Discussion
The protozoan parasite *Leishmania* spp. is the causative agent of a clinically diverse disease known as leishmaniasis that affects an estimated 12 million people (Desjeux, 2001 and Herwaldt, 1999) and which is currently listed by the World Health Organization as the second most important protozoan disease after malaria in terms of patient mortalities (WHO Fact Sheet No. 116, 2000; WHO Report, 1999 (http://www.who.org).

The role played by the host immune system in healing of these diseases is well established, however, a vaccine for leishmaniasis seems to be far in the future, a more immediate solution would be the development of new antileishmanial chemotherapy. The current scenario for the chemotherapy of leishmaniasis is highly alarming and is limited to handful of drugs with high toxicity that bound to compromise with the existing drugs. Science still has no magic bullet for this disease and many doubt that such a single solution will ever exist. Under this point of view molecular biology techniques and drug discovery must walk together in order to find new targets for chemotherapy intervention.

Recent advancements in the identification and understanding of genome sequence of *Leishmania* parasite have provided new impetus to find novel drug targets. *L. major* genome sequencing showed that its 32.8-megabase haploid genome had 36 chromosomes, which predict 911 RNA genes, 39 pseudogenes, and 8272 protein-coding genes, of which 36% can be ascribed a putative function. The mean length of genes was approximately 2045bp and total G+C content was 57.3 % (Ivens et al, 2005). The availability of the entire genetic content of one *Leishmania* species provides the foundation for the identification and in-depth functional analysis of virulence factors, critical enzymes in key metabolic pathways, and potential vaccine candidates. Beside this it also provide crucial information for the development of new therapies for the leishmaniasis.

As better understanding of novel biochemical pathways would be helpful in the development of new antileishmanial we exploited folate metabolic pathway of *Leishmania*. The folate metabolic pathway has been exploited for the development of antimicrobial and antineoplastic agents successfully, however, this has not been exploited against *Leishmania* and other trypanosomatids.
SHMT catalyzes the interconversion of serine and glycine, with THF serving as the one-carbon acceptor; the latter is required for the biosynthesis of purines, thymidylate, methionine, and formylmethionyl-tRNA in yeast, fungi, plants, and mammals. SHMT activity has been detected in trypanosomatids, like *Trypanosoma cruzi*, *C. fasciculate* and *Leishmania* spp., but the enzyme has not been cloned, over-expressed and purified from these sources. The function of the enzyme in these parasites is still unknown.

As substantial differences with the mammalian host are found, SHMT might become a good target for chemotherapy. The results presented herein represent a first step towards a better understanding of the biochemical, biophysical and structural properties of SHMTs in trypanosomatids. Our findings further support the feasibility of specifically targeting *Leishmania* SHMT through rational drug design.

5.1. Cloning and sequencing of *ldSHMT*

The full length coding sequence of the SHMT gene was amplified by PCR from *L. donovani* genomic DNA using primers designed from *L. major* SHMT sequence annotated in GenBank. An amplicon of 1443 bp PCR fragment was then amplified from *L. donovani* gDNA and sequenced. The nucleotide sequence of *ldSHMT* has been deposited in GenBank under Accession No. AY189738. Translation of *ldSHMT* nucleotide sequence revealed ORF of 1443 bp encoding 480 amino acids with predicted molecular mass of 53,040 Da. *ldSHMT* nucleotide sequences was enriched in G and C residues (61.9%) as for other *Leishmania* gene. The average hydropathy of *ldSHMT* is suggestive of a hydrophilic protein. No signal peptide sequence or trans-membrane domains were observed in putative *ldSHMT*. Multiple sequence alignment of *L. donovani* SHMT with published sequences for other SHMTs showed that a main difference lies on N terminal and C terminal of the protein. At N terminal *ldSHMT* has extension of 25 amino acids in comparison with *Trypanosomal* SHMT, 23 with *Plasmodium* SHMT, 4 with human SHMT, 10 with mouse SHMT, 26 with *B. subtiliss* and *B. stearothermophilus* SHMT and 22 with *E. coli* and
Mycobacterium SHMT. At C terminal Trypanosomal SHMT has 8 amino acid extension where as human, mouse, B stearothermophilus, B. subtilis, E. coli, Mycobacterium and Plasmodium has 4,4,19,21,28,22 and 9 amino acid deletion respectively. Another important feature of all SHMTs is octapeptide having VTTTTHKT is conserved in ldSHMT also. This octapeptide differs in M. tuberculosis and T. cruzi SHMT where threonine is replaced by serine or isoleucine.

5.2. Bioinformatic Analysis of ldSHMT

Elucidation of the 3D structure of enzymes and correlating it to their function is one of the prime aims of modern biologists. In order to understand the structural features of the ldSHMT and compare with crystal structure of hSHMT to find some differences in substrate binding, we first carried out homology modeling of ldSHMT.

The quality of our ldSHMT model has been checked using three different criteria. Our studies suggest that the backbone conformation (PROCHECK), the residue interaction (Prosall) and, the residue contact (WHATIF) are well within the limits established for the reliable structures. These tests suggest that we have obtained a good model for ldSHMT to further characterize its binding site and explore its interactions with the substrate.

Site directed mutagenesis studies on sheep liver SHMT and crystal structure of hSHMT revealed that His134 of sheep SHMT and His135 of hSHMT corresponding to His139 in ldSHMT are involved in tetramer formation. Since this His139 is conserved in ldSHMT and based on previously reported structural information, we predicted that ldSHMT is a tight dimer of identical monomers and these tight dimers are associated more loosely with another dimer forming a tetramer. This was experimentally proved with size exclusion chromatography. Considering that all SHMT are active as dimers and to reduce the computation time we decided to restrict our study to a dimeric structure.

When ldSHMT model was compared with hSHMT we found that the C and N terminal of ldSHMT has an extension of 7 and 10 amino acid residues
respectively. Moreover, in IdSHMT there is a five amino acid deletion (SVDPA) creating an extended loop in hSHMT. Since we were more interested in finding key differences in active sites between hSHMT 3D structure and IdSHMT model, the probable active site was determined on the basis of its alignment to the template and comparing it with previously reported structural information on SHMT's. Analysis of the active site revealed that it has a well-defined pocket at the interface of the two monomers of the dimer and comprised of residues from both monomer A and monomer B.

Further docking of substrates PLG and THF were performed with IdSHMT and hSHMT. Docking of PLG with IdSHMT showed three more interacting residues in comparison hSHMT. Out of these three residues Ser233 is unique in IdSHMT as it is replaced by Alanine in hSHMT at the corresponding position. The OG atom of Ser233 was found to involve in hydrogen bond interaction with N1 atom of PLG, which is not possible in hSHMT due to the presence of methyl group of Ala237.

Unlike PLG binding, which is almost similar in IdSHMT and hSHMT, except for a few important differences, the THF binding differs significantly in both IdSHMT and hSHMT. These significant differences in the binding mode of THF may be attributed to the large size and flexibility of the molecule. The other factors contributing to this result may be the different orientations of the side chains of the THF binding site residues in the two proteins.

5.3. Over-expression and purification of IdSHMT

Leishmania proteins are generally insoluble in nature and tend to form aggregate, i.e., inclusion bodies upon expression in prokaryotic hosts, e.g., adenylate kinase 2 of L. donovani (Villa et al, 2003), methionine adenosyl transferase (MAT 2) of L. donovani (Pertejoy et al, 2003), cysteine protease of L. infantum (Rafati et al, 2003), glucose regulating protein 94 (GRP 94) of L. infantum (Larreta et al, 2003), myristoyl-CoA N-myristoyl transferase of L. major (Price et al, 2003). To get active protein from inclusion bodies is a tedious process and requires lot of laboratory work and time. Many researchers have
tried several conditions to get active protein in the soluble form but the yield is too low to work upon. Thus, the conditions were developed for the efficient expression and single step purification of IdSHMT using pQE60 expression vector. We have tried some vectors having T7 promoters like pET21d and pET30. Though the expression of protein increased in pET21d and pET30 but we were not able to get the protein in soluble fraction. Conditions were standardized to get the protein in soluble form. We were able to achieve the soluble protein only in pQE60 vector having weak T5 promoter, low temperature 25°C, induction with 0.1mM IPTG and 12 hours induction period. We were not able to get high yield more than 0.5 mg/l and concentrated protein of more than 0.25mg/ml. In spite of getting low yield, we were lucky enough to get the IdSHMT in soluble form. In other protozoans like plasmodium, Rathod et al, 2000 were able to over-express the protein but there is no report till date by the same author about the soluble recombinant SHMT protein. Recovery of soluble protein at lower concentration restricted our studies to some extent. This is the first report to achieve soluble and active SHMT protein from any protozoan source.

5.4. Oligomeric properties of IdSHMT

SHMT is present as a dimer in all prokaryotes like E. coli, S. typhimurium, Campylobacter jejuni, B. stearothermophilus (Plamann et al 1983; Urbanowski et al 1984, Chan et al 1991, Trivedi et al, 2002), whereas it occurred as a tetramer in eukaryotes like human, rabbit, sheep, mouse, Zebra fish (Renwick et al 1998; Scarsdale et al 1999; Talvar et al 1997; Szebenyi et al 2000 and Chang et al 2006). Recently, it was shown that the Trypanosoma cruzi SHMT was present as a catalytically active monomer (55 kDa) (Capelluto et al 2000). It has so far not been possible to obtain a fully active dimeric form of the eukaryotic enzyme either by mutation or dissociation. On the other hand, the enzyme from prokaryotes is invariably present as a dimer, although there is some evidence to suggest that a tetrameric form is present in the crystal structure of the ternary complex of eSHMT with Gly and 5-formyl H4-folate (Scarsdale et al 2000) and bsSHMT.
The SDS–PAGE of purified recombinant \textit{ldSHMT} showed a single band with an estimated size of 54 kDa. This compares to the calculated size of 54 kDa based on the amino acid sequence of the recombinant protein. Thus confirming that the monomer of recombinant \textit{ldSHMT} is of 54 kDa. Purified recombinant \textit{ldSHMT} protein was subjected to size exclusion chromatography on Pharmacia Superdex-200. The protein migration was compared with molecular weight markers suggested that \textit{ldSHMT} recombinant protein exist as a tetramer as observed for other eukaryotic SHMTs but differs from \textit{T. cruzi} SHMT which was shown to exist as catalytic active monomer.

5.5. Generation of \textit{ldSHMT} antibodies and its cross reactivity with SHMT from different sources

Till now selective liberation of enzymes contained in different subcellular compartments by detergents like digitonin were done for localization of SHMT. In our studies we tried to localize SHMT in \textit{Leishmania} by immuno-fluorescence microscopy. For this purpose polyclonal \textit{ldSHMT} antibodies were raised against purified recombinant \textit{ldSHMT} protein in female rabbit. The antisera recognized only a single protein of 53 kDa of western blot of crude \textit{L. donovani} lysate and the size of protein matched with the predicted size of the putative SHMT in the \textit{Leishmania}. \textit{ldSHMT} antibodies reacted strongly with \textit{L. major} and UR6 cell lysates but fail to recognize any protein in BHK cells and Plasmodium. This study indicates that antibodies raised against purified recombinant shmt protein are specific to \textit{Leishmania}. Different strains of \textit{Leishmania} contain SHMT of approximately same size and have similar antigenic determinants.

Crossreactivity of \textit{ldSHMT} antibodies were also checked with the purified recombinant SHMT of \textit{L. donovani}, \textit{B. subtilis}, \textit{B. stearothermophilus} and \textit{M. tuberculosis}. \textit{ldSHMT} antibodies only recognized the recombinant \textit{ldSHMT} but failed to react with other recombinant SHMTs of different organisms. The sequence identity of \textit{L. donovani} SHMT with \textit{B. subtilis}, \textit{B. stearothermophilus} and \textit{M. tuberculosis} are 42.1\%, 43.1\% and 37.1\% respectively. This indicates the
IdSHMT not only differs in primary sequence of SHMT of *B. subtilis*, *B. stearothermophilus* and *M. tuberculosis* but has antigenic variations also.

5.6. Localization of IdSHMT

SHMT is distributed mainly in cytosol and mitochondria of yeast, fungi, and mammalian cells (Chen et al, 1997; Shane, 1989 and Schirch, 1982). In some plant cells SHMT is present in chloroplast where as it is also found in glycosomes of *C. fasciculata*. The apparent presence of a SHMT in the glycosomes of *C. fasciculata* suggests that this isoform could be involved in pyrimidine metabolism in the organelle. Since *Crithidia*, *Trypanosoma* and *Leishmania* all are closely related organism it was interesting to see the localization of SHMT in *Leishmania*. Our immuno-fluorescence studies revealed that IdSHMT is present predominantly in the cytoplasm of *L. donovani* promastigotes. However we were not able to detect any isoform of SHMT in mitochondria and glycosomes. Our result is in agreement with *Trypanosoma*, which also had only cytoplasmic isoform but differs from *Crithidia* SHMT. It has been reported that different genes encode different forms of SHMT. *L. major* genome sequence is published and it also indicates that only one SHMT gene is present. However it had also mentioned one more putative sequence of mitochondrial SHMT but it lacks the proper start codon. This sequence needs further validation to see whether this is a functional mitochondrial SHMT gene or pseudogene of SHMT as found in human (Snell et al, 1996).

5.7. Spectroscopic characterization of the recombinant IdSHMT

The secondary and tertiary structures of the recombinant IdSHMT were evaluated by circular dichroism and intrinsic tryptophan fluorescence spectroscopy respectively. The far-UV circular dichroism spectrum of the recombinant IdSHMT was similar to the far-UV-CD spectra reported for other SHMTs. Most of the reported SHMT have characteristic far UV CD spectra of protein having both α-helix and β-sheet secondary structure. IdSHMT shows that
it also contains α-helix and β-sheet secondary structure like other reported SHMTs.

The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic. Most of the emissions are due to excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine. According to the primary aminoacid sequence, IdSHMT has four tryptophan molecules at positions 157, 212, 321 and 358. The buried tryptophan residues in folded protein show fluorescence emission maxima at 330–335 nm, whereas on unfolding of protein the tryptophan fluorescence emission maxima shift to about 350 nm (Angelaccio et al, 1992). The fluorescence spectra IdSHMT showed a peak at 340 nm indicating that tryptophan molecule(s) are not completely buried in the native protein but partially exposed to the solvent. This result is in agreement with mycobacterium SHMT that gives peak at 339 nm in fluorescence spectra due to having partially exposed tryptophan molecules in native protein.

5.8. Enzyme activity

Like most of the SHMTs, IdSHMT showed activity in alkaline pH (capelluto et al, 1999, capelluto et al, 2000). The pH optimum for the IdSHMT was observed as 7.8 and enzyme retained more than 50% of activity at pH 9. The pH optimum was little lower as compared to Crithidia and Trypanosoma the other member to same family, which have 8.2.

Thermal stability of SHMT varies in different organisms. The temperature optimum may be high as 80°C in B. stearothermophilus (Jala et al, 2002) and as low as 40°C in H. methylovorum (Miyazaki et al, 1987), where as 2 temperature optima at 35°C and 55°C are reported for Vigna Radiata (Rao et al, 1982). IdSHMT showed maximum activity at 40°C, where as it exhibited 84% and 85% activity on temperature 37°C and 45°C. IdSHMT was found to be temperature sensitive as compared with sheep SHMT as it looses its activity on 65°C.

The effect of denaturants like urea and GdmCl was observed on IdSHMT by incubating the enzyme at different concentrations of denaturants. IdSHMT
was found to be more sensitive to GdmCl in comparison with urea. The enzyme looses its 81% and 86% activity at concentration of 1M urea and 0.25 M GdmCl respectively. The reason for loosing activity at low concentration of denaturants may be due to removal of PLP from active site. PLP is loosely bound at the active site and on addition of denaturants partial unfolding of the enzyme occurs which might resulted to the release of PLP from active site.

The $K_m$ and $V_{max}$ for the substrate L-serine and THF was higher in \textit{Id}SHMT as observed in other SHMTs but can be comparable. The \textit{Id}SHMT catalyzed the $H_4$- folate dependent retro aldol cleavage of L-serine to form glycine and 5,10-CH$_2$-$H_4$-folate with a specific activity 1.79 U/mg. This value was significantly lower in comparison with other SHMTs (sheep SHMT 4.2 U/mg, \textit{B. subtilus} 6.7 U/mg) but was higher in comparison with mycobacterium SHMT 1.5 U/mg specific activity. When L-serine was used as the variable substrate, the plot was hyperbolic. THF saturation patterns were observed with two concentration of serine (2 mM saturating or 0.2 mM non-saturating) as reported in Crithidia. When varied concentration of THF was used at the non-saturating concentration of serine, THF was found to be inhibitory at the concentration above 1.5 mM. This result was in agreement with Crithidia SHMT, which also showed THF as inhibitor at unsaturated concentration of serine. This result might be explained by the hypothesis that the serine might have two different binding sites in \textit{Id}SHMT. SHMT active site is present at the inter phase of both the dimer. In all the tetrameric SHMT and \textit{Id}SHMT, which are, dimer of dimer had four active sites. As reported in mouse SHMT, all the active sites are not identical, similar case might happen with \textit{Id}SHMT also. All the active sites may differ in affinity for serine, one being saturated at low concentrations of L-serine and the another at higher concentrations of the serine.

The results presented herein represent a first step towards a better understanding of the properties of SHMT in \textit{Leishmania}. \textit{Id}SHMT is the third major enzyme of folate pathway of \textit{Leishmania} which has been characterized after DHFR and TS. The further studies will also help in determining the complexities of folate metabolic pathway in \textit{Leishmania}. These primary studies

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will help in evaluating this enzyme as drug target in *Leishmania*. If substantial difference with human and leishmanial SHMT can be exploited then the availability of leishmanial SHMT in catalytically active form should facilitate the search for antileishmanial agents directed at this enzyme.