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The tricarboxylic acid cycle in L_3 *Teladorsagia circumcincta*: metabolism of acetyl CoA to succinyl CoA

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ABSTRACT

Nematodes, like other species, derive much of the energy for cellular processes from mitochondrial pathways including the TCA cycle. Previously, we have shown L_3 *Teladorsagia circumcincta* consume oxygen and so may utilise a full TCA cycle for aerobic energy metabolism. We have assessed the relative activity levels and substrate affinities of citrate synthase, aconitase, isocitrate dehydrogenase (both NAD^+ and $NADP^+$ specific) and α-ketoglutarate dehydrogenase in homogenates of L_3 *T. circumcincta*. All of these enzymes were present in homogenates. Compared with citrate synthase, low levels of enzyme activity and low catalytic efficiency was observed for NAD^+ isocitrate dehydrogenase and especially α-ketoglutarate dehydrogenase. Therefore, it is likely that the activity of these to enzymes regulate overall metabolite flow through the TCA cycle, especially when [NAD^+] limits enzyme activity. Of the enzymes tested, only citrate synthase had substrate affinities which were markedly different from values obtained from mammalian species. Overall, the results are consistent with the suggestion that a full TCA cycle exists within L_3 *T. circumcincta*. While there may subtle variations in enzyme properties, particularly for citrate synthase, the control points for the TCA cycle in L_3 *T. circumcincta* are probably similar to those in the tissues of their host species.

1. Introduction

Assays of enzyme activity suggest that nematode species utilise the tricarboxylic acid (TCA) cycle to varying degrees in their life cycle, possibly depending upon tissue oxygen availability. Analysis of the kinetics of the nematode TCA cycle has generally focussed on the anaerobic pathway in the adult stage, especially in *Ascaris suum*, and the activity of the succinate:quinone oxidoreductase/fumarate reductase (SQR/FR) complex (Kita et al., 2002, 1997; Tielens, 1994; Tielens and Van Hellemond, 1998). As the activity in this enzyme complex is very different from that observed with mammalian succinate:quinone oxidoreductase, it may be a potential target for new anthelmintics (Kita et al., 2003). However, oxygen consumption by an electron transfer chain resembling those of mammals has been demonstrated in larvae (Fry and Jenkins, 1984a,b; Takamiya et al., 1993), and in adults of some species (Fry and Jenkins, 1984a,b). Similarly, oxygen consumption by cytochrome oxidase occurs in both L_3 and adult *Teladorsagia circumcincta* (Simcock et al., 2006). In the species and stages in which aerobic energy metabolism occurs, a full TCA cycle may also be functional. Fry and Jenkins (1984a,b) suggested that an electron transfer chain resembling those in mammals may be dependent on the size of the worm and whether its habitat is close to or in the tissue of the host. This may be true also for the presence of a full TCA cycle.

There have been few studies of the TCA cycle in either the larvae of nematodes, or in the adults of species in which oxygen consumption occurs. The few studies in larvae have shown that a complete TCA cycle exists in the infective (usually L_3) larvae of *Ascaris lumbricoides*, *Haemonchus contortus* (Ward and Schofield, 1967), *Ancylostoma tubaeforme*, *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis* (Onwuliri, 1985; Singh et al., 1992; Ward and Fairburn, 1970) and in the soil nematode *Caenorhabditis elegans* (O'Riordan and Burnell, 1989). While the relative levels of enzyme activity in homogenates have been reported, no attempt has been made to characterise the kinetics of the enzymes, so critical insight into the operation of the pathway has yet to be obtained. This is also true of many studies of the TCA cycle in the adult stage of parasitic nematodes, including those performed with *H. contortus* (Ward and Schofield, 1967), *A. lumbricoides* muscle (Barrett, 1978) and *Obeliscoides cuniculi* (Hutchison and Fernando, 1975).

When the full TCA cycle is present in non-nematode animal species, flux through the cycle is controlled by regulation of citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Gibala et al., 2000; Popova and Pinheiro de Carvalho,

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1998; Williamson and Cooper, 1980). The kinetic properties of these enzymes have not been studied in nematode species, though kinetics have been reported for purified α-ketoglutarate dehydrogenase from *Fasciola hepatica* (Diaz and Komuniecki, 1996). For accurate assessment of relative rate of catalysis, K_m values, as well as activity levels, are necessary. Estimates of K_m for TCA cycle enzymes can vary greatly between species. For example, NAD^+ isocitrate dehydrogenase from *Saccharomyces cerevisiae* has a K_m for isocitrate of 0.11 mM (Plaut, 1963), but the human enzyme has a K_m for isocitrate of 2.2 mM (Soundar et al., 2003). While it may appear that variable substrate affinity can be overcome by adding the substrate in high concentrations, this approach may also result in underestimation of activity due to substrate inhibition, as reported for bacterial citrate synthase (Johnson and Hanson, 1974) and pig heart α-ketoglutarate dehydrogenase (Kanzaki et al., 1969).

Ideally, the examination of the properties of an enzyme is conducted with a purified protein, or, for TCA cycle enzymes at least, a mitochondrial separation. Such preparations have been performed for enzyme analysis concerning the adult stages of *H. contortus* and *A. suum*, but for smaller species and life cycle stages, obtaining enough tissue becomes difficult. Fortunately, citrate synthase, NAD^+ isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are typically only present in mitochondria (Beeckmans, 1984; Qi et al., 2008); therefore activity in whole tissue homogenates is likely to be from mitochondrial enzyme only. In contrast, aconitase (Matasova and Popova, 2008) and $NADP^+$ isocitrate dehydrogenase have both cytosolic and mitochondrial forms, so interpretation of activity requires more caution.

We report the activity kinetic properties of the enzymes of the decarboxylation phase of the TCA cycle in homogenates of L_3 *T. circumcincta*. Specifically, citrate synthase (E.C. 2.3.3.1), aconitase (E.C. 4.2.1.3), NAD^+ and $NADP^+$ isocitrate dehydrogenase (E.C. 1.1.1.41 and E.C. 1.1.1.42, respectively) and α-ketoglutarate dehydrogenase (E.C. 1.2.4.2) were characterised using a range of substrate concentrations and in a range of buffer conditions. Results were compared with the characteristics of these enzymes in other species, particularly mammalian host tissues, and identify the likely limiting steps in this phase of the TCA cycle.

2. Methods

2.1. Parasite culture and collection

A pure strain of L_3 *T. circumcincta* was maintained by regular passage through sheep. Animals were housed and parasites collected in accordance with Massey University Animal Ethics protocol AEC 06/117.

2.1.1. Sheep

Male Romney cross lambs between 3 and 6 months of age were housed indoors fed on lucerne chaff and water *ad libitum*. To remove any parasites from previous grazing, the animals were treated with anthelmintic when bought to housing via oral administration of 2 ml/5 kg of a Matrix® (Ancare New Zealand Ltd., Auckland, New Zealand) containing 1 g/l Abamectin, 40 g/l Levamisole and 22.7 g/l Oxfendazole, followed by 1 ml/5 kg of the same treatment the next day. Prior to infection with a monoculture of *T. circumcincta*, faecal examinations were conducted on two subsequent days at varying times of the day, to ensure lambs were parasite free.

2.1.2. Faecal examinations

Faecal testing by egg float involved weighing 2 g of faeces into a sieve over a plastic container, then adding 30 ml of saturated saline solution and grinding the faecal material to a slurry using a pastille. Large particulate material was retained by the sieve but eggs and smaller particles passed through the sieve into the plastic container. The slurry solution was poured into a 30 ml glass flask and covered with a glass cover slip ensuring contact with the solution. After 10 min allowing the eggs to float to the surface and stick to the cover slip, the cover slip was carefully removed onto a glass slide and examined under a standard light microscope (Olympus OM2, Olympus, Japan) for the presence of eggs.

2.1.3. Infection of sheep and L_3 culture

Parasite free lambs were infected orally with 50,000 L_3 *T. circumcincta*. After 18 days of infection, a faecal egg count was conducted to confirm a positive egg count of a minimum of 400 eggs/g. The faecal egg count was conducted using the modified McMaster method (Stafford et al., 1994). If the egg count had reached the threshold of 400 eggs/g, the faeces were collected into a canvas bag.

On the day of collection, faeces were immersed in water and incubated at 27 °C. The next day, faeces were mixed with Vermiculite (Farmlands, NZ) and then incubated for a further 10 days at 27 °C. During incubation the faecal material was turned by hand to aerate twice a week. During incubation, the faeces were placed on tissue paper on a metal sieve, and immersed into a bowl of reverse osmosis filtered (RO) water and incubated at room temperature over night. The faeces were then discarded and the water filtered through a 20 μm sieve to collect L_3, then the collected larvae were Baermannised in RO water for 24 h. The recovered larvae were stored at 4 °C in deionised water until used.

2.2. Preparation of homogenates

Homogenates were prepared by centrifuging 50,000 larvae at 1000*g*, which, after washing, were resuspended in about 0.5 mL MilliQ water. The suspension was frozen at −18 °C for 1 h, then the frozen pellet was broken into small particles and then ground manually using a mortar and pestle until a liquid slurry formed.

A supernatant fraction of the homogenate was prepared by centrifuging the 0.5 ml sample in a 1 ml Eppendorf tube at 10,000*g* for 20 s in a microfuge and collecting the supernatant for use in enzyme assays. The pellet of the 10,000*g* centrifugation was also tested for α-ketoglutarate dehydrogenase activity, after resuspension in 50 mM phosphate buffer pH 7.0. In those cases where isocitrate dehydrogenase or in some cases where α-ketoglutarate dehydrogenase was to be assayed, 50 mM dithiothreitol (DTT) was included in the homogenisation buffer. The volume of the original suspension, the pellet and the supernatant were recorded and the protein concentration determined for each by the Bradford method (Bradford, 1976), then the activity of each enzyme was expressed on the basis of the total protein in the homogenate.

2.3. Enzyme assays

All enzymes were assayed using either an Ultrospec III (LKB Instruments, Mt Waverley, Australia) or a CE 599 (Cecil Instruments Ltd., Cambridge, UK) UV/Visible light spectrophotometer. All assays were performed at 30 °C and at pH 7.50, unless otherwise stated. All chemicals were purchased from Sigma Chemical Co. (St. Louis, USA) unless otherwise stated.

Separate homogenates were used for each replicate datum for each of the enzymes tested. For each method, assays were performed by first measuring the rate of absorbance change in the reaction mixture which contained buffer, homogenate or supernatant along with all cofactors and saturating concentrations of all reactants except for the rate limiting chemical being tested. This rate change in absorbance was regarded as the 'zero' rate. Next, the rate limiting chemical was added, the absorbance rate change