Original Article

Urea Output by L₃ Teladorsagia circumcincta and some Properties of Two Urea Producing Enzymes

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(Received 20 Oct 2012; accepted 09 Dec 2012)

ABSTRACT

Background: Like several other parasites, Teladorsagia circumcincta secretes or excretes urea, but neither the rate of efflux nor the possible metabolic sources of the urea has been considered.

Methods: Parasites were maintained by passage through sheep. Urea efflux was measured using phenol/hypochlorite after treatment with urea aminohydrolase. The kinetics of creatine amidinohydrolase and arginine amidinohydrolase were characterised by coupling the reactions with urea aminohydrolase and glutamate dehydrogenase.

Results: Infective L₃ T. circumcincta secreted or excreted urea at 25% of the rate of NH₃/NH₄⁺. The rate of urea efflux was about 84 pmol h⁻¹ (10³ larvae)⁻¹ over 4 hours, corresponding to about 11 nmol h⁻¹ mg⁻¹ protein. We could not detect urea aminohydrolase activity, but urea production by both creatine amidinohydrolase and arginine amidinohydrolase was characterised by coupling the reactions with urea aminohydrolase and glutamate dehydrogenase.

Conclusion: The activity of creatine amidinohydrolase and arginine amidinohydrolase was sufficient to account for the rate of urea secretion or excretion.

Keywords: Kinetics, Teladorsagia circumcincta, Urea
Introduction

A number of nitrogenous small molecules are known to be secreted or excreted by nematodes. Of these, \( \text{NH}_3/\text{NH}_4^+ \) and urea are probably the most commonly observed. Urea secretion or excretion has been reported in *Ascaris lumbricoides*, *A. galli*, *Nematodirus* spp. and *Panagrellus redivivus*, but was not observed in *Trichinella spiralis*, *Nippostrongylus brasiliensis*, *Ditylenchus triformis*, or, to any significant extent, in *Caenorhabditis briggsae* (1). In the case of *A. lumbricoides* the rate of urea secretion or excretion is slower than that of \( \text{NH}_3/\text{NH}_4^+ \) (2-4). We have shown previously that L, *Teladorsagia circumcincta* secretes or excretes \( \text{NH}_3/\text{NH}_4^+ \) at 0.18-0.6 pmol h \(^{-1} \) larva \(^{-1} \) (5, 6) and, while urea secretion or excretion has also been reported for L, *T. circumcincta*, no supporting data were shown and no indication of the rate was given (7).

The source of the urea secreted or excreted by *T. circumcincta* has not yet been investigated in any detail. Of the enzyme reactions known to involve urea, Rogers (2) reported activity of urea aminohydrolase in *A. lumbricoides* and *Nematodirus* spp. and a much lower activity in *Haemonchus contortus*. He also reported arginine amidohydrolase activity in *A. lumbricoides*, *Nematodirus* spp. and *A. galli* (2) and it has been reported in *T. circumcincta* although no data were given (8, 9), but not in *Toxoplasma gondii* (10, 11). Paltridge and Janssens (4) confirmed that arginine amidohydrolase activity could be observed in *A. lumbricoides*, but of the remaining urea cycle enzymes, only ornithine carbamoyltransferase (E. C. 2.1.3.3) was detectable.

Here we report on the initial kinetics of secretion or excretion of urea by L, *T. circumcincta*. We also attempted to assay urea aminohydrolase (E. C. 3.5.1.5)

\[
\text{urea} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3 \quad 1)
\]

to determine whether the apparent rate of urea secretion or excretion might have been influenced by the activity of this enzyme. We also quantified some of the properties of two urea producing enzymes: arginine amidohydrolase (E. C. 3.5.3.1)

\[
\text{arginine} + \text{H}_2\text{O} \rightarrow \text{ornithine} + \text{urea} \quad 2)
\]

and creatine amidohydrolase (E. C. 3.5.3.3)

\[
\text{creatine} + \text{H}_2\text{O} \rightarrow \text{sarcosine} + \text{urea}. \quad 3)
\]

We show that the activity of either of these amidohydrolases (eqns 2-3) would be sufficient in the absence of any detectable activity of urea aminohydrolase (eqn 1) to account for the observed rate of secretion or excretion of urea.

Materials and Methods

Parasite culture and homogenate preparation

Pure strains of *T. circumcincta* were maintained by regular passage through sheep to provide L. Larvae were concentrated by centrifugation at approximately 1000 × g, washed and then resuspended in 100 mM KH\(_2\)PO\(_4\)-KOH pH 7.5. Where necessary adult *T. circumcincta* were obtained from the abomasal contents of donor sheep as described previously (12). It was not practicable to culture sufficient L, or obtain enough adults to purify the enzymes and so all the work described here was carried out using nematode homogenates. In order to homogenise the nematodes, the suspension was frozen at -20°C and then ground manually at 4°C as described previously (13).

Ammonia and urea determination

The concentration of \( \text{NH}_3/\text{NH}_4^+ \) in was determined spectrophotometrically at 635 nm after reaction with hypochlorite and phenol (14). Concentrations were determined by reference to an \( \text{NH}_4\text{Cl} \) standard.

Ammonia and urea excretion or secretion

The excretion or secretion of \( \text{NH}_3/\text{NH}_4^+ \) and urea was monitored at 37°C in capped Eppendorf tubes. The tubes containing 50000 L, mL \(^{-1} \) in 1 mL 0.8 mM NaH\(_2\)PO\(_4\)-NaOH pH 7.0. Before the concentration of \( \text{NH}_3/\text{NH}_4^+ \)
was determined, the incubation tubes were centrifuged briefly to pellet the larvae and the supernatant was adjusted to pH 7.

To determine the efflux of urea, 1 U urea aminohydrolase was added to 0.5 mL of the supernatant which was incubated at 37°C for 30 min, after which total NH$_3$/NH$_4^+$ was determined. The urea produced was estimated as half of the difference between the total NH$_3$/NH$_4^+$ measured after urea hydrolysis and the NH$_3$/NH$_4^+$ determined without enzymatic treatment.

**Enzyme activities**

By using urea aminohydrolase to hydrolise the urea produced by creatine or arginine aminohydrolase and then employing the reductive amination reaction of glutamate dehydrogenase to incorporate the NH$_4^+$ into glutamate it was possible to monitor the oxidation of NADH at 340 nm. Using S to denote the substrate (arginine or creatine) and P to indicate the corresponding product (ornithine or sarcosine) of the aminohydrolase (eqns 2-3), these reactions can be written

\[ S + H_2O = P + \text{urea} \]

\[ \text{urea} + H_2O = CO_2 + 2NH_3 \]

\[ 2 \alpha\text{-ketoglutarate} + 2NH_3 + 2\text{NAD}^+ = 2\text{glutamate} + 2\text{NADH} + 2H_2O, \]

so the observed rate of NADH oxidation was assumed to be twice the rate of the amidohydrolase reaction. Details of the assays are provided in the relevant figure legends. Two approaches were employed to attempt to measure the activity of endogenous urea aminohydrolase. The first coupled the production of NH$_4^+$ to the reductive amination reaction of glutamate dehydrogenase in which the oxidation of NADH was monitored at 340 nm. The second approach involved detecting the NH$_3$/NH$_4^+$ directly using the chemical assay described in section 2.2.

The protein concentration of homogenates was determined using the Bradford method (15) and bovine serum albumin as the standard. No detergents were employed to solubilise the nematode homogenate as they can interfere with protein assays (16).

**Analysis**

While it is likely that the kinetics of L$_3$ *T. circumcincta* creatine aminohydrolase and arginine aminohydrolase can be modelled using the same mechanism as that of other species (17-19), we have not attempted to employ these models in the analysis presented here. Instead, apparent $K_m$s and $V_{max}$s were obtained by fitting the standard Michaelis-Menten expression to the activity data for each substrate. This approach is consistent with that adopted previously (20, 21). Estimates of apparent $V_{max}$ depend on the enzyme concentration, so comparisons between L$_3$ and adult should be made with caution, but such comparisons can be made more reliably for estimates involving the same lifecycle stage. Estimates of apparent $K_m$s are independent of enzyme concentration, so comparisons between values for L$_3$ and adult preparations can be made with more confidence.

All nonlinear regression and the other analyses described below were carried out using R (22).

**Results**

**Accumulation of NH$_3$/NH$_4^+$ and urea**

As we have previously reported (5, 7) NH$_3$/NH$_4^+$ accumulated in a medium containing L$_3$ *T. circumcincta*. However, treatment of the medium with urea aminohydrolase increased the total concentration of NH$_3$/NH$_4^+$ (Fig. 1), from which we infer that urea was also secreted or excreted by the larvae.

Within 5 min of setting up the suspensions of larvae, the concentration of NH$_3$/NH$_4^+$ was 8 ± 3 μM ($P = 0.03$), which was increased to 24 ± 7 μM ($P = 0.005$) by treatment with urea aminohydrolase (Fig. 1). From this we estimated that the corresponding concentration of urea was about 8 μM. Thereafter, the rate of NH$_3$/NH$_4^+$ accumulation was 0.336 pmol h$^{-1}$ larva$^{-1}$ and this was increased by about 0.17 pmol h$^{-1}$ larva$^{-1}$ by urea aminohydrolase treatment, both of which were approximately con-
stant for the first four hours of incubation. From the difference we infer that there was a constant rate of urea accumulation of 0.084 pmol h<sup>-1</sup> larva<sup>-1</sup> over this time (Fig. 1).

**Enzymatic reactions involving urea**

Sustained attempts to measure endogenous urea aminohydrolase activity were unsuccessful. Neither the enzymatic assay, in which NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> production was monitored using glutamate dehydrogenase, nor the chemical method, involving the measurement of NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> production using hypochlorite and phenol (14), yielded any evidence of the activity of this enzyme. Of course, this was important in our efforts to monitor urea secretion or excretion and the activity of both arginine and creatine amidinohydrolase.

The activity of creatine amidinohydrolase was small (V<sub>max</sub> = 48 ± 2 nmol min<sup>-1</sup> mg<sup>-1</sup> protein) and the apparent K<sub>m</sub> was 1.1 ± 0.1 mM (Figure 2A).

**Fig. 1:** Initial accumulation of total NH<sub>3</sub> (■), NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> (□) and urea (○) in suspensions of 50000 larvae mL<sup>-1</sup> in 0.8 mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 7 incubated in sealed Eppendorf tubes at 37ºC. Total NH<sub>3</sub> is the concentration of NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> measured in the medium after treatment with urea aminohydrolase. Half of the difference between total NH<sub>3</sub> and the concentration of NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> observed without enzyme treatment was used to estimate the urea concentration. The solid lines are the least squares fit to all of the appropriate data. The error bars represent ± SEM (n = 3).

**Fig. 2:** Rate of creatine hydrolysis in homogenates of L<sub>3</sub> T. circumcincta as a function of creatine concentration (A) and pH (B). In (A) the smooth curve is the least squares fits of the Michaelis-Menten equation to all the data. The error bars represent ± SEM for n = 4 (A) or n = 2-5 (B). The reactions were carried out at 30ºC using 50 μM α-ketoglutarate, 0.2 mM NADH, 1 U urea aminohydrolase, 1 U glutamate dehydrogenase and 50 μg of homogenate protein in in 100 mM phosphate buffer. In (B) 5 mM creatine was used.

The activity was greatest at pH 8 and declined at more acidic and alkaline pHs with apparent pK<sub>s</sub> of 7.1 and 8.9 (Figure 2B). The activity of creatine amidinohydrolase was not affected by Fe(II), but was strongly inhibited by Fe(III), Mg(II), and Cu(II) (Table 1). The activity was slightly stimulated by 2 mM EDTA, but neither ATP nor ADP had any significant effect (Table 1).
In homogenates of adult nematodes \((n = 2)\), creatine amidinohydrolase appeared to have kinetic properties that were very similar to those of the \(L_3\) enzyme shown in Figure 2A. For the adult enzyme, the apparent \(V_{\text{max}}\) was \(45 \pm 3 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}\) and the apparent \(K_m\) was \(0.6 \pm 0.1 \text{ mM}\) (data not shown).

In \(L_3\) \textit{T. circumcincta} the activity of arginine amidinohydrolase was also small \((V_{\text{max}} = 62 \pm 4 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein})\) and the apparent \(K_m\) was \(0.7 \pm 0.2 \text{ mM}\) (Figure 3A), although for adults the \(V_{\text{max}}\) was \(126 \pm 6 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}\) and the apparent \(K_m\) was \(1.4 \pm 0.2 \text{ mM} \ ((n = 2)\). The activity in homogenates of \(L_3\) nematodes was greatest at pH 7.9 and declined at more acidic and alkaline pHs with apparent \(pK_a\)s of 7.4 and 8.7 (Figure 3B). The rate of arginine hydrolysis was halved by 0.1 mM Fe(II) and Mn(II) stimulated the activity slightly (Table 2), consistent with there being no significant loss from the enzyme of the Mn(II) required for activity (23). Low concentrations of Cu(II) or EDTA stimulated the activity of the enzyme, but slightly higher concentrations were inhibitory (Table 2).

**Table 1:** Effect of ions and adenine nucleotides on the activity of creatine amidinohydrolase in homogenates of \(L_3\) \textit{T. circumcincta}

| Conditions | Rate of creatine hydrolysis (nmol min\(^{-1}\) mg\(^{-1}\) protein) (%) |
|------------|-------------------------------------------------------------|
| Control    | 42 (100)                                                   |
| + 0.5 mM FeSO\(_4\) | 40 (95)                                                  |
| + 0.5 mM FeCl\(_3\) | 7 (17)                                                    |
| + 0.5 mM CuSO\(_4\) | 5 (12)                                                    |
| + 0.5 mM MgCl\(_2\) | 12 (28)                                                   |
| + 2 mM EDTA  | 52 (124)                                                   |
| + 0.5 mM ATP  | 40 (95)                                                    |
| + 0.5 mM ADP  | 43 (102)                                                   |

**Table 2:** Effect of ions on the activity of arginine hydrolysis by \(L_3\) arginine amidinohydrolase in homogenates of \(L_3\) \textit{T. circumcincta}

| Conditions | Rate of arginine hydrolysis (nmol min\(^{-1}\) mg\(^{-1}\) protein) (%) |
|------------|-------------------------------------------------------------|
| Control    | 41 (100)                                                   |
| + 0.1 mM FeSO\(_4\) | 19 (46)                                                   |
| + 1 mM CuSO\(_4\) | 30 (73)                                                   |
| + 0.1 mM CuSO\(_4\) | 48 (117)                                                  |
| + 0.1 mM MnCl\(_2\) | 43 (105)                                                  |
| + 1 mM MnCl\(_2\) | 46 (112)                                                   |
| + 0.1 mM EDTA  | 45 (124)                                                   |
| + 1 mM EDTA  | 32 (78)                                                     |

**Fig. 3:** Rate of arginine hydrolysis in homogenates of \(L_3\) \textit{T. circumcincta} as a function of arginine concentration (A) or pH (B). The smooth curve in (A) is the least squares fit of the Michaelis-Menten equation to all the data. The error bars represent ± SEM for \(n = 3\) (A) or \(n = 2-5\). The reactions were carried out at 30°C using 100 μM \(\alpha\)-ketoglutarate, 0.2 mM NADH, 1 U urea amidinohydrolase, 1 U glutamate dehydrogenase and 50 μg of homogenate protein in 100 mM phosphate buffer. In (B) 5 mM arginine was used.

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Discussion

We have shown that the rate of urea secretion or excretion by L₃ T. circumcincta was similar to those reported by Rogers (2) for Nematodirus spp., but higher than those he reported for A. lumbricoides and A. galli (Table 3). The rate of urea secretion or excretion by T. circumcincta was about 25% of that of NH₃/NH₄⁺, which is similar to previous reports (2-4) for A. lumbricoides, Nematodirus spp. and A. galli (Table 3). We have also shown that L₃ T. circumcincta have both arginine and creatine amidinohydrolase activity, but we were unable to detect any urea aminohydrolase activity.

Table 3: Rates of secretion or excretion of urea and NH₃/NH₄⁺ reported for various parasites

| Species             | Rate of secretion or excretion (nmol h⁻¹ g⁻¹ WW) | Relative rate | Reference |
|---------------------|------------------------------------------------|---------------|-----------|
|                     | urea | NH₃/NH₄⁺ | (urea: NH₃/NH₄⁺)  |
| A. lumbricoides     | –    | 200      | –           | (29)      |
| A. lumbricoides     | 17   | 80       | 0.21        | (3)       |
| A. lumbricoides     | 52   | 524      | 0.10        | (4)       |
| Nematodirus spp.    | 312  | –        | –           | (2)       |
| A. galli            | 89   | –        | –           | (2)       |
| T. circumcincta     | 252  | 1008     | 0.25        | this work |

The presence of arginine amidinohydrolase activity is consistent with the observations of activity in A. lumbricoides, Nematodirus spp. and A. galli (2, 4). It is possible that arginine deiminase (E. C. 3.5.3.6), which hydrolyses arginine to citrulline and NH₃/NH₄⁺, might also be active in L₃ T. circumcincta homogenates. However, we think this is unlikely for two reasons. First, control reactions in which the urea aminohydrolase was omitted yielded very little activity, perhaps consistent with the low rates of chemical hydrolysis previously reported (24). Second, while there is also an orthologue of the human arginine amidinohydrolase gene in the Caenorhabditis elegans genome (T24F4.1 or NP_508948), we were unable to identify an arginine deiminase homologue in the C. elegans genome.

Our observation of creatine amidinohydrolase activity may be novel. We have been unable to identify either a previous report of this activity in a nematode or a creatine amidinohydrolase homologue in the C. elegans genome or among the sequences currently available for other nematodes. The maximum activity of creatine amidinohydrolase was observed at the same pH as that of the enzyme from Pseudomonas putida (25) and Alcaligenes sp. (20). However, the apparent Kₘ for creatine is much more similar to that of the P. putida enzyme (Kₘ = 1.33 mM, (26)) than that of the Alcaligenes sp. enzyme (Kₘ = 17.2 mM, (20)), although Schumann et al. (27) gives an estimate of 14.3 mM for the P. putida enzyme. The effects of Fe(II), Cu(II) and EDTA were similar to those reported for the enzyme from Alcaligenes sp. and Arthrobacter ureafaciens (20, 28), although Mg(II) had no effect on the Alcaligenes sp. enzyme (20). The crystal structure of the P. putida enzyme shows that the creatine is stabilised at the carboxyl end by a pair of arginines and at the guanidino nitrogen end by a pair of glutamate residues, and the substrate is sandwiched between a phenylalanine residue and a catalytically significant histidine (18). So, the lower pKₐ in the pH dependence (Fig. 2B) might reflect changes in the protonation of either the glutamates or the histidine and the upper pKₐ probably reflects pH-induced changes in the polarity of the guanidinium group.

Our inability to detect urea aminohydrolase activity may simply reflect a failure to identify the right assay conditions or test the parasite at the appropriate stage in its life cycle. Urea

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aminohydrolase activity has been identified in *A. lumbricoides*, *H. contortus* and *Nematodirus* spp. (2) and nucleotide sequence similar to that encoding the β subunit of the enzyme from *Pseudomonas fluorescens* has been identified in the *Caenorhabditis elegans* genome (hypothetical protein F40G9.5, GenBank accession code: ACO15784.1). However, Rogers (2) reported that the urea aminohydrolase activity was much lower in *H. contortus* than in *A. lumbricoides* and *Nematodirus* spp. and concluded that it was unlikely to protect the nematode from urea in the host. If urea aminohydrolase is present, we conclude that the activity is unlikely to have significant impact on the rate at which urea is secreted or excreted by *L.3 T. circumcincta*.

The rate of urea secretion or excretion was about 0.084 pmol h⁻¹ larva⁻¹ which corresponds to about 11 nmol h⁻¹ mg⁻¹ protein (5). The apparent *V*ₘₐₓ-s for creatine and arginine amidinohydrolase are 48 and 62 nmol h⁻¹ mg⁻¹ protein, respectively, so either enzyme could account for the observed urea output. Moreover, the enzymes have similar apparent *Kₘ*-s, so the most significant factor in determining their relative contribution to urea efflux is the intracellular concentration of each substrate.

**Conclusion**

The activity of creatine amidinohydrolase and arginine amidinohydrolase was sufficient to account for the rate of urea secretion or excretion.

**Acknowledgements**

This work was funded by Beef and Lamb New Zealand, AGMARDT, Universiti Malaysia Sarawak and the Public Service Department, Malaysia. The authors declare that there is no conflict of interest.

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