enzyme forms are, in most instances, either conserved or less affected by temperature change if pH is allowed to change with decreasing temperature than if pH is held constant. These results agree with the findings for enzymes from vertebrate poikilotherms. For example, Yancey & Somero (1978) found that K_m values for pyruvate of M4-lactate dehydrogenases are conserved when the pH of the imidazole assay buffer is allowed to vary with changing temperature. The present study, in looking at a wide range of kinetic parameters, shows that alphastat regulation affects not just enzyme-substrate affinity but influences a variety of enzyme functions involving both active and allosteric sites on the enzyme protein.

TEMPERATURE EFFECTS ON PK

The present study also analyses the effect of temperature on the properties of aerobic and anoxic forms of PK from whelk organs. The results have relevance to the general reduction of activity and feeding that occurs during the winter months for many temperate zone marine invertebrates. Animals may enter a type of dormancy that for many species can also involve anaerobic metabolism. Murphy (1977, 1983) demonstrated both a strong reduction in oxygen consumption and an accumulation of fermentative end products in the bivalve *Modiolus demissus* held $< 5\,°C$. Stickle et al. (1985) analyzed ingestion and absorption rates for the dogwhelk *Thais lapillus* (L.) at various temperatures and these indicated that cold torpor existed at $5\,°C$. Growth is suppressed at $5\,°C$ (Stickle & Bayne, 1987) and oxygen consumption by *T. lapillus* is lower in the winter than in the summer, the seasonal difference being attributable to both lower temperature and reduced feeding in winter (Bayne & Scullard, 1978). The present results show a steeper drop in the activity of PK-anoxic from *B. canaliculatum* foot when temperature drops below $5\,°C$ and both forms of the gill enzyme show a sharp decrease in activity at temperatures below $10\,°C$ (Fig. 1). Indeed, for the gill enzyme Q_{10} for PK maximal velocity was 8.4 over the range between 10 and $0\,°C$. Similar discontinuous Arrhenius plots have been reported for the effect of temperature on muscle PK from bats with the temperature at which the "break" in the Arrhenius plot occurred being higher ($17\,°C$) for summer vs. winter ($4\,°C$) animals (Borgmann & Moon, 1976). $S_{0.5}$ for PK-anoxic from gill was also strongly affected by low temperature, rising 3–6 fold at $5\,°C$ compared to $20\,°C$. This did not occur for the aerobic enzyme form, however. With $S_{0.5}$ values of 7.5–15.5 mM, activity of the anoxic enzyme would be extremely restricted at low temperature.

For foot PK other kinetic parameters showed high sensitivity to temperature change. The sensitivity of foot PK-aerobic to FBP activation was changed radically at low temperature, K_a rising from $0.046\,\mu M$ at $20\,°C$ to 1.6–2.1 mM at $5\,°C$; at physiological FBP concentrations (Storey et al., 1990) this would virtually remove the enzyme from feed-forward control by the product of phosphofructokinase. For the anoxic form of foot PK, temperature change most strongly affected the I_{50} value for ATP; the enzyme at $20\,°C$ showed very little ATP inhibition (I_{50} 26 mM) whereas the enzyme at $5\,°C$ was inhibited in the range of 4–8 mM ATP, a level that has physiological relevance. These data indicate that the effects of temperature alone can alter the metabolic expression of PK activity. In each case where temperature had such differential effects, the result at $5\,°C$ was a change that would reduce or restrict enzyme activity, as compared to $20\,°C$. Direct temperature effects on enzyme kinetic properties (probably the result of conformational changes at low temperature) may, therefore, be one way that metabolism can be changed or reorganized to respond to differing seasonal needs, including winter dormancy.

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