Mammalian Folylpoly-γ-glutamate Synthetase. 2. Substrate Specificity and Kinetic Properties†

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ABSTRACT: The specificity of hog liver folylpolyglutamate synthetase for folate substrates and for nucleotide and glutamate substrates and analogues has been investigated. The kinetic mechanism, determined by using aminopterin as the folate substrate, is ordered Ter-Ter with MgATP binding first, folate second, and glutamate last. This mechanism precludes the sequential addition of glutamate moieties to enzyme-bound folate. Folate, dihydrofolate, and tetrahydrofolate possess the optimal configurations for catalysis (k_{cat} = 2.5 s^{-1}) while 5- and 10-position substitutions of the folate molecule impair catalysis. k_{cat} values decrease with increasing glutamate chain length, and the rate of decrease varies depending on the state of reduction and substitution of the folate molecule. Folate binding, as assessed by on rates, is slow. Dihydrofolate exhibits the fastest rate, and the rates are slightly reduced for tetrahydrofolate and 10-formyltetrahydrofolate and greatly reduced for 5-methyltetrahydrofolate and folic acid. The on rates for most pteroyldiglutamates are similar to the rates for their respective monoglutamate derivatives, but further extension of the glutamate chain results in a progressive decrease in on rates. Tetrahydrofolate polyglutamates are the only long glutamate chain length folates with detectable substrate activity. The specificity of the L-glutamate binding site is very narrow. L-Homocysteate and 4-*threo*-fluoroglutamate are alternate substrates and act as chain termination inhibitors in that their addition to the folate molecule prevents or severely retards the further addition of glutamate moieties. The K_m for glutamate is dependent on the folate substrate used. MgATP is the preferred nucleotide substrate, and β,γ-methylene-ATP, β,γ-imido-ATP, adenosine 5′-*O*-(3-thiotriphosphate), P^1,P^5-di(adenosine-5′) pentaphosphate, and free ATP^{4-} are potent inhibitors of the reaction.

In the preceding paper (Cichowicz & Shane, 1987), the purification and general properties of hog liver folylpolyglutamate synthetase are described. The enzyme catalyzes the reaction

$$MgATP + H_4PteGlu_n + glutamate \rightarrow H_4PteGlu_{n+1} + MgADP + P_i \quad (1)$$

and metabolizes $H_4PteGlu_n$[1] to the types of folylpolyglutamates that are found in vivo.

A major interest of our laboratory is to understand how folylpolyglutamate synthesis is regulated, to devise inhibitors of the synthetase, and to study the effects of these compounds on one-carbon metabolism. As a prelude, the specificity of the enzyme for a variety of folate derivatives, and for glutamate and nucleotide substrates and analogues, and the kinetic mechanism of the enzyme have been investigated. These studies are described in this report.

Experimental Procedures

Materials. Substrates and reagents were synthesized or obtained from commercial sources as described in the accompanying paper (Cichowitz & Shane, 1987). 3-Methyl-DL-glutamate and 4-methyl-DL-glutamate were gifts from Dr. Owen Griffith, Cornell University Medical College. Folate derivatives were purified by chromatography on DEAE-cellulose or Sephadex G-25 just prior to use (Shane, 1980a). The concentrations of folate derivatives were calculated from their absorption spectra (Blakley, 1969).

Folylpolyglutamate Synthetase Assay. Enzyme activity was assayed by using the standard assay conditions [(6*RS*)-H_4PteGlu (40 μM), ATP (5 mM), [^{14}C]glutamate (250 μM), 2 h at 37 °C] described in the accompanying paper (Cichowicz & Shane, 1987). All assays utilized purified enzyme.

Folate and nucleotide substrate specificities were compared by using standard assay conditions modified by the replacement of H_4PteGlu or ATP with various concentrations of the folate derivative or nucleotide under study. The specificity of glutamate analogues for the glutamate binding site was assessed by adding unlabeled analogue (usually 5 mM) to the standard assay mixture (containing 250 μM [^{14}C]glutamate) and measuring the inhibition of labeled glutamate incorporation into folate products.

Kinetic parameters for folate substrates and for nucleotide and glutamate substrates and inhibitors were measured by using a modified assay. Fixed substrates were (6*RS*)-H_4PteGlu (40 μM), ATP (1 mM), and [^{14}C]glutamate (2 mM, 2.5 mCi/mmol), where appropriate, and the assay time was reduced to 1 h.

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[1] Abbreviations: PteGlu, pteroylmonoglutamic acid (folic acid); $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylpoly-γ-glutamate, *n* indicating the number of glutamate moieties; *p*AbaGlu$_n$, *p*-aminobenzoylpoly-γ-glutamate; Pte, pteroic acid; ZTP, 5-amino-4-imidazolecarboxamide riboside 5′-triphosphate; Ap_nA, P^1,P^n-di(adenosine-5′) polyphosphate; HPLC, high-performance liquid chromatography; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate).

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Table I: Kinetic Constants of Glutamate Analogues for Folylpolyglutamate Synthetase[a]

analogue	K_m (mM)	K_i(app) (mM)	V_{max} [μmol h^{-1} (mg of protein)$^{-1}$]
L-glutamate	0.35	0.32	74
L-homocysteate	*b*	4.0	*b*
4-*erythro*-fluoro-DL-glutamate	*b*	>100	*b*
4-*threo*-fluoro-DL-glutamate	*b*	2.0	*b*

[a] Enzyme activity was assayed as described under Experimental Procedures using 1 mM ATP and 40 μM (6*RS*)-H_4PteGlu and various concentrations of labeled L-glutamate (0.1–2 mM). K_i(app) values were assessed by adding fixed concentrations of the indicated unlabeled analogues to the assay mixtures. [b] Not determined.

Substrate Activity of Glutamate Analogues. The ability of glutamate analogues to act as substrates was measured by using an assay procedure modified by the replacement of labeled glutamate with the unlabeled analogue under study and H_4PteGlu with H_4[^{3}H]PteGlu. Labeled folate substrate and products were cleaved to *p*AbaGlu and *p*AbaGlu analogue derivatives and were purified and separated by HPLC as described in the previous paper (Cichowicz & Shane, 1987).

Results

Specificity for L-*Glutamate.* The specificity of the glutamate binding site was evaluated by the ability of unlabeled amino acids and analogues (5 mM) to inhibit the incorporation of L-[^{14}C]glutamate (250 μM) into folate products. Unlabeled L-glutamate inhibited the incorporation of label by 90%, 4-*threo*-fluoro-DL-glutamate by 62%, L-homocysteate by 40%, 3-*erythro*,*threo*-methyl-DL-glutamate (20 mM) by 36%, L-2-hydroxyglutarate by 21%, 4-*erythro*,*threo*-methyl-DL-glutamate (20 mM) by 18%, and 4-*erythro*-fluoro-DL-glutamate by 12%. A wide variety of other glutamate analogues, including D-glutamate, L-aspartate, DL-aminoadipate (10 mM), L-glutamine, *N*-acetylglutamate, 4-aminobutyrate, L-2-aminobutyrate, DL-2-methylglutamate (10 mM), 4-carboxy-L-glutamate, 4-methylene-DL-glutamate (10 mM), D-2-hydroxyglutarate, 2-oxoglutarate, glutarate, 2-oxoproline, glutaconate, 5-aminolevulinate, kainate, cysteate, carboxymethyl-L-cysteine, and L-ornithine, had little, if any, apparent affinity for the glutamate binding site (0–10% inhibition).

With (6*RS*)-H_4PteGlu as the folate substrate, the apparent K_m for L-glutamate was 346 μM, and the K_i(app) for unlabeled L-glutamate, when measured by its ability to inhibit activity with labeled glutamate as the substrate, was 323 μM (Table I). The K_i(app) was increased about 3-fold for 4-*threo*-fluoroglutamate, assuming that the D isomer did not bind to the protein, and about 12-fold for L-homocysteate.

Under conditions where MgATP and the folate substrate were at saturating levels, the K_m for glutamate was 335 μM with (6*S*)-H_4PteGlu as the folate substrate, 336 μM with (6*R*)-10-formyl-H_4PteGlu, and 962 μM with PteGlu.

The ability of glutamate analogues to act as alternative substrates for the enzyme was measured by using (6*S*)-H_4-[^{3}H]PteGlu as the labeled substrate and separating the labeled substrate and products by HPLC following their cleavage to *p*AbaGlu and *p*AbaGlu analogues. With 10 μM H_4PteGlu and 5 mM L-glutamate as the substrates, polyglutamates of chain length up to five were detected after 2 h with the diglutamate predominating. Under similar conditions, a single homocysteate residue was added to the folate substrate, and the major product obtained with 4-fluoroglutamate as a substrate also incorporated only one residue although a small amount of product equivalent to the tripeptide was detected. In each case, about 80% of the folate substrate was converted to products (data not shown). The *p*AbaGlu–homocysteate and *p*AbaGlu–4-fluoroglutamate products eluted later than the corresponding *p*AbaGlu$_2$, as predicted by their increased electronegativity, and could be resolved completely from mixtures containing the *p*AbaGlu analogue and *p*AbaGlu$_2$.

With 0.1 μM H_4PteGlu as the labeled substrate, a high enzyme concentration, and a 24-h incubation time, the major product detected with L-glutamate as the substrate was the hexaglutamate derivative (Table II). Under similar conditions, a single homocysteate moiety was added to the folate molecule, and further chain elongation was prevented. Although most of the labeled folate was converted to products with 4-fluoroglutamate as the substrate, the products obtained, with the triglutamate analogue predominating, were much shorter than was observed with glutamate as the substrate. Similar products were obtained with 4-*threo*-fluoro-DL-glutamate and 4-*erythro*-fluoro-DL-glutamate. Weak substrate activity was observed with 3-methylglutamate and trace activity with the 4-methyl derivative.

Other analogues tested, such as D-glutamate, L-aspartate, L-2-aminoadipate, L-2-aminopimelate, 4-carboxy-L-glutamate, 6-diazo-5-oxo-L-norleucine, DL-2-methylglutamate, and γ-L-glutamyl-L-glutamate, were inactive as substrates, or the trace amounts of products formed eluted at the position of *p*AbaGlu$_n$ and not at the expected positions of the *p*AbaGlu analogues, suggesting contamination with trace amounts of L-glutamate.

Specificity for Nucleotides. Table III shows the effectiveness of various Mg salts of nucleotides as substrates for folylpolyglutamate synthetase. Under the standard assay conditions, the apparent K_m for $MgATP^{2-}$ was about 10 μM.

Table II: Glutamate Analogues as Substrates for Folylpolyglutamate Synthetase[a]

glutamate analogue	time (h)	glutamate analogue distribution (%)						
		1[b]	2	3	4	5	6	7
5 mM L-glutamate	3	8	2	3	3	58	23	3
5 mM L-glutamate	24	6	2	2	3	23	54	9
10 mM 4-*threo*,*erythro*-fluoro-DL-glutamate	3	10	42[c]	37[c]	6[c]	5[c]	0	0
10 mM 4-*threo*,*erythro*-fluoro-DL-glutamate	24	8	11[c]	55[c]	24[c]	3[c]	0	0
10 mM 4-*threo*-fluoro-DL-glutamate	24	10	8[c]	43[c]	38[c]	2[c]	0	0
10 mM 4-*erythro*-fluoro-DL-glutamate	24	11	8[c]	46[c]	31[c]	3[c]	0	0
5 mM L-homocysteate	24	10	90[c]	0	0	0	0	0
5 mM D-glutamate	24	85	7	6	2	0	0	0
10 mM 2-CH_3-DL-glutamate	3	100	0	0	0	0	0	0
20 mM 3-CH_3-DL-glutamate	3	88	12	0	0	0	0	0
20 mM 4-CH_3-DL-glutamate	3	95	5	0	0	0	0	0

[a] Assay conditions and the cleavage of folate products to *p*AbaGlu$_n$ and their separation by HPLC are described under Experimental Procedures. Assay mixtures contained 0.1 μM (6*S*)-H_4[^{3}H]PteGlu as the labeled substrate and 4 units of enzyme. The glutamate substrate was replaced by the indicated unlabeled glutamate analogues. Assay mixtures were incubated for 3 or 24 h. In 24-h incubation studies, additional enzyme (4 units) was added at 3 h. [b] Unmetabolized labeled substrate. [c] Radioactive peak eluted slightly later than the corresponding *p*AbaGlu$_n$ standard.

Table III: Nucleotide Analogues as Substrates for Folylpolyglutamate Synthetase[a]

analogue	relative activity 0.1 mM	5 mM
ATP	100	96
dATP	58	81
ADP	8.2	21
AMP	1.2	0.1
GTP	7.4	19
ITP	28	65
XTP	3.8	6.0
UTP	11	58
CTP	0.3	8.7
dTTP	0	0
ZTP	9.6	13[b]

[a] Enzyme activity was assayed as described under Experimental Procedures using 1.2 units of purified enzyme, except ATP was replaced by the indicated concentration of ATP analogue. Substrate activity is expressed relative to that obtained with 100 μM ATP. [b] 500 μM.

Table IV: Nucleotide Analogues as Inhibitors of Folylpolyglutamate Synthetase[a]

analogue	relative activity 100 μM	1 mM	5 mM
none	100	*b*	*b*
ADP	84	*b*	40
AMP	93	*b*	74
phosphate	*b*	93	92
pyrophosphate	*b*	91	58
ATPγS	9.4	1.3	*b*
β,γ-methylene-ATP	5.8	0.5	*b*
β,γ-imido-ATP	13	1.8	*b*
[(fluorosulfonyl)benzoyl]adenosine	91	87	*b*
Ap_2A	104	*b*	*b*
Ap_3A	101	*b*	*b*
Ap_4A	95	*b*	*b*
Ap_5A	2.4	*b*	*b*
Ap_6A	2.7	*b*	*b*

[a] Enzyme activity was assayed as described under Experimental Procedures using 1.2 units of purified enzyme, except assay mixtures contained 100 μM ATP and the indicated concentration of ATP analogue. Activity is expressed relative to that obtained in the absence of analogue. [b] Not determined.

Although ATP was the most effective nucleotide substrate, dATP, ITP, and UTP were alternate substrates. GTP, XTP, ZTP, and ADP were less effective, and the possibility that these compounds were contaminated with ATP was not excluded.

ADP, AMP, and pyrophosphate were weak inhibitors of the reaction when measured with MgATP as the nucleotide substrate, while little inhibition was observed with phosphate (Table IV). ATPγS, β,γ-methylene-ATP, β,γ-imido-ATP, Ap_5A, and Ap_6A were effective inhibitors of the reaction, and none of these compounds was a substrate.

Kinetic parameters for nucleotide substrates and inhibitors are shown in Table V. The glutamate concentration in the assay mixture was increased to 2 mM, which is near-saturating with H_4PteGlu as substrate, to allow a more accurate measure of K_m and V_{max}. MgATP had the lowest K_m and highest V_{max} of any of the nucleotide substrates. Ap_5A and β,γ-methylene-ATP were the most potent nucleotide analogue inhibitors of the enzyme, while ATPγS and β,γ-imido-ATP were almost as effective. Phosphate and pyrophosphate were weak inhibitors. In each case, inhibition was competitive with respect to MgATP.

Specificity for Folates. The substrate abilities of different folates were compared by using subsaturating levels of glutamate (standard assay conditions, 250 μM L-[^{14}C]glutamate),

Table V: Kinetic Constants of Nucleotide Analogues for Folylpolyglutamate Synthetase[a]

analogue	K_m (μM)	K_i (μM)	V_{max} [μmol h^{-1} (mg of protein)$^{-1}$]	V_{max}/K_m (rel)[b]
ATP	10	*c*	73	100
dATP	50	*c*	63	18
ITP	136	*c*	45	5
UTP	190	*c*	34	3
β,γ-methylene-ATP	*c*	0.9	*c*	
β,γ-imido-ATP	*c*	3.0	*c*	
ATPγS	*c*	2.0	*c*	
Ap_5A	*c*	0.6	*c*	
pyrophosphate	*c*	4200	*c*	
phosphate	*c*	29000	*c*	

[a] Enzyme activity was assayed as described under Experimental Procedures using 40 μM (6*RS*)-H_4PteGlu and 2 mM L-[^{14}C]glutamate and various concentrations of nucleotide substrates. K_i values were assessed by adding fixed concentrations of the indicated analogues to assay mixtures containing ATP as the variable substrate. [b] Values are relative to those obtained with ATP as the substrate. [c] Not determined or not applicable.

Table VI: Folate Compounds as Substrates for Folylpolyglutamate Synthetase[a]

substrate	relative activity 10 μM	50 μM	100 μM	1 mM
H_2Pte[b]	3.1	11	*c*	24
PteGlu	5.7	17	33	47
H_2PteGlu	128	122	83	22
(6*RS*)-H_4PteGlu	62	100	89	33
(6*RS*)-5-methyl-H_4PteGlu	10	36	*c*	61
(6*RS*)-5-formyl-H_4PteGlu	0.8	5.3	*c*	61
(6*RS*)-5,10-methylene-H_4PteGlu	10	38	*c*	36
(6*RS*)-10-formyl-H_4PteGlu	18	29	*c*	13
$PteGlu_2$	*c*	45	116	120
$PteGlu_3$	*c*	*c*	52	80
$PteGlu_4$	*c*	*c*	4.4	5.7
$PteGlu_5$	*c*	*c*	0.3	1.0
$PteGlu_6$	*c*	*c*	0	0.5
$PteGlu_7$	*c*	*c*	0.2	0.6
$H_2PteGlu_2$	*c*	55	47	*c*
$H_2PteGlu_5$	0.4	*c*	0.4	0.4
(6*RS*)-$H_4PteGlu_2$	86	127	139	127
(6*RS*)-$H_4PteGlu_3$	39	56	63	65
(6*RS*)-$H_4PteGlu_5$	3.4	1.4	1.7	1.7
(6*RS*)-$H_4PteGlu_7$	2.6	1.8	0.4	0.9

[a] The assay mixture was as described under Experimental Procedures and contained 5 mM ATP and 250 μM L-[^{14}C]glutamate. Folate substrates were added at 10, 50, 100, and 1000 μM, as indicated. Activities are expressed relative to that obtained with (6*RS*)-H_4PteGlu (50 μM) as substrate. [b] Activity due to <0.2% contamination with H_2PteGlu. [c] Not determined.

which mirrors the physiological situation in most tissues. At low concentrations, the most effective pteroylmonoglutamate substrate was H_2PteGlu (Table VI). Other pteroylmonoglutamates were less effective than (6*RS*)-H_4PteGlu. Variable activity was observed with 5,10-methylene-H_4PteGlu as the substrate, presumably reflecting removal of formaldehyde from this compound by the thiols in the assay mixture. The substrate activity found for H_2Pte was artifactual and was due to trace contamination with H_2PteGlu. The only product found with H_2Pte as the substrate was the diglutamate derivative, and no pteroylmonoglutamate product was detected. Significant substrate inhibition was observed with all effective folate substrates of the enzyme. PteGlu was a relatively poor substrate with a high apparent K_m, while $PteGlu_2$ and $PteGlu_3$ were more effective. Further elongation of the $PteGlu_n$ chain led to a sharp loss in substrate effectiveness. Similarly, (6*RS*)-$H_4PteGlu_2$ was a better substrate than the monoglutamate derivative while further glutamate chain elongation

Table VII: Pteroylpolyglutamate Substrates of Folylpolyglutamate Synthetase[a]

n	(6S)-H_4PteGlu$_n$	(6R)-5,10-methylene-H_4PteGlu$_n$	(6R)-10-formyl-H_4PteGlu$_n$	(6S)-5-methyl-H_4PteGlu$_n$
1	100	20	31	20
2	63	18	13	1.7
3	15	4.0	1.7	*b*
4	7.0	3.5	0	*b*
5	2.2	1.5	0	*b*
6	1.3	0	0	*b*
7	0.3	0	0	*b*

[a] The assay mixtures were as described in the legend to Table VI except the naturally occurring diastereoisomers of the indicated reduced folate substrates (50 μM) were used. Activities are expressed relative to that obtained with (6S)-H_4PteGlu. [b] Not determined.

led to a drop in substrate effectiveness. Under the assay conditions used, the low activity observed with H_4PteGlu polyglutamates appeared to be due to a drop in the apparent V_{max} of these compounds. H_2PteGlu$_2$ was a less effective substrate than the monoglutamate derivative while the pentaglutamate was essentially inactive.

The substrate activities of the naturally occurring diastereoisomers of reduced folates are shown in Table VII. (6S)-H_4PteGlu was a more effective substrate than its 5- and/or 10-substituted derivatives. The substrate activity of longer chain length derivatives decreased with increasing glutamate chain length. Although (6R)-10-formyl-H_4PteGlu was a reasonable substrate, only low levels of activity were detected with its triglutamate derivative, and no activity was detected with the tetraglutamate. The activity of (6S)-5-methyl-H_4PteGlu derivatives dropped to low levels as the glutamate chain length increased from one to two.

Kinetic Constants of Folates. Kinetic parameters for folates were determined under conditions where MgATP was saturating. To prevent high background, the glutamate concentration used (2 mM) was not quite saturating (86–92%, depending on the folate under study). Higher glutamate concentration (5 mM) was used with PteGlu to achieve this level of saturation. Enzyme concentration and incubation times were reduced to minimize the addition of more than one glutamate moiety to the folate substrate and to ensure initial rate conditions. Marked substrate inhibition was observed with many of the folate substrates tested, particularly those that displayed high affinity for the enzyme. Substrate concentrations were chosen that minimized this effect. The apparent affinities of folates that displayed little or no substrate activity were determined by their ability to inhibit activity with PteGlu or (6S)-H_4PteGlu as the variable folate substrate. At high concentrations, noncompetitive inhibition was observed. At lower concentrations, competitive inhibition was observed, and these conditions were used to determine K_i(app) values. The reason for the substrate inhibition and the noncompetitive inhibition with higher concentrations of inhibitors was not studied further. However, as the substrates of this enzyme resemble its products, the simplest explanation of the data would be the binding of folates to the product site on the enzyme.

Kinetic constants for a variety of folate compounds are shown in Table VIII. Also shown are V_{max}/K_m relative to the value obtained with (6S)-H_4PteGlu. This ratio is a comparison of the reaction rates with the different substrates at identical concentrations when these concentrations are considerably below the K_m for the substrate. Total intracellular folate levels in hog liver are approximately 30 μM, of which less than 1% are monoglutamate derivatives (Cichowicz & Shane, 1987).

Table VIII: Kinetic Constants of Folates for Folylpolyglutamate Synthetase[a]

folate	K_m (μM)	K_i(app) (μM)	V_{max}(rel)[b]	V_{max}/K_m-(rel)[b]
PteGlu[c]	115	*d*	95	6.4
PteGlu$_2$	62	*d*	60	7.4
PteGlu$_3$	119	*d*	33	2.2
PteGlu$_4$	*d*	147	*d*	*d*
PteGlu$_5$	*d*	227	*d*	*d*
PteGlu$_6$	*d*	380	*d*	*d*
PteGlu$_7$	*d*	527	*d*	*d*
H_2PteGlu	5.0	*d*	101	156
H_2PteGlu$_2$	2.6	*d*	51	152
H_2PteGlu$_4$	*d*	14	*d*	*d*
H_2PteGlu$_5$	*d*	17	*d*	*d*
H_2PteGlu$_6$	*d*	33	*d*	*d*
H_2PteGlu$_7$	*d*	74	*d*	*d*
(6*RS*)-H_4PteGlu	6.0	*d*	79	101
(6S)-H_4PteGlu	7.7	*d*	100	100
(6R)-10-formyl-H_4PteGlu	2.2	*d*	27	95
(6R)-5,10-methylene-H_4PteGlu	57	*d*	39	5.2
(6S)-5-methyl-H_4PteGlu	54	*d*	39	5.6
(6S)-H_4PteGlu$_2$	3.4	*d*	45	102
(6S)-H_4PteGlu$_3$	1.1	*d*	8.8	62
(6S)-H_4PteGlu$_4$	2.0	11	4.5	17
(6S)-H_4PteGlu$_5$	2.7	14	1.6	4.6
(6S)-H_4PteGlu$_6$	*d*	34	*d*	*d*
(6S)-H_4PteGlu$_7$	*d*	47	*d*	*d*

[a] Enzyme activity was assayed as described under Experimental Procedures using 1 mM ATP and 2 mM L-[^{14}C]glutamate as the fixed substrates and various concentrations of the folate substrate. K_i(app) values were assessed by adding fixed concentrations of the indicated derivatives to assay mixtures containing PteGlu or (6S)-H_4PteGlu as the variable substrate. [b] Values are relative to those obtained with (6S)-H_4PteGlu as the substrate. For V_{max}(rel), 100 equals 123 μmol h^{-1} mg^{-1} (k_{cat} = 2.1 s^{-1}). For V_{max}/K_m(rel), 100 equals 28 × 10^4 s^{-1} M^{-1}. [c] 5 mM L-glutamate used as the fixed substrate. [d] Not determined.

Consequently, the V_{max}/K_m ratio can be used to directly compare the effectiveness of the various folate derivatives as substrates for the hog liver enzyme under physiological conditions except for the penta–, hexa–, and heptaglutamates that accumulate in vivo. This ratio also compares on rates for the substrates (see Discussion).

V_{max} values dropped with increasing glutamate chain length. It was not possible to obtain K_m and V_{max} values for long-chain folylpolyglutamates due to their low activity. However, (6S)-H_4PteGlu polyglutamates of chain length at least up to the heptaglutamate are substrates for the enzyme (Cook et al., 1987). The activity obtained with (6S)-5-methyl-H_4PteGlu$_2$ at concentrations up to 250 μM was too low to measure accurately.

At a limiting glutamate concentration (Table VI), most pyeroyldiglutamates were more effective substrates than pteroylmonoglutamates. Although this is consistent with the lowered K_m values for diglutamates obtained in kinetic studies at higher glutamate concentrations (Table VIII), the lowered V_{max} of the diglutamates suggests that the diglutamates would be less effective substrates at the concentrations that were tested. To examine this anomaly further, apparent K_m values for L-glutamate were determined by using PteGlu (100 μM) and PteGlu$_2$ (50 μM) as the folate substrates at concentrations that approximated their K_m values. Under these conditions, the K_m for L-glutamate was 904 μM with PteGlu as the substrate and 141 μM with PteGlu$_2$, and V_{max} values were about 3-fold higher with the monoglutamate substrate. At limiting glutamate concentrations, PteGlu$_2$ was the more effective substrate while at high glutamate concentrations PteGlu was more effective.

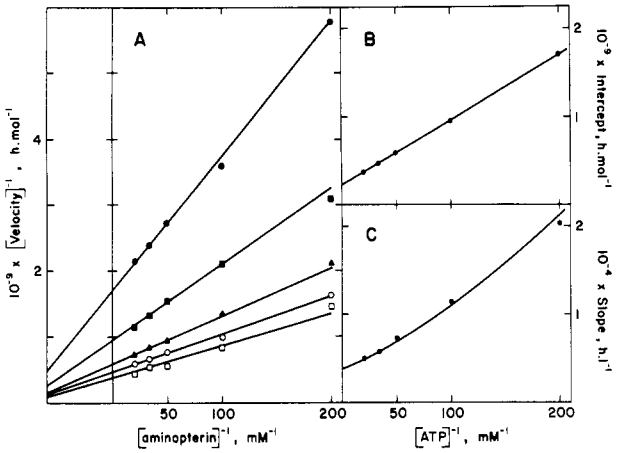


FIGURE 1: Effect of aminopterin concentration on folylpolyglutamate synthetase activity. (A) Double-reciprocal plot of velocity against aminopterin concentration at various fixed levels of ATP and glutamate. Enzyme activity was measured as described under Experimental Procedures using 39 ng of purified folylpolyglutamate synthetase and the indicated folate concentration. Assay mixtures (0.5 mL) were incubated at 37 °C for 1 h. The respective concentrations of ATP and glutamate were 5 μM and 200 μM (●), 10 μM and 400 μM (■), 20 μM and 800 μM (▲), 30 μM and 1.2 mM (○), and 50 μM and 2 mM (□). (B) Intercept replot. The intercepts of the Lineweaver–Burk plots with the ordinate (A) were plotted against reciprocal concentrations of one of the fixed substrates (ATP). (C) Slope replot. The slopes of the Lineweaver–Burk plots (A) were plotted against reciprocal concentrations of one of the fixed substrates (ATP).

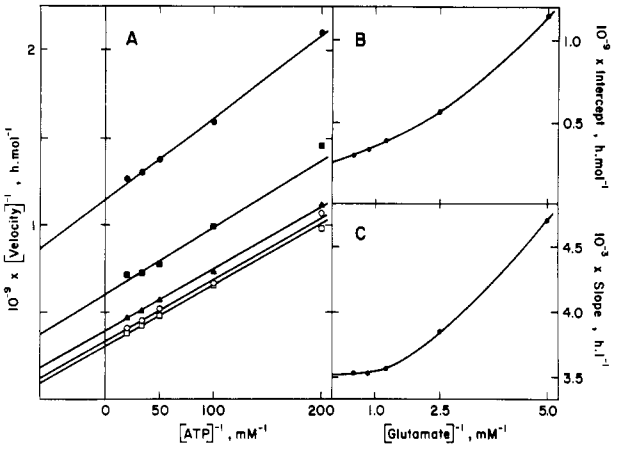


FIGURE 2: Effect of ATP concentration on folylpolyglutamate synthetase activity. (A) Double-reciprocal plot of velocity against ATP concentration. Experimental details are as described in the legend to Figure 1 except the indicated ATP concentrations were used and aminopterin and glutamate concentrations were held constant at the following fixed concentrations, respectively: 20 μM and 200 μM (●), 40 μM and 400 μM (■), 80 μM and 800 μM (▲), 120 μM and 1.2 mM (○), and 200 μM and 2 mM (□). (B) Intercept replot. The intercepts of the Lineweaver–Burk plots with the ordinate (A) were plotted against reciprocal concentrations of one of the fixed substrates (glutamate). (C) Slope replot. The slopes of the Lineweaver–Burk plots (A) were plotted against reciprocal concentrations of one of the fixed substrates (glutamate).

Kinetic Mechanism. Initial velocity studies were carried out as described by Fromm (1975). The concentration of one substrate was varied while holding the other two substrates at fixed nonsaturating levels. This process was repeated at various concentrations of the two fixed substrates, keeping the ratio of concentrations of the two fixed substrates constant.

Aminopterin was used as the folate substrate as this compound was effectively converted to the diglutamate derivative but further chain elongation proceeded only slowly (Cichowicz & Shane, 1987). Under the assay conditions used, the only folate product formed was the diglutamate derivative. Kinetic analyses were not conducted with (6*S*)-H_4PteGlu as the products detected in this case were di- and triglutamate derivatives. All assay mixtures contained 10 mM Mg^{2+} to ensure complete conversion of ATP to the substrate form, $MgATP^{2-}$, and to prevent inhibition by ATP^{4-}.

The effect of varying the aminopterin concentration at different fixed concentrations of MgATP and glutamate is shown in Figure 1A. The Lineweaver–Burk plots showed a series of intersecting lines indicative of a sequential mechanism. Intersecting plots were also seen with MgATP (Figure 2A) and glutamate (Figure 3A) as the variable substrates, suggesting that the overall mechanism is sequential.

Intercepts of the Lineweaver–Burk plots with the ordinate, plotted against reciprocal concentrations of one of the fixed substrates, are shown in Figures 1B, 2B, and 3B. Slopes of the Lineweaver–Burk plots, plotted against reciprocal concentrations of one of the fixed substrates, are shown in Figures 1C, 2C, and 3C. All secondary replots, with the exception of the intercept replot for aminopterin, were hyperbolic and did not intersect the origin at infinite concentrations of the fixed substrates. The intercept replot for aminopterin (Figure 1B) was linear and did not intersect the origin. These patterns are consistent with those expected for an ordered Ter-Ter mechanism with aminopterin as the second substrate binding to the enzyme (Fromm, 1975). As phosphate was a competitive inhibitor of the nucleotide substrate, MgATP was probably the first substrate to bind, and phosphate the last product to leave, the enzyme. Kinetic constants obtained in this analysis are shown in Table IX.

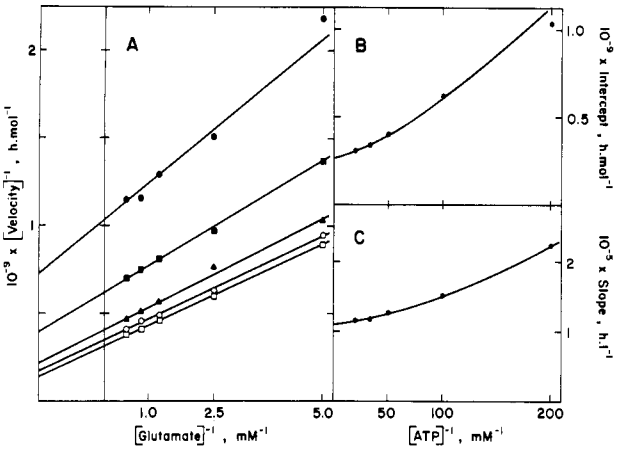


FIGURE 3: Effect of glutamate concentration on folylpolyglutamate synthetase activity. (A) Double-reciprocal plot of velocity against glutamate concentration. Experimental details are as described in the legend to Figure 1 except the indicated glutamate concentrations were used and aminopterin and ATP concentrations were held constant at the following fixed concentrations, respectively: 20 μM and 5 μM (●), 40 μM and 10 μM (■), 80 μM and 20 μM (▲), 120 μM and 30 μM (○), and 200 μM and 50 μM (□). (B) Intercept replot. The intercepts of the Lineweaver–Burk plots with the ordinate (A) were plotted against reciprocal concentrations of one of the fixed substrates (ATP). (C) Slope replot. The slopes of the Lineweaver–Burk plots (A) were plotted against reciprocal concentrations of one of the fixed substrates (ATP).

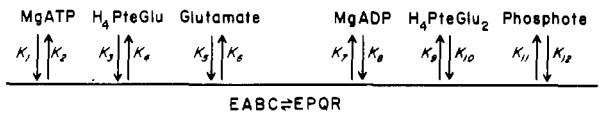
Table IX: Kinetic Constants of Hog Liver Folylpolyglutamate Synthetase[a]

compound	K_m (μM)	K_{is} (μM)	k_{cat} (s^{-1})	k_{on} ($\times 10^{-5}$ s^{-1} M^{-1})	k_{off} (s^{-1})
MgATP	14	~3	1.7	1.3	0.4
aminopterin	17	~10	2.0[b]	1.0	1.0
L-glutamate	417	[c]	1.7	[c]	[c]

[a] Kinetic analyses were carried out as described under Experimental Procedures and in the legends to Figures 1–3. [b] Equivalent to V_{max} of 114 μmol h^{-1} mg^{-1}. [c] Not estimated or not applicable.

Discussion

The kinetic mechanism of hog liver folylpolyglutamate synthetase was investigated by using aminopterin as the folate substrate to prevent the addition of more than one glutamate moiety to the folate molecule. The data are consistent with the following steady-state ordered Ter-Ter mechanism:



This type of mechanism, previously suggested in more detailed studies with the purified *Corynebacterium* (Shane, 1980c) and *Lactobacillus* (Bognar & Shane, 1983) enzymes, precludes the sequential addition of glutamate to enzyme-bound folate and is consistent with the observed metabolism of labeled folate doses in mammalian tissues. For instance, hepatic labeled folate in the first few hours after administering [^{3}H]PteGlu to the rat is primarily of short glutamate chain length while longer glutamate chain length derivatives predominate at longer times (Brody et al., 1979). Although it is possible that the kinetic mechanism might be different with folylpolyglutamate substrates, an identical mechanism was observed with the *Lactobacillus* enzyme when (6*R*)-5,10-methylene-$H_4PteGlu_2$ was used as the substrate. The folylpolyglutamate synthetase reaction resembles the glutamine synthetase reaction with folate replacing the glutamate substrate and glutamate replacing ammonia (Meister, 1974, 1978). By analogy, it has been suggested that the folylpolyglutamate synthetase reaction proceeds via phosphorylation of the γ-carboxyl of folate followed by nucleophilic attack by the free amine of glutamate on the acylphosphate intermediate (Shane, 1980c; Tang & Coward, 1983). This type of mechanism is consistent with the ordered addition of substrates, the requirement for the free base of glutamate as the substrate for the reaction (Cichowicz & Shane, 1987), and the potent inhibition by nonhydrolyzable ATP analogues. Recent studies demonstrating transfer of ^{18}O from [α,γ-^{18}O]methotrexate to phosphate provide more direct evidence for an acyl phosphate intermediate (Banerjee et al., 1986).

The turnover number of the hog liver enzyme with aminopterin (1.7 s^{-1}) or (6*S*)-$H_4PteGlu$ (2.5 s^{-1} at saturating glutamate concentrations) as the substrate is slightly higher than that found with the preferred substrates of the *Corynebacterium* (Shane, 1980c), *Lactobacillus* (Bognar & Shane, 1983) and *Escherichia coli* folylpolyglutamate synthetases (Bo[illegible] et al., 1985). The low levels of folylpolyglutamate synt[illegible]e activity commonly found in mammalian tissues, esp[illegible] when compared to most bacterial sources, are due to t[illegible] concentration of this protein in mammalian sources rat[illegible]an a decreased catalytic rate for the mammalian pro[illegible]

F[illegible]te Specificity. The specificity of the folate binding site of hog liver folylpolyglutamate synthetase was investigated by using a variety of naturally occurring folates. k_{cat} values are a function of the off rates of the products of the reaction. k_7, the off rate of the first product, MgADP, is a composite rate term which also includes the interconversion of the quaternary catalytic complex, i.e., EABC → EPQR. For reasons discussed below, k_7 is probably rate limiting for turnover. For an ordered mechanism, K_m values are a function of k_{cat} and k_3, the on rate of the folate substrate, and cannot be used as a measure of affinity for the enzyme. k_{cat}/K_m, the pseudo-first-order rate constant, which is used to compare substrate effectiveness at concentrations considerably below the K_m, is also the on rate (k_3) for the folate substrate (Fromm, 1975). The apparent affinities of folates that lacked detectable substrate activity or were very poor substrates were assessed by their ability to inhibit activity with pteroylmonoglutamate substrates. Under the conditions used (high MgATP and glutamate concentrations), the K_i(app) values obtained do not reflect dissociation constants (K_{is}) but should approximate the K_m values for these poor alternate substrates. True dissociation constants would only be obtained if the compounds were acting as dead-end inhibitors, and the only folate substrate for which a K_{is} has been obtained is aminopterin (10 μM).

k_{cat} values for (6*S*)-$H_4PteGlu_n$ decrease with increasing glutamate chain length. The value drops about 2-fold for each glutamate moiety added up to the pentaglutamate except for a 5-fold drop between the di- and triglutamates. k_{cat} values for $H_2PteGlu_n$ are almost identical with the $H_4PteGlu_n$ series for the mono- and diglutamates but fall off faster with longer glutamate derivatives such that the pentaglutamate is essentially inactive as a substrate. The $PteGlu_n$ series also shows a drop in k_{cat} with extension of the polyglutamate chain. However, although the k_{cat} for PteGlu is identical with that of (6*S*)-$H_4PteGlu$, the value for $PteGlu_2$ is slightly higher than for $H_4PteGlu_2$, and the value for $PteGlu_3$ is 4-fold higher than for $H_4PteGlu_3$. k_{cat} is decreased about 20% for (6*RS*)-$H_4PteGlu$, 60% for (6*S*)-5-methyl-$H_4PteGlu$ and (6*R*)-5,10-methylene-$H_4PteGlu$, and 70% for (6*R*)-10-formyl-$H_4PteGlu$ compared to (6*S*)-$H_4PteGlu$.

Although differences in k_{cat} could reflect differences in off rates of the folate product (k_9), this is unlikely as it would require that the off rate decrease with increasing glutamate chain length of the product, the converse of which appears to be the case with folate substrates (see below). The data suggest that the interconversion of the quaternary catalytic complex is rate limiting and imply that PteGlu, $H_2PteGlu$, and (6*S*)-$H_4PteGlu$ represent the optimal configurations for catalysis and that the 5- and 10-position substitutions of naturally occurring folates hinder catalysis. Increases in the glutamate chain length of folates decrease the catalytic rate.

Although affinities of folate substrates were not assessed, the initial binding or on rates could be obtained from k_{cat}/K_m. At saturating MgATP and glutamate concentrations, folate binding to the enzyme would be rate limiting at low folate concentrations. The on rates for (6*S*)-$H_4PteGlu$, (6*RS*)-$H_4PteGlu$ and (6*R*)-10-formyl-$H_4PteGlu$ are identical while $H_2PteGlu$ binds about 50% faster. The on rates for (6*S*)-5-methyl-$H_4PteGlu$, (6*R*)-5,10-methylene-$H_4PteGlu$, and PteGlu are about 20-fold lower than for $H_4PteGlu$. Addition of glutamate moieties to (6*S*)-$H_4PteGlu$ does not affect the on rate for the diglutamate and causes only a 40% reduction in rate for the triglutamate, while the rate is reduced 3–4-fold with each additional glutamate residue with a slightly larger drop for the hexaglutamate. For the hexa- and heptaglutamate derivatives, the K_i(app) values obtained when these compounds were used as inhibitors of the reaction give an approximate measure of their K_m values. As K_m values increase beyond the triglutamate, the on rate decreases at a faster rate than k_{cat}. $H_2PteGlu_2$ and $PteGlu_2$ also have on rates identical with their monoglutamates, while the rate decreases for $PteGlu_3$. These data demonstrate little, if any, discrimination in initial binding between these pteroylmono- and -diglutamates and moderate decreases in initial binding with longer chain length derivatives. The fastest on rate for any folate substrate, 4.3 × 10^5 M^{-1} s^{-1}, was observed with $H_2PteGlu_{1,2}$. This slow rate is several orders of magnitude lower than the diffusion-controlled limit and may reflect a conformational change in the protein on substrate binding.

These data show that the conformation of the folate molecule and substitutions of the pterin moiety optimal for catalysis can be quite different from that required for binding to the protein and that increasing the glutamate chain length of the folate molecule causes an increased difficulty in positioning the γ-carboxyl of the terminal glutamate residue at the active site(s). In addition, the substrate specificity of this enzyme for pteroylmonoglutamate derivatives may provide information on the folate binding site requirements but is not necessarily a good indicator of which compounds are the most effective substrates in vivo. An effective in vivo substrate would have to be converted to long-chain polyglutamate derivatives. Little or no substrate activity was detected with (6*S*)-H_4PteGlu$_6$, (6*R*)-10-formyl-H_4PteGlu$_3$, and H_2PteGlu$_4$ although their monoglutamate derivatives were execellent substrates. Detailed kinetic analyses were not carried out with polyglutamate derivatives of 5- and 10-substituted folates because of their low substrate activity. However, the limited studies carried out suggest that the low activities of 5-methyl-H_4PteGlu$_2$ and 10-formyl-H_4PteGlu$_3$ are due to large decreases in k_{cat} rather than increases in K_m. Similarly, the 4-amino substitution of aminopterin increases the on rate of this compound for the enzyme and its substrate effectiveness compared to PteGlu. However, its diglutamate derivative is a very poor substrate such that aminopterin is converted primarily to the diglutamate form under conditions where PteGlu is converted to the tetraglutamate (Cook et al., 1987). The accumulation of hexaglutamate derivatives in vivo in hog liver is due primarily to the very low k_{cat} values with hexaglutamate substrates.

Long chain length folylpolyglutamates comprise over 95% of the folates in mammalian tissues. Although they have very low catalytic activity, they do act as inhibitors of the reaction in vitro with K_i(app) values similar to their in vivo concentrations. Consequently, they may act as inhibitors of folylpolyglutamate synthesis in vivo by competing with incoming pteroylmonoglutamate for folylpolyglutamate synthetase, and consequently act as regulators of intracellular folate stores. This potential for regulation, which was previously suggested by studies with partially purified enzyme from rat liver (McGuire et al., 1980) and Chinese hamster ovary cells (Foo & Shane, 1982), will be discussed in more detail in an accompanying paper (Cook et al., 1987).

The specificity for pteroylmonoglutamate derivatives reported here is generally similar to that previously reported for partially purified enzyme preparations from hog liver (Bognar et al., 1983), rat liver (McGuire et al., 1980), and mouse liver (Moran & Colman, 1984; Moran et al., 1985), except that 5,10-methylene-H_4PteGlu was reported to be a good substrate for the rat and mouse liver enzymes. Although this may reflect the different sources of the enzymes used, it is possible that 5,10-methylene-H_4PteGlu was converted to H_4PteGlu during the course of the enzyme assays in some of these studies. Substitution of PteGlu with a 10-methyl group increased the K_m of the compound for the mouse liver enzyme while 10-formylation of H_4PteGlu decreased the K_m (Moran et al., 1985). These data have been interpreted to suggest that folates bind to the mammalian enzyme in a conformation in which the N-5, C-6, C-9, and N-10 atoms approximate the shape of the imidazolidine ring introduced by the 5,10-methylene substitution (Moran et al., 1985). Dipole interactions between the formyl group and either the N-5 or the N-10 atom would explain why 5-formyl- and 10-formyl-H_4PteGlu are good substrates while 5-methyl- and 10-methyl-substituted folates are poor substrates. However, this is not consistent with the excellent binding of H_4PteGlu and H_2PteGlu and the poor binding of 5,10-methylene-H_4PteGlu reported in the current study. Tight binding of 10-formyl-H_4PteGlu was inferred in previous studies from the K_m value (Moran & Colman, 1984; Moran et al., 1985), which is not necessarily an accurate reflection of affinity. The data presented here indicate that the lowered K_m is generated by a lowered k_{cat} and that the on rate is unaffected by the 10-formyl substitution. In addition, as reported previously for the rat liver enzyme (McGuire et al., 1980), (6*R*)-H_4PteGlu is an effective substrate with an identical on rate and an impaired k_{cat} compared to the 6*S* isomer. This would argue against a rigid conformation around the N-5, C-6, C-9, and N-10 atoms being required for binding or catalysis and suggests flexibility around this region.

The pteroylmonoglutamate substrate specificity of the hog liver enzyme differs markedly from that observed with bacterial folylpolyglutamate synthetases. Bacterial enzymes utilize a variety of reduced pteroylmonoglutamates as their preferred substrates, depending on the source of the enzyme, and oxidized folates have little affinity for these proteins. However, in each case, the preferred, and sometimes only, polyglutamate substrates are the 5,10-methylene-H_4PteGlu$_n$ derivatives (Shane, 1980b, 1982; Cichowicz et al., 1981; Shane & Cichowicz, 1983; Bognar & Shane, 1983; Ferone et al., 1983). The lack of substrate activity of H_4PteGlu$_n$ derivatives is primarily due to low k_{cat} values. In these cases, the specificity for the 5,10-methylene derivatives may be ascribed to the rigid conformation introduced into the folate molecule by the imidazolidine ring (Poe et al., 1979) which hinders the rotation of the *p*Aba moiety around the C-1′ to C-4′ axis and distorts the angle between the planes of the *p*Aba and pyrazine rings (Fontecilla-Camps et al., 1979).

The specificity of the folate binding site of the hog liver enzyme and the regulatory implications of the differences in substrate effectiveness of different folates will be discussed in more detail in accompanying papers (George et al., 1987; Cook et al., 1987).

Glutamate Specificity. The affinity ($K_{is.}$) of glutamate, the third substrate to bind to the enzyme, cannot be obtained by initial rate kinetic analysis. The K_m value equals $k_{cat}[(k_6 + k_7)/k_5k_7]$. If k_7 is rate limiting for the reaction, then the upper limit for $K_{is.}$ is the K_m value ($K_m = K_{is.} + k_{cat}/k_5$). Regardless of whether k_7 is rate limiting, competition of labeled glutamate incorporation into polyglutamates by unlabeled glutamate analogues will provide an apparent K_i that will be equivalent to the dissociation constant for the analogue if it is a dead-end inhibitor but will be equivalent to the K_m for the analogue if it is an alternate substrate.

Competition studies with the rat liver (McGuire et al., 1980) and *Corynebacterium* (Shane, 1980b) enzymes demonstrated that a wide variety of glutamate analogues, including the D isomer, lack apparent affinity for these proteins. Later studies demonstrated that homocysteate is an alternate substrate for the *Corynebacterium* (Shane & Cichowicz, 1983) and *Lactobacillus* (Bognar & Shane, 1983) enzymes and that 4-fluoroglutamate is a substrate for the *Lactobacillus* (Bognar & Shane, 1983) and rat liver (McGuire & Coward, 1985) enzymes. Both of these glutamate analogues appear to act as chain termination inhibitors in that their incorporation into the folate molecule prevents or greatly retards the further addition of glutamate moieties. Galivan et al. (1985) have recently shown that γ-fluoromethotrexate is a very poor substrate for rat liver folylpolyglutamate synthetase.

A similar tight specificity was found for the glutamate binding site of the hog liver enzyme. Almost all modifications

of the L-glutamate molecule lead to complete loss of apparent affinity. The only exceptions are compounds that are alternate substrates. The tightest binding nonsubstrate, L-2-hydroxyglutarate, has a K_i in excess of 10 mM. If k_7 is rate limiting, this would indicate that the specificity suggested by the competition studies is a true reflection of the very poor affinity of nonsubstrate glutamate analogues for the enzyme. However, if k_7 is not rate limiting, and k_6, the off rate for glutamate, is very high, the $K_{is.}$ for glutamate would also be high. Although we consider this unlikely, the possibility that lack of inhibition by glutamate analogues is due to low affinity for the analogues and glutamate, and consequently does not indicate specificity of the glutamate binding site, cannot be excluded at present.

When unlabeled L-glutamate was used as an alternate substrate inhibitor of the reaction, the inhibition constant obtained was similar to the K_m for L-glutamate. Replacement of the γ-carboxyl group with a sulfonate group (homocysteate) leads to a 12-fold increase in K_m while introduction of a florine atom at the 4 carbon in the *threo* configuration increases the K_m about 3-fold. The apparent K_m values for the *erythro* isomer and for 4-methylglutamate are very high. Methylation at carbon 3 is tolerated better than at the 4-position although 3-methylglutamate is still a very poor substrate. An increase in K_m can be caused by an increase in the off rate (k_6) and/or a decrease in the on rate (k_5) of the analogue i.e., a decrease in affinity and/or an increase in k_{cat}. Metabolism studies suggest similar k_{cat}'s for glutamate, homocysteate, and 4-*threo*-fluoroglutamate and possibly decreased values for the other alternate substrates. The increased K_m values for these analogues most likely reflect decreased affinities.

In short-term incubation studies with homocysteate and 4-fluoroglutamate, only one residue was added to the folate molecule. However, in extended incubation studies, more than one 4-fluoroglutamate residue was incorporated. Under conditions where (6*S*)-H_4PteGlu was converted primarily to the hexaglutamate with L-glutamate as a substrate, the major products found with 4-*threo*-fluoroglutamate as the substrate were (6*S*)-H_4PteGlu–(fluoroglutamate)$_{2,3}$. This is consistent with the weak substrate activity reported for γ-fluoromethotrexate (Galivan et al., 1985). Although 4-fluoroglutamate cannot be considered a chain terminating inhibitor in the strict sense, it would be expected to act as such in vivo. The slow metabolism of folates to modified polyglutamate derivatives in the presence of this compound would result in a decreased ability of cells to retain these folates. Surprisingly, similar products were detected regardless of whether *erythro*- or *threo*-fluoroglutamate was used as the substrate, despite large differences in their K_m values. However, McGuire et al. (1986) have recently shown that, although the *threo* isomer is the preferred alternate glutamate substrate, the *erythro* isomer of pteroylfluoroglutamate is the preferred alternate folate substrate.

The K_m for L-glutamate with (6*S*)-H_4PteGlu as the folate substrate is about 350 μM, which is similar to that reported for other mammalian and bacterial folylpolyglutamate synthetases (Bognar & Shane, 1983; Bognar et al., 1985; Masurekar & Brown, 1975; McGuire et al., 1980; Pristupa et al., 1984; Shane, 1980c). The K_m with other pteroylmonoglutamate substrates is similar except for a 3-fold increase with PteGlu. This almost certainly reflects a decreased affinity for glutamate with this folate substrate. The K_m for glutamate decreases with $PteGlu_2$ as the substrate. Although this may reflect increased affinity of glutamate for the enzyme–nucleotide–pteroyldiglutamate complex, it should be noted that a decrease in k_{cat}, which occurs with pteroyldiglutamate substrates, can generate a lowered K_m for glutamate in the absence of any change of affinity for glutamate. The K_m also varies with pH, which reflects the proportion of unprotonated glutamate present (Cichowicz & Shane, 1987).

Nucleotide Specificity. The specificity of the nucleotide binding site is less stringent. MgATP is the best substrate. MgdATP, ITP, and UTP are also fairly good substrates with reduced k_{cat} values and greatly reduced on rates (k_{cat}/K_m). ZTP is also a substrate. This unusual nucleotide has been reported to be an alarmone for folate deficiency in bacteria (Bochner & Ames, 1982) and may play a role in the regulation of mammalian metabolism (Sabina et al., 1984). The substrate effectiveness of MgUTP is surprising and differs from that reported for the rat liver enzyme (McGuire et al., 1980). However, MgUTP is also a substrate for the *Corynebacterium* and *Lactobacillus* enzymes (Shane, 1980b; Bognar & Shane, 1983). The slow on rate for MgATP (1.3×10^5 M^{-1} s^{-1}) may be due to a conformational change on nucleotide binding. A conformational change would explain why MgATP protects the *Corynebacterium* enzyme from proteolytic inactivation (Shane, 1983).

A variety of nucleotide derivatives are potent inhibitors of the reaction with affinities similar to that of MgATP ($K_{is.}$ = 3 μM). These include β,γ-methylene-ATP, β,γ-imido-ATP, ATPγS, and Ap_5A and Ap_6A. Ap_nA compounds have been proposed to be pleiotropically acting alarmones in mammalian cells (Varshavsky, 1983; Zamecnik, 1983).

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Registry No. ZTP, 82989-82-0; ATP, 56-65-5; dATP, 1927-31-7; ITP, 132-06-9; UTP, 63-39-8; PteGlu, 59-30-3; $PteGlu_2$, 19360-00-0; $PteGlu_3$, 89-38-3; $PteGlu_4$, 29701-38-0; $PteGlu_5$, 33611-85-7; $PteGlu_6$, 35409-55-3; $PteGlu_7$, 6484-74-8; H_2PteGlu, 4033-27-6; $H_2PteGlu_2$, 52723-92-9; $H_2PteGlu_4$, 73358-95-9; $H_2PteGlu_5$, 53749-53-4; $H_2PteGlu_6$, 105881-92-3; $H_2PteGlu_7$, 105857-99-6; (6*RS*)-H_4PteGlu, 135-16-0; (6*S*)-H_4PteGlu, 71963-69-4; (6*R*)-10-formyl-H_4PteGlu, 74644-66-9; (6*R*)-5,10-methylene-H_4PteGlu, 31690-11-6; (6*S*)-5-methyl-H_4PteGlu, 31690-09-2; (6*S*)-$H_4PteGlu_2$, 105879-64-9; (6*S*)-$H_4PteGlu_3$, 88903-89-3; (6*S*)-$H_4PteGlu_4$, 105816-60-2; (6*S*)-$H_4PteGlu_5$, 88903-90-6; (6*S*)-$H_4PteGlu_6$, 88904-13-6; (6*S*)-$H_4PteGlu_7$, 105816-61-3; β,γ-methylene-ATP, 3469-78-1; β,γ-imido-ATP, 25612-73-1; ATPγS, 35094-46-3; Ap_5A, 2068-77-1; MgATP, 1476-84-2; folylpoly-γ-glutamate synthetase, 63363-84-8; L-glutamate, 56-86-0; L-homocysteate, 14857-77-3; 4-*erythro*-fluoro-DL-glutamate, 91383-48-1; 4-*threo*-fluoro-DL-glutamate, 91383-47-0; pyrophosphate, 2466-09-3; phosphate, 14265-44-2; aminopterin, 54-62-6.

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