Tight Binding of Folate Substrates and Inhibitors to Recombinant Mouse Glycinamide Ribonucleotide Formyltransferase†

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ABSTRACT: The binding of the prototypical folate inhibitor of *de novo* purine synthesis, 5,10-dideazatetrahydrofolate (DDATHF), and its hexaglutamate to recombinant trifunctional mouse glycinamide ribonucleotide formyltransferase (rmGARFT) was studied by equilibrium dialysis and by steady-state kinetics using sensitive assays that allowed initial rate calculations. rmGARFT was expressed in insect cells infected with a recombinant baculovirus and purified by a two-step procedure that allowed production of about 25 mg of pure protein/L of culture. The binding of DDATHF to GARFT was ≈50-fold tighter than previously reported, with K_d and K_i values of 2−9 nM, making the parent form of this antifolate a tight-binding inhibitor. The binding of the hexaglutamate of DDATHF to rmGARFT had K_d and K_i values of 0.1−0.3 nM, consistent with the view that polyglutamation enhances binding of antifolates to GARFT. Kinetic analyses using either mono- or hexaglutamate substrate did not yield different values for the K_i for the hexaglutamate form of DDATHF, in contradiction with previous reports. Both the folate substrate commonly used to study GARFT, 10-formyl-5,8-dideazafolate, and its hexaglutamate were found to have very low K_m values, namely, 75 and 7.4 nM, respectively, and the folate reaction products for these substrates were equally potent inhibitors, results which modify the interpretation of previous kinetic experiments. The product analog DDATHF and β-glycinamide ribonucleotide bound to enzyme equally well in the presence and absence of the other, an observation at variance with the concept that GARFT obeys an ordered sequential binding of the substrates. We conclude that the kinetics of mouse GARFT are most consistent with a random order of substrate binding, that both the inhibitor DDATHF and the folate substrate are tight-binding ligands, and that polyglutamate forms enhance the affinity of both substrate and inhibitor by an order of magnitude.

Mammalian glycinamide ribonucleotide formyltransferase (GARFT)[1] is a trifunctional protein which catalyzes not only the GARFT reaction but also the glycinamide ribonucleotide synthetase and aminoimidazole ribonucleotide synthetase reactions (*2*). That is, GARFT carries out the second, third, and fifth sequential reactions of the *de novo* purine synthesis pathway. We became acutely interested in the GARFT protein and its genetics since the discovery (*3*−*5*) of a class of folate antimetabolites that block purine synthesis as a result of direct inhibition of the catalytic activity of GARFT. The first known potent antifolate which specifically targeted GARFT was 5,10-dideazatetrahydrofolate (DDATHF). DDATHF was shown to have negligible activity against the traditional target for antifolates, dihydrofolate reductase, or the more recently targeted enzyme, thymidylate synthase (*3, 4*).

The 6*R* diastereomer of DDATHF was found to be antiproliferative and cytotoxic against mammalian tumor cells in culture and to have broad spectrum activity against transplantable mouse tumors and human tumor xenografts (*6*). The compound has undergone a series of Phase I clinical trials in cancer patients, and a number of often striking antitumor responses were noted in these trials (*7*−*10*). The toxicity profile of DDATHF was significantly different from that of previous antifolates advanced to human trials, and the combination of DDATHF with oral folic acid has dramatically altered the toxicity of this compound (*11, 12*). The biochemical basis of the toxicity profile is not currently understood nor is the mechanism of its prevention by oral folic acid. Second generation compounds are currently undergoing clinical trial.

Studies in this laboratory previously established DDATHF as a moderately strong but reversible competitive inhibitor of GARFT purified from L1210 mouse leukemia cells and revealed that the polyglutamates of DDATHF were much more active inhibitors of this enzyme (*5, 13*). Subsequently, others reported (*14*) that the K_i of a DDATHF polyglutamate was much higher when studied by steady-state kinetics using a polyglutamate substrate than when a monoglutamate substrate was used. This was very puzzling, because it defied the expectations of basic enzyme kinetics; yet others have observed this apparent phenomenon with other folate-dependent enzymes, namely, aminoimidazole carboxamide ribonucleotide formyltransferase (*15*) and thymidylate synthase (*16*). These observations remain unexplained. Given the clinical promise of this class of agents, and the several

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[1] Abbreviations: GARFT, glycinamide ribonucleotide formyltransferase; rmGARFT, recombinant mouse GARFT; DDATHF, (6*R*)-5,10-dideazatetrahydrofolate; GAR, glycinamide ribonucleotide.

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surprising phenomena uncovered by the administration of DDATHF to cell cultures, animals, and humans, we set out to understand the binding of DDATHF to GARFT in more detail.

We recently constructed a recombinant baculovirus which allowed high-level expression of mouse GARFT in insect cells (*17*). With the high levels and purity of rmGARFT that this recombinant enzyme allowed, it was possible to study the interaction of this enzyme with DDATHF and its polyglutamates as well as with the reaction substrates and products both by equilibrium dialysis and by initial velocity steady state-kinetics. In this paper, we report that DDATHF itself is a tight-binding inhibitor of GARFT. We also note that the tightness of binding of both folate substrates and products has clouded the interpretation of previous kinetic analyses of this reaction, and that the data are more consistent with a random addition, rather than an ordered sequential binding mechanism. Finally, we note that the kinetics of inhibition of the GARFT reaction by DDATHF hexaglutamate were identical when driven by either monoglutamate or polyglutamate folate substrates if initial rate conditions are imposed.

MATERIALS AND METHODS

Materials. 10-CHO-Dideazafolic acid was initially a gift from Dr. Homer Pearce of Lilly Research Laboratories (Indianapolis, IN) and, along with 5,8-dideazafolate, was subsequently obtained from Dr. John Hynes of the Medical University of South Carolina. 2,4,6-Trimethoxy-*s*-triazine and both (6*R*,*S*)- and (6*R*)-DDATHF were gifts from Dr. Chuan Shih of Lilly Research Laboratories. α,*β*-GAR was also a gift from Dr. Pearce. All other chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemicals (Milwaukee, WI).

Preparation of Affinity Chromatography Matrix. *ω*-(Aminoethyl)agarose (Sigma Chemical Co.) containing 3.7 *μ*mol of diaminoethane/mL of resin was washed with water, and 7.5 mL of resin was mixed with 31 *μ*mol of (6*R*,*S*)-DDATHF and 1.2 mmol of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and the mixture was incubated at 25 °C for 12 h. An additional 0.5 mmol of carbodiimide was added, and incubation was continued for 8 h. The resin was washed thoroughly with water, and then with 0.1 M sodium bicarbonate. The DDATHF−agarose could be stored at 4 °C in this form for at least 3 years. The 10-CHO-5,8-dideazafolic acid−agarose was prepared by the same procedure.

Recombinant Expression of Mouse GARFT. A full-length cDNA for mouse trifunctional GARFT (*18*) was partially digested with *Nco*I and *Hin*dIII, and a 3.24 kb fragment from the start ATG codon including the 3′ untranslated region and polyadenylation signal was inserted into the pBlueBac III transfer vector (InVitroGen Corp., San Diego, CA). This transfer vector was cotransfected with linearized wild-type AcMNPV baculoviral DNA into Sf9 insect cells, and recombinant virus in which the polyhedrin gene had been replaced by the mouse GARFT sequence was selected. Recombinant viruses were plaque-purified, and a high-producing plaque (recombinant SS9) was identified. Virus SS9 was titered on monolayers of Sf9 cells and used for expression of protein in log phase spinner cultures of Sf9 insect cells.

Purification of Recombinant Mouse GARFT. rmGARFT was purified to >98% purity (Figure 1) by a two-step procedure involving ion exchange followed by affinity chromatography on DDATHF−agarose. In a typical purification, 800 mL of insect cell culture at a density of 1−1.5 × 10^6 cells/mL was infected with the recombinant virus SS9 at a multiplicity of infection of 2, and the cells were harvested at 60 h. The cell pellet was suspended in 60−80 mL of 10 mM phosphate, pH 7.5, containing 10 mM 2-mercaptoethanol, 1 mM leupeptin, 1.4 mM pepstatin, and 0.1% Triton X-100 at 4 °C (lysis buffer), and the cells were broken by five 10 s pulses of sonication. The lysate was centrifuged at 160000*g* for 1 h, and the supernatant was loaded onto a 1.5 × 20 cm column of DEAE-Sephacel in 10 mM phosphate buffer. The column was washed with 50 mL of 10 mM phosphate buffer, pH 7.5, and then a linear gradient of 0−0.4 M KCl in phosphate buffer was applied; the volume of the mixer used was 200 mL. rmGARFT eluted from this column as a sharp peak centered on 0.12 M KCl. Fractions containing GARFT activity were identified, and the pooled fractions (≈40 mL) were mixed overnight with 7.5 mL of DDATHF−agarose resin at 4 °C. The resin was then packed into a 10 cm^3 syringe and washed with 400 mL of 10 mM phosphate buffer, pH 7.5 (buffer A), followed by 150 mL of 0.2 M phosphate, pH 7.5, containing 0.5 M KCl, (buffer B), with a final 50 mL wash of 1.5 M KCl in 10 mM phosphate buffer; all of these buffers contained 10 mM 2-mercaptoethanol and 0.1% Triton X-100. In the purification described under Results, rmGARFT was eluted from the DDATHF−agarose affinity column with 5 mL of 19 mM 10-CHO-5,8-dideazafolate followed by 10 mL of 9 mM 10-CHO-5,8-dideazafolate. Subsequent purifications eluted rmGARFT with 10 mL of 20 mM 10-CHO-5,8-dideazafolate. The fractions containing enzyme were pooled and passed down a 2.5 × 12 cm column of Sephadex G-50 to remove excess folate, glycerol was added to the desalted rmGARFT to a final content of 50%, and the enzyme was stored at −20 °C. rmGARFT purified and stored in this way was found by HPLC to contain 5,8-dideazafolic acid at a stoichiometry of about 1, presumably reflecting tightly bound 10-CHO-5,8-dideazafolate which was converted to product during storage. This stoichiometric 5,8-dideazafolic acid contaminant was removed for the studies reported herein by chromatography of enzyme as needed on a 7.5 mL column of DEAE-Sephacel equilibrated as described above. The column was washed with 6 mL of starting buffer, and enzyme was then removed with 15 mL of 0.2 M KCl; enzyme was concentrated by Amicon filtration using a Centricon 30 filter, and glycerol was added to 25%. The enzyme was stored in this solution at −20 °C and was stable for at least 2 years.

Purification of L1210 GARFT. Typically, 14 DBA/2 female mice were innoculated ip with 1 × 10^6 cells/mouse, and the tumor was harvested 7 days later. The cells were resuspended in 20 mL of lysis buffer and broken by sonication. A high-speed supernatant resultant from 1 h of centrifugation at 160000*g* was loaded directly onto 4.5 mL of DDATHF−agarose, and the resin was washed with 40 mL of buffer A, followed by 100 mL of buffer B. GARFT was eluted by 10 mL of 9 mM 10-CHO-5,8-dideazafolic acid. The folate was removed as described above. Active fractions were loaded onto a 4 mL column of 10-CHO-5,8-dideazafolate−agarose, and the resin was washed with 30

mL of buffer A, and enzyme was eluted with 10 mL of 4 mM 10-CHO-5,8-dideazafolic acid. Excess folate was again removed as above, and the active fractions were stored at −20 °C in 50% glycerol.

Synthesis of β-Glycinamide Ribonucleotide (β-GAR). High specific activity tritium-labeled biologically active β-GAR was synthesized enzymatically utilizing the GAR synthetase activity of trifunctional mouse GARFT. [2-^{3}H]-Glycine (4.46 nmol; 43 Ci/mmol) was incubated at 37 °C with 16.5 mM NH_4Cl, 23 mM $MgCl_2$, 1 mM adenosine 5′-triphosphate, 1.1 mM ribose 5-phosphate, and 3 μg of rmGARFT in a total volume of 30 μL of 0.1 M Tris buffer, pH 8.0. The reaction was stopped after 90 min by the addition of 50 μL of 0.2 M HCl. ^{3}H-β-GAR was purified by cation exchange column chromatography on a 6 mL bed volume of Dowex 50-x8 using 0.2 M HCl as the mobile phase. The elution position of β-GAR from this column was determined by chromatography of a sample of α,β-GAR and assay of fractions with GARFT; ^{3}H-product cochromatographed with this standard. Fractions containing β-GAR were lyophilized and reconstituted in water. ^{3}H-β-GAR was quantitated by using the ^{3}H-product and 10-CHO-5,8-dideazafolate as substrates for the GARFT reaction and determination of the amount of 5,8-dideazafolate formed using the HPLC system described below. The specific activity of the ^{3}H-β-GAR formed by this method was determined to be 46 Ci/mmol.

Synthesis of ^{3}H-DDATHF. High specific activity ^{3}H-DDATHF was prepared by coupling [3,4-^{3}H]glutamic acid diethyl ester to (6*R*)-5,10-dideazapteroic acid in a procedure developed by Mr. Archie Tse of this laboratory. In a typical preparation, 7 mg of (6*R*)-DDATHF was hydrolyzed with 500 μL of 6 N HCl by incubation at 100 °C in a sealed tube to yield the pteroic acid derivative. After hydrolysis, the precipitated product was dissolved by the dropwise addition of 10 N ammonium hydroxide, and the product was purified on a 5 mL column of DEAE-cellulose. A 0.06−1.5 M convex gradient of ammonium formate was applied to this column using a 250 mL mixer volume; 5,10-dideazapteroic acid eluted after 80−100 mL of this gradient. Fractions of this peak containing only the pteroic acid analog were identified by spectrophotometry followed by reverse phase HPLC and were pooled; the pteroic acid derivative was precipitated by the addition of formic acid to pH 4. The diethyl ester of [^{3}H]glutamic acid was prepared by reacting 18 μmol of [3,4-^{3}H]glutamic acid (1 mCi; 55 Ci/mmol) (Amersham Corp., Arlington Heights, IL) with 0.1 M ethyltosylate in dry ethanol in a total volume of 200 μL at 77 °C for 24 h. The reaction mixture was dried under a stream of N_2, dissolved in 100 μL of 5% methanol/chloroform, and applied to a 5 mL column of silica gel equilibrated with 5% methanol in chloroform. The diethyl ester of glutamic acid was eluted as a broad peak by 30−50 mL of this same solvent; the pooled fractions were dried under N_2. After purification on silica gel, excess [^{3}H]-diethylglutamate (16 μmol) was added to 5,10-dideazapteroic acid (3.4 μmol), and the two compounds were coupled by incubation at room temperature for 8 h in 25 μL of dry DMSO in the presence of 36 μmol of 4-methylmorpholine and 3.4 μmol of 2,4,6-trimethoxy-*s*-triazine. ^{3}H-DDATHF was obtained by hydrolysis of the [^{3}H]diethyl ester of (6*R*)-DDATHF by the addition of 30 μL of 1 N sodium hydroxide and further incubation for 8 h at room temperature. The product was purified by chromatography on a 5 mL column of DEAE-cellulose under the same conditions described above. Radioactive material which chromatographed at the position of marker DDATHF was pooled and further purified on a reverse phase HPLC using a 3 μm VeloSep RP-18 100 × 3.2 mm column (Applied Biosystems, Inc., Foster City, CA) and a multiphase gradient from 27 to 42% methanol in tetrabutylammonium hydrogen sulfate over 35 min. To generate this gradient (chromatography system A), the methanol concentration was initially 27%, and then changed in a linear gradient to 35% over 10 min; subsequently, a less steep linear gradient was initiated which reached a methanol concentration of 42% after an additional 15 min; the methanol concentration was then held at 42% for the next 10 min. Pooled HPLC fractions containing ^{3}H-DDATHF were dried under vacuum, dissolved in 300 μL of water, and passed through a 1 mL column of AG 50W-x8 (Bio-Rad Laboratories, Hercules, CA) to remove the ion-pairing reagent; the product was concentrated and stored at −20 °C in 20% ethanol. The purity and specific activity of the final product were determined by ion-paired HPLC and scintillation counting; coinjection of ^{3}H-DDATHF with standard DDATHF allowed purity to be estimated at >98%. A typical yield of [^{3}H]DDATHF obtained by this procedure was 10% relative to the amount of [^{3}H]glutamic acid used, which was purposely added in excess.

Enzymatic Preparation of Pteroyl Hexaglutamates. Hexaglutamate forms of DDATHF, 10-CHO-5,8-dideazafolic acid, and 5,8-dideazafolic acid were synthesized enzymatically using recombinant human folylpoly-γ-glutamate synthetase (rhFPGS). rhFPGS (*19*) was a generous gift from Dr. Paresh Sanghani of this laboratory. High specific activity radiolabeled pteroyl hexaglutamates were synthesized typically by incubating 0.5 mM [^{3}H]glutamic acid (5.4 nmol; 40−60 Ci/mmol) at pH 9.0 at 37 °C with 25 μM folate substrate, 20 mM 2-mercaptoethanol, 10 mM ATP, 20 mM $MgCl_2$, 30 mM KCl, and 1 μg of rhFPGS in a total volume of 10 μL of 0.2 M Tris, pH 9.0, containing 0.2 mg/mL bovine serum albumin. Intially, [^{3}H]glutamic acid was the only source of glutamic acid in these reactions to maximize the specific activity of the folate polyglutamate; however, this low concentration of glutamic acid did not allow facile synthesis of hexaglutamate derivatives, although the product was predominantly pentaglutamate. Hence, fresh enzyme and reagents were added after 8−12 h, and 15−30 mM unlabeled glutamic acid was added to force the formation of hexaglutamates. The reaction was monitored by HPLC and stopped by heating at 100 °C once the major product was the pteroyl hexaglutamate derivative. The hexaglutamate product was purified by HPLC reverse phase paired-ion chromatography using chromatography system A (see above). The folate hexaglutamate product was quantitated against the area of a known amount of monoglutamate standard. This separation required knowledge of the retention times of each of the polyglutamate derivatives of a given folate compound; they were identified in a separate experiment in which a similar FPGS reaction was performed using 50 μM folate compound and 30 mM glutamic acid spiked with 10 μCi of [^{3}H]glutamic acid. The ratio of peak area to cpm was determined on this same HPLC column system; this ratio increased as an integer when normalized to the ratio for the folate diglutamate, allowing calibration of the retention times for the individual polyglutamates. The above

procedure allowed the synthesis of both ^{3}H-DDATHF and ^{3}H-10-CHO-5,8-dideazafolic acid hexaglutamate derivatives. However, to efficiently synthesize the tritium-labeled hexaglutamate form of 10-CHO-5,8-dideazafolic acid, it was necessary to decrease the initial concentration of this folate analog to 20 μM.

Unlabeled polyglutamates of DDATHF, 10-CHO-5,8-dideazafolic acid, and 5,8-dideazafolic acid were prepared by a modification of this method. The reaction volume and FPGS were increased to 5 mL and 0.5−1.2 mg, and glutamic acid and folate substrates were maintained at 30 mM and 50 μM, respectively. Aliquots of reaction mixture were injected onto the HPLC, and the fractions corresponding to hexaglutamate were pooled and concentrated. The HPLC method used for purification of the unlabeled hexaglutamate form of 5,8-dideazafolic acid involved use of a 220 × 4.6 mm Spheri-5 column (Applied Biosystems, Inc.) at a flow rate of 0.8 mL/min with a monophasic concave gradient of 33−41% methanol containing tetrabutylammonium hydrogen sulfate over a period of 30 min. This methanol gradient again was adjusted slightly for other folate substrates to allow optimum separation of their polyglutamates, as follows: for 10-CHO-5,8-dideazafolic acid, the initial methanol concentration was 31%, and the final concentration was 39%; for DDATHF, the initial methanol concentration was 32%, and the final was 38.5%.

Equilibrium Dialysis. The binding constants for the interaction of rmGARFT with DDATHF and the DDATHF hexaglutamate derivative were determined in 50 mM Tris buffer, pH 7.4 (at 4 °C), containing 0.1% Triton X-100 and 20 mM 2-mercaptoethanol using a Spectra/Por equilibrium dialyzer rotating at 15 rpm. In initial experiments, it was noted that the equilibration of these folates across the dialysis membrane was slow; the first-order rate constant for diffusion in the absence of GARFT was estimated to be 0.03 min^{-1} for DDATHF and 0.0045 min^{-1} for DDATHF hexaglutamate[2] at 4 °C. The time required to reach equilibrium was decreased by the addition of part or all of the ligand on the enzyme side of the dialysis cell. Hence, the equilibrium times allowed for the binding of DDATHF and its hexaglutamate to rmGARFT were 5 h and 18 h, respectively, at 4 °C. Samples were withdrawn from both sides of the cells, and the radioactivity in duplicate 50 μL aliquots was determined by scintillation counting. In a typical experiment, five dilutions of ligand of a constant specific activity were added to a series of dialysis cells along with a constant amount of rmGARFT; duplicate cells were used for each dilution. The volume of each half-cell was 200 μL. The fraction of ligand bound at equilibrium was estimated, and Scatchard analysis was applied to the data to allow estimation of the dissociation constant and the number of binding sites per mole of enzyme. Each experiment was performed at least twice.

Steady-State Kinetics Using 10-CHO-5,8-Dideazafolic Acid as Substrate. GARFT assays were carried out using a HP 8452A spectrophotometer. The spectrophotometric assay previously reported (*13*, *20*), which followed the rate of conversion of 10-CHO-5,8-dideazafolate to 5,8-dideazafolate at 295 nm, was modified by the use of a 25 mL volume, 10 cm path-length cuvette. The buffer used was 75 mM HEPES, pH 7.4, containing 50 μg/mL bovine serum albumin. 10-CHO-5,8-Dideazafolate was used as a substrate in these studies, rather than the naturally-occurring substrate 10-formyltetrahydrofolate, to circumvent the problems caused by the sensitivity of the latter to oxidation. Initial velocity was calculated using the Hewlett-Packard kinetics software, version HP89531A revision A.03.00, from the slope measured usually on a 10 s window within the first 15 s after the initiation of reaction. For a typical experiment, these conditions allowed rate determination with less than 20% substrate consumption at all concentrations used and the accumulation of only (0.1−0.5) × K_i of product. Analyses of the K_m value for 10-CHO-5,8-dideazafolate and the K_i for DDATHF were carried out at 25 °C with 10-CHO-5,8-dideazafolate ranging from 0.05 to 10 μM, and α,β-GAR was held at 5 μM in 75 mM HEPES, pH 7.5, containing 50 μg/mL bovine serum albumin; the rmGARFT concentration used in these experiments was 0.75 nM. Enzfitter software was used to fit the data to the Michaelis−Menten equation for estimation of K_m, and the inhibition constants for the inhibitors were determined either by Lineweaver−Burk plot or by Dixon analysis assuming competitive inhibition. For tight-binding inhibitors, Morrison's equation (*21*) was applied for estimation of inhibition constants assuming competitive inhibition and using Enzfitter software (Elsevier Sciences, Amsterdam) as reported earlier (*13*). Analysis of the inhibition of rmGARFT by DDATHF analogs and product inhibitors used 5 μM α,β-GAR, 1 μM 10-CHO-5,8-dideazafolic acid, and 0.75 nM rmGARFT, and data were analyzed using Dixon plots.

Steady-State Kinetic Studies Using 10-CHO-5,8-Dideazafolic Acid Hexaglutamate as Substrate. The reaction of 10-CHO-5,8-dideazafolate hexaglutamate with GAR catalyzed by rmGARFT was studied by quantitation of the production of [^{3}H]-5,8-dideazafolate hexaglutamate using HPLC and scintillation counting. Reactions were performed at 25 °C with [^{3}H]-10-CHO-5,8-dideazafolate hexaglutamate set at 30 nM, α,β-GAR at 5 μM, and rmGARFT at 10 pM. Reactions were terminated by placing them in a boiling water bath after a 15 min incubation; the samples were then dried under vacuum in a Speed-Vac concentrator and reconstituted in 15 μL of reagent-grade tetrabutylammonium hydrogen sulfate (low UV PicA reagent; Waters Corp., Milford, MA). Unlabeled 5,8-dideazafolic acid hexaglutamate standard (20 pmol) was added to each reaction mixture to help locate the HPLC elution peak corresponding to the product, and the samples were injected onto a 5 μm 250 × 4.6 mm Beckman Ultrasphere ODS column. A gradient of methanol in Pic A initially at 38% methanol increasing as a linear gradient to 42.8% over 20 min, followed by a second linear gradient to 48% over the next 10 min, was applied at 0.9 mL/min. The product peak was collected and quantitated in a Beckman LS 5000 CE scintillation counter. The kinetic constants were determined using the Enzfitter software package.

RESULTS

Expression and Purification of Recombinant Mouse GARFT. Because of the size of the trifunctional GARFT protein (107 kDa), an insect cell system was chosen for the production of large amounts of recombinant enzyme. Using a recombinant baculovirus to infect Sf9 cells and with an optimized

[2] The folate molecule itself contains 1 mol of glutamate, making the nomenclature somewhat imprecise. The folyl polyglutamate compounds synthesized enzymatically and used through this paper all contained a total of 6 mol of glutamate per mole of dideazapteroate.

Table 1: Purification of Recombinant GARFT[a]

purification step	total act. (μmol min^{-1})	total protein (mg)	sp act.[b] (μmol min^{-1} mg^{-1})	purification factor
cell lysate	300	250	1.19	1.0
ion exchange	230	62	3.7	3.1
DDATHF−agarose	146	21	7.0	5.9

[a] The purification listed in this table was performed using Sf9 cells from a 800 mL culture at an initial density of 1.2×10^6 cells/mL. [b] Assayed using 11 μM 10-CHO-5,8-dideazafolic acid and 10 μM α,β-GAR as substrates at 37 °C in a 1 cm cuvette. For details, see Materials and Methods.

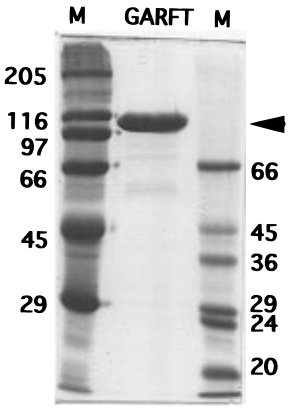


FIGURE 1: SDS−polyacrylamide gel electrophoresis of purified rmGARFT. Five micrograms of rmGARFT was applied to a 10% SDS−PAGE gel under reducing conditions, and the gel was stained with Coomassie Blue after electrophoresis. The lanes marked M were loaded with protein standards of the indicated molecular masses (in kilodaltons). The molecular mass calculated from the cDNA sequence of rmGARFT was 107 600 daltons (arrow).

purification scheme, about 20 mg of highly pure rmGARFT (Figure 1) was routinely obtained from an 800 mL culture of cells. The yield of rmGARFT from this purification scheme was about 50%. Purified enzyme prepared by this method had a specific activity of 7 μmol min^{-1} (mg of protein)$^{-1}$ when assayed at 37 °C with 10 μM α,β−GAR and 11 μM 10-CHO-5,8-dideazafolate as substrates; assayed at 25 °C using a 10-cm cuvette, 1 μM 10-CHO-5,8-dideazafolate, and 5 μM α,β-GAR, the specific activity of pure rmGARFT was 2.8 μmol min^{-1} (mg of protein)$^{-1}$.

The experience that we (*13*) and others (*22*) have had with purification of this enzyme from endogenous sources indicated that proteolysis can be a major problem with preparation of this enzyme; hence, the purification scheme designed for recombinant protein attempted to rapidly process crude fractions. Ammonium sulfate fractionation, which had been the initial step in our previous purifications, was not used, and crude enzyme was directly applied to a DEAE-Sephacel column, which proved to be a rapid method for removal of the majority of contaminating proteins. The purification factor obtained with this column (Table 1) underestimated the value of the DEAE-Sephacel column to the processing of large amounts of protein (although we have purified small batches of recombinant rmGARFT by a one-step process using affinity chromatography by itself). In particular, the DEAE-Sephacel column efficiently separated a low molecular mass (28 kDa) protein with GARFT activity that was observed in one preparation, apparently the result of proteolysis during initial steps. This 28 kDa protein eluted from DEAE-Sephacel at 50 mM KCl and was well separated from 107 kDa rmGARFT by this column. A fragment of 28 kDa also accumulated in a batch of highly purified rmGARFT during storage at 4 °C, so this fragment clearly did not represent a separate species of GARFT. This presumed proteolytic fragment was found to have a much higher k_{cat} than the 107 kDa GARFT, an observation in agreement with previous literature (*22*) that GARFT can be activated by limited proteolysis.

The DDATHF−agarose column proved to be more useful than the 10-CHO-5,8-dideazafolate affinity column that we (*13*) and others (*23*) have routinely used for purification of GARFT. The binding of rmGARFT to the DDATHF−agarose column was much tighter than that to the 10-CHO-5,8-dideazafolate column, allowing more stringent conditions for washing. The DDATHF−agarose column was also very stable and could be used repetitively; in contrast, 10-CHO-5,8-dideazafolate−agarose columns often bound enzyme poorly even after a single use.

Properties of rmGARFT. The cDNA used to construct the recombinant virus for expression of GARFT in insect cells was originally isolated from a mouse leukemic cell cDNA library (*18*), and the rmGARFT described herein was found to be identical to enzyme purified from L1210 cells by several criteria. The migrations of recombinant and L1210 proteins were compared on a 10% SDS−PAGE gel; both traveled at an apparent molecular mass of 115 kDa, and only a single band was detected in the lane in which both proteins were loaded as a mixture. Both proteins had an isoelectric point of 6.7 when examined on a BioRad Rotophor apparatus. When 300 pmol of each protein was subjected to automated Edman microsequencing, both proteins were found to be blocked at the amino terminus. The Michaelis constants for α,β-GAR measured in the presence of 10 μM 10-CHO-5,8-dideazafolate for the GARFT purified from L1210 cells and for rmGARFT were both 1.5 μM,[3] and the K_ms for 10-CHO-5,8-dideazafolate for the two enzymes were, likewise, indistinguishable (see below).

Binding of DDATHF and Its Hexaglutamate to rmGARFT. The binding of DDATHF to rmGARFT was examined directly using equilibrium dialysis. In order to have a range of free and total drug concentrations that were easily and accurately measurable, high specific activity (≈35 Ci/mmol) tritium-labeled DDATHF was synthesized, and a total enzyme content of 4 pmol per 200 μL dialysis half-cell was used. Under these conditions, Scatchard analysis of the binding of DDATHF to rmGARFT (Figure 2A) indicated a dissociation constant of 1.8 ± 0.3 nM and a total binding of 1.08 ± 0.15 mol/mol (4 experiments). The total enzyme content was varied during these experiments from 5 to 55 nM in order to detect a second weaker binding site, but, in all of these experiments, the data were fit by a linear Scatchard plot with an intercept corresponding to a stoichiometry of approximately 1. The hexa-γ-glutamate of

[3] Measured in 1 cm cuvettes at 37 °C; when measured in 10 cm cuvettes at 25 °C, the K_m for α,β-GAR was 0.76 μM.

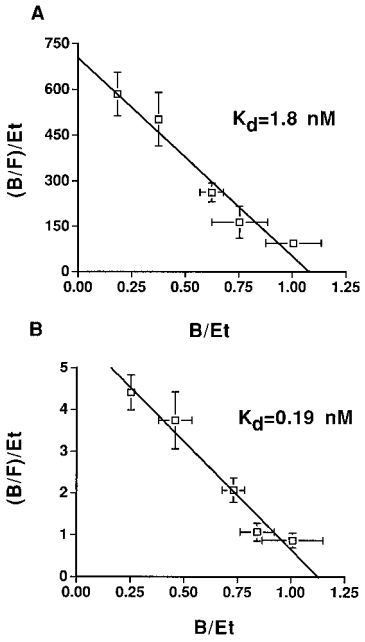


FIGURE 2: Scatchard analysis of the binding of DDATHF (A) and of DDATHF hexaglutamate (B) to rmGARFT. (A) ^{3}H-DDATHF (4−40 nM initial concentration in a 200 μL half-cell) and rmGARFT (16−20 nM per 200 μL half-cell) were added to the sides of a 400 μL dialysis cell in 50 mM Tris buffer, pH 7.4 (at 4 °C), containing 0.1% Triton X-100 and 20 mM 2-mercaptoethanol. After mixing for 5 h at 0−4 °C, the radioactivity on each side of the dialysis membrane was determined. (B) DDATHF hexaglutamate was used in the range of 0.4−4 nM initial concentration in 200 μL; rmGARFT was used at 1 nM, and samples were withdrawn from each side of the membrane after 18 h of mixing; other conditions were the same as those in (A). Each data point is an average of 6−8 observations drawn from 4 separate experiments, and the error bars depict ±1 standard deviation. *B*, *F*, and E_t represent the concentrations of bound and free DDATHF and total enzyme, respectively.

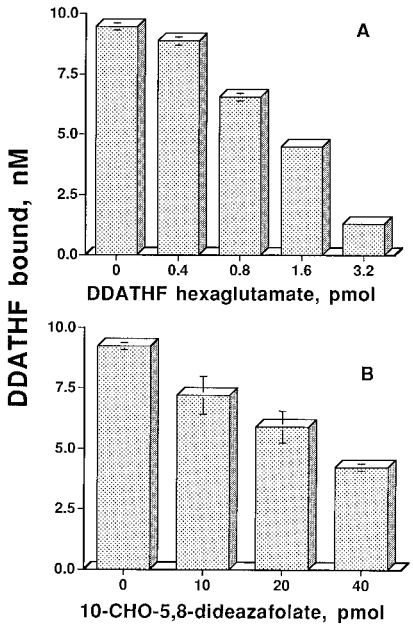


FIGURE 3: Inhibition of the binding of DDATHF to rmGARFT by DDATHF hexaglutamate and 10-CHO-5,8-dideazafolate. ^{3}H-DDATHF (4.8 pmol, 18 Ci/mmol) and rmGARFT (2.8 pmol) were added to 200 μL half-cells of a Spectro/Por equilibrium dialysis unit as in Figure 2, and each half-cell was sampled after rotating for 18 h (A) or 5 h (B) at 4 °C after the addition of the indicated amounts of either DDATHF hexaglutamate (A) or 10-CHO-5,8-dideazafolate (B).

DDATHF was synthesized enzymatically using recombinant human folylpoly-γ-glutamate synthetase (*19*), incorporating [^{3}H]glutamic acid to a specific activity of 110 Ci/mmol. Using this material, equilibrium dialysis allowed the K_d for the binding of DDATHF hexaglutamate to rmGARFT to be estimated at 0.19 ± 0.04 nM; the stoichiometry of this binding was measured to be 1.12 ± 0.09 mol/mol (Figure 2B).

The K_d measured for the binding of DDATHF hexaglutamate to rmGARFT was comparable to the K_i previously reported for the inhibition of L1210 mouse GARFT by DDATHF pentaglutamate [0.5 nM; (*13*)], but there was a major discrepancy between the K_d measured by equilibrium dialysis and the K_i previously reported for the inhibition of mouse and human GARFT by DDATHF [100 and 300 nM, respectively (*13, 24*)]. This was surprising, in light of the fact that the interactions of DDATHF and of several structural analogs were previously found to be strict competitive inhibitors of L1210 GARFT with respect to variable folate substrate, and noncompetitive with respect to variable glycinamide ribonucleotide (GAR) (*13*). Although this could be caused by tight binding of DDATHF to a site on the enzyme different from the active site, a second, weaker site was not detected in our equilibrium dialysis experiments (see above). In order to examine this possibility further, competition for the binding of ^{3}H-DDATHF to rmGARFT during equilibrium dialysis was used to determine whether these three ligands were binding to the same site on the protein. Both DDATHF hexaglutamate and 10-CHO-5,8-dideazafolate displaced DDATHF from its binding site on a limiting amount of rmGARFT (Figure 3A,B, respectively). Because both DDATHF and DDATHF hexaglutamate bound to rmGARFT with a 1:1 stoichiometry, it was clear that all three ligands were competing for binding to the same site on rmGARFT, presumably the substrate binding site. The binding constant calculated from the data in Figure 3A for the interaction of rmGARFT and DDATHF hexaglutamate was 0.4 nM, consistent with the results obtained for direct binding of labeled DDATHF hexaglutamate to the enzyme (0.2 nM) as well as the K_i values obtained from steady-state kinetics [0.5 nM (*13*), and 0.11 nM (see below)]. It was noted that the K_d calculated[4] for 10-CHO-5,8-dideazafolic acid from these data, 30 nM, was much lower than the K_m of this substrate for mouse GARFT [reported to be 1.3 μM (*13*) or higher (*25, 26*)).

Kinetic Reevaluation of the Interaction of DDATHF with rmGARFT. Although it was clearly possible that the concentration range at which 10-CHO-5,8-dideazafolic acid competed for DDATHF binding to rmGARFT (Figure 3B) and the K_m for this compound as a substrate for GARFT (*13, 25−27*) be different, the magnitude of this difference prompted us to reexamine the kinetics of the GARFT reaction. When the dependency of the rate of the rmGARFT reaction on 10-CHO-5,8-dideazafolate concentration was studied spectrophotometrically using 10 cm cuvettes to

[4] The dissociation constant for the unlabeled competing ligand in these experiments was calculated using the equation: $K_{d,U} = U_{50}/(1 + L/K_{d,L})$, where $K_{d,U}$ and $K_{d,L}$ are the dissociations constants for unlabeled and labeled ligands, respectively, L is the concentration of labeled ligand, and U_{50} is the concentration of unlabeled ligand that decreases the binding of labeled ligand by 50% (*1*).

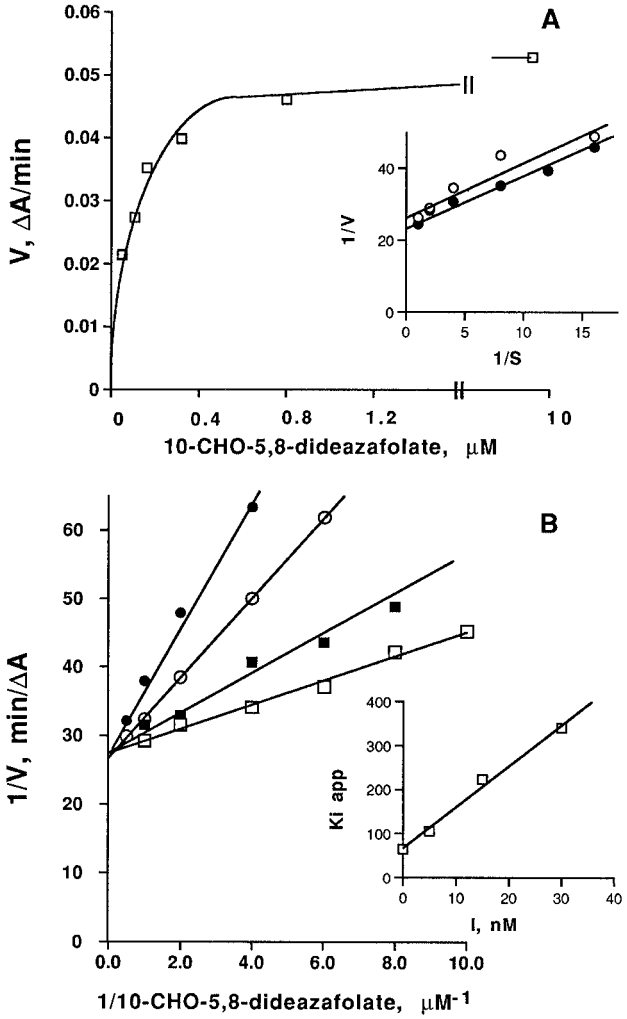


FIGURE 4: Kinetics of the reaction catalyzed by rmGARFT using 10-CHO-5,8-dideazafolic acid as a substrate. (A) Saturation of the rmGARFT reaction by 10-CHO-5,8-dideazafolate. The rate of the GARFT reaction was followed spectrophotometrically at 295 nm in 10 cm cuvettes at the indicated concentrations of 10-CHO-5,8-dideazafolate and 5 μM α,β-GAR. The buffer used was 75 mM HEPES, pH 7.4, containing 50 μg/mL bovine serum albumin. The inset compares the kinetics of this reaction carried out by rmGARFT (○) with enzyme purified from mouse L1210 cells (●). The slopes calculated for the two lines in the inset were not significantly different. (B) Kinetics of inhibition of rmGARFT by DDATHF. The saturation of the rmGARFT reaction by 10-CHO-5,8-dideazafolate was followed as in (A) in the presence of 0 (□), 5 (■), 15 (○), and 30 (●) nM DDATHF. The inset plots the apparent K_i calculated at each of the indicated concentrations of DDATHF. For details, see text.

amplify the signal and measuring the rate of the reaction during the first 10−15 s as an estimate of the initial velocity and to minimize the effects of product accumulation (see below), the K_m was found to be 75 ± 14 nM. The raw data from a representative experiment are shown in Figure 4A to document this surprising but important result. The inset in Figure 4A compares data using enzyme purified from L1210 leukemia cells and with recombinant mouse enzyme purified from insect cells; a similar low K_m was found for either enzyme. In light of the identical behavior of endogenously expressed and recombinant mouse GARFT in these experiments, it was concluded that this low K_m was not an anomalous characteristic of recombinant enzyme, but rather that the K_m for 10-CHO-5,8-dideazafolic acid had been overestimated in previous studies (see Discussion).

The kinetics of inhibition of rmGARFT by DDATHF were studied using this enhanced spectrophotometric assay and using 5 μM α,β−GAR and 10-CHO-5,8-dideazafolic acid concentrations over the range of the measured K_m. rmGARFT was held below 0.75 nM in the (25 mL) cuvette to prevent the complexities of what has been called zone B kinetic behavior (*28*) when the K_i for an inhibitor is below enzyme concentration. Under these experimental conditions, DDATHF itself was found to be a competitive inhibitor of rmGARFT with a K_i of 5.6 ± 1.2 nM; replots of the slope of double reciprocal plots were linear (Figure 4B). Hence, previous studies appear to have underestimated the strength of this interaction, and the K_i measured under conditions of low substrate consumption (5.6 ± 1.2 nM) was consistent with the K_d measured by equilibrium dialysis (1.8 ± 0.3 nM).

Product Inhibition of the GARFT Reaction. There have been reports that the K_i s for inhibition of several folate-dependent enzymes (*15*, *16*), including GARFT (*14*, *29*), by an antifolate polyglutamate were much weaker when measured by enzyme kinetics using a polyglutamate substrate as a probe of active site occupancy than if the interactions were studied with a monoglutamate substrate. This suggests either an interesting deviation from theory, a more complex kinetic interaction than apparent from the data, or an experimental artifact. Because of the surprisingly low K_m found in our studies for 10-CHO-5,8-dideazafolic acid (Figure 4A), and for its hexaglutamate (see below), the products of the GARFT reaction with these two substrates were studied as inhibitors, using a spectrophotometric assay, 10 cm cuvettes, and near initial rate (10−15 s) conditions. The K_i for inhibition of the rmGARFT reaction by 5,8-dideazafolic acid was 94 nM, a value equivalent to the K_m for 10-CHO-5,8-dideazafolic acid (see above). The K_i for inhibition of rmGARFT by 5,8-dideazapteroyl hexaglutamate was 1.6 nM, a value lower than the K_m for 10-CHO-5,8-dideazafolic acid hexaglutamate (see below), and 4 times lower than the K_i measured for DDATHF. Hence, even low levels of accumulation of product from either of these substrates during steady-state kinetic experiments would severely complicate the analysis of data (see Discussion).

Interaction of rmGARFT with DDATHF Hexaglutamate. The interaction of rmGARFT with DDATHF hexaglutamate was directly studied by steady-state kinetics using both a monoglutamate and a polyglutamate substrate to probe the occupancy of the active site by the inhibitor. A Dixon analysis of the inhibition of rmGARFT by (6*R*)-DDATHF hexaglutamate, which was performed using 1 μM 10-CHO-5,8-dideazafolate, 5 μM α,β-GAR, an enzyme concentration of 0.75 nM, and initial velocity conditions in 10 cm cuvettes, allowed a K_i estimate of 0.11 nM. Reanalysis of these data sets with the Morrison equation (*21*) for tight-binding inhibition gave the same value for K_i.

In order to perform kinetic studies using 10-CHO-5,8-dideazafolate hexaglutamate as a substrate, high specific activity (110 Ci/mmol) [^{3}H]-10-CHO-5,8-dideazafolate hexaglutamate was made, and an HPLC method was used to assay for the enzymatic synthesis of product 5,8-dideazafolate hexaglutamate by rmGARFT. The purity of the labeled substrate required for this method is demonstrated in Figure 5 A, as is the enzymatic complete conversion of this labeled substrate to product. The chromatographic conditions used in this method allowed the complete separation of product from substrate even at very low levels of substrate conversion

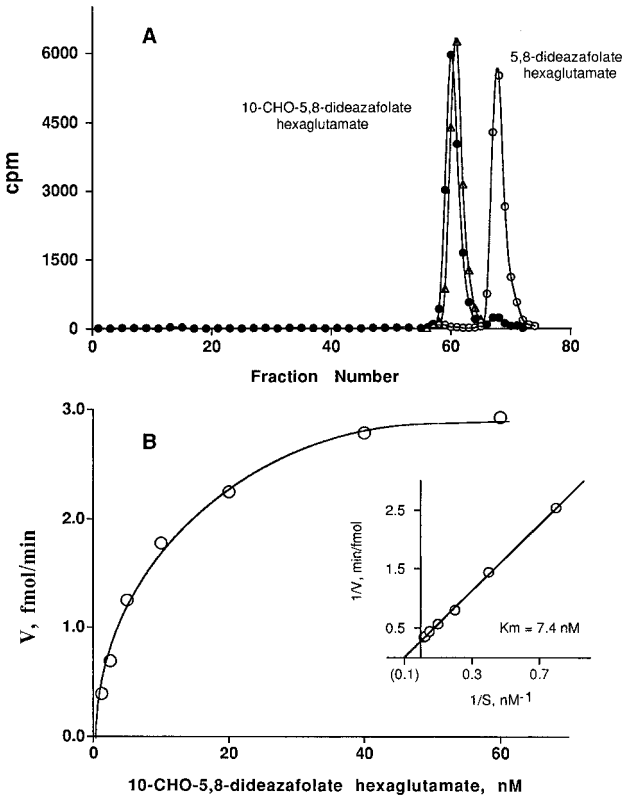


FIGURE 5: Kinetics of the reaction catalyzed by rmGARFT using 10-CHO-5,8-dideazafolate hexaglutamate as a substrate. (A) HPLC separation of the folate substrate and product of the GARFT reaction using 10-CHO-5,8-dideazafolate hexaglutamate as a substrate. [^{3}H]-10-CHO-5,8-Dideazafolate hexaglutamate was prepared enzymatically, and an aliquot was applied to a reverse phase HPLC column under paired ion conditions (△). Also shown is the same material after complete consumption during the rmGARFT reaction (○) or after a reaction that resulted in <2% consumption of substrate (●), typical of the reactions followed in the experiments described in (B). (B) 10-CHO-5,8-dideazafolate hexaglutamate saturates the rmGARFT reaction at very low concentrations. The reactions followed in these experiments used 2 pM enzyme and 5 μM α,β-GAR. The Michaelis constant can be estimated from the replot shown in the inset. For other conditions, see Figure 4 and the text.

(Figure 5A), a requirement for these experiments, because product inhibition was anticipated to be a potential problem at ≥5% consumption of substrate. The K_m measured for 10-CHO-5,8-dideazafolate hexaglutamate with this assay using 5 μM α,β-GAR was 7.4 nM (Figure 5B) ($n = 2$), about 10-fold lower than the K_m measured for the monoglutamate. The K_i values for DDATHF and its hexaglutamate were measured by Dixon analysis using a concentration of 10-CHO-5,8-dideazafolic acid hexaglutamate equivalent to 4 × K_m (3 nM) and 5 μM α,β-GAR. These values were estimated to be 8.6 ± 1.0 nM and 0.30 nM (two experiments; values = 0.285 and 0.305 nM) for the mono- and hexaglutamates of DDATHF, respectively, in agreement with the values for K_d obtained by equilibrium dialysis and also by steady-state kinetics using 10-CHO-5,8-dideazafolic acid as substrate (see above). Hence, the deviations from theory reported by others for the interaction of GARFT with DDATHF polyglutamates which resulted in reports of different K_is using monoglutamate and polyglutamate forms of substrates (*14*) were not observed when initial velocity conditions were maintained.

Binding of GAR to rmGARFT. Previous kinetic investigations on vertebrate GARFT (*26*), based on the use of dead-end inhibitors, have supported an ordered sequential pathway for the addition of substrates with folate binding preceding that of GAR. The K_m values found in the present studies would predict that the concentrations of the folate substrate would be saturating at all concentrations used in previous studies, a condition which could mask a random order binding mechanism, which would appear to be ordered sequential. Hence, we reexamined the binding of GAR to rmGARFT directly, using equilibrium dialysis. The binding of [^{3}H]-β-GAR to rmGARFT was studied in the presence of α,β-GAR and in the presence or absence of 2 μM DDATHF. Scatchard analysis of the results of these experiments indicated that β-GAR bound equally well in the absence ($K_d = 1.1$ μM) or presence of DDATHF ($K_d = 0.9$ μM) with an observed stoichiometry of 0.89 and 0.62 mol of GAR bound per mole of rmGARFT, respectively ($n = 2$). In a series of complementary experiments, equilibrium dialysis was used to study the binding of ^{3}H-DDATHF to enzyme in the presence of GAR; neither the K_d nor the stoichiometry of binding of DDATHF to GARFT was affected by the presence of α,β-GAR ($K_d = 1.61$ nM and intercept = 1.1 mol/mol in the presence of 12 μM α,β-GAR). If the binding of substrates to rmGARFT followed an ordered sequential pattern with folate binding first, then it would be expected that GAR would not form a stable complex in the absence of folate. In addition, the binding of DDATHF to enzyme should be enhanced in the presence of some concentration of GAR as the apparent rate of dissociation of DDATHF from a ternary complex decreased due to kinetic trapping in an ordered sequential binding mechanism. This did not prove to be the case; as increasing concentrations of GAR were added to equilibrium dialysis cells containing DDATHF and an equimolar amount of rmGARFT, binding did not increase with increasing GAR over the range of GAR concentrations equivalent to the K_d for the rmGARFT−GAR binary complex (Figure 6). However, binding of DDATHF to enzyme decreased at very high GAR concentrations. This decreased binding of DDATHF in the presence of α,β-GAR at concentrations ≥30 μM was reminiscent of the substrate inhibition of GARFT activity previously observed in kinetic experiments using enzyme purified from L1210 mouse leukemia cells and also using a recombinant monofunctional form of the human GARFT (*13*, *30*).

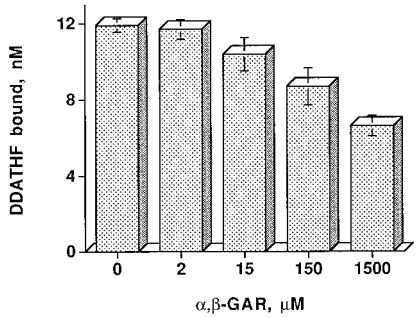
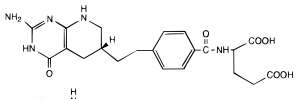
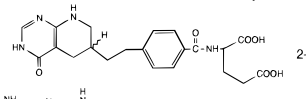
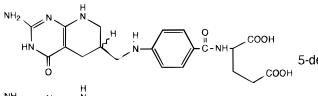
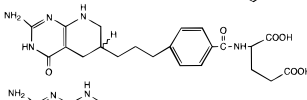
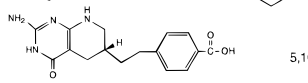
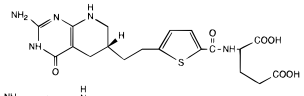
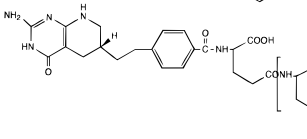
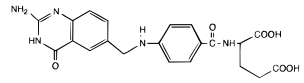
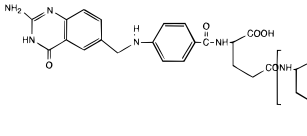


FIGURE 6: Inhibition of binding of ^{3}H-DDATHF to rmGARFT by α,β-GAR under equilibrium dialysis conditions. ^{3}H-DDATHF (4.8 pmol, 18 Ci/mmol) and rmGARFT (2.8 pmol) were added to 200 μL half-cells of a Spectro/Por equilibrium dialysis unit as in Figure 2, and each half-cell was sampled after rotating for 5 h at 4 °C after the addition of GAR to the indicated final concentrations in the 400 μL dialysis cell. Other conditions are described in Figure 2 and the text.

Structural Determinants of Inhibition of GARFT by DDATHF. In an earlier study (*13*), the inhibition of mouse

Table 2: Inhibition of rmGARFT by DDATHF Analogs

Compound	K_i, nM	Previous Value, nM (ref)
 DDATHF	5.6 ± 1.1	100 (*13,24*)
 2-Desamino-DDATHF	1200	27,000 (*13*)
 5-deazatetrahydrofolate	5.1	65 (*13*)
 Homo-DDATHF	1.6	19 (*13*)
 5,10-dideazapteroic acid	21	150 (*13*)
 LY309887	0.6	6.5 (*30*)
 DDATHF hexaglutamate	0.12	0.6 (*13*)
 5,8-dideazafolic acid	107	
 5,8-dideazafolic acid hexaglutamate	1.6	

L1210 GARFT by several analogs of DDATHF was used as an indicator of which features of the structure of DDATHF were important for inhibition of this enzyme. Because the previously reported experiments (*13*) were performed at concentrations of 10-CHO-5,8-dideazafolate corresponding to a range from 14 to 850 × K_m, the results of those studies were reassessed at much lower concentrations of folate substrate using a spectroscopic assay and initial rate conditions in 10 cm cuvettes. The K_i values derived from these studies (Table 2) confirm the earlier deductions concerning the structural requirements for inhibition of GARFT by DDATHF analogs, but the values of K_i are uniformly lower than in previous studies by a factor of about 12-fold, consistent with the difference in K_m values reported herein and in the previous structure−activity studies (*13*). Previous studies have led to the conclusion that it was the isosteric replacement of a methylene group for the nitrogen at N-5, rather than that at N-10, that explained the inhibition of GARFT by DDATHF. This conclusion is again supported by the observation that 5-deazatetrahydrofolate is as good an inhibitor of rmGARFT as DDATHF (Table 2). Previous kinetic (*13*) and structural studies (*31*) had drawn the conclusions that the 2-amino group of DDATHF contributed an important hydrogen bond to the interaction of drug with enzyme surface, that the glutamic acid side chain minimally interacted with the enzyme surface, and that there was flexibility in the bound conformation so that changes in the bridge region of the DDATHF molecule were compatible with efficient binding to GARFT. In agreement with these conclusions, the binding of 2-desamino-DDATHF to rmGARFT was >200-fold weaker than that of DDATHF, but the binding of 5,10-dideazapteroic acid was only 4-fold lower and that of homo-DDATHF was somewhat stronger than that of DDATHF. Since the original publication describing the structural requirements for inhibition of GARFT by DDATHF analogs (*13*), a second-generation GARFT inhibitor, LY309887, has been advanced to clinical trial which was reported to bind to GARFT at a lower concentration than did DDATHF (*30*). This compound differs from DDATHF only by the replacement of the phenyl ring by a thiophene group (Table 2). When the inhibition of rmGARFT by this compound was studied, it was found to bind much tighter than DDATHF, with a K_i of 0.6 nM, 10-fold lower than previous (*30*) reports.

DISCUSSION

The availability of large amounts of very pure mouse recombinant GARFT allowed us to evaluate several puzzling aspects of the mechanism of this enzyme and of its interaction with DDATHF and its analogs. Thus, DDATHF had appeared to be a only a moderate enzyme inhibitor when studied *in vitro* [K_i = 100−300 nM; (*13, 24*)], yet it was a very potent inhibitor of purine synthesis *in vivo* with half-maximal inhibition of purine synthesis at 5−30 nM in intact mammalian tumor cells (*4, 5*). Likewise, there had been reports that the K_i values measured for DDATHF polyglutamates as inhibitors of GARFT were much higher when the competing substrate was a polyglutamate than when a monoglutamate substrate was used (*14*). If so, it implied that the reported K_i values were not synonymous with true dissociation constants and that the K_i values previously reported from this and other laboratories (*5, 13, 14, 24*) were flawed due to some unrecognized factor in the kinetics of this enzyme. In an attempt to sidestep any such effects, the binding of DDATHF and its polyglutamates to rmGARFT was directly studied in the absence of substrates. These experiments indicated that the previously reported K_is were not equivalent to K_ds, a result which prompted us to reevaluate the kinetics of this interaction. The results of our reappraisal of the kinetic properties of GARFT indicated that the problem lay in two interacting factors: (1) The K_m values of the folate substrates were much lower than previously recognized. As a result, preceding studies had been performed under saturating conditions with respect to this substrate. Using standard spectrophotometric conditions with a 1 cm path-length, the initial velocity of the GARFT reaction would be difficult to measure at concentrations of folate substrate in the range of 1 μM (15 × K_m). A similar situation was true for the dihydrofolate reductase reaction, for which the K_m of dihydrofolate was found to be too low to be easily estimated using spectrophotometric assays (*32*). (2) The folate products, either monoglutamate or hexaglutamate, were very potent inhibitors of the reaction, so that inhibitory concentrations of product accumulated at very low levels of substrate consumption. Thus, both substrate and product in substantial excess over their respective K_ms and K_is were competing with DDATHF for binding to GARFT in previous studies.

The results of this study suggest that either substrate can bind to GARFT equally well in the presence or absence of saturating levels of the other substrate, implying that the folate substrate analogs and GAR bind to the active site in a random sequential order. This is directly supported by equilibrium binding experiments with DDATHF or GAR

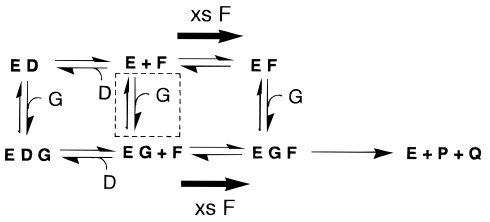


FIGURE 7: Order of binding of substrates and inhibitors to rmGARFT. DDATHF was found to bind to rmGARFT equally well in the presence or absence of saturating concentrations of GAR, and GAR was found to bind equally well to enzyme in the presence or absence of 2 μM (>1000 K_ds) DDATHF, leading to the conclusion that rmGARFT binds these ligands in a random order. The previous experiments (*26*), which suggested an ordered sequential binding mechanism, were performed at high concentrations of 10-CHO-5,8-dideazafolate, relative to the K_m reported in this paper, so that binding reactions involving folate substrate would become essentially irreversible (heavy arrows) and the contribution of the competing reactions shown in the dotted box would be negligible. Under such conditons, an enzyme operating by a random addition pathway will behave like an ordered sequential system. In this diagram, E represents enzyme (rmGARFT), F is folate substrate, G is GAR, D is DDATHF (or any other folate inhibitor), and P and Q represent the reaction products.

performed in the presence or absence of the second ligand, which demonstrate that each binary complex exists and which do not support the formation of a dead-end GARFT−DDATHF−GAR complex. The crystal structure of the *E. coli* GARFT supports free access of GAR to the active site in the presence or absence of bound folate; the active site binding positions for the two substrates are separate and lie in a shallow groove on the enzyme (*31, 33*). If one superimposes the crystal structures for the ternary complex of *E. coli* GARFT, GAR, and 5-deazatetrahydrofolate with that of the apo enzyme (*31*), the shape of the GAR binding pocket does not change, suggesting that the binding of folates does not alter the binding site for GAR. It is noted that the previously reported steady-state kinetic patterns suggesting an ordered sequential binding order (*26*) would have been performed under uniformly saturating folate conditions, under which condition any binding step which competed with binding of folate would have a negligible impact on the applicable kinetic equations (Figure 7). We have not been able to pursue steady-state experiments to examine the order of binding by a parallel approach due to the low signal at K_m levels of substrate even with 10 cm cuvettes and the level of product accumulation at higher substrate concentrations in the 25 mL volume of these cuvettes.

Substrate inhibition has been observed at higher concentrations of α,β-GAR with mouse (*13*) and with human GARFT (*30, 34*) but not with chicken GARFT (*35*). An ordered sequential binding mechanism often results in substrate inhibition by the second substrate to bind to enzyme, due to the formation of a dead-end complex (*36*). However, the effect of excess α,β-GAR on rmGARFT was clearly of a partial substrate inhibition pattern, which is inconsistent with formation of a dead-end complex; in our hands, even at an α,β-GAR concentration of 750 μM, only 50% inhibition was observed (data not shown). The results of Figure 6 would agree with this reasoning, given that they exclude the formation of a dead-end complex but support the concept of competition for binding to enzyme between excess GAR and the folate ligand; again, only a partial binding inhibition pattern was seen. Partial substrate inhibition might well be due to a "preferred pathway" (*37*) binding mechanism, but the effect might also be due to the presence of α-GAR in the chemically synthesized α,β-GAR, which would increase proportionally with β-GAR in inhibition experiments.

A major conclusion from this study is that DDATHF is itself a tight binding inhibitor of GARFT, with a K_i approximately 3 orders of magnitude lower than the estimated concentration of GARFT in mammalian cells which express high levels of this enzyme, such as the L1210 mouse leukemia. Hence, inhibition of *de novo* purine synthesis would only require the transport of sufficient drug to titrate available enzyme, and then a small excess to keep the GARFT inhibited. During this time course, drug would also be metabolized to long-chain polyglutamates, which would bind even tighter to the target GARFT (Figure 1B) and would efflux poorly from mammalian cells (*38*). Recently, a second-generation GARFT inhibitor has gone to clinical trial, LY309887 (*39*). This compound is a more powerful GARFT inhibitor than DDATHF [(*30*) and Table 2] and is, in fact, equivalent in inhibitory potency to the hexaglutamate of DDATHF. LY309887 is a substrate for polyglutamation and would probably have pharmacologic characteristics very similar to DDATHF, given that both are tight-binding inhibitors of GARFT. However, the ability to develop resistance to DDATHF via a decrease in polyglutamation would probably be significantly less for LY309887.

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