Characterization and Physiological role of Tauropine Dehydrogenase and Lactate Dehydrogenase from muscle of abalone, Haliotis discus hannai

Characterization and Physiological role of Tauropine Dehydrogenase and Lactate Dehydrogenase from muscle of abalone, *Haliotis discus hannai*

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Summary

Tauropine dehydrogenase was detected mainly in the upper part of the columella muscle of abalone, whereas lactate dehydrogenase was detected homogeneously in all muscle tissues. Total enzyme activity of tauropine dehydrogenase was 3-fold higher than that of lactate dehydrogenase in both the columella and foot muscles of abalone. Both enzymes were purified by chromatography using Blue Sepharose CL-6B, Toyopearl HW-50, DEAE-Toyopearl 650 M and TSK SW2000G. Tauropine dehydrogenase was finally purified about 900-fold with a yield of 16.7%. The Mr of tauropine dehydrogenase obtained by HPLC using TSK SW2000G in its native form and by SDS-polyacrylamide gel electrophoresis in its denatured form, was coincident as 42000. This means tauropine dehydrogenase has the monomeric structure. The Mr of lactate dehydrogenase, though it had small impurities, was estimated with HPLC method to be 51000. Amino acid specificity of tauropine dehydrogenase is characteristic for taurine. This enzyme requires amino acid having all of C_2 carbon chain lenght, alpha amino group and beta sulfonic acid group for enzyme activity. The apparent Km values of tauropine dehydrogenase for taurine, pyruvate and NADH were 44.4, 3.6 and 0.071 mM, respectively. On the other hand, lactate dehydrogenase showed a higer affinity for both pyruvate (Km=0.50 mM) and NADH (Km=0.014 mM) than tauropine dehydrogenase, and synthesized mainly D-lactate. However the tauropine content in the columella muscle was 5-fold higher than D-lactate in its stationary stage and increased from 4.89 to 9.60 μmol/g fresh wt during columella muscle exercise, whereas D-lactate increased from 0.94 to 1.14 μmlo/g fresh wt. The data obtained from this study indicate that tauropine dehydrogenase plays an important physiological role in energy production during shell fixation activity in the columella muscle, whereas D-lactate dehydrogenase may be related to the slow locomotive activity in abalone.

Introduction

We have recently isolated tauropine (D-rhodoic acid) from the foot and columella muscles of abalone *Haliotis discus hannai* and also demostrated that the muscle tissue possessed the ability to biosynthesize tauropine from taurine and pyruvate (1-3). This biosynthesis appears to be mediated by the tauropine dehydrogenase which catalyzes the reductive condensation reaction :

$$\text{taurine} + \text{pyruvate} + \text{NADH} \rightleftarrows \text{tauropine} + \text{NAD}^+ + \text{H}_2\text{O}.$$
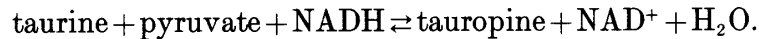
Tauropine has a structure analogous to octopine, alanopine, strombine and β-alanopine, which are all referred to as "opines" in comparative biochemistry literature (4). Opines are biosynthesized by octopine dehydrogenase, alanopine dehydrogenase, strombine dehydrogenase, and β-alanopine dehydrogenase and are generally considered to be end products of anaerobic glycolysis of molluscs. It is widely accepted that the opine dehydrogenasses have an important physiological role in the maintenance of cytoplasmic redox balance during anaerobic glycolysis in the muscle tissue of molluscs like lactate dehydrogenase in vertebrates (5-10). The fact of coexistence of tauropine dehydrognease and lactate dehydrogenase in the muscle of abalone *Haliotis discus hannai* which compete for available pyruvate (11), call us forth to investigate the properties and physiological roles of tauropine dehydrogenase and lactate dehydrogenase from the view point of comparative biochemistry.

Materials and Methods

Animals

Abalone *Haliotis discus hannai* were purchased from the shellfish nursery, Sanriku, Iwate, and were maintained in well aerated aquaria prior to use.

Materials

Tauropine was prepared by synthesis from D-alanine and 2-bromoethanesulfonic acid, in 2 N NaOH solution (12). Homotaurine (3-aminopropanesulfonic acid) was prepared by the method of Sen (13). Proteins used as molecular mass markers for analytical size exclusion and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Serva. All coenzymes, NAD (H) and NADP (H), were obtained from the Boehringer Mannheim. All other chemicals were of reagent grade quality. Blue Sepharose was obtained from Pharmacia. Toyopearl HW-50, DEAE-Toyopearl 650M and TSK-SW2000G prepacked column (7.5 mm × 600 mm) were obtained from Toso (Japan).

Enzyme assays

Enzyme assays were performed in Shimazu UV-200 spectrophotometer. Standard assay conditions for tauropine dehydrogenase were : 100 mM Tris/HCl buffer, pH 7.2, 6 mM sodium pyruvate (pH 7.2), 100 mM taurine (pH 7.2) and 0.3 mM NADH in the forward direction, and 100 mM Tris/HCl buffer, pH 9.0, 20 mM tauropine (pH 9.0) and 1 mM NAD$^+$ in the reverse direction. In both assays, the final volume was 750 μl. The reaction mixture less enzyme was preincubated at 30°C for 2 min and the assay was started by the addition of the enzyme. Assays of other opine dehydrogenases were conducted by replacing taurine with corresponding amino acid substrates (L-arginine, L-alanine, glycine and β-alanine) at 10 mM (L-arginine) and 100 mM (other amino acids) final concentrations. Lactate dehydrogenase was assayed by omitting taurine from the standard assay mixture of tauropine dehydrogenase. One enzyme unit is defined as the amount of enzyme utilizing 1 μmol of coenzyme per 1 min.

Protein assay

The protein concentration was assayed by Coomassie Blue G-250 binding method (14), with bovine serum albumin as the standard.

Preparation of crude enzyme solution

All steps in the extraction procedures were carried out at 0-4°C. Prior to the enzyme purification, the muscle tissue of abalone *Haliotis discus hannai* was divided into three parts : the upper part of the columella and lower part of columella muscle and the foot muscle, as shown in Fig. 1. Each part was cut into small pieces and homogenized with 3 volumes (v/w) of 20 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 10 mM 2-mercaptoethanol using a Ystral disperser (F.R.G.). The resulting homogenate was centrifuged at
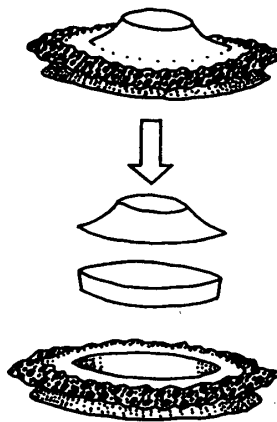

FIG. 1. Rough sketch of the sampling parts of the columella and foot muscles of abalone for enzyme assay.

10,000 × g for 30 min, and the pellet was discarded. The resulting supernatant was subjected to ammonium sulphate fractionation. The precipitate obtained between 40 and 80% ammonium sulphate saturation was dissolved in a small volume of extracting buffer and dialyzed against the same buffer. The dialyzed sample was used as a crude enzyme solution and was used to determine the enzyme activities of opine dehydrogenases and lactate dehydrogenase. The columella muscles (50 g) obtained from five pieces of abalone *Haliotis discus hannai* were used for enzyme purification. Crude enzyme solution was prepared in the same manner as mentioned above.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on 7% tube gel at pH 8.9 by the method of Davis (15). Tauropine dehydrogenase and lactate dehydrogenase were detected on gels with a mixture of 1 mM NAD^+, 0.1 mM phenazine methosulphate, 1 mM nitroblue tetrazolium, and 10 mM tauropine (pH 9.0) for tauropine dehydrogenase and 20 mM DL-lactate (pH 9.0) for lactate dehydrogenase in 100 mM Tris/HCl buffer, pH 9.0. Control gels, stained with the above reaction mixture less tauropine or lactate, showed no bands of activity. A protein band was stained on the gels with Coomassie Brilliant Blue R250.

Determination of molecular mass

Molecular mass values were determined by two methods, analytical size exclusion and SDS-PAGE. Analytical size exclusion was perfored on a TSK SW2000G column using 250 mM potassium phosphate buffer (pH 7.2) containing 1.0 mM EDTA and 10 mM 2-mercaptoethanol. The subunit molecular mass was determined by SDS-PAGE (1% SDS containing 7.5% acrylamide gel) by the method of Weber & Osborn (16). Molecular mass markers were bovine catalase (Mr 240000, subunit Mr 60000), egg albumin (Mr 45000), rabbit muscle aldorase (Mr 160000, subunit Mr 40000) and chymotrypsinogen (Mr 25000).

Kinetic studies

Tauropine dehydrogenase and lactate dehydrogenase kinetics were assayed using the purified enzymes. The apparent Km values were determined from Hanes plots, S/V versus S, using constant, saturating levels of pyruvate and NADH. Inhibitor constants (Ki) were determined from Dixon plots.

Determination of tauropine and lactate

Abalones were immediately frozen in liquid nitrogen and perchloric acid extracts were obtained by homogenizing the tissues in 3 volumes (v/w) of 1 M perchloric acid using a Ystral disperser. The neutralized extracts were used for tauropine and lactate determinations. Tauropine was determined by the HPLC

method after phenylthiocarbamyl derivatization (17). Lactate was determined by enzymatic determination (18).

Results

Opine dehydrogenases and lactate dehydrogenase activities in the muscle tissues

Both tauropine and lactate dehydrogenase activities were detected in each of the muscle tissues, whereas octopine, alanopine, strombine and β-alanopine dehydrogenases were not present. Total tauropine dehydrogenase activity of all muscle tissues was greater, about 3-fold, than that of lactate dehydrogenase under standard assay condition. The greater part of tauropine dehydrogenase, about 70% of total activity, was presented in the upper part of the columella muscle, and only 17% in the foot muscle. On the other hand, lactate dehydrogenase activity was distributed equally in each of the muscle tissues (Table 1).

Enzyme purification

The crude enzyme solution was first applied on a Blue Sepharose CL-6B column (2 cm × 50 cm) which had been previously equilibrated with extracting buffer. After washing the column with the same buffer to remove unbound protein, tauropine dehydrogenase and lactate dehydrogenase were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer (total 400 ml) and 5 ml fractions were collected. Each fraction was assayed for enzyme activities. Tauropine dehydrogenase eluted prior to lactate dehydrogenase was concentrated by pressure filtration using Amicon YM-10, and applied on a Toyopearl HW-50 column (2 cm × 100 cm), which had been previously equilibrated with extracting buffer containing 0.2 M NaCl. Tauropine dehydrogenase fractions were pooled, concentrated, dialyzed for extracting buffer and then applied to a DEAE-

TABLE 1. *Tauropine dehydrogenase and D-lactate dehydrogenase activities in each part of the columella and foot muscles of abalone*

Muscle part		Fresh we (g)	Total enzyme activity (units)				Specific activity (units g⁻¹ fresh wt)	
			Tauropine dehydrogenase	(%)	D-Lactate dehydrogenase	(%)	Tauropine dehydrogenase	D-Lactate dehydrogenase
Columella	upper part	1.5	67.7	(70.4)	7.9	(25.2)	45.1	5.3
	lower part	3.0	11.9	(12.4)	8.4	(26.8)	4.0	2.8
Foot		5.0	16.5	(17.2)	15.1	(48.1)	3.3	3.0

Muscle tissues were homogenized with 3 volumes of 20 mM sodium phosphate buffer containing 10 mM mercaptoethanol and 1 mM EDTA. The protein fraction obtained from 40-80% ammonium sulphate saturation was used for enzyme assay as stated in MATERIALS AND METHODS.
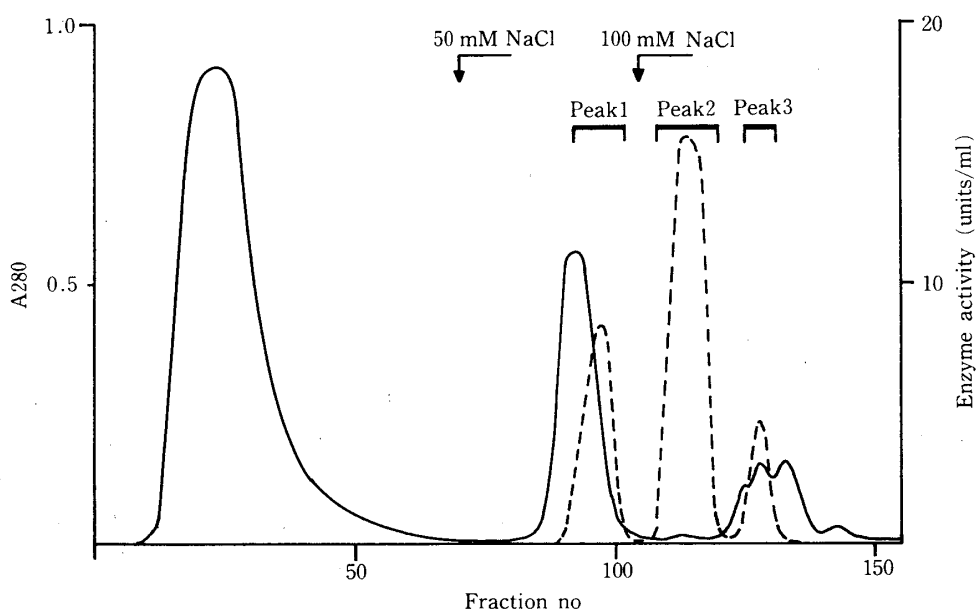
FIG. 2. DEAE-Toyopearl 650 M column chromatography of the abalone tauropine dehydrogenase.

The tauropine dehydrogenase fraction obtained from Toyopearl HW-50 column chromatography was applied to a column (3 cm × 30 cm) of DEAE-Toyopearl previously equilibrated with 20 mM sodium phosphate buffer, pH 7.2, containing 10 mM mercaptoethanol and 1 mM EDTA. Material was eluted stepwise with 200 ml of the same buffer, each containg 50 mM and 100 mM NaCl. The flow rate was 60 ml/h and 5 ml fractions were collected. The absorbance at 280 nm (———) and tauropine dehydrogenase activity (- - -) were determined.

Arrows show the starting position of each solvent. The three fractions indicated by thin bars were pooled as peak 1, 2 and 3, respectively. The peak 2 fraction was then purified by a TSK SW2000G column and used for further study.

Toyopearl 650 M column (2 cm × 30 cm) which had been previously equilibrated with the same buffer. Three tauropine dehydrogenase active fractions were eluted by stepwise elution as shown in Fig. 2. The activities of each fraction were 22.7, 64.1 and 13.1%, respectively. The main enzyme peak (Peak 2) was finally purified by HPLC using a TSK SW2000G column. These purification procedures resulted in a single tauropine dehydrogenase band on a polyacrylamide gel and a single protein band on SDS-polyacrylamide gel (Fig. 3). An overall purification of 895-fold was achieved with a yield of 16.7% (Table 2). This purified enzyme (Peak 2) was used for further enzyme studies.

Lactate dehydrogenase, which was separated from tauropine dehydrogenase by Blue Sepharose, was further purified in the same manner as tauropine dehydrogenase using Toyopearl HW-50, DEAE-Toyopearl 650 M and TSK SW2000 G. Lactate dehydrogenase thus obtained, though it contained small impurities, was used for further enzyme studies.
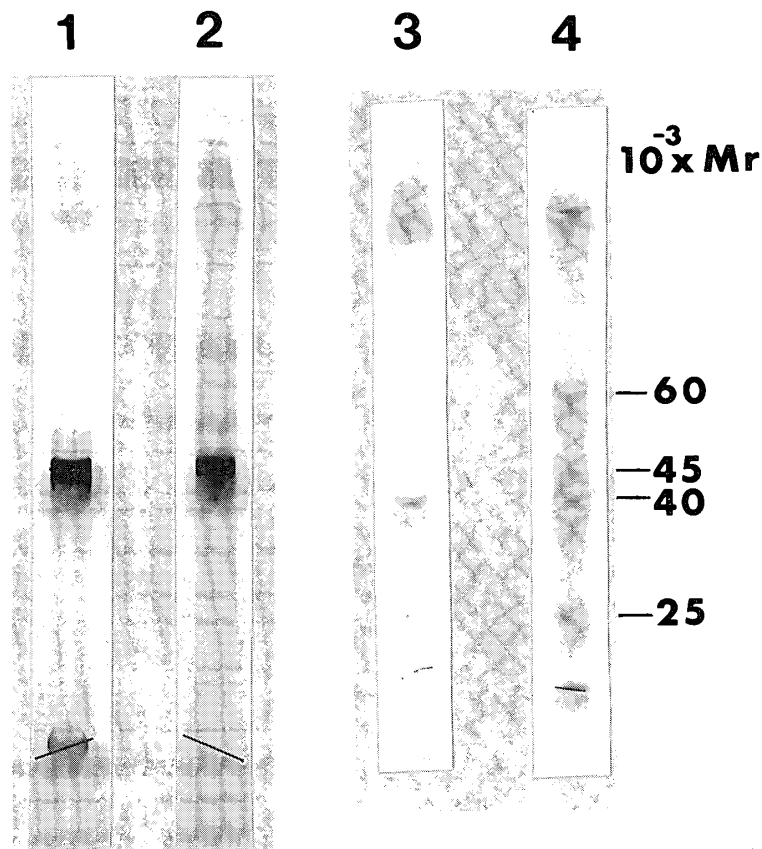
Fig. 3. Polyacrylamide gel electrophoresis of the purified abalone tauropine dehydrogenase.
Track 1 and 2 were polyacrylamide gels of the enzyme activity stain and protein stain, respectively. Track 3 and 4 were SDS-polyacrylamide gels of the enzyme and marker proteins. The marker proteins were bovine catalase (subunit Mr 60000), egg albumin (Mr 45000), rabbit muscle aldolase (subunit Mr 40000) and chymotrypsinogen (Mr 25000).

Molecular mass of the abalone tauropine dehydrogenase and lactate dehydrogenase

The molecular mass of tauropine dehydrogenase determined by the means of analytical size exclusion and SDS-PAGE, was 42000 ± 1000. Its molecular mass and monomeric structure are quite identical to that of ormer *Haliotis lamellosa* tauropine dehydrogenase (19) and also other opine dehydrogenases (20-24). The molecular mass of lactate dehydrogenase, though it contained small impurities, was estimated by the HPLC method to be 51000 ± 1000. This value differed from D-lactate dehydrogenase of black abalone, *Haliotis cracherodii*, Mr 80000 (25).

General and Kinetic properties of abalone tauropine dehydrogenase

Abalone tauropine dehydrogenase was specific for NAD (H), exhibiting no activity for NADP (H) as a coenzyme. This enzyme showed pH optima 7.2 for

TABLE 2. *Purification of tauropine dehydrogenase from columella muscle of abalone*

Purification step	Activity (units)	Protein (mg)	Sp. Activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	2605	1447	1.8	100	1
40-80%-satn.-$(NH_4)_2SO_4$ fraction	2006	340	5.9	77	3
Blue Sepharose CL-6B column eluate	1355	16	84.7	52	47
Toyopearl HW-50 column eluate	1250	10	125.0	48	69
DEAE-Toyopearl 650 M column eluate					
Peak 1	216	2.58	83.7	8.3	46
Peak 2	610	0.44	1386.4	23.4	770
Peak 3	125	0.89	140.4	4.7	78
TSK-SW2000G column eluate					
Peak 2	435	0.27	1611.1	16.7	895

For full experimental details see MATERIALS AND METHODS.   Enzyme activity was determined in the direction of tauropine formation reaction with standard assay condition.   One enzyme unit corresponds to the utilization of 1 μmol of NADH per min at 30°C.

forward tauropine formation reaction and about 10.0 for the reverse reaction.   In the physiological range, around pH 7.2, the quotient of tauropine synthesis versus oxidation was 11.   This enzyme was most active at about 30°C, and loss its activity over 35°C.

Abalone tauropine dehydrogenase showed a characteristic amino acid specificity for taurine.   There was weak activity with L-alanine (about 2% of taurine).   On the other hand, other amino acids such as L-arginine, L-cysteic acid,

TABLE 3. *Amino acid specificity of the abalone tauropine dehydrogenase*

Amino acid	Relative activity (%)
Taurine	100
L-Alanine	2.1 ± 0.1
L-Arginine	—*
Glycine	—
Homotaurine	—
Aminomethanesulfonic acid	—
Hypotaurine	—
L-Cysteic acid	—
β-Alanine	—

* under the level of 1%
Assay condition was as follows : 6 mM sodium pyruvate, 0.3 mM NADH, 100 mM Tris/HCl buffer (pH 7.2) and 100 mM amino acid.
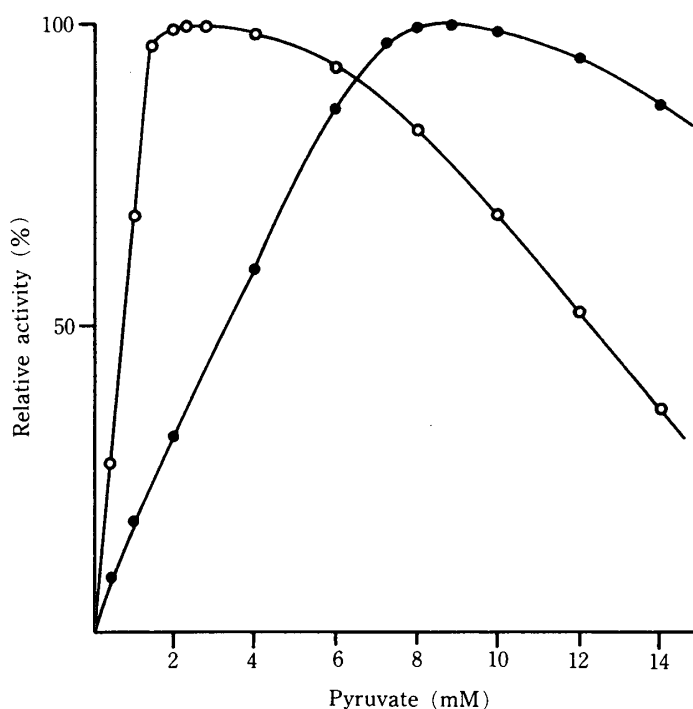
FIG. 4. Pyruvate saturation kinetics for the purified tauropine dehydrogenase and
D-lactate dehydrogenase from abalone *Haliotis discus hannai*.
The purified tauropine dehydrogenase (peak 2, ●——●) and D-lactate dehydrogenase
(○——○) were assayed at various pyruvate concentrations with 0.3 mM NADH for
both enzymes and 100 mM taurine for tauropine dehydrogenase.

homotaurine (3-aminopropanesulfonic acid), aminomethanesulfonic acid,
hypotaurine, β-alanine and glycine were not used (Table 3). This enzyme
showed a high degree of specificity for pyruvic acid, but not for oxaloacetic acid,
α-ketobutyric acid and glyoxylic acid. There was no evidence of substrate
inhibition by taurine at concentrations up to 100 mM. In contrast, pyruvate
showed substrate inhibition at concentrations over 8 mM (Fig. 4). Apparent Km

TABLE 4. *Apparent Km values for abalone tauropine dehydrogenase*

Substrate	Apparent Km (mM)	Cosubstrate and buffer system
Taurine	44.4	6 mM sodium pyruvate, 0.3 mM NADH and 100 mM Tris/HCl, pH 7.2
Pyruvate	3.57	100 mM taurine, 0.3 mM NADH and 100 mM Tris/HCl, pH 7.2
NADH	0.071	100 mM taurine, 6 mM pyruvate and 100 mM Tris/HCl, pH 7.2
Tauropine	4.17	1 mM NAD⁺ and 100 mM Tris/HCl, pH 9.0
NAD⁺	0.072	20 mM tauropine and 100 mM Tris/HCl, pH 9.0

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values for the substrates are listed in Table 4. The apparent Km value for taurine was influenced by the pyruvate concentration. As the pyruvate concentration was increased, there was a decrease in the apparent Km value for taurine.

Fluorescein mercuric acetate and 5, 5-dithiobis (2-nitro benzoate) inhibited the enzyme activity completely at 0.1 mM, but other thiol attacking reagents such as iodoacetate, iodoacetamide, N-ethylameimide and p-chloromercuribenzoate, showed no effect under standard assay condition. Sulfhydryl reagents, L-cysteine, dithiothreitol and 2-mercaptoethanol, did not show significant activation or inhibition up to 10^{-3}M. However, these sulfhydryl reagents showed the remarkable ability to restore enzyme activity to 70-80% of initial activity after inactivation by fluorescein mercuric acetate and 5, 5-dithiobis (2-nitro-benzoate). Hg^{+2}, Zn^{+2}, Cu^{+2} and Fe^{+2} showed a strong inhibition at 1 mM, whereas Ba^{+2}, Ca^{+2}, Mg^{+2} and Mn^{+2} showed no effect. All five chelating agents (acetylacetone, EDTA, EGTA, and nitrilotriacetic acid) showed no effect.

Product inhibition for tauropine formation reaction was observed for both NAD^+ (Ki = 3.5 mM) and tauropine (Ki = 18 mM). D-Lactate, which very likely accumulates in the tissue, was not inhibitory at concentrations up to 10 mM.

Kinetic properties of abalone lactate dehydrogenase

The apparent Km values for lactate dehydrogenase were 0.50 mM for pyruvate and 0.014 mM for NADH. Abalone lactate dehydrogenase showed a higher affinity for pyruvate but was more strongly inhibited by this substrate than tauropine dehydrogenase (Fig. 4).

TABLE 5. *Tissue levels of tauropine and D-lactate in the muscle and internal tissues of abalone, Haliotis discus hannai before (Resting) and after (Exercised) electrical stimulation.*

Tissue	Tauropine		D-Lactate	
	Resting	Exercised	Resting	Exercised
Columella muscle	4.89	9.60	0.94	1.14
Foot muscle	ND*	0.10	0.22	0.39
Internal tissue	ND	ND	0.36	0.53

* ND : under the level of 0.05 μmol of tauropine g^{-1} fresh wt.
In the exercised treatment, abalones were placed in an aquarium (35 cm × 65 cm × 25 cm) containg a 15 cm depth of well aerated sea water. Platinum electrodes were set in diagonal corners. Alternating current (50 volts, about 100 mA) was applied at 10 second interval in the sea water for 10 min. Both resting and exercised abalones were immediately frozen with liquid nitrogen. Two individuals were used in each experiments. Values are expressed in terms of μmol g^{-1} fresh wt.

Tauropine and lactate content in the abalone tissues

The results of tauropine and lactate determinations in the columella muscle, foot muscle and internal tissues of resting and exercised abalone *Haliotis discus hannai* were shown in Table 5. The lactate presented in the abalone tissues was D-lactate. This proves the existence of D-specific lactate dehydrogenase in abalone *Haliotis discus hannai* like other invertebrates (25). Tauropine was detected exclusively in the columella muscle, and increased approximately 2-fold from 4.89 to 9.60 μmol/g fresh wt during muscular exercise by electrical stimulation. D-Lactate was detected in relatively higher concentrations in the columella muscle (0.94 μmol/g fresh wt), but was also detected in the foot muscle and internal tissue (about 0.22-0.36 μmol/g fresh wt). The increase of D-lactate during muscular exercise, about 0.17 μmol/g fresh wt in each tissues was apparently smaller than that of tauropine (4.71 μmol/g fresh wt in columella muscle).

Discussion

This study of abalone *Haliotis discus hannai* revealed the presence of high activity of tauropine dehydrogenase (D-rhodoic acid dehydrogenase) in the upper part of the columella muscle tissue (45.1 unit/g fresh wt) and low activity in the foot muscle (3.3 unit/g fresh wt). D-Lactate dehydrogenase was present in each of the muscle tissues (2.8-5.3 unit/g fresh wt). Other opine dehydrogenases, shuch as octopine, alanopine, strombine and β-alanopine dehydrogenases were not detected. All these data were similar to that of ormer *Haliotis lamellosa* tauropine dehydrogenase (19).

The abalone tauropine dehydrogenase has many similarisies not only in the physical properties such as the molecular mass and monomeric structure, but also in some catalytic properties such as coenzyme specificity and pH optima, with other opine dehydrogenases from various sources (6, 19-24, 26-28). However, it showed a remarkable difference in amino acid specificity. This enzyme showed a strict amino acid specificity for taurine. Namely, this enzyme required all of the alpha amino group, C_2 carbon chain length and beta sulfonic acid group for active site binding of the amino acid. The sulfonic acid group in beta position could not be substituted for a sulfurous acid group (hypotaurine) or a carboxylic acid group (β-alanine). Second, a carbon chain length of C_2 is absolutely necessary. There was no enzyme activity with C_1 and C_3 amino sulfonic acid, aminomethanesulfonic acid and 3-aminopropanesulfonic acid (homotaurine). The narrow amino acid specificity of this enzyme is similar to gastropod tauropine dehydrogenase, alanopine dehydrogenase (19, 22, 29) and cephalopod octopine dehydrogenase (30), but apparently differs from that of bivalve alanopine and strombine dehydrogenases which have a wide degree of amino acid specificity (20, 27, 31). Thus,

there seems to be a phylogenetic trend in terms of amino acid specificity of molluscan opine dehydrogenases.

Finally, the role of the two pyruvate competing dehydrogenases, tauropine dehydrogenase and D-lactate dehydrogenase, can be questioned. The confined distribution of tauropine dehydrogenase in the columella muscle suggests that this enzyme may play the key role in energy production during rapid and strong muscle activity when fixing the shell to a rock. The lower affinity for pyruvate and lower inhibition by pyruvate indicate that abalone tauropine dehydrogenase can be classified as an M-type opine dehydrogenase (32). On the other hand, D-lactate dehydrogenase presenced in all muscle tissues equally may be related to slow energy production during slow locomotion. The higher affinity for pyruvate and strong inhibition by pyruvate indicate that abalone D-lactate dehydrogenase can be classified as an H-type enzyme. Higher tauropine dehydrogenase activity and tauropine contents in the columella muscle showed that abalone may consume a lot of energy during shell fixation.

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