Review of Literature
2.1. Folates

Folate and folic acid are forms of a water-soluble vitamin B. Folate occurs naturally in food. Folic acid is the synthetic form of this vitamin that is found in supplements and fortified foods. Folate is a generic name given to several structurally similar compounds that serve as co-factors in a variety of one-carbon transfer reactions. Folates play an essential role in the metabolism of the biosynthesis of purines and pyrimidines for all living organisms; they are therefore directly or indirectly involved in the processes of cell reproduction, in which numerous enzymes that use folates either as cofactor or as substrate are involved. The reduced form of folate can exist as various one-carbon derivatives including 5-methyl-, 10-formyl-, 5,10-methylene-, and 5,10-methenyltetrahydro folate. Folic acid consists of three building blocks: a pterin, p-aminobenzoic acid (pABA) and glutamic acid (Fig. 2.1).

![Fig. 2.1. Structure of folic acid. Folic acid is composed of three building blocks: a pterin moiety, p-aminobenzoic acid (pABA) and glutamic acid (Glu).](image)

Most bacteria, fungi and several parasites such as Plasmodium are capable of synthesizing folates de novo. Organisms that can synthesise folates de novo need to conjugate the pterin to pABA to form dihydropteroate. This is catalysed by the enzyme dihydropteroate synthase. Organisms incapable of folate synthesis, including mammals and Leishmania, rely on folates from the environment and have a need for specific transport systems and receptors to satisfy their folate requirements. Intracellular folates in microorganisms and animal cells occur primarily as polyglutamates with four to six residues (Moran, 1999; Shane, 1989).

Folate metabolism can be broadly divided into two parts. First that leads to the formation of the pteridine nucleus and the second that regards the use of the
already synthesized folate by means of simple functional modification or oxidation/reduction of the preformed pteridine ring. The first part is present in autotrophic microorganisms, such as bacteria and parasites that possess their own enzymes able to synthesize the folate by precursors, while the second part essentially regards mammals that are heterotrophic and need to obtain folic acid from external sources.

![Diagram of folate metabolism]

Fig. 2.2. De novo biosynthesis of pterins and folates.

### 2.2. Folate metabolism

Folate is present in cells as a family of coenzymes that carry one-carbon units and function in both the mitochondrial and cytoplasmic compartments (Appling, 1991; Wagner, 1995; Chen, 1996). Mitochondrial folate metabolism is necessary for the conversion of serine to glycine and formate (a one-carbon unit) (Appling, 1991; Shane, 1995), whereas cytoplasmic folate metabolism utilizes mitochondria-derived formate for the biosynthesis of purines (supplies the #2 and #8 carbons of the purine ring), thymidine (conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and for the
generation of methionine from homocysteine (Appling, 1991). Methionine, in turn, can be converted to S-adenosylmethionine (SAM), a cofactor for many methylation reactions, including the methylation of proteins, phospholipids, neurotransmitters, RNA, and DNA (Selhub, 1999; Clarke et al., 2001). Serine hydroxymethyltransferase (SHMT) catalyzes the reversible transfer of the hydroxymethyl group of serine to tetrahydrofolate (THF) to form methyleneTHF and glycine (Schirch et al., 1989). The enzyme is present in both the mitochondria and cytoplasm (Stover et al., 1997, Girgis et al., 1998). This reaction is a major source of THF-activated one carbon units in mammalian cells (Schirch et al., 1989). Loss of mitochondrial SHMT (mSHMT) function cannot be rescued by the activity of the cytoplasmic SHMT (cSHMT) isozyme in cultured cells; some evidence suggests that cSHMT may be a serine synthase in the cytoplasm (Stover et al., 1997, Pfendner et al., 1980).

Cellular folate derivatives are sequestered by a variety of proteins collectively called folate binding proteins (Wagner, 1995; Yeo, et al., 1999). The cellular concentration of folate binding proteins exceeds that of folate derivatives, and therefore the concentration of free folate in the cell is negligible (Schirch, et al., 1989; Strong, et al., 1990; Suh, et al., 2001). This implies that folate dependent biosynthetic pathways must compete for folate cofactors (Suh, et al., 2001; Scott, et al., 1981). This competition for folate cofactors is most pronounced for reactions that utilize methyleneTHF, a derivative that serves as a cofactor in three known enzymatic reactions in the cytoplasm. It is required for the conversion of dUMP to dTMP, catalyzed by thymidylate synthase (TS); for the conversion of glycine to serine, catalyzed by cSHMT; and for the synthesis of 5-methylTHF, catalyzed by methylenetetrahydrofolate reductase (MTHFR), a reaction that commits one-carbon units to the methionine cycle. Because the MTHFR reaction is virtually irreversible in vivo (Appling, 1991; Wagner, 1995), methionine synthase (MS) activity is essential for recycling 5-methylTHF to other folate cofactor forms. Otherwise, 5-methylTHF accumulates at the expense of all other folate derivatives, impairing folate-dependent deoxyribonucleotide synthesis. This phenomenon is known as the "methyl trap," a state of functional
folate deficiency and impaired DNA synthesis (Scott, et al., 1981). SAM inhibits MTHFR and thereby provides feedback regulation that protects against a folate methyl trap.

2.3. Folate Pathway of *Leishmania donovani*

Although *Leishmania* is probably incapable of carrying out de novo synthesis of folates, they are nonetheless essential cofactors for several enzymes (Fig. 2.3).

![Folate Pathway Diagram](image)

Fig. 2.3. Cellular functions of reduced folates. Folic acids in their reduced forms are involved in the biosynthesis of thymidine, the conversion of serine to glycine, methionine biosynthesis, histidine catabolism, and purine biosynthesis. The first three functions were shown to occur in *Leishmania* (or are likely to occur since the *Leishmania* genome contains the relevant genes), the histidine catabolic pathway has not been studied and purine biosynthesis does not occur in trypanosomatids.
Early studies have suggested that *Leishmania* includes a number of folate metabolizing enzymes including dihydrofolate reductase, thymidylate synthase, serine hydroxymethyltransferase and methylenetetrahydrofolate reductase (Avila and Nosei, 1983; Nosei and Avila, 1985; Scott *et al.*, 1987). When *Leishmania* cells are incubated with [3H] folic acid, it is metabolised and 10-formyltetrahydrofolate and 5-methyltetrahydrofolate are the only intermediates that can be detected (Scott *et al.*, 1987). The main enzymes of folate Pathway of *Leishmania donovani* are as follows

### 2.3.1. Dihydrofolate reductase–thymidylate synthase

Dihydrofolate reductase (DHFR) and thymidylate synthase (TS) in *Leishmania* exist as bifunctional enzyme. The main role of DHFR in thymidine biosynthesis is the reduction of dihydrofolate (DFH) to tetrahydrofolate (THF) using the cofactor NADPH. Following this reduction, THF is methylated to form methylene-tetrahydrofolate (methylene-THF) by SHMT. Methylene-THF then methylates deoxyuridine monophosphate (dUMP) to give TMP in a reaction catalysed by TS. During this reaction, methylene-THF is converted back to DHF, completing the cycle. Therefore, inhibition of DHFR probably leads to build up in levels of dUMP and hence to a biosynthetic precursor, deoxyuridine triphosphate (dUTP). High levels of dUTP lead to incorporation of uracil into DNA to a level beyond which DNA repair enzyme (uracil-DNA-glycosylase) can cope, leading to cell death.

The Crystal structure of bifunctional DHFR-TS indicates that DHFR domain is present at N-terminal where as TS domain is present at C terminal of the enzyme. Both domains are attached with a peptide linker with each other. The native protein is composed of a dimer of two such subunits and is of 110 kDa (Meek *et al.*, 1985). There is substrate channelling between the DHFR-TS domains of the enzyme, by a positively charged electrostatic channel on the protein surface, which interacts with the negatively charged substrate. Detailed kinetic studies (Liang and Anderson, 1998) also support this model and, in addition, interaction between the DHFR-TS domains was also observed. DHFR-
TS null mutants are thymidine auxotrophs (Cruz et al., 1991), confirming that the main role of the protein is in thymidine biosynthesis. It has been suggested that additional enzyme might be associated with TS-DHFR of protozoa (Ferone and Rowland, 1980). However, in L. tropica TS-DHFR and SHMT are readily separable upon MTX-Sepharose chromatography and if an association does exist, it must be weak. Further, SHMT levels are not increased in MTX resistant cells, indicating that the gene that codes for SHMT is not present in the amplified region of DNA that posses the TS-DHFR gene.

2.3.2. Serine hydroxymethyltransferase

Serine hydroxymethyltransferase (SHMT) catalyses the inter conversion of serine and glycine, with tetrahydrofolate serving as the one carbon acceptor (Fig. 2.3.). This activity has been described in a number of trypanosomatid (Capelluto et al., 1999; Nosei and Avila, 1985; Scott et al., 1987). The Leishmania genome project clearly indicates that the corresponding gene is present in Leishmania and shares more than 50% identity with yeast and mammalian SHMT. The SHMT enzyme has been characterized in detail in C. fasciculata and T. cruzi. Three isoforms of SHMT were observed in C. fasciculata (Capelluto et al., 1999) and a single form was detected in epimastigotes of T. cruzi (Capelluto et al., 2000). Crithidia fasciculata subcellular fractionation data suggest that the isoforms of SHMT are located in the cytosol, the mitochondria and possibly the glycosome (Capelluto et al., 1999). In eukaryotes, folate-mediated one-carbon metabolism is compartmentalized between the cytosol and mitochondria (Appling, 1991) but in trypanosomatids especially in Leishmania these studies were not carried in detail. This work is based on the study of SHMT of L. donovani, which has been discussed in detail.

2.3.3. Methionine biosynthesis

The folate cofactor 5,10-methylenetetrahydrofolate produced via SHMT is a substrate for methylenetetrahydrofolate reductase leading to 5-methyltetrahydrofolate (Fig. 2.3.). Methionine synthase catalyses the transfer of a
carbon group from this intermediate to produce methionine (Fig. 2.3). Very little is known about the methionine biosynthetic pathway in trypanosomatids. *Leishmania* genome project has revealed that *Leishmania* have both methylenetetrahydrofolate reductase and methionine synthase homologues. Thus, it is probable that reduced folates are important for methionine biosynthesis in *Leishmania*.

### 2.3.4. Folate transporters

Folate transport in *Leishmania* is known to be regulated with the growth stage of the parasite with high activity in the logarithmic stage of the parasite and residual activity in the stationary phase (Ellenberger, *et al*., 1987; Cunningham, *et al*., 2001). *Leishmania* cycles between sand flies and host macrophages, and through its life cycle, the parasite may encounter environments with variable essential nutrient concentrations. For example, to take advantage of the high folate concentrations in the insect midgut where intensive *Leishmania* replication occurs, the parasite could have evolved a high capacity transporter like FT1. In conditions where the nutrient is scarce, the higher affinity transporter FT5 may play a more prominent role. *Leishmania* have a common folate/methotrexate transporter and mutations in the gene for this transporter lead to methotrexate resistance (Dewes *et al*., 1986; Ellenberger *et al*., 1987 and Kaur *et al*., 1988). Although these cells can now resist antifolates, they must compensate for the lack of folate uptake. *L. tarentolae* methotrexate resistant mutants with profound defects in folate/methotrexate transport did overexpress BT1 through which some folate can be transported (Kündig *et al*., 1999a). Since *Leishmania* is a folate auxotroph, mutants with markedly reduced folate uptake must compensate for this decrease so the parasite can meet its folate requirements. In *Leishmania tarentolae*, the mechanism of compensation involves the over-expression of the biopterin transporter 1 (BT1), which transports folates but not MTX to meet the folate requirements of the cell.
Leishmania has developed transport systems for both biopterin (B) and folic acid (FA) and the appropriate enzymes for their reduction to their bioactive form. The completely reduced forms of biopterin and folic acid, BH4 and THF, are involved in a number of metabolic and cellular functions (Kundig et al., 1999). BT1 is a biopterin transporter (Kundig, et al., 1999; Lemley, et al., 1999) that is part of a family of transmembrane proteins. Fourteen putative proteins belonging to this family are part of the Leishmania genome (www.genedb.org). FT1 activity accounted for more than 75% of all folate transport in Leishmania. The remaining transport was possibly due to FT5 or other BT1 family transporters. FT5 has shown that it corresponds to a high affinity but relatively low capacity folate transporter.

2.4. Serine hydroxymethyltransferase

SHMT, a member of the α-class of the PLP-dependent enzyme, is ubiquitous for generating one carbon fragment for the synthesis of nucleotides, methionine, thymidylate, choline etc (Rao et al., 2003). Increased activity coupled with enhanced DNA synthesis in neoplastic tissues has suggested SHMT as a possible target for cancer chemotherapy (Volm, 1998). SHMT has been isolated from a variety of sources and the properties of the enzyme have been reviewed (Appaji Rao, et al., 2000). The enzyme from prokaryotic sources is a dimer (Schirch et al., 1985), whereas the eukaryotic SHMT exists as a tetramer (Appaji Rao et al., 2003). The reasons for the evolution of the same enzyme as a
tetramer in higher organisms and dimer in prokaryotes are unknown. The isolated SHMT are in the form of homodimers or of homo-tetramers in which each monomer weighs 45-54 kDa. Although the secondary and tertiary structures are similar to other PLP-dependent enzymes, the amino acid sequences show very little similarity (Usha et al., 1994). SHMT catalyzes to some extent all of the reactions in this group, such as hydroxymethyltransferase, transamination, decarboxylation and racemization. Apart from physiological reaction (H$_4$-Folate dependent hydroxymethyl transfer from L-Ser), SHMT catalyzes the aldol-cleavage of a number of β-hydroxy amino acids such as L-Thr and L-alloThr, (Malkin et al., 1964) L-threo and L-erythro-β-phenyl-serine (Ulevitch et al., 1977) and β-hydroxyvaline (Webb, 1995).

2.4.1. SHMT --- a member of PLP dependent enzymes

SHMT is a PLP dependent enzyme. The main characteristics of these enzymes are as follows

2.4.1.1. PLP dependent enzymes

Pyridoxal-5-phosphate (PLP) is a versatile cofactor able to catalyze a spectrum of reactions on a variety of amino acid substrates but there are some other PLP-dependent enzymes like glycogen phosphorylase that also acts on other substrate, such as glycogen. Two broad classes include those enzymes, which break a bond only on the α-carbon of the amino acid substrate (transamination, decarboxylation, racemization) and enzymes, which catalyze elimination or replacement reactions on either β- or γ-carbons.

2.4.1.2. Classification of PLP dependent enzymes

Based on sequence comparison, family profile analysis and profile analysis combined with available structural information, PLP enzymes have been categorized into four different families (Mehta and Christen, 2000). These four classes have a unique fold, but these folds are indeed unrelated to each other.
2.4.1.2.1. \(\alpha\)-Family enzymes

The \(\alpha\)-family is the largest and functionally most diverse family. All crystal structures of \(\alpha\)-enzymes that have been determined, share a similar fold I. It is a typical \(\alpha\beta\) type of structure. Majority of \(\alpha\)-class of enzymes show specific common functional features. In the reaction they catalyze, the covalency changes are limited to carbon atom (C\(\alpha\)), which is involved in imine bond formation with PLP. However, this is not a universal rule. The important exception is SHMT, which catalyzes reactions at C\(\alpha\)-C\(\beta\) bond. Other examples are \(\alpha\) -lyases, which catalyzes the covalency changes extend from C\(\alpha\)-C\(\beta\). It has been suggested that this divergence may have occurred prior to the establishment of substrate specificity in these enzymes (Mehta and Christen, 2000).

2.4.1.2.2. \(\beta\)-Family enzymes

The members of this family are structurally similar to each other. The enzymes of this family are \(\alpha_2\beta_2\) proteins. Tryptophan synthase is the prototype enzyme of this family. InTryptophan synthase \(\alpha_2\beta_2\) complex is a dimer of two folds related (\(\alpha\beta\)) pairs, in which \(\beta\)-subunit lies back to back. The \(\alpha\)-subunit is a Tim barrel enzyme, \(\beta\)-subunit is a PLP enzyme, catalyzing the \(\beta\)-replacement. All \(\beta\)-family enzymes share a conspicuous functional feature, as they are lyases, catalyzing reactions in which not only C\(\alpha\) but also C\(\beta\) participates in the covalency changes. Threonine synthase is an exception as it catalyzes \(\beta\gamma\) -replacement reactions.

2.4.1.2.3. D-Alanine aminotransferase family enzymes

The D-alanine aminotransferase family, named after its prototype enzyme, represents a third independent evolutionary lineage. These enzymes have a unique fold III with both \(\alpha/\beta\) and \(\alpha+\beta\) feature. These enzymes mostly exist as dimer. The unique feature of the active site is that the A face of the cofactor is solvent exposed, whereas in the \(\alpha\) and \(\beta\) family enzymes, the B face is exposed. Apart from D-alanine amino transferase, this family consists of two additional
enzymes, that is, branched chain amino acid aminotransferase and 4-amino-4-deoxychorismate lyase, which are acting on L-amino acids.

2.4.1.2.4. Alanine racemase family enzymes

The alanine racemase family is a small family. The enzymes of the alanine racemase family are $\beta/\alpha_8$ proteins. The enzymes of the alanine racemase family are the only PLP enzymes whose fold is related to that of non-B6 proteins such as Ornithine decarboxylase. Family profile analysis (FPA) also indicates that many other $\beta/\alpha_8$ barrel proteins might be evolutionarily related with each other (Wilmanns et al., 1991; Mehta et al., 1999).

2.4.2. Importance of SHMT in cellular pathway

Folate derivatives are not thought to be transported across the mitochondrial membrane to any significant degree (Cybulski et al., 1981; Barlowe et al., 1989; Horne et al., 1989). Transport of one-carbon units between mitochondria and cytoplasm occurs via one-carbon donors such as serine, glycine, or formate (Pasternack et al., 1992; Chappell et al., 1967; Cybulski et al., 1976; Cybulski et al., 1977). Consistent with these metabolite transport constraints, eukaryotic cells contain distinct SHMT isozymes in both the cytoplasm and mitochondria (Schirch, 1977; Chasin et al., 1974; Garrow et al., 1993; McNeil et al., 1994). The existence of isozymes in separate compartments and the reversible nature of the reaction raise questions regarding the roles of each isozyme in providing serine, glycine, and one-carbon units for the cell.

The role of SHMT isozymes depends upon the nutritional requirement of the cell. Due to this difference the role and the activity of SHMT differs from cell to cell and organism to organism. SHMT knock out studies are carried out in some organisms to find the dependence of cell towards different isoforms of SHMT. For example, mammalian cells with active cSHMT, but lacking mSHMT, are glycine auxotrophs (Chasin et al., 1974), implicating mitochondrial SHMT as the primary route of glycine synthesis. Bacterial strains deficient in SHMT and CHO cells defective in mitochondrial SHMT exhibit an auxotrophy for glycine,
while Neurospora strains lacking cytoplasmic SHMT require formate for growth. Surprisingly, yeast mutants with both SHMT genes inactivated do not require glycine for normal growth (McNeil et al., 1994) indicating the existence of an alternative pathway for glycine synthesis. Mutation at a third gene, designated GLY1, was required to render the double SHMT-disrupted strain completely auxotrophic for glycine. The GLY1 gene was recently shown to encode a protein with threonine aldolase activity (Liu et al., 1997; Monschau et al., 1997) suggesting threonine as the source of glycine in this pathway. The GLY1-dependent pathway, rather than SHMT, appears to be the primary glycine source in yeast (McNeil et al., 1994).

SHMT isoforms were also reported from chloroplast and glycosomes. But not much work has been carried out. The presence of a SHMT in the glycosomes of C. fasciculata suggests that this isoform could be involved in pyrimidine metabolism in the organelle. In summary, the roles of the SHMT isozymes change as the nutritional requirements of the cell changes.

### 2.4.3. Catalytic properties of SHMT

The major physiological reaction of SHMT involves the retro-aldol cleavage of L-serine to glycine in the presence of 5,6,7,8-tetrahydrofolate (H₄-folate) converting it to 5,10-methylene H₄-folate. SHMT also catalyzes non-physiological reactions, such as transamination, racemization and decarboxylation.

**Table 2.1: Substrates cleaved by a retro-aldol mechanism by SHMT**

(Schirch, 1998)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ser + H₄-folate</td>
<td>Gly + 5, 10-CH₂- H₄-folate</td>
</tr>
<tr>
<td>α-Methyl-L-Ser + H₄-folate</td>
<td>D-Ala + 5, 10-CH₂- H₄-folate</td>
</tr>
<tr>
<td>L-alloThr</td>
<td>Gly + Acetaldehyde</td>
</tr>
<tr>
<td>L-Thr</td>
<td>Gly + Acetaldehyde</td>
</tr>
<tr>
<td>L-erythroβ-Phenyl Ser</td>
<td>Gly + Benzaldehyde</td>
</tr>
<tr>
<td>L-threoβ-Phenyl Ser</td>
<td>Gly + Benzaldehyde</td>
</tr>
<tr>
<td>β-Hydroxyvaline</td>
<td>Gly + Acetone</td>
</tr>
</tbody>
</table>
In addition, SHMT catalyzes other reactions with amino acids, such reactions include transamination, racemization and decarboxylation (Table 2.2).

**Table 2.2: Some of the alternative reactions catalyzed by SHMT**

(Schirch, 1998)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Ala + PLP</td>
<td>Pyruvate + ammonia + PMP</td>
</tr>
<tr>
<td>L-Ala + PLP</td>
<td>Pyruvate + ammonia + PMP</td>
</tr>
<tr>
<td>L-Ala</td>
<td>D-Ala</td>
</tr>
<tr>
<td>Aminomalonate</td>
<td>Gly + CO₂</td>
</tr>
<tr>
<td>Gly + oxidized lipoate</td>
<td>NH₂CH₂-lipoate + CO₂</td>
</tr>
</tbody>
</table>

**2.4.4. Possible mechanism of catalysis**

Several mechanisms were proposed for the physiological reaction catalyzed by SHMT (Dunathan, 1966) The most favoured mechanism involves the retro-aldol cleavage of L-Ser (Scarsdale et al., 1999) but some groups also proposed another mechanism known as direct displacement method (Trivedi et al., 2002). Recent observation by Szebenyi et al., suggested that a combination of direct displacement and retroaldol cleavage could be more probable mechanism (Szebenyi et al., 2004).

**2.4.4.1. Mechanism I**

This mechanism was originally proposed by Jordan and Akthar (Jordan, et al., 1970) and was strongly supported by the observation of substantial racemization of the transferred carbon (Fig.4.5.). This mechanism is attractive because it involves an aldol cleavage, entirely analogous to the aldol cleavages seen with other β-hydroxyamino acid substrates. In the retro-aldol cleavage of L-ser, starting with serine aldime as the external aldime. The first step is the cleavage of the α-β bond of serine, to generate the formaldehyde and quinonoid intermediate. Addition of a proton to α-carbon of the amino acid in this quinoid intermediate forms the external Aldemine of glycine. The formaldehyde is
Fig. 4.5. Mechanism I of SHMT catalysis involves the retro-aldol mechanism. Serine is depicted as forming a Schiff base imine with the pyridoxal phosphate cofactor. Free formaldehyde is liberated by general-base-catalyzed aldol cleavage and trapped by one of two possible isomers of H4 folate, which are related by nitrogen inversion transferred to THF to form methyl-THF and the glycine aldemine is concerted to glycine, returning the enzyme to its original state internal aldemine (PLP attached with lysine).

2.4.4.2. Mechanism II

The first step is proposed to be a direct, reversible and nucleophilic attack by N5 of tetrahydrofolate at the β-carbon of the serine aldimine with the loss of water. The intermediate formed is directly comparable to the characterized intermediate in the transfer of a methylene group from CH2-H4-folate to dUMP by
Figure 4.6. Mechanism II of SHMT catalysis involving direct displacement. Serine is depicted as forming a Schiff base imine but N5 of H4-folate is proposed function as a nucleophile to displaces a water in a reversible reaction. The covalently coupled intermediate is resolved by anti elimination of glycine quinoid aldime, and 5-iminium cation so generated cyclizes to form CH2-H4-folate. The quinonoid is reprotonated to give the glycine Schiff base form of the enzyme.

thymidylate synthase. Since free formaldehyde is not an intermediate, the reaction proceeds with complete stereospecificity, provided that aldol cleavage of the serine aldime is not kinetically competitive, and the enzyme would not be expected to catalyze the condensation of H4-folate with formaldehyde. Lack of precedence for such nucleophilic dehydration is one major reservation about such mechanism. Direct displacement mechanism explain the catalysis mechanism in few SHMT crystal but this mechanism does not address the folate independent cleavage of allothreonine and β-phenyl serine, which is presumed to proceed via the retroaldol mechanism.

One more mechanism are also proposed for catalysis i.e thiohemiacetal (Matthews et al., 1990) mechanism1. However, absence of a cysteine residue at
the active site of the enzyme ruled out this reaction mechanism (Scarsdale et al., 2000 and Trivedi et al., 2002).

2.4.5. Functional group at the Active site of SHMT

Elucidation of the crystal structure of enzymes and correlating it to their function is one of the prime aims of modern biologists. Identifying the conserved amino acid residues with a functional group from the known sequences of SHMT and analyzing the 3D structure of the enzymes in detail have unraveled the structure–function relationships in SHMT up to some extent. There are various conserved amino acids residues, which play significant role in catalysis and maintaining the secondary/tertiary structure of protein. The mutational analysis of SHMT has sorted out the possible role of these amino acids.

The residue numbers indicated in the table is according to sheep liver cytosolic SHMT (scSHMT).

Table 2.3. : Amino acid residues important for the structure of SHMT

<table>
<thead>
<tr>
<th>Residues</th>
<th>Possible role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y73</td>
<td>PLP binding</td>
<td>(Krishna, 2002)</td>
</tr>
<tr>
<td>E74</td>
<td>‘OPEN’ to ‘CLOSED’ structural form</td>
<td>(Krishna, 2000)</td>
</tr>
<tr>
<td>R80</td>
<td>inter subunit interaction</td>
<td>(Krishna, 2002)</td>
</tr>
<tr>
<td>Y82</td>
<td>stabilizing quinoid iontermediate</td>
<td>(Krishna, 2000)</td>
</tr>
<tr>
<td>D89</td>
<td>inter subunit interaction</td>
<td>(Krishna, 1999)</td>
</tr>
<tr>
<td>R98</td>
<td>enzyme folding</td>
<td>(Krishna, 2002)</td>
</tr>
<tr>
<td>W110</td>
<td>maintenance of oligomeric structure</td>
<td>(Krishna, 2002)</td>
</tr>
<tr>
<td>H134</td>
<td>inter subunit interaction</td>
<td>(Jagath, 1997)</td>
</tr>
<tr>
<td>H147</td>
<td>cofactor binding/ stacking interaction</td>
<td>(Jagath, 1997)</td>
</tr>
<tr>
<td>D227</td>
<td>PLP interaction</td>
<td>(Krishna, 2002)</td>
</tr>
<tr>
<td>H230</td>
<td>enhance proton abstraction</td>
<td>(Talwar, 200)</td>
</tr>
<tr>
<td>K256</td>
<td>PLP binding/ maintenance of oligomeric structure</td>
<td>(Talwar, 1997)</td>
</tr>
</tbody>
</table>
R 262 distal interactions with PLP (Krishna, 2002)
P297 imparting substrate specificity (Talwar, 2000)
H 304 inter subunit interaction (Krishna, 2000)
H356 charge relay (Krishna, 2000)
R401 substrate binding (Jagath, 1997)

2.4.5.1. Amino acids involved in maintenance of oligomeric structure

Mutation of D89 in scSHMT weakened the subunit interactions resulting in the formation of mixture of dimers and tetramers. However, at a higher concentration of PLP, a fully active tetramer could be formed (Krishna, 1999). Though mutation of D89 is deleterious but interaction of PLP at the interface of the tight dimer overcomes the destabilizing effect of mutation. The mutation of R80 that interacts with D89 resulted in inactive dimer with complete loss of PLP (Krishna, 2002). Since R80 is having lot of crucial interactions in it's vicinity and being on β-sheet2, it is stabilizing the α-helix4 by these interactions. Therefore, it is not surprising that the mutation of R80A had resulted in a dislocation of multiple interactions, thereby leading to the formation of inactive dimer with loss of PLP (Krishna, 2002).

R98 is conserved in all eukaryotic SHMTs and in 21 out of 28 prokaryotic sequences. The corresponding residue R99 in hcSHMT is located at α-helix 4 and interacts with D30 (located at α-helix 2) of the neighboring subunit (across the subunits of the tight dimer). The mutation of R98 to A in scSHMT led to the disruption of interactions at the tight dimer interface, which was probably deleterious for the folding of the enzyme (Krishna, 2002).

H304 is conserved in eukaryotes but not in prokaryotic organisms, suggesting a possible role for the residue in the evolution of the tetrameric enzyme. H305 (H304 in scSHMT) is positioned to interact with S119 (S118 in scSHMT) from a neighboring subunit and is in H-bonding distance with phosphate group of PLP. Mutation of this residue to 'A' in scSHMT resulted in the formation of inactive dimers emphasizing the importance of inter subunit
interactions in maintaining the tetrameric structure of scSHMT. H134 is another residue, which is conserved only amongst the eukaryotic SHMTs. Mutation of this H134 residue to N resulted in the formation of loose dimers which retained 50% activity, suggesting that interaction of the loose dimers may not be as critical as in the tight dimer for catalysis (Jagath, 1997). Apart from the residues discussed above, the N-terminal arm and PLP stabilizes the quaternary structure in tetrameric SHMTs (Jagath, 1997).

2.4.5.2. Residues involved in catalysis

2.4.5.2.1. Lysine Residues

All PLP enzymes appear to have the coenzyme bound to the $\varepsilon$-amino group of a lysyl residue as an internal aldimine. This Lys has been changed to another amino acid by site-directed mutagenesis in several PLP enzymes (Bhavani et al., 2005; Bhatia et al., 1993; Lu et al., 1993; Ziak et al., 1993; Ilag and Jahn, 1992; Yoshimura et al., 1992; Grimm et al., 1992; Toney and Kirsch, 1991, 1992; Nishimura et al., 1991; Planas and Kirsch, 1991; Smith et al., 1989). In most cases, the mutant enzymes contained low observable catalytic activity.

In aspartate aminotransferase, considerable evidence indicates that the active site Lys plays a dual role in the mechanism of the reaction (Toney and Kirsch, 1991). First, it is the base that accepts and donates the proton in the interconversion of the aldimine and ketimine intermediates. Second, it is required to expel the product amino acid from the external aldimine intermediate by forming an internal aldimine. The mutational studies in E. coli SHMT suggest that Lys-229 is not the base that removes the proton but only required to expel the product amino acid from active site. Similar results were also observed with other SHMTs indicating that this residue is crucial for product expulsion.

2.4.5.2.2. Histidine Residues

The histidine residues are conserved in prokaryotic and eukaryotic SHMTs (Usha et al., 1994) and were suggested to be essential for catalysis. Mutation of H147 affected cofactor binding/stacking interactions while H150 was indirectly
involved in proton abstraction (Jagath, 1997). The crystal structure of bsSHMT in the external aldimine complex suggests that the H122 (H147 in scSHMT) also has a role in substrate binding and might function as a base for proton abstraction (Trivedi et al., 2002). H230 is directly linked to O3 of PLP and facilitates the proton abstraction step (Talwar et al., 2000 a). Interestingly, a change in reaction specificity from a hydroxymethyl transfer to NADH oxidase reaction occurred when H230 was mutated to Y (Talwar et al., 2000 b).

2.4.5.2.3. Aspartic Acid Residues

D228, which is H bonded to the pyridinium N is conserved not only in SHMT but also in all fold type I PLP-dependent enzymes (Grishin et al., 1995). It is, therefore, not surprising that mutation of this residue (D227 in scSHMT) to N in scSHMT has serious consequences on the oligomeric structure of the tetrameric enzyme and catalytic activity (Jagath, 1997). The negative charge of D227 stabilizes the positive charge of N1 of PLP and thereby enhances the function of PLP as an electron sink. This is a key feature in the function of all PLP-dependent enzymes irrespective of the reaction catalyzed. It is likely that D227 in scSHMT has a similar function. It could be postulated that the mutation of the corresponding residue in prokaryotic enzymes that would not result in loss of oligomeric structure would establish its role in catalysis.

2.4.5.2.4. Tryptophan Residues

Chemical modification studies had indicated that Tryptophan (W) residues were essential for the activity. W110 is located in the hydrophobic pocket, surrounded by the hydrophobic residues and is conserved in all eukaryotic enzymes. When Trp. is mutated to Ala. resulted in the improper folding of the enzyme due to disruption of the hydrophobic core. However, when it was mutated to Phe. the mutant enzyme was present in soluble form as tetramer and was catalytically active.
2.4.5.2.5. Threonine Residues

In addition to the conserved histidine preceding the active site lysine, another unique feature of the sequence is the presence of a conserved stretch of T residues (224–T–T–T–H–K–T–230) around the active site lysine. Attempts at assigning a specific function for these threonine residues have not been successful. An interesting observation with the T226A mutant of eSHMT was that the formation of the geminal diamine was faster and the formation of the external aldimine became a rate-determining step (Angelaccio et al., 1992). These results were interpreted to suggest a function for this residue in an early step in catalysis although the effects on the overall rate were not dramatic.

2.5. 3-D structures of SHMT

The first SHMT structure was solved from human (hcSHMT) in 1998 by S.B. Renwick and from that date many structures of SHMT were solved from different sources. For example human liver cytosolic recombinant SHMT (HcSHMT) (Renwick et al., 1998), rabbit liver cytosolic recombinant SHMT (RcSHMT) (Scarsdale et al., 1999), E. coli SHMT (EcSHMT) (Scarsdale et al., 2000) and murine cytoplasmic SHMT (McSHMT) (Szebenyi et al., 2000) and Rabbit cytosolic SHMT in complex with FTHF (Scarsdale et al., 2003). These structures have enabled us a more critical examination of the role of specific amino acid residues in the different steps of catalysis.

2.5.1. Human liver cytosolic SHMT

The monomer of the enzyme bears considerable fold similarity to other PLP enzymes of the α-class. The monomer is composed of three domains; the N-terminal, the large domain (53-321) and the small domain (322-480) and a C-terminal domain. The N-terminal region, which folds into 2 α helices and 1 β strand, is involved in inter subunit interactions and is crucial for strengthening the overall tetrameric structure. The large domain is comprised of 9 α helices and 7 β sheets in antiparallel arrangement and has the PLP binding domain. The C-terminal small domain folds into a αβ sandwich. Small stretches of amino acids
143-146, 175-180, 204-207 and 354-359 mediate interdomain contacts. The monomers interact with each other to different extent to form the final tetrameric structure. The tetramer is actually a dimer of dimers. The dimers A-D and B-C overlap considerably, thus forming very strong contacts with each other and are aptly called "tight dimers". The "loose dimers" made by the overlap between A-B and C-D subunits and are not held very strongly. The crystal structure indicates that PLP is present at the interface of tight dimers and the active site is made up of residues from two different subunits of the tetrameric enzyme (Renwick et al., 1998).

2.5.2. Rabbit liver cytosolic SHMT

The structure of Rabbit cytosolic SHMT consists of tight dimers of identical monomers and these tight dimers are associated more loosely with another dimer forming a tetramer. The structure of Rabbit cytosolic SHMT is described in two forms; one with the PLP covalently bound as an aldimine to the Nε-amino group of the active site lysine and second with the aldimine reduced to a secondary amine. This structure for the first time explains hydrogen bonding between K229 and T226 which is responsible for formation of gem-diamine intermediate, absorbing at 343 nm and the reaction specificity of the enzyme (Schirch et al., 1991).

2.5.3. Ternary complex of E. coli SHMT

The crystal structure of a ternary complex of E. coli SHMT was analysed with bound glycine product (substrate) and the stable H₄PteGlu₈ analogue. In this structure, the glycine product/substrate is covalently linked to the C4’ carbon atom of the PLP ring as either the external aldimine or quinoid intermediate of the SHMT reaction pathway. Crystal structure of E. coli suggests that the rcSHMT tetramer plays a role in binding the extended, polyglutamylated folates, which is absent in the dimeric eSHMT under physiological in vitro conditions. This distinction may be related to different charge surface requirements for binding the chemically distinct polyglutamate tails of folates. In eukaryotes, the polyglutamate
tail is entirely γ-linked, while in *E. coli*, only the first three glutamates are γ-linked, subsequent ones being α-linked (Scarsdale *et al.*, 2000). The tetrameric quaternary structure of liganded *E. coli* SHMT also differs in symmetry and relative disposition of the functional tight dimers from that of the unliganded eukaryotic enzymes. SHMT tetramers have surface charge distributions that suggest distinctions in folate binding between eukaryotic and *E. coli* enzymes.

### 2.5.4. Ternary complex of Murine SHMT

The structure of McSHMT permitted identification of amino acid residues involved in substrate binding and catalysis, although the structure was determined in the presence of Gly and 5-formyl H$_4$-folate, a tight binding inhibitor. One important conclusion drawn from the structure determined in the presence of Gly and 5-formyl H$_4$-folate was negative co-operativity observed in the interactions and that only two of the four catalytic sites are catalytically competent. The most notable feature of the McSHMT ternary complex is lack of structural symmetry and the corresponding difference in substrate binding between the obligate dimers. One of the dimer binds folate tightly and the other dimer binds the folate either more loosely or not at all (Szebenyi *et al.*, 2000).

### 2.6. Inhibitors of SHMT

The study of specific irreversible inhibition of SHMT has attracted considerable attention because of the potential therapeutic value of these inhibitors and also in view of their usefulness as probes to study the physiological role of this enzyme.

#### 2.6.1. D-cycloserine

D-Cycloserine (D-4-amino-3-isoxazolidone (DCS) (Sammon, 1999), which is a cyclic structural analogue of D-alanine (D-Ala) and is produced by *Streptomyces garyphalus* and *Streptomyces lavendulae*, is a clinical medicine for the treatment of tuberculosis. The antibiotic is an effective anti-mycobacterial agent (Zygmunt, 1963). The antibiotic inhibits the enzymatic activities of both Ala
racemase (ALR,) and D-Ala-D-Ala ligase. These enzymes are expressed ubiquitously in bacteria and are indispensable because both D-Ala synthesis and the linkage of two D-Ala molecules are essential for peptidoglycan synthesis in the bacterial cell wall.

Although very effective against Mycobacterium tuberculosis, it is seldom employed in the management of this infection due to its high toxicity. The antibiotic is an effective anti-mycobacterial agent, but it is rarely prescribed and is used only in combined therapies because of its serious side effects (Heifets and Iseman, 1991).

\[
E + DCS \xrightarrow{k_1} E-DCS \xrightarrow{k_2} E-DCS^* \xrightarrow{k_3} E' + DCS-PLP
\]

D-Cycloserine initially binds rapidly and irreversibly to the enzyme to form an enzyme D-cycloserine complex (E-DCS). This step can be reversed neither by dilution of the reaction mixture nor by addition of excess substrate, L-serine. This step of interaction of D-cycloserine with SHMT differs from its interaction with other PLP –enzymes such as aspartate-glutamate aminotransferase. (Khomutov et al., 1963, Karpeiskii et al., 1964) and cystathionase (Brown et al., 1969). In the second step a PLP-D-cycloserine Schiff’s base complex is formed resulting into complete loss of enzyme activity (E-DCS*). The final step results in an almost complete dissociation of a PLP-D-cycloserine Schiff’s base complex from the active site, resulting in formation of apoenzyme.

2.6.2. Mimosine

Mimosine is a naturally occurring rare amino acid derivative isolated from Leucaena seeds. It is an iron/zinc chelator. It is an extremely effective inhibitor of DNA replication in mammalian cells that may act by preventing the formation of replication forks (Lalande, 1990). Some studies indicate that mimosine prevents the initiation of DNA replication, whereas other studies indicate that mimosine disrupts elongation of the replication fork by impairing deoxyribonucleotide
synthesis by inhibiting the activity of the iron-dependent enzyme ribonucleotide reductase and the transcription of the cytoplasmic SHMT genes (Oppenheim et al., 2000). Studies in human SHMT indicate that it is a zinc-inducible gene. A mimosine-responsive transcriptional element was localized within the first 50 base pairs of the human SHMT1.

Inhibition of serine hydroxymethyltransferase is moderated by a zinc responsive unit located in front of the SHMT gene. A mimosine responsive transcriptional element was localized within the first 50 base pairs of the human SHMT1. MRE elements are present in the promoters of zinc-activated genes and are bound by the transcription factor MTF-1. Mimosine inhibits MRE binding activity leading to low transcription of the SHMT gene (Lin et al., 1996).

2.6.3. Aminooxy compounds

O-amino-D-serine (OADS) is a hydrolytic product of DCS and also an aminooxy analogue of serine. Studies with sheep liver SHMT indicates that OADS is a reversible noncompetitive inhibitor when the serine was varied substrate. The kinetic studies showed that OADS interacts with the enzyme rapidly and reversibly by disrupting the enzyme-lysine-PLP Schiff's base to form PLP. In second step PLP-OADS oxime gets formed, followed by third step where PLP-OADS oxime is released from the enzyme, and apoenzyme formed could be partially reactivated by PLP (Baskaran et al., 1989a).

Other aminooxy compounds such as aminooxyacetate (AAA) and L-canaline as well as the parent compound hydroxylamine inhibits the SHMT activity. Aminooxy compounds that are structural analogues of serine formed PLP as an intermediate prior to the formation of oxime, but hydroxylamine, which did not have much structural similarity, formed PLP oxime without any intermediate (Baskaran et al., 1989a).

2.6.4. Thiosemicarbazide

Thiosemicarbazide (TSC) is a slow binding inhibitor of sheep liver SHMT. Its slow binding with the enzyme results in the formation of a hitherto undetected
intermediate. This intermediate has not been detected in interaction of TSC with other PLP dependent enzymes. The intermediate was converted very slowly to the final products, apoenzyme and the thiosemicarbazone of PLP (Acharya et al., 1992).

2.6.5. 4-Chloro-L-threonine

4-Chloro-L-threonine is a serine analogue. It is substrate for SHMT and it is cleaved to form chloroacetaldehyde and glycine. 4-Chloro-L-threonine inactivates SHMT in a time and concentration dependent manner. 4-Chloro-L-threonine undergoes aldol cleavage and generation of chloroacetaldehyde at the active site of the enzyme results in inactivation. Serine or glycine can protect the inactivation but while THF does not.

Further work needs to be carried out to evaluate the potential of these compounds as a drug and more studies in crystal structures, molecular modeling is required for the development of new inhibitors which can be targeted to inhibit SHMT (Webb et al., 1995).

2.7. Scope of the study

Control of leishmaniasis is very difficult and challenging. Despite impressive advances in science, technology and medicine, we have until now not been successful in allocating sufficient resources to fight this dreadful disease that particularly affects the poor. Although drug management in leishmaniasis has evolved rapidly and with success, but obstacles continue to limit the impact of these advances in regions of endemcity (Murray, 2001). Lack of affordable new drugs, still a basic unsolved problem, has been joined by additional therapeutic obstacles including large scale resistance to pentavalent antimony (SbV) in India and coinfection with human immunodeficiency virus in all endemic regions. Available treatment options have actually expanded and includes successful application of less expensive generic SbV; rediscovery of the high level efficacy of amphotericin B; implementation of short course parenteral regimens (lipid formulations of amphotericin B); potential to replace SbV and
amphotericin B with price capped paromyomycin; and identification of the first effective oral agent, miltefosine. How to sustain and move this progress ahead remain difficult next steps in the treatment of leishmaniasis. There is an urgent need for more selective and efficacious drugs, for that matter, identification of potential drug targets for anti-leishmanial therapy that are unique to the parasite.

The folate metabolic pathway has been exploited successfully for the development of antimicrobial and antineoplastic agents. Inhibitors of this pathway, however, are not useful against *Leishmania* and other trypanosomatids. Work on the mechanism of methotrexate resistance in *Leishmania* has dramatically increased our understanding of folate and pterin metabolism in this organism. The metabolic and cellular functions of the reduced form of folates and pterins are beginning to be established. Moreover, the currently sequencing efforts on trypanosomatid genomes had suggested the presence of several gene products that are likely to require folates and pterins. A number of the properties of folate and pterin metabolism are unique suggesting that these pathways are valid and worthwhile targets for drug development.