RESEARCH LETTER

Characterization of *Trypanosoma cruzi* L-cysteine transport mechanisms and their adaptive regulation

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Abstract

L-Cysteine and methionine are unique amino acids that act as sulfur donors in all organisms. In the specific case of Trypanosomatids, L-cysteine is particularly relevant as a substrate in the synthesis of trypanothione. Although it can be synthesized *de novo*, L-cysteine is actively transported in *Trypanosoma cruzi* epimastigote cells. L-Cysteine uptake is highly specific; none of the amino acids assayed yield significant differences in terms of transport rates. L-Cysteine is transported by epimastigote cells with a calculated apparent K_m of 49.5 μM and a V_{max} of about 13 pmol min^{-1} per 10^7 cells. This transport is finely regulated by amino acid starvation, extracellular pH, and between the parasite growth phases. In addition, L-cysteine is incorporated post-translationally into proteins, suggesting its role in iron–sulfur core formation. Finally, the metabolic fates of L-cysteine were predicted *in silico*.

Introduction

Chagas disease, a triatominean vector-borne disease, is a major public health problem in the Americans affecting more than 15 million people. Caused by the parasite *Trypanosoma cruzi*, this disease is not only a health problem but also creates financial and social burdens. Despite the significant decreases in infection and mortality that have been achieved through vector- and transfusion-related control, the therapeutic resources remain unsatisfactory (Tarleton *et al.*, 2007). The metabolism of *T. cruzi* along its life cycle offers insights into conspicuous voids in survival with the potential of therapeutic intervention. The sequencing of the *T. cruzi* genome provides essential insights into the metabolism of this parasite (El-Sayed *et al.*, 2005). The amino acid requirements of *T. cruzi* cells represent an important feature, which was the subject of multiple studies during the last century (Camargo, 1964; Hampton, 1970). In fact, it was shown that *T. cruzi* have catabolic pathways connected to an operative Krebs cycle and respiratory chain, allowing the utilization of amino acids as fuel (Nowicki & Cazzulo, 2007). Thus, transport systems and enzymes downstream of these processes become interesting targets for drug design. L-Cysteine plays an important role in the stability, structure, catalytic activity, and regulation of numerous proteins due to the unique properties of sulfur and thiol. In addition, an oxidized derivative of L-cysteine, cystine, forms disulfide bonds that stabilize the tertiary structure of proteins (Nozaki *et al.*, 2005). L-Cysteine is also utilized for the production of ubiquitous iron–sulfur clusters, which participate in electron transfer, redox regulation, nitrogen fixation, and sensing for regulatory processes, and are probably present in all living organisms (Johnson *et al.*, 2005).

Parasite-related L-cysteine metabolism was the subject of many studies. Because of its high reactivity, in *Trypanosoma* and *Leishmania*, L-cysteine is involved in the synthesis of glutathione and trypanothione (a spermidine–glutathione conjugate, unique to trypanosomatids), which play important roles in protection against oxidative stress (Oza *et al.*, 2002). Moreover, L-cysteine was proposed to be an essential growth factor in *Trypanosoma brucei* bloodstream form (Duszenko *et al.*, 1992).

In *T. cruzi*, little is known about L-cysteine metabolic pathways. Transsulfuration and assimilatory L-cysteine

biosynthetic pathways have been identified. Cystathionine β-synthase is a key enzyme of the transsulfuration pathway. In addition to this activity, the enzyme also has serine sulfhydrylase and L-cysteine synthase activities. A serine acetyltransferase, an enzyme related to sulfate assimilation in the L-cysteine biosynthetic pathway, was also reported in *T. cruzi* (Nozaki *et al.*, 2001). The *T. cruzi* genome project also showed the presence of other putative L-cysteine-related enzymes. In contrast, only a few studies have been carried out with respect to L-cysteine catabolism.

In addition to L-cysteine biosynthetic pathways, the present study shows that *T. cruzi* can also incorporate L-cysteine from the extracellular via transport processes, and the metabolic derivatives from the uptaken L-cysteine have been studied.

Materials and methods

Parasite cultures

Epimastigotes of the CL Brener strain were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 mL of LIT medium (starting with 10^6 cells mL^{-1}) supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Camargo, 1964). The parasites were subcultured with passages every 7 days, unless otherwise indicated. At the indicated times, cells were counted using a hemocytometric chamber.

L-Cysteine transport assays

Aliquots of epimastigote cultures (10^7 parasites) were grown for the indicated periods. The parasites were centrifuged at 8000 ***g*** for 30 s, and washed twice with phosphate-buffered saline (PBS) at pH 7.0. Cells were resuspended in 0.1 mL PBS and then 0.1 mL of the transport mixture containing 300 μM [^{35}S]-L-cysteine (Perkin Elmer; 2 μCi per assay) or the indicated concentrations was added. Following incubation for the indicated time at 28 °C, cells were centrifuged as indicated above, and washed twice with 1 mL of ice-cold PBS. Pellets were then resuspended in 0.2 mL of 0.2 N NaOH and counted for radioactivity in an UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meridien, CT). Nonspecific transport and carryover were measured in transport mixtures containing 10 mM L-cysteine. Assays were run at least in triplicate. Cell viability was assessed by direct microscopic examination. Kinetic constants were calculated following the procedures of Lineweaver and Burk as described by Dixon & Webb (1964).

Post-translational incorporation of [^{35}S]-L-cysteine into proteins

To assess post-translational incorporation of L-cysteine into *T. cruzi* epimastigotes, translational arrest was performed by adding cycloheximide at a final concentration of 0.5 mg mL^{-1} to a 5-mL culture grown at 28 °C for 16 h. After treatment, cells were harvested, washed twice with PBS, and incubated with [^{35}S]-L-cysteine for 2 h. [^{3}H]-Arginine and [^{3}H]-glutamate (5 μCi per assay) incorporations were used as controls. After incubation with the radiolabeled amino acids, trichloroacetic acid (TCA) protein precipitation was carried on and protein pellets were counted for radioactivity as mentioned above.

Results

Biochemical properties of L-cysteine transport

The total [^{35}S]-L-cysteine influx in *T. cruzi* epimastigotes was found to be roughly proportional to the incubation time up to 5 min. In addition, uptake continuously increases for 25 min and did not reach a complete plateau (Fig. 1a, inset). The transport rate was dependent on the L-cysteine concentration and was saturable over 400 μM (Fig. 1a) up to 1 mM (data not shown). The data obtained from the concentration-dependent L-cysteine influx curve were analyzed using Lineweaver–Burk plots; the maximum velocity (V_{max}) and the apparent Michaelis–Menten constant (K_m) value were 13 pmol min^{-1} per 10^7 cells and 49.5 μM, respectively (Fig. 1b). Considering that *T. cruzi* possess large families of amino acid transporters (Bouvier *et al.*, 2004), competition studies were carried out to demonstrate the specificity of the L-cysteine transport system using 20-fold molar excess of other amino acids such as methionine, arginine, glutamate, proline, glycine, and serine. However, none of the tested amino acids had an effect on the L-cysteine uptake (Fig. 2a). Moreover, the sulfur-containing amino acid methionine did not have any effect on L-cysteine uptake. Hence, like other amino acid transport systems in *T. cruzi*, L-cysteine is specifically incorporated (Silber *et al.*, 2002, 2006; Canepa *et al.*, 2004, 2005).

Regulation mechanisms of L-cysteine transport

Trypanosoma cruzi epimastigote cells were assayed for L-cysteine transport during the exponential and stationary phases of culture growth. During the interval between the 4th and the 14th day of culture, L-cysteine transport increased about 15-fold (Fig. 2b, left panel). The effect of media and L-cysteine deprivation on transport was also investigated. Epimastigote cells were starved in PBS and PBS/L-cysteine for 3 h and the rate of L-cysteine transport was determined. L-Cysteine transport was increased 2.3-fold in cells starved for 3 h in PBS and 1.8-fold in cells incubated with PBS/L-cysteine (Fig. 2b, right panel). Therefore, it can be hypothesized that energy is not a limiting factor of this transport system, but transient alterations in the intracellular environment may be affected by the L-cysteine pool requirements. Several studies to date have determined the

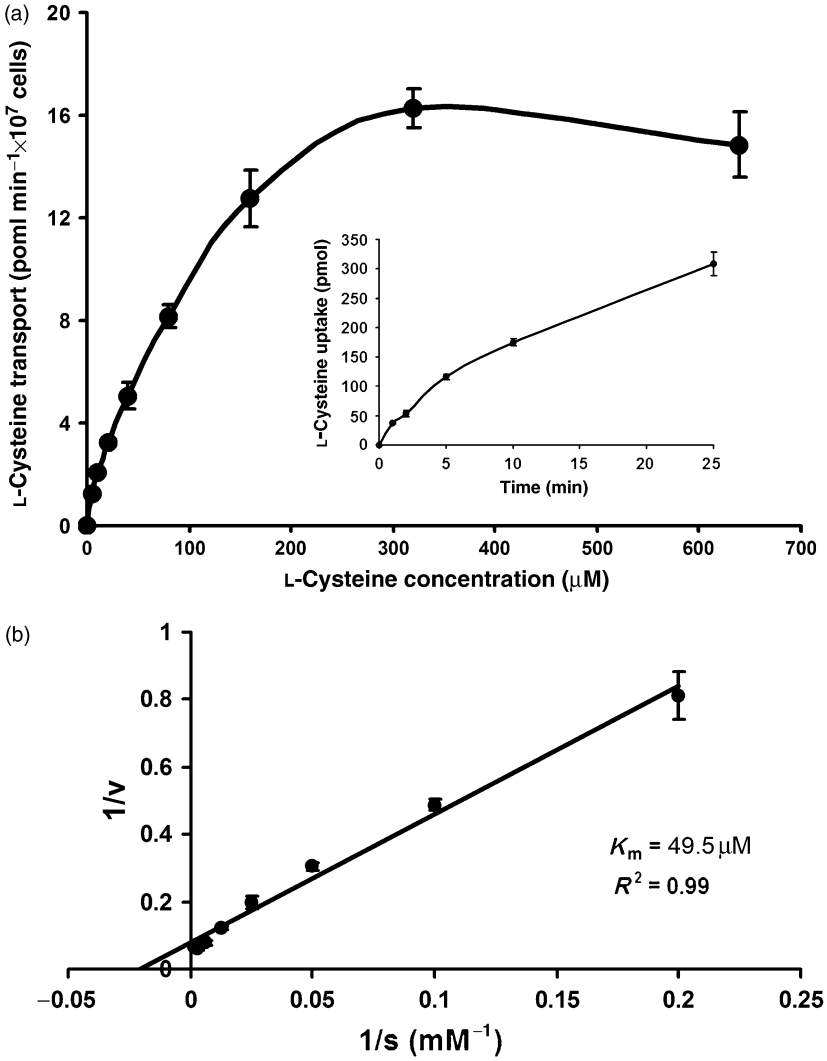

Fig. 1. Kinetic analysis of L-cysteine uptake in *Trypanosoma cruzi* epimastigote cells. The concentration-dependent [^{35}S]-L-cysteine uptake rate was determined as described under Materials and methods (a). Initial velocities (V_0) were determined by measuring the uptake rate during the first 5 min at 0.3 mM L-cysteine (inset). The kinetic parameters were calculated from a Lineweaver–Burke plot; K_m and the regression coefficient are indicated (b). Velocity values were calculated from triplicates of three independent experiments.

pH dependence on amino acid transport in *T. cruzi* epimastigote cells (Silber *et al.*, 2002; Canepa *et al.*, 2005). L-Cysteine uptake was strongly dependent on pH, increasing with the decrease of pH from 8 to 5. The transport activity reached maximum values at pH 5, about 1.7-fold higher than the activity measured at pH 7, or 2.2-fold when compared with pH 8 (Fig. 2c, left panel).

L-Cysteine metabolism in *T. cruzi* epimastigotes

In order to study *in vivo* post-translational incorporation of L-cysteine into proteins (e.g. sulfur–protein cores), *T. cruzi* epimastigotes cells were treated with the translation inhibitor cycloheximide. After protein translation inhibition, [^{35}S]-L-cysteine incorporation was expressed as a ratio: c.p.m. with cycloheximide/c.p.m. without cycloheximide and compared with controls using [^{3}H]-arginine and [^{3}H]-glutamate in three independent assays. After a 2-h labeling period, TCA protein precipitation was performed and the total radiolabeled macromolecules were quantified. The incorporation of [^{35}S]-L-cysteine into proteins was about 50% higher than that labeled with the amino acid glutamate and five times higher than amino acid arginine (Fig. 2c, right panel). These results suggest that the organic sulfur of L-cysteine is incorporated into proteins perhaps in the form of iron–sulfur clusters or other protein modifications. The presence of a basal protein translation was evidenced by the incorporation of control amino acids.

Discussion

This study reveals the existence of a high-affinity L-cysteine-specific transport system in *T. cruzi*. Several amino acids were used to evaluate the substrate preference by measuring the percent of inhibition of L-cysteine uptake, but no compound affected the inward transport of L-cysteine, demonstrating the high specificity of this transport system. Some adaptive changes in the L-cysteine transport process

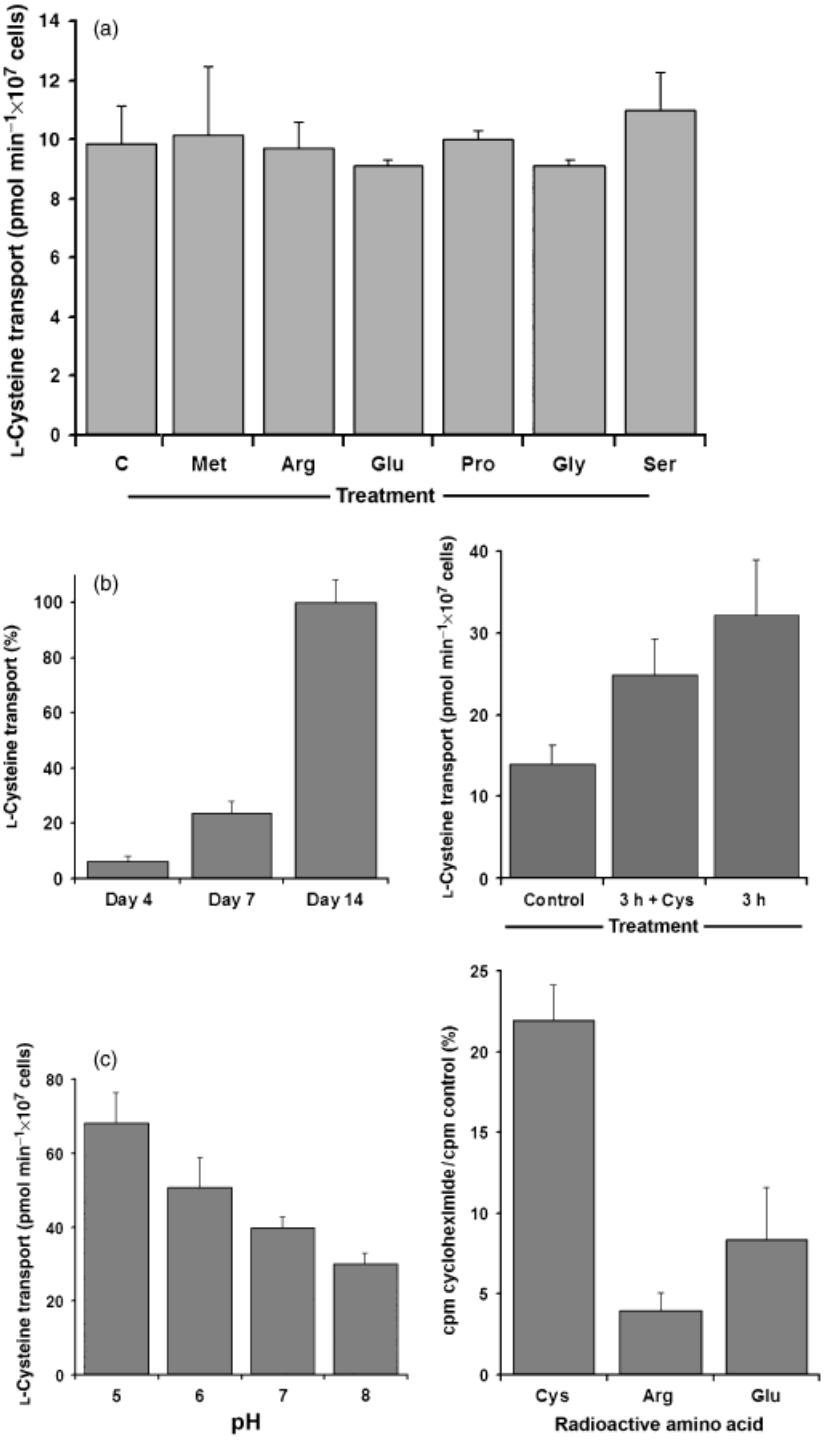

Fig. 2. Regulation of L-cysteine uptake and incorporation into proteins. The specificity of L-cysteine transport was evaluated by assaying the uptake rate in the presence of different competitor amino acids at 20-fold excess (a). Parasite samples from different growth phases, early logarithmic (4th day), late logarithmic (7th day) and stationary phase (14th day), were evaluated for L-cysteine transport. Results are expressed as percentage from the highest value corresponding to day 14 (b, left panel). Parasite samples were subjected to 3 h of starvation with (3 h+cys) or without 10 mM L-cysteine. Control parasites were maintained without starvation. After the treatment, L-cysteine transport rates were measured (b, right panel). Epimastigote cells were incubated at different extracellular pH in the range 5–8 for 30 min and assayed for L-cysteine transport (c, left panel). Incorporation of L-cysteine (Cys) into proteins was evaluated with and without cycloheximide treatment. Arginine (Arg) and glutamate (Glu) were used as translation-negative controls. Results were expressed as the ratio between c.p.m. with cycloheximide and c.p.m. without cycloheximide (c, right panel).

were also studied. When parasites from different days of culture were compared, an increase in L-cysteine uptake was observed as a consequence of the amino acids/nutrients depletion. The dependence on extracellular pH was also observed in this transport system. Similar regulation mechanisms have been observed for other *T. cruzi* amino acid systems, such as arginine, proline, and aspartate, suggesting that all permeases involved in these transport systems may belong to the same family of amino acid transporters (Silber *et al.*, 2002, 2006; Bouvier *et al.*, 2004; Canepa *et al.*, 2004, 2005). The metabolic fate of the radiolabeled sulfur was also investigated. Our results showed that after translational

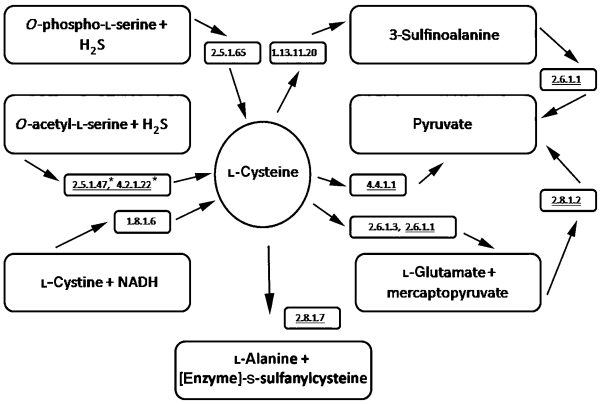

Fig. 3. Schematic representation of L-cysteine metabolism. General L-cysteine metabolism was summarized in the figure; Enzyme Commission numbers correspond to *O*-phosphoserine sulfhydrylase (2.5.1.65), cystine reductase (1.8.1.6), L-cysteine synthase (2.5.1.47), cystathionine β-synthase (4.2.1.22), L-cysteine desulfurase (2.8.1.7), L-cysteine transaminase (2.6.1.3), aspartate transaminase (2.6.1.1), cystathionine gamma-lyase (4.4.1.1), 3-mercaptopyruvate sulfurtransferase (2.8.1.2) and L-cysteine dioxygenase (1.13.11.20). Underlined numbers correspond to genes found in the *Trypanosoma cruzi* genome, but not experimentally confirmed. The asterisk indicates a previously reported pathway (Nozaki *et al.*, 2001).

arrest, the sulfur group of L-cysteine was still incorporated into proteins perhaps due to post-translational modification of proteins such as incorporation of the ^{35}S into iron–sulfur clusters into proteins by L-cysteine desulfurases, found in the *T. cruzi* genome. The pathway shown in Fig. 3 summarizes the L-cysteine biosynthetic and degradation pathways found in many organisms. L-Cysteine can be obtained from phosphoserine by *O*-phosphoserine sulfhydrylase or from cystine by cystine reductase, but none of these enzymes were found in the *T. cruzi* genome. Interestingly, *T. cruzi* epimastigotes are the only trypanosomatids capable of synthesizing L-cysteine *de novo*, possessing two apparent redundant pathways for L-cysteine production by L-cysteine synthase and cystathionine β-synthase with L-cysteine synthase activities (Nozaki *et al.*, 2001). Both pathways should provide L-cysteine to fulfill the cell's physiological demands. However, the high rates of L-cysteine transport indicate the importance of maintaining the homeostasis of intracellular L-cysteine pools. This can be explained by the low concentration of sulfur-containing amino acids in the guts of *Reduviidae* vectors (Harington, 1956), and so a large supply of L-cysteine might be required to satisfy the demands of trypanothione synthesis in epimastigote cells. On the other hand, L-cysteine degradation, followed by the formation of pyruvate, was the subject of many studies (Cooper *et al.*, 1982; Wróbel *et al.*, 2000). Mercaptosulfurtranferase converts mercaptopyruvate to pyruvate with the release of the sulfur group. This enzyme was cloned and characterized in *Leishmania*, and two copies of this gene were found in the *T. cruzi* genome (Williams *et al.*, 2003). Mercaptopyruvate can be obtained from L-cysteine by L-cysteine aminotransferase, despite the fact that *T. cruzi* lacks this gene; in other organisms, it was observed that this transamination could be produced by an aspartate aminotransferase (Taniguchi *et al.*, 1984), an enzyme that is represented by three copies in the *T. cruzi* genome (Marciano *et al.*, 2008). Another way to obtain pyruvate from L-cysteine is by the cystathionine gamma-lyase reaction (Cellini *et al.*, 2005). Genome data mining revealed the presence of a high number of putative cystathionine gamma-lyase genes. On the other hand, no L-cysteine dioxygenase genes were found. The biological significance of *T. cruzi*, being the only kinetoplastid with multiple L-cysteine biosynthesis routes, and their putative overlapped functions, present only in *T. cruzi* among kinetoplastid organisms, are currently unknown. Moreover, L-cysteine transport systems seem to be redundant mechanisms in addition to such pathways. One could speculate on the existence of regulation mechanisms to maintain the intracellular L-cysteine levels. Extracellular L-cysteine availability could trigger different pathways to obtain a major elemental nutrient that is also a source of nitrogen and sulfur; transport mechanisms are most efficient in terms of energetic economy. However, little is known about the elements involved in regulatory mechanisms. Another possible explanation for the apparent requirement of extracellular L-cysteine is that L-cysteine synthesized *de novo* may not be enough for the maintenance of the redox state of the surface thiol molecules. Hence, cysteine may serve not only as fuel but also as a redox controller in *T. cruzi* epimastigotes.

Because amino acids participate in a wide variety of metabolic routes leading to many crucial compounds for the survival of *T. cruzi*, transporters and associated enzymes become interesting targets for drug design.

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References

Bouvier LA, Silber AM, Galvao Lopes C, Canepa GE, Miranda MR, Tonelli RR, Colli W, Alves MJ & Pereira CA (2004) Post

genomic analysis of permeases from the amino acid/auxin family in protozoan parasites. *Biochem Bioph Res Co* **321**: 547–556.

Camargo EP (1964) Growth and differentiation in *Trypanosoma cruzi*. Origin of metacyclic trypanosomes in liquid media. *Rev I Med Trop* **6**: 93–100.

Canepa GE, Silber AM, Bouvier LA & Pereira CA (2004) Biochemical characterization of a low-affinity arginine permease from the parasite *Trypanosoma cruzi*. *FEMS Microbiol Lett* **236**: 79–84.

Canepa GE, Bouvier LA, Urias U, Miranda MR, Coli W, Alves MJ & Pereira CA (2005) Aspartate transport and metabolism in the protozoan parasite *Trypanosoma cruzi*. *FEMS Microbiol Lett* **247**: 65–71.

Cellini B, Bertoldi M, Montioli R & Borri Voltattorni C (2005) Probing the role of Tyr 64 of *Treponema denticola* cystalysin by site-directed mutagenesis and kinetic studies. *Biochemistry* **44**: 13970–13980.

Cooper AJ, Haber MT & Meister A (1982) On the chemistry and biochemistry of 3-mercaptopyruvic acid, the alpha-keto acid analog of cysteine. *J Biol Chem* **257**: 816–826.

Dixon M & Webb EC (1964) *Enzymes*, 2nd edn. Longmans, Green and Co., London, pp. 67–70.

Duszenko M, Mühlstädt K & Broder A (1992) Cysteine is an essential growth factor for *Trypanosoma brucei* bloodstream forms. *Mol Biochem Parasit* **50**: 269–273.

El-Sayed NM, Myler PJ, Bartholomeu DC *et al.* (2005) The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* **309**: 409–415.

Hampton JR (1970) Lysine uptake in cultured *Trypanosoma cruzi*: interactions of competitive inhibitors. *J Protozool* **17**: 597–600.

Harington JS (1956) Histamine and histidine in excreta of the blood-sucking bug *Rhodnius prolixus*. *Nature* **178**: 268.

Johnson DC, Dean DR, Smith AD & Johnson MK (2005) Structure, function, and formation of biological iron–sulfur clusters. *Annu Rev Biochem* **74**: 247–281.

Marciano D, Llorente C, Maugeri DA, de la Fuente C, Opperdoes F, Cazzulo JJ & Nowicki C (2008) Biochemical characterization of stage-specific isoforms of aspartate aminotransferases from *Trypanosoma cruzi* and *Trypanosoma brucei*. *Mol Biochem Parasit* **161**: 12–20.

Nowicki C & Cazzulo JJ (2007) Aromatic amino acid catabolism in trypanosomatids. *Comp Biochem Phys A* **151**: 381–390.

Nozaki T, Shigeta Y, Saito-Nakano Y, Imada M & Kruger WD (2001) Characterization of transsulfuration and cysteine biosynthetic pathways in the protozoan hemoflagellate, *Trypanosoma cruzi*. Isolation and molecular characterization of cystathionine beta-synthase and serine acetyltransferase from *Trypanosoma*. *J Biol Chem* **276**: 6516–6523.

Nozaki T, Ali V & Tokoro M (2005) Sulfur-containing amino acid metabolism in parasitic protozoa. *Adv Parasit* **60**: 1–99.

Oza SL, Tetaud E, Ariyanayagam MR, Warnon SS & Fairlamb AH (2002) A single enzyme catalyses formation of trypanothione from glutathione and spermidine in *Trypanosoma cruzi*. *J Biol Chem* **277**: 35853–35861.

Silber AM, Tonelli RR, Martinelli M, Colli W & Alves MJ (2002) Active transport of L-proline in *Trypanosoma cruzi*. *J Eukaryot Microbiol* **49**: 441–446.

Silber AM, Rojas RL, Urias U, Colli W & Alves MJ (2006) Biochemical characterization of the glutamate transport in *Trypanosoma cruzi*. *Int J Parasitol* **36**: 157–163.

Taniguchi M, Hosaki Y & Ubuka T (1984) Transaminative metabolism of L-cysteine in guinea pig liver and kidney. *Acta Med Okayama* **38**: 375–380.

Tarleton RL, Reithinger R, Urbina JA, Kitron U & Gürtler RE (2007) The challenges of Chagas disease – grim outlook or glimmer of hope. *PLoS Med* **4**: e332.

Williams RA, Kelly SM, Mottram JC & Coombs GH (2003) 3-Mercaptopyruvate sulfurtransferase of *Leishmania* contains an unusual C-terminal extension and is involved in thioredoxin and antioxidant metabolism. *J Biol Chem* **278**: 1480–1486.

Wróbel M, Sura P & Srebro Z (2000) Sulfurtransferases and the content of cysteine, glutathione and sulfane sulfur in tissues of the frog *Rana temporaria*. *Comp Biochem Phys B* **125**: 211–217.