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SUBCELLULAR DISTRIBUTION OF AMINOTRANSFERASES, AND PYRUVATE BRANCH POINT ENZYMES IN GILL TISSUE FROM FOUR BIVALVES*

KENNEDY T. PAYNTER, GEORGE A. KARAM, LEHMAN L. ELLIS and STEPHEN H. BISHOP
Department of Zoology, Iowa State University, Ames, IA 50011, USA (Tel: 515-294-3316)

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Abstract—1. Aspartate aminotransferase (AAT), alanine aminotransferase (ALAT), malic enzyme (ME), malate dehydrogenase (MDH), pyruvate kinase (PK), and phosphoenolpyruvate carboxykinase (PEPCK) activities in cytosolic and mitochondrial fractions of gill tissue from *Modiolus demissus* (ribbed mussel), *Mytilus edulis* (sea mussel), *Crassostrea virginica* (oyster) and *Mercenaria mercenaria* (quahog) were determined using enzyme assay and starch gel electrophoresis combined with subcellular fractionation.

2. AAT showed distinct mitochondrial and cytosolic isozymes in gills of all these animals.

3. Although ALAT showed distinct mitochondrial and cytosolic isozymes in the gills of oysters, sea mussels and quahogs, only the mitochondrial ALAT was evident in ribbed mussel gill tissue.

4. PK and PEPCK were cytosolic in all these preparations.

5. ME was found only in the mitochondrial fraction of ribbed mussel and quahog gill tissue whereas sea mussel gills showed distinct cytosolic and mitochondrial ME isozymes.

6. With oyster gills, the "cytosolic ME" was electrophoretically identical to the mitochondrial ME indicating that *in vivo*, the ME is probably mitochondrial.

7. MDH showed distinct cytosolic and mitochondrial isozymes in all bivalve gills tested.

INTRODUCTION

The subcellular distribution and properties of isozymes associated with the metabolism of the amino acids and the organic acids of the TCA cycle seem critical to the metabolic processes regulating the high cellular free amino acid concentrations in tissues from ribbed mussels and other euryhaline bivalves. For instance, the levels of alanine, glycine, proline, and glutamate in ribbed mussel gill tissue appear to be controlled by specific enzymes within the mitochondria. The alanine aminotransferase (ALAT) activity (Paynter *et al.*, 1984b) and the enzymes regulating the catabolism of glutamate (Reiss *et al.*, 1977; Hoffmann *et al.*, 1978), proline and pyruvate (Burcham *et al.*, 1984; Bishop *et al.*, 1981) and glycine (Ellis *et al.*, 1985) are mitochondrial. On the other hand, the lactate, and opine dehydrogenases are cytosolic (Bishop *et al.*, 1980; Nicchitta and Ellington, 1984). Recently Paynter *et al.* (1984a) have shown that the kinetic behavior of the cytosolic aspartate aminotransferase (AAT) activity differs considerably from that of the mitochondrial AAT activity.

An essential aspect of the studies from this laboratory is the combining of subcellular fractionation studies with metabolic and electrophoretic studies to establish the identity, heterogeneity within the compartment of origin, the relative activity and the physiological roles of these isozymic forms. The study reported here compares the subcellular distribution of six enzyme activities, malic enzyme (ME), malate dehydrogenase (MDH), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), AAT and ALAT in ribbed mussel gill tissues to that in the gill tissue of three other euryhaline bivalves often used in the study of cellular osmoregulation.

MATERIALS AND METHODS

Ribbed mussels (*Modiolus demissus*), purchased from Northeast Environmental Laboratories (Monument Beach, MA), were kept in artificial sea water (Jungle Laboratories, Inc., Sanford, FL) and maintained as described by Greenwalt and Bishop (1980). Quahogs (*Mercenario mercenaria*) and oysters (*Crassostrea virginica*) were obtained from Dr. Michael Castagna (Virginia Institute of Marine Science, Wachapreague, VA) and kept in the same tank with the ribbed mussels. Sea mussels (*Mytilus edulis*) were obtained from the New England coast through a local seafood supplier and maintained in artificial sea water (850 m0smols) at approximately 10°C. Except when noted, all reagents were purchased from Sigma Chemical Co., St. Louis, MO. Enzyme grade ammonium sulfate was obtained from Schwartz-Mann (Orangeburg, NY).

Tissue fractionation

Gills were removed from the bivalves and collected in chilled, tared beakers. Approximately 20 g of tissue was typically collected. The tissue was homogenized in a mitochondrial isolation buffer consisting of 0.4 M sucrose, 20 mM HEPES and 1 mM EGTA (pH 7.5) using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH) with three 10-sec bursts on a setting of "40". The homogenates were filtered through Miracloth (Calbiochem) to remove large particulate debris. The filtrate was centrifuged at 1500 *g* for 8–10 min; the supernatant collected and recentrifuged at 9000 *g* for 15 min. This procedure has been used previously (Burcham *et al.*, 1983, 1984; Ellis *et al.*, 1985; Paynter *et al.*, 1984b). The supernatant from this second centrifugation constituted the cytosolic fraction. The pellet of the second centrifugation was resuspended in mitochondrial isolation buffer (10 ml) and extracted with recrystallized digitonin. Digitonin (Sigma grade) was recrystallized according to Kun *et al.* (1979). The mitochondrial suspension was extracted by addition of 1 mg digitonin/mg mitochondrial protein and stirred with a magnetic stirring bar for at least 30 min on ice. The resulting suspension was centrifuged at 10,000 *g* for 20 min and the

supernatant collected as the mitochondrial extract (Paynter *et al.*, 1984b). Protein was determined using the biuret method (See Burcham *et al.*, 1984).

Enzyme assays

All enzyme activities were assayed spectrophotometrically by measuring the oxidation/reduction of NADH/NAD as a function of absorption at 340 mm. The PK and ALAT assays were coupled with LDH, and the PEPCK and AAT assays were coupled with MDH. The six reaction mixtures used for the assays consisted of the following: AAT–100 mM Tris–HCl (pH 8.0), 20 mM aspartate, 10 mM α-ketoglutarate, 70 μM NADH, 5 units MDH, and enzyme in 2 ml; ALAT—50 mM Tris–HCl (pH 8.0), 20 mM alanine, 10 mM α-ketoglutarate, 70 μM NADH, 5 units LDH, and enzyme in 2 ml; PEPCK—50 mM Tris–HCl (pH 8.0), 70 μM NADH, 1 mM $MnCl_2$, 1.5 mM IDP, 1 mM $MgCl_2$, 2 mM PEP, 20 mM $NaHCO_3$, 5 units MDH and enzyme in 2 ml; ME–1 mM malate, 0.1 mM NADP, 1 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0) and enzyme in 2 ml; MDH—10 mM OAA, 70 μM NADH, 50 mM Tris–HCl (pH 8.0) in 2 ml. All enzyme assays were performed at the time of tissue sample preparation.

Starch gel electrophoresis

Starch gels were prepared as described by Paynter *et al.* (1984b). Samples of the cytosolic and mitochondrial fractions were loaded into the gel slots and electrophoresed in a 4°C refrigerator at 35 mA until the bromophenol blue marker reached the sponge bridge. The gels were removed from the refrigerator, sliced in half and immediately stained. AAT, ALAT, PK, ME, and MDH were stained on individual gel halves using the methods of Harris and Hopkinson (1976).

RESULTS

The data for the six enzyme activities in the four bivalve gill tissues are presented in Table 1. The use of the digitonin treatment of the mitochondria appears to have been particularly useful in obtaining preparations that showed full activities and no staining at the origin after electrophoresis. The percentages of the activities in the mitochondrial and cytosolic fractions are presented in parentheses in Table 1. Isozyme patterns are described (Table 1) to indicate the presence or absence of different isozymes between compartments. Fractions exhibiting no electrophoretically common activities were labelled as distinct. Where both fractions exhibited identical isozyme patterns, the patterns were labelled identical. Where both fractions exhibited activities which contained both distinct and identical bands, the patterns were labelled mixed. Although identical electrophoretic banding patterns may not indicate identity between the two activities, different banding patterns do indicate different or modified proteins and hence different isozymes.

The gill tissue AAT activity in pooled samples of tissue from representatives of the four bivalve species studied exhibited different electromorphs in both cellular compartments of the gills of all four species. The mitochondrial AAT was the major form in the oyster gill tissue, whereas the mitochondrial and cytosolic AAT distribution was more equal in the gill tissue from the other species. Although the ALAT activity showed distinct mitochondrial and cytosolic forms in oyster, quahog, and sea mussel gill tissue, only the mitochondrial form was found in the ribbed mussel gill tissue (See Paynter *et al.*, 1984b). The ME activity showed distinct mitochondrial and cytosolic forms in sea mussel gills; gill tissue from the other bivalves showed the single mitochondrial form.

The MDH activity showed distinct cytosolic and mitochondrial forms in gills from all bivalves tested. The majority of the total MDH activity was cytosolic in oyster, quahog, and sea mussel gill tissue. The cytosolic and mitochondrial MDHs showed multiple, nonidentical electromorphs in gill tissue from all the bivalves tested. Both the PK and the PEPCK activities were cytosolic in the gills from all four bivalves.

Table 1. Mitochondrial and cytosolic distribution of enzyme activities in gill tissue from four euryhaline bivalves

	Specific activity (percentage of total activity in tissue fraction preparations)*					
Bivalve	AAT†	ALAT	ME	MDH	PK	PEPCK
Crassostrea virginica						
Mitochondria	1.7 (92)	0.10 (56)	0.010 (49)	0.06 (10)	0.008 (2)	N.D.‡
Cytosol	0.035 (8)	0.02 (44)	0.002 (51)	0.13 (90)	0.093 (98)	0.11 (100)
Isozyme pattern§	Distinct	Distinct	Identical	Mixed	Identical	—
Mercenaria mercenaria						
Mitochondria	0.16 (43)	0.053 (80)	0.018 (100)	0.50 (16)	0.09 (6)	N.D.
Cytosol	0.19 (57)	0.001 (20)	N.D.	0.13 (84)	0.12 (94)	0.004 (100)
Isozyme pattern	Distinct	Distinct	—	Distinct	Identical	—
Mytilus edulis						
Mitochondria	0.086 (25)	0.034 (44)	0.073 (76)	0.034 (15)	0.084 (18)	N.D.
Cytosol	0.088 (75)	0.015 (56)	0.080 (24)	0.068 (85)	0.10 (82)	0.065 (100)
Isozyme pattern	Distinct	Distinct	Distinct	Mixed	Identical	—
Modiolus demissus						
Mitochondria	0.18 (56)	0.10 (77)	0.02 (100)	0.14 (30)	0.031 (8)	N.D.
Cytosol	0.030 (44)	0.006 (23)	N.D.	0.068 (70)	0.075 (92)	0.007 (100)
Isozyme pattern	Distinct	Identical	—	Mixed	Identical	—

*Specific activity in μmol/min/mg protein. The standard error in all determinations was $< 10\%$. Values represent averages obtained from duplicate measurements on two (PK, PEPCK) to five (AAT, ALAT, ME, MDH) preparations. See Paynter *et al.* (1984a, b) for more data on AAT and ALAT.

†AAT, Aspartate aminotransferase; ALAT, alanine aminotransferase; ME, malic enzyme; MDH, malate dehydrogenase; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase.

‡N.D. Activity below detectable units.

§Isozyme pattern: distinct—activities in the mitochondrial and cytosolic fractions were electrophoretically different; identical—no electrophoretic differences were found between fractions; mixed—fractions were neither completely distinct nor identical, but shared at least one common electrophoretic band.

DISCUSSION

AAT activity has been detected in the cytosol and mitochondria of all bivalve species investigated by a number of investigators (see Bishop *et al.*, 1983; Burcham *et al.*, 1983; Paynter *et al.*, 1984a). The general distribution of AAT activity in sea mussel, ribbed mussel and quahog gill tissue (Table 1) would seem to fit the pattern seen in most other bivalves. However, the high proportion of mitochondrial AAT compared to cytosolic AAT in oyster gill tissue (Table 1) may be of some importance in terms of population genetics. For instance, Buroker (1983, 1984) reports two AAT isozyme groups in American oyster populations from the Cheasapeake Bay area where these animals were collected. The slower moving isozyme is apparently the mitochondrial form (Table 1) and is essentially monomorphic. The other faster moving cytosolic form can show considerable polymorphism. The relatively low tissue level of the cytosolic AAT isozyme compared to the mitochondrial AAT isozyme may account for some of the difficulties in determining polymorphism for these isozymes in some bivalve tissues (see Fujio *et al.*, 1983; Kartavtsev and Zaslavskaya, 1983; Johnson and Utter, 1973; Johnson *et al.*, 1972).

The finding of distinct cytosolic and mitochondrial ME isozymes in *M. edulis* tissues supports other studies on ME in sea mussel tissues (de Zwaan and van Marrewijk, 1973; de Zwann, 1977; de Zwann *et al.*, 1981; Fujio *et al.*, 1983; Blanc, 1983). A single monomorphic ME activity has been reported for the giant clam *Tridacna* by Ayala *et al.* (1973). However, Fujio *et al.* (1983) report both polymorphic and monomorphic forms of ME in the oyster *C. gigas* and some other bivalves. Although the "cytosolic ME" activity from *C. gigas* adductor muscle has been partially characterized by Hochachka and Mustafa (1973), there are no studies on the mitochondrial MEs from the tissues of these other bivalves. The appearance of the mitochondrial ME form in the cytosolic fraction of the oyster gill preparations (Table 1) probably indicates a loose binding within the mitochondria and release to the cytosol during homogenization. The ME in the other bivalve gill tissues is apparently more tightly bound within the mitochondria.

The ALAT activity is usually found in both the cytosol and mitochondria of bivalve tissues (Chambers *et al.*, 1975; Burcham *et al.*, 1983; de Zwann, 1977; Addink and Veenhof, 1975; Bishop *et al.*, 1983). With oyster, sea mussel and quahog gill tissues these two ALAT activities are associated with separate isozyme groups. Ribbed mussel gill tissue lacks the cytosolic ALAT activity (Paynter *et al.*, 1984b).

The cytosolic localization of PK and PEPCK is common to most bivalves (deZwann, 1977). Although no clear indication of polymorphism for PK was observed in this study, cytosolic isozymes of PK have been shown in different sea mussel tissues and some variations in electrophoretic migration may occur with phosphorylation or dephosphorylation (Siebenaller, 1980; Holwerda *et al.*, 1983).

A complex MDH isozyme distribution pattern is found in most bivalve tissues (Ayala *et al.*, 1973; Tracey *et al.*, 1975; Koehn and Mitton, 1972; Fujio *et al.*, 1983; Buroker, 1983, 1984). Both the cytosolic and mitochondrial MDH activities in the gill tissues of these bivalves showed polymorphism (data not shown).

From the physiological viewpoint; the mitochondrial localization of ME in most species and the unusual AAT and ALAT distribution in oyster gill and ribbed mussel gill, respectively, may be helpful in rationalizing some of the species and tissue differences in amino acid accumulation during osmotic and anaerobic stress (see Paynter *et al.*, 1984a,b).

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