Thermodynamic Stabilization of Nucleotide Binding to Thymidylate Synthase by A Potent Benzoquinazoline Folate Analogue Inhibitor†

Chang-Hwei Chen,‡ R. Alan Davis,§ and Frank Maley*,‡

Wadsworth Center, New York State Department of Health, and Department of Biomedical Sciences, The University at Albany, State University of New York, P.O. Box 509, Albany, New York 12201-0509, and College of Pharmacy, University of South Carolina, Columbia, South Carolina 29208

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ABSTRACT: The stabilization of dUMP, FdUMP, and dGMP binding to *Escherichia coli* thymidylate synthase (TS) in the presence and absence of a folate analogue inhibitor of TS, 1843U, was determined by differential scanning calorimetry. When the enzyme is thermally unfolded in the presence of dUMP, *two separate temperature transitions are evident,* although only one binding site/dimer was detected in equilibrium dialysis experiments. In the absence of dUMP, TS shows a major peak of unfolding at 45 °C with a shoulder at 47 °C. In the presence of increasing amounts of dUMP progressive changes in the size of each peak occur, each associated with a higher temperature of unfolding. At a ratio of dUMP/TS of 100, a major peak predominates with an unfolding temperature (T_d) of 60 °C. FdUMP shows a similar profile, while dGMP does not alter the T_d of the enzyme since dGMP alone does not bind to TS. Despite the fact that 1843U binds tightly to TS in the absence of nucleotide ligands [Dev, I. K., Dallas, W. S., Ferone, R., Hanlon, H., McKee, D. D., & Yates, B. B. (1994) *J. Biol. Chem. 269*, 1873−1882], it exhibits only a small effect on the T_d profile of TS. However, when 1843U is present, in addition to the nucleotides (dUMP, FdUMP, or dGMP), a T_d of 72 °C is achieved and the enthalpy of unfolding is increased by one-third. The stabilizing effect of substrate binding to TS by 1843U examined by thermodynamic parameters can be attributed to the considerable extra amount of free energy released on formation of the ternary complex of TS−1843U−nucleotide. The tightness of this complex is due to the stacking energy that results from Van der Waals contacts between the nucleotide purine or pyrimidine ring and the benzoquinazoline ring of 1843U [Weichsel, A., Montfort, W. R., Cieśla, J., & Maley, F. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3493−3497], which induces a local conformational change in the protein. This conformational change is associated with a significant positive entropy change, which suggests that water is expelled from the active site region.

The primary function of thymidylate synthase (TS)[1] (EC2.1.145) in the cell is to catalyze the synthesis of dTMP, a nucleotide that is essential for DNA synthesis. Because of this enzyme's location at a critical juncture in its metabolic pathway, it has become a logical chemotherapeutic target (Heidelberger, 1970).

TS is a homodimer that has been remarkably conserved during the course of evolution, with each of its subunits ranging from 30 to 35 kDa over a wide range of animal and bacterial species (Carreras & Santi, 1995). The mechanism of the reaction, one of the more unique ones in nature, utilizes a nucleophile in the protein to initiate the reaction by saturating the 5,6-double bond of dUMP (Pogolotti & Santi, 1977). The nucleophile, in this instance, was discovered to be a cysteinyl residue in a highly conserved region of TS (Bellisario *et al*., 1976; Pogolotti *et al*., 1976), which, by adding to the 6-position of dUMP, sets the stage for the addition of the methylene residue of CH_2H_4PteGlu to the 5-position of this nucleotide. What makes this reaction so unique is that the hydrogen required to reduce the methylene to a methyl group is stereochemically transferred from the 6-position of the pyrazine ring of CH_2H_4PtEGlu, followed subsequently by the ordered release of H_2PteGlu and dTMP (Lorenson *et al*., 1967). As suggested in this study, substrate binding is also ordered, which was shown subsequently to be asymmetric in that one subunit of the enzyme was preferred over the other by its substrates and substrate analogues (Galivan *et al*., 1976, 1977b). This aspect of substrate binding has been confirmed by many groups in both bacterial and mammalian species of TS (Leary *et al*., 1975; Galivan *et al*., 1976; Langenbach, 1976; Danenberg & Danenberg, 1979; Beaudette *et al*., 1980; Santi & Danenberg, 1984; Moore *et al*., 1986; LaPat-Polasko *et al*., 1990; Dev *et al*., 1994). It appeared from these studies, and was surmised from CD (Galivan *et al*., 1975) and more specifically from proteolysis protection studies (Galivan *et al*., 1977a), that a conformational change in the enzyme protein was associated with substrate binding, which has now been

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* Tel: (518) 474-4184. FAX: (518) 473-2900.

‡ Wadsworth Center.

§ University of South Carolina.

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[1] Abbreviations: TS, thymidylate synthase; dUMP, 2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; dGMP, 2′-deoxyguanosine 5′-monophosphate; 3′-dGMP, 2′-deoxyguanosine 3′-monophosphate; dTMP, 2′-deoxythymidine monophosphate; dIMP, 2′-deoxyinosine 5′-monophosphate; dAMP, 2′-deoxyadenosine 5′-monophosphate; 1843U, (*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo[*f*]quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindolinyl)glutamic acid; PDDF, 10-propargyl 5,10-dideazafolate; DSC, differential scanning microcalorimetry.

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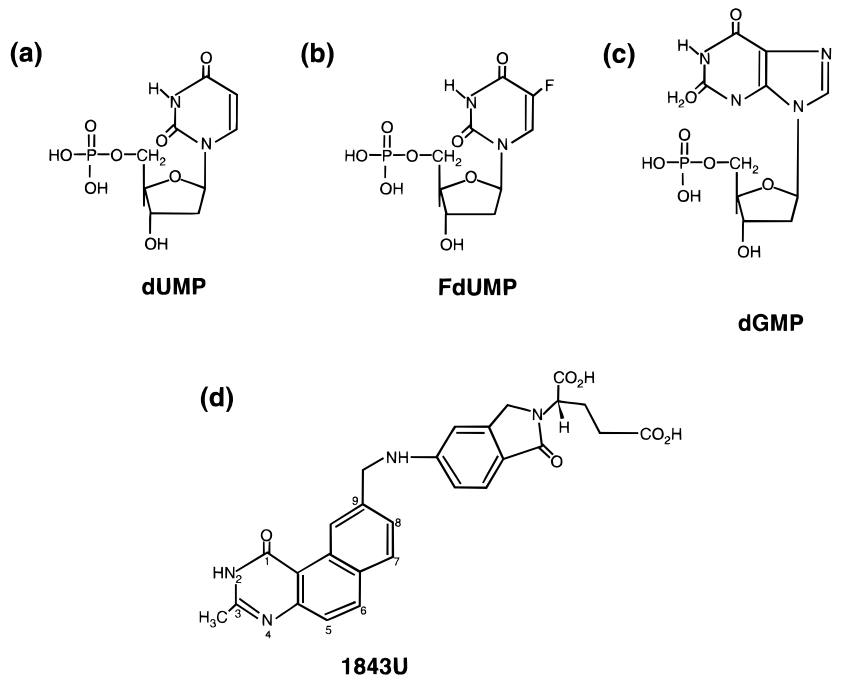

FIGURE 1: Structures of compounds used in the DSC experiments: (a) dUMP, (b) FdUMP, (c) dGMP, and (d) 1843U.

clearly shown in X-ray diffraction analyses (Montfort *et al*., 1990; Matthews *et al*., 1990a,b).

Of interest is the finding that each folate analogue elicits a unique CD spectrum in the presence of TS and FdUMP or dUMP (in preparation). This effect is particularly pronounced in the presence of 1843U (Weichsel *et al*., 1995), a potent folate analogue inhibitor of TS (Duch *et al*., 1993). 1843U was found not only to potentiate the binding of dUMP and FdUMP by about 2 orders of magnitude, it also enhanced unexpectedly the binding of such purine nucleotides as dGMP and dIMP but had no effect on the binding of neither 3′-dGMP nor dAMP (Weichsel *et al*., 1995). X-ray diffraction studies revealed that the binding of the purine nucleotides was accomplished by a distortion of the active site by 1843U to accommodate the larger purine ring, with the binding energy being provided by extensive stacking between the purine ring and the benzoquinazoline ring of 1843U and hydrogen bonds from H_2O to both the N_7 of dUMP and the hydroxyl of Tyr94 (Weichsel *et al*., 1995; Weichsel & Montfort, 1995; Stout & Stroud, 1996).

The purpose of this paper is to investigate the thermodynamics of this stacking energy and that of substrate binding to TS by DSC.

MATERIALS AND METHODS

Chemicals. 1843U was generously provided by Robert Ferone of the Glaxo-Wellcome Co., Research Triangle Park, NC. The nucleotides used, dUMP, FdUMP, and dGMP, were of the highest purity available commercially. Dithiothreitol and Hepes buffer were purchased from Sigma. Ethylene glycol (>99% pure) was obtained from Fisher. The chemical structures of dUMP, FdUMP, dGMP, and 1843U are presented in Figure 1.

Enzyme Preparation. *Escherichia coli* TS was amplified using a high-expression system, which yielded 40−50% of the cellular protein of *E. coli* as TS (in preparation). The enzyme was purified to homogeneity on DE-52 by a procedure similar to that we described previously (Maley & Maley, 1988), but with omission of the affinty-column step. Enzymatic activity was assayed at 30 °C by measuring formation of the product, dihydrofolate, by the increase in absorbance at 340 nm (Wahba & Friedkin, 1961).

Differential Scanning Microcalorimetry (DSC). The stabilization of TS as a result of nucleotide binding to TS in the absence and presence of 1843U was measured using a Hart Scientific DSC (Pleasant Valley, UT), which contained three sample cells and one solid ampule cell. TS (20 to 25 mg) was dialyzed twice against 100 mL of a solution containing 30 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% ethylene glycol. Each sample cell contained 109 μM (2.32 mg) TS with the various additions of nucleotides and 1843U as indicated in the legends of Figures 2−5. The reaction solutions in the sample cells were brought to 0.35 mL with the same buffer used to dialyze the enzyme. The Hart DSC software was used for base line subtraction, peak integration, and unit conversion as described previously (Roth & Chen, 1992). In nucleotide binding and stabilization experiments, samples of the above dialyzed TS were scanned from 10 or 20 °C to 85 °C at a heating rate of 60 °C/h in the presence and absence of an appropriate amount of substrate or substrate analogue. Buffer solution, with or without substrate, as indicated in the legends, or the second DSC scan after protein denaturation, was used as a control. Subtraction of the control from the sample run yielded a corrected thermogram for the temperature of thermal unfolding and the enthalpy of unfolding of TS. To determine the stabilization effect of 1843U on the binding of nucleotide to TS, the thermal unfolding of nucleotide bound TS in the presence of 1843U was examined. Buffer solution in the presence of substrate and 1843U or the second DSC scan after protein denaturation was used as the control from which the thermal unfolding temperature and the enthalpy of unfolding of TS were determined. The enthalpy change in thermal unfolding of TS was determined by peak area integration, while the heat capacity change of unfolding was determined from the initial and final base lines

Table 1: Thermodynamic Parameters for the Binding of dUMP and FdUMP to TS at T_d

TS	T_d [a] (K)	ΔH_d [a] (kcal/mol)	K_b [b] ($\times 10^5$ M^{-1})	ΔG_b (kcal/mol)	ΔH_b (kcal/mol)	ΔS_b (cal/mol/K)
alone	318.1 ± 0.3[c]	156 ± 4[d]				
+ dUMP	323.7 ± 0.5	158 ± 6	3.1 ± 0.8	-8.1 ± 0.2	-9.8 ± 1.6	-5.3 ± 1.0
+ FdUMP	323.0 ± 1.0	154 ± 4	1.1 ± 0.1	-7.4 ± 0.1	-8.8 ± 1.7	-4.3 ± 1.0
+ dGMP	318.3 ± 0.6	157 ± 4				
+ 1843U	320.6 ± 0.9	159 ± 5				

[a] The reported values of T_d and ΔH_d are the average of three to five measurements. [b] Data are taken from equilibrium dialysis measurements (Weichsel *et al*., 1995), as compared to 4.0×10^5 and 1.8×10^5 M^{-1} as determined by eq 1, for the respective binding of dUMP and FdUMP to TS. [c] Taken as T_o for eq 2. [d] Taken as $\Delta H(T_o)$ for eq 3.

Table 2: Thermodynamic Parameters for the Formation and Stabilization at the T_d of the Ternary Complexes

TS	T_d [a] (K)	ΔH_d [a] (kcal/mol)	K_b [b] ($\times 10^7$ M^{-1})	ΔG_b (kcal/mol)	ΔH_b (kcal/mol)	ΔS_b (cal/mol/K)	$\Delta\Delta H_b$[c] (kcal/mol)	$\Delta\Delta G_b$[c] (kcal/mol)	$\Delta\Delta S_b$[c] (cal/mol/K)
+ dUMP + 1843U	346.0 ± 0.8	220 ± 5	2.5 ± 0.1	-11.7 ± 0.1	4.9 ± 1.1	48 ± 3	15 ± 2	-3.6 ± 0.2	54 ± 7
+ FdUMP + 1843U	345.1 ± 1.0	217 ± 7	7.7 ± 1.2	-12.5 ± 0.1	3.8 ± 0.5	47 ± 2	13 ± 2	-5.1 ± 0.1	52 ± 8
+ dGMP + 1843U[d]	345.1 ± 1.0	206 ± 5	1.3 ± 0.3	-11.2 ± 0.2	-7.2 ± 1.5	12 ± 4	−	−	−

[a] The reported values of T_d and ΔH_d are the average of three to five measurements. [b] Data are taken from equilibrium dialysis measurement (Weichsel *et al*., 1995). [c] $\Delta\Delta H_b$, $\Delta\Delta G_b$, or $\Delta\Delta S_b = \Delta H_b$, ΔG_b, or ΔS_b (in the presence of 1843U) − ΔH_b, ΔG_b, or ΔS_b (in the absence of 1843U), respectively. [d] No values for $\Delta\Delta H_b$, $\Delta\Delta G_b$ or $\Delta\Delta S_b$ are reported, since dGMP does not bind to TS in the absence of 1843U.

of the thermogram (Privalov & Khechinashvili, 1974; Chen *et al*., 1984).

Data Analysis of Binding Parameters. Thermal unfolding data obtained by DSC measurements were used to determine the binding constant (K_b) and the enthalpy of binding (ΔH_b) of inhibitor to enzyme (Schellman, 1975; Schwartz, 1988; Brandts & Lin, 1990; Pace & McGrath, 1980). The binding constant (K_b) at the unfolding temperature (T_d) can be determined from an equation derived from Schellman (1975),

$$K_b = \{\exp[(T_d - T_o)\Delta H_d/(RT_dT_o)] - 1.00\}/[I] \quad (1)$$

where ΔH_d is the enthalpy of thermal unfolding of the TS binary complex at T_d. The thermal unfolding temperature of unbound TS is represented by T_o; $R = 1.987$ cal/mol/K, and [I] is the concentration of free inhibitor or substrate. Through the use of eq 1 binding constants (K_b) for the binding of dUMP and FdUMP to TS were determined.

The use of DSC to determine the thermodynamics of the stability of ligand−TS complexes assumes that the thermal denaturation transition of enzyme is reversible. Many, if not most systems, although not reversible on the second heating, do in fact conform closely to thermodynamic behavior during the brief period they spend in the denaturation transition during the first heating (Manly *et al*., 1985). Although the unfolding of TS does not reappear upon rescanning in DSC, this thermodynamic assumption is supported by the following observations. First, the determined values of K_d (4.0×10^5 M^{-1} and 1.8×10^5 M^{-1} at T_d) based on eq 1 for the binding of dUMP and FdUMP to TS, respectively, are close to the corresponding K_d values obtained by equilibrium dialysis measurements (3.1×10^5 M^{-1} and 1.1×10^5 M^{-1}, respectively), implying that a reversible thermodynamic model is applicable to interpret thermal unfolding of TS. Second, the T_d and ΔH_d values for TS remained unchanged, within experimental error, as the scan rate was increased from 50 °C/h to 70 °C/h, which further supports the assumption of a thermodynamic model (Manly *et al*., 1985).

Table 1 also lists the values of ΔH_d, T_o, and T_d. However, eq 1 could not be used to determine the values of K_b for the ternary complexes (dUMP−TS−1843U, FdUMP−TS−1843U, and dGMP−TS−1843U) because of difficulty in determining [I] in the presence of the second ligand in the reaction. Therefore, the values of K_b given in Table 2 are taken from our previously reported data (Weichsel *et al*., 1995) by assuming that the temperature dependency is not large.

From the values of K_b, the free energy of binding (ΔG_b) at T_d can be determined according to

$$\Delta G_b = -RT_d \ln K_b \quad (2)$$

The obtained values of ΔG_b are also listed in Table 2. The heat of binding may be estimated from the DSC data. Using the unfolding enthalpy ($\Delta H(T_d)$) of unbound TS and the enthalpy for bound TS ($\Delta H_d(T_d)$) in binary or ternary complex at T_d, the enthalpy of binding (ΔH_b) at the unfolding temperature (T_d) as the TS ligand binding sites are saturated was obtained by

$$\Delta H_b = \Delta H_d(T_d) - \Delta H(T_d) \quad (3)$$

$$= \Delta H_d(T_d) - [\Delta H(T_o) + \Delta C_p(T_d - T_o)] \quad (4)$$

(Schwartz, 1988; Fukada *et al*., 1983; Brandts & Lin, 1990), where $\Delta H(T_d) = \Delta H(T_o) + \Delta C_p(T_d - T_o)$, assuming that ΔC_p is independent of temperature. In eq 4, $\Delta H(T_o)$ is the unfolding enthalpy of unbound TS at T_o. ΔC_p is the heat capacity change in the thermal unfolding of TS, which is determined from the initial and final base lines of the thermogram (Privalov & Khechinashvili, 1974; Chen *et al*., 1984), and was found to be 2.12 kcal/mol/K. From the calculated values of ΔG_b and ΔH_b, the entropy of binding (ΔS_b) at the denaturation temperature (T_d) can be determined according to

$$\Delta S_b = (\Delta H_b - \Delta G_b)/T_d \quad (5)$$

RESULTS

Effect of dUMP on Thermal Unfolding of TS. Typical DSC measurements of the thermal unfolding of TS in the absence and presence of increasing amounts of dUMP are shown in Figure 2. It is rather clear from the data in Figure 2 that there are two transition temperatures associated with the unfolding of *E. coli* TS, perhaps reflecting the asymmetric

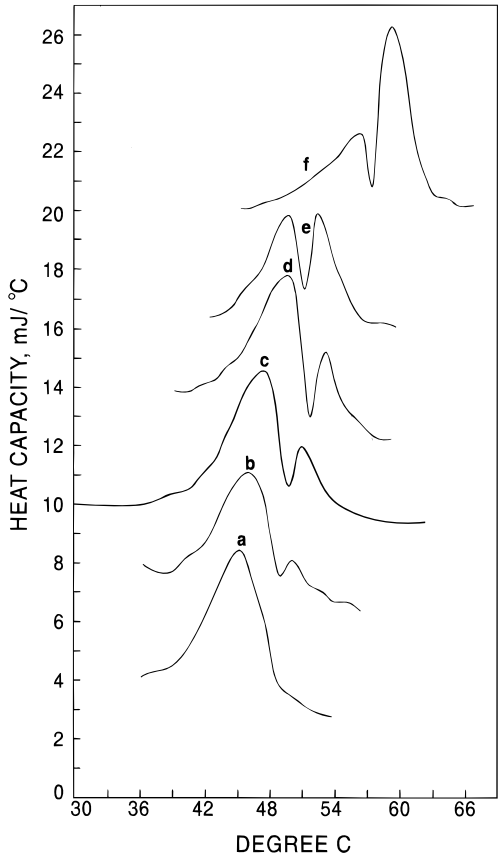

FIGURE 2: Thermal unfolding of TS as a function of the concentration of dUMP. The final concentrations of dUMP in each of the DSC reaction solutions (see Materials and Methods for the procedures used) were as follows: (a) zero, (b) 25.7 μM, (c) 37.1 μM, (d) 100 μM, (e) 240 μM, and (f) 14.3 mM.

interaction of its two subunits under a variety of conditions. In the absence of dUMP the thermogram reveals a major peak (t_a in °C) at 45 °C with a small shoulder (t_b in °C) at 47 °C (Figure 2a). As the dUMP concentration is increased the shoulder becomes a separate high-temperature peak (Figure 2b−f) distinct from a lower temperature peak. Both peaks represent two domains (A and B) that are not equivalent in structure, being that their peak temperatures (t_a and t_b) of unfolding are about 2−3 °C apart. This result is somewhat surprising in that only one binding site on TS is evident for dUMP (Galivan *et al*., 1976; Weichsel *et al*., 1995), that is, unless its binding to one subunit site cooperatively affects the conformation of the second domain. Interestingly, when the dUMP level is increased 60-fold above that in Figure 2e, the unfolding temperature of the major peak is increased to about 60 °C (Figure 2f). It is not clear whether both subunits are saturated with dUMP at this high concentration of nucleotide, which could account for the higher temperature of unfolding, or that saturation of the main binding site makes domains A and B more symmetric. Similar types of temperature transitions have been seen with other proteins (Ghosaini *et al*., 1988; Lin *et al*., 1994; Blandamer *et al*., 1994). Previous studies have shown that the change in the temperature of a transition process, as a solute is added or the solvent is perturbed, is related to its free energy change (Chen, 1981; Wang & Chen, 1993). In unfolding thermograms consisting of high- and low-temperature peaks, T_d is taken as the temperature at which one-half of the total area under the unfolding thermogram is

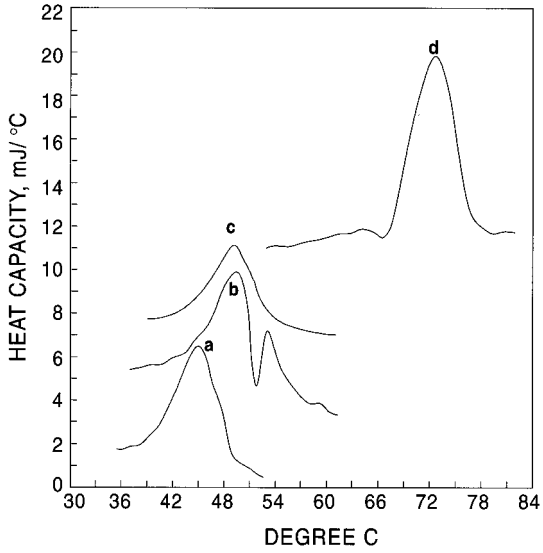

FIGURE 3: DSC measurements of the effects of dUMP and 1843U on the thermal unfolding of TS. The final concentrations of each compound in the DSC reaction solutions (see Materials and Methods) were as follows: (a) no additions, (b) dUMP, 100 μM, (c) 1843U, 263 μM, and (d) dUMP, 100 μM, and 1843U, 263 μM.

generated (Lin *et al*., 1993). An increase in T_d as dUMP binds to TS as shown in Table 1 implies that the free energy of unfolding of TS is increased and that substrate binding stabilizes the structure of TS.

Table 1 reveals that, although the temperature of unfolding (T_d) increases from 318 K to almost 324 K as dUMP binds to the enzyme, the enthalpy of unfolding (ΔH_b) shows no significant increase upon dUMP binding (156 vs 158 kcal/mol). A small negative value (−5 cal/mol/K) is found for ΔS_b. These results suggest that hydrophobic interactions, which are associated with the release of water, are not involved in the binding, since solute−solute hydrophobic interactions are associated with a large enthalpy or entropy change (Chen, 1980, 1981).

Effect of 1843U on Binding of dUMP to TS. The stabilizing effect of dUMP on the thermal unfolding of TS is greatly enhanced by the presence of 1843U (Figure 3d). Thus, at a molar ratio of dUMP to TS of one and with 1843U present, peak A essentially disappears, and t_d is shifted to 72 °C. In the control experiment, Figure 3c reveals that 1843U by itself has a small effect on the unfolding thermogram of TS; the shape of the thermogram is essentially unchanged and t_d is increased by only 2.5 °C above that of the enzyme alone (Figure 3a), while the enthalpy of unfolding (ΔH_b) shows no significant increase. In addition to an effect on t_d (28 °C higher), Table 2 reveals that ΔH_d is greatly increased by the presence of 1843U (from 156 to 220 kcal/mol). These findings confirm that 1843U strongly enhances the binding of dUMP to TS, resulting in the formation of a dUMP−TS−1843U ternary complex (Dev *et al*., 1994). Meanwhile, 1843U increases the entropy of binding (ΔS_b) from −5 to 48 cal/mol/K.

Effect of 1843U on the Binding of FdUMP to TS. The effect of FdUMP on the unfolding thermogram of TS is demonstrated in Figure 4. Similar to the case of dUMP, as the molar ratio of FdUMP to TS is increased to 2.6, domain B increases from a small shoulder to a peak which is comparable in size to domain A (Figure 4b). Simultaneously, the values of t_a and t_b shift upward. Table 1 reveals that the

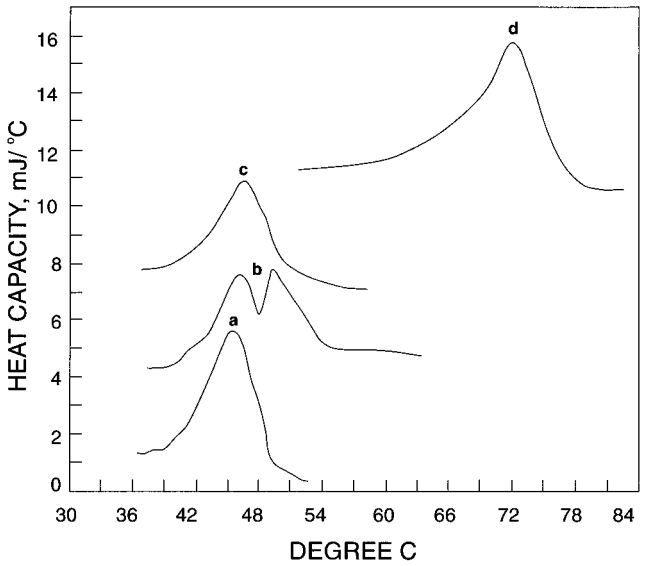

FIGURE 4: DSC measurements of the effects of FdUMP and 1843U on the thermal unfolding of TS. The final concentrations of each compound in the DSC reaction solutions were as follows: (a) no additions, (b) FdUMP, 286 μM, (c) 1843U, 263 μM, and (d) FdUMP, 286 μM, and 1843U, 263 μM.

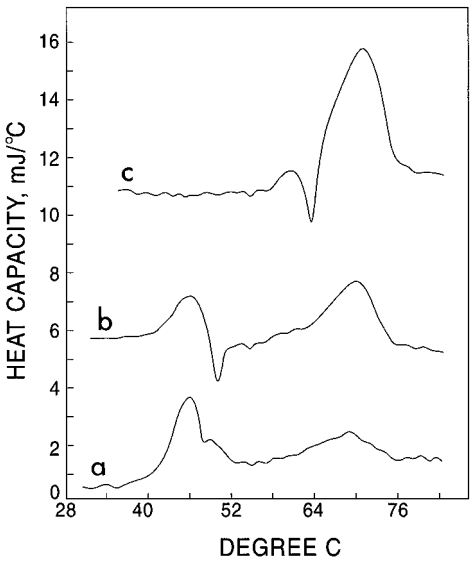

FIGURE 5: The effect of 1843U on the thermogram of TS in the presence of dGMP. The concentrations of 1843U in each of the DSC reaction solutions were 263 μM with dGMP varied as follows: (a) 28.6 μM, (b) 71.4 μM, and (c) 286 μM.

T_d increases from 318 to 323 K, similar to dUMP, while ΔH_d shows little effect due to the presence of FdUMP (154 vs 156 kcal/mol) and ΔS_b is slightly negative (−4 cal/mol/K). Similar to the case of dUMP, these observations confirm that the binding of FdUMP to TS, like dUMP, does not involve hydrophobic interactions or the release of water.

A comparison of the thermal unfolding profiles of TS in the presence of FdUMP (Figure 4b) versus those in the presence of dUMP (Figure 2e) at similar molar ratios of nucleotide to TS, reveals that t_a and t_b for FdUMP are about 4 °C lower than those for dUMP. These results indicate that the effect of FdUMP on thermal unfolding of TS is less profound than that of dUMP, which is a consequence of the fact that FdUMP binds more weakly to TS than dUMP (see K_b values in Table 1). This finding agrees with *Lactobacillus casei* TS (Galivan *et al*., 1976) and *E. coli* TS (Weichsel *et al*., 1995) binding studies, which indicate that FdUMP has a lower affinity for TS although it appears to be more disposed to bind to two sites in contrast to dUMP. This latter degree of binding has been reported also for human TS (Dev *et al*., 1994) and earlier for *L. casei* (Galivan *et al*., 1976).

Similar to the case of dUMP, 1843U causes a dramatic effect on the thermogram of TS in the presence of FdUMP (Figure 4d). When a combination of FdUMP and 1843U is used, the peak of domain B becomes dominant over that of domain A, and T_d is shifted upward by 22 K (Table 2) relative to FdUMP alone (Table 1). These observations confirm that 1843U strongly increases the binding of FdUMP to TS, resulting in the formation of an FdUMP−TS−1843U complex as previously reported (Weichsel *et al*., 1995). In addition to domain and temperature effects, a comparison of the results in Tables 1 and 2 shows that the combination of FdUMP and 1843U greatly enhances the values of ΔH_d (217 vs 154 kcal/mol for FdUMP alone) and ΔS_b (47 vs −4 cal/mol/K).

Effect of 1843U on Binding of dGMP to TS. In contrast to the results in Figures 3 and 4, dGMP essentially does not affect the unfolding thermogram of TS, as evidenced by the lack of change in the values of t_a and t_b (data not shown). This observation supports the finding that dGMP by itself does not bind to TS (Weichsel *et al*., 1995). However, when 1843U is added to dGMP and TS in solution, the unfolding temperature of TS is dramatically increased (Figure 5), an effect associated with the binding of dGMP to TS (Weichsel *et al*., 1995). The extent of this response (at a fixed amount of 1843U) appears dependent on the molar ratio of dGMP to TS. Thus, at a molar ratio of dGMP to TS of 0.3/1.0, two separate peaks (A and B) are obtained, with the area under A greater than that under B. Peak A occurs at a t_a of 46 °C, about the same as the t_a of TS alone, while peak B attains a t_b of 70 °C. As the molar ratio of dGMP to TS is raised to 0.66/1.0 (Figure 5b), peak B increases in area while peak A decreases, although t_a and t_b do not change. When the molar ratio is increased to 2.6/1.0 (Figure 5c), peak A essentially disappears and the t_b of peak B increases slightly to 72 °C, with ΔH_d increasing to 206 kcal/mol (Table 2). Thus, unlike dUMP and FdUMP, T_d is not altered by dGMP alone, indicating that dGMP binding does not occur until 1843U is added, resulting in the formation of a dGMP−TS−1843U ternary complex with its elevated value of T_d. This latter complex has been clearly seen in X-ray diffraction studies (Weichsel *et al*., 1995). Table 2 also shows that dGMP binding to TS by 1843U is associated with a positive ΔS_b (12 cal/mol/K).

Stabilization of Nucleotide Binding to TS by 1843U. The stabilizing effect of 1843U on the structure of TS associated with the binding reaction can be evaluated by defining the parameter $\Delta\Delta G_b$ as ΔG_b (in the presence of 1843U) − ΔG_b (in the absence of 1843U), assuming that the value of ΔG_b does not change significantly in the temperature range between T_d of binary binding and T_d of ternary binding. Table 2 shows that the values of $\Delta\Delta G_b$ are −3.6 and −5.1 kcal/mol in the cases of dUMP and FdUMP, respectively, indicating a significant stabilization of substrate binding reaction by 1843U. A value for $\Delta\Delta G_b$ is not determined for dGMP, since it does not bind to TS in the absence of 1843U. However, a similar negative value of ΔG_b for dGMP (−11 kcal/mol) as compared with dUMP and FdUMP reveals

that the presence of 1843U is essential for binding to occur. Similarly, $\Delta\Delta H_b$ and $\Delta\Delta S_b$ are defined as the difference between the presence and absence of 1843U for the binding of dUMP and FdUMP. A modest positive value is found for $\Delta\Delta H_b$, and a significant positive value is found for $\Delta\Delta S_b$.

DISCUSSION

These studies show that the effect of specific ligands on the unfolding thermogram of TS is rather significant (Table 1). This result might have been expected from earlier findings which show that dUMP stabilizes TS activity to heat inactivation (Lorenson *et al*., 1967; Galivan *et al*., 1976). It is possible to rationalize some of the ligand-associated responses of *E. coli* TS on the basis of their known crystal structures (Weichsel *et al*., 1995). Nothing remarkable is observed in the dUMP or FdUMP binary structures with respect to their conformational states relative to unliganded TS, but the observed charge and Van der Waals contacts between the nucleotides and amino acids associated with the active site undoubtedly play a role in limiting the thermal unfolding of the ligand containing protein. Particularly significant are the two contacts involving charged arginines from the second subunit that hydrogen-bond to the phosphate of the nucleotide bound to the active site of the first subunit. Table 1 reveals a modest negative value for ΔH_b associated with the binding of dUMP and FdUMP, which suggests that the binding reaction is driven by electrostatic forces (Beaudette *et al*., 1980). These results agree with the crystallographic data of the TS−dUMP binary complex, which show that the nucleotide phosphate group is involved in hydrogen bonding to four arginines and a serine (Montfort *et al*., 1990). The relatively small negative value of ΔS_b for the binding of dUMP and FdUMP to TS (Table 1) suggests that the binding of these nucleotides is likely to affect local changes around the binding site rather than cause a significant change in subunit conformation and is consistent with the X-ray crystallography studies (Matthews *et al*., 1990a,b; Montfort *et al*., 1990). Surprisingly, although 1843U binds tightly to TS (Dev *et al*., 1994), this folate analogue by itself does not increase T_d as much as dUMP and FdUMP do (Figures 3 and 4). It is also noteworthy that the above mentioned arginines are not involved in 1843U binding. The contacts with these inter-subunit or shared arginines could thus be the cement that holds the subunits together and restricts the thermal unfolding of the TS nucleotide binary complex.

As noted in Figure 2, the thermal unfolding is concentration-dependent, which might be expected if this parameter is related to the amount of dUMP (or FdUMP) associated with the active site. Of interest is the appearance of two domains (A and B) as the concentration of dUMP is raised, one obviously more stable than the other (Figure 2b−f). In the absence of nucleotide, the thermogram in Figure 2a shows a hump in the TS unfolding profile of what is believed to be domain B. It is not clear whether this domain is a consequence of localized asymmetry in the two subunits or of the association of inorganic phosphate, an anion that has been observed in the crystals of TS (Montfont *et al*., 1990; Perry *et al*., 1990). The presence of inorganic phosphate, a competitive inhibitor of dUMP (Galivan *et al*., 1976, 1977a; Lewis *et al*., 1978), in the active site of TS is not surprising, since the enzyme is purified in the presence of potassium phosphate buffer. As indicated earlier (Galivan *et al*., 1976), binding at the two active sites shows asymmetry in that dUMP appears to prefer one site over the other. Whether binding at one site affects the conformational stability of the other subunit or whether the second site is gradually filled after the first is filled (Figure 2f), as suggested by Danenberg and Danenberg (1979), is not known.

While 1843U by itself has only a small effect on TS stability, it is clear from the results in Figures 3−5 that the thermal stability of binary complexes of TS and nucleotide is increased even further by the presence of 1843U. This marked increase in T_d (Table 2) is associated with a corresponding increase in the binding of dUMP and FdUMP by 2 orders of magnitude [Weichsel *et al*. (1995) and Tables 1 and 2]. Of even more interest is the fact that the binding of dGMP occurs, which is not evident in the absence of 1843U (Weichsel *et al*., 1995). A comparison of the combined effects of dGMP and 1843U (Figure 5) with those obtained for 1843U and dUMP (Figure 3d) or 1843U and FdUMP (Figure 4d) reveals significant differences between them. Thus, in the case of dUMP and FdUMP, two distinct peaks are observed initially for each, which became single peaks as the binding domain(s) was saturated with 1843U and nucleotide (Figures 3d and 4d). By contrast, since dGMP does not bind in the absence of 1843U, it is seen (Figure 5a,b) that of the two peaks present, one represents unbound TS (t_a, 46 °C), while the other represents a ternary complex of TS, 1843U, and dGMP (t_b, 72 °C). This is supported by the fact that, as the dGMP concentration is increased, the former peak diminishes and the latter increases.

The broadening of the transition at subsaturating dGMP levels in the presence of the strong binding ligand 1843U (curves a and b in Figure 5) is in accord with a thermodynamic model formulated for interpreting DSC data on proteins that have interacting domains (Brandts *et al*., 1989). In this model, the interactions were quantified by the inclusion of an interface free energy in the thermodynamics of the unfolding of multidomain proteins, and a broadening of the transition is observed at subsaturating ligand concentrations. This would appear to be the case for ligand−TS binding where the separation of its two domains occurs, implying that the binding of dGMP and 1843U to one domain in TS is stabilized by the participation of the second domain. A similar transition broadening was observed for the binding of dUMP and FdUMP to TS when present at subsaturating concentration in the presence of 1843U (data not presented).

From X-ray data, the nature of these ternary complexes is clearer and can provide a rationale for the enhanced stability of TS. Thus, similar to other TS ternary complexes including TS−dUMP−PDDF (Matthews *et al*., 1990a; Montfort *et al*., 1990) and TS−FdUMP−CH_2H_4PteGlu (Matthews *et al*., 1990b), TS−dUMP−1843U undergoes extensive conformational changes but with numerous local changes to accommodate the binding of the larger 1843U molecule (Weichsel *et al*., 1995). In addition, a stacking arrangement occurs between the benzoquinazoline ring of 1843U and the pyrimidine ring of dUMP (and FdUMP), which no doubt cintributes to the enhanced binding of this nucleotide. Similarly, in the case of dGMP the purine ring is located parallel to the benzoquinazoline ring at a distance of 3.5 Å, with favorable Van der Waals contacts to N_7, C_8, and N_9 of dGMP relative to dUMP, which does not contain these atoms. To accommodate the larger dGMP in the active site, localized rearrangements occur including a 0.4 Å shift of the J helix

away from the active site, a 0.8 Å shift of the deoxyribose moiety in a direction opposite the J helix, and a shift of the (Gln19)−(Thr24) loop about 0.5 Å outward from the active site. The Arg21 residue is also altered in its local conformation so as to remain bound to the phosphate of dGMP. A new hydrogen bond, not present in the dUMP ternary structure, is mediated by a water molecule between N_7 of dGMP and the hydroxyl of Tyr94 (Weichsel *et al*., 1995). It appears, therefore, that the enlargement of the active site pocket by 1843U, which is associated with extensive stacking between the purine and benzoquinazoline rings, makes the binding of dGMP and other related purines (GMP, IMP, dIMP) possible.

It is worthwhile to point out that the value of ΔH_b in the TS−dGMP−1843U complex is −7.2 kcal/mol relative to 4.9 kcal/mol in TS−dUMP−1843U and 3.8 kcal/mol in TS−FdUMP−1843U (Table 2). This negative value of ΔH_b for the TS−dGMP−1843U complex is comparable to those in the binary complexes of TS−dUMP (−9.8 kcal/mol) and TS−FdUMP (−8.8 kcal/mol) (Table 1), indicating that, as in TS−dUMP or TS−FdUMP binary binding, the binding of dGMP to TS (in the presence of 1843U) is driven by electrostatic forces. This suggestion is consistent with the finding that polar groups (Gln, Thr, and Arg) and hydrogen bonding contribute to the binding of dGMP to TS in its ternary complex as described above. In contrast, the positive values found for ΔH_b and ΔS_b (Table 2) suggest that hydrophobic interactions play a role in the binding of dUMP and FdUMP to TS in their respective ternary complexes, resulting in a conformational change in TS that is associated with the exclusion of water from its active site. This finding is supported by a significant positive $\Delta\Delta S_b$ change (52−54 cal/mol/K), a positive change in the enthalpy ($\Delta\Delta H_b = 13-15$ kcal/mol), and a negative change in the free energy ($\Delta\Delta G_b = -4$ to -5 kcal/mol). As shown earlier, an opposite sign for the change in enthalpy and free energy ($\Delta\Delta H_b > 0$ and $\Delta\Delta G_b < 0$) is characteristic of solute−solute (protein) hydrophobic interactions (Chen, 1980, 1981). The exclusion of water may occur as the active site pocket is enlarged by 1843U to accommodate dUMP or FdUMP. It should be noted that these thermodynamic parameters were determined from protein unfolding data at T_d. In future studies, we hope to determine thermodynamic binding parameters at room temperature using isothermal titration microcalorimetry to further support the present conclusions.

Negative values of $\Delta\Delta G$, shown in Table 2, reveal that a considerable extra free energy of stabilization of TS appears in the formation of these complexes, as compared with the binding of substrate to TS alone. This extra stabilization energy is most likely due to the stacking interaction between dUMP, FdUMP, or dGMP and 1843U, as revealed by X-ray analyses (Weichsel *et al*., 1995). Since 1843U does not significantly stabilize the enzyme against thermal unfolding, although binding tightly to TS (Dev *et al*., 1994), it appears that the stacking energy resulting from the interaction of the pyrimidine ring (or purine ring in the case of guanosine and inosine nucleotides) and the benzoquinazoline ring of 1843U (Weichsel *et al*., 1995) is a major force in restricting the thermal unfolding of the TS subunits.

Interestingly PDDF alone (and its polyglutamate derivatives) can induce most of the conformational changes in TS observed in its ternary complex (dUMP−TS−PDDF) (Kamb *et al*., 1992). Consistent with this finding is our observation that the T_d of PDDF − TS increases to 61 °C (data not shown). The most striking conformational transition, however, occurs on ternary complex formation, which is associated with the sealing off of the active site by the carboxy terminal end of TS (Matthews *et al*., 1990a; Montfort *et al*., 1990). In this instance, the T_d of the PDDF ternary complex increased to over 70 °C (data not shown), which is similar to that obtained for the 1843U ternary complex. But, the T_d of the binary complex of 1843U−TS is lower than that of PDDF−TS. The reason for this is not clear at present, since 1843U binds to TS even more tightly than PDDF does, but this may be due to the fact that the latter binds to both active site subunits while the former appears to bind primarily to only one (Dev *et al*., 1994).

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