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Exclusion of 2′-Deoxycytidine 5′-Monophosphate by Asparagine 229 of Thymidylate Synthase†

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ABSTRACT: In thymidylate synthase (TS, EC 2.1.1.45), the only side chain in direct hydrogen bonding with the pyrimidine ring of the substrate dUMP is asparagine 229 (N229). In binary and ternary complexes, the carboxamide moiety of the side chain of N229 forms a cyclic hydrogen bond network bridging N-3 and O-4 of the uracil heterocycle. Most of the N229 mutants of TS bind dUMP and catalyze dTMP formation as well as the wild-type enzyme; thus, N229 does not contribute to binding of dUMP. Wild-type TS binds dCMP weakly and does not accept dCMP as a substrate. Mutations at N229 of TS modify the interaction of TS with dCMP. TS N229D and TS N229E catalyze the methylation of dCMP [Liu, L., & Santi, D. V. (1992) *Biochemistry 31*, 5010–5014]. With the exception of the TS N229Q, most of the N229 mutants bind dCMP as well as or tighter than dUMP and bind dCMP 300–3000-fold tighter than wild-type TS. We conclude that TS discriminates binding of dUMP *versus* dCMP by a 3–4 kcal mol^{-1} difference in binding energy by exclusion of dCMP from the active site. We propose that this exclusion is a consequence of untoward interactions between dCMP and the side-chain carboxamide group of the Asn or Gln at position 229 of TS. We speculate that exclusion of cytosine *versus* uracil by Asn or Gln may account for specificity observed in other protein–pyrimidine interactions.

Thymidylate synthase (TS,[1] EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2-H_4folate to dTMP and H_2-folate. TS has been extensively studied and is the most conserved enzyme known (Perry et al., 1990; Perryman et al., 1986). Three-dimensional structures of free and bound enzyme forms have been determined (Hardy et al., 1987; Matthews et al., 1990; Montfort et al., 1990; Finer-Moore et al., 1993), and the basic features of the catalytic mechanism of TS have been established (Santi & Danenberg, 1984).

The crystal structure of the TS–dUMP complex shows that the completely conserved residue N229[2] contributes the only side chain in direct hydrogen bonding with the pyrimidine ring of the substrate dUMP (Figure 1). In the binary and ternary complexes, the carboxamide moiety of the side chain of N229 forms a cyclic hydrogen bond network bridging N-3 and O-4 of the uracil heterocycle. The carboxamide group of N229 is further restricted by a highly structured water molecule which bridges the carboxamide nitrogen to the completely conserved Glu 60 of TS.

TS effectively discriminates between the related deoxyribonucleotides dUMP and dCMP as substrates. dUMP is a relatively tight binding substrate, with a K_m of $\sim$ 5 μM. In contrast, dCMP is not a substrate but is a competitive inhibitor

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[1] Abbreviations: TS, thymidylate synthase; dUMP, 2′-deoxyuridine 5′-monophosphate; dCMP, 2′-deoxycytidine 5′-monophosphate; dTMP, thymidine 5′-monophosphate; dUrd, 2′-deoxyuridine; dCyd, 2′-deoxycytidine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; CH_2-H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PLP, pyridoxal 5′-phosphate.

[2] The amino acid numbering used is for *Lactobacillus casei* TS.

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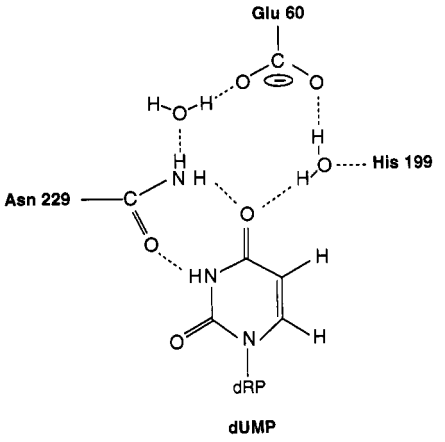

FIGURE 1: Schematic diagram of the hydrogen bond interactions among the pyrimidine ring of the substrate dUMP and the amino acid residues at the active site (Montfort et al., 1990; Finer-Moore et al., 1993).

of dUMP with a K_i some 1000-fold poorer than the K_m of dUMP (Liu & Santi, 1992; Rode et al., 1990). It has recently been proposed that the substrate specificity of TS for dUMP *versus* dCMP resides in the single amino acid residue N229. When this residue is converted to Asp, the enzyme loses TS activity toward dUMP and becomes a dCMP methylase (Hardy & Nalivaika, 1992; Liu & Santi, 1992). In a recent paper, we showed that 15 of 19 mutants at position 229 of *Lactobacillus casei* TS were catalytically active, some having K_m and k_{cat} values approaching those of the wild-type enzyme (Liu & Santi, 1993). It was concluded that N229 assists but is not essential for dUMP binding or for catalysis.

How does one accommodate the finding that N229 provides the only side chain that is in contact with the pyrimidine of dUMP yet is not essential for binding with the notion that this residue is the sole determinant of specificity for dUMP *versus* dCMP? In the present work, we address the question of how TS distinguishes between dUMP and dCMP by studies of binding of these nucleotides to wild-type TS and N229 mutants of TS. We have reached the conclusion that the discrimination is due to exclusion of dCMP by the carboxamide group of N229.

MATERIALS AND METHODS

Materials, Mutagenesis, Protein Purification, and Enzyme Assays. These were as described previously (Kealey & Santi, 1992; Liu & Santi, 1992, 1993).

Enzyme Inhibition Assay. Studies of inhibition of TS activity by dCMP were performed in the standard TES buffer (Liu & Santi, 1992). The competitive inhibition pattern for wild-type TS and the N229C mutant was determined by Lineweaver–Burk plots (Segel, 1975). We assumed that dCMP competes with the dUMP binding site for the other mutants and inhibition constants were obtained by nonlinear least squares fit of the percent inhibition of TS activity as a function of dCMP concentration using the appropriate equation for competitive inhibition (Segel, 1975).

Fluorescence Quenching Assay. According to a modified method of Lewis et al. (1981), fluorescence measurements were performed using a Spex Fluorolog-2 spectrofluorometer (Spex Industrial Inc., Edison, NJ) equipped with a magnetic stirring device. Cuvettes (0.4 × 1.0 cm with 1.0 cm for the emission path) contained slowly stirred solutions of 50 mM TES, pH 7.4, 2 mM DTT, 25 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, and about 0.5 μM enzyme at ambient temperature. Excitation was at 290 nm (slit width 2.5 mm), and emission spectra were recorded from 250 to 450 nm (slit width 2.5 mm). There were no shifts in the emission spectra upon addition of ligands, and quenching was monitored at 340 nm. dUrd and dCyd, which do not bind to TS, were used to correct for absorption at the excitation wavelength of corresponding nucleotide ligands; fluorescence quenching was calculated as the quenching caused by dUMP or dCMP minus that caused by equimolar concentrations of dUrd or dCyd, respectively.

Determination of Dissociation Constants by Displacement of the TS–PLP Complex. The K_d values for enzyme–PLP complexes were determined from the increase in differential absorbance at 328 nm which accompanies formation of a thiohemiacetal complex between PLP and C198 of TS (Chen et al., 1989); K_d values for dUMP and dCMP were determined by displacement of PLP from the complex, as monitored by loss of absorbance at 328 nm. All measurements were obtained using a Hewlett-Packard 8452A diode array spectrophotometer in buffer containing 50 mM TES, pH 7.4, and 50 μM DTT. Spectra were corrected for light scattering using the Scatwav program supplied by Hewlett-Packard. Dissociation constants for enzyme–PLP complexes were obtained by titration of about 3 μM enzyme with equal increments of a concentrated solution of PLP; final concentrations of PLP were 50 μM for TS N229E and 25 μM for other mutants. UV–vis difference spectra were obtained by subtracting the spectrum of equimolar concentrations of PLP from that of the TS–PLP complex. Spectra between 300 and 500 nm were collected, corrected for dilution and light scattering, and assessed for thiohemiacetal formation. K_d values were obtained by nonlinear least squares fit of the increase in differential absorbance at 328 nm *versus* PLP concentration to an equation which corrects for ligand depletion by protein (Segel, 1975).

Binary complexes of PLP with wild-type TS or N229 mutants were formed using 20 μM PLP and 2.3 μM protein, except for TS N229E where 40 μM PLP was used. Equal increments of solutions of dUMP or dCMP were added to the cuvette, and spectra (300–500 nm) were recorded. At high dUMP concentrations (>500 μM), there was interfering absorbance above 300 nm which was corrected for by difference spectroscopy. Dissociation constants for dUMP or dCMP were obtained by nonlinear least squares fit of the decrease in absorbance at 328 nm to an equation for competitive inhibition (Segel, 1975) and utilization of the predetermined K_d values for the enzyme–PLP complexes.

RESULTS AND DISCUSSION

N229 is completely conserved in TS and is the only side chain in direct hydrogen bonding with the pyrimidine ring of the substrate dUMP (Matthews et al., 1990; Montfort et al., 1990; Perry et al., 1990; Finer-Moore et al., 1993). In addition to possible roles in catalysis (Hardy et al., 1987), N229 must play the sole role in the recognition of dUMP *versus* dCMP.

Whereas dUMP binds tightly to TS with a K_d of ~1 μM, dCMP inhibits TS with a K_i of ~3000 μM, corresponding to an apparent difference of about 4 kcal mol^{-1} in binding energy. In the context of the structure of the TS–dUMP complex, one might attribute much of this difference in binding energy to disruption of the two hydrogen bonds of the network between the side chain of N229 and dUMP. However, several mutants at position 229 catalyzed the TS reaction with K_m values comparable to the wild-type enzyme (Liu & Santi, 1993), suggesting that N229, and thus the hydrogen bond network, may not contribute to the binding of dUMP.

Table I: Steady-State Kinetic Parameters of N229D and N299E Mutants of TS[a]

229 residue	K_{cat}[b] (s^{-1})	K_m (μM) dUMP or dCMP	K_m (μM) CH_2-H_4folate	k_{cat}/K_m (s^{-1} μM^{-1}) dUMP or dCMP	k_{cat}/K_m (s^{-1} μM^{-1}) CH_2-H_4folate
(1) TS activity					
D[c]	0.0037	50.4	35.4	7.3×10^{-5}	1.0×10^{-4}
E	0.0043	66.7	80.8	6.5×10^{-5}	5.0×10^{-5}
(2) dCMP methylase activity					
D[c]	0.45	150	170	3.2×10^{-3}	2.6×10^{-3}
E	0.018	392	141	4.6×10^{-5}	1.3×10^{-4}

[a] Assays are as described in Materials and Methods. For kinetic measurements of TS N229E catalyzed dCMP methylation, 340 μM CH_2-H_4folate or 800 μM dCMP was used as the fixed substrate concentration. The standard errors from nonlinear least squares fit of the experimental data are less than 15% for all values. [b] Calculation of k_{cat} was based on the molecular weight of the dimer. [c] N229D mutant data were taken from Liu and Santi (1992).

Table II: dCMP Inhibition Constants (K_i) and dUMP Michaelis Constants (K_m) of Wild-Type TS and N229 Mutants[a]

229 residue	K_i (μM), dCMP	K_m (μM),[b] dUMP	affinity ratio dUMP/dCMP
N (wt)	3000	5.0	600
Q	5400	12	450
C	5.0	17	0.29
G	10	15	0.67
D	150[c]	50	3.0
E	390[c]	67	5.8
T	1.7	100	0.017
V	79	91	0.87
L	59	220	0.26
W	260	580	0.45
M	7.8	150	0.051
I	100	150	0.67

[a] Assays are as described in Materials and Methods. The standard errors from nonlinear least squares fit of the experimental data are less than 15% for all values. [b] K_m data are taken from Table 1 of Liu and Santi (1993). [c] K_m for dCMP methylation from Table I.

Table III: Dissociation Constants of Wild-Type TS and N229 Mutant Complexes with PLP, dUMP, and dCMP[a]

229 residue	K_d (μM) PLP	K_d (μM) dCMP	K_d (μM) dUMP	affinity ratio dUMP/dCMP
N (wt)	0.78	160	0.38	430
Q	1.9	270	1.0	270
C	0.51	0.49	1.6	0.3
G	0.36	0.55	0.85	0.65
S	0.28	0.4	0.87	0.46
D	3.9	2.8	2.7	1.0
E	7.3	20	4.5	4.4
T	0.37	0.71	81	0.0088
V	0.43	5.0	62	0.081
A	0.44	0.36	1.7	0.21

[a] Assays are as described in Materials and Methods. The standard errors from nonlinear least squares fit of the experimental data are less than 20% for all values.

The N229D mutation converts TS into a dCMP methylase, presumably because of the ability of Asp to protonate N-3 of the dCMP heterocycle (Liu & Santi, 1992). In the present work, we first assayed all of the other N229 mutants for their ability to catalyze the methylation of dCMP and found that TS N229E also catalyzed the reaction (Table I), albeit not with the efficiency of TS N229D. None of the other mutants had detectable dCMP methylase activity ($k_{cat} < 0.001$ s^{-1}; data not shown), although as shown below, most bind well to dCMP.

We next measured K_i values for dCMP inhibition of dTMP formation by the active N229 mutants and compared these to K_m values for dUMP (Table II). Although K_m values may not reflect dissociation constants and thus preclude comparisons of dUMP and dCMP binding, K_i values of dCMP should reflect relative affinities of dCMP to the wild-type and mutant proteins. dCMP is a poor competitive inhibitor of wild-type TS ($K_i = 3000$ μM) and the N229Q mutant ($K_i = 5400$ μM). In contrast, dCMP was a potent inhibitor of dTMP formation by all other mutants, with K_i values comparable to or lower than K_m values of dUMP. Moreover, with the exception of the wild-type homolog N229Q, the mutants bound dCMP with much higher apparent affinity (10–1000-fold) than wild-type TS.

Kinetically determined constants may not reflect true binding constants; therefore, we sought confirmation of these results by direct determination of K_d values by equilibrium binding methods. We initially investigated the utility of fluorescence quenching of TS which occurs upon formation of the TS–dUMP complex (Lewis et al., 1981; Mittelstaedt & Schimerlik, 1986). Titration of TS with dUMP resulted in quenching of about 10% of the intrinsic fluorescence of TS and gave a K_d value of 0.98 ± 0.36 μM, in accord with reported values and about 5-fold lower than K_m. Likewise, dUMP caused quenching of fluorescence of TS N229Q with a K_d of 0.58 ± 0.06 μM, some 20-fold lower than K_m. Surprisingly, a series of other mutants tested (TS N299A, -C, -G, -D, -L, -I, -M, and -S) showed no quenching of fluorescence upon treatment with up to 100 μM dUMP, which was more than sufficient to form binary complexes (see below). These results suggest that the carboxamide groups of Asn and Gln interact with dUMP in a unique way that perturbs the environment of one or more tryptophan residues in the TS. Consequently, fluorescence quenching could not be used to assess binding of nucleotides to these mutants.

The dissociation constants of dUMP and dCMP to TS and most of the N229 mutants were determined by a competitive binding assay which measures displacement of PLP from the binary enzyme–PLP complex. PLP binds to TS at the phosphate binding site of dUMP and forms a reversible thiohemiacetal with the active site C198 (Chen et al., 1989) (Santi et al., unpublished data). The TS–PLP complex is conveniently quantified by the unique absorbance of the thiohemiacetal at 328 nm. dUMP and dTMP completely displace PLP in a competitive manner (Santi et al., unpublished data), and the loss of absorbance at 328 nm provides a convenient assay for the formation of TS–nucleotide complexes.

Most of the N229 mutants formed complexes with PLP with K_d values in the micromolar range, with the TS N229E and N229D complexes having K_d values at the high end of the range (Table III). Using an extinction coefficient of 4700 cm^{-1} M^{-1} at 328 nm from the PLP titration of wild-type TS (Santi et al., unpublished data), the mole ratio of PLP/TS monomer for mutants which formed complexes was calculated to be 1.0 ± 0.1 except for the N229E mutant which had a

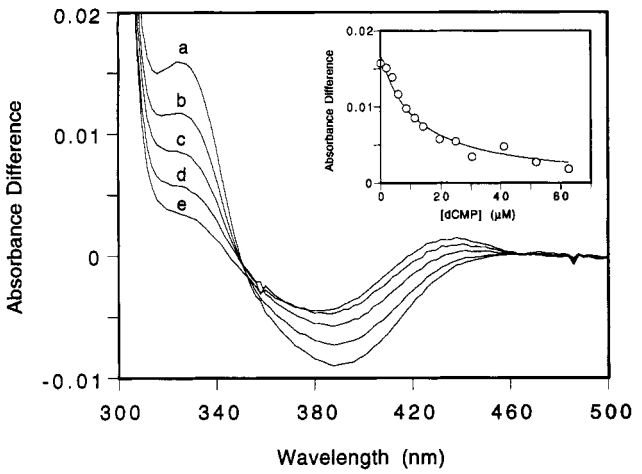

FIGURE 2: Ultraviolet–visible difference spectra of the PLP–TS N229C complex in the presence of different concentrations of dCMP (a, 0.0 μM; b, 6.0 μM; c, 11 μM; d, 20 μM; e, 30 μM). The insert shows the change of absorbance at 328 nm *versus* dCMP concentration. The solid curve was obtained after the data were fit to an equation which described the competitive exchange between PLP and dCMP from the enzyme (Segel, 1975).

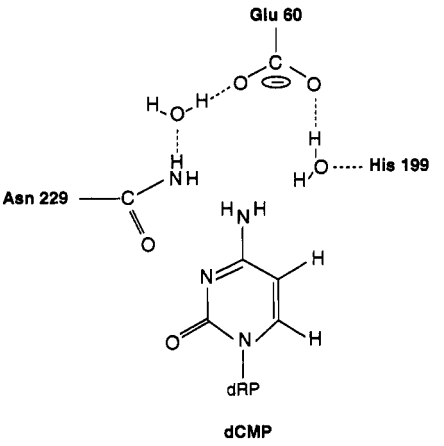

FIGURE 3: Schematic diagram showing that the hydrogen bonding once formed between dUMP and N229 (Figure 1) now is disrupted between dCMP and N229. Although rotation of the carboxamide group of N229 could once again form a hydrogen bond network between dCMP and N229, it would also destroy the hydrogen bonding among N229 and the ordered water molecule as well as E60.

ratio of 0.45. For reasons unknown, mutants with large hydrophobic side chains at position 229 (TS N229L, -W, -M, and -I) did not show the spectral changes characteristic of the TS–PLP complex, even though they catalyze the normal reaction and therefore have an intact active site. These mutants also have the highest K_m values for dUMP in the TS reaction, and it is possible that sufficiently high concentrations of PLP could not be attained for binding without causing interfering absorbance.

When dUMP or dCMP is added to the preformed enzyme–PLP complex, there is a concentration-dependent decrease in absorbance at 328 nm which fits well to a binding equation for two equivalent, noninteracting sites (Figure 2). Further, PLP was completely displaced from TS–PLP complexes by either dUMP or dCMP, which shows that the binding of PLP and nucleotide is mutually exclusive.

The K_d values for dUMP in wild-type TS and most of the mutants were in the micromolar range (Table III). It is clear that the K_m values of dUMP for wild-type TS and N229 mutants are significantly higher than the K_d values for dUMP. However, mutants with the highest K_m values ($\geq$50 μM) also showed the highest K_d values. Importantly, many of the mutants, including TS N229G, bind dUMP as well as the wild-type TS, which verifies that the side chain of N229 is neither necessary nor contributes significantly to binding of dUMP.

For dCMP, the K_d values determined by displacement of PLP were significantly lower than the K_i values for inhibition in the normal enzymic reaction, although the trend of K_i and K_d is similar. The differences may be due to the fact that the K_d values reflect dissociation of the binary complex, whereas the K_i values reflect binding of nucleotide in the presence of cofactor. Wild-type TS and the N229Q mutant bind poorly to dCMP, with K_d values 300–400-fold higher than for dUMP. In most other mutants, binding of dCMP is comparable to or tighter than that of dUMP. In one case, TS N229T, dCMP binds 100-fold tighter than dUMP and about 280-fold tighter than the wild-type enzyme or the N229Q mutant. The binding preference for dUMP *versus* dCMP ($K_{d,dUMP}/K_{d,dCMP}$) varies about 50 000-fold between the wild-type TS and the N229T mutant.

It is clear that the carboxamide side chain at position 229 of wild-type or N229Q TS does not contribute directly to the binding of dUMP, yet dCMP binds poorly to the wild-type enzyme. Thus, the apparent increase in binding energy in mutants which bind dCMP tightly must be attributed to a release of repulsive interactions between Asn or Gln at 229 and dCMP. Replacement of dUMP by dCMP into the active site of TS or the N229Q mutant would place the carboxamide oxygen of the side chain adjacent to the heterocyclic nitrogen of dCMP and the amide nitrogen adjacent to the 4-NH_2 (Figure 3); presumably, the juxtaposition of relatively negative groups of the enzyme and dCMP would lead to a repulsion of the ligand from the enzyme and account for the poor binding. Rotation of the Asn or Gln carboxamide group at 229 to better accommodate the dCMP binding would require disruption of the hydrogen bond network with other important amino acid residues, which could likewise be manifested in a loss of binding.

We conclude that wild-type TS and the N229Q mutant discriminate binding of dUMP *versus* dCMP by the exclusion of dCMP from the active site and that the exclusion results from untoward interactions between the carboxamide group of Asn or Gln at position 229 and dCMP. There are several important ramifications of this finding. First, there are a large number of enzymes that bind uracil in preference to cytosine, and these enzymes may utilize a mechanism for discrimination similar to that described here. Second, the phenomenon of uracil acceptance and cytosine exclusion by Asn or Gln may also apply to proteins which distinguish guanine from adenine, since the hydrogen-bonding properties of the carboxamide of Asn or Gln to the relevant positions of uracil and guanine, and to cytosine and adenine, are expectedly similar. Finally, the uracil *versus* cytosine exclusion could play a role in interactions of proteins with single-strand nucleic acids. The high discrimination of Asn for uracil *versus* cytosine (some 3–4 kcal mol^{-1} in binding energy) could account for much of the specificity seen in such interactions. Using methods of site-directed mutagenesis, this hypothesis could be tested by appropriate substitution of Asn or Gln.

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