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Article Type: Regular Paper

Keywords: glycolytic enzyme; trematode; drug target; vaccine target; neglected tropical disease; G3PDH

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Order of Authors: Veronika L Zinsser; Elizabeth M Hoey; Alan Trudgett; David Julian Timson, BSc, PhD

Abstract: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyses one of the two steps in glycolysis which generate the reduced coenzyme NADH. This reaction precedes the two ATP generating steps. Thus, inhibition of GAPDH will lead to substantially reduced energy generation. Consequently, there has been considerable interest in developing GAPDH inhibitors as anti-cancer and anti-parasitic agents. Here, we describe the biochemical characterisation of GAPDH from the common liver fluke Fasciola hepatica (FhGAPDH). The primary sequence of FhGAPDH is similar to that from other trematodes and the predicted structure shows high similarity to those from other animals including the mammalian hosts. FhGAPDH lacks a binding pocket which has been exploited in the design of novel antitrypanosomal compounds. The protein can be expressed in, and purified from Escherichia coli; the recombinant protein was active and showed no cooperativity towards glyceraldehyde 3-phosphate as a substrate. In the absence of ligands, FhGAPDH was a mixture of homodimers and tetramers, as judged by protein-protein crosslinking and analytical gel filtration. The addition of either NAD+ or glyceraldehyde 3-phosphate shifted this equilibrium towards a compact dimer. Thermal scanning fluorimetry demonstrated that this form was considerably more stable than the unliganded one. These responses to ligand binding differ from those seen in mammalian enzymes. These differences could be exploited in the discovery of reagents which selectively disrupt the function of FhGAPDH.

Response to Reviewers: Manuscript No.: BBAPRO-14-17

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General comments: We thank both reviewers for their positive and constructive comments. Below, we set out how we address these comments.

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We have also taken the opportunity to correct a small number of typographical errors in the paper.
Dr David J Timson  
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Profs Goto, Lee and Meyer  
Executive Editors  
BBA - Proteins & Proteomics

8th January 2014

Dear Profs Goto, Lee and Meyer

We would like you to consider our paper entitled "Biochemical characterisation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the liver fluke, Fasciola hepatica" for publication in BBA Proteins & Proteomics.

We believe that this paper is suitable for publication in BBA because:

1. It documents some biochemical properties of GAPDH from a pathogen which causes a neglected tropical disease in humans and billions of pounds of damage to the global agricultural industry. GAPDH has been proposed as a drug target in a number of pathogens, including other parasites. It is also a target of the anti-cancer drug, 3-bromopyruvate.

2. We reveal that the protein lacks a key cleft which has been exploited in the discovery of novel anti-trypanosomal agents. Therefore, alternative approaches will be required to target this enzyme.

3. We show that, compared to mammalian homologues, the protein's oligomeric state responds differently to ligands. This suggests a possible route to selectively antagonizing the enzyme's function.

We hope that you will agree that this paper is suitable for publication and look forward to hearing from you in the near future.

Yours sincerely

David J Timson
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Graphical Abstract (for review)
Biochemical characterisation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the liver fluke, *Fasciola hepatica*

Veronika L. Zinsser, Elizabeth M. Hoey, Alan Trudgett and David J. Timson*

**Highlights**

- FhGAPDH lacks an inhibitor binding pocket present in *Trypanosoma spp* GAPDH
- In the absence of ligands FhGAPDH is a mixture of tetramers and two forms of dimer
- NAD$^+$ and glyceraldehyde 3-phosphate shift the equilibrium towards a compact dimer
- FhGAPDH’s responses to these ligands differ from mammalian GAPDH enzymes
- These ligands greatly stabilise FhGAPDH towards thermal denaturation ($\Delta T_{m}$≈20 K)
Biochemical characterisation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the liver fluke, *Fasciola hepatica*

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Abstract
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyses one of the two steps in glycolysis which generate the reduced coenzyme NADH. This reaction precedes the two ATP generating steps. Thus, inhibition of GAPDH will lead to substantially reduced energy generation. Consequently, there has been considerable interest in developing GAPDH inhibitors as anti-cancer and anti-parasitic agents. Here, we describe the biochemical characterisation of GAPDH from the common liver fluke *Fasciola hepatica* (FhGAPDH). The primary sequence of FhGAPDH is similar to that from other trematodes and the predicted structure shows high similarity to those from other animals including the mammalian hosts. FhGAPDH lacks a binding pocket which has been exploited in the design of novel antitrypanosomal compounds. The protein can be expressed in, and purified from *Escherichia coli*; the recombinant protein was active and showed no cooperativity towards glyceraldehyde 3-phosphate as a substrate. In the absence of ligands, FhGAPDH was a mixture of homodimers and tetramers, as judged by protein-protein crosslinking and analytical gel filtration. The addition of either NAD$^+$ or glyceraldehyde 3-phosphate shifted this equilibrium towards a compact dimer. Thermal scanning fluorimetry demonstrated that this form was considerably more stable than the unliganded one. These responses to ligand binding differ from those seen in mammalian enzymes. These differences could be exploited in the discovery of reagents which selectively disrupt the function of FhGAPDH.

**Keywords:** glycolytic enzyme; trematode; drug target; vaccine target; neglected tropical disease; G3PDH
1. Introduction

In recent years, there has been renewed interest in targeting metabolic enzymes in the treatment of infectious diseases [1]. This presents considerable challenges – most significantly the highly conserved nature of these enzymes which makes the discovery of reagents which discriminate between host and pathogen enzymes difficult. However, the essential nature of pathways such as glycolysis mean that inhibition is likely to lead to growth inhibition and subsequent death of the pathogen. Thus, increased efforts are being made to characterise the biochemistry of metabolic enzymes from pathogens with the long term aim of investigating their potential as drug targets. A key goal of these studies is to identify biochemical differences between the pathogen enzyme and the host enzyme. These differences may then provide the basis for the discovery of compounds which selectively disrupt the activity of the pathogen enzyme.

_Fasciola hepatica_ (the common liver fluke) is a trematode which infects humans and other mammals. It is estimated that approximately 7 million humans are infected with millions more at risk; the majority of these people live in the developing world and fascioliasis is classified as a neglected tropical disease by WHO [2]. In addition, the organism causes a significant economic burden to agriculture globally since it infects domestic herbivores such as cows and sheep. Over ten years ago global losses due to infection were estimated to be several billion US dollars [3]. _F. hepatica_ infections can be treated with the modified benzimidazole drug triclabendazole. This compound is highly effective and kills both mature and juvenile worms. However, resistance to this drug is emerging worldwide and resistance to alternative benzimidazoles such as albendazole has also been reported [4-6]. It is inevitable that triclabendazole resistance will spread and the agricultural burden of _F._
hepatica infections is likely to increase if the trend to warmer, wetter summers in temperate regions continues [7;8].

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is a key enzyme in the glycolytic pathway. It catalyses the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in a reaction which requires a phosphate ion and the redox cofactor NAD⁺ as reactants (scheme 1). Therefore, it performs two functions: it generates reduced cofactor (NADH) which can be reoxidised through oxidative phosphorylation under aerobic conditions and it produces the “high energy” molecule 1,3-bisphosphoglycerate. This compound donates one of its phosphate groups to ADP in the subsequent reaction of the pathway resulting in the direct generation of ATP, a reaction which is critical for the organism’s survival under anaerobic conditions. Thus it is likely that inhibition of GAPDH would result in reduced energy production and reduced growth of the organism. Adenosine derivatives and 1,4-dihydro-4-oxoquinoline ribonucleosides which target the GAPDH of Trypanosoma spp have been shown to kill the organisms at micromolar concentrations [9;10]. Antitrypanosomal compounds which target GAPDH have also been isolated from Keetia leucantha leaves [11]. The antitumour compound 3-bromopyruvate targets human GAPDH resulting in altered energy metabolism and cell death [12-14].

The majority of GAPDH proteins which have been characterised are homotetrameric [15;16]. However, there are some reports of dimeric and trimeric forms of the enzyme [17-19]. There is intra-molecular communication between the four active sites resulting in complex, cooperative ligand binding behaviour. Saccharomyces cerevisiae GAPDH showed positively cooperative NAD⁺ binding (h=1.91) up to half-saturation of the active sites and mildly negative cooperativity (h=0.92) at higher concentrations of ligand [20]. The kinetic
mechanism of the enzyme requires the sequential addition of NAD$^+$, glyceraldehyde 3-phosphate and phosphate and the sequential release of 1,3-bisphosphoglycerate and NADH [21;22]. Following the binding of glyceraldehyde 3-phosphate to the enzyme, a cysteine residue (Cys-149 in the lobster enzyme) reacts with the substrate generating a covalent enzyme-substrate complex. Oxidation by NAD$^+$ then occurs in a reaction which requires a histidine residue to act as a base; the resulting NADH molecule is then exchanged for a new molecule of NAD$^+$. Inorganic phosphate reacts with, and displaces, the bound substrate generating 1,3-bisphosphoglycerate and regenerating the enzyme for a further round of catalysis [16;21;23].

There is also considerable evidence for GAPDH proteins having functions other than their catalytic role in glycolysis. These functions include gene regulation, vesicle transport and the prevention of telomere shortening [24]. In some bacteria, for example, Bacillus anthracis and pathogenic strains of Escherichia coli, the protein is present on the extracellular surface and can interact with the mammalian plasma protein plasminogen [25;26]. Similar interactions occur between the extracellular GAPDH of the protozoan parasite Trichomonas vaginalis and plasminogen and fibronectin [27]. GAPDH is also released into the extracellular environment by F. hepatica and by the blood flukes Schistosoma japonicum and Schistosoma mansoni [28-30]. In Schistosoma bovis, GAPDH is one of ten extracellular proteins which were identified as plasminogen binders [31]. It has been suggested that the plasminogen binding role of bacterial GAPDHs is important in pathogenesis [25]. This may also be the case in trematodes. The extra-cellular location of the enzyme has resulted in trematode GAPDHs receiving considerable attention as a vaccine candidate [32-36]. Vaccination with peptides derived from Schistosoma mansoni GAPDH conferred some protection on mice infected under laboratory conditions [32;34]. Immunisation of goats with
a DNA vaccine encoding *Haemonchus contortus* GAPDH resulted in partial protection against this parasite [37].

The development of novel drugs or vaccines for the treatment of *F. hepatica* infection will require a greater understanding of the biochemistry of this organism. In light of the progress being made in the identification of antitrypanosomal and antitumour compounds which target this enzyme, we cloned, recombinantly expressed and biochemically characterised the GAPDH from *F. hepatica* (FhGAPDH).

2. Materials and Methods

2.1 Cloning, expression and purification of FhGAPDH

The coding sequence for FhGADH was amplified from *F. hepatica* cDNA by PCR. The amplicon was inserted into the expression vector pET-46 Ek/LIC (Merck, Nottingham, UK) by ligation independent cloning according to the manufacturer’s protocol and the insert was sequenced (GATC Biotech, London).

FhGAPDH was expressed in *E. coli* Rosetta(DE3) cells (Merck). The expression vector was transformed into this strain and a single colony used to inoculate 100 ml of LB media (supplemented with 100 μgml⁻¹ ampicillin and 34 μgml⁻¹ chloramphenicol). This culture was grown overnight, shaking at 30 °C. The following day, this was diluted into 1 l of LB (supplemented with 100 μgml⁻¹ ampicillin and 34 μgml⁻¹ chloramphenicol) and the culture grown, shaking at 30 °C until A₆0₀nm=0.6-1.0 (typically ~5 h). The culture was then induced with 0.2 g IPTG and grown at 16 °C for 20-24 h. Cells were harvested by centrifugation (4200g for 15 min), resuspended in ~20 ml of buffer R (50 mM Hepes-OH, pH 7.4, 150 mM NaCl, 10%(v/v) glycerol) and stored, frozen at -80 °C. The protein was purified from these
cells source using nickel-agarose resin (His-Select, Sigma, Poole, UK) as previously described for *F. hepatica* triose phosphate isomerase (FhTPI) [38].

2.2 *Bioinformatics and molecular modelling*

The protein sequence of FhGAPDH was aligned to other, selected GAPDH sequences using ClustalW [39]. This alignment was used to construct a maximum likelihood tree using MEGA [40-42]. ProtParam from the EXPasY suite of programs was used to estimate the molecular mass and isoelectric point of the protein [43]. An initial molecular model of a FhGAPDH monomer was generated using Phyre2 [44]. Four of these monomeric structures were superimposed onto the subunits of human liver GAPDH (PDB: 1ZNQ [45]) using PyMol (www.pymol.org) to generate an initial model of tetrameric FhGAPDH. This initial model was energy minimised using YASARA [46] to generate the final model which is provided as supplementary data to this paper.

2.3 *Analytical gel filtration*

FhGAPDH (250 μl of 120 μM solution) was resolved (flow rate 1 ml min⁻¹) on a Sephacryl-S300 (Sigma, Poole, UK) column of total volume (*Vₜ*) 49.5 ml in 50 mM TrisHCl, 17 mM Tris base, 150 mM sodium chloride, pH 7.4 [47]. The elution volume (*Vₑ*) was determined from the peak in absorbance at 280 nm. The void volume (*V₀*) was determined to be 17 ml by measuring the elution volume of blue dextran. The column was calibrated by determining *Vₑ* for four standards (β-galactosidase, 116 kDa; BSA, 66 kDa; chymotrypsinogen A, 25 kDa; ribonuclease A, 14 kDa). The partition constant (*Kₐᵥ*) was calculated for these standards according to the equation *Kₐᵥ*=(*Vₜ*-*Vₑ*)/(*Vₑ*-*V₀*). These values were used to construct a standard curve (*Kₐᵥ* against the logarithm of the molecular mass) which was used, along with the *Kₐᵥ* for FhGAPDH, to estimate that protein’s molecular mass.
2.4 Other analytical methods

Protein concentrations were estimated using the method of Bradford [48] with BSA as a standard. Protein-protein crosslinking with bis(sulfosuccinimidyl)suberate (BS³) and the determination of protein melting points (T_m) by thermal scanning fluorimetry (TSF) were carried out as previously described [38;49].

2.5 Enzyme kinetics

The rate of reaction catalysed by FhGAPDH was determined by measuring the increase in A₃₄₀nm resulting from the reduction of NAD⁺ to NADH [50]. Reactions (150 µl) were monitored at 37 ºC in a Multiskan Spectrum spectrophotometric plate reader (Thermo Scientific) for 15 min and contained 1 mM NAD⁺, 30 mM sodium arsenate and 100 mM triethanolamine-HCl, pH 7.6. They were initiated by the addition of FhGAPDH (12 nM, monomer). Initial rates were estimated from the linear parts of the progress curves and plotted against substrate concentration. These data were fitted to both the Michaelis-Menten and Hill equations by non-linear curve fitting as implemented in GraphPad Prism 5.0 (GraphPad Software, CA, USA); an F-test was used to select the better fit.

3 Results and Discussion

3.1 A GAPDH from Fasciola hepatica

The primary sequence of FhGAPDH (derived from the cDNA sequence, GenBank: KF700239) encodes a polypeptide of 338 amino acid residues, a calculated, monomeric molecular mass of 37 kDa and an estimated isoelectric point of 7.1. Analysis of the protein sequence showed that FhGAPDH has greatest similarity to other trematode GAPDH enzymes and was well differentiated (bootstrap values >90) from GAPDH enzymes from potential
host species (Supplementary Figure 1). The protein can be expressed in, and purified from, *E. coli* with a typical yield of ~4.5 mg per litre of bacterial culture (Figure 1a). The recombinant protein was active and showed Michaelis-Menten kinetics with glyceraldehyde 3-phosphate as a substrate (Figure 1b).

### 3.2 *FhGAPDH* has a similar overall fold to mammalian GAPDHs

The *F. hepatica* GAPDH protein sequence was used to model the three dimensional structure of the protein in a tetrameric form (Figure 2a). Overall, the predicted fold and oligomeric arrangement was similar to that of the human enzyme (PDB: 1U8F [51]; rmsd 0.994 Å over 8416 similar atoms). The fold is also similar to that from other parasites, for example *Plasmodium falciparum* (PDB: 1YWG [52]; rmsd 1.226 Å over 8358 equivalent atoms) *Trypanosoma brucei* (PDB: 2X0N, [53]; rmsd 2.009 Å over 8522 equivalent atoms) and *Trypanosoma cruzi* (PDB: 4LSM, note that this structure only contains a dimer; rmsd 1.133 Å over 4079 equivalent atoms). Comparison to the structure of the human enzyme also enabled the prediction of the catalytically critical cysteine residue which reacts covalently in the reaction mechanism as Cys-152.

A cleft in the protein, close to the 2'-OH on the NAD⁺, which is present in *Trypanosoma spp* GAPDH has been exploited in the structure based drug design of adenosine analogues. This space is largely filled with the sidechain of Ile-37 in the human enzyme, thus leading to selectivity of these drugs for the parasite enzyme over the human one [54]. Ile-37 is conserved in the FhGAPDH (as Ile-38) and the backbone conformation in this region is similar to the human enzyme and not the *T. brucei* one (Figure 2b). Therefore, this line of drug discovery is unlikely to be fruitful in *F. hepatica*. 

9
3.3 The equilibrium between FhGAPDH tetramers and dimers is affected by ligands

Addition of the crosslinker BS\textsuperscript{3} to FhGAPDH, followed by SDS-PAGE analysis resulted in the appearance of three additional bands at >120 kDa, ~70 kDa and ~80 kDa (Figure 3a). The highest molecular mass band is most likely a fully crosslinked tetramer and the two smaller bands may represent different conformations of dimers. Interestingly, when glyceraldehyde 3-phosphate or NAD\textsuperscript{+} or the two substrates together were added, the largest band was not detected and the intensity of the ~70 kDa band increased relative to the ~80 kDa one (Figure 3a). The most likely explanation for this is that substrate binding induces conformational and oligomerisation changes in FhGAPDH.

Analytical gel filtration of FhGAPDH in the absence of ligands resulted in a single peak at \( V_\text{e} = 19 \text{ ml} \) corresponding to an estimated molecular mass of 113 kDa, with a small “shoulder” at approximately 22 ml (70 kDa) (Figure 3b). This is most consistent with a trimeric solution structure with some dimers also present although it should be noted that no trimers were detected by crosslinking (Figure 3a). It is, therefore, possible that the major species detected by gel filtration is a tetramer with an unusually compact structure. When this experiment was repeated using FhGAPDH mixed with NAD\textsuperscript{+} (9 \mu M), the estimated molecular mass dropped to 70 kDa (\( V_\text{e} = 22 \text{ ml} \)) (Figure 3b).

Both methods provide evidence for a ligand-induced change in FhGAPDH’s conformation and oligomeric state. Both glyceraldehyde 3-phosphate and NAD\textsuperscript{+} appear to favour the formation of dimers over tetramers. Mammalian GAPDH’s oligomerisation states can also be affected by ligands. Rabbit GAPDH dissociates into dimers in the presence of NAD\textsuperscript{+} and glyceraldehyde 3-phosphate; however, in contrast to FhGAPDH, both substrates are required [55]. In some mammalian species, addition of NAD\textsuperscript{+} alone stabilises the tetramer [56;57].
3.4 FhGAPDH is greatly stabilised by substrates

In the absence of ligands, FhGADH had a melting temperature of 46±1 °C. This is substantially lower than that measured for *F. hepatica* triose phosphate isomerase (67.0 °C) under similar conditions [38]. The value is also lower than those for rabbit GAPDH (54.7 °C, determined by turbidity measurements) [58;59]. Addition of glyceraldehyde 3-phosphate or NAD$^+$ increased the melting temperature in a saturatable, concentration-dependent manner (Figure 4). In previous studies on liver fluke and human enzymes using this technique ligand-induced stabilisation typically increased the melting temperature by 2-5 K [38;49;60]. However both FhGAPDH substrates caused changes in $T_m$ of more than 20 K (Figure 4). The effect of glyceraldehyde 3-phosphate fit well to a simple, one site binding curve ($K_{D,app}$=4.2±0.4 mM). However, the data obtained with NAD$^+$ fitted better to a cooperative binding curve, with a Hill coefficient ($h$) of 0.48±0.02 and a $K_{D,app}$ of 0.67±0.16 mM. Since the effects of substrate on thermal denaturation are complex, there is a risk of over-interpretation of these findings. However, these results suggest that the binding of the two substrates has different effects on the protein and that NAD$^+$ may bind in a negatively cooperative manner.

3.5 Dynamic behaviour of the oligomeric forms of FhGAPDH

Taken together, data from oligomerisation and stability experiments suggest the following model for FhGAPDH’s behaviour. In the absence of substrates, the enzyme exists as an equilibrium mixture of tetramers and two forms of dimer. Either glyceraldehyde 3-phosphate or NAD$^+$ shifts this equilibrium towards one of the two dimer forms (Figure 3). This form is substantially more resistant to thermal denaturation than either the other dimeric form or the tetramer (Figure 4). By analogy with GAPDHs from other species, which often adopt more
compact forms on binding ligands, we hypothesise that this more stable form is also a more compact one. This hypothesis is consistent with the faster migration of its corresponding crosslinked product in SDS-PAGE (Figure 3a).

3.6 Conclusions and future prospects

FhGAPDH has a similar sequence and predicted three-dimensional structure to mammalian GAPDHs (Figures S1, 2a). It lacks a key binding cleft which has been exploited in the design of anti-parasitic drugs targeting GAPDH enzymes from unicellular parasites (Figure 2b). However, FhGAPDH does show an important biochemical difference when compared to mammalian GAPDH: its tetramer-dimer equilibrium responds differently to ligands. Further understanding of this process by, for example, molecular dynamics simulations may suggest ways to perturb this equilibrium. It would also be desirable to determine if FhGAPDH, like GAPDHs from other species, has functions other than its catalytic role in glycolysis. Understanding these roles may also suggest possible avenues for therapeutically relevant disruption or vaccine development.

Acknowledgements

We thank Prof Aaron Maule (Queen’s University, Belfast) for access to the qPCR machine used in the TSF assays.
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Figure legends

Figure 1: Expression, purification and activity of FhGAPDH. (a) Expression and purification of FhGAPDH was monitored by 10% SDS-PAGE. The purified protein is indicated by an arrow to the right of the gel. M, molecular mass markers (masses to the left of the gel in kDa); U, total protein extract from uninduced E. coli cells just prior to induction; I, total protein extract from E. coli cells 2 h after induction; S, soluble proteins released on sonication and after centrifugation to remove solid matter; W₁, material which passed through the column following application of the sonicate; W₂, material washed from the column by washing in buffer A (50 mM Hepes-OH, pH 7.4, 500 mM NaCl, 10%(v/v) glycerol); E₁, E₂ and E₃ three times 2 ml elutions in buffer B (buffer A supplemented with 250 mM imidazole). (b) Activity of FhGAPDH was monitored by measuring the rate of production of NADH (see Materials and Methods) as a function of glyceraldehyde 3-phosphate concentration. The data were fitted to the Michaelis-Menten equation with an apparent Michaelis constant (Kₘ,app) of 1.01±0.15 mM and an apparent maximum rate (Vₘₐₓ,app) of 0.044±0.002 μMs⁻¹. Each point represents the mean of three independent determinations of the rate and the error bars the standard errors of these means. The line is a non-linear fit to the Michaelis-Menten equation.

Figure 2: Predicted structure of FhGAPDH. (a) The overall fold of FhGAPDH was modelled as a tetramer by analogy to other GAPDH enzymes (described in Materials and Methods). The quaternary structure is shown as a “dimer of dimers” arrangement. The figure was produced using PyMol and each polypeptide chain of the tetramer is shown in a different colour. (b) A close up of the structure near the binding pocket which has been exploited in the design of novel anti-trypanosomal drugs. FhGAPDH (lime green; residues Val-33 to Met-43) and human GAPDH (blue; residues Phe-33 to Met-43) show high similar
folds in this region, whereas *T. brucei* GAPDH (pink; residues Val-35 to Phe-45) has a different backbone conformation creating a binding pocket close to the hydroxyl groups on the ribose of NAD⁺. An NAD⁺ molecule from *T. brucei* GAPDH is also shown.

**Figure 3: Oligomerisation of FhGAPDH.** (a) Crosslinking of FhGAPDH (30 μM) with BS³ (800 μM) revealed ligand-induced changes in the oligomeric state. Proteins were incubated with ligands (5 mM) as shown for 15 min at 37 ºC prior to the addition of crosslinker. After 30 min of reaction, the products were analysed by 8% SDS-PAGE. The first lane contains molecular mass markers (masses to the left of the gel in kDa) and the second FhGAPDH in the absence of ligands or crosslinker. (b) Analytical gel filtration of FhGAPDH (250 μl of 122 μM) showed a different elution profile in the presence and absence of NAD⁺ (9 μM). A calibration curve (inset) was constructed using proteins of known molecular mass and used to estimate the molecular mass of FhGAPDH. That the major peaks contained FhGAPDH was verified by SDS-PAGE (data not shown).

**Figure 4: Thermal stability of FhGAPDH.** The effect of varying the concentrations of substrates on the melting temperature of FhGAPDH (3 μM) was measured. Each point represents the mean of three independent determinations of the rate and the error bars the standard errors of these means. The lines are non-linear fits to simple, one site binding (glyceraldehyde 3-phosphate, upper panel) and negatively cooperative binding (NAD⁺, lower panel).
Biochemical characterisation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the liver fluke, *Fasciola hepatica*

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Abstract
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyses one of the two steps in glycolysis which generate the reduced coenzyme NADH. This reaction precedes the two ATP generating steps. Thus, inhibition of GAPDH will lead to substantially reduced energy generation. Consequently, there has been considerable interest in developing GAPDH inhibitors as anti-cancer and anti-parasitic agents. Here, we describe the biochemical characterisation of GAPDH from the common liver fluke *Fasciola hepatica* (FhGAPDH). The primary sequence of FhGAPDH is similar to that from other trematodes and the predicted structure shows high similarity to those from other animals including the mammalian hosts. FhGAPDH lacks a binding pocket which has been exploited in the design of novel antitrypanosomal compounds. The protein can be expressed in, and purified from *Escherichia coli*; the recombinant protein was active and showed no cooperativity towards glyceraldehyde 3-phosphate as a substrate. In the absence of ligands, FhGAPDH was a mixture of homodimers and tetramers, as judged by protein-protein crosslinking and analytical gel filtration. The addition of either NAD$^+$ or glyceraldehyde 3-phosphate shifted this equilibrium towards a compact dimer. Thermal scanning fluorimetry demonstrated that this form was considerably more stable than the unliganded one. These responses to ligand binding differ from those seen in mammalian enzymes. These differences could be exploited in the discovery of reagents which selectively disrupt the function of FhGAPDH.

**Keywords:** glycolytic enzyme; trematode; drug target; vaccine target; neglected tropical disease; G3PDH
1. Introduction

In recent years, there has been renewed interest in targeting metabolic enzymes in the treatment of infectious diseases [1]. This presents considerable challenges – most significantly the highly conserved nature of these proteins which makes the discovery of reagents which discriminate between host and pathogen enzymes difficult. However, the essential nature of pathways such as glycolysis mean that inhibition is likely to lead to growth inhibition and subsequent death of the pathogen. Thus, increased efforts are being made to characterise the biochemistry of metabolic enzymes from pathogens with the long term aim of investigating their potential as drug targets. A key goal of these studies is to identify biochemical differences between the pathogen enzyme and the host enzyme. These differences may then provide the basis for the discovery of compounds which selectively disrupt the activity of the pathogen enzyme.

_Fasciola hepatica_ (the common liver fluke) is a trematode which infects humans and other mammals. It is estimated that approximately 7 million humans are infected with millions more at risk; the majority of these people live in the developing world and fascioliasis is classified as a neglected tropical disease by WHO [2]. In addition, the organism causes a significant economic burden to agriculture globally since it infects domestic herbivores such as cows and sheep. Over ten years ago global losses due to infection were estimated to be several billion US dollars [3]. _F. hepatica_ infections can be treated with the modified benzimidazole drug triclabendazole. This compound is highly effective and kills both mature and juvenile worms. However, resistance to this drug is emerging worldwide and resistance to alternative benzimidazoles such as albendazole has also been reported [4-6]. It is inevitable that triclabendazole resistance will spread and the agricultural burden of _F._
hepatica infections is likely to increase if the trend to warmer, wetter summers in temperate regions continues [7;8].

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is a key enzyme in the glycolytic pathway. It catalyses the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in a reaction which requires a phosphate ion and the redox cofactor NAD⁺ as reactants (scheme 1). Therefore, it performs two functions: it generates reduced cofactor (NADH) which can be reoxidised through oxidative phosphorylation under aerobic conditions and it produces the “high energy” molecule 1,3-bisphosphoglycerate. This compound donates one of its phosphate groups to ADP in the subsequent reaction of the pathway resulting in the direct generation of ATP, a reaction which is critical for the organism’s survival under anaerobic conditions. Thus it is likely that inhibition of GAPDH would result in reduced energy production and reduced growth of the organism. Adenosine derivatives and 1,4-dihydro-4-oxoquinoline ribonucleosides which target the GAPDH of Trypanosoma spp have been shown to kill the organisms at micromolar concentrations [9;10]. Antitrypanosomal compounds which target GAPDH have also been isolated from Keetia leucantha leaves [11]. The antitumour compound 3-bromopyruvate targets human GAPDH resulting in altered energy metabolism and cell death [12-14].

The majority of GAPDH proteins which have been characterised are homotetrameric [15;16]. However, there are some reports of dimeric and trimeric forms of the enzyme [17-19]. There is intra-molecular communication between the four active sites resulting in complex, cooperative ligand binding behaviour. Saccharomyces cerevisiae GAPDH showed positively cooperative NAD⁺ binding (h=1.91) up to half-saturation of the active sites and mildly negative cooperativity (h=0.92) at higher concentrations of ligand [20]. The kinetic
mechanism of the enzyme requires the sequential addition of NAD$^+$, glyceraldehyde 3-phosphate and phosphate and the sequential release of 1,3-bisphosphoglycerate and NADH [21;22]. Following the binding of glyceraldehyde 3-phosphate to the enzyme, a cysteine residue (Cys-149 in the lobster enzyme) reacts with the substrate generating a covalent enzyme-substrate complex. Oxidation by NAD$^+$ then occurs in a reaction which requires a histidine residue to act as a base; the resulting NADH molecule is then exchanged for a new molecule of NAD$^+$. Inorganic phosphate reacts with, and displaces, the bound substrate generating 1,3-bisphosphoglycerate and regenerating the enzyme for a further round of catalysis [16;21;23].

There is also considerable evidence for GAPDH proteins having functions other than their catalytic role in glycolysis. These functions include gene regulation, vesicle transport and the prevention of telomere shortening [24]. In some bacteria, for example, Bacillus anthracis and pathogenic strains of Escherichia coli, the protein is present on the extracellular surface and can interact with the mammalian plasma protein plasminogen [25;26]. Similar interactions occur between the extracellular GAPDH of the protozoan parasite Trichomonas vaginalis and plasminogen and fibronectin [27]. GAPDH is also released into the extracellular environment by F. hepatica and by the blood flukes Schistosoma japonicum and Schistosoma mansoni [28-30]. In Schistosoma bovis, GAPDH is one of ten extracellular proteins which were identified as plasminogen binders [31]. It has been suggested that the plasminogen binding role of bacterial GAPDHs is important in pathogenesis [25]. This may also be the case in trematodes. The extra-cellular location of the enzyme has resulted in trematode GAPDHs receiving considerable attention as vaccine candidates [32-36]. Vaccination with peptides derived from Schistosoma mansoni GAPDH conferred some protection on mice infected under laboratory conditions [32;34]. Immunisation of goats with
a DNA vaccine encoding *Haemonchus contortus* GAPDH resulted in partial protection against this parasite [37].

The development of novel drugs or vaccines for the treatment of *F. hepatica* infection will require a greater understanding of the biochemistry of this organism. In light of the progress being made in the identification of antitrypanosomal and antitumour compounds which target this enzyme, we cloned, recombinantly expressed and biochemically characterised the GAPDH from *F. hepatica* (FhGAPDH).

2. Materials and Methods

2.1 Cloning, expression and purification of FhGAPDH

The coding sequence for FhGADH was amplified from *F. hepatica* cDNA by PCR using primers: 5’-GACGACGACAAGATGTCCAAACCAAAGTG-3’ (forward) and 5’-GAGGAGAAGCCCGGTTCACAATACCTTTTGCTTCCA-3’ (reverse). The amplicon was inserted into the expression vector pET-46 Ek/LIC (Merck, Nottingham, UK) by ligation independent cloning according to the manufacturer’s protocol. This method uses the proof-reading activity of T4 DNA polymerase to generate long overhangs at the 5’- and 3’-ends of the insert, thus avoiding the need for restriction digestion and ligation. The vector adds sequence coding for an N-terminal hexahistidine tag (MAHHHHHHVDDDDK). The presence of the insert was verified by PCR and then the insert was sequenced (GATC Biotech, London).

FhGAPDH was expressed in *E. coli* Rosetta(DE3) cells (Merck). The expression vector was transformed into this strain and a single colony used to inoculate 100 ml of LB media (supplemented with 100 μgml⁻¹ ampicillin and 34 μgml⁻¹ chloramphenicol). This culture was
grown overnight, shaking at 30 °C. The following day, this was diluted into 1 l of LB (supplemented with 100 μgml⁻¹ ampicillin and 34 μgml⁻¹ chloramphenicol) and the culture grown, shaking at 30 °C until $A_{600nm}=0.6-1.0$ (typically ~5 h). The culture was then induced with 0.2 g IPTG and grown at 16 °C for 20-24 h. Cells were harvested by centrifugation (4200g for 15 min), resuspended in ~20 ml of buffer R (50 mM Hepes-OH, pH 7.4, 150 mM NaCl, 10%(v/v) glycerol) and stored, frozen at -80 °C. The protein was purified from these cells source using nickel-agarose resin (His-Select, Sigma, Poole, UK) as previously described for *F. hepatica* triose phosphate isomerase (FhTPI) [38].

2.2 *Bioinformatics and molecular modelling*

The protein sequence of FhGAPDH was aligned to other, selected GAPDH sequences using ClustalW [39]. This alignment was used to construct a maximum likelihood tree using MEGA [40-42]. ProtParam from the EXPasY suite of programs was used to estimate the molecular mass and isoelectric point of the protein [43]. An initial molecular model of a FhGAPDH monomer was generated using Phyre2 [44]. Four of these monomeric structures were superimposed onto the subunits of human liver GAPDH (PDB: 1ZNQ [45]) using PyMol (www.pymol.org) to generate an initial model of tetrameric FhGAPDH. This initial model was energy minimised using YASARA [46] to generate the final model which is provided as supplementary data to this paper.

2.3 *Analytical gel filtration*

FhGAPDH (250 μl of 120 μM solution) was resolved (flow rate 1 ml min⁻¹) on a Sephacryl-S300 (Sigma, Poole, UK) column of total volume ($V_t$) 49.5 ml in 50 mM TrisHCl, 17 mM Tris base, 150 mM sodium chloride, pH 7.4 [47]. The elution volume ($V_e$) was determined from the peak in absorbance at 280 nm. The void volume ($V_0$) was determined to be 17 ml
by measuring the elution volume of blue dextran. The column was calibrated by determining $V_e$ for four standards ($\beta$-galactosidase, 116 kDa; BSA, 66 kDa; chymotrypsinogen A, 25 kDa; ribonuclease A, 14 kDa). The partition constant ($K_{av}$) was calculated for these standards according to the equation $K_{av} = (V_t - V_e)/(V_t - V_0)$. These values were used to construct a standard curve ($K_{av}$ against the logarithm of the molecular mass) which was used, along with the $K_{av}$ for FhGAPDH, to estimate that protein’s molecular mass.

2.4 Other analytical methods

Protein concentrations were estimated using the method of Bradford [48] with BSA as a standard. Protein-protein crosslinking with bis(sulfosuccinimidyl)suberate (BS3) and the determination of protein melting points ($T_m$) by thermal scanning fluorimetry (TSF) were carried out as previously described [38;49].

2.5 Enzyme kinetics

The rate of reaction catalysed by FhGAPDH was determined by measuring the increase in $A_{340\text{nm}}$ resulting from the reduction of NAD$^+$ to NADH [50]. Reactions (150 µl) were monitored at 37 ºC in a Multiskan Spectrum spectrophotometric plate reader (Thermo Scientific) for 15 min and contained 1 mM NAD$^+$, 30 mM sodium arsenate and 100 mM triethanolamine-HCl, pH 7.6. They were initiated by the addition of FhGAPDH (12 nM, monomer). Initial rates were estimated from the linear parts of the progress curves and plotted against substrate concentration. These data were fitted to both the Michaelis-Menten and Hill equations by non-linear curve fitting as implemented in GraphPad Prism 5.0 (GraphPad Software, CA, USA); an F-test was used to select the better fit.

3 Results and Discussion
3.1 A GAPDH from Fasciola hepatica

The primary sequence of FhGAPDH (derived from the cDNA sequence, GenBank: KF700239) encodes a polypeptide of 338 amino acid residues, a calculated, monomeric molecular mass of 37 kDa and an estimated isoelectric point of 7.1. Analysis of the protein sequence showed that FhGAPDH has greatest similarity to other trematode GAPDH enzymes and was well differentiated (bootstrap values >90) from GAPDH enzymes from potential host species (Supplementary Figure 1). The protein can be expressed in, and purified from, *E. coli* with a typical yield of ~4.5 mg per litre of bacterial culture (Figure 1a). The recombinant protein was active and showed Michaelis-Menten kinetics with glyceraldehyde 3-phosphate as a substrate (Figure 1b).

3.2 FhGAPDH has a similar overall fold to mammalian GAPDHs

The *F. hepatica* GAPDH protein sequence was used to model the three dimensional structure of the protein in a tetrameric form (Figure 2a). Overall, the predicted fold and oligomeric arrangement was similar to that of the human enzyme (PDB: 1U8F [51]; rmsd 0.994 Å over 8416 similar atoms). The fold is also similar to that from other parasites, for example *Plasmodium falciparum* (PDB: 1YWG [52]; rmsd 1.226 Å over 8358 equivalent atoms) *Trypanosoma brucei* (PDB: 2X0N, [53]; rmsd 2.009 Å over 8522 equivalent atoms) and *Trypanosoma cruzi* (PDB: 4LSM, note that this structure only contains a dimer; rmsd 1.133 Å over 4079 equivalent atoms). Comparison to the structure of the human enzyme also enabled the prediction of the catalytically critical cysteine residue which reacts covalently in the reaction mechanism as Cys-152.

A cleft in the protein, close to the 2'-OH on the NAD⁺, which is present in *Trypanosoma spp* GAPDH has been exploited in the structure based drug design of adenosine analogues. This
space is largely filled with the sidechain of Ile-37 in the human enzyme, thus leading to selectivity of these drugs for the parasite enzyme over the human one [54]. Ile-37 is conserved in the FhGAPDH (as Ile-38) and the backbone conformation in this region is similar to the human enzyme and not the T. brucei one (Figure 2b). Therefore, this line of drug discovery is unlikely to be fruitful in F. hepatica.

3.3 The equilibrium between FhGAPDH tetramers and dimers is affected by ligands

Addition of the crosslinker BS³ to FhGAPDH, followed by SDS-PAGE analysis resulted in the appearance of three additional bands at >120 kDa, ~70 kDa and ~80 kDa (Figure 3a). The highest molecular mass band is most likely a fully crosslinked tetramer. The two smaller bands correspond to molecular masses close to that predicted for a dimer (74 kDa). It is possible that these represent two different conformations, one more open (and thus more slowly migrating) and one more compact (see also Section 3.5). Interestingly, when glyceraldehyde 3-phosphate, NAD⁺ or the two substrates together were added, the largest band was not detected and the intensity of the ~70 kDa band increased relative to the ~80 kDa one (Figure 3a). The most likely explanation for this is that substrate binding induces conformational and oligomerisation changes in FhGAPDH.

Analytical gel filtration of FhGAPDH in the absence of ligands resulted in a single peak at 

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when this experiment was repeated using FhGAPDH mixed with NAD⁺ (9 μM), the
estimated molecular mass dropped to 70 kDa ($V_e=22$ ml) (Figure 3b). Under these conditions two additional peaks were observed, a small one corresponding to an approximate molecular mass of 7 kDa ($V_e=30$ ml) which we assumed to result from degradation and a broad peak ($V_e$ approximately 41 ml) which corresponded to unbound NAD$^+$ (Figure 3b).

Both methods provide evidence for a ligand-induced change in FhGAPDH’s conformation and oligomeric state. Both glyceraldehyde 3-phosphate and NAD$^+$ appear to favour the formation of dimers over tetramers. Mammalian GAPDH’s oligomerisation states can also be affected by ligands. Rabbit GAPDH dissociates into dimers in the presence of NAD$^+$ and glyceraldehyde 3-phosphate; however, in contrast to FhGAPDH, both substrates are required [55]. In some mammalian species, addition of NAD$^+$ alone stabilises the tetramer [56;57].

3.4 FhGAPDH is greatly stabilised by substrates

In the absence of ligands, FhGADH had a melting temperature of 46±1 °C. This is substantially lower than that measured for *F. hepatica* triose phosphate isomerase (67.0 °C) under similar conditions [38]. The value is also lower than those for rabbit GAPDH (54.7 °C, determined by turbidity measurements) [58;59]. Addition of glyceraldehyde 3-phosphate or NAD$^+$ increased the melting temperature in a saturatable, concentration-dependent manner (Figure 4). In previous studies on liver fluke and human enzymes using this technique ligand-induced stabilisation typically increased the melting temperature by 2-5 K [38;49;60]. However both FhGAPDH substrates caused changes in $T_m$ of more than 20 K (Figure 4). The effect of glyceraldehyde 3-phosphate fitted well to a simple, one site binding curve ($K_{D,app}=4.2±0.4$ mM). However, the data obtained with NAD$^+$ fitted better to a cooperative binding curve, with a Hill coefficient ($h$) of 0.48±0.02 and a $K_{D,app}$ of 0.67±0.16 mM. Since the effects of substrate on thermal denaturation are complex, there is a risk of over-
interpretation of these findings. However, these results suggest that the binding of the two substrates has different effects on the protein and that NAD$^+$ may bind in a negatively cooperative manner.

3.5 Dynamic behaviour of the oligomeric forms of FhGAPDH

Taken together, data from oligomerisation and stability experiments suggest the following model for FhGAPDH’s behaviour. In the absence of substrates, the enzyme exists as an equilibrium mixture of tetramers and two forms of dimer. Addition of either glyceraldehyde 3-phosphate or NAD$^+$ shifts this equilibrium towards one of the two dimer forms (Figure 3). This form is substantially more resistant to thermal denaturation than either the other dimeric form or the tetramer (Figure 4). By analogy with GAPDHs from other species, which often adopt more compact forms on binding ligands (see, for example, [17]), we hypothesise that this more stable form is also a more compact one. This hypothesis is consistent with the faster migration of its corresponding crosslinked product in SDS-PAGE (Figure 3a).

3.6 Conclusions and future prospects

FhGAPDH has a similar sequence and predicted three-dimensional structure to mammalian GAPDHs (Figures S1, 2a). It lacks a key binding cleft which has been exploited in the design of anti-parasitic drugs targeting GAPDH enzymes from unicellular parasites (Figure 2b). However, FhGAPDH does show an important biochemical difference when compared to mammalian GAPDH: its tetramer-dimer equilibrium responds differently to ligands. Further understanding of this process by, for example, molecular dynamics simulations may suggest ways to perturb this equilibrium. It would also be desirable to determine if FhGAPDH, like GAPDHs from other species, has functions other than its catalytic role in glycolysis. We postulate that these roles could be similar to those seen in some bacterial pathogens and other
parasites, i.e interaction with plasma proteins during pathogenesis [25-27]. By analogy to higher eukaryotes, it may also have cell signalling roles in *F. hepatica* [24]. Understanding these various roles may also suggest possible avenues for therapeutically relevant disruption or vaccine development.

**Acknowledgements**

We thank Prof Aaron Maule (Queen’s University, Belfast) for access to the qPCR machine used in the TSF assays.
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**Figure 1:** Expression, purification and activity of FhGAPDH. (a) Expression and purification of FhGAPDH was monitored by 10% SDS-PAGE. The purified protein is indicated by an arrow to the right of the gel. M, molecular mass markers (masses to the left of the gel in kDa); U, total protein extract from uninduced *E. coli* cells just prior to induction; I, total protein extract from *E. coli* cells 2 h after induction; S, soluble proteins released on sonication and after centrifugation to remove solid matter; W_1, material which passed through the nickel agarose column following application of the sonicate; W_2, material washed from the column by washing in buffer A (50 mM Hepes-OH, pH 7.4, 500 mM NaCl, 10%(v/v) glycerol); E_1, E_2 and E_3 three times 2 ml elutions in buffer B (buffer A supplemented with 250 mM imidazole). (b) Activity of FhGAPDH was monitored by measuring the rate of production of NADH (see Materials and Methods) as a function of glyceraldehyde 3-phosphate concentration. The data were fitted to the Michaelis-Menten equation with an apparent Michaelis constant (*K_m,app*) of 1.01±0.15 mM and an apparent maximum rate (*V_{max,app}* ) of 0.044±0.002 μM s⁻¹. Each point represents the mean of three independent determinations of the rate and the error bars the standard errors of these means. The line is a non-linear fit to the Michaelis-Menten equation.

**Figure 2:** Predicted structure of FhGAPDH. (a) The overall fold of FhGAPDH was modelled as a tetramer by analogy to other GAPDH enzymes (described in Materials and Methods). The quaternary structure is shown as a “dimer of dimers” arrangement. The figure was produced using PyMol and each polypeptide chain of the tetramer is shown in a different colour. (b) A close up of the structure near the binding pocket which has been exploited in the design of novel anti-trypanosomal drugs. FhGAPDH (lime green; residues Val-33 to Met-43) and human GAPDH (blue; residues Phe-33 to Met-43) show high similar
folds in this region, whereas *T. brucei* GAPDH (pink; residues Val-35 to Phe-45) has a different backbone conformation creating a binding pocket close to the hydroxyl groups on the ribose of NAD$^+$. An NAD$^+$ molecule from *T. brucei* GAPDH is also shown.

**Figure 3: Oligomerisation of FhGAPDH.** (a) Crosslinking of FhGAPDH (30 μM) with BS$_3$ (800 μM) revealed ligand-induced changes in the oligomeric state. Proteins were incubated with ligands (5 mM) as shown for 15 min at 37 ºC prior to the addition of crosslinker. After 30 min of reaction, the products were analysed by 8% SDS-PAGE. The first lane contains molecular mass markers (masses to the left of the gel in kDa) and the second FhGAPDH in the absence of ligands or crosslinker. (b) Analytical gel filtration of FhGAPDH (250 μl of 122 μM) showed a different elution profile in the presence and absence of NAD$^+$ (9 μM). A calibration curve (inset) was constructed using proteins of known molecular mass and used to estimate the molecular mass of FhGAPDH. That the major peaks contained FhGAPDH was verified by SDS-PAGE (data not shown).

**Figure 4: Thermal stability of FhGAPDH.** The effect of varying the concentrations of substrates on the melting temperature of FhGAPDH (3 μM) was measured. Each point represents the mean of three independent determinations and the error bars the standard errors of these means. The lines are non-linear fits to simple, one site binding (glyceraldehyde 3-phosphate, upper panel) and negatively cooperative binding (NAD$^+$, lower panel).
Scheme 1

Glyceraldehyde 3-phosphate + NAD$^+$ + P$_i$ → 1,3-bisphosphoglycerate + NADH
Figure 1

(a)

(b)
Figure 3

(a) +BS³ + + NAD⁺ + Glyceraldehyde 3-Phosphate

(b) A₂₈₀

Volume (ml)

FhGAPDH
FhGAPDH+NAD⁺
Figure 4

The top graph shows the relationship between the change in temperature ($\Delta T$) and the concentration of Glyceraldehyde 3-phosphate (mM). The bottom graph depicts the similar relationship with NAD (mM).
Supplementary Figure S1: Maximum Likelihood tree showing similarity in GAPDH protein sequences from selected organisms.

The tree was calculated using the Maximum Likelihood method based on the JTT matrix-based model [S1]. The tree with the highest log likelihood (-6149.4229) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (5000 bootstraps). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 330 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [S2].

Species abbreviations: Fh, Fasciola hepatica; Hs, Homo sapiens; Bt, Bos Taurus; Ss, Sus scrofa; La, Loxodonta Africana; Gg, Gallus gallus; Dm, Drosophila melanogaster; Ha, Homarus americanus; Sb, Schistosoma bovis; Sm, Schistosoma mansoni; Cs, Clonorchis sinensis; Ce, Caenorhabditis elegans; Hc, Haemonchus contortus; Bm, Brugia malayi; Ts, Taenia solium; Tb, Trypanosoma brucei; Pf, Plasmodium falciparum; Eh, Entamoeba histolytica; Sc, Saccharomyces cerevisiae.


3D molecular models (PDB, PSE or MOL/MOL2)

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