Characterization of the glutamate dehydrogenase activity of *Gigantocotyle explanatum* and *Gastrothylax crumenifer* (Trematoda: Digenea)

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Abstract Glutamate dehydrogenase (GLDH) (EC 1.4.1.3) is a ubiquitous enzyme, which is present at the protein and carbohydrate metabolism crossroads. The enzyme activity was investigated in biliary and rumen amphistomes, *Gigantocotyle explanatum* and *Gastrothylax crumenifer*, respectively, infecting the Indian water buffalo *Bubalus bubalis*. The enzyme activity was consistently higher in *G. explanatum* as compared to *G. crumenifer*, where NAD(H) was utilized as coenzyme and the pH optima was recorded at 8. The K_m and V_{max} values for α -ketoglutarate were 2.1 mM and 9.09 units in *G. explanatum*, whereas 3.03 mM and 1.90 units in *G. crumenifer*, respectively. Among the allosteric modulator nucleotides, AMP, ADP, ATP, GMP, CMP and UMP, only AMP enhanced GLDH activity in *G. crumenifer* while ADP was stimulatory in *G. explanatum*. The amino acid leucine stimulated the GLDH activity in both the amphistomes while alanine was stimulatory only in *G. crumenifer*. Pronounced interspecific differences in response to different metabolic inhibitors like diethyldithiocarbamate, semicarbazide hydrochloride and mercurial ions were also observed. The osmotic stress alters the enzyme activity, particularly in hypertonic saline the GLDH activity increased significantly ($p < 0.01$) in *G. explanatum*, while insignificant effects were observed in rumen dwelling *G. crumenifer*. Histochemistry revealed region/tissue specific distribution of GLDH with prominent staining in tissues like vitellaria,

lymph system and tegument/subtegument, thus showing specific distribution of GLDH indicating differential metabolic state. Such intergeneric differences in GLDH activity could also be a consequence of occupying different microenvironments within the same host.

Keywords Glutamate dehydrogenase, Kinetics, Allosteric modulators, Osmotic stress, *Gigantocotyle explanatum*, *Gastrothylax crumenifer*

Introduction

Glutamate dehydrogenase (GLDH) is responsible for reversible reductive amination of α -ketoglutarate and because of its position at the crossroads to protein and carbohydrate metabolism, GLDH is considered an important enzyme playing a vital role in amino acid metabolism and ammonia fixation. The enzyme has been widely used as a clinical marker for various parasitic infections (Yang et al. 1998; Schuster et al. 2003; Phiri et al. 2007), and it has been characterized in a number of parasitic species as well (Mustafa et al. 1978; Cutillas et al. 1992; Krauth-Siegel et al. 1996; Dominguez and Rodriguez-Acosta 1996; Skuce et al. 1999; Vilas et al. 2002). GLDH from a wide variety of sources responds differently to various metabolic stimulators or inhibitors (Frieden 1959; McNeil and Hutchinson 1971; Stephen et al. 1978; Rao and Husain 1979). In a particular environment the GLDH function is reported to be under strict allosteric control (Mustafa et al. 1978). The enzyme activity particularly in the aquatic organisms is greatly influenced by different osmolarities of the medium through which an

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organism traverses (Florkin and Schoffeniels 1969). In spite of the physiological significance of GLDH very limited information is available on this enzyme in helminths and almost nothing is known about amphistomes.

The present study was undertaken to investigate various kinetic and functional aspects of GLDH in two amphistomes (Trematoda) inhabiting two different microhabitats within the same host.

Materials and methods

The *Gigantocotyle explanatum* and *Gastrothylax crumenifer* were collected from infected bile duct and rumen, respectively, of Indian water buffalo, *Bubalus bubalis*, slaughtered at the local abattoir. Fresh 10% (w/v) cell free total tissue extract of each parasite was separately prepared at 4°C in 70 mM phosphate buffered saline (PBS), pH 7.4, containing 0.25 M sucrose using a glass teflon tissue homogenizer, centrifuged at 3000 x g for 10 minutes, debris was discarded and clear supernatant was used for different studies.

Enzyme assay

The GLDH activity was determined spectrophotometrically at room temperature (25°C) by monitoring NADH utilization at 340 nM using the method of Schmidt (1974) in the direction of glutamate formation. The assay mixture of 1 ml contained 0.2 mM NADH, 50 mM triethanolamine-HCl buffer (pH 8.0), 100 mM ammonium acetate, and 7 mM α -ketoglutarate and enzyme preparation. Controls were run either by omitting the substrate or inactivating the enzyme at 80°C.

Substrate concentration

Different concentrations of α -ketoglutarate ranging from 0.5 to 10 mM were used in order to see their effect on the enzyme activity. The Michaelis constant, K_m and the V_{max} values were determined from the Lineweaver-Burk (1934) double reciprocal plots.

pH optima

To determine the pH optima, following buffers each at 70 mM concentration was used: phosphate buffer (pH 6.0–8.0),

triethanolamine-HCl (pH 7–8.5), glycine-NaOH (pH 8–9.5) and phosphate-NaOH (pH 9.5–10).

Effect of nucleotides and amino acids

The stock solutions of nucleotides adenosine mono-, di- and triphosphates (AMP, ADP, ATP), cytidine monophosphate (CMP), guanosine monophosphate (GMP) and uridine monophosphate (UMP); and the amino acids DL-alanine, L-proline, L-arginine, L-glycine and L-glutamine were prepared in the assay buffer taking care to maintain the pH, and a final concentration of 1 mM each in the assay mixture was used to study their effect on the enzyme activity.

Metabolic inhibitors

The assay mixture contained various inhibitors like diethyldithiocarbamate, semicarbazide hydrochloride, iodoacetate and mercuric chloride each at 1 mM final concentration, whereas 10 mM ammonium sulfate and sodium sulfate were tested to see their influence on enzyme activity.

Osmotic and ionic stress

To study the effect of osmotic and ionic changes on the GLDH activity, ten worms each were incubated in 10 ml of different concentrations of Tyrode's solution, premainained at $37 \pm 2^\circ\text{C}$ (Siddiqi et al. 1975) for 2 hours in a metabolic shaker and thereafter the enzyme activity was determined as described earlier. The osmolarity of normal Tyrode was determined by Osmomat-030 and the instrument was calibrated with double distilled water.

Enzyme unit

The specific activity of enzyme is expressed as micromoles NADH oxidized/mg protein/hour. An extinction coefficient of 6.22 for NADH at 340 nM was employed for calculations.

Protein estimation

Protein content of both the parasites was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as standard. The data was subjected to statistical analysis as described in Sokal and Rohlf (1981).

Histochemical localization

The mature flukes of both the species were embedded in Tissue Tek II (Lab-Tek products, Illinois, USA) at -20°C and sectioned at $10\text{ }\mu\text{M}$ on a cryostat (American Optical Corp., USA). The sections were stained by the method of Lojda et al. (1979). For controls, the sections were either heated at 80°C or the substrate was omitted from the staining solution and processed simultaneously.

Result

It is evident from the data summarized in Table 1, that the level of GLDH in biliary amphistome, *G. explanatum* was significantly higher ($p < 0.01$) as compared to the rumen amphistome, *G. crumenifer*. The Michaelis constant (K_m) and $V_{\max}$ values for α -ketoglutarate were calculated from Lineweaver-Burks (1934) double reciprocal plots (Fig. 1). It can be seen from Table 1, that the $V_{\max}$ values for α -ketoglutarate is considerably higher ($p < 0.01$) in *G. explanatum* while K_m is slightly higher in *G. crumenifer*, whereas, peak enzyme activity was recorded at pH 8.0 in both the amphistomes.

The enzyme modulators, particularly, nucleotides and amino acids produced varying degree of alterations in the enzyme activity (Fig. 2). In *G. explanatum* the GLDH was slightly stimulated by 1 mM ADP while AMP, GMP, CMP and UMP produced inhibitory effects. In *G. crumenifer*, AMP significantly ($p < 0.05$) stimulated the GLDH activity, while other nucleotides under study produced different level of inhibitory effects (Fig. 2A). Among various amino acids used, only leucine significantly ($p < 0.01$) enhanced the GLDH activity of *G. explanatum*. In *G. crumenifer*, leucine, alanine and proline significantly stimulated the enzyme activity, whereas influence of other amino acids was insignificant ($p < 0.05$) (Fig. 2B).

The GLDH of both the amphistomes was found to be sensitive to a wide array of metabolic inhibitors (Table 2), which produced different levels of inhibition of enzyme activity. Among various inhibitors used, the enzyme was found to be very sensitive to iodoacetate, semicarbazide hydrochloride and mercury ions as compared to other inhibitors indicating that cationic specificity and anionic efficiency are different according to the origin of enzyme. The differential enzyme response to various metabolic inhibitors or stimulators also reflects the differences in

enzyme moieties, possibly due to their polymorphic nature, which needs to be worked out.

Beside allosteric modulators there was a considerable influence of the osmotic and ionic stress on the GLDH activity of amphistomes, particularly the effect was more pronounced in *G. explanatum* (Fig. 3). The enzyme activity was retarded in the hypotonic medium while the reverse phenomenon was observed in hypertonic saline. In distilled water, *G. explanatum* do not survive, hence enzyme activity was not determined. However, in *G. crumenifer*, absolute levels of the enzyme were not affected by differences in osmolarity over a period of 2 hours, suggesting a great tolerance of the GLDH to wide fluctuations in the osmolarity of incubation medium of rumen amphistome.

The histoenzymological study exhibited differential distribution of the GLDH in various regions of the two parasites (Figs. 5–9). In *G. explanatum* intense enzyme reaction was recorded in vitellaria and lymph system followed by oral sucker, subtegument and uterine tube, while in other regions it was moderately present except intestinal caecae where formazan deposits were almost totally lacking, indicating the absence of enzyme activity (Figs. 5, 7, 9). Contrary to this, in *G. crumenifer* intense enzyme reaction was recorded in intestinal caecae and also in lymph system, subtegument, oral sucker and vitellaria followed by moderate activity in gonads, muscular lining of the acetabulum and parenchyma. The formazan deposits were lacking from the ventral pouch lining of *G. crumenifer* (Figs. 4, 6, 8).

Discussion

The amphistomes under study inhabiting two different microenvironments of the same host showed considerable differences in their GLDH activity. In rumen amphistome, the GLDH activity was found much lower as compared to the biliary amphistome despite the fact that rumen contains a high level of free ammonia due to the activity of intraruminal flora. This study is in agreement with Krvavica et al. (1967) who also reported lower GLDH activity in rumen amphistome, *Paramphistomum cervi* as compared to the liver fluke. The kinetics of GLDH of both the amphistomes under study differed markedly from the mammalian enzyme (Frieden 1959). Higher $V_{\max}$ values of GLDH in *G. explanatum* as compared to *G. crumenifer* indicate that the ammonium ions could

Table 1 Kinetic parameters of glutamate dehydrogenase of amphistome

Parasites	Specific activity*	$V_{\max}$	K_m (mM)	$V_{\max}/K_m$	pH optima
<i>Gigantocotyle explanatum</i>	1.91 ± 0.02 (5)	9.09	2.12	4.28	8
<i>Gastrothylax crumenifer</i>	0.79 ± 0.01 (4)	1.90	3.03	0.63	8

*The specific enzyme activity is expressed as micromoles NADH oxidized/mg protein/hour $\pm$ SEM

Number of experiments is indicated in the parentheses.

Fig. 1 The enzyme activity as a function of α -ketoglutarate concentration (A, C) and the double reciprocal plots of Lineweaver-Burk (B, D) for glutamate dehydrogenase of *G. explanatum* (A, B) and *G. crumenifer* (C, D). Each point represents a mean of at least three replicates $\pm$ SEM

Fig. 2 The effect of allosteric modulators nucleotides (A) and amino acids (B) on the glutamate dehydrogenase of *G. explanatum* and *G. crumenifer*. AMP, ADP, ATP: Adenosine 5'-mono-, -di-, -triphosphate; GMP: Guanosine 5'-monophosphate; CMP: Cytidine 5'-monophosphate; and UMP: Uridine 5'-monophosphate; LEU: Leucine; ALA: Alanine; PRO: Proline; ARG: Arginine; GLY: Glycine; GLU: Glutamine. Atleast three replicates of each experiment were performed

be efficiently salvaged in the biliary amphistomes for the production of glutamate, which in turn may serve as precursor for the synthesis of various non-essential amino acids. The pH optima (pH 8.0) as recorded in the present study is found similar in both the amphistomes indicating

that the acidic rumen (Czerkawski 1986) and alkaline bile duct microenvironments do not influence the pH optima of the parasite GLDH. Similar pH optima for GLDH have also been reported for *Hartmanella culbertsoni* (Rao and Husain 1979).

Table 2 Effect of metabolic inhibitors on the activity of glutamate dehydrogenase of amphistomes

Inhibitors	<i>Gigantocotyle explanatum</i>		<i>Gastrothylax crumenifer</i>	
	Specific activity*	% inhibition	Specific activity*	% inhibition
Control	1.906 ± 0.024 (5)		0.786 ± 0.008 (4)	
Diethyldithiocarbamate	1.217 ± 0.163 (4)	36 (p < 0.05)	0.426 ± 0.39 (4)	54 (p < 0.01)
Semicarbazide hydrochloride	0.00 (3)	100.00	0.016 ± 0.10 (3)	98 (p < 0.001)
Iodoacetate	0.00 (3)	100.00	0.016 ± 0.009 (4)	98 (p < 0.001)
Mercuric chloride	0.016 ± 0.003	99 (p < 0.001)	0.00 (4)	100
Ammonium sulfate	1.569 ± 0.098 (4)	18 (p < 0.01)	0.316 ± 0.79 (4)	40 (p < 0.01)
Sodium sulfate	1.189 ± 0.201 (4)	38 (p < 0.05)	0.719 ± 0.137 (4)	9 (p < 0.05)

*The specific enzyme activity is expressed as micromoles NADH oxidized/mg protein/hour ±SEM

p values found <0.05 were significant.

Figures in parentheses of specific activity column represent number of experiments.

Fig. 3 The effect of osmotic and ionic changes on the activity of GLDH of amphistomes. The biliary amphistome *G. explanatum* did not survive in distilled water (dH₂O), while rumen amphistome *G. crumenifer* remained activess

Figs. 4–9 Histochemical localization of glutamate dehydrogenase in different regions of *G. crumenifer* (4,6,8) and *G. explanatum* (5,7,9). Magnification: x 50. Al, Acetabular lining; Es, Egg shell; Gp, Gonopore; Ic, Intestinal caeca; Os, oral sucker; Pa, parenchyma; Ph, Ventral pouch; STG, subtegument; Tg, Tegument; Ts, Testis; Ut, Uterine tube; Vs, Ventral sucker; Vit, Vitellaria.

The nucleotides, which are known to be allosteric modulators, produced differential effects on the GLDH of two amphistomes. Since 1 mM ADP stimulated the GLDH of *G. explanatum*, it can be inferred that the GLDH of liver amphistome behaves more like mammalian enzyme, which is activated by ADP and inhibited by GTP and ATP (Smith et al. 1975). However, 1 mM AMP produced significant increase in GLDH of *G. crumenifer* but contrary to this the enzyme of *Hymenolepis diminuta* was not affected

by 1 mM AMP, ADP, ATP, IDP, GDP or GTP (Mustafa et al. 1978), whereas the GLDH of *Haemonchus contortus* was inhibited by AMP, ADP, ATP, aspartate and thyroxine (Barrett 1981). The *Plasmodium falciparum* enzyme was not affected by GTP and ADP (Wagner et al. 1998). Beside nucleotides, amino acids also influence the enzyme activity of amphistomes. The stimulatory effect of leucine on amphistome GLDH could be physiologically important as also found for mammalian GLDH (McGivan and

Chappell 1973). The amino acids are known to regulate the intracellular osmotic and ionic balance, which in turn influences the GLDH activity (Florkin and Schoffeniels 1969). A similar situation appears to exist in amphistomes under study where both nucleotides and amino acids act as modulators of GLDH activity. The differential response of amphistome GLDH to the changes in osmotic and ionic balance of the incubation medium reflects physiological adaptation, and possible existence of polymorphic forms as reported for other parasites (Cutillas et al. 1992; Krauth-Siegel et al. 1996; Vilas et al. 2002). Since *G. explanatum* inhabits bile duct where more or less constant environment prevails, the GLDH of this parasite was found to be more sensitive as compared to the rumen amphistomes in which enzyme activity fluctuates in a very narrow range. The enormous food and water intake by the host, and the activity of intraruminal microbial fauna could be responsible for continuous change in the rumen environment; hence the GLDH of *G. crumenifer* appeared to be more tolerant to the osmotic and ionic changes.

The differences in GLDH response to various metabolic inhibitors also reflect interspecific variation in the enzyme moieties of the two amphistomes. The inhibition of GLDH particularly by sodium-dithiocarbamate containing sulphahydryl group may presumably be due to the presence of sulphahydryl (-SH) groups in the enzyme molecules of both the amphistomes as also reported in case of crystalline beef liver GLDH (Olson and Anfinsen 1953). The data on the relevant effect of metabolic inhibitors on the GLDH activity revealed that the liver enzyme was more sensitive as compared to the GLDH of rumen amphistomes.

Intense localization of enzyme activity in lymph system of amphistomes indicates that it could be possibly involved in the amino acid metabolism. According to Dunn et al. (1985), the lymph system of amphistomes appears to function in storage and mobilization of amino acids for subsequent transport to tissues undergoing active protein synthesis. Vitellaria are an active site of protein metabolism and also provide precursor for yolk and egg shell; therefore, intense localization of GLDH in these organs of amphistomes is not surprising. The presence of enzyme activity in the intestinal caecae of *G. crumenifer* and its absence in *G. explanatum* reflects the differences in the nature of gut contents and the nutritional requirements of the two parasites. Enzyme was also found to be conspicuously absent from the microvillar surface of *H. contortus* (Skuce et al. 1999). The strong enzyme activity in subtegument and intestinal caecae of the

rumen amphistomes, *G. crumenifer* may, however, be due to the availability of large amount of free ammonia and other amino compounds in the surrounding ruminal contents. There is a possibility that enzyme might be existing in the polymorphic form, hence showed characteristic localization and also responding differentially to various metabolic inhibitors used in the present study.

Hence, it can be concluded that the similarities found in the nature of GLDH of these amphistomes could be due to their taxonomic proximity but the variations in kinetic properties, response to different enzyme modulators, osmotic stress and differential distribution could be a biochemical adaptation of amphistomes in response to different physico-chemical conditions of the microenvironment. However, further studies are required on the GLDH heterogeneity to better understand habitat-parasite relationship leading to the understanding of physiology of host-parasite interactions.

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