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SUBSTRATE SPECIFICITIES OF OCTOPINE DEHYDROGENASES FROM MARINE INVERTEBRATES

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Abstract—1. Amino acid, keto acid and imino acid substrate specificities of octopine dehydrogenase (ODH) from seven marine invertebrate sources were investigated.

2. Three groups of ODH enzymes were identified determined, largely, by their use of L-lysine as an alternative amino acid substrate.

3. The broadly specific ODH from the sea anemone, *Calliactes parasitica*, utilized L-arginine and L-lysine at equal rates showing a substrate site able to utilize both guanidino and non-guanidino amino acids. Keto acid specificity was also broad; apparent K_m's for pyruvate, oxaloacetate and α-ketobutyrate were similar. Both D-octopine and D-lysopine were oxidized by the enzyme.

4. ODH from 3 bivalves, *Mytilus edulis*, *Cerastoderma edule* and *Glycymeris glycymeris* and from the cephalopod, *Sepia officinalis* showed lower rates of enzyme activity with L-lysine or L-ornithine (3–79% of L-arginine activity), lower rates with alternative guanidino amino acids, L-homoarginine and L-canavanine, and higher apparent K_m's for alternative keto acids compared to the sea anemone enzyme. Mantle muscle ODH from *S. officinalis*, with its major physiological role in glycolytic energy production during burst swimming, showed the highest specificity for L-arginine of all enzymes examined.

5. ODH from the bivalve, *Arctica islandica*, showed no activity in the presence of non-guanidino amino acids.

6. The evolutionary development of the ODH enzyme appears to have led from a broadly specific imino acid dehydrogenase in sea anemones to enzymes increasingly specific for the substrates L-arginine and pyruvate only. This trend is correlated with an increasing importance of ODH in glycolytic redox balance in working muscle and an increased dependence on muscle arginine phosphate reserves for rapid energy generation in higher invertebrate groups.

INTRODUCTION

Octopine dehydrogenase (ODH) (EC 1.5.1.11), catalyzing the reaction

$$\text{pyruvate} + \text{L-arginine} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{D-octopine} + \text{NAD}^+ + \text{H}_2\text{O}$$

is widely distributed amongst many marine invertebrate phyla (Regnouf Thoai, 1970; Haas *et al.*, 1973; Zammit & Newsholme, 1976; Gade, 1980) where it plays a role analogous to that of lactate dehydrogenase as the terminal enzyme of anaerobic glycolysis. The accumulation of octopine as the end product of glycolytic muscle work has now been well established particularly amongst mollusc species (Grieshaber & Gade, 1976; Hochachka *et al.*, 1977; Storey & Storey, 1979a; Gade, 1980). Purified ODH has been characterized from a number of sources (Thoai *et al.*, 1969; Haas *et al.*, 1973; Fields *et al.*, 1976; Storey & Storey, 1979b; Walsh, 1981). Substrate specificity of the enzyme was first investigated by Thoai & Robin (1961) for *Pecten maximus* adductor muscle ODH. The enzyme was specific for guanidino amino acids only and could utilize oxaloacetate and α-ketobutyrate as alternative substrates to replace pyruvate. Similar specificities were noted for two other bivalve species, *Cerastoderma edule* (Gade & Grieshaber, 1976) and the freshwater *Anodonta cygnea* (Gade & Grieshaber, 1975) although in neither case was oxaloacetate utilized as a keto acid substrate. Haas *et al.* (1973) demonstrated, however, that ODH from the sipunculid, *Sipunculus nudus*, showed a significant activity (15% of the arginine activity) with the non-guanidino amino acid, lysine, while Walsh (1981) found that ODH from the sea anemone, *Metridium senile*, utilized arginine and lysine at nearly equal rates. Ellington (1979a) described an imino acid dehydrogenase with an even broader amino acid specificity. An enzyme preparation from the sea anemone, *Bunodosoma cavernata*, catalyzed, apparently by a single enzyme protein, the reductive condensation of pyruvate with L-arginine, L-lysine, L-alanine and glycine.

In the present study we have characterized the substrate specificities of ODH purified from seven marine invertebrate sources: the sea anemone, *Calliactes parasitica* (Couch), the bivalve molluscs, *Mytilus edulis* L., *Cerastoderma edule* (L.), *Glycymeris glycymeris* (L.) and *Arctica islandica* (L.) and the mantle muscle and brain isozymes from the cephalopod mol-

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lusc. *Sepia officinalis* (L.). The data suggest an evolutionary trend to narrow the substrate specificity of ODH amongst the higher invertebrate groups.

MATERIALS AND METHODS

Chemicals and animals

D-Octopine was purified from the mantle muscle of *Loligo* sp. by the procedure of Robin & Guillou (1977). L-Bromopropionic acid was synthesized immediately before use (Fu *et al.*, 1954) and used to synthesize D-lysopine by the method of Biemann *et al.* (1960). Sodium pyruvate and oxaloacetate were from Boehringer Mannheim while L-arginine, L-ornithine and L-lysine were the chromatographically homogeneous grade from BDH Chemicals. Other biochemicals were from Sigma Chemical Co.

All animals were collected by dredge or trawl from the waters near Plymouth, *M. edulis* and *C. edule* from beds in the Tamar estuary.

Enzyme purification

The buffers used were: buffer A, 5 mM triethanolamine pH 5.8; buffer B, 10 mM triethanolamine pH 6.4; buffer C, 25 mM imidazole pH 6.0; buffer D, 25 mM imidazole pH 7.2 (containing 2 mM phenylmethyl sulphonyl fluoride when homogenizing *C. parasitica* muscle); buffer E, 10 mM Tris pH 8.5; buffer F, 200 mM Tris pH 8.7 containing 200 mM NaCl; buffer G, 1 M Tris pH 8.7 containing 1 M NaCl; buffer H, 50 mM diethanolamine pH 9.0; buffer I, 100 mM diethanolamine pH 9.0; buffer J, 500 mM diethanolamine pH 9.0. All buffers contained 2 mM 2-mercaptoethanol and 1 mM disodium EDTA and were adjusted to pH with HCl.

Fresh tissues were homogenized in 2–4 vol (w/v) of ice-cold buffer using a Polytron homogenizer. Buffer A was used for *A. islandica* foot and *S. officinalis* mantle muscle, buffer B for *M. edulis* foot, buffer C for *G. glycymeris* foot, buffer D for *C. parasitica* pedal plus side wall muscle and *S. officinalis* brain and buffer E for *C. edule* foot plus adductor muscle. Homogenates were centrifuged for 45 min at 30,000 ***g*** at 2 C and supernatants were removed and dialyzed against the same buffers with 4 changes over 2 hr. Dialyzed supernatants were filtered through a 1.0 μm pore size glass fibre filter and then loaded onto a column (1.5 × 30 cm) of Procion Red HE 3B-agarose (Amicon Corp.) pre-equilibrated in the appropriate homogenizing buffer. The column was then washed with buffer until $A_{280\,nm}$ dropped to near zero. With the exception of the *C. edule* enzyme, all ODH's bound to the column. *C. edule* ODH was retarded on the column and eluted after the major protein fractions. The column binding *M. edulis* ODH was further washed with buffer E while the one binding *A. islandica* ODH was washed with buffer F. ODH's were then eluted from the column using linear pH or salt gradients into buffer E for *S. officinalis* mantle muscle ODH, buffer G for *M. edulis* and *A. islandica* ODH, buffer H for *G. glycymeris* ODH, buffer I for *C. parasitica* ODH and buffer J for *S. officinalis* brain ODH. After this stage, the *M. edulis* preparation still contained malate dehydrogenase and the *A. islandica* preparation contained malate dehydrogenase and strombine dehydrogenase. The *M. edulis* preparation was dialyzed against buffer E and then passed through a column (1 × 13 cm) of Procion Red agarose equilibrated in the same buffer. Under these conditions, malate dehydrogenase, but not ODH, was retained on the column. The *A. islandica* preparation was dialyzed against buffer B and passed through a column (1 × 12 cm) of Cibacron Blue F3G-A-Sepharose (Pharmacia Fine Chemicals) pre-equilibrated in the same buffer. ODH was not retained by the column. The step was repeated, the resulting preparation showing only trace amounts of malate dehydrogenase and strombine dehydrogenase.

Peak fractions from the triazine-dye columns were pooled and concentrated against solid polyethylene glycol (M.W. 6000) to a volume of 1–2 ml. The preparations were then chromatographed on a column (2.5 × 98 cm) of Sephadex G-150 (Pharmacia Fine Chemicals) eluted with buffer D. Peak fractions containing ODH activity were pooled and used for kinetic studies.

Most of the resulting ODH preparations contained no detectable D- or L-lactate dehydrogenase, malate dehydrogenase, alanopine dehydrogenase or strombine dehydrogenase activities. ODH from *S. officinalis* brain and from *C. parasitica* contained up to 1% malate dehydrogenase activity at saturating substrate concentrations while *M. edulis* ODH contained 1% lactate dehydrogenase.

Enzyme assay

Standard assay conditions for ODH, in a final volume of 1 ml, were: in the direction of octopine production, 100 mM imidazole–HCl buffer, pH 7.0, 10 mM L-arginine, 1 mM pyruvate and 0.1 mM NADH and for octopine oxidation, 100 mM diethanolamine–HCl buffer, pH 9.0, 10 mM D-octopine and 0.2 mM NAD. All assays were performed at 20 C using a Pye Unicam recording spectrophotometer or a Perkin Elmer 3000 spectrophotofluorometer.

Electrophoresis

Both crude tissue extracts and purified enzymes were examined by starch gel electrophoresis at pH 8.7 and 7.7. Substrate specificities were examined by differential staining (Dando *et al.*, 1981). ODH was detected on gels using a 1% (w/v) agar overlay containing 20 mM D-octopine, 1 mM NAD, 0.3 mM MTT tetrazolium, 7 μM Meldola's blue and 50 mM diethanolamine pH 9.0. Amino acid or keto acid specificities of ODH were detected by staining with an overlay containing 2 mM keto acid, 20 mM amino acid, 0.1 mM NADH and 50 mM triethanolamine pH 7.0 with visualization under ultraviolet light.

RESULTS

Tissue ODH activities and purifications

Table 1 shows the activities of ODH in the six species studied. ODH activity in mantle and brain of *S. officinalis* is similar to that reported by Storey (1977). ODH was the major glycolytic dehydrogenase present in four species; the activity of strombine dehydrogenase was approx. four fold higher in *A. islandica* foot muscle while alanopine dehydrogenase and ODH showed similar activities in *C. parasitica*. Low activities of lactate dehydrogenase were found in all species and alanopine dehydrogenase was also present in *C. edule* and *M. edulis*. The techniques used resulted in purifications of 30–78 fold producing partially pure enzyme preparations which were free of contaminating enzyme activities which would interfere with the substrate specificity studies.

Substrate specificities

Table 2 outlines the substrate specificities of the seven ODH preparations with data for *P. maximus* and *S. nudus* (Haas *et al.*, 1973), *A. cygnea* (Gade & Grieshaber, 1975) and *M. senile* (Walsh, 1981) ODH included for comparison. The structural relationships between L-arginine and alternative amino acid substrates of ODH are shown in Fig. 1. ODH from all sources utilized both L-homoarginine (for *A. cygnea* the compound was not tested) and L-canavanine, two guanidino amino acids. Only eight of the eleven

Table 1. Tissue enzyme activities and purification of octopine dehydrogenase from marine invertebrates

Species	Tissue	Enzyme activity U/g wet weight ODH	LDH	ADH/SDH	Protein mg/g wet weight	Fold Purification	Final specific activity U/mg protein
Sepia officinalis	mantle muscle	96.5	0.3	0.0	37.1	40	102.4
	brain	30.0	4.0	0.0	75.1	78	31.2
Mytilus edulis	foot	12.2	0.8	1.6	28.0	70	30.3
Cerastoderma edule	adductor + foot	24.3	7.1	2.1	51.6	70	32.9
Glycymeris glycymeris	foot	66.7	1.1	0.0	44.4	45	67.5
Arctica islandica	foot	8.4	0.2	34.4	36.6	78	18.0
Calliactes parasitica	pedal + side wall muscle	1.2	—	0.9	7.1	30	5.0

Enzyme activities were measured in the dialyzed crude supernatants with assay conditions: 100 mM imidazole buffer, pH 7.0, 0.1 mM NADH and 1 mM pyruvate for lactate dehydrogenase with 10 mM L-arginine, 150 mM L-alanine or 150 mM glycine added for octopine dehydrogenase, alanopine dehydrogenase or strombine dehydrogenase, respectively. One unit of enzyme activity is defined as the amount of enzyme using 1 μmol of NADH per min at 23°C. Protein was measured by the method of Bradford (1976) using the Bio-Rad Laboratories reagent. Of species displaying ADH or SDH, SDH is present in *A. islandica* while ADH is present in the other species.

enzymes, however, showed significant enzyme activities with L-lysine and/or L-ornithine. None of the ODH preparations were active with agmatine or L-citrulline as a substrate nor were the enzymes prepared in the present study active with L-alanine, glycine, L-histidine or L-glutamic acid. For keto acid specificity, all enzymes used α-ketobutyrate and all except *A. cygnea* ODH used oxaloacetate. None of the ODH's was active with α-ketoglutarate. Most of the ODH preparations showed similar activities with either oxaloacetate or α-ketobutyrate except for *A. cygnea* ODH and the *M. edulis* enzyme which showed a much higher activity with oxaloacetate than with α-ketobutyrate. For *C. edule*, our findings run contrary to those of Gade & Grieshaber (1976) who reported that the cockle enzymes did not use either oxaloacetate or L-ornithine as alternative substrates. ODH's showing significant activity with L-lysine as a substrate also oxidized D-lysopine while those utilizing L-ornithine could oxidize D-octopinic acid. However, oxidation of D-lysopine by *S. officinalis* mantle muscle ODH or oxidation of D-octopinic acid by *M. edulis* or *C. parasitica* ODH's was not detected.

Electrophoresis confirmed that the enzyme activities seen with alternative substrates were, in fact, catalyzed by ODH. For each species examined in the present study the zone staining on starch gels with octopine plus NAD was identical with the zone staining with NADH + pyruvate + alternative amino acid or NADH + L-arginine + alternative keto acid.

The eleven ODH preparations can be loosely divided into three groups based upon amino acid specificities, in particular specificities for L-arginine and L-lysine. These two amino acids are probably the only potential substrates of the enzyme *in vivo*. In group I are the two sea anemone ODH's. These enzymes have essentially equal activities with either L-arginine or L-lysine as the amino acid substrate. This indicates that the enzyme substrate site of these ODH's does not require the presence of a guanidino group for

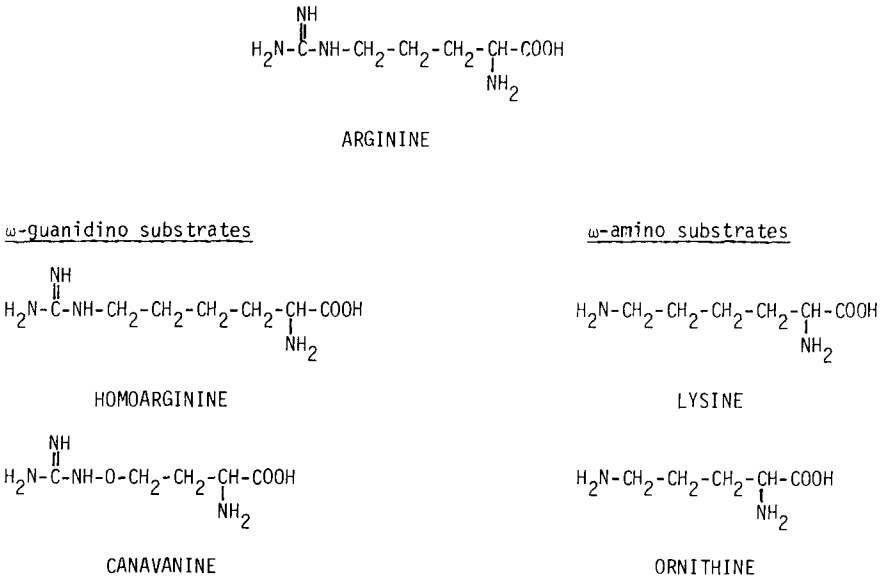

Fig. 1. Structural relationships between amino acid substrates of octopine dehydrogenase.

Table 2. Substrate specificities of octopine dehydrogenase from various marine invertebrate sources

Substrate	Relative velocity											
	S. officinalis										*M. senile*‡	
	Mantle	Brain	*M. edulis*	*C. edule*	*G. glycymeris*	*A. islandica*	*P. maximus**	*A. cygnea*†	*S. nudus**	*C. parasitica*	ss	ff
L-Arginine	100	100	100	100	100	100	100	100	100	100	100	100
L-Lysine	1.6	6.8	78.8	0	28.0	0	0	0	15	106.7	91.4	92.6
L-Ornithine	0	5.7	1.5	8.0	1.0	0	0	0	3	5.3	ND	ND
L-Homoarginine	13.0	11.8	11.3	46.2	55.2	169.4	7	ND	17	343.8	58.6	66.1
L-Canavanine	15.0	35.0	56.4	22.6	39.0	34.8	5	33	11	22.5	57.6	92.6
Pyruvate	100	100	100	100	100	100	100	100	100	100	100	100
Oxaloacetate	57.6	62.7	51.5	33.8	43.4	66.3	53	0	21	35.6	14.4	18.1
α-Ketobutyrate	32.0	44.6	8.6	43.1	47.4	82.6	34	40	15	25.4	20.6	29.0
D-Octopine	100	100	100	100	100	100	100	100	100	100	100	100
D-Lysopine	0	17.8	24.6	0	43.9	0	ND	ND	ND	85.5	ND	ND
D-Octopinic acid	0	1.0	0	1.0	0.6	0	ND	ND	ND	0	ND	ND

Amino acid and keto acid specificities are expressed relative to the velocity with pyruvate + L-arginine which is set at 100; imino acid specificities are relative to D-octopine. Enzyme activities for each substrate were determined at constant substrate concentrations: 10 mM amino or imino acid, 1 mM keto acid, 0.1 mM NADH or 0.2 NAD with 100 mM imidazole buffer, pH 7.0 in the forward direction and 100 mM diethanolamine buffer pH 9.0 in the reverse direction. Muscle tissues used are listed in Table 1. Data are the means of determinations on 3 preparations of enzyme with variability ±10%.

* Data from Haas *et al.* (1973) for *P. maximus* adductor muscle and *S. nudus* muscle ODH.

† Data from Gade & Grieshaber (1975) for adductor muscle ODH.

‡ Data from Walsh (1981) for ODH from whole animal. Data for the two homozygous phenotypes, ss and ff, are shown.

ND = Not determined.

recognition of the amino acid substrate. The enzymes do show a preference for chain length of the amino acid; for *C. parasitica* L-homoarginine gave the highest enzyme activity while L-ornithine gave the lowest activity. In the oxidative direction, *C. parasitica* ODH utilized both D-octopine and D-lysopine with comparable facility. The high lysine/lysopine activities of the sea anemone ODH's suggest that lysine, as well as arginine, may be a physiologically important substrate of ODH *in vivo*.

Group II ODH's, including the enzymes from *M. edulis*, *G. glycymeris*, *S. nudus* and *S. officinalis*, showed significant enzyme activity with lysine as an alternative substrate but not the high rates seen with the sea anemone enzymes. L-Lysine activities ranged from 1.6 to 79% of the activities with L-arginine. The enzymes, with the exception of *S. officinalis* mantle muscle ODH, also showed oxidation of D-lysopine at 18–44% of the rate with D-octopine. *C. edule* ODH, which is also included in this group, did not utilize L-lysine but showed the highest activities of all the enzymes with L-ornithine and D-octopinic acid. ODH's of this second group, then, show a decreased activity with non-guanidino amino acids with the two ODH isozymes from *S. officinalis* showing the narrowest substrate specificity. Mantle muscle ODH not only shows the lowest activity with L-lysine (only 1.6% of L-arginine activity) but also has the lowest activities with L-homoarginine and L-canavanine of group II enzymes.

Group III enzymes are the highly arginine-specific ODH's. *A. islandica*, *P. maximus* and *A. cygnea* ODH were inactive with non-guanidino amino acids. For *P. maximus* ODH, the specificity was narrowed further, this enzyme showing the lowest activities of all the enzymes with L-homoarginine and L-canavanine.

Enzyme affinity constants

Table 3 shows the apparent K_m's for substrates of the seven partially purified ODH preparations. The apparent K_m's for L-arginine varied from 1.3 mM (*M. edulis*) to 7.7 mM (*A. islandica*), differences which may reflect *in vivo* levels of the arginine/arginine phosphate pool in the different species. In the presence of alternative keto acids the apparent K_m for arginine increased for many of the molluscan ODH's. Of enzymes which utilized L-lysine as a substrate, apparent K_m's for this amino acid were significantly higher than the apparent K_m for L-arginine, with the exception of the *S. officinalis* enzymes. The apparent K_m's for L-ornithine of the *S. officinalis* brain and *C. edule* enzymes were 25 and 28 mM, respectively, about 10 fold higher than the K_m's for L-arginine. L-Ornithine is unlikely, therefore, to be an important physiological substrate for ODH. However, the apparent K_m for L-ornithine of *C. parasitica* ODH was similar to that for arginine and lysine providing a further indication of a rather flexible amino acid substrate site for the sea anemone enzyme.

The apparent K_m's for pyruvate varied from 0.6 to 2.5 mM, lowest and highest K_m's again belonging to *M. edulis* and *A. islandica*, respectively. The apparent K_m's for oxaloacetate were similar to the K_m's for pyruvate for ODH from four species but were markedly higher for the *S. officinalis* and *A. islandica* enzymes. The apparent K_m for α-ketobutyrate was 3–10 fold higher than K_m pyruvate for all species suggesting that this compound is unlikely to be a physiologically important substrate of ODH.

The apparent K_m for D-octopine was lowest for the *S. officinalis* brain isozyme of ODH, an enzyme which is kinetically suited for the oxidation of octopine as an aerobic fuel for brain (Storey & Storey, 1979b). The apparent K_m's for octopine of the bivalve ODH's were all significantly higher. The apparent K_m's for D-lysopine were in all cases higher than the corresponding K_m's for D-octopine, even for sea anemone ODH, suggesting that octopine is the preferred substrate of the enzyme.

The apparent K_m's for NADH varied from 2.4 to 18 μM and were lowest amongst many of the bivalve ODH's. The apparent K_m for NAD ranged from 15 to 100 μM for most species but was very high (3.35 mM) for *A. islandica* ODH. The K_m was reduced by more than 20 fold at pH 7.5, however (P. R. Dando, unpublished work). None of the enzymes utilized NADP or NADPH as coenzymes.

DISCUSSION

The substrate specificities for ODH suggest some of the requirements for active site binding of the amino acid and keto acid substrates of the enzyme. For amino acid binding an alpha carboxyl group is required (agmatine was not a substrate) and a carbon chain length of C_5 or C_6 is needed. The R side chain group of the amino acid must terminate in a guanidino group or, for ODH from some sources, in an amino group but a terminal carbamyl group (L-citrulline) renders a substrate inactive. For keto acid binding C_3 or C_4 keto acids can be utilized but C_5 acids (α-ketoglutarate) are not. Despite the close structural similarity between C_3 pyruvate and C_4 α-ketobutyrate, the C_4 dicarboxylic acid, oxaloacetate, appears to be the preferred alternative C_4 substrate showing higher enzyme velocities (at 1 mM keto acid) for most ODH's and lower apparent K_m's than those for α-ketobutyrate. This could be a result of enzyme modification to allow the use of oxaloacetate as a possible alternative physiological substrate in some circumstances. Most of these findings were first demonstrated by the work of Thoai & Robin (1961) and Haas *et al.* (1973) for *P. maximus* and *S. nudus* ODH but the present study suggests that they apply generally to ODH from many invertebrate sources.

The data in the present study provide a picture of the evolutionary and phylogenetic development of octopine dehydrogenase. The major evolutionary modification of the enzyme has been in its amino acid specificity, first in the elimination of the ability to utilize non-guanidino amino acids and secondly in the narrowing of the guanidino amino acid specificity in some species. Accompanying this has been a lesser trend narrowing the keto acid specificity as shown by the increasingly greater apparent K_m's for alternative keto acids relative to $K_{m(\text{pyruvate})}$ amongst some mollusc species.

The broadly specific ODH of sea anemones (Group I ODH) uses both L-arginine and L-lysine at similar rates and probably represents the ancestral ODH enzyme. The enzyme may have evolved from a more

Table 3. Kinetic constants for octopine dehydrogenase from various marine invertebrates

Substrate	Apparent K_m (mM)						
	S. officinalis		*M. edulis*	*C. edule*	*G. glycymeris*	*A. islandica*	*C. parasitica*
	Mantle	Brain					
L-Arginine							
at 1 mM pyr	5.5 ± 0.61	2.0 ± 0.18	1.3 ± 0.02	3.0 ± 0.40	1.8 ± 0.30	7.7 ± 1.28	2.2 ± 0.18
at 1 mM OXA	8.0 ± 1.10	12.0 ± 1.50	5.0 ± 1.69	4.0 ± 0.80	2.5 ± 0.42	8.0 ± 0.44	2.1 ± 0.30
at 1 mM α-KB	6.2 ± 0.57	5.3 ± 0.70	3.7 ± 0.51	3.8 ± 0.30	2.2 ± 0.30	30.2 ± 3.00	2.0 ± 0.22
L-Lysine	6.1 ± 0.55	2.5 ± 0.20	3.3 ± 0.20	NA	3.7 ± 0.30	NA	4.8 ± 0.33
L-Ornithine	NA	25.0 ± 1.00	ND	28.0 ± 1.0	ND	NA	1.6 ± 0.30
Pyruvate	1.4 ± 0.10	0.6 ± 0.09	0.6 ± 0.03	0.8 ± 0.06	0.8 ± 0.04	2.5 ± 0.01	1.3 ± 0.08
Oxaloacetate	2.6 ± 0.30	1.6 ± 0.30	0.6 ± 0.14	0.9 ± 0.11	1.0 ± 0.11	9.0 ± 0.44	1.4 ± 0.10
α-Ketobutyrate	7.8 ± 0.80	6.1 ± 0.05	7.3 ± 0.94	6.2 ± 0.17	8.0 ± 0.71	16.9 ± 1.12	3.8 ± 0.41
D-Octopine	0.9 ± 0.06	0.1 ± 0.01	3.1 ± 0.23	5.2 ± 0.60	1.5 ± 0.10	22.1 ± 1.36	0.8 ± 0.04
D-Lysosine	NA	2.6 ± 0.30	6.4 ± 0.30	NA	3.2 ± 0.40	NA	3.1 ± 0.34
NADH (μM)	18.0 ± 1.9	15.0 ± 1.2	5.8 ± 0.6	13.0 ± 1.4	2.4 ± 0.25	3.6 ± 0.26	12.0 ± 1.40
NAD (μM)	110 ± 17	48.0 ± 6.0	65.0 ± 10.0	110 ± 9.0	75.0 ± 6.0	3350 ± 290	15.0 ± 1.3

Assay conditions for determining apparent K_m's used constant co-substrate concentrations: 10 mM L-arginine, 1 mM keto acid, 0.1 mM NADH, 10 mM imino acid and 0.2 mM NAD in 100 mM imidazole buffer, pH 7.0 for the forward and 100 mM diethanolamine buffer, pH 9.0 for the reverse direction. Results are means ± S.E.M. for at least 3 determinations of each K_m.

NA = Not applicable, ND = Not determined.

broadly specific imino acid dehydrogenase capable of catalyzing the reductive condensation between pyruvate and a variety of amino acids. This enzyme, described by Ellington (1979a) could represent the primitive imino acid dehydrogenase from which other animal octopine, alanopine and strombine dehydrogenases have evolved. The role of ODH in sea anemones is still unclear. Two studies have failed to demonstrate appreciable accumulations of octopine during anoxia (Ellington, 1979b, 1980) while the role of ODH in muscle work in sea anemones has not been studied. Intracellular free arginine levels are very low in sea anemones averaging 0.24 μmol/g wet weight in two species while lysine levels are similar at 0.43 μmol/g (Severin *et al.*, 1972). If these concentrations are good estimates of amino acid levels in *C. parasitica*, then *in vivo* levels of these two amino acids are about 10 fold lower then the respective apparent K_m's of ODH for these substrates. Conditions under which appreciable octopine would be produced *in vivo* would, therefore, be difficult to imagine. However, it is likely that any ODH function in sea anemones would make use of both arginine and lysine as physiological substrates.

ODH's belonging to group II have a considerably decreased enzyme activity with L-lysine as an alternative substrate and also show a tendency towards decreased utilization of alternative guanidino amino acids. Overall, then, active site specificity for L-arginine and D-octopine has been strengthened. This alteration correlates well with an increased physiological importance of ODH and increased intracellular arginine and arginine phosphate pools amongst species of group II. Amongst these species (and also amongst group III species, except *A. islandica*), ODH has become the major cytosolic dehydrogenase acting as the terminal enzyme of anaerobic glycolysis (Table 1). Arginine phosphate stores are considerable in these species, ranging from 3 to 6 μmol/g wet weight in *M. edulis* foot (Zurburg, 1981) to more than 30 μmol/g wet weight in *S. officinalis* mantle muscle (Storey & Storey, 1979a) and provide a high energy phosphagen store for use during "burst" muscular work. But while arginine and arginine phosphate levels have increased, particularly in muscle tissues, lysine levels remain low, often 10 fold lower than the arginine/arginine phosphate pool (Shumway *et al.*, 1977; Storey & Storey, 1978; Suyama & Kobayashi, 1980). Lysine is unlikely, therefore, to be a physiological substrate for ODH amongst these molluscan species.

Amongst cephalopod molluscs, ODH occurs in two tissue specific isozymes (Storey & Storey, 1979b; Gade, 1980; Dando *et al.*, 1981). The two enzymes function in different aspects of octopine metabolism *in vivo*; the muscle enzyme is geared for rapid octopine production during glycolytic muscle work while the brain isozyme appears to be poised to allow exogenous octopine to be utilized as an aerobic substrate for brain metabolism (Storey & Storey, 1979b). In line with these functions, brain ODH shows the lowest apparent K_m for octopine of any of the enzymes examined. Muscle ODH, for its key role in maintaining cytoplasmic redox balance during muscle work by coupling the products of glycolysis and arginine phosphate breakdown, has developed a very high specificity for L-arginine as its preferred substrate. The lysine activity of the enzyme is very low and activities with alternative guanidino amino acids are also amongst the lowest seen in the species examined suggesting that enzyme substrate affinity has been finely tuned to fit enzyme function.

Octopine dehydrogenases belonging to group III are completely specific for guanidino amino acids, activity with lysine or ornithine having been lost. ODH from *P. maximus* has, in addition, greatly narrowed its guanidino amino acid specificity to become highly specific for only L-arginine. ODH in pectinids, like the enzyme in cephalopods, has a highly developed physiological role. The enzyme occurs in high activities [45 U/g wet weight in phasic adductor of *P. maximus* (K. B. Storey and P. R. Dando, unpublished data)] in adductor muscle and octopine is produced in muscle during the recovery period following arginine phosphate fuelled contraction (Gade *et al.*, 1978; Livingstone *et al.*, 1981). In the subtidal clam, *A. islandica*, ODH is not the major dehydrogenase activity; strombine dehydrogenase occurs at four fold higher levels. Although highly specific for guanidino amino acids as substrates, the enzyme shows the lowest affinities (highest apparent K_m's) for substrates (arginine, pyruvate, octopine and NAD) of any of the enzymes examined. These low affinities may restrict ODH function in favour of strombine dehydrogenase activity, strombine dehydrogenase forming the primary means of cytoplasmic redox balance. ODH may be called into play only during very strenuous muscle work when intracellular levels of both pyruvate and arginine (from arginine phosphate breakdown) are very high.

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