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Mouse thymidylate synthase: High-level expression and protein engineering

Zhang, Haichao, Ph.D.
The Ohio State University, 1990
MOUSE THYMIDYLATE SYNTHASE:
HIGH LEVEL EXPRESSION AND PROTEIN ENGINEERING

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Haichao Zhang, B.S.

*****

The Ohio State University

1990

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Biochemistry
To my mother
ACKNOWLEDGEMENTS

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PUBLICATIONS

Site-directed Mutagenesis of Mouse Thymidylate Synthase: Alteration of Arg-44 to Val-44 in A Conserved Loop Guarding the Active Site Has Striking Effects on Catalysis and Nucleotide Binding.


Efficient Synthesis of Mouse Thymidylate Synthase in Escherichia coli.


FIELDS OF STUDY

Major field: Biochemistry
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<th>Full Form</th>
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<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>dUMP</td>
<td>2'-deoxyuridine 5'-monophosphate</td>
</tr>
<tr>
<td>FdUMP</td>
<td>5-fluoro-2'-deoxyuridine 5'-monophosphate</td>
</tr>
<tr>
<td>H2folate</td>
<td>dihydrofolate</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxylamine</td>
</tr>
<tr>
<td>CH2H4folate</td>
<td>N5,10-methylenetetrahydrofolate</td>
</tr>
<tr>
<td>oligo</td>
<td>oligodeoxyribonucleotide</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>H4folate</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
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LISTS OF PLASMIDS

Parental plasmids

pBluescribe M13 (+) or (-): general cloning vector or ss DNA producing vector (Stratagene).

pBluescript M13 (pBST) (+) or (-): general cloning vector or ss DNA producing vector (Stratagene).

pET-3a: expression vector (provided by Dr. Studier).

pKK223-3: expression vector (Pharmacia).

pUC8/pUC18: general cloning vectors (NEB).

Constructs—expression vectors

pKTS: TS cDNA was cloned into pKK223-3.

pUTS: the expression cassette from pKTS (PvuI/SalI) was cloned into pUC8.

pKR44V: same as pKTS except that the arg 44 was replaced by a val in the coding region.

pETS: TS gene was cloned into pET-3a.

pETSM: same as pETS except that the second codon CUG was changed to CUU.

pETSMU: the expression cassette from pETSM (PvuI/SalI) was cloned into pUC8.

pETSMMM: same as pETSM except that the EcoRV fragment in the vector was deleted.
Constructs—ss DNA producing vectors (for site-directed mutagenesis)

pRegionI: Region I [PstI (+27) to Apa I (+380)] covering the N-terminal region of the TS coding region was cloned to pBST(+).

pRegionII: Region II [ApaI (+380) to PstI (+714)] covering the middle part of TS coding region was cloned into pBST(+).

pC-Term: the Clal in the C-terminal region of TS to Clal in the expression vector from pETSM was cloned into pBST(-). The orientation was such so that the noncoding strand was produced (the ATG strand is named a coding strand).

pBTSM: the expression cassette (Sall/EcoRV) from pETSM was cloned into pBST(-). The possibility of using this construct as an expression vector was investigated. It was found that bacteria harboring the plasmid grow more slowly than those with pETSM. Thus, this plasmid was not used as an expression vector but rather as a ss DNA producing vector in which the whole TS coding region was present.

Constructs with mutants

Plasmid with a particular mutant was named after the wild type plasmid except TS in the wild type vector was replaced by the specific mutant. For example, mutant R44V or R44Val in pETSM was named pER44VM or pER44ValM; mutant R44V or R44Val in pETSMM was named pER44VMM or pER44ValMM.
Chapter I
Structural and Mechanistic Aspects of Thymidylate Synthase

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP, Figure 1-1a) to thymidylate (Figure 1-1c) with concomitant conversion of N5,10-methylenetetrahydrofolate (CH$_2$H$_4$folate or cofactor, Figure 1-1i) into dihydrofolate (Figure 1-1h). This enzyme catalyzes the only reaction which provides the de novo biosynthesis of thymidylate, a necessary precursor for DNA biosynthesis. Thus, a cell will not survive if thymidylate synthase is inactive. For this reason, thymidylate synthase has become one of the target enzymes in cancer chemotherapy. Another unique property of TS is that it is the only enzyme which uses a folate cofactor and catalyzes the oxidation of tetrahydrofolate during the one carbon transfer. Due to these unique properties, TS has been subjected to extensive and numerous investigations. It is therefore not surprising that the knowledge about the enzyme has been well documented and reviewed (Lewis and Dunlap, 1981; Heidelberger et al., 1983; Santi and Danenberg, 1984; Cisneros et al., 1988). Here, only some of the well established facts will be reviewed and the recent progress in the enzymatic research will be discussed.
Figure 1-1. Structures of some molecules.

(a), 2'-deoxyuridylate (dUMP);
(b), 5-fluoro-2'-deoxyuridylate (FdUMP);
(c), thymidylate (same as dUMP except there is a methyl group in C-5 instead of a hydrogen atom);
(d), Friedkin intermediate;
(e), structure of inhibitory ternary complex in native enzyme;
(f), pteroyl acid (parental compound for all folate derivatives);
(g), folic acid or folate;
(h), 7,8-dihydrofolate;
(i), N5,10-methylenetetrahydrofolate (CH₂H₄folate, cofactor);
Figure 1-1
Figure 1-1
**Enzyme sources**

Since thymidylate synthase is the enzyme that catalyzes the *de novo* synthesis of thymidylate, it is generally believed to be ubiquitous to all wild type organisms. Because TS provides thymidylate for DNA biosynthesis, it is not surprising that genes encoding thymidylate synthases are expressed at a higher level in proliferating cells than in quiescent cells (Navalgund et al., 1980; Storms et al., 1984; Ayusawa et al., 1986). The low level of TS protein present in organisms made it very difficult to study the enzyme properties until the development of amethopterin (Dunlap et al., 1971) and dichloromeihotrexate (Crusberg et al., 1970) resistant *L. casei* strains. These strains overproduce TS proteins up to 400-fold as compared with the sensitive strain. This breakthrough led to a large increase in the number of investigations into the structure and function of the enzyme, resulting in most of our current concepts about the bacterial TS enzymes. However, the in-depth studies of mammalian TS proteins have been frustrated due to poor sources of TS, even with drug resistant mammalian cell lines which overproduce TS up to 100-fold (Rossana et al., 1982; Berger et al., 1985; Jackman et al., 1986). Recently, the development of molecular cloning and expression systems has led to the expression of bacterial or phage TS genes in a variety of hosts, especially the easily cultured bacterial host (Belfort et al., 1983a; 1983b; Pinter et al., 1988; Climie and Santi, 1990).
With the help of affinity chromatography, TS has been purified from a variety of sources including _L. casei_ (Dunlap et al., 1971), _E.coli_ (Haertle et al., 1979), _S.faecium_ (Rao and Kisliuk, 1983), _P. carinii_ (Edman et al., 1989), T2-phage (Galivan et al., 1974), T-4 phage (Belfort et al., 1983b), _S. cerevisiae_ (Bisson and Thorner, 1981), mouse liver (Priest et al., 1981), regenerating rat liver (Nakata et al., 1987), Ehrlich ascites carcinoma cells (Jastreboff et al., 1982), pig and calf thymus (Gupta and Meldrum, 1972; Horinishi and Greenberg, 1972), pig liver (Lu et al., 1984), chick embryo (Lorenson et al., 1967), mouse cell lines (Rossana et al., 1982; Shimizu et al., 1985; Rode et al., 1979), and human cell lines (Lockshin et al., 1979b; Dolnick and Cheng, 1977; Rode et al., 1980). In protozoan sources, it has been shown that TS exists in a bifunctional protein form, which is associated with DHFR (Ivanetich and Santi, 1990). The bifunctional protein has been purified from _C. fasciculata_ (Ferone and Roland, 1980), _L.tropica_ (Coderre et al., 1983), and blue algae (Bachmann and Follmann, 1987).

**Affinity chromatography**

Since affinity chromatography has the ability to specifically bind a desired target protein, it is widely used to purify proteins when specific ligands of a protein can be coupled to a resin. The specific interaction between a protein and an affinity resin makes purification from low abundance sources possible. Before the development of expression systems,
the small amount of TS present in an organism was purified with the help of affinity chromatography. Based on the properties of affinity ligands developed so far, the affinity chromatography for TS purification can be divided into two groups, nucleotide based, and folate based. The first group includes dUMP (Danenberg et al., 1972), 5'-fluoro-2'-dUMP (Whitley et al., 1974), UDP-glucuronate (Nakata et al., 1987), and poly U (Haertle et al., 1979). TS binds the affinity resin and thus is retained in the column in the presence of low salt or no salt and is eluted from the column by washing with high salt solutions. These affinity resins lack specificity, and have a lower binding capacity. As special examples of this group, phosphocellulose (Bisson and Thorner, 1981; Gupta and Meldrum, 1972) and Cibacron blue-agarose (Bisson and Thorner, 1981; Nakata et al., 1987; Bachmann and Follmann, 1987) were used to purify TS.

The ligands for the folate based group includes methotrexate (Dolnick and Cheng, 1977), tetrahydromethotrexate (Slavik et al., 1976), N-10-formyldeideazafolate (Rode et al., 1979), N-10-methyldeideazafolate (Priest et al., 1981), Na-[pteroyl(tetraglutamyl)]-lysine (Plante et al., 1978), and 10-formylfolate (Banerjee et al., 1982). The principle involved in this group is believed to be that in the presence of dUMP, TS is able to form a pseudoternary complex with dUMP and the folate derivatives coupled to the affinity resins. TS is dissociated from the pseudoternary complex under some conditions (for example, high salt solution without dUMP). However, some studies suggest that hydrophobic interactions between TS and the linker
used to link the folate derivative to the affinity resin may play some roles (Slavik et al., 1976). Several lines of evidence have suggested that the interaction is not necessarily covalent since some mutant forms of TS that are known to be incapable of forming the covalent complexes bind the affinity resin (Dev et al., 1988).

**Methodology**

I. Activity measurements

1. Spectrophotometric method. In the presence of both substrates, dUMP and cofactor, TS catalyzes the formation of thymidylate and the concomitant formation of dihydrofolate. Dihydrofolate has an absorbance at 340 nm and can be monitored spectrophotometrically (Wahba and Friedkin, 1962, Dunlap et al., 1971). This method is very quick and the most convenient, but its sensitivity is limited by the molar extinction coefficient of dihydrofolate (E = 6400).

2. Tritium release assay. It has been observed that with the formation of thymidylate, the release of tritium from [5-\(^3\)H]dUMP into water is quantitative. Based on this principle, two sensitive methods were developed. After incubation of enzyme with the radiolabeled dUMP and cofactor for a certain time, the unreacted tritium labeled dUMP is separated from the tritium labeled water by allowing dUMP to adsorb onto charcoal or by evaporation (Roberts, 1966; Lomax and Greenberg, 1967). Then, the tritium labeled water is
counted in a scintillation counter and the activity is calculated. The tritium release method is by far the most sensitive one. However, due to isotope effects and some unknown factors, the activity detected by the tritium release method, is generally 30-50% of that determined by the spectrophotometric method (Shapiro et al., 1965).

II. Stoichiometry studies

Since TS can form complexes with nucleotide, dUMP (the substrate), or 5'-fluoro-2'-dUMP, an analog of dUMP and a mechanism based inhibitor (FdUMP, Figure 1-1b) and the cofactors (folate derivatives), binding stoichiometry studies are important to understand the enzyme mechanisms. The term "binary complex" generally refers to a TS-nucleotide complex, while the term "inhibitory ternary complex" refers to the TS-FdUMP-cofactor complex. The principle used to determine the stoichiometry is based on the interaction between TS protein and a radiolabeled ligand under proper conditions. The bound protein is then separated from the free ligand by a variety of methods as described below and the binding ratio is determined by the molar ratio of the ligands bound to the amount of protein.

1. Gel filtration (Santi et al., 1974b). The free ligand is separated from the bound complex by a gel filtration process in which free ligands move faster than the complexes. The binding ratio is determined by measuring the radioactivity associated with the protein.
2. Nitrocellulose filter assay (Santi et al., 1974a; Lockshin et al., 1979). The complexes and free ligands are separated by adsorption of protein (and the complexes) to nitrocellulose filters.

3. Charcoal assay (Moran et al., 1979; Spears et al., 1982). Similarly, the unbound free ligands are adsorbed by charcoal, and the charcoal is then separated from the bound complex by centrifugation. However, the adsorption time is very critical due to the shift of equilibrium of bound and free ligand.

4. Equilibrium Dialysis (Galivan et al., 1976b). At equilibrium, the concentrations of free ligands on both sides of a semipermeable membrane are the same. Thus the concentration of bound ligands are calculated by determining the total radioactivity in the sample channel, and subtracting the radioactivity due to the free ligand.

5. Centrifugal column chromatography (Priest et al., 1980a). Ternary complexes pass through a gel filtration column faster than the uncomplexed protein and other small molecules under the centrifugal forces.

6. TCA assay. Recently, a TCA procedure has been developed to rapidly denature the covalent protein-ligand complexes. The complexes are collected on a nitrocellulose paper (Moore et al., 1984; Ahmed et al., 1985; Moore et al., 1986a; 1986b) and the free ligands are washed away with TCA. Alternatively, the complexes are also collected by centrifugation and solubilized by ethanolic NaOH and transferred into a scintillation vial (Cisneros and Dunlap, 1990).
7. Native gel electrophoresis (Aull et al., 1974b). When the inhibitory ternary complexes of TS protein, FdUMP, cofactor are subjected to native gel electrophoresis, three bands are observed-- Form I (FdUMP:Cofactor:TS 0:0:1), Form II (FdUMP:Cofactor:TS 1:1:1), and Form III (FdUMP:Cofactor:TS 2:2:1). By calculating the relative percentage of each band, a binding ratio can be obtained.

All of the methods mentioned above, except the TCA and the TCA with filter assays, are used to determine the total binding ratio, including both covalent and noncovalent. Noncovalent binding of FdUMP and cofactor has recently been shown to survive the gel electrophoresis method.

III Immunoassay

The assay methods described above require the enzyme to be fully active. This requirement is not met in several cases, for example, in the up and down regulation of the enzyme activity (Greenwood et al., 1986), site-directed mutants with decreased or abolished enzyme activity (Dev et al., 1988) and proteolytic cleavage of the C-terminal amino acid (Aull et al., 1974a). In order to accurately estimate the presence of TS protein, an immunoassay has been developed for mouse TS (Jehn, 1985, Ph.D thesis, The Ohio State University). Recently, an ELISA reaction has been developed for L. casei TS. About 1.4 femoles of TS from L.casei can be detected by this method (Tolleson, personal communication).
**General properties of TS**

By studying the properties of thymidylate synthase from different sources, some general properties have been observed.

I. Primary structure

TS consists of two identical monomers. The molecular weight of the whole molecule is in the range of 60-75 kD. The primary amino acid sequences of several TS proteins have been determined by either DNA sequencing or by direct chemical methods (Maley et al., 1979a). When the known amino acid sequences are compared, all TS proteins show remarkable homology (Perryman et al., 1986; Deng, Ph.D thesis, The Ohio State University). Shown in Figure 1-2, is part of a comparison of amino acid sequences of the folate and nucleotide binding sites and the C-terminus. Clearly, they are highly conserved. The mouse and human sequences share a 90% overall identity. The two substrate binding sites and the C-terminal region are almost identical. TS protein purified from mouse also shows that the N-terminal amino acid is blocked (Perryman et al., 1986). The functions of the modified N-terminus, if any, are not known.
Figure 1-2. Partial amino acid sequence comparison of TS from different sources.

Sequence information is obtained from the following sources: Mouse (Perryman et al., 1986), human (Takeishi et al., 1985), yeast (Taylor et al., 1987), L.c (L. casei, Maley et al., 1979a), E.c (E. coli, Belfort et al., 1983a), B.s (B. subtilis, Iwakura et al., 1988), T4 (Chu et al., 1984), 3T (B. subtilis thy P3 phage, Kenny et al., 1985), VZV (Varicella zoster virus, Thompson et al., 1987), P.c (P. carinii, Edman et al., 1989), L.m (L. major, Beverley et al., 1986), P.f (P. falciparum, Bzik et al., 1987), L.t (L. tropica, Grumont et al., 1986), H.s (H. saimiri, Honess et al., 1986), C.f. (C. fasciculata, Hughes et al., 1989).
**Folate binding site**

Mouse

Human

Yeast

L.c

E.c

B.s

T4

3T

VZV

P.c

L.m

P.f

L.t

H.s

C.f

**dUMP binding site**

Mouse

Human

Yeast

L.c

E.c

B.s

T4

3T

VZV

P.c

L.m

P.f

L.t

H.s

C.f

**G-terminal**

Mouse

Human

Yeast

L.c

E.c

B.s

T4

3T

VZV

P.c

L.m

P.f

L.t

H.s

C.f

Figure 1-2
II. Interaction with other proteins in vivo

It has been shown that the TS protein, dihydrofolate reductase, and dihydropteroylhexaglutamate in T-even bacteriophages are associated with each other in the phage base plate. The protein complexes are believed to be involved in deoxyribonucleotide biosynthesis (Reddy and Matthews, 1978). Similar complexes involved in DNA biosynthesis have been isolated from mammalian cells and demonstrated to have the capability to perform DNA synthesis in vitro (Reddy, 1982). These aggregates are sometimes called replittases and are believed to function only in the period of DNA synthesis (Reddy and Pardee, 1980). Among many other enzymes, TS is one of the components. Some evidence suggests that TS protein is involved in the protein-protein interaction since inhibitors of other enzymes can successfully inhibit the function of TS only in the S-phase (Reddy and Pardee, 1983).

Recently, it has been observed that FdUrd, a TS inhibitor, could not inhibit the TS activity in a cell line with an elevated TS activity due to the resistance to folate inhibitors regardless of the concentration of FdUrd used (Danenberg and Danenberg, 1989). This observation further supports the idea that TS may be compartmentalized or in a complex form which is not accessible to the inhibitor.

In some protozoans, TS exists in a form of a bifunctional protein which contains the DHFR protein domain in the N-terminus and the TS domain in the C-terminus (Ferone and Roland, 1980; Bachmann and Follmann, 1987;
Beverley et al., 1986; Grumont et al., 1986; Ivanetich and Santi, 1990). The nature of these bifunctional proteins is poorly understood, although it is hypothesized that the bifunctional protein may stimulate the overall enzymatic catalytic efficiency due to an internal channeling of product/substrate movements (Ivanetich and Santi, 1990).

III. Structural information

The primary structures of TS from a variety of sources shows that the protein sequences are highly conserved. The secondary structure of L. casei TS has been predicted by several methods and shown to be about 23-41% $\alpha$-helix, and 17-33% $\beta$-sheet structure (Manavalan et al., 1986). When CD spectra of L. casei TS were analyzed, 33% of $\alpha$-helix, 25% $\beta$-sheet, 20% turns, and 16% other structures were predicted (Manavalan et al., 1986). Although large amounts of pure TS protein could be easily purified from bacterial sources, attempts to determine the X-ray structure have failed until recently. Now, the 3-D structures of native L. casei TS (Hardy et al., 1987; Figure 1-3A) and E. coli TS (Matthews et al., 1989) have been determined. The most impressive feature of the TS ternary structure is that each monomer has five stranded $\beta$-sheets. The back side of the sheet primarily consists of hydrophobic side chains, and is folded face to face against the corresponding side of the sheet in the second monomer to form the subunit-
Figure 1-3. X-ray structures of TS and TS ternary complex.

A. X-ray structure of the native L._casei_ TS (from Hardy et al., 1986). The nucleotide binding site is outlined in red. The folate binding site is outlined in blue. The C-terminus is outlined in yellow.

B. X-ray structure of the _E.coli_ TS complexed with FdUMP and PDDF (from Matthews et al., 1990).

C. Detailed hydrogen bonding network formed among the C-terminus, the conserved arginine, and the substrate analogues in the _E.coli_ complexed TS (from Matthews et al., 1990).
Figure 1-3C
subunit interaction (Matthews et al., 1989). Another interesting structural feature is that the nucleotide binding pocket is a large funnel-shaped cleft which is extended into the interior of each subunit. Recently, both the x-ray structures of *E. coli* TS protein, with the substrate analogues FdUMP and 10-propargyl-5,8-dideazafolate (PDDF) or cofactor substrate CH$_2$H$_4$folate, have been solved (Matthews et al., 1990; Matthews, personal communication; Figure 1-3B). Comparison of the structures of the native TS and the complexed TS revealed that there is a remarkable structural change after the substrate analogs bind to the enzyme (Matthews et al., 1990). This will be further discussed later.

**Mechanistic Aspects of Thymidylate Synthase**

I. Amino Acids Involved in Catalysis

1. Active site residue. Numerous studies in the past have strongly suggested the involvement of cysteine residue(s) in the active site(s), although the number of the cysteine residues varies from 0.8 to 1.8 (see Lewis and Dunlap, 1981 for a review). Direct evidence to show that cysteine residues are involved in the binding of nucleotides came from the trapping of the covalent catalytic ternary complex of dUMP-cofactor-TS. Peptide sequencing suggested that cysteine residues were covalently attached to the catalytic complex (Moore et al., 1986b). Recently, by using site directed mutagenesis, replacement of the active site cysteine-146 in *E. coli* TS with an Ala
completely inactivated the enzymes (Dev et al., 1988). The X-ray crystal 
structure of *E. coli* TS, with both of its substrate analogues, confirmed that 
the side chain of cysteine-146 is covalently linked to the nucleotide substrate 
(Matthews et al., 1990). It has also been inferred from the 3-D structure that 
at least 8 conserved residues in *E. coli* TS control the precise orientation of 
the active site by forming hydrogen bonds (Matthews et al., 1989). The 
precise orientation of the active cysteine residue in *L. casei* TS is also 
determined by precise hydrogen bonds between the backbone carbonyl 
group and the HN group of residue of 218, and between the side chain of the 
cysteine residue and the side chains of highly conserved His-199 or Asn-229 
(Hardy et al., 1987).

2. Other amino acids involved in catalysis. By chemical modifications and 
other chemical methods, the following residues have been identified or 
inferred to have some roles in catalysis or substrate binding.

a. Arginine residue(s). TS from *L. casei* can be inactivated by arginine 
specific reagents (Cipollo and Dunlap, 1978; 1979; Belfort et al., 1980), 
although the number of arginine residues involved is not clear. From the X-
ray structure of *L. casei* TS, two arginine residues, one from each subunit, 
are within a reasonable distance (5.5A) of the active site thiolate anion of 
Cys-198, and were proposed to function as the cationic locus for the 5'-
phosphate of dUMP (Hardy et al., 1987). Arg-218 in *L. casei* TS (Arg-166 in 
*E. coli* TS and Arg-209 in mouse TS) forms a hydrogen bond or an ion pair 
with the catalytic thiol of the active cysteine. The interaction may lower the
pK\textsubscript{a} and thus enhance the nucleophilicity for attacking at the 6-position of dUMP. The third closest arginine residue Arg-21 in \textit{E.coli}, which is in a conserved loop guarding the active site cleft, was thought to be too far away to be involved. However, this conserved Arg in \textit{E. coli} TS forms hydrogen bonds with the α-carboxylate of the C-terminal amino acid residue and the phosphate of the nucleotide substrate analog (Matthews et al., 1990; Figure 1-3C), thus, providing evidence of the importance of this arginine in catalysis.

b. Tyrosine residue(s). A tyrosine residue(s) was first revealed to be involved in catalysis or in maintaining overall structural features of the enzyme by chemical modification studies (Rosson et al., 1980). The loss of enzyme activity correlated with the modification of two tyrosine residues per dimer when the tyrosine specific reagents were used. Although the roles of these residues are not clear, it was hypothesized that the tyrosine residues may function as a general base either to abstract the proton from C-5 of the pyrimidine ring when the ternary complex undergoes breakdown or to activate the sulfhydryl group of the active site cysteine residues to enhance its nucleophilicity before formation of covalent complex between the enzyme and dUMP (Hardy et al., 1987).

c. Histidine residues. When a His specific reagent was used to modify the protein, most of the histidine residues reacted and the activity was lost (Daron and Aull, 1981). This was the first evidence to shown that histidine residues may play a structural role rather than a specific functional role. However, when all of known TS protein sequences are compared (Figure 1-
2), the nucleotide active site Pro-Pro-Cys-His is very conserved. This conservation suggests that the histidine residue in the active site may be important. From the X-ray structure of *L. casei* TS, it was found that the side chain of histidine-199 is linked to the two sides of the substrate binding site by forming hydrogen bonds with Asn-229 and Tyr-146, and it is proposed that His-199 might function as a base that assists in the removal of the weakly acidic proton at C-5 of 2'-deoxyuridylate while it was part of a ternary complex with the enzyme and cofactor (Hardy et al., 1987). Site-directed mutagenesis in which this highly conserved residue was changed to other residues suggested that this residue is not absolutely necessary for the activity but is involved in an initial catalytic step and alters the pK_a value of a catalytically important Lys or Arg residue (Dev et al., 1989; Climie and Santi, 1990).

d. C-terminal valine residue. It has been shown that carboxypeptidases, for example, Carboxypeptidase C, inactivate TS from *L. casei* even when only one valine per dimer is released (Aull et al., 1974a). The inactivated TS still retains its ability to bind dUMP (Galivan et al., 1977), but it no longer binds pteroylfolylpolyglutamate (Galivan et al., 1976a; Galivan et al., 1977) unless in the presence of dUMP or FdUMP, and the ability to form covalent binary and ternary complex is reduced by a factor of 2 compared with the wild type enzyme (Cisneros and Dunlap, personal communication). The importance of the C-terminal residue is further appreciated when the X-ray structures of TS in complex with FdUMP and PDDF or cofactor were solved. Once the
substrates are bound, the four C-terminal residues close down on the top of
the active site and sequester the bound ligands from bulk solvent by forming
hydrogen bond networks with Arg 21 of *E. coli* TS (Matthews et al., 1990;
Figure 1-3C).

e. Lysine residues. By using the folylpolyglutamyl derivatives of cofactor
as the substrates for TS, it was concluded that the folylpolyglutamyl forms of
cofactor are considerably better substrates (Kisliuk et al., 1974; 1981;
Matthews et al., 1987). This observation is supported by the fact that (1),
without dUMP, folylpolyglutamyl forms can bind to the enzyme (Galivan et al.,
1976a), (2), folylpolyglutamyl forms can successfully compete at the binding
site with the monoglutamyl form; (3), folylpolyglutamyl forms, with up to 5 Glu
residues, form much tighter complexes with the enzyme than the monoform
of folate (Priest and Mangum, 1981). Early studies also suggested the
presence of separate nucleotide and folate binding sites (Galivan et al.,
1976a). Thus, based on the above facts, it was suggested that the folate
binding site may contain several positively charged residues, for example,
lysine and/or arginine residues. By using the folylpolyglutamyl form of
cofactor, the folate binding site has been identified and shown to have 3
lysine residues which could have electrostatic interactions with the carboxyl
anions of the pteroylheptaglutamate (Maley et al., 1982, Figure 1-2 for the
sequences of the binding site of folate).

f. Other amino acid residues. Although there is no direct evidence to
suggest that any other amino acid residues may play a direct role in
catalysis, there is indirect evidence to suggest that some conserved residues are very likely involved. Some of the residues have been considered to be functional from the X-ray structure. For example, the conserved proline and histidine residues in the active site sequence of Pro-Pro-Cys-His. The Asn-229 and Gln-217 residues of L.casei TS may form a H-bonds to O-4 and N-3 of dUMP (Hardy et al., 1987).

II. Order of substrate binding and product releasing

TS catalyzes the conversion of dUMP (with a $K_m$ about 1-10 $\mu$M) and cofactor (with a $K_m$ about 10-50 $\mu$M) into thymidylate and dihydrofolate. All studies suggest that the reaction follows an ordered Bi-Bi mechanism. Early studies using TS enzymes from S. faecalis (Blakley, 1963) and Ehrlich ascites (Reyes and Heidelberg, 1965) suggested that TS binds cofactor first, then binds dUMP. However, the experimental designs of these studies have been questioned (Danenberg and Lockshin, 1982). Carefully designed experiments using TS from bacterial (Daron and Aull, 1978; Danenberg and Danenberg, 1978), yeast (Bisson and Thorner, 1981) and mammalian (Dolnick and Cheng, 1977; Lu et al., 1984, Radparvar et al., 1988) sources established that the reaction is ordered and that dUMP binds first, cofactor binds second, whereas the product dihydrofolate releases before thymidylate.

However, the intracellular folates have been shown to exist as the folylpolyglutamyl derivatives. Thus, further studies were performed on the order of substrate bindings. Confusing results were obtained regarding the
number of glutamyl residues required to reverse the binding order. Results obtained by using the partially purified TS from fetal pig liver show that use of folylpolyglutamyl substrates with four glutamyl residues led to the reversal in the order of substrate binding and product release (Lu et al., 1984), whereas, studies using the TS from human colon adenocarcinoma revealed that use of folylpolyglutamyl substrates did not lead to a change in the order of substrate binding or product release (Radparvar et al., 1988).

III. Detailed mechanism: Biochemistry of substrate-TS complexes

Back in 1959, Friedkin proposed a reaction pathway based on the early observations that (a) cofactor serves as both a one carbon donor and a reductant in the dTMP biosynthesis and (b) tritium labeled in the 6 position of folate cofactor is quantitatively transferred to the methyl group of dTMP. This pathway basically consists of two consecutive reaction parts: the first half requires the condensation of dUMP and cofactor to form a so-called Friedkin intermediate, 5-thymidylyl-tetrahydrofolate (Figure 1-1d), in which dUMP retains its double bond between C-5 and C-6 and is linked to N-5 position of the folate via a methylene bridge through C5 of the pyrimidine ring. The intermediate breaks down to produce the final two products. Extensive studies using model compounds have confirmed the accuracy of this pathway, with different intermediates in which dUMP is also covalently linked to thymidylate synthase (see Lewis and Dunlap, 1981 for a review).
With the development of new techniques, and increased understanding in other areas concerning TS, especially the establishment of the ordered bi-bi mechanism and the observation that FdUMP serves as a mechanism-based inhibitor (see later), much knowledge has been accumulated and the original pathway has been greatly expanded.

1. The binary complex

The order of the bi-bi mechanism requires that nucleotide binds the enzyme to form a binary complex as the first step. However, the research in this area resulted in some controversies. Studies using a variety of methods detected the presence of binary complexes (for a review, see Lewis and Dunlap, 1981) and it is generally believed that only one site per dimer bound dUMP to form the TS-dUMP complex (Leary et al., 1975; Galivan et al., 1976b; Beaudette et al., 1980), while studies using other methods such as the charcoal assay (Moran et al., 1979), gel filtration (Donato et al., 1976) and gel electrophoresis (Aull et al., 1974b) have failed to detect the presence of the binary complex. This conflict may be explained by the survivability of the binary complex upon application of different methods. Another controversy is about the nature of the binary complex: is it covalent or noncovalent? The filter binding assay experiments performed in some laboratories failed to detect the binary complex (Santi et al., 1974a; Galivan et al., 1974). Based on these observation it has been explicitly stated that the presence of cofactor was a prerequisite for the covalent attachment of nucleotide to the enzyme (Danenberg, 1977; Moran et al., 1979). However, $^{19}$F-NMR studies have
suggested that both covalent and noncovalent binary complexes exist in equilibrium in the enzyme-FdUMP solution (Lewis et al., 1981a). Similar results were obtained from the stopped flow method (Mittelstaedt and Schimerlik, 1986). Recently, the TCA method has been used to trap any covalent intermediate if present (Moore et al., 1984; Ahmed et al., 1985; Cisneros and Dunlap, 1990). With this method, the nucleotide binary complex has been detected from a variety of sources of TS with covalent binding stoichiometry for dUMP, about 0.3, and for FdUMP, about 0.6-0.8.

From X-ray studies of bacterial enzymes, it is believed that the funnel-shaped active site of TS is in an open conformation for the binding of nucleotides while the folate binding site is not open. It has been argued that some folates which bind the enzyme in the absence of nucleotide must bind in other locations (Matthews, personal communication). It is also clear that from the X-ray structures of bacterial enzymes and TCA trapping of the catalytic ternary complex and the inhibitory ternary complex, the nucleotide is covalently linked to the sulfhydryl group of the active site cysteine through C-6.

By study of model compounds and X-ray studies, a mechanism that leads to the formation of binary complex has been proposed. In the native protein, the position of the active site cysteine is maintained by hydrogen bond networks in the active site cleft (Hardy et al., 1986; Matthews et al., 1989; 1990). The nucleophilicity of the sulfhydryl group of the active site cysteine is believed to be enhanced by a general base in the active site,
possibly by the invariant Arg 166 in *E. coli*. The positively charged guanidinium group may electrostatically stabilize the anionic form of the thiol group. When the nucleotide binds, the anionic form of the thiol group attacks the C6 of the pyrimidine ring, making the C5 a stronger nucleophile in the form of an enolate anion which is further stabilized by hydrogen bonding with a water molecule and the carboxamide of Asn-177 of *E. coli* TS. It is interesting to note that the guanidinium group of the Arg-166 also forms a hydrogen bond with the phosphate group of the nucleotide. Thus, the nucleophilicity of the thiol group is also modulated by the nucleotide (Hardy et al., 1986; Matthews et al., 1990). It has also been noticed that the binding of nucleotide is accompanied by some conformational change (Leary et al., 1975; Lewis et al., 1981b; Mittelstaedt and Schimerlik, 1986, Santi et al., 1974b), which is thought to be involved in the opening of the folate binding sites.

2. The role of nucleotide-cofactor-TS ternary complex

A. Catalytic dUMP-cofactor-TS ternary complex

The binding of dUMP opens the binding site for cofactor. The binding of the cofactor triggers the reaction to progress so fast that the intermediates involved can not be monitored except by using chemical quenching. TCA has been used to quench the reaction and the catalytic ternary complex has been isolated and studied. These studies show that the dUMP is covalently bound to the cysteine-198 (Moore et al., 1986b). The difficulties in the studies of the catalytic ternary complex led the researcher to study the FdUMP-cofactor-TS
complex, which is stable, and in some cases, can be studied by handling the sample manually.

B. FdUMP-cofactor-TS ternary complex

a. Formation. Once the FdUMP is bound in the active site, the pyrimidine ring, which is believed to be parallel to the interface of subunit contact, provides a docking surface for the folate. The folate binding process is a very complicated process in which major conformational change is involved. First, the glutamate tail of the folate binds to the folate binding site in the opening of the active site while the tetrahydropyrazine-imidazolidine fused ring is on the top of the pyrimidine ring. Once the folate is in position, the conformational changes bring the free carboxyl group of the C-terminal residue and the invariant Arg-21 (in E.coli) together to form a hydrogen bond network to close up the active cleft (Matthews et al., 1990). By some unknown mechanism, the folate is converted to a N5 iminium ion, which is attacked by the active enolate anion of the pyrimidine ring underneath. A ternary complex (Figure 1-1e) is formed. The ternary complex is much more stable because the β-elimination reaction leading to the collapse of the ternary complex is prevented due to the uneliminated fluoro atom at the 5' position of FdUMP. The presence of cofactor has been shown to enhance the covalent binding of the nucleotides and vice versa, suggesting that the affinity for both cofactor and nucleotide is increased. The mechanism for the enhanced covalent nucleotide binding is the formation of an inhibitory ternary
complex among TS, FdUMP, and cofactor. In this complex, both nucleotide and folate (through nucleotide) are covalently linked to the enzyme. The covalent complex is believed to represent the "capture" of a transient intermediate occurring during the normal enzyme reaction pathway. The kinetic parameters for the formation of the ternary complex have been characterized. The formation parameter $k_{on}$ has been determined to be $2 \times 10^8 \text{M}^{-1}\text{min}^{-1}$ (Danenberg and Danenberg, 1978).

b. Structure. Isolation of the ternary complex and subsequent peptide mapping has shown that the complex is covalently attached to the sulfhydryl group of cysteine-198 of the L. casei enzyme (Bellisario et al., 1976). This has also been proved by the site-directed mutagenesis experiments in which replacement of the cysteine with an Ala results in no formation of the ternary complex (Dev et al., 1988). The ternary complex is covalently attached to the thiol group of the enzyme at C-6 of FdUMP (Bellisario et al., 1976; Bellisario et al., 1979). The cofactor is not covalently linked to the enzyme but covalently linked to C-5 of the pyrimidine ring of FdUMP by a methylene bridge (Moore et al., 1986; Pellino and Danenberg, 1980). The stereochemistry of the ternary complex has been examined by $^{19}$F NMR with either native TS (Byrd et al., 1978) or a peptide derived from the complex (James et al., 1976). It has been shown that the enzyme imposes certain conformational restrictions on the complex. But the restriction is different in native and denatured forms of TS. In the native complex, the cysteinyl thioester bond to C-6 and the methylene bridge between the C-5 of FdUMP
and tetrahydrofolate are in a trans-diaxial relationship as shown in Figure 1-1e (Byrd et al., 1978).

c. Conformational change. It is well documented that there is a large conformational change upon formation of the ternary complex. UV/VIS spectrophotometric analyses of the ternary complex results in two new peaks at 375 and 322 nm. UV/VIS difference spectroscopy has shown that the ternary complex yields a spectrum with a new maximum peak at 325 nm and a minimum peak at 280 nm. CD spectra give characteristic positive ellipticity maxima at 360 and 290 nm and a negative ellipticity maximum at 327 nm (Galivan et al., 1975; Donato et al., 1976; Lewis et al., 1981b). Also the formation of the ternary complex cause a 5% to 6% decrease in β-sheet and β-turns, and about an 8% increase in other structures (Manavalan et al., 1986). Protein fluorescence of TS is quenched markedly when the ternary complex is formed (Donato et al., 1976). Direct evidence comes from hydrodynamic methods, which have shown that the Stokes radius of the human and L. casei enzymes undergoes a 3.5% decrease after the formation of the complex (Lockshin and Danenberg, 1980). The conformational change may be related to the capping of the active site after substrate binding as evidenced by X-ray studies.

d. Decomposition. Although the inhibitory ternary complex is very stable with $K_a$ in the range of $10^{11} \text{ M}^{-1}$ (Danenberg, 1977) and $K_d$ about $10^{-13} \text{ M}$ (Santi et al., 1974b), it has been shown that the complex undergoes a very slow
degradation process (Spears et al., 1989). The $k_{off}$ for human TS is about $5 \times 10^3 \text{ min}^{-1}$ (Fernandes and Cranford, 1984) and for *L. casei* TS, $1.39 \times 10^4 \text{ min}^{-1}$. The dissociate rates from both FdUMP binding sites has been shown to be the same (Danenberg and Danenberg, 1979).

In the case of the catalytic ternary complex, the reaction leads to the collapse of the ternary complex which has been hypothesized based on limited information from kinetic and X-ray studies. The reaction precedes by concomitant elimination of the tertiary amine (N5) in the cofactor and the 5-H of dUMP (possibly transferred to a water molecule in the active site), resulting in enzyme bound 5-CH$_2$dUMP and H$_4$folate. This is followed by transfer of a hydride (C-6 hydrogen) from H$_4$folate to the methylene group in dUMP, yielding products, thymidylate and H$_2$folate (Matthews et al., personal communication). Since tertiary amines are generally poor leaving groups, a conserved Asp-169 is proposed to function as a general acid catalyst in the same manner as it does in DHFR (Villafranca et al., 1983; Benkovic et al., 1988). Once the products are formed, the capping structure of the active site is reopened and the products are ejected in an ordered manner. This process is poorly understood.

3. TS-nucleotide-folate

Not only does the cofactor have the ability to enhance the covalent binding of nucleotide (FdUMP and dUMP), but other folate derivatives also have the ability to enhance the nucleotide binding (Moore et al., 1986a). Since these folates generally do not have a functional group to covalently link
to FdUMP in the ternary complex, it has been suggested that these folate derivatives could mimic the cofactor and cause a conformational change which enhances the nucleotide binding. The covalent link between folate and nucleotide may not be required for the enhancement. The covalent binding of dUMP is also enhanced by a variety of folate derivatives (Danenberg and Lockshin, 1982). It has been shown that pteroylfolylpolyglutamates bind significantly tighter to TS than the corresponding monoglutamyl form (Galivan et al., 1976a; Kisliuk et al., 1981). The folylpolyglutamyl form has also been shown to enhance the binding of dUMP to TS (Lockshin and Danenberg, 1979). It seems that the tightness of the ternary complex is directly related to the number of the glutamate residues (Priest and Mangum, 1981).

IV. Current proposed mechanism

Numerous investigations have led to a general mechanism proposed for the catalytic process of thymidylate synthase as summarized in Figure 1-4. The mechanism has been subjected to extensive testing and has been proved correct. However, details of the scheme still remain to be solved. In this scheme, the nucleotide substrate dUMP (A) binds enzyme first both noncovalently and covalently (through C-5 of dUMP and sulfhydryl group of the active site Cys residue) in an equilibrium (B, C, D). The folate substrate (5,10-methylenetetrahydrofolate, A') which may exist in the form of the 5-iminium cation (5-CH⁺-tetrahydrofolate, A") when binding to the enzyme, then
Figure 1-4. Current mechanism for thymidylate synthase.

Nucleotide substrate, dUMP (A), binds enzyme both covalently (by attacking of the thiol group of active site cysteine to C-5 of the pyrimidine ring) and noncovalently (B,C,D). The binding of nucleotide opens the folate binding site to which cofactor (A',A'') is bound. After severe conformational change, a covalent linkage is formed connecting C-6 of the nucleotide and N-5 of cofactor through a methylene group (E,F). The ternary complex is collapsed by $\beta$-elimination of the N-5 amine and C6-H of nucleotide (F), forming tetrahydrofolate and enzyme bound methylene dUMP. After a hydride transfer from tetrahydrofolate to the methylene group (G), the dihydrofolate is released before the enzyme bound thymidylate undergoes a bond breakage with the enzyme (J). Finally, thymidylate is released (K). The structures are from Lewis and Dunlap (1981).
Figure 1-4
binds to the folate binding site of TS noncovalently, causing a large conformational change which not only enhances the covalent binding of dUMP but also buries the two substrates inside the protein. A covalent bond is then formed between the C-6 of dUMP and N-5 of folate via a methylene bridge, forming the so-called covalent catalytic ternary complex (E). Then β-elimination from the tertiary amine of the folate and 6-H in the pyrimidine ring happens resulting in 5-methylene dUMP and tetrahydrofolate (F). Then, a hydride is transferred to the methylene group forming thymidylate and dihydrofolate (G). However, the order of product releasing is not clear.

**Current Investigations**

I. Molecular Approaches

Rapid development in molecular cloning has led to the isolation of TS genes from a variety of sources, including, *E.coli* (Belfort et al., 1983a), *B. subtilis* Thy P3 phage (Kenny et al., 1985), T4 phage (Chu et al., 1984; Purohit and Mathews, 1984), human (Takeishi et al., 1985), mouse (Perryman et al., 1986), *L. casei* (Pinter et al., 1988), yeast (Taylor et al., 1987), *B. subtilis* (Iwakura et al., 1988), *P. carinii* (Edman et al., 1989), varicella zoster virus (Thompson et al., 1987), and also from such sources such as *H. saimiri* (Honess et al., 1986), *L. major* (Beverley et al., 1986), *L. tropica* (Coderre et al., 1983; Grumont et al., 1986), *P. falciparum* (Bzik et al., 1987), human malaria parasites (Snewin et al., 1989), and *C. fasciculata* (Hughes et al., 1989).
1989) for bifunctional TS proteins. The coding DNA sequences obtained from the cloned genes could be used to decipher the amino acid sequences of proteins from different sources. The comparison of the amino acid sequences not only reveals that TS is one of the most conserved proteins studied thus far, but also provides information on the secondary structure. Recently, the development of expression systems has allowed the TS genes to be efficiently expressed in bacteria in large quantities, and also allowed the use of protein engineering to characterize roles of a particular amino acid in enzyme structure and function.

1. Expression systems.

Several expression systems have been established that express a particular gene in a particular environment. The bacterial expression system is especially attractive for enzymatic studies of TS because it is inexpensive and easy to perform large scale culture and provides large amounts of protein. However, in order to establish an expression system, a proper expression vector and a proper bacterial host need to be carefully selected. Early experiments on E.coli (Belfort et al., 1983a) and T4 phage (Belfort et al., 1983b) genes used the phage lamda pL promoter and Thy- bacteria. Later, it was found that the L. casei TS promoter could direct the expression of the L. casei TS gene in E. coli (Pinter et al., 1988). Promoters of lac and tac were used to direct the expression of P. carinii (Edman et al., 1989). However, attempts to express the mammalian TS genes in bacteria have failed until recently.
Other expression systems have been developed to study the regulatory roles of DNA or RNA sequences of the TS genes. For example, mouse TS minigenes have been established and expressed in TS-V79 cell line (DeWille et al., 1988). The mammalian expression system could potentially become important for enzymatic studies, providing that enough TS can be expressed, since this would allow posttranslational modifications to be analyzed. The low expression problem can be partially overcome by including introns 5 and 6, which stimulate gene expression up to 10 fold (Deng et al., 1989). Recently, it was found that introns 1 and 2 stimulate the expression up to 20 fold (Y.Li, personal communication). Still greater expression was obtained by using the SV40 promoter and poly(A) signal.

2. Site-directed mutagenesis

The development of the expression systems for the TS genes and the commercially available, highly efficient in vitro site-directed mutagenesis methods, makes it possible to specifically alter an amino acid in the TS protein to another. Thus, the functional and/or structural roles of this particular residue can be examined.

Several reports have been published to probe the functional roles of particular amino acid residues in TS. In one case, the active site cysteine residue was changed to alanine or serine. The results were surprising in that the serine mutant still retained some catalytic activity, although it is 4000-fold decreased (Dev et al., 1988). Another report characterized a natural mutant in which the cysteine 50 is replaced by a tyrosine (Maley and Maley, 1988). Two
recent reports suggested that the conserved histidine in the active site of *E. coli* TS and the Arginine-179 in *L. casei* are not absolutely important for substrate binding (Dev et al., 1989; Santi et al., 1990). These reports have already shown the usefulness of site-directed mutagenesis in solving important questions of enzymatic kinetics.

II. Communications

1. Communication between subunits. There are at least three observations that strongly suggest that inter-subunit communication may play an important role in substrate binding and catalysis. First; it has been long established that the two active sites, especially the sulfhydryl groups of the cysteine residues, are not identical with regard to substrate binding affinity or stoichiometry (Danenberg and Danenberg, 1979), although they are identical in amino acid sequence. The asymmetrical arrangement clearly imposes the question of communication between these two monomers. Second; it has been shown that when only one C-terminal valine residue from one monomer of *L. casei* TS is removed, the enzyme completely loses its catalytic activity, although half the covalent binding stoichiometry was still retained (Aull et al., 1974a; R. Cisneros, personal communication). This observation suggests that both "complete monomers" are needed for complete activity. Third; from 3-D structure of *L. casei* TS, it appears that an arginine from one subunit is functionally involved in the active site of the other subunit (Hardy et al., 1986). This is by far the most direct evidence to show the cooperation of the two
monomers. However, ambiguity exists in whether the two subunits are arranged asymmetrically in the native enzyme or whether there is a negative cooperation after binding or modification of one site, although the former is preferred (Danenberg and Danenberg, 1979).

2. Communication within monomer. Enhancement of one substrate binding by the presence of the other substrate suggested the presence of internal communications. Evidence has shown that the binding of substrates will introduce a major conformational change, which may be used as a signal in communication.

Drugs of the Future

The ultimate goal of any study in enzymology is to completely understand the structure-function relationships. In the case of TS, due to its important role in cancer chemotherapy, there is another important goal-- to find powerful inhibitors to be used in cancer treatment. With the understanding of the enzyme mechanism and structure, many inhibitors have been developed and are reviewed by Cisneros et al., (1988). Principally, these inhibitors could be divided into three groups: nucleotide analogues, which mimic the nucleotide substrate; folate derivatives, which are the analogues of cofactor; and catalytic ternary complex analogues, which are the analogues of the catalytic ternary complex.
The nucleotide analog inhibitors, primarily, FUrd and FdUrd have been used in the treatment of solid tumors of the gastrointestinal tract, the liver and breasts. It was originally thought that FUrd and FdUrd inhibit cells by forming covalent complexes with TS by their active form, FdUMP, in the presence of cofactor. Other effects include causing fragmentation of DNA at replication forks, induction of fragile sites in the chromosomes, and rearrangement of chromosomes, all of which result in the death of cells (Schuetz et al., 1984; Ayusawa et al., 1983). Recently, it was found that FdUMP could also be incorporated into DNA (Akazawa et al., 1986), damaging the DNA, thus causing cell death.

The promise of the folate derivative inhibitors lies in the fact that some folylpolyglutamyl forms of folate, with a different number of glutamate residues, exhibit preferential inhibition of enzymes from different origins (Maley et al., 1979b; Bellisario et al., 1979). Also, even the same folylpolyglutamates may differentially inhibit TS from different sources (Maley et al., 1979b).

However, one of the major limitations to the effective clinical treatment is the development of resistance, which overcomes the effects of a drug. It is believed that cells develop resistance to FdUrd by the following pathways: amplifying the TS gene, thus, increasing the amount of TS (e.g. Jehn et al., 1985; Ali Imam et al., 1987); increasing the TS level by unknown mechanisms (Wilkinson et al., 1977; Priest et al., 1980b; Washtien et al., 1984); rapid disappearance of FdUMP (Klubes et al., 1978); decreasing the activity of
thymidine kinase which catalyzes FdUrd to FdUMP (Mulkins and Heidelberger, 1982); enhancing the activity of a phosphatase which degrades FdUMP (Fernandes and Cranford, 1985); changing the TS properties (Heidelberg et al., 1960, Bapat et al., 1983; Jastreboff et al., 1983; Barbour et al., 1990). Thus, the development of drugs of the future may have two directions: one is to design more powerful drugs based on the structure of TS; another is to use drugs in combination to reduce the possibility of TS overproduction and other possible pathways leading to the resistant phenotype.
Chapter II
Materials and Methods

Part I
DNA Manipulations

General procedures

All standard procedures were performed according to Maniatis et al., 1982, Sambrook et al., 1989 and Ausubel et al., 1987.

Bacterial cell culture

All liquid bacterial cell cultures were grown at 37 °C in a shaker (250 rpm/min) and were derived from a single colony. For bacteria containing no plasmid, LB, YT, or 2XYT liquid, or the corresponding plates (1.7% agar) were used. For bacteria harboring plasmids, proper drugs (ampicillin at a final concentration of 50 µg/ml, chloramphenicol at 10 µg/ml, or tetracycline at 15 µg/ml) were used. Bacteria were stored at -70 °C in medium containing 20% glycerol.
Some constants used for calculations

Some of the simple calculations in molecular biology were based on the following constants: DNA, 50 µg/ml at unit absorption at 260nm; RNA, 40 µg/ml at unit absorption at 260nm; oligonucleotide, 36 µg/ml at unit absorption at 260nm. Also 1 pmol of oligonucleotide was assumed to equal to 10 ng.

Phenol extraction/ethanol precipitation

Very often, the combination of phenol extraction followed by ethanol precipitation was used to remove protein components in DNA samples. Approximately the same volume of TE- or water-saturated phenol was added to the sample (final volume of 100 µl to 450 µl). After vortexing for 30 sec, the two layers (DNA containing aqueous layer on the top and the phenol layer on the bottom) were separated by centrifugation and the phenol layer was removed. The sample was then extracted with water-saturated ether to remove the residual phenol. Salt (half volume of 7.5 M NH₄OAc or 0.1 M NaCl final concentration) was then added (if there was no salt at the beginning of phenol extraction), and 2.5 volumes of 95% ethanol were added and mixed completely. The sample was centrifuged immediately if there was a large amount of DNA, or stored at -70 °C for 30 min before centrifugation. The DNA pellet was washed with 70% ethanol and was dried in a Speed Vac.
DNA fragment isolation from gels

Occasionally, color selection or shotgun cloning was used for DNA subcloning. In most cases, one of the following methods was used to purify a DNA fragment from an agarose or polyacrylamide gel.

1. Gene Clean. The principle for this method was described by Vogelstein and Gillespie (1979) and the purification procedure was performed using a commercially available kit. Briefly, digested DNA was separated on a 1% agarose gel and the band of interest was cut to small pieces and transferred to an eppendorf tube. Saturated Nal solution (provided in the kit) was added to the tube at 2.5 ml per gram of the gel. Then, the tube was placed in a 55 °C waterbath until all of the gel pieces dissolved, after which about 1 μl of "glass milk" per μg of DNA (estimated) was added to the tube. The tube was vortexed briefly, and kept at room temperature for 5 min. The tube was then centrifuged for 30 sec in an eppendorf centrifuge. The pellet was washed 3 times with NEW washing solution (10 mM Tris-Cl, pH 7.2, 0.1 M NaCl, 1 mM EDTA, 50% ethanol) by mixing/centrifugation. Finally, DNA was eluted from the pellet by suspending the pellet in water or TE buffer. After 30 sec. of centrifugation, the supernatant was transferred and stored. DNA fragments isolated in this way are ready for use in DNA cloning and many other purposes. The DNA isolated can also be further purified by a phenol extraction/ethanol precipitation procedure if necessary.
2. D-gel. The apparatus and supplies were purchased from Epigene company. This method is especially efficient when small fragments of DNA are to be dealt with. Basically, gel pieces (agarose or polyacrylamide) containing a particular DNA fragment were cut into small pieces and loaded onto a column packed with DEAE anion exchange resin. The column was then put in the elution apparatus with other blank columns (as required) and electroelution was started in TBE buffer at 10 mA for 3 hrs (for small fragments <500 bp, 1 hr is sufficient). Then, the sample column was taken out and allowed to drain, and washed with 15 ml of 0.1 M NaCl or 0.25 M ammonium acetate. The residual washing solution in the resin was forced out by a rubber bulb before eluting DNA with 2X0.4 ml of 1 M NaCl or 4 M ammonium acetate. Then, 2.5 vol excess of 95% ethanol was added to the eluant (add carrier tRNA if necessary), and the sample was stored at -70 °C for at least half an hour. DNA was collected by centrifugation.

3. Elutip. Isolation of DNA fragments by this method is based on the principle that DNA will be selectively bound to the DEAE anion exchange resin packed in a small tip, which is commercially available from S&S company. Briefly, DNA was separated on a LOW MELTING gel. The gel slice containing a DNA band was dissolved in 2 ml of low salt buffer (20 mM Tris-Cl, pH 7.4, 0.2 M NaCl, 1 mM EDTA) at 65 °C for 20 min. The melted solution was passed through a low salt buffer-equilibrated tip at the rate of approximately 1 drop per second. After the tip was washed with 2 ml of low salt buffer, DNA was eluted from the tip slowly with 0.4 ml of high salt buffer.
(20 mM Tris-Cl, pH 7.4, 1 M NaCl, 1 mM EDTA). 2.5 vol of 95% ethanol was then added to precipitate DNA.

**ss DNA Isolation**

ss DNA was isolated by helper phage rescue techniques. All DNA inserts were cloned into one of the following ss DNA producing vectors: pBluescribe M13 (+) or (-) (Stratagene), pBluescript M13 (+) or (-) (Stratagene). They were then transformed into either NM522 or XL-1 blue or CJ236 bacteria, depending on the purpose: for DNA sequencing, NM522 or XL-1 Blue was used; for isolation of U-containing ss DNA for site-directed mutagenesis, CJ236 was used.

A single colony containing the vector was transferred to a 5 ml 2XYTA medium, and cultured at 37 °C for 10-12 hrs. About 1 ml of the culture was then transferred to 20 ml of the same medium and cultured at the same conditions until OD_{600} nm reached 0.3. Then, 40 μl of M13K07 or VCS M13 helper phage (2X10^{11} pfu/ml) was added and the culture was continued overnight. The supernatant containing the helper phage was collected by centrifugation at 10,000Xg for 10 min at 4 °C, and PEG solution (20% PEG-8000, 3.5 M NaCl) was added (0.25 ml/ml). After incubating on ice for 40 min, the precipitated phage were collected by centrifugation at 10,000Xg for 15 min at 4 °C. The tube was carefully drained and the phage pellet was dissolved in TE buffer. Proteins were removed by repeated phenol (saturated with TE) extractions until no interphase protein was visible. DNA which was in
the aqueous layer was precipitated by adding 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of 95% ethanol and then dried in a Speed Vac.

**Oligo synthesis, purification and phosphorylation**

All oligonucleotides (oligos) were synthesized at the University of South Carolina and the OSU Biochemical Instrument Center.

Three methods were used to purify detritylated oligos. Briefly, an oligo was heated at 55 °C overnight, dried, and dissolved in different buffers, depending on the purification method.

1. The oligo was dissolved in 0.1 M TEAB, pH 7.5 (Triethylammonium bicarbonate), and passed through a DEAE gel column. The oligo was adsorbed to the column and then eluted by washing with 1 M TEAB (pH 7.1). The solution was dried and the oligo was dissolved in water or TE buffer.

2. The oligo was dissolved in water and passed though a PD-10 gel filtration column (Pharmacia) equilibrated and eluted with water. The amount of oligo recovered was determined by measuring the absorbance at 260 nm (Jayaraman, 1987).

3. The oligo was dissolved in a low salt buffer (20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 10 mM NaCl), and passed through an Elutip column (S&S), the oligo was eluted with 400 μl of high salt buffer (1 M NaCl in low salt buffer). The oligo was recovered by adding 2.5 volume of 95% ethanol, stored at -70 °C for > 1 hr, and collected by centrifugation. The oligo pellet was dried and
dissolved in water or TE buffer.

Phosphorylation of an oligo was carried out in 20 μl final volume which contains 20 pmol of oligo, 2 μl 10X kinase buffer (1 M Tris-Cl, pH 8.3, 0.1 M MgCl₂, 0.1 M DTT), 1 μl of 10 mM ATP and 20-30 units of T4 polynucleotide kinase. The reaction was performed at 37 °C for 1 hr and was terminated by heating at 65 °C for 10 min.

Site-directed mutagenesis

All of the site-directed mutagenesis procedures were performed according to Kunkel (1985), using ss DNA isolated from bacteria CJ236 as the template.

The standard annealing process was performed by mixing 100 ng of template, 1 pmol of oligo, and 1 μl of 10X annealing buffer (0.2 M Tris-Cl, pH 7.4, 0.1 M MgCl₂, 0.5 M NaCl) in a final volume of 10 μl. The temperature of the mixture was first raised to 75 °C, and was then gradually decreased to room temperature over a period of at least 40 min. Then, 2.5 units of T4 DNA ligase, 2.5 units of T4 DNA polymerase, and 1.3 μl of 10X synthesis buffer (0.1 M Tris-Cl, pH 7.4, 50 mM MgCl₂, 20 mM DTT, 10 mM ATP, 5 mM each dNTP) were added and the reaction was continued at 37 °C for 1 hr. The mutagenesis result was checked directly on a 1% agarose gel, in which 1 μl of the reaction mixture was loaded. If the ss DNA in the control lane was shifted in the reaction lane, successful mutagenesis was indicated. Then, 1-2 μl of the reaction mixture was directly used to transform XL-1 Blue. Mutants
were identified by restriction mapping (if applicable) and DNA sequencing.

**DNA sequencing**

DNA sequencing was performed according to the dideoxy method (Sanger et al., 1977).

1. ss DNA sequencing. ss DNA was isolated from either NM522 or XL-1 blue as described earlier and the sequencing was performed using a kit from USB (Sequenase) according to the manufacturer's specifications.

2. Double strand DNA sequencing. Miniprep DNA was used as the template to perform ds DNA sequencing. For this to be successful, two points are critical: the quality of DNA and the conditions for denaturation. Basically, a single colony was transferred to 3 ml of medium and cultured overnight at 37 °C. 1.5 ml was centrifuged and the pellet was suspended in 0.1 ml of Solution 1 (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, 50 mM Glucose). After sitting at room temperature for 5 min, 0.2 ml of 0.2 M NaOH/1% SDS was added. The solution was mixed by inversion and put on ice for 5 min. Then, 0.15 ml of 3 M KOAc, pH 4.8 was added, mixed by inversion and incubated on ice for another 5 min. Proteins and cell debris were precipitated by centrifugation, and the supernatant containing plasmid DNA was transferred to a new eppendorf tube. DNA was precipitated by ethanol and the precipitate was dissolved in 50 μl of TE containing 50 μg/ml RNase. After 1 hr at 37 °C, the volume was adjusted to 200 μl with water and the solution was extracted once with phenol (saturated with TE buffer, pH 8.0). The
phenol layer was removed after centrifugation and the residual phenol was removed by ether extraction. Finally, the plasmid in the aqueous layer (200 μl) was precipitated by adding 120 μl of 20% PEG-2.5 M NaCl solution and incubating on ice for 1 hr. The pellet was collected by centrifugation and dried. In alkaline denaturation, DNA was dissolved in 50 μl of 0.2 M NaOH, 0.2 mM EDTA, pH 8.0 and was denatured at room temperature for 30 min. The solution was then neutralized by adding 6 μl of 3 M ammonium acetate, pH 4.5. The denatured DNA was precipitated by adding 2.5 vol of 95% ethanol, and stored at -70 °C for at least 30 min before centrifugation. The annealing reaction and subsequent DNA sequencing were performed according the manual from USB.

**Strategies for constructing mutant proteins**

Mouse cDNA (Perryman et al., 1986; DeWille et al., 1988) was used to construct expression vectors and will be described in detail in Chapter 3. However, mutant coding regions were constructed in a ss DNA producing vector, and must be cloned back into the expression vector in order to produce mutant proteins. The following strategies were used, as described in Figure 2-1. The TS gene in the expression vector pETSM (see Chapter 3) was dissected into 3 parts, the 354 bp of the initial PstI/Apal (+27 to +380) covering the folate binding region I (Region I), the middle 330 bp of Apal/PstI
TS coding region in the expression vector, pETSM, was dissected into three parts for subcloning into a ss DNA producing vector, pBluescript M13 (pBST) (+) or (-). The Pstl (P)/Apal (Ap) fragment covering the N-terminal of the coding region (Region I) was cloned into pBST(+). The Apal/Pstl fragment covering the middle portion of the coding region (Region II) was also cloned into pBST(+). The C-terminal region (Clal in the coding region to Clal in the expression vector) was cloned into pBST(-). After ss DNA isolations from CJ236 and subsequent site-directed mutagenesis, the mutated Region I was cloned back to the expression vector to replace the wild type Bsu36I (Bs)/Apal fragment while the mutated Region II was cloned back to replace the wild type Apal/Stul (St) fragment. The C-terminal region was cloned back by a simple replacement of the wild type Clal (C)/Clal fragment. All mutants were ds DNA sequenced before being used for protein expression. Other abbreviations: B, BamHI; Bg, BglII. R44X, mutants replacing the arginine 44; P188X, C189X, R170X, R209X, mutants created in the region II at position of 188, 189, 170, and 209. TS-MAX, mutants changing the valine 307; TS-MV, deletion of an Ala residue before the valine 307; TS-MAAV, addition of an Ala before the valine 307.
Single strand DNA
Site-directed mutagenesis

R44X
P188X
C189X
R170X
R209X

V307X
V-A
V+A

Replacement of corresponding Fragment in pETSM

Figure 2-1
(+380 to +714) covering the nucleotide binding site (Region II), and finally the Clal fragment covering the C-terminal region (+846 to the Clal in the vector). These three parts were cloned into pBluescript M13 (+) or (-) to yield plasmid, pRegionI, pRegionII, and pC-term respectively. Single stranded DNA was isolated and site-directed mutagenesis was performed. The mutant DNA was cloned back into the expression vector using unique restriction enzymes of Bsu36I/Apal, Apal/Stul for region I and II, and replacement of the wild type Clal fragment by the mutant Clal fragment. The mutant sequence was confirmed by ds DNA sequencing.

The above strategy was used in most of this study. However, this strategy requires two rounds of DNA sequencing and subcloning processes in which error-prone substitutions of wild type fragments were carried out. In addition, some other regions in the coding region were not covered. In order to solve these problems, the expression cassette from pETSM (Sall to EcoRV) was subcloned to pBluescript M13 (-) to yield a plasmid, pBTSM. Thus, site-directed mutagenesis was directly performed on the vector. After sequencing the whole gene, it was cloned to an expression vector, pET-3a (Sall/EcoRV), which yielded a slightly different expression vector, pETSMM, in which the EcoRV fragment in the pETSM vector was deleted.

**Bacterial cell culture for expressing protein**

A single colony of the proper *E. coli* host strain transformed with the expression plasmid was used to inoculate 10 ml of LA (1% tryptone, 0.5%
yeast extract, 0.5% NaCl, 0.1% glucose, 50 μg/ml Ampicillin, pH 7.5) which was grown overnight at 37 °C. The culture was then diluted into 0.5 liter of LA, which was similarly grown to stationary phase and used to inoculate 19 liters of LA in a glass carboy. The 19.5 liter culture was maintained at 37 °C and subjected to continuous aeration and stirring. When the absorbance of the culture at 600 nm reached 0.5, IPTG was added to a final concentration of 1 mM. Samples (10 ml) were withdrawn at various times, centrifuged to pellet the cells, which were then suspended in 1.0 ml of Lysis Buffer A (50 mM Tris-HCl, pH 7.4, 12 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100). The cells were lysed by repeated extrusion (3 min) of the cell suspension through the tip of an 18 gauge needle until the initially viscous fluid flowed freely. Following centrifugation, TS enzyme levels in the extracts were determined by a modified ternary complex assay in which TCA was added to quench the reaction mixture after a 5 min incubation period. When the enzyme level reached a maximum (usually 3-4 hr after induction), the culture was rapidly cooled to 4 °C in an ice bath. Bacteria were concentrated by ultrafiltration and harvested by centrifugation at 6,000xg at 4 °C. A typical large scale culture (19.5 liter) yielded 40 g of cells which were divided into 5 x 8 g lots and stored at -20 °C.
Part II
Protein chemistry

Concentration calculations for some chemicals

The concentrations of some of the chemicals were calculated using their specific absorption at a particular wavelength. dUMP, \( E = 8300 \, \text{cm}^{-1}\text{M}^{-1} \) at 263nm, H\(_2\)folate, \( E = 6400 \, \text{cm}^{-1}\text{M}^{-1} \) at 340nm, cofactor, \( E = 30000 \, \text{cm}^{-1}\text{M}^{-1} \), FdUMP, \( E = 8900 \, \text{cm}^{-1}\text{M}^{-1} \) at 263nm.

Materials

Tritium labeled nucleotides, [6-\(^3\)H]-dUMP (20 Ci/mmol), [6-\(^3\)H]-FdUMP (20 Ci/mmol), [5-\(^3\)H]-dUMP (15 Ci/mmol) were purchased from Moravek Biochemicals, Inc. Dilutions with the corresponding nucleotides were made to achieve about \( 10^5 \) dpm/nmol before use. Tetrahydrofolate was synthesized according to Hatefi et al., 1960, and was kindly provided by Drs. Cisneros and Dunlap. Tetrahydrofolate is also available commercially. Methylene tetrahydrofolate was synthesized by dissolving tetrahydrofolate in 25 fold molar excess of formaldehyde. For pH profile measurements, the following buffers (at 100 mM final concentration) were used: acetate buffer (\( pK_a \) 4.75), pH range from 3.5 to 6.0; citrate buffer (\( pK_a \) 5.40), pH range from 3.5 to 6.0; HEPES buffer (\( pK_a \) 7.5), pH range from 6.5 to 8.5; Bis-Tris buffer
(pKₐ 6.4), pH range from 5.2 to 7.6; phosphate buffer (pKₐ 6.70), pH range from 6.0 to 8.0; Tris buffer (pKₐ 8.14), pH range from 6.5 to 9.0.

**Preparation of 10-formylfolate aminoethyl Sepharose**

The affinity resin, 10-formylfolate aminoethyl sepharose, used for affinity purification of mouse recombinant TS, was prepared using a modified procedure of Benerjee et al., 1982. Three grams of cyanogen bromide activated Sepharose 4B was washed with 350 ml of chilled 1 mM HCl, filtered to dryness (Whatman Gf/A filter), and suspended in 4 ml of 0.1 NaHCO₃, pH 8.5 containing 5 g of ethylenediamine dihydrochloride. This suspension was stirred for 16 hr at room temperature in a 50 ml flask attached to a Labline orbit shaker (100 rpm).

The product was filtered and washed with 400 ml of 0.1 M NaHCO₃ and 400 ml double distilled water. The resulting aminoethyl Sepharose was reacted with 150 mg of 10-formylfolate dissolved in 10 ml of 0.1 M NaHCO₃. The pH was adjusted to 6.0 with 6 N HCl, and 0.2 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride was added as an aqueous suspension (0.2 g/ml of H₂O). The mixture was stirred for 6 hr at room temperature and the pH was maintained at pH 6.0 by addition of 2 N NaOH. The incubation was then continued for an additional 18 hr.

The final product was washed with 500 ml 10% potassium bicarbonate and 500 ml double distilled water, and was stored in 75% ethanol.
**TS purification by conventional chromatography**

Frozen cells (generally 2 g) were suspended in Lysis Buffer (50 mM Tris-Cl, pH 7.0, 1 mM EDTA, 12 mM β-mercaptoethanol, 0.1% Triton X-100, and 10% glycerol), and the resulting suspension was sonicated in an ice bath for 2 min using a Heat-Systems model W-200F sonicator, followed by centrifugation at 10,000 xg for 20 min. The supernatant (cell-free extract) was loaded on a 2.4 cm x 15 cm DEAE-Trisacryl (IBF Biotechnics, France) column preequilibrated with Lysis Buffer. All column chromatography steps were performed with a Pharmacia FPLC system. The bound proteins were eluted with 2 bed volumes of a NaCl linear gradient (0 to 150 mM) in Lysis Buffer. Fractions containing TS were pooled and loaded directly onto a 2.4 cm x 9 cm Q-Sepharose (Pharmacia) fast flow column equilibrated with Lysis Buffer. The column was first washed with 1 bed volume of a 0 to 90 mM NaCl gradient, followed by 2 bed volumes of a 90 to 150 mM NaCl gradient in Lysis Buffer. Fractions containing TS activity at different levels of purity were pooled separately and dialyzed against 6 liters of Lysis Buffer. The purified enzyme was stable (>90% activity for at least 6 weeks) when stored in Lysis Buffer at -20 °C, and could be maintained in dialysis for several days at 4 °C in Lysis Buffer without significant loss of activity.
TS purification by formylfolate affinity chromatography

DEAE-Trisacryl fractions (about 25 mg protein) containing TS activity were made 50 μM in dUMP and loaded on a 10-formylfolate aminoethyl Sepharose 4B column (1 cm x 8 cm) equilibrated in Lysis Buffer containing 50 μM dUMP (Benerjee et al., 1982). The column was washed in Lysis Buffer containing 50 μM dUMP and 0.2 M NaCl. TS was eluted with a 0 to 1 M NaCl gradient in Lysis Buffer in the absence of added dUMP. The pure protein was stored at 4 °C or -20 °C directly. Before use, TS was dialyzed against Lysis buffer at 4 °C.

TS purification by dideazafolate affinity chromatography

This purification procedure was first developed to isolate small amounts of TS. The bacterial pellet was dissolved in buffer A. The mixture was sonicated at 30-40 cycles for 2 min using the microtip and centrifuged. The supernatant was first brought to 30% ammonium sulfate saturation. After incubation on ice for 15 min, it was centrifuged and the supernatant was further brought to 70% ammonium sulfate saturation, incubated on ice for 15 min, and cleared by centrifugation. The pellet containing TS activity was dissolved in buffer B (30 mM Tris-Cl, pH 7.5, 2 mM DTT), and the solution was dialyzed against several liters of buffer B at 4 °C. The sample was then applied to a DEAE-cellulose column (equilibrated with buffer B) and fractioned according to Dolnick and Cheng (1977). After washing with buffer B, TS
protein was eluted from the column by a linear gradient of buffer B to buffer C (0.5 M Tris-Cl, pH 7.5, 2 mM DTT), and fractions containing TS activity were pooled and subjected to purification by affinity chromatography on 10-formyl-5,8-dideazafolate-ethyl-Sepharose as described previously (Rode et al., 1979) [The folate derivative was provided by Dr. Hynes at Univ. of South Carolina and was linked to Sepharose resin by Dr. Johnson in a similar manner as described earlier]. Basically, the enzyme fraction from a DEAE cellulose column (similar to DEAE Trisacryl) was dialyzed against affinity buffer B (200 mM sodium phosphate buffer, pH 7.5, 0.5 M KCl, 10 mM 2-mercaptoethanol, 20 μM dUMP, 0.1% Triton), and was then loaded to an affinity buffer B-equilibrated affinity column. The column was washed with affinity buffer A (Buffer B without KCl, and 10 mM sodium phosphate). TS was eluted by affinity buffer C (Buffer A without dUMP). TS activity was monitored by the filter binding assay, and the active fractions were combined and concentrated in a centricon ultrafiltration unit, and stored at -70 °C.

**Protein determination**

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard. Generally, 200 μl of dye solution, 750 μl of water, and 50 μl of protein sample was added to a cuvette and mixed. After incubating at room temperature for 5 min, the absorbance at 595 nm was monitored, and the protein concentration was obtained from the standard curve.
Gel electrophoresis techniques

1. SDS polyacrylamide gel electrophoresis (10%). This was performed according to the method of Laemmli (1970). The running gel contained 10% acrylamide, 0.27% N,N-methylene bisacrylamide, 0.16% SDS, 0.375 M Tris-Cl pH 8.8, and was polymerized by adding 1:2000 (v/v) TEMED and 0.25 mg/ml ammonium persulfate. The stacking gel contained 4% acrylamide, 0.08% bisacrylamide, 0.16% SDS, 0.0625 M Tris-Cl pH 6.2 and was polymerized as described above. The electrophoresis buffer contained 0.025 M Tris-Cl pH 8.0, 0.192 M glycine and 0.1% SDS. Protein samples were dissolved in sample buffer containing 0.063 M Tris-Cl pH 7.2, 2% SDS, 10% glycerol, 0.6% mercaptoethanol and 0.001% bromophenol blue. All samples were incubated in boiling water for 5 min before loading. The gel was run at 25 mA overnight, and was fixed for 2 hrs in 9% acetic acid and 50% methanol. Proteins were detected by staining with 0.25% Coomassie Blue R for 2 hrs and visualized after destaining in 7.5% acetic acid/50% methanol for several hrs. The protein band could be quantitated by scanning on a Hoeffer densitometer.

Radiolabeled TS inhibitory ternary complexes were detected by fluorography using Fluoro-Hance (Research Products International).

2. Native gel electrophoresis (10%). Same as the SDS PAGE except that there was no SDS in any of the buffers and there was no stacking gel.

3. PhastGel native and SDS gel electrophoresis. The PhastGel electrophoresis was performed by using a Pharmacia PhastGel system.
according to the instructions given by the manufacture. Approximately 1 μl of sample was loaded onto the native gradient (8-25%) polyacrylamide gel or 10 % SDS polyacrylamide gel.

**Activity assay by spectrophotometric method**

TS catalyzes the conversion of dUMP to thymidylate with the concomitant conversion of methylenetetrahydrofolate to dihydrofolate. The formation of dihydrofolate was monitored by a spectrophotometer at 25 °C using its absorption at 340nm as described (Dunlap et al., 1971). The standard reaction mixture contained 100 mM Tris buffer, pH 7.4, 100 mM NaCl, 100 μM dUMP, and 200 μM cofactor (prepared as 2 mM solution of H₄folate in 50 mM NaHCO₃, pH 8.5, 65 mM HCHO, 250 mM 2-mercaptoethanol) in 0.9 ml. The reaction was initiated by addition of 100 μl of TS. The change in the absorbance at 340 nm due to the formation of dihydrofolate was monitored for 30 seconds on a Hewlett-Packard 8450A UV/VIS spectrophotometer and converted into OD/min by an online computer. The units of the enzyme activity (μmol/min) were calculated by dividing the OD/min by 6.4 (E for H₂folate is 6400 cm⁻¹M⁻¹).

**Activity by tritium release method**

The more sensitive method for determining the activity of TS was based on the observation that a tritium labeled in the 5 position of dUMP was quantitatively transferred to water during the reaction. The tritium release
assay was performed according to the method of Roberts (1966). The standard reaction mixture contained 50 mM Tris-Cl, pH 7.4, 50 mM NaCl, 0.2 mM cofactor solution, 50 mM NaF, TS and 0.22 mM 5-$^{3}$H]dUMP (Moravek Biochemicals, 2.37x10$^5$ dpm/nmole) in 100 µl. The reaction was incubated at 25°C for 30 min. Duplicate 40 µl samples were withdrawn and mixed with 20 µl of 25% trichloroacetic acid to stop the reaction. Unreacted $[^3H]$FdUMP was removed by the addition of charcoal and the amount of tritiated water formed during the incubation was determined in a scintillation counter.

**TCA assay for covalent complex formation**

The formation of binary covalent complexes between TS and nucleotides (dUMP and FdUMP) and ternary covalent complexes among TS, cofactor and FdUMP were determined by a TCA assay in which the covalently bound complexes were separated from the free labeled nucleotides by TCA precipitation. The standard reaction mixture (0.5 ml) for binary complexes contained $[^3H]$FdUMP (Moravek Biochemicals, diluted to a final specific activity of 10$^5$ dpm/nmol), 0.1 µM enzyme, 100 mM Tris-Cl, pH 7.0, 100 mM NaCl, and was incubated at 37°C for 30 min before quenching the reaction by adding 125 µl of 50% TCA. For ternary complexes, the incubation mixture contained either $\text{H}_4\text{folate}$ or $\text{CH}_2\text{H}_4\text{folate}$ (at concentrations indicated in a particular experiment) in addition to the other components. The denatured protein was collected by centrifugation, washed 5 times with 10% TCA, and dissolved in ethanolic NaOH before counting in a
Beckman LS 7500 liquid scintillation counter as described by Cisneros and Dunlap (1990). The binding ratio was defined as nmols of nucleotide bound per nmol of TS. To investigate the dependence of covalent nucleotide binding stoichiometry on pH, the same buffers (100 mM final concentration) were employed as cited above for the pH activity profile studies.

**TS enzyme level assay by filter binding assay**

It was found that under proper conditions, TS protein, tritium labeled FdUMP and methylenetetrahydrofolate or tetrahydrofolate could form a tight ternary complex which could be collected by filtration (Lockshin et al., 1979). The complex was formed in 100 μl (final volume) of 20 nM [6-3H]-FdUMP, 0.75 mM tetrahydrofolate, 10 mM DTT, and 100 mM Tris-Cl, pH 7.0, and TS. The complex was formed at room temperature for 30 min. The ternary complex was precipitated by 10% TCA, and collected on a filter, which was counted in a scintillation counter.

**Overall Binding ratio determination**

The total binding stoichiometry (both covalent and noncovalent) of TS was determined by running a native gel of the complex formed with TS, FdUMP and cofactor as described (Aull et al., 1974b). The inhibitory ternary complex was formed at room temperature for 5 min by incubating a 500 fold excess of FdUMP and cofactor. Native PAGE was performed with a PhastGel gradient (8-25%) PAGE on a Pharmacia PhastSystem. The overall binding
stoichiometry of FdUMP in the inhibitory ternary complexes was determined, after scanning the gel with a Soft Laser densitometer (Biomed Instruments Inc.), by calculating the relative amounts of Form I (native enzyme), Form II (FdUMP:Cofactor:TS, 1:1:1), and Form III (FdUMP:Cofactor:TS, 2:2:1).

Preparation of Triton X-100 free TS

For some experiments, it was necessary to obtain Triton X-100-free TS. Two different methods were used. The first was a modification of the regular affinity purification procedure where Triton X-100 was omitted from all solutions. The second method utilized chromatography on a Bio/Rad Econo-Pac 10 adsorbent column packed with CM-2 Bio-Beads. The column was initially washed with Lysis Buffer without Triton X-100, and loaded with TS protein purified in the presence of Triton X-100. TS protein was washed out with Lysis Buffer without Triton X-100 while the detergent remained in the column.

Consecutive centrifugal column chromatography

This type of chromatography was originally designed to remove small molecules from macromolecules. It was used in this study to remove FdUMP and cofactor after the formation of inhibitory ternary complex between TS, FdUMP and cofactor and was performed as described (Bradshaw et al., 1988). Buffer equilibrated G-50 Sephadex was packed fully into a 5 ml regular syringe with glass wool on the bottom as the filter. The syringe was put in a
centrifugation tube and was centrifuged at 2000 rpm for 2 min in a desktop centrifuge to pack the resin. Then, about 1 ml of sample was added to the column, and an eppendorf tube was hooked to the syringe to collect flow through, which was applied to a second column to repeat. The final flow through was free of small molecules.

**UV/VIS spectra of native TS and ternary complex**

The UV/VIS spectrum of homogeneous TS (purified in the absence of Triton X-100) was obtained on a Hewlett-Packard UV/VIS diode array spectrophotometer at four different dilutions using Lysis Buffer (without Triton X-100) as the blank. Inhibitory ternary complexes were formed in solutions containing about 4 μM TS, 54 μM FdUMP and 60 μM CH$_2$H$_4$folate using enzyme purified in either the presence or absence of Triton X-100. After incubation at 37 °C for 10 min, excess FdUMP and cofactor were removed by consecutive centrifugal column chromatography on Sephadex G-50 columns. The UV/VIS spectra of the isolated inhibitory ternary complexes and their native enzyme controls were obtained as indicated above. Lysis Buffer was used as the blank for those samples containing Triton X-100. In all cases, the sample was scanned from 220 nm to 850 nm.
Chapter III

Efficient synthesis of mouse thymidylate synthase in *E. coli*

SUMMARY

The coding region of mouse thymidylate synthase cDNA was inserted downstream of two different promoters and translation initiation signals of the expression vectors, pKK223-3 and pET-3a, and transformed into *E. coli* bacterial hosts. TS protein expression was monitored and several factors affecting the gene expression were examined. It was found that induction time, DMSO, and Triton X-100 had some effect on the expression of TS protein while differences in the promoter and second codon had dramatic effects on gene expression. When the wild-type cDNA sequence was used, mouse TS was synthesized in the bacterial cells in response to induction, but the level of expression was low. About 30-fold higher expression was observed when the T7 promoter was used as compared with that of the tac promoter. When the second codon (Leu) in the TS gene in pETS was changed from CUG, found in the normal mRNA, to CUU, as in the expression vector, pETSM, the level of expression increased 17-fold, and TS represented 5-10% of total cell protein as revealed by one dimensional SDS polyacrylamide gel electrophoresis.
INTRODUCTION

The strategic role of TS in metabolism and cancer chemotherapy has made TS the subject of extensive kinetic, mechanistic, and structural studies [including nuclear magnetic resonance spectroscopy (Byrd et al., 1978) and X-ray crystallography (Hardy et al., 1987; Matthews et al., 1989; Matthews et al., 1990)] during the past two decades. Since many of these studies require tens of milligrams of protein, these investigations have necessarily been performed on TS of bacterial origin, such as that from amethopterin-resistant L. casei, which overproduces TS up to 400-fold (Dunlap et al., 1971). For purposes of conducting detailed biochemical analyses of the mammalian enzyme, the development of a source of mammalian TS which provides quantities of enzyme at least comparable to those of amethopterin-resistant L. casei is a major research objective.

As the first step in solving this problem, 5-fluorodeoxyuridine- or N10-propargyl-5,8-dideazafolic acid-resistant mammalian cell lines were developed and were found to produce 50-100-fold more TS than the corresponding sensitive cell lines (Rossana et al., 1982, Jackman et al., 1986). Unfortunately, the overproducing cell lines are not convenient sources of the required amounts of mammalian TS since they are expensive to culture in the large quantities needed for structural and mechanistic studies, and since TS still represents less than 0.5% of the total cell protein.
Perhaps the best approach to facilitate the production of large amounts of mammalian TS is to construct an expression vector that directs the synthesis of normal mouse TS in an easily and inexpensively cultured host cell. Such a vector would also permit the analysis of the enzyme by protein engineering techniques. In order to express mammalian proteins in bacteria, the following problems must be solved. First, mammalian genes generally contain introns which can not be spliced by bacteria. This has been solved by using cDNAs or synthetic genes without introns. Second, promoters (including both transcriptional and translational control signals) of mammalian genes can not be recognized by the transcription systems of bacteria. Thus, bacterial promoters must be used to direct the transcription and translation of mammalian genes. This is solved by using commercially available bacterial expression vectors or by fusing bacterial promoters to an eucaryotic gene. Third, proteins, with mammalian cell origins, produced in bacteria are generally unmodified posttranslationally and the folding may not be correct. The folding problem can be solved by an in vitro denaturation and renaturation process, while the posttranslational modifications generally do not affect protein functions. The fourth major problem with the recombinant protein synthesis in bacteria is that the presence of these unusual proteins is occasionally harmful or even lethal to the cell. A solution to this problem is to use a transient expression system in which the gene expression is controllable.
I have constructed and expressed in *E. coli* a plasmid that incorporates the full length coding region of mouse TS cDNA downstream of a tac promoter or a T7 phage promoter in the expression vectors, pKK223-3 (de Boer et al., 1983) and pET-3a (Rosenberg et al., 1987). The resulting recombinant mouse TS produced from the expression vector, pETSM, represented 5-10% of total cell protein. The expression system should greatly facilitate the detailed biochemical analysis of the mouse enzyme.

**RESULTS AND DISCUSSION**

*Rationales for choosing expression systems*

The most commonly used expression systems currently available are those of pKK223-3 and its derivatives which can grow in a variety of bacterial hosts, and those of pET-3a and derivatives which need specialized bacterial hosts in which bacterial phage T7 RNA polymerase is expressed (Studier et al., 1986). The pKK223-3 vector was first chosen for the expression of the mouse TS gene in bacteria for the following reasons. First, this vector has a very strong promoter (tac) which consists of the -10 region of the lac UV5 promoter and the -35 region of the trp promoter (de Boer et al., 1983). The hybrid promoter has proved to be better than both trp and lac promoters (de Boer et al., 1983) and is one of the strongest promoters available (Mulligan et al., 1984). In a lac^Q^ host, such as JM105, the tac promoter is repressed by the repressor and can be derepressed by addition of IPTG, and thus is
controllable. Second, it has a very strong rRNA ribosomal RNA transcription terminator which is used to terminate the transcription. It is believed that a strong promoter needs a strong terminator in order to get maximal gene expression (D. Dean, personal communication). Third, it has multicloning sites and is commercially available (from Pharmacia). Finally, it has been used to express a variety of mammalian genes in bacteria.

Another expression vector, pET-3a, was also chosen to express the TS gene because of its unique features. First, the plasmid contains a T7 phage promoter, a Shine-Dalgarno sequence (SD, Shine and Dalgarno, 1975), several cloning sites (including a Ndel site) and a transcriptional termination signal. The promoter, the SD sequence and the terminator are very strong signals which enable the plasmid to express foreign genes efficiently. The utilization of the T7 promoter enables the expression to be highly specific due to the specificity of the promoter. Second, this plasmid needs a special bacterial host, *E. coli* BL21(DE3)pLysS (Studier et al., 1986) which contains a plasmid with the gene for T7 RNA polymerase under the control of the lacUV5 promoter, which is inducible by IPTG. Thus, the host not only provides the specific polymerase needed for the specific expression but also in a controllable manner. In addition, this strain also expresses a low level of T7 lysozyme, which acts not only as a specific inhibitor of T7 RNA polymerase, thus lowering the basal activity of the polymerase that may be present prior to induction with IPTG (Moffatt et al., 1987), but also renders the cells susceptible to lysis under very mild conditions, such as a single freeze-
thaw cycle (Moffatt et al., 1987).

Construction of expression vectors

1. Construction of pKTS and pUTS

The first expression vector constructed was named pKTS using the expression vector pKK223-3. The strategy for the construction is described in Figure 3-1. Most of the mouse TS coding and 3' flanking regions were derived from pTSMG3 (Dewille et al., 1988). The initial portion of the coding region extending from a pseudo EcoRI site (right before the ATG codon) to the first PstI site in the coding region was derived from oligonucleotides which were synthesized in an Applied Biosystems model 380B DNA synthesizer at the University of South Carolina Biochemical Instrument Center (kindly provided by Dr. Dunlap) and were cloned into the pKK223-3. Next, the TS coding region (Pst I partial fragment) was inserted at the PstI site, and the correct orientation was selected. Since pKK223-3 is derived from pBR322, the copy number for the vector is relatively low. In order to optimize the gene expression, the expression cassette (PvuI to Sall) from the pKTS vector was cloned into the plasmid pUC8, which has been shown to have a higher copy number (Miki et al., 1987). The resulting expression vector was named pUTS.
Figure 3-1. Constructions of pKTS.

A pair of oligos were synthesized to cover the N-terminal portion (a pseudoEcoRI and a PstI site flanking the two ends) and were cloned into EcoRI/PstI cut pKK223-3, the rest of the TS gene was derived from the TSMG3 (the PstI partial fragment). The correct orientation was selected by restriction mapping and was named pKTS. DNA sequences of the oligos were as follows.

```
AATTATGCTGGTGTTGGCTCCGCAGCTGCA
TACGACCACCAACCGAGGCTCG
```
Select correct orientation

Figure 3-1
2. Construction of pETS, pETSM, pETSMU, pBTSM.

As mentioned early in this Chapter, the pET-3a expression vector was also used to drive TS gene expression. The expression vectors pETS and pETSM (in which the second codon was changed) were constructed in a similar manner as the construction of pKTS. Described in detail in Figure 3-2 is the construction of pETSM. The initial portion of the coding region extending from a Ndel site (covering the ATG codon) to the first PstI site in the coding region which has an altered Leu second codon and contains a BamHI site as a marker was derived from oligonucleotides which were synthesized in an Applied Biosystems model 380B DNA synthesizer at the OSU Biochemical Instrument Center and were cloned into pUC8. Next, the TS coding region was inserted at the PstI site in two steps. Finally, the 1.15 kB Ndel fragment representing the whole TS coding region was cloned into the Ndel site of pET-3a to yield pETSM (in the correct orientation). The construction of pETS is similar to the pETSM, but the initial part (synthetic oligo) was exactly the same as in the wild type TS gene. The relative low copy number associated with pETSM vector was solved by cloning the whole expression cassette (PvuI to Sall) into pUC8 (yielding pETSMU). pBTSM was constructed by cloning the fragment from Sall/EcoRV cut pETSM, which contains the entire TS coding region and the promoter and terminator, into Sall/EcoRV sites of pBluescript M13(-). This vector was used alternatively
Figure 3-2. Construction of pETSM.

Synthetic oligonucleotides corresponding to the sequence of mouse TS from the ATG codon (Ndel site) to the first PstI site in the coding region were cloned into the corresponding restriction sites of pUC8 to form pSYN. The 0.7 kb PstI fragment of TSMG3 was cloned into the PstI site of pSYN. A plasmid that contained the insert in the correct orientation was identified by restriction analysis. The small (0.5 kb) SphI to EcoRI fragment of this plasmid was replaced by the large (1 kb) SphI to EcoRI fragment from TSMG3. Finally, the 1.15 kb Ndel fragment was cloned into the Ndel site of pET-3a. A plasmid (pETSM) that contained the fragment in the correct orientation was used for subsequent analyses. The following abbreviations are used: B, BamHI; E, EcoRI; G, BglII; H, HindIII; I, ATG initiation codon; N, Ndel; P, PstI; S, SstI; Sp, SphI; T, UAG termination site; lig., ligation; fr., fragment.
pUC8

TATGCTTGTGGTTGGATCCGAGCTGCA
ACGAACACCAACCTAGGCTCG

pSYN

TSMG3

P. 0.7 kb fr.

NP

Sp B B G PE

Sp+E, 3kb fr.

pET-3a

N, 1.15 kb fr.

pETSM

Figure 3-2
to isolate ss DNA for site-directed mutagenesis. Once a mutant was formed, the fragment was cloned into pET-3a to yield a slightly different expression vector, pETSMM, which is identical to pETSM, except that a small EcoRV fragment is deleted.

Effect of IPTG, cell density, and induction time

The expression vector pKTS was constructed and the TS gene expression was monitored and optimized. First, the expression vector was transformed into a variety of bacterial hosts with the lacI<sup>q</sup> genotype. In all cases, before induction by IPTG, a very low background of TS activity was observed as determined by the filter binding assay or TCA assay. The residual TS activity was likely due to leaky expression of the vector and at least in part by the wild type bacteria which were used in this study. TS protein was produced in response to the addition of IPTG, which turns on the tac promoter and leads to the expression of the protein. The IPTG concentrations seemed to have no effect on the expression in the range of 0.1-5 mM. Cell density at which the IPTG was added has little effect on the level of expression. It has been found that differences in the cell density (OD 600 nm) ranging from 0.5-1.0 resulted in similar expression of the protein. However, the induction time was found to be critical. TS level was at its maximum after 2-5 hrs of induction. Induction times longer than 5 hrs decreased TS activity as measured by the FdUMP binding assays (filter binding and TCA). Since the FdUMP binding assay is only useful for fully
active enzyme, it is not known at this time if the low binding activity results from the degradation or the inactivation of the TS activity (e.g. due to the proteolytic degradation or the formation of inclusion bodies). Although the expression vector pKTS responded to the induction of IPTG, the level of the expression was very low. No significant protein band was observed on protein gel (data not shown).

**Effect of Proteases**

The low level expression of TS from pKTS may result from the presence of proteases in bacteria. The proteolytic effect has been observed in several studies to be the major factor leading to the degradation of recombinant proteins (e.g. Charney et al., 1980). Protein degradation may occur two ways, an in vivo degradation of the protein and an in vitro degradation during the purification process. In order to overcome these effects, Lon protease deficient bacteria were used initially to host the expression vector. The Lon strains are deficient in one of the ATP-dependent proteases that are responsible for the destruction of certain proteins. It has also found that the lon mutation does not interfere with the normal growth properties of the bacteria, thus, it should not interfere the gene expression directly. However, the same low level of gene expression was observed when the Lon- bacteria were used as host bacteria. Thus, in vivo degradation is probably not responsible for the low expression.
In order to solve the problem of \textit{in vitro} degradation during the purification process, PMSF, a serine proteinase inhibitor, was added to the extract. 2-3 fold enhancement of the binding specific activity was observed regardless of the concentrations of PMSF (data not shown). Thus, this 2-3 fold enhancement is contributed by the solvent, DMSO, in which the PMSF was dissolved (see next).

\textbf{Effect of DMSO and Triton X-100}

From the PMSF enhancement experiment, DMSO was found to increase the FdUMP binding specificity in the crude extract. In order to further optimize gene expression, Both Triton X-100 and DMSO were used and both were found to increase the binding specificity in the crude extract by a factor of 2-3 fold, as shown in Figure 3-3. Since Triton X-100 and DMSO could be used to solubilize membrane proteins or disrupt inclusion bodies (a common phenomenon when expressing foreign proteins in bacteria), it is reasonable to suspect that the increase in the binding specificity in the crude extract may have resulted from the solubilization and/or stabilization of TS (also see chapter 4 for the effect of Triton X-100).
Figure 3-3. Effect of Triton X-100 and DMSO.

Equal amounts of crude extracts from the pKTS expression system were used for each determination. Triton X-100 or DMSO was added to a final concentration indicated. Water was used to bring the final volumes of all samples same. TS binding specificity was determined by the [6-³H]FdUMP filter binding assay.

(A) Effects of Triton X-100.
(B) Effect of DMSO.
Enhancement (fold)

![Graph A]

Enhancement (fold)

![Graph B]

Figure 3.3

(DMSO (%))

Triton X-100 (%)

0 10 20 30 40 50 60

0 2 4 6 8 10 12
Effect of copy number

Although pKK223-3 is a well used and highly efficient expression vector, low or no expression of foreign proteins in bacteria has been observed in several cases. One of the strategies used to improve the gene expression is to increase the copy number of the vector so that a relative high level of expression can be achieved (Easton and Rownd, 1982). It has been shown that the copy numbers of pBR322-based vectors are not very high, usually 15-28 copies per chromosome (Balbas et al., 1986). In order to determine whether the expression can be enhanced by using a high copy number vector, the whole pKTS expression cassette was cloned into pUC8 vector, which has been shown to give up to 400 copies of the plasmid per chromosome (Miki et al., 1987). When both expression vectors were transformed into a variety of bacterial hosts to express TS protein at identical conditions except that the bacteria containing pKTS were cultured at 37 °C while the bacteria containing pUTS were cultured at 42 °C. When the expression based on pKTS and pUTS were compared, there was no difference with respect to the binding specificity as measured by the filter binding assay (Table 3-1, pUTS vs pKTS). The same conclusion was reached when the expression of the low copy number vector, pETSM, (see later) and high copy number pETSMU were compared (Table 3-1, pETSM vs pETSMU), indicating that the copy number is probably not responsible for the low expression associated with the pKTS vector. However, since the experiment
was performed at 42 °C, the possibility that TS protein is inactivated at a higher temperature can not be ruled out.

**Effect of promoter**

One of the major factors affecting gene expression is the strength of a promoter. Many sequences upstream of the ATG codon have been shown to have dramatic effects on the gene expression in bacteria. Examples include the -10, -35, SD sequences, the spacers separating them, the spacer sequences, and the potential secondary structure in the mRNA level which may hamper translation. In this study, the low expression of TS protein based on the tac promoter in pKTS and pUTS could not be explained by any of the above factors because the expression vector, pKK223-3 is commercially available and the promoter region has been optimized. Thus, a totally different expression vector, pET-3a, was used. As mentioned before, this vector contains a T7 phage promoter, a T7 phage terminator, and is also widely used to express foreign proteins in bacteria. Based on the vector pET-3a, a third expression vector pETS was constructed. The vector was transformed into bacteria BL21(D3)pLysS for expression. The host bacterial cells were wild type with regard to TS. Preliminary analyses indicated that the vector stimulated the synthesis of mouse TS in the bacterial cells following IPTG induction. However, even under optimal conditions, the level of expression was still low. It was not possible to detect a significant protein band that corresponded to mouse TS when extracts were analyzed by
1-dimensional SDS polyacrylamide gel electrophoresis (see Figure 3-5). However, when compared with the pKTS expression vector, expression was generally about 30 fold higher (Table 3-1, pKTS vs pETS).

Effect of second codon

Since the sequence upstream of the ATG initiation codon is derived from the original vector and thus was not altered during the construction, it was likely that the low level expression was the result of a problem in the coding or 3' flanking sequences. It has been well documented that the DNA sequences encoding the N-terminus of protein can have a dramatic effect on gene expression (Sharp and Li, 1986; Ernst, 1988). The second codon was shown to have some effect on the efficiency of mRNA translation in bacterial cells (Looman et al., 1987). However, the second codon in normal TS mRNA (CUG) is by far the most common leucine codon in E. coli (Ikemura et al., 1985, Sharp and Li., 1986). In spite of this, a mRNA that has CUG as the second codon is translated about 6 times less efficiently than the same mRNA with a different leucine codon (such as CUU) at that position (Looman et al., 1987). Although the mechanism for this observation has not been established, it is possible that the interaction of different isoacceptor tRNAs with the A site of the ribosome can differ considerably during the initiation versus the elongation reactions of protein synthesis. An alternative explanation has been proposed that there is another interaction site between the first 21 nucleotides in a bacterial mRNA and the 16S rRNA (Petersen et
al., 1988). In the TS gene, there is only one perfect match sequence near the end of the first 21 nucleotides. However CUU, as in the pETSM as the second codon, could form a perfect match with the 16 sRNA. Thus, use of an alternative second codon may increase the level of TS gene expression.

To determine if alteration of the second leucine codon from CUG to CUU would lead to a significant increase in the production of TS in bacterial cells, the pETSM expression vector described in Figure 3-2 was constructed. In this vector, the second codon CUU (leu) was changed into CUG (leu).

To measure the expression of the constructed ts gene in bacterial cells, pETSM was transformed into BL21(DE3)pLysS. Cells were grown in liquid culture until the A_{600} reached 0.5, IPTG was then added to stimulate TS production. Cells were harvested at various times and the TS enzyme level was determined by the TCA ternary complex assay. Figure 3-4 shows that in the absence of IPTG, the TS enzyme level was very low. The small amount of activity detected corresponded to the E. coli TS present in the host cells. However, TS enzyme level increased rapidly following addition of IPTG and reached a maximum about 2-5 hr after induction (in the late log phase of bacterial growth). The maximum TS enzyme level achieved by pETSM in the induced cells was about 17 times greater than that observed with pETS (Table 3-1, pETSM vs pETS), which is even larger than the increase reported by Looman et al., (1987).
To determine if TS was an abundant protein in the induced bacteria, cell extracts were prepared from uninduced and induced cells that contained pETSM as well as from induced cells that contained pETS. One-dimensional SDS polyacrylamide gel electrophoretic analysis of the extracts (Figure 3-5) demonstrated that there was one abundant protein band in the induced cells that contained pETSM but not in the uninduced cells. Densitometric scanning of the gel revealed that TS represented about 5-10% of total cell protein.
Figure 3-4. Induction of TS expression with IPTG.

Bacteria containing pETSM were grown in liquid culture until the absorbance at 600 nm reached 0.5 at which IPTG (at a final concentration of 0.5 mM) was added. Samples were withdrawn at the indicated times for determinations of cell density (OD, 600nm) and the TS enzyme level (cpm, [3H]FdUMP bound). Equal amounts of protein were assayed to determine the enzyme level at each time point.
Figure 3-4
Figure 3-5. Electrophoretic analysis of bacterial cell extracts.

80 μg of total bacterial cell proteins from induced or uninduced cells, prepared by a cycle of freezing and thawing, followed by a 2 min sonication, were analyzed by SDS polyacrylamide gel electrophoresis.

Lane A: uninduced cells that contained pETSM;
Lane B: induced cells that contained pETS;
Lane C: induced cells that contained pETSM;
Lane D: 10 μg of TS purified by affinity chromatography (on 10-formyl-5,8-dideazafolate-ethyl-Sepharose) from induced cells that contained pETSM.
Figure 3-5
Table 3-1  Comparison of TS expression vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>cpm/ug</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109 TS-</td>
<td>&lt;0.06</td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>pKTS</td>
<td>15</td>
<td>&lt;0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pUTS</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>pETS</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>pETSM</td>
<td>8000</td>
<td>5-20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pETSMU</td>
<td>8000</td>
<td></td>
</tr>
<tr>
<td>LU 3-7</td>
<td>250</td>
<td>0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by theoretical calculation in which the amount of TS is calculated based on the activity and the specific activity.

<sup>b</sup> Measured by scanning protein gels.
Chapter IV

Purification and Characterization of Mouse Recombinant Thymidylate Synthase

SUMMARY

Two rapid and efficient purification procedures were developed to purify recombinant mouse thymidylate synthase that was expressed at high levels in E. coli. The properties of the recombinant protein were investigated and the following conclusions were made: (1) The recombinant TS had similar properties as the enzyme from a mouse cell line, LU 3-7, from which the original cDNA was synthesized, as judged by kinetic parameters and molecular weight determination. (2) It was found that both Triton X-100 and glycerol were required to stabilize the enzymatic activity. (3) Maximal binding ratios for the covalent binary complexes of dUMP and FdUMP were found in a narrow range centered at pH 7.0. The pH profiles for the enzyme activity and the formation of the covalent complex exhibited a broad optimum ranging from pH 5.5 to pH 8.0. (4) Scatchard analysis of covalent binary complex formation gave a dissociation constant $K_d$ of about 26 $\mu$M and a binding ratio of 0.38 for dUMP and a $K_d$ of about 9 $\mu$M and a binding stoichiometry of 0.65 for FdUMP. (5) The extent of covalent binding of
FdUMP was substantially enhanced by the presence of $H_4$ folate and $CH_2H_4$ folate. The effect of temperature on the TS activity was determined. The activation energy for catalysis was determined to be about 25 KJ/mol ($6$ kcal/mol). The UV/VIS spectrum of the enzyme exhibited a maximum at 280 nm, with a molar extinction coefficient of $117,000\text{ M}^{-1}\text{cm}^{-1}$.

**INTRODUCTION**

Thymidylate synthases of bacterial origin are well studied both kinetically and structurally due to the availability of large amounts of pure protein. Limited studies of mammalian thymidylate synthases have shown that some of the properties of bacterial TS are the same as those of mammalian enzymes. Since TS is a target for cancer chemotherapy, it is important to study the properties of mammalian TS so that improved drugs can be developed for human cancers. However, the investigation of mammalian TS has been hampered due to the relatively low levels of TS protein and/or the labile nature of the protein.

Recently, Davisson et al., (1989) have constructed an expression vector which directs the synthesis of human TS in *E. coli*. The human enzyme represents about 1.6% of the soluble protein in the bacteria. In Chapter 3, I have established a high level expression system to direct the expression of mouse cDNA in *E. coli*. TS protein produced in this system represented about 10% of total cell protein. Thus, this expression system provides a convenient system to study mammalian TS. In addition, such a system will permit the
introduction of specific alterations into the primary structure of the enzyme by protein engineering techniques.

In this Chapter, several properties of the recombinant mouse TS were studied. I have: compared the properties of the recombinant TS produced from bacteria and the authentic TS; developed facile methods for the purification of 10-20 mg quantities of mouse recombinant TS; determined the optimum pH for catalytic activity as well as nucleotide complex formation; analyzed the enzyme (free and complexed with substrates and inhibitors) by UV/VIS spectroscopy; measured other parameters which are important to the enzymatic properties. The properties associated with mouse TS were also compared with the those from other sources.

RESULTS AND DISCUSSION

Comparison of recombinant TS with TS from LU 3-7

One of the common problems associated with gene expression of mammalian proteins in bacteria is the incorrect folding of proteins and subsequently the degradation of the proteins by bacterial host proteases (Charney et al., 1980). Thus, if possible, protein produced by this method must be first compared with the protein produced from the mammalian sources. In order to confirm that the recombinant TS has the same or similar properties as the TS from a mouse cell line LU 3-7 from which the mouse cDNA was synthesized, several parameters were considered.
First, the molecular weights of the recombinant enzyme and that of TS from mouse cells were compared. Extracts prepared from the induced bacteria and from mouse LU3-7 cells (a TS overproducing cell line derived from mouse 3T6 fibroblasts) were incubated with \(^{3}\text{H}\)FdUMP under conditions that would allow the formation of a covalent inhibitory complex. Following one-dimensional SDS polyacrylamide gel electrophoresis, the location of the labeled protein in the gel was determined by fluorography. The electrophoretic mobility (and thus the molecular weight) of the recombinant enzyme synthesized by the bacterial cells was indistinguishable from that of the enzyme synthesized by the mouse cells (Figure 4-1).

Next, some of the kinetic parameters of the recombinant enzyme and of the enzyme from the mouse LU 3-7 extract were compared. The activity was measured by using the spectrophotometric assay for the recombinant TS since large amounts of protein were available, and the tritium release assay for mouse extract due to the sensitivity of the method. The apparent \(K_m\) values for dUMP and CH\(_2\)H\(_4\) folate were determined using recombinant mouse TS purified by the conventional chromatography procedure (see later) and TS from unfractionated extracts from mouse LU3-7. The values for the recombinant TS were found to be 10.5 ± 1.5 \(\mu\)M and 22 ± 2 \(\mu\)M, respectively using the spectrophotometric assay. These values are very close to those determined using unfractionated extracts from mouse LU3-7 cells when assayed by the tritium release assay (13 ± 0.5 \(\mu\)M and 27 ± 0.6 \(\mu\)M, respectively) and are very similar to those determined for TS isolated
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<td>4</td>
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**Figure 4-1** Molecular weight comparison of wild type and recombinant mouse TS.

The ternary complex between TS, [6-³H]-FdUMP and methylenetetrahydrofolate was formed using affinity purified recombinant mouse TS or crude extracts from bacteria transformed with pETSM or crude extracts from cultured mouse LU3-7 cells as the source of enzyme. 25,000 cpm of complex was analyzed by SDS polyacrylamide gel electrophoresis followed by fluorography. Lane 1, pure recombinant mouse TS; lane 2, a mixture of equal amounts (cpm) of pure recombinant TS and TS from LU3-7 cells; lane 3, 4, TS from LU 3-7 cells; lane 5, a mixture of equal amounts (cpm) of TS from unfractionated bacterial and LU 3-7 cell extracts; lane 6, TS from the bacterial extract.
from other organisms. Using the tritium release assay, the \( K_i \) for FdUMP was about 2 nM for both the mouse enzyme from LU3-7 cells and for the recombinant enzyme isolated from \textit{E. coli} cells. Thus, it appears that except that the N-terminal amino acid of the mouse TS is blocked (Perryman et al., 1986), the recombinant TS seems to be reflecting the authentic TS in mouse LU3-7.

**Purification of recombinant TS**

The high level expression of the mouse TS gene in bacteria made the purification process much easier. However, it was found that pure protein could not be obtained without affinity chromatography. Several conventional column chromatography procedures including, Hydroxylapatite (widely used in bacterial TS purification), Phenyl Sepharose, CM-cellulose, Cibacron Blue agarose (used for bacterial TS purification), were used to purify the recombinant mouse TS under standard conditions. TS was not retained in any of these columns under the condition I employed (data not shown). Thus, other alternative large scale purification schemes were developed.

1. Using the Pharmacia FPLC system, a rapid (24 hr) and efficient purification procedure was devised that involved fractionation of the cell-free extract on DEAE-Trisacryl followed by chromatography on Q-Sepharose (Figure 4-2). This sequence was selected for two reasons. First, since TS had moderate affinity for DEAE-Trisacryl, it was readily fractionated from those proteins which bound tightly to the resin as well as those proteins
Figure 4-2. Chromatograms of DEAE-Trisacryl and Q-Sepharose.

(A). Cell free extract was loaded to a DEAE-Trisacryl column and the experiment was performed on a FPLC machine. Only part of the elution profile is presented. Only 10 μl of the sample was used to measure the amount of protein (A594nm) in a final volume of 1 ml by the Bradford assay.

(B). The fractions containing TS activity from the DEAE-Trisacryl column were loaded directed on a Q-Sepharose column and fractionation was performed on a FPLC machine. Only part of the elution profile is presented.

In both cases, 10 ml of elution was collected per fraction. Activity was measured by the spectrophotometric assay (A340nm, •). Protein concentration (••).
Figure 4-2

A

Protein (mg/ml)

Activity (A340nm)

Fraction Number

B

Protein (mg/ml)

Activity (A340nm/min)

Fraction Number
Table 4-1 Large scale purification process.

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Protein (mol)</th>
<th>Vol. (ml)</th>
<th>TS units (umol/min)</th>
<th>Sp. Activ. (units/mol)</th>
<th>Yield (%)</th>
<th>Purif. (fold)</th>
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<tr>
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<td>C.F.E.</td>
<td>205.4</td>
<td>58</td>
<td>15.4</td>
<td>0.075</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>DEAE-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trisacryl</td>
<td>30.8</td>
<td>44</td>
<td>12.4</td>
<td>0.40</td>
<td>81</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>Q-column</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>0.48</td>
<td>39</td>
<td>6.4</td>
</tr>
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<td></td>
<td>Pool 2</td>
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<td>20</td>
<td>6.4</td>
<td>0.86</td>
<td>42</td>
<td>11.5</td>
</tr>
<tr>
<td>3'</td>
<td>Affinity chromatography</td>
<td>12.2</td>
<td>--</td>
<td>11.2</td>
<td>0.92</td>
<td>73</td>
<td>12.2</td>
</tr>
</tbody>
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which bound weakly or not at all. Second, since TS was eluted from DEAE-Trisacryl by 50 mM NaCl, it could be loaded directly on Q-Sepharose, thus avoiding a time-consuming dialysis step. TS was eluted from the Q-Sepharose column at 120 mM NaCl. As shown in Table 4-1 and Figure 4-3, TS of the highest specific activity (>0.8 units/mg) was recovered with an overall yield of 42% in the later eluting fractions (pool 2) of the major protein peak from the Q-Sepharose column. As shown in lane 3 of Figure 4-3, this enzyme fraction exhibited >95% purity when subjected to native polyacrylamide gel electrophoresis. Pool 1 from the Q-Sepharose column (0.3-0.8 units/mg) was about 50% pure and contained 39% of the original TS activity present in the cell-free extract. Therefore the overall yield after this column was greater than 80%. Pool 1 can be rechromatographed on Q-Sepharose to yield more highly purified TS.

2. Affinity chromatography on 10-formylfolate resin. The conventional chromatography procedure was intentionally developed for later studies of mutant forms of the recombinant mouse enzyme, which may not behave predictably on affinity chromatography. Nevertheless, a purification scheme based on affinity chromatography was also developed. It was found that enzyme from the DEAE-Trisacryl column can be successfully purified to apparent homogeneity by affinity chromatography on 10-formylfolate aminoethyl Sepharose. The affinity-purified enzyme exhibited a specific activity of 0.92 units/mg (Table 4-1), which is higher than the specific activities of TS purified from most mammalian sources (Dolnick and Cheng,
1977; Rode et al., 1980; Davisson et al., 1989), but is lower than that of the enzyme purified from human leukemic cells (Lockshin et al., 1979) while comparable to that of enzyme isolated from FdUMP-resistant mouse Ehrlich ascites carcinoma (Jastreboff et al., 1983).

The purification procedures used to isolate TS from other mammalian sources have yielded small amounts (usually < 1 mg) of pure TS after a multistep purification and/or a single affinity chromatography step. The conventional and affinity chromatography procedures developed here are faster (as little as 6 hrs) and have substantially higher yields (10 to 20 mg of pure TS from 2 g of bacteria containing pETSM). Recently, a system yielding moderate expression of the human enzyme in bacterial cells (Davisson et al., 1989) has been developed. However, a six-step purification procedure (81-fold purification with 41% recovery of enzyme activity) was required to obtain 3.7 mg of pure enzyme from a cell free extract containing 631 mg of protein (Davisson et al., 1989).

3. Affinity chromatography on dideazafolate resin. This procedure was developed to purify protein by open column chromatography because the above affinity procedure did not consistently give pure protein when used for open column chromatography instead of FPLC. This procedure consisted of an ammonium sulfate cut, a DEAE-cellulose column, and a dediazafo late Sepharose affinity column (Rode et al., 1979). Although the yield was relatively low, this combination resulted in apparent homogeneity (as shown in lane D of Figure 3-6).
**Electrophoretic analyses of purified TS**

The electrophoretic behavior of the recombinant mouse TS was compared with that of TS purified from *L. casei* and *E. coli* on the same native gel as shown in Figure 4-3. The mobility of the mouse enzyme was significantly slower than that of either bacterial TS. This observation is interesting in view of the fact that the size of mouse TS (307 amino acids) (Perryman et al., 1986) is intermediate between that of the *L. casei* enzyme (316 amino acids) (Maley et al., 1979a) and the *E. coli* enzyme (264 amino acids) (Belfort et al., 1983).

Analysis of these three enzymes by SDS-gel electrophoresis confirmed that the molecular weight of the recombinant mouse TS was smaller than that of the *L. casei* enzyme but larger than that of the *E. coli* enzyme (data not shown). These observations suggest that there may be important charge and/or structural differences between the mouse and bacterial enzymes. The above data also substantiate that we have purified the recombinant mouse TS without (detectable) contamination by the host *E. coli* enzyme.

Previous studies with TS isolated from bacteria revealed that TS, FdUMP and cofactor interact to form inhibitory ternary complexes [Form II (1:1:1) and Form III (1:2:2)] which exhibit greater mobilities on native gels than the free enzyme (Form I) (Aull et al., 1974b). Comparison of lanes 3 and 6 in Figure 4-3 indicates that the mouse enzyme also forms a ternary
Figure 4-3. Native gel electrophoresis of TS.

Protein samples were subjected to electrophoresis on a native gradient PhastGel (Pharmacia). About 1 \( \mu l \) of proteins were loaded.

Lane 1: cell free extract;
Lane 2: after DEAE-Trisacryl;
Lane 3: after Q-Sepharose;
Lane 4, *L. casei* TS (kindly provided by J. Zapf, Univ. of So. Carolina);
Lane 5: *E. coli* TS (kindly provided by J. Zapf, Univ. of So. Carolina);
Lane 6: inhibitory ternary complex between recombinant mouse TS (after Q-Sepharose), cofactor and FdUMP.
complex which migrates more rapidly than the native enzyme. Furthermore, since all of the free enzyme was converted to the ternary complex (lane 6, Figure 4-3), it is apparent that all of the recombinant TS was capable of binding substrates and that no proteins with the same electrophoretic mobility as the free enzyme had been copurified with the recombinant enzyme.

**TS stability**

Originally, TS was purified without Triton X-100 or glycerol. TS was very unstable under these conditions. It was found that both Triton X-100 and glycerol were needed to stabilize the mouse recombinant TS. In the presence of 0.1% Triton and 10% glycerol, mouse TS showed no loss of activity when stored for several weeks in Lysis buffer at -20 °C (Figure 4-4) and 4 °C (data not shown). However, when either Triton X-100 or glycerol was removed from the Lysis buffer, the enzyme was much less stable. For example, about 50% of the enzyme activity was lost within 5 days in both cases. Use of Triton X-100 and/or glycerol has recently been applied in the purification protocol of TS from mammalian sources. In fact, neutral detergents, such as Triton X-100, have been shown to stabilize TS isolated from mouse L1210 cells (Rode et al., 1979). Perhaps, the labile nature of mammalian TS reported in the early studies (Fridland et al., 1971; Gupta and Meldrum, 1971; Horinishi and Greenberg, 1972; Jastreboff et al., 1982) may be explained by the absence of Triton X-100 or glycerol during the purification process. Furthermore, TS purified from several mammalian sources was reported to associate tightly
Figure 4.4  Stability of TS at -20 °C.

Newly purified enzyme in the presence of 0.1% Triton (curve a) was made in 10% glycerol (curve b), 7.5 M ammonium sulfate (curve c), and was stored at -20 °C. At indicated time point (days), the frozen aliquot was thawed on ice and the activity was measured by spectrophotometric assay.
with hydrophobic adsorbents (Slavik et al., 1976; Davisson et al., 1989).

The strong interaction between the enzyme and various hydrophobic adsorbents suggested that there may be significant hydrophobic regions in the protein. When the hydrophobic plot of Kite and Doolittle (1982) was applied to the amino acid sequence of the mouse recombinant TS, most of the primary structure was found to be hydrophilic. However, two strong hydrophobic regions were observed. One is from Leu 181 to Leu 203, which covers the nucleotide binding site of TS. The other region covers Leu 215 to Ile 234. The significance of these two hydrophobic regions is not clear at this point. It is clear that the nucleotide binding site is exposed in the native, uncomplexed state.

**pH and buffer effect on activity**

As shown in Figure 4-5, recombinant mouse TS was active over a broad pH range. Activity was at a maximum between pH 5.5 and 6.0, and gradually declined to 70% of its maximum value at pH 8.5. Activity declined sharply at pH values less than 5.5. When the enzyme was preincubated at pH 5.0, 5.5, 6.0, 7.0, and 7.5 at 25°C for 30 min, the same activities were observed with enzyme that was not preincubated. However, preincubation of TS in acetate buffer pH 4 even for 2 min completely inactivated the TS activity. The inactivation of TS in acetate buffer pH 4.5 was a relative slow process, which
Figure 4-5. Effects of pH on TS catalytic activity.

The experiment was performed at 20 °C as described in Materials and Methods by the spectrophotometric assay. In each measurement, 100 μl of TS purified by conventional chromatography was used in different buffers. At least two determinations for one particular pH for one buffer. There was no preincubation at a particular pH before measuring the activity.
took 30 min. Apparently, the decrease in activity when pH was below 5.5 was caused by denaturation. These results compare favorably with those for human TS which functions in a broad pH range from 6.9 to 8.8 (Dolnick and Cheng, 1977) while enzymes from other mammalian sources have yielded narrower pH ranges for their activities (Gupta and Meldrum, 1971; Horinushi and Greenberg, 1972). The $pK_a$ obtained from the curve was about 4.7, which suggested the involvement of $\alpha$-carboxyl(s) ($pK_a$ 3.0), aspartyl carboxyl(s) ($pK_a$ 3.9), or glutamyl carboxyl(s) ($pK_a$ 4.1), or histidine imidazole(s) ($pK_a$ 6.0) in the catalytic or substrate binding process. However, the results of pH studies must be treated cautiously, since the $V_{max}$ or more accurately the $k_{cat}$, may be a composite function of several rate constants, not all of which may be affected by pH to the same extent (Fersht, 1985). Besides, the microenvironment can severely alter the apparent $pK_a$ (e.g. Sierks et al., 1990), especially in the case of TS, which has been shown to undergo large conformational changes during the catalytic process.

**pH and buffer effect on covalent TS complexes**

TS catalyzes the formation of thymidylate and dihydrofolate from dUMP and cofactor through an ordered bi-bi mechanism. In the absence of cofactor, dUMP and FdUMP (a mechanism based inhibitor) can covalently bind to the enzyme, while in the presence of cofactor, FdUMP can form an inhibitory ternary complex with the enzyme. This ternary complex is
Figure 4-6. Effect of pH and buffer on covalent nucleotide binding.

(A) dUMP binary complex; the reaction mixture contained 0.108 μM TS, and 6.12 μM dUMP.
(B) FdUMP binary complex; the reaction contained 0.173 μM TS and 6.88 μM FdUMP.
(C) FdUMP-CH₂H₄folate-TS ternary complex; the reaction mixture contained 0.108 μM TS, 1.858 μM FdUMP and 80 μM CH₂H₄folate.

Buffers used were: acetate (□); citrate (◊); Tris (□); phosphate (◊); HEPES (∞); and bis-Tris (◊). The TCA assay was used to measure the extent of covalent binding stoichiometries.
thought to be the "capture" of the catalytic ternary complex. Thus, by studying the conditions affecting the formation of the complex, useful information about the substrate binding can be obtained. The maximum stoichiometry for the formation of covalent binary complexes between TS and dUMP (Figure 4-6A) or FdUMP (Figure 4-6B) occurred over a relatively narrow range centered at pH 7.0. In both cases, covalent nucleotide binding was highly dependent on the nature of the buffer. Maximum binding of FdUMP occurred in Tris and Bis-Tris buffers while maximum dUMP binding occurred in Tris buffer. As expected, phosphate buffer partially inhibited the formation of binary complexes presumably by interacting with the side chains of arginine residues at the nucleotide binding site (Lockshin and Danenberg, 1981). Since it has been shown that the nucleotide binds enzyme both covalently and noncovalently in an equilibrium, it is not known that whether there is any noncovalent binding in the pH area where covalent binding is undetectable.

Figure 4-6C shows the effects of pH and buffer composition on the formation of FdUMP-CH₂H₄folate-enzyme ternary complexes. The results are in marked contrast to those for the binary complexes. Between pH 6 and 9, the binding ratios showed little dependence on either pH or buffer composition, though phosphate buffer did yield somewhat lower values. At pH values lower than 5.5, the extent of covalent inhibitory ternary complex formation decreased precipitously, but in a manner which was highly dependent on the nature of the buffer. In many ways, this segment of the
profile is similar in shape to the same region in the pH activity profile (Figure 4-5). Remarkably, the mouse enzyme is capable of forming covalent ternary complexes as well as catalyzing the complete reaction (at a low rate) even at pH 4.5. When the FdUMP binary and ternary complex formations were compared, some interesting observations were obtained. First, the results indicate that there is no covalent FdUMP binary binding at pH 4.5 while the covalent FdUMP binding in the presence of cofactor (ternary binding) at the same pH gives a binding stoichiometry of about 1.6. Thus, it appears that the cofactor may also bind to the enzyme first to open the binding site for the nucleotide. This is clearly contradictory to the previous observation that cofactor can not bind the enzyme without nucleotide binding. Another explanation is that there is some noncovalent nucleotide binding at pH 4.5, the binding of the cofactor somehow changes the protein structural conformation so that the pH effect is overcome and the covalent nucleotide binding is enhanced. The effect of cofactor will be further discussed later.

**Nucleotide concentration effect on binding ratio**

As shown in Figure 4-7, under the optimum pH and buffer, at least a 50-fold excess of FdUMP relative to enzyme was necessary to reach maximum covalent binding in an enzyme-nucleotide binary complex while maximum covalent binding of dUMP required substantially higher ratios of dUMP relative to the enzyme. Scatchard analysis of these data gave a $K_d$ of 26 μM for the covalent binding of dUMP and a binding ratio of 0.38. The $K_d$ for the
Figure 4-7. Nucleotide titration of TS.

The reaction mixture (0.5 ml) contained about 0.1 μM of TS and varying concentrations of nucleotide (dUMP or FdUMP), in Bis-Tris buffer, pH 7.0 for the measurements of binary complexes for dUMP (c) or FdUMP (b). The formation of the inhibitory ternary complex (a), FdUMP-CH_2H_4folate-TS, was measured in Tris buffer, pH 7.0 in the presence of 80 μM methylenetetrahydrofolate. All reactions were incubated at 37 °C for 30 min before stopping the reactions by TCA as described in TCA assay method.
covalent binary complex with FdUMP was 9 μM and the binding stoichiometry was 0.65. Also shown is the titration curve for the inhibitory ternary complex which attained a maximum binding ratio of 1.5 at a 5 fold excess of FdUMP (in the presence of 80 μM cofactor).

Folate effect on activity and nucleotide binding

CH₂H₄folate is not only required as a substrate, but also appears to have one more function: enhancement of the covalent binding of nucleotides. In the case of FdUMP covalent binding, the presence of cofactor not only increased the covalent nucleotide binding ratio from 0.7 to 1.5 (Figure 4-7) at optimum pH and buffer, but also dramatically broadened the pH range for maximum binding (compare Figure 4-6B and 4-6C).

It has long been established that thymidylate synthase first binds to the nucleotide. The binding of dUMP is believed to change the conformation of enzyme to open the folate binding site (because folate can not bind the enzyme without dUMP). The binding of cofactor, in turn, could stimulate the covalent nucleotide binding, as revealed by the enhanced covalent binding stoichiometry. In this study, the covalent nucleotide binding at pH 5 was basically undetectable, which may suggest that either the covalent nucleotide binding is not necessarily required for the opening of the folate binding sites or the folate binding sites are open at this pH. Other folate derivatives such as H₄folate also showed the enhancement of FdUMP covalent binary complex (a final binding ratio of 1.5) and dUMP covalent binary complex (final
binding ratio of 0.8). Thus, it appears that the binding of folate introduces a conformational change which enhances the covalent nucleotide binding. Since covalent nucleotide binding is critical to catalysis, the cofactor not only provides the one carbon transfer, but also enhances the enzyme reaction itself presumably by changing the enzyme conformation to a form which is more suitable for the binding of both substrates (Matthews et al., 1990).

**Temperature effect on enzyme activity**

The effect of temperature on enzyme activity was examined by the spectrophotometric assay. The enzyme and the nucleotide substrate (dUMP) were incubated at the experimental temperatures for 15 min before triggering the reaction by adding the cofactor. As shown in Figure 4-8(A), TS was inactivated at temperature higher than 44 °C. In order to determine the activation energy of the reaction, the data from Figure 4-8(A) were used for the Arrhenius plot as shown in Figure 4-8(B). The activation energy was calculated to be 25 KJ/mol (6 kcal/mol). This result differed from the observations made thymidylate synthases from human leukemia, Hela cells, and E.coli where it was found that the Arrhenius plots gave biphasic curves with a transitional temperature around 35 °C (Dolnick and Cheng, 1977; Rode et al., 1980; Haertle et al., 1979; Jastreboff et al., 1983). Thus, the temperature-dependent conformational change at 35 °C observed in human and bacterial TS was not observed with the mouse TS.
Figure 4-8. Effect of temperature on TS activity.

All components except cofactor were incubated at the specified temperatures for 15 min, and the activity was measured by the spectrophotometric assay (A). The data were used to calculate the activation energy as shown in (B). Different symbols represent two different experiments.
UV/VIS spectra of native TS and FdUMP ternary complex

Determination of the UV/VIS spectrum of native TS purified in the presence of Triton X-100 was prevented due to a large absorption peak centered at 274 nm, which is attributed to the neutral detergent. In order to examine the UV/VIS spectrum of the enzyme, it was necessary to obtain enzyme that was free of Triton X-100. At first, an attempt was made to remove Triton X-100 from the enzyme by chromatography on a BioRad Econo-Pac 10 adsorbent column packed with CM-2 Bio-Beads. Only 25% of the protein loaded on the column was recovered, but the absorption spectrum obtained was that of a simple protein, featuring a maximal absorbance at 280 nm. When the native enzyme was purified in the absence of Triton X-100, the absorption spectrum (Figure 4-9, dashed curve) was similar to that of a pure protein. There was no indication of a tryptophan shoulder at 290 nm, as was observed in bacterial proteins (Lyon et al., 1975). Based on the absorbance at 280 nm at several concentrations of protein, the molar extinction coefficient for mouse recombinant TS was calculated to be 117,000±9%. Also shown in Figure 4-9 is the UV/VIS spectrum of the FdUMP-CH$_2$H$_4$folate-TS inhibitory ternary complexes obtained with TS purified in the absence of Triton X-100 (solid curve). A new peak was observed at 320 nm which is known to be characteristic of the inhibitory ternary complexes formed with the enzyme isolated from L. casei (Donato et al., 1976), with A$_{320nm}$/A$_{280nm}$ about 0.5.
Figure 4-9. UV spectra of native TS and FdUMP ternary complex.

About 4 μM TS (1 ml in each determination) purified in the absence of Triton X-100 was used for obtaining the UV/VIS spectrum of the native protein (dashed curve). After which, the FdUMP inhibitory ternary complex was formed, isolated, and the UV/VIS spectrum was obtained (solid curve). In both cases, Lysis Buffer was used as the blank.
Chapter V

Site Directed Mutagenesis of Thymidylate Synthase: Arg-44 to Val-
44 Mutant Produces Multiple Effects on Catalysis and Binding

SUMMARY

The arginine residue located at position 44 of mouse thymidylate synthase is in a highly conserved loop that is in close proximity to the active site cleft of the enzyme. This residue has been changed to a variety of amino acid residues by site directed mutagenesis to probe the functions of this highly conserved residue. It was found that the activity of the R44V mutant enzyme was at least 100-fold lower than that of the wild type enzyme, suggesting that this arginine is important for catalysis. The mutant enzyme also showed a much reduced binary binding stoichiometry when compared with the wild type enzyme, showing that the arginine side chain was involved in the covalent nucleotide binding. The involvement of this arginine in maintaining the overall enzyme structure was evidenced by a dramatic change of pH profile on the covalent FdUMP ternary complex formation.
INTRODUCTION

The importance of arginine residues in maintaining bacterial TS activity was originally appreciated by chemical modification experiments, in which arginine specific reagents were used to modify the protein (Cipollo and Dunlap, 1978; 1979; Belfort et al., 1980). It was found that this modification led to the total inactivation of TS. However, the number of arginine residues involved was not clear. In one report, modification of one arginine residue was enough to inactivate the activity (Belfort et al., 1980) while others reported that several arginine residues were involved (Cipollo and Dunlap, 1979). From other experiments, positively charged residues were implicated in catalysis (Dev et al., 1989). Arginine residues could be the ones involved, although the possibility that lysine residues may responsible for can not be excluded. Sequences comparisons revealed that several arginine residues are highly conserved, for example, Arg-44, Arg-170, Arg-209 (Perryman et al., 1986).

The importance of arginine residues was also revealed when the X-ray structures of bacterial thymidylate synthases were solved (Hardy et al., 1987; Matthews et al., 1989; Matthews et al., 1990; see Figure 1-3). Examination of the native, uncomplexed three dimensional structures of the dimeric thymidylate synthases from *L. casei* and *E. coli* revealed several important arginine residues which may be involved in the catalytic pathway. For example, Arg-170 in *L. casei* is one of the residues at the active site which appears to stabilize the phosphate group of dUMP. Even more important is
the observation that this arginine residue comes from the other subunit. Thus, the long proposed intrasubunit communication has proved to be true.

Arg-44 is located in a loop of amino acids residues (located between \(\beta\)-strands A and B, residues 20-26 in \textit{L. casei}, residues 19-24 in \textit{E. coli}), which appears to guard the active site cleft. The fact that these residues and their neighbors exhibit a high degree of homology suggests this rather exposed segment of the amino terminal region of thymidylate synthase plays a non-trivial role in the function of the enzyme. Indeed, the crystal structure of the \textit{E. coli} thymidylate synthase-FdUMP-N10-propargyl-5,8-dideazafolate complex reveals that an extensive hydrogen bonding network exists which ties together this loop and the carboxyl terminus while holding in place the 5' phosphate of FdUMP (Matthews et al., 1990; see Figure 1-3C). In particular, a conserved arginine at position 21 in \textit{E. coli} thymidylate synthase is found to make hydrogen bonds both to the carboxyl terminal \(\alpha\)-carboxylate anion (Ile-264 in \textit{E. coli}) and to the 5' phosphate of FdUMP. Since the C-terminal residues have been shown to be important for \textit{L. casei} thymidylate synthase activity (Aull et al., 1974a, Chapter 6), these hydrogen bond interactions are suggestive of a central role for Arg-21 in the formation of the ternary complex.

Due to the high degree of homology of bacterial and mammalian thymidylate synthases, site-directed mutagenesis was used to introduce amino acid substitutions at the mouse arginine 44, which corresponds to arginine 21 in the \textit{E. coli} thymidylate synthase. The mutant proteins were
purified and their properties were examined. Only the results from the valine substitution are discussed.

RESULTS

Construction of mutants

The mutant coding region for mouse TS in which Arg-44 was replaced was created by site-directed mutagenesis by using the following oligonucleotides: GAAGGAGGACGTCACGGGCACTG for the valine replacement; GAAGGAGGAC(A/G)AAACGGGCACTG for Lys and Glu replacements; GAAGGAGGAC(A/G)(C/G)CACGGGCACTG for Thr, Ala, Ser, Gly replacements; GAAGGAGGACCAAAACGGGCACTG for Gln replacement. All of the mutants except the Gln mutant were constructed by using ss DNA isolated from pRegionI in which Region I was cloned into pBluescript M13(+) (Chapter 2). The mutant DNA was sequenced, cloned back to the expression vector and was further confirmed by ds DNA sequencing. For the Gln construction, ss DNA was isolated from pBTSM, and the mutant DNA was cloned into the expression vector, pET-3a (Chapter 2).

Described in Figure 5-1A is the construction of the valine 44 substitution. The sequences of wild type and mutated coding regions corresponding to residues 40 through 50 of the mouse enzyme are shown in Figure 5-1B. A new Aat II site was created in the mutant DNA.
Figure 5-1. Construction of R44V.

Panel A. A 350 bp PstI to Apal fragment from the coding region of wild type mouse TS was isolated from pTSMG3, subcloned into pBluescribe M13(+) and transformed into E. coli CJ236. ss DNA was isolated and site-directed mutagenesis was performed. The mutants were confirmed by DNA sequencing. A restriction fragment containing the mutated region and bounded by unique Bsu36I and Apal restriction sites was used to replace the corresponding fragment of the wild type expression vector, pETSM, to create the mutant plasmid. The mutant region in the expression vector was further confirmed by ds DNA sequencing and by testing for the presence of the newly created AatII restriction site (GACGTC).

Panel B. The sequences of the wild type and mutated coding regions.
**Figure 5-1**

**A**

- pPBS(+) through mutagenesis
- pETSM
- pTSR44V

**B**

40 lys lys glu asp arg thr gly thr gly thr gly
45 wild type: AAG AAG GAG GAC CGC ACG GCC ACT GCC ACC GGC
50

**Mutant:** AAG AAG GAG GAC GTC ACG GCC ACT GCC ACC GGC

lys lys glu asp val thr gly thr gly thr gly
So the mutant fragment could be monitored by restriction enzyme digestions, and its existence in the expression vector was established by the presence of the newly introduced AatII site (data not shown).

**Purification of mutant TS (R44V)**

In Chapter 2 (Materials and Methods), a purification procedure for the wild type enzyme was described based on an ion-exchange chromatography on DEAE-Trisacryl followed by an affinity chromatography on 10-formylfolate-aminoethyl-Sepharose 4B. This procedure was also used to purify the mutant enzyme. It was found that both the mutant and wild type enzymes behaved similarly during ion exchange chromatography. However, the mutant enzyme bound less tightly than the wild type enzyme to the affinity column. Wild type enzyme was completely retained by the column when washed at 200 mM NaCl, while the mutant enzyme was eluted under these conditions. Since the affinity chromatography procedure is dependent on the formation of a ternary complex between dUMP, enzyme and the 10-formylfolate affiant, these results provided the first indication that the mutant enzyme had a lower affinity for its substrates.

**Total binding stoichiometry**

The lower affinity of R44V mutant TS for its substrates was further confirmed by native gel electrophoresis in which the total binding stoichiometry (covalent plus noncovalent) of FdUMP in the presence of
CH₂H₄folate was determined. After removing excess dUMP and salt by dialysis, the native uncomplexed enzymes were subjected to native gel electrophoresis on a Pharmacia PhastGel system. Both the native wild type and mutant enzymes revealed single bands (Form I) indicating their purity (data not shown). When incubated in the presence of FdUMP and cofactor, both enzymes formed ternary complexes as evidenced by the appearance of two additional, faster migrating bands (Form II and Form III) on the native gel, similar to the situation for TS isolated from bacterial amethopterin-resistant L. casei (Aull et al., 1974b). Previous work with the bacterial enzyme established that Forms II and III are inhibitory ternary complexes of FdUMP, cofactor and TS, with binding stoichiometries of 1:1:1 and 2:2:1, respectively (Aull et al., 1974b). Scanning of the ternary complex lanes yielded 0% Form I, 10% Form II and 90% Form III for the wild type enzyme as compared to 12% Form I, 56% Form II, and 32% Form III for the mutant enzyme (Figure 5-2). The latter pattern was observed consistently after each purification process of the mutant enzyme and did not change during several weeks of storage of the mutant enzyme at 4 °C. These data provided a second indication of the diminished ability of the mutant enzyme to bind ligands.
Figure 5-2. Gel scanning profile of R44V

The mutant enzyme was incubated with a 10 fold molar excess of FdUMP and a 100 fold molar excess of cofactor at room temperature for 5 min to form the inhibitory ternary complex. About 1 μl of the mixture was loaded on a native gradient gel. The gel was stained and destained and was scanned in a densitometer to determine the relative amount of Form I, Form II, and Form III.

(A). A computer printout of the gel scanning.
(B). The relative peak areas calculated by the computer
Figure 5-2
Activity of the mutant enzyme

The ability of the mutant enzyme to catalyze the conversion of substrates into products was assessed by both spectrophotometric and tritium release assays. When concentrations of the mutant enzyme of 5 μM (100 times the usual concentration of wild type enzyme) were employed, activity close to background was detected by the spectrophotometric assay (which measures the formation of dihydrofolate). When the more sensitive tritium release method (which measures the enzyme catalyzed removal of the C-5 proton from [5-^3H]-dUMP) was used, the specific activity (μmoles of tritium released/min/mg) of the mutant enzyme was about 100 fold lower than that of the wild type enzyme.

The lower activity measured from the tritium release assay may result from the exchange of the tritium in dUMP with hydrogens of water (Pogolotti et al., 1979). For example, the 5-H exchange reaction in the Lcasei TS occurs in the absence of any folate and is stimulated by tetrahydrofolate (Pogolotti et al., 1979). The effect is very small in the wild type enzyme. However, when a mutant enzyme is dealt with, the natural exchange reaction may be mistakenly considered to represent the enzyme activity. Thus, further analyses have been performed by our collaborators at Univ. of South Carolina. The results showed that the mutant TS still retained a residual capacity to catalyze the formation of dihydrofolate when very high concentrations of TS were used. Another decisive experiment to show that the enzyme had some activity came from an experiment in which the tritium
labeled product ([6-³H]-TMP) was separated from substrate ([6-³H]-dUMP) by TLC.

**Covalent nucleotide binding**

Titrations of the mutant and wild type enzymes with FdUMP in the presence or absence of CH₂H₄folate and with dUMP alone were performed by the TCA assay, which measures only the covalent binding stoichiometry (Cisneros and Dunlap, 1990). The results are compared in Figure 5-3. The extent of covalent FdUMP binding by the mutant enzyme in both the binary complex and the inhibitory ternary complex with CH₂H₄folate was dramatically reduced as compared with the wild type enzyme. Scatchard analyses of the data shown in Figure 5-3 yielded estimates of the number of covalent FdUMP binding sites in the binary (0.1) and ternary (0.9) complexes for the mutant enzyme, which were substantially lower than for the wild type enzyme (0.7 and 1.6, respectively). Similarly, the binding ratio for dUMP to the mutant enzyme was at least 5-10-fold lower than that for the wild type enzyme.

**Effect of pH on the covalent binding of FdUMP**

When the effect of pH on the binding stoichiometries for the inhibitory ternary complex of mutant and wild type enzymes was compared (Figure 5-4). The results demonstrated that the mutant enzyme exhibited a narrower pH profile for the ternary complex formation with substantially lower covalent binding ratios at each pH. Furthermore, the binding ratios of the inhibitory ternary complex with the mutant enzyme appeared to be much more
Figure 5-3. Nucleotide titrations of both wild and mutant TS.

The reaction mixture (0.5 ml) contained 0.1-0.2 μM TS and varying concentrations of FdUMP for binary complex formation in Bis-Tris buffer pH 7.0 (wild type, panel A) and Tris-Cl buffer pH 7.0 (mutant, panel B). For ternary complex formation, same conditions were used except that CH2H4folate was included at 80 μM. The formation was performed at 37 °C for 30 min and the binding stoichiometry was assayed by TCA assay.
The reaction mixture (0.5 ml) contained about 80 $\mu$M $\text{CH}_2\text{H}_4\text{folate}$, and 0.108 $\mu$M of TS and 1.858 $\mu$M FdUMP for wild type TS (Panel A), or 0.1686 $\mu$M mutant TS and 5.574 $\mu$M FdUMP for mutant TS (Panel B) in 100 mM acetate ( ), citrate ( ), Tris ( ), phosphate ( ), HEPES ( ), and Bis-Tris ( ) buffers. The formation was performed at 37 °C for 30 min and stopped by adding 10% TCA and the binding stoichiometry was assayed by TCA assay.
sensitive to buffer composition and were especially pronounced with acetate, citrate, and Bis-Tris buffers.

**DISCUSSION**

Arg 44 is involved in covalent nucleotide binary binding

The importance of the arginine 44 in maintaining TS activity was hypothesized from the observation of the X-ray structures of the complexed TS, in which the guanidinium group of the arginine 44 forms hydrogen bonds with the free carbonyl group of the C-terminal residue, with the phosphate group of FdUMP, and with the side chain of folate cofactor via a water molecule (Matthews et al., 1990). It was proposed that this hydrogen bonding network happened during or after the ternary complex formation. Thus, it was suspected that the change of the arginine may not have an effect on the covalent nucleotide binary binding. Here it was clearly shown that when the conserved arginine was changed to a valine, the binding stoichiometry of the covalent binary complex of dUMP or FdUMP was severely decreased.

Although it is not clear at this point whether the total binding stoichiometries for the binary complex are changed, it is safe to speculate that the Arg is involved in the covalent binary binding of nucleotides. Thus, I propose that the hydrogen bond formed between the guanidinium group of Arg 44 and the phosphate group of nucleotides exists before the guanidinium group is hydrogen bonded to the carboxyl group of the C-terminal residue. The hydrogen bond may help position the nucleotide and thus provide the correct
geometry for the attack by the sulfhydryl group at the active site.

Arg 44 is involved in catalysis

Although the covalent binary binding stoichiometry of nucleotides were greatly hampered, the covalent binding of FdUMP in the ternary complex was only reduced from 1.6 to 0.9. If the inhibitory ternary complex resembles the real catalytic ternary complex formed between TS, dUMP, and cofactor, I expected that the covalent binding of dUMP in the catalytic ternary complex would not be severely reduced (the real binding stoichiometry can only be obtained by pre-steady state methods).

However, when the activity of the mutant enzyme was compared with that of the wild type enzyme, the activity was decreased at least 100 fold. This suggested that the catalytic process was somehow blocked. There are two possibilities to explain the observed results. First, the breakdown of the ternary complex may be blocked due to an incorrect conformation of protein (which is caused by the inability to form the hydrogen bonding network). The second possibility is that the ternary complex can break down but the release of the products is blocked, although I consider this to be less likely.
Arg 44 is involved in the intersubunit communication

In the presence of cofactor, the binding stoichiometry for FdUMP was reduced from 2 for the wild type TS to 1 for the mutant TS. When the total binding stoichiometry of FdUMP in the ternary complex was determined by native gel electrophoresis, about 12% of the mutant enzyme was not accessible for FdUMP binding at all. This result was very reproducible. Thus, it was not the result of inactive enzyme. Most of the mutant enzymes maintained only one binding site for FdUMP, only 30% of the mutant enzyme maintained 2 binding sites, compared with the wild type enzyme which has a majority with 2 FdUMP binding sites (90%).

From early studies based on kinetics, it has been proposed that the two active sites are not same; one site is not open to the substrate until the other site is occupied by the substrate. Thus, in the wild type enzyme, the binding sites for FdUMP will reach 2 per dimer. In the case of this mutant enzyme, the communication between the subunits is hampered and the majority of the mutant enzyme has lost this cooperative effect. This communication is more noticeable when the pH effects were compared as shown in Figure 5-4. The tolerance of the mutant enzyme to different pHs is narrowed down to a optimum pH around 7.0.
Discussion of conformational change

It is possible that introducing a valine in the conserved loop to replace positively charged arginine residue may have a dramatic effect on the native protein conformation. However, due to the highly flexible nature of the loop, it is difficult to imagine that the replacement will have a big effect on the interior region of the native protein.
Chapter VI

Functional Studies of Carboxyl Terminal Valine Residue of Mouse Recombinant Thymidylate Synthase by Site-Directed Mutagenesis

SUMMARY

Several mutant coding regions were constructed by site-directed mutagenesis in order to probe the functional roles of the carboxyl terminal (C-terminal) residues of the dimeric mouse recombinant thymidylate synthase (TS-MAV). Deletion of the Ala-306 (TS-MV) resulted in an almost functionless enzyme, as judged by the covalent nucleotide binding stoichiometries and the enzyme activity, while addition of an Ala (TS-MAAV) before the valine residue on each monomer decreased the enzyme's ability to bind covalently both 2'-deoxyuridylate (dUMP) and 5-fluoro-2'-deoxyuridylate (FdUMP). By tritium release assay, this mutant showed a 43 fold lower activity than that of wild type. When Val-307 was changed to Glu-307 (TS-MAE), the covalent binding of dUMP was reduced to some extent, while the covalent binding of FdUMP in binary and ternary complex remained similar to that of the wild type. The
catalytic activity to produce dihydrofolate was decreased 13 fold. Changing Val-307 to Gly-307 (TS-MAG) resulted in a reduced covalent binding of both dJUMP and FdUMP and a 42 fold lower activity to catalyze the conversion of 5,10-methylenetetrahydrofolate to dihydrofolate. All the mutant enzymes seemed to have a normal overall (covalent plus noncovalent) stoichiometry for the FdUMP ternary complex. Kinetic studies on the TS-MAG and TS-MAE mutants showed that the hydrophobic side chain at the C-terminus was favored over both hydrogen and hydrophilic side chains. These results suggested that (1) the C-terminal residues are not involved in the noncovalent FdUMP binding in the ternary complex; (2) the C-terminal carboxylate group is involved in the covalent binding of both dUMP and FdUMP; (3) the hydrophobic side group of the C-terminal valine may be involved in hydrophobic interactions.

INTRODUCTION

The first report to show that the C-terminal residue may have some functional role in catalysis came from studies in which TS protein was treated with a variety of carboxypeptidases. The results showed that TS was inactivated by these carboxypeptidases. Surprisingly, it was also revealed that TS was inactivated by the loss of only one of two C-terminal valines per enzyme dimer (Aull et al., 1974a). Further studies suggested that the carboxypeptidase A inactivated TS still had the ability to bind substrates or
FdUMP (Galivan et al., 1976a; Galivan et al., 1977). However, the covalent nucleotide binding was reduced to half of its maximal stoichiometry (R.Dunlap, personal communication). Furthermore, the treated enzyme had a reduced ability to form noncovalent binary complexes with polyglutamyl forms of folate (Galivan et al., 1976a; Galivan et al., 1977).

From these experiments, the relative 3-dimensional position of the C-terminal residue has been hypothesized. The accessibility of the C-terminal residue for carboxypeptidase cleavage suggested that the C-terminal residue in the native enzyme is exposed to the solvent. The fact that the cleavage effect can be protected to some extent when the enzyme has formed an inhibitory ternary complex (Galivan et al., 1976a), suggested that the C-terminus is buried inside once the substrates or substrate analogues are bound. The position change of the C-terminal residue is in line with the observation that there is a major conformational change upon the binding of both substrates. The hypotheses were confirmed later by the X-ray structures of the L. casei TS (Hardy et al., 1987) and the E.coli TS (Matthews et al., 1989) and the structure of E. coli TS complexed with FdUMP and 10-propargyl-5,8-dideazafolate (PDDF) (Matthews et al., 1990) or with FdUMP and cofactor (Matthews, personal communication). Comparison of the X-ray structures indicates that when the substrates or analogues are bound, the enzyme undergoes a conformational change which brings the C-terminal residue and a conserved arginine residue together to form an extensive
hydrogen bonding network between the carboxylate group of the C-terminal amino acid, the phosphate group of FdUMP, the quinazoline of PDDF, a water molecule, and the arginine within a highly conserved loop that guards the mouth of the active site (Matthews et al., 1989; 1990). The importance of this hydrogen bonding network has been discussed in Chapter 5. Detailed comparisons of the uncomplexed, native structure with the complexed structure also revealed that after the substrate binds, the last four C-terminal residues move, up to 6Å in distance, to form hydrogen bonds with the arginine residue, thus capping the active site (Matthews et al., 1990). The conformational change and the subsequent capping of the active site have also been observed in E.coli aspartate transcarbamylase (Kantrowitz and Lipscomb, 1988).

In Chapter 3, a bacterial expression vector which directed mouse cDNA to produce relatively large amounts of mouse recombinant TS in E.coli was constructed. The expression system was also suitable for site-directed mutagenesis as described in Chapter 5. Here, further employment of the expression system to introduce specific mutants in the C-terminal region of mouse TS is described and the function of the C-terminal amino acid residues are proposed.
RESULTS

Construction of mutations

In order to study the importance of the C-terminal amino acid residue of mouse TS enzyme, several altered enzymes were created using the technique of site-directed mutagenesis as described in Figure 6-1. The 0.9 kb Clal-Clal fragment from the expression vector pETSM (Chapter 3), which encodes the C-terminal 25 amino acids of mouse TS and the 3' untranslated region was cloned into a ss DNA-producing vector, pBluescript M13(-). The orientation was checked by restriction digestions. The construct was transformed into bacteria CJ236, from which ss DNA was isolated. Site-directed mutagenesis was performed using the method of Kunkel (1985) by using the following oligos: AAAATGGAAATGGTTTAGAGTGCT for the TS-MV mutation; ATGGAAATGGCTGCAGTTTAGAGTGCT for the TS-MAAV mutation; ATGGAAATGGCT(A/G/T)(A/G)GTAGAGTGCTTTC for the TS-MAG and TS-MAE mutations; ATGGAAATGGCTT(A/C)TTAGAGTGCTTTC for the TS-MAS and TS-MAF, and TS-MAY. In order to facilitate the detection of mutants, a PstI site was introduced into TS-MAAV construction. Subsequent mutagenesis was done by using single strand DNA from the latter mutant as template. Thus, either the formation or loss of the PstI site indicated that a mutated coding region had been formed. The sequences of the mutated
Figure 6-1. Construction of C-terminal mutants.

The 0.9 kb of Clal-Clal fragment from the expression vector pETSM was inserted into a Clal-cut pBluescript M13(-). The recombinant was identified and was in such an orientation that the coding strand was produced as the ss DNA. ss DNA was isolated from CJ236 and site-directed mutagenesis was performed as described in Chapter 2. The mutant was screened by restriction digestions (if applicable) and ss DNA sequencing. The mutated Clal-Clal fragment was finally cloned back to the expression vector to replace the wild type Clal-Clal fragment and the correct orientation was selected. The mutants in the final expression vector were further confirmed by ds DNA sequencing.
Figure 6-1
coding regions were determined by the method of Sanger (Sanger et al., 1977) and were cloned back into the expression vector to replace the C-terminal region of the wild type cDNA. The mutation in the final construction was confirmed by ds DNA sequencing before used for the expression of the mutant protein.

**Enzyme purification**

To study the importance of the C-terminal amino acid sequence for mouse TS enzyme functions, several different altered enzymes were created. The alterations changed either the spatial configuration or the nature of the side chain of the terminal amino acid and are named according to the amino acid sequence at the C-terminus. The wild type mouse enzyme ends in MAV and is designated TS-MAV. The first alteration deletes the penultimate Ala (TS-MV); the second introduces an additional Ala prior to the terminal Val (TS-MAAV); the third changes the terminal Val to a Gly (TS-MAG); the fourth alters the terminal Val to a Glu (MAE). Other changes which were made but were not investigated include the changing of the valine to hydrophobic residues of phenylalanine (TS-MAF) and tyrosine (TS-MAY), and to a serine (TS-MAS).

In Chapter 4, a rapid procedure for purification of wild type mouse recombinant TS, which is based on consecutive chromatography on DEAE-Trisacryl and 10-formylfolate Sepharose was described. Using the same approach, all four mutants exhibited the same chromatographic properties on
the DEAE-Trisacryl column, and were capable of binding to the affinity column in the presence of 150 μM dUMP.

The purity of the mutant enzymes was checked on a native gel electrophoresis. For all mutants studied in this Chapter, a single band was observed, indicating a high degree of purity (data not shown).

**Nucleotide binding**

The ability of each enzyme to form a ternary complex (both covalent and noncovalent) with the mechanism-based inhibitor, FdUMP and the cofactor was determined first. The pattern of the complex formation was analyzed by native gel electrophoretic separation followed by densitometric analyses (Aull et al., 1974b), and the relative amounts of Form I, Form II, and Form III for each of the enzymes was determined. The overall stoichiometry (both noncovalent and covalent) of the inhibitory ternary complex of each of the mutant enzymes, was calculated to be 1.9, with about 10% Form II and 90% Form III, values which were similar to those of the wild type mouse recombinant TS. One of the scanning profiles of the mutant, TS-MV, is shown in Figure 6-2. All other mutants gave similar scanning profiles.

To study the covalent nucleotide binding of these mutants, the TCA precipitation assay was used to determine the ability of each altered enzyme to form covalent complexes with dUMP and FdUMP. The titrations of wild type and mutant enzymes by dUMP and FdUMP in both binary and ternary complexes are shown in Figure 6-3, 4, 5. Analyses of
Figure 6-2. Gel scanning profile of the mutant TS-MA.

The mutant enzyme was incubated with a 10 fold molar excess of FdUMP and a 100 fold molar excess of cofactor at room temperature for 5 min to form the inhibitory ternary complex. About 1 μl of the mixture was loaded on a 8-25% native gradient polyacrylamide PhastGel. The gel was run, stained, destained, and analyzed by a densitometer to determine the relative amounts of Form I, Form II and Form III. The scanning map and the calculated results were automatically printed out by an online computer.

A. Computer scanning profile of the relative amounts of different forms of TS-MV.
B. The relative peak areas calculated by the computer.
<table>
<thead>
<tr>
<th>PKID</th>
<th>Peak Start (minutes)</th>
<th>Peak End (minutes)</th>
<th>Retention (minutes)</th>
<th>Type</th>
<th>Peak Area (microvolt-sec)</th>
<th>Peak Height (microvolt)</th>
<th>Area Percent</th>
<th>Height Percent</th>
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<td>0.172</td>
<td>0.168</td>
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<td>39828.438</td>
<td>115643.99</td>
<td>12.16</td>
<td>13.12</td>
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<tr>
<td>2</td>
<td>0.172</td>
<td>0.190</td>
<td>0.180</td>
<td>PB</td>
<td>287779.60</td>
<td>765568.94</td>
<td>37.84</td>
<td>36.88</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>327608.03</td>
<td>891212.94</td>
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<td></td>
</tr>
</tbody>
</table>

Figure 6-2
Figure 6-3. Covalent binary dUMP complex formation.

Formation of the covalent binary complex between dUMP and the wild type (a), TS-MAE (b) and TS-MAG (c) enzymes was measured by the TCA assay as described in Chapter 2. TS concentration in all experiments was about 1 μM.
Figure 6-4. Covalent binary FdUMP complex formation.

Formation of the covalent binary complex between FdUMP and the wild type (a), TS-MAE (b), TS-MAG (c) and TS-MAAV (d) enzymes was measured by the TCA assay. TS concentration in all experiments was about 2 μM.
Formation of the covalent ternary complex between FdUMP, cofactor, and the wild type (a), TS-MAE (b), TS-MAG (c) and TS-MAAV (d) enzymes was measured by the TCA assay. TS concentration in all experiments were about 2 μM and cofactor concentration was 80 μM.
### Table 6-1. Summary of covalent nucleotide binding ratios (B.R.) and $K_d$ values.

<table>
<thead>
<tr>
<th></th>
<th>dUMP binary</th>
<th>FdUMP binary</th>
<th>FdUMP ternary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d^a$</td>
<td>B.R.</td>
<td>$K_d$</td>
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<tr>
<td>W.T.</td>
<td>26</td>
<td>0.37</td>
<td>8.8</td>
</tr>
<tr>
<td>TS-MV</td>
<td>--</td>
<td>&lt;0.01$^b$</td>
<td>--</td>
</tr>
<tr>
<td>TS-MAAV</td>
<td>0.12$^b$</td>
<td>106</td>
<td>0.4</td>
</tr>
<tr>
<td>TS-MAE</td>
<td>46</td>
<td>0.24</td>
<td>7.2</td>
</tr>
<tr>
<td>TS-MAG</td>
<td>85</td>
<td>0.11</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ in μM

$^b$ [Nucleotide]/[E] up to 250

$^c$ [Nucleotide]/[E] up to 250, [cofactor]/[E] up to 1000
these data by Scatchard plot yielded $K_d$ values and covalent binding ratios listed in Table 6-1. With regard to both the covalent binary and ternary complex titration curves and binding ratios for FdUMP, the TS-VAE mutant enzyme is clearly similar to wild type TS. However, by each criterion, the extent of covalent binding of dUMP by the TS-MAE mutant is less than that of the wild type enzyme. With the TS-MAE mutant enzyme, the binding ratios for FdUMP are decreased by 50%, while the binding ratio for dUMP is only 30% that for the wild type enzyme. Corresponding increases in $K_d$ are found for the TS-MAG mutant enzyme which are corroborated by the shapes of the titration curves in Figure 6-3. These results show that the extent and tightness of covalent nucleotide binding is closely linked to the nature of the side chain of the carboxyl terminal amino acid residue.

When the carboxyl terminal region of both subunits was shortened by one residue but valine was retained as the C-terminal amino acid (TS-MV), the covalent binding ratios are all less than 0.01, even when a 250-fold excess of nucleotides over enzyme were used. When both C-terminal regions were increased in length by adding an additional alanine residue at position 307, as in the TS-MAAV mutant, the $K_d$ values increased substantially and the extent of covalent FdUMP and dUMP binding decreased about 30 and 70%, respectively when compared to the wild type enzyme.
Catalytic activity

The catalytic activity of each mutant enzyme was assayed both by the spectrophotometric and tritium release assays as shown in Table 6-2. By the spectrophotometric method, there was no measurable activity when up to 0.12 mM solutions of TS-MV and TS-MAAV mutant enzymes were used. When TS-MAE and TS-MAG were analyzed, measurable dihydrofolate was produced, although the specific activities were 13 and 43 fold respectively lower than that of wild type enzyme.

When the more sensitive tritium release assay was used, the specific activities of TS-MV and TS-MAAV were found to be reduced 1900- and 43-fold, respectively, while the activities of TS-MAE and TS-MAG were reduced 15- and 23-fold respectively relative to that of wild type TS. The ratio of the specific activity values (tritium release/spectrophotometric) was 0.35 for wild type TS, 0.28 for TS-MAG, and 0.64 for TS-MAE, while the ratios for wild type enzymes from other sources have been reported to vary between 0.3 to 0.5 due to the isotope and/or other unknown effects (Shipiro et al., 1965).

In order to further investigate the effects of altering the side chain of the carboxyl terminal residue on enzyme function, $K_m$ of the TS-MAE and TS-MAG mutant enzymes were measured by the tritium release assay (Table 6-3). Since $k_{cat}$ values would be underestimated by the tritium release assay, the $k_{cat}$ values from the spectrophotometric assay were used to calculate the specific constants ($k_{cat}/K_m$). The $K_m$ values for cofactor for TS-MAE and TS
Table 6-2. Summary of the mutant enzyme activities.

<table>
<thead>
<tr>
<th></th>
<th>H₂folate production</th>
<th>³H₂O released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.A.ᵃ</td>
<td>F.D.ᵇ</td>
</tr>
<tr>
<td>TS-MAV</td>
<td>0.94</td>
<td>1ᶜ</td>
</tr>
<tr>
<td>TS-MV</td>
<td>--ᵈ</td>
<td>--</td>
</tr>
<tr>
<td>TS-MAAV</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TS-MAE</td>
<td>0.074</td>
<td>13</td>
</tr>
<tr>
<td>TS-MAG</td>
<td>0.022</td>
<td>43</td>
</tr>
</tbody>
</table>

ᵃ Specific activity (units per mg).
ᵇ Fold decreased.
ᶜ The value for the wild type enzyme was considered as 1.0. Other values were the fold decreased compared with the wild type.
ᵈ Not measurable.
Table 6-3. Summary of kinetic results.

<table>
<thead>
<tr>
<th></th>
<th>$K_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)$^a$</th>
<th>$K_{\text{cat}}/K_m$ (s$^{-1}$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS-MAV</td>
<td>0.372</td>
<td>10.5</td>
<td>3.54X104</td>
</tr>
<tr>
<td>TS-MAE</td>
<td>0.024</td>
<td>94</td>
<td>2.55X102</td>
</tr>
<tr>
<td>TS-MAG</td>
<td>0.016</td>
<td>2.7</td>
<td>5.93X103</td>
</tr>
</tbody>
</table>

$^a$ Value for dUMP while keeping cofactor at saturated concentrations.
MAG were increased 5- and 9-fold, respectively. The $K_m$ for dUMP for TS-MAE was 9-fold higher than that of wild type enzyme. However, when Gly replaced Val, the $K_m$ for dUMP was surprisingly 4-fold lower than that for wild type TS. The results and the specific constants ($k_{cat}/K_m$) for dUMP are summarized in Table 6-3.

**DISCUSSION**

**Importance of overall C-terminal geometry**

Previous studies with the carboxypeptidase-inactivated *L. casei* TS demonstrated that it retained only one intact C-terminal valine residue per dimer revealed that the altered TS was no longer performing the catalytic functions as studied by both the spectrophotometric method (Aull et al., 1974a) and the tritium release assay, although the overall nucleotide binding was unchanged (Galivan et al., 1976a). Further investigations showed that the covalent nucleotide binding still occurred, but only half stoichiometry was detected by the TCA assay and $^{19}$F NMR experiments (R. Dunlap, personal communication). All these results show that the overall geometry plays a very important role in mediating the covalent nucleotide binding and subsequently activity. In view of the X-ray structures of the native TS and the ternary complex, the importance of exact geometric positioning of the C-terminal residues becomes obvious. The hydrogen bonds between the free carbonyl group and the guanidinium group of the conserved arginine could not be formed if the last residue is removed.
In this study, site-directed mutagenesis was used to shorten both carboxyl-terminals one amino acid residue (deletion of the Ala before the terminal valine), while still keeping the valine as the last amino acid residue. The enzyme seems to be almost completely inactivated as judged by the covalent nucleotide binding and activity. By the tritium release assay, the activity is at least 1900-fold lower. The results are consistent with the x-ray structure of the TS-FdUMP-PDDF in that the length of TS-MV is too short to allow the formation of the hydrogen bonding network with the substrates and the conserved arginine residue. The length of TS-MAAV may be too long but a suboptimal hydrogen bond network may still be formed, with reduced covalent dUMP and FdUMP binding and decreased activity.

However, the overall nucleotide binding stoichiometry (both covalent and noncovalent) for FdUMP in the FdUMP ternary complex is normal in both TS-MV and TS-MAAV mutants as revealed by native gel electrophoresis (e.g. Figure 6-2). Thus it appears that the overall position is important for the covalent nucleotide binding, but it is not involved in the noncovalent binding. Based on the information obtained from the X-ray structure and this study, I propose that noncovalent ternary complex could be formed first without the involvement of the C-terminal residue. After the substrate binds, a conformational change is introduced leading to the repositioning of the C-terminus and formation of a hydrogen bond network which enhances the covalent nucleotide binding of dUMP or FdUMP.
Advantage of hydrophobic side chain at the terminus

When 15 known TS sequences were compared at the C-terminus, it is interesting to note that all but one (ended with Ala) are ended with hydrophobic side chains (valine, leucine and isoleucine, Figure 1-2). This high degree of conservation raises the possibility that the side chain as well as the carboxylate group of the C-terminal amino acid may be important for enzyme function. Here, I have examined this possibility by replacing the C-terminal valine residue with an amino acid residue that lacks a side chain (glycine) or with an amino acid residue with an acidic side chain (glutamic acid). When the carboxyl-valine residue is replaced by a glutamic acid residue, the covalent FdUMP binding in the ternary complex formation is essentially the same as that for the wild type. Although all the potential hydrogen bonding networks could be formed in a manner similar to the wild type TS, the covalent dUMP binary binding is decreased to some extent and the activity is decreased even more. When a glycine residue is introduced to replace the hydrophobic side chain, only about half of the normal covalent nucleotide binding stoichiometry is achieved, and the activity is about 23 fold lower than that of wild type.

These observations strongly suggest that the hydrophobic side chain of the terminal valine residue is important for enzyme function. The side chain may participate in hydrophobic interactions after the binding of both substrates, or decrease the conformational flexibility by imposing steric
constraints that are not possible with glycine. Introduction of a second carboxylate group would prevent these hydrophobic interactions and would probably lead to restructuring of the hydrogen bonding network in the vicinity of the active site, which would have significant effects on catalytic activity. Although the roles of the hydrophobic side chain is not clear from these two mutant studies, it was very likely that the side chain is involved. The constructed mutants TS-MAS, TS-MAF or TS-MAY, and TS-MAA will further probe the functions of the C-terminal side chain.

Effect of mutations on structural conformation

When a mutation is introduced into a protein, conformational changes may disrupt the normal protein functions (Ackers and Smith, 1985). In this study, I believe that in the native (uncomplexed) protein, the introduction of the C-terminal mutants are very unlikely to cause conformational changes in the native enzyme for the following reasons. First, the mutation is introduced in the C-terminus which is exposed to the solvent. X-ray structural analysis shows that the terminus is very flexible. In fact, it is so flexible that the C-terminus can not be precisely resolved (Hardy et al., 1986). Second, all mutant enzymes showed normal total substrate binding as determined by native gel electrophoresis.
Possible roles of the C-terminal residue

From Table 6-1, and 6-2, it is clear that changes in the C-terminal residue had effects on covalent nucleotide binding and catalytic activity. However, the real question is in which step(s) is the C-terminal residue involved. The fact that the total binding stoichiometries for all the mutants are unchanged suggests that the C-terminal amino acid is involved after both substrates bind. This observation is strongly supported by the comparison of X-ray structures of both native and complexed E.coli TS. In the native TS, the nucleotide binding pocket is open. Once the nucleotide is bound, the pyrimidine ring provides a docking surface for the binding of folate. These binding processes do not require the involvement of the C-terminal residue. After both substrates are bound, conformational changes occurred during which the C-terminal residue and the conserved arginine residue are brought together and form a hydrogen bond. The C-terminal residue and the arginine also form hydrogen bonds with the two substrates. Since all mutants have a different degree of reduced covalent nucleotide binding, it is speculated that the C-terminal residue must be involved in promoting the covalent binding of the nucleotide to the enzyme. Other lines of evidence come from kinetic studies, in which it was found that the $K_m$ for both dUMP were changed (Table 6-3). Although the $K_m$ for dUMP of TS-MAE is lower that the wild type enzyme, indicating a stronger binding, the weaker binding of the cofactor (data not shown) would offset the effect due the decreased ability of folate to promote covalent binding of dUMP. As a conclusion, the C-terminal residue is
involved in the covalent substrate binding. However, whether the C-terminal residue is involved in direct catalytic processes remains to be investigated.
Appendix A

Second codon effect in mammalian cell system

The effect of the second codon on gene expression in bacteria led me to study the possibility of the second codon effect in a mammalian cell system. Some preliminary studies suggested that there may be some differences in gene expression when synonymous codons are used as the second codon in mammalian systems (Kozak, 1989). Since a 17 fold difference in gene expression was observed in the bacterial system, it was of interest to investigate whether there is a similar effect in mammalian systems.

The expression vector used was pl56 in which the TS promoter, the TS coding region plus introns 5 and 6 in their normal locations, and the TS polyadenylation signal were cloned into pUC18 (Deng et al., 1988). pl56 contains the normal second codon (CUG). This second codon was changed into CUU by site-directed mutagenesis, yielding plasmid, pl56sec. The wild type or mutant plasmid (40 μg per plate) was cotransfected (Wigler et al., 1979) along with CAT plasmid (5 μg per plate) into mouse fibroblast V79 TS-cells (Nussbaun et al., 1985). The cells were harvested after 48 hrs of transfection, and the cellular proteins were isolated and TS enzyme level (by the filter binding assay), total protein level (assayed by Bradford method), and CAT activity (Gorman et al., 1982) were determined. Data is shown in the
table. It was found that unlike the bacterial system, the second codon has no effect on the TS gene expression in mammalian cell system.

Effect of second codon on mammalian gene expression.

<table>
<thead>
<tr>
<th>Construction</th>
<th>Relative TS level</th>
<th>TS/CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pl56</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pl56sec</td>
<td>1.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Appendix B

Other site-directed mutants constructed

Through the course of this investigation, many site-directed mutants were constructed in order to probe the functional roles of a particular amino acid residue. Some of the mutants were studied kinetically as described in Chapters 5 and 6. Here, other mutants which were constructed but not investigated are described.

1. Active site mutants. The active site residue in TS has been determined to be a cysteine residue (Cys 189 in mouse TS). This residue was replaced by an alanine or a serine residue in E.coli TS (Dev et al., 1988). It was found that the alanine replacement lost the activity as expected due to the inability of enzyme to form a covalent ternary complex with dUMP and cofactor. However, it was unexpected that the serine mutant still retained a small amount of activity. In this study, it was unclear whether the serine mutant could form a weak covalent ternary complex. I constructed the same mutants at the same time in mouse TS. Preliminary studies showed that the ability to form the covalent inhibitory ternary complex was unmeasurable for both mutant enzymes by the filter binding assay. These mutants, especially the serine mutant, will be further analyzed in our collaborators at Univ. of South Carolina by $^{19}$F NMR to monitor the active site of the enzyme.

2. R170. This residue was mutated because it was hypothesized that this residue was in the active site to stabilize the phosphate group of
nucleotide. A more important factor was that this arginine was contributed by the other subunit (Hardy et al., 1986). The mutants created in this position of mouse TS were based on site-directed mutagenesis in Region II by using an oligo, TGATGATC(A/T)(T/G)TCTGTCA, which could theoretically result in the following replacements, Lys, Met, Gin, Leu. By DNA sequencing, the Lys replacement was obtained.

3. R209. This arginine was chosen because it was found from the X-ray structure studies that the residue is involved in stabilizing the phosphate group of the nucleotide. The mutants created at this position were also based on site-directed mutagenesis in Region II by using an oligo, TCCTGAC(T/A)(T/G)CTGGTAA, which could result in the following replacements, Lys, Met, Gln, Leu. By DNA sequencing, only Lys and Leu replacements were obtained.
References


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