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Variation in Human Thymidylate Synthase Is Associated with Resistance to 5-Fluoro-2′-deoxyuridine

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SUMMARY

Two human colorectal tumor cell lines are differentially sensitive to growth inhibition by 5-fluorodeoxyuridine (FdUrd); cell line RCA is less sensitive to FdUrd than is cell line C. Thymidylate synthase (TS), a target of FdUrd, has been purified to homogeneity from both cell lines. Because of differences in the avidity for a folate ligand affinity matrix, TS forms from the cells were purified by two different procedures. Relative to the enzyme from C cells, the enzyme from RCA cells demonstrated higher K_m values for the substrates deoxyuridylate and 5,10-methylenetetrahydrofolate, a lower rate of association of the inhibitor 5-fluorodeoxyuridylate (FdUMP), a similar rate of FdUMP dissociation, and lower enhancement of covalent FdUMP binding by folate derivatives. The activities of the enzymes *in situ* and the catalytic efficiencies of the purified enzymes were similar. Thus, a cell line that is naturally resistant to FdUrd has been identified that expresses a TS with reduced affinity for FdUMP and 5,10-methylenetetrahydrofolate, relative to the enzyme expressed in a FdUrd-sensitive cell line.

The enzyme TS (EC 2.1.1.45) catalyzes the reaction in which the substrates dUMP and CH_2H_4PteGlu are converted to dTMP and H_2PteGlu. The enzymatic reaction provides the sole *de novo* source of dTMP, an essential precursor required for DNA biosynthesis. During enzyme catalysis, dUMP is localized at the active site before CH_2H_4PteGlu (1, 2). CH_2H_4PteGlu binding at the active site is postulated to promote covalent binding of the nucleotide to the enzyme (2, 3). During the enzyme reaction, a methylene group and hydride ion from CH_2H_4PteGlu are transferred to the 5-position of dUMP and a proton is abstracted from the 5-position of dUMP (4, 5). The folate product, H_2PteGlu, dissociates before release of dTMP (1). The enzyme is a dimer of identical polypeptides that apparently are arranged in a manner that generates functional asymmetry; thus, it is postulated that the two active sites differ in substrate affinity and that binding of substrates at one active site positively influences binding at the second site (2, 6).

Because of its pivotal role in DNA biosynthesis, TS is a target for chemotherapeutic intervention in neoplastic and parasitic disease. The 5-fluoropyrimidine drugs 5-fluorouracil and FdUrd have been used in the therapy of cancers of the breast, gastrointestinal tract, and head and neck. Although these agents are cytotoxic through several mechanisms of action, a major action associated with clinical response is inhibition of TS (7). The fluoropyrimidines are metabolized to the nucleotide FdUMP, which is an analog of dUMP. It is postulated that FdUMP proceeds along the same reaction pathway as dUMP and that CH_2H_4PteGlu functions similarly with both nucleotides (8, 9). At the step in which a proton at the 5-position of dUMP is abstracted to initiate product formation and release, the difference between dUMP and FdUMP is manifested. The substitution of a fluorine atom at this position in FdUMP precludes further catalytic progress, resulting in the formation of a ternary complex devoid of catalytic activity. The ternary complex is highly stable because it is composed of two covalent linkages, one between an enzyme cysteine residue and the 6-position of FdUMP and the second between the methylene group of CH_2H_4PteGlu and the 5-position of FdUMP. Dissociation of the complex occurs at a rate inversely dependent upon the concentration of CH_2H_4PteGlu (10, 11). Thus, CH_2H_4PteGlu, which stimulates the covalent binding of FdUMP to TS and reduces the dissociation of the ternary complex, is an important determinant of TS inhibition and of response to fluoropyrimidine agents.

Heterogeneity in drug response has been associated with variation in drug-metabolizing enzymes and in drug targets. Numerous studies have been conducted to identify systemic and cellular factors that influence tumor cell response to 5-fluoropyrimidines. A plethora of phenotypic alterations have

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ABBREVIATIONS: TS, thymidylate synthase; CH_2H_4PteGlu, 5,10-methylenetetrahydrofolic acid; H_2PteGlu, dihydrofolic acid; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; dUrd, 2′-deoxyuridine; PteGlu, folic acid; CF, folinic acid (5-formyltetrahydrofolic acid); $PteGlu_4$, pteroyltetraglutamic acid; H_4PteGlu, tetrahydrofolic acid; 10-CHO-PteGlu, 10-formylfolic acid.

been identified that encompass nearly every conceivable determinant of fluoropyrimidine metabolism (12). Expression of TS with altered affinity for FdUMP has been observed in mammalian cells selected for FdUrd resistance (13, 14). The molecular basis for the TS variation was not identified in these studies. In recent studies in this laboratory, a structural variation in TS has been identified that confers resistance to FdUrd (15–17). The TS variation was identified in a human colonic tumor cell line not previously exposed to 5-fluoropyrimidines and thus represents a naturally occurring variation. Additional evidence for the existence of naturally occurring TS variations was obtained from investigations of two human colonic tumor cell lines that exhibit differing FdUrd-response phenotypes (18). In the present study, the existence of TS variation has been established by functional characterization of the enzymes expressed in these cell lines.

Experimental Procedures

Materials. [6-^{3}H]FdUMP (20 Ci/mmol), [5-^{3}H]dUMP (25 Ci/mmol), and [5-^{3}H]dUrd (20 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). PteGlu, CF, FdUMP, dUMP, Sephadex G-50-80, thiopropyl-Sepharose 6B, DEAE-cellulose microgranular anion exchanger, Cibacron blue 3GA-agarose (type 3000-CL), dextran (M_r 70,000), and acid-washed activated charcoal were purchased from Sigma Chemical Co. (St. Louis, MO). Cyanogen bromide-activated Sepharose 6B was purchased from Pharmacia (Piscataway, NJ). Bradford protein dye-binding reagent and Gel Silver Rapid Stain were purchased from Bio-Rad Laboratories (Richmond, CA). Trichloroacetic acid was purchased from Fisher Scientific (Fairlawn, NJ). $PteGlu_4$ was purchased from C. Krumdieck (University of Alabama, Birmingham, AL). (6*RS*)-H_4PteGlu was prepared from PteGlu by a previously described method (19). (6*S*)-H_4PteGlu was enzymatically synthesized from PteGlu using purified *Lactobacillus casei* dihydrofolate reductase (20). (6*RS*)- and (6*R*)-CH_2H_4PteGlu were prepared from the H_4PteGlu forms as described previously (21). Concentrations of the (6*RS*)-form are reported in terms of active isomer. 10-CHO-PteGlu was prepared from PteGlu and linked to cyanogen bromide-activated Sepharose 6B as described previously (22). RPMI 1640 medium, folate-free 1640 medium, and fetal bovine serum were purchased from GIBCO (Grand Island, NY). Bovine calf serum supplemented with iron was purchased from Hyclone Laboratories (Logan, UT).

Cell culture and growth conditions. Human colorectal tumor cell lines RCA and C (obtained from M. Brattain, Medical College of Ohio, Toledo, OH) were maintained as monolayers in RPMI 1640 medium supplemented with 5% fetal bovine serum. The cells were routinely monitored for the absence of *Mycoplasma* by the Mycoplasma T.C. Rapid Detection System (Gene-Probe, San Diego, CA). Cells were depleted of folates by growth in folate-free medium supplemented with 5% charcoal-stripped fetal bovine serum, 100 μM hypoxanthine, 30 μM thymidine, and 30 μM glycine, as described previously (18). For large-scale cultivation, cells were grown in suspension in maintenance medium supplemented with 8% bovine calf serum, in spinner flasks (Bellco Biotechnology, Vineland, NJ), with RCA requiring the inclusion of microcarrier beads.

TS purification. All procedures were conducted at 4°. Centrifugations were carried out for 30 min at 15,000 × *g*, using a Sorvall RC5B centrifuge. All buffers contained 10% glycerol and 0.1% Triton X-100 unless otherwise indicated. Protein in column eluents was detected by absorbance at 280 nm. Protein in column fractions was analyzed by the modified Bradford dye-binding assay (23). Purified protein was subjected to electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate, according to a previously described method (24), and was detected by silver staining.

Cell-free extracts of tumor cell lines (10–12 g) were prepared in 50 mM Tris·HCl, 100 mM KCl, 1 mM EDTA, 4 mM dithiothreitol, pH 7.4 (3 volumes), by sonication (6 × 15 sec, using a Biosonik IV cell disrupter) and centrifugation. The 30–70% ammonium sulfate fraction was dissolved in 10 ml of buffer A (100 mM Tris·HCl, 100 mM KCl, 4 mM dithiothreitol, 1 mM EDTA, pH 7.4) and dialyzed. The dialysate was applied to a Cibacron blue agarose column (6 × 0.8 cm) and protein elution was carried out as described previously (25). Briefly, the column was washed with buffer A until eluent protein was undetectable. TS was eluted using a linear gradient of 0.2–0.7 M KCl in buffer A. Fractions containing enzyme activity were combined and dialyzed against buffer B (50 mM Tris·HCl, 50 mM KCl, 4 mM dithiothreitol).

Protein from cell line C was dialyzed against buffer B, pH 7.2. To the dialysate, dUMP was added to a final concentration of 50 μM. The protein (0.2 mg/ml) was applied to a 10-CHO-PteGlu-Sepharose column (8 × 1.5 cm) and eluted by a modification of a previously described procedure (22). The column was washed with buffer B, pH 7.2, containing dUMP, until eluent protein was undetectable. TS was eluted from the column with buffer B, pH 7.2, without dUMP. Fractions containing enzyme activity were pooled and dialyzed against buffer B, pH 7.5. Dialyzed protein was applied to a DEAE-cellulose column (5 × 0.75 cm) and eluted by a previously described procedure (26). Briefly, the column was washed with buffer B, pH 7.5, until eluent protein was undetectable. TS was eluted using a linear gradient of 50–300 mM KCl in buffer B, pH 7.5. Fractions containing enzyme activity were combined and dialyzed against storage buffer (50 mM Tris·HCl, 100 mM KCl, 4 mM dithiothreitol, 15% glycerol, pH 7.4). Bovine serum albumin was added to a final concentration of 100 μg/ml before storage at −20°.

Protein from cell line RCA was dialyzed against buffer B, pH 7.5, and separated on DEAE-cellulose as described for protein from cell line C. Fractions containing TS activity were pooled and concentrated by ultrafiltration. Thiopropyl-Sepharose sulfhydryl affinity chromatography was used for further resolution of the fractions.[2] All buffers were deoxygenated with argon gas for 30 min before use. Thiopropyl-Sepharose was equilibrated in a column (5 × 0.5 cm) with buffer C (50 mM Tris·HCl, 50 mM KCl, 1 mM EDTA, pH 6.8). Excess buffer was removed by centrifugation for 5 min at 1000 × *g*. The protein fractions were dethiolated by two successive centrifugations (5 min, 1000 × *g*) on Sephadex G-50 equilibrated with 50 mM Tris·HCl, pH 7.4. Dethiolated protein was applied to the column and the column was tightly sealed under argon gas. The protein was incubated with the resin for 3 hr. The column was eluted with buffer C until eluent protein was undetectable. To eliminate unreacted 2′,2-dithiopyridyl groups, the column was washed with buffer C containing 5 mM L-cysteine. TS was eluted by using a linear gradient of 0.1–0.5 M KCl in buffer C, pH 8.0, containing 20 mM 2-mercaptoethanol. Fractions containing enzyme activity were concentrated by ultrafiltration and dialyzed against storage buffer. The enzyme was stored at −20° in the presence of 100 μg/ml bovine serum albumin.

Enzyme activity analyses. TS activity was assayed by a modification of a tritium release procedure described previously (27). Activity was determined at 37° in reactions containing 50 mM Tris·HCl, pH 7.4, 150 mM KCl, 4 mM dithiothreitol, 100 mM NaF, 150 μM (6*RS*)-CH_2H_4PteGlu, and 60 μM [5-^{3}H]dUMP (0.4 Ci/mmol). For termination, dUMP and trichloracetic acid were added to final concentrations of 3 mM and 0.9 N, respectively, and unreacted radiolabel was adsorbed with 7% activated charcoal. After charcoal removal by centrifugation, tritium release was determined by liquid scintillation counting. One unit is defined as the amount of enzyme required to release 1 nmol of ^{3}H/min. For determination of kinetic constants, initial velocities were measured by utilizing 2–3 mU of purified TS, 2–60 μM dUMP, and 12–150 μM (6*RS*)-CH_2H_4PteGlu. The data were fit to the hyperbolic form of the Henri-Michaelis-Menten rate equation using the HYPER program (28). Calculation of k_{cat} values was based on enzyme quantitation using [6-^{3}H]FdUMP and CH_2H_4PteGlu, as described previously (29).

TS activity *in situ* was determined by a modification of a previously

[2] T. P. Bradshaw and R. B. Dunlap, unpublished observations.

described procedure (30). Folate-depleted cells (1.5×10^5) were incubated for 24 hr with either 10 nM or 10 μM CF; controls were incubated with 2 μM FdUrd for 20 min. Enzyme activity was measured in medium supplemented with 1 μM [5-^{3}H]dUrd (2.5 Ci/mmol), during a 2-hr period. Reactions were terminated with trichloracetic acid (final concentration, 0.2 N) and tritium release was determined as described above. The data were expressed as dpm of ^3H_2O formed/min/mg of total cell protein.

Ligand binding analyses. All studies were conducted with purified TS and at 37° unless otherwise indicated. Ternary complex association was analyzed in reactions containing 1.7–6.0 nM ligand binding sites (assuming 1.7 mol of FdUMP/mol of protein dimer), 0.4–18 nM [6-^{3}H] FdUMP, and 1.0–175 μM (6*RS*)-CH_2H_4PteGlu. Reactions were terminated at 4° by the addition of KCl and FdUMP at final concentrations of 0.3 M and 75 μM, respectively (11). Unbound radiolabel was adsorbed with 2% activated charcoal/0.5% bovine serum albumin/0.05% dextran. After charcoal removal by centrifugation at 4°, the amount of protein-bound FdUMP was determined by liquid scintillation counting. The rate of FdUMP binding was determined at intervals during 1.5 min of incubation. Apparent bimolecular rate constants for FdUMP association (k_{on}) were calculated from a second-order rate equation described previously (11). For equilibrium studies, 2.3 nM ligand binding sites were incubated with 0.03–40 nM [6-^{3}H]FdUMP and 150 μM (6*RS*)-CH_2H_4PteGlu for 6 hr at 24°. Protein-bound FdUMP was determined as described above. Apparent dissociation constants were determined by computer-assisted linear regression analysis of data graphed according to the Scatchard equation (31). Ternary complex dissociation was analyzed after formation of complexes in reactions using 3.0 nM ligand binding sites, 60 nM [6-^{3}H]FdUMP, and 150 μM (6*RS*)-CH_2H_4PteGlu. Complexes were isolated by Sephadex G-50 centrifugal elutriation at 4° and were incubated for varying periods in the presence of 0.1 mM FdUMP and 0–500 μM (6*RS*)-CH_2H_4PteGlu. Protein-bound FdUMP was determined as described above. Apparent rate constants for dissociation of FdUMP from ternary complexes (k_{off}) were calculated from a first-order rate equation described previously (11).

Enhancement of FdUMP binding to TS by folate derivatives was determined by a modification of a previously described procedure (32). TS (2.3–3.5 nM ligand binding sites) was incubated for 1 hr with 0.5 μM [6-^{3}H]FdUMP and either (6*RS*)-CH_2H_4PteGlu or $PteGlu_4$ (0.001–300 μM) in 50 mM Tris, pH 7.4, containing 0.15 M KCl, 2 mM dithiothreitol, and 100 μg/ml bovine serum albumin. Protein-bound FdUMP was precipitated with 10% trichloroacetic acid, and the precipitate was solubilized with 0.5 N NaOH/50% ethanol and quantitated by liquid scintillation counting. The EC_{50} value, the concentration of folate derivative required for half-maximal FdUMP binding, was determined by computer-assisted linear regression analysis of binding data obtained between 20% and 80% saturation.

Results

Characterization of TS enzymes. Two different schemes, summarized in Table 1, were utilized to purify the enzymes expressed in C and RCA cells. The approach used for TS expressed in C is similar to that developed for other mammalian enzymes. An alternative procedure was developed for purification of the enzyme expressed in RCA, because approximately 70% of the enzyme eluted in the void volume of the 10-CHO-PteGlu affinity column even in the presence of 100 μM dUMP. The novel step in the second approach is thiopropyl-Sepharose affinity chromatography. Resolution of TS derives from the presence of the catalytic cysteine, a potent nucleophile that covalently binds to the 2′,2-dithiopyridyl moiety of the thiopropyl ligand, releasing 2-thiopyridone. To establish the validity of the alternative scheme, human enzyme isolated from HEp-2/500 cells, which overproduce TS by 100-fold with respect to other human cells, was used (33). Enzyme purified by either scheme demonstrated similar specific activities and K_m values for substrates. The apparent homogeneity of TS purified from HEp-2/500 cells by the modified procedure is shown in Fig. 1. Purification results for enzymes from C and RCA cells are shown in Table 1. Electrophoretic analysis of purified protein revealed the presence of a single polypeptide of approximately 36 kDa, consistent with the calculated value of M_r 35,706 for the human enzyme (Fig. 1) (34).

Kinetic constants defining substrate interactions with the enzymes were determined (Table 2). All studies were conducted with (6*RS*)-CH_2H_4PteGlu because substitution with the active form resulted in a marginal (10%) increase in enzyme activity

TABLE 1
Purification of TS from C and RCA

Step	Total units[a]	Total protein	Specific activity	Purification	Yield
		mg	*units/mg*	*fold*	%
A. C					
Cell-free extract	31	630	0.05	1.0	100
$(NH_4)_2SO_4$ fraction	30	475	0.06	1.2	97
Cibacron Blue	21	19	1.1	23	68
10-CHO-PteGlu-Sepharose/ DEAE-cellulose	10	<0.10	>100	>2000	32
B. RCA					
Cell-free extract	2.1	470	0.004	1.0	100
$(NH_4)_2SO_4$ fraction	1.7	250	0.007	1.5	80
Cibacron Blue	1.2	30	0.04	10	58
DEAE-cellulose	1.1	13	0.09	19	52
Thiopropyl-Sepharose	0.7	<0.05	>13.7	>3000	33

[a] 1 unit = 1 nmol of ^{3}H released/min at 37°.

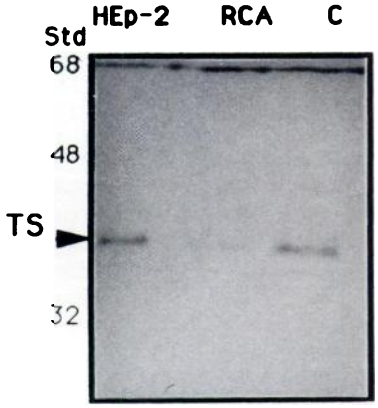

Fig. 1. Electrophoretic analysis of human TS. TS was purified from RCA and C cells as described in Experimental Procedures. TS was purified from the human tumor cell line HEp-2/500 by the procedure used to purify the enzyme expressed in RCA. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Experimental Procedures and were detected by silver staining.

TABLE 2
Kinetic constants of C and RCA TS

Constant	C	RCA
K_m for dUMP	*3.0 ± 0.5* μM (4)[a]	6.0 ± 0.7 μM (4)
K_m for CH_2H_4PteGlu	17.5 ± 1.5 μM (4)	77.7 ± 11.1 μM (4)
k_{cat}	150 min^{-1}	132 min^{-1}
k_{on} for FdUMP	$6.3 \pm 0.3 \times 10^7$ M^{-1} min^{-1} (6)[b]	$1.6 \pm 0.3 \times 10^7$ M^{-1} min^{-1} (6)[b]
k_{off} for FdUMP	$1.90 \pm 0.04 \times 10^{-2}$ min^{-1} (6)[b]	$1.50 \pm 0.05 \times 10^{-2}$ min^{-1} (6)[b]

[a] Numbers in parentheses, number of separate determinations.
[b] Determined at 75 μM CH_2H_4PteGlu.

and had no effect on FdUMP binding.[3] The enzyme isolated from RCA had higher K_m values for both dUMP and $CH_2H_4PteGlu$, relative to the enzyme isolated from C. The k_{cat} values of the two enzymes were similar.

The apparent rate constants for FdUMP binding in a ternary complex are presented in Table 2. The rate of FdUMP association to the enzyme from RCA was 4-fold lower than that to the enzyme from C (Fig. 2). Similar results were obtained when either FdUMP or TS was varied. The apparent rate constants for FdUMP dissociation from the ternary complex were similar for the enzymes isolated from RCA and C. The concentration of $CH_2H_4PteGlu$ affected both FdUMP association and dissociation. The rate of FdUMP binding increased with increasing $CH_2H_4PteGlu$ at concentrations below 10 μM; at concentrations between 10 and 175 μM the rate of FdUMP binding was relatively constant (Fig. 3). The rate of FdUMP dissociation from the ternary complex was inversely related to $CH_2H_4PteGlu$ concentration (data not shown).

The ratio of the rate constants (k_{off}/k_{on}) provided an estimate of the K_d for FdUMP binding. K_d values for the enzymes from

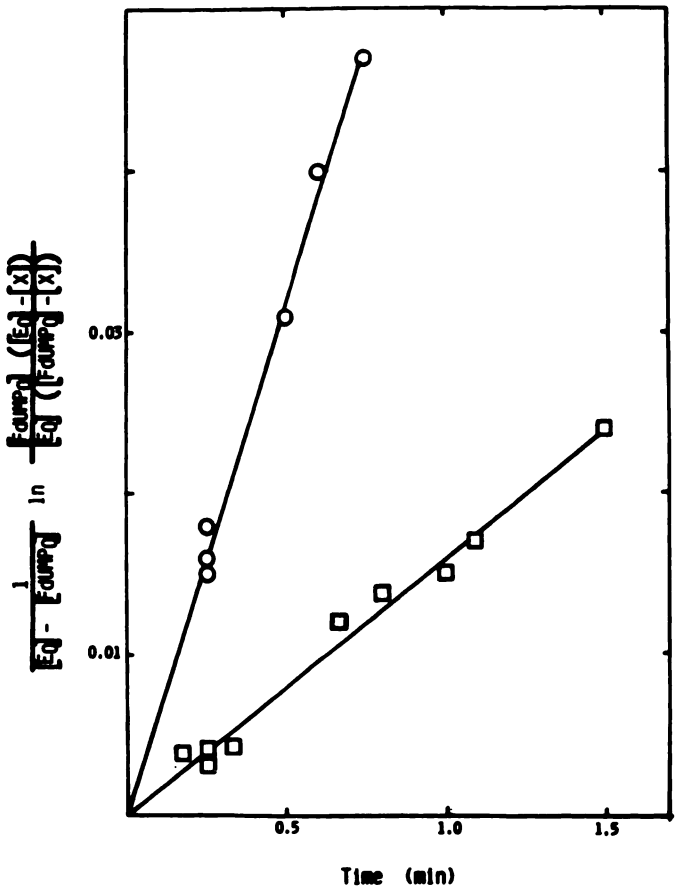

Fig. 2. Apparent rate constant (k_{on}) for FdUMP binding. TS (1.7–6.0 nM binding sites) purified from RCA (□) or C (○) was incubated for varying times with 0.4–18 nM [6-^{3}H]FdUMP and 75 μM (6*RS*)-$CH_2H_4PteGlu$, as described in Experimental Procedures. Protein-bound FdUMP, [*X*], was determined as described in Experimental Procedures. [E_0] and [*FdUMP*$_0$], initial concentrations of enzyme binding sites and FdUMP, respectively. The data were plotted according to the second-order rate equation described previously (11) and the k_{on} values were determined from the slope of the plot. Each data point is the average of two or three separate determinations.

[3] S. T. Davis and S. H. Berger, unpublished observations.

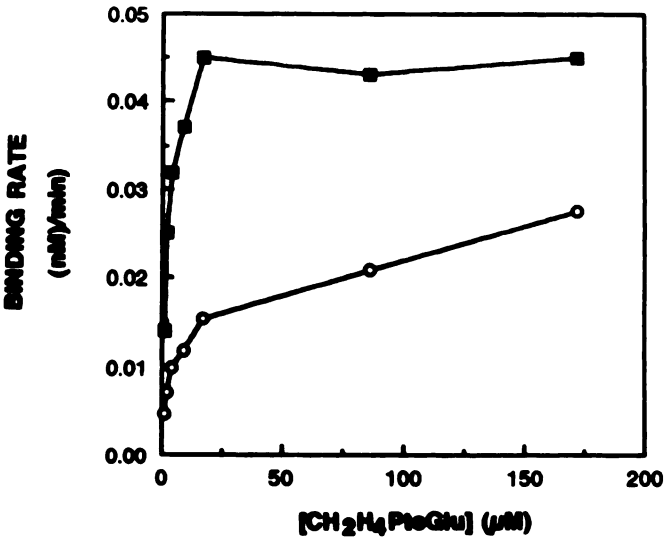

Fig. 3. Effect of $CH_2H_4PteGlu$ on the rate of FdUMP binding to TS. TS (2.3 nM ligand binding sites) purified from RCA (○) or C (■) was incubated with 1.6 nM [6-^{3}H]FdUMP and varying concentrations of $CH_2H_4PteGlu$ as described in Experimental Procedures. Protein-bound FdUMP was determined as described in Experimental Procedures, in aliquots removed at the indicated time points. Each value represents the slope of a linear plot of six to eight determinations relating protein-bound FdUMP and time.

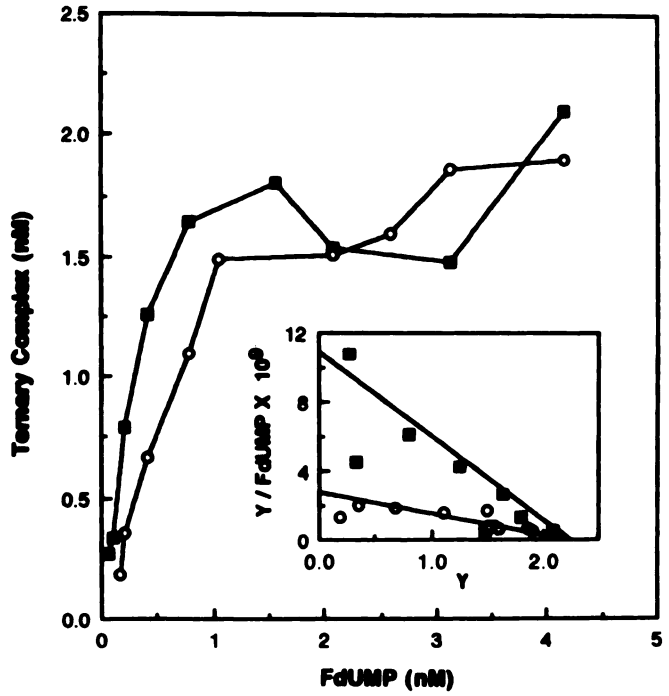

Fig. 4. FdUMP binding in a ternary complex. TS (2.3 nM binding sites) purified from RCA (○) or C (■) was incubated for 6 hr at 24° with 0.03–4.0 nM [6-^{3}H]FdUMP and 150 μM $CH_2H_4PteGlu$ as described in Experimental Procedures. Protein-bound FdUMP was determined as described in Experimental Procedures. The data are the mean of three separate determinations. *Inset*, Scatchard plot of the binding data. Y designates the moles of FdUMP bound/mol of TS.

C and RCA were 3.0×10^{-10} and 1.0×10^{-9} M, respectively. The K_d for FdUMP association into a ternary complex was also determined by ligand binding studies (Fig. 4). Graphical analysis of the data revealed the existence of a single class of binding sites that were saturated under the conditions of the experi-

ments. The K_d values are apparent because the concentration of ligand binding sites requisite for complex detection exceeded the observed K_d. The values determined for FdUMP binding were essentially identical to those calculated from rate constants (Table 3).

The effect of folate ligand on the covalent binding of FdUMP to the enzymes was determined with $CH_2H_4PteGlu$, which covalently binds to the FdUMP-enzyme binary complex, and with $PteGlu_4$, which does not (Fig. 5). The EC_{50} values for enhancement of FdUMP binding to the enzymes from C and RCA by $CH_2H_4PteGlu$ and PteGlu are presented in Table 3. The concentration of folate derivative required for half-maximal FdUMP binding was higher for the enzyme isolated from RCA than for that from C.

TS activity *in situ*. Because enzymes purified from RCA and C were observed to be functionally distinct, the effect of enzyme variation on the formation of dTMP *in situ* was assessed. In the assay, cells are incubated with [5-^{3}H]dUrd, which is converted intracellularly to [5-^{3}H]dUMP. Tritium released from [5-^{3}H]dUMP during the enzyme reaction exchanges with H_2O protons and equilibrates with cell medium. No difference in ^3H_2O formation was observed between the cells at either 10 nM or 10 μM CF (data not shown). Furthermore, no difference in ^3H_2O formation was detected in either cell line over the 1000-fold range of exogenous CF. In cells exposed to FdUrd before activity determination, no ^3H_2O was detected during the assay period.

TABLE 3
FdUMP binding to TS

Cell line	K_d for FdUMP[a]	EC_{50} for $CH_2H_4PteGlu$[b]	EC_{50} for $PteGlu_4$[b]
	M	*M*	*M*
C	3.1×10^{-10}	$1.8 \pm 0.5 \times 10^{-8}$	$0.9 \pm 0.1 \times 10^{-6}$
RCA	1.1×10^{-9}	$1.0 \pm 0.2 \times 10^{-7}$	$3.3 \pm 0.4 \times 10^{-6}$

[a] Apparent equilibrium binding constant for FdUMP, determined as described in the legend to Fig. 4.
[b] Concentration of folate derivative required for half-maximal FdUMP binding, determined as described in the legend to Fig. 5.

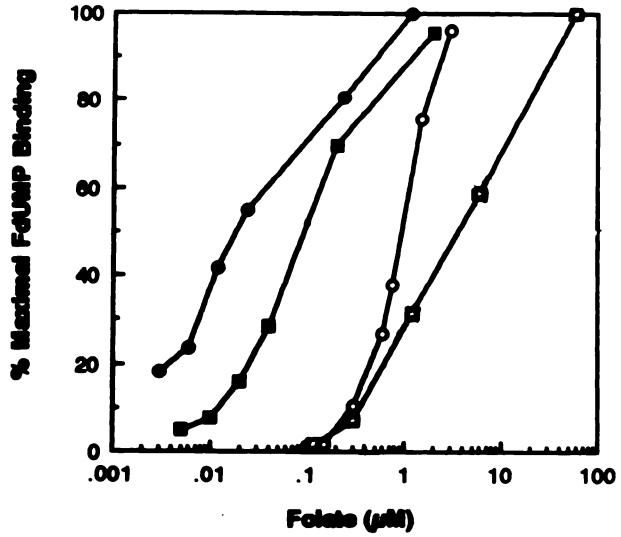

Fig. 5. Enhancement of covalent FdUMP binding to TS by $CH_2H_4PteGlu$ and $PteGlu_4$. TS (3.5 nM binding sites) purified from RCA (*squares*) or C (*circles*) was incubated for 1 hr at 37° with 0.5 μM [6-^{3}H]FdUMP and either 0.001–1 μM (6*RS*)-$CH_2H_4PteGlu$ (*closed symbols*) or 0.1–200 μM $PteGlu_4$ (*open symbols*). Protein-bound FdUMP was precipitated with trichloroacetic acid and determined as described in Experimental Procedures. The data are the mean of three separate determinations.

Discussion

Previous studies revealed that RCA is 10-fold less sensitive to growth inhibition by FdUrd than is C (35). The addition of CF in pharmacological concentrations (10 μM) eliminated the response difference (18). Intracellular folate pool analyses indicated that TS in RCA responded differently to intracellular $CH_2H_4PteGlu$ than did the enzyme expressed in C (18). Furthermore, preliminary ligand binding studies with partially purified enzymes revealed that enhancement of FdUMP binding by $CH_2H_4PteGlu$ was 3-fold lower for the enzyme from RCA than for that from C (18). These data suggested that TS is functionally variant in C and RCA. The enzymes were purified to apparent homogeneity; the specific activities of the purified enzymes were within the range reported for other human enzymes (25, 26). A novel purification strategy was developed for TS isolated from RCA because the majority of enzyme failed to bind to 10-CHO-PteGlu-Sepharose. TS enzymes isolated from mammalian sources have demonstrated an avidity for chromatographic matrices derivatized with folate ligands (36); thus, the poor avidity of the enzyme isolated from RCA was indicative of functional uniqueness.

The finding that the enzymes from C and RCA exhibit distinct properties was confirmed by kinetic and ligand binding studies. K_m values for dUMP and $CH_2H_4PteGlu$ were higher for the enzyme from RCA. The rate of FdUMP association into a ternary complex was 4-fold lower for the enzyme from RCA. A 3-fold difference was observed between the enzymes in the K_d for FdUMP binding. Thus, the major factor contributing to the difference in K_d values for ternary complexes was FdUMP association. In studies of FdUMP binding in the formation of FdUMP-enzyme binary complexes, the enzyme purified from RCA had a 4-fold lower affinity for FdUMP than did the enzyme from C (37). It is of interest that the enzymes from RCA and C differed in the rate of association of FdUMP into a ternary complex. The RCA and C cell lines were derived from the tumors of patients not previously exposed to 5-fluoropyrimidines (38). If these cell lines are representative of the tumors from which they were established, the ligand binding studies indicate that human TS is functionally variant. Additional evidence for the existence of TS variation has been obtained in studies utilizing enzymes isolated from human tumors (39). The enzymes from 11 tumors varied by 150-fold in the binding of FdUMP in a ternary complex. The major factor contributing to the differences among the enzymes in FdUMP binding was the rate of association of FdUMP into a ternary complex.

K_d values calculated from rate constants were essentially identical to those determined by ligand binding. Graphical analysis of equilibrium binding studies revealed that the human enzymes exhibited a single class of FdUMP binding sites. This observation is similar to that reported for the enzyme derived from human CCRF-CEM cells but differs from the report of two classes of FdUMP binding sites for the enzyme purified from *Lactobacillus casei* (11, 40). It has been suggested that the apparent difference in FdUMP binding between the enzymes derived from bacterial and human sources is due to the use of different diastereomers of $CH_2H_4PteGlu$ (11). Regardless of whether (6*R*)- or (6*RS*)-$CH_2H_4PteGlu$ was used, a single class

of FdUMP binding sites was observed for the enzymes derived from C and RCA. The basis for the apparent species difference is unknown.

The role of folate ligand in covalent binding of FdUMP to TS was examined with the enzymes from C and RCA. Previous studies revealed that derivatives of PteGlu, regardless of oxidation state, promote the covalent association of FdUMP to TS (3). The extent of partitioning of FdUMP between covalent and noncovalent complexes may be an important factor in enzyme inhibition. Both CH_2H_4PteGlu and $PteGlu_4$ enhanced the covalent binding of FdUMP to the enzymes from C and RCA. Significantly higher EC_{50} values were observed for complexes composed of the enzyme derived from RCA, independently of the nature of the folate ligand. The enzymes exhibited 6-fold and 4-fold differences in the enhancement of FdUMP binding by CH_2H_4PteGlu and $PteGlu_4$, respectively. Thus, at low folate ligand concentrations significantly less FdUMP was covalently bound to the enzyme purified from RCA than to that from C. This observation is consistent with the growth response of the cells to FdUrd and CF (18). At 10 nM CF, RCA was 4-fold less sensitive to FdUrd than was C; at 10 μM CF, the cells exhibited similar sensitivity to FdUrd. A similar growth response to FdUrd at pharmacological CF concentrations is unexpected, given the 3-fold difference between the purified enzymes in affinity for FdUMP. It is likely that other factors beside TS affinity for FdUMP have an impact upon the response of the cells to FdUrd. Two factors that may reduce the difference in FdUrd response between the cells are TS concentration, which is 2.5-fold lower in RCA than in C, and thymidine kinase activity, which is 5-fold lower in C than in RCA (35).

In addition to enhancement of FdUMP binding, folate ligands promote the covalent binding of dUMP to TS (41). It is proposed that CH_2H_4PteGlu enhances the covalent association of dUMP to TS in a manner similar to that for FdUMP. Thus, at low CH_2H_4PteGlu concentrations, less dUMP may be bound in covalent linkage to the enzyme expressed in RCA than to that in C, which could have an impact on catalytic activity. Endogenous TS activity was measured in the cells at different CF concentrations. No difference was observed in catalytic efficiency of the enzymes expressed in RCA and C cells grown in the presence of 10 nM CF. To eliminate artifacts arising from the experimental protocol, activity was determined in cells exposed to 10-fold higher dUrd concentrations and in cells synchronized by serum deprivation. The *in situ* activities remained unaffected by these modifications (data not shown). Furthermore, no increase in *in situ* activity was observed when CF was increased to 10 μM, a concentration that elevated intracellular CH_2H_4PteGlu pools in both cells by >10-fold (18). Obviously, CH_2H_4PteGlu pools are adequate for maximal TS activity at 10 nM CF, a concentration at the threshold for optimal cell growth in these cells (18). Moreover, the results suggest that the pools of CH_2H_4PteGlu formed in the cell lines after exposure to 10 nM CF are optimal for maximal TS activity but not for maximal enzyme inhibition.

The similar *in situ* activities are a reflection of the similar intrinsic activities of the enzymes purified from C and RCA. Thus, enzymes with differing affinities for nucleotide and folate derivatives exhibited similar catalytic efficiencies but differed in the concentration of ternary complexes at equilibrium. The data indicate that RCA colonic tumor cells express an enzyme that is catalytically efficient yet a poor target for 5-fluoropyrimidines. Expression of this enzyme is associated with the resistance of RCA to FdUrd at low CF concentrations (18). Because the RCA cell line was derived from the tumor of a patient with no previous exposure to 5-fluoropyrimidines, the enzyme expressed in this cell line may be representative of variant TS segregating in the human population (38). Interestingly, polymorphism in the TS gene among the Japanese population has been reported recently (42). Alternatively, the variation may have arisen during tumor progression or maintenance of the tumor cells in culture. The molecular basis for the functional variation between the enzymes expressed in RCA and C is under investigation.

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