Proc. NIPR Symp. Polar Biol., 7, 103–109, 1994

KINETIC PROPERTIES OF PYRUVATE KINASE PURIFIED FROM THE FLESH MUSCLE OF THE ANTARCTIC KRILL *EUPHAUSIA SUPERBA* DANA*

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Abstract: In previous experiments, BACILA, ROSA, and RODRIGUES showed that enolase, partially purified from the flesh muscle of the krill *Euphausia superba* DANA, has not been inhibited by F^- in concentrations as high as 2 mM, a property which is paralleled by the enolase from the penguins liver. This finding led to the study of the structure and the kinetic properties of other enzymes from the carbohydrate metabolism of the krill's flesh muscle. In the present communication some data on the kinetic properties of the pyruvate kinase (PK) purified from the krill's flesh muscle are shown. L-Phenylalanine inhibits this preparation of PK both in the presence and in the absence of 1 mM L-alanine. However, the inhibition of PK by Mg-ATP was almost completely reversed in the presence of 1 mM Fru-P_2, but not in the presence of 1 mM L-alanine. The Km values for PK have been established to be 0.03 μM PEP and 0.038 μM PEP respectively in the presence and in the absence of Fru-P_2. This flesh muscle krill's preparation of PK shows clear sigmoidal kinetics. However, in the presence of Fru-P_2, PK shows a Michaelian kinetics. The effect of ADP has also been established and found to have a Km value of 0.15 μM ADP. Cations inhibit the krill's pyruvate kinase in the following order: $Mn^{2+} > Ca^{2+} > Ni^{2+} > Co^{2+}$ while Li^{2+} did not show any inhibitory effect.

1. Introduction

In spite of the krill's meaning for the Antarctic ecosystem, only few studies on the metabolic activity of *Euphausia superba* DANA, a most important member of the trophic chain from the Antarctic seas, have been reported in the litera-

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Abbreviations used: LDH, lactate dehydrogenase; Fru-P_2, fructose-1, 6-bisphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase.

ture. The finding by SOEVIC and BRAEKHAN (1979) of abnormally high concentrations of fluoride in the krill was a very important contribution to this field. BOONE and MANTHEY (1983) established the anatomical distribution of fluorine. Within various segments and organs of the Antarctic krill, F^- was found to be mostly concentrated in the cephalotorax. CHRISTIANS and LEINEMAN (1980, 1983) and CHRISTIANS *et al.* (1981) found that fluorine migrates from the shell into the flesh muscle of the dead krill. Following this, BACILA *et al.* (1985, 1989) disclosed a very interesting biochemical adaptation by Antarctic organisms in regard to the susceptibility of enolase from the flesh muscle of the krill and the penguin liver to fluorine inhibition. It has been found that these preparations of enolase are not inhibited by concentrations of fluorine as high as 1 mM, still leaving a significant residual activity, even in higher concentrations.

Due to these finding, we undertook to study the structure and function of the krill enzymes related to the energetic metabolism. A first account of the levels of the glycolytic enzymes in the flesh muscle of *Euphausia superba* has been given by ROSA *et al.* (1991). It is the aim of these paper to present results obtained on the kinetic parameters of pyruvate kinase purified from the flesh muscle of the krill *Euphausia superba* DANA.

2. Materials and Methods

This research work was carried out at the Brazilian Antarctic Station Commander Ferraz, King George Island, South Shetlands and at the Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, Brasil.

Krill was collected at Admiralty Bay, King George Island, in front of the Brazilian Antarctic Station Commander Ferraz. The animals were immediately taken alive to the laboratory and carefully dissected in order to obtain the flesh muscle. The dissected muscle was immediately frozen and then taken to our laboratories at the University of São Paulo. For enzyme purification, all steps were carried out at 4°C following then the technique described by ZAMORA *et al.* (1992). Frozen flesh muscle was homogenized in a Potter-Elvehjem homogenizer with 6 volumes of a solution containing 33 mM phosphate buffer, pH 6.5, 1 mM $MgCl_2$, and 0.1 mM DTT. The homogenate was then centrifuged at 10000 rpm for 20 min in a Sorvall RC5B refrigerated centrifuge. The supernatant was brought to 70% saturation by the gradual addition of a saturated solution of $(NH_4)_2SO_4$, pH 6.5, stirred for 30 min and spun down for 20 min at 10000 rpm. The supernatant was discarded and the precipitate dissolved in a minimum volume of the same extraction buffer, and the excess salt subsequently removed by overnight dyalisis against 10 mM phosphate buffer, pH 6.5, containing 10 mM β-mercaptoethanol and 20% glycerol (v/v). The dyalized enzyme solution was then used for enzyme assay. Pyruvate kinase activity was assayed by using a modification of a coupled assay technique (BUCHER and PFLEIDERER, 1955). The standard assay solution containing 1 mM ADP, 1.6 mM PEP, 7 mM $MgCl_2$, 50 mM KCl, 0.12 mM $NADH+H^+$, 10 mM EDTA and 3 U/m*l* LDH in 50 mM

imidazole-HCl buffer, pH 6.5. Assays were carried out at 30°C. One unit of the enzyme activity was defined as the amount of enzyme which produced 1μmol of pyruvate per min under these conditions. Protein was measured by the method of LOWRY *et al.* (1951). Substrates, coenzyme, lactate dehydrogenase and other chemicals were obtained from Sigma Chemical Company. All common chemicals were reagent grade.

3. Results and Discussion

Experiments in which the effect of PEP concentration on the activity of the PK from the krill's flesh muscle were carried out (Fig. 1). The Km values for PEP were 0.043 mM and of 0.038 mM in the absence and in the presence of 1 mM Fru-P_2, respectively. This preparation of PK shows sigmoidal kinetics which becomes hyperbolic upon addition of 1 mM Fru-P_2. The effect of ADP concentration on the activity of krill's PK, was also established (Fig. 2). The Km value obtained was 0.15 mM. Table 1 and Fig. 3 show the effect of different metabolites, —L-alanine, L-phenylalanine and $MgATP^{-2}$—, on the PK activity. It can be seen that the PK of krill is not inhibited by L-alanine. However, inhibition of the enzyme was found in the presence of L-phenylalanine and of MgATP.

Cations inhibit the krill's PK in the following order: $Mn^{2+} > Ca^{2+} > Ni^{2+} > Co^{2+} >$ while Li^{2+} did not show any inhibitory effect (Fig. 4). ZAMORA *et al.* (1992) established nH values of 11.35 at pH 6.5 and of 1.9 at pH 7.4 for the binding capacity of Mg^{2+} to the ice-fish PK. On the other hand, Mn^{2+} also acts as an activator of the ice-fish PK, showing, however, a very neat inhibitory effect on the krill's PK. In both PK preparations, Ca^{2+} acts as an inhibitor. However, the ice-fish PK is more sensitive to Ca^{2+} than the krill's enzyme. On the other

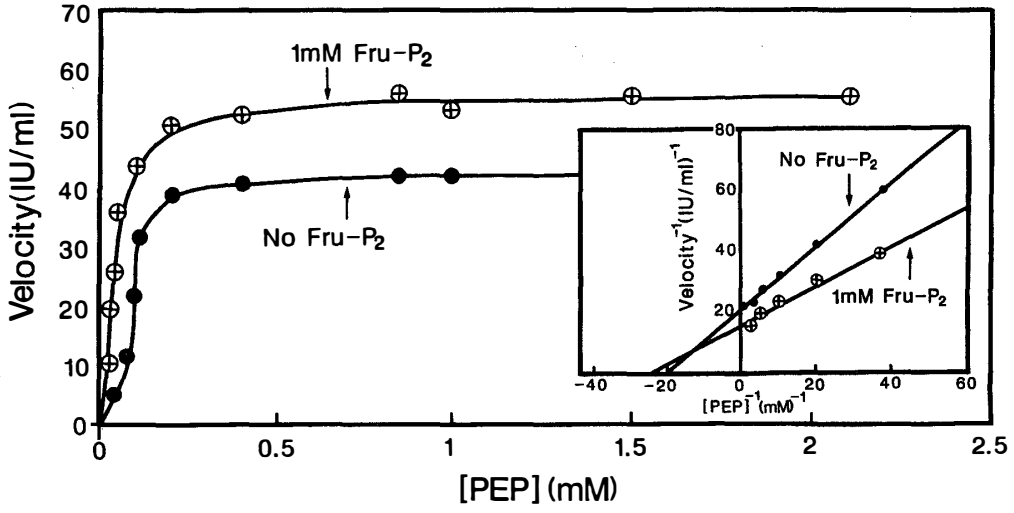

Fig. 1. Effect of PEP concentration and of fructose-1,6-bisphosphate on the activity of piruvate kinase from the krill's flesh muscle. System: Tris-HCl, pH 7.4, 50 mM; PEP, in suitable concentrations; $MgCl_2$, 8 mM KCl, 70 mM; NADH, 0.12 mM LDH, 3 U/ml; 10 µl of the enzyme solution. Incubation at 30°C.

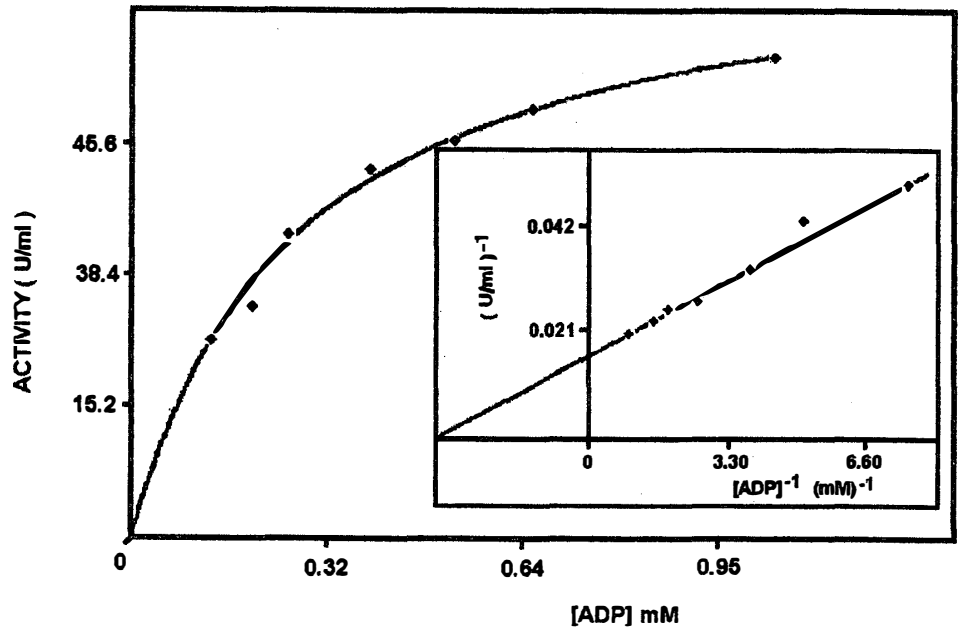

Fig. 2. Effect of ADP concentration on the activity of pyruvate kinase from the krill's flesh muscle. System: Tris-HCl, pH 7.4, 50 mM; PEP, 1 mM; ADP in suitable concentrations; $MgCl_2$, 8 mM KCl, 70 mM; NADH, 0.12 mM LDH, 3 U/ml; 10 µl of the enzyme solution. Incubation at 30°C.

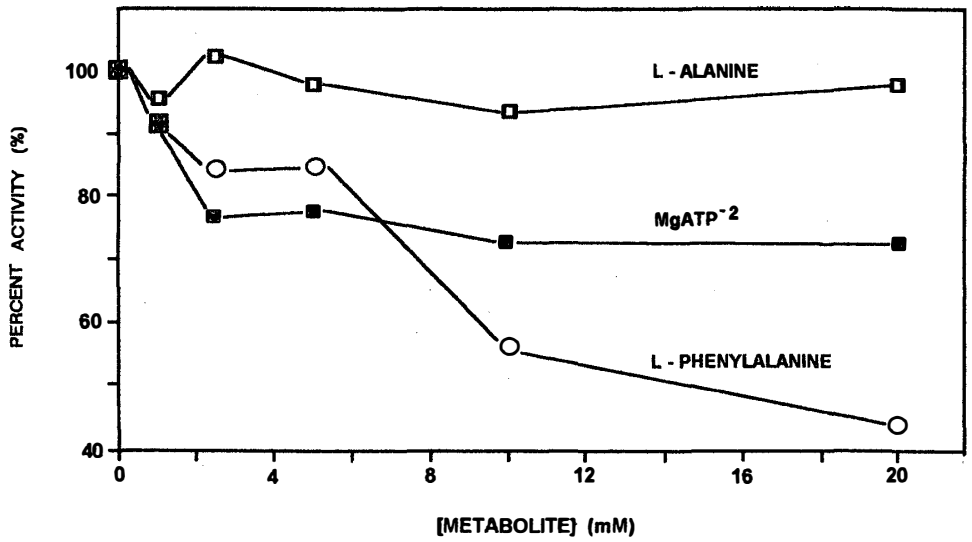

Fig. 3. Effect of L-alanine, L-phenylalanine and Mg ATP on the activity of pyruvate kinase from the krill's flesh muscle. System: Tris-HCl, pH 7.4, 50 mM; PEP, 1 mM; ADP, 1 mM $MgCl_2$, 8 mM KCl, 70 mM; NADH, 0.12 mM LDH, 3 U/ml; and suitable concentrations of L-alanine, L-phenylalanine and Mg ATP; piruvate kinase preparation, 10 µl. Incubation at 30°C.

hand, in the presence of Mn^{2+}, which is it self an inhibitor for the krill's enzyme, the inhibition caused by 10 mM Ca^{2+} on the ice-fish PK decreases by 14%.

Due to its important role in the glycolytic pathway, pyruvate kinase has been isolated from several animal sources and had its molecular properties established.

Table 1. Effect of metabolites on the activity of krill's flesh muscle pyruvate kinase. System: Tris-HCl pH 7.4, 50 mM; PEP, 1 mM; $MgCl_2$ 8 mM; KCl, 70 mM; NADH, 0.12 mM; LDH, 31 U/ml; and suitable concentrations of metabolites. Values are given in terms of percent activity.

Metabolite	No Fru-P_2 No L-Ala	1 mM Fru-P_2	1 mM Fru-P_2 1 mM L-Ala
Ala	100.0	109.0	-
L-Phe	75.5	78.3	93.4
MgATP	86.8	92.5	88.6

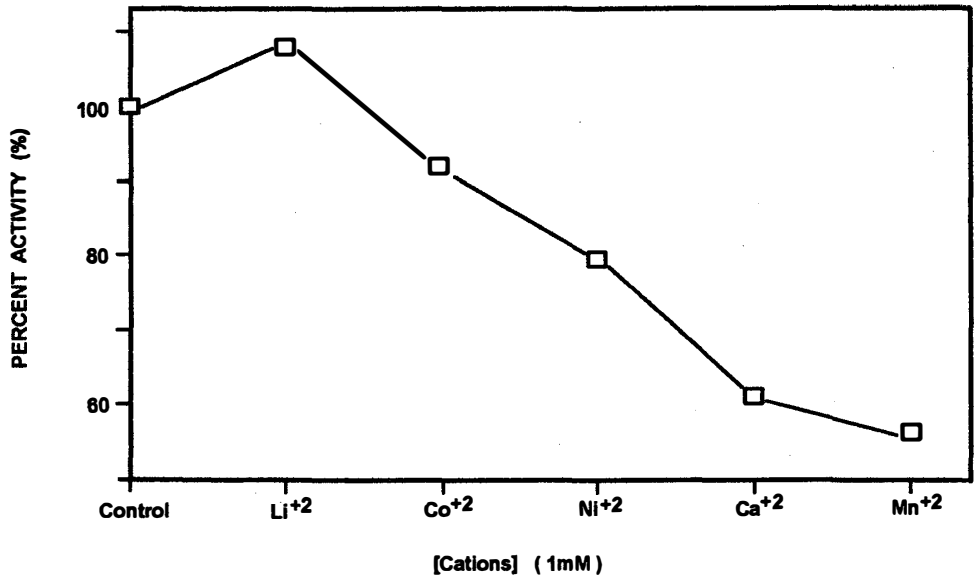

Fig. 4. Effect of cations on the activity of piruvate kinase from the krill's flesh muscle. System: Tris-HCl, pH 7.4, 50 mM; PEP, 1 mM; ADP, 1 mM; $MgCl_2$, 8 mM KCl, 70 mM; NADH, 0.12 mM; LDH, 3 U/ml; cation, 1 mM; piruvate kinase preparation, 10 µl. Incubation at 30°C.

In mammals, there are known at least four types of PK isoenzymes IMAMURA and TANAKA (1982) named M1, M2, L and R. These four isoenzymes are different in physical, chemical, immunological, catalytic and regulatory properties. However, several studies have shown that muscle PKs (M1) from lower vertebrates, including fish, possess kinetic properties different from those of mammals. In fact, they are activated by Fru-P_2 and may show a sigmoidal kinetic with PEP (SOMERO and HOCHACHKA, 1968; RANDALL and ANDERSON, 1975; GUDERLEY and CARDENAS, 1980; OCAMPOS *et al.*, 1987).

Pyruvate kinase from the krill's flesh muscle shows clear Michaelian behavior for ADP, at pH 7.4 Km 0.15 mM. With respect to PEP, however, the krill's PK displays allosteric behavior, showing a slightly sigmoidal curve in the absence of Fru-P_2. However, in the presence of Fru-P_2, the activity of the enzyme reaction is stimulated and no sigmoidicity of the curve can be detected. Thus, in both cases, ADP and PEP, the krill's flesh muscle PK parallels the kinetic properties of the PK from the eppaxial muscle of the ice-fish *Chaenocephalus aceratus*

(ZAMORA *et al.*, 1989), resembling in this way the pyruvate kinase type Mm or K from mammalian tissue (HALL and COTTAM, 1978). The enzyme possesses properties in between of those of the two major forms found in adult mammals (m1 and L), particularly the Michaelian kinetics with respect to ADP and PEP, and no inhibition in the presence of L-alanine, displaying, on the other hand, allosteric properties with Fru-P_2; and is inhibited by MgATP. Furthermore, L-phenylalanine inhibits the krill's PK in the absence of L-alanine, but such inhibition was almost completely reversed in the presence of 1mM Fru-P_2 and 1 mM alanine.

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(*Received March 29, 1993; Revised manuscript received September 27, 1993*)