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Trypanothione-dependent glyoxalase I in *Trypanosoma cruzi*

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bis(glutathionyl)spermidine

SYNOPSIS

The glyoxalase system, comprising glyoxalase I and glyoxalase II, is a ubiquitous pathway that detoxifies highly reactive aldehydes such as methylglyoxal, using glutathione as a co-factor. Recent studies of *Leishmania major* glyoxalase I and *Trypanosoma brucei* glyoxalase II have revealed a unique dependence upon the trypanosomatid thiol trypanothione as a co-factor. This difference suggests that the trypanothione-dependent glyoxalase system may be an attractive target for rational drug design against the trypanosomatid parasites. Here we describe the cloning, expression and kinetic characterisation of glyoxalase I from *Trypanosoma cruzi*. Like *L. major* glyoxalase I, recombinant *T. cruzi* glyoxalase I showed a preference for nickel as its metal co-factor. In contrast to the *Leishmania* enzyme, *T. cruzi* glyoxalase I was far less fastidious in its choice of metal co-factor efficiently utilising cobalt, manganese and zinc. *T. cruzi* glyoxalase I isomerised hemithioacetal adducts of trypanothione more than 2400-times more efficiently than glutathione adducts with the methylglyoxal adducts 2-3 fold better substrates than the equivalent phenylglyoxal adducts. However, glutathionylspermidine hemithioacetal adducts were most efficiently isomerised and the glutathionylspermidine-based inhibitor S-4-bromobenzylglutathionylspermidine was found to be a potent linear competitive inhibitor of the *T. cruzi* enzyme with a K_i of $5.4 \pm 0.6 \mu\text{M}$. Prediction algorithms, combined with subcellular fractionation, suggest that *T. cruzi* glyoxalase I localises to the cytosol but also the mitochondria of *T. cruzi* epimastigotes. The contrasting substrate specificities of human and trypanosomatid glyoxalase enzymes, confirmed in this study, suggest that the glyoxalase system may be an attractive target for anti-trypanosomal chemotherapy.

INTRODUCTION

Chagas' disease, or American trypanosomiasis, is caused by the flagellated, protozoan parasite *Trypanosoma cruzi*. Despite a marked reduction in rates of transmission in countries such as Brazil, Chile and Uruguay over the last 20 years, the disease remains a major health risk in many Latin American countries with more than 15 million infected and an annual death toll of approximately 13,000 [1]. The efficacy of the current nitro-drugs, nifurtimox and benznidazole is poor, particularly in the chronic stage of infection. Both drugs have a high incidence of side effects [2] so that many patients are unable to complete a full course of treatment. Consequently, identification of novel drug targets and more effective replacement drug therapies has now become a matter of urgency.

In the search for anti-parasitic drug targets, biochemical pathways that are both essential for parasite survival and absent from the host are sought after [3]. One such pathway is the thiol metabolism of trypanosomes and *Leishmania*. These parasites are uniquely dependent upon trypanothione [T[SH]₂, N¹,N⁸-bis(glutathionyl) spermidine] as their principal low molecular-mass thiol, which, together with trypanothione reductase, assumes many of the anti-oxidant functions commonly undertaken by glutathione [GSH] and glutathione reductase in their human hosts [4]. In related trypanosomatids, T[SH]₂ has already been implicated in the mode of action as well as the mechanism of resistance of antimonial drugs in *Leishmania spp* [5-7] and in defence against chemical and oxidant stress induced by arsenicals and nifurtimox in African trypanosomes [8-10]. Therefore, in an attempt to exploit this essential and unique metabolic pathway, trypanothione-dependent enzymes have become a major focus for drug discovery against these neglected tropical diseases.

In our previous study [11] we reported the characterisation of the T[SH]₂-dependent enzyme glyoxalase I (GLO1) in *L. major*. This finding, combined with the identification of T[SH]₂-dependent glyoxalase II in both *Trypanosoma brucei* [12] and *L. donovani* [13], provide compelling evidence for a unique T[SH]₂-dependent glyoxalase system in certain trypanosomatids. The glyoxalase system is a ubiquitous pathway for the detoxification of highly reactive oxoaldehydes. The metalloenzymes glyoxalase I (lactoylglutathione lyase) and glyoxalase II (hydroxyacylglutathione lyase) catalyse the step-wise dismutation of 2-oxoaldehydes into the corresponding 2-hydroxyacids, using glutathione as a co-factor [14,15]. The principal role of the glyoxalase system is thought to be the detoxification of methylglyoxal (2-oxopropanal), a highly toxic α -oxoaldehyde

produced as a by-product of glycolysis and possessing cytotoxic, cytostatic and mutagenic properties [16]. In addition, methylglyoxal is also produced by the catabolism of threonine via aminoacetone or hydroxyacetone [17]. The inherent toxicity of this molecule stems from its propensity to react with the nucleophilic centres of DNA, RNA and proteins. In particular, the oxoaldehyde reacts with the side chains of arginine, lysine, cysteine and with the base guanine.

Inhibitors of GLO1 exhibit anti-cancer and anti-malarial properties and are selectively toxic for rapidly proliferating cells [18]. The trypanothione-dependent glyoxalase system may provide an ideal target for Chagas' disease drug development. With this in mind, here we describe the expression, purification and kinetic characterisation of GLO1 from *T. cruzi*.

EXPERIMENTAL

Materials

Methylglyoxal was prepared from methylglyoxal dimethylacetal and confirmed to be free of formaldehyde, as previously described [19]. All other reagents were standard commercial products of high purity.

Cloning and Expression of *TcGLO1*

A putative *T. cruzi* GLO1 gene (Tc00.1047053510659.240) was identified in the *T. cruzi* genome database (www.genedb.org). This gene was amplified by PCR from *T. cruzi* CL Brener genomic DNA using the sense primer

5'-CATATGTCAACACGACGACTTATGCACACGATG-3' with an additional *NdeI* site (underlined) and the antisense primer

5'-GGATCCGGATCCTTAAGCCGTTCCCTGTTC-3' with an additional *BamHI* site (underlined). The PCR product was then cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The pCR-Blunt II-TOPO-GLO1 constructs were then digested with *NdeI* and *BamHI* and the fragments cloned into the pET15b expression vector (Novagen) resulting in a pET15b- *TcGLO1* construct.

Transformed BL21(DE3)pLysS *E. coli* were grown in LB medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin and 12.5 $\mu\text{g ml}^{-1}$ chloramphenicol to an OD_{600} of ~ 0.6 . Expression was induced with 1 mM isopropyl- β -D-galactopyranoside for 4 h at 25 °C. Following expression, cells were pelleted (3000 x g, 10 min, 4 °C), resuspended in lysis buffer [75 mM (Na^+) phosphate, pH 7.5, 500 mM NaCl, 10 mM 2-mercaptoethanol and protease inhibitor cocktail (Roche)] and then lysed by sonication (four 30 s bursts at 15 microns amplitude), with cooling between pulses. The lysate was centrifuged (30 000 x g, 30 min) and the supernatant loaded at 2 ml min^{-1} onto a 5-ml HiTrap chelating Sepharose column (Amersham Pharmacia) in 50 mM (Na^+) phosphate, 200 mM NaCl pH 7.5. *TcGLO1* was eluted with a gradient of 0 – 500 mM imidazole in the same buffer. Eluted protein was then dialysed against 25 mM Bis-Tris, pH 6.5 and loaded onto a 5-ml HiTrap Q-Sepharose column (Amersham Pharmacia) at 2 ml min^{-1} and eluted with a gradient of 0 – 500 ml NaCl in the same buffer. Finally, the N-terminal 6-histidine tag was removed by thrombin cleavage (30 Units mg^{-1} for 12 h at 25 °C) and untagged *TcGLO1* recovered using a HiTrap Q-Sepharose column, as previously described [11].

Physical properties of *TcGLO1*

The native M_r of *TcGLO1* was determined using Superdex 200 HR 10/30 size exclusion column chromatography (Amersham Pharmacia) against gel filtration standards (BioRad). Molecular mass was determined by matrix-assisted laser desorption ionisation time-of-flight spectroscopy in linear mode using sinapinic acid as a matrix on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems).

Production of thiols

Trypanothione disulphide (Bachem) was reduced using *tris*(2-carboxyethyl)phosphine (TCEP) agarose, as previously described [11].

Apoenzyme production and metal reconstitution

Apoenzyme was prepared by dialysis of *TcGLO1* for 24 h [11] against 1 L of 20 mM imidazole, 1 mM EDTA, pH 7.0 containing 5 g Chelex resin (2 volume changes). The enzyme was then reconstituted by incubation with a 10-fold molar excess of metal chloride in Chelex-treated 50 mM Hepes pH 7.0 for 10 min at room temperature.

Kinetic analysis of *TcGLO1* activity

The pH optimum for the enzyme was determined over the pH range 4-10 using a mixed buffer system of 50 mM each of CHES [2-(*N*-cyclohexylamino)ethanesulphonic acid], HEPPS and MES (pH adjusted using KOH). The effect of ionic strength on enzyme activity was determined as above in 50 mM HEPES pH 7.0 with varying concentrations (0.8-0 M) NaCl and $(\text{NH}_4)_2\text{SO}_4$. To ensure that NiCl_2 co-factor was not a limiting factor for enzyme activity in these assays, *TcGLO1* was pre-incubated with a 10-fold molar excess of NiCl_2 .

Kinetic constants (K_m and k_{cat}) and pH optimization studies were determined by measuring the rate of isomerised hemithioacetal adduct formation at 240 nm using a continuous spectrophotometric assay (Shimadzu UV-2401 PC dual beam Spectrophotometer) [11]. The rate of phenylglyoxal adduct formation was measured at 263 nm. Hemithioacetal concentrations were calculated using the previously established K_d values of 3.0 mM for the methylglyoxal-thiol adduct and 0.6 mM for the phenylglyoxal-thiol adduct [20], in the presence of 0.1 M reduced thiol. Assays (0.5 ml) were performed in 50 mM HEPES pH 7.0 with 25 mM NaCl at 27 °C. With the

methylglyoxal and phenylglyoxal glutathione hemithioacetals, k_{cat}/K_m values were determined from the slope of rates plotted against enzyme concentration.

Inhibitor studies

Inhibition constants for *Tc*GLO1 were determined over a range of substrate concentrations (0.5 - 2.5 times K_m with the hemithioacetal of trypanothione) at three fixed concentrations of S-4-bromobenzylglutathionylspermidine [21]. Linear Lineweaver-Burk transformations of each data set were inspected for inhibition pattern and found to be competitive. Data sets were fitted by non-linear regression using the programme GraFit and kinetic parameters determined.

Western analyses of cell extracts

Polyclonal antisera against *L. major* GLO1 were raised in adult male Wistar rats. An initial injection of 100 μg of purified antigen [11], emulsified in complete Freund's adjuvant, was followed by two identical booster injections of antigen emulsified in Freund's incomplete adjuvant at 2 week intervals.

L. major promastigotes (Friedlin strain; WHO designation: MHOM/JL/81/Friedlin) ($2 \times 10^7 \text{ ml}^{-1}$, 1 L) and *T. cruzi* epimastigotes (CL Brener, genome project standard clone) ($3 \times 10^7 \text{ ml}^{-1}$, 1 L) were pelleted by centrifugation ($1600 \times g$, 10 min, 4 °C), washed twice in 20 mM Tris, pH 7.0 containing 0.1 mM sucrose and re-suspended in cell lysis buffer (10 mM HEPES, pH 7.0). For biological safety, parasites were inactivated by three cycles of freezing in liquid nitrogen and thawing in a 37 °C water bath, before lysis under pressure (30 kpsi) using a one shot cell disruptor (Constant Systems). Following centrifugation ($20,000 \times g$ for 40 min), cell supernatant was collected and protein concentration determined. Whole cell extracts (30 μg) were then separated on SDS-PAGE and subsequently transferred onto nitrocellulose. After blocking with 5% skimmed milk in PBS at room temperature for 1 h, the blot was incubated with *L. major* GLO1 polyclonal antiserum (1/700 dilution) at room temperature for 1 h, washed in PBS containing 0.1% (v/v) Tween 20 and then incubated with a secondary rabbit anti-rat (IgG) antibody (Dako; 1/10,000 dilution). The immunoblot was subsequently developed using the ECL® plus (enhanced chemiluminescence) system from Amersham Biosciences.

Subcellular fractionation

Subcellular fractions of *L. major* promastigotes and *T. cruzi* epimastigotes were prepared essentially as previously described [22]. Each fraction (30 µg) was then separated on SDS-PAGE and transferred onto nitrocellulose. After blocking, the blot was probed with *L. major* GLO1 polyclonal antiserum and detected by chemiluminescence, as described above. Following analysis, the immunoblot was stripped using Restore Western Blot Stripping Buffer (Pierce) and reprobed using the following primary antibodies in turn: anti-Hsp60 monoclonal antibody (1/5000 dilution; Stressgen); anti-*L. major* trypanothione synthetase antiserum (1/1000 dilution, [22]) and anti-*T. brucei* BiP antiserum (1/5000, [23]). Rabbit anti-rat (IgG) antibody (Dako; 1/10,000 dilution) and anti-mouse (IgG-HRP) antibody (Sigma; 1/10000 dilution) were used as secondary antibodies where appropriate and immunoblots were once again developed by enhanced chemiluminescence.

RESULTS AND DISCUSSION

Identification of *T. cruzi* GLO1 sequences

The identification of a unique trypanothione-dependent glyoxalase system within *Leishmania spp.* and *Trypanosoma brucei* [11-13] has raised hopes that the enzymes of this pathway may provide suitable targets for rational drug design against all trypanosomatids. However, despite having certain unique metabolic features in common [24], the evolutionary distance between *Leishmania* and *Trypanosoma* is large, approaching that of fly and human [25]. With this in mind, we have determined the physicochemical and kinetic properties of GLO1 from *Trypanosoma cruzi*.

Blast searches of the *T. cruzi* genome database, using *E. coli* and *L. major* GLO1 sequences as templates, revealed a 426 bp sequence (Tc00.1047053510659.240) which shared 72 % and 44% sequence identity to the *L. major* and *E. coli* GLO1 sequences, respectively. Alignment of the putative *Tc*GLO1 along with other trypanosomatid, eukaryotic and bacterial GLO1 amino acid sequences (Figure 1) illustrated a high degree of similarity between the trypanosomatid and bacterial GLO1 proteins. Similarity to the eukaryotic GLO1 sequences was between 39.4 and 32.4 % with mammalian enzymes differing in having a number of unique insertions and an N-terminal extension. The conservation of a histidine residue near the N-terminus of the trypanosomatid proteins (residue 8 in *T. cruzi*) is suggestive of a nickel co-factor binding site. However, in eukaryotic sequences a conserved glutamine in the same position is indicative of a zinc co-factor binding site [26]. Conserved acidic residues (Asp 100 and Tyr 101) in the trypanosomatid enzymes are believed to be involved in the specific binding of T[SH]₂ [21].

Cloning and expression of *Tc*GLO1 in *E. coli*

Sequence-specific sense and anti-sense primers were used to amplify the 426-bp *Tc*GLO1 gene from *T. cruzi* CL Brener genomic DNA by PCR. The product was sub-cloned into the pCR-Blunt II-TOPO vector, sequenced to verify identity and cloned into pET15b giving the plasmid pET15b-*Tc*GLO1. The resulting plasmid contains the entire open reading frame of *Tc*GLO1 fused in frame with a cleavable hexahistidine [(His)₆] tag at the N-terminus. *E. coli* BL21(DE3)pLysS transformed with pET15b-*Tc*GLO1 produced soluble and enzymatically active protein. *Tc*GLO1 was purified to homogeneity via nickel affinity and anion exchange chromatography. The N-terminal (His)₆ tag was then

removed by thrombin cleavage followed by a second anion exchange chromatography step (Figure 2). No residual (His)₆ tagged enzyme could be detected either by SDS-PAGE or Western analysis with a anti-(His)₆ antibody. Both (His)₆ tagged and untagged *TcGLO1* readily formed dimers which were resistant to SDS denaturation and reduction with dithiothreitol, TCEP or 2-mercaptoethanol. Typical yields were between 5 and 7 mg L⁻¹ of starting culture.

Determination of *TcGLO1* physical properties

SDS-PAGE analysis of *TcGLO1* revealed a recombinant protein with an apparent molecular mass of 17 kDa (18.5 kDa with the (His)₆ tag), in good agreement with the predicted size (16,647 Da). The nominal molecular mass of recombinant *TcGLO1* was determined to be 16,650 Da by MALDI-TOF analysis. Size exclusion chromatography and analytical ultracentrifugation showed native *TcGLO1* to actively form dimers in solution with a M_r of 31,300 (not shown). These physiological properties are in good agreement with those for *LmGLO1* [11].

Metal specificity of *TcGLO1*

Sequence analyses suggested that nickel was likely to be the preferred metal co-factor of *TcGLO1*. To test this assumption, a range of divalent cations were analysed for their ability to reactivate enzyme activity in the *TcGLO1* apo-protein (Figure 3). Like the *Leishmania* enzyme, nickel was found to be the most effective in reactivating glyoxalase I enzyme activity. However, reactivation by cobalt was found to be only marginally less effective than nickel. Indeed, in comparison to the *L. major* enzyme, *TcGLO1* was found to be far less fastidious in its choice of metal co-factor with both zinc and manganese capable of significant levels of reactivation. The versatility of *TcGLO1* in utilizing a wider range of metal co-factors might be due to differing bioavailability in the mammalian host cells parasitized or in their respective insect vectors.

Kinetic characterisation

Using a mixed buffer system, the optimum pH range for *TcGLO1* activity was determined to be 7.0 (Figure 4A). Interestingly, a rapid decline in enzyme activity was noted in mildly alkaline conditions. This loss in *TcGLO1* activity was subsequently shown to be reversible indicating that it is not due to denaturation at alkaline pH (data not

shown). In 50 mM HEPES buffer at the optimum pH the optimum ionic conditions of the assay were found to be 25 mM NaCl (Figure 4B).

*Lm*GLO1 has a high degree of substrate specificity for trypanothione hemithioacetals in preference to glutathione hemithioacetals[11], the substrate of choice for human GLO1 [16]. Similarly, *Tc*GLO1 isomerised hemithioacetal adducts of trypanothione and glutathionylspermidine 2,400 and 14,000 times more efficiently than glutathione adducts, respectively (Table 1). Of these two preferred substrates, *Tc*GLO1 had a significantly higher affinity for the glutathionylspermidine adducts with either methylglyoxal or phenylglyoxal as demonstrated by a markedly lower K_m value, resulting in a 6-fold difference in k_{cat}/K_m . The *T. cruzi* enzyme displayed substrate specificity based not only upon thiol co-factor, but also on the oxoaldehyde component of the hemithioacetal. Methylglyoxal adducts were found to be between 2-3 fold better substrates than the equivalent phenylglyoxal adducts. The most striking difference between the *L. major* and *T. cruzi* enzymes is the 5-fold greater efficiency with which the *Leishmania* enzyme utilizes trypanothione hemithioacetals. This observation may be explained by the significantly lower intracellular levels of trypanothione in *L. major* promastigotes [27].

While glutathionylspermidine adducts appear to be the most efficient substrate for *Tc*GLO1, 6-fold more efficient than trypanothione hemithioacetals, it should be noted that trypanothione levels are 8-fold higher than glutathionylspermidine in *T. cruzi* epimastigotes [27]. Based on their relevant ratios it would seem that both glutathionylspermidine and trypanothione hemithioacetal adducts may act as the physiological substrates of the *T. cruzi* enzyme *in vivo*. The mutually exclusive substrate specificities of the human and parasitic GLO1 enzymes have been directly related to major differences in active site architecture [28]. It is hoped that these differences can be exploited in the structure-based design of selective inhibitors against the trypanosomatids.

Inhibitor studies

The high affinity of the trypanosomatid GLO1 enzymes for glutathionyl spermidine adducts may be useful in the future design of substrate-mimic competitive inhibitors. S-4-bromobenzylglutathionylspermidine was found to be a linear competitive inhibitor of GLO1 with respect to trypanothione hemithioacetal, as shown by diagnostic

Lineweaver-Burk plots (K_i of $5.4 \pm 0.6 \mu\text{M}$, Figure 5) which is 10-fold higher than for the *L. major* enzyme [28]. Comparison of LmGLO1 with a homology model of TcGLO1 did not identify any striking differences between the active sites of both enzymes that could account for differences in inhibitor potency (Ariza and Bond personal communication). In contrast to the glutathionylspermidine analogue, S-4-bromobenzylglutathione is inactive as an inhibitor of both trypanosomatid enzymes (data not shown).

Substrate analogues have been successfully used as inhibitors against GLO1 in several other organisms [29-31]. For example, *S*-(*N*-aryl-*N*-hydroxycarbamoyl)-glutathione and *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)-glutathione compounds have been shown to inhibit human GLO1 with K_i values in the nM range [32] and in the form of a diester to rapidly kill proliferating cancer cells [33]. It is tempting to suggest that the glutathionylspermidine version of these compounds may selectively inhibit trypanosomatid GLO1 activity and in their diester forms may prove to be toxic for parasites.

Western analyses of cell extracts and subcellular fractions

Immunoblots of *L. major* promastigote and *T. cruzi* epimastigote whole cell lysates were probed with a *L. major* GLO1-specific polyclonal antiserum (Figure 6A). As expected, a 17 kDa protein equivalent to the predicted molecular mass of the GLO1 enzymes, reacted strongly with the antiserum in both the *L. major* and the *T. cruzi* lysates. Crude subcellular fractions of *L. major* promastigotes were also prepared and analysed by Western blot (Figure 6B). The purity of each fraction was demonstrated using antibodies against marker proteins in each subcellular location. The endoplasmic reticulum-localised BiP protein (immunoglobulin heavy chain-binding protein) was used as a marker for the microsomal fraction while Hsp60 and trypanothione synthetase were used as marker proteins for the large granular and cytosolic fractions, respectively. Interestingly, GLO1 was found to localise not only in the cytosol, but also in the large granular fraction from these parasites. Similar GLO1 localisation was observed in lysates of *T. cruzi* epimastigotes (data not shown). Subsequent analysis of the TcGLO1 and LmGLO1 primary sequences with MitoProt II [34], used to predict targeting of proteins to the mitochondria, suggested that both enzymes are likely to be exported to the mitochondria with a probability of 0.909 and 0.8704, respectively. However, no cleavage site was predicted for the mitochondrial targeting sequences of either protein.

While comparatively little is known about the cellular location of GLO1 enzymes in other organisms, glyoxalase II has been extensively studied and detected in the cytoplasm and mitochondria of several higher eukaryotes including rat [35], human [36], yeast [37] and *Arabidopsis thaliana* [38]. In higher plants and yeast, distinct genes encode the alternate forms of GLO2, whereas, analysis of GLO2 mRNA sequences in vertebrates indicates that dual initiation from alternative AUG codons is responsible [36]. In our future studies we hope to explore the targeting mechanism of GLO1 to trypanosomatid mitochondria.

In conclusion, the contrasting substrate specificities of human and trypanosomatid glyoxalase enzymes suggest that the glyoxalase system may be an attractive target for anti-trypanosomal chemotherapy. Gene knockout studies are underway to determine the role this pathway plays in parasite growth, survival or virulence.

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FIGURE LEGENDS

Figure 1. Alignment of the predicted amino acid sequences of glyoxalase I. Gaps introduced into sequences to optimise alignments are represented by dashes. Conserved and similar residues are indicated by asterisks and dots respectively. The circles indicate conserved metal binding residues in the various sequences. Squares indicate conserved residues implicated in the binding of γ -glutamate moieties of thiol co-factors while triangles highlight conserved acidic residues (Asp 100 and Tyr 101) in the trypanosomatid enzymes thought to be involved in the binding of T[SH]₂ [28]. Protein sequences are from *T. cruzi* (TcGLO1; Tc00.1047053510659), *L. major* (LmGLO1; LmjF35.3010), *E. coli* (Swiss-Prot accession no. P77036), *Synechococcus* sp. WH 8102 (TrEMBL accession no. Q7U3T2), human (Swiss-Prot accession no. P78375) and mouse (Swiss-Prot accession no. Q9CPU0).

Figure 2. Purification of recombinant *T. cruzi* GLO1. Lane 1, soluble fraction of BL21 (DE3)pLysS [pET15b-*TcGLO1*] – uninduced; lane 2, soluble fraction of BL21 (DE3)pLysS [pET15b-*TcGLO1*] – induced; lane 3, pooled fractions from nickel affinity chromatography; lane 4, pooled fractions from anion exchange chromatography (Resource-Q); lane 5, pooled fractions from anion exchange chromatography (Resource-Q) after removal of the His₆ tag with thrombin protease.

Figure 3. Metal reconstitution of *T. cruzi* GLO1 in various divalent metal chlorides. The apo form of *TcGLO1* was incubated with a 10-fold molar excess of divalent metal ions for 10 min at room temperature. Assays contained 25 μ M trypanothione hemithioacetal and 0.1 mM free T[SH]₂. *L. major* GLO1 reconstituted activity [11] is overlaid (black bars) on the *T. cruzi* GLO1 data (white bars). All assays were performed in triplicate, with the mean activity relative to the nickel-activated enzymes displayed.

Figure 4. Enzymatic characterisation of *T. cruzi* GLO1. (A) Determination of assay pH optimum. Assays were performed with 50 μ M trypanothione hemithioacetal in a mixed buffer system, as described in the methods. Activity is expressed as a percentage relative to the maximum activity determined for *TcGLO1*. Values are the means with standard deviations from three measurements. (B) Effect of buffer concentration and ionic strength on *TcGLO1* activity. The assay mixtures contained either various amounts

of HEPES buffer, pH 7.0 (closed circles), NaCl (open circles), or $(\text{NH}_4)_2\text{SO}_4$ (open squares). Activity is expressed as a percentage of the activity determined in 50mM Hepes buffer, 25 mM NaCl. The inset shows the effect on activity as a function of buffer or salt concentration.

Figure 5. S-4-bromobenzylglutathionylspermidine inhibition of *T. cruzi* GLO1. *T. cruzi* GLO1 inhibition constants were determined at three fixed concentrations of the inhibitor (S-4-bromobenzylglutathionylspermidine) and over a range of substrate concentrations (25-125 μM trypanothione hemithioacetal). Data sets for each inhibitor concentration were fitted by non-linear regression and are presented as Lineweaver-Burk transformations. Trypanothione hemithioacetal was varied in the presence of 0 (\circ), 3.15 (\blacksquare), 6.3 (\square) and 12.6 μM (\bullet) S-4-bromobenzylglutathionylspermidine.

Figure 6. Immunoblot analysis of cell lysates and cellular localisation of *T. cruzi* GLO1. (A) Blots of whole cell extracts from *T. cruzi* epimastigotes (lane 1) and *L. major* promastigotes (lane 2) (30 μg of protein in each lane) were probed with an anti-*L. major* GLO1 antiserum. (B) Subcellular fractions of *L. major* promastigotes, containing the large granule (LG), cytosol (C), and microsomal fractions (MF), were prepared as described in the methods. Western blots of these equally loaded fractions (30 μg of protein in each lane) were probed with anti-serum to *L. major* GLO1. In addition, blots were stripped and reprobed with antiserum to marker proteins for each subcellular fraction to demonstrate the purity of each fraction (LG; anti-Hsp60, C; anti-*L. major* trypanothione synthetase and MF; anti BiP).

Table 1**Kinetic properties of *T. cruzi* GLO1**

Kinetic constants (K_m and k_{cat}) were determined as previously described [11]. Data are means of triplicate measurements \pm S.D. ND, not determined; S.D, standard deviation.

Hemithioacetal substrate	K_m , μM	k_{cat} , s^{-1}	$k_{cat}/K_m \times 10^6$, $\text{M}^{-1} \text{s}^{-1}$	k_{cat}/K_m (relative)
Glutathionylspermidine				
Methylglyoxal	8.0 ± 0.4	161 ± 12	20	100
Phenylglyoxal	14 ± 2.1	105 ± 28	7.5	37.5
Trypanothione				
Methylglyoxal	109 ± 10	363 ± 33	3.3	16.5
Phenylglyoxal	207 ± 23	265 ± 31	1.5	7.5
Glutathione				
Methylglyoxal	>1800	ND	0.0014	0.007
Phenylglyoxal	>1800	ND	0.0005	0.0025



Figure 1, Greig *et al.*

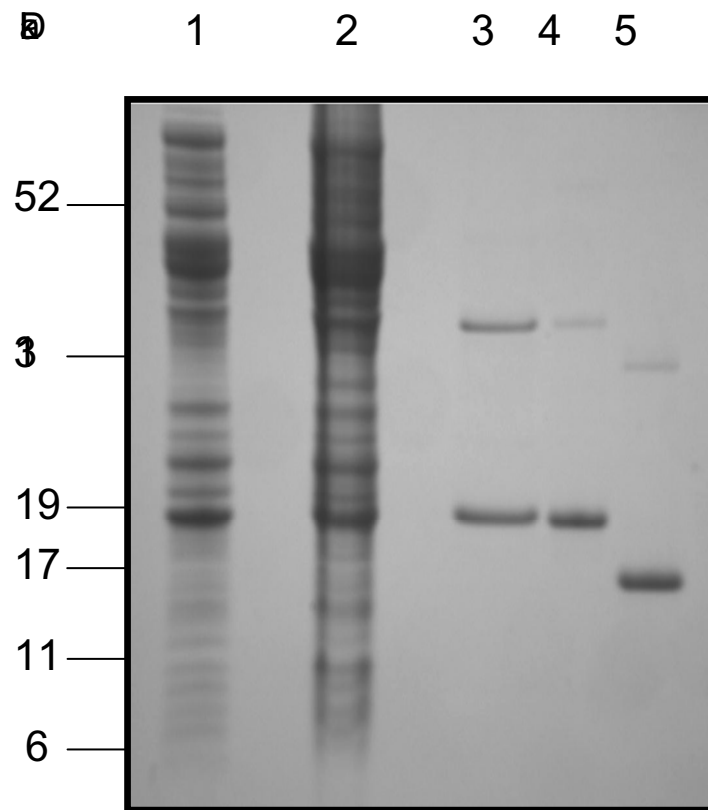


Figure 2, Greig *et al.*

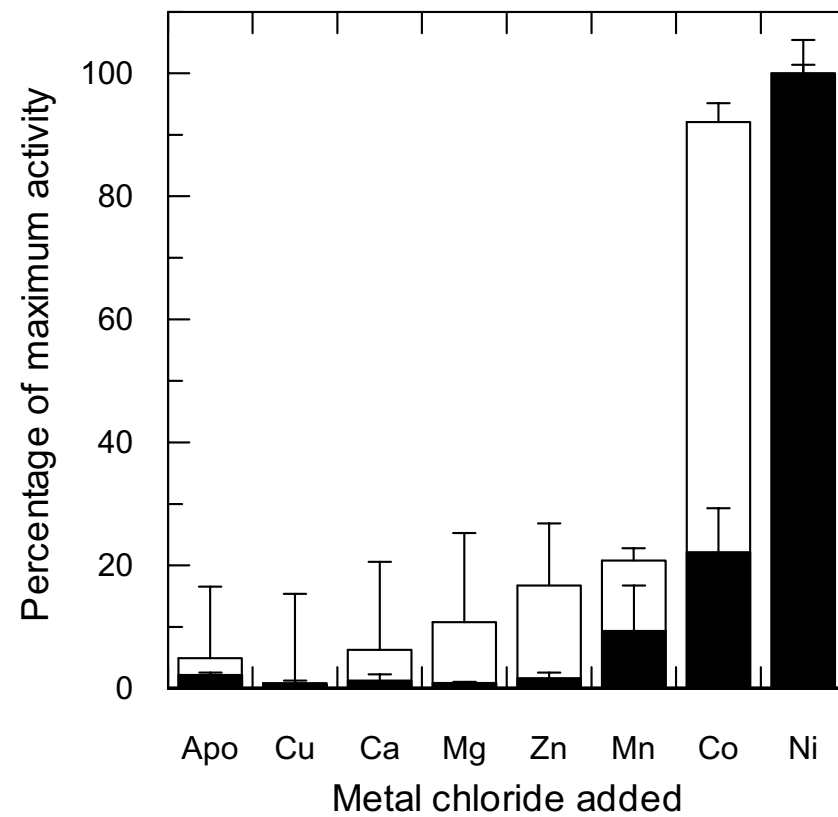


Figure 3, Greig *et al.*

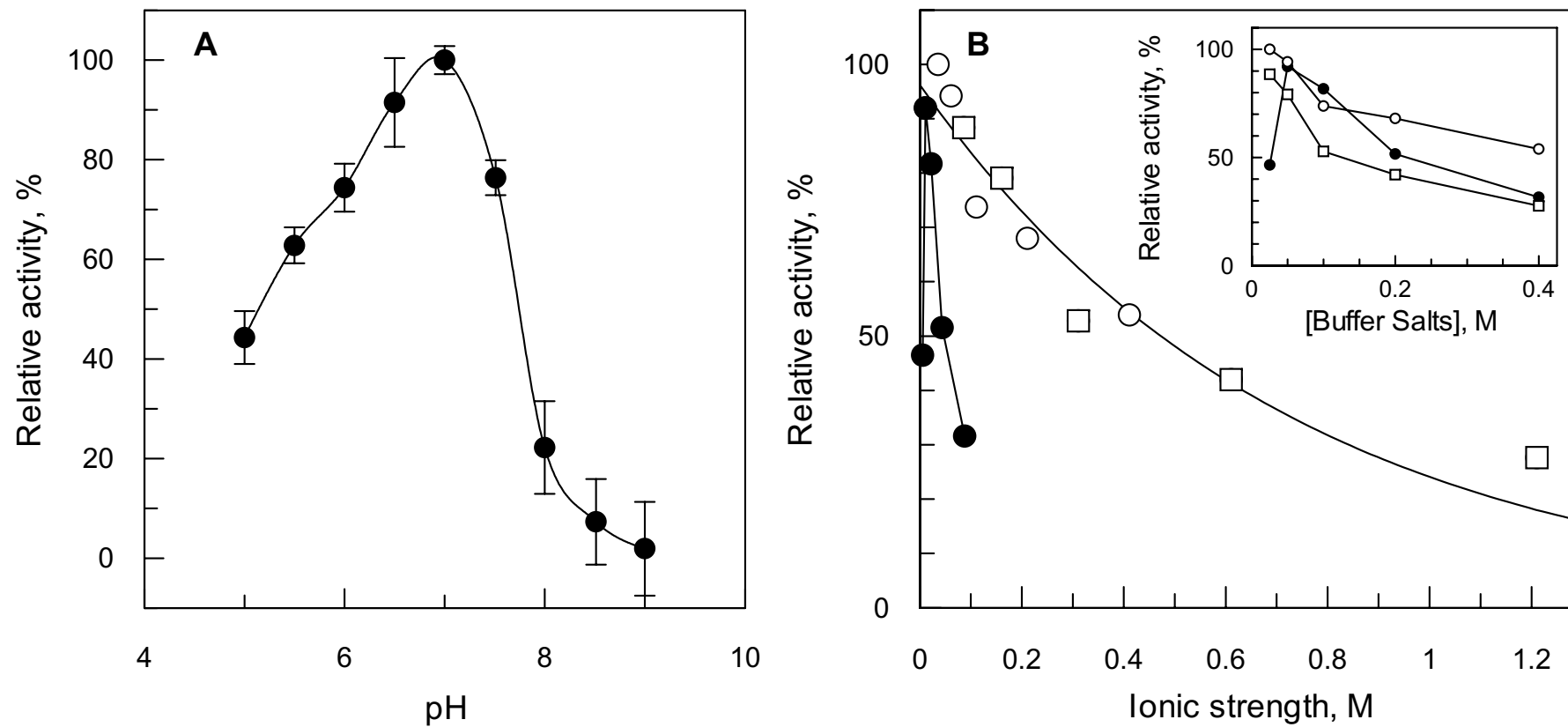


Figure 4, Greig *et al.*

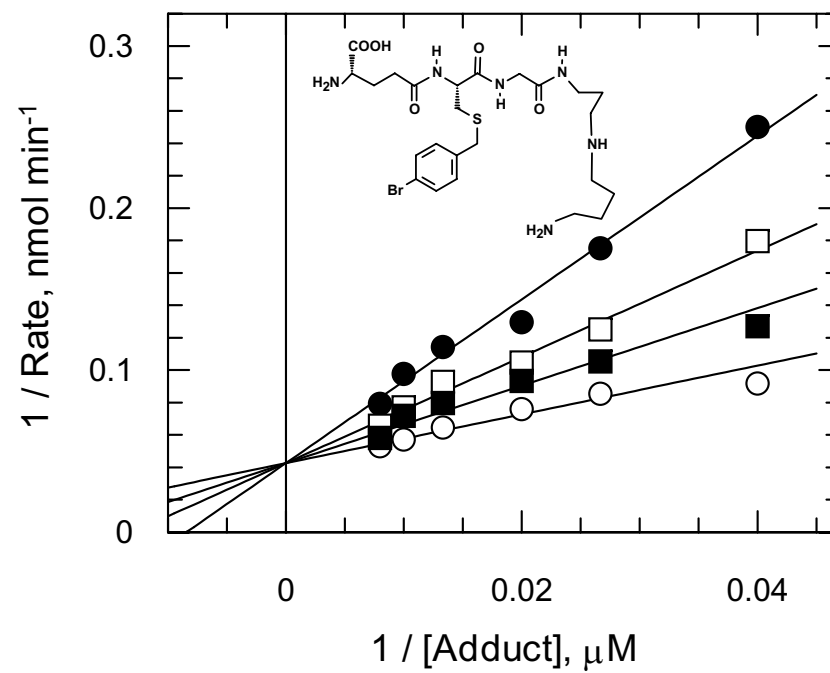


Figure 5, Greig *et al.*

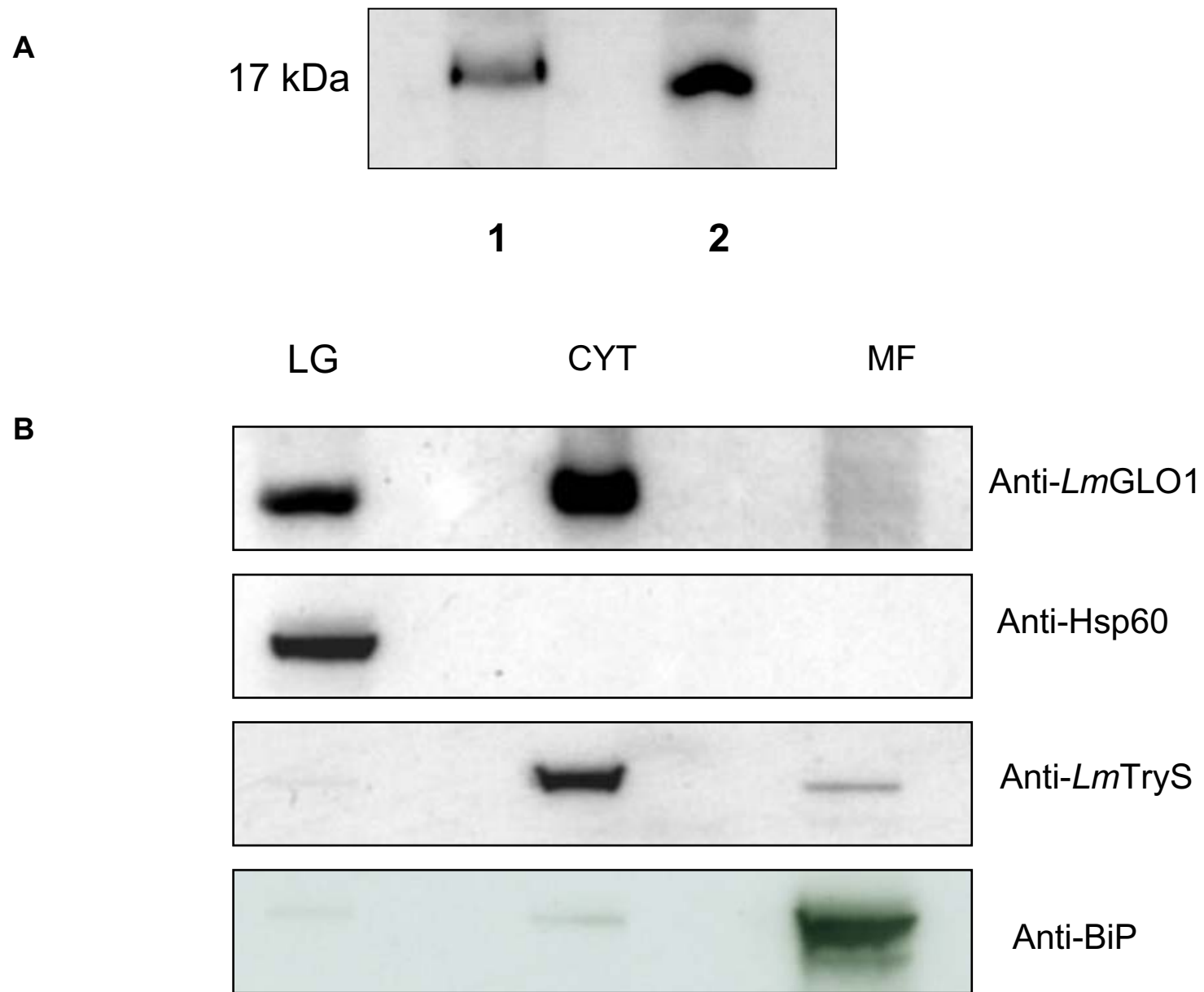


Figure 6, Greig *et al.*