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Pyruvate Kinase Variants of the Alaskan King-Crab

EVIDENCE FOR A TEMPERATURE-DEPENDENT INTERCONVERSION BETWEEN TWO FORMS HAVING DISTINCT AND ADAPTIVE KINETIC PROPERTIES

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1. Pyruvate kinase of Alaskan king-crab leg muscle exists in two kinetically distinct forms, each of which displays a different temperature-dependence in the K_m for phosphoenolpyruvate. 2. A 'cold' variant of the enzyme has hyperbolic kinetics and exhibits a minimal K_m for substrate at 5°. At physiological concentrations of phosphoenolpyruvate the 'cold' enzyme is active only below 10°. A 'warm' pyruvate kinase has a minimal K_m for substrate at about 12°. This enzyme displays sigmoidal kinetics and is likely to be inactive, at physiological substrate concentrations, at temperatures below 9°. 3. The combined activities of these two pyruvate kinases yield highly temperature-independent rates of catalysis, at physiological substrate concentrations, over the range of habitat temperatures encountered by the organism, namely 4–12°. 4. The two variants of pyruvate kinase do not appear to be isoenzymes in the conventional sense. Electrophoretic and electrofocus analyses revealed only single peaks of activity. 5. The results suggest that the 'warm' pyruvate kinase and the 'cold' pyruvate kinase are formed by a temperature-dependent interconversion of one protein species. This interconversion has major adaptive significance: as the temperature is lowered the 'warm' enzyme is converted into the 'cold' enzyme; the opposite situation obtains when the temperature is raised. Temperature changes thus mimic the effects noted for fructose 1,6-diphosphate on certain mammalian pyruvate kinases.

Enzymes of poikilothermic organisms are uniquely adapted to promote highly stable rates of catalysis over the range of temperatures normally encountered by the organism in its habitat. Thus, at physiological concentrations of substrate, temperature coefficients, Q_{10}, approximating unity have been reported for a large number of reactions catalysed by enzymes of poikilothermic organisms (Zydowo, Makarewicz, Umiastowski & Purzicka, 1965; Cowey, 1967; Makarewicz, 1968; Hochachka & Somero, 1968; Somero & Hochachka, 1968, 1969; Somero, 1969). The basis of this temperature-independence of enzymic reaction rates at physiological substrate concentrations is as follows: for all enzymes of poikilothermic organisms we have examined, decreases in the assay temperature promote concomitant decreases in the K_m for substrate. This temperature–K_m effect is observed over most of the range of temperatures normally experienced by the organism, although sharp increases in K_m are frequently noted at temperatures near the lower extreme of the organism's habitat temperature (Hochachka & Somero, 1968; Somero & Hochachka, 1968; Somero, 1969). Thus, in terms of contemporary enzyme regulation theory (Atkinson, 1966), decreases in temperature through the organism's physiological range can be said to act analogously to positive modulators of enzymes. This 'positive thermal modulation' of catalysis is, in some instances, sufficient to counteract completely the decelerating effects of decreased temperature.

Previous studies in this laboratory of temperature–K_m effects were performed with single enzymes. In many poikilotherms, however, several variants (isoenzymes) of a given enzyme may be present in a single tissue. We therefore initiated studies of the effects of temperature on the kinetics of complex isoenzyme systems (Somero & Hochachka, 1969). The present paper discusses the results of a study of Alaskan king-crab (*Paralithodes camtschatica*) leg-muscle PyK,* which, on the basis of initial kinetic observations, appeared to exist in two isoenzyme forms. One PyK, termed the 'cold' variant, appeared to function only at low

* Abbreviations: PyK, pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40); PEP, phosphoenolpyruvate.

temperatures, whereas a 'warm' variant seemed to be active only at high temperatures. Together, these two PyK variants promote highly temperature-independent rates of activity through the king-crab's physiological temperature range (4–12°). The probable molecular basis of these two PyK variants is discussed.

MATERIALS AND METHODS

Experimental animals. The large Alaskan king-crab is distributed in colder waters across the northern Pacific Ocean. Specimens used in these studies were captured in the vicinity of Kodiak Island, Alaska, U.S.A., during Phase III of the Bering Sea Expedition of the R/V Alpha Helix (University of California, San Diego, Calif., U.S.A.). Water temperatures during the time of study (July, 1968) ranged from 7° at the bottom to about 12° at the surface. As king-crabs exhibit a considerable degree of migration through depth (Mr Guy Powell, personal communication), it is possible that a single king-crab may experience temperatures in the range 4–12° during the year.

King-crabs were captured in crab pots kindly loaned by Mr Andrew Tobor and Mr Oscar Dyson of Kodiak, Alaska, U.S.A. King-crabs were held aboard ship in running-sea-water tanks at approx. 8°.

Preparation of enzyme. Leg muscle from a freshly killed king-crab was homogenized with 4–6 vol. of cold 10mM-tris–HCl buffer, pH7·5, containing 1mM-EDTA. The crude homogenate was stirred for 1hr. and then centrifuged at 5000*g* for 20min. The pellet was discarded and the supernatant was brought to 35% saturation with solid $(NH_4)_2SO_4$ (209g./l.). The precipitate was collected by centrifugation and discarded. The supernatant was then brought to 75% saturation with solid $(NH_4)_2SO_4$ (278g./l.) and the precipitate was collected and saved. Samples of this 35–75%-saturated-$(NH_4)_2SO_4$ fraction were exhaustively dialysed against the above buffer for use in the assays.

Assay of PyK activity. The spectrophotometric assay system of Bücher & Pfleiderer (1955) was used. Lactate dehydrogenase activity was coupled to pyruvate formation, and PyK activity was measured by following the decrease in E_{340} due to NADH oxidation. Care was taken to maintain pH7·5 at all assay temperatures.

The assay mixture contained, in a total volume of 2·0ml., 50mM-tris–HCl buffer, pH7·5, 0·15mM-NADH, 0·23mM-ADP, 8mM-$MgSO_4$, 75mM-KCl, excess of lactate dehydrogenase, various concentrations of PEP, and PyK, added last. The PyK was maintained at a temperature approximating the assay temperature before addition to the reaction cuvette. This was not a necessary precaution, however; identical kinetics were observed with enzyme previously incubated at 0°.

Electrophoresis of PyK. Electrophoretic analysis of king-crab PyK was kindly performed by Dr W. A. Susor of the University of Washington, Seattle, Wash., U.S.A., according to the procedures given by Susor & Rutter (1968).

Electrofocussing of PyK. The technique of electrofocussing (Haglund, 1967) was used to further purify the enzyme and to determine whether isoenzymes having different p*I* values were present. In electrofocussing, an ampholyte solution (Ampholine) is placed in an electric field to generate a pH gradient; proteins migrate to their isoelectric points. Two pH gradients were used in these studies: pH3–10 (LKB Ampholine 8141) and pH5–8 (Ampholine 8133). The former gradient was run at 300v for 48hr.; the pH5–8 gradient was run at 700v for 53hr. The temperature of the apparatus was maintained at approx. 8°.

RESULTS

Temperature effects on PyK activity. The complex saturation curves obtained in the initial examination of king-crab PyK kinetics (Fig. 1) seemed to indicate that two isoenzyme forms of the enzyme are present. A 'cold' PyK displays a minimal K_m for PEP near 5°, and a 'warm' PyK has a minimal K_m for PEP near 10–12° (Figs. 1 and 2). A further important difference distinguishes the two PyK variants: the 'cold' PyK has hyperbolic kinetics (Figs. 1 and 3), and the 'warm' PyK has sigmoidal (substrate-activated) kinetics (Figs. 1 and 3).

On the basis of our observations of temperature–K_m effects (Hochachka & Somero, 1968; Somero & Hochachka, 1968, 1969; Somero, 1969), the following model of king-crab PyK function is proposed: at temperatures below 9°, only the 'cold' PyK makes a significant contribution to pyruvate kinase activity at physiological PEP concentrations (0·013–0·034m-mole/l. of total water in king-crab leg muscle; Mr James Freed, personal communication). The K_m for PEP of the 'warm' PyK is so high at these lower temperatures that the enzyme's contribution to activity at physiological substrate concentrations is probably negligible.

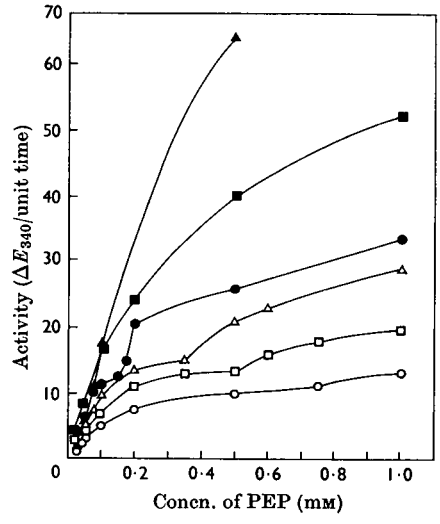

Fig. 1. Substrate (PEP) saturation curves for king-crab leg-muscle PyK at a series of assay temperatures. ○, 2°; □, 5°; △, 8°; ●, 10°; ■, 15°; ▲, 20°.

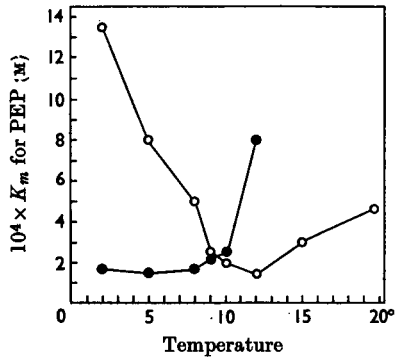

Fig. 2. Estimates of the Michaelis constants for PEP of the two forms of king-crab PyK. ●, 'Cold' PyK; ○, 'warm' PyK. K_m values were determined from Lineweaver–Burk plots and Hill plots (see Fig. 3) for the 'cold' PyK and the 'warm' PyK respectively.

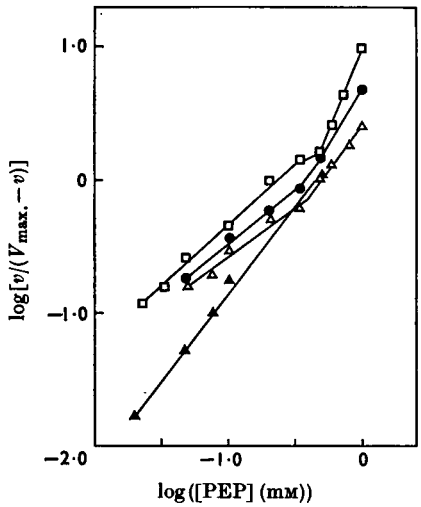

Fig. 3. Hill plots for king-crab PyK at a series of temperatures. The co-operativity of substrate binding with the 'warm' PyK and the absence of co-operativity with the 'cold' PyK (see Fig. 1) should be noted. □, 5°; △, 8°; ●, 10°; ▲, 20°.

However, as the temperature is raised to 10° and above the opposite situation obtains. At these higher temperatures the 'cold' PyK ceases to be of importance because of its high K_m for PEP, whereas the 'warm' enzyme becomes highly activated by means of a sharp decrease in its apparent K_m for PEP (Fig. 2).

The adaptive significance of these two forms of PyK is obvious. If the king-crab were dependent

Table 1. *Temperature coefficients for the king-crab PyK reaction at a series of substrate (PEP) concentrations*

Physiological substrate concentrations are 0·013–0·034m-mole/l. of total water (Mr James Freed, personal communication).

Concn. of PEP (mM)	Q_{10} (5–15°)
1·00	2·7
0·10	2·5
0·05	1·9
0·02	1·6

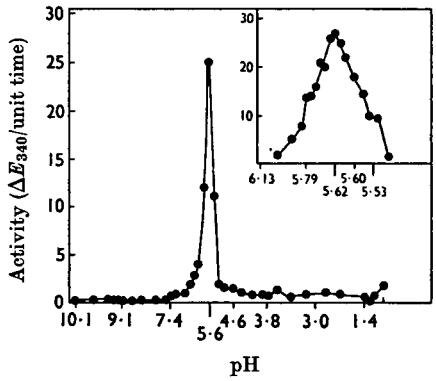

Fig. 4. Electrofocus analysis of king-crab PyK. Activity is plotted against fraction number; the numerical values plotted on the abscissa indicate the pH of selected fractions. The inset illustrates the same phenomenon for the narrow pH gradient (see the Materials and Methods section).

on either the 'cold' PyK or the 'warm' PyK alone, then its ability to convert PEP into pyruvate would be severely limited at one extreme of the habitat temperature. Instead, the combined activities of the two PyK variants allow a high degree of temperature-independence in the rate of PyK activity, at physiological PEP concentrations, over the organism's entire range of habitat temperatures (Table 1).

Lack of evidence for PyK isoenzymes. Classically, isoenzymes have most frequently been defined on the basis of non-kinetic criteria such as electrophoretic patterns. Attempts to demonstrate isoenzymes of king-crab PyK by such techniques were unsuccessful.

In electrophoretic analysis of the enzyme(s), 2hr. of electrophoresis on cellulose acetate at 250v revealed only a single band of PyK activity (Dr W. A. Susor, personal communication).

The electrofocussing experiments also failed to demonstrate isoenzymes; over both pH gradients

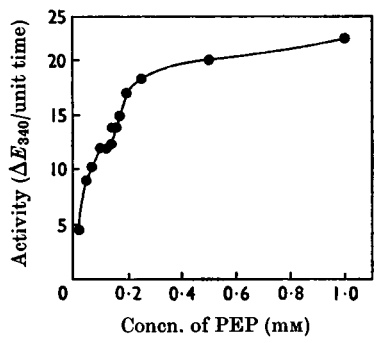

Fig. 5. Substrate (PEP) saturation curve for PyK purified by electrofocussing. The PyK was obtained from the pooled peak fractions from the pH 5–8 gradient. The assay temperature was 10°.

only single peaks of PyK activity were observed (Fig. 4). Thus, if isoenzymes of PyK are present, they apparently have identical isoelectric points.

A second important result was obtained in the electrofocussing experiments. The purified enzyme from the peak fractions of the pH 5–8 gradient exhibited the same complex kinetics found with the less-purified enzyme used to obtain the curves in Fig. 1 (Fig. 5). It is therefore unlikely that the complex kinetics are due to contaminants in the enzyme preparation.

DISCUSSION

These studies have yielded one biologically important result and have raised one interesting biochemical question. In the first case, a highly effective mechanism for decreasing the temperature coefficient of an enzymic reaction has been demonstrated. In the second case, one is faced with the need to explain the molecular basis of the two PyK variants. There appears to be only one explanation for the two PyK variants that is consistent with the results presented here, namely that the 'warm' PyK and the 'cold' PyK are formed by a temperature-dependent interconversion of one protein species. Decreases in temperature favour the conversion into the 'cold' PyK; increases in temperature shift the equilibrium in favour of the 'warm' PyK. Two strong arguments for this hypothesis can be made on the basis of the results presented here.

First, the relative contributions made by the 'warm' PyK and the 'cold' PyK to the overall $V_{max.}$ of the reaction (at PEP concentrations of 1 mM and above) (Fig. 1) should be noted. If there were no temperature-dependent interconversion of enzyme types, then it seems likely that the relative contributions of the two PyK variants to the overall $V_{max.}$ of the reaction would remain the same at all temperatures. Clearly this is not the case (Fig. 1); the relative contribution of the 'warm' PyK greatly increases as the temperature is raised. Thus the $V_{max.}$ of the 'warm' reaction increases more than fivefold as the temperature is raised from 2° to 10°, whereas the $V_{max.}$ of the 'cold' reaction scarcely doubles over this temperature range. This finding strongly suggests that, as the temperature is increased, 'warm' PyK is formed at the expense of 'cold' PyK.

A second line of evidence favouring the interconversion hypothesis is the change from hyperbolic (substrate-insensitive) kinetics to sigmoidal (substrate-activated) kinetics as the temperature is raised (Figs. 1 and 3). At intermediate temperatures (5–12°) both kinetic types of PyK can be detected in Hill plots (Fig. 3). At 20°, however, only a substrate-activated PyK is present. It should be stressed that decrease in Q_{10} at physiological PEP concentrations is favoured by this conversion of an enzyme with hyperbolic kinetics into an enzyme with sigmoidal kinetics as the temperature is raised.

Similar interconversions of PyK forms have been observed with mammalian liver PyK (Bailey, Stirpe & Taylor, 1968) and adipose-tissue PyK (Pogson, 1968*a*,*b*). With the mammalian enzymes, fructose 1,6-diphosphate is effective in converting a substrate-activated (high-K_m) form of the enzyme into a (low-K_m) form of the enzyme with hyperbolic kinetics. The coexistence of the two forms of mammalian liver PyK is evident in the shapes of saturation curves (Bailey *et al.* 1968), which greatly resemble the curves of Figs. 1 and 5. Unlike the mammalian enzymes, king-crab PyK does not exhibit a fructose diphosphate-mediated interconversion between PyK types with hyperbolic and sigmoidal kinetics.

The similarity between the mammalian PyK interconversions and the proposed interconversion in the king-crab PyK system allows us to extend our earlier analogy between the effects of temperature decreases and positive modulators on enzymes of poikilothermic organisms. Thus temperature decreases not only mimic positive modulators by lowering K_m values, but in addition they may also promote interconversions between substrate-activated and substrate-insensitive enzyme forms.

Finally, if the interconversion hypothesis is correct, then king-crab PyK seems a most elegantly adapted enzyme for function in a varying thermal environment. Not only does the animal possess PyK variants that function well over the entire range of habitat temperatures, but in addition the actual amounts of these two PyK variants are adaptively regulated in immediate response to

changes in habitat temperature. It is relevant to mention that a functionally analogous PyK system is found in rainbow trout acclimatized to high and to low temperatures. Cold-acclimatized trout have a PyK isoenzyme with a minimal K_m for PEP near 5° (G. N. Somero, unpublished work). Warm-acclimatized trout possess a PyK isoenzyme with a K_m minimum near 15° (Somero & Hochachka, 1968). Similar isoenzyme changes are known for trout lactate dehydrogenases (Hochachka & Somero, 1968) and brain acetylcholinesterase (Mr J. Baldwin, personal communication). In the trout system, the time-course of appearance of these changes is measured in weeks. In comparison with these isoenzyme-induction changes, the rapid interconversion of PyK forms found in the king-crab PyK system would appear to be a model of biochemical adaptability and efficiency.

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