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[SHORT COMMUNICATION]

L-Cysteine is a Competitive Inhibitor of Pyruvate Kinase from the Intertidal Sipunculan, *Phascolosoma arcuatum*

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ABSTRACT—L-Cysteine is a competitve inhibitor of the pyruvate kinase (PK) isozymes from the body wall and introvert of the sipunculan *Phascolosoma arcuatum* exposed to normoxia or anoxia. Firstly, the $1/V$ versus $1/$[phosphoenolpyruvate] plot shows that the V_{max} values of PK isozymes from these body parts were unaffected by L-cysteine with exception of the body wall from the anoxic worm. Secondly, the Dixon plot shows that the percentage inhibition of PK activity by L-cysteine decreased with increasing concentrations of phosphoenolpyruvate. Kinetic properties of PK isozymes from the body wall and introvert of *P. arcuatum* could be altered by anoxic exposure. In anoxia, the modified PK isozymes from these body parts had lower affinity to phosphoenolpyruvate and L-cysteine. Despite the increase in K_i(L-cysteine) value upon anoxic exposure, the $[I]_{0.1}$, $[I]_{0.5}$ and $[I]_{0.9}$ values of L-cysteine for these PK isozymes were lower than those obtained for the normoxic worms. L-Cysteine was a more effective inhibitor than alanine for PK isozymes from the body wall and introvert. However, the effect of L-cysteine on PK was tissue specific and it had no effect on the isozyme obtained from the internal organs.

INTRODUCTION

Many studies have been conducted to elucidate the effects of amino acids on the kinetic properties of pyruvate kinase (PK, EC 2.7.1.40) from various animals. The inhibitory effect of alanine was tested on the PK from muscles of the sea mussel (Holwerda and De Zwaan, 1973), sea anemone, scallop, oyster, crab, lobster and frog (Zammit and Newsholme, 1978). Those of alanine and phenylalanine were demonstrated on PK from the cow (Cardenas *et al.*, 1975), *Patella caerulea* L. (Michaelidis *et al.*, 1985), and *Concholepas concholepas* (Leon *et al.*, 1982). The effects of alanine, tryptophan, serine and phenylalanine were tested on chicken PK (Ibsen and Marles, 1976). The effects of phenylalanine, valine, proline, tryptophan, glutamate, alanine and threonine on the muscle of *C. concholepas* were investigated by Moran *et al.* (1983). Ibsen and Trippet (1974) investigated the effects of eighteen amino acids, including phenylalanine, isoleucine, valine, proline, tryptophan, alanine, threonine, histidine, leucine, arginine, citrulline, lysine, glutamine, glutamate, aspartate,

hydroxyproline, glycine and serine, on the three basic non-interconvertible forms of rat PK. However, none of these studies has mentioned the effect L-cysteine on PK, with the exception of Lim and Ip (1991a) who worked on the covalent modification of PK from *Phascolosoma arcuatum* during anoxia.

P. arcuatum (Rice and Stephen, 1970) belongs to a group of benthic, marine worms known as sipunculans or peanut worms (Rupert and Barnes, 1994). The majority of sipunculans live in shallow waters, but *P. arcuatum* is distinctive, with its ability to thrive in the mud of mangrove swamps. This is a relatively more physiologically stressful habitat and the worm has to adapt to the cyclical changes in salinity (Moran *et al.*, 1983; Peng *et al.*, 1994) and oxygen tension (Lim and Ip, 1991b). Lim and Ip (1991a) reported the presence of tissue specific PK isozymes in *P. arcuatum*. Those in the body wall and introvert could be modified by covalent modification during anoxic exposure, and became much less active (Lim and Ip, 1991a). Consequently, the flow of carbon through phosphoenolpyruvate carboxykinase led to the formation of succinate (Lim and Ip, 1991b).

Since the inhibitory effect of L-cysteine on PK has never been reported before for other animals, and since nothing is

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known about the nature of its inhibition, the present studies were undertaken to elucidate: (1) the type of inhibition exerted by L-cysteine on the PK from the body wall, introvert and internal organs of *P. arcuatum*, (2) the effects of anoxia on the PK from the body wall and introvert of *P. arcuatum* towards L-cysteine inhibition, (3) whether there were differences between effects of L-cysteine and alanine inhibition, and (4) whether the effects of L-cysteine on PK was tissue specific.

MATERIALS AND METHODS

P. arcuatum were collected from the mud flats of the mangrove swamp at Mandai, Singapore between June 1996 and July 1997, and maintained at 25°C in the laboratory in plastic aquaria with aerated 50% (15‰ salinity) seawater. No attempt was made to feed the worms and experiments were performed 5 days after the acclimatization of the worms to the laboratory conditions.

The procedures of Lim and Ip (1991a,b) were followed. Groups of 20 worms were placed in shaded 1000-ml flasks containing 300 ml 50% seawater which had been flushed with 100% N_2 for 30 min prior to the start of the experiment. Flushing was continued for another 1 hr after the introduction of the worms before the flasks were sealed and left undisturbed at 25°C for 48 hr. Worms kept in 50% seawater saturated with air were used as aerobic controls for comparison.

P. arcuatum was dissected, drained of coelomic fluid and then dissected into three major body parts. These were the body wall, the introvert with the attached retractor muscles (presented as the introvert hereafter) and the internal organs (which comprised oesophagus, intestine, rectum and rectal diverticulum). They represented 68%, 17% and 18% of the total tissues, respectively. These body parts were blotted dry and frozen in liquid N_2.

For PK assay, the frozen samples were initially ground to a powder. They were then homogenized three times in 5 vol (w/v) of ice-cold 50 mM imidazole-HCl buffer (pH 7.5) containing 50 mM NaF, 3 mM EGTA, 3 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulphonyl-fluoride (PMSF) using an Ultra-Turrax homogenizer (Janke and Kunkel Co., Germany) at maximum speed for 20 s each with 10 s off intervals. The homogenized sample was centrifuged at 27 000 × g and 4°C for 20 min. After centrifugation, 3 ml of the supernatant fluid was passed through a 10 ml column of Econo-Pac 10DG desalting column (Bio-Rad Laboratories, USA) and eluted out with 4 ml of extraction buffer without PMSF. Preliminary experiment carried out on the filtrate from different body part of *P. arcuatum* showed that there was minimum background activities thus no further purification was required. The filtrate obtained after eluting with the Econo-Pac 10DG desalting column was thus used as the source of enzyme for subsequent assays.

PK activities were determined by following the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH), at 340 nm and 25°C using a Shimadzu UV 160A spectrophotometer. Specific activities were expressed as μmol NADH oxidized per min per mg protein.

PK was assayed by a modified method of Gutmann and Bernt (1974). The reaction mixture contained optimal concentrations of the following components: 120 mM triethanolamine-HCl buffer (pH 7.5), 16 mM $MgSO_4$, 90 mM KCl, 5 mM ADP, 0.3 mM NADH and 3 IU per ml lactate dehydrogenase (Sigma Chemical, USA) in a final volume of 1.71 ml. The enzyme activity (V) was measured after initiation of the reaction mixture with phosphoenolpyruvate (PEP) at final concentrations of 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 mM. Reaction mixture with PEP omitted served as the control. Kinetic constants, K_m and V_{max} values were determined using double reciprocal plot of (1/V versus 1/[S]). Each point in the graph represent means of 3 determinations on separate preparations of enzyme extracts from different specimens. The effects of different concentrations of L-cysteine (0.025–0.1 mM) or alanine (0.5–1.0 mM) were also examined. The inhibitor constant, K_i value was determined from the Dixon plot (1/V against [I]). The concentrations of inhibitor required for 10%, 50% and 90% inhibition were calculated from the following equations (Segel, 1975): $[I]_{0.1}=1/9(1+[S]/K_m)K_i$, $[I]_{0.5}=(1+[S]/K_m)K_i$, and $[I]_{0.9}=9(1+[S]/K_m)K_i$.

Protein content was determined by the method of Bradford (1976). Bovine-γ-globulin dissolved in 25% glycerol was used as a standard for comparison.

For L-cysteine assay, the frozen tissues were homogenised in 7 vol (w/v)of 6% TCA. The homogenised samples were then centri-
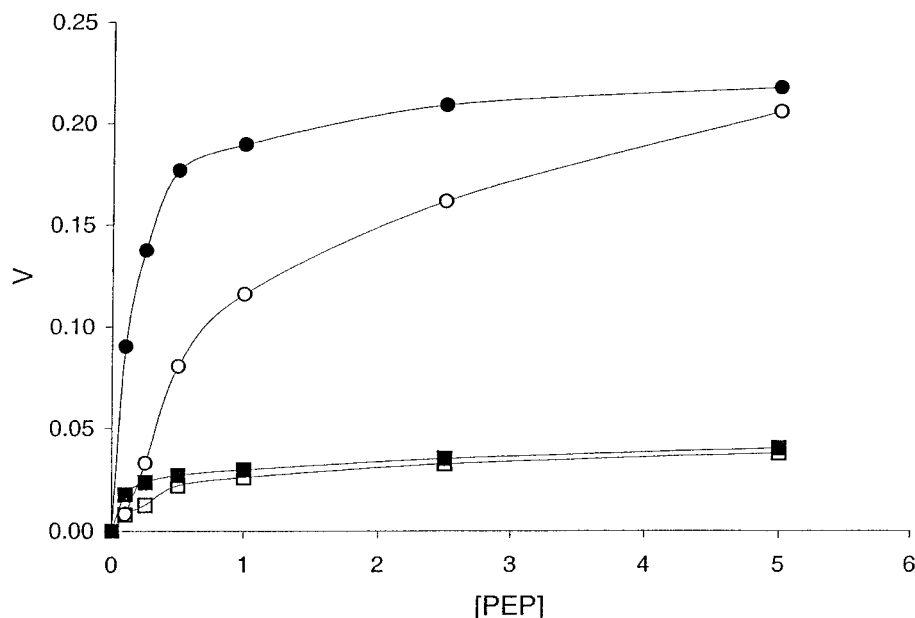

Fig. 1. Michaelis-Menten plot of V (μmol NADH oxidized per min per mg protein) against [phosphoenolpyruvate] (mM) for PK from the body wall of normoxic *P. arcuatum* in the absence (   ) or presence of a final concentration of 0.3 mM (   ) of cysteine and for PK from the introvert of normoxic *P. arcuatum* in the absence (   ) or presence of a final concentration of 0.3 mM (   ) of cysteine

fuged at 12,000 x g for 10 min. The supernatant fluid obtained was adjusted to pH 2.2 with 4 M LiOH and diluted appropriately with 0.2 M lithium citrate buffer(pH 2.2). The L-cysteine present was analysed using a Shimadzu LC-6A Amino acid Analysis System with a Shim-pack ISC-07/S1504 Li type column.

Results were presented as mean ± SEM. Student's *t* test was used to compare differences between means. Differences with P < 0.05 were regarded as statistically significant. Data presented graphically were plotted as least square regression lines wherever applicable.

RESULTS

L-Cysteine exhibited inhibitory effect on the PK isozymes extracted from the body wall and introvert of *P. arcuatum* (Fig. 1). In the presence of various concentrations of L-cysteine, the double reciprocal plot of 1/V versus 1/[phosphoenolpyruvate] for the PK extracted from the body wall of the normoxic *P. arcuatum* shows all the lines intersecting at the Y-axis (Fig. 2). Hence, the V_{max} of this PK isozyme from *P. arcuatum* was unaffected by L-cysteine. However, only two of the lines intersect at the Y-axis for the double reciprocal plot of PK from the body wall of the anoxic worms (Fig. 2).

On the other hand, the Dixon plots for PK extracted from the body wall of the normoxic worms (Fig. 3) and the anoxic worms (Fig. 4) are similar with all the lines for the various concentrations of phosphoenolpyruvate intersecting to the left of the Y-axis.

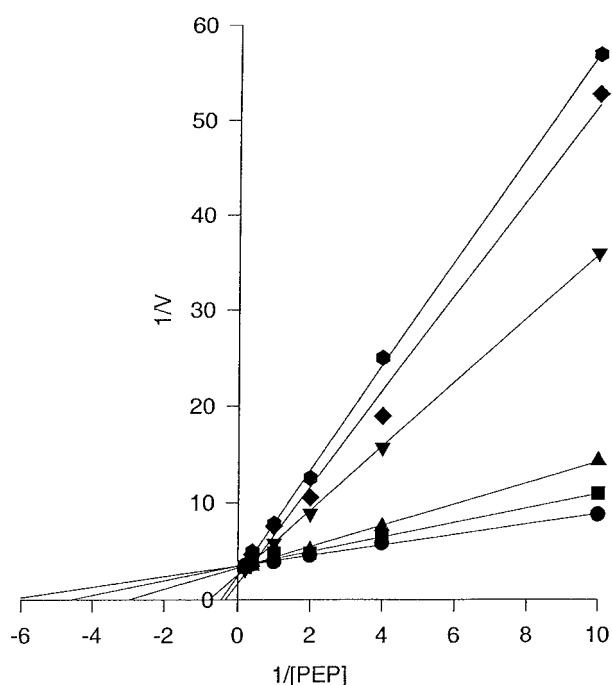The K_m value of phosphoenolpyruvate for the anoxic form

Fig. 3. Dixon plot or 1/V against [cysteine] (mM) for PK from the body wall of normoxic *P. arcuatum* in the presence of a final concentration of 5.0 mM ( ), 0.5 mM ( ) or 0.25 mM ( ) of phosphoenolpyruvate

Fig. 2. Double reciprocal plot of 1/V against 1/ [Phosphoenolpyruvate] for PK from the body wall of normoxic *P. arcuatum* in the absence ( ) or presence of a final concentration of 0.05 mM ( ) or 0.1 mM ( ) of cysteine and anoxic *P. arcuatum* in the absence ( ) or presence of a final concentration of 0.025 mM ( ) or 0.05 mM (●) of cysteine
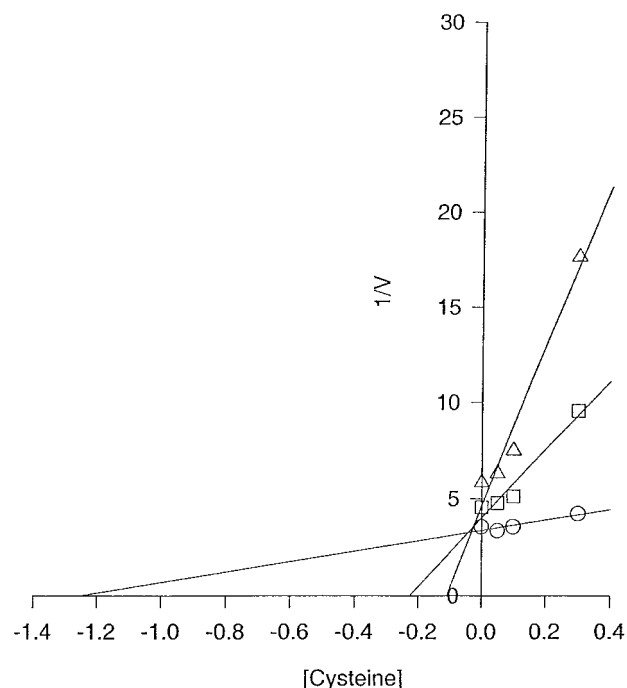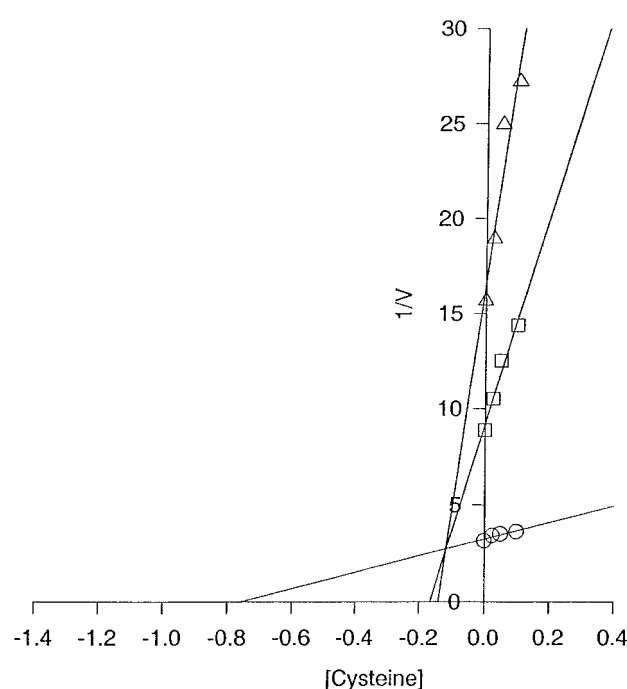

Fig. 4. Dixon plot or 1/V against [cysteine] (mM) for PK from the body wall of anoxic *P. arcuatum* in the presence of a final concentration of 5.0 mM ( ), 0.5 mM ( ) or 0.25 mM ( ) of phosphoenolpyruvate

of PK was significantly greater than that for the normoxic one (Table 1). Similarly, the K_i value of L-cysteine for the PK from the body wall of the normoxic worm was significantly lower than that for the anoxic one (Table 2).

The $[I]_{0.1}$, $[I]_{0.5}$ and $[I]_{0.9}$ values of L-cysteine for the PK from the body wall of normoxic worm were 5 times that of the corresponding values of the anoxic worm (Table 3).

The double reciprocal plot of $1/V$ versus $1/[\text{phospho-enolpyruvate}]$ on the effect of alanine on PK from the body wall of the normoxic worm shows all the lines intersecting at the Y-axis (Fig. 5). However, a similar plot of results obtained for the anoxic worm indicates that both K_m and V_{max} were affected (Fig. 6).

The K_i values of alanine for the PK isozymes from the body wall of the normoxic or anoxic worm were generally greater than those of L-cysteine (Table 2). Exposure of the worm to anoxia increased the K_i of both inhibitors (Table 2). Consequently, the $[I]_{0.1}$, $[I]_{0.5}$ and $[I]_{0.9}$ values of alanine were approximately 3–4 times higher than the corresponding values of L-cysteine (Table 3).

As for the introvert, the double reciprocal plots on the effects of L-cysteine on both the normoxic and anoxic forms

of PK show all the lines intersecting at the Y axis. However, different from the body wall, anoxic exposure did not affect the K_i(L-cysteine) value (Table 2). Different from the PK from the body wall, the isozyme from the anoxic introvert exhibited a lower K_i of alanine (Table 2). The $[I]_{0.1}$, $[I]_{0.5}$ and $[I]_{0.9}$ of L-cysteine for the PK isozyme from the introvert were generally greater than those from the body wall, and these values were decreased by anoxic exposure (Table 3). Similar results were obtained for alanine, although it did not appear to be an effective inhibitor of the PK isozyme from the introvert as compared to that from the body wall (Table 3).

The PK isozymes extracted from the internal organs of both anoxic and normoxic worms were neither inhibited by L-cysteine nor alanine.

We have tried to determine the concentration of L-cysteine in the body wall, introvert and internal organs of the sipunculan, *P. arcuatum* using the Shimadzu LC-6A Amino acid Analysis System with a Shim-pack ISC-07/S1504 Li type column. However, we were not able to separate the L-cysteine and proline present in the different body parts and they both appear as a single peak in the chromatogram. This could most probably be due to the limitation in the instrument used.

Table 1. Effects of 48 hr environmental anoxia on the V_{max} (µmol NADH oxidized per min per mg protein) and K_m (mM PEP) of PK extracted from the body wall, introvert and internal organ of *P. arcuatum*. Data represents means ±SEM of 3 determinations on separate preparations of enzyme extracts from different animals

Condition	Body Wall		Introvert		Internal Organ	
	V_{max}	K_m (PEP)	V_{max}	K_m (PEP)	V_{max}	K_m (PEP)
Normoxic	0.27±0.02	0.16±0.06	0.030±0.006	0.053±0.007	0.33±0.02	1.1±0.1
Anoxic	0.22±0.02	5.2 ±0.3[*]	0.068±0.011	0.48 ±0.04[*]	0.28±0.05	1.2±0.1

[*] significantly different from corresponding normoxic value

Table 2. Effects of 48 hr environmental anoxia on the K_i (cysteine or alanine) of PK extracted from the body wall and introvert of *P. arcuatum*. Data represents means ±SEM of 3 determinations on separate preparations of enzyme extracts from different animals

Condition	Inhibitor	K_i	
		Body Wall	Introvert
Normoxic	Cysteine	0.050±0.011	0.12±0.02
Anoxic	Cysteine	0.12 ±0.01[a]	0.11±0.02
Normoxic	Alanine	0.16 ±0.08	0.59±0.05[b]
Anoxic	Alanine	0.44 ±0.07[ab]	0.30±0.05[ab]

[a] significantly different from corresponding normoxic value
[b] significantly different from corresponding value for cysteine

Table 3. Concentrations of inhibitor (mM) required for 10%, 50% and 90% inhibition of PK isozymes in the body wall and introvert of *P. arcuatum*

Condition	Inhibitor	Body Wall			Introvert		
		$[I]_{0.1}$	$[I]_{0.5}$	$[I]_{0.9}$	$[I]_{0.1}$	$[I]_{0.5}$	$[I]_{0.9}$
Normoxic	Cysteine	0.096	0.86	7.8	0.66	5.9	53
Anoxic	Cysteine	0.020	0.18	1.6	0.078	0.70	6.3
Normoxic	Alanine	0.30	2.7	23	3.2	28	256
Anoxic	Alanine	0.072	0.65	5.9	1.9	17	152

Our results showed that there was a 4 fold increased in this peak in the body wall and introvert of worms exposed to anoxic condition but no such changes were observed in the internal organ. Actually, the concentration (μmol per g wet weight$\pm$SE; n=4) of proline +L-cysteine in the body wall, introvert and internal organs of normoxic worm was 0.25$\pm$0.03, 0.19$\pm$0.04 and 0.11$\pm$0.01, respectively while the concentration in the anoxic worm was 1.07$\pm$0.25, 0.78$\pm$0.21 and 0.14$\pm$0.03, respectively.

DISCUSSION

P. arcuatum has to survive periods of environmental anoxia in its burrows when the tide rises in the mangrove swamp. It is able to tolerate anoxia through the regulation of PK at the phosphoenolpyruvate branch point (Lim and Ip, 1991a, b). This branch point controls the partitioning of carbon flow into pyruvate-derived (lactate, opines and alanine) versus oxaloacetate-derived (succinate and propionate) endproducts (De Zwaan 1983; De Zwaan and Dando 1984). Anoxia induces the phosphorylation of PK, thereby converting it to a much less active form (Lim and Ip, 1991a). Besides covalent modification, other mechanisms involved in PK regulation include effects of pH and allosteric inhibitors (Lim and Ip, 1991b). The lower activity of PK in the anoxic animal results in the formation of mainly oxaloacetate-derived endproducts. This would decrease the degree of tissue acidosis in the animal, but at the same time, generate more adenosine triphosphate (ATP) than utilizing only the traditional glycolytic pathway (Hochachka, 1980).

Similar to studies on other organisms (Leon *et al.*, 1982; Michaelidis *et al.*, 1985), Lim and Ip (1991b) showed that the PK obtained from *P. arcuatum* was inhibited by alanine; the anoxic enzyme being more sensitive to alanine inhibition than the normoxic one. During the course of investigation to find out if the PK from the anoxic *P. arcuatum* was phosphorylated, Lim and Ip (1991a) discovered that L-cysteine could inhibit not only the activity of the added phosphatase but also the activity of PK extracted from *P. arcuatum*.

We confirm in this study that the PK isozymes from the body wall and introvert of *P. arcuatum* were inhibited by L-cysteine. Since the V_{max} values as confirmed by the Dixon plot remained relatively constant regardless of the concentrations of L-cysteine present, and since the K_m(phosphoenolpyruvate) increased linearly with increasing L-cysteine concentrations, L-cysteine acted neither as an uncompetitive nor a noncompetitive inhibitor. An uncompetitive inhibitor causes changes in both the V_{max} and K_m values, whereas a noncompetitive inhibitor affects only the V_{max} but not the K_m value. The Dixon plot shows that the percentage inhibition by L-cysteine decreased with increasing concentrations of phosphoenolpyruvate. It can thus be concluded that L-cysteine is a competitive inhibitor of PK isozymes from the body wall and introvert of *P. arcuatum*. A competitive inhibitor combines with the free enzyme in a manner that prevents substrate binding (Segel, 1975). L-Cysteine and phosphoenolpyruvate might compete

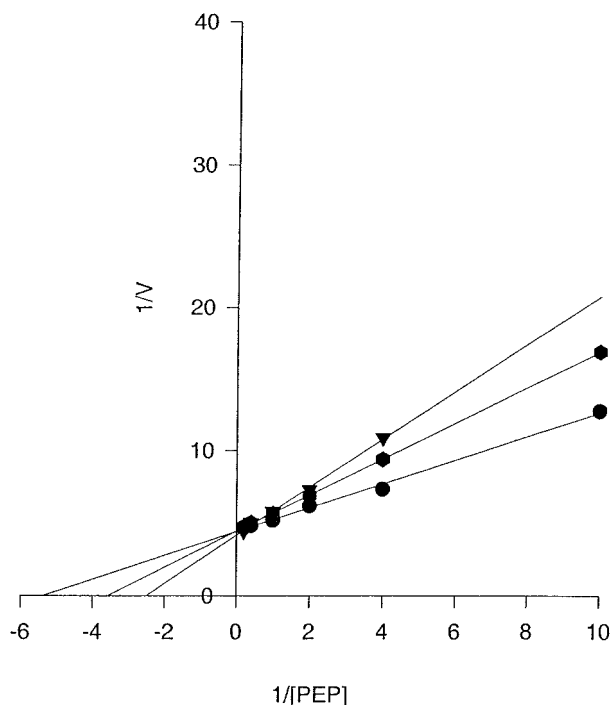**Fig. 5.** Double reciprocal plot of 1/V against 1/ [phosphoenolpyruvate] for PK from the body wall of normoxic *P. arcuatum* in the absence ( ) or presence of a final concentration of 0.5 mM (●) or 1.0 mM ( ) of alanine
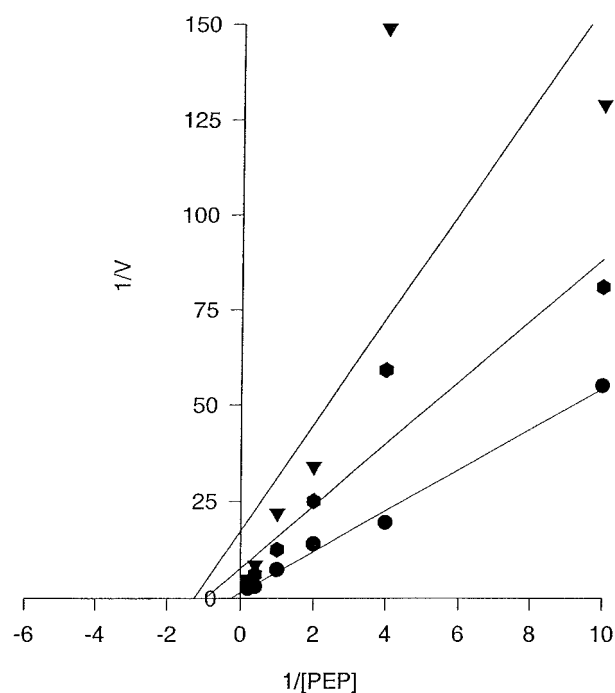

Fig. 6. Double reciprocal plot of 1/V against 1/ [phosphoenolpyruvate] for PK from the body wall of anoxic *P. arcuatum* in the absence ( ) or presence of a final concentration of 0.5 mM (●) or 1.0 mM ( ) of alanine

for the same binding site on PK isozymes from the body wall and introvert of *P. arcuatum*, hence affecting the K_m but not the V_{max} value of the enzyme.

After 48 hr of anoxic exposure, PK isozymes from these body parts show decreases in affinities for phosphoenolpyruvate, manifested by increases in K_m values. The PK isozymes from the body wall of the anoxic worm also exhibited lower affinities for L-cysteine as compared to those of the normoxic one. Despite the increase in K_i(L-cysteine) value upon anoxic exposure, the $[I]_{0.1}$, $[I]_{0.5}$ and $[I]_{0.9}$ values of PK from the body wall of the anoxic worm were lower than the respective values of the normoxic one due to the decrease in K_m(phosphoenolpyruvate). Hence, the effect of anoxia on K_m(phosphoenolpyruvate) overrides that on K_i(L-cysteine), suggesting that substrate affinity plays a more important role in the regulation of PK activity than the inhibitor affinity.

These results also indicate that the anoxic form of PK from these two body parts were more sensitive to the inhibitory effect of L-cysteine. The greater inhibitory effect probably facilitates the re-routing phosphoenolpyruvate via phosphoenolpyruvate carboxykinase into succinate formation at the phosphoenolpyruvate branched point during periods of anoxia, such as when the tides come into the mangrove swamps. Succinate formation not only prevents tissue acidosis but also generates more ATP as compared to the traditional glycolytic pathway (Hochachka, 1980).

Our results confirmed that the effect of L-cysteine on PK from *P. arcuatum* was tissue specific, and the PK isozyme from the internal organs was unaffected by L-cysteine.

The PK isozymes from the body wall and introvert of *P. arcuatum* had greater affinities for L-cysteine than alanine. The $[I]_{0.1}$, $[I]_{0.5}$ and $[I]_{0.9}$ values were also lower for L-cysteine than for alanine. Hence, in comparison to alanine, L-cysteine is a more powerful inhibitor for PK isozymes from these two body parts. Alanine was a competitive inhibitor of the PK from the body wall of the normoxic worms. However, it appeared not to be so for the corresponding anoxic form of PK. Since L-cysteine acted as a competitive inhibitor for both the normoxic and anoxic form of PK from this body part of *P. arcuatum*, the inhibitory properties of L-cysteine and alanine appeared to be different.

Ibsen and Trippet (1974) suggested that the PK isozyme from the rat has two different binding sites for amino acids. Ibsen and Marles (1976) further hypothesised that the carboxyl, α and β carbon of amino acids fit into the phosphoenolpyruvate binding site. Bound amino acids with small polar side chains further interact with a nonpolar group on PK isozymes in the T conformation. This interaction stabilizes the T conformation and thus enhances the effectiveness of these amino acids as inhibitors. On the other hand, the amino acids with non-bulky and polar side chains repel the nonpolar region of the T conformation because of their polarity. This repulsion causes the enzyme to favour the R conformation. L-Cysteine is a polar amino acid due to the presence of a sulphur atom in its side chain. Alanine is a nonpolar amino acid with a non-bulky methyl side chain. The difference in interaction of the

polar side chain of L-cysteine and the nonpolar side chain of alanine with the nonpolar group on the enzyme probably explains the difference in their inhibitory properties. Therefore, it can be concluded that L-cysteine is a novel inhibitor of PK and not just a substitute for alanine.

An analysis of the physiological concentration of L-cysteine revealed that there was most probably an increase in L-cysteine in the body wall and introvert of the anoxic worms since there was a 4 fold increase in the concentration of L-cysteine + proline in these two body parts of the anoxic worms. Thus, further supporting that L-cysteine, being an inhibitor of PK may serve as an important regulator in these body parts during anoxia. However, no such increased in L-cysteine was detected in the internal organs since no change in the concentration of L-cysteine + proline was observed. Further studies would have to be carried out in order to elucidate the physiological significance of the inhibitory role of L-cysteine on PK in this worm.

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