Thymidylate synthase (TS)  catalyzes the reductive methyl-


dation of 2'-deoxyuridine 5'-monophosphate (dUMP) to thymi-
dine 5'-monophosphate (dTMP), using the co-substrate, 5,10-
methylenetetrahydrofolate (CH$_2$H$_4$folate) as a 1-carbon donor and reductant. The physical structures of bacterial TSs have been relatively well defined, and crystallographic data, in con-
cert with data derived from kinetic, spectroscopic, and site-
directed mutagenesis studies, have led to a detailed under-
standing of the catalytic mechanism of these enzymes (1). In
contrast, relatively few investigations of mammalian TS struc-
ture and catalysis have been conducted. The three-dimensional
structure of the native human TS (hTS) has been reported
previously (2). The data showed a surprising feature not ob-
served in TSs from other sources: loop 181–197 containing the
catalytic cysteine, Cys-195, was in an inactive conformation,
rotated ~180° with respect to its orientation in bacterial TSs,
with the sulfhydryl of Cys-195 over 10 Å from the location of
sulfhydrys of corresponding cysteine residues in bacterial en-
zymes. Subsequent determination of the structure of a ternary
inhibitory complex between closely related ratTS (rTS) and
dUMP and Tomudex (3) has shown that the ligands bind to the
enzyme in the active conformation. Recently, it was found that
also in the hTS-dUMP-Tomudex complex hTS is in the active
conformation (4). The inactive conformation has not been ob-
served in TSs other than human.

TS has been a primary target for chemotherapy aimed at
cancers of the gastrointestinal tract and head and neck (5)
despite moderate response in 30–40% of patients. A major
problem affecting TS-directed treatments is that tumor cells
often react to an exposure to TS inhibitors by raising levels of
intracellular TS activity about 2- to 4-fold, which may lead to
resistance. The levels of TS mRNA do not change significa-
tly; the increase is predominantly at the protein level. Two effects
have been found that can explain the observed increased TS
levels. TS was observed to bind to its own mRNA, which is
proposed to act as a feedback inhibition mechanism regulat-
ing TS levels (6). When TS is bound by its physiological substrates,
dUMP and/or CH$_2$H$_4$folate, or inhibitors, FdUMP or antifo-
lates, it is unable to interact with its own mRNA. The end-
result of this disruption in RNA binding is relief of transla-
tional repression with resultant synthesis of new TS. Other

Thymidylate synthase (TS) catalyzes the reductive methyl-


tylene synthesis; ecTS, Escherichia coli thymidylate synthase;
lcTS, Lactobacillus casei thymidylate synthase; NTE-hTS, N-termi-
nally extended human thymidylate synthase; dUMP, 2'-deoxyuridine 5'-monophosphate; dTMP, 2'-deoxythymidine 5'-monophosphate; CH$_2$H$_4$folate, 5,10-methylenetetrahydrofolate; raltitrexed, Tomudex or ZD 1694, N-[5-[[4-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl]-N-methylamino]-2-phenyl]-1-glutamic acid; FdUMP, 5-fluoro-2'-de-
oxuridine 5'-monophosphate; 2-ME, 2-mercaptoethanol; H$_2$folate, (+)-tetrahydrofolate; PEG, polyethylene glycol; r.m.s.d., root mean square
deviation.
researchers (7), using different cell lines, did not observe changes in the extent of ribosome binding to TS mRNA in the presence of TS inhibitors but have shown in an elegant way that ligand-mediated induction of TS occurs by lowering protein turnover.

The primary structure of hTS differs from that of bacterial TSs in three regions: the N terminus of hTS is extended (by 29 amino acids) (8). Recent studies of hTS isolated from bacteria as a recombinant protein and TS isolated from human cells suggested that catalytic and some RNA binding activities of these enzymes are dependent upon the N terminus (8). The sole difference between the proteins was the presence of a blocked N terminus, probably acetylation, in the enzyme isolated from human cells. In addition, a recombinant hTS in which the N terminus was blocked with a polyhistidine peptide exhibited catalytic and RNA binding activity similar to that of the native, blocked protein (8). These data suggested that the N terminus influences enzyme function. Interestingly, the recombinant human enzyme that was utilized as the source for the originally studied crystals was heterogeneous; 80% of the protein was a fusion protein containing an additional 13 amino acids at the N terminus (see Fig. 1) that are not present in human TSs (2).

The original structure determination of the native hTS was at medium (3.0 Å) resolution, and the solvent structure could not have been analyzed. We report here the first determination of high resolution, 2.0 Å, structures of recombinant unblocked hTS and NTE-hTS, both with well-defined solvent structures, and kinetic and ligand binding studies for NTE-hTS. Analysis of the solvent structure suggested that phosphate ions stabilize the inactive conformation. We have conducted fluorescence studies that support this hypothesis.

**Experimental Procedures**

**Materials**—Nucleotides, salts, isopropyl-β-D-thiogalactopyranoside, 2-mercaptoethanol (2-ME), phenylmethylsulfonyl fluoride, ethanediol, polyethylene glycol (PEGs), and folic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Ultra-pure ammonium sulfate was from J.T. Baker (Phillipsburg, MD). The bacterial strain X2913, which has a deletion of the gene from the plasmid pDHTS-1, was a gift from M. Chirgwin (12). Transformants were grown in Luria broth with 100 μg/ml ampicillin. At t = 0 hr, cells were washed with buffer X. The cell pellet was resuspended in buffer Y (20 mM Tris, 1 M KCl, pH 7.4), and NTE-hTS buffer Z (20 mM Tris, 100 mM KCl, 100 mM imidazole, and 14 mM 2-ME, pH 8.5). Purified NTE-hTS was dialyzed overnight against buffer A (10). Purified NTE-hTS was analyzed by 12% SDS-polyacrylamide gel electrophoresis for purity and stored in buffer A containing 15% glycerol at −20 °C.

**Steady-state Kinetic Measurements**—Kinetic data describing the binding of ligands with hTS were obtained using a stopped-flow fluorimeter/spectrometer (Bi-Tech SX.18MV, Applied Photophysics, UK) as described previously (10, 16). Kinetic and thermodynamic constants for nucleotide binding to NTE-hTS were determined by titrating the enzyme with various concentrations of dUMP, FdUMP, or dTMP. Data were collected over 20- or 50-ms time periods. Final concentrations of dUMP, FdUMP, and dTMP were 5–200, 10–250, and 5–250 μM, respectively. Transient-state kinetic constants for CH2H4folate binding to the binary complex of NTE-hTS and dUMP were measured by titration of the binary complex with various concentrations of CH2H4folate. Data were collected over a 1-s time period. The concentration range of CH2H4folate was 5–250 μM. The final concentration of dUMP was 0.50 mM.

**Crystallization of Human Thymidylate Synthase**—Crystals of unblocked hTS and NTE-hTS were grown by the vapor diffusion method in the hanging-drop setup at conditions similar to those reported previously (2). Typically, 5 μl of enzyme solution (18 mg/ml) containing 1 mM EDTA, 10 mM 2-ME, 0.1–1 mM potassium chloride, 100 mM imidazole (NTE-hTS), 50 mM Tris-HCl (pH 7.5–8.5) was mixed with an equal volume of precipitant solution containing 38–50% saturated ammonium sulfate, 40 mM potassium phosphate, 20 mM 2-ME, and 100 mM Tris-HCl (pH 7.8), and allowed to equilibrate with 0.6 ml of precipitant solution present in the well. The culture plates were stored undisturbed at temperatures of 20 °C, and crystals appeared after 3 weeks and grew to the size of 0.1–0.7 mm within 1 week.

**X-ray Diffraction Data Measurement and Processing**—Data were collected on a single crystal of wild type hTS and NTE-hTS with approximate dimensions of 0.45 × 0.50 × 0.50 and 0.15 × 0.15 × 0.15 mm, respectively. The crystals were transferred to a cryoprotectant solution containing 45% saturated ammonium sulfate, 30% concentrated ethylene glycol, 0.1 M sodium chloride, and 100 mM Tris-HCl (pH 7.8), and flash-cooled to −175 °C. Crystallographic diffraction experiments were carried out at the SBC insertion device beamline of the advanced photon source at Argonne National Laboratory using x-rays of 0.9793 Å wavelength.

The data for unblocked hTS and NTE-hTS were collected at a crystal to detector distance of 225 and 180 mm, respectively, and processed with the HKL 2000 suite of programs (17). At the initial stage of our analysis, the coordinates of hTS were not available; therefore, a structure of ecTS (Protein Data Bank code 1qqq (18)) with appropriate modifications was used as the search model in the molecular replacement method carried out with the CNS software (19). The final structure of unblocked hTS replaced ecTS as the initial model for NTE-hTS. The data were refined and optimized with the CNS software (19) using simulated annealing with torsional dynamics and positional and temperature refinements. Electron density maps calculated with 2Fo − Fc and Fc − Fo coefficients were utilized to introduce manual corrections to the model with the interactive graphics program CHAIN (20). Ribbon diagrams and wire- and basket-models were prepared with the programs MOLSCRIPT (21) and CHAIN (20), respectively.

**Fluorescence Studies**—Evidence for the existence of the inactive loop conformation in solution was obtained utilizing a spectrophotometer (Model 8100, SLM-Aminco Inc., Urbana, IL). The excitation wavelength was set at 295 nm, and the emission was scanned from 305 to 450 nm at a rate of 5 nm/s. An Ultra-Vu polystyrene cuvette (Baxter Diagnostics Inc., Deerfield, IL) was used to hold a 3-ml sample containing 4 μM protein in 50 mM Tris-base (pH 7.4). The purity of the protein (recombinant wild type TS) was better than 95%. The titration was carried out
by addition of potassium phosphate (pH 7.4) or dUMP in increments up to 10% of the sample volume. No fluorescence signal was observed from the buffer (Tris-HCl), phosphate, or dUMP in the experiments. All measurements were done in triplicate to eliminate errors introduced by non-homogeneity of the sample and instrumental drift.

RESULTS

Determination of Steady-state Kinetic Constants for NTE-hTS and hTS—The specific activities, determined at 25 °C, for NTE-hTS and hTS were 1.1 and 1.2 units/mg, respectively, whereas catalytic rates (kcat) were 1.5 and 1.6 s⁻¹. These values were very similar to previously published values of 1.2 units/mg for the specific activity and 1.5 s⁻¹ for kcat of hTS at 25 °C (10, 22).

Determination of Kinetic and Thermodynamic Constants for Nucleotide Binding to NTE-hTS—The time courses of fluorescence quenching upon nucleotide binding were analyzed using single-exponential curve fits (23). Kinetic and thermodynamic constants for nucleotide binding were determined as previously described (16, 23). A linear relationship was observed between kobs and nucleotide concentration. The kinetic and thermodynamic constants for nucleotide binding to NTE-hTS are listed in Table I. The kinetic and thermodynamic constants describing nucleotide binding to NTE-hTS are nearly identical to those determined for hTS by transient-state kinetic analysis (16).

Determination of Kinetic and Thermodynamic Constants for CH₂H₄folate Binding to the Binary Complex of NTE-hTS—Fluorescence quenching due to CH₂H₄folate binding to the binary complex of NTE-hTS-dUMP was analyzed using a single-exponential curve with a steady-state parameter to account for product formation as previously described (23). Mixing NTE-hTS with substrates resulted in a fluorescence burst (kburst) at 340 nm. The dependence of kburst on CH₂H₄folate concentration was hyperbolic as previously observed for hTS (13), and the kinetic and thermodynamic constants for CH₂H₄folate binding to NTE-hTS-dUMP were determined using the expression:

$$k_{\text{burst}} = k_{\text{obs}}(L)(L + K_M) + k_{\text{r, iso}} + k_{\text{chem}}$$

(23). The kinetic and thermodynamic constants determined for NTE-hTS are shown in Table II and are similar to those determined for hTS by transient-state kinetic analysis (16).

Crystallization—The typical high salt conditions used by Schiffer et al. (2) and in the present report exclusively support the growth of hTS crystals in which the active-site loop adopts the inactive conformation. We have investigated other crystallization conditions, especially those with low ionic strength, and obtained several systems that were able to grow hTS crystals. A semi-factorial approach was employed to screen a wide variety of conditions arranged into matrices containing polyethylene glycol, different buffers, and a multitude of salts at varying concentrations (data not shown). At 38% (weight/volume) PEG 4000, 100 mM Tris-HCl (pH 7.6), 20 mM 2-ME, and 2% saturated ammonium sulfate, thin and irregularly shaped crystals were observed after 3 weeks measuring ~0.1 x 0.2 x 0.2 mm. Despite their fair size, these crystals did not scatter x-rays at all. However, we succeeded in growing trigonal crystals, similar to those reported here and by Schiffer et al. (2) with the PEG/ammonium sulfate biphasic system (24). The optimum conditions were at 15–30% (weight/volume) PEG 4000, 42–50% saturated ammonium sulfate, and 100 mM Tris-HCl (pH 8.5). The results were similar for hTS and NTE-hTS.

Despite the extra 42 residues at the N terminus, the crystals of hTS and NTE-hTS are isomorphous and isomorphous to those reported by Schiffer et al. (2). The crystals belong to the space group P3₁21 with one subunit in the asymmetric part of the unit cell. The parameters and statistics of crystallographic refinements are summarized in Table III.

Determination of hTS and NTE-hTS—The protein model reported by the Stroud group (2) was, in general, excellent; however, stretches of additional residues were fit and some side chains were rebuilt to fit to the more resolved density. The hTS data, which were refined first, having a mosaicity of 0.22 and an overall Rmerge of 0.033, yielded excellent electron density maps. The structural differences between hTS and NTE-hTS in unliganded form appear to be insignificant and limited to occasional shifts in the solvent structure. The root-mean-square deviations of the positions of Cα between hTS and NTE-hTS, was 0.16 Å. A significantly smaller number of structured water molecules observed in the NTE-hTS structure is likely to be related to the smaller crystal used and resulting quality of the data. The discussion below refers to the wild type enzyme but is valid as well for the NTE-hTS.

The N terminus is largely disordered; good density starts at Pro-26, and this region does not change between hTS, NTE-hTS, and an inhibitory ternary complex of hTS with dUMP and Tomudex (ZD1894 or raltitrexed) (4) or analogous rat TS complex (PDB entry 2tsr (3)). A least-squares superposition of the Cα plots of hTS and the inhibitory complex is shown in Fig. 2. The extra N-terminal residues of NTE-hTS occupy intermolecular space in multiple positions without affecting crystal packing. The main chain follows closely the fold of ecTS starting from Glu-30, which is equivalent to Met-1 of ecTS. As previously observed in a Lactobacillus casei TS (lCTS) structure (25), the carboxylate moiety of the N-carbamoylated ecTS occupies the same position as the carboxylate of a glutamic acid (Glu-30 in hTS), apparently forming a structural lock that isolates the rest of the TS molecule from the presence and/or conformational state of the N terminus. Indeed, a glutamic acid at this relative position is conserved in all TSs that contain additional N-terminal sequences with one exception, which contains aspartic acid. In the region preceding the first insert, there is reasonable electron density for residues 99–106, which was not observed previously. Region 107–128, which includes the insert, is disordered in the crystals. This is in contrast to the structures of hTS-dUMP-Tomudex and rTS-dUMP-Tomudex complexes, in which this region has interpretable electron density (3, 4). The second insert, residues 146–153, has a well-defined structure in our crystals. A short loop, Thr-103 to Arg-107 in ecTS, which is solvent-exposed, is replaced in hTS with a longer loop, Ala-144 to Gln-156, which is directed in the opposite direction toward the hTS surface (Fig. 2, top). The insert loop is quite polar with the exception of Met-149, which forms hydrophobic contacts with Thr-96 and Asn-97, and Tyr-...
The occurrence of such cavity must destabilize the folded state, so it is likely that it has some function compensating for the reduced stability. It appears that its presence is linked to the inactive conformation; in the ternary inhibitory complex hTS-dUMP-Tomudex the cavity does not exist. It may be speculated that the cavity facilitates loop 181–197 flipping. The glycol binding energy must stabilize the inactive conformation and thus it was possible that glycol might be a noncompetitive inhibitor. However, measurements of catalytic activity of hTS in the presence and absence of up to 200 mM glycol did not show significant differences. The cryoprotectant, from which the bound phosphate/sulfate ions map the 2.0 M in sulfate. Because sulfate ions often occupy phosphate-binding sites in crystals, the bound glycol originated, was about 5 mM and moreover, synergism with the crystallographic and refinement statistics.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{\text{dUMP}}$</th>
<th>$k_{\text{iso}}$</th>
<th>$k_{\text{r,iso}}$</th>
<th>$K_{\text{c}}$</th>
<th>$k_{\text{chem}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTE-hTS</td>
<td>78 s$^{-1}$</td>
<td>47 s$^{-1}$</td>
<td>1.6 s$^{-1}$</td>
<td>29</td>
<td>1.0 s$^{-1}$</td>
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<tr>
<td>hTS$^a$</td>
<td>89 s$^{-1}$</td>
<td>49 s$^{-1}$</td>
<td>1.6 s$^{-1}$</td>
<td>31</td>
<td>1.1 s$^{-1}$</td>
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</tbody>
</table>

$^a$ Determined from the ratio of $k_{\text{iso}}$ to $k_{\text{r,iso}}$.

$^b$ Calculated using the expression $k_{\text{chem}} = k_{\text{chem}}(1 + k_{\text{chem}}/k_{\text{iso}})$, assuming a $k_{\text{chem}}$ of 1.0 s$^{-1}$ at 20°C.

$^c$ Reprinted from Steadman et al. (16) for comparison.

### TABLE II

<table>
<thead>
<tr>
<th>X-ray Source</th>
<th>Space Group</th>
<th>a (Å)</th>
<th>c (Å)</th>
<th>Mosaicity</th>
<th>Resolution (Å)</th>
<th>Reflections, measured</th>
<th>Reflections, independent</th>
<th>Completeness (%)</th>
<th>Rmerge (highest shell)</th>
<th>Rmerge (overall)</th>
<th>r.m.s.d. bond angles (degrees)</th>
<th>r.m.s.d. bond lengths (Å)</th>
<th>No. solvent molecules</th>
<th>No. ligand molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synchrotron</td>
<td>P3$\text{1}$, P3$\text{1}$, P3$\text{1}$, P3$\text{1}$</td>
<td>95.52</td>
<td>95.68</td>
<td>0.22</td>
<td>2.0</td>
<td>144,310</td>
<td>29,971</td>
<td>99.2</td>
<td>0.310</td>
<td>0.0033</td>
<td>0.0060</td>
<td>1.3</td>
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<td>5</td>
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<tr>
<td>Synchrotron</td>
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<td>93.21</td>
<td>83.00</td>
<td>0.21</td>
<td>2.0</td>
<td>156,435</td>
<td>27,460</td>
<td>99.6</td>
<td>0.373</td>
<td>0.051</td>
<td>0.0064</td>
<td>1.3</td>
<td>102</td>
<td>4</td>
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</table>

$^d$ Reference molecule.

$^e$ Root-mean-square deviation.

$^i$ Reference molecule.

$^j$ Calculated using the expression $k_{\text{chem}} = k_{\text{chem}}(1 + k_{\text{chem}}/k_{\text{iso}})$, assuming a $k_{\text{chem}}$ of 1.0 s$^{-1}$ at 20°C.

$^k$ Reprinted from Steadman et al. (16) for comparison.

### TABLE III

<table>
<thead>
<tr>
<th>Crystallographic and refinement statistics</th>
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<tr>
<td><strong>TSS</strong></td>
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<td>Space Group</td>
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<tr>
<td>a (Å)</td>
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<tr>
<td>c (Å)</td>
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<tr>
<td>Mosaicity (degree)</td>
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<tr>
<td>r.m.s.d. bond angles (degrees)</td>
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<tr>
<td>r.m.s.d. bond lengths (Å)</td>
</tr>
<tr>
<td>Average B (Wilson plot)</td>
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<tr>
<td>Most favored ($\phi$ (%)</td>
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<td>Reference$^d$</td>
</tr>
<tr>
<td>No. solvent molecules</td>
</tr>
<tr>
<td>No. ligand molecules</td>
</tr>
</tbody>
</table>

153 interacting with Phe-137. These interactions likely anchor the loop to the rest of the molecule and induce the observed ordered conformation. Although Met-149 is not conserved, equivalent residues are usually hydrophobic and probably function in a similar manner. The presence of the loop creates a cavity in which four ordered water molecules are present. The structure of this loop is in excellent agreement with that observed in the hTS-dUMP-Tomudex and rTS-dUMP-Tomudex complexes (3, 4), indicating that its function is not related to catalysis.

The active site loop 181–197 is in the inactive conformation that was reported in previous investigations (2). The loop forms only limited intermolecular interactions: hydrophobic contacts between the side chain of Leu-189 and Pro-305 and a hydrogen bond between Pro-188 O and Arg-274 NH1. A major intramolecular interaction stabilizing the inactive conformation is the hydrogen bond between the guanidinium group of Arg-163 and the carbonyl oxygen of Pro-188 (26). In the structure of a complex between the active site loop 181–197 and the catalytic Cys-195, the loop forms a hydrogen bond between the side chain of Leu-192. These interactions must stabilize the inactive conformation of this loop is in excellent agreement with that observed in previous investigations (2).

Solvent Structure and Ligand Binding—The high resolution and low temperature of the data collection resulted in well-resolved solvent structure. The positions of 211 water molecules, 4 phosphate/sulfate ions, and 1 glycol molecule were identified per asymmetric unit of the D-dimensional data set (27). The cryoprotectant, from which the bound glycol originated, was located together with a water molecule in a large cavity (Fig. 3, top). This cavity is lined mostly with hydrophobic residues, although some hydrogen-bonding functions are present. The cavity is elongated and connects to the molecular surface on one side, the other end being blocked by the side chain of Gln-36, preventing the cavity from becoming a channel (Fig. 3, bottom). The occurrence of such cavity must destabilize the folded state, so it is likely that it has some function compensating for the reduced stability. It appears that its presence is linked to the inactive conformation; in the ternary inhibitory complex hTS-dUMP-Tomudex the cavity does not exist. It may be speculated that the cavity facilitates loop 181–197 flipping. The glycol binding energy must stabilize the inactive conformation and thus it was possible that glycol might be a noncompetitive inhibitor. However, measurements of catalytic activity of hTS in the presence and absence of up to 200 mM glycol did not show significant differences. The cryoprotectant, from which the bound glycol originated, was about 5 mM and moreover, synergism between phosphate and glycol binding is likely.

There are four tetrahedrally shaped peaks in the ordered solvent region with environment suitable for bound phosphate/sulfate ions. The mother liquor of the crystal used to collect data was 40 mM in phosphate and ~2.0 mM in sulfate. Because sulfate ions often occupy phosphate-binding sites in crystals obtained with ammonium sulfate due to the similarity of their shape and charge, it is likely that the bound ions are some mixture of the two species. Three of them are located within or close to each other and in vicinity of the active site (Fig. 4). It is not unlikely that the bound phosphate/sulfate ions map the contacts between the enzyme molecule and the phosphate moieties of a nucleic acid. Indeed, in the structure of a complex between mRNA and a sequence-nonspecific mRNA binding protein, all protein-mRNA interactions are through the sugar phosphate backbone and most of them through arginine-phosphate ion pairs (26). In the structure between the Sex-lethal protein isolated from Drosophila melanogaster and...
12-nucleotide, single-stranded RNA, which is an example of mRNA sequence-specific binding, many arginine-phosphate ion pairs are observed (27). In hTS, one distance between two phosphate/sulfate sites, 6.5 Å, is the same as phosphate-phosphate distances in single-stranded RNA helices (26). The other distance is 10.0 Å and may correspond to a second neighbor phosphate-phosphate. Indeed, in the Sex-lethal protein-RNA complex, several second neighboring phosphates are separated by distances of 10.4 Å (27).

Fluorescence Measurements—The observed involvement of the residues of loop 181–193 in phosphate ion binding sites suggested that phosphate concentration might affect the loop conformation. The loop has a tryptophan at position 182 and our modeling, based on the structure of the hTS-dUMP-Tomu-dex complex, showed that the position of the indole moiety differs between the active and inactive conformations by about 5 Å. It appeared likely that even with five tryptophans per subunit such change would be reflected in the enzyme fluorescence. This hypothesis turned out to be true, because there is a strong phosphate ion concentration-dependent increase in the intensity of hTS fluorescence (Fig. 5, top). Subsequent dUMP binding effectively reverses this signal enhancement as fluorescence falls below the intensity level observed for the unliganded hTS (Fig. 5, center). This indicates that even without phosphate ions present a significant fraction of the enzyme is in the inactive conformation and eliminates the possibility that the inactive conformation is an “artifact of the crystal field.” Such effects were not observed in ecTS (Fig. 5, bottom) for which the inactive conformation has not been observed.

DISCUSSION

Previous studies indicated that modifications at the N terminus of hTS alter catalytic activity and RNA binding activity, presumably through effects on the enzyme conformation (8). In the present investigation, the reaction mechanisms and crystal structures of recombinant hTS with an unmodified N terminus and hTS with an extended N terminus have been determined. Our data indicate that the presence of an additional 42 amino acids at the N terminus of hTS exerts a minimal effect on ligand binding and on the kinetically measurable steps in the reaction pathway. These results are supported by crystallographic data, which indicate no significant differences in the arrangement of atoms that were ordered in crystals. It appears that the modification of the N terminus induces only local
FIG. 3. Top, stereo view of the binding site for a cryogen molecule, ethanediol (EDO), is shown with $2F_o - F_c$ density maps contoured at the 1.2σ level. This cavity is not present in bacterial TSs in which bulky groups replace Ala-63 and His-250. Bottom, ribbon representation (21) of the glycol-binding cavity, which is not far from the putative RNA binding site in hTS. The glycol molecule is in the center, between the side chains of His-250 and Gln-36. The cavity is surrounded by hydrophobic and polar residues, which form a channel leading to the active site where three phosphate/sulfate ions are bound.

FIG. 4. Stereo diagram of the phosphate/sulfate binding sites. Electron density presented as basket contouring is at the 1.2σ level. The distances between the phosphate ions are very similar to those between the phosphate moieties in RNAs, possibly mapping the sites of TS-RNA binding.
differences that do not propagate to the rest of the molecule. The poor conservation of the amino acid sequence at the N-terminus strongly suggests that the physiological function of the N-terminal extension, if such indeed exists, is strongly species-specific and thus is unlikely to be related to catalysis, in agreement with our experimental data.

Several new structural features of hTS have been deduced from the high resolution model reported here. Of interest is the possibility that they may represent sites for interactions of TS with macromolecules. Evidence that TS is involved in protein-protein interactions was derived from studies indicating that it is a component of a multi-enzyme DNA-biosynthesizing complex termed the replisome (29). The identification of two cavities specific for mammalian TSs suggests that they are related to interaction between hTS and other macromolecules and opens a new field for studies of this amazingly complex enzyme.

A large body of evidence indicates that hTS interacts with ribonucleic acids (6, 30–32). At least 11 ribonucleoprotein complexes involving hTS have been identified in vivo, including complexes with TS mRNA and mRNA encoding the tumor suppressor gene product, p53 (30–32). No consensus sequences or structures have been identified among the RNAs interacting with hTS. The binding of hTS to TS mRNA and to p53 mRNA inhibits translation in vitro (6, 32). That translational regulation by TS occurs in vivo is supported by recent studies of interactions between TS and p53 mRNA (33). Binding motifs of 36 and 70 nucleotides have been identified for the interaction between TS and TS mRNA (30, 34); however, the site(s) of interaction on the human protein have not been identified. The crystal structures analyzed in the present investigation provide the first suggestion of the location of a region in which multiple phosphate substituents are predicted to bind whereas fluorescence studies correlate it with the inactive conformation of loop 181–197. It is possible that the inactive conformation makes hTS less vulnerable to an oxidative stress or other factors that derivate thiols, however, it appears more probable that the inactive conformation facilitates the binding of nucleic acids, because Arg-185 of the loop binds one of the closely positioned phosphate/sulfate ions.

The existence of the inactive conformation offers a novel mechanism for inhibition of hTS. Its stabilization should be an effective manner of inhibiting hTS and providing an alternative to active site-directed inhibitors that are currently used in cancer therapy. Inhibitors stabilizing the inactive conformation and binding away from the active site may be of significant advantage, because they would be expected not to interfere with mRNA binding to hTS. Active site-directed inhibitors, such as FdUMP, inhibit the binding of hTS to TS mRNA in vitro, reducing the repression by TS of TS mRNA translation (6). In addition, exposure of tumor cells to active site-directed inhibitors results in a significant increase in steady-state levels of TS protein (35–37). Two mechanisms underlying the elevation in protein have been documented in different cell lines. The first emphasizes translational derepression by TS binding to TS mRNA, the second the increased stabilization of the protein upon ligand binding (6, 7). Our structural data strongly support both mechanisms. The putative mRNA binding site involves the inactive conformation of loop 181–197 and overlaps the active site. Thus, the formation of inhibitory complexes must eliminate mRNA binding. The ternary inhibitory complex has a much more compact structure than the native TS (4). Disordered loop 107–128 is likely to be more prone to degradation than the same residues in the compact conformation observed in the inhibitory complex.

Therapeutic approaches aimed at the stabilization of the inactive conformer should not activate those mechanisms that lead to TS accumulation and may improve the outcome of cancer chemotherapy in which TS is the target. Such approaches may utilize more powerful inhibitors binding in the glycol cavity or cross-linking of the cluster of four thiols from Cys-195, Cys-180, and their equivalents from the other subunit.

Acknowledgments—We thank Dr. Cathy Murphy for making her fluorimeter available and Dr. Trent Spencer for many helpful discussions.
REFERENCES
