Energetic Contributions of Four Arginines to Phosphate-Binding in Thymidylate Synthase Are More than Additive and Depend on Optimization of "Effective Charge Balance" †,‡

Richard J. Morse,§ Shido Kawase,|| Daniel V. Santi,§,|| Janet Finer-Moore,*,§ and Robert M. Stroud*,§,||

Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, The University of California at San Francisco, San Francisco, California 94143-0448

Received August 9, 1999; Revised Manuscript Received October 29, 1999

ABSTRACT: In thymidylate synthase, four conserved arginines provide two hydrogen bonds each to the oxygens of the phosphate group of the substrate, 2′-deoxyuridine-5′-monophosphate. Of these, R23, R178, and R179 are far removed from the site of methyl transfer and contribute to catalysis solely through binding and orientation of ligands. These arginines can be substituted by other residues, while still retaining more than 1% activity of the wild-type enzyme. We compared the kinetics and determined the crystal structures of dUMP complexes of three of the most active, uncharged single mutants of these arginines, R23I, R178T, and R179T, and of double mutants (R23I, R179T) and (R178T, R179T). The dramatically higher K_m for R178T compared to the other two single mutants arises from the effects of R178 substitution on the orientation of dUMP; 10−15-fold increases in $K_m^{CH_2H_4\,folate}$ for R23I and R178T reflect the role of these residues in stabilizing the closed conformation of TS in ternary complexes. The free energy for productive dUMP binding, ΔG_S, increases by at least 1 kcal/mol for each mutant, even when dUMP orientation and mobility in the crystal structure is the same as in wild-type enzyme. Thus, the four arginines do not contribute excess positive charge to the PO_4^{-2} binding site; rather, they ideally complement the charge and geometry of the phosphate moiety. More-than-additive increases in ΔG_S seen in the double mutants are consistent with quadratic increases in ΔG_S predicted for deviations from ideal electrostatic interactions and may also reflect cooperative binding of the arginines to the phosphate oxygens.

The phosphate-binding pocket of thymidylate synthase (TS)[1] is a paradigm for an electrostatic interaction in a protein-binding site. Four conserved arginines, two from each protomer of the dimeric enzyme, form the binding site for the phosphate moiety of the substrate, dUMP. Inorganic phosphate, or phosphoribose binds at the same site in competition with dUMP (*1*, *2*). When any arginine is altered for another residue, K_d for dUMP increases at least 20-fold, even when the number of hydrogen bonds to the phosphate is preserved. This corresponds to an electrostatic contribution to the arginine−phosphate interaction of greater than 1.8 kcal/mol (*3*).

Despite being totally conserved, three of the four arginines, arginines 23, 178′,[2] and 179′, can be substituted by several other residues without loss of function as reflected by the ability of such mutations to complement a TS deficient (thy^-) strain (*Escherichia coli* χ2913recA) (*4*, *5*). Such complementation generally implies activity above ∼1% of wild-type TS. This result reflects the fact that these three arginines (unlike R218) are too distant from the catalytic cysteine and the pyrimidine ring of dUMP to have a direct impact on the chemical steps involved in methylation. Thus, mutations of these arginines evoke their effects, clearly seen in both k_{cat}, and K_m, primarily by altering the binding affinity (K_d), orientation, and vibrational entropy of the bound ligands. Like R23, R178′, and R179′, the fourth arginine at the phosphate site, R218, makes two donor hydrogen bonds to the phosphate; however, it cannot be mutated without loss of activity (*4*, *5*). R218 is adjacent to the catalytic cysteine, C198, and we postulate that the guanidinium group of R218 polarizes C198 for nucleophilic attack on the pyrimidine ring (*6*). In addition, it stabilizes the conformation of the loop containing C198 by hydrogen bonding to the carbonyls of P196 and P197, thus it may be essential for the orientation and reactivity of the active-site cysteine.

All four arginines make hydrogen bonds to the protein and/or cofactor, besides their two donor hydrogen bonds to the phosphate moiety of dUMP. Consequently, they not only provide electrostatic binding energy for dUMP but also

† This work was supported by NIH grants CA-41323 to J.F.-M. and R.M.S. and CA-14394 to D.V.S. S.K. was supported by UNITKA Ltd.

‡ Coordinates of R23I, R178T, R179T, (R23I, R179T) and (R178T, R179T) are deposited in the PDB with accession codes 1BP0, 1B08, 1B07, 1BP6, and 1BPJ, respectively.

§ Department of Biochemistry and Biophysics.

|| Department of Pharmaceutical Chemistry.

[1] Abbreviations: TS, thymidylate synthase; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_2H_4folate or THF, 5, 10-methylene-5,6,7,8-tetrahydrofolate; 5-bromo-dUMP, 5-bromo- 2′-deoxyuridine-5′-monophosphate; F_o, measured structure factor amplitude; F_c, calculated structure factor amplitude; $(2F_o-F_c)\alpha_{calc}$ map, electron density map calculated with coefficients $(2F_o-F_c)$ and phases derived from the coordinates of the structure; $(F_o-F_c)\alpha_{calc}$ map, electron density map calculated with coefficients (F_o-F_c) and phases derived from the coordinates of the structure; ΔG_S, free energy of dUMP binding during the TS reaction; R178T, variant in which R178 has been mutated to threonine; ES, enzyme−substrate complex.

[2] Residues from the "second" monomer that enter into discussion of the "first" monomer are indicated with a prime, e.g., R178′.

10.1021/bi9918590 CCC: $19.00
Published on Web 01/11/2000

Table 1: Kinetic Constants for Arginine Mutants

mutant	k_{cat} (s^{-1})	K_m^{dUMP} (μM)	K_d^{dUMP} (μM)	k_{cat}/K_m^{dUMP} [a] (μM^{-1} s^{-1})	K_m^{THF} (μM)	$\Delta\Delta G_S$ [b] (kcal mol^{-1})	H-bonds to PO_4
native	5.1	2.6	0.20	2.0	20		10
R179T	4.6	10		4.6×10^{-1}	44	0.87	9
R178T	2.8	900		3.0×10^{-3}	332	3.8	7
R23I	0.55	44	4.0	1.3×10^{-2}	224	3.0	8
R178TR179T	0.02	33000		6.0×10^{-7}	250	8.9	5
R23IR179T	0.04	2500		1.6×10^{-5}	900	6.9	7

[a] Under conditions of saturating folate used, the on-rate for dUMP is given by $k_1 = k_{cat}/K_m^{dUMP}$. [b] $\Delta\Delta G_S = -RT \ln [(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$ where subscripts "mut" and "wt" refer to the mutant and wild-type enzyme, respectively (*30*).

contribute to orientations of ligands and ligand-binding residues and to the stability of the active site. Because of conformational changes in both the protein and its ligands during catalysis, these arginines come into play in binding and orienting the substrate and cofactor at different times during the multistep reaction (*7*). Steady-state kinetic and binding constants for "replacement sets" of all 20 mutations of each of the arginines at the phosphate binding site are dramatically different for each phosphate-binding arginine and reflect the distinct structural roles these arginines have during the course of the reaction (Kawase et al., in preparation). On the basis of structures of wild-type apo-TS, TS binary complexes with dUMP and ternary complexes with dUMP and the cofactor, CH_2H_4folate, we have rationalized the structural basis for the consequences of mutation on the thermodynamic and kinetic properties of the enzyme (Kawase et al., in preparation).

To assess in a quantitative way the contributions of arginines 23, 178, and 179 to substrate and cofactor binding, we have now examined the effects of the mutations on the structure of the active-site cavity and on ligand-binding contacts and have correlated these effects with functional parameters. Active mutants were chosen so that kinetic constants and structures could be compared to determine the energetic contributions of each residue to binding and catalysis. Specifically, we selected mutations of R23 and R178 that removed the positive charge and were among the most active, with activity (k_{cat}/K_m ~1% of wild-type). R179 is less sensitive to mutation, with only one mutant (R179P) being inactive (*4*). We have previously analyzed structures of several R179 variants (R179A, R179E, and R179K) and determined that the protein can accommodate mutations at R179 through reorganization of water structure or movement of other side chains (*3*). For comparison to the threonine variant of R178, we have selected R179T for structure determination and analysis. Double mutants (R178T, R179T) and (R23I, R179T) were characterized to address the question of whether the individual roles of the arginines are simply additive in effect or are cooperative in impairing function. We determined structures of binary ES complexes (without cofactor) to isolate the first binding step in the ordered reaction. This structure−function analysis of the phosphate-binding arginines is one key vignette in our attempt to achieve a complete, quantitative description of the stereochemical roles of each component of the protein and ligands in the reaction.

EXPERIMENTAL PROCEDURES

Preparation of the Protein, Crystallization, and Data Collection. The pSCTS9 expression vector was used to generate single-stranded DNA for mutagenesis and sequencing (*4*). Mutant *Lactobacillus casei* TS was expressed in *E. coli* χ2913 (thy^-), which lacks a gene for TS, and purified by sequential chromatography on phosphocellulose and hydroxyapatite (*8*). Activity of the TS mutants was monitored by disappearance of CH_2H_4folate, spectrophotometrically, as described (*9*). K_d's for dUMP binding to the mutant enzymes were determined by competition with the competitive binder, pyridoxal 5′-phosphate, an assay that is independent of cofactor binding (*10*).

The proteins were cocrystallized with the substrate ([dUMP] = 38 mM) at pH 7.0 as described (*1*). Hexagonal bipyramidal crystals isomorphous with the native binary complex crystal form in space group $P6_122$, grew to full size (0.5 × 0.5 × 0.75 mm) in three days. X-ray diffraction data were collected on an RAXIS-II image plate detector using X-rays from a Rigaku rotating anode generator operating at 50 kV and 300 mA. Data were collected at room temperature as a series of 1.5° or 2.0° oscillation frames of 20- and 25-minute exposure duration, respectively. Crystals did not survive transfer to cryosolvents and did not decay appreciably during the time required to collect data. Intensity data were processed with Denzo/Scalepack (*11*) and the French and Wilson truncation algorithm (*12*), with converted resulting average intensities to scaled structure factors.

Structure Solution and Refinement. Since the mutants were isomorphous to wild-type TS, structures were solved by difference Fourier maps (*13*). Several alternating rounds of simulated annealing (T_{start} = 1500°K) (*14*), positional, and individual atomic B-factor refinement were followed by manual rebuilding. Waters were placed in the $(F_o-F_c)\alpha_{calc}$ density maps, based also on favorable chemical environments. In the later stages of refinement, the occupancy of the substrate was refined for the poorer binding mutants, R178T, R23I, (R178T, R179T), and (R23I, R179T). Convergence of refinement in X-PLOR resulted in crystallographic R-factors ~19.5% and free R-factors ~25% (*15*) shown in Table 2.

Analysis of Structures. Structures were compared using difference maps, calculated with coefficients $(F_o1-F_o2)\alpha_{calc}$. Solvent accessibility of individual atoms in the apo-TS and dUMP-bound dimers were calculated with a probe sphere with radius 1.4 Å, using the program SURFACE, written by M. D. Handschumacher and F. M. Richards, as implemented in the CCP4 suite of programs.

RESULTS

Of the three single mutants whose structures we determined, R178T shows the most extensive structural changes in the active site as well as the greatest perturbation in dUMP

Table 2: Crystallographic Statistics

statistic	R179T	R178T	R23I	(R178, R179T)	(R23I, R179T)	native[a]
unit cell *a, b*	78.7	78.5	79.1	78.8	78.5	78.6
(Å) *c*	240.9	241.7	243.3	243.8	243.4	240.2
resln. (Å)	2.4	2.4	2.4	2.5	2.4	2.5
R_{merge} (%)[b]	7.4	8.1	8.4	8.9	9.3	
completeness (%)	96.2	94.7	87.6	95.5	87.0	
no. reflns. $\times 10^3$	17.5	17.3	16.2	15.6	15.9	
R_{cryst} (%)[c]	20.5	19.6	19.5	20.0	19.7	
R_{free} (%)	25.1	24.7	25.6	26.7	25.1	
$RMSD_{bonds}$ (Å)	.007	.006	.006	.007	.007	
$RMSD_{angles}$ (deg)	1.4	1.4	1.4	1.4	1.4	
$RMSD_{dihedrals}$ (deg)	24.3	24.6	24.1	24.4	24.3	
$RMSD_{impropers}$ (deg)	1.4	1.3	1.1	1.2	1.2	
$\langle B \rangle$ all (Å^2)	38	34	34	26	31	29
$\langle B \rangle$ dUMP (Å^2)	29	49	25	63	48	26
occ(dUMP) (%)	100	64	60	90	66	100
no. waters	55	67	52	59	56	47

[a] Structure determined previously. [b] $R_{merge} = \Sigma|I-\langle I\rangle|/\Sigma|\langle I\rangle|$; negative intensities included as zero. [c] $R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$.

orientation. As a consequence of these structural changes, four hydrogen bonds to the phosphate are abrogated: two from the R178 itself, at least one from R23, and another from S219; the affinity for dUMP as well as the rate of dUMP binding are severely compromised. Structural changes revealed in the binary complex of R178T with dUMP would interfere with alignment of the cofactor and closure of the active site during ternary complex formation, thus explaining the 30-fold higher K_m for CH_2H_4folate. In R23I and R179T where protein changes are more localized to the vicinities of the mutations and dUMP orientations in the binary complexes are unchanged, K_m^{dUMP}'s are closer to wild-type values. The double mutants show distinctly greater-than-additive effects on binding energy even though in (R23I, R179T) the substrate remains oriented in the wild-type position. The structures allow for an assessment of the effects of vibrational entropy and charge complementarity in an enzyme-binding site.

R178T: A 700-fold Decrease in On-rate for Productive Binding is Associated with Altered dUMP Orientation ($\Delta\Delta G_S$ = 3.8 kcal/mol). R178T has a 350-fold effect on K_m^{dUMP}. In TS, K_m^{dUMP} is related to both k_{cat} and to the productive on-rate for dUMP binding, k_1, by $K_m^{dUMP} = k_{cat}/k_1$ (*16*). Since k_{cat} in R178T is only 2-fold less than wild-type TS, the increase in K_m^{dUMP} is mostly due to 700-fold reduction in the productive on-rate, k_1. A difference map reveals extensive small differences between the structures of R178T−dUMP and wild-type TS−dUMP (Figure 1).

Substitution of R178′ by threonine is accompanied by movement of the loop that contains a second dUMP−phosphate-binding arginine, residue R23, away from the phosphate moiety. The entire R23 loop, residues 21−26, is more disordered, with average atomic B-factors of 58 Å^2 compared to 44 Å^2 in wild-type TS−dUMP. The hydroxyl of the substituted residue, T178′ Oγ, is hydrogen bonded to a conserved phosphate-binding water molecule, Wat8. A new water occupies the space vacated by the guanidinium group of R178′ and hydrogen bonds to the phosphate oxygen (Figure 2b). Several other ordered waters surround the space formerly occupied by R178′ and are hydrogen bonded to residues in the R23 loop.

In R178T, dUMP shows higher vibrational entropy ($\langle B \rangle$ = 49 Å^2, occupancy = 0.64) (Table 2) but still is extensively hydrogen bonded to the protein (Table 3, Figure 2b). However the orientation of dUMP in the active site is

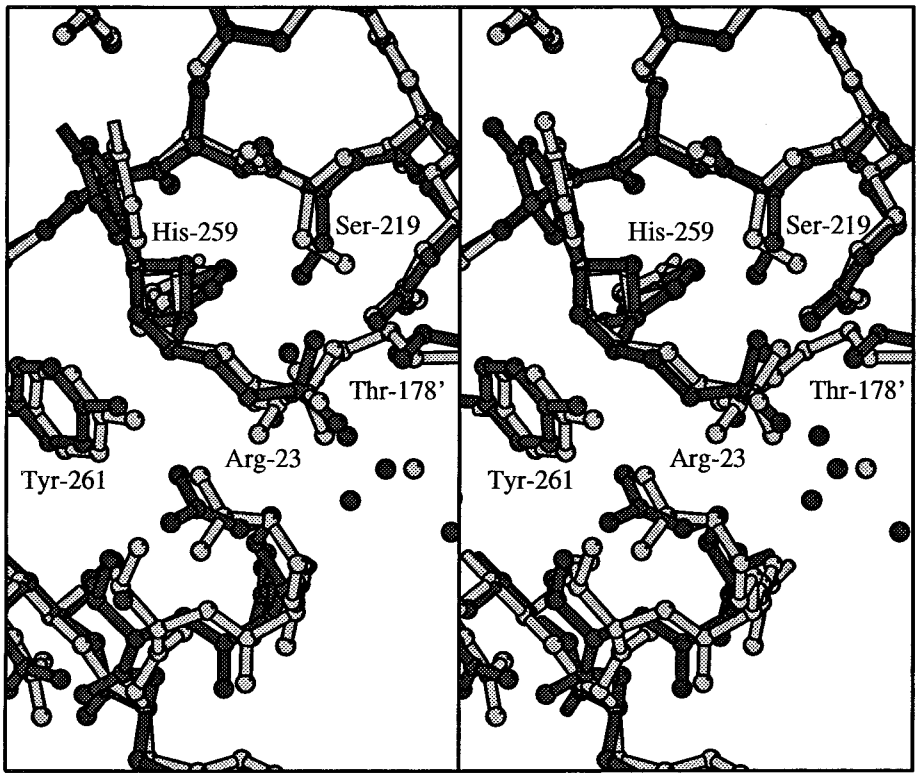

FIGURE 1: Stereo MOLSCRIPT (*37*) plot of R178T−dUMP superposed on the structure of wild-type TS, showing a shift in dUMP orientation as well as numerous small shifts in dUMP binding residues, including residues Y261, H259, and R23. These changes were also evident in the $(F_o1 - F_o2)\alpha_{calc}$ difference map (not shown).

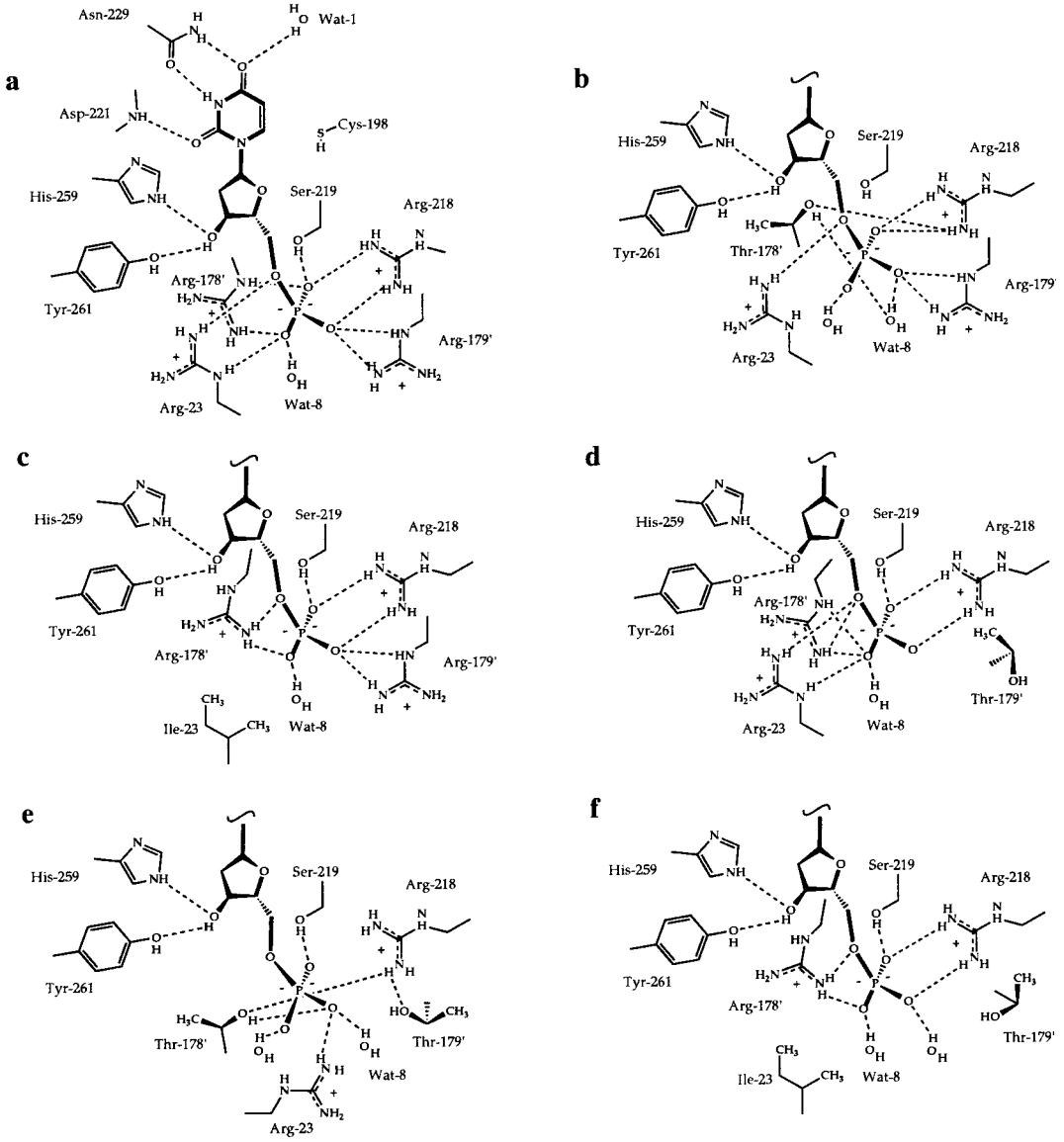

FIGURE 2: Diagrams showing hydrogen bonding interactions (dashed lines) between dUMP (or just the phosphate and ribose groups of dUMP) and the protein or ordered water for (a) wild-type *L. casei* TS, (b) R178T, (c) R23I, (d) R179T, (e) (R178T, R179T), and (f) (R23I, R179T).

different than in wild-type TS and the hydrogen bonds made to the phosphate by R218 and S219 are altered (Figure 2b). Many of the other residues that hydrogen bond to dUMP, including, R179′, H259, and Y261, shift by small amounts (0.5 Å or less) in a concerted fashion (Figure 1), tracking the shift in dUMP. As a result, all hydrogen bonds between the protein and the pyrimidine and ribose moieties that are seen in wild-type TS−dUMP are present in R178T−dUMP. The side chain of C198 is rotated slightly about the Cα−Cβ bond, a change that is visible in the $(F_o1-F_o2)\alpha_{calc}$ map, and the distance between dUMP-C6 and C198-Sγ is 3.8 ± 0.15 Å, compared to 3.3 ± 0.15 Å in wild-type (*17*).The subtle but extensive adaptations in protein and ligand structure seen for this mutant profoundly underlie the need for structure determination in any assessment of the contribution of protein side chains to substrate binding and orientation.

The water network in the active-site cavity surrounding the dUMP pyrimidine base is generally conserved in the R178T−dUMP structure; this network is postulated to be important for catalysis (*18*−*20*). Wat1, which stabilizes the transition-state enolate, is not visible in the density maps for R178T−dUMP, indicating lower occupancy for this water that parallels the lower occupancy and greater disorder of dUMP. (Whereas Wat1 coordinates an important hydrogen bond network in ternary complexes, it is hydrogen bonded only to O4 of dUMP in binary dUMP complexes and has a high thermal factor). The small change in k_{cat} for R178T indicates that this catalytically important water may be better ordered in ternary complex structures. Since the mutation of R178 to threonine induces no important structural changes in residues or waters implicated in catalysis, the 700-fold decrease in k_{cat}/K_m for R178T is largely accounted for by the 0.5 Å change in minimum energy orientation of the dUMP against the catalytic cysteine.

R23I: A 150-fold Effect in Productive On-rate ($\Delta\Delta G_S$ = 3.0 kcal/mol) Where dUMP Orientation and Mobility Are Not Perturbed Suggests Cooperative Contributions to k_1. Structural changes resulting from replacement of R23 with an isoleucine are limited to the loop, residues 21−26, containing the mutation. This loop is more disordered than in wild-type TS−dUMP and moved farther from the active

Table 3: Hydrogen Bonds to dUMP, Distances Between Heavy Atom Donor to Acceptor in Å[a]

dUMP atom	protein atom	Wild-type	R179T	R178T	R23I	R178T, R179T	R23I, R179T
OP1	R23Nϵ	3.0	3.1	−	X	−	X
	R178Nη1	3.0	3.0	X	3.1	X	3.1
	R178Nϵ	3.2	3.1	X	−	X	−
	Wat8	2.9	2.7	−	2.6	−	3.1
	(water)	X	X	3.1	X	2.7	X
OP2	S219Oγ	2.8	2.8	−	2.9	3.2	3.1
	R218Nη2	2.8	2.7	2.7	3.0	−	2.8
	R218Nη1	−	−	2.9	−	−	−
OP3	R218Nη1	2.9	2.9	−	2.8	-	2.9
	R179Nϵ	2.9	X	2.6	3.3	X	X
	R179Nη1	3.0	X	2.7	3.0	X	X
	Wat8	−	−	3.0	−	3.1	−
	R23Nη1	−	−	−	X	2.9	X
	T178Oγ1	X	X	−	X	2.8	X
	(water)	X	X	X	X	X	3.3
OR5	R23Nη1	3.0	2.9	3.0	X	−	X
	R178Nη1	3.2	3.1	X	3.1	X	3.0
OR3	H259Nϵ2	2.9	2.7	2.9	2.8	2.7	2.8
	Y261Oη	2.8	2.9	3.0	3.3	3.2	3.1
O4	N229Nδ2	2.9	2.8	2.7	3.1	2.7	2.8
	H199Cϵ1	3.0	3.1	−	3.1	−	−
O2	D221N	2.8	2.7	2.7	2.8	2.7	2.8
N3	N229Oδ1	3.1	2.8	2.9	3.1	2.8	2.9

[a] An "X" indicates the protein atom is not present in the mutant, while a "−" indicates the atom is present but not in a favorable position for forming a hydrogen bond to the dUMP atom.

site. dUMP itself is well-ordered and in the same conformation and binding site as in wild-type TS (Figure 3). The dUMP-C6−C198-Sγ distance is 3.3 Å, the same as in wild-type TS−dUMP, and the angle C198 Cβ−Sγ−C6 is 114°, compared to 108° in wild-type TS−dUMP. K_d^{dUMP} is only 20-fold higher for R23I than for the wild-type enzyme; therefore, the binding affinity is less impaired by mutation than is the free energy of productive dUMP binding. This suggests that the role of R23 in promoting closure of the active site during ligand binding (*1*, *21*) may be as important a determinant of k_{cat}/K_m as R23 interactions with the phosphate moiety of dUMP. It is clear that changes in entropy, like misorientation and reduction in affinity, can affect the productive on-rate for dUMP.

R179T: Mutation Has No Effect on dUMP Orientation, Mobility, or Occupancy, and Minimal Change in Productive On-rate ($\Delta\Delta G_S = 0.9$ kcal/mol). The dUMP-binding site in R179T−dUMP is fully occupied and identical to that seen in wild-type TS (Figures 4, 2a, and 2d). The average atomic B-factors for the protein and ligand are similar in the two structures (Table 2). Differences between the structures of the mutant and wild-type complexes are confined to the substituted side chain and those of neighboring residues, L195, W85, and R23. The $(F_o1-F_o2)\alpha_{calc}$ difference map between R179T−dUMP and wild-type TS−dUMP indicates that the R23 side chain may adopt two, statistically disordered conformations in the crystal, both of which retain hydrogen bonds to the phosphate moiety and water (Figures 4 and 2d).

(R178T, R179T): Most Hydrogen Bonds between dUMP and Phosphate-binding Arginines are Absent, and dUMP is Misoriented ($\Delta\Delta G_S = 8.9$ kcal/mol). Structural changes resulting from the mutation R178T were more exaggerated in (R178T, R179T)−dUMP (Figure 5). Both dUMP and the loop containing residues 21−26 are more disordered than in R178T−dUMP (Table 2) and the pyrimidine of dUMP is shifted even further from the catalytic cysteine: dUMP-C6−C198-Sγ = 4.4 Å. As in R178T−dUMP, the loop containing residues 21−26 is shifted away from the active site. R23 lies under the phosphate group of dUMP and makes, at most, a single hydrogen bond to a phosphate oxygen (Table 3,

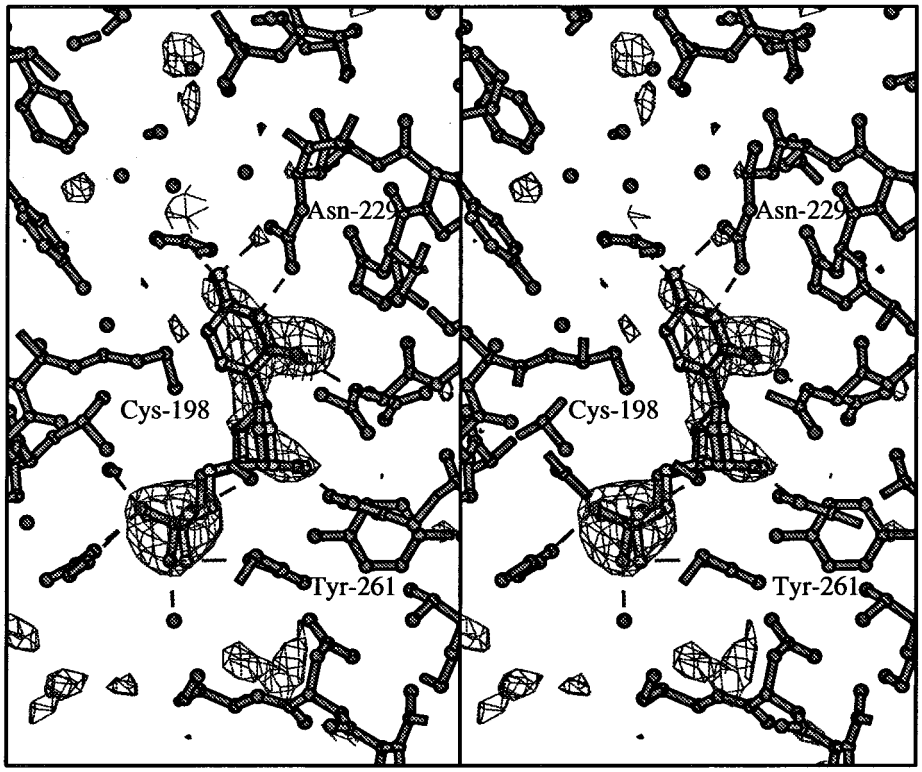

FIGURE 3: Stereo plot (*37*) of the $(F_o - F_c)\alpha_{calc}$ omit map for dUMP-bound R23I where dUMP was omitted from simulated annealing refinement and calculation of α_{calc}s. The map is contoured at 2.5σ.

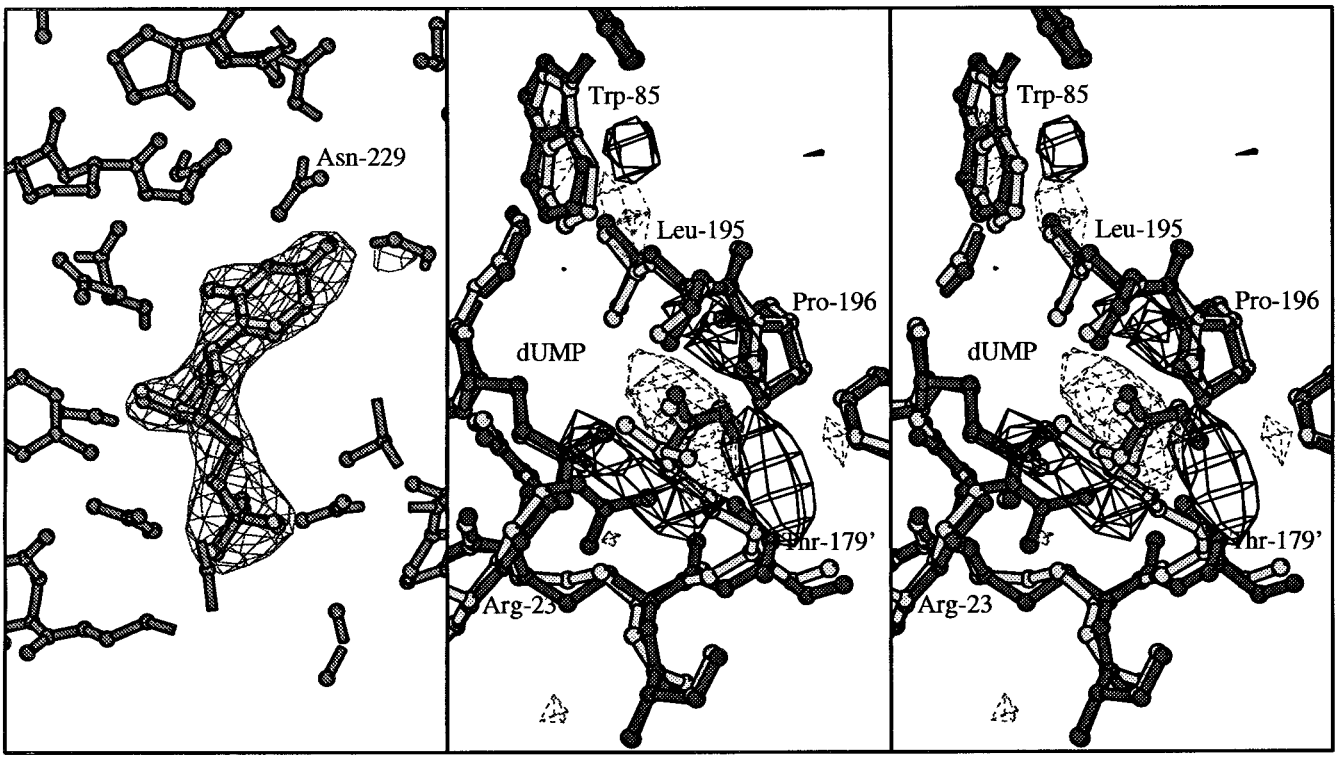

FIGURE 4: Simulated annealing omit map (left panel) showing difference density for dUMP in R179T−dUMP, and stereo ($F_o1 - F_o2)\alpha_{calc}$ difference map (right panels), where F_o1 is an amplitude from the R179T-dUMP data set and F_o2 is an amplitude from the wild-type TS−dUMP data set. The contour level is $+3\sigma$ for the omit map and $\pm 3\sigma$ for the direct difference map. Positive contours are drawn with solid lines and negative contours are drawn with broken lines. In contrast to the ($F_o1 - F_o2)\alpha_{calc}$ difference map for R178T, the direct difference map for R179T shows only changes to residues in the immediate vicinity of T179, none of which contact any part of dUMP beyond the phosphate moiety. MOLSCRIPT (*37*) plots of R179T-dUMP (dark bonds) and either wild-type dUMP or TS−dUMP (light bonds) are superposed on the density maps and show that the dUMP occupies the identical binding site in wild-type TS.

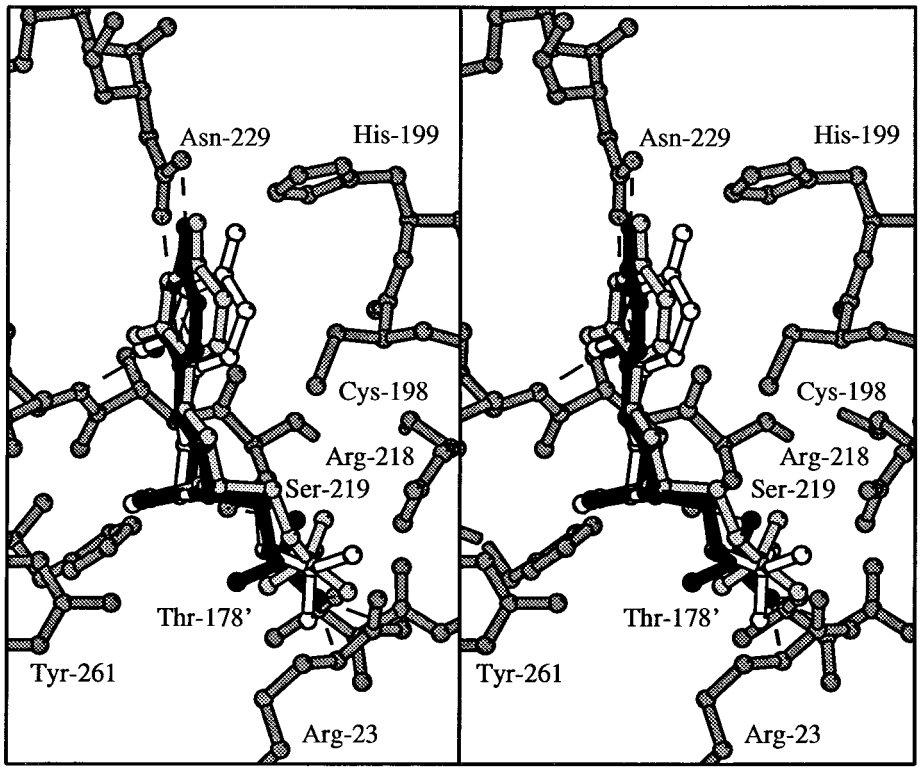

FIGURE 5: Stereo MOLSCRIPT (*37*) plot of (R178T, R179T) (black and dark gray bonds) superposed with dUMP from wild-type *L. casei* TS (white bonds) and R178T (light gray bonds).

Figure 2e). Aside from R23, major changes in the orientations of dUMP binding residues are not observed in (R178T, R179T)−dUMP. The functionally important water network surrounding the pyrimidine base is conserved, except for

lower occupancy of Wat1, in (R178T, R179T)−dUMP. The positions of other residues involved in catalysis are also conserved. This result suggests that the nonadditivity of k_{cat} and K_m values for (R178T, R179T) vs the R178T and R179T single mutants (Table 1) is the result of the 1.1 Å shift in minimum energy position of dUMP-C6 against the nucleophile, C198. We would expect this kind of cooperativity in general wherever orientation changes are evoked in an enzyme.

(R23I, R179T): "Extra" Free Energy due to Entropy or Electrostatic Imbalance, Since dUMP Orientation is Identical to Wild-type ($\Delta\Delta G_S$ = 6.9 kcal/mol). Remarkably, dUMP in (R23I, R179T)−dUMP is in the same conformation and binding site as dUMP in wild-type TS. However, it is in higher libration around the same ground-state position; it has an average B-factor that is approximately twice the average B-factor for dUMP in R23I. The distance between dUMP C6 and C198 Sγ is 3.3 Å, exactly as in wild-type TS. dUMP makes the same hydrogen bonds to the protein as in R23I, except that in place of two hydrogen bonds between one of the phosphate oxygens and the guanidinium of R179′, there is a single hydrogen bond between the same phosphate oxygen and a new, ordered water (Figure 2f). The R23-loop, residues 21−26, is poorly ordered and shifted away from the active site, as in R23I−dUMP. Once again, the double mutant has ∼150-fold greater effect on productive on-rate than that expected from additivity of $\Delta\Delta G_S$ of the single mutants. The progressive increase in entropy of bound dUMP on going from wild-type enzyme, to R23I, to the double mutant, seen as increased thermal vibration, and the need to pay the price of ordering the substrate for catalysis may contribute to the "extra" $\Delta\Delta G_S$ that results from the second mutation. Nonadditive effects of altering the charge distribution at the phosphate-binding site can also account for the larger than expected $\Delta\Delta G_S$, as discussed below.

DISCUSSION

The Increase in K_{mdUMP} Differs for Each Arginine and Is Related to the Importance of the Arginines to the Structure of the Binding Cavity. On the basis of the wild-type TS−dUMP structure, one might expect that mutation of any of the three arginines, R23, 178′, or 179′, would have similar effects on dUMP binding since each contributes two hydrogen bonds and one positive charge to the dUMP-phosphate moiety. In fact, mutants of R178′ have K_m^{dUMP}s that are 40−2000-fold higher than wild-type, while K_m^{dUMP}s for R23 and R179′ mutants are 2−20-fold higher. R178′ mutations have a larger effect on dUMP-binding rate because R178′ is more buried than R23 or R179′ and it interfaces with several other dUMP binding residues. In wild-type TS−dUMP, the side chain of R178′ is largely buried by protein, dUMP, and ordered solvent, with only two side chain atoms with a small amount (<0.5 Å) of surface exposed to bulk solvent. An illustration of the more buried state of R178′ compared to R23 or R179′ is that substitution of Glu at R179′ is easily accommodated by rearrangement of water and other side chains, whereas substitution by Glu at R178′ blocks the dUMP-binding site and reduces activity by compromising the ability of the enzyme to form a ternary complex *(22)*. R178′ packs against S219, H259, and Y261 and is hydrogen bonded to D22, adjacent to R23. R178′ is the platform against which the loop comprised of residues 21−26 packs in the "closed" ternary complex conformation. R178′ is an integral component of the wall of the dUMP-binding cavity that contains most of the residues that hydrogen bond to the ribose and pyrimidine ring of dUMP.

Threonine is a relatively conservative substitution for R178 in that it does not introduce incompatible charge or steric clashes with the protein. Unlike arginine, threonine is branched at its β-carbon. In the R178−dUMP structure, the methyl branch, Cγ, is easily accommodated in the arginine space and is not in close contact with other residues in the R178T−dUMP complex. However, in the closed ternary complex conformation of the enzyme, R178′ is in closer contact with surrounding residues, and it is possible that T178 Cγ would interfere with closure of the active site. In the crystal structure of R178T−dUMP, the orientations of the dUMP-binding residues H259 and Y261, both in contact with R178′ in wild-type TS−dUMP, show small shifts in position as a result of the mutation but both still form the normal hydrogen bonds to dUMP. The unusually large decrease in k_{cat}/K_m^{dUMP} then, cannot be attributed primarily to structural changes relayed to these residues by the threonine substitution, rather, to the greater mobility of dUMP and its suboptimal orientation in the equilibrium structure with respect to both the catalytic cysteine and the other phosphate-binding arginines. The net result of the aberrant dUMP orientation is elimination of four or five hydrogen bonds to the dUMP phosphate moiety in the binary complex structure.

In contrast to mutations of R178′, mutations of the two most external arginines at the phosphate site, R23 and R179′, in which more than half of the atoms in the side chains are exposed to bulk solvent, do not affect the orientation of dUMP in the binary complex structures. By virtue of having one face of their side chain interfacing with solvent, these two arginines are not part of a rigid binding cavity wall. The enzyme can accommodate mutations of these residues by local rearrangements of side chains or waters that do not contact dUMP, or only contact the dUMP phosphate moiety, and that do not affect its orientation. Even in (R23I, R179T) where both R23 and R179′ are mutated, the orientation of dUMP in the binary complex is the same as in the wild-type enzyme, although dUMP has higher vibrational entropy than in the wild-type complex. These results are consistent with structures of R179A, R179E, and R179K substrate complexes, in which the conformations and orientations of dUMP in the substrate-binding sites are very similar to that seen in the wild-type complex (*3*). They indicate that the orientation of dUMP is determined primarily by the shape of the binding cavity, and by hydrogen bonds from Asn-229 to the pyrimidine moiety (*23*), but not by the hydrogen bonds from R23 and R179′. Since these two more external arginines do not determine dUMP orientation and are not in contact with other dUMP binding residues, the rate of binding dUMP in the optimum mode for catalysis is less sensitive to their mutation.

The debromination of 5-bromo-dUMP assays the ability of the enzyme to bind dUMP and form a covalent bond to the catalytic cysteine in the absence of cofactor and has proven to be a sensitive indicator of dUMP orientation in binary complexes (*23*, *24*). Disordering of the dUMP base, or small shifts in its position prevent the reaction from

occurring. While R178T does not catalyze debromination of 5-bromo-dUMP, indicating that its orientation against the catalytic cysteine is compromised, R23I and R179T undergo the debromination reaction with only small changes in kinetics with respect to the wild-type enzyme (Kawase et al., in preparation). Binary complexes of R23I and R179T show the pyrimidine rings of bound dUMP optimally positioned for covalent addition to C198 Sγ. Therefore, the results of the debromination assay are in full agreement with crystallographic results.

Cofactor Binding: Key Roles of R23 and R178′ in Stabilization of Ternary Complexes Reflected in Higher K_m's for Cofactor. The perturbations to the active site and dUMP orientation seen in R178T significantly increase $K_m^{CH_2H_4\,folate}$ even though R178′ is not in direct contact with the cofactor in ternary complexes. Mutations of residues that contact dUMP but not cofactor have been shown to indirectly affect cofactor binding by perturbing the orientation of dUMP (*25*). dUMP orientation is not affected by mutations of R179′, and predictably, neither is $K_m^{CH_2H_4\,folate}$ (Table 1) (*3*). In R178T, and (R178T, R179T) where substrates are misoriented, $K_m^{CH_2H_4\,folate}$ increases 17-fold and 11-fold, respectively. During catalysis, dUMP normally binds to TS first and provides the surface on which cofactor binds. Therefore, small perturbations in dUMP orientation usually result in increases in $K_m^{CH_2H_4\,folate}$ (*23*, *25*, *26*).

R178 may also contribute indirectly to cofactor binding by stabilizing the closed conformation of the enzyme. Only in the closed conformation can the enzyme form the network of hydrogen bonds to the cofactor pterin ring that is most likely to be critical for cofactor binding and orientation. In closed ternary complexes, the side chain of R178 is completely buried and close-packed against the R23 loop (residues 21−26). Thus, R178 contributes even more to the stability of the ternary complex than to the stability of the enzyme−dUMP complex. In R178T, and (R178T, R179T), the arginine side chain is replaced with the shorter, branched threonine side chain and ordered water, resulting in a more disordered R23-loop, which is shifted away from the active site. Mutations of R178 may interfere with the conformational change that is required to bring R23 and the C-terminus into contact with the cofactor, and so be responsible for the increase in $K_m^{CH_2H_4\,folate}$.

R23 mutations also increase $K_m^{CH_2H_4\,folate}$, largely because of the direct role R23 has in cofactor binding. In the ternary complex, the R23 side chain is in a single well-ordered conformation with its guanidinium group hydrogen bonded to the dUMP phosphate, to the C-terminus, and to an ordered water that mediates hydrogen bonds to the cofactor pterin ring (*18*, *21*, *27*). The C-terminus of the enzyme is poorly ordered in the apoenzyme and dUMP complex, but in the ternary complex, swings 5 Å into the active site to hydrogen bond to the pterin ring of the cofactor (*21*, *28*). The hydrogen bond between R23 and the C-terminus may stabilize this important interaction, and so be responsible for the 10-fold higher $K_m^{CH_2H_4\,folate}$ for R23I.

dUMP-binding Energetics: Alone, Each Arginine−Phosphate Hydrogen Bond is worth 0.9−1.3 kcal/mol. Kinetic and binding data for the mutants, interpreted in the light of the mutant TS−dUMP crystal structures, provide insight into the energetic contributions of the arginine side chains to dUMP binding. The crystal structures represent minimum energy conformations of dUMP-bound enzymes, which are best compared to equilibrium binding constants, K_d^{dUMP}. We have previously correlated K_d^{dUMP} values for R179 mutants with hydrogen bonding changes in the mutant−substrate complexes to estimate an electrostatic contribution of a single arginine to dUMP binding affinity of almost 3 kcal/mol (*3*). Now, we seek to use the structures to interpret changes in transition-state-binding energy, as assessed by changes in k_{cat}/K_m^{dUMP} (*29*). The crystal structures of the binary complexes are essential for our analysis because they isolate the first binding step in the reaction, and because the plasticity of the dUMP-binding site frequently leads to unpredictable rearrangements of side chains or water in compensation for a mutation (*3*).

As a consequence of the Ordered Bi Bi mechanism used by TS in binding first substrate, then cofactor during catalysis, k_{cat}/K_m^{dUMP} under saturating concentrations of folate is equal to the rate of dUMP binding, k_1 (effectively k_{-1} is ~0 under these conditions) (*16*). Therefore, changes in k_{cat}/K_m^{dUMP} under saturating cofactor concentrations are related to changes in free energy of dUMP binding $\Delta\Delta G_S = -RT \ln [(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$ (*30*). Table 1 lists the kinetic constants and $\Delta\Delta G_S$ for the five mutants. As shown for R179T, net loss of a single arginine−phosphate hydrogen bond, without significant structural perturbation to the rest of the system, is worth only about 0.9 kcal/mol in binding energy, which is in the range of the energies for a hydrogen bond between two uncharged groups (*29*). TS undergoes some small conformational changes when dUMP binds, involving mainly the ordering of the loop 21−26 and movement of R23 into the active site and the $\Delta\Delta G_S$s should include the energy for these conformational adjustments. The apparent greater contribution of R178 to ΔG_S, as measured by the $\Delta\Delta G$ in Table 1, may reflect its role in stabilizing the dUMP-bound conformation of the loop. However, even for R178T, the change in $\Delta\Delta G$ per phosphate hydrogen bond lost on mutation is only 1.2 kcal/mol. This energy is much less than the 3.5−4.5 kcal/mol binding energy contributed by hydrogen bonds involving charged groups in tRNA synthetase (*29*). In our case, removal of an arginine with two hydrogen bonds to phosphate, can be partially compensated for by water, as explicitly seen in our crystal structures. This was not thought to be the case in tRNA synthetase.

dUMP-binding Energetics: Loss of Two Arginines Evokes a Much Greater Loss Than the Sum of Each Arginine Contribution Separately. Loss of a second arginine, however, has a much larger impact on ΔG_S, even when compensating hydrogen bonds to the phosphate are formed by ordered water. For example, in (R23I, R179T) the hydrogen bonds to the phosphate moiety are the same as in R23I except that two hydrogen bonds from R179′ are replaced by a single hydrogen bond from an ordered water. In this case, the net loss of one charged hydrogen bond relative to R23I TS−dUMP ($\Delta\Delta G_S$ = 3.0 kcal/mol) and the entropy cost of ordering one water result in a further 3.9 kcal/mol increase in ΔG_S for (R23I, R179T) with respect to R23I. The impact of R179T alone is only $\Delta\Delta G_S$ = 0.9 kcal/mol. Therefore, taken separately the sum of the two $\Delta\Delta G_S$ for the individual mutations is 3.0 kcal/mol less than the $\Delta\Delta G_S$ when both mutations are made together, even though the orientation of

dUMP in the site remains the same. The nonadditive energetic effects of the arginine mutations underscore the sensitivity of dUMP binding to the electrostatic environment of the phosphate-binding site.

We discuss three effects that could contribute to the much more-than-additive free energetic effects of the mutations. Another way of looking at the results is that the presence of one or the other member of an arginine pair led to substantially greater binding enhancement in the absence (rather than the presence) of the other member of the pair. First, the nonadditive effects may result from synergistic binding of the phosphate by the four arginines. That is, binding of one arginine to the phosphate oxygens helps position the other oxygens in an ideal orientation for hydrogen bonding to the remaining arginines, so reducing the entropic cost of forming the additional hydrogen bonds. In the double mutant (R178T, R179T), for example, the equilibrium orientation of dUMP is shifted such that the dUMP-phosphate moiety no longer makes well-ordered hydrogen bonds to the remaining two arginines at the phosphate-binding site and dUMP-C6 is over 4 Å from the catalytic cysteine; k_{cat}/K_m^{dUMP} for (R178T, R179T) is 3×10^6-fold lower than for wild-type TS ($\Delta\Delta G_s = 8.9$ kcal/mol), compared to $\Delta\Delta G_s = 0.9$ kcal/mol for R179T, and 3.8 kcal/mol for R178T. Binding of one arginine fixes the orientation of phosphate so that the others can form hydrogen bonds with reduced entropic cost for each increment in enthalpy.

A second effect contributing to the nonadditivity could be enhanced repulsion between pairs of arginine side chains on binding. Unfavorable intramolecular electrostatic repulsions between arginines is expected to be screened more by solvent in the unbound than the bound form. This enhanced arginine repulsion on binding would be expected to lead to nonadditive effects for one- versus two-arginine replacements. This type of effect has been shown to produce substantial free energetic contributions in calculations made of binding events in other systems (*31*, *32*).

A third effect that could also be responsible for the nonlinear relationship between charged side chains at the active site and binding free energy involves a new theory of electrostatic complementarity and optimization at binding interfaces. Electrostatic effects on binding involve a tradeoff between favorable intermolecular interactions (whose magnitude increases linearly with increases in the magnitude of individual point charges, $\Delta G_s = -Bq_0q/\epsilon r$) and unfavorable desolvation effects (whose magnitude increases with terms including the square of individual charges, $\Delta G_s = Aq^2$) (*31*, *33*−*35*). When Coulombic attraction is maximized without an excess of buried charge, the electrostatic contribution to binding can be favorable (*31*, *35*). Deviations of charge distributions on either ligand or receptor from optimal complementarity increases ΔG_s proportionally to the square of the deviations, as described by a term of the form $\Sigma_{i=1,n}$ $\Sigma_{j=1,n}$ $[B_{ij}(q_i - qopt_i)(q_j - qopt_j)]$ where q_i and $qopt_i$ are actual and ideal charges, respectively (*33*). The term will always be positive, reflecting the unfavorable extra desolvation energy for any increase in buried charge, and the relative loss of Coulombic energy for a decrease in charge relative to optimal.

In TS, tightest binding is found to the wild-type enzyme with four-arginines at the active site, and the affinity drops off in a greater than linear fashion for the three-arginine and two-arginine variants, respectively. These results are consistent with the theoretical treatment. In this view, the four-arginine active site is closest to the complement for dUMP followed by the active three-arginine active sites, with the two-arginine active sites furthest from optimal. The quadratic term in the active-site desolvation penalty could be responsible for the greater-than-linear binding decrements, although calculations will need to be done to examine these suggestions in a more quantitative manner. Note that in the absence of bound phosphate or dUMP, the guanidinium groups of the four arginines are partially (in the case of R178 or R218) or fully (for R23 or R179) solvent accessible (*36*). Some desolvation penalty was paid upon protein folding; however, a further major desolvation penalty must be paid upon binding of phosphate (similar effects have been computed in other systems (*32*)). After dUMP binds, the two external arginines, R23 and R179, could readily adopt conformations in which their guanidinium groups remain fully solvated and do not interface with the phosphate. Yet R23 and R179 nearly always cluster around the phosphate, each making two hydrogen bonds to phosphate rather than to water, in *L. casei* TS substrate complexes. This is further evidence for the surprising result that four charged arginines, with a net charge of +4 apparently balance a phosphate moiety with a −2 charge.

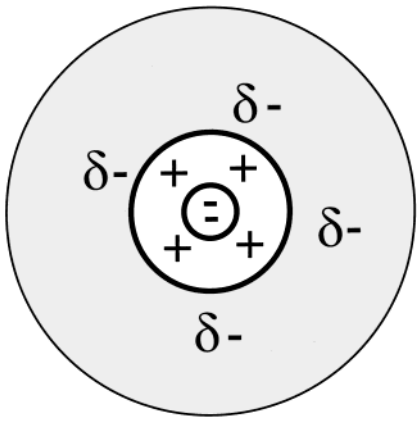

FIGURE 6: Any mutation that replaces arginine by an uncharged, or oppositely charged side chain reduces the on-rate for binding dUMP, i.e., it increases the activation energy for binding dUMP in the productive orientation. Therefore the optimum for binding phosphate in the site involves four positive charges in the first shell around the doubly charged phosphate anion. There is only one negative charged side chain in the second shell around the arginines, however the dielectric of the protein may also serve to buffer the "effective charge" in the first shell making it close to optimum electrostatically. Any reduction in effective charge is expected to rise as the square of the deviation from the optimum, a factor that could explain the more than additive $\Delta\Delta G_S$s for the double mutants.

The fact that four arginines are more optimal than two in TS reflects the influence of a second shell of residues surrounding the arginines on the electric field at the phosphate site (Figure 6). These include residues that are negatively charged or directly affect the charge distribution on the guanidinium groups of the arginines through hydrogen bonding. (Since the resulting "effective" positive charge at the phosphate-binding site need not be an integral number, the ratio of change in free energy caused by the second arginine vs the first could be quite large, consistent with the nonadditive effects of mutation.) An illustration of putative second shell effects on the charge distribution at the

phosphate-binding site is the crystal structure of an *E. coli* TS ternary complex with dUMP and an inhibitor that blocks two negatively charged groups, the C-terminus and the invariant residue Asp22 (20), from the active-site cavity (Fritz and Stroud, unpublished). In this structure, dUMP binds in its wild-type orientation and is coordinated in the usual way to R126 (178) and R166 (218). However, R23 (*21*) is disordered and R179 (127) adopts a conformation in which its side chain is fully solvated, far removed from the phosphate moiety, and not hydrogen bonded to any protein atom. The change in R179 (127) conformation is a particularly striking result since it is on the opposite wall of the active-site cavity from the C-terminus, and illustrates cooperative interactions at the phosphate binding site.

CONCLUSION

Three of the four phosphate-binding arginines in TS, R23, R178, and R179, are not essential for activity and contribute to catalysis through ligand binding. These three arginines have seemingly equivalent and redundant roles in dUMP binding in that each provides two hydrogen bonds to the phosphate moiety of dUMP. These hydrogen bonds contribute significantly to dUMP affinity. However, K_m^{dUMP} increases more dramatically for R178 mutants compared to mutants of the more solvent-exposed R23 and R179, since substitutions at R178 alter the orientation of dUMP with respect to the catalytic cysteine and the other phosphate-binding arginines.

To assess the energetic contribution of each arginine to the dUMP-binding step in catalysis, changes in the free energy for binding dUMP in its catalytically competent orientation ($\Delta\Delta G_S$) have been evaluated from changes in k_{cat}/K_m^{dUMP}. Double mutants (R23I, R179T) and (R178T, R179T) show increases in ΔG_S that are 3−4 kcal/mol greater than the sum of the increases for the individual single mutants. The structure of (R178T, R179T) indicates that the more-than-additive effects may be partly the result of cooperative binding of dUMP by the four arginines, while the structure of (R23I, R179T) indicates that these nonadditive effects may also arise from enhanced electrostatic repulsion on binding and from quadratic increases in the ΔG_S resulting from perturbation of an electrostatic environment finely tuned for binding phosphate anion.

ACKNOWLEDGMENT

We thank Professor Peter Kollman and Professor Bruce Tidor for valuable discussions regarding binding energetics and critical reading of parts of the paper.

REFERENCES

1. Finer-Moore, J., Fauman, E. B., Foster, P. G., Perry, K. M., Santi, D. V., and Stroud, R. M. (1993) *J. Mol. Biol. 232*, 1101−1116.
2. Stout, T. J., Sage, C. R., and Stroud, R. M. (1998) *Structure 6*, 839−848.
3. Finer-Moore, J. S., Fauman, E. B., Morse, R. J., Santi, D. V., and Stroud, R. M. (1996) *Protein Eng. 9*, 69−75.
4. Climie, S., Ruiz-Perez, L., Gonzalez-Pacanowska, D., Prapunwattana, P., Cho, S. W., Stroud, R., and Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776−18779.
5. Michaels, M. L., Kim, C. W., Matthews, D. A., and Miller, J. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3957−3961.
6. Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V., and Stroud, R. M. (1987) *Science 235*, 448−455.
7. Stroud, R. M., and Finer-Moore, J. S. (1993) *FASEB J. 7*, 671−677.
8. Kealey, J. T., and Santi, D. V. (1992) *Protein Exp. Purif. 3*, 380−385.
9. Pogolotti, A. L., Jr., Danenberg, P. V., and Santi, D. V. (1986) *J. Med. Chem. 29*, 478−482.
10. Santi, D. V., Ouyang, T. M., Tan, A. K., Gregory, D. F., Scanlon, T., and Carreras, C. W. (1993) *Biochemistry 32*, 11819−11824.
11. Otwinowski, Z. (1993) in *Proceedings of the CCP4 Study Weekend* (Sawyer, L., Isaacs, N., and Bailey, S., Eds), pp 56−62, SERC Daresbury Laboratory, Warrington, U.K.
12. French, G. S., and Wilson, K. S. (1978) *Acta Crystallogr. Sect A A34*, 517.
13. Chambers, J. L., and Stroud, R. M. (1977) *Acta Crystallogr. Sect. B B33*, 1824−1837.
14. Brunger, A. T. (1988) *J. Mol. Biol. 203*, 803−816.
15. Brunger, A. T. (1992) *Acta Crystallogr. Sect. D D49*, 24−36.
16. Santi, D. V., Pinter, K., Kealey, J., and Davisson, V. J. (1990) *J. Biol. Chem. 265*, 6770−6775.
17. Stroud, R. M., and Fauman, E. B. (1995) *Protein Sci. 4*, 2392−2404.
18. Matthews, D. A., Villafranca, J. E., Janson, C. A., Smith, W. W., Welsh, K., and Freer, S. (1990) *J. Mol. Biol. 214*, 937−948.
19. Fauman, E. B., Rutenber, E. E., Maley, G. F., Maley, F., and Stroud, R. M. (1994) *Biochemistry 33*, 1502−1511.
20. Sage, C. R., Rutenber, E. E., Stout, T. J., and Stroud, R. M. (1996) *Biochemistry 35*, 16270−16281.
21. Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., and Stroud, R. M. (1990) *Biochemistry 29*, 6964−6977.
22. Strop, P., Changchien, L., Maley, F., and Montfort, W. R. (1997) *Protein Sci. 6*, 2504−2511.
23. Finer-Moore, J., Liu, L., Birdsall, D. L., Brem, R., Apfeld, J., Santi, D. V., and Stroud, R. M. (1998) *J. Mol. Biol. 276*, 113−129.
24. Birdsall, D. L., Huang, W., Santi, D. V., Stroud, R. M., and Finer-Moore, J. (1998) *Protein Eng. 11*, 101−113.
25. Agarwalla, S., LaPorte, S., Liu, L., Finer-Moore, J., Stroud, R. M., and Santi, D. V. (1997) *Biochemistry 36*, 15909−15917.
26. Finer-Moore, J. S., Liu, L., Schafmeister, C. E., Birdsall, D. L., Mau, T., Santi, D. V., and Stroud, R. M. (1996) *Biochemistry 35*, 5125−5136.
27. Hyatt, D. C., Maley, F., and Montfort, W. R. (1997) *Biochemistry 36*, 4585−4594.
28. Matthews, D. A., Appelt, K., Oatley, S. J., and Xuong, N. H. (1990) *J. Mol. Biol. 214*, 923−936.
29. Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., and Winter, G. (1985) *Nature 314*, 235−238.
30. Wilkinson, A. J., Fersht, A. R., Blow, D. M., and Winter, G. (1983) *Biochemistry 22*, 3581−3586.
31. Chong, L. T., Dempster, S. E., Hendsch, Z. S., Lee, L.-P., and Tidor, B. (1998) *Protein Sci. 7*, 206−210.
32. Hendsch, Z. S., and Tidor, B. (1999) *Protein Sci. 8*, 1381−1392.
33. Lee, L.-P., and Tidor, B. (1997) *J. Chem. Phys. 106*, 8681−8690.
34. Kangas, E., and Tidor, B. (1998) *J. Chem. Phys. 109*, 7522−7545.
35. Kangas, E., and Tidor, B. (1999) *Physical Review E 59*, 5958−5961.
36. Stout, T. J., Schellenberger, U., Santi, D. V., and Stroud, R. M. (1998) *Biochemistry 37*, 14736−14747.
37. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

BI9918590