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THE EFFECT OF TEMPERATURE ON THE KINETIC PROPERTIES OF PHOSPHOFRUCTOKINASE AND HEXOKINASE FROM THE ANTARCTIC FISH *TREMATOMUS BERNACCHII**

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Abstract: Phosphofructokinase (PFK) and hexokinase (HK) were concentrated by ammonium sulfate precipitation from the *Trematomus bernacchii*'s epaxial and cardiac muscles respectively. The apparent Km of PFK for ATP and F6P was established at pH 8.0, at four different temperatures: 5°, 11°, 20° and 30°C. No significant effect of the temperature on the enzyme activity was observed, due to the fact that PFK does not exhibit allosteric properties at pH 8.0. However, at pH 7.0, in which condition the allosteric properties of PFK are displayed, it was observed that the rate of inhibition of the enzyme by ATP at 5°C is lower than at 20°C. Kinetic data of the allosteric behavior of PFK at pH 7.0, were assayed at 5° and 20°C in the presence of fixed concentrations of ATP and variable concentrations of F6P. According to the results obtained it was possible to conclude that the enzyme affinity with the substrate was 20 times higher at 5°C than at 20°C. This result seems to possess high significance in regard to the mechanisms of biochemical adaptation of those organisms at low temperatures. Values for energy of activation for both PFK and HK were also established. The apparent KM for HK from the cardiac muscle was equal to 384 μM and 599μM at 5°C and 20°C, respectively.

1. Introduction

The mechanism of adaptation to extreme temperatures is one of the main issues in biological Antarctic research. The most comprehensive and fruitful

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Abbreviations used: ATP, adenosine triphosphate; DTT, dithiothreitol; F6P, fructose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; LDH, lactate dehycrogenase; NADH, nicotinamide adenine dinucleotide, reduced form; NADP, nicotinamide adenine dinucleotide phosphate; Na4-EDTA, sodium ethylene diamine tetra acetate; PMSF, phenylmethanesulfonyl fluoride.

progress toward understanding this important behavioral aspect of the life on the Antarctic Continent is being made by studies of the mechanisms of biochemical adaptation, particularly at molecular level.

In 1968, SOMERO and HOCHACHKA analyzed the effects of temperature on the catalytic and regulatory properties of pyruvate kinase from the rainbow trout, a typical fish from the temperate zone, and the Antarctic fish *Trematomus bernacchii*. LOW and SOMERO (1976) studied the kinetic and structural properties of muscle pyruvate kinase from species of fish adapted to different temperatures and hydrostatic pressures, including *T. bernacchii*. LUCCHIARI *et al.* (1989) introduced a polarograph adapted with a micro electrode for the assay of the effect of temperature on the oxygen uptake by the epaxial muscle of *Notothenia neglecta*. BACILA *et al.* (1989) carried out studies on the effect of temperature on the respiration and oxidative phosphorylation of sarcosomes isolated from the cardiac muscle of the ice-fish *Chaenocephalus aceratus*.

More recent data on the effect of temperature on the kinetic properties of enzymes from fish endemic to the Antarctic Ocean have been obtained by RODRIGUES *et al.* (1991), and ROSA *et al.* (1992) by studying a nonallosteric muscle enzyme, lactate dehydrogenase, purified from *N. neglecta* epaxial muscle, and hexokinase and phosphofructokinase from the cardiac muscle and epaxial muscle from *T. bernacchii*. The fish used in these experiments were caught in different environments: *N. neglecta* specimens were from the Admiralty Bay, King George Island, South Shetlands, while *T. bernacchii* were caught at the Japanese Antarctic Station, Syowa (69°00'S–39°35'E), Lützow-Holm Bay. A preliminary account of the experiments with HK and PFK from *T. bernacchii* has been given by RODRIGUES *et al.* (1992).

In the present paper data will be shown on the kinetic properties of PFK and HK in regard to the effect of temperature, in preparations obtained from striated muscle and cardiac muscle, respectively.

2. Materials and Methods

This research work was carried out at the Japanese Antarctic Station of Syowa, during the 33rd Japanese Antarctic Research Expedition (JARE), summer 1991/92, and at the Laboratório de Piscicultura, Universidade Federal do Paraná Curitiba, Brasil.

Specimens of *Trematomus bernacchii* were caught by hooks lowered through ice-holes of about 2–3 m depth, near Syowa Station, during January 1992. After being caught, the fishes were immediately dissected. Striated muscle, cardiac muscle, liver and encephalon were thus obtained, washed in cold saline, maintained on ice and taken to the laboratory on board the icebreaker SHIRASE.

Whole blood was obtained by cardiac puncture by means of a syringe containing 10 U of heparin per m*l* of blood. The whole blood was then spun down 10 min at 3000 rpm. The plasma and the layer of white cells from the surface of the packed cells were discarded by aspiration. The packed RBC was washed twice by the same procedure. After the last centrifugation, the packed

RBC was homogenized in a Potter-Elvehjem homogenizer with 10 volumes of distilled water, and spun down 40 min at 15000 rpm in a Hitachi Refrigerated Centrifuge Model Himac CR 15. The supernatant obtained was then used for enzyme assays.

Striated muscle, cardiac muscle, liver and encephalon were processed as follows. For determination of the levels of HK, a crude extract of each organ or tissue was prepared by using an extraction medium containing Tris-HCl, 50 mM, NaF, 50 mM, ammonium sulfate, 3 mM, DTT, 4 mM, Na4EDTA, 1 mM, PMSF, 1 mM, glucose, 10 mM, final pH, 8.0. Homogenates from those organs and tissues (1 volume of tissue or organ / 3 volumes of extraction solution) were obtained in a Potter-Elvejhem homogenizer by disrupting the tissues by hand shaking twice for 60 s each time. After this step, the homogenates were spun down 40 min at 15000 rpm in a Hitachi Refrigerated Centrifuge Model Himac CR 15. The supernatants thus obtained were then used as the source of enzymes.

The crude cell free extract from striated epaxial muscle, obtained as described above, has been used as starting material for the preparation of phosphofructokinase (PFK). For this purpose, the cell free extract was heated for 3 min at 50°C in a water bath and spun down for 40 min at 15000 rpm. The supernatant containing the enzyme was saturated with 60% ammonium sulfate from a solution previously adjusted to pH 7.7 with ammonium hydroxide. The precipitate was then spun down for 40 min at 15000 rpm, the supernatant discarded and the sediment resuspended in a 50 mM Tris-HCl, pH 8.0 solution containing 100 mM KCl, 20 mM DTT, 0.1 mM Na4EDTA and 30% (v/v) glycerol. This preparation contained PFK activity and was used for kinetic studies.

A preparation of hexokinase (HK) was obtained from the cardiac muscle of *T. bernachii* as follows. Homogenates (9.35 g of cardiac muscle per 3 volumes of extraction medium) were prepared in a 50 mM Tris-HCl, pH 8.0 solution containing 50 mM Na4EDTA, 3 mM ammonium sulfate, 4 mM DTT, 1 mM PMSF and 10 mM glucose and then spun down 40 min at 15000 rpm. Then the HK from the supernatant was precipitated by the gradual addition of a saturated solution of ammonium sulfate (pH 7.7) up to 60% saturation. The whole preparation was kept in a cold room (0 to 4°C) for 60 min. After this step, the preparation was spun down 40 min at 15000 rpm, the supernatant being discarded and the precipitate resuspended in 0.5 ml of 5 mM phosphate buffer, pH 7.4, containing 5 mM glucose, 1 mM EDTA, 5 mM β-mercaptoethanol and 20% glycerol (v/v) and used as the source of enzyme for kinetic studies. All steps of enzyme preparation were carried out in a cold room, at temperatures from 0 to 4°C.

Phosphofructokinase was assayed at pH 7.0, for allosteric studies, and at pH 8.0, for nonallosteric analysis. The assay procedures followed the method of LAYZER *et al.* (1969). Hexokinase was assayed by the method of UYEDA and RACKER (1965). Protein assays were performed by the method of LOWRY *et al.* (1951). All spectrophotometric assays were carried out with a Shimadzu UV 1200 spectrophotometer adapted with a water-circulator made by Fanem do Brasil,

containing a thermostat with a sensitivity of ±0.1°C.

Considering that the assays carried out in the present paper used partially purified preparation and that the enzymatic determinations were carried out by using coupled enzyme systems, all the measurements were performed under all possible controlled conditions of temperature and substrates in the coupled systems.

3. Results and Discussion

Effect of temperature on the apparent Km of PFK and HK: Table 1 shows data obtained when PFK activity was assayed in nonallosteric conditions (pH 8.0)

Table 1. Apparent Km (μM) of phosphofructokinase from the epaxial muscle of T. bernacchii, *for ATP and F6P, assayed at different temperatures. System: 1.0 ml of medium containing 50 mM Tris-HCl buffer, pH8.0: 100 mM KCl; 20 mM $(NH_4)_2SO_4$; 0.2 mM DTT; 0.1 mM EDTA; 0.2 mM NADH; 1 mM PEP; 2 U/ml LDH; suitable concentrations of substrates.*

System	5°C	11°C	20°C	30°C
ATP (216 μM)	91.7	55.6	104	104
F6P (1Mm) for ATP	57.2	43.7	38.6	33.2

Table 2. Apparent Km of Hexokinase from the cardiac muscle of T. bernacchii assayed at different temperatures. System: 125 mM glycylglycine, pH 7.4; 0.3 mM NADP+; 3 mM DTT; 15 mM MgCl; 0.3 U/ml G-6-PDH; 10 μM EDTA; suitabule concentration of suitable concentrations of substrates.

System	5°C	20°C
ATP (2 mM)+ Glucose	143	208
Glucose (5 mM)+ ATP	384	599*

* Values for HK type I from birds and mammals are around 100 μM at 25°C.

Table 3. Values of Ea (Kcal/mol) for striated muscle phosphofructokinase and for cardiac muscle hexokinase from T. bernacchii. *Ea was assayed at the interval between 5°C−35°C.*

Enzymes	Above transition point	Below transition point	Inflexion (°C)
PFK	9.16	14.41	15-18
HK	5.44	13.60	16

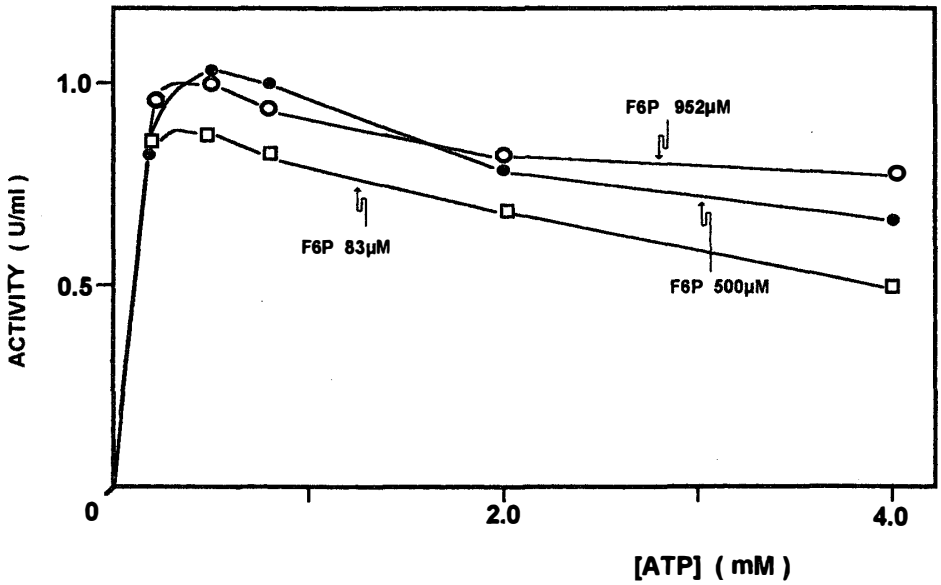

Fig. 1. Kinetic of phosphofructokinase from the striated muscle of Trematomus bernacchii *in the presence of ATP and F6P, assayed at 5°C. System: 50 mM Tris-HCl, 100 mM, KCl, 20 mM ammonium sulfate, 2 mM dithiotreitol, 0.1 mM Na4EDTA, 0.2 mM NADH, 2U/ml PK, 2 U/ml LDH, 1 mM PEP, 5 mM non-complexed Mg as $MgCl_2$. Final pH, 7.0. Temperature: 5°C. Concentrations of ATP and F6P as shown in the experiment.*

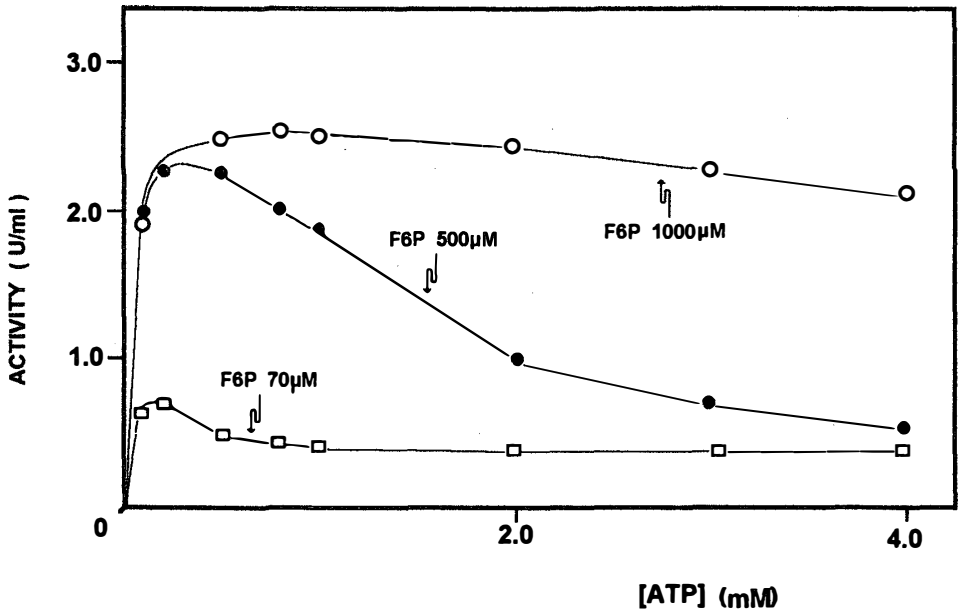

Fig. 2. Kinetic of phosphofructokinase from the striated muscle of Trematomus bernacchii *in the presence of ATP and F6P, assayed at 20°C. System: 50 mM Tris-HCl, 100 mM KCl, 20 mM ammonium sulfate, 2 mM dithiotreitol, 0.1 mM Na4EDTA, 0.2 mM NADH, 2 U/ml PK, 2 U/ml LDH, 1 mM PEP, 5 mM non-complexed Mg as $MgCl_2$. Final pH, 7.0. Temperature: 20°C. Concentrations of ATP and F66P as shown in the experiment.*

at different temperatures. Table 2 shows data on the effect of temperature on HK activity assayed at 5° and 20°C.

Energy of activation: Values for energy of activation (Ea) for PFK and HK were assayed in the temperature range of 5°–35°C (Table 3).

Table 4. Kinetic date of the allosteric behavior of phosphofructokinase from the epaxial muscle of T. bernacchii *assayed at 5°C and 20 °C in the presence of fixed concentration of ATP and variable concentration of F6P.*

System	Kinetic constants					
	n	n	K 0.5 (μM)	K 0.5 (μM)	V max	V max
	5°C	20°C	5°C	20°C	5°C	20°C
ATP(1 mM)+F6P	1.65	1.80	41	820	0.85	0.95
ATP(0.5 mM)+F6P*	1.44	3.00	58	940	0.85	0.95
ATP(0.1 mM)+F6P*	2.40	1.82	45	1100	0.62	0.90

*F6P from 0-2000 μM.

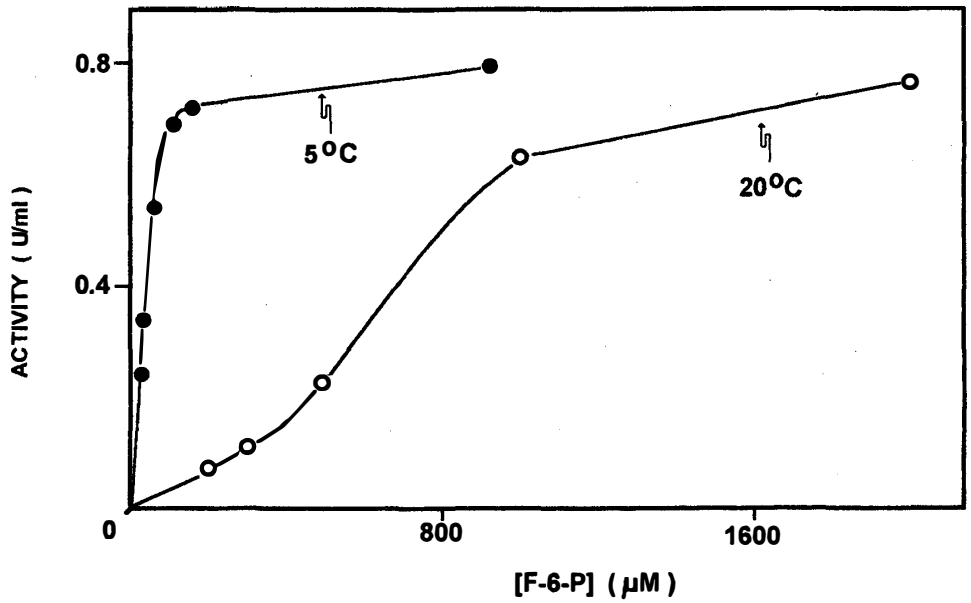

Fig. 3. The effect of temperature on the allosteric properties of phosphofructokinase from the striated muscle of Trematomus bernacchii. *System: The reaction medium contained 50 mM Tris-HCl, 100 mM KCl, 20 mM ammonium sulfate, 2 mM dithiotreitol, 0.1 mM Na4EDTA, 0.2 mM NADH, 2 U/ml PK, 2 U/ml LDH, 1 mM PEP, 5mM of noncomplexed Mg as $MgCl_2$. Final pH, 7.0. Substrates in the system: ATP, 1 mM and F6P, in concentrations varying from 0–2000 μM.*

Effect of temperature on the allosteric behavior of phosphofructokinase: The kinetic behavior of PFK from the striated muscle of *T. bernacchii* was established at 5° and 20°C (Fig. 1 and Fig. 2). At 5°C (Fig. 1) PFK activity reached its maximum in the presence of 0.5 mM F6P and 0.5 mM ATP. However, in concentrations of ATP more than 0.5 mM, the inhibitory effect of ATP became very clear. Furthermore, at 20°C the activity of PFK reached its maximum in the presence of 0.2 mM ATP and of 1000 μM F6P (Fig. 2). On the other hand, the inhibition of PFK activity by ATP is much more evident at 20°C than at 5°C (Fig. 2) toward concentrations of F6P lower than 1 mM. In the presence of 1 mM F6P, the inhibitory effect of ATP becomes much less evident.

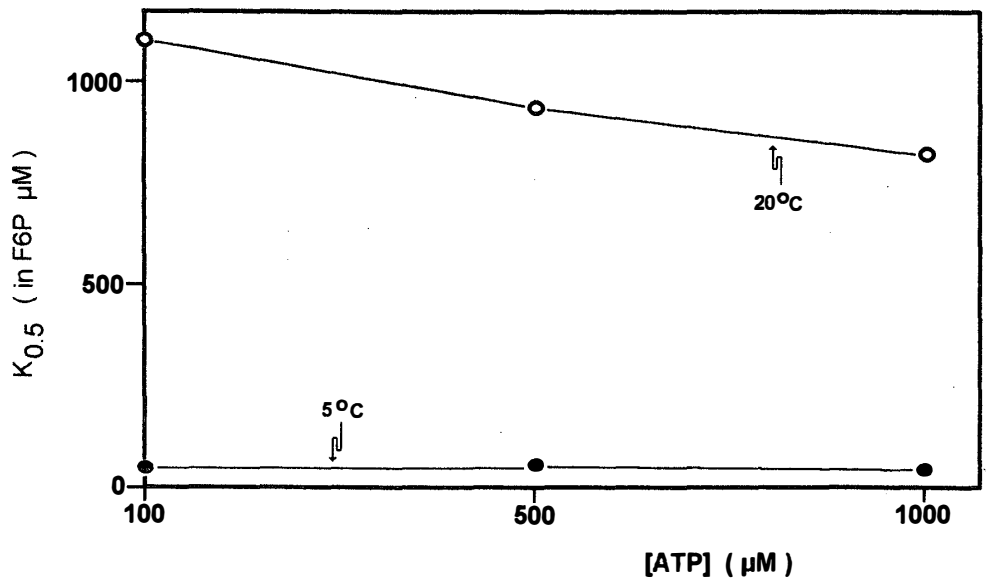

Fig. 4. Correlation among K 0.5 for phosphofructokinase from the striated muscle of Trematomus bernacchii *assayed in terms of concentrations of F6P and ATP at 5°C and 20°C.*

Experiments were also carried out in order to establish the allosteric behavior of this preparation of PFK at 5 and 20°C, (pH 7.0), in the presence of fixed concentrations of ATP (1 mM, 0.5 mM, 0.1 mM) and concentrations of F6P varying from 0 to 2000 μM (Table 4, Figs.3, 4). These experiments show that temperature is very meaningful for the allosteric behavior of PFK from the striated muscle of *T. bernacchii* in terms of the affinity between the enzyme and its substrate, F6P. Thus, at 5°C (Table 4, Fig.4) the values of K 0.5 are about 20 times smaller than the ones obtained at 20°C. On the other hand, Fig. 3 shows the sigmoidal correlation between the concentration of F6P and the activity of PFK in the presence of a fixed concentration of 1 mM ATP. Furthermore, at 5°C the enzyme affinity is enhanced about 20 times, reaching its maximum activity at low concentrations of F6P. In this way, at 5°C PFK becomes much more efficient, from the kinetic point of view, than at 20°C.

The cooperative effect of this preparation of PFK gives its highest value (n=2.4) at 5°C, when incubated with 0.1 mM ATP in the presence of variable concentrations of F6P.

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