- There is a galactokinase-like protein in *F. hepatica*
- The protein lacks enzymatic activity with galactose and N-acetylgalactosamine
- The protein binds ATP
- Some trematodes may lack a *bona fide* galactokinase
- Therefore, they may be unable to metabolise galactose via the Leloir pathway
Galactose + ATP $\rightarrow$ Galactose 1-phosphate + ADP
A galactokinase-like protein from the liver fluke *Fasciola hepatica*

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Abstract

Galactokinase catalyses the ATP-dependent phosphorylation of galactose. A galactokinase-like sequence was identified in a Fasciola hepatica EST library. Recombinant expression of the corresponding protein in Escherichia coli resulted in a protein of approximately 50 kDa. The protein is monomeric, like galactokinases from higher animals, yeasts and some bacteria. The protein has no detectable enzymatic activity with galactose or N-acetylgalactosamine as a substrate. However, it does bind to ATP. Molecular modelling predicted that the protein adopts a similar fold to galactokinase and other GHMP kinases. However, a key loop in the active site was identified which may influence the lack of activity. Sequence analysis strongly suggested that this protein (and other proteins annotated as “galactokinase” in the trematodes Schistosoma mansoni and Clonorchis sinensis) are closer to N-acetylgalactosamine kinases. No other galactokinase-like sequences appear to be present in the genomes of these three species. This raises the intriguing possibility that these (and possibly other) trematodes are unable to catabolise galactose through the Leloir pathway due to the lack of a functional galactokinase.

Keywords: GHMP kinase; Leloir pathway; fascioliasis; galactokinase; N-acetylgalactosamine; trematode metabolism
1. Introduction.

Galactokinase (EC 2.7.1.6) catalyses the ATP-dependent phosphorylation of α-D-galactose (Caputto, et al., 1949, Cardini and Leloir, 1953, Holden, et al., 2004, Trucco, et al., 1948). The reaction is required in the Leloir pathway of galactose catabolism. This pathway enables galactose to be converted into the glycolytic intermediate glucose 6-phosphate (Frey, 1996, Holden, et al., 2003). Thus, the reaction catalysed by galactokinase is essential for the utilisation of the six carbon atoms in galactose in energy metabolism. In humans and some other mammals, mutations in the gene encoding galactokinase can result in the inherited metabolic disease type II galactosemia (Holden, et al., 2004, Stambolian, 1987, Timson, 2016, Timson, et al., 2009). The symptoms of this disease are considered to be relatively mild: early onset cataracts which can be resolved by surgery or diet (Bosch, et al., 2002). In recent years, a number of promising and selective inhibitors of human galactokinase have been identified (Lai, et al., 2014, Liu, et al., 2015, Odejinmi, et al., 2011, Tang, et al., 2012, Tang, et al., 2010, Wierenga, et al., 2008).

Sequence and structural analysis shows that galactokinase is part of the GHMP (galactokinase, homoserine kinase, mevalonate kinase, phosphomevalonate kinase) family (Bork, et al., 1993, Timson, 2007). The enzyme’s structure is formed from two lobes, with the active site in the cleft between the lobes (Hartley, et al., 2004, Holden, et al., 2004, Thoden and Holden, 2003, Thoden, et al., 2005, Thoden, et al., 2005). Despite the solution of high resolution structures of the enzymes from several species and numerous kinetic studies, the catalytic mechanism of galactokinase remains controversial. Initially, it was believed that an aspartate
residue in the active site abstracts a proton from the substrate and the resulting, strongly nucleophilic, alkoxide ion attacks the γ-phosphate of ATP. Site-directed mutagenesis studies on rat galactokinase showed that alteration of the potentially catalytic active site reduced the activity to undetectable levels (Chu, et al., 2009). Similar results were seen in the human enzyme, although these were partly due to protein misfolding rather than any direct effect on catalysis (Megarity, et al., 2011, Tang, et al., 2010). Recently further mutagenic studies combined with careful measurement of the pH effects on activity have suggested that the pKₐ of Asp-183 in *Lactococcus lactis* galactokinase is raised to approximately 7, and that the pKₐ of the C₁-OH on galactose is lowered by the proximity of Arg-36. These radically altered pKₐ values would facilitate the proton transfers required for a base-catalysed mechanism (Reinhardt, et al., 2013). Molecular dynamics studies suggested a mechanism in which the aspartate polarises, but does not break, the bond between the hydrogen and oxygen atoms of C₁-OH on galactose (Huang, et al., 2013, Megarity, et al., 2011). This is aided by a number of other, charged residues in the active site and facilitates a direct reaction between the galactose and ATP (Huang, et al., 2013).

Interestingly, some fungi, including the budding yeast *Saccharomyces cerevisiae*, have two galactokinase-like proteins. Gal1p is a functional galactokinase with similar structure and enzymology to the human enzyme (Thoden, et al., 2005, Timson and Reece, 2002). In contrast, Gal3p appears to be catalytically inactive; however, insertion of two additional amino acid residues into the active site gives the protein a low level of activity (Platt, et al., 2000). Its overall structure is similar to Gal1p (Lavy, et al., 2012). Gal3p functions in gene regulation, acting as ligand sensor and gene
activator for the GAL genetic switch which controls the expression of genes associated with galactose metabolism (Sellick, et al., 2008, Timson, 2007). This demonstrates that galactokinase-like structures can act in non-catalytic roles, including signal transduction.

The common liver fluke (*Fasciola hepatica*) is parasitic organism is of considerable medical and veterinary importance since it infects millions of humans (primarily in the developing world) and also farm animals causing billions of dollars of agricultural losses annually (Boray, 1994, Robinson and Dalton, 2009, Schweizer, et al., 2005). Resistance is emerging to current drugs and so there is interest in characterising liver fluke proteins in order to help assess their potential as drug targets (Brennan, et al., 2007). We postulated that galactokinase might be an interesting potential target given the occurrence of galactose moieties in tegumental glycoproteins of the liver fluke (Hanna, 1976). Furthermore, the existence of several groups of compounds with proven ability to inhibit human galactokinase suggests that it would be possible to design inhibitors for the fluke enzyme (Lai, et al., 2014). Here we describe the characterisation of a galactokinase-like protein from *F. hepatica* (*FhGALK*). Interestingly this protein has no detectable enzymatic activity although it does bind to ATP. Our findings have wide implications for the mechanisms and functions of galactokinases.

2. Materials and Methods

2.1 Cloning, expression and purification of *F. hepatica* galactokinase-like protein
The coding sequence for a *F. hepatica* galactokinase-like protein was identified in a part-annotated liver fluke EST library (Ryan, et al., 2008) and amplified using PCR. The amplicon was inserted into pET46 Ek/LIC (Merck, Nottingham, UK) according to the manufacturer's instructions such that FhGALK would be expressed with an N-terminal hexahistidine tag.

Recombinant FhGALK was expressed using a method based on that used for human N-acetylgalactosamine kinase (Agnew and Timson, 2010). The recombinant plasmid was transformed into *Escherichia coli* Rosetta(DE3) and a colony resulting from this transformation picked and grown overnight in 100 ml LB supplemented with 100 μg ml⁻¹ ampicillin and 34 μg ml⁻¹ chloramphenicol with shaking at 37 °C. This culture was diluted into 1 l of LB (supplemented with 100 μg ml⁻¹ ampicillin) and grown, shaking at 37 °C until mid-log phase (as judged by A₆₀₀ nm=0.6-1.0, typically 4 h). At this point the culture was cooled to 20 °C and induced with 2 mM IPTG and grown, shaking at 20 °C for 18 hours. The cells were harvested by centrifugation (4200 g for 15 min), resuspended in buffer R (50 mM Hepes-OH, pH 7.4, 150 mM NaCl, 10%(v/v) glycerol) and frozen at -80 °C.

The protein was purified using the method previously described for *F. hepatica* triose phosphate isomerase (Zinsser, et al., 2013). Briefly, cells were thawed and then disrupted by sonication on ice (three pulses at 100 W for 30 s with 30-60 s gaps for cooling). The extract was clarified by centrifugation (27,000 g for 20 min at 4 °C). The supernatant was applied to a 1 ml nickel-agarose column (His-Select, Sigma) which had been equilibrated in buffer A (50 mM Hepes-OH, pH 7.4, 500 mM NaCl,
10%(v/v) glycerol). The column was washed with 20 ml of buffer A and the protein eluted with three aliquots (2 ml) of buffer C (buffer A supplemented with 250 mM imidazole). Protein containing fractions were identified by SDS-PAGE and dialysed overnight at 4 °C against buffer D (buffer R supplemented with 2 mM DTT). Where SDS-PAGE indicated the presence of some impurities, the protein was repurified on a nickel-agarose column (1 ml). The dialysed protein was applied to the column, which had been pre-equilibrated in buffer A. The column was then washed with 2 ml buffer supplemented with 10 mM imidazole and then twice with 2 ml of buffer A supplemented with 20 mM imidazole. The protein was the eluted with two 2 ml washes with buffer C. The elutions were dialysed overnight at 4 °C against buffer D. The protein was stored frozen at -80 °C in aliquots of 20-100 µl.

2.2 Galactokinase assay

Galactokinase activity was measured by coupling the production of ADP to the reactions catalysed by pyruvate kinase and lactate dehydrogenase, essentially as previously described for human galactokinase (McAuley, et al., 2017, Megarity, et al., 2011, Timson and Reece, 2003, Timson and Reece, 2003). Reactions (150 µl in 96-well plates) contained 20 mM Hepes-OH, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, 0.2 mM NADH, 5 mM phosphoenolpyruvate, 25 mM MgCl₂, 3.4 U pyruvate kinase, 5.0 U lactate dehydrogenase and variable concentrations of ATP (0-5 mM) and monosaccharide (0-5 mM). They were pre-incubated at 37 °C for 10 min before being initiated with 0.5-8000 nM FhGALK. The rate of change in concentration of NADH (which is equivalent to the rate of production of ADP) was monitored at 340
nm in a Multiskan Spectrum platereader (Thermo Scientific) for 40 min with readings taken every 30 s.

2.3 Analytical methods

Analytical gel filtration was carried out using a Sephacryl S300 (Sigma) column of total volume \( V_t \) 49.5 ml and void volume \( V_0 \) 17.0 ml. Approximately 250 µl of a 10 µM solution of protein was applied to this column; the flow rate was approximately 1 ml min\(^{-1}\) and 1 ml fractions were collected. The elution volume \( V_e \) of FhGALK was estimated by measurement of \( A_{280\text{nm}} \) and by SDS-PAGE of the fractions. The partition coefficient \( K_{av} \) was calculated according to the equation: 
\[
K_{av} = \frac{(V_e-V_0)}{(V_t-V_0)}
\]

The elution volumes of four proteins of known molecular mass were determined and these used to determine the linear relationship between \( K_{av} \) and the logarithm of the molecular mass as described previously (Zinsser, et al., 2013, Zinsser, et al., 2014, Zinsser, et al., 2014). This relationship was used to estimate the native, solution molecular mass of FhGALK.

Crosslinking with bis(sulphosuccinimidyl) suberate (BS\(^3\)) were carried out as previously described using 16 µM FhGALK (Zinsser, et al., 2013, Zinsser, et al., 2014, Zinsser, et al., 2014) and 2-1600 µM BS\(^3\) in a final volume of 10 µl. Reactions were allowed to proceed for 30 min, terminated by the addition of SDS-PAGE loading buffer (100 mM tris-HCl, pH 6.8, 24%(v/v) glycerol, 8% (w/v) SDS, 0.02%(w/v) bromophenol blue, 3%(w/v) DTT) and heating to 95 °C for 3 min.
Differential scanning fluorimetry (DSF) was performed as previously described using 3.5 μM FhGALK mixed with 50× Sypro Orange (Sigma; manufacturer’s concentration definition) (Zinsser, et al., 2013, Zinsser, et al., 2013, Zinsser, et al., 2014, Zinsser, et al., 2014). Where required ligands (ATP and/or monosaccharides) were added to a final concentration of up to 5 mM. This concentration is consistent with that used in similar studies on human galactokinase (McAuley, et al., 2017, McAuley, et al., 2018). An apparent dissociation constant \(K_{d,\text{app}}\) for ATP was determined by plotting the change in melting temperature \(\Delta T_m\) against concentration of the ligand \([\text{ATP}]\). These data were then fitted to the equation \(\Delta T_m = (\Delta T_{m,\text{max}} \times [\text{ATP}])/(K_{d,\text{app}} + [\text{ATP}])\), where \(\Delta T_{m,\text{max}}\) is the maximum possible value of \(\Delta T_m\), using non-linear curve fitting as implemented in GraphPad prism 6.0 (Graphpad Software, CA, USA).

Protein concentrations were determined by the method of Bradford, using BSA as a standard (Bradford, 1976).

### 2.4 Bioinformatics, molecular modelling and molecular dynamics

Similar proteins were identified using BLAST searches (Altschul, et al., 1990). The draft genomes for F. hepatica and other trematode species were searched using WormBase ParaSite (http://parasite.wormbase.org/) (Howe, et al., 2016, Howe, et al., 2017). Sequence alignments were calculated using ClustalW as implemented in MEGA5 (Kumar, et al., 2008, Larkin, et al., 2007, Tamura, et al., 2011). Phylogenetic trees were also calculated in MEGA5. Protein molecular masses and isoelectric points were estimated using ProParam from the Expasy suite of programs (http://web.expasy.org/protparam/) (Gasteiger, et al., 2005).
Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) (Kelley, et al., 2015) was used in the intensive mode to generate a model of FhGALK. This model was then computationally solvated and energy minimised using YASARA (http://www.yasara.org/minimizationserver.htm) (Krieger, et al., 2009) to produce the final model of the apo-protein. Ligand binding sites were predicted using 3DLigandSite (http://www.sbg.bio.ic.ac.uk/~3dligandsite/) (Wass, et al., 2010). A model with galactose and MgATP bound was generated by aligning the structure to S. cerevisiae Gal3p (PDB: 3V2U (Lavy, et al., 2012)) and saving a new pdb file which contains FhGALK and the ligands. This structure was then minimised using YASARA. The two models are presented as supplementary data to this paper.

3. Results and Discussion

3.1 F. hepatica expresses a galactokinase-like protein

A DNA sequence with similarity to galactokinase-encoding genes from other species was identified through BLAST searches carried out on a F. hepatica EST library (Ryan, et al., 2008). This enabled the design of specific primers, amplification of the full length DNA sequence and verification of the sequence which was named FhGALK. This sequence has been submitted to GenBank with the accession number **KF700238**. The predicted protein has 494 amino acids, a molecular mass of 53.5 kDa and an estimated isoelectric point of 5.93. The protein sequence showed greatest similarity to a galactokinase-like protein from Clonorchis sinensis (77% similarity). A similar sequence is coded for by a transcript predicted by the draft F. hepatica genome (Cwiklinski, et al., 2015). This predicted transcript, a product of gene
BN1106_s1298B000178 is somewhat longer (688 amino acids; 72 kDa) than the actual transcript in the EST library. This longer sequence has a 38 amino insert between Asn-217 and Ala-217 and a 33 amino acid insert between Met-351 and Thr-352. One exon also appears to be repeated in the sequence predicted from the draft genome. Therefore, the different lengths of the two sequences presumably results either from incorrect prediction of introns or the existence of alternatively spliced variants. The two sequences also differ at a small number of points. In the EST sequence, residue 351 is methionine (compared to isoleucine in the genome-derived sequence), residue 376 is leucine in the EST library and arginine in the genome-derived sequence and residue 447 is serine in the EST library and glycine in the genome-derived sequence.

The coding sequence for FhGALK was inserted into the expression vector pET46 Ek/LIC. This vector directed the expression of a protein of approximately 50 kDa (Figure 1a). Approximately 0.5 mg of protein was produced per litre of bacterial culture. No protein-protein crosslinking was observed with BS3 (data not shown). In an analytical gel filtration experiment, FhGALK eluted with a $K_{av}$ of 0.23 which corresponds to an apparent molecular mass of 48 kDa (Figure 1b). Taken together these results show that FhGALK is a monomeric protein like galactokinases from most bacteria, yeast, trypanosomes and mammals, but unlike those from plants and some unicellular eukaryotes, which are dimeric (Ballard, 1966, Dey, 1983, Foglietti and Percheron, 1974, Lavine, et al., 1982, Lobo-Rojas, et al., 2016, Schell and Wilson, 1977, Sherman and Adler, 1963, Thoden and Holden, 2003, Thoden, et al., 2005, Timson and Reece, 2003, Walker and Khan, 1968).
3.2 *FhGALK* binds ATP but does not catalyse the phosphorylation of galactose or other common sugars

When *FhGALK* (0.5-8000 nM) was assayed with ATP (5 mM) and D-galactose (0.5-16 mM), no activity was detected. Activity has been previously detected with yeast and human galactokinases under the same conditions with enzyme concentrations at the lower end of this range (15-200 nM) (Megarity, et al., 2011, Timson and Reece, 2002). This equates to a detection limit for the specific activity of 4 nmol min⁻¹ µg⁻¹ (assuming an absorbance change of at least 0.1 over 40 min). Thus the activity is either zero or negligible with this substrate.

*FhGALK* also lacked detectable activity with N-acetylgalactosamine, D-galactosamine, 2-deoxy-D-galactose, D-fucose (6-deoxy-D-galactose), D-mannosamine, L-arabinose, N-acetyl-D-mannosamine and N-acetyl-D-glucosamine. This suggests that either the protein has activity with a substrate not tested, or that it does not function as an enzyme.

In the absence of ligands, the protein had a melting temperature (*T_m*) of 67.8±0.4 °C (Figure 2a). No significant difference was seen when D-galactose was added (1mM, 5mM or 10 mM). Similar results were observed with N-acetyl-D-galactosamine, 2-deoxy-D-galactose and N-acetyl-D-glucosamine (data not shown). However, addition of ATP increased the *T_m* of *FhGALK* in a concentration dependent manner, demonstrating that this molecule binds to, and stabilises, the protein (Figure 2b).

The apparent dissociation constant (*K_{d,app}*), derived from this method was 1.4 ± 0.3
mM. Care should be taken in interpreting this value as it is an indirect measure of the affinity based on the effect of the ligand on the protein’s thermal stability. However, it is one to three orders of magnitude greater than the Michaelis constants \( K_m \) reported for fungal and animal galactokinases which are typically in the range 10-500 µM (Ballard, 1966, Lobo-Rojas, et al., 2016, Timson and Reece, 2002, Timson and Reece, 2003, Walker and Khan, 1968). However, it is similar to the \( K_m \) values reported for plant galactokinases, which tend to be in the low millimolar range (Dey, 1983, Foglietti and Percheron, 1976). As with all enzymes, it cannot be assumed that \( K_m \) is equal to the dissociation constant. Nevertheless, this suggests that, while \( FhGALK \) does interact with ATP, it does so with lower affinity than some functional galactokinases. Galactose and ATP together (both 5 mM) had no greater effect than ATP alone. The same result was seen with \( N \)-acetyl-\( \alpha \)-galactosamine, 2-deoxy-\( \alpha \)-galactose and \( N \)-acetyl-\( \alpha \)-glucosamine (data not shown).

### 3.3 The predicted structure of the galactokinase-like protein reveals a key difference at the active site

A molecular model of \( FhGALK \) was built and this predicted that the protein adopts a typical GHMP kinases fold. Two globular domains are orientated in a “v”-shape, with a cleft between the two. The predicted MgATP binding site is in this cleft (Figure 3a). Interestingly, 3DLigandSite did not predict a binding site for galactose or any other monosaccharide (data not shown). The highest ranked template used by Phyre2 to build the model was the galactokinase-like protein from \( Saccharomyces cerevisiae \), Gal3p (PDB: 3V5R; (Lavy, et al., 2012)). Alignment of these two structures resulted in a root-mean square deviation (rmsd) of 3.045 Å over 1865 equivalent atoms.
Alignment with the human galactokinase structure (1WUU; (Thoden, et al., 2005)) identified Asp-205 as the structurally equivalent residue to the putative active site base in human galactokinase (Asp-186). Interestingly this residue is also conserved in the catalytically inactive Gal3p (as Asp-209). An adjacent arginine (Arg-37 in the human enzyme) is also considered to be important in catalysis. This is conserved in FhGALK as Arg-45 and Gal3p as Arg-47. Therefore, the presence of these residues is necessary, but not sufficient, for galactokinase activity. In addition to these two residues, the structure of human galactokinase revealed that the sidechains of Glu-43, Asp-46 and Try-236 and the backbone groups of His-44 and Gly-183 are involved in sugar recognition and binding (Thoden, et al., 2005). Analysis of the model of FhGALK suggests that these are conserved as Glu-51, Asp-46, Tyr-257, His-52 and Gly-202. Thus, the lack of suitable binding residues does not explain the lack of detectable interaction with galactose. It is, of course, possible that the methods used here were not sensitive enough to detect interaction. It is also possible that subtle differences in the conformation of the putative binding site (or orientation of the side chains of these conserved residues) may account for the lack of affinity. It should be noted that such subtle structural differences would not be predicted by homology modelling since this technique uses known, experimentally determined structures as templates and thus tends to replicate conformations in flexible regions (such as active sites) and the orientations of side chains. The three residues (Met-351, Leu-376 and Ser-447) which differ from the sequence derived from the draft genome sequence are all distant from the active site.
Gal3p functions as a ligand sensor in a genetic switch which responds to galactose (Bajwa, et al., 1988, Murthy and Jayadeva Bhat, 2000, Platt and Reece, 1998, Sil, et al., 1999, Suzuki-Fujimoto, et al., 1996, Torchia and Hopper, 1986, Yano and Fukasawa, 1997). In this switch, Gal3p binds to the same ligands as Gal1p (galactose and MgATP). S. cerevisiae also expresses a functional galactokinase, Gal1p (Miyajima, et al., 1984, Vollenbroich, et al., 1999). A key sequence difference between Gal1p and Gal3p lies in the active site. Sequence alignments show that two residues (Ser-171 and Ala-172 in Gal1p) are “missing” in Gal3p. Insertion of a serine and alanine into the corresponding location in the Gal3p sequence converted this enzymatically inactive protein into a galactokinase, albeit one with lower activity compared to Gal1p (Platt, et al., 2000). Inspection of Gal1p structure showed that the Ser-Ala dipeptide lies in a helical segment adjacent to the adenine ring of the bound ATP. The sequence immediately before this helix forms an extended loop, approximately in the shape of a “w”. Unexpectedly, the helical segment is structurally conserved in Gal3p, despite the absence of the Ser-Ala dipeptide. In effect the helix draws residues in from the extended loop and is thus formed from different residues to the structurally equivalent helix in Gal1p. As a consequence, the loop becomes “stretched” and changes in conformation from “w”-shaped to “u”-shaped (Figure 3b). Based on the available structural evidence, it therefore seems that alteration of the shape and length of this loop rather than loss of the helix are responsible for the lack of catalytic activity in Gal3p. In both Gal1p and Gal3p, the loop and helical segment of the protein begin with a proline residue and end with a phenylalanine; in Gal1p this segment spans 12 amino acids and in Gal3p it spans 10 amino acid residues. In FhGALK, the segment contains 11 residues. Many N-
acetylgalactosamine kinases (GALK2; EC 2.7.1.157) also have 11 residues in this segment (Thoden and Holden, 2005). Thus, in terms of structural similarity in this segment, FhGALK lies between the inactive Gal1p and the active Gal3p and appears to be more similar to GALK2, despite lacking activity with N-acetylgalactosamine in our experiments.

3.5 Conclusions

The absence of detectable catalytic activity in FhGALK suggests that either that the protein works with substrate(s) not tested as part of this study or that it does not have a role in catalysis. There are no known examples of Gal3p-like proteins in multicellular eukaryotes (and the protein is not present in all fungi). Nevertheless it is tempting to hypothesise that FhGALK has some role in ligand sensing, perhaps linked to transcription and/or intracellular signalling. The interaction with ATP may be important in this role and we cannot rule out the possibility of other (as yet unidentified) ligands also binding to FhGALK and modulating its function. It is tempting to speculate that the binding of ATP by FhGALK means that it plays some role in sensing the energy status of cells in which it is expressed. If so it would be expected to have binding partners involved in the regulation of energy metabolism. In this context it should be noted that there is a known example of a GHMP-kinase family member acting as a signalling molecule. The Caenorhabditis elegans protein XOL-1 is structurally similar to other GHMP kinase family members; however, it has no known enzymatic activity or small molecule binding partners (Luz, et al., 2003). It functions in the control of development, regulating the choice between male or

Based on the evidence presented here, FhGALK is not the galactokinase of F. hepatica. Since the organism is known to have at least one other Leloir pathway enzyme (UDP-galactose 4′-epimerase) it seems possible that may be a bone fide galactokinase (Zinsser, et al., 2014). This has yet to be identified. Searches of the proteins encoded by the F. hepatica draft genome on WormBase ParaSite using human galactokinase (NP_000145) and Drosophila melanogaster galactokinase (NP_729438) only produced the protein coded for by F. hepatica transcript BN1106_s1298B000178 as a hit. As shown above, the protein product of this transcript has high similarity to FhGALK. Similar results were produced when the proteins annotated as galactokinase from the trematodes Schistosoma mansoni (XP_018654315) and Clonorchis sinensis (GAA52165) were used. Interestingly when these two proteins were used in a BLAST search against human proteins, the top match was N-acetylgalactosamine kinase (GALK2) and not galactokinase (GALK1). This suggests that the proteins annotated as galactokinase in these two species may actually be N-acetylgalactosamine kinases. This conclusion is supported by neighbour joining analysis of the protein sequences. The two trematode “galactokinases” and FhGALK clearly cluster with N-acetylgalactosamine kinases and not with galactokinases (Fig. 4).

Given the lack of detectable enzymatic activity with FhGALK, it would be interesting to test the galactokinase-like proteins in these other trematode species for their
ability to catalyse the phosphorylation of galactose and/or N-acetylglactosamine. 

**As far as we are aware, this has not yet been done.** Furthermore, searches using human or *D. melanogaster* galactokinase produced no other hits in *S. mansoni* and *C. sinensis*. This strongly suggests that all three species of trematode lack a functional galactokinase. Interestingly there appears to be no evidence in the literature for metabolism of galactose by the Leloir pathway by any of these species. Indeed, some earlier studies suggest that glucose and glycogen are the sole energy sources for adult *F. hepatica* (Lloyd, 1986). However, there is evidence for galactose uptake and its incorporation into glycoproteins and glycolipids (Dalton and Joyce, 1987, Hanna, 1976, Podesta and Dean, 1982, Wuhrer, et al., 2004, Wuhrer, et al., 2003). This suggests the intriguing possibility that the Leloir pathway does not operate in some (or all) trematode species, primarily due to the absence of the enzyme which catalyses the first committed step.

This work also has implications for the mechanism of galactokinases. It suggests that the length of a key loop in the active site of the protein will control whether, or not, it is enzymatically active. The shortening of the loop by two residues in Gal3p appears to be correlated with loss of activity in this protein. In *FhGALK*, the loop is shortened by one residue and this may contribute to the lack of activity in this protein.

While the disruption of galactose metabolism (by, for example, the inhibition of enzymes involved in glycoprotein synthesis) may be a viable anthelmintic strategy, it is not obvious how *FhGALK* could be exploited in novel therapies. However, if it
does prove to have other enzymatic activity, this may change if the pathway(s) it contributes to is vital. Similarly, if the protein is subsequently shown to have a role in transcriptional regulation or signalling then antagonism may prove to be pharmacologically interesting.

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**Figure legends**

**Figure 1:** Expression, purification and characterisation of *FhGALK*. (a) Recombinant expression and purification of *FhGALK* was monitored by SDS-PAGE. The upper gel shows the initial purification and the lower gel the re-purification (see Materials and Methods). M, molecular mass markers (masses shown to the left of the gel in kDa); U, cell extract prior to induction; I, cell extract prior to harvesting; W₁, material passing through the column following application of the supernatant following sonication; W₂, material passing through the column following the wash with buffer A; E₁, E₂, E₃ three elutions with buffer B; E, protein containing elutions from the first purification; W₃, material passing through the column following the application of the protein solution; W₄, W₅ material passing through the column following two washes with buffer A; E₄, E₅, two elutions with buffer B. (b) Analytical gel filtration analysis of *FhGALK*. The main graph shows the elution of the protein as a single major peak with an elution volume (*Vₑ*) of 24.7 ml. The inset shows the calibration curve. The gel shows the material present in fractions 24 and 25. M, molecular mass markers (masses shown to the left of the gel in kDa); C, material applied to the column.

**Figure 2:** Stability of *FhGALK* is increased by ATP, but not galactose. (a) First derivative curves from the thermal denaturation of *FhGALK*. The “melting temperature” (*Tₘ*) corresponds to the temperature at the peak of the curve. Note how this is shifted to the right in the presence of ATP. (b) The dependence of the change in “melting temperature” (*ΔTₘ*) on ATP concentration. The points represent the mean of the three experimental determinations of the value and the error bars.
the standard errors of these means. The line is a non-linear fit to the equation

\[ \Delta T_m = (\Delta T_{m,\text{max}} \times [ATP]) / (K_{d,\text{app}} + [ATP]) \] (see Materials and Methods).

**Figure 3:** Predicted structure of FhGALK. (a) The overall fold of the protein is shown in cartoon format with MgATP and shown in space filling format. (b) Close up of the helix and loop structure in the ligand binding site for *F. hepatica* galactokinase-like protein (FhGALK; Trp-154 to Phe-163), human galactokinase (HsGALK1; Pro-134 to Leu-145), *S. cerevisiae* Gal1p (ScGal1p; Pro-163 to Phe-174) and *S. cerevisiae* Gal3p (ScGal3p; Pro-157 to Phe-166).

**Figure 4:** Protein sequence analysis of FhGALK. A neighbour-joining tree with 2000 bootstraps was calculated. *F. hepatica* galactokinase-like protein (FhGALK) was compared with the following species. Galactokinase (GALK): Sm: Schistosoma manso; Fh: Fasciola hepatica; Cs: Clonorchis sinensis; As: Ascaris suum; Rn: Rattus norvegicus; Hs: Homo sapiens; Mm: Macaca mulatta; Bt: Bos taurus; Cf: Canis familiaris; N-acetylgalactosamine kinase (GALK2): Hs: Homo sapien; Pa: Pongo abelii; Mm: Macaca mulatta; Bt: Bos taurus; Rn: Rattus norvegicus; Gg: Gallus gallus; and As: Ascaris suum.
References:


