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Relative Substrate Activities of Structurally Related Pteridine, Quinazoline, and Pyrimidine Analogs for Mouse Liver Folylpolyglutamate Synthetase

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SUMMARY

Several structurally related series of folate analogs were studied as substrates for mouse liver folylpolyglutamate synthetase (FPGS). A comparison of the kinetics of the interaction of this enzyme with folate analogs that contained the quinazoline ring in place of the pteridine ring with those of the analogous pteridines demonstrated that the quinazoline derivatives were more efficient substrates for and tighter binding inhibitors of this enzyme. A series of 2,4-diaminopyrimidine dihydrofolate reductase inhibitors were found to be substrates for FPGS; these are the first known compounds without a fused ring system analogous to the pteridine ring of the folate molecule that are substrates for FPGS. Several 5,8-dideazafolate derivatives that lack the 2-amino group had activity as substrates for FPGS equivalent to that of the corresponding 5,8-dideazafolates. When a homologous series of 5,8-dideazafolic acid analogs with hydrocarbon substituents on the 10-nitrogen were studied, these substituents were found to diminish the efficiency of utilization of these analogs as substrates for FPGS; this effect increased with increasing chain length of the hydrocarbon. It was concluded that neither the 2-amino group nor an intact pyrazine ring of folates and folate analogs are essential for the binding of folates to the active site of mouse liver FPGS but that the pyrazine ring probably serves to position other regions of the folate molecule that interact with amino acid residues in the active site. It was also inferred from these observations that the volume within the active site of FPGS above/below the pyrazine ring or near the 10-position of folate derivatives are regions of limited bulk tolerance; binding of folate analogs with substituents at these positions probably distorts the active site.

Mammalian FPGS catalyzes the formation of an amide bond between the α-amino group of L-glutamic acid and the γ-carboxylic acid of the side chain of the naturally occurring folate compounds (1–3) and, also, that of a number of folate antimetabolites (1, 3–6). In recent years, it has become clear that FPGS plays an important role in the cytotoxicity of MTX and of other folate antimetabolites (7–9) and that this enzyme per se represent an attractive target enzyme for cancer chemotherapy (10, 11). The intracellular metabolism of folate monoglutamates to polyglutamates ensures the retention of folate cofactors in cells, a function now known to be essential for the continued survival of proliferating mammalian cell populations (12). Likewise, polyglutamation converts folate antimetabolites such as MTX, which are readily transported into and out of mammalian cells by a specific carrier-mediated process, into derivatives that efflux from cells much more slowly than do the parent drugs (13). For those cases that have been studied to date (9, 14, 15), antifolate polyglutamates bind to their target enzymes more tightly than do the parent compounds. In addition, polyglutamation of MTX enhances binding to folate-dependent enzymes other than dihydrofolate reductase to such a degree that some investigators (16, 17) have concluded that MTX polyglutamates inhibit several folate-dependent enzymes whereas MTX itself inhibits only dihydrofolate reductase. The importance of FPGS to cancer chemotherapy is obvious, in view of the evidence that inhibition of the function of FPGS would be lethal to proliferating (tumor) cell populations and that polyglutamation of folate antimetabolites increases their intracellular retention and activity as enzyme inhibitors.

The active site of mammalian FPGS binds a wide variety of compounds with structures similar to those of the naturally occurring folates (1, 3–6). Reduced and unreduced pteridines, some quinazolines, and pyridopyrimidines with various substituents at different positions have been shown to be either substrates or inhibitors of this enzyme (1–6). In previous studies, we have attempted to isolate the contribution of each

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ABBREVIATIONS: FPGS, folylpolyglutamate synthetase; MTX, methotrexate, PteGlu, folic acid; H_4PteGlu, tetrahydrofolic acid.

position of the folate molecule to the binding of folates to the active site of FPGS in a configuration compatible with catalysis. We have previously concluded (5) that mouse liver FPGS preferred a folate substrate for which (a) there was a four-amino substituent, (b) the pyrazine ring was reduced, and (c) the 10-nitrogen was either unsubstituted or had a formyl substituent. In this paper, we compare the kinetics of the FPGS reaction for several series of folate analogs that differ in the fused ring system or in the substituents at the N-10; C-2; and C-4-positions and we use these data to refine our previous conclusions. We analyze the activity of some of the best substrates of FPGS yet reported and attempt to dissect why these compounds are so efficiently utilized by this enzyme.

Materials and Methods

PteGlu, MTX, and aminopterin were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without purification. *N*-[4-(4-(2,4-Diamino-6-alkyl-5-pyrimidinyl)-1-piperazinyl)benzoyl]-L-glutamic acid derivatives were synthesized at Warner-Lambert/Park Davis and have been previously reported (18). Among the analogs studied in this work that were made at the Institute of Cancer Research at Sutton, Surrey, the 2-amino-4-hydroxyquinazoline analogs of folic acid substituted at N-10 have been previously described (19), as have the N-10-substituted 2-desamino-5,8-dideazafolic acid derivatives (20); these compounds have been kindly supplied by the Institute for Cancer Research. 2,4-Diaminopteroyl-L-aspartic acid and its 2,4-diaminoquinazolinyl counterpart were obtained from the National Cancer Institute and were purified on a column of DEAE-cellulose that was eluted with a convex gradient of ammonium acetate.

5,6,7,8-Tetrahydromethotrexate was prepared from MTX by reduction with dimethylamine-borane complex, as described by Martinelli and Chaykovsky (21). The product gave a single peak on reverse phase paired-ion high pressure liquid chromatography (approximately 95% purity) and have a UV spectrum (single peak at 294 nm at pH 7) identical to that of 5,6,7,8-tetrahydromethotrexate (21). It was stored as a lyophilized dry powder in sealed ampoules, at −25°, protected from light. 5,6,7,8-Tetrahydroaminopterin was also prepared by reduction of aminopterin in glacial acetic acid (21). After anaerobic precipitation with ether containing 1% β-mercaptoethanol, the product was dissolved in 1% aqueous β-mercaptoethanol and purified on a 0.9 × 50 cm column of DEAE-cellulose that was eluted with a convex gradient of ammonium acetate. The product was lyophilized and stored under dry N_2 in ampoules. It had the UV spectrum (single peak, λ_{max} = 290 nm at pH 7) previously reported for 5,6,7,8-tetrahydroaminopterin (21).

Preparation of mouse liver FPGS. B6D2F$_1$ or Swiss-Webster female mice were obtained from Simonsen Laboratories (Gilroy, CA). Livers were removed and homogenized, the homogenate was centrifuged at 165,000 × *g* for 1 hr, and a 30% $(NH_4)_2SO_4$ fraction was prepared as previously described (22). This $(NH_4)_2SO_4$ fractionation purified FPGS activity 8–15-fold and these preparations had a typical specific activity of 1.2 nmol of product/hr/mg of protein when assayed (22) with 500 μM PteGlu at pH 8.6 in Tris buffer. We have previously shown that 90% of the conjugase activity of the high speed supernatant fraction was removed by this salt fractionation and that conjugase activity was undetectable when assayed under the conditions of the FPGS assays (2).

Enzyme assays. Compounds were incubated with FPGS for 60 min at 37° in a mixture containing, in addition to a folate or folate analog (at 0–1000 μM), 1 mM [^{3}H] glutamic acid (4 mCi/mmol), 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 20 mM α-thioglycerol, and 200 mM Tris, pH 8.6, in a total volume of 0.25 ml. The tritium-labeled diglutamyl product was isolated by adsorption onto charcoal, as previously described (22). The characteristics of this assay have been described in detail elsewhere (2, 5, 22). In the experiments reported here, there were 2.8–3.8 cpm/pmol of glutamate incorporated into product. Saturation curves used in these experiments were restricted to substrate concentrations ≤1000 μM to avoid saturation of the charcoal (22). No more than 15% of substrate was consumed at the lowest substrate concentration in these experiments. Under these conditions, reaction rates approximated initial velocities and the reaction being measured was restricted to the formation of a folyldiglutamate. This experimental design limited the amount of product formed to levels compatible with the assumption of constant substrate concentration even for excellent substrates. For some such substrates, the specific activity of the glutamate used was increased 4-fold, less enzyme was used, and reaction time was limited to 30 min in order to meet these criteria. For analysis of the kinetics of product formation, FPGS was incubated with substrate at at least five concentrations ranging from 0.2 to 3.0 K_m; duplicate enzyme assays were performed for each condition in each experiment and all experiments were performed at least twice on different days. Data were analyzed by weighted nonlinear fit to a rectangular hyperbola using a standard statistical program for enzyme data (23). Values for K_i of competitive inhibitors were determined from a replot of the slopes of double-reciprocal lines calculated by this program. For substrates that showed significant substrate inhibition, kinetic constants were derived by extrapolation from data points that were linear on a double-reciprocal plot. In all experiments, a saturation curve for PteGlu was performed as an internal standard in each experiment to allow the standardization of V_{max} and V_{max}/K_mvalues among experiments.

Results

Comparison of the interaction of analogs with pteridine and quinazoline ring systems with FPGS. Mouse liver FPGS utilized 5,8-dideazafolic acid as a substrate more efficiently than PteGlu, with substantially lower concentrations of the former compound saturating the enzyme (Fig. 1). A somewhat higher V_{max} value was also observed for 5,8-dideazafolate, although this factor was obscured by a distinct substrate inhibition (Fig. 1; Table 1). The relative efficiency of utilization of these two compounds, as measured by the apparent first-order rate constants (V_{max}/K_m) (Table 1), indicated that the enzyme reaction using the derivative containing the quinazoline ring system was favored over that containing the pteridine ring by a factor of about 30. It is interesting to note that the kinetics of this reaction with 5,8-dideazafolic acid were identical to those using (6*S*)-H_4PteGlu as a substrate (Table 1), except that the substrate inhibition seen with the former (Fig. 1A) was not observed with (6*S*)-H_4PteGlu (not shown). A comparison of the substrate activity of 10-methyl-5,8-dideazafolate with that of 10-methylfolate leads to a similar conclusion. Thus, the V_{max}/K_m ratio for 10-methyl-5,8-dideazafolate was 9 times higher than that of PteGlu (Fig. 1; Table 1) when measured in the same experiment, whereas as we have previously reported (5) that 10-methylfolate was a poor substrate relative to PteGlu (ratio of first-order rate constants = 0.048) for mouse liver FPGS. From a series of experiments in which PteGlu was used as an internal standard, we conclude that V_{max}/K_m for 10-methyl-5,8-dideazafolate was 180 times greater than for 10-methylfolic acid.

These kinetic comparisons suggest that apparent K_m values for otherwise equivalent pteridine and quinazoline ring-containing folate analogs would favor reaction of the quinazoline at substantially lower concentration but do not necessarily imply tighter binding of the quinazoline to the enzyme. This question was approached by a quantitative comparison of the inhibition of FPGS by 2,4-diaminopteroyl-L-aspartate and by 2,4-diamino-5,8-dideazapteroyl-L-aspartate. These two folate

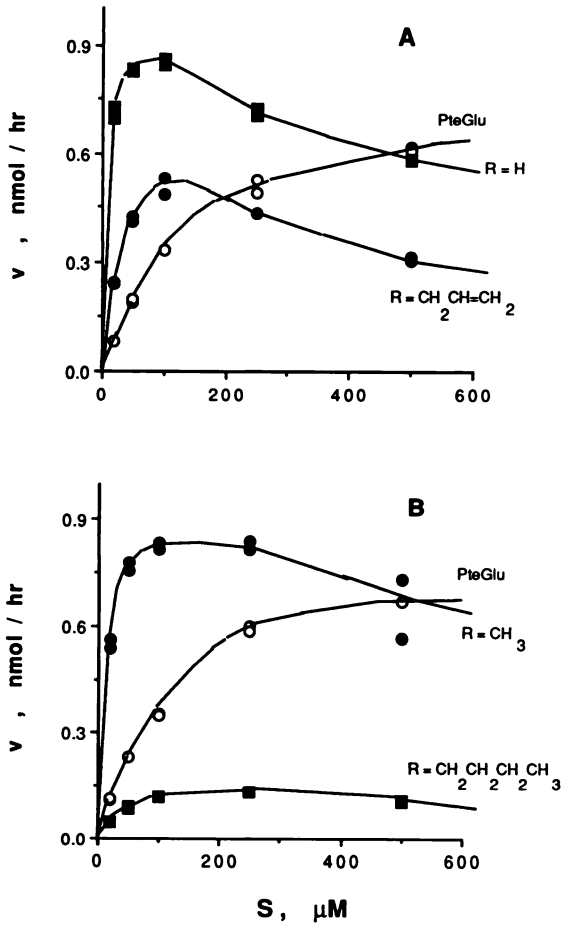


Fig. 1. Relative activities of N^{10}-substituted analogs of 5,8-dideazafolic acid as substrates for mouse liver FPGS. The indicated concentrations of 5,8-dideazafolic acid (A, ■) or its 10-methyl (B, ●), 10-allyl (A, ●), or 10-butyl (B ■) derivative were incubated with enzyme for 60 min and product was isolated and quantitated as described in the text. In each experiment, PteGlu (○) was used as an internal control. Each *point* represents a single incubation, although single points indicate superimposed data.

analogs were previously found to be devoid of substrate activity with FPGS, as would be expected for folate analogs with aspartate as a side chain (5). As shown in Fig. 2, they were both strict competitive inhibitors of mouse liver FPGS with respect to the folyl substrate, with K_{is} values of 133 ± 15 μM (two experiments) and 24.2 ± 2.2 μM (two experiments), respectively. Under such circumstances, the K_{is} values represent dissociation constants and, hence, reflect binding energies. The data of Fig. 2 indicate about 6-fold tighter binding of the quinazoline ring-containing analog to mouse liver FPGS than of the compound containing the pteridine ring.

Analogs of 5,8-dideazafolate with hydrocarbon substituents at N-10 had lower V_{max}/K_m and higher K_m values than did the unsubstituted compound, as can be seen from the lower initial slopes of v versus S plots for these compounds (Fig. 1). However, any of the methyl, ethyl, allyl, or propargyl groups at N-10 had similar effects on V_{max}/K_m, with apparent K_m values differing only slightly among these compounds (Table 2). Alkylation at N-10 with the bulkier butyl group appeared to affect V_{max} (Table 2). Substrate inhibition was observed with all of these compounds (Fig. 1); this effect was particularly obvious from Lineweaver-Burk plots (data not shown). For the determination of K_m values under these conditions, data were obtained for the range of concentrations for which substrate inhibition was negligible.

Substrate activity of 2,4-diamino-5,6,7,8-tetrahydrofolyl compounds. We previously reported (5) that substitution of an amino function for the 4-oxo group of folates resulted in a major decrease in the apparent K_m value of several compounds as substrates for mouse liver FPGS and that reduction of PteGlu to H_4PteGlu has a similar effect. It was unclear whether such effects would be additive if both structural modifications were incorporated into a single molecule. To test this question, tetrahydromethotrexate and tetrahydroaminopterin were prepared and the substrate activities of these compounds were compared with those of MTX, aminopterin, and H_4PteGlu. Both tetrahydromethotrexate and tetrahydroaminopterin were excellent substrates for FPGS, with K_m values[1] of 25 ± 13 and 5.4 ± 0.32 μM, respectively, and apparent first-order rate constants (relative to Pte Glu) of 16 ± 4.5 and 45 ± 4, respectively. As such, tetrahydroaminopterin represents one of the best substrates for FPGS reported to date. Comparison of the data for tetrahydromethotrexate with those for methotrexate and for tetrahydroaminopterin with aminopterin (Table 1) indicates that reduction of these 4-aminofolates enhances utilization by FPGS by a substantial factor. This would lead us to conclude that, at least in these cases, the presence of two structural modifications, each of which enhanced substrate activity, in a single molecule led to better substrate activity than was introduced by either modification alone.

Role of the pyrazine ring of the folate molecule in binding of folates to FPGS. A unique class of folate analogs has recently be reported by others, in which the distances between a benzoylglutamate moiety and a pyrimidine ring are maintained fixed by a piperazine ring rather than by a ring fused to the pyrimidine ring (as is the case for folates). The structures of these *N*-[4-(4-(2,4-diamino-6-alkyl-5-pyrimidinyl)-1-piperazinyl)benzoyl]-L-glutamic acid derivatives are shown in Table 3. These novel analogs were all substrates for mouse liver FPGS (Fig. 3; Table 3) and represent the first compounds demonstrated to be accepted as substrates for this enzyme that do not have the pteridine ring structure or a related fused heterocyclic ring, such as quinazoline or a pyridopyrimidine. Substrate saturation experiments indicated that the reaction velocity was a rectangular hyperbolic function of substrate concentration for most of these compounds (Fig. 3). However, the 6-unsubstituted parent compound of this series displayed substrate inhibition at high concentrations (>250 μM), an effect not observed at achievable concentrations of the

[1] Our previous experiments on 10-formyltetrahydrofolate used a mixture of diastereomers about the 6-position (2). Because both diastereomers of tetrahydrofolate (1, 24), of 5-formyltetrahydrofolate (24), and of 5,10-dideazatetrahydrofolate (R. G. Moran, S. Baldwin, E. C. Taylor, and C. Shih. The 6-*S* and 6-*R* diastereomers of 5,10-dideaza-5,6,7,8-tetrahydrofolate are equiactive inhibitors of *de novo* purine synthesis. *J. Biol. Chem.* in press.) have been shown to be substrates for mammalian FPGS, any folate analog with a chemically reduced 5,6,7,8-tetrahydro structure may prove to be a mixture of two compounds, both of which are active as substrates for FPGS. The K_m and V_{max}/K_m values reported in this paper for any such derivatives assume that all compound (i.e., both diastereomers about the 6-position) is accepted and equivalent as substrates for FPGS. This assumption is needed to calculate these values but the resultant K_m values could be as much as a factor of 2 too high if only one isomer is a substrate. The K_m values were calculated assuming that both 6*R*- and 6*S*-diastereomers were active as substrates and were equivalent in this respect. If only one diastereomer were active, then tetrahydroaminopterin and tetrahydromethotrexate would have K_m values of 2.7 and 12.4 μM, respectively, and V_{max}/K_m ratios 90 and 32 times higher than that of PteGlu.

TABLE 1

Superior kinetic properties of 5,8-dideazafolates and 2-aminotetrahydrofolates as substrates for mouse liver FPGS

Compound	No. of experiments	K_m	V_{max}[a]	V_{max}/K_m[a]
		μM		
PteGlu[b]	49	140 ± 47	1.0	1.0
Aminopterin (4-amino-folic acid)	13	21 ± 4.8	1.59 ± 0.01	10.1 ± 4.2
Methotrexate (4-amino-10-methyl-folic acid)[b]	12	166 ± 49	0.99 ± 0.16	0.90 ± 0.05
10-Methyl-folic acid[b]	2			0.048 ± 0.001
(6S)-Tetrahydrofolate[c]	2	7.0 ± 1.4	1.31 ± 0.07	30 ± 7
5,8-Dideazafolic acid[d]	2	6.4 ± 1.8	1.27 ± 0.20	29 ± 4.7
10-Methyl-5,8-dideazafolic acid[d]	3	17.1 ± 4.2	1.19 ± 0.14	8.7 ± 2.2
(6RS)-Tetrahydroaminopterin	2	5.4 ± 0.32	1.11 ± 0.04	45 ± 4
(6RS)-Tetrahydromethotrexate	2	25 ± 13	1.20 ± 0.11	15.8 ± 4.6

[a] Relative to that measured for PteGlu in the same experiment.
[b] Data taken from Ref. 5.
[c] Data taken from Ref. 2.
[d] Kinetic constant values for compounds that displayed substrate inhibition were derived by using data points at low enough concentrations that Lineweaver-Burk plots were linear.

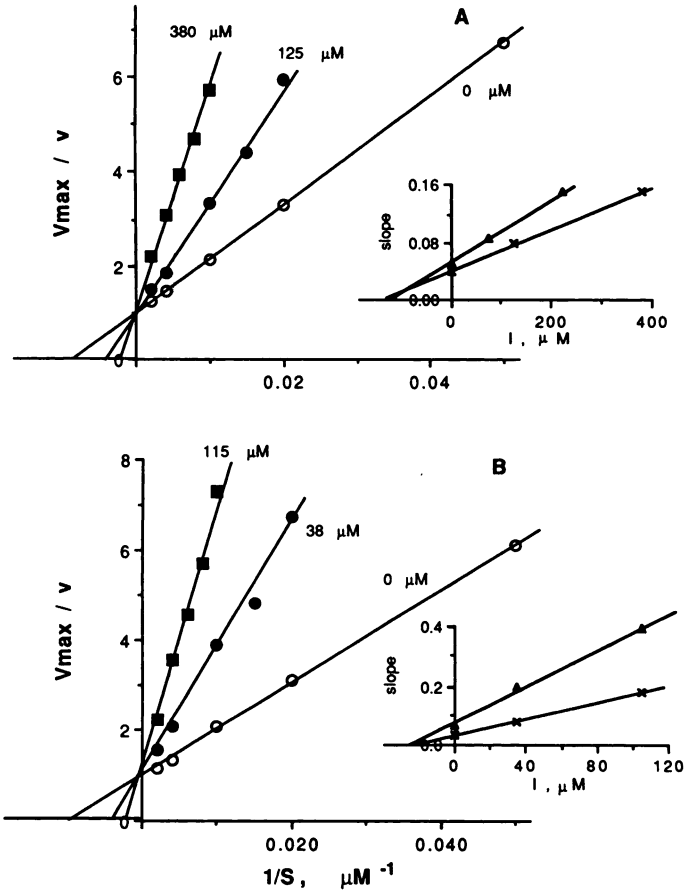


Fig. 2. Kinetics of inhibition of mouse liver FPGS by 2,4-diaminopteroyl-L-aspartate (A) and by 2,4-diamino-5,8-dideazapteroyl-L-aspartate (B). Enzyme was incubated with concentrations of PteGlu on the range of 20 to 500 μM and the indicated concentrations (*numerals over lines*) for 1 hr and product was measured as described in the text. *Insets*, replots of the slopes of the lines such as those in the main figures; each *symbol* represents a separate experiment.

other members of this series (data not shown). The 6-unsubstituted derivative of this series was an exceptionally efficient substrate for mouse liver FPGS, with an apparent K_m value intermediate between those of aminopterin and of tetrahydroaminopterin (Tables 1 and 3). The presence of a methyl group at position 6 of the pyrimidine ring of this structure resulted in a major increase (60-fold) in the apparent K_m for FPGS with no detectable change in V_{max} (Table 3; Fig. 3). Substitution at position 6 with bulkier aliphatic groups further decreased the apparent K_m and also decreased V_{max}. However, these decrements were minor when compared with the substantial change in substrate efficiency observed upon introduction of the methyl group at this position (Table 3; Fig. 3). These results seem to indicate that steric interaction between the hydrogens of the 6-methyl group and those of the methylene groups in the piperazine ring restrict the rotation of the piperazine relative to the pyrimidine and this is unfavorable to binding of the analogs to enzyme. It was of interest that a 2-amino-4-oxopyrimidine analogous to this series was utilized as a substrate for FPGS equally well as the corresponding 2,4-diamino derivative (Table 3).

2-Amino group does not affect FPGS activity. A series of 2-desamino-5,8-dideazafolate compounds have recently been reported (20) that allowed us to test the involvement of the 2-amino group of the fused ring system on the interaction of folate and folate analogs with FPGS. As shown by the data of Table 4, replacement of the 2-amino group by hydrogen in *N*-10-methyl-, ethyl-, allyl-, or propargyl-5,8-dideazafolic acid did not have a major effect on the ability of these compounds to serve as substrates for mouse liver FPGS.

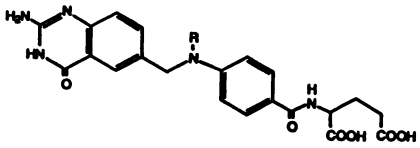
Discussion

The active site of an enzyme can be probed by the systematic comparison of the activity of inhibitors that are structurally related (25, 26). This approach has been adapted for the study of the active site of FPGS by utilizing the fact that this enzyme accepts a variety of folates and folate analogs as substrates. Using a series of comparisons of the substrate activities of pairs of compounds that differ in structure at single positions, we (5, 27) and others (3, 4) have attempted to deduce the type of interaction between various positions in the folate molecule and the adjacent residues on this protein.

The three kinetic parameters that were measured in this study give different information about the interaction of folate analogs with FPGS. The V_{max} should be proportional to k_{cat} and, hence, measures the rate of enzyme reaction once saturation of the active site has occurred. It is remarkable that this parameter changes so little among folate analogs with often major structural changes in the pteridine, bridge region, and

TABLE 2

Effect of N-10 Substitution of quinazoline analogs on substrate activity for mouse liver FPGS

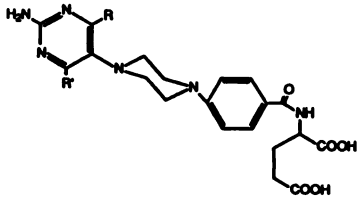


—R	CB No.	No. of experiments	K_{m-pg}	V_{max}[a]	V_{max}/K_m[a]
			μM		
—H	3705	2	6.4 ± 1.8	1.27 ± 0.20	29 ± 4.7
—CH_3	3713	3	17.1 ± 4.2	1.19 ± 0.14	8.7 ± 2.2
—CH_2CH_3	3714	2	31 ± 5.9	1.15 ± 0.06	5.3 ± 0.7
—$CH_2CH{=}CH_2$	3716	2	29 ± 2.5	0.79 ± 0.00	4.3 ± 0.5
—$CH_2C{\equiv}CH$	3717	3	40 ± 2.0	0.88 ± 0.01	2.26 ± 0.04
—$CH_2CH_2CH_2CH_3$	3719	2	40 ± 0.8	0.175 ± 0.009	0.63 ± 0.04

[a] Relative to PteGlu in the same experiment. In these experiments, the K_m for PteGlu was 145 ± 7.3 μM (five experiments).

TABLE 3

Activity of several 2,4-diaminopyrimidine dihydrofolate reductase inhibitors as substrates for mouse liver FPGS



R	R′	K_m	V_{max}[a]	V_{max}/K_m[a]
		μM		
—H[b]	—NH_2	16.7 ± 2.8	1.51 ± 0.11	13.0 ± 2.9
—CH_3	—NH_2	950 ± 280	1.47 ± 0.29	0.21 ± 0.014
—CH_2CH_3	—NH_2	1390 ± 230	0.89 ± 0.07	0.095 ± 0.022
—$CH_2CH_2CH_3$	—NH_2	2070 ± 1240	0.81 ± 0.25	0.053 ± 0.014
—CH_2CH_3	—OH	2280 ± 650	0.94 ± 0.31	0.063 ± 0.001

[a] Relative to PteGlu in the same experiment. The K_m for PteGlu in the five experiments reported in this table was 134 ± 13.2 μM. For all of these values, two experiments were performed.

[b] Substantial substrate inhibition was seen with this compound. Kinetic constants were derived using only data points for which substrate inhibition was negligible (see Materials and Methods).

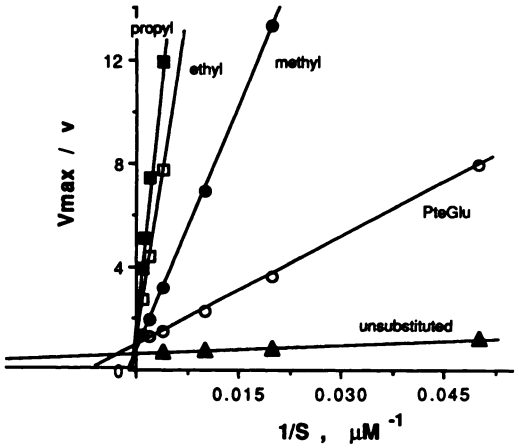


Fig. 3. Relative substrate activities of a series of N-[4-(4-(2,4-diamino-6-alkyl-5-pyrimidinyl)-1-piperazinyl)benzoyl]-L-glutamic acid derivatives as substrates for mouse liver FPGS. The substituent at position 6 of these molecules, whose structures are shown in Table 3, is indicated above each line. The rate of reaction was measured during a 1-hr incubation with increasing concentrations of each compound . Duplicate rate determinations at each concentration in each of two experiments were combined for this figure by averaging the velocities at each data point, expressed relative to the V_{max} of folic acid, which was determined in each experiment.

phenyl ring region of the folate molecule (Tables 1–4 and Refs. 2, 5, and 27). This seems to indicate that the enzyme binding site for the pteridine ring of folates has little communication with the residues involved in catalysis unless the binding site is perturbed by very bulky substituents, e.g., a butyl group on N-10 (Table 3). Many modifications in the structure of the side chain of folate analogs result in analogs that not only are not substrates but also are poor inhibitors and, hence, simply do not bind to enzyme (3, 5, 28). This leads to the conclusion that both the pterin ring and the glutamic acid side chain of folates are involved in the binding of folates to the active site residues of mammalian FPGS. Related results on pure hog liver FPGS have lead George *et al.* (3) to reason that the initial binding of pterin and side chain to FPGS is followed by a conformational change that results in a tighter mode of binding of the folate that is, presumably, necessary for further steps leading to catalysis. The first-order rate constant, V_{max}/K_m, is often useful as an index of the comparative efficiency of utilization of alternate substrates for an enzyme because it reflects reaction rates at low substrate concentrations. George *et al.* (3) have interpreted the first-order rate constant as a measure of the rate constant for the association of the FPGS-ATP complex with the folate substrate. The K_m values reported here, of course, are kinetic constants and not dissociation constants. It is interesting to speculate on the degree to which K_m values determined for mammalian FPGS deviate from dissociation constants. The catalytic steps for the mammalian FPGS reation are slow, with turnover numbers for pure hog liver (29) and calf thymus[2] enzymes found to be on the range of 1–4 catalytic events/active site/sec. This may well be slow enough, relative to the rates of association and dissociation of folate substrates, to allow the assumption of pseudoequilibrium, a condition under which substrate K_m valves become identical to dissociation constants.

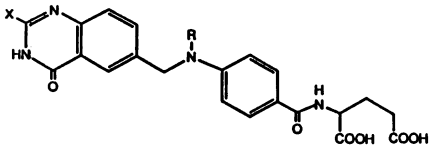
Within these limitations, some generalizations[3] should be

[2] B. Antonsson and R. G. Moran, unpublished results.

[3] These generalizations are derived from our data on mouse liver FPGS. Although limited evidence (5, 29) suggests some species differences in the substrate specificity of FPGS, it appears that enzyme of human origin has a pattern of substrate specificity that closely resembles that of mouse liver enzyme (5, 29, 30).

TABLE 4

Similarity of the activities of a series of 2-amino- and 2-desamino-quinazoline folate antimetabolites as substrates for mouse liver FPGS



R	X	CB No.	K_m	V_{max}[a]	V_{max}/K_m[a]
			μM		
—CH_3	NH_2	3713	18.7 ± 4.1	1.17 ± 0.11	8.7 ± 2.2
—CH_3	H	3824	19.9 ± 0.78	1.30 ± 0.10	8.2 ± 0.81
—CH_2CH_3	NH_2	3714	31 ± 5.9	1.15 ± 0.06	5.3 ± 0.7
—CH_2CH_3	H	3822	51 ± 4.1	1.37 ± 0.08	3.5 ± 0.33
—$CH_2CH{=}CH_2$	NH_2	3716	26 ± 4.2	0.78 ± 0.02	4.3 ± 0.35
—$CH_2CH{=}CH_2$	H	3826	74 ± 2.6	1.10 ± 0.014	1.87 ± 0.04
—$CH_2C{\equiv}CH$	NH_2	3717	36 ± 6.9	0.87 ± 0.02	3.0 ± 1.3
—$CH_2C{\equiv}CH$	H	3804	48 ± 7.2	0.90 ± 0.10	3.2 ± 0.5

[a] Relative to PteGlu in the same experiment. The PteGlu controls from this series had a K_m of 138 ± 11 μM (12 experiments). For this series, two or three experiments were performed.

made that may prove stimulating to the design of folate analogs that inhibit FPGS or that are activated to polyglutamates efficiently by this enzyme. (a) A folate analog containing a quinazoline ring will be activated to polyglutamates by FPGS more efficiently than the corresponding pteridine (Table 1). This conclusion has also been reached by others (31, 32) on the basis of experiments with FPGS from other mammalian species. Likewise, an inhibitor of FPGS that is competitive with the folate substrate[4] would be more potent if the fused ring system used for drug design were a quinazoline rather than a pteridine ring (Fig. 2). (b) Folate analogs with a reduced 5,6,7,8-ring system will probably be more readily utilized as substrates for FPGS than the corresponding nonreduced compounds. This has proven to be the case for both the 4-oxo- and 4-aminopteridines (Table 1) and for the 4-oxo- and 4-amino-5,10-dideazapteridine (33) series. George *et al.* (3) have reported that several reduced folate analogs that are inhibitors of hog liver FPGS are 10–30-fold more potent than are the corresponding nonreduced compounds. Thus, this generalization seems to hold for the interaction of mammalian FPGS with both folate substrates and inhibitors that are competitive[4] with the folate substrate. (c) The 2-amino group is not essential for binding of folate substrates within the active site. Results to be reported elsewhere demonstrate that the 2-position represents a region of the folate molecule at which at least small substituents can be placed without affecting polyglutamation and that major changes in the pyrimidine ring can be tolerated. (d) In some (Table 1 and Refs. 5 and 27), but not all (Table 3) series, the presence of a 4-amino group on a folate analog allows polyglutamation to proceed at lower substrate concentrations than with the corresponding compound containing a 4-oxo- group. It is also noted that 5,10-dideazafolate and 5,10-dideazaaminopterin are equivalent substrates for mouse liver FPGS, as are the 5,6,7,8-tetrahydro forms of these two analogs (33). (e) The pyrazine ring of the folate structure is probably not required as such for binding of folates to FPGS. It may only serve to position the 10-nitrogen and the benzoylglutamate moiety within the active site relative to the position of the pyrimidine ring. The activity of *N*-[4-(4-(2,4-diamino-5-pyrimidinyl)-1-piperazinyl) benzoyl]-L-glutamic acid as a substrate implies that (at least the C-7 and N-8 of) the pyrazine ring of classical folate analogs does not participate in bonds essential for substrate positioning. (f) The data of Table 3 suggest that the active site of mouse liver FPGS has limited bulk tolerance above or below the volume filled by the tetrahydropyrazine ring of folates or by the piperazine ring of the compounds shown in this table. The latter structure would probably be in the active site in a position spacially similar to that occupied by the imidazolidine ring of 5,10-methylenetetrahydrofolate. It is interesting to note that this latter compound has been reported to be a poor substrate for hog liver FPGS (3) but a good substrate for mouse liver FPGS (2). There have been other suggestions (5, 30) based on somewhat limited data that FPGS from different sources have different structural requirements for optimal substrate activity. (g) The substrate activities of either folic acid analogs (Table 1) or of 5,8-dideazafolates (Table 2) with hydrocarbon substituents at N-10 are substantially less than those of the corresponding unsubstituted compounds. The pattern of change in the kinetic parameters shown in Table 2 suggests that alkylation of N-10 with short chain alkyl groups may limit the conformational opportunities available to the molecule due to the restriction of the torsion angle of the bond between the 10 nitrogen and the phenyl ring. This is also suggested by the observation that a N to C change at position 10 of aminopterin alters the kinetics of utilization by mouse liver FPGS exactly the same as does methylation of the 10-nitrogen of aminopterin (5). Longer aliphatic substituents at this position may encounter steric hindrance from amino acid residues in the active site.

Several of the folate derivatives reported in this study are among the best substrates for FPGS yet reported among synthetic folate analogs. Thus, tetrahydroaminopterin, 5,8-dideazafolate, 5,10-dideazatetrahydrofolate (33), and the 6-unsubstituted-2,4-diaminopyrimidine shown in Table 3 were all activated to polyglutamates half-maximally at low concentrations (3–17 μM) and have V_{max} values equivalent to that of H_4PteGlu. These compounds have in common: (a) a 4-oxo- or 4-aminopyrimidine ring, (b) a structure spatially equivalent to the tetrahydropyrazine ring of H_4PteGlu, (c) the same distance of the 10-position from the pyrimidine ring and also from the L-glutamate side chain as is found in the naturally occurring reduced folates, (d) a phenyl ring at the position equivalent to that in H_4PteGlu, and (e) L-glutamate as a side chain. These structural features seem to be sufficient for efficient utilization as a substrate by mouse liver FPGS. Another important structural modification that optimized FPGS substrate activity was reported by Cichowicz *et al.* (32), who demonstrated that a 5-chloro substituent results in a major decrease of the K_m value of 5,8-dideazafolates for hog liver FPGS.[5]

Clearly, what is needed is an understanding of the physical basis of these empirically derived structure-activity relationships. For instance, we herein show that the quinazolines are better substrates than are the corresponding folates and the former display kinetic constants closer to those expected for reduced folates such as tetrahydrofolate. Results published

[4] This relationship does not seem to hold for inhibitors of FPGS with mixed, noncompetitive, or other more complex patterns of inhibition relative to the folate substrate (unpublished data).

[5] A publication (34) appeared while this manuscript was being reviewed that reported that 5-chloro-5,8-dideazapteroyl-L-ornithine was an extremely potent inhibitor of hog liver FPGS.

elsewhere indicate that, for reduced folate analogs, the presence or absence of N-8 has little effect on interaction with FPGS but that 5-deazatetrahydrofolate is a better substrate than tetrahydrofolate.[6] It is difficult to reconcile these facts into a unified physical model of the forces involved in binding of the pyrazine ring of folates to the enzyme active site. It could be proposed that, for aromatic folates, interaction of substrates with aromatic amino acid residues above/below the pyrazine ring is impeded until the sp^2 azine nitrogens are stripped of water molecules, whereas this same energy cost is not necessary for the corresponding quinazolines [the drying out effect (20)]. For reduced folate analogs, energy costs of drying out N-5 could be balanced by energy gains from hydrogen bonds. However, such explanations must remain speculative and the physical basis of these observations, a puzzle, without more direct information on the binding of substrates to enzyme.

The selective cytotoxicity of MTX and other classical dihydrofolate reductase inhibitors for some tumors under conditions that do not cause excessive toxicity to dividing host tissues seems attributable to intracellular retention of drug in tumor (reviewed in Refs. 37 and 38). Because of the role of FPGS in metabolically trapping folates and antifolates inside mammalian cells, the proposal has been made (37) that FPGS plays a major role in the selective cytotoxicity of folate antimetabolites. Recent results suggest cautious appraisal of this concept. Fry *et al.* (18) have reported that the 6-unsubstituted and 6-methyl-substituted 2,4-diaminopyrimidine dihydrofolate reductase inhibitors shown in Table 3 have equivalent therapeutic activities (at optimal dosages of each) against a murine leukemia *in vivo*, in contrast to the 65-fold superior utilization of the 6-unsubstituted analog by FPGS (Table 3). However, maximal therapeutic effects of the 6-unsubstituted analog of this series were attained at a 50-fold lower dose than those optimal for the 6-methylated compound (18). Hence, in this series, efficient utilization as a substrate for FPGS *in vitro* correlates with the dosage at which therapeutic activity was seen *in vivo* rather than with the degree of selective cytotoxicity obtained at optimal dosage. These data support the concept that, among members of a series of analogs, substrate activity for FPGS is a major factor predictive for the cytotoxic *potency* of folate antimetabolites.

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