Identification and Biochemical Characterization of Serine Hydroxymethyl Transferase in the Hydrogenosome of Trichomonas vaginalis*†

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Serine hydroxymethyl transferase (SHMT) is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and methylenetetrahydrofolate. We have identified a single gene encoding SHMT in the genome of Trichomonas vaginalis, an amitochondriate, deep-branching unicellular protist. The protein possesses a putative N-terminal hydrogenosomal presequence and was shown to localize to hydrogenosomes by immunofluorescence analysis, providing evidence of amino acid metabolism in this unusual organelle. In contrast to the tetrameric SHMT that exists in the mammalian host, we found that the T. vaginalis SHMT is a homodimer, as found in prokaryotes. All examined SHMT contain an 8-amino-acid conserved sequence, VTTTTHKT, containing the active-site lysyl residue (Lys 251 in TvSHMT) that forms an internal aldimine with PLP. We mutated this Lys residue to Arg and Gln and examined structural and catalytic properties of the wild-type and mutant enzymes in comparison to that reported for the mammalian protein. The oligomeric structure of the mutant K251R and K251Q TvSHMT was not affected, in contrast to that observed for comparable mutations in the mammalian enzyme. Likewise, contrary to that observed for mammalian SHMT, the catalytic activity of K251R TvSHMT was unaffected in the presence of PLP. The K251Q TvSHMT, however, was found to be inactive. These studies indicate that the active site of the parasite enzyme is distinct from its prokaryotic and eukaryotic counterparts and identify TvSHMT as a potential drug target.

MATERIALS AND METHODS

Parasite cell culture. Trichomonas vaginalis strains T1 and G3 were grown in Diamond’s medium supplemented with 10% (vol/vol) horse serum and iron as described previously (22).

Plasmid construction. We designed primers SHMTNF and SHMTXR (see the table in the supplemental material) to amplify the complete open reading frame (ORF) of the gene encoding the SHMT protein from the T. vaginalis genomic DNA for in-frame cloning into the pET-29B expression vector (Novagen) using NdeI and XhoI restriction sites. The resulting recombinant C-terminally His-
tagged fusion protein was expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen). For expressing the protein in *T. vaginalis*, we PCR amplified the gene with primer pairs SHMTNF/SHMTTvKR (see the table in the supplemental material). The PCR fragment was then cloned into master-neo-(HA)2 plasmid (15) to generate the construct to transfect *T. vaginalis*. Point mutations were introduced (K251R and K251Q) at Lys-251 (Fig. 1) by site-directed mutagenesis using the primer sets K251RFP/K251RRP and K251QFP/K251QRP (see the table in the supplemental material), respectively. These mutant proteins were expressed in *T. vaginalis* and in bacteria to purify the recombinant proteins.

Selectable transfection of *T. vaginalis*. Electroporation of *T. vaginalis* strain T1 was carried out as described previously (14) with 50 μg/H9262 g of circular plasmid DNA. Transfectants were selected with 100 μg/ml of G418 (Sigma) prior to crude fractionation and organelle purification.

Immunofluorescence microscopy. Live *T. vaginalis* cells were washed with warm Diamond’s media and allowed to attach to coverslips for 30 min in a humidifying chamber. The cells were then washed with warm 1/11003 phosphate-buffered saline and fixed with 3.5% formalin for 20 min at room temperature. The C-terminally hemagglutinin (HA)-tagged SHMT protein and Hsp70 were visualized using mouse anti-HA monoclonal antibody (Sigma) and rabbit anti-Hsp70 polyclonal antibody as primary antibodies and secondary Alexa Fluor-488 donkey anti-mouse (green) and Alexa Fluor-594 donkey anti-rabbit (red) antibodies (Invitrogen). Images were processed with Axiovision v. 3.2 software (Zeiss).

Isolation of hydrogenosomes. Hydrogenosomes were prepared as previously described by Bradley et al. (9). The organelles were then resuspended in a buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 2% sucrose, 250 mM NaCl, 5 mg/ml leupeptin, and 25 mg/ml N-p-tosyl-L-lysine chloromethyl ketone (TLCK) and incubated on ice for 30 min, with occasional vortexing every 5 min. The clear hydrogenosomal lysate was finally obtained after centrifugation at 16,000 × *g* for 30 min at 4°C.

Sucrose density gradient analysis. Hydrogenosomal extracts (0.5 mg) from transfected *T. vaginalis* cells (1.0 ml) were loaded into linear sucrose gradients (2 to 20%) in 20 mM Tris-HCl, pH 7.5, 2% sucrose, 250 mM NaCl, TLCK, and leupeptin prepared in 25- by 89-mm centrifugation tubes (Beckman) and were immediately subjected to centrifugation at 38,000 rpm for 16 h at 4°C with an SW41 rotor (Beckman). Ten serial fractions were collected from the top of the gradient and analyzed for the presence of SHMT and Hsp70.

FIG. 1. Alignment of SHMT amino acid sequences. The alignment of amino acid sequences deduced from the gene encoding SHMT from *T. vaginalis* (**Tv**, TVAG_109540), *Homo sapiens* (**mHs**, P34897), and *E. coli* (**Ec**, POA825) using the software CLUSTALX is shown. Asterisks and dots represent identities and similarities, respectively. The black line indicates an 8-amino-acid conserved sequence containing the active-site lysine residue (boxed). The predicted hydrogenosomal precursor is marked in boldface type in the *T. vaginalis* protein sequence.
The purity of the purified proteins was checked by SDS-PAGE. 

7.6, containing 50 mM NaCl, 1 mM EDTA, 8% glycerol, and 1 mM DTT. The excess imidazole was removed by dialysis against 25 mM potassium phosphate buffer, pH 7.6, containing 50 mM NaCl, 1 mM EDTA, 8% glycerol, and 1 mM DTT. The purity of the purified proteins was checked by SDS-PAGE.

Enzyme activity. SHMT activity of the purified recombinant proteins (wild type and mutants) were assayed as reported earlier (2). The enzyme activity was tested directly by monitoring conversion of radioactive carbon from serine to methylenetetrahydrofolate. Briefly, the assay mixture consisted of 50 mM Tris–HCl, pH 8.0, 1 mM DTT, 0.25 mM PLP, 2.5 mM EDTA, 0.2 mM L-Ser, 0.6 nmol [14C]Ser, and varied concentrations of THF. The reaction was started by adding ~2 μl of the enzyme. After incubation at 37°C for 20 min, the reaction mixture was streaked onto 2-cm2 Whatman DE-81 paper. Unreacted serine was desorbed by washing the filter for 30 min (with three changes) in distilled water. The radioactivity associated with methylenetetrahydrofolate was determined by drying the washed filter and subjecting it to liquid scintillation spectroscopy.

Expression of recombinant proteins. The pET-SHMTwt, pET-K251R, and pET-K251Q plasmids were transformed into E. coli BL21(DE3) cells and selected with 30 μg/ml kanamycin in LB media. For the expression of the wild-type and mutant proteins, 500-ml cultures were grown at 37°C to an A600 of 0.8 before induction with 0.5 mM isopropyl-1-thio-D-galactopyranoside for 3 ha at 37°C. Harvested cells were then resuspended in a buffer containing 1 mg/ml of lysosyme, 0.05 M NaH2PO4, 0.3 M NaCl, 50 μM PLP, 10 mM imidazole, protease inhibitor cocktail (Roche Diagnostics), 5 mg/ml leupeptin, and 25 mg/ml TLCK for 20 min at 4°C and sonicated. The insoluble material was pelleted at 10,000 × g for 30 min at 4°C, and the resulting lysate was applied to a nickel-nitrilotriacetic acid-agarose column. The column was washed four times with a buffer containing 0.05 M NaH2PO4, 0.3 M NaCl, and 20 mM imidazole, pH 8.0. Specifically bound proteins were eluted with the above buffer containing 0.25 M imidazole. The fractions containing SHMT were pooled, and glycerol and dithiothreitol (DTT) were added to final concentrations of 8% and 1 mM, respectively. Excess imidazole was removed by dialysis against 25 mM potassium phosphate buffer, pH 7.6, containing 50 mM NaCl, 1 mM EDTA, 8% glycerol, and 1 mM DTT. The purity of the purified proteins was checked by SDS-PAGE.

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Identification of a putative gene encoding SHMT. To identify the T. vaginalis SHMT, we searched the ~7× coverage of the Trichomonas vaginalis genome database (http://tigrblast.tigr.org/er-blast/index.cgi?project=tvg) with both prokaryotic and eukaryotic SHMT sequences, and only one gene (locus TVAG_109540 [83992.m00192]) was found. Comparison of deduced amino acid sequences of this TvSHMT revealed 49%
T. vaginalis enzymes were expressed in to its human counterpart. Lys-251 residue to Arg or Gln to ascertain whether differences with PLP, are conserved in TvSHMT (Fig. 1). We mutated the acids known to be associated with the catalytic site of other for a drug target to treat trichomoniasis. Most of the amino acids known to be associated with the catalytic site of other SHMT, such as Lys-251 (31), which is predicted to interact with PLP, are conserved in TvSHMT (Fig. 1). We mutated the Lys-251 residue to Arg or Gln to ascertain whether differences exist in structural and catalytic properties of TvSHMT relative to its human counterpart. Wild-type TvSHMT (SHMTwt) and mutant (K251R and K251Q) enzymes were expressed in T. vaginalis with a C-terminal HA tag. Hydrogenosomes isolated from the three T. vaginalis transfectants were solubilized in nonionic, nondenaturing detergent, Triton X-100, and the cleared lysates were subjected to 2 to 20% sucrose gradient ultracentrifugation. The sucrose gradient fractions were then analyzed by SDS-PAGE, followed by immunoblotting using anti-HA antibody. SHMTwt, K251R, and K251Q were all found to fractionate in 8 to 10% sucrose, between 67-kDa and 140-kDa markers, indicating that TvSHMT exists as a dimer in the hydrogenosomes and that mutating the Lys residue to Arg or Gln does not alter its oligomeric structure (Fig. 3). Analytical ultracentrifugation results also indicate that recombinant SHMTwt, K251R, and K251Q proteins form dimers (data not shown).

Catalytic activity of the wild-type and mutant recombinant proteins. To determine whether TvSHMT is active and to compare the activity of SHMTwt, K251R, and K251Q, these recombinant proteins were expressed in BL21(DE3) cells as C-terminally HA-tagged proteins. The proteins were purified using Ni-nitrilotriacetic acid column chromatography and as- sessed to be >98% pure by Coomassie blue-stained SDS-PAGE (data not shown). We then determined the catalytic activity of the recombinant SHMTwt, K251R, and K251Q proteins in the presence and absence of PLP, using a fixed concentration of L-Ser (0.2 mM) and variable concentrations of 50 kDa. Expression of the TvSHMT gene was confirmed by reverse transcription-PCR (data not shown).

SHMT localizes in the hydrogenosomes of T. vaginalis. To investigate the subcellular localization of the SHMT protein, C-terminally HA-tagged proteins were expressed in T. vaginalis and localized using immunofluorescence analyses. The protein was found to colocalize with Hsp70, a marker for the hydrogenosomes, demonstrating that the protein is found in this unusual organelle (Fig. 2A). Western analyses of hydrogenosomes isolated from T. vaginalis cells expressing the tagged protein with anti-HA antibody also indicated that SHMT is localized to the hydrogenosomes (data not shown). Moreover, SHMT activity was detected in hydrogenosomal extracts prepared from untransfected cells, further confirming this result (Fig. 2B).

Characterization of TvSHMT. We have characterized TvSHMT to determine whether it might be a good candidate for a drug target to treat trichomoniasis. Most of the amino acids known to be associated with the catalytic site of other SHMT, such as Lys-251 (31), which is predicted to interact with PLP, are conserved in TvSHMT (Fig. 1). We mutated the Lys-251 residue to Arg or Gln to ascertain whether differences exist in structural and catalytic properties of TvSHMT relative to its human counterpart.

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or the “large” domain (residues 50 to 300) binds PLG, an analogue of PLP and FFO (5-formyl tetrahydrofolate), an analogue of THF, has most of the active-site residues, and folds into a 7-stranded mixed β-sheet. Finally, the C-terminal domain (residues 301 to 451) folds into an α-β complex. The proposed amino acid residues interacting directly with PLP of TvSHMT are Ser-113, His-142, Asp-222, His-225, and Lys-251. His-145 is found to be hydrogen bonded to Asp-222, which in turn affects the interaction of Asp-222 with PLP (Fig. 5B).

**DISCUSSION**

We have identified and characterized a homolog of SHMT from the eukaryotic pathogen *T. vaginalis*. This protein is encoded by a single gene with an ORF of 1,353 bp and a predicted protein of 451 amino acid residues. The protein is found to be localized to the hydrogenosome (Fig. 2A and B). Our earlier studies (20a) indicate the presence of components of glycine decarboxylase complex in the hydrogenosomes providing the first evidence of amino acid metabolism in this organelle. Both glycine decarboxylase complex and SHMT are vital for the interconversion of glycine and serine (27). Thus, the presence of hydrogenosomal SHMT further supports this new predicted function of this unusual organelle in trichomonads.

SHMT is a highly conserved protein and exists in different isoforms encoded by different genes. In most eukaryotes, both cytosolic and mitochondrial isoforms are found. However, only a cytosolic isoform is found in the parasite *Trypanosoma cruzi* (12). Likewise, only a single gene encoding the cytosolic isoform of the malarial SHMT has been identified (2). On the other hand, the trypanosome *Crithidia fasciculata* has three isoforms found in the mitochondrion, cytosol, and glycosome (11). Cytosolic (SHMT-S) and mitochondrial (SHMT-L) isoforms are also found in the kinetoplastid parasite *Leishmania* (17). We found only one SHMT gene in *T. vaginalis* that encodes a hydrogenosomal protein. The apparent lack of a cytosolic form of SHMT implies that the interconversion of glycine to serine occurs exclusively in hydrogenosomes in the parasite. However, we cannot exclude the possibility that another gene, encoding a cytosolic TvSHMT, is simply missing from the 7× coverage of the *T. vaginalis* genome sequence. If so, the gene is likely to be highly divergent, as only one gene copy is detected in the genome by hybridization analyses (data not shown).

SHMT has been widely studied in many living systems (e.g., bacteria, humans, sheep, kinetoplastids, and plants) with regard to its expression, subunit composition, and structural folding patterns. Mitochondrial SHMTs studied to date are usually found to exist as tetramers. Sucrose density gradient analysis of the hydrogenosomal TvSHMT indicates that the protein exists as a dimer, as is found in *E. coli* and *Bacillus stearothermophilus* (8), which differentiates it from its human counterparts that exist as tetramers.

Structural features that are fundamental to the maintenance of the tetrameric structure of all the mammalian SHMTs include the His-158 residue (Fig. 1, human SHMT) which hydrogen bonds to another His-158 residue to create the second dimeric interface, facilitating the assembly of the symmetric tetrameric quaternary structure. TvSHMT and *E. coli* SHMT (EcSHMT) lack this histidine residue and instead have a glycine that would not facilitate hydrogen bonding between the dimers to form tetramers (23). Other important structural features to the tetramer-stabilizing interactions are two insertions present in the mammalian enzymes. The first insertion is KR ISATSI beginning at residue Arg-293. This insertion is incomplete in both TvSHMT and EcSHMT (Fig. 1). Scarsdale et al. (25) proposed that EcSHMT could not form the

**TABLE 1. Kinetic properties of SHMTwt and K251R**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>0.25 mM PLP</th>
<th>No PLP</th>
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<tbody>
<tr>
<td></td>
<td><em>k</em>&lt;sub&gt;c&lt;/sub&gt;</td>
<td><em>K</em>&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>(mM)</td>
</tr>
<tr>
<td>SHMTwt</td>
<td>0.89</td>
<td>1.3</td>
</tr>
<tr>
<td>K251R</td>
<td>1.16</td>
<td>1.13</td>
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* a The enzymatic assay was performed as described in Materials and Methods. Kinetic parameters were calculated using varying concentrations of THF in the presence of 0.25 mM PLP or in the absence of externally added PLP.
same tetrameric quaternary structure observed for mammalian SHMT because, besides not having a residue equivalent to His-158, it lacks the tetramer-stabilizing interactions. We observed that TvSHMT possesses the first tetramer stabilizing insertion; however, 4 of the 8 amino acid residues are different. The parasite enzyme was also found to lack the second tetramer-stabilizing insertion along with the central histidine residue. These data are consistent with the proposed role of these insertions in tetramer stabilization. Moreover, our experimental evidence together with primary sequence analyses demonstrate that the oligomeric structure of the \textit{T. vaginalis} protein is distinctly different from its counterpart in the human host.

All characterized SHMT contain an 8-amino-acid conserved sequence, VTTTTHKT, containing the active-site lysyl residue (K) that forms the internal aldimine with PLP (Fig. 1) (e.g., Lys-251 in TvSHMT and Lys-229 in EcSHMT). Mutation of this Lys to Arg or Gln in TvSHMT did not alter the oligomeric structure of the proteins (Fig. 3), nonetheless a complete loss of activity was observed in the K251Q mutant. The K251R mutant retained activity comparable to that of the wild-type enzyme in the presence of 0.25 mM PLP (Fig. 4A) but showed a very low activity in the absence of PLP (Fig. 4B; Table 1). These data are consistent with the oligomeric structure of the protein not requiring K251 for stabilization and indicate that the effect on activity when this residue is mutated can be overcome by interaction of other residues with PLP (Fig. 5B). In contrast, mutation of Lys-229 to His or Arg in dimeric \textit{E. coli} recombinant SHMT resulted in the loss of catalytic activity and the inability to bind PLP, except in its free aldehyde form (26). When the EcSHMT Lys-229 was mutated to Gln (K229Q), binding to external aldimine and catalysis of a single turnover was observed. Finally, with regard to differences between the mammalian and \textit{T. vaginalis} enzymes, mutation of the corresponding Lys to Gln or Arg in scSHMT converted the tetrameric enzyme to an inactive dimer with no bound PLP (19). Taken together, these data reveal structural differences between the active sites of mammalian and trichomonad SHMT that might be exploited in drug design to specifically inhibit the parasite enzyme.

Although detailed structural analysis of the parasite protein awaits the resolution of its crystal structure, the identity of the \textit{T. vaginalis} SHMT and initial characterization of its domain structure and active site are first steps toward deciphering differences between the parasite protein and its human counterpart. Moreover, further analyses of the catalytically active form of TvSHMT will facilitate the search for specific inhibitors that may allow the enzyme to be exploited as a potential drug target. Detailed analysis of the structural changes induced in K251Q, resulting in a catalytically inactive enzyme, should also be useful for screening drugs which stabilize its structure, thus reducing the activity of the enzyme.

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