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Tissue specific isozymes of pyruvate kinase in the channelled whelk *Busycotypus canaliculatum*: enzyme modification in response to environmental anoxia

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Summary. 1. Isoelectrofocusing and cellulose acetate electrophoresis demonstrated the presence of three tissue specific isozymic forms of pyruvate kinase (PK) occurring in red muscle (ventricle, radula retractor), white muscle (foot, mantle) and soft tissues (gill, kidney, hepatopancreas) of the channelled whelk, *Busycotypus canaliculatum*.

2. Under the assay conditions used the three isozymes differed kinetically with respect to fructose-1,6-bisphosphate activation: red muscle PK was insensitive, white muscle PK was slightly activated and soft tissue PK was strongly activated.

3. Kinetic and physical properties of the enzyme in all tissues were modified when PK was isolated from whelks exposed to 21 h of anoxia stress. Compared to PK from aerobic tissues, the enzymes from anoxic animals showed a decrease in pI of 0.4 pH unit and an increase in anodal migration on cellulose acetate. Anoxia reduced the maximal activity of PK in all tissues and increased the activity ratios at high versus low phosphoenolpyruvate (PEP) concentrations. White muscle and soft tissue PK from anoxic whelks also showed increased activation by fructose-1,6-biphosphate compared to aerobic animals.

4. Maximal activities and activity ratios at high versus low PEP for phosphoenolpyruvate carboxykinase (PEPCK) were not altered as a result of anoxia stress.

5. The anoxia-induced in vivo modification of PK appears to be the result of enzyme phosphorylation during anoxia and this covalent modification is probably a major factor limiting PK activity during anoxia and promoting the carboxylation of PEP via PEPCK.

Introduction

Pyruvate kinase (PK) (E.C. 2.7.1.40) is a key regulatory enzyme of the glycolytic pathway. Tissue specific isozymic forms of PK are found in mammals, the two major forms being L (liver) and M (muscle) type PK (Munday et al. 1980). L-type PK exhibits cooperative kinetics with respect to phosphoenolpyruvate (PEP), is activated by fructose-1,6-bisphosphate (Fru-1,6-P_2) and is subject to phosphorylation by hormone stimulated protein kinase (Engstrom 1978). The properties of L-type PK suit it for function in a tissue where flux through the locus must be severely restricted to allow other routes of PEP metabolism; in liver this allows glycolysis to be restricted in favour of gluconeogenesis. M-type PK occurs in non-gluconeogenic tissues, exhibits Michaelis-Menten kinetics with respect to PEP, is insensitive to Fru-1,6-P_2 and has never been shown to be phosphorylated in vivo.

Electrophoretically distinct tissue specific isozymes of PK have also been identified in birds, fish and frogs (Guderley et al. 1978; Guderley and Cardenas 1979; Schloen et al. 1969) and amongst invertebrates in bivalve and cephalopod molluscs (Mustafa and Hochachka 1971; Guderley et al. 1976) and in the cockroach (Storey 1985). Enzymes with kinetic properties analogous to those of L-type PK are found in crustacean hepatopancreas and hypodermis (Giles et al. 1976; Guderley and Hochachka 1977), insect fat body (Storey 1985) and cephalopod hepatopancreas (Guderley et al.

Abbreviations: *IDP* inosine diphosphate; *MES* 2-(N-morpholino)ethanesulfonic acid; *PEP* phosphoenolpyruvate; *PEPCK* phosphoenolpyruvate carboxykinase; *PK* pyruvate kinase; *PMSF* phenylmethylsulphonyl fluoride

1976) while the enzyme from aerobic invertebrate muscles such as crustacean leg muscle, insect flight muscle and cephalopod mantle muscle shows properties analogous to those of mammalian M-type PK (Guderley et al. 1976; Storey and Hochachka 1975; Storey 1985). Muscles of anoxia tolerant bivalve molluscs, however, have an allosteric PK (Mustafa and Hochachka 1971; de Zwaan 1972). Properties of this enzyme including reduced activity at low pH and inhibition by alanine have been thought to regulate the shut-down of carbon flow through PK during anaerobiosis and promote instead the carboxylation of PEP by phosphoenolpyruvate carboxykinase (PEPCK) (E.C. 4.1.1.32) and the resultant formation of succinate as an anaerobic end product (Hochachka and Mustafa 1972). Recent studies have also reported differences in enzyme properties for PK isolated from muscle and mantle of aerobic versus anaerobic bivalves (Holwerda et al. 1981, 1983, 1984), differences which suggest covalent modification of the enzyme during long term anaerobiosis.

The present study reports the presence of three isozymic forms of PK in the channelled whelk, *Busycotypus canaliculatum*, specific for red muscle, white muscle and soft tissues. All three isozymes show distinct kinetic and electrophoretic changes as a result of long term anoxia stress suggesting anoxia-induced covalent modification of the enzyme in all tissues. Studies reported elsewhere (Plaxton and Storey 1984a, b) confirm that the aerobic and anaerobic forms of red muscle PK are respectively the dephosphorylated and phosphorylated forms of the enzyme.

Materials and methods

Chemicals and animals. All biochemicals were purchased from Sigma Chemical Co.; Ampholines (pH 3.5–10) were from LKB Products. Coupling enzymes were from Boehringer, Mannheim, and cellulose acetate strips (Seprapore III, $1 \times 6''$) were from Gelman Instrument Co. Specimens of the channelled whelk, *Busycotypus canaliculatum*, were obtained from the Marine Biological Laboratory, Woods Hole, MA. and were held in recirculating sea water (1000 mOsm) at 12–15 °C without feeding until use. Control, aerobic animals were sampled directly from the sea water tank. To impose anoxia, animals were placed in large jars containing sea water and nitrogen gas was continuously bubbled through the sea water for 21 h. Anoxic animals were held at room temperature (20 °C).

Tissue preparation and enzyme assay

Tissues from aerobic and anoxic whelks were rapidly dissected out, blotted and frozen in liquid nitrogen until use. No loss in enzyme activity in frozen tissue was seen for periods of at least one month. Frozen tissues were ground to a powder under liquid nitrogen using a mortar and pestle and were then weighed and homogenized for 2×30 sec in ice cold 50 mM imidazole-HCl buffer (pH 7.5) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM 2-mercaptoethanol, 50 mM NaF, 3 mM EDTA and 3 mM EGTA, using a Polytron PT 10 homogenizer. The ratios of tissue weight to volume of homogenization buffer were: hepatopancreas (1/3), gill and kidney (1/4), foot and mantle (1/5), and heart and radula retractor (proboscis muscle) (1/10). Homogenates were centrifuged at 27,000 *g* for 30 min at 4 °C, and the supernatant was removed and dialyzed overnight at 4 °C against homogenization buffer minus PMSF.

Tissue extracts used for cellulose acetate electrophoresis were prepared as described above except the homogenization buffer was 15 mM Tris-HCl buffer (pH 7.2) containing 250 mM sucrose, 0.1 mM PMSF, 1.5 mM EDTA, 1.5 mM EGTA, 25 mM NaF and 10 mM 2-mercaptoethanol and ratios of tissue weights to volume of homogenization buffer used were: kidney, gill and hepatopancreas (1/1), mantle and foot (1/2), and ventricle and radula retractor (1/10).

Activities of enzymes in the dialyzed preparations were determined by following NADH utilization in a coupled reaction at 340 nm using a Gilford recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Assay temperature was maintained at 21 °C. Standard assay conditions for PK were: 50 mM imidazole-HCl buffer (pH 7.0), 5 mM PEP, 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH and excess dialyzed lactate dehydrogenase. Standard assay conditions for PEPCK were: 50 mM MES-HCl buffer (pH 7.0; extensively degassed), 3 mM PEP, 1.5 mM IDP, 20 mM $KHCO_3$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 15 mM NaF, 0.15 mM NADH and excess dialyzed malate dehydrogenase. A blank was conducted without $KHCO_3$ correcting for PK which may show some activity under the assay conditions employed. All assays were initiated by addition of enzyme preparation. One unit of enzyme activity is defined as the amount of enzyme resulting in the utilization of 1 µmol of NADH per min at 21 °C.

Isoelectrofocusing. Isoelectrofocusing was performed by the method of Vesterberg (1971) using an LKB 8101 column (110 ml) and a pH 3.5 to 10 gradient. Dialyzed enzyme preparations were run at 300 V for 20 h at 4 °C. The column was then drained and 1.5 ml fractions were collected and assayed for PK activity. Typical results led to 80% yields of enzyme activity following overnight dialysis and isoelectrofocusing.

Electrophoresis. Prior to sample application cellulose acetate strips were soaked for 2 h in electrophoresis buffer (15 mM Tris-HCl, pH 7.2, containing 250 mM sucrose, 10 mM 2-mercaptoethanol and 1.5 mM EDTA) containing 0.1% (w/v) bovine serum albumin. Aliquots (kidney, gill and hepatopancreas = 1 µl; mantle and foot = 0.7 µl; ventricle and radula retractor = 0.5 µl) were applied to the center of the strips. Electrophoresis was carried out for 1 h at 4 °C with 18 V/cm. Detection of PK activity was by a modification of the method of Susor and Rutter (1971). Following electrophoresis the strips were laid on a plexiglass ruler and were covered with another strip which had been soaked in a solution of 50 mM imidazole-HCl buffer (pH 7.0) containing 5 mM PEP, 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 20 µM fructose-1,6-bisphosphate, 1 mM NADH and 10 I.U/ml lactate dehydrogenase. Opposing strips were then placed in a humidified box and PK activity was visually observed under UV light owing to the loss of fluorescence associated with NADH oxidation. Incubations were carried out for 5 to 10 min at room temperature. Migration distances were noted immediately after the initial appearance of the enzyme band. Electrophoresis was performed a minimum of three times to confirm the observed staining patterns.

Table 1. *B. canaliculatum* pyruvate kinase: enzyme activities and enzyme activity ratios at high (5 mM) versus low (0.5 and 0.25 mM) PEP concentrations and plus or minus 1 mM Fru-1,6-P_2

Tissue	Status	Enzyme activity at 5 mM PEP units / g wet weight	Enzyme activity ratios		
			$\frac{v_{5\text{ mM PEP}}}{v_{0.5\text{ mM PEP}}}$	$\frac{v_{5\text{ mM PEP}}}{v_{0.25\text{ mM PEP}}}$	$\frac{v_{1\text{ mM Fru-1,6-P}_2}}{v_{control}}$
Radula retractor	Aerobic	116 ±33	1.4± 0.1	1.9± 0.1	1.03±0.01
	Anoxic	39 ± 7	36 ±13	88 ±20	1.00±0.03
Ventricle	Aerobic	75 ±12	1.5± 0.1	1.9± 0.2	1.01±0.01
	Anoxic	28 ± 5	27 ± 8	67 ± 7	1.05±0.04
Foot	Aerobic	45 ± 4	1.5± 0.2	1.7± 0.2	1.19±0.04
	Anoxic	17 ± 7	30 ± 5	79 ±10	1.34±0.05
Mantle	Aerobic	4.4 ± 0.9	1.6± 0.2	2.3± 0.4	1.22±0.06
	Anoxic	1.6 ± 0.6	20 ± 4	53 ± 8	1.43±0.08
Gill	Aerobic	1.7 ± 0.02	3.2± 0.5	5.5± 1.5	2.71±0.60
	Anoxic	0.1 ± 0.03	∞[a]	∞[a]	4.36±0.60
Kidney	Aerobic	1.1 ± 0.2	3.5± 0.6	4.4± 0.3	2.64±0.12
	Anoxic	0.05± 0.01	∞[a]	∞[a]	4.78±0.36
Hepatopancreas	Aerobic	1.6 ± 0.1	2.9± 0.4	6.9± 0.8	2.37±0.20
	Anoxic	0.18±0.03	5.5± 2	24 ± 9	3.94±0.46

All assays were performed at 21 °C and pH 7.0 using otherwise standard assay conditions as described in Materials and methods. Values are means ± s.e.m. of $n=3$ preparations of each tissue obtained from aerobic animals and whelks subjected to 21 h of anoxic stress

[a] PK activity ratios for extracts prepared from gill and kidney tissue of anoxic animals = ∞ because PK at 0.5 and 0.25 mM PEP was undetectable

Results

Pyruvate kinase

PK was present in all tissues tested; highest activities occurred in muscle tissues, whereas much lower activities were found in the soft tissues (Table 1). In all cases, when enzyme activity was measured in tissues taken from animals subjected to 21 h of anoxic stress, maximal activity was decreased significantly. Maximal activity of PK from anoxic whelks averaged 36.3% of that of aerobic controls in radular retractor, ventricle, foot and mantle and 5.9, 4.5 and 11.3% in gill, kidney and hepatopancreas, respectively. Table 1 also shows the ratio of enzyme activity at high (5 mM) versus low (0.5 and 0.25 mM) PEP concentrations. This ratio was dramatically increased in tissues of anoxic animals, indicative of a higher $K_{0.5}$ for PEP. A similar kinetic response to 21 h of anoxic stress was noted for whelk PKs when assayed at pH 8.0 (data not shown). Such an increase in the $K_{0.5}$ for PEP is characteristic of the phosphorylated form of mammalian L-type PK compared to the dephosphorylated form (Engstrom 1978).

Table 1 also shows the effect of the activator Fru-1,6-P_2 on PK from the various tissues. Activity ratios in the presence versus absence of 1 mM Fru-1,6-P_2 (at saturating PEP and ADP) are shown for PKs prepared from tissues of aerobic and anoxic animals. PK from white muscle tissues (foot, mantle) showed slight Fru-1,6-P_2 activation while the enzyme in soft tissues (gill, kidney, hepatopancreas) was strongly activated by Fru-1,6-P_2 under the assay conditions used. Anoxia stress increased the Fru-1,6-P_2 sensitivity of the white muscle and soft tissue enzymes, the activity ratio rising by an average of 15% and 69%, respectively, for the two groups compared to the aerobic state. Under these assay conditions PK from the red muscle tissues (ventricle and radula retractor) of both aerobic and anoxic animals is Fru-1,6-P_2 insensitive.

Phosphoenolpyruvate carboxykinase

Like PK, PEPCK activity was also found in all tissues tested of the whelk (Table 2). However, although muscle tissues contained the highest maximal activities, differences in maximal activities with respect to the soft tissues were not nearly so dramatic as was the case for PK. In contrast to PK, in no case was maximal PEPCK activity or the ratio of PEPCK activity at high (3 mM) versus

Table 2. *B. canaliculatum* PEPCK: enzyme activities and enzyme activity ratios at high (3 mM) vs low (0.3 and 0.15 mM) PEP concentrations

Tissue	Status	Enzyme activity at 3 mM PEP units / g wet weight	Enzyme activity ratios $v_{3\text{ mM PEP}}/v_{0.3\text{ mM PEP}}$	$v_{3\text{ mM PEP}}/v_{0.15\text{ mM PEP}}$
Radula retractor	Aerobic	2.9±0.2	2.3±0.2	3.2±0.6
	Anoxic	3.1±0.8	2.8±0.6	3.1±0.7
Ventricle	Aerobic	4.7±1.1	2.5±0.9	2.4±0.5
	Anoxic	3.4±0.8	3.4±0.8	3.7±0.6
Foot	Aerobic	2.7±0.3	3.1±0.3	5.3±0.9
	Anoxic	2.1±1.0	3.0±0.8	5.2±1.0
Mantle	Aerobic	1.0±0.1	3.0±0.7	4.4±1.0
	Anoxic	0.9±0.2	2.7±0.9	4.7±0.7
Gill	Aerobic	1.2±0.5	1.7±0.2	2.9±0.2
	Anoxic	0.8±0.3	2.0±0.3	3.2±0.3
Kidney	Aerobic	0.8±0.2	2.1±0.5	3.0±0.5
	Anoxic	0.6±0.3	1.9±0.7	2.9±0.5
Hepatopancreas	Aerobic	0.4±0.1	1.4±0.2	1.9±0.1
	Anoxic	0.4±0.2	1.3±0.2	2.0±0.4

All assays were performed at 21 °C and pH 7.0 using otherwise standard assay conditions as described in Materials and methods. Values are means ± s.e.m. of $n=3$ preparations of each tissue obtained from aerobic animals and whelks subjected to 21 h of anoxic stress

Table 3. Effect of 21 h of environmental anaerobiosis on isoelectric points of pyruvate kinase from seven tissues of the channelled whelk, *B. canaliculatum*

Tissue	Average pI	
	Aerobic	Anoxic
Radula retractor	5.81	5.42
Ventricle	5.84	5.43
Foot muscle	4.21	3.81
Mantle	4.28	3.72
Gill	4.91	4.52
Kidney	4.92	4.56
Hepatopancreas	4.98	4.53

Results are an average of at least two separate runs and are reproducible to within ± 0.10 pH units

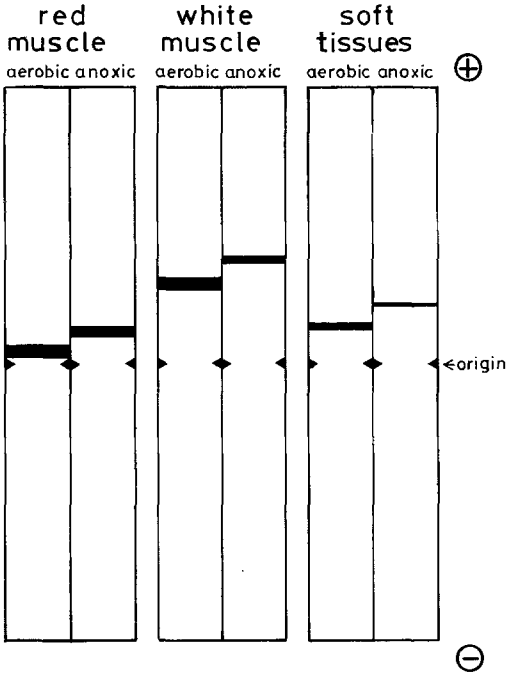

Fig. 1. Composite figure showing electrophoretic resolution of pyruvate kinase isozymes from red muscle (radula retractor and ventricle), white muscle (foot and mantle) and soft tissues (gill, kidney and hepatopancreas) of aerobic and 21 h anoxic specimens of *B. canaliculatum*. Cellulose acetate electrophoresis was carried out for 1 h at 18 V/cm as described in Materials and methods

low (0.3 and 0.15 mM) PEP levels significantly altered when the enzyme from aerobic tissues was compared with that from animals subjected to 21 h of anoxic stress at either pH 6.0 (data not shown) or pH 7.0 (Table 2).

Isoelectrofocusing and electrophoresis of whelk pyruvate kinase

Table 3 shows the isoelectric points of PK from seven tissues in aerobic and 21 h anoxic whelks. Three isozymic forms differing in pI were identified, corresponding to red muscle tissue (ventricle and radular retractor), white muscle tissue (foot and mantle) and soft tissues (gill, kidney and hepatopancreas). In each case the enzyme from anoxic animals showed a decrease in pI of about 0.4 pH unit when compared to the corresponding enzyme from aerobic tissues.

Cellulose acetate electrophoresis showed similar results (Fig. 1). PK from all tissues migrated towards the anode; PK from white muscle tissues

showed a fast migrating band of activity, red muscle PK showed a slow migrating band of activity, and soft tissue PK showed an intermediate migrating band of activity. Moreover, the extent of anodal migration of PK from all tissues was increased to the same degree when analyzed from extracts prepared from animals subjected to 21 h of anoxic stress.

Discussion

Three tissue specific isozymic forms of pyruvate kinase occur in *B. canaliculatum*. Isoelectrofocusing and cellulose acetate electrophoresis identified a red muscle isozyme occurring in the two myoglobin-containing tissues, ventricle and radula retractor muscle. A second form was specific for the two white muscle tissues, foot and mantle, and a third form occurred in the soft tissues, gill, kidney and hepatopancreas. This is the first demonstration of tissue specific isozymes of PK in a gastropod mollusc. The data suggest that the three isozymes have distinct kinetic properties. Under the conditions used the isozymes showed distinct responses to the activator, Fru-1,6-P_2 : red muscle PK was not affected, white muscle PK was slightly activated and soft tissue PK was strongly activated. The soft tissue enzyme also differed from the enzyme from other tissues in showing higher activity ratios ($v_{\text{high PEP}}/v_{\text{low PEP}}$) suggesting differences in enzyme affinity for PEP between the isozymes.

The data clearly show that PK in all tissues is modified in vivo during anoxia stress. All tissues showed a decline in the maximal activity and a large increase in the activity ratios ($v_{\text{high PEP}}/v_{\text{low PEP}}$) for the enzymes from anoxic whelks compared to those from aerobic whelks. A decrease in PK activity at subsaturating PEP levels has been demonstrated for adductor muscle and mantle of the bivalve, *Mytilus edulis* as a result of anoxia (Holwerda et al. 1981, 1983; de Zwaan and Dando 1984). Whelk white muscle and soft tissue PK's also showed increased sensitivity to Fru-1,6-P_2 as an activator for the enzyme from anoxic tissues.

Physical differences between the enzymes from aerobic and anaerobic whelks were also found. The enzyme from all tissues showed a decrease in pI of 0.4 pH unit in anoxic compared to aerobic animals and this was reflected in an increase in the anodal mobility of the isozymes on cellulose acetate. These results suggest that the molecular nature of the anoxia-induced modification of PK is identical for the three isozymes.

A larger $K_{0.5}$ for PEP reflecting a higher activity ratio ($v_{\text{high PEP}}/v_{\text{low PEP}}$) (Engstrom 1978) and a lower pI (Blair et al. 1982) are typical of the phosphorylated form of mammalian L-type PK as compared to the dephosphorylated form. This suggests that whelk PK's can undergo reversible phosphorylation and that the phosphorylated form, typically the less active form in mammals (Engstrom 1978), occurs in anoxic tissues. Our studies have recently confirmed this suggestion for PK from the radula retractor muscle of the whelk. Aerobic and anoxic forms of the enzyme were purified to homogeneity. The anoxic form showed many kinetic properties typical of mammalian L-type PK when phosphorylated by cyclic AMP-dependent protein kinase including: (a) decreased affinity for PEP, (b) increased cooperativity in binding of PEP, (c) increased inhibition by ATP and alanine, and (d) decreased sensitivity to activation by Fru-1,6-P_2 (Plaxton and Storey 1984a). The covalent incorporation of injected [^{32}P]-orthophosphate into the enzyme under anoxic, but not under aerobic, conditions demonstrated that the anoxic form of the enzyme is a phosphoprotein (Plaxton and Storey 1984b). By analogy it is probable that the anoxia-induced changes in the properties of PK from white muscle and soft tissues of the whelk are also the result of enzyme phosphorylation.

Until recently it was thought that the controlling factors promoting PK inactivation during anoxia and the resulting diversion of carbon flow via PEPCK into the succinate pathway were a) the decrease in tissue pH during anoxia and b) the elevated levels of the inhibitor alanine (Hochachka and Mustafa 1972; Holwerda et al. 1973; Wijsman 1975; de Zwaan 1977). It is now apparent, however, that tissue intracellular pH reductions during anoxia are not as dramatic as was first believed (Ellington 1983) and that this factor is perhaps of limited importance. Instead, anoxia-induced phosphorylation of PK resulting in a much less active enzyme form is probably the key mechanism reducing PK activity in the anoxic state.

The data for PEPCK show no evidence for an alteration of enzyme properties as a result of anoxia. Holwerda et al. (1981) reported similar results for PEPCK in aerobic versus anaerobic adductor muscle of *M. edulis*. Unlike PK then, PEPCK does not appear to undergo in vivo modification during anoxia. Changes in the levels of substrates and effectors of the enzyme, coupled with the strong depression of PK activity as a result of enzyme phosphorylation, are probably sufficient to promote the rerouting of PEP via PEPCK into the succinate pathway.

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