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Nucleotide allosteric regulation of the glutamate dehydrogenases of *Teladorsagia circumcincta* and *Haemonchus contortus*

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ABSTRACT

The expression of glutamate dehydrogenase (GDH; EC 1.4.1.3) in L3 of the nematode *Haemonchus contortus* was confirmed by detecting GDH mRNA, contrary to earlier reports. The enzyme was active in both L3 and adult *H. contortus* homogenates either with NAD^+/H or $NADP^+$/H as co-factor. Although it was a dual co-factor GDH, activity was greater with NAD^+/H than with $NADP^+$/H. The rate of the aminating reaction (glutamate formation) was approximately three times higher than for the deaminating reaction (glutamate utilisation). GDH provides a pathway for ammonia assimilation, although the affinity for ammonia was low. Allosteric regulation by GTP, ATP and ADP of L3 and adult *H. contortus* and *Teladorsagia circumcincta* (Nematoda) GDH depended on the concentration of the regulators and the direction of the reaction. The effects of each nucleotide were qualitatively similar on the mammalian and parasite GDH, although the nematode enzymes were more responsive to activation by ADP and ATP and less inhibited by GTP under optimum assay condition. GTP inhibited deamination and low concentrations of ADP and ATP stimulated weakly. In the reverse direction, GTP was strongly inhibitory and ADP and ATP activated the enzyme.

1. Introduction

The nematodes *Teladorsagia circumcincta* and *Haemonchus contortus* are economically important gastric parasites of sheep for which new control measures, either chemical or vaccines, are urgently needed. Essential metabolic enzymes, such as those involved in glutamate metabolism, are potential targets, particularly if they are absent from the host or if there are unusual properties or significant structural differences in the protein. Glutamate dehydrogenase (GDH) is a universal enzyme which reversibly catalyses the formation of glutamate from ammonia and 2-oxoglutarate (2-OG) (Hudson and Daniel, 1993). Unlike mammals, abomasal parasites have both glutamate dehydrogenase activity (Rhodes and Ferguson, 1973; Muhamad et al., 2011; Umair et al., 2011b) and also the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway (Umair et al., 2011a), which is used for ammonia incorporation into glutamate by plants and bacteria (Masclaux-Daubresse et al., 2006; Labboun et al., 2009). Although the GS–GOGAT pathway is functional in adult *H. contortus* and L3 and adult *T. circumcincta* (Umair et al., 2011a), no GOGAT activity was detected in homogenates of sheathed L3 *H. contortus*, even after incubation with ammonia, which up-regulated GOGAT activity in L3 *T. circumcincta* (Umair et al., 2011a). Surprisingly, a developmental expression study suggested GDH was also not active in L3 *H. contortus*, whereas it was present in L4 and adult worms (Skuce et al., 1999). There are no reports of studies involving enzymatic assay of GDH in L3 *H. contortus* which could confirm the absence of both ammonia assimilatory pathways in L3.

Despite the similarity of the GDH genes in *H. contortus* and *T. circumcincta* (Umair et al., 2011b), there may be differences in the properties of *H. contortus* and *T. circumcincta* GDH activity in L3 and adult worm homogenates (Muhamad et al., 2011) and recombinant *Tc*GDH (Umair et al., 2011b). Rhodes and Ferguson (1973) reported that purified adult *H. contortus* GDH had an absolute requirement for NAD^+/H and less than 2% of that activity with $NADP^+$/H, which would classify it as NAD^+ specific (E.C. 1.4.1.2) and not a dual co-factor (E.C. 1.4.1.3) or $NADP^+$ specific enzyme (E.C. 1.4.1.4). This contrasts with GDH activity in homogenates of L3 and adult *T. circumcincta* (Muhamad et al., 2011) and recombinant *Tc*GDH (Umair et al., 2011b) which were active with either co-factor, consistent with a dual co-factor GDH, as are mammalian enzymes (Hudson and Daniel 1993). Recombinant *Tc*GDH had almost equal activity with the two co-factors, whereas activity in homogenates was greater with NAD^+ than $NADP^+$. The lack of activity of *H. contortus* GDH with $NADP^+$ is unexpected, since the predicted amino acid sequences of *H. contortus* and *T. circumcincta* (Umair et al., 2011b, Supplementary Fig. 1) had 96% similarity and 91% identity and the binding sites and

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conserved residues were largely identical in the proteins of the two nematode species.

Nematode GDH appeared to differ from mammalian enzymes in the response to short-term allosteric regulators, which are not directly involved in the reaction catalysed by GDH. In contrast to mammalian GDH (Fang et al., 2002; Kim et al., 2003), *T. circumcincta* GDH activity in both directions has been reported to be stimulated by 1 mM ATP (Muhamad et al., 2011; Umair et al., 2011b), while *H. contortus* GDH was slightly inhibited in the deaminating direction (Rhodes and Ferguson, 1973). In mammals, inhibition of GDH by GTP and ATP is believed to result from increased binding affinity and reduced rate of release of the product (Koberstein and Sund, 1973), whereas ADP acts as an enzyme activator by aiding product release (Smith and Stanley, 2008). ATP can be either stimulatory or inhibitory to mammalian GDH (Fang et al., 2002; Kim et al., 2003), depending on the concentration, as ATP can bind to either the GTP or ADP binding site.

In the present study, first the expression of GDH in L3 *H. contortus* was confirmed by PCR, followed by a comparison of the kinetic properties, including the role of the co-factors in both L3 and adult *H. contortus* homogenates. The concentration-dependent effects of each of the three nucleotide regulators on GDH activity in both the aminating and deaminating directions of L3 and adult worm GDH revealed differences in sensitivity to nucleotide regulators of mammalian and nematode GDH.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless stated otherwise. Use of experimental animals has been approved by the Massey University Animal Ethics Committee.

2.1. Parasites

L3 *H. contortus* or *T. circumcincta* were cultured from the faeces of sheep infected with pure strains of parasites and stored in reverse osmosis (RO) water at 10 °C for the former and 4 °C for the latter species. Sheathed larvae were either freshly collected or prior to each experiment; L3 were baermannised in RO water to remove inactive worms and re-suspended in assay buffer. Adult worms were recovered from the abomasa of infected sheep as described previously (Umair et al., 2011c). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and placed in assay buffer.

2.2. Expression of H. contortus GDH mRNA

The expression of *H. contortus* mRNA in L3 and adults was accessed by reverse transcriptase PCR. Total RNA was extracted from L3 and adult worms using Trizol (Invitrogen) according to the manufacturer's instructions and the first strand cDNA synthesised using the iScript select cDNA synthesis kit (BioRad). 20 ng cDNA was used as template in a PCR using the primers and amplification conditions described by Skuce et al. (1999). The resulting L3 amplification product was sequenced after cloning into pCR4 using the TOPO PCR TA cloning kit for sequencing (Invitrogen).

2.3. Preparation of homogenates

Approximately 50,000 L3 were centrifuged at 600 *g* for 5 min, washed twice by re-suspending in buffer followed by centrifugation, then finally re-suspended in 1 mL buffer. The concentrated L3 suspension or approximately 5 mg of clumped adult worms was transferred to a chilled mortar, frozen at −20 °C for at least 15 min and homogenised with a chilled pestle and mortar. The protein concentrations of homogenates were determined by the method of Bradford (1976).

2.4. Enzyme assays

2.4.1. Kinetic properties of H. contortus GDH

All assays were performed at 30 °C on three homogenates of sheathed L3 and two homogenates of adult worms unless stated otherwise, using 50 μg homogenate protein and 100 mM phosphate buffer in a total volume of 1 mL assay mixture.

GDH activity in L3 and adult worms was determined both for the deamination and amination reactions. Routine assays were carried out in 100 mM phosphate buffer (pH 8.5 for the deamination reaction and pH 8 for the amination reaction). Enzyme activity (V_{max}) was determined by the rate of production of NADH/NADPH in the deamination reaction or by the rate of utilisation of NADH/NADPH in the amination reaction, measured spectrophotometrically at 340 nm.

(1) In homogenates of sheathed L3, the optimum pH was determined over the pH range 5.5 to 9.5 in both direction, with substrate concentrations of 5 mM glutamate in the deaminating direction and with 1 mM 2-OG and 50 mM NH_4Cl in the opposite direction. Subsequent assays were carried out at pH 8.5 for the deamination reaction and pH 8 for the amination reaction.
(2) The apparent K_m for co-factors was determined in homogenates of sheathed L3 and adult worms with $NAD(P)^+$ concentrations from 0–3 mM with 5 mM glutamate (in the deamination reaction) or 0–0.5 mM NAD(P)H with 1 mM 2-OG and 50 mM NH_4Cl in the amination reaction.
(3) The apparent K_m for glutamate was determined in homogenates of sheathed L3 and adult worms in reaction mixtures containing 0–15 mM glutamate and 2 mM NAD^+ or $NADP^+$. The apparent K_m for 2-OG was determined with 0–3 mM 2-OG, 50 mM NH_4Cl and 0.2 mM NADH or NADPH and the apparent K_m for ammonium with 0–250 mM NH_4Cl, 1 mM 2-OG and 0.2 mM NADH or NADPH.

2.4.2. Nucleotide allosteric regulation of H. contortus and T. circumcincta GDH

The effects of the addition of increasing concentrations of GTP, ATP or ADP were determined in homogenates ($n = 2$) of sheathed L3 and adult *H. contortus* and *T. circumcincta* both in the deamination and amination reactions with NAD^+/H as co-factor The reactions were carried out with concentrations of substrates and optimum buffer pH known to produce maximal enzyme activity. The buffer pH for the deamination and amination reactions were pH 8.5 and pH 8 for *H. contortus* and pH 7.5 for both reactions for *T. circumcincta* homogenates. The reaction mixtures contained 1 mM 2-OG, 50 mM NH_4Cl, 0.2 mM NADH and 0–5 mM ATP, GTP or ADP in the amination reaction and 5 mM glutamate, 2 mM NAD^+ and 0–5 mM ATP, GTP or ADP in the deamination reaction.

2.5. Data analysis

Replicate data are presented as mean ± SEM. Graph Prism v5 was used to plot kinetic data and estimate K_m and V_{max}. Student's t-test was used to compare K_m and V_{max} values.

3. Results

3.1. Expression of H. contortus GDH mRNA

H. contortus GDH cDNA was detected in both L3 and adults using reverse transcriptase PCR. The amplification products were both of