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Alanopine Dehydrogenase: Purification and Characterization of the Enzyme from *Littorina littorea* Foot Muscle

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Summary. 1. Alanopine dehydrogenase from the foot muscle of the common periwinkle, *Littorina littorea* was purified to homogeneity using a combination of ammonium sulphate fractionation, gel filtration and chromatofocusing.

2. The enzyme has a molecular weight of 42,200 ± 500 and is a monomer.

3. L-Alanine and pyruvate are the preferred substrates. Alternate amino acids (glycine > L-α-aminobutyrate > L-serine > L-cysteine) are used at rates less than 37% of enzyme activity with L-alanine. Alternate keto acids used include oxaloacetate, α-ketobutyrate and glyoxylate. The enzyme is specific for meso-alanopine in the reverse direction; D-strombine is not oxidized.

4. Apparent K_m's for both pyruvate and L-alanine decrease with increasing co-substrate (L-alanine or pyruvate) concentration or with decreasing pH.

5. Absolute K_m's for pyruvate and L-alanine are 0.17 ± 0.02 and 14.9 ± 0.85 mM at pH 6.5 rising to 0.26 ± 0.01 and 23.8 ± 0.52 mM at pH 7.5, respectively. Apparent K_m's for meso-alanopine are 6.5 mM at pH 6.5 and 50 mM at pH 8.5 while apparent K_m's for NADH (9 ± 0.1 μM) and NAD^+ (0.18 ± 0.03 mM) are pH independent.

6. Substrate inhibition by pyruvate (I_{50} = 8 mM) and L-alanine (I_{50} = 450–550 mM) occurs at saturating co-substrate levels while NAD^+ (K_i = 0.16 ± 0.012 mM) and meso-alanopine (K_i = 35 ± 0.4 mM) are product inhibitors of the forward reaction. ATP and ADP are competitive inhibitors with respect to NADH while L-lactate, D-strombine and succinate inhibit with respect to pyruvate and L-alanine.

7. The kinetic properties of alanopine dehydrogenase favour enzyme function in cytoplasmic redox balance during anoxia stress in this intertidal gastropod. In particular the effects of rising co-substrate levels (pyruvate and alanine are products of glycolysis) and decreasing pH (occurring during anoxia) on enzyme apparent K_m's for substrates would favour alanopine accumulation as a product of anaerobic glycolysis.

Introduction

Anaerobic energy production in vertebrate tissues depends upon the action of lactate dehydrogenase to maintain cytoplasmic redox balance, lactic acid accumulating as the product of carbohydrate fermentation. The tissues of many marine invertebrates, however, contain activities of one or more imino acid dehydrogenases:

pyruvate + amino acid + NADH + H^+ ⇌ imino acid + NAD^+ + H_2O, which functionally replace lactate dehydrogenase as the terminal enzyme of anaerobic glycolysis (Regnouf and Thoai 1970; Fields 1976; de Zwaan and Zurburg 1981; Dando et al. 1981). Octopine dehydrogenase, catalyzing the reductive condensation of pyruvate and L-arginine, has been well characterized (Thoai et al. 1969; Fields et al. 1976; Storey and Storey 1979a); amongst cephalopod molluscs, high speed swimming, supported by glycogenolysis and arginine phosphate breakdown, results in the accumulation of octopine as the end product of mantle muscle metabolism (Grieshaber and Gäde 1976; Storey and Storey 1979b; Baldwin and England 1980).

Alanopine dehydrogenase, catalyzing the reaction:

pyruvate + L-alanine + NADH + H^+ ⇌ meso-alanopine + NAD^+ + H_2O, was first identified in 1976 in the muscle tissues of the oyster, *Crassostrea gigas* (Fields 1976) while alanopine (2,2′-iminodi-

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propionic acid) and/or strombine (2-methyliminodiacetic acid) accumulate as end products of ^{14}C-glucose fermentation during anoxia in oyster ventricle (Collicutt and Hochachka 1977). Subsequently alanopine dehydrogenase and strombine dehydrogenase (utilizing glycine as the amino acid substrate) activities have been identified in the tissues of a variety of marine bivalve and gastropod molluscs as well as in sea anemones and other groups (de Zwaan and Zurburg 1981; Dando et al. 1981; Ellington 1979, 1981). Some kinetic properties of purified oyster adductor muscle alanopine dehydrogenase have recently been reported (Fields and Hochachka 1981); the enzyme has a broad amino acid specificity, utilizing L-alanine, glycine and several other amino acids at nearly equal rates and probably produces both alanopine and strombine in vivo.

In the present study we have examined the kinetic properties of alanopine dehydrogenase purified from foot muscle of the intertidal gastropod mollusc, *Littorina littorea*. The gastropod enzyme, unlike bivalve, alanopine dehydrogenase, is highly specific for L-alanine and meso-alanopine as substrates, a specificity which may link enzyme function in vivo to anaerobiosis, the production of alanopine coupling two products, pyruvate and L-alanine, of anaerobic glycolysis. Other kinetic characteristics of the enzyme including strong pH effects on enzyme affinity for pyruvate and alanine also appear designed to promote alanopine synthesis during anoxia.

Materials and Methods

Animals and Chemicals. Specimens of the common periwinkle, *Littorina littorea,* were obtained from local fish markets. Foot muscle was dissected out and stored at −80 °C until use. No loss of enzyme activity was seen during storage for periods of at least one month.

ATP, ADP, D- and L-lactate and NADH were purchased from Boehringer Mannheim Corp. while all other biochemicals were from Sigma Chemical Co., Sephadex G-100, PBE 94 chromatofocusing exchanger and Polybuffer 74 were obtained from Pharmacia Fine Chemicals, Ampholines (pH 4–6) were from LKB Products, and molecular weight standard proteins were from Sigma and Pharmacia.

All buffers used in the study were adjusted to the respective pH at 23 °C.

Enzyme Assay and Kinetic Studies. Alanopine dehydrogenase activity was assayed by monitoring NAD(H) utilization at 340 nm using a Gilford recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Standard assay conditions for alanopine dehydrogenase were: 50 mM imidazole buffer, pH 7.5, 1.3 mM pyruvate, 130 mM L-alanine and 0.1 mM NADH in the forward direction, and 50 mM Tris-HCl buffer, pH 9.2, 50 mM meso-alanopine and 2 mM NAD^+ in the reverse direction in a final volume of 1 ml. Assays were started by the addition of enzyme preparation. Standard assay conditions for lactate dehydrogenase were: 50 mM imidazole buffer, pH 7.5, 1.3 mM pyruvate and 0.1 mM NADH. One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol NADH per minute at 23 °C.

Apparent K_m values were determined from Hanes plots under conditions of constant, saturating concentrations of co-substrate(s) while absolute K_m values for L-alanine and pyruvate were derived from secondary plots using data from apparent K_m determinations at 3 subsaturating co-substrate concentrations (Cornish-Bowden 1979). Inhibitor constants (K_i) were determined from Dixon plots and the effect of inhibitors was evaluated using double reciprocal plots. I_{50}'s (inhibitor concentration producing 50% inhibition of enzyme activity) were determined by the method of Job et al. (1978). All kinetic parameters are the means of duplicate determinations on three separate preparations of the purified enzyme and are reproducible within at least ±10%. In most cases, results are expressed as means ± s.e.m.

Purification of Alanopine Dehydrogenase. Foot muscle was minced with scissors and homogenized (1:5 w/v) for 2 × 1 min in ice-cold 50 mM imidazole buffer, pH 7.5 containing 15 mM β-mercaptoethanol, using a Polytron PT 10–35 homogenizer. The homogenate was centrifuged at 27,000 × *g* for 20 min at 4 °C and the supernatant used as the source of crude enzyme.

The crude supernatant was brought to 2.95 M (60% saturation) with ammonium sulphate, stirred at room temperature for 30 min, and then centrifuged as above. The protein pellet was discarded and the resulting supernatant was then adjusted to 4.03 M (80% saturation) with ammonium sulphate and again stirred and centrifuged. The pellet was resuspended in 2–3 ml 20 mM imidazole buffer, pH 7.4 containing 10 mM β-mercaptoethanol and centrifuged to remove insoluble material. The partially purified enzyme solution was then layered onto a column (60 × 0.9 cm) of Sephadex G-100 equilibrated in 20 mM imidazole buffer, pH 7.4 containing 10 mM β-mercaptoethanol. The column was eluted at 20 ml/h using the same buffer and 1 ml fractions were collected. Peak fractions, containing alanopine dehydrogenase activity were pooled and layered onto a column (30 × 0.9 cm) of PBE 94 chromatofocusing exchanger pre-equilibrated in 20 mM imidazole buffer, pH 7.4 containing 10 mM β-mercaptoethanol. The column was eluted at 20 ml/h with 200 ml Polybuffer 74 (stock diluted 1:8 with distilled water) adjusted to pH 4.0 and 1 ml fractions were collected. Alanopine dehydrogenase activity eluted in a single sharp peak at pH 5.7 (±0.05, $n=3$) on the linear pH gradient. Peak fractions from chromatofocusing were pooled and applied to a second, larger G-100 column (90 × 1.5 cm) equilibrated in 50 mM imidazole buffer, pH 7.5 containing 10 mM β-mercaptoethanol and 0.04% sodium azide. The column was eluted with this same buffer and peak fractions, containing purified alanopine dehydrogenase, were combined and stored at 4 °C for use in kinetic studies. The purified enzyme was stable in this form, with no loss of activity or change in kinetic parameters, for at least 2 weeks.

Molecular Weight Determination. Molecular weight determinations were made on a column (90 × 1.5 cm) of Sephadex G-100 using 50 mM imidazole buffer, pH 7.5 containing 10 mM β-mercaptoethanol and 0.04% sodium azide as the equilibration/elution buffer. One ml fractions were collected and assayed for absorbance at 280 nm and alanopine dehydrogenase activity. The molecular weight of alanopine dehydrogenase was determined from a plot of K_{av} (partition coefficient) versus log M_r for the standard proteins: ribonuclease ($M_r=13{,}700$), chy-

motrypsinogen ($M_r = 25{,}000$), ovalbumin ($M_r = 45{,}000$) and bovine serum albumin ($M_r = 67{,}000$).

Electrophoresis. Polyacrylamide gel electrophoresis using 10% slab gels was carried out at 4 °C with 40 mA constant current for approximately 4 h. Tris/glycine (46 mM/35 mM), pH 8.0 (±1% SDS) was used as the electrode buffer with bromophenol blue as the tracker dye. Duplicate non-SDS gels were stained for protein using the silver stain of Switzer et al. (1979) or for alanopine dehydrogenase activity. To detect enzyme activity, gels were incubated in the dark at room temperature for 30 min in a mixture of 50 mM Tris-HCl buffer, pH 8.5 (8 ml), 50 mM meso-alanopine (2 ml), 10 mg/ml NAD^+ (2 ml) and 1 mg/ml nitroblue tetrazolium (5 ml) followed by visualization of the enzyme band by the addition of 0.5 ml 1 mg/ml phenazine methosulfate. For SDS gels, protein samples were preincubated in 1% SDS for at least 2 h at 37 °C, then run and stained for protein as described above. For the determination of subunit molecular weight using SDS electrophoresis, a plot of R_f versus molecular weight was constructed using the following standard proteins: trypsinogen (24,000), ovalbumin (45,000) and bovine serum albumin (66,000).

Isoelectrofocusing. Isoelectrofocusing was performed by the method of Vesterberg (1971) using an LKB 8101 column (110 ml) and a pH gradient of 4.0 to 6.0. Crude enzyme supernatant was run at 400 V for 14 h at 4 °C. The column was then drained and 1 ml fractions collected and assayed for alanopine dehydrogenase activity.

Protein Assay. Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad prepared reagent and bovine gamma globulin as the standard.

Results

Foot Muscle Enzyme Activities

L. littorea foot muscle contains two cytoplasmic dehydrogenases acting at the pyruvate branchpoint: alanopine dehydrogenase (11.1 units/g wet weight) and D-lactate specific lactate dehydrogenase (12.2 units/g wet weight). Octopine dehydrogenase is not present.

Purification and Physical Properties

The purification of alanopine dehydrogenase is summarized in Table 1. The enzyme was purified about 1,300-fold to a final specific activity of 240 units/mg protein and was judged to be homogeneous by SDS polyacrylamide gel electrophoresis.

The molecular weight of *L. littorea* alanopine dehydrogenase, determined by gel filtration, was found to be 42,200±500 ($n=4$) similar to that reported for oyster muscle alanopine dehydrogenase (47,000) (Fields and Hochachka 1981) and for octopine dehydrogenases (38,000 to 43,000) from various sources (Fields et al. 1976; Olomucki et al. 1972). Like other imino acid dehydrogenases, *L. littorea* alanopine dehydrogenase was a monomer; SDS gel electrophoresis showed a subunit size of 42,400.

Isoelectrofocusing showed an isoelectric point of 5.6±0.02 ($n=3$).

In all preparations only a single band of alanopine dehydrogenase activity was seen on either SDS or non-SDS gels stained for protein or for enzyme activity. Isoelectrofocusing of the crude enzyme preparation showed only a single peak of alanopine dehydrogenase activity. There was no evidence, therefore, for multiple molecular forms of alanopine dehydrogenase in foot muscle.

Table 1. Summary of the purification of alanopine dehydrogenase from the foot muscle of the gastropod mollusc, *Littorina littorea*

Step	Total activity (units)	Total protein (mg)	Yield (%)	Purification (-fold)	Specific activity (units/mg protein)
Crude homogenate	340	1,728	–	–	0.19
Ammonium sulphate	238	216	70	6	1.10
Sephadex G-100	200	6.9	59	153	29.0
Chromatofocusing	167	1.9	49	463	88.0
Sephadex G-100	116	0.48	34	1,263	240.0

Table 2. Substrate specificity of *Littorina littorea* alanopine dehydrogenase. Assay conditions were 50 mM imidazole buffer, pH 7.5 with constant co-substrate concentrations, 1.3 mM pyruvate, 130 mM L-alanine and 0.1 mM NADH. Relative velocities were measured at saturating levels of each of the amino or keto acids and are expressed relative to the apparent V_{max} with L-alanine and pyruvate as substrates

Substrate	Apparent K_m (mM)	Relative velocity
L-Alanine	47	100
Glycine	1,267	37
L-α-Aminobutyrate	67	33
L-Serine	220	13
L-Cysteine	35	4
Pyruvate	0.55	100
Oxaloacetate	5.7	94
α-Ketobutyrate	9.4	37
Glyoxylate	10.0	10

Substrate Specificity

The substrate specificity of purified ADH was assessed in terms of apparent K_m values and relative velocities (Table 2). L-Alanine and pyruvate were

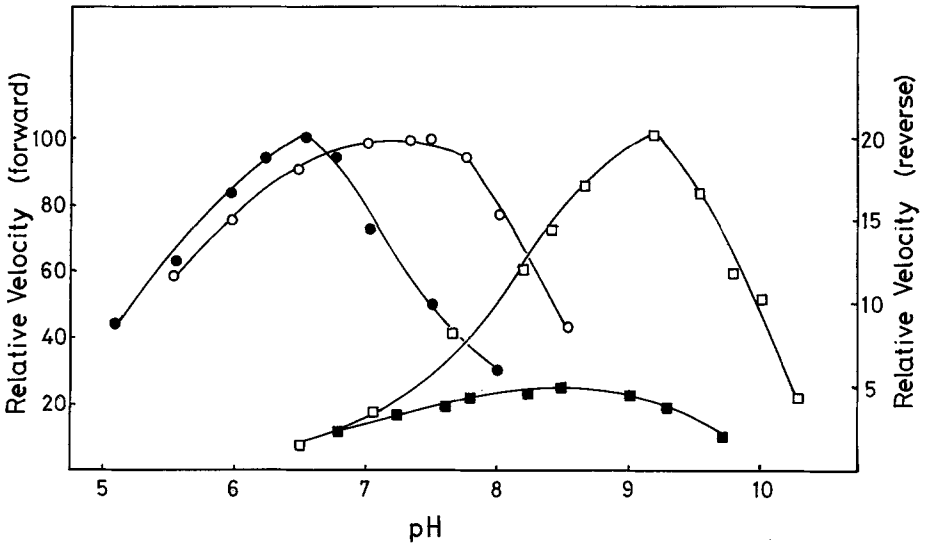

Fig. 1. pH profiles for *L. littorea* alanopine dehydrogenase in the forward and reverse directions. Conditions are: ●, 1 mM pyruvate, 100 mM L-alanine, 0.1 mM NADH, 50 mM imidazole buffer (saturating conditions at pH 6.5); ○, 1.3 mM pyruvate, 130 mM L-alanine, 0.1 mM NADH, 50 mM imidazole buffer (saturating conditions at pH 7.5); ■, 10 mM meso-alanopine, 2 mM NAD^+, 50 mM Tris-HCl buffer; and □, 50 mM meso-alanopine, 2 mM NAD^+, 50 mM TAPS (N-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid) buffer. Results are expressed relative to V_{max} for the forward direction which is set at 100

the preferred substrates of the enzyme. Low activity was found with four alternative amino acid substrates while three alternative keto acid substrates were found. Relative activity with oxaloacetate as an alternative substrate was high but the apparent K_m for this keto acid was too great to suggest a physiological role for this substrate. No enzyme activity was observed with other L-amino acids including arginine, aspartate, glutamate, histidine, isoleucine, leucine, methionine, phenylalanine, proline, taurine, tyrosine, asparagine, glutamine, lysine, threonine, tryptophan or valine, with D-alanine, with NH_4^+, or with hydroxypyruvate, α-ketoglutarate, or α-ketovalerate. In the reverse direction, activity was observed with meso-alanopine only. No activity was apparent with up to 30 mM D-strombine (2-methyliminodiacetic acid) or 200 mM iminodiacetic acid. Similarily, gels stained for alanopine dehydrogenase activity showed no band of enzyme activity when D-strombine was substituted for meso-alanopine. The enzyme was specific for NAD(H) showing no activity in the presence of NADP(H).

pH Effects

pH profiles of periwinkle muscle alanopine dehydrogenase are shown in Fig. 1. In the forward, alanopine-producing direction, the enzyme has a pH optimum of 7.5 at high substrate concentrations (130 mM L-alanine, 1.3 mM pyruvate, 0.1 mM NADH: saturating substrate conditions at pH 7.5) but this is shifted to about 6.5 at lower substrate concentrations (100 mM L-alanine, 1 mM pyruvate, 0.1 mM NADH: saturating substrate concentrations at pH 6.5). This effect appears to be related to the pyruvate and alanine substrate inhibition characteristics of the enzyme. In the reverse direction, the pH optimum was 9.2 at 50 mM meso-alanopine but dropped to 8.5 at lower (10 mM) levels of meso-alanopine. The ratio of the maximal activities in the forward (at pH 7.5) *versus* the reverse (at pH 9.2) directions was about 5:1.

Kinetic Constants

L. littorea alanopine dehydrogenase followed Michaelis-Menten saturation kinetics for all substrates. Kinetic constants for alanopine dehydrogenase are given in Table 3. Due to the marked effect of pH on the K_m's for pyruvate and L-alanine and the importance of intracellular pH in regulating anaerobic metabolism in marine invertebrates, the kinetics of the enzyme in the forward direction were examined at two pH's 6.5 and 7.5. The apparent K_m's for pyruvate and L-alanine were strongly influenced by co-substrate concentration (L-alanine or pyruvate), the apparent K_m for either substrate being reduced with increasing co-substrate concentrations. This effect is well known for octopine dehydrogenase from a variety of sources (Thoai et al. 1969; Fields et al. 1976; Storey and Storey 1979b). Table 3 shows the absolute K_m's

Table 3. Michaelis constants for *Littorina littorea* alanopine dehydrogenase at two pH's. Kinetic constants were derived as outlined in Materials and Methods from analyses of three separate preparations of the purified enzyme. Values are means ± s.e.m. Assays were performed at 23 °C in 50 mM imidazole buffer with constant co-substrate concentrations, where appropriate, of 1.3 mM pyruvate, 130 mM L-alanine, 0.1 mM NADH, 30 mM meso-alanopine, and 2 mM NAD^+ at pH 7.5 and 1.0 mM pyruvate, 100 mM L-alanine, 0.1 mM NADH, 15 mM meso-alanopine, and 2 mM NAD^+ at pH 6.5

Substrate	pH 6.5	pH 7.5
Absolute K_m (mM)		
L-Alanine	14.9 ±0.85	23.8 ±0.52
Pyruvate	0.17±0.02	0.26±0.01
Apparent K_m (mM)		
NADH	0.009±0.0001	0.009±0.0001
Meso-Alanopine	6.5 ±0.59	16.9 ±1.73
NAD^+	0.15 ±0.03	0.18 ±0.003

for pyruvate and L-alanine. K_m's for both substrates were influenced by pH, decreasing by 35–37% when pH was lowered from 7.5 to 6.5. During anoxia, then, when intracellular pH drops due to an accumulation of acidic end products, CO_2 and succinate, production of alanopine as the product of anaerobic glycolysis could be enhanced by this pH effect on enzyme kinetics. The apparent K_m for NADH was not affected by pH nor was the apparent K_m for NAD^+ in the reverse direction. However, the apparent K_m for meso-alanopine, like those for pyruvate and L-alanine, was strongly dependent on pH. While enzyme activity in the reverse direction was highest at pH 8.5, the K_m at this pH was also very high (50 mM) but K_m's fell with decreasing pH, reaching 6.5 mM at pH 6.5.

Pyruvate and L-Alanine Substrate Inhibition

The effect of pyruvate concentration on the activity of alanopine dehydrogenase is shown in Fig. 2. At saturating concentrations of L-alanine, pyruvate substrate inhibition was apparent at pyruvate concentrations above 1.0 mM at pH 6.5 and above 1.3 mM at pH 7.5. An I_{50} of about 8 mM pyruvate was observed at both pH's, similar to I_{50}'s of about 10 mM reported for pyruvate substrate inhibition of H type lactate dehydrogenases and of the brain specific isozyme of octopine dehydrogenase (Long and Kaplan 1973; Eichner and Kaplan 1977; Storey and Storey 1979a). Muscle forms of lactate dehydrogenase and octopine dehydrogenase typically do not show pyruvate substrate inhibition. However, muscle alanopine dehydrogenases differ in this property, both *L. littorea* and *Crassostrea gigas* (Fields and Hochachka 1981) muscle alanopine dehydrogenase showing substrate inhibition by pyruvate. Substrate inhibition at high pyruvate levels decreased, however, as L-alanine concentrations were lowered to subsaturating levels (Fig. 2).

Comparable effects were found when L-alanine substrate inhibition was examined. At saturating concentrations of pyruvate, substrate inhibition by L-alanine was apparent above 100 mM at pH 6.5 and above 130 mM at pH 7.5. The apparent I_{50}'s were 450 mM at pH 6.5 and 550 mM at pH 7.5. At subsaturating pyruvate concentration, however, L-alanine substrate inhibition was lost.

Metabolite and Salt Effects

A variety of metabolites was tested for effects on alanopine dehydrogenase activity in the forward direction at subsaturating substrate concentrations (50 mM imidazole buffer, pH 7.5, 0.5 mM pyruvate, 40 mM L-alanine, and 0.02 mM NADH). The following compounds (at 10 mM) had no effects on alanopine dehydrogenase activity: glucose-6-P, fructose-6-P, phosphoenolpyruvate, D-lactate, malate, fumarate, succinate, arginine phosphate, carnitine, acetyl-CoA and the L-amino acids, proline, histidine, methionine and tyrosine. The pyruvate analogue, oxamate (10 mM), the glycolytic intermediates, fructose-1,6-biphosphate, 3-phosphoglycerate and dihydroxyacetone-P (all 10 mM) and the Krebs cycle intermediates, citrate and α-ketoglutarate (both 10 mM), all produced 10–25% inhibition of alanopine dehydrogenase activity. Other L-amino acids, arginine, glutamate, lysine (all 100 mM), leucine, isoleucine (both 30 mM) and tryptophan (3 mM) inhibited enzyme activity by 25–40% compared to controls. However, the effects of these inhibitors were not further investigated due to the non-physiological concentrations needed to produce the observed inhibitory effects.

ATP, ADP, NAD^+, meso-alanopine and L-lactate were inhibitors of alanopine dehydrogenase (Table 4). Product inhibition by NAD^+ was competitive with respect to NADH while meso-alanopine was a competitive inhibitor with respect to both pyruvate and L-alanine, the K_i for alanopine being the same (35 mM) with respect to either substrate. L-Lactate inhibition is probably a result of the structural similarity between L-lactate and the substrates, pyruvate and L-alanine. Inhibition by L-lactate has no physiological relevance, however, as *L. littorea* tissues contain a D-lactate dehydrogenase. The adenylates, ATP and ADP, as well as their Mg^{2+} complexes (ratio 1:1 Mg:adenylate) were competitive inhibitors with respect to

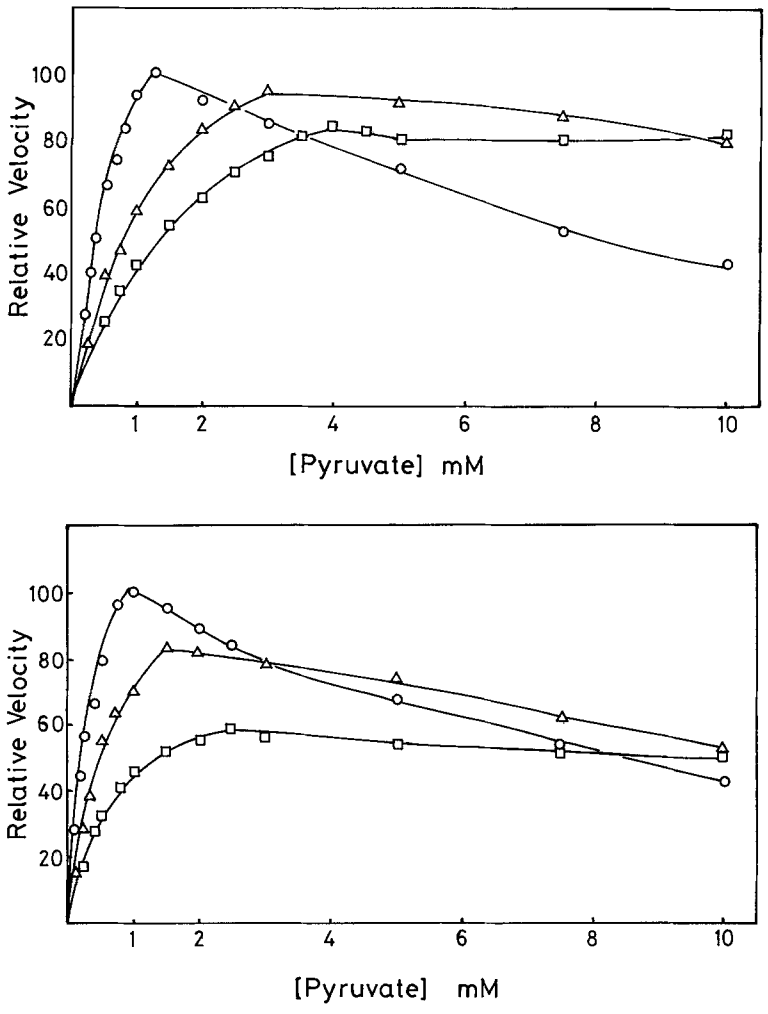

Fig. 2. Effect of pyruvate substrate concentration on reaction velocity for alanopine dehydrogenase at saturating and subsaturating, L-alanine concentrations and two pH's. Assays were performed at 23 °C with 0.1 mM NADH and 50 mM imidazole buffer. Top panel: pH 6.5, alanine concentrations: □, 15 mM; △, 30 mM; ○, 100 mM. Bottom panel: pH 7.5, alanine concentrations: □, 30 mM; △, 50 mM; ○, 130 mM

Table 4. Inhibitor effects on *Littorina littorea* alanopine dehydrogenase. Inhibitor constants were determined from Dixon plots with type of inhibition evaluated by double reciprocal plots. Assay conditions were 50 mM imidazole buffer, pH 7.5 with constant concentrations of co-substrates, 1.3 mM pyruvate, 130 mM L-alanine and 0.1 mM NADH. Values are means ± s.e.m. for $n=3$ determinations on separate preparations of purified enzyme. Mg^{2+}:adenylate ratio was 1:1

Inhibitor	Apparent K_i	Inhibition	
		Type	With respect to
NAD^+	0.16 ± 0.012	Competitive	NADH
ATP	5.6 ± 0.25	Competitive	NADH
Mg·ATP	3.0 ± 0.32	Competitive	NADH
ADP	1.7 ± 0.25	Competitive	NADH
Mg·ADP	4.2 ± 0.32	Competitive	NADH
Meso-Alanopine	35.0 ± 0.35	Competitive	L-alanine and pyruvate
L-Lactate	3.2 ± 0.12	Competitive	L-alanine
	5.0 ± 0.21	Mixed Competitive	Pyruvate

NADH. ATP effects probably occur within the physiological range of ATP concentrations in vivo although ATP inhibition of dehydrogenases is probably due to structural rather than regulatory effects, reflecting the presence of an adenine nucleotide domain in the coenzyme binding site (McPherson 1970).

To examine interactions between inhibitors and pH, inhibitor effects (I_{50}) were quantitated at pH 6.5 and 7.5 using sub-saturating substrate levels (Table 5). The inhibitory effects of ATP, ADP and NAD^+ on alanopine dehydrogenase activity decreased (I_{50} increased) at pH 6.5 compared to pH 7.5 while inhibition by meso-alanopine, D-strombine and L-lactate showed the opposite response. Succinate, which had no effect on enzyme activity at pH 7.5, was inhibitory, at high concentrations, at pH 6.5.

Monovalent and divalent ions had inhibitory effects on alanopine dehydrogenase activity. Inhibition by 200 mM salt was 16, 23, 33 and 38%

Table 5. Inhibitor effects on *Littorina littorea* alanopine dehydrogenase at two pH's. Inhibition constants (I_{50}) were determined by the method of Job et al. (1978). Assays were performed in 50 mM imidazole buffer at constant subsaturating substrate conditions: 0.2 mM pyruvate, 20 mM L-alanine, 0.025 mM NADH at pH 6.5 and 0.25 mM pyruvate, 25 mM L-alanine, 0.025 mM NADH at pH 7.5. Values are means of n = 2 determination with variation ± 10%

Inhibitor	I_{50} (mM)	
	pH 6.5	pH 7.5
NAD^+	0.50	0.38
ATP	2.50	1.50
ADP	3.65	2.50
Meso-Alanopine	9.5	25.0
D-Strombine	14.5	61.0
L-Lactate	0.90	3.15
Succinate	19.0	No inhibition

for $(NH_4)_2SO_4$, NH_4Cl, KCl and NaCl, respectively, at pH 7.5 and subsaturating substrate concentrations (0.4 mM pyruvate, 40 mM alanine, 0.03 mM NADH). Mg^{2+} ions were also inhibitory; at 50 mM salt, inhibition was 30 and 45% in the presence of $MgSO_4$ and $MgCl_2$, respectively.

Discussion

Alanopine dehydrogenase, purified from *L. littorea,* differs most strongly from purified oyster muscle alanopine dehydrogenase in the substrate specificities of the enzyme. *L. littorea* alanopine dehydrogenase is very specific for L-alanine and pyruvate as substrates; maximal activities with other amino acids were no more than $^1/_3$ of that found with L-alanine while alternate keto acids showed high (and unphysiological) apparent K_m values and/or low maximal velocities. By contrast, oyster alanopine dehydrogenase showed virtually equal maximal activities with L-alanine, glycine, L-serine or L-α-aminobutyrate as well as equal apparent K_m values for L-alanine and glycine (Fields and Hochachka 1981). The amino acid substrate specificities of imino acid dehydrogenases may be, in part, related to the predominant free amino acids available in vivo in each species; in *L. littorea* alanine is one of the most abundant free amino acids in foot muscle (at 18 μmol/g wet weight in animals acclimated to 100% seawater; Hoyaux et al. 1976). However, it is now becoming apparent, that of imino acid dehydrogenases using L-alanine and/or glycine as substrates, two distinct enzymes can be identified. One enzyme has a high specificity for L-alanine as its substrate and shows very low activity with glycine. This enzyme is exemplified by alanopine dehydrogenase from *L. littorea* and also occurs in the tissues of another gastropod, the whelk, *Busycotypus canaliculatum* (Plaxton and Storey 1982). The other enzyme, which is now called strombine dehydrogenase by most authors (de Zwaan and Zurburg 1981; Dando et al. 1981; Dando 1981) shows glycine activity ≥ L-alanine activity. The enzyme from oyster adductor muscle, called alanopine dehydrogenase by Fields and Hochachka (1981), is actually a good example of this enzyme type. Both of these enzymes occur in the tissues of *Mytilus edulis* where they are tissue specific and are electrophoretically separable (Dando et al. 1981; Dando 1981). Alanopine dehydrogenase occurs in many of the soft tissues of *M. edulis* while strombine dehydrogenase is restricted to muscle tissues which have high intracellular levels of glycine as the major free amino acid.

Substrate specificities and inhibitor effects provide information about the substrate site of alanopine dehydrogenase. Carbon chain length is important in determining substrate utilization. C_2 (glycine, glyoxylate) and C_4 (L-α-aminobutyrate, α-ketobutyrate) substrates were utilized but only at reduced rates and with high apparent K_m values compared to pyruvate and L-alanine. Alterations to a C_3 substrate (the hydroxyl groups of serine and hydroxypyruvate, the sulfhydryl group of cysteine) also reduced enzyme activity indicating the importance of the methyl groups of L-alanine and pyruvate in substrate binding. Compounds of chain length greater than C_4 or with substitutions on the C_4 chain (threonine, methionine, valine, leucine, isoleucine, α-ketovalerate, α-ketoglutarate) were inactive. The enzyme was also inactive with D-alanine. D-lactate, the product of the D-lactate dehydrogenase of *L. littorea*, had no effect on alanopine dehydrogenase but L-lactate was inhibitory with respect to both L-alanine and pyruvate. This suggests the importance of the L configuration at the substrate site.

The kinetic properties of *L. littorea* alanopine dehydrogenase can suggest a physiological role for the enzyme in redox regulation during anaerobiosis. Two properties are particularly persuasive. Firstly, alanopine dehydrogenase shows a dependence of the apparent K_m for pyruvate of L-alanine on co-substrate (L-alanine or pyruvate) concentration, rising levels of either the amino or keto acid co-substrate decreasing the apparent K_m for keto or amino acid, respectively. This effect is well known for octopine dehydrogenase and it has been suggested that this property is a key factor influencing enzyme activity in vivo and promoting

the synthesis of octopine as the product of glycolytic muscle work (Fields et al. 1976). In *L. littorea*, where alanine is a major end product of anaerobic metabolism (Wieser 1980), the activation of glycolysis during anoxia could lead to both alanine and alanopine accumulation as end products. Indeed, studies in the oyster have shown that alanopine/strombine are substantial products of anaerobiosis in ventricle accounting for 35% of ^{14}C-glucose catabolized during one hour of anoxia (Collicutt and Hochachka 1977). Secondly, *L. littorea* alanopine dehydrogenase is strongly affected by pH. The K_m's for both pyruvate and L-alanine decrease with decreasing pH, an effect which would promote alanopine synthesis during anoxia when intracellular pH declines. For oyster muscle alanopine dehydrogenase, this effect of pH of K_m's for alanine and pyruvate was not found although pH had a slight effect on K_m for NADH (Fields and Hochachka 1981).

Alanopine or strombine production as products of anaerobic metabolism in marine molluscs has now been reported by several authors (de Zwaan and Zurburg 1981; Fields 1977; Collicutt and Hochachka 1977). In *Mytilus edulis*, accumulation of strombine was greatest during the first six hours of anoxia (de Zwaan and Zurburg 1981) suggesting that the activation of anaerobic glycolysis to produce products such as alanine and alanopine/strombine is the primary response of these animals to anoxia. Long term anoxia, however, is met by a secondary response in which glycolytic carbon flow is channeled into the production of succinate and volatile fatty acids. Thus the primary function of alanopine dehydrogenase in vivo appears to be in redox regulation of glycolysis during the initial hours of anaerobic function.

Imino acid dehydrogenases can also function, like lactate dehydrogenase, in providing glycolytic redox balance during 'burst' muscle work when oxygen supplies are insufficient to meet energy demand by contracting muscle. This function appears to be the primary function of octopine dehydrogenase in the species in which the enzyme occurs (Grieshaber and Gäde 1976; Storey and Storey 1979b) and must also apply to alanopine or strombine dehydrogenases in certain species. In the oyster, for example, neither lactate dehydrogenase nor octopine dehydrogenase are present, so alanopine dehydrogenase must play a dual role in the response to both muscle work and anoxia stress in vivo. In *L. littorea* foot muscle, however, both alanopine dehydrogenase and lactate dehydrogenase occur in approximately equal activities suggesting distinct roles for the two enzymes. Lactate dehydrogenase may function during muscle work.

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