Cysteine biosynthesis in *Trichomonas vaginalis* involves cysteine synthase utilizing O-phosphoserine

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**Key words:** *Trichomonas*, parasite, antioxidant, cysteine synthase, desulphurase.

**The abbreviations used are:** kb, kilobase(s); bp, base pairs; CS, gene encoding cysteine synthase; CS, protein encoded by CS; rTvCS1, recombinant *T. vaginalis* CS1; similar nomenclature for other genes/proteins (details given in text); ORF, open reading frame; PCR, polymerase chain reaction; RT PCR, reverse transcriptase PCR; PAGE, polyacrylamide gel electrophoresis.

**Running title:** Cysteine biosynthesis in *Trichomonas*

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*Trichomonas vaginalis* is an early divergent eukaryote with many unusual biochemical features. It is an anaerobic protozoan parasite of humans that is thought to rely heavily on cysteine as a major redox buffer, as it lacks glutathione. We report here that for synthesis of cysteine from sulphide, *T. vaginalis* relies upon cysteine synthase. The enzyme (TvCS1) can use as substrates either O-acetylserine or O-phosphoserine. The $K_m$ of the enzyme for sulphide is very low (0.02 mM), suggesting that the enzyme may be a means of ensuring that sulphide in the parasite is maintained at a low level. *T. vaginalis* appears to lack serine acetyltransferase, the source of O-acetylserine in many cells, but has a functional 3-phosphoglycerate dehydrogenase and an O-phosphoserine aminotransferase that together result in the production of O-phosphoserine, suggesting that this is the physiological substrate. TvCS1 can also use thiosulphate as substrate. Overall, TvCS1 has substrate specificities similar to those reported for cysteine synthases of *Aeropyrum pernix* and *Escherichia coli* and this is reflected by sequence similarities around the active site. We suggest that these enzymes are classified together as type B cysteine synthases and we hypothesise that the use of O-phosphoserine is a common characteristic of these cysteine synthases. The level of cysteine synthase in *T. vaginalis* is regulated according to need, such that parasites growing in an environment rich in cysteine have low activity, whereas exposure to propargylglycine results in elevated cysteine synthase activity. Humans lack cysteine synthase, thus this parasite enzyme could be an exploitable drug target.

The parasite itself is an unusual protozoon that may be one of the earliest branching organisms (4,5). Undoubtedly it is an unusual eukaryote, both at the molecular and cellular levels (6-10). It is adapted for an environment containing only low oxygen concentrations by being a fundamentally fermentative organism, with oxygen apparently not making a significant contribution to energy metabolism (11). Nevertheless, the cells are exposed to oxygen in the natural environment (12) and have to withstand oxidant challenge. Some of the parasite’s enzymes are inactivated by oxygen itself, notably key proteins of the hydrogenosomes (9,10), and various metabolites likely to arise from the metabolism of oxygen (such as hydrogen peroxide and hydroxyl free radical) are generally harmful to cells and so need to be counteracted. Most eukaryotes have glutathione as a key redox buffer and antioxidant, but trichomonads lack this and related thiols (13). *T. vaginalis* is able to generate various thiols (methanethiol, propanethiol and hydrogen sulfide) which have been postulated to have antioxidant roles (13) and also contains thioredoxin reductase, which functions together with thioredoxin and thioredoxin peroxidase to detoxify potentially damaging oxidants (14). Nevertheless, it has been generally believed that cysteine is the major cellular reducing agent and antioxidant (15). However, the source of cysteine for *T. vaginalis* had not been elucidated. We have analysed the genome sequence of *T. vaginalis* ([http://www.tigr.org/tdb/e2k1/tvg/](http://www.tigr.org/tdb/e2k1/tvg/)) in an attempt to predict how the parasite obtains the cysteine that it requires and then tested the hypotheses arising experimentally.

Cysteine can be generated from homocysteine using the trans-sulphuration pathway (as occurs in mammals) or from serine and inorganic sulphide. The latter pathway, which occurs in bacteria and plants and just a few protozoa, incorporates the multistep synthesis of sulphide from inorganic sulphate and a final reaction in which O-acetylserine and sulphide are used to generate cysteine. This final step is catalysed by cysteine synthase (CS). Two types of
bacterial CSs have been categorised (types A and B), and the key differences between the two types are beginning to emerge (16-20). Both types can use O-acetylserine and inorganic sulphide to generate cysteine using a ping-pong bi bi catalytic mechanism and proceeding by two half reactions: β-elimination of acetate to form the α-aminoacrylate intermediate and addition of H₂S to form cysteine (16,17).

Analysis of the type A CS of *Salmonella typhimurium* has shown it to be a homodimer with each monomer composed of two domains each with a similar αβ structure (18). The active site pocket, containing pyridoxal 5'-phosphate, is located in a deep cleft between the C-terminal and N-terminal domains. The first half reaction is facilitated by conformational changes, involving a sub-domain of the N-terminal domain (19). This closes the active site pocket, leaving only a narrow channel that allows the product, acetate, to leave and the second substrate, hydrogen sulphide, to enter, but excludes larger molecules such as thiosulphate.

The three dimensional structure of the type B CS of *Escherichia coli* closely resembles that of *S. typhimurium* type A CS in many ways (20). The enzymes undergo similar conformational changes during the catalytic cycle and residues lining the catalytic pocket are highly conserved. There are significant differences, however, in the flexible loop located opposite the pyridoxal 5'-phosphate molecule and these account for differences in substrate specificity, notably its ability to use thiosulphate in place of hydrogen sulphide. Small residues Gly230, Ala231 and Gly232 in *Salmonella* type A CS are replaced by bulky residues Arg210, Arg211 and Trp212 in *E. coli* type B CS and the type B enzyme has an insertion of 3 amino acid residues. The loop in the *E. coli* type B CS (residues 210-216) bulges outward, enlarging the active site pocket. This allows access to thiosulphate, which is excluded from the type A enzyme. It has been proposed that the side chain of Arg210 stabilises interaction with thiosulphate.

This same region has been identified as a determinant of substrate specificity in the CS of hyperthermophilic archaeon *Aeropyrum pernix*. This CS appeared to be unusual in that it uses O-phosphoserine rather than O-acetylserine, which is unstable at very high temperatures (21). The CS of *A. pernix* has an additional N-terminal domain but otherwise has a similar conformation and dimer structure to the type A and type B enzymes (22). The flexible loop of *A. pernix* CS (residues 297-302) resembles the loop (residues 210-216) of the *E. coli* type B enzyme. Arg297 of *A. pernix* CS, equivalent to Arg210 of *E. coli* type B CS, is thought to interact with the phosphate group of O-phosphoserine. The importance of this was demonstrated using an Arg297Ala mutant, which had no activity with O-phosphoserine as a substrate but retained high activity with O-acetylserine.

We report here that a CS of *T. vaginalis* has similarities to both *E. coli* type B enzyme and the CS of *A. pernix*. Analysis of the *T. vaginalis* enzyme’s activity has provided more insight into key residues and the specificities they determine. This allows us to hypothesise the existence of a group of CSs (designated type B CSs) that are characterised by common features. The data on *T. vaginalis* also allow us to postulate a scheme for the unusual metabolism of cysteine that occurs in this parasite. Central to this is CS using O-phosphoserine and the provision of sulphide by homocysteine desulphurase (also known as methionine γ-lyase (MGL) (23)). As humans lack both CS and MGL, this pathway represents a distinct parasite-specific feature that could be an exploitable drug target.

**EXPERIMENTAL PROCEDURES**

**Growth and harvesting of parasites and preparation of lysates -** A clonal cell line (G3) of *T. vaginalis* was routinely grown axenically in modified Diamond's medium and harvested as previously described (23). Parasites were either stored as cell pellets at -70°C for the Western blot analyses and genomic DNA isolation or suspended in Trizol reagent (Life Technologies) for the RNA preparations and analyses.

*T. vaginalis* lysates were prepared by resuspending cells in ice-cold lysis buffer containing peptidase inhibitors (0.25 M...
sucrose, 0.25 % (v/v) Triton X-100, 10 μM N-trans-epoxysuccinyl-L-leucine-4-guanidinobutylamide (E-64), 2 mM 1,10 phenanthroline, 4 μM pepstatin A and 1 mM phenylmethane sulphonyl fluoride (PMSF)) to the equivalent of $10^8$ cells/ml and repeated aspiration via a 1 ml micropipette. Cell debris was removed by centrifugation at 12000 g for 10 min at 4°C and soluble protein in the supernatant was quantified using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin (BSA) as standard.

**In silico analysis of T. vaginalis genes involved in cysteine metabolism** - The T. vaginalis genome database (7.2X genome sequence, April 1st, 2005) ([http://www.tigr.org/tdb/e2k1/tvg/](http://www.tigr.org/tdb/e2k1/tvg/)) was interrogated by BLAST search using gene model sequences from the KEGG GENES database. Potential homologs were further analysed by BLAST search of the GenBank database, CD search of the NCBI Conserved Domain database, multiple sequence alignment with AlignX (Vector NTI, Invitrogen) and phylogenetic analysis using MEGA 3.0 (24). Gene function was assigned on the basis of sequence similarity, the presence of conserved domains, conservation of key active site residues and phylogenetic relationships to model proteins. Genes were deemed to be absent if sequences identified in the initial BLAST search had in minimum sum probability of $\geq 1.0 \times 10^{-3}$ or if sequences identified were assigned an alternative function by the above criteria.

**Cloning of CS, PGDH and PSAT of T. vaginalis** - A gene apparently corresponding to CS was first identified in an EST database of T. vaginalis G3 [1]. Blast search of the T. vaginalis genome database identified the identical sequence and confirmed that EST 215 contained the complete TvcSI open reading frame.

The TvcSI coding region was amplified from EST 215 using primers NT194 (GCCGATATGATCTACGACACATCTTCG) and NT195 (GTTGTCGACTTCGTGTCGAAGACCTTCTCG) and the Expand High Fidelity PCR system (Roche) (94°C for 2 min, 25 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min, 72°C for 7 min). The PCR product was digested with Ndel and Sall and the 0.92 kb fragment containing the full length TvcSI sequence was ligated into pET21a+ previously digested with Ndel and XhoI to give pBP158. A similar procedure was used to clone the coding regions for TvpGDH1 and TvpPSAT1, identified by Blast search of the T. vaginalis genome database (Table 1). T. vaginalis genomic DNA was used as a template for 30 cycles of PCR using primers NT268 (TACATGAAGATTCTCATTGCAAGACAC TCTCGCAC) and NT269 (CGCTCGAGTTAATCAAACATCTTAC AGAAACACC) for TvpGDH1 and primers NT296 (GCAAGCTTAAAGCCTTGCCATTCCT TCATTG) and NT297 (GCCATATGTCTGCAC ACGCCATAC AAC) for TvpPSAT1. The PCR conditions were modified by increasing the extension time to 2 min. PCR products encoding TvpGDH1 and TvpPSAT1 were cloned into pGEM®-T Easy vector (Promega) to give pBP171 and pBP208, respectively. The 1.2 kb Ndel and XhoI fragment from pBP171, encoding TvpGDH1, was ligated into vector pET28a+ previously digested with the same enzymes to give pBP175. Similarly, the TvpPSAT1 gene, contained in the 1.1 kb Ndel and HindIII fragment from pBP208, was cloned into pET28a+ to give pBP222. The sequences of all constructs were confirmed on both strands by the University of Glasgow MBSU Sequencing Facility. Sequence analysis was carried out using Vector NTI software (Informax, Invitrogen). Plasmids were introduced into strain BL21/DE3 for protein expression. The recombinant proteins have N-terminal (rTvpGDH1 and rTvpPSAT1) or C-terminal (rTvcSI) 6 x histidine tags, which facilitated purification using Ni$^{2+}$-NTA agarose.

**Mutagenesis of CS** - Mutations of TvcSI were incorporated into pBP158 using the QuickChangeTM site-directed mutagenesis kit (Stratagene) The complementary primers used to construct pBP233 encoding the K43A mutant were NT345
Northern blot analyses - Total RNA from 2 x 10^7 T. vaginalis was fractionated by electrophoresis on 1.2 % (w/v) agarose formaldehyde gels and transferred to Hybond-N membranes (Amersham). [α-^32P] dATP-labeled DNA probes were prepared from agarose gel-purified restriction endonuclease fragments using Prime-IT II Random Primer Kit (Stratagene) and purified on Microspin S-200 HR columns (Amersham). The probes used were: TvCS1, a 0.8 kb XhoI fragment of EST 215; α-actin, a 1.2 kb EcoRI/XhoI fragment of EST 197. Hybridisations were performed at 42°C overnight in 5 X SSPE, 5 X Denhardts, 50% formamide, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. Filters were washed twice for 10 min at room temperature in 2 X SSC, 0.5% (w/v) SDS and twice for 30 min at 55°C in 0.1 X SSC, 0.1% SDS. Storage phosphor screens were exposed to the labeled filters and scanned using a Typhoon 8600 Imager (Amersham). Levels of mRNA were quantified using ImageQuant image analysis software (Amersham). Levels of mRNA were quantified using ImageQuant image analysis software (Amersham). Filters were stripped with boiling 0.1% SDS, rinsed with 2 X SSC and re-probed. The T. vaginalis α-actin mRNA levels were used to normalise TvCS1 mRNA levels. Normalised mRNA levels in the treated cultures were expressed relative to the control culture, which was given an arbitrary value of 1.0 unit.

Production of recombinant enzymes - Single colonies of BL21/DE3 harboring pBP158, pBP233, pBP236, pBP174 and pBP222 were grown at 37°C in Luria Bertani medium and the expression of the recombinant proteins, rTvCS1, rTvPGDH1 and rTvPSAT1 were induced for 4 h with 1 mM isopropyl thio-β-D-galactoside (IPTG). The soluble rTvCS1 protein was purified using Ni^{2+}-NTA agarose (Qiagen) according to the manufacturer’s recommendations using a BioCad FPLC system. Elution was in 50 mM sodium phosphate, 300 mM NaCl, 20 μM pyridoxal 5’-phosphate, 500 mM imidazole, pH 7.9. The eluted protein (rTvCS1) was dialysed against 50 mM potassium phosphate, 1 mM EDTA, 0.2 mM pyridoxal 5’-phosphate, pH 7.8 and stored at 4°C with 0.02% sodium azide.

Soluble rTvPGDH1 and rTvPSAT1 were purified with Ni^{2+}-NTA agarose using a batch purification method. All buffers were supplemented with 10% (v/v) glycerol for rTvPGDH1 purification and with 20 μM pyridoxal 5’-phosphate for rTvPSAT1 purification. Cells from a 500 ml culture were harvested by centrifugation at 4,000 g for 20 min, resuspended in 10 ml buffer A (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication in a Soniprep 150 sonicator (MSE) for 6 bursts of 30 sec at maximum output with a 30 sec cooling interval between each burst. The lysates were cleared by centrifugation at 10,000 g for 30 min at 4°C. 5 ml of Ni^{2+}-NTA agarose was equilibrated in buffer A by two cycles of centrifugation at 1000 g for 5 min at 4°C and resuspension in 50 ml buffer A. The agarose was mixed with 10 ml cleared lysate and rotated overnight to bind the histidine-tagged protein. The agarose was washed two times in buffer A and two times in buffer B (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0). For each wash, the agarose was pelleted by centrifugation at 1000 g for 5 min at 4°C, resuspended in 50 ml buffer and rotated at 4°C for 30 min. Recombinant protein was eluted by resuspending the agarose pellet in 5 ml buffer C (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and rotating at 4°C for 2 h. The eluted rTvPSAT1 protein was stored at 4°C with 0.02 % (w/v) sodium azide and the rTvPGDH1 protein was stored at –20°C with 50 % (v/v) glycerol.

The rTvCS1, rTvPGDH1 and rTvPSAT1 remained highly active, as assessed by enzymatic analysis, for more than 2 months. The concentrations of the recombinant proteins were determined using
the Bio-Rad protein assay (Bio-Rad), with BSA as standard.

**Western blot analyses** - Polyclonal antiserum was raised in rabbits against purified rTvCS1 by the Scottish Antibody Production Unit (Carluke, UK) using standard immunisation protocols. Soluble proteins from *T. vaginalis* lysates were fractionated on 12% (w/v) polyacrylamide SDS PAGE and electroblotted onto ECL nitrocellulose membranes (Amersham). Membranes were blocked for 2 h at room temperature in Tris-buffered saline (TBS) containing 5% (w/v) milk and 0.2% Tween 20 and subsequently incubated overnight at 4°C with specific polyclonal antiserum diluted 1:10,000 in TBS containing 1% (w/v) milk powder, ECL reagents (Pierce) and Hyperfilm™ ECL (Amersham).

**Enzyme assays** - The sulfhydrylase activity of rTvCS1 was determined by measuring cysteine formation in 0.5 ml reactions containing 200 mM potassium phosphate, 1 mg/ml bovine serum albumin (BSA), 1 mM EDTA, 0.2 mM pyridoxal 5′-phosphate, 100 mM O-acetylserine, 10 mM methionine, 5 µg rTvMGL1, 4 µg rTvCS1, pH 7.8. The reaction was started by addition of O-acetylserine after pre-incubating all other components for 3 min. Reactions were stopped after 2 to 20 min. The rTvMGL1 was produced as described previously (23) and had a specific activity of 101.2 µmol/min/mg protein in production of hydrogen sulphide from homocysteine. The activity of rTvMGL1 for methionine, producing methanethiol, was 30-fold lower (23).

Desulphurase activity was determined by measuring lead sulphide formation at 360 nm in 1 ml reactions containing 200 mM potassium phosphate, 1 mg/ml BSA, 0.2 mM pyridoxal 5′-phosphate, 5 mM cysteine, 15 mM β-mercaptoethanol (BME), 0.33 mM lead acetate, 2 µg rTvCS1, pH 7.8. Reactions were started by addition of the rTvCS1.

The Kₘ of rTvCS1 for O-acetylserine was determined with a fixed concentration of sodium sulphide (3 mM) and variable concentrations of O-acetylserine (1 mM to 100 mM). The Kₘ for sodium sulphide was measured with 100 mM O-acetylserine and 0.1 mM to 10 mM sodium sulphide or with 80 mM O-phosphoserine and 0.01 mM to 2.0 mM sodium sulphide. Reactions in 0.2 M potassium phosphate buffer with 2 mM sodium sulphide showed that the Kₘ for O-phosphoserine was >100 mM. The enzyme activity was inhibited at higher concentrations of O-phosphoserine due to reduction in the pH of the buffer. Thus the phosphate buffer was replaced by 0.5 mM Tris HCl, pH 7.8 and the Kₘ for O-phosphoserine was >100 mM. The two buffers gave identical rates with 80 mM O-phosphoserine and 1 mM sodium sulphide. The Kₘ of rTvCS1 catalyzing the desulphurase reaction with cysteine was determined with a fixed concentration of β-mercaptoethanol (15 mM) and variable concentrations of cysteine (0.1 mM to 10 mM). The Kₘ for β-mercaptoethanol was measured with 5 mM cysteine and 1 mM to 50 mM β-mercaptoethanol.

Cystathione β-synthase activity was
determined in 0.5 ml reactions containing 100 mM potassium phosphate, 25 mM homocysteine, 100 mM serine, 0.2 mM pyridoxal 5’-phosphate and 20 μg of enzyme. Reactions were started by addition of the enzyme and terminated after 5 to 20 min at 37°C. Cystathionine formed was quantified as described by Kashiwamata and Greenberg (27).

Potential inhibitors were investigated using 500 μl reactions containing 100 mM O-acetylserine, 3 mM sodium sulphide, 0.2 μg rTvCS1 and various concentrations of the inhibitor. The enzyme was pre-incubated with the inhibitor for 3 min at 37°C before addition of O-acetylserine. After a further 3 min at 37°C, the reaction was started by addition of 3 mM sodium sulphide. Reactions were terminated after 2 to 20 min and the cysteine formed was quantified (25).

rTvPGDH1 and rTvPSAT1 activities were analysed using linked assays with both enzymes. The forward reaction from 3-phosphoglycerate to O-phosphoserine was measured using a fluorimetric assay to detect the NADH generated in the first reaction. Reactions were performed using 1 ml 100 mM potassium phosphate, 5 mM 3-phosphoglycerate, 0.2 mM NAD+, 5 mM glutamate, 1 mM DTT, 0.5 μg rTvPGDH1 and 50 μg rTvPSAT1, pH 7.8. Reactions were started by addition of 3-phosphoglycerate and the rate of NADH formation was monitored by measuring fluorescence intensity using a PerkinElmer LS 55 Luminescence Spectrometer (excitation 340 nm, emission 470 nm). With 50 μg/ml rTvPSAT1, the rate of NADH formation was proportional to rTvPGDH1 concentration (0.2-0.8 μg/ml). No activity was observed in the absence of 3-phosphoglycerate.

The combined activity of the two enzymes in the reverse direction was measured by monitoring the oxidation of NADH. The reaction were carried out using 1 ml of 100 mM potassium phosphate, 0.25 mM NADH, 5 mM α-ketoglutarate, 5 mM O-phosphoserine, 1 mM DTT, 25 μg rTvPGDH1, 2 μg rTvPSAT1, pH 7.8. The reactions were started by addition of O-phosphoserine and the oxidation of NADH determined by monitoring the absorbance at 340 nm for 5 min. With 25 μg rTvPGDH1, the rate of NADH oxidation was proportional to rTvPSAT1 concentration (2-12 μg/ml).

All assays were performed at 37°C. Kinetic calculations were performed using the computer program Grafit (Erithacus Software).

**The effects of growth conditions upon the expression of CS in T. vaginalis** - Parasites were grown in 25 ml medium in tightly capped universal tubes with little gas phase. The standard modified Diamond’s medium was varied by removal of ascorbate (normally present at 5.7 mM) or addition of cysteine to 10 mM or propargylglycine (PAG) to 5 μM. None of these significantly affected growth over 18 h. Cultures were initiated at 10^5 parasites/ml and incubation was for 18 h at 37°C, whereupon the parasites were harvested, washed and stored as pellets at –70°C until analysis (23).

**RESULTS**

**Identification of genes potentially involved in cysteine biosynthesis** - The genome analysis revealed (see Table 1) four genes encoding proteins possibly involved in cysteine biosynthesis: cysteine synthase (CS, EC 2.5.1.47, 6 gene copies); 3-phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95, 3 gene copies); O-phosphoserine aminotransferase (PSAT, EC 2.6.1.52, 3 gene copies); O-phosphoserine aminotransferase (PSAT, EC 2.6.1.52, 3 gene copies); and mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2). The previously identified methionine γ-lyases (MGL) (23) may also be involved. Genes searched for but not found were ones encoding serine acetyltransferase (SAT, EC 2.3.1.30), the usual source of O-acetylserine for CSs, and the enzymes of the trans-sulphuration pathway (cystathionine β-synthase, CBL, EC 4.2.1.22; cystathionine γ-lyase, CGL, EC 4.4.1.1; cystathionine γ-synthase, CGS, EC 2.5.1.48; cystathionine β-lyase, CBL, EC 4.4.1.8). Interestingly, a gene encoding O-phosphoserine phosphatase (PSP, EC 3.1.3.3), which catalyses the conversion of O-phosphoserine to serine in the final step of the phosphorylated serine pathway, also could not be identified. Searches of the genome database for enzymes of the sulphide...
de novo synthesis pathway identified a possible homologue of 3’phosphoadenylylsulfate reductase (PAPSR, EC 1.8.4.8) in the genome. However, the *T. vaginalis* predicted protein lacks the conserved C-terminal motif of PAPSR that contains the redox active cysteine (28), and the *T. vaginalis* protein has similarity to other related enzymes such as FAD synthetase. Additional genes encoding enzymes required for reduction of sulphate to sulphide (sulphate adenylyl transferase, adenylyl sulphate kinase and sulphite reductase(NADPH)) are also apparently absent from the *T. vaginalis* genome (Table 1). These data together suggest that sulphide is not synthesized de novo in *T. vaginalis*. The results of the genome analyses enabled us to draw up an hypothetical metabolic map for cysteine biosynthesis in *T. vaginalis* (Fig. 1; pathway shown in bold) The main features postulated were that CS uses O-phosphoserine as substrate rather than O-acetylserine and that the sulphide it requires is provided either from homocysteine or mercaptopyruvate through the action of MGL or MST, respectively. We tested the hypotheses arising from these predictions experimentally.

**Analysis of CS of *T. vaginalis* - CS of *T. vaginalis* was originally identified as an EST containing the full-length coding region. The same sequence was subsequently identified in the *T. vaginalis* genome database (95238.m00105) and designated TvCS1. Five additional sequences (TvCS2-6) encoding proteins with between 89 and 98% identity to TvCS1 were also found in the database. TvCS1 of *T. vaginalis* is predicted to encode a protein (TvCS1) of 299 amino acids, with a subunit molecular mass of 32.7 kDa.

Analysis of the predicted amino acid sequences of TvCS1 confirmed that it contains all of the key active site residues identified for the CSs of spinach (29), *S. typhimurium* (30) and *A. thaliana* (31). Comparisons suggest that the predicted active site lysine of TvCS1, which covalently binds to pyridoxal 5’-phosphate, is Lys43, whereas residues Asn73, Gln144, His154, Gly178, Thr179, Ser180, Thr182 and Ser259 are also linked to the co-factor by hydrogen bonding. Similarly, residues Asn73 and Gln144 together with Thr70 and Ser71 are predicted to be involved in binding the α-carboxylate group of the substrate of the first half reaction.

Plants produce mitochondrial and chloroplast isofor**ms of cysteine synthase that contain targeting sequences in long N-terminal extensions. The TvCS1 protein, as well as TvCS2-6 predicted proteins, lack an extension and amino acid residues close to the N-terminus are conserved in bacterial and plant CSs (Fig. 3a). Analysis of TvCS1 and the other CS sequences from *T. vaginalis* failed to detect a targeting sequence. These results suggest that all the CSs of *T. vaginalis* are cytosolic.

The levels of identity of TvCS1 with other CSs are: *Geobacter sulfurreducens* type B (AAR36549), 51%; *Entamoeba histolytica* (BAA93051), 47%; *E. coli* type B (NP_311319), 43%; *S. typhimurium* type A (NP_461365), 41%; *A. thaliana* (P47998), 41%; *Leishmania major* (LmjF36.3590), 38%; *Aeropyrum pernix* (NP_148041), 30%. Phylogenetic analysis with the conserved 207 amino acids of the CSs using MEGA 3.0 (24) showed that the 6 CSs of *T. vaginalis* are more similar to each other than any other CSs and fall within a clade with bacterial type B CSs (Fig. 2) The TvCSs clearly aligned more closely with the type B CSs of *S. typhimurium* and *Eschericia coli* than the type A CSs from the same organisms.

The key differences between the bacterial types A and B CSs (including the ability of the type B enzymes to use thiosulphate as an alternative substrate and the type A CSs to form a regulatory complex with SAT in which the CS activity is decreased and SAT activity increased) are mediated by a loop that is located between β8 and β9 of *S. typhimurium* CS type A (StCS-A), *E. coli* CS type B (EcCS-B) and *Arabidopsis thaliana* CS (AtCS). The corresponding region of *Aeropyrum pernix* CS (ApCS) is between β13 and β14. Fig. 3b shows an alignment of this region from different CS enzymes. This region contains a short segment that lines the entrance of the active site pocket, Gly230-Phe233 in StCS-A and Arg210-Tyr216 in EcCS-B. In the
bacterial type A enzymes (group 1 in Fig. 3b), this region forms part of a highly conserved sequence motif that also occurs in plant CSs and CSs known from other protozoa (groups 2 and 3, respectively, in Fig. 3b). This sequence motif is absent from bacterial type B enzymes (group 4, Fig. 3b), known CSs of Archea (group 6, Fig. 3b) and also TvCS1. Residues adjacent to the active site pocket implicated in binding of SAT to Arabidopsis thaliana CS by site directed mutagenesis (Lys217, His221, Lys222, (31)) are included in this motif. These residues are conserved in StCS-A and other CSs of groups 1-3, except for the E. histolytica CS in which residues corresponding to Lys217 and Lys222 of A. thaliana CS are Ala228 and Gly233, respectively. The SAT binding motif is not found in EcCS-B, which is not thought to form a complex with SAT (32), archeaon CSs and TvCS1. The sequences in this region of the bacterial type B CSs have their own characteristics. Residues 210-216 in EcCS-B comprise an extended flexible loop that bulges outward, enlarging the active site pocket and allowing access to larger substrates which are excluded from type A CSs (20). Moreover, the side chain of Arg210 of EcCS-B stabilises interaction with thiolsulphate (20). Interestingly, the equivalent residue of the CS of Aeropyrum pernix (Arg297) has been reported to be crucial for interaction with O-phosphoserine (22) and the CS of A. pernix apparently also has the extended loop. Sequence similarities suggest that TvCS1 also has the extended loop characteristic of the type B enzymes and archeaon CSs. The TvCS1 residue equivalent to the conserved arginines of EcCS-B and ApCS is Lys214, which could also interact the negatively charged side chain of substrates such as thiolsulphate and O-phosphoserine. Two other typically bulky residues in bacterial type B CSs (Arg211 and Trp212 in EcCS-B) are Ser215 and Met216 in TvCS and Arg298 and Val299 in ApCS. These analyses allowed us to postulate that TvCS1 may have substrate specificities similar to EcCS-B and ApCS and that all could be classified together as type B CS enzymes.

CS of T. vaginalis catalyses the synthesis of cysteine from O-phosphoserine - Soluble recombinant CS of T. vaginalis (designated rTvCS1) was produced in large quantities (~14 mg/l) in E. coli with a C-terminal 6 x histidine tag. Denaturing SDS-PAGE analysis confirmed that the histidine-tagged rTvCS1 was of the correct size (~33 kDa) and showed that it had a high degree of purity (Fig. 4). It was stable for several weeks when stored at 4 °C.

Recombinant TvCS1 showed high activity in the synthesis of cysteine using O-acetylsereine and sodium sulphide as substrates. O-phosphoserine was used as a substrate with a similarly high activity (Table 2), but the rTvCS was inactive towards O-acetylmethionoserine, O-succinylmethionoserine and methionine (the detection limit was 0.2 μmol/min/mg protein). The enzyme was highly active over the pH range 7.0-8.5, but not at acid pH. RTvCS1 also showed activity with thiolsulphate as substrate, but the maximal rates were considerably less than with sulphide (Table 2). No activity was detected with methanethiol and O-acetylmethioninder (limit of detection, 0.6 μmol/min/mg protein).

Substrate saturation curves and double reciprocal plots used to determine the kinetic parameters of the cysteine synthase reaction with O-acetylmethionoserine (O-acetylmethionoserine sulphydrylase) and O-phosphoserine (O-phosphoserine sulphydrylase) are shown in Fig. 5.

The apparent Kms of rTvCS towards its substrates were in the range of other CSs and was relatively high towards O-acetylmethionoserine compared with that for sulphide (Table 3). The Km for sulphide was reduced 40-fold with O-phosphoserine as substrate in place of O-acetylmethionoserine, however the apparent Km towards O-phosphoserine was very high.

rTvCS was also active as a desulphurase in hydrolyzing cysteine to yield sulphide, but only in the presence of β-mercaptoethanol (Table 2). DTT could not substitute for β-mercaptoethanol. Again the activity was highest at alkaline pHs (pH 7.0-8.5). The products of the reaction in addition to sulphide were not analysed, but as there was no release of sulphide in the absence of
the second substrate the likelihood is that the reaction mechanism is the same as the sulphhydrase reaction, as proposed for E. coli CS (33), in which cysteine binds to the enzyme and sulphide is released by β-elimination to form the α-aminoacrylate intermediate. The β-mercaptoethanol acts as the nucleophile in the second half reaction to produce S-2-hydroxyethyl-L-cysteine. Unlike our findings with rTvCS1, the E. coli enzyme was also able to use DTT. The $K_m$ towards cysteine in this reverse reaction was much lower than that towards O-acetylserine in the sulphhydrase reaction, although the turnover number ($k_{cat}$) was also much lower (Table 3). rTvCS1 was inactive towards homocysteine (Table 2). rTvCS1 showed no cystathionine β-synthase activity (limit of detection, 1 µmol/min/mg protein) unlike the A. pernix CS (21).

rTvCS1 was relatively insensitive to trifluoroalanine (F3Ala) and propargylglycine, inhibitors of various pyridoxal 5'-phosphate-dependent enzymes (34,35), with 14 mM F3Ala inhibiting the activity by 40 % (with 100 mM O-acetylserine) and 50 mM propargylglycine inhibiting only 4 %.

**Lys214 is important for O-phosphoserine sulphhydrase activity** – Mutant cysteine synthases were constructed by site-directed mutagenesis. In TvCS(K43A), the active site lysine has been substituted by alanine. The conserved positively charged residue, Lys214, predicted to play a role in substrate specificity was replaced by alanine in TvCS1(K214A). Both mutants expressed in similar levels as rTvCS1 and were purified in the same way as rTvCS1. The purified rTvCS(K43A) protein was yellow, indicating that it bound pyridoxal 5'-phosphate, but had no detectable activity as a cysteine synthase with O-acetylserine or O-phosphoserine and no detectable cysteine desulphurase activity (Table 2). These data confirm Lys43 as the active site residue. The rTvCS(K214A) mutant retained high activity with O-acetylserine and sulphide (40% of the activity of the wild type enzyme) but its activity with O-phosphoserine and sulphide was reduced by more than 100-fold (Table 2). The ability to use thiosulphate as an alternative nucleophile in the sulphhydrase reaction was also greatly reduced, but the mutant showed no change in cysteine desulphurase activity.

**CS expression in T. vaginalis is modulated by exogenous cysteine concentration** - Northern blots with T. vaginalis total and poly [A]+ RNA revealed a single CS transcript of 1.1 kb, which corresponds well with the size of the full length EST. Western blots using the antiserum raised against rTvCS1 revealed that it recognized the recombinant protein itself and just a single protein of the expected size, of about 33 kDa, in a T. vaginalis soluble fraction (Fig. 6, lane 1). No proteins were detected when duplicate blots were probed with the pre-immune serum (not shown).

* T. vaginalis was cultured under conditions that were predicted to alter the parasite’s requirement for cysteine biosynthesis. Gene expression was assessed via measuring mRNA levels by Northern blotting and protein levels by Western blotting and activity assays. The conditions chosen were: (i) minus ascorbate, by removal of the antioxidant ascorbate from the medium with the rationale that the parasite would need to adapt to the greater oxidant stress, perhaps by up-regulating cysteine biosynthesis; (ii) plus cysteine, by addition of an extra 10 mM cysteine as a redox buffer and potential source of the amino acid for the parasite; (iii) plus 5 µM propargylglycine, by addition of the compound, which is an inhibitor of many pyridoxal 5'-phosphate-dependent enzymes, including methionine γ-lyase of *T. vaginalis* (23), but a poor inhibitor of TvCS1. The protein, activity and mRNA levels of CS were greatly reduced in cells grown in the presence of additional cysteine (Fig. 6a, b and c, respectively, lane 3). There was also, surprisingly, a marked reduction when the parasites were grown in medium lacking ascorbate (Fig. 6, lane 2). This suggests that the ascorbate in the medium does not help protect the parasite from oxidant stress intracellularly, perhaps because it is not transported in. The reason that the lack of ascorbate resulted in down-regulation of CS is unclear, but perhaps could reflect ascorbate interfering with cysteine salvage by the parasite. In contrast, when the parasites were
exposed to propargylglycine there was a great increase in the level of protein and also enzyme activity (Fig. 6, lane 4).

3-phosphoglycerate dehydrogenase and O-phosphoserine aminotransferase together synthesis O-phosphoserine - A BlastP search of the T. vaginalis genome database with the E. coli K12 serA encoded protein sequence (3-phosphoglycerate dehydrogenase, PGDH) identified 5 predicted proteins. Phylogenetic analysis showed that three branched with PGDH, one with glyoxylate reductase and one with glyceraldehyde dehydrogenase. The three putative PGDH proteins had between 68% and 98% amino acid identity. One of these was expressed in E. coli and purified (designated rTvPGDH1) (Fig. 4, lane 3; Table 1). The yield was 22.3 mg/l.

Similarly, 3 potential PSAT proteins were identified by BlastP search of the T. vaginalis genome database with the E. coli PSAT sequence. The putative T. vaginalis PSAT proteins showed between 92% and 99% amino acid identity. In contrast to a recent report (36), the predicted proteins did not contain N-terminal extensions or any potential mitochondrial-targeting signal. One of these genes was expressed in E. coli and purified (designated rTvPSAT1) (Fig. 4, lane 2; Table 1). The yield was 21.3 mg/l.

The forward reactions from 3-phosphoglycerate to O-phosphoserine via phosphohydroxypyruvate (PHP) were measured by monitoring the formation of NADH fluorimetrically. With 50 μg/ml rTvPSAT1, the rate of NADH formation was proportional to rTvPGDH1 concentration. An initial rate of 1.47 ± 0.46 μmol/min/mg rTvPGDH1 was obtained. No activity was observed in the absence of phosphoglycerate. In the absence of glutamate, the reaction stalled after 1-2 min, when the NADH concentration reached 2 μM. Removal of PHP by the action of PSAT therefore increases the extent of the reaction but not the initial rate, as also observed for the E. coli PGDH (37). Thus the two trichomonad proteins together are able to synthesise O-phosphoserine, which confirms their identity and activity as PSAT and PGDH.

In general, the PGDH forward reaction is more than 10-fold slower (for example, 70-fold for E. coli PGDH (37) and 20-fold for Entameoba histolytica PGDH (38)) than the reverse reaction (37,38). In vivo, the forward reaction is favoured because the intracellular concentration of NAD⁺ is much higher than that of NADH and the product PHP is removed by the action of PSAT. Thus the forward reaction of PGDH by itself is not readily analysed. Experimentally, the activity of PGDH can be analysed in the reverse reaction but the substrate PHP is not commercially available. In order to determine whether the T. vaginalis PGDH1 is active in the reverse direction, we provided PHP by the reverse reaction of PSAT1 and measured the combined activity of the two enzymes by monitoring the oxidation of NADH. With 25 μg rTvPGDH1, the rate of NADH oxidation was proportional to rTvPSAT1 concentration. The complete reaction showed a rate of 4.78 ± 0.08 μmol/min/mg rTvPSAT1. No activity was detected when PGDH1, PSAT1 or O-phosphoserine were omitted. These data show that both T. vaginalis enzymes are active in the reverse direction.

Escherichia coli PGDH is subject to allosteric regulation by serine (37,39), the end product of the biosynthetic pathway. Serine binds to the N-terminal regulatory domain. The N-terminal region of T. vaginalis PGDH1 does not have obvious homology to the E. coli regulatory domain. We used the PGDH1/PSAT1 combined forward assay to investigate possible regulators. The activity was not inhibited by serine (up to 20 mM). There was some inhibition with cysteine. Inhibition was proportional to cysteine concentration, but peaked at 50% inhibition and this required 10 mM cysteine. E. coli PGDH is inhibited by μM concentrations of serine, whereas a range of amino acids also inhibit when at mM concentrations. Thus the T. vaginalis PGDH1 may not be regulated tightly.

DISCUSSION

We have shown that T. vaginalis has a gene that encodes a functional CS. The recombinant enzyme is active in vitro both in
the synthetic and desulphurase directions, but the finding that the enzyme level is greatly reduced when cysteine is available in abundance exogenously (Fig. 5) is strongly supportive of the enzyme operating in the synthesis of cysteine in the parasite. These data also suggest that *T. vaginalis* can salvage cysteine from its host. Whether this is salvaged as such or is oxidised and then salvaged as cystine is unknown. However the parasite contains abundant thioredoxin reductase which is able to reduce cystine (14) and so the latter would be effective in generating cysteine intracellularly. The level of exogenous cysteine and cystine available to the parasite in its host is unknown, and so it is not possible to predict the extent to which this can satisfy the parasite’s requirement for cysteine *in vivo*. Irrespective of this, the presence of CS in *T. vaginalis* suggests that *de novo* biosynthesis certainly plays an important role. A 7.2 X coverage of the ~180 Mb *T. vaginalis* genome has now been finished (http://www.tigr.org/tdb/e2k1/tvg/) and we have identified 6 CS genes. The significance of this multiplicity is uncertain, but the relatively minor differences between the predicted proteins (they are 89-98% identical) is suggestive that they perform similar functions.

The O-acetylserine used by CS enzymes results from the action of serine acetyltransferase (SAT) in most cells that synthesise cysteine, whereas the sulphide required is produced in many cases via a *de novo* synthesis pathway. This is reported to be the case, for instance, in another anaerobic protozoon *Entamoeba histolytica* (40,41). However, analysis of the *T. vaginalis* genome sequence data has failed to detect a gene that could encode a SAT homologue. The genome sequence analysis is not 100%, so we cannot completely discount the possibility that the gene is present. The possibility that the source of O-acetylserine in *T. vaginalis* is unusual also deserves consideration, but we investigated the possibility that CS of *T. vaginalis* uses a different substrate. The extreme thermophile *A. pernix* has a CS, but lacks a SAT gene (42). There is evidence that the physiological substrate of *A. pernix* CS is O-phosphoserine, produced as an intermediate in the phosphorylated serine pathway (43). The hypothesis suggested was that this thermophile uses O-phosphoserine as it, unlike O-acetylserine, is stable at high temperatures (43). Our studies have now shown that *T. vaginalis* CS1 is also able to use O-phosphoserine as a substrate and indeed suggest that perhaps this is a common characteristic of type B CSs. *Trichomonas* lives at 37°C, so in this case there is no correlation between lack of stability of O-acetylserine and use of O-phosphoserine.

Phylogenetic analysis showed that TvCSs form a clade with bacterial type B CSs (Fig. 2), and sequence comparison indicates that these enzymes and those of thermophiles have a conserved loop (Lys214-Ile220 in TvCS1) which includes a conserved basic residue (Lys214) near the active site. Modifying this conserved basic residue in *A. pernix* CS (to generate a R297A mutant) resulted in an enzyme with 74% of wild type sulphydrylase activity with O-acetylserine but with no detectable activity with O-phosphoserine (22). The K214A mutation of TvCS1 had a similar affect on the specific activities with O-acetylserine (40% of wild type) and phosphoserine (0.5% of wild type). In addition, the K214A mutant showed only 3% of wild type activity in its reaction with O-phosphoserine and thiosulphate. The *A. pernix* CS was shown to use thiosulphate as an alternative nucleophile (43), but the effect of the R297A mutation on this activity was not reported. However, the equivalent basic residue of *E. coli* type B CS (Arg210) was implicated in binding of thiosulphate by molecular modeling of the 3D structure (20), although O-phosphoserine binding was not considered. Thus our studies have shown that Lys214 of *T. vaginalis* CS has an important role in the substrate specificity, conferring the ability to use phosphoserine, as reported for Arg297 of *A. pernix* CS (22), and thiosulphate, as reported for Arg210 of *E. coli* type B CS (20). It will be interesting to see if this is a common of this group of CSs and if other type B enzymes can use O-phosphoserine as substrate and, if so, the implications of this for the organisms.

The explanation for the similarity between the CSs of *T. vaginalis* and the
bacterial CSs is unclear, especially as the known CSs of other protozoa appear to be type A (Fig. 3). The possibility that the *Trichomonas* CS arose through lateral gene transfer from a bacterium deserves consideration, especially in light of previous observations of likely bacterial-like genes in this organism that may have been acquired through gene transfer from bacteria.

The type A CSs form a macromolecular complex with SAT in which CS activity is inhibited and SAT activity is enhanced. A conserved sequence motif within the β8-β9 surface loop important for this association (30) is absent from the type B CSs of *E. coli* and *S. typhimurium*, supporting the suggestion that these enzymes do not form a regulatory complex with SAT (32). This appears to be a property shared by TvCS and the thermophile CSs because these enzymes also lack the SAT binding motif and there is no evidence of a SAT homologue in the genomes of *T. vaginalis* or thermophiles. The *E. histolytica* CS is unusual in that it is related to type A CSs by phylogeny, the structure of the β8-β9 loop and by its inability to use thiosulphate as a substrate (44), but does not appear to associate with SAT (41). However, a pair of Lys residues found to be critical for SAT binding in *A. thaliana* CS (30) are not conserved in the *E. histolytica* enzyme.

The ability to use thiosulphate as a substrate rather than sulphide is a characteristic of bacterial type B CSs (45). rTvCS1 also has activity towards thiosulphate, although much lower than towards sulphide (Table 2). This raises the possibility that thiosulphate is of physiological significance as a substrate for TvCS *in vivo*. The sulphocysteine produced in such a reaction could feasibly be converted to cysteine. For instance, the parasite’s thioredoxin, which is able to reduce cystine (14), perhaps could reduce the sulfocysteine. The type B CSs of *S. typhimurium* is also able to use methanethiol as a substrate, forming methycysteine in its reaction with O-acetylserine – although at a 100-fold lower rate than the reaction with thiosulphate and sulphide (45). rTvCS1 showed no activity with methanethiol, showing that it does not share all of the features of bacterial type B CSs. Similarly, TvCS1 differs from the CS of *A. pernix* in not using serine as a substrate (Table 2).

The apparent *K*ₘ of TvCS1 for O-phosphoserine is extremely high, similar to that of the CS of *A. pernix* (43), but the *V*ₘₐₓ is high and notably the *K*ₘ for sulphide is very low when O-phosphoserine is used. This combination could relate both to the physiological concentration of O-phosphoserine (reported to be 0.98 mM by Knodler et al. (46)) and the need for the parasite to salvage sulphide very efficiently to avoid any toxic effects. *T. vaginalis* is unusual in containing high activity of methionine γ-lyase (MGL), which hydrolys very efficiently both methionine and, more relevantly, homocysteine, with the release of hydrogen sulphide (23). The latter is known to be toxic to cells and it is likely that *T. vaginalis* has to have some means of negating such an effect. The low *K*ₘ of CS for sulphide could be this mechanism. The large difference in the *K*ₘ for sulphide with O-phosphoserine compared with O-acetylserine is surprising. There is no binding site for sulphide in the CS active site, thus the decrease in the apparent *K*ₘ for sulphide with O-phosphoserine is likely to be the result of an increase in the rate of the first half reaction, to form the α-aminoacrylate. There is a conformational change triggered by the binding the first substrate for *E. coli* type B CS (20), perhaps this is induced more rapidly by O-phosphoserine than O-acetylserine with *Trichomonas* CS.

O-phosphoserine is synthesised in most cells by the phosphorylated serine pathway that has as its initiating metabolite 3-phosphoglycerate, an intermediate of glycolysis. We have shown in this study that in *T. vaginalis* PGDH and PSAT can function to generate O-phosphoserine. *T. vaginalis* relies heavily upon glycolysis, both for energy production *per se* and also to generate pyruvate that feeds into the hydrogenosomes to produce additional ATP (47). Glycolytic flux in *T. vaginalis* is controlled by regulation of pyruvate kinase (47). The phosphorylated serine pathway feeds off glycolysis before this point and so potentially has an unlimited source of initiating metabolite. In mammals,
the first two steps of the phosphorylated serine pathway, catalysed by PGDH and PSAT, are reversible and uncontrolled, the flux from 3-phosphoglycerate to serine is regulated by the final step of the pathway catalysed by O-phosphoserine phosphatase (PSP) (48). Thus the biosynthesis of serine is controlled by the rate of serine utilisation rather than the availability of 3-phosphoglycerate. PSP appears to be absent from *T. vaginalis*, thus it seems likely that the synthesis of O-phosphoserine may be relatively uncontrolled and so, potentially, reach high levels. A concentration of 0.98 mM has been reported (46). The high $K_m$ of TvCS for O-phosphoserine could ensure that the parasite can obtain the level of cysteine that it needs without disrupting energy production in the parasite by draining 3-phosphoglycerate from the glycolytic pathway.

The source of the sulphide required by CS is enigmatic, especially as genome mining provides no evidence for the de novo pathway. This led us to postulate that the sulphide required by CS in *T. vaginalis* is provided by MGL. The enzyme’s physiological function is yet to be discovered but its presence must have implications for the availability of sulphur amino acids in the parasite. For instance, exogenous methionine is rapidly catabolised. Moreover, the parasite uses methionine to produce $S$-adenosylmethionine (SAM) for methylation reactions (49) with the resulting $S$-adenosylhomocysteine (SAH) being hydrolysed by SAH hydrolase (50) to homocysteine, which is even more rapidly catabolised by MGL. This would provide sulphide for the biosynthesis of cysteine using CS. MGL is inhibited by propargylglycine and growth of the parasites in 5 μM propargylglycine would have resulted in the majority of MGL being inhibited. This could correlate with the observed increase in *T. vaginalis* CS activity that resulted from growth with propargylglycine, in that the reduced availability of sulphide would necessitate an increased CS activity in order to ensure a sufficient cysteine biosynthetic flux to satisfy the parasite’s requirement.

*T. vaginalis* appears to lack all four enzymes of both the forward and reverse trans-sulphuration pathway, suggesting that direct methionine-cysteine interconversions do not occur. This is also thought to be the case in the anaerobic protozoon *E. histolytica* (51), which has sulphur amino acid somewhat similar to that of *T. vaginalis*. Moreover, methionine synthase (which converts homocysteine to methionine) is apparently absent from *T. vaginalis* (Table 1), and so the only fate of homocysteine in the parasite may well be catabolism by MGL, with the provision of sulphide. Another possibility source of sulphide may be from the *T. vaginalis* MST, as has been suggested for *Leishmania* (52).

The metabolic scheme arising from these studies is outlined in Fig. 1. The findings suggest that *T. vaginalis* depends heavily upon cysteine biosynthesis involving CS, with MGL possibly providing the required sulphide. MGL has been postulated as a drug target as it has no counterpart in mammals, we have solved its structure (15) and we have designed a pro-drug that is activated by the parasite-specific enzyme and has antitrichomonal activity in vivo (53). CS is also absent from humans and so could represent a good drug target. Unfortunately, there appear to have been few attempts to date to obtain specific inhibitors of CS from any source, and so there are few known inhibitors that could be used to investigate whether inhibition of the enzyme is toxic to the parasite. We did investigate 1,2,4-triazole, a reported inhibitor of bacterial CSs (54), but at 20 mM it neither inhibited the rTvCS1 of *T. vaginalis* nor parasite growth in vitro. However, we are currently investigating the structure of CS of *T. vaginalis* with an aim of being able to discover ways of exploiting its importance to *T. vaginalis*.

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FOOTNOTES

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**FIGURE LEGENDS**

Fig. 1. Cysteine biosynthesis in *T. vaginalis*. Enzymes present in *T. vaginalis* and reactions catalysed by them (this study and previous published work) are shown in bold, enzymes predicted to be present are given in normal type. Enzymes apparently absent from *T. vaginalis* are in grey type with the reactions the enzymes catalyse shown as dotted lines. See Table 1 for key to abbreviations. MGL, methionine γ-lyase; SAM-MT, S-adenosylmethione-dependent methyltransferase (49); SAHH, S-adenosylhomocysteine hydrolase (50).

Fig. 2. Phylogenetic tree of CS sequences. Sequences were aligned with AlignX (Vector NTI 9.0, Invitrogen) and all regions with gaps and inserts/extensions were removed. Phylogenetic analyses were conducted using MEGA version 3.0 (24) on 207 conserved positions. A bootstrap test was performed with the Neighbour-joining method (500 replicates, seed 74307). Bootstrap values for interior branches of < 95% are shown. Protein accession numbers are shown in brackets. The bar shows the number of substitutions per amino acid position.

Fig. 3. Multiple alignments of protein sequences of various cysteine synthases. Sequences were aligned with AlignX (Vector NTI 9.0, Invitrogen) and edited by reference to published alignments based on 3D structures of *S. typhimurium* CS type A (StCS-A) (18), *E. coli* CS type B (EcCS-B) (20), *A. pernix* CS (22) and *A. thaliana* CS (32). Blocks of conserved residues are indicated by white on a black background and blocks of similar residues by white on a grey background. Sequences are arranged into 6 groups: 1, bacterial CS type A sequences; 2, protozoan CS, type A-related; 3, plant CSs; 4, bacterial CS type B sequences; 5, CS, type B-related; 6, archaeon CSs. EcCS-A, *E. coli* CS type A (NP-311319); StCS-A, *S. typhimurium* CS type A (NP_461365); EhCS, *E. histolytica* CS (BAA21916); LmCS, *L. major* CS (LmjF36.3590); AtCS, *A. thaliana* CS (P47998); AtCS chlor., *A. thaliana* chloroplast CS (P47999); AtCS mito., *A. thaliana* mitochondrial CS (Q43725); EcCS-B, *E. coli* CS type B (NP_311319); StCS-B, *S. typhimurium* CS type B (NP_461375); GsCS-B, *G. sulfurreducens* CS type B (AAR36549); TvCS1, *T. vaginalis* CS ([95238.m00105); ApCS, *A. pernix* CS (NP_148041); PfCS, *P. furiosus* CS (CAB49296). (a), Conserved N-terminal amino acid residues. Active site lysine is indicated by symbol ▲. (b), loop region amino acid residues (located between β8 and β9). Structural features identified in plant and bacterial type A CS proteins are indicated by symbols above the top sequence: *, highly conserved sequence; †, residues implicated (by mutagenesis of AtCS) in binding of serine acetyltransferase to plant CS proteins. Structural features identified in bacterial type B and thermophile CS proteins are indicated by symbols below the bottom sequence: •, loop forming part of active site pocket (20,22); †, conserved positively charged residue implicated (by mutagenesis) in binding of O-phosphoserine to thermophile CS proteins (22).

Fig. 4. Recombinant proteins used in activity analyses. Lane 1, rTvCS1 (34.7 kDa); lane 2, rTvPSAT11 (44.1 kDa); lane 3, rTvPGDH11 (45.1 kDa). All ~5 μg/lane.
Fig. 5. Variation of cysteine synthase activity of rTvCS1 with substrate concentration. Inset: double reciprocal plot. a, 1-100 mM O-acetylserine with 3 mM sodium sulphide (Na₂S); b, 0.1–10 mM sodium sulphide with 100 mM O-acetylserine; c, 50–270 mM O-phosphoserine with 1 mM sodium sulphide; d, 0.01–1 mM sodium sulphide with 80 mM O-phosphoserine.

Fig. 6: Changes in CS protein, enzymic activity and mRNA levels in *T. vaginalis* grown under different conditions. (a) The protein expression levels determined by western blotting: TvCS, cysteine synthase; TvTR, thioredoxin reductase (14). (b) CS activities, means ± SD from 3 experiments in μmol/min/mg protein, determined at 37°C in 0.2 ml reactions containing 15 mM O-acetylserine, 3 mM sodium sulphide and 10 μg soluble protein from *T. vaginalis*. (c) The levels of mRNA assessed by quantitation of 32P hybridization using a phosphoimager and shown relative to the levels in *T. vaginalis* grown under standard conditions. The variation in growth conditions were: lane 1, control; lane 2, without ascorbate, lane 3; with added 10 mM cysteine; lane 4, with 5 μM propargylglycine. Northern blot analysis was not carried out for the propargylglycine-treated cells.
| EC          | Protein                                | Gene Model\(^1\) Sequence | TIGR Gene id                    | Name         | P\(^2\)  |
|-------------|----------------------------------------|----------------------------|---------------------------------|--------------|---------|
| 1.1.1.95    | Phosphoglycerate dehydrogenase (PGDH)  | eco:b2913                  | 96252.m00274 85506.m00072 95284.m00068 | TvPGDH1      | 2.1e-30 |
|             |                                        |                            | 88466.m00220 91003.m00104 85232.m00146 | TvPGDH2      | 5.1e-29 |
|             |                                        |                            | 94219.m00121 97048.m00021 114984.m00002 | TvPGDH3      | 8.3e-29 |
| 2.6.1.52    | Phosphoserine aminotransferase (PSAT)  | eco:b0907                  | 83680.m00049 87835.m00217 86485.m00650 | TvPSAT1      | 1.6e-71 |
|             |                                        |                            | 70585.m00106 70585.m00106 70585.m00106 | TvPSAT2      | 1.6e-71 |
|             |                                        |                            | 70585.m00106 70585.m00106 70585.m00106 | TvPSAT3      | 1.5e-70 |
| 3.1.3.3     | Phosphoserine phosphatase (PSP)        | eco:b4388 sce:YGR208w hsa:5723 | Not found | Not found |         |
| 2.3.1.30    | Serine O-acetyltransferase (SAT)       | eco:b3607 13.0033\(^3\) ath:At1g55920 | Not found | Not found |         |
| 2.5.1.47    | Cysteine synthase (CS)                | EST 215                    | 95238.m00105 118212.m00003 93857.m00319 | TvCS1        | 6.6e-155|
|             |                                        |                            | 93088.m00099 93792.m00191 81660.m00131 | TvCS2        | 4.3e-151|
|             |                                        |                            | 93792.m00191 81660.m00131            | TvCS3        | 1.1e-147|
|             |                                        |                            | 93792.m00191 81660.m00131            | TvCS4        | 1.8e-145|
|             |                                        |                            | 93792.m00191 81660.m00131            | TvCS5        | 1.4e-143|
|             |                                        |                            | 93792.m00191 81660.m00131            | TvCS6        | 1.5e-141|
| 2.7.4       | Sulphate adenylyl-transferase          | eco:b2751 pae:PA4442 see:YJR010W | Not found | Not found |         |
| 2.7.1.25    | Adenylyl-sulphate kinase               | eco:b2750 pae:PA1393 see:YKL001C | Not found | Not found |         |
| 1.8.4.8     | Phosphoadenylyl-sulphate (PAPS) reductase (PAPSR) | eco:b2762 pae:PA1756 see:YPR167C | Not found | Not found |         |
|             |                                        |                            | 85889.m00484 85889.m00484            |              | 6.5e-9  |
|             |                                        |                            | 85889.m00484 85889.m00484            |              | 2.0e-4  |
| 1.8.1.2     | Sulfite reductase (NADPH)              | eco:b2763 pae:PA1838 see:YFR030W | Not found | Not found |         |
| 2.8.1.2     | Mercaptopuruvate sulfurtransferase (MST) | eco:b2521                  | 84511.m00289 | TvMST      | 4.2e-25 |
| 4.2.1.22    | Cystathionine β-synthase (CBS)         | hsa:875 sce:YGR155W        | Not found | Not found |         |
| 4.4.1.1     | Cystathionine γ-lyase (CGL)            | hsa:1491 sce:YAL012W       | Not found | Not found |         |
| 2.5.1.48    | Cystathionine γ-synthase (CGS)         | eco:b3939 scr:YJR130C ath:At3g01120 | Not found | Not found |         |
| 4.4.1.8     | Cystathionine β-lyase (CBL)            | eco:b3008 sce:YFR055W ath:At3g57050 | Not found | Not found |         |
| 2.1.1.13    | Methionine synthase (MS)               | eco:b4019 pae:PA1843 hsa:4548 | Not found | Not found |         |
| 2.5.1.6     | Methionine adenosyltransferase (MAT)   | eco:b2942                  | 88428.m00062 88666.m00220 91003.m00104 | TvMAT1       | 1.1e-118|
|             |                                        |                            | 85232.m00146 94219.m00121 97048.m00021 | TvMAT2       | 1.2e-109|
|             |                                        |                            | 114984.m00002 | TvMAT3      | 1.0e-108|
|             |                                        |                            | 88428.m00062 88666.m00220 91003.m00104 | TvMAT4       | 9.4e-108|
|             |                                        |                            | 85232.m00146 94219.m00121 97048.m00021 | TvMAT5       | 2.5e-107|
|             |                                        |                            | 114984.m00002 | TvMAT6      | 3.2e-107|
|             |                                        |                            | 114984.m00002 | TvMAT7      | 4.7e-90 |

Table 1. Genes involved in cysteine metabolism in *T. vaginalis*
1Organism code and gene name from the KEGG GENES database (http://www.genome.jp/kegg/genes.html). Organism codes: eco, Escherichia coli; pae, Pseudomonas aeruginosa; sce, Saccharomyces cerevisiae; ath, Arabidopsis thaliana; hsa, human.

2Minimum sum probability

3Genes were classed as Not found when the Minimum sum probability was < 1.0e-03 or sequences identified by BLAST search were assigned an alternative function on basis of amino acid homology, conserved domains and phylogeny.

4TIGR E. histolytica database.
Table 2. Activities of *T. vaginalis* cysteine synthase

| Activity       | Substrate 1        | Substrate 2 | Specific activity (μmol/ min/mg protein) |
|----------------|--------------------|-------------|----------------------------------------|
|                |                    |             | T\textsubscript{v}CS1 | T\textsubscript{v}CS1(K43A) | T\textsubscript{v}CS1(K214A) |
| Sulphydrylase  | O-acetylserine     | sulphide    | 180 ± 28 | <0.2 | 72.6 ± 40.2 |
|                | O-phosphoserine*   | sulphide    | 98.5 ± 20.8 | <0.2 | 0.5 ± 1.0 |
|                | O-acetylserine     | thiosulphate | 0.99 ± 0.02 | <0.02 | 0.10 ± 0.02 |
|                | O-phosphoserine    | thiosulphate | 2.44 ± 0.38 | <0.02 | 0.07 ± 0.02 |
| Desulfurase    | cysteine           | BME         | 25.1 ± 4.8 | <0.05 | 28.9 ± 3.4 |

Specific activities of *T. vaginalis* cysteine synthase TVCS1 and the amino acid substitution mutants TVCS1(K43A) and TVCS1(K214A) were determined at 37°C. Sulphydrylase activities were determined using 100 mM of the named substrate and 3 mM sodium sulfide or 20 mM sodium thiosulfate. Desulfurase activities were determined using 5 mM cysteine and 15 mM β-mercaptoethanol (BME). Data are means ± SD from 3 experiments.

*Attempts to use saturating concentrations of O-phosphoserine (>0.5 M) resulted in inhibition of the reaction due to acidification of buffer.*
Table 3. Kinetic parameters of *T. vaginalis* cysteine synthase

| StCS-B2 | StCS-B2 | ApCS1 | AtCS3 |
|---------|---------|-------|-------|
| K<sub>m</sub> OAS (mM) | K<sub>m</sub> Na<sub>2</sub>S (mM) | k<sub>cat</sub> (s<sup>-1</sup>) | K<sub>m</sub> OPS (mM) | K<sub>m</sub> Na<sub>2</sub>S (mM) | k<sub>cat</sub> (s<sup>-1</sup>) | K<sub>m</sub> Cys (mM) | K<sub>m</sub> BME (mM) | k<sub>cat</sub> (s<sup>-1</sup>) |
| 0.9 ± 0.1 | 0.01 ± 0.00 | 115 ± 12 | 1.4 ± 0.2 | 0.22 ± 0.09 | 1780 ± 280 |

Kinetic parameters of TvCS1 compared with other CSs. O-acetylserine sulfhydrylase activity: K<sub>m</sub> O-acetylserine (OAS) and K<sub>m</sub> sodium sulphide (Na<sub>2</sub>S) were determined using 3 mM sodium sulphide and 100 mM O-acetylserine, respectively. K<sub>cat</sub> shown is the mean calculated from values for V<sub>max</sub> obtained from both the OAS and Na<sub>2</sub>S saturation curves. O-phosphoserine sulfhydrylase activity: K<sub>m</sub> O-phosphoserine (OPS) and K<sub>m</sub> sodium sulphide (Na<sub>2</sub>S) were determined using 2 mM sodium sulphide and 100 mM O-phosphoserine, respectively. K<sub>cat</sub> was calculated from the O-phosphoserine saturation curves only, because it was not possible to use saturating amounts of O-phosphoserine (>500 mM) for the Na<sub>2</sub>S saturation curves. Cysteine desulphurase activity: K<sub>m</sub> cysteine (Cys) and K<sub>m</sub> β-mercaptoethanol (BME) determined using 15 mM BME and 5 mM L-cysteine, respectively. Data were determined at 37°C and are means ± SD from 3 experiments.

1Data from Mino and Ishikawa, 2003 (43). Kinetic parameters for O-acetylserine sulfhydrylase reaction determined at 60°C and for O-phosphoserine sulfhydrylase reaction determined at 85°C.

2Data from Tai et al., 1993 (55). Kinetic parameters for O-acetylserine sulfhydrylase reaction determined at 25°C.

3Data from Bonner et al., 2005 (30). Kinetic parameters for O-acetylserine sulfhydrylase reaction determined at 25°C.
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Fig. 3a
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Fig. 3b
Fig. 4
Fig. 5

(a) O-acetylserine (mM) vs. μmol/min/mg protein
(b) Na₂S (mM) vs. μmol/min/mg protein
(c) O-phosphoserine (mM) vs. μmol/min/mg protein
(d) Na₂S (mM) vs. μmol/min/mg protein
Fig. 6

(a) Western blots showing the expression of TvCS and TvTR.

(b) Graph showing CS Activity across different samples (1-4).

(c) Graph showing Relative mRNA Levels across different samples (1-3).
Cysteine biosynthesis in Trichomonas vaginalis involves cysteine synthase utilizing O-phosphoserine

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