Tissue specific isozymes of alanopine dehydrogenase in the channeled whelk *Busycotypus canaliculatum*

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Received November 18, 1981

PLAXTON, W. C., and K. B. STOREY. 1982. Tissue specific isozymes of alanopine dehydrogenase in the channeled whelk *Busycotypus canaliculatum*. Can. J. Zool. **60**: 1568–1572.

The tissues of the channeled whelk *Busycotypus canaliculatum* displayed activities of three glycolytic dehydrogenases, alanopine dehydrogenase (ADH), octopine dehydrogenase (ODH), and lactate dehydrogenase (LDH). ADH and ODH were present in all seven tissues (hepatopancreas, gill, kidney, and mantle, ventricle, foot, and proboscis muscles) tested. ADH was the major cytosolic dehydrogenase activity in all tissues except proboscis, while ODH was present in high activities only in the four muscular tissues. Significant LDH activity occurred only in the two muscles which perform sustained work, ventricle, and proboscis.

Tissue specific isozymes of ADH were identified. Three forms, specific for hepatopancreas, gill–kidney, and muscle tissues, were separable by isoelectrofocusing (pI's 5.69, 5.58, and 5.93, respectively), chromatofocusing, and polyacrylamide gel electrophoresis. The three isozymes differed kinetically showing significant differences in apparent K_m's for L-alanine (e.g., 8.84 ± 0.03, 10.64 ± 0.40, and 13.12 ± 0.65 m*M* for hepatopancreas, ventricle, and gill, respectively) and even greater differences in apparent K_m's for glycine (619 ± 55, 1412 ± 115, and 2542 ± 230 m*M* for the above three tissues, respectively). The ratios $K_{m(gly)}/K_{m(ala)}$ averaged 70, 192, and 132 for the three isozymes, hepatopancreas, gill–kidney and muscle, respectively.

The physiological functions of ADH isozymes in the whelk may be similar to those of the M and H isozymes of LDH in vertebrates or the muscle and brain specific isozymes of ODH in cephalopod molluscs.

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Trois déshydrogénases glycolytiques sont actives dans les tissus du buccin *Busycotypus canaliculatum*, l'alanopine déshydrogénase (ADH), l'octopine déshydrogénase (ODH) et la lactate déshydrogénase (LDH). L'ADH et l'ODH sont présentes dans les sept tissus examinés (hépatopancréas, branchies, rein, muscles du manteau, du ventricule, du pied et du proboscis). L'ADH est la déshydrogénase qui manifeste le plus d'activité cytosolique dans tous les tissus sauf le proboscis, alors que l'ODH ne se manifeste fortement que dans les quatre tissus musculaires. L'activité de la LDH n'est élevée que dans les deux muscles qui travaillent de façon continue, le ventricule et le proboscis.

Des isozymes tissulaires spécifiques de l'ADH ont pu être identifiés. Trois formes spécifiques de l'hépatopancréas, du complexe branchies–rein et des muscles ont été reconnues par focalisation isoélectrique (pI = 5,69, 5,58 et 5,93, respectivement), par focalisation chromatographique et par électrophorèse sur gel de polyacrylamide. Les trois isozymes ont des comportements kinétiques différents; les constantes apparentes K_m diffèrent significativement pour la L-alanine (p. ex. 8,84 ± 0,03, 10,64 ± 0,40 et 13,12 ± 0,65 m*M* pour l'hépatopancréas, le ventricule et les branchies, respectivement) et les constantes apparentes K_m diffèrent encore plus pour la glycine (619 ± 55, 1412 ± 115 et 2542 ± 230 m*M* pour les trois mêmes tissus, respectivement). Les rapports $K_{m(gly)}/K_{m(ala)}$ sont en moyenne de 70, 192 et 132 pour les trois isozymes hépatopancréas, complexe branchies–rein et muscles, respectivement.

Les fonctions physiologiques des isozymes de l'ADH du buccin ressemblent peut-être à ceux des isozymes M et H de la LDH des vertébrés ou à ceux des isozymes de l'ODH spécifiques au muscle et au cerveau chez les mollusques céphalopodes.

[Traduit par le journal]

Introduction

The tissues of many marine invertebrates display activities of one or more imino acid dehydrogenases. Enzymes of this group, which catalyze the reductive condensation between an alpha–keto acid (generally pyruvic acid *in vivo*) and an amino acid, often replace or coexist with lactate dehydrogenase (LDH) and perform functions analogous to that of LDH in maintenance of cytoplasmic redox balance. Octopine dehydrogenase (ODH), which utilizes L-arginine as its amino acid substrate, is widely distributed amongst marine molluscs and other groups (Regnouf and Thoai 1970; Gade 1980) and has been characterized from several sources (Thoai *et al.* 1969; Fields *et al.* 1976; Storey and Storey 1979*a*). Amongst cephalopod molluscs, ODH occurs in tissue specific isozymic forms. The muscle and brain isozymes display kinetic properties analogous to those of M_4 and H_4 LDH, respectively, (Storey and Storey 1979*a*) and appear to be involved in tissue specific aspects of octopine metabolism *in vivo* (Storey and

0008-4301/82/071568-05$01.00/0

Storey 1979*b*). Alanopine dehydrogenase (ADH), catalyzing the reaction:

pyruvate + L-alanine + NADH + H^+ $\rightleftharpoons$ *meso*-alanopine + NAD^+ + H_2O

has only recently been identified (Fields 1976). The enzyme occurs amongst various marine invertebrate groups including bivalve and gastropod molluscs, polychaetes, sea anemones, and sponges (Dando *et al.* 1981; Barrett and Butterworth 1981; K. B. Storey, unpublished data). Purified muscle ADH from the oyster *Crassostrea gigas* (Fields and Hochachka 1981) and from the periwinkle *Littorina littorea* (Plaxton and Storey 1982) has been characterized.

In the present study we turned our attention to the question of the occurrence of tissue specific isozymic forms of ADH. The channeled whelk *Busycotypus* (formerly *Busycon*) *canaliculatum* is a large gastropod with well defined tissues. Three cytosolic dehydrogenases, ADH, ODH, and LDH, occur with distinct tissue specific relative activity profiles. Kinetic data, as well as isoelectrofocusing, chromatofocusing, and electrophoretic analyses, show the presence of tissue specific isozymic forms of ADH, the first demonstration of isozymes of this enzyme. Three forms, specific for muscle tissue, hepatopancreas and kidney–gill, occur.

Materials and methods

Chemical and animals

Ampholines were obtained from LKB Products, chromatofocusing materials were from Pharmacia Fine Chemicals, *meso*-alanopine was synthesized by the method of Abderhalden and Haase (1931), and oxamate-Sepharose affinity gel was supplied by Dr. T. Moon. All other biochemicals were purchased from Sigma Chemical Co. Specimens of the whelk *Busycotypus canaliculatum* were obtained from Woods Hole Marine Biological Laboratory, MA, and kept in recirculating, aerated seawater at 12°C without feeding until use.

Tissue preparation and enzyme assay

Tissues were excised from freshly killed whelks, blotted, weighed, and homogenized (1:5 w/v) in ice-cold 50 m*M* imidazole buffer, pH 7.5, containing 15 m*M* 2-mercaptoethanol using a Polytron homogenizer. For kidney and hepatopancreas, 3 m*M* PMSF (phenylmethylsulfonyl fluoride), a protease inhibitor, was included in the homogenizing buffer. 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 20 m*M* imidazole buffer, pH 7.5, containing 15 m*M* 2-mercaptoethanol to free the preparations of endogenous amino acids. Enzyme activities were stable to dialysis.

Activities of enzymes in the dialyzed preparations were measured by following NADH utilization at 340 nm using a Gilford recording spectrophotometer. Standard assay conditions were 50 m*M* imidazole buffer (pH 7.5), 2.0 m*M* pyruvate, and 0.15 m*M* NADH for LDH, with additions of 4.0 m*M* L-arginine for ODH and 50 m*M* L-alanine or 300 m*M* glycine for the alanine versus glycine dependent ADH activities. For tissues containing LDH, an LDH blank was subtracted from the ADH or ODH activity.

Michaelis constants (apparent K_m's) for L-alanine and glycine were determined from Hanes plots, $[S]/v$ versus $[S]$, using constant, saturating levels of pyruvate and NADH. For kinetic studies of ADH in tissues containing LDH activity, the dialyzed supernatant was first passed through an oxamate-Sepharose column to remove the LDH. A column (0.7 × 10 cm) was packed with 2 mL oxamate-Sepharose equilibrated in 10 m*M* imidazole buffer, pH 7.5, containing 10 m*M* 2-mercaptoethanol and 0.4 m*M* NADH. Under these conditions, LDH was bound to the column while ADH was eluted, with the same buffer, in a single peak with a yield of 70–80%.

Isoelectrofocusing

Isofocusing was performed by the method of Vesterberg (1971) using an LKB column (110 mL) and LKB ampholines, pH 4 to 6 for hepatopancreas, kidney, and gill preparations and pH 5 to 8 for all other tissues. Preliminary runs using a pH 3.5 to 10 gradient were used for all tissues to establish the best narrow range gradient for use. Enzyme preparations were run at 450 V for 16 h at 4°C. The column was then drained and 1-mL fractions were collected and assayed for ADH, ODH, and LDH activities.

Chromatofocusing

Enzyme preparations were layered onto a column (0.7 × 50 cm) of PBE 94 chromatofocusing exchanger preequilibrated in 25 m*M* imidazole buffer, pH 7.3, containing 10 m*M* 2-mercaptoethanol. The column was eluted at 17 mL/h with 200 mL Polybuffer 74 (a 1:8 dilution of stock), adjusted to pH 4.0.

Polyacrylamide gel electrophoresis

Electrophoresis of dialyzed enzyme preparations using 7.5% slab gels was carried out at 4°C with 50 mA constant current for 4 h with Tris–glycine (46:35 m*M*), pH 7.3, as the electrode buffer and bromophenol blue as the tracker dye. To detect ADH activity, gels were incubated in the dark at room temperature for 30 min with a mixture of 50 m*M* Tris–HCl buffer (pH 8.4; 24 mL), 50 m*M* *meso*-alanopine (6 mL), 10 mg/mL NAD^+ (6 mL), and 10 mg/mL nitro blue tetrazolium (2 mL), followed by visualization of the bands by the addition of 2 mL 5 mg/mL phenazine methosulfate. Control gels, omitting *meso*-alanopine, showed no stained bands.

Results

Enzyme distribution and activities

Table 1 shows the activities of LDH, ODH, and ADH in seven tissues of *B. canaliculatum*. ADH and ODH were present in all tissues tested; highest activities of both enzymes were found in muscle tissues although ADH occured in substantial activities in all of the soft tissues (hepatopancreas, gill, kidney) as well. In all tissues except proboscis muscle, ADH activity exceeded that of ODH. LDH activity was detected in three muscle

TABLE 1. Distribution and activities of lactate dehydrogenase, octopine dehydrogenase, and alanopine dehydrogenase in the tissues of the whelk *B. canaliculatum*

	Enzyme activity (μmol NADH utilized·min^{-1}·g wet weight^{-1})				
			ADH		
Tissue	LDH	ODH	V_{ala}	V_{gly}	V_{ala}/V_{gly}
Hepatopancreas	—	1.9±0.52	26.4±2.23	11.9±0.70	2.2
Gill	—	1.3±0.65	9.6±0.47	2.2±0.25	4.3
Kidney	—	1.8±0.48	6.5±0.70	1.4±0.15	4.6
Mantle	—	7.2±0.33	17.8±0.93	4.8±0.31	3.7
Ventricle	28.9±2.58	53.1±5.47	83.7±7.39	23.3±2.41	3.6
Foot muscle	0.4±0.13	40.6±2.85	47.2±2.01	12.7±0.53	3.7
Proboscis muscle	22.5±0.82	150.6±12.4	74.5±1.35	21.4±0.98	3.5

NOTE: Enzyme activities were measured under standard assay conditions as described in Materials and methods. Values are the means ± SEM of determinations on at least three individual preparations of each tissue.

tissues only with substantial activities of the enzyme found in ventricle and proboscis muscle, two "aerobic" muscle types.

Busycotypus canaliculatum ADH, like ADH from other sources, utilizes glycine as an alternate amino acid substrate. Table 1 shows the V_{ala} versus V_{gly} for ADH from the seven tissues. The activity ratios (V_{ala}/V_{gly}) fall into three groups. The ratio is highest (4.3 to 4.7) in gill and kidney, lower in muscle tissues (3.5 to 3.7), and lowest (2.2) in hepatopancreas. This suggests three kinetically distinct forms of ADH in the whelk.

Enzyme kinetics

Table 2 shows the apparent K_m's for alanine and glycine for ADH from the seven tissues. Again, the enzymes can be grouped into three kinetically distinct forms, a muscle-type enzyme, a gill–kidney form, and a hepatopancreas form. Apparent K_m's for both amino acids were lowest for the hepatopancreas enzyme. The apparent K_m for L-alanine was significantly higher in muscle tissues than in hepatopancreas and was higher still in gill and kidney. Kinetic differences between the tissue ADH forms were even more strongly evident when comparing the apparent K_m's for glycine, the $K_{m(gly)}$ of the muscle and gill–kidney forms being two- and four-fold higher, respectively, than the $K_{m(gly)}$ of hepatopancreas ADH. The very high apparent K_m's for glycine found for *B. canaliculatum* ADH's suggest that the enzyme from this species, like that of another gastropod, *L. littorea* (Plaxton and Storey 1982), but unlike ADH from bivalve molluscs (Fields and Hochachka 1981), is highly specific for L-alanine as the physiological substrate *in vivo*.

TABLE 2. Apparent K_m's for L-alanine and glycine of alanopine dehydrogenase from tissues of the whelk *B. canaliculatum*

	Apparent K_m		Ratio
Tissue	L-Alanine	Glycine	$K_{m(gly)}/K_{m(ala)}$
Hepatopancreas	8.84±0.03	619±55	70
Gill	13.12±0.65	2542±230	194
Kidney	13.35±0.26	2518±209	189
Mantle	10.87±0.58	1367±84	126
Ventricle	10.64±0.40	1412±115	133
Foot muscle	10.31±0.10	1395±122	135
Proboscis muscle	10.91±0.23	1443±91	132

NOTE: Assays were performed at 24°C with 50 m*M* imidazole buffer (pH 7.5), 2.0 m*M* pyruvate, and 0.15 m*M* NADH. Values are the means ± SEM of determinations on $n = 3$ enzyme preparations of each tissue. Based on kinetic properties, three groups of ADH enzymes are apparent: (1) hepatopancreas; (2) gill and kidney; and (3) mantle, ventricle, foot, and proboscis muscle. Apparent K_m's for both alanine and glycine are significantly different (using Student's *t*-test), $P < 0.05$, between the three groups but are not statistically different within each group.

Isofocusing, chromatofocusing, and electrophoresis

In addition to their kinetic differences, the tissue specific forms of ADH were distinctly separable by three techniques: isoelectric focusing, chromatofocusing, and polyacrylamide gel electrophoresis. Each technique showed three distinct forms of ADH present in the tissues of *B. canaliculatum*, specific for muscle tissue, gill–kidney, and hepatopancreas.

Table 3 shows the isoelectric points of ADH from the seven tissues of the whelk and indicates three isozymic forms, differing in pI, corresponding to hepatopancreas, gill and kidney, and the four muscular tissues. Parenthetically, isofocusing also revealed tissue specific isozymes of ODH.

Polyacrylamide gel electrophoresis showed similar results. ADH migrated cathodally under the conditions used. Gill and kidney each showed a single, fast-migrating band of ADH while muscle tissues had two bands of activity, a major slower moving band and a very minor band corresponding to the gill–kidney enzyme. This minor band was evident in the isoelectro-

TABLE 3. Isoelectric points of alanopine dehydrogenase from seven tissues of the whelk *B. canaliculatum*

Tissue	pI
Hepatopancreas	5.69±0.05
Gill	5.58±0.04
Kidney	5.57±0.03
Mantle	5.93±0.01
Ventricle	5.95±0.02
Foot muscle	5.91±0.01
Proboscis muscle	5.94±0.04

NOTE: Values are means ± SEM of $n = 3$ preparations of each tissue. pI's of hepatopancreas and gill–kidney are significantly different, $P < 0.1$, and are each statistically different from the pI's of the four muscular tissues, $P < 0.05$.

focusing as a small shoulder on the acidic side of the muscle ADH peak. A well-defined band of hepatopancreas ADH was not achieved, probably as a result of proteolysis.

Chromatofocusing resolved the major and minor ADH peaks in muscle with a ratio of activity in the two peaks of 8:1. The major peak, eluting at pH 6.3, had a K_m for glycine of 1250 m*M* while the minor peak had a K_m for glycine of 2600 m*M*. The minor form of ADH in muscle tissue appears, therefore, to be identical with the gill–kidney isozyme by both electrophoretic and kinetic criteria. Chromatofocusing of gill and hepatopancreas ADH showed only a single peak of enzyme activity in each case, gill ADH eluting at pH 6.95 (elution pH in chromatofocusing is dependent upon enzyme pI but is not a quantitative measure of pI) and hepatopancreas ADH eluting at pH 6.15, with apparent K_m's for glycine of 2480 and 500 m*M*, respectively.

Discussion

Three tissue specific isozymes of alanopine dehydrogenase occur in the whelk identified by differences in their kinetic and electrophoretic properties. A muscle specific form occurred in ventricle, foot, and proboscis muscle and also in mantle. A second form was specific for kidney and gill, two very aerobic tissues. A third form was found in hepatopancreas. A similar distribution of ODH isozymes was found in the cephalopod mollusc *Sepia officinalis*; muscle, brain, and hepatopancreas specific isozymes were identified (Storey 1977). Soft tissues, such as gill and kidney, contained both brain and muscle specific isozymes in varying ratios (Storey 1977; Dando *et al.* 1981).

The occurrence of tissue specific isozymes of ADH implies a tissue specific metabolism of alanopine in the whelk, similar perhaps to the tissue specific metabolism of lactate in vertebrates or of octopine in cephalopods. The M and H forms of LDH in vertebrates appear to gear tissues for roles in lactate production versus lactate oxidation, respectively. H_4 LDH (occurring in heart and brain) displays kinetic properties, including substrate inhibiton at elevated levels of pyruvate and a low K_m for lactate, which appear to limit the enzyme's function in anaerobic glycolysis but enhance the oxidation of lactate as an aerobic substrate (Everse and Kaplan 1973). M_4 LDH, on the other hand, is kinetically well suited for catalyzing the rapid production of lactate during glycolytic muscle work. Similar kinetic differences characterize the muscle versus brain forms of ODH in cephalopods (Storey and Storey 1979*a*). Studies of tissue metabolism of lactate and octopine back up the roles suggested for LDH or ODH by enzyme studies. Both products are accumulated rapidly during muscle work but are not effectively oxidized in muscle. Instead the end products are released into the bloodstream to be cycled to tissues capable of utilizing them as substrates for aerobic oxidation or for gluconeogenesis (Everse and Kaplan 1973; Storey and Storey 1979*b*). A parallel situation may exist for the ADH–alanopine system in *B. canaliculatum*. Further kinetic studies of the purified isozymes of ADH, plus experiments to test the tissue specific metabolism of alanopine, are needed.

The tissue ditributions of ADH, ODH, and LDH in *B. canaliculatum* suggest differential functions for the three cytosolic dehydrogenases. ADH was present in quite high activities in all tissues of the whelk suggesting a function for ADH in all tissues. Such a function could be a role in redox balance during anaerobiosis, anoxia survival in these burrowing gastropods being associated with the accumulation of end products such as alanine, alanopine, and succinate. ODH, however, was much more strongly associated with muscle tissues suggesting that octopine, which couples the products of phosphagen (arginine phosphate) breakdown and glycogenolysis, may be the principal product of glycolytic muscle work. LDH was present in substantial activities in only two tissues, both of which perform sustained work, the ventricle in pumping blood and the proboscis muscle in fueling the long-term grinding action of the radular teeth needed to penetrate the shell or exoskeleton of prey. The presence of high activities of all three dehydrogenases in these two tissues shows that sustained work by these tissues is not always aerobic. A sustained demand for glycolytic energy production could result in a depletion of tissue arginine–arginine-P reserves and alanine pools, thereby halting glycolytic redox regulation by ODH or ADH and necessitating the action of LDH.

Acknowledgements

The authors thank Dr. T. W. Moon, Department of Biology, University of Ottawa for supplying the oxamate-Sepharose; Dr. P. R. Dando, Marine Biological Association of the United Kingdom for advice in the

synthesis of alanopine; and Miss C. O'Grady for technical assistance in the preparation of alanopine. Supported by an NSERC operating grant and by NRCC contract No. OSU80-00218 to K.B.S.

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