FOLYLPOLYGLUTAMATES AS SUBSTRATES AND INHIBITORS OF FOLATE-DEPENDENT ENZYMES

ROWENA G. MATTHEWS,* CHANDRALEKHA GHOSE,* JACALYN M. GREEN,* KEITH D. MATTHEWS* and R. BRUCE DUNLAP†

*The Department of Biological Chemistry and the Biophysics Research Division, The University of Michigan, Ann Arbor, MI 48109

†Department of Chemistry, University of South Carolina, Columbia, SC

INTRODUCTION

While folate is normally transported from cell to cell as CH_3–H_4PteGlu$_1$*, a monoglutamate derivative of tetrahydrofolate, once CH_3–H_4PteGlu$_1$ enters the cell it is rapidly converted to other folate derivatives (e.g. tetrahydrofolate) and additional glutamyl residues are added by the enzyme folylpolyglutamate synthase (EC 6.3.2.17). In mammalian cells the concentrations of monoglutamate folate derivatives are very low and most folate derivatives bear multiple glutamyl residues (1–3), with the predominant forms being penta- and hexaglutamates.

Studies in many laboratories have established that folylpolyglutamates are substrates for folate-dependent enzymes and in many cases they have lower K_m values than the corresponding monoglutamate substrates. Both folyl- and antifolylpolyglutamates have also been shown to be potent inhibitors of folate-dependent enzymes. Methotrexate, an effective antineoplastic agent, is considered to produce its cytotoxic effects by inhibition of dihydrofolate reductase (EC 1.5.1.3). Methotrexate is a substrate for folylpolyglutamate synthetase and polyglutamylation results in enhanced efficacy and toxicity of this agent (4–7). Due to its direct effect on dihydrofolate reductase, administration of methotrexate results in profound shifts in cellular folate pools (8). Most importantly, cellular levels of dihydrofolate polyglutamates rise in response to methotrexate administration, e.g. from less than 1% to

*The abbreviations used are: AICAR, aminoimidazolecarboxamide ribonucleotide; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_3–H_4PteGlu$_n$, methyltetrahydropteroylpolyglutamate with n glutamyl residues; CH_2–H_4PteGlu$_n$, 5,10-methylenetetrahydropteroylpolyglutamate with n glutamyl residues; H_2PteGlu$_n$, dihydropteroylglutamate with n glutamyl residues; CHO–H_4PteGlu$_n$; formyltetrahydropteroylpolyglutamate with n glutamyl residues; PteGlu$_n$, pteroylpolyglutamate with n glutamyl residues; H_4PteGlu$_n$, tetrahydropteroyl polyglutamate with n glutamyl residues; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; CH_2–H_4folate, 5,10-methylenetetrahydrofolate; MTX, methotrexate (4-amino-10-methylpteroylglutamate); MTX-Glu$_n$, methotrexate with a total of n glutamyl residues; CH_3–H_4folate, 5-methyltetrahydrofolate; HCy, homocysteine; Met, methionine; FAD, flavin adenine dinucleotide; CH=H_4folate, 5,10-methenyltetrahydrofolate.

greater than 30% of the total folate pool of human MCF-7 breast cancer cells (8). *In vitro* studies suggest that a number of folate-dependent enzymes will be inhibited by elevated concentrations of either methotrexate or dihydrofolate polyglutamates. These enzymes include thymidylate synthase (EC 2.1.1.45) (9–11), AICAR transformylase (EC 2.1.2.3) (12) and methylenetetrahydrofolate reductase (EC 1.5.1.20) (13).

Our ability to predict the effects of antifolate drugs and to manipulate them for maximal clinical efficacy depends on a detailed understanding of the specificity of folate-dependent enzymes for folylpolyglutamate substrates and inhibitors, both those enzymes which are targets for chemotherapy such as dihydrofolate reductase, thymidylate synthase, AICAR transformylase, and GAR transformylase (EC 2.1.2.2), and those which are not, but whose inhibition might result in toxicity due to interference with cellular processes in non-dividing cells.

MATERIALS AND METHODS

Isolation and assay of thymidylate synthase from Lactobacillus casei. Thymidylate synthase was purified from amethopterin-resistant *L. casei* by the method of Lyon *et al.* (14). Enzyme was stored prior to use at -70°C in 50 mM Tris chloride buffer, pH 7.3/20% glycerol in aliquots sufficient for one day's use. Under these conditions enzyme activity was stable for several months. For kinetic studies spectrophotometric assays were performed at 25°C and the absorbance changes associated with the conversion of CH_2–H_4-folate to H_2folate were measured at 340 nm. The CH_2–H_4folate was generated in the cuvet by condensation of (6-*R,S*)H_4folate and formaldehyde. The assay mixture (1 ml) contained 100 mM potassium phosphate buffer, pH 6.8, 100 μM dUMP, 50 mM 2-mercaptoethanol, 6 mM formaldehyde and $PteGlu_n$ and H_4folate as indicated. Prior to addition of H_4folate, the assay solution was equilibrated with nitrogen for 3 min. After addition of H_4folate, incubation was continued for another 5 min under nitrogen to ensure complete formation of CH_2–H_4folate. The cuvet was sealed with parafilm and the assay was initiated by addition of 10 μl of enzyme.

Preparation of folylpolyglutamate substrates and inhibitors. For studies with monoglutamate substrate (6-*R,S*)H_4folate was prepared by catalytic hydrogenation of $PteGlu_1$ and purified as described by Ross *et al.* (15). For studies with polyglutamate substrates (6-*S*)$H_4PteGlu_1$ and (6-*S*)CH_3–$H_4PteGlu_6$ were prepared by enzymatic reduction of $PteGlu_1$ as described by Matthews *et al.* (16).

Preparation and assay of methionine synthase from pig liver. Crude extracts of methionine synthase from pig liver were prepared by homogenization in a

Waring blendor in 50 mM potassium phosphate buffer, pH 7.2. The enzyme was absorbed onto DEAE cellulose equilibrated with the same buffer and the DEAE cellulose was collected using filtration through a Buchner funnel. The DEAE cellulose was rinsed with 100 mM phosphate buffer and the enzyme was batch-eluted with 400 mM phosphate buffer. After dialysis against 50 mM phosphate buffer, the enzyme was subjected to ammonium sulfate fractionation, and the fraction precipitating between 25 and 45% saturation was collected and dialyzed against 50 mM phosphate buffer. The enzyme was then applied to a column of DEAE-52, rinsed with 100 mM phosphate buffer containing 2 μM adenosylmethionine, and eluted with a linear gradient of 100–500 mM phosphate buffer containing 2 μM adenosylmethionine. Fractions containing methionine synthase activity were pooled, dialyzed against 50 mM phosphate buffer containing 2 μM adenosylmethionine, brought to 10% glycerol and stored until use at –70°C in small aliquots. The enzyme used for these studies had a specific activity of 0.019 μmoles $min^{-1}mg^{-1}$ and was purified 210-fold from the crude extract.

Methionine synthase was assayed by measuring the conversion of $^{14}CH_3$–$H_4PteGlu_1$ and homocysteine to $H_4PteGlu_1$ and [^{14}C-*methyl*]methionine by a modification of the procedure used by Taylor and Weissbach (17). Assay mixtures (1 ml) contained 100 mM potassium phosphate buffer, pH 7.2, 500 μM homocysteine, 95 μM adenosylmethionine, 25 mM dithiothreitol, 5 μM cyanocobalamin, and methionine synthase. After incubation for 5 min at 37°C, 125 μM (6-*S*)$^{14}CH_3$–$H_4PteGlu_1$, added as a 6-*R*,*S* racemic mixture, was introduced to initiate the reaction. After incubation for up to 20 min at 37°C, the reaction was terminated by heating for 2 min at 96°C, and the cooled assay mixture was passed over a 0.5 × 3 cm column of Bio-Rad AG1-X8 to separate labeled CH_3–$H_4PteGlu_1$ from labeled methionine. The formation of labeled methionine was determined by scintillation counting. Where tritiated (6-*S*)CH_3–$H_4PteGlu_6$ was used as the substrate, the same general protocol was followed, except that lower concentrations of this substrate were employed for the assays, as indicated below.

RESULTS AND DISCUSSION

Affinity of Folate-Dependent Enzymes for Folylpolyglutamates

To provide a firm basis for an understanding of the specificity of folate-dependent enzymes for folylpolyglutamylated substrates and inhibitors it is desirable to compare dissociation constants for folates which differ only in the number of glutamyl residues in the polyglutamate chain. We have elected to characterize the four enzymes involved in the metabolism of CH_2–H_4 folate (Fig. 1); viz. serine hydroxymethyltransferase (EC 2.1.2.1) (16), methylenetetrahydrofolate reductase (13), thymidylate synthase (18), and methylenetetra-

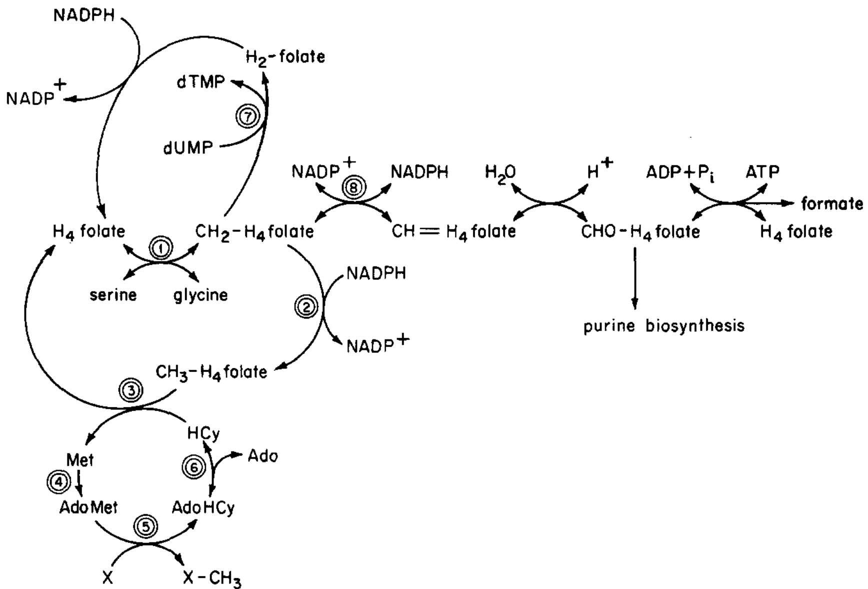


FIG. 1. Major pathways of folate metabolism in mammalian cells. The enzymes indicated are 1, serine hydroxymethyltransferase; 2, methylenetetrahydrofolate reductase; 3, methionine synthase; 4, adenosylmethionine synthetase; 5, adenosylmethionine-dependent methyl transferases (there are many, with different methyl acceptor specificities); 6, adenosylhomocysteine hydrolase; 7, thymidylate synthase; and 8, methylenetetrahydrofolate dehydrogenase (one activity of a trifunctional protein which catalyzes the three steps required for interconversion of CH_2-H_4-folate and H_4folate by way of CH=H_4folate and CHO-H_4folate).

hydrofolate dehydrogenase (EC 1.5.1.5) (15). In each case we have used the enzyme purified from pig liver, a readily available mammalian tissue, so that species variation in folylpolyglutamate specificity will not complicate the comparison. Our approach has been to measure the K_i values for a series of inhibitory polyglutamates which are not metabolized by the enzyme in question (dead-end inhibitors) and which exhibit competitive inhibition with respect to the folate substrate. In general such K_i values can be equated with dissociation constants characterizing the equilibria between the competitive inhibitors and the enzyme forms to which they bind. Kinetic determination of K_d values has the advantage that nonspecific binding of the inhibitor to the enzyme is generally undetectable. Results from studies of these four enzymes are shown in Figure 2. It is apparent that these folate-dependent enzymes all bind folylpolyglutamate inhibitors more tightly than the corresponding monoglutamates. However, the chain length which is bound most tightly is different for each of these enzymes, and the ratio of enzyme affinity for the monoglutamate and for the most tightly bound polyglutamate is highly variable. Thus methylenetetrahydrofolate reductase binds $H_2PteGlu_6$ 430-

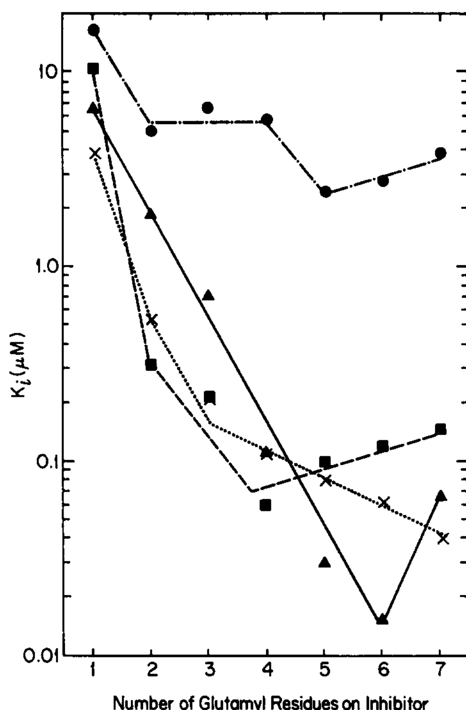


FIG. 2. Comparison of the affinities of the folylpolyglutamate inhibitors of four folate-dependent enzymes from pig liver. Semilogarithmic plots of K_i vs. the number of glutamyl residues on the inhibitor are shown for methylenetetrahydrofolate dehydrogenase (●), methylenetetrahydrofolate reductase (▲), serine hydroxymethyltransferase (×), and thymidylate synthase (■). For methylenetetrahydrofolate reductase, K_i values were determined kinetically for inhibition of enzyme by $H_2PteGlu_n$ inhibitors in the presence of saturating NADPH and varied CH_2-$H_4PteGlu_1$ (13). For thymidylate synthase, K_i values were determined kinetically for inhibition of enzyme by $PteGlu_n$ inhibitors in the presence of saturating dUMP and varied CH_2-$H_4PteGlu_1$ (18). For serine hydroxymethyltransferase, K_d values for the dissociation of CH_3-$H_4PteGlu_n$ from E·CH_3-$H_4PteGlu_n$·glycine ternary complexes were determined spectrophotometrically (16). For methylenetetrahydrofolate dehydrogenase, K_i values were determined kinetically for inhibition of the enzyme by $H_2PteGlu_n$ inhibitors in the presence of saturating $NADP^+$ and varied CH_2-$H_4PteGlu_1$ (15).

fold more tightly than $H_2PteGlu_1$, while methylenetetrahydrofolate dehydrogenase binds $H_2PteGlu_5$ only 7-fold more tightly than $H_2PteGlu_1$. Based on extrapolations from these data, we would expect cellular folate-dependent enzymes to differ markedly in their susceptibility to inhibition by folyl- or antifolylpolyglutamates. Antifolates which are not substrates or which are poor substrates for folylpolyglutamate synthetase may differ in their cellular effects from derivatives which are polyglutamylated. Studies from several laboratories (19–21) have established that introduction of a methyl group of N^5 on H_4folate greatly reduces the affinity of folylpolyglutamate

synthetase for this compound and suggests possibilities for alteration of structurally analogous antifolates in order to manipulate their degree of polyglutamylation.

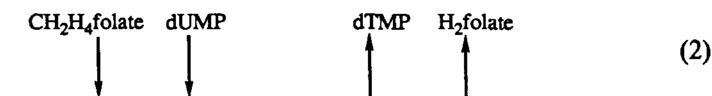
Effects of Substrate Polyglutamylation on Catalysis

If we assume that the interaction between the polyglutamate chain and an enzyme will be the same for substrates and for inhibitory substrate analogues, this binding energy may be expressed in a variety of ways. The increased affinity of an enzyme for polyglutamate substrates may manifest itself in a lower K_m for the folate substrate. Tighter binding of both polyglutamate substrates and products to an enzyme may be due to decreased rates of dissociation of these compounds from the enzyme surface and may result in product release becoming rate limiting in catalysis with a concomitant decrease in V_{max}. Alternatively, binding of a folate polyglutamate may enforce a conformational change of the enzyme which alters the affinity of the binary complex for a non-folate substrate. In such case, the K_m for the folate polyglutamate substrate might be the same as or even higher than the K_m for the monoglutamate, but the K_m for the non-folate substrate would be decreased in the presence of a folylpolyglutamate substrate (enhanced ligand synergism). A third possibility is that the interaction between the polyglutamate chain and the enzyme will not be optimal in the Michaelis complexes but only in the transition state of the reaction (22). In such case, the increased affinity of the enzyme for polyglutamate substrates will appear as an increase in V_{max} rather than as a decrease in K_m for the folate substrate. Finally, polyglutamate substrates may be channeled from one active site to another in multifunctional proteins, or in macromolecular complexes of enzymes while the more weakly bound monoglutamate substrates dissociate and then rebind (23).

In the case of methylenetetrahydrofolate reductase (13) the enhanced affinity of the enzyme for folylpolyglutamate inhibitors is mirrored in lower values for the K_m of CH_2–H_4PteGlu$_n$ substrates with the lowest K_m values observed with CH_2–H_4PteGlu$_6$. In contrast, while PteGlu$_4$ is bound 175-fold more tightly to porcine thymidylate synthase than is PteGlu$_1$, the K_m for CH_2–H_4PteGlu$_4$ is only 2.7-fold lower than that for CH_2–H_4PteGlu$_1$ and changes in V_{max} and in the K_m for dUMP are also very small (18). In this case steady-state kinetic studies suggest an ordered sequential mechanism with dUMP binding prior to CH_2–H_4PteGlu$_1$ (equation 1). With CH_2–H_4PteGlu$_4$,

(1)

the order of substrate binding and product release is reversed (equation 2).



(2)

The alteration in the kinetic mechanism presumably results because the off-constants characterizing the dissociation of both substrate and product from binary complexes with the enzyme are decreased with polyglutamates as compared with the monoglutamate. The K_m value for the second substrate in an ordered sequential mechanism is a complicated function of rate and equilibrium constants, and when the mechanism changes, the resultant alterations in the rate constants obscure the contribution of K_d to the observed K_m values for the folate substrates.

Such changes in kinetic mechanism on going from monoglutamate to polyglutamate substrates may be the rule rather than the exception. When the kinetic mechanism changes, such that the form of enzyme which binds substrate is different for monoglutamate and polyglutamate substrates, the K_i value measured for folylpolyglutamate inhibitors may vary depending on which folate substrate is used. Allegra *et al.* (12) have reported that the K_i value for MTX–Glu_5 interacting with AICAR transformylase is 57 nM when measured in competition with CHO–$H_4PteGlu_1$, but is only 6 μM when measured in competition with CHO–$H_4PteGlu_5$. We have made similar observations when measuring K_i values for $PteGlu_n$ inhibitors of thymidylate synthase from *L. casei* that are summarized in Table 1. Our studies of thymidylate synthase from *L. casei* suggest that this enzyme too undergoes a change in kinetic mechanism with polyglutamate as compared to monoglutamate substrates. Product inhibition studies with CH_2–$H_4PteGlu_1$ and CH_2–$H_4PteGlu_6$ substrate are summarized in Tables 2 and 3. The results are

TABLE 1. INHIBITION OF THYMIDYLATE SYNTHASE BY $PteGlu_n$*

Inhibitor	Substrate	K_i^{app} (μM)	Substrate	K_i^{app} (μM)
$PteGlu_1$	CH_2–$H_4PteGlu_1$	29	CH_2–$H_4PteGlu_1$	29
$PteGlu_2$	CH_2–$H_4PteGlu_1$	6	CH_2–$H_4PteGlu_2$	13
$PteGlu_3$	CH_2–$H_4PteGlu_1$	1	CH_2–$H_4PteGlu_3$	9
$PteGlu_4$	CH_2–$H_4PteGlu_1$	0.8	CH_2–$H_4PteGlu_4$	22
$PteGlu_6$	CH_2–$H_4PteGlu_1$	0.07	CH_2–$H_4PteGlu_6$	2

*Thymidylate synthase from *L. casei* was used for these experiments. Assays contained 100 μM dUMP and varied CH_2–$H_4PteGlu_n$. In all cases the observed inhibition was competitive with respect to the folate substrate except where $PteGlu_1$ was the inhibitor — in this latter case a small effect on the intercepts of double reciprocal plots was also observed.

TABLE 2. PRODUCT AND DEAD END INHIBITION PATTERNS WITH CH_2-H_4PteGlu$_1$ SUBSTRATE

Inhibitor	Varied substrate	Fixed substrate	Pattern Expected*	Observed
PteGlu$_6$	dUMP	CH_2-H_4PteGlu$_1$	uncomp	uncomp
dTMP	dUMP	CH_2-H_4PteGlu$_1$	comp	comp
dTMP	CH_2-H_4PteGlu$_1$	dUMP (sat.)	noncomp	noncomp
H_2PteGlu$_1$	CH_2-H_4PteGlu$_1$	dUMP	noncomp	noncomp
H_2PteGlu$_1$	dUMP	CH_2-H_4PteGlu$_1$ (sat.)	uncomp	uncomp

*Expected patterns are those for a kinetic mechanism in which addition of dUMP precedes addition of CH_2-H_4PteGlu$_1$ and dissociation of H_2PteGlu$_1$ precedes that of dTMP. Thymidylate synthase from *L. casei* was used for these experiments.

TABLE 3. PRODUCT AND DEAD END INHIBITION PATTERNS WITH CH_2-H_4PteGlu$_6$ SUBSTRATE

Inhibitor	Varied substrate	Fixed substrate	Pattern Expected*	Observed
PteGlu$_6$	dUMP	CH_2-H_4PteGlu$_6$	noncomp	noncomp
dTMP	dUMP	CH_2-H_4PteGlu$_6$	noncomp	comp
dTMP	CH_2-H_4PteGlu$_6$	dUMP (sat.)	uncomp	uncomp
H_2PteGlu$_6$	CH_2-H_4PteGlu$_6$	dUMP	comp	comp
H_2PteGlu$_6$	dUMP	CH_2-H_4PteGlu$_6$ (sat.)	noncomp	noncomp

*Expected patterns are those for a kinetic mechanism in which addition of CH_2-H_4PteGlu$_6$ precedes addition of dUMP and dissociation of dTMP precedes that of H_2PteGlu$_6$. Thymidylate synthase from *L. casei* was used for these experiments.

fully consistent with those obtained using fetal pig liver, and suggest that dUMP is bound prior to CH_2-H_4PteGlu$_1$ but after CH_2-H_4PteGlu$_6$ and dTMP is released after H_2PteGlu$_1$ but before H_2PteGlu$_6$. Our studies also suggest that reversal in the order of substrate addition and product release has occurred with CH_2-H_4PteGlu$_4$ (data not shown). Our studies with di- and triglutamate substrates suggest that these substrates utilize a random order mechanism, where either dUMP or CH_2-H_4 folate may bind first and either H_2 folate or dTMP may be released last, and the product inhibition patterns seen are inconsistent with either ordered sequential mechanism.

Incorporation of Plasma Folate Derivatives into the Cellular Folate Pool

The normal circulating folate derivative is CH_3-H_4PteGlu$_1$. Dietary folates are generally degraded to the monoglutamate level by intestinal folylpoly-glutamate-γ-hydrolases, and the resulting derivatives converted to CH_3-H_4PteGlu$_1$. It is of interest to ascertain how CH_3-H_4PteGlu$_1$ is incorporated into the cellular folate pool after transport across the cell membrane. CH_3-H_4PteGlu$_1$ is a poor substrate for folylpolyglutamate synthase (19–21) and is

probably not polyglutamylated under *in vivo* conditions. Thus the first step towards incorporation into the cellular folate pool must be metabolic conversion to another folylmonoglutamate derivative. As shown in Scheme I, only two enzymes catalyze reactions involving CH_3–$H_4PteGlu_1$ in mammalian cells, namely methylenetetrahydrofolate reductase and cobalamin-dependent methionine synthase (EC 2.1.1.13). Both these enzymes show higher affinity for polyglutamate than for monoglutamate substrates (13, 24, and data presented below). The metabolism of CH_3–$H_4PteGlu_n$ can be impaired by treatment of animals with N_2O, which inhibits methionine synthase and leads to secondary declines in the cellular levels of methionine and AdoMet. Under these conditions an increased percentage of the total folate pool is transiently present as CH_3–$H_4PteGlu_n$ derivatives but the total folate pool is depleted (25). This depletion of intracellular folate presumably results because CH_3–$H_4PteGlu_1$ is neither polyglutamylated nor converted to other folate derivatives in N_2O-treated animals and is not then retained by the cell.

The reaction catalyzed by methylenetetrahydrofolate reductase is shown in equation 3. This reaction is irreversible both *in vitro* and *in vivo* (26).

$$\text{NADPH} + CH_2\text{–}H_4\text{folate} \rightarrow \text{NADP}^+ + CH_3\text{–}H_4\text{folate} \quad (3)$$

Oxidation-reduction of the substrates is mediated by the enzyme-bound flavin which is alternately reduced by NADPH and reoxidized by CH_2–H_4folate (26, 27). The reduction of the enzyme-bound flavin by NADPH constitutes the irreversible segment of the reaction, while there is a freely reversible equilibrium between enzyme-bound flavin and CH_2–H_4folate and CH_3–H_4-folate. The enzyme catalyzes exchange between $^{14}CH_3$–H_4folate and CH_2–H_4-folate according to equations 4 and 5 (27). Thus in principle, an exchange

$$\text{E·FAD} + {}^{14}CH_3\text{–}H_4\text{folate} \rightarrow \text{E·FADH}_2 + {}^{14}CH_2\text{–}H_4\text{folate} \quad (4)$$

$$\text{E·FADH}_2 + CH_2\text{–}H_4\text{folte} \rightarrow \text{E·FAD} + CH_3\text{–}H_4\text{folate} \quad (5)$$

reaction catalyzed by methylenetetrahydrofolate reductase could be used to convert CH_3–$H_4PteGlu_1$ to CH_2–$H_4PteGlu_1$, with the accompanying reduction of CH_2–$H_4PteGlu_n$ to CH_3–$H_4PteGlu_n$. Two factors mediate against such an exchange reaction playing an important role in cellular incorporation of CH_3–$H_4PteGlu_1$. NADPH, which is present at high levels (~200 μM) in the cytoplasm of mammalian cells, should compete favorably for the oxidized enzyme with CH_2–$H_4PteGlu_1$, which is bound with roughly the same affinity (27) and which is present in much lower concentration in cells. Secondly, the activity of methylenetetrahydrofolate reductase is regulated by adenosylmethionine (28) and we have now shown that

adenosylmethionine inhibits both the reduction of the enzyme-bound flavin by CH_3–H_4folate and the reoxidation of reduced enzyme by CH_2–H_4folate (Green, Ballou and Matthews, unpublished data).

Alternatively, cellular incorporation of CH_3–$H_4PteGlu_1$ may be mediated by cobalamin-dependent methionine synthase according to equation 6.

$$CH_3\text{–}H_4\text{folate} + \text{HCy} \rightarrow H_4\text{folate} + \text{Met} \qquad (6)$$

Coward and coworkers (24) report a decrease in K_m for the folate substrate of only 2.6-fold on going from CH_3–$H_4PteGlu_1$ to CH_3–$H_4PteGlu_5$ using the enzyme from bovine brain. However our observations with the partially purified enzyme from pig liver are quite different, as shown in Figures 3 and 4. Using a mixture of (6-*R,S*)$^{14}CH_3$–$H_4PteGlu_1$ and unlabeled (6-*S*)CH_3–$H_4PteGlu_6$, a lag phase in the consumption of CH_3–$H_4PteGlu_1$ is seen which is proportional to the amount of CH_3–$H_4PteGlu_6$ added. This suggests that the K_m for CH_3–$H_4PteGlu_6$ is much lower than that of CH_3–$H_4PteGlu_1$, such that the unlabeled substrate is almost completely consumed before appreciable amounts of CH_3–$H_4PteGlu_1$ are converted to product. This conclusion has been tested using a mixture of tritiated CH_3–$H_4PteGlu_6$ and $^{14}CH_3$–$H_4PteGlu_1$ and measuring the $^3H/^{14}C$ ratio of the methionine product as a function of the time of reaction. The results of this experiment are shown in Figure 4. Direct measurements of the K_m values of CH_3–$H_4PteGlu_1$ and CH_3–$H_4PteGlu_6$ substrates were also determined separately, and the results of these

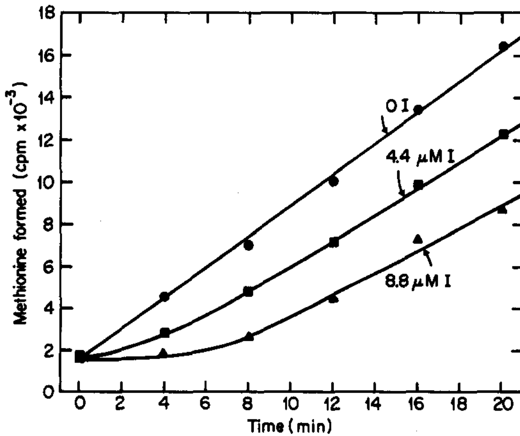


FIG. 3. Rate of formation of methionine catalyzed by pig liver methionine synthase from $^{14}CH_3$–$H_4PteGlu_1$ in the presence of the indicated amounts of unlabeled CH_3–$H_4PteGlu_6$ (I). The concentration of (6-*S*)CH_2–$H_4PteGlu_1$ was initially 21 μM, added as a racemic (6-*R,S*) mixture. The CH_3–$H_4PteGlu_6$ was prepared enzymatically as the (6-*S*) diastereomer.

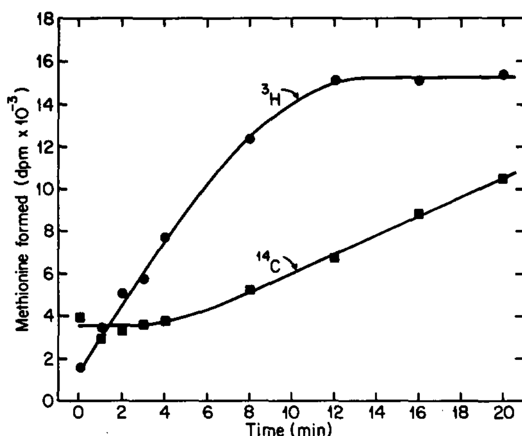


FIG. 4. Rate of formation of methionine catalyzed by pig liver methionine synthase from a mixture of [^{3}H-*methyl*]-CH_3-H_4PteGlu$_6$ (4200 dpm per mol) and $^{14}CH_3$-H_4Pte-Glu$_1$ (2000 dpm per nmol). CH_3-H_4PteGlu$_1$ was added as a racemic (6-*R*,*S*) mixture and the concentration of the (6-*S*) diastereomer was 21 μM. The CH_3-H_4PteGlu$_6$ was prepared enzymatically as the (6-*S*) diastereomer and was present at a concentration of 4.2 μM.

experiments are shown in Figure 5. A double-reciprocal plot of v vs. [CH_3-H_4PteGlu$_1$] is linear, but a similar plot of v vs. [CH_3-H_4PteGlu$_6$] is markedly

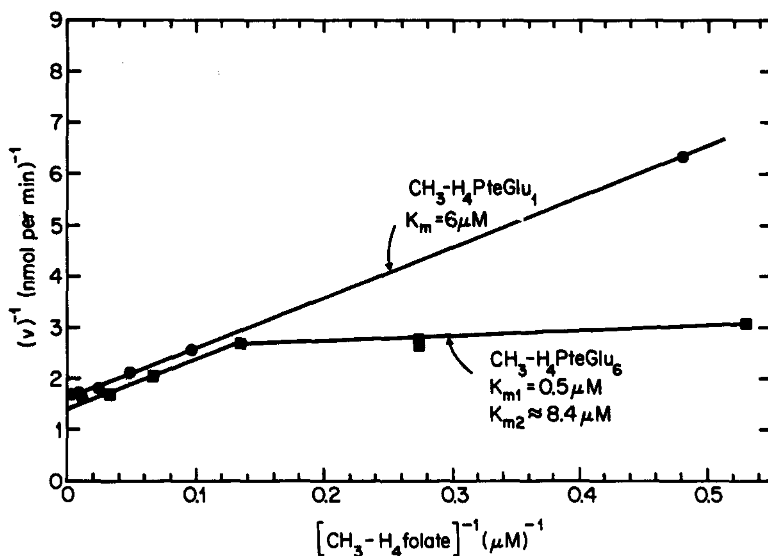


FIG. 5. Determination of K_m values for CH_3-H_4PteGlu$_1$ (•) and CH_3-H_4PteGlu$_6$ (■) substrates using methionine synthase from pig liver.

nonlinear and suggests that methionine synthase has two classes of binding sites for folylpolyglutamates which differ markedly in their affinity. It is quite possible that these apparent differences in affinity may indicate negative cooperativity associated with substrate binding.

These preliminary data suggest that methionine synthase will only use CH_3-H_4PteGlu$_1$ as a substrate when the cellular pool of CH_3-H_4PteGlu$_n$ is largely depleted. The pool size of CH_3-H_4PteGlu$_n$ is primarily regulated by methylenetetrahydrofolate reductase activity, which in turn is regulated by adenosylmethionine levels. Thus incorporation of CH_3-H_4PteGlu$_1$ into the cellular folate pool appears to require active methionine synthase and adequate cellular levels of adenosylmethionine. Studies of Fujii and Huennekens (29) have shown that CH_3-H_4 folate pools are greatly expanded in cobalamin-depleted L1210 cells, and that these cells are unable to use CH_3-H_4PteGlu$_1$ as an extracellular source of folate. Methionine administration partially restores the deficient cellular folate uptake characteristic of cells of animals which are cobalamin-deficient or which have been treated with N_2O (30, 31).

SUMMARY

The true intracellular substrates for folate-dependent enzymes are folylpolyglutamates. We have used measurements of the K_i values of folylpolyglutamate dead end inhibitors to assess the relative affinities of folate-dependent enzymes for folate derivatives of different polyglutamate chain lengths. Studies of four enzymes from pig liver, methylenetetrahydrofolate reductase, serine hydroxymethyltransferase, methylenetetrahydrofolate dehydrogenase and thymidylate synthase, have indicated that folylpolyglutamate inhibitors are bound 3–500 fold more tightly than the corresponding monoglutamates. The individual enzymes differ in their selectivity for polyglutamate vs. monoglutamate inhibitors, and in the chain length associated with the greatest affinity of enzyme for inhibitor. We have also examined the effect of polyglutamate chain length on the catalytic parameters associated with folate substrates. Two enzymes, methylenetetrahydrofolate reductase and serine hydroxymethyltransferase, show decreases in K_m values for folypolyglutamate substrates. Methylenetetrahydrofolate dehydrogenase shows no detectable differences in the catalytic parameters of polyglutamate vs. monoglutamate substrates and no change in the order of substrate addition or product release. Thymidylate synthase shows small effects of K_m and V_{max} values, but the order of addition of substrates and of release of products is reversed with polyglutamate as compared with monoglutamate substrates. Our studies with thymidylate synthase from *L. casei* have shown that the bacterial enzyme also exhibits a greatly increased affinity for polyglutamate vs. monoglutamate derivatives of folic acid, and

that reversal in the order of substrate addition and product release also occurs with polyglutamate as compared with monoglutamate substrates. We have also studied the polyglutamate specificity of methionine synthase, which is responsible for the conversion of CH_3–$H_4PteGlu_1$ into $H_4PteGlu_1$. This reaction is required for the incorporation of plasma folate into the cellular folate pool, because methyltetrahydrofolate is a poor substrate for folylpolyglutamate synthetase. Our studies demonstrate that CH_3–$H_4PteGlu_1$ metabolism is potently inhibited in the presence of CH_3–$H_4PteGlu_6$, and suggest that incorporation of plasma CH_3–$H_4PteGlu_1$ will only occur when methylenetetrahydrofolate reductase is inhibited by adenosylmethionine and cellular pools of CH_3–$H_4PteGlu_6$ are at very low levels.

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REFERENCES

1. I. ITO and C. L. KRUMDIECK, Improved methods of folylpolyglutamate analysis: application to the study of central nervous system folates, pp. 284–296 in *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates* (I. D. GOLDMAN, ed.), Praeger Publishers, New York (1985).
2. S. K. FOO and B. SHANE, Regulation of folylpoly-γ-glutamate synthesis in mammalian cells: *In vivo* and *in vitro* synthesis of pteroylpoly-γ-glutamates by Chinese hamster ovary cells, *J. Biol. Chem.* **257**, 13587-13592 (1982).
3. D. PRIEST, K. K. HAPPEL, M. MANGUM, J. M. BEDNAREK, M. T. DOIG and C. M. BAUGH, Tissue folylpolyglutamate chain-length characterization by electrophoresis as thymidylate synthetase-fluorodeoxyuridylate ternary complexes, *Anal. Biochem.* **115**, 163–168 (1981).
4. C. M. BAUGH, C. L. KRUMDIECK and M. G. NAIR, Polygammaglutamyl metabolites of methotrexate, *Biochem. Biophys. Res. Commun.* **52**, 27–34 (1973).
5. V. M. WHITEHEAD, Synthesis of methotrexate polyglutamates in L-1210 murine leukemia cells, *Cancer Res.* **37**, 408–412 (1977).
6. R. L. SCHILSKY, B. D. BAILEY and B. A. CHABNER, Methotrexate polyglutamate synthesis by cultured human breast cancer cells, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2919–2922 (1980).
7. D. W. FRY, J. C. YALOWICH and I. D. GOLDMAN, Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high pressure liquid chromatography in the Ehrlich ascites tumor cell *in vitro*, *J. Biol. Chem.* **257**, 1890–1896 (1982).
8. C. J. ALLEGRA, R. L. FINE, J. C. DRAKE and B. A. CHABNER, The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells, *J. Biol. Chem.* **261**, 6478–6485 (1986).
9. C. J. ALLEGRA, B. A. CHABNER, J. C. DRAKE, R. LUTZ, D. RODBARD and J. JOLIVET, Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates, *J. Biol. Chem.* **260**, 9720–9726 (1985).
10. R. L. KISLIUK, Y. GAUMONT and C. M. BAUGH, Polyglutamyl derivatives of folate as substrates and inhibitors of thymidylate synthetase, *J. Biol. Chem.* **249**, 4100–4103 (1974).
11. D. J. FERNANDES and J. R. BERTINO, 5-Fluorouracil-methotrexate synergy: enhancement of 5-fluorodeoxyuridylate binding to thymidylate synthase by dihydropteroylpolyglutamates, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5663–5667 (1980).

12. C. J. ALLEGRA, J. C. DRAKE, J. JOLIVET and B. A. CHABNER, Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4881–4885 (1985).
13. R. G. MATTHEWS and C. M. BAUGH, Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroylpolyglutamate substrates and with dihydropteroylpolyglutamate inhibitors, *Biochemistry* **19**, 2040–2045 (1980).
14. J. A. LYON, A. L. POLLARD, R. B. LOEBLE and R. B. DUNLAP, Thymidylate synthetase: an improved purification procedure and description of some spectral properties, *Cancer Biochem. Biophys.* **1**, 121–128 (1975).
15. J. ROSS, J. GREEN, C. M. BAUGH, R. E. MACKENZIE and R. G. MATTHEWS, Studies on the polyglutamate specificity of methylenetetrahydrofolate dehydrogenase from pig liver, *Biochemistry* **23**, 1796–1801 (1984).
16. R. G. MATTHEWS, J. ROSS, C. M. BAUGH, J. D. COOK and L. DAVIS, Interactions of pig liver serine hydroxymethyltransferase with methyltetrahydropteroylpolyglutamate inhibitors and tetrahydropteroylpolyglutamate substrates, *Biochemistry* **21**, 1230–1238 (1982).
17. R. T. TAYLOR and H. WEISSBACH, N^5-Methyltetrahydrofolate-homocysteine transmethylase: partial purification and properties, *J. Biol. Chem.* **242**, 1502–1508 (1967).
18. Y.-Z. LU, P. D. AIELLO and R. G. MATTHEWS, Studies on the polyglutamate specificity of thymidylate synthase from fetal pig liver, *Biochemistry* **23**, 6870–6876 (1984).
19. J. J. McGUIRE, P. HSIEH, J. K. COWARD and J. R. BERTINO, Enzymatic synthesis of folylpolyglutamates: characterization of the reaction and its products, *J. Biol. Chem.* **255**, 5776–5788 (1980).
20. R. G. MORAN and P. D. COLMAN, Mammalian folyl polyglutamate synthetase: partial purification and properties of the mouse liver enzyme, *Biochemistry* **23**, 4580–4589 (1984).
21. D. CICHOWICZ, J. COOK, S. GEORGE and B. SHANE, Hog liver folylpolyglutamate synthetase: substrate specificity and regulation, pp. 7–13 in *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates* (I. D. GOLDMAN, ed.), Praeger Publishers, New York (1985).
22. W. P. JENCKS, Binding energy, specificity, and enzymic catalysis: the Circe effect, *Adv. Enzymol. Relat. Areas Mol. Biol.* **43**, 219–410 (1975).
23. R. E. MACKENZIE and C. M. BAUGH, Tetrahydropteroylpolyglutamate derivatives as substrates of two multifunctional proteins with folate-dependent enzyme activities, *Biochim. Biophys. Acta* **611**, 187–195 (1980).
24. J. K. COWARD, P. L. CHELLO, A. R. CASHMORE, K. N. PARAMESWARAN, L. M. DEANGELIS and J. R. BERTINO, 5-Methyl-5,6,7,8-tetrahydropteroyl oligo-γ-L-glutamates: synthesis and kinetic studies with methionine synthetase from bovine brain, *Biochemistry* **14**, 1548–1552 (1975).
25. M. LUMB, R. DEACON, J. PERRY, I. CHANARIN, B. MINTY, M. J. HALSEY and J. F. NUNN, The effect of nitrous oxide inactivation of vitamin B_{12} on rat hepatic folate: implications for the methylfolate-trap hypothesis, *Biochem. J.* **186**, 933–936 (1980).
26. M. A. VANONI and R. G. MATTHEWS, Kinetic isotope effects on the oxidation of reduced nicotinamide adenine dinucleotide phosphate by the flavoprotein methylenetetrahydrofolate reductase, *Biochemistry* **23**, 5272–5279 (1984).
27. M. A. VANONI, D. P. BALLOU and R. G. MATTHEWS, Methylenetetrahydrofolate reductase: steady-state and rapid reaction studies on the NADPH-methylenetetrahydrofolate, NADPH-menadione, and methyltetrahydrofolate-menadione oxidoreductase activities of the enzyme, *J. Biol. Chem.* **258**, 11510–11514 (1983).
28. C. KUTZBACH and E. L. R. STOKSTAD, Mammalian methylenetetrahydrofolate reductase: partial purification, properties and inhibition by S-adenosylmethionine, *Biochim. Biophys. Acta* **250**, 459–477 (1971).
29. K. FUJII, T. NAGASAKI and F. M. HUENNEKENS, Accumulation of 5-methyltetrahydrofolate in cobalamin-deficient L1210 mouse leukemia cells, *J. Biol. Chem.* **257**, 2144–2146 (1982).
30. J. M. NORONHA and M. SILVERMAN, On folic acid, vitamin B_{12}, methionine, and formiminoglutamate metabolism, pp. 728–736 in *Vitamin B_{12} and Intrinsic Factor, Second European Symposium* (H. C. HEINRICH, ed.), Enke Verlag, Stuttgart (1962).

31. J. M. GAWTHORNE and E. L. R. STOKSTAD, The effect of vitamin B_{12} and methionine on folic acid uptake by rat liver, *Proc. Soc. Exp. Biol. Med.* **136**, 42–46 (1971).