Characterization of Transsulfuration and Cysteine Biosynthetic Pathways in the Protozoan Hemoflagellate, *Trypanosoma cruzi*

ISOLATION AND MOLECULAR CHARACTERIZATION OF CYSTATHIONINE β-SYNTHASE AND SERINE ACETYLTRANSFERASE FROM *TRYPANOSOMA cruzi*

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Sulfur-containing amino acids play an important role in a variety of cellular functions such as protein synthesis, methylation, and polynuclear glutathione synthesis. We cloned and characterized cDNA encoding cystathionine β-synthase (CBS), which is a key enzyme of transsulfuration pathway, from a hemoflagellate protozoan parasite *Trypanosoma cruzi*. *T. cruzi* CBS, unlike mammalian CBS, lacks the regulatory carboxyl terminus, does not contain heme, and is not activated by S-adenosylmethionine. *T. cruzi* CBS mRNA is expressed as at least six independent isoforms with sequence microheterogeneity from tandemly linked multicopy genes. The enzyme forms a homotetramer and, in addition to CBS activity, the enzyme has serine sulfhydrylase and cysteine synthase (CS) activities in vitro. Expression of the *T. cruzi* CBS in Saccharomyces cerevisiae and *Escherichia coli* demonstrates that the CBS and CS activities are functional in vivo. Enzymatic studies on *T. cruzi* extracts indicate that there is an additional CS enzyme and stage-specific control of CBS and CS expression. We also cloned and characterized cDNA encoding serine acetyltransferase (SAT), a key enzyme in the sulfate assimilatory cysteine biosynthetic pathway. Dissimilar to bacterial and plant SAT, a recombinant *T. cruzi* SAT showed allosteric inhibition by L-cysteine, L-cysteine, and, to a lesser extent, glutathione. Together, these studies demonstrate the *T. cruzi* is a unique protist in possessing both transsulfuration and sulfur assimilatory pathways.

Various sulfur compounds, especially cysteine, methionine, and S-adenosylmethionine, are essential for the growth and activities of all cells (1, 2). Methionine initiates the synthesis of proteins, whereas cysteine plays a critical role in the structure, stability, and catalytic function of many proteins. S-Adenosylmethionine plays a crucial role in methyl group transfer and in polynuclear biosynthesis. Cysteine is also involved in the synthesis of the major antioxidant glutathione.

In the filamentous fungi, *Aspergillus nidulans* and *Neurospora crassa*, the major route for the synthesis of cysteine is the condensation of O-acetylserine (OAS) with sulfide, catalyzed by cysteine synthase (CS, OAS sulfhydrase) (3, 4). This pathway has been shown to be present in prokaryotes, plants, and enteric protozoan *Entamoeba* and is generally called assimilatory cysteine biosynthetic pathway since this process involves reduction and fixation of inorganic sulfate to organic amino acids. Cysteine can also be synthesized by an alternative pathway: the sulfurylation of O-acetylhomoserine to give homocysteine, which can then be converted to cysteine via cystathionine by the transsulfuration pathway. In vertebrates, cysteine is synthesized from methionine via cystathionine by the transsulfuration pathway. This pathway is believed to be the sole route for cysteine synthesis in vertebrates with cystathionine β-synthase (CBS) acting as the flux-controlling enzyme (1). In mammals, this pathway also functions as catabolic pathway of methionine and its toxic intermediates including homocysteine. Genetically determined impairment of the activities of CBS and/or cystathionine γ-lyase results in serious clinical disorders (1, 5, 6). In contrast to vertebrates, prokaryotes, fungi, and plants synthesize methionine from cysteine via the reverse transsulfuration pathway employing the complementary enzymes cystathionine γ-synthase and cystathionine β-lyase (7).

We have shown previously that the enteric protist parasite *Entamoeba histolytica*, a causative agent of amebic colitis and extraintestinal abscesses (8), and a related parasite *Entamoeba dispar* possess sulfur assimilatory cysteine biosynthetic pathway by cloning and characterization of genes encoding three key enzymes: CS, serine acetyltransferase (SAT), and ATP sulfurylase (9–12). Since *Entamoeba* apparently lack enzymes necessary to produce cysteine from methionine in transsulfuration pathway, assimilatory cysteine biosynthesis appears to be the sole biosynthetic pathway of this amino acid. To better understand sulfur-containing amino acid metabolism and cysteine biosynthesis in parasitic protists, we have identified and characterized transsulfuration and assimilatory cysteine biosynthetic pathways from the parasitic hemoflagellate *Trypanosa*.
Trypanosoma CBS and SAT

We also cloned two additional full-length CBS cDNA clones based on possible partial CBS sequences deposited in the T. cruzi data base (GenBank®/EMBL/DDJB accession nos. A0445417 and A0445365). We obtained a 0.5-kb fragment by polymerase chain reaction (PCR) using CBS/CS-specific sense and antisense primers, a lystate of the T. cruzi phase cDNA library as template, and Taq polymerase with the following cycling parameters: 1) denaturation at 94 °C for 1 min; 2) annealing at 50 °C for 1 min; 3) elongation at 72 °C for 2 min; 4) 30 cycles. To obtain a full-length CBS cDNA, the T. cruzi cDNA library was screened by hybridization with the partial CBS cDNA fragment as described (27). Two CBS cDNA clones were randomly chosen and sequenced.

Isolation of SAT cDNA—Based on possible SAT sequences deposited in the T. cruzi data base (A0445417 and A0445365), we amplified a partial SAT cDNA fragment by PCR. Since screening of the cDNA library with this partial SAT cDNA probe was unsuccessful presumably due to a low representation of SAT mRNA, we cloned SAT cDNA by 5'- and 3'-rapid amplification of cDNA end (RACE). A 1.100-bp SAT cDNA, which lacked only three amino acids at the carboxyl terminus, was obtained by 5'-RACE using a set of the T3 primer and the SAT-specific antisense primer (5'-gtggtgctgctcagcata-3'), a lystate of the T. cruzi phase cDNA library as template, and Taq polymerase under the condition described above. A 400-bp COOH-terminal portion of the SAT cDNA was obtained by 3'-RACE essentially as described for 5'-RACE using the SAT-specific sense primer (5'-ggcggctgctcagcata-3') and the T7 primer were used. Sequencing of the 5'- and 3'-RACE cDNA clones revealed that the overlapped region of these sequences were completely identical, suggesting that they were derived from a single gene.

Bacterial Expression and Purification of Recombinant Trypanosomal CBS—A plasmid was constructed to produce a glutathione S-transferase (GST)-T. cruzi CBS fusion protein (GST-TcCBS). An open reading frame (ORF) encoding TcCBS was amplified by PCR using a plasmid containing TcCBS1 cDNA (see “RESULTS AND DISCUSSION”) as template and appropriate oligonucleotide primers including Sma1 site at the end. PCR was performed as described except that Pfu polymerase was used instead of Taq polymerase. The 1.2-kb PCR fragment was 5'-HI-digested and cloned into E. coli NK3 strain transformed with pGEX-TcCBS and cultivated in the presence of 1 mM isopropyl-β-thiogalactoside at 37 °C for 2 h. The GST-TcCBS fusion protein was purified using glutathione-Sepharose 4B column (Amersham Pharmacia Biotech, Tokyo, Japan). The purified GST-TcCBS was dialyzed against phosphate-buffered saline at 4 °C for 1 h and then transferred onto a glutathione-Sepharose 4B column. The bound proteins were eluted in the same orientation as the tac promoter to produce pGEX-TcCBS. E. coli NK3 strain was transformed with pGEX-TcCBS, and cultivated in the presence of 1 mM isopropyl-β-thiogalactoside at 37 °C for 2 h. The recombinant TcCBS, which contained TcCBS and extra four amino acids (GSPG) at the amino terminus, was eluted from the column and used for further analyses.

Biochemical Characterization of TcCBS—Heme concentration was determined by the pyridine hemochrom assay as described previously (28). The absorption spectrum of recombinant TcCBS was recorded by use of a visible/UV spectrometer Beckman model DU530 thermostatted at 25 °C.

Bacterial Expression and Purification of Recombinant Trypanosomal SAT—A plasmid was constructed to produce a GST-T. cruzi SAT fusion protein. An ORF encoding SAT from T. cruzi (TcSAT) was amplified, as described above, by PCR using a sense and an antisense primer containing BamHI and Sma1 sites, respectively. The 1.1-kb PCR fragment was double-digested with BamHI and Sma1 and cloned into BamHI-Sma1 double-digested pGEX-4T-3 to produce pGEX-TcSAT. The SAT-deficient E. coli strain JM395 (F'*, cyS51, recA56) (29), a gift by Masaaki Noji and Kazuki Saito, was transformed with pGEX-TcSAT and cultivated as described above. The GST-TcSAT was produced and purified as described for the GST-TcCBS. The purified rGST-TcSAT was free from bacterial contamination, as judged by SDS-polyacrylamide gel electrophoresis. The purified GST-TcSAT was further analyzed by a gel filtration column for unknown reasons.

Chromatographic Characterization of CBS and CS from T. cruzi—Epimastigote Lysate and Recombinant TcCBS—After 105 epimastigotes were washed twice with ice-cold phosphate-buffered saline, the cell pellet was resuspended in 10 ml of 50 mM Tris-HCl, pH 7.5, containing 0.1 mM sodium ethylenediaminetetraacetate, 1 mM each of dithiothreitol, phenylmethylsulfonyl fluoride, and trans-epoxysuccinyl-L-leucylamidomethane (L-tert-butyl)-phosphinic acid (TPBPA).

soma cruzi, a causative agent of Chagas’ disease that affects more than 15 million people in Central and South America (13). Members of the family Trypanosomatidae are characterized by complex life cycles involving both vertebrate and invertebrate hosts (14). During its developmental transitions, T. cruzi encounters numerous nutritional and environmental changes both in the alimentary tract of insect hosts and in the cytoplasm of mammalian cells. In addition, in trypanosomes and their related organisms, the majority of glutathione is conjugated with spermidine to form a kinetoplastid-specific antioxidant, trypanothione (N4,N8-bis(glutathionyl)spermidine), which constitutes about 70% of total glutathione (15). Trypanothione and trypanothione-dependent enzymes seem to replace glutathione and glutathione-dependent enzymes present in a variety of prokaryotic and eukaryotic cells. Trypanothione system plays a vital role in cellular metabolism, especially maintaining the intracellular redox balance and protecting the cells from oxidative damage of free radicals and peroxides in trypanosomatids (16, 17). Therefore, understanding sulfur amino acid metabolism in trypanosomes may allow us to further exploit trypanothione metabolism to develop new drugs against trypanosomiasis. In this work, we show, by cloning and molecular characterization of the genes encoding the major enzymes associated with cysteine biosynthesis, that T. cruzi is capable of producing cysteine by two independent pathways: sulfur assimilatory cysteine biosynthesis and transsulfuration from methionine via cystathionine. In the literature, this is the first demonstration of a unicellular protist possessing both of these two pathways.

EXPERIMENTAL PROCEDURES

Microorganisms and Cultivation—Epimastigotes (the insect form) of T. cruzi YNIH (18) and Tulahuen strain (19) were grown at 26 °C in liver infusion tryptose liquid medium, supplemented with 20 µg/ml hemin, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Tissue-culture-adapted amastigotes and trypanastigotes were obtained from COS7 cultures. COS7 cell cultures were grown and infected with trypanastigotes of Tulahuen strain as described previously (20). The amastigotes and trypanastigotes (the mammalian forms) were harvested 5–7 days after inoculation (50–95% parasitemia).

Enzyme Assays—The enzymatic activity of CBS was assayed by measuring cystathionine production as described (21). The enzymatic activity of CS was assayed by measuring cysteine production (22). The enzymatic activity of SAT was measured by measuring hydrogen sulfide production (23) in the mixture of 50 mM Tris-HCl, pH 8.0, 10 mM L-cysteine, 20 mM L-mercaptoethanol, 0.1 mM pyridoxal phosphate, 0.075 mM lead acetate. For all assays using a recombinant CBS, bovine serum albumin was included at 1 mg/ml. The enzymatic activity of SAT was measured by monitoring the decrease in absorbance at 232 nm (A232) due to the thioester bond of acetyl-CoA cleaved or 1 µmol of L-cysteine, L-cystathionine, or hydrogen sulfide synthesized at 25 °C for 1 min.

Isolation of CBS cDNA—Isolation of poly(A)+ RNA and construction of phage and phagemid cDNA library from epimastigotes were described previously (25). To obtain CBS/CSS cDNA encoding functional enzymes, we took advantage of functional rescue of the cysteine-auxotrophic Escherichia coli NK3 strain (ΔtryE5 leu-6 thi hsdR hsdM+ cysK cysM) (26), a gift by Masaaki Noji and Kazuki Saito (Chiba University), which is defective of the two CS genes, with putative CBS/CSS cDNA from T. cruzi. Procedures for functional rescue were described previously (9). Ten colonies out of 5 × 106 grew in the absence of cysteine. After screening twice, all these cDNA clones were confirmed to complement cysteine auxotrophy of NK3 cells. Six clones were randomly chosen, and fully sequenced.
(4-guanidino)butane, and 10 µg/ml each of antipain, aprotinin, leupeptin, and pepstatin (Buffer A). The suspension was then subjected to three cycles of freezing and thawing. The cell lysate was centrifuged at 15,000 × g for 15 min at 4 °C and filtrated with 0.45-µm cellulose acetate membrane. For gel filtration chromatography, the lysate was applied to a column of TOYOPEARL HW 55S (Toyo Corp., Tokyo, Japan) that had been equilibrated with Buffer A. Elution was carried out with the same solution and each fraction was subjected to CBS, CS, and SS assays. For anion exchange chromatography, DEAE-TOYOPEARL 650S was pre-equilibrated with Buffer A. The pre-equilibrated DEAE column was loaded with the parasite lysate, washed extensively with Buffer A, and eluted with a 300-ml linear gradient of 0–0.5 M KCl in Buffer A.

**Functional Rescue of the Yeast CBS-deficient Mutant Strain with Expression of TcCBS—** The ORF of TcCBS1 was PCR-amplified as described above, end-trimmed, and cloned into EcoRI-digested and end-trimmed pKT10 (25) to produce the plasmid construct pyTcCBS to express the trypanosomal CBS under the control of the TDH3 promoter in yeast. Each of the three plasmid constructs: pyTcCBS, pKT10, and leupeptin, and pepstatin (Buffer A). The suspension was then subjected to three cycles of freezing and thawing. The cell lysate was centrifuged at 15,000 × g for 15 min at 4 °C and filtrated with 0.45-µm cellulose acetate membrane. For gel filtration chromatography, the lysate was applied to a column of TOYOPEARL HW 55S (Toyo Corp., Tokyo, Japan) that had been equilibrated with Buffer A. Elution was carried out with the same solution and each fraction was subjected to CBS, CS, and SS assays. For anion exchange chromatography, DEAE-TOYOPEARL 650S was pre-equilibrated with Buffer A. The pre-equilibrated DEAE column was loaded with the parasite lysate, washed extensively with Buffer A, and eluted with a 300-ml linear gradient of 0–0.5 M KCl in Buffer A.

**RESULTS AND DISCUSSION**

**Isolation of Six Isotypes of TcCBS cDNA—** We attempted to obtain functional CS cDNA from *T. cruzi* epimastigotes by using the strategy of functional rescue of the CS-deficient *E. coli* strain. Ten cDNAs obtained by this strategy apparently encoded a protein with high sequence similarity to CBS from a variety of species. This suggests that trypanosomal CBS functions as CS in *E. coli in vivo*. Although the native and recombinant yeast and mammalian CBS showed CS and CBS activities in vitro (23, 34), this is, in the literature, the first demonstration that a eukaryotic CBS physiologically functions as CS in prokaryotes. Sequencing of six randomly chosen cDNA clones obtained by rescue, and two additional CBS cDNA clones obtained by hybridization revealed that all of these clones contained an insert with a 1155-bp ORF, encoding a protein of 384 amino acids with a calculated molecular mass of 42.0 kDa. These cDNA sequences were heterogeneous; most of these individual cDNAs were independent. Based on nucleotide sequences, the eight cDNAs encoding CBS from *T. cruzi* (TcCBS) were grouped into six isotypes, TcCBS1–TcCBS6 (Fig. 1). Nucleotide variations among the cDNA isotypes were found at one, eight, and one nucleotide positions in 5’-untranslated region (UTR), the protein coding region, and 3’-UTR, respectively. Each cDNAs contained an identical length (191 bases) of 3’-UTR. Two splice acceptor sites, resulting in heterogeneity of the length of 5’-UTR (14 or 16 bases), were identified; 5’-UTR of TcCBS3 was two bases shorter than the rest of the isotypes.

**Features of the Deduced Amino Acid Sequences of TcCBS—** Five of the six cDNA isotypes encoded identical proteins, while one (TcCBS6) contained two nucleotide substitutions that resulted in amino acid alterations (Val14 → Ala and Tyr261 → Cys) (Fig. 1). TcCBS revealed significant identities to CBS and CS from a variety of organisms. Both TcCBS isotypes show higher identities to CBS from eukaryotic organisms (47–66%) than to CS from bacteria and plants (32–39%). Specifically, TcCBS shows a 66% identity to CBS from *Leishmania tarentola*, a 54% identity to CBS from *Dictyostelium discoideum*, and a 50% identity to Rat CBS. Sequence alignment (Fig. 2) shows that TcCBS lacks the 90–120-amino acid COOH-terminal extension present in CBS from all other organisms except with *Leishmania*. Thus, trypanosomal CBS is one of the shortest CBS enzymes reported to date. The consensus sequence for the putative pyridoxal phosphate-binding domain and three of four lysine residues shown to be important for the catalytic activity of spinach CS-A by site-directed mutagenesis (35) are very well conserved in TcCBS.

**Genomic Organization and Significance of Heterogeneity of TcCBS—** Digestion of *T. cruzi* genomic DNA with restriction enzymes that cut once within TcCBS ORF gave 2.5-kb ladders, suggesting that TcCBS genes are tandemly linked in a head-to-tail fashion (Fig. 3). The hybridization patterns of different enzymes were not identical, suggesting that there is heterogeneity of these enzyme recognition sites in the different TcCBS isotypes. However, these differences must be within the intergenic region, as all three enzymes tested, BamHI, EcoRI, and HindIII, cut once in each of the isotype cDNAs. Differences in the ladder patterns were not due to incomplete digestion since hybridization of the identical blot with a single-copy gene, e.g., gp72 (36), gave single bands (data not shown). The premise that TcCBS genes are tandemly linked in a head-to-tail fashion was also supported by the fact that a genomic fragment containing the 10-bp carboxyl end of ORF and the 191-bp 3’-UTR of TcCBS4, the 1.2-kb intergenic region, and the 160-bp aminoterminal end of TcCBS2 or TcCBS3 or TcCBS4, was obtained by PCR (data not shown). Assuming that each hybridizing band of the ladder in the BamHI-digest represents an identical number of molecules, TcCBS genes appeared to be present as >15 copies per haploid. The fact that XhoI and XhoI digest gave large hybridizing bands suggests that TcCBS genes likely clustered at a single or two loci.

There is, to our knowledge, no precedent for an example of heterogeneous CBS expressed in a single organism although expression of alternatively spliced CBS mRNA isoforms from a single gene was reported (37). Whether a single cell expresses...
Fig. 3. Southern blot analysis of TcCBS genes. Ten μg of the total DNA from T. cruzi epimastigotes was digested with a variety of restriction endonucleases, separated by agarose gel electrophoresis, transferred onto a nylon membrane, and then hybridized with the [32P]dCTP-labeled TcCBS cDNA probe, corresponding to an entire open reading frame, as described (27). Restriction endonucleases used were EcorI (lane 1), BamHI (lane 2), HindIII (lane 3), Clal (lane 4), XbaI (lane 5), and XhoI (lane 6).
ote lysate, a soluble parasite lysate was fractionated by DEAE anion exchange chromatography, and each fraction was assayed for CBS, SS, and CS activities (Fig. 5A). CBS and SS activities were eluted as a symmetrical overlapped peak (fractions 43–44). To confirm that this peak was actually due to TcCBS, rTcCBS was loaded on the same column and was shown to elute with the same fractions that contained CBS, SS, and CS activities.

Altogether, these data have extended the previous findings suggesting the presence of the transsulfuration pathway in trypanosomes (45, 46). The presence of an intact transsulfuration pathway in T. cruzi is further bolstered by our finding that another important enzyme in transsulfuration pathway, cystathionine γ-lyase, which forms cysteine, ammonia, and 2-oxobutyric acid from cystathionine, was eluted at fractions 39–40 on DEAE anion exchange chromatography (data not shown, Fig. 5A).

Identification of the Putative CS—CS activity was eluted on the DEAE column as two distinct peaks (fractions 43–44 and 60–62). The first CS peak overlapped those of CBS and SS, suggesting that this activity was likely attributed to TcCBS. However, the fractions from the second peak solely showed CS activity; neither CBS nor SS activity was detected in these fractions (see below). The presence of this putative CS activity, lacking both CBS and SS activities, together with that of SAT (see below), strongly indicates the presence of the bacteria/plant-like assimilatory cysteine biosynthetic pathway in trypanosomes. It is possible that, although TcCBS can function as a CS in E. coli, this other putative CS enzyme is the key player in assimilatory cysteine biosynthesis in vivo in T. cruzi. The fact that we did not obtain cdNA encoding this putative CS by functional rescue of the CS-deficient E. coli strain suggests that either T. cruzi CS does not function as CS in a prokaryote, or that CS mRNA was not abundant in epimastigotes, comparing to CBS mRNA. The former possibility indicates the presence of a novel CS in trypanosomes.

Multimerization State of the Native and Recombinant Tc-CBS—Multimerization state of TcCBS was examined by isolating the first peak from the DEAE chromatography, containing CBS/SS/CS activities, then loading and eluting on the gel filtration column. Fractions were then assessed for CS activity (Fig. 5B). More than 70% of total CS activity was eluted at 155 ± 25 kDa, and the rest of the activity was eluted at molecular mass of 65 ± 7 and 30 ± 5 kDa. These data are consistent with the majority of rTcCBS being a homotetramer, and a small proportion of the protein existing as a homodimer and monomer. In contrast to rTcCBS, the native TcCBS was eluted as a single peak at 155 ± 35 kDa, suggesting that the native TcCBS also forms a homotetramer. Formation of homotetramer of the native and recombinant TcCBS implies that the COOH-terminal region, present in yeast and mammalian CBSs and absent in trypanosomal CBS, is not exclusively responsible for tetrameric formation as suggested for the mammalian enzyme (47).

Functional Rescue of the Yeast CBS-deficient Mutant Strain with TcCBS and In Vivo Function of TcCBS—To see if TcCBS also functions as a CBS enzyme in vivo, we attempted to rescue the growth defect of the CBS-deficient yeast strain, WB63yCBSA by the expression of TcCBS (Fig. 6). As predicted, introduction of a plasmid containing either yeast or trypanosomal CBS gene suppressed growth defect of WB63yCBSA in either the absence or presence of cysteine, whereas the transformant harboring the vector control grew only in the presence of cysteine. Together with the fact that TcCBS functions as CS in E. coli, this result suggests that trypanosomal CBS may be

Fig. 4. CBS, SS, and CS activities in the lysate of T. cruzi epimastigotes and tissue culture-derived amastigotes/trypomastigotes, and of the recombinant TcCBS. Open bars, gray bars, and filled bars represent CBS, SS, and CS activities, respectively. All activities are expressed in units/mg of protein from three to five separate triplicate experiments. Error bars represent standard error of mean.

Fig. 5A, elution profile of the native CBS, SS, and CS activities from the T. cruzi epimastigote lysate by DEAE anion exchange chromatography. CBS, SS, and CS activities in the parasite lysate were separated on a DEAE-TOYOPEARL 650S column. The trypanosomal lysate was eluted with Buffer A containing a linear gradient of 0–0.5 mM KCl. Circles and a thick solid line represent CBS activities. Squares and a thin solid line represent SS activities. Triangles and a thick dashed line represent CS activities. A thin dotted line represents absorbance at 280 nm. B, elution profile of the recombinant TcCBS and the native CBS and CS from the T. cruzi epimastigote by gel filtration chromatography. The recombinant rTcCBS and a mixture (2 ml) of fractions 43–44, corresponding to the native CBS, and a mixture of fractions 61–62, corresponding to the native CS, from DEAE anion exchange chromatography, were separated on TOYOPEARL HW 55S gel filtration chromatography. Each fraction was assayed for CS activity. Circles and a dashed line represent an elution profile of the recombinant rTcCBS. Triangles and a solid line represent that of the native CBS. Squares and a dotted line represent that of the native CS. Note that the peaks for rTcCBS and native TcCBS were completely overlapped. Molecular standards shown above, purchased from Bio-Rad (Tokyo, Japan), are aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and chymotrypsin (25 kDa).
a bifunctional enzyme involved in the two cysteine-synthesizing pathways \textit{in vivo}. As mentioned above, TcCBS is one of the shortest "prototype" CBS, based on the primary protein sequence, no interaction with heme, and no activation with S-adenosylmethionine. This might contribute, at least in part, to the fact that the trypanosomal CBS functions as CS in a prokaryotic organism.

**Significance of the Two Independent Pathways for Cysteine Production in \textit{T. cruzi}**—\textit{T. cruzi} is the first protist, in the literature, shown to possess two independent pathways for cysteine production: sulfur-assimilation sequence from sulfate and transsulfuration sequence from methionine via cystathionine. Extracellular L-cysteine, but not L-cystine or any reducing agents, has been shown to be essential for growth of \textit{Trypanosoma brucei} bloodstream forms (48). Since trypanosomes require cysteine not only for protein biosynthesis, but also for formation of glutathione and trypanothione, which are present in high amounts (15), trypanosomes may require multiple cysteine-acquiring pathways to ensure there is cysteine available to fulfill these needs. The presence of two independent cysteine-synthetic pathways in a single protist may be unique for \textit{T. cruzi} or trypanosomatids, since the other parasitic protozoans \textit{Entamoeba} contain only the sulfur assimilation pathway and not the transsulfuration pathway (9–11).

**Stage-specific Expression of Transsulfuration and Assimilatory Cysteine Biosynthetic Pathways**—We next examined whether expression of CBS, SS, and CS activities is stage-specific using the lysate of the insect-form (epimastigotes) and the mammalian form parasites (tissue culture-derived amastigotes and trypomastigotes) (Fig. 4). Both CBS and SS activities were 8–9 times higher in the lysate of epimastigotes than those in the lysate of amastigotes and trypomastigotes. The ratio of CBS to SS activity in the lysates was 1.3–1.5, which was comparable to that of rTcCBS (1.6), indicating that a majority of CBS activity in the parasite lysate is attributable to TcCBS. In contrast, significant CS activity was detected in both epimastigotes and amastigotes/trypomastigotes. CS activity in amastigotes and trypanomastigotes was about half of that in epimastigotes. Based on the fact that rTcCBS showed comparable SS and CS activities (1:1.0), we attribute ~50% of CS activity in the epimastigote lysate to TcCBS and the other half of CS activity to the second putative CS, whereas we attribute about 90% of CS activity in the amastigote/trypomastigote lysate to the putative CS. Thus, the putative CS activity is expressed in both epimastigotes and amastigotes/trypomastigotes at a comparable level.

The stage-specific regulation of transsulfuration pathway are likely associated with the complex life cycle of \textit{T. cruzi}. Within the cytoplasm of mammalian cells, where amastigotes...
actively replicate, sulfur-containing amino acids and their intermediates are readily available, whereas these compounds are scarce in the insect alimentary tract, where epimastigotes and metacyclic trypomastigotes reside. Thus, abundance of sulfur-containing amino acids may be a major cause of the down-regulation of TcCBS expression in the mammalian forms. It was shown that CBS activity was up-regulated 3-fold when yeast cells were cultured under aerobic condition (49). However, cultivation of amastigotes/trypomastigotes in liver infusion tryptose medium, which presumably provides a less aerobic condition than the condition for amastigote/trypomastigote cultivation, resulted in an 4–6-fold increase of CBS activity in the parasite lysates (data not shown). The contradiction between the yeast and the T. cruzi data could be explained if the anaerobic up-regulation was due to factors other than oxygen tension, e.g. temperature, nutrients, or a lack of nutrients.

In contrast to the down-regulation of CBS activity in the mammalian forms of T. cruzi, CBS and other activities involved in transsulfuration pathway were detected in the mammalian forms (bloodstream trypomastigotes) of T. brucei (45, 46). This indicates that substantial differences in metabolism of sulfur-containing amino acids exist between T. cruzi and T. brucei. Entamoeba, which involves only mammalian hosts, solely possesses sulfate assimilatory pathway (9–11), but not transsulfuration pathway. This may suggest that the complexity of life cycles of parasitic protozoa and the redundancy of cysteine biosynthetic pathways are causally connected.

Cloning of TcSAT cDNA and Features of the Deduced Amino Acid Sequence of TcSAT—Sequencing of a putative SAT cDNA obtained by RACE revealed that this cDNA contained a 20-bp 3′ end of spliced leader sequence, a 17-bp 5′-UTR, a 1047-bp ORF, and a 86-bp 3′-UTR, followed by poly(A) tail. The ORF of TcSAT cDNA encoded a protein of 348 amino acids with a calculated molecular mass of 38.4 kDa. The deduced protein sequence of TcSAT showed 8–34% identities to SAT from a variety of organisms. Among them TcSAT encoded highest identities to a plasmid-encoded SAT in Synechococcus (Srph (Ref. 50); 34%) and SAT from enteric protozoan parasites E. histolytica and E. dispar (Refs. 10 and 11; 31%). Sequence alignment (Fig. 7) showed several important aspects of TcSAT. First, TcSAT contains a conserved structural domain, called a left-handed parallel β-helix (51), found in a variety of acetyl- and acyltransferases (see references in Ref. 51). Second, TcSAT contains an amino-terminal extension that is absent in cytosolic isoforms of SAT from other species. Third, the COOH-terminus of TcSAT is 20–70 amino acids shorter than SAT from other species. Finally, TcSAT contains a unique 10-amino acid insertion (amino acids 264–273) that is only found in Entamoeba SATs and Synechococcus Srph. These data reinforce the hypothesis that protozoan and cyanococcal plasmid-borne SAT shared a common ancestor as suggested previously (10).

Enzymatic Characterization of the Recombinant TcSAT—We produced a recombinant GST-TcSAT fusion protein (rGST-TcSAT) for enzymatic studies since identification and biochemical characterization of TcSAT is the second piece of evidence supporting the significance of sulfate assimilatory cysteine biosynthesis in T. cruzi. The enzymatic reaction of rGST-TcSAT followed the Michaelis-Menten kinetics and showed an apparent Km of 0.24 ± 0.1 mM for L-serine (in the presence of 0.1 mM acetyl-CoA) and 0.92 ± 0.3 mM for acetyl-CoA (10 mM L-serine). Enzymatic activity of TcSAT was inhibited by L-cysteine in a competitive manner with both L-serine and acetyl-CoA (Ki was 12 ± 4 μM for L-serine, and 0.14 ± 0.03 μM for acetyl-CoA) (Fig. 8). This inhibitory effect was specific for stereoisomeric structure of L-cysteine, but not specific for the redox state of this amino acid. In the presence of 0.02 mM acetyl-CoA and 0.2 mM L-serine, 0.2 mM L-cysteine showed no inhibition whereas 0.2 mM L-cysteine showed 85 ± 7% inhibition. An oxidized form of L-cysteine (L-cystine) also showed 70 ± 10% reduction Only slight or moderate inhibition was observed at 0.2 mM with structurally similar amino acids, L-homocysteine (11 ± 3%), L-homoserine (13 ± 5%), and N-acetyl-L-cysteine (7 ± 4%). Surprisingly, the activity of rTcSAT was also inhibited in the presence of 0.2 mM glutathione by 36 ± 8%. Allosteric inhibition by glutathione has not been shown for SAT (e.g. Refs. 52 and 53). Therefore, trypanosomal SAT may play an important role in the monitoring of total thiol concentration and perhaps the cellular redox state. A comparable level of inhibition by L-cysteine and L-cysteine was also observed for SAT from E. histolytica (10). Therefore, this biochemical property may be a common feature shared by the protozoan SATs.

Possible in Vivo Function of TcSAT—Why do trypanosomes need SAT when they possess CBS, which can form cysteine from serine and hydrogen sulfide (SS activity), and therefore, compensates the reactions catalyzed by SAT and CS? In yeasts, where cysteine is exclusively synthesized through transsulfuration pathway (54), OAS solely serves as a coinducer of the sulfate assimilation pathway (55). In contrast, OAS serves as both a metabolic intermediate and a regulatory element in enteric bacteria (56). Taken together, we propose that the trypanosomal SAT plays a major role to regulate sulfate incorporation, activation, and assimilation pathway, by controlling OAS abundance, as a part of a complex mechanism to balance thiol concentration. The fact that the putative CS activity and TcSAT mRNA are constitutively expressed in both the insect and mammalian stages of T. cruzi (Fig. 4, data not shown) also indicates that this pathway plays a housekeeping role. A biological role of individual enzymes in the assimilatory cysteine biosynthetic pathway in trypanosomes shall be unequivocally demonstrated by gene replacement or disruption (e.g. Refs. 57 and 58) of TcSAT, which is a single copy gene (data not shown), and the putative CS gene.

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