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The terminal step in vitamin C biosynthesis in *Trypanosoma cruzi* is mediated by a FMN-dependent galactonolactone oxidase.

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Short title: Vitamin C biosynthesis in trypanosomes

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SYNOPSIS

Humans lack the ability to synthesise vitamin C (ascorbate), due to the absence of gulonolactone oxidase, the last enzyme in the biosynthetic pathway in most other mammals. The corresponding oxidoreductase in trypanosomes therefore represents a target that may be therapeutically exploitable. This is reinforced by our observation that *Trypanosoma cruzi*, the causative agent of Chagas disease, lacks the capacity to scavenge ascorbate from its environment and is therefore dependent on biosynthesis to maintain intracellular levels of this vitamin. Here, we show that *T. cruzi* galactonolactone oxidase (TcGAL) can utilize both L-galactono-γ-lactone and D-arabinono-γ-lactone as substrates for synthesis of vitamin C, in reactions that obey Michaelis-Menten kinetics. It is >20-fold more active than the analogous enzyme from the African trypanosome *Trypanosoma brucei*. Flavin mononucleotide (FMN) is an essential cofactor for enzyme activity and binds to TcGAL non-covalently. In other flavoproteins, a histidine residue located within the amino terminal flavin-binding motif has been shown to be crucial for cofactor binding. Using site-directed mutagenesis, we show that the corresponding residue in TcGAL (Lys-55) is not essential for this interaction. In contrast, we find that histidine and tryptophan residues (His-447 and Trp-448), localised within a carboxyl terminal motif (HWXK) that is a feature of ascorbate-synthesising enzymes, are necessary for the FMN-association. The conserved lysine residue within this motif (Lys-450) is not required for cofactor binding, but its substitution by glycine renders the protein completely inactive.

Key words: Ascorbate, trypanosomes, uptake, biosynthesis, site-directed mutagenesis

Abbreviations: ALO, arabinolactone oxidase; APX; ascorbate-dependent peroxidase; CHEFE, contour-clamped homogeneous electric field electrophoresis; GAL, galactonolactone oxidase; GALDH, galactonolactone dehydrogenase; GLO, gulonolactone oxidase; GULDH, gulonolactone dehydrogenase; IPTG, isopropyl β-D-thiogalactoside; Ni-NTA, Ni^{2+}-nitrilotriacetic acid

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INTRODUCTION

The insect-transmitted protozoan *Trypanosoma cruzi* is the aetiological agent of Chagas disease. There are more than 10 million people infected with this parasite in 21 countries across Central and South America, with another 40 million at risk. Around 14,000 deaths annually are attributed to Chagas disease [1,2]. There is no immediate prospect of a vaccine and the development of improved chemotherapy is a priority. Nifurtimox and benznidazole are the only drugs currently available for *T. cruzi* infections. However, both have toxic side effects and are largely non-effective against the chronic stage of the disease, which can occur years after the initial infection. The activation of nifurtimox within *T. cruzi* can lead to the production of reactive oxygen species, a process that has been implicated in trypanocidal activity [3]. As a consequence, oxidative defence in trypanosomes has been a major focus of research.

In aerobic organisms, endogenous biochemical processes result in the generation of reactive oxygen species, which include superoxide anions, hydrogen peroxide and hydroxyl radicals. Complex enzymatic and non-enzymatic pathways have evolved to protect cells from these toxic oxygen metabolites. In trypanosomatid parasites, many aspects of oxidative defence are distinct from those in the mammalian hosts. For example, trypanosomes lack catalase, their superoxide dismutases use iron as a cofactor and peroxide metabolism involves a complex series of overlapping pathways linked to the unique thiol trypanothione, a glutathione-spermidine conjugate [4-8]. In the *T. cruzi* endoplasmic reticulum, detoxification of hydrogen peroxide is dependent on an unusual plant-like hemoperoxidase (TcAPX) [5]. The activity of this enzyme is linked to trypanothione, via ascorbate reduction, in a process that involves non-enzymatic interaction, with ascorbate acting as an electron shuttle. *APX* genes are present in *T. cruzi* and the related protozoan *Leishmania*, both of which have an obligatory intracellular mammalian stage. However, the gene is absent from the African trypanosome, *Trypanosoma brucei*, which is exclusively extracellular. Ascorbate may therefore be more important to oxidative defence in *T. cruzi* and *Leishmania*, parasites which are exposed to reactive nitrogen/oxygen species when they invade host macrophages [9-12]. In line with this, deletion of both *TcAPX* genes

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has only been possible in parasites containing an episomal copy (MCT, unpublished data).

Ascorbate is a water-soluble vitamin that has multiple cellular functions, both as a cofactor and as an antioxidant molecule [13]. Two major properties enable ascorbate to detoxify reactive oxygen species directly. First, the low reduction potential of ascorbate and the ascorbyl radical allows them to reduce almost all radicals and oxidants [14]. Second, the ascorbyl radical, formed when ascorbate scavenges reactive nitrogen or oxygen species is very stable and has a low reactivity [15,16]. Most mammals and plants are able to synthesise ascorbate. Exceptions include humans and other primates, guinea pigs, and several species of fish [17]. Humans rely entirely on their diet for ascorbate, and as such, are susceptible to scurvy, a syndrome caused by vitamin C deficiency [18,19]. The final stage in ascorbate biosynthesis is common to all organisms where this pathway is active, and involves the conversion of an aldonolactone substrate to ascorbate. The flavin-dependent oxidoreductases responsible utilise a range of substrates in different cell types, and are classified accordingly; gulonolactone oxidase (GLO) in mammalian cells [20], arabinonolactone oxidase (ALO) in fungal cells [21], galactonolactone dehydrogenase (GALDH) in plant cells [22,23], and gulonolactone dehydrogenase (GULDH) in bacteria [24,25]. In most flavoproteins, the cofactor (FAD or FMN) is bound non-covalently. However in mammalian GLO and fungal ALO [21,22], this interaction is covalent, with the cofactor attached to a histidine residue, which is conserved in many other oxidoreductases where this type of linkage has been identified. Covalent binding of flavin cofactors typically involves histidine, although there are instances where linkage to cysteine or tyrosine residues has been identified [26].

In humans, the terminal enzyme in ascorbate biosynthesis is missing, although a pseudogene remains [27]. This absence raises the possibility that specific-inhibitors targeted at this biosynthetic step could have potential as antimicrobial or antiparasitic agents, in situations where this pathway is essential for pathogen viability. However, inhibitor design has so far been restricted by the lack of a three-dimensional structure for this class of enzyme and the absence of detailed information on the precise mechanism of action. Recently, we demonstrated that *T. brucei* has an ascorbate biosynthetic capacity and that this reaction occurs in the glycosome, a kinetoplastid-

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specific organelle related to peroxisomes [28]. The enzyme which catalyses the final step in this pathway can use both galactonolactone and arabinonolactone as substrates. It has a slight preference for the latter and was initially designated TbALO. Here, we describe the properties of the corresponding enzyme from *T. cruzi.* We show that activity of this plant-like enzyme is dependent on the non-covalent binding of an FMN cofactor. Furthermore, using site-directed mutagenesis, we identify residues required for flavin-binding, and consequently for enzyme activity.

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EXPERIMENTAL

Parasites

T. cruzi epimastigotes (MHOM/BR/78/Silvio - X10/6) were cultured at 28 °C in RPMI supplemented medium (Sigma) [29]. Culture-derived amastigotes were obtained from Vero cells (ATCC CRL-1586) infected with metacyclic stationary-phase trypomastigotes. Genomic DNA was isolated from parasites by the phenol/chloroform method, and chromosomes for contour-clamped homogenous electric field electrophoresis (CHEFE) analysis were extracted using an agarose-embedding technique [30] and separated by a BioRad CHEFE mapper. Total RNA was extracted from parasites using the RNeasy® mini-kit (Qiagen).

Ascorbate uptake

L-[carboxyl-^{14}C] ascorbate (13 mCi mmol^{-1}) and D-[2-^{3}H] glucose (14 Ci mmol^{-1}) were supplied by GE Healthcare UK Ltd. For assays of dehydroascorbate uptake, radiolabelled ascorbate was oxidised to dehydroascorbate by the addition of 0.5 % (v/v) bromine. The resulting solution was vortexed for 30 seconds, and then nitrogen bubbled through for 10 minutes to remove the bromine. Aliquots of dehydroascorbate were prepared freshly on each occasion, and used immediately. Epimastigotes, in the exponential phase of growth, were pelleted and washed 3 times in assay buffer (90 mM Tris.HCl pH 7.5, 3.1 mM KCl, 96.9 mM NaCl, 5 mM $MgCl_2$, 2 mM Na_2HPO_4, 2 mM glycerol), then resuspened at 2 x 10^8 cells ml^{-1}. To initiate uptake, 50 µl parasites was added to an equal volume of assay buffer containing radiolabelled ascorbate (0.625 µCi) or glucose (5 µCi), giving final concentrations of 480 µM and 3.6 µM, respectively. The cell suspension was layered over a 150 µl cushion of oil (1-bromododecane) in a 1.5 ml eppendorf tube. Reactions were incubated at 22 °C and terminated by the addition of 200 µl 10mM unlabelled substrate in assay buffer (Quench buffer) at <0 °C. The cells were then separated from the uptake buffer by centrifugation at 13,000 ***g*** for 30 seconds and the tubes flash-frozen using liquid nitrogen. The parasite pellet was cut from the bottom of the tube into a scintillation vial, and lysed in 250 µl 2 % (w/v) SDS. 3 ml scintillation fluid (Ecoscint A, National Diagnostics) was added to vials, which were incubated overnight. Incorporated radioactivity was then measured using a Beckman liquid scintillation spectrometer.

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Vero-cell derived amastigotes were treated in a similar manner, except that cells were not layered over oil, and the assay was stopped by the addition of 2 % (v/v) paraformaldehyde, followed by pelleting of the cells as before. Pellets were washed 3 times in 1 x PBS, followed by lysis in 250 µl 2 % (w/v) SDS in a scintillation vial. As a control, quench buffer was added to the assay prior to the cells. The tube was then immediately centrifuged, and the pellet treated as for the experimental samples.

Isolation of the *T. cruzi* galactonolactone oxidase (*Tcgal*) gene

Tcgal was amplified from *T. cruzi* (X10/6 clone) genomic DNA using primers based on the sequence of the corresponding gene from the genome reference clone CL Brener (GeneDB; accession number Tc00.1047053509179). Primers 5'-AGAggtaccTTGTGACGTTTCCATGCGGCCC and 5'-GGGaagcttTTACAAATGACTATTGGTGCT were used for amplification. Lower case letters indicate restriction sites for *Kpn* I and *Hin*d III, respectively, incorporated to facilitate cloning of the resulting fragment. Amplifications were performed under the following conditions: 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 2 minutes. The 1.5 kb product was ligated into the *E. coli* expression vector pTrcHis-C (Invitrogen).

Expression and purification of His-tagged TcGAL

E. coli BL-21+ were transformed with the plasmid pTrcHisCTcGAL. Optimal expression of the soluble protein was obtained by induction with 100 µM isopropyl β-D-thiogalactoside (IPTG) for 6 hours at 37 ºC. The induced cells were pelleted and lysed by sonication in 20 ml buffer A (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) + 10 mM imidazole. The lysate was centrifuged at 7,000 ***g***, and the supernatant applied to a 2 ml Ni^{2+}-nitrilotriacetate (Ni-NTA) column (Qiagen), pre-equilibriated in buffer A. Initially, the column was washed with 40 ml buffer A + 20 mM imidazole, and the recombinant protein then eluted in buffer A + 250 mM imidazole. Each step was performed in the presence of protease inhibitors (Roche Molecular Biochemicals). Fractions were analysed by SDS/PAGE, and protein concentrations determined by the bicinchoninic acid protein assay system (Pierce). Approximately 3 mg purified recombinant protein was recovered per litre of *E. coli* culture.

Site-directed mutagenesis

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Oligonucleotide-directed *in vitro* mutagenesis was performed using the Stratagene QuikChange® mutagenesis kit, following the manufacturer's protocol. Briefly, amplifications were performed in 50 µl reaction volumes, with pTrcHisCTcGAL as template. The reaction consisted of 1 cycle of 95 °C for 30 seconds, then 16 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 68 °C for 6 minutes. Following this, 10 units *Dpn* I were added to the reaction mixture to digest parental double-stranded DNA. 1µl *Dpn* I-digested PCR product was then used to transform *E. coli* XL1-Blue supercompetent cells. The following sense primers (along with their antisense counterparts) (Sigma Genosys), were used to generate each of the desired mutations. The relevant substitution sites, incorporating the required base change, are underlined.

K55G: 5'-TGCCGGGGCCGGAGGAAGCCCCAATGC,
K55H: 5'-TGCCGGGGCCGGACATAGCCCCAATGC,
K55L: 5'-TGCCGGGGCCGGACTAAGCCCCAATGC,
H447G: 5'-GGGCGGTAGGCCGGGATGGGCGAAGTACTACACATGG,
W448G: 5'-TAGGCCGCACGGTGCGAAGTACTACACATGG,
K450G: 5'-GCACTGGGCGGGATACTACACATGGGG.

Tcgal mutants were confirmed by sequencing using a BigDye® terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM® 377 automated sequencer.

Enzyme activity

Activity was measured by monitoring ferricytochrome *c* reduction, using an absorbance of 550 nm. A standard reaction mixture (1 ml) contained 50 mM KH_2PO_4 pH 8.0, 1 mM EDTA, 100 µl cytochrome *c* and 12.2 µg purified TcGAL. The background rate of cytochrome c reduction was determined, and the reaction initiated by the addition of D-arabinono-γ-lactone (Dextra Lab), L-gulono-γ-lactone (Acros), or L-galactono-γ-lactone (Sigma). Enzyme activity was calculated using a ε value of 17.3 mM^{-1}, and assumes that two molecules of ferricytochrome *c* are reduced per molecule of ascorbate oxidised [31]. Data were analysed using GraphPad Prism software. Inhibitory compounds [21,32] were added to the reaction mixture prior to the reading of the background rate of cytochrome *c* reduction.

High performance liquid chromatography analysis

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All HPLC equipment and software were from Dionex (UK) Ltd. Separations were carried out as outlined by Birch *et al.* [33] utilising an ODS Hypersil 5 µm column (250 x 4.6 mm) (Thermo Electron Corp.), eluting with acetonitrile/water/trifluoracetic acid (10 % w/v) in water/phosphoric acid (14 : 84 : 1.5 : 0.09) passing through the fluorescence detector (Dionex model RF 2000) set at the excitation wavelength of 430 nm and emission wavelength of 525 nm, at a flow rate of 0.5 ml min^{-1}. Peak identity in the protein fraction was confirmed by measuring the retention time, and comparison with commercially available FMN and FAD. On an assumption of one molecule of flavin per molecule of TcGAL, 12 mg ml^{-1} of purified protein was used to generate 0.1 mg ml^{-1} of cofactor. TcGAL was trypsin digested in the dark for 3 hours at 37 ^{o}C prior to mixing with a solution of ice cold 5 % (w/v) trichloroacetic acid in water, and the precipitated protein removed by centrifugation at room temperature. A 20 µl aliquot of the resulting supernatant was injected onto the column, and the chromatograms were compared with those from the standard samples of FMN and FAD.

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RESULTS

Ascorbate uptake capacity of *T. cruzi*

To determine if *T. cruzi* epimastigotes and amastigotes can scavenge ascorbate from their environment, uptake assays were performed on both life-cycle stages. Parasites were incubated with radiolabelled ascorbate or glucose and the extent of uptake measured (Experimental section). Both parasite life-cycle stages took up glucose rapidly, reaching equilibrium within 5-10 minutes, indicating that they were competent for transport (Figure 1). Epimastigotes transported 20 times as much glucose as amastigotes (V_{max} 10.95 and 0.49 pmoles min^{-1} per 10^7 cells, respectively), partly reflecting their larger cell volume (2-3-fold; [34]). In contrast, neither cell type displayed any capacity to take up ascorbate, even when followed for more than an hour (Figure 1). Mammalian cells can also transport dehydroascorbate, the oxidised form of the vitamin [35]. Radiolabelled ascorbate was therefore converted to dehydroascorbate by the addition of bromine (Experimental section), and uptake assays carried out as before. Neither cell type showed any capacity to take up dehydroascorbate over a 60 minute time course (data not shown). These experiments imply that both the insect and intracellular mammalian stages of *T. cruzi* are incapable of obtaining ascorbate from their environment, and as such, must acquire this vitamin by *de novo* synthesis.

Isolation and expression of *T. cruzi* galactonolactone oxidase

A putative galactonolactone/arabinonolactone oxidase gene was identified in the *T. cruzi* database [36]. DNA primers were designed and used to amplify the corresponding sequence from parasite genomic DNA (X10/6 clone). The 1.5 kb gene (designated *Tcgal*) has the potential to encode a 57 kDa protein (Figure 2) and is expressed as a 2.0 kb transcript (Supplementary Figure 1). In CHEFE analysis, the *Tcgal* probe hybridised to a chromosome band of 1100 kb (Supplementary Figure 2). With the genome reference strain CL Brener, bands of ~3000, 1100, and 600 kb were detected. These differences reflect the hybrid nature of the CL Brener clone [37], and the fact that chromosome homologues in *T. cruzi* often vary considerably in size.

Proteins that synthesise the final step in ascorbate biosynthesis do not display a high level of cross-species sequence conservation (~15 %). One feature they do share is an

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amino terminal flavin-binding motif (Figure 2A), which often contains a histidine residue implicated in the covalent attachment of FAD. Substitution of this residue with leucine in plant GALDHs has been interpreted as the reason that flavin binding occurs non-covalently. Unusually, in *T. cruzi*, the corresponding amino acid is a lysine (Lys-55). A second histidine residue, which is widely conserved in aldonolactone oxidases/dehydrogenases, has also been implicated in FAD-binding in the flavoprotein vanillyl-alcohol oxidase [38]. In TcGAL, this residue is also a histidine (His-447) and is found within the motif HWXK (highlighted, Figure. 2A), a feature that is completely conserved in ascorbate-synthesising enzymes. The *T. cruzi* enzyme also contains the carboxyl terminal tripeptide SHL (black bar, Figure 2A), which has been implicated in glycosomal targeting [28].

To investigate the biochemical properties of TcGAL, the gene was cloned into the vector pTrcHis-C and expressed in *E. coli* BL-21+ (Experimental section). In this system, recombinant TcGAL is tagged at its amino terminus with a histidine-rich sequence and an epitope-tag detectable with the anti-Xpress monoclonal antibody (Invitrogen). After IPTG induction, the recombinant protein could be readily purified by one round of affinity chromatography on a Ni-NTA column, following washing with imidazole (Experimental section). SDS/PAGE gels and western blotting using the anti-Xpress antibody confirmed the purification of the 55 kDa protein (Figure 3).

Characterisation of TcGAL activity

Assays were carried out using purified recombinant TcGAL (Figure 3) and, as a control, extracts of non-transformed *E. coli* BL-21+ that had been affinity purified in parallel. Activity was assessed in the presence of various aldonolactone substrates. We found that recombinant TcGAL could synthesise vitamin C using the substrates L-galactono-γ-lactone and D-arabinono-γ-lactone, but no significant activity was detected with L-gulono-γ-lactone, the preferred substrate for the mammalian enzyme. Recombinant TcGAL was active across a wide range of temperatures, but was heat inactivated above 50 °C. The optimal pH was between 7.5 and 8.0. Kinetic studies were carried out using L-galactono-γ-lactone and D-arabinono-γ-lactone as substrates, measuring the TcGAL-mediated ascorbate-dependent reduction of ferricytochrome c. Double-reciprocal plots of 1/TcGAL activity vs 1/[substrate] were linear for substrate

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concentrations up to 4 mM (Figure 4), allowing kinetic constants for both metabolites to be calculated (Table 1A). Based on apparant V_{max} values, TcGAL is >20-fold more active towards both substrates than the *T. brucei* homologue TbALO [28]. TcGAL exhibited a slightly higher turnover constant and catalytic efficiency value for L-galactono-γ-lactone over D-arabinono-γ-lactone, the normal substrates of the plant and fungal enzymes, respectively (Table 1A). This, together with an analysis of the *T. cruzi* genome sequence [36], which suggests that L-galactono-γ-lactone is the normal physiological substrate (FJL, unpublished), led us to designate the enzyme as TcGAL. The apparant K_M value for L-galactono-γ-lactone (Table 1A) was in a similar range to that previously established for plant GALDHs (60–150 μM) (Table 3).

It has previously been suggested that cysteine residues may play a role in aldonolactone oxidoreductase activity [21]. To establish if this was the case for TcGAL, we carried out assays in the presence of P-chloromercuribenzoate (pCMB), N-ethyl maleimide (NEM), zinc and mercury ions. pCMB and NEM form thioether linkages with cysteine sulphydryl groups, specifically inhibiting them, whilst zinc and mercury ions, lead to toxicity mainly due to sulphydryl group inhibition [39]. The inhibition profile in each case suggested that cysteine residue(s) are essential for the activity of this enzyme (Table 1B). These results are similar to those seen with the *Candida albicans* ALO [21], which shares several cysteine residues (Cys-27 and Cys-249) with TcGAL.

To determine the nature of the interaction between TcGAL and its flavin cofactor, we added 5 % trichloroacetic acid to trypsin digested recombinant enzyme and subjected the extract to HPLC analysis (Experimental section). This treatment resulted in release of the flavin cofactor from the protein, indicating that it was noncovalently bound [40]. FAD and FMN standards were fractioned on the HPLC with retention times of 8.13 and 12.26 minutes, respectively (Figure 5). The supernatant from the TcGAL extraction gave a retention time of 12.26 minutes for the flavin peak, identifying the cofactor as FMN. The *T. brucei* enzyme, which had previously been assumed to covalently bind FAD [28], also had a profile consistent with noncovalently bound FMN (data not shown). Therefore, the trypanosome enzymes show a significant difference to the situation in the mammalian GLO and the fungal

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ALO, where the cofactor is covalently bound FAD [20,21], but similarity to plant GALDHs, which also noncovalently bind FMN [22]. In the case of bacterial GULDH, the cofactor has yet to be identified, but is a noncovalently bound flavin [25].

Effects of mutated residues

The flavin-binding motif toward the amino terminus of trypanosome GAL/ALO (residues 22–57) (Figure 2) lacks the histidine residue which in mammalian GLOs and fungal ALOs, covalently binds FAD via an 8-α-(N1-histidyl)-riboflavin linkage [20,26]. Instead, it contains a lysine (Lys-55) at this position. In plant GALDHs, where the corresponding residue is a leucine (Figure 2B), the flavin cofactor is FMN, and it is bound noncovalently [22]. However, with bacterial GULDHs, where the flavin cofactor also binds non-covalently, there is a histidine at this position [25]. Therefore, rather than influencing the type of cofactor interaction, this residue could be a determinant of cofactor specificity, with the histidine residue being associated with FAD binding, and the lysine/leucine residues with FMN binding. In the absence of information on the nature of the flavin cofactor in the bacterial enzyme, we therefore determined the functional importance the corresponding residue in TcGAL.

Using site-directed mutagenesis (Experimental section), we investigated the role of key residues (Lys-55, His-447, Trp-448, and Lys-450; Figure 2B), proposed to be involved in cofactor binding (Lys-55), and enzyme activity. We first converted the lysine in TcGAL to glycine (Lys-55-Gly). This had only a minor inhibitory effect (~25 %) on both FMN binding (Table 2) and biochemical activity (Figure 6). By inference, the lysine at this position in TcGAL does not have an essential role in either cofactor binding or enzyme activity. When the lysine was converted to a histidine (Lys-55-His), as in mammals, bacteria, and fungi, FMN binding was dramatically reduced (>90 %) and enzyme activity fell by a similar amount (Table 2, Figure 6). When the lysine was converted to leucine (Lys-55-Leu) as in plants, flavin was not detectable, and the enzyme was inactive. It may be that the bulkier side chains in these amino acids provide steric hindrance that blocks FMN binding, with subsequent loss of enzyme activity. The conversion of Lys-55 to a histidine, as in mammals, did not appear to alter the non-covalent nature of the residual FMN binding.

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The 'HWXK' domain in the carboxyl terminus of TcGAL (Figure 2B) is highly conserved amongst enzymes that synthesize ascorbate and is specific to this group. There are no detailed reports on the mechanism by which these enzymes function, and the role of this region has yet to be elucidated. When residues His-447 and Trp-448 in TcGAL were converted individually to glycine, we found that the mutated enzyme was inactive (Figure 6) and FMN binding not detectable (Table 2). Assuming that the amino acid changes that we introduced do not affect protein folding, these experiments therefore suggest that the histidine and tryptophan residues participate in non-covalent FMN binding, directly or indirectly. When the conserved lysine was replaced with glycine (Lys-450-Gly), this substitution resulted in an enzyme that retained an ability to bind FMN (~50 % of the wild-type enzyme), but which exhibited no activity. The result is consistent with Lys-450 having an important role in activity that is independent of flavin binding.

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DISCUSSION

Humans do not have the capacity to synthesise ascorbate, and rely on their diet to provide sufficient levels of this vitamin. In contrast, *T. cruzi* appears to lack the ability to scavenge ascorbate (Figure 1), and is dependent on biosynthesis to maintain intracellular levels. Ascorbate plays an important function in the *T. cruzi* oxidative defence system. In addition to its role as a free radical scavenger, it acts as an electron shuttle which transfers reducing equivalents from trypanothione to the ER-localised hemoperoxidase TcAPX [5], an enzyme that appears to be essential for parasite survival (MCT, unpublished). In both *T. brucei* and *T. cruzi*, the enzyme responsible for the terminal step in ascorbate biosynthesis is a glycosomal flavin-dependent oxidoreductase (Table 3). Despite the widespread distribution of ascorbate-synthesizing enzymes, surprisingly little is known about their structure or the precise location of amino acids critical to function.

The mammalian and fungal ascorbate-synthesizing flavoproteins utilize covalently linked FAD as a cofactor [20,21]. In contrast, FMN is the cofactor found in plant enzymes, where it binds non-covalently [22]. Here, we have shown that the corresponding enzymes from *T. cruzi* and *T. brucei* resemble the plant enzymes with regard to flavin specificity and binding. Similar to both the fungal arabinonolactone oxidases and the plant galactonolactone dehydrogenases, the *T. cruzi* enzyme appears to require sulphydryl groups for biochemical activity, as inhibition is observed in the presence of reagents which specifically inactivate these groups. The trypanosome enzymes, like those of fungi, can utilise either arabinonolactone or galactonolactone as substrates, compounds in which the hydroxyl groups on the lactone ring are arranged in a *trans*-configuration. Whereas the *T. brucei* enzyme has a preference for arabinonolactone over galactonolactone (K_M 55 and 154 μM, respectively), TcGAL has a slightly higher affinity for galactonolactone over arabinolactone (K_M 161 and 285 μM, respectively). In terms of specific activity however, that of the *T. cruzi* enzyme is more than 20-fold higher than that of the *T. brucei*. This may reflect a greater requirement for ascorbate in a parasite that has an obligatory intracellular stage, and can be exposed to reactive oxygen and nitrogen species generated as part of

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the immune response. Consistent with this, *T. cruzi,* but not the extracellular parasite *T. brucei*, contains an ER-localised ascorbate-dependent peroxidase [5].

Ascorbate-synthesising enzymes, including those from trypanosomes, contain two conserved domains - the amino terminal flavin binding region and the carboxyl terminal HWXK motif. Since there is a paucity of structural data for this group of enzymes, we used site-directed mutagenesis to explore the functional significance of specific amino acids in these conserved regions. Within the amino terminal flavin binding domain, residue 55 had been linked to cofactor binding. In the mammalian and fungal enzymes, this amino acid is a histidine which is covalently linked to FAD [20,21]. In trypanosomes, the corresponding residue is a lysine, in plants a leucine, and in bacteria a histidine. Enzymes from each of these groups bind their cofactors non-covalently [21,25], although the identity of the flavin cofactor in bacteria is not yet known. One possibility is that the residue at this position could be a determinant of cofactor specificity, with histidine being associated with FAD and leucine/lysine with FMN. When Lys-55 in the *T. cruzi* enzyme was replaced with glycine, this had only a minor effect on enzyme activity and the extent of FMN interaction. In contrast, its replacement with histidine greatly reduced cofactor binding and enzyme activity, although there was no shift in the preference of FMN over FAD. Therefore, our results show that in TcGAL, Lys-55 does not have a direct influence on flavin specificity, although its replacement by residues with bulky side chains does reduce FMN binding, with a resultant loss of enzyme activity.

In the conserved carboxyl terminal 'HWXK' motif, we observed that mutations led to a loss of enzyme activity. In the case of mutants His-447-Gly and Trp-448-Gly, this was associated with an inability to bind FMN. With the Lys-450-Gly mutation however, the enzyme retained a capacity to bind the flavin cofactor, although all activity was lost. These results highlight the significance of this conserved domain for ascorbate biosynthesis. In the flavoprotein vanillyl-alcohol oxidase [38], a carboxyl terminal histidine residue covalently links the FAD cofactor, whilst an amino terminal histidine residue promotes this covalent linkage. If either histidine residue was mutated however, the enzyme retained the ability to bind FAD non-covalently, although enzyme activity was reduced. It has been suggested, at least in the case of vanillyl-alcohol oxidase, that the carboxyl terminal histidine residue could form part

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of a second flavin binding domain, although a scenario where it might be involved in 'docking' the cofactor has been postulated. This has also been considered a possibility with TcGAL and TbALO [28]. Our findings indicate that in the case of TcGAL, His-447 and Trp-448 appear to a have a role, directly or indirectly, in FMN binding, with a concomitant effect on enzyme activity. Furthermore, the observation that the Lys-450-Gly mutant retained a capacity for FMN binding in the absence of enzyme activity, may point to this residue having an alternative role in the some other aspect of the biochemical mechanism, distinct from the cofactor interaction. Certainly the loss of activity associated with these mutations supports the hypothesis that the conserved HWXK motif plays an important role in the active site of the enzyme.

TcGAL shares many characteristics with the plant and fungal enzymes (Table 3), even though there is not a high degree of conservation at the level of primary sequence. The enzymes are of similar size and contain sulphydryl groups that are necessary for full activity, as inferred from inhibition profiles. The trypanosomal enzyme shares with the plant enzyme a preference for noncovalently bound FMN as its prosthetic group. This contrasts with the fungal and mammalian enzymes which are reliant on covalently bound FAD. Although the trypanosomal and plant enzymes appear to be more closely related, they do not share all of the same characteristics. Both TcGAL and mammalian GLO are microsomally located, whereas the plant GALDH and fungal ALO are mitochondrial. The natural substrate of the *T. cruzi* enzyme is not yet known. TcGAL has the ability to use arabinonolactone or galactonolactone, which would give rise to D-erythroascorbic acid or L-ascorbic acid, respectively. These products have indistinguishable biochemical properties. Fungi synthesize D-erythroascorbate from arabinose, whereas plants synthesize ascorbate in several ways, the best characterized pathway being from glucose via mannose and galactose to reach galactonolactone and then ascorbate. Many of the genes in this pathway, such as galacturonate reductase, and GDP-mannose-3,5-epimerase, have been described previously [41]. The mammalian vitamin C biosynthetic pathway also starts with glucose, but from there it follows a different path, through glucoronate to gulonolactone oxidase [42]. Putative homologues of each of the genes from the plant pathway can be identified in the *T. cruzi* genome, suggesting that ascorbate biosynthesis in trypanosomes could proceed by way of a plant-like pathway, via L-galactose and L-galactono-γ-lactone [28].

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In summary, this study shows that trypanosomes are unable to obtain ascorbate from the environment, and that the terminal step in vitamin C biosynthesis in *T. cruzi* involves an enzyme that is significantly different from the mammalian equivalent. Furthermore, we have demonstrated for the first time, the importance of the HWXK motif to this reaction. Genetic-based approaches, aimed at resolving the biological importance of TcGAL, should now provide additional experimental evidence for the validation of this pathway as a candidate drug target.

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Figure Legends

Figure 1. *T. cruzi* lack an ascorbate uptake capacity.

Epimastigotes (A) and Vero cell-derived amastigotes (B) (10^7 parasites per assay) were incubated with ^{3}H-labelled glucose (squares) or ^{14}C-labelled ascorbate (circles) for the times indicated. Cells were prepared and uptake of the corresponding radiolabel measured by scintillation counting (Experimental section). The data are expressed in disintegrations per minute (d.p.m.). Similar results were obtained with dehydroascorbate.

Figure 2. Organisation of TcGAL.

(A) Schematic of TcGAL showing the flavin-binding motif (residues 22-57) (PFAM designation PF01565), catalytic region (residues 436-452) (PFAM designation PF04030) and glycosomal targeting signal (black bar) [28]. Residues selected for site-directed mutagenesis are highlighted in bold, with arrows indicating new amino acids that were generated. Numbers refer to position of amino acids in the TcGAL sequence (GenBank accession no. Tc00.1047053509179.100). (B) Alignment of selected regions of TcGAL (Tc), with the corresponding sequences from *Mus musculus* GLO (Mm) (AAH28828), *Arabidopsis thaliana* GALDH (At) (T06690), and *Mycobacterium tuberculosis* GULDH (Mt) (D70989).

Figure 3. Purification of recombinant TcGAL.

Lanes 1-6; 10 % SDS-PAGE gel stained with coomassie blue. Lane 1, size standards; lane 2, crude extract loaded on a Ni-NTA column and flow through (lane 3). The column was washed extensively with 10 mM imidazole (lane 4, first wash; lane 5, last wash). Recombinant protein was eluted with 200 mM imidazole (lane 6). Lane 7; Western blot of samples from the 200 mM imidazole elution stage in TcGAL purification. Samples were western blotted and probed with anti-Xpress antibody (Invitrogen) to identify the recombinant protein. Markers are in kiloDaltons.

Figure 4. Lineweaver Burke plot of TcGAL activity

The activity of TcGAL was assayed by following the ascorbate-dependent reduction of ferricytochrome c in the presence of 12.2 μg TcALO and D-arabinono-γ-lactone (0.025–4 mM) or L-galactono-γ-lactone (0.025–4 mM) (Experimental section).

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Double reciprocal plots of 1/TcGAL activity (nmol ascorbate formed min^{-1} mg^{-1}) against 1/[galactonolactone] or 1/[arabinonolactone] were linear for substrate concentrations up to 4 mM.

Figure 5. TcGAL noncovalently binds FMN

HPLC analysis was performed as described (Experimental section). Under these conditions, FAD (dotted line) and FMN standards (dashed line) could be separated with retention times of 8.13 and 12.26 minutes, respectively. The supernatant from TCA precipitated wild type TcGAL (122 μg) had a retention time of 12.26 (solid line), confirming FMN as the flavin cofactor.

Figure 6. Activity of TcGAL mutant proteins.

12.2 μg TcGAL (WT) and six mutant proteins (Figure 2) were assayed for the production of ascorbate (Experimental section) in the presence of 2 mM arabinonolactone (arab) or galactonolactone (gala). Activity is given in nmoles ascorbate formed min^{-1} mg^{-1} protein. Assays were carried out in duplicate, with standard errors indicated.

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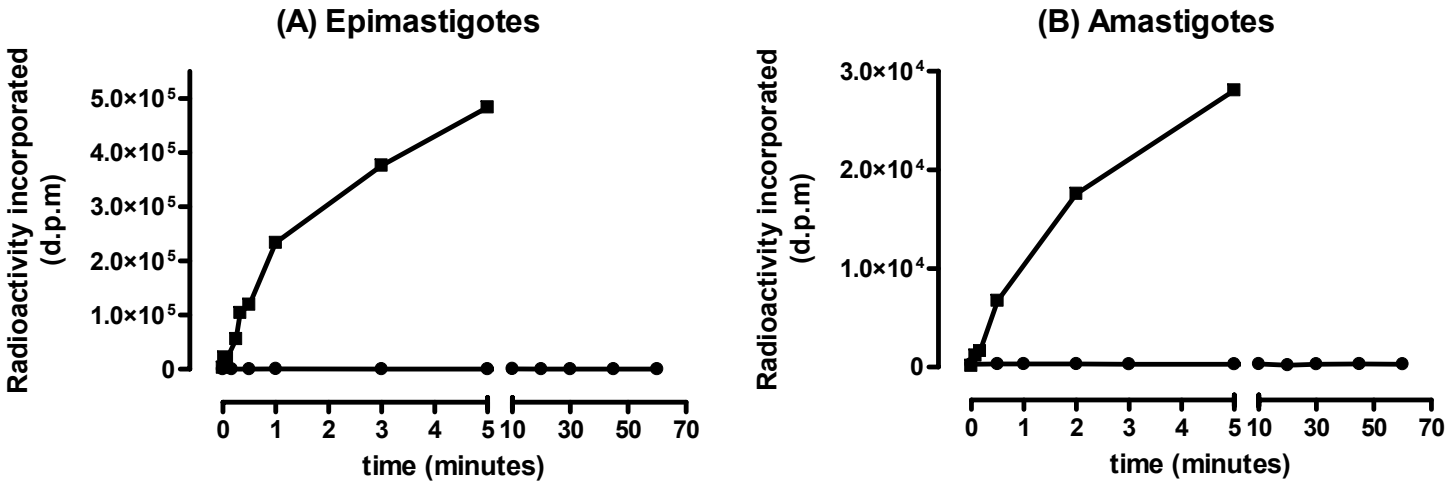

Figure 1.

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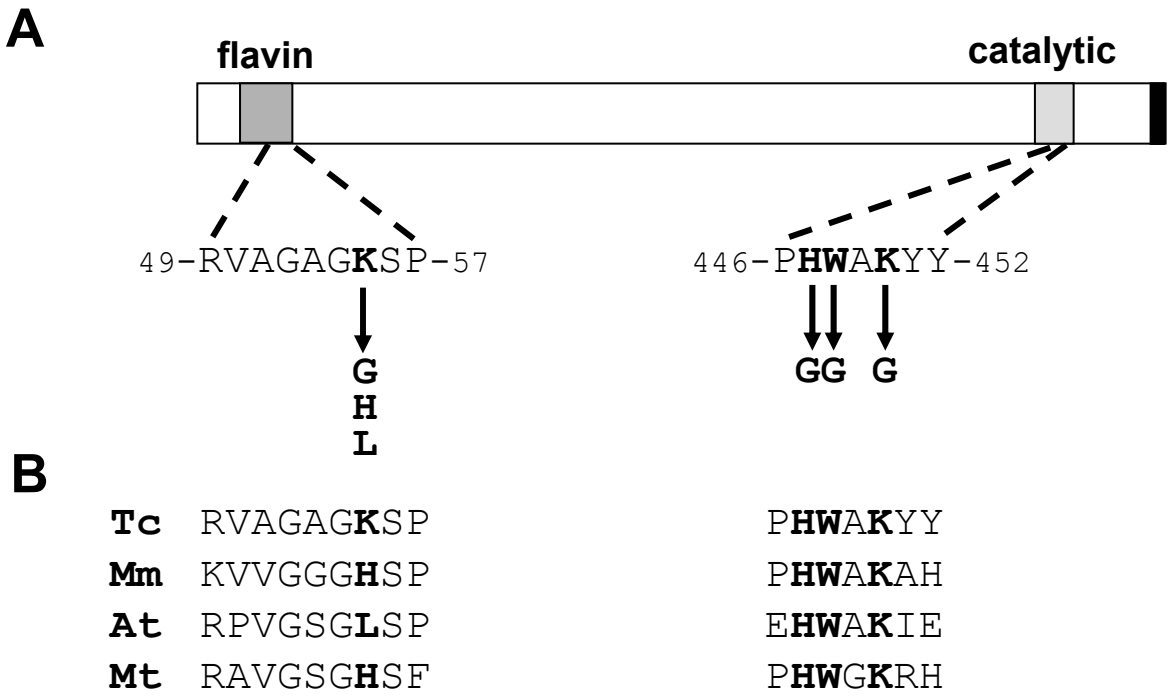

Figure 2.

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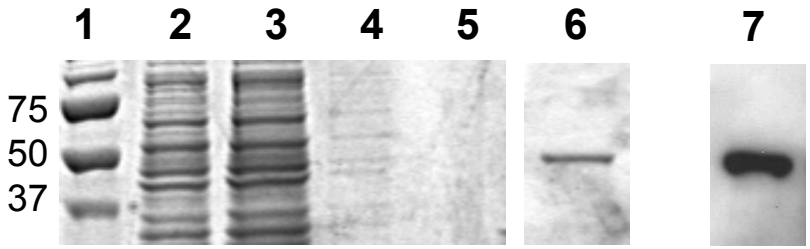

Figure 3.

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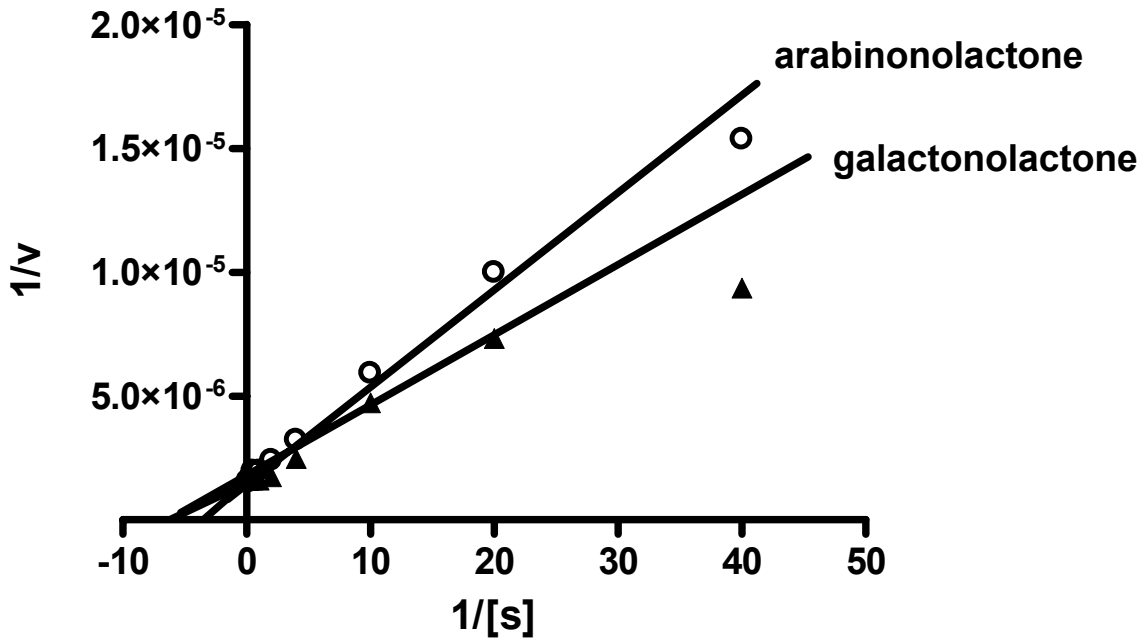

Figure 4.

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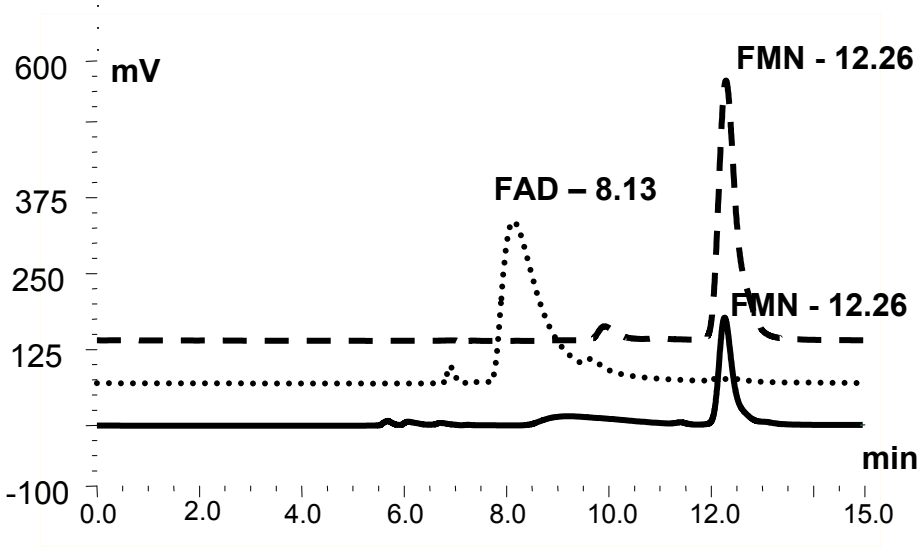

Figure 5.

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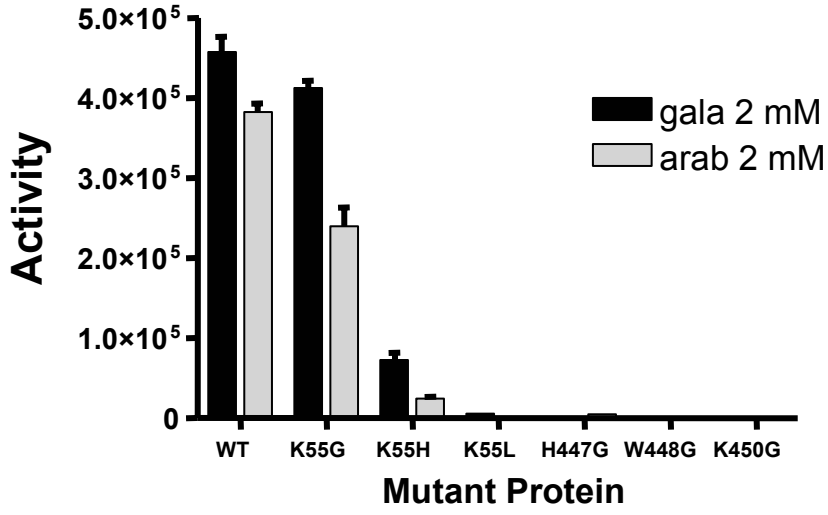

Figure 6.

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A

Substrate	Michaelis-Menten constant (K_M) (μM)	Activity (V_{max}) (μmol ascorbate formed $min^{-1}mg^{-1}$)	Turnover constant (K_{cat}) (s^{-1})	Catalytic efficiency (K_{cat}/K_M) ($M^{-1}s^{-1}$)
arabinonolactone	285 ± 36 (55 ± 3)	651 ± 20 (29.6 ± 0.4)	649 (27.2)	2.3×10^6 (4.9×10^5)
galactonolactone	161 ± 24 (154 ± 24)	675 ± 22 (22.8 ± 0.8)	673 (20.9)	4.2×10^6 (1.4×10^5)

B

INHIBITOR (1 mM)	% INHIBITION
$HgCl_2$	100 ± 0
p-chloromercuribenzoate (pCMB)	100 ± 5.0
N-Ethylmaleimide (NEM)	77 ± 2.4
$ZnSO_4$	63 ± 3.2

Table 1. Kinetic parameters of vitamin C formation and inhibition of TcGAL.

(A) Enzyme activity (*V*max) was calculated using an ε value of 17.3 mM^{-1} [31]. *K*cat assumes 1 catalytic site per 60 kDa monomer. Numbers in brackets are for the corresponding *T. brucei* enzyme [28]. **(B)** Enzyme activity was determined at pH 8.0 and 21 °C, with 2 mM arabinonolactone. Inhibition was calculated as a percentage of activity of the untreated wild type enzyme. Experiments were performed in triplicate and the values shown are ± the standard deviation from the mean.

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Mutant	FMN peak height (%WT)
K55G	74.6 ± 1.3
K55H	8.1 ± 0.7
K55L	0
H447G	0
W448G	0
K450G	52.4 ± 0.9

Table 2. FMN binding properties of mutated TcGAL proteins.
Supernatants from TCA precipitated TcGAL mutants (Figure 2) were analysed by HPLC (Experimental section). The resulting peaks had the same retention time as the FMN control (Figure 5). The extent of cofactor binding is expressed as a percentage of the peak height obtained with the same amount of wild type (WT) enzyme (122 μg). Assays were performed in duplicate, and the standard errors are indicated.

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Properties	GALDH (Plant)[a]	GLO (Mammal)[a]	ALO (Fungi)[a]	GULDH (Bacteria)[b]	GAL/ALO (Trypanosomes)[c]
Product	L-Ascorbic acid	L-Ascorbic acid	D-Erythroascorbic acid	L-Ascorbic acid	Substrate-dependent
Cellular compartment	Mitochondria	Microsomes	Mitochondria	N/A	Glycosome
Molecular Mass (kDa)	56	51	56 - 60	61	57 (*T. cruzi*) 59 (*T. brucei*)
Cofactor	Noncovalent flavin FMN	Covalent flavin FAD	Covalent flavin FAD	Noncovalent flavin (proposed)	Noncovalent flavin FMN
Relative substrate specificity:					
L-Galactonolactone	100	87	87	0	100 (*T. cruzi*) 74 (*T. brucei*)
L-Gulonolactone	0 - 20	100	24	100	3 - 9
D-Arabinonolactone	-	-	100	-	96 (*T. cruzi*) 100 (*T. brucei*)
K_M (preferred substrate)	0.12 - 3.3 mM	0.066 mM (rat) 0.15 mM (goat)	44.1 mM	5.5 mM	0.16 mM (*T. cruzi*) 0.05 mM (*T. brucei*)
Essential sulphydryl groups	Yes	Yes	Yes	Yes	Yes

Table 3. Comparison of the properties of the enzyme that mediates the final step in ascorbate (or erythroascorbate) biosynthesis across five kingdoms.
Adapted from [41][a] with incorporation of data from [24,25][b], [28][c] and this paper

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