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CO_2-fixing enzymes during moulting from third larval to fourth larval stage of *Anisakis simplex* and *Hysterothylacium aduncum* (Nematoda: Anisakidae)

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Abstract The fixing of CO_2 is an important metabolic process for many organisms. In the anisakid nematodes, CO_2 has been shown to be necessary for their development, at least in vitro. The presence of CO_2 stimulates the moulting (M3) of the larvae from the third (L3) to the fourth (L4) stage and prolongs the survival, at least, in vitro. We determined the activity of CO_2-fixing enzymes, common to many organisms, in two anisakids: *Anisakis simplex*, a parasite of cetaceans, and *Hysterothylacium aduncum*, a parasite of fish. Although no activity was detected for pyruvate carboxylase or carboxylating-malic enzyme, we detected phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC) activity. In *A. simplex*, PEPCK was clearly higher than that of PEPC throughout the moulting process studied. In *H. aduncum*, although the activity of both enzymes was of similar magnitude, they showed different behaviour; PEPCK activity decreased after the moulting to L4, PEPC activity increased so that the ratio PEPCK/PEPC activity decreased from 1.90 before moulting to 0.59 after.

Introduction

Anisakis simplex and *Hysterothylacium aduncum* are anisakid nematodes which parasitize cetaceans and fish, respectively, although their infective form, third stage larvae (L3), is found almost worldwide in fish and aquatic crustaceans. When placed in an in vitro culture, the presence of CO_2 stimulates the moulting (M3) of the larvae from the third (L3) to the fourth (L4) stage and prolongs the survival (Sommerville and Davey 1976; Iglesias et al. 1997, 2001, 2002). However, the mode of action of CO_2 in moulting from L3 to L4 (M3) is not known and no information is available on the CO_2-fixing enzymes of these parasites. Two enzymes, which can fix CO_2, have been detected and purified in *Ascaris suum*; phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme, the latter functioning mainly by decarboxylation (Van den Bossche and Borgers 1973; Landsperger and Harris 1976; Wilkes et al. 1982; Park et al. 1984; Rohrer et al. 1986; Mallick et al. 1991). Phosphoenolpyruvate carboxylase (PEPC), a typical enzyme in plants, has been detected in filariae such as *Molinema dessetae* (Loisseau et al. 1993). Although infrequent in helminths, pyruvate carboxylase (PC) has been detected in *Moniezia expansa* and *Echinococcus granulosus* but not in *Ascaris* (Barrett 1981). The aim of the present study is to determine the occurrence and variations in activity of these CO_2-fixing enzymes during M3.

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Materials and methods

Collection of L3 larvae and cultivation

The L3 of *Hysterothylacium aduncum* were collected from horse mackerel (*Trachurus trachurus*) while the L3 of *Anisakis simplex* were obtained from blue whiting (*Micromesistius poutassou*). All of the latter were either free in the body cavity or encapsulated on the viscera. After washing the larvae with saline solution (NaCl, 0.9%), samples were frozen (−80°C) for enzyme determination, separating the free specimens from those which were encapsulated in the case of *A. simplex*. The rest of the non-encapsulated larvae were axenized and individually placed in culture, in a modified RPMI-1640 medium at 13°C (*H. aduncum*) and 37°C (*A. simplex*) (Iglesias et al. 2001, 2002), in order to determine the exact stage of development of each larva.

Preparation of the extract and protein determination

The following samples were taken and frozen (−80°C) for enzyme determination: L3 after 48 h of cultivation, L4 recently moulted and L4 24 and 48 h after moulting (10 larvae per sample for *H. aduncum* and 5 for *A. simplex*). For enzyme determination, the samples were thawed, and, after three freezing-thawing cycles, were homogenized in a potter with 0.5 ml of Tris-saccharose buffer (0.01 M:0.25 M) pH 7.4 with addition of 1 mM reduced glutathione and 1 mM EDTA (ethylene diamine tetraacetic acid). Once homogenized, the crude extract was centrifuged at 45,000×g (30 min, 4°C). After protein determination by the method of Lowry et al. (1951), using crystalline bovine serum albumin as standard, the supernatant was used for the spectrophotometric measurement of the activity of the enzymes assayed.

Enzyme determination

This was followed spectrophotometrically by the disappearance of NADH from the assay mixture at 340 nm. Assay temperature was 37°C for the *A. simplex* extracts and 13°C for those of *H. aduncum*. The techniques described by Cataldi de Flombaum et al. (1977) were modified and used to determine enzyme activity. The assay mixture to determine PEPCK activity contained: 100 mM Tris-HCl pH 7.4, 1 mM $MnSO_4$, 0.25 mM β-NADH (disodium salt), 1.65 mM IDP (sodium inosine diphosphate), 2.5 U malic dehydrogenase (MDH, E.C. 1.1.1.37), extemporaneous solution of 16 mM $NaCO_3H$ bubbled with CO_2 and extemporaneous solution of 2 mM PEP (sodium phosphoenolpyruvate, monohydrate) and 40 µg of protein from the *A. simplex* extract, or 25 µg from *H. aduncum*, in a final volume of 1.555 ml. The assay mixture for measuring the activity of the carboxilating-malic enzyme (ME) contained: 100 mM Tris-HCl pH 7.4, 2 mM $MnSO_4$, 0.25 mM β-NADH (disodium salt), extemporaneous solution of 16 mM $NaCO_3H$ bubbled with CO_2 and 8 mM sodium pyruvate and 25 µg of protein from the *A. simplex* or *H. aduncum* extract, in 1 ml of mixture. The assay mixture for the measurement of PC activity contained: 100 mM Tris-HCl pH 7.4, 2 mM $MnSO_4$, 0.5 mM β-NADH (disodium salt), 1.5 mM ATP (disodium salt), 5 U malic dehydrogenase (MDH, E.C. 1.1.1.37), extemporaneous solution of 16 mM $NaCO_3H$ bubbled with CO_2 and extemporaneous solution of 16 mM sodium pyruvate and 100 µg of protein extract from *A. simplex*, or 50 µg from *H. aduncum*, in 1 ml of mixture. The assay mixture to measure PEPC activity contained: 100 mM Tris-maleate pH 6.0, 30 mM $MgCl_2$, 0.5 mM β-NADH (disodium salt), 10 U malic dehydrogenase (MDH, E.C. 1.1.1.37), extemporaneous solution of 16 mM $NaCO_3H$ bubbled with CO_2 and extemporaneous solution of 10 mM PEP (sodium salt, monohydrate) and 100 µg of protein from the *A. simplex* or *H. aduncum* extract, in 1 ml of mixture. One unit (U) of enzyme is defined as the quantity of enzyme required to consume 1 µmol of NADH of the reaction mixture in 1 min at the temperature of the assay.

Statistical method

Treatment values were tested for significance by Student's *t* distribution.

Chemicals

Culture medium, fetal bovine serum, substrates, enzymes and other reagents were purchased from Sigma Chemical Co., Boehringer Mannhein and Merck.

Results

Figures 1 and 2 show the velocity of the CO_2-fixing enzymes determined throughout M3 of *A. simplex*. The high velocity of PEPCK with respect to the other enzymes measured can be seen. Under our experimental conditions no significant ME or PC activity was detected at any moment of the process. In Fig. 1, it can be seen that there is no significant difference between the L3, collected free in the general cavity of the fish (L3F), and those which were found encapsulated in the viscera (L3E). It is noteworthy that after 48 h of cultivation (L3–48), the velocity of the enzymes showed different trends: PEPCK (Fig. 1) velocity reduced by 62%

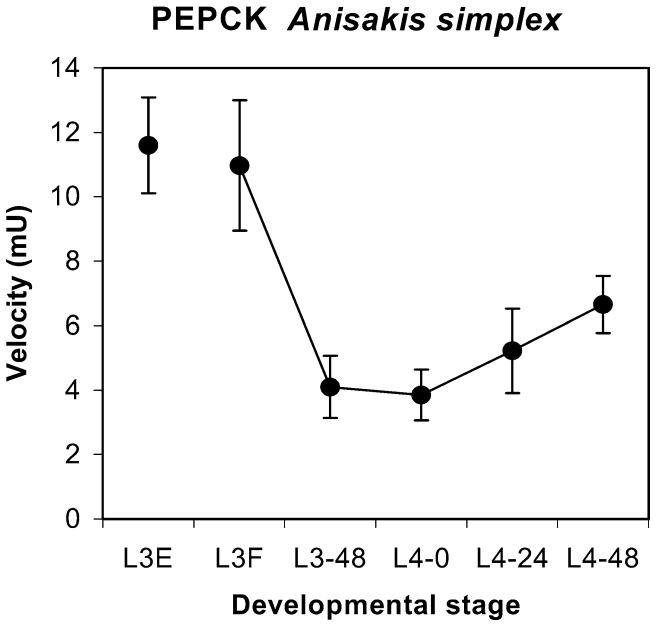

Fig. 1 PEPCK (*filled circle*) velocity during the *Anisakis simplex* M3. Each point is the mean of three to five experiments in triplicate ± SEM

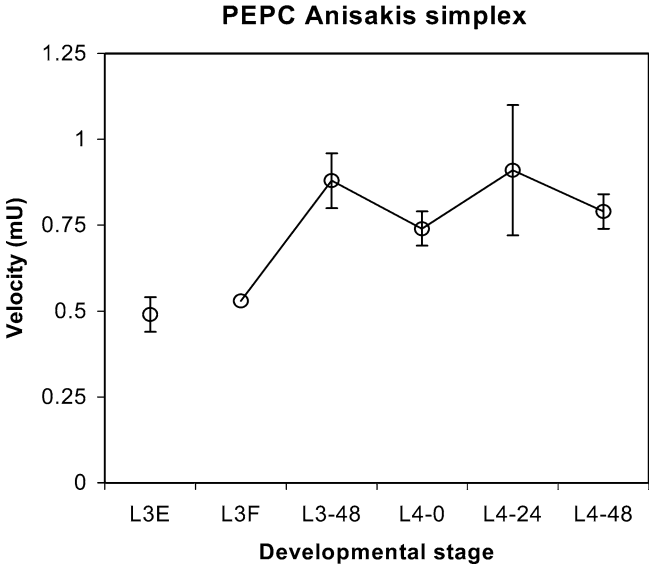

Fig. 2 PEPC (*circle*) velocity during the *Anisakis simplex* M3. Each point is the mean of three to five experiments in triplicate ± SEM

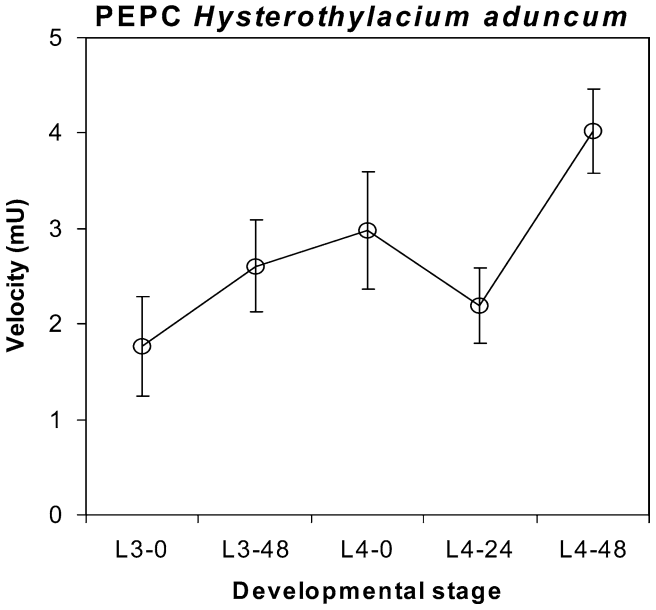

Fig. 4 PEPC (*circle*) velocity during the *Hysterothylacium aduncum* M3. Each point is the mean of three to five experiments in triplicate ± SEM

($P < 0.02$) while that of PEPC (Fig. 2) increased by 66% ($P < 0.04$). The fixing of CO_2 by PEPCK activity (specific activity always between ca. 100 mU/mg and 300 mU/mg protein) is clearly greater than that of PEPC (always between ca. 5 mU/mg and 10 mU/mg protein), increasing slightly following moulting to L4. Finally, the low activity of PEPC does not seem to change significantly throughout the moulting from L3 to L4.

Figures 3 and 4 show the velocity of the CO_2-fixing enzymes determined throughout the M3 of *H. aduncum*. Under our experimental conditions, no significant ME or PC activity was detected in any sample. The PEPCK specific activity decreased slightly when the parasite was placed in the culture (from 54 mU/mg to 49 mU/mg protein after 48 h of cultivation) and only fell significantly 24 h after M3 (11 mU/mg protein; $P < 0.05$ with respect to L3-0, L3-48 and L4-0). However, PEPC tended to show an increase in specific activity throughout the cultivation, this becoming significant 48 h after M3 (from 18 in L3-0 to 40 mU/mg protein in L4-48; $P < 0.03$), only interrupted with a minimum 24 h after M3 (22 mU/mg protein).

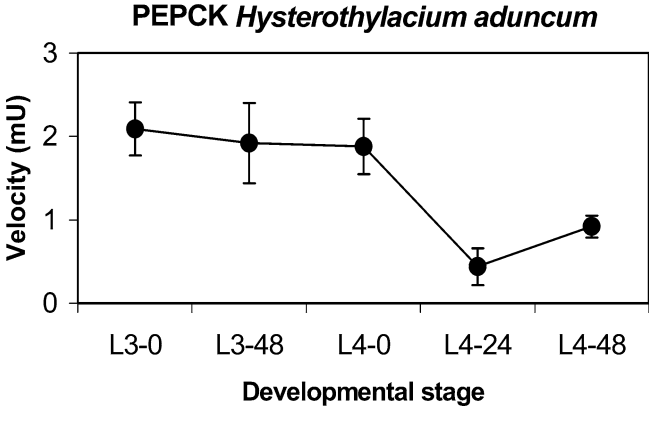

Fig. 3 PEPCK (*filled circle*) velocity during the *Hysterothylacium aduncum* M3. Each point is the mean of three to five experiments in triplicate ± SEM

Discussion

The adults of *Ascaris suum* live in the microaerobic environment of the intestinal lumen of the pig. The main metabolic route in the metabolism of these helminths, typical of gastrointestinal worms, involves the fixing of CO_2 by transforming the phosphoenolpyruvate into oxalacetate, which is then reduced to malate and introduced into the mitochondria to obtain energy. The main CO_2-fixing enzyme, PEPCK, has been purified and its activity determined in adults of *A. suum* by several authors (Van den Bossche and Borgers 1973; Wilkes et al. 1982; Rohrer et al. 1986). Vanover–Dettling and Komuniecki (1989) determined the activity of this enzyme at various developmental stages of the parasite, observing higher activity in L4 (160% of L3) and lower activity in L2 (17% of L3). In *Ascaris*, moulting to L4 takes place in the presence of a high pCO_2. Furthermore, in vivo, it normally takes place after the larva has entered the intestine, where pCO_2 is high (Vanover–Dettling and Komuniecki 1989). These authors have reported that the formation of branched chain fatty acids, the final products of anaerobic metabolism, only occurs when pCO_2 is high (5% or more). Thus, the fixing of CO_2 forms an essential part of the anaerobic metabolism of *Ascaris* and of gastrointestinal anaerobic helminths in general.

In the current study, we have determined the activity of PEPCK and other CO_2-fixing enzymes of two aquatic ascaridoids, *A. simplex* and *H. aduncum*, from their isolation from the fish until 2 days after moulting to L4 in in vitro cultivation. This usually takes place between days 3 and 5 of cultivation. Thus, the study spans the period from pre- to post-moulting to L4.

In *A. simplex*, maximum PEPCK activity was detected in L3, recently extracted from fish (L3F and L3E), and activity in the culture was clearly lower, especially in the pre- and post-moulting to L4 samples (Fig. 1). In *H. aduncum*, the decrease occurred 1 day after M3 (Fig. 3). It is not only interesting to observe variation in activity during the development of the parasite but also the different behaviour of the two parasites studied. In *A. simplex*, it seems that PEPCK is the enzyme responsible for the CO_2-fixing activity. This is notable in the L3 found in the fish (L3E and L3F), although, on being placed in the culture (L3-48), their activity falls to 37% of L3F activity, only increasing in the L4-48 (61% of L3F). On comparing L3-48 and L4-48, a non-significant increase of 162%, similar to the increase of 160% found on comparing L3 and L4 in *A. suum* (Vanover–Dettling and Komuniecki 1989).

The enzyme PEPC is an alternative to PEPCK for the carboxylation of PEP and to obtain oxalacetate. In the case of parasites such as *Molinema dessetae*, it represents a genuine alternative (Loisseau et al. 1993). In *A. simplex*, the activity detected was low (Fig. 2) while in *H. aduncum*, (Fig. 4) activity was low but increased with the development of the parasite, with a decrease in activity in L4-24. This also occurred with PEPCK, suggesting that, at this point, CO_2-fixing is minimal in this nematode but doubles only 24 h later.

The significant decrease in PEPCK activity of L3 after 48 h of cultivation may be due to the change in environmental conditions of the larva or to the activation of the development of the larva induced by these changes. The fact that the decrease in PEPCK activity does not occur in *H. aduncum* when placed in culture leads us to believe that these changes in enzymatic activity must be related more to the activation and posterior development of the worms than to their placing in the culture medium.

On the other hand, whether these changes in the enzyme-specific activities are due to different expression rates of the enzymes, or to varying ratios between possible isoforms of the enzymes, has still to be explained.

In short, these parasites of the digestive tract require CO_2 for their development and obtain this, at least partly, from the environment in which they are found (where there is abundant CO_2) through the fixing activity of enzymes such as PEPCK (in *A. simplex* and *H. aduncum*) and PEPC (in *H. aduncum*). Further study is required in order to determine whether the fixing of CO_2 occurs throughout the other stages of the life cycle of these nematodes and whether this takes place with the help of these or other enzymes.

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