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ASPARTATE ACTIVATION OF PYRUVATE KINASE IN ANOXIA TOLERANT MOLLUSCS

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Abstract—1. The effects of the amino acid, aspartate, as an allosteric modifier of pyruvate kinase (PK) were surveyed in tissue extracts from 18 species of animals.

2. Aspartate was a strong activator of PK in selected tissues of gastropod and bivalves molluscs, particularly amongst anoxia-tolerant species; with one exception, a polychaete worm, aspartate had no effect on PK from non-molluscan species tested including marine, freshwater and terrestrial forms.

3. Amongst gastropods, aspartate activation of PK was confined to selected soft tissues, most often the hepatopancreas; in the whelk, *Busycotypus canaliculatum*, hepatopancreas, gill and kidney PK all showed the effect.

4. Amongst bivalves, aspartate activated PK in all tissues tested of the oyster, *Crassostrea virginica*, but activated only hepatopancreas PK in *Mytilus edulis* and foot and phasic adductor muscle PK in *Mercenaria mercenaria*.

5. Kinetic properties of *C. virginica* adductor and *M. mercenaria* foot muscle PK were further examined; for *C. virginica* adductor PK aspartate (5 mM) raised enzyme maximal velocity by 2.8-fold and lowered $S_{0.5}$ for P-enolpyruvate by 36%.

6. Aspartate ($K_a = 4.14 \pm 1.15$ mM) and fructose-1,6-P_2 ($K_a = 0.075 \pm 0.005$ mM) showed strong synergistic effects in their activations of *C. virginica* adductor PK.

7. Proposed functions for the unique activation of PK by aspartate in anoxia tolerant molluscs are (a) in gluconeogenic tissues (hepatopancreas), allosteric activators (aspartate, fructose-1,6-P_2) are key to regulation of the aerobic (dephosphorylated) form of PK with respect to glycolytic vs gluconeogenic carbon flux; and (b) aspartate activation of PK provides co-ordinated control of the coupled anaerobic fermentations of aspartate → succinate and glycogen → alanine.

INTRODUCTION

Pyruvate kinase (EC 2.7.1.40) (PK) is one of the key regulatory enzymes of glycolysis. Allosteric and non-allosteric forms of the enzyme occur (Ibsen, 1977; Hall and Cottam, 1978). The L type (liver) isozyme of PK in mammals is regulated by effectors such as fructose-1,6-P_2 and alanine and modified by protein kinase dependent phosphorylation such that enzyme activity can be strictly controlled to allow glycolytic vs gluconeogenic flux in multifunctional tissues. The non-allosteric M type PK occurs in tissues such as muscle which basically require only unidirectional carbon flow through glycolysis. The same distribution of PK isozyme types typifies most invertebrate animals (Lesicki, 1976; Guderley *et al.*, 1976; Munday *et al.*, 1980; Storey, 1985a).

A number of anoxia-tolerant marine molluscs (mostly intertidal inhabitants), however, have co-opted the use of the allosteric enzyme (Mustafa and Hochachka, 1971; Holwerda and de Zwaan, 1973), now known to be covalently modified (Siebenaller, 1979; Holwerda *et al.*, 1983; Plaxton and Storey, 1984a, b, 1985, 1986), to provide all tissues (both muscle and soft tissues) with a mechanism for regulating PK activity and glycolytic flux in response to long term environmental anoxia stress. Anoxia-induced phosphorylation of the enzyme, producing kinetic changes including a susceptibility to powerful inhibition by alanine, leads to a strong depression of enzyme activity and allows a redirection of glycolytic carbon flow, via P-enolpyruvate carboxykinase, into the production of succinate and propionate as anaerobic end products (Plaxton and Storey, 1985).

The use of covalent modification of PK to regulate the aerobic vs anaerobic disposal of glycolytic carbon may pose a problem for tissues with a high gluconeogenic capacity. How do these tissues regulate glycolytic vs gluconeogenic carbon flow in an aerobic tissue if covalent modification of PK can no longer be put to this use? Plaxton and Storey (1986) undertook an investigation of this problem using PK purified from the hepatopancreas of the whelk, *Busycotypus canaliculatum*. Studies revealed that the aerobic (dephosphorylated) form of PK was highly sensitive to and highly dependent upon the actions of allosteric activators, acting largely on enzyme V_{max}, for expression of substantial enzyme activity. Thus allosteric control, by changing levels of activators, appears to be the method for regulating PK activity with respect to the requirements for gluconeogenesis. However, in addition to the well known enzyme activator, fructose-1,6-P_2, a novel activator of PK was found: the amino acid, aspartate. Aspartate, as a major substrate of anaerobic metabolism in marine invertebrates, could, as an activator of PK, add an important new dimension to the regulatory control of carbon flow in anoxia tolerant animals. Present in high concentrations in the aerobic state the amino acid would promote an active PK. During anoxia, however, the depletion of this activator (to produce succinate) would slow PK activity (and with it alanine

production) and might also promote the phosphorylation of the enzyme.

The present study was undertaken to assess the effects of aspartate as an effector of PK in a variety of animal and tissue types. Tissues from 18 species were examined with results indicating that the unique regulation of PK by aspartate is almost exclusively limited to PK from certain tissues of anoxia-tolerant marine molluscs.

MATERIALS AND METHODS

Animals and chemicals

Cherrystone clams, *Mercenaria mercenaria* and oysters, *Crassostrea virginica*, were purchased from a local seafood retailer and were held for at least 1 week before use in aerated, recirculating seawater at 10°C. Other species used fresh (and acclimated as above if marine animals) originated from: Marine Biological Laboratory, Woods Hole (whelks, crabs, starfish, sea anemones), Boreal Laboratories, Mississauga (crayfish, land snails), local seafood retailers (periwinkles, mussels, lobsters), Carleton University colonies (cockroaches, freshwater snails, trout) and outdoor collections (slugs). For remaining species frozen tissues stored at −80°C were used.

Biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp.; Sephadex G-25 was from Pharmacia Fine Chemicals.

Enzyme preparation

Tissues were homogenized 1:3 (w/v) in ice-cold 20 mM imidazole–HCl buffer, pH 7.0 (pH of all solutions adjusted at 23°C) containing 15 mM 2-mercaptoethanol, 100 mM NaF, 5 mM EDTA, 5 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using a Polytron PT 10 homogenizer. Homogenates were centrifuged at 28,000 *g* for 15 min at 4°C. Supernatants were removed and used as the source of enzyme for initial survey studies.

For kinetic studies on *C. virginica* adductor muscle and *M. mercenaria* foot muscle PK, supernatants, obtained as above, were passed through a column of Sephadex G-25 (7 × 1 cm) equilibrated in homogenizing buffer minus PMSF before use to remove low mol. wt metabolites.

Enzyme assay

Standard assay conditions utilized 25 mM imidazole–HCl buffer, pH 7.0, 2 mM ADP, 10 mM $MgSO_4$, 50 mM KCl, 0.15 mM NADH, excess lactate dehydrogenase and P-enolpyruvate at varying concentrations (see Table 1).

Enzymes were assayed using a Pye Unicam SP8-100 recording spectrophotometer with water-jacketed cell holder for temperature control of assays at 23°C. Assays were started by the addition of enzyme preparation. Blanks (minus P-enolpyruvate) were tested for both control and metabolite modulated (fructose-1,6-P_2 or aspartate addition) cuvettes.

RESULTS

Survey of aspartate effects on PK

The effect of aspartate on the activity of PK, measured at subsaturating (approx. $S_{0.5}$) levels of P-enolpyruvate, was tested on tissue extracts from 18 species of animals (Tables 1 and 2). Data is presented as the ratio of enzyme velocities in the presence vs absence of added aspartate. For comparison, the effects of fructose-1,6-P_2, a well known activator of PK in many systems, on these PK preparations are also shown.

As Table 1 shows, PK from three soft tissues of the whelk, *B. canaliculatum*, was strongly activated by aspartate; under the conditions used the amino acid was as effective in increasing enzyme velocity as was fructose-1,6-P_2. Aspartate activation of PK was first discovered during an investigation of whelk hepatopancreas PK (Plaxton and Storey, in press) and the present evidence indicates that other tissues of the whelk which contain the same isozymic form of PK, the gill and kidney (Plaxton and Storey, 1985), also show aspartate activation of PK. Aspartate did not activate PK in whelk muscle however. Aspartate activation of hepatopancreas PK was also found in two other gastropod molluscs, the intertidal marine periwinkle, *L. littorea* and the terrestrial snail, *H. aspersa*. Hepatopancreas PK in two other gastropod species was not activated by aspartate but the enzyme from other soft tissues of the terrestrial slug and freshwater snail did show aspartate activation.

The effects of aspartate on PK from tissues of other marine molluscs are also shown in Table 1. The three bivalve species tested are all intertidal animals with well developed capacities for anaerobiosis. The oyster, *C. virginica* and the mussel, *M. edulis*, both showed aspartate activation of PK in hepatopancreas. The effect was also evident in all other tissues of *C. virginica* tested with greatest activation found for adductor muscle PK. *M. edulis*, however, showed aspartate activation of PK in no other tissue. The cherrystone clam, *M. mercenaria*, showed a different pattern of aspartate activation of tissue PK's with only the foot and phasic adductor muscles having the effect and no activation occurring for PK in any of the soft tissues. PK from hepatopancreas and gonad of the chiton, *K. tunicata*, was slightly activated by aspartate while of tissues tested in the cephalopod, *S. officinalis*, only brain showed a minor indication of aspartate effects.

Results for other marine invertebrates as well as for freshwater and terrestrial animals are shown in Table 2. In general aspartate was without effect on PK from tissues of the 8 species selected. The one exception to this was aspartate activation of PK in preparations of the pharynx muscle from the polychaete, *A. aculeata*.

To further investigate aspartate effects on PK and the possible role of this amino acid in the control of enzyme activity and glycolytic flux in marine molluscs, a study of some of the kinetic properties of PK was undertaken using two tissues which had shown high activation by aspartate: oyster adductor muscle and cherrystone clam foot muscle.

PK from oyster (C. virginica) *adductor muscle*

Some kinetic properties of PK from oyster adductor (phasic and catch combined) muscle were examined using crude preparations of the enzyme treated by passage through a column of G-25 to remove low mol. wt metabolites.

Both aspartate and fructose-1,6-P_2 activated the enzyme affecting both maximal velocity and enzyme affinity for P-enolpyruvate. At saturating P-enolpyruvate concentrations (5 mM) aspartate (5 or 20 mM) raised V_{max} by 2.8-fold while fructose-1,6-P_2 (0.1 or 0.5 mM) increased V_{max} 8.9-fold. In the absence of effectors, the affinity constant for P-enolpyruvate ($S_{0.5}$) was 2.8 ± 0.3 mM ($n_H = 1.5$)

Table 1. Activity ratios for pyruvate kinase from tissues of molluscs assayed in the presence vs absence of added aspartate or fructose-1,6-P_2

Species	Tissue	Activity ratio (plus effector/minus effector) 5 mM Aspartate	10 mM Aspartate	0.5 mM Fructose-1,6-P_2
Gastropoda				
Busycotypus canaliculatum	Hepatopancreas	2.6	2.6	2.6
(whelk)	Kidney	3.7	4.1	4.2
	Gill	4.5	8.3	2.1
	Foot	1.0	1.0	1.0
	Ventricle	1.0	1.4	1.1
	Radular retractor	1.0	1.1	1.2
Littorina littorea	Hepatopancreas	2.1	2.7	7.6
(periwinkle)	Foot	1.1	1.1	2.6
	Remaining body	1.0	1.3	2.2
Lymnaea stagnalis	Hepatopancreas	1.0	1.0	2.4
(snail)	Foot	1.1	1.1	1.1
	Remaining body	1.3	1.6	1.1
Helix aspersa	Hepatopancreas	1.4	1.3	1.2
(snail)	Foot	1.1	1.2	1.6
	Remaining body	1.3	1.4	1.6
Deroceras laeve	Hepatopancreas	1.0	1.0	3.7
(slug)	Gut	1.3	1.7	2.4
	Remaining body	1.0	1.0	1.5
Bivalvia				
Mercenaria mercenaria	Hepatopancreas	1.0	1.0	11.0
(cherrystone clam)	Kidney	1.0	1.0	6.0
	Gill	1.0	1.0	3.0
	Mantle	1.0	1.0	4.6
	Foot	2.4	2.7	3.5
	Phasic adductor	1.6	2.1	5.4
	Catch adductor	1.0	1.0	6.3
Crassostrea virginica	Hepatopancreas	1.4	1.6	3.8
(oyster)	Gill	1.3	1.7	2.5
	Mantle	1.3	1.3	3.0
	Adductor (combined)	2.3	4.5	9.8
	Ventricle	1.2	1.5	1.3
Mytilus edulis	Hepatopancreas	1.4	1.7	1.5
(mussel)	Gill	1.0	1.0	1.8
	Mantle	1.1	1.1	1.7
	Foot	1.0	1.1	1.5
	Posterior adductor	1.0	1.0	1.5
Amphineura				
Katarina tunicata	Hepatopancreas	1.0	1.6	2.9
(chiton)	Mantle	1.0	1.0	1.4
	Foot	1.0	1.0	1.0
	Gonad	1.2	1.3	1.8
Cephalopoda				
Sepia officinalis	Hepatopancreas	1.0	1.0	4.4
(cuttlefish)	Gill	1.0	1.0	2.5
	Mantle	1.0	1.0	1.4
	Brain	1.2	1.4	3.2
	Ventricle	1.0	1.0	2.7

Crude extracts of tissues were prepared as described in Materials and Methods. Except for P-enolpyruvate concentrations, all other assay conditions remained constant: 25 mM imidazole–HCl buffer, pH 7.0, 2 mM ADP, 10 mM $MgSO_4$, 50 mM KCl, 0.15 mM NADH and excess lactate dehydrogenase. For each tissue extract the P-enolpyruvate concentration chosen was one which gave suboptimal (approximately the $S_{0.5}$ value) rates of enzyme activity based upon an initial V vs [P-enolpyruvate] curve in the absence of added effectors. The choice of suboptimal P-enolpyruvate concentrations would allow detection of activation by aspartate or fructose-1,6-P_2 whether the effector altered enzyme $S_{0.5}$ or V_{max}. Results present the ratio of enzyme activity in the presence vs the absence of added effector and are the means of duplicate determinations for each parameter and tests on two individual animals for each species. Blank determinations (leaving out P-enolpyruvate) were done for all assays, both with or without an added effector, and where appropriate were subtracted from enzyme rates.

(determined from Hill plots); addition of 5 mM aspartate lowered this to 1.8 ± 0.4 mM ($n_H = 1.2$) while 0.1 mM fructose-1,6-P_2 decreased $S_{0.5}$ to 0.4 ± 0.04 mM ($n_H = 1.0$). Apparent activation constants, K_a (measured at 0.1 mM P-enolpyruvate), were 4.15 ± 1.15 mM for aspartate and 0.075 ± 0.005 mM for fructose-1,6-P_2.

Effects of activators were tested at substrate (0.1 mM P-enolpyruvate) and activator (5 mM aspartate, 0.1 mM fructose-1,6-P_2) concentrations close to physiological levels. Under these conditions the relative velocities of PK were 1.0 (control), 2.4 ± 0.3 ($\pm$aspartate), 17 ± 0.6 (+fructose-1,6-P_2) and 54 ± 7.2 (+ both activators) (all $n = 4$). Effects of aspartate and fructose-1,6-P_2 on enzyme velocity are therefore synergistic. Addition of aspartate (at 5 mM) did not reverse inhibitions of the enzyme by alanine or Mg·ATP.

PK from clam (M. mercenaria) *foot muscle*

Comparable kinetic properties were examined using PK from crude preparations of clam foot muscle, again after treatment by passage through G-25.

Table 2. Activity ratios for pyruvate kinase from tissues of selected animals assayed in the presence vs absence of added aspartate or fructose-1,6-P_2

Species	Tissue	Activity ratio (plus effector/minus effector) 5 mM Aspartate	10 mM Aspartate	0.5 mM Fructose-1,6-P_2
Metridium senile (sea anemone)	Pedal muscle	1.0	1.0	1.9
	Internal soft tissues	1.0	1.0	2.7
Aphrodite aculeata (polychaete worm)	Longitudinal muscle	1.0	1.2	1.7
	Pharynx	1.8	1.9	2.7
	Nerve	1.1	1.2	1.8
Homarus americanus (lobster)	Hepatopancreas	1.0	1.1	1.0
	Gill	1.2	1.2	1.3
	Hypodermis	1.0	1.0	1.1
	Leg muscle	1.2	1.2	1.0
	Ventricle	1.0	1.1	1.0
	Gonad	1.3	1.3	1.4
Callinectes sapidus (crab)	Hepatopancreas	1.0	1.1	1.2
	Gill	1.2	1.2	1.3
	Hypodermis	1.1	1.1	1.0
	Leg muscle	1.0	1.1	1.2
Asterias forbesi (starfish)	Pyloric caecum	1.0	1.0	1.0
	Eggs	1.0	1.0	1.3
	Tube feet	1.1	1.1	1.1
Orconectes virilis (crayfish)	Hepatopancreas	1.1	1.3	1.5
	Gill	1.1	1.2	1.3
	Leg muscle	1.0	1.1	1.1
Salmo gairdneri (rainbow trout)	Liver	1.2	1.2	2.9
Periplaneta americana (cockroach)	Fat body	1.0	1.0	1.1
	Flight muscle	1.0	1.0	1.4
	Gut	1.0	1.0	1.2

Conditions for assays are as described in Table 1.

Effects of both aspartate and fructose-1,6-P_2 were less pronounced than for the oyster enzyme. At saturating concentrations of P-enolpyruvate (5 mM), neither compound had a large effect on enzyme maximal velocity; V_{max} in the presence of 7 mM aspartate was increased only 1.3-fold while 0.8 mM fructose-1,6-P_2 increased V_{max} 1.5-fold. Enzyme affinity for P-enolpyruvate ($S_{0.5}$) was 0.72 ± 0.08 mM in the absence of activators. $S_{0.5}$ decreased slightly in the presence of 7 mM aspartate (0.55 ± 0.05 mM) and dropped 2.4-fold to 0.30 ± 0.04 mM with the addition of 0.8 mM fructose-1,6-P_2. Apparent activation constants, K_a (at 0.1 mM P-enolpyruvate), were 2.5 ± 0.2 mM for aspartate and 0.16 ± 0.06 mM for fructose-1,6-P_2.

Relative enzyme velocity was examined at near physiological concentrations of substrate (0.1 mM P-enolpyruvate) and activators (3 mM aspartate, 0.2 mM fructose-1,6-P_2). Relative rates were 1.0 (control), 1.8 ± 0.2 (+aspartate), 4.9 ± 0.8 (+fructose-1,6-P_2) and 5.1 ± 1.0 (+both activators). Again activator effects were not as pronounced as were seen for the oyster enzyme and effects of the two activators were not additive or synergistic.

Aspartate (3 mM) did not reverse inhibitions of the enzyme by alanine or Mg·ATP; inhibitor constants, I_{50}, in the presence or absence of activator remained unchanged at 5 mM for alanine and 1.2 mM for Mg·ATP. Fructose-1,6-P_2 (0.2 mM), however, strongly reversed inhibitions raising I_{50} for alanine to greater than 100 mM and I_{50} for Mg·ATP to 18 mM.

DISCUSSION

The amino acid aspartate is a very powerful activator of pyruvate kinase from the hepatopancreas of the whelk (Plaxton and Storey, 1986). Aspartate increases the maximal velocity of the aerobic (dephosphorylated) form of the enzyme by 13-fold (compared to 9-fold for fructose-1,6-P_2) and reduces $S_{0.5}$ for P-enolpyruvate by 55%. The amino acid also reduces the $S_{0.5}$ of the anoxic (phosphorylated) form by 75% but in this case does not affect V_{max}. In addition both aspartate and fructose-1,6-P_2 strongly override the effects of inhibitors, including alanine, on the aerobic enzyme. The function of aspartate activation of the aerobic form of PK in hepatopancreas may be two-fold. Firstly, the very strong effect of activators on the aerobic enzyme may allow the presence/absence of allosteric activators to be the key factor modulating PK activity in response to the needs for glycolytic vs gluconeogenic flux in the aerobic tissue. Secondly, aspartate may modulate the routing of carbon during anoxia through its effects on PK. Thus early stages of anoxia are characterized by the coupled conversion of glycogen to alanine (requiring PK activity) and aspartate to succinate (de Zwaan, 1983). Later when aspartate reserves are exhausted glycolytic carbon is rerouted via P-enolpyruvate carboxykinase into succinate synthesis and alanine production is stopped (PK shut down). Aspartate could act therefore to maintain an active PK during the early stage of anaerobiosis and closely regulate the link (via amino group transfer) between aspartate depletion and alanine accumulation. As aspartate reserves are exhausted, activity of PK would decrease and the loss of activator might even promote enzyme phosphorylation to produce the relatively inactive anoxic form of the enzyme.

Such functions for aspartate as an activator would lead to the prediction that aspartate effects on PK would occur in anoxia-tolerant animals which utilize

the coupled conversion of glycogen → alanine and aspartate → succinate and particularly in tissues which either (a) need additional regulation of PK to accomplish a gluconeogenic function, or (b) rely on the linked catabolism of glycogen and aspartate throughout anoxia and lack the capacity for glycogen conversion to succinate. This indeed seems to be the pattern revealed by the present results. Thus with the exception of the polychaete worm, major effects by aspartate on PK were confined largely to gastropod and bivalve molluscs. Amongst gastropod molluscs aspartate effects on PK were found mainly in the hepatopancreas although PK in some other soft tissues, including kidney and gill of the whelk, was also aspartate activated. Greatest effects (activity ratios of 2.6 and 2.7) were found in the anoxia-tolerant marine gastropods, *B. canaliculatum* and *L. littorea*. The presence of the effect in *H. aspersa* may be related to the anoxia tolerance displayed by this animal during periods of aestivation (Storey, 1977). Amongst the marine bivalves the tissue distribution of aspartate effects on PK was more varied. PK from all tissues of the oyster showed the effect, in the mussel only hepatopancreas PK was affected and in the cherrystone clam two muscle tissues were the only ones affected. The activation of PK by aspartate in the bivalves does not correlate well with the ability to covalently modify PK in response to anoxia stress. Thus both *M. edulis* (Siebenaller, 1979; Holwerda *et al.*, 1983) and *C. virginica* (W. C. Plaxton, unpublished results) show changes in the kinetics of PK during anoxia which imply phosphorylation of the enzyme; however aspartate activation of PK occurs in all oyster tissues but only in hepatopancreas of *M. edulis*. By contrast there is no evidence for covalent modification of PK in *M. mercenaria* during anoxia (Korycan, 1984) but two muscle tissues show a PK which is aspartate activated. In these bivalves there is probably a much better correlation of aspartate effects on PK with the way in which aspartate is utilized as a substrate during anaerobiosis. Results of studies of anaerobic metabolism in oysters show that aspartate utilization is not just confined to the early hours of anoxia but continues throughout anoxia (as long as 96 hr) with succinate accumulation equalling aspartate depletion (Eberlee *et al.*, 1983). This suggests no switch-over to the glycogen → succinate pathway and parenthetically implies that the phosphorylation of PK in anoxia which occurs does not necessarily cause the rerouting of glycolytic carbon into succinate synthesis; rather the purpose of protein phosphorylation during anoxia, which affects PK and many other enzymes/proteins (Storey, 1985; Plaxton and Storey, 1984b), may be simply to greatly reduce metabolic rate. Muscle tissues of *M. mercenaria* show the same pattern of anaerobic metabolism; aspartate depletion equals succinate accumulation throughout anoxia (Korycan and Storey, 1983) and again aspartate effects on PK may be used to optimize the coupled catabolism of glycogen and aspartate and co-ordinate the synthesis of alanine with the utilization of aspartate.

Kinetic studies of PK from oyster adductor and clam foot muscle revealed that aspartate effects on V_{max} and $S_{0.5}$ for P-enolpyruvate were not as great as those seen for the whelk hepatopancreas enzyme. However for the oyster enzyme, aspartate had very substantial effects, increasing V_{max} by 2.8-fold and decreasing $S_{0.5}$ by 36%. Effects occurred well within physiological levels of aspartate ($K_a = 4.15$ mM compared to concentrations in aerobic adductor of 6.5 μmol/g wet wt (Eberlee *et al.*, 1983). Additionally the activating effects of aspartate and fructose-1,6-P_2 showed a strong synergism, an effect which was also seen for whelk hepatopancreas PK (Plaxton and Storey, 1986). Effects of aspartate on *M. mercenaria* foot muscle PK were much less pronounced although at near physiological levels of substrate (0.1 mM P-enolpyruvate) aspartate (3 mM) significantly increased enzyme velocity (by 1.8-fold). *In vivo* levels of aspartate [10 μmol/g wet wt in aerobic foot muscle (Korycan and Storey, 1983)] could therefore have significant regulatory effects on PK activity.

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