Folate Analogues as Substrates of Mammalian Folylpolyglutamate Synthetase

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ABSTRACT

The antifolate drugs methotrexate (MTX) and aminopterin (AM) have been tested as substrates for folypolyglutamate synthetase (FPGS) partially purified from beef liver. The Km for MTX, 100 μM, and that for AM is 25 μM. These values are considerably higher than those for either tetrahydrofolate or folinic acid. Based on their ratios of Vmax to Km, AM is a better substrate than is MTX for the beef liver FPGS. Both are poorer substrates than tetrahydrofolate. The 7-hydroxy metabolites of MTX and AM are also substrates for FPGS. The reactivity of 7-hydroxymethotrexate is similar to that of MTX, but 7-hydroxyaminopterin is a poorer substrate than AM.

Folic acid, often used as the rescue agent in high-dose MTX therapy, has a low Km with mammalian FPGS (7 μM). Its activity is comparable to that of the best substrate, tetrahydrofolate. Low concentrations of folic acid prevent the formation of polyglutamates of MTX. This inhibition is competitive, presumably because folic acid and MTX are competing substrates for FPGS.

The activities of folate and antifolate substrates also have been determined with rat liver FPGS. With near-saturating concentrations of AM, MTX, or 7-hydroxymethotrexate, the reaction velocity exceeds that with an optimal concentration of tetrahydrofolate. However, the Km values of the folate analogues all are greater than those of the tetrahydrofolate coenzymes.

In contrast to the formation of long-chain polyglutamates observed when tetrahydrofolate or folic acid was the substrate, beef liver FPGS, under our reaction conditions, cannot catalyze the formation from MTX monoglutamate of polyglutamates longer than the triglutamate. MTX di- and triglutamates are poorer substrates than is MTX itself. Longer polyglutamates of MTX, while having no activity as substrates, must bind to the enzyme, because they are inhibitors.

Our observations using MTX and AM with the enzymatic FPGS system help to rationalize the therapeutic use of antifolates.

INTRODUCTION

Folate coenzymes occur in cells principally as pteroyl-γ-glutamate conjugates (1, 2). The number of glutamic acid residues present in the coenzymes in mammalian tissues varies between 2 and 7. The predominating species are the penta- and hexaglutamates (2–5). 4-Amino-4-deoxy analogues of folic acid, including MTX and AM, are potent inhibitors of dihydrofolate reductase, the major target of these drugs (6). These antifolates also can form polyglutamates in mammalian cells (7, 8). For example, tissues from animals receiving weekly doses of MTX contain polyglutamates at least as long as MTXGlun (9), and liver cells grown in culture add from one to 4 residues of glutamate to MTX in 24 h (10).

FPGS has a wide specificity for folate compounds. The addition of glutamate residues both to a number of the folate coenzymes and to antifolate analogues is catalyzed by FPGS. This enzyme catalyzes the glutamylatation of MTX, for example, by one or more cycles of this reaction.

MTXGlun + glutamate + ATP → MTXGlun+1 + ADP + P

FPGS requires the activators Mg2+ and K+ and a thiol. It has been partially purified from Chinese hamster ovary cells (11), rat liver (12), pig liver (13), and mouse liver (14). Other workers (12–14) have reported that these mammalian synthetases are extremely unstable. Recently, we have reported the partial purification and stabilization of FPGS from beef liver (15). In addition, we described a modification to previous assay methods that allows more accurate results to be obtained.

Folic acid antagonists are metabolized in mammels by at least 2 other pathways. MTX, AM, and DCM all can be converted to their 7-hydroxy derivatives (16). This conversion is catalyzed by liver aldehyde oxidase, but only to a significant extent in some mammalian species. Although MTX and AM are poor substrates for human hepatic aldehyde oxidase compared to DCM (16), 7-OH-MTX has been detected in the plasma and urine of patients receiving MTX, under either high-dose or therapeutic conditions (17, 18). MTX can also undergo loss of its glutamate residue. This conversion is thought to be metabolism of the drug by intestinal bacteria (19). However, recently, it has also been shown that a carboxypeptidase termed “folate-hydrolyzing enzyme” present in mammalian cells can catalyze the hydrolysis of folate to glutamate and pteric acid, or MTX to glutamate and 4-amino-4-deoxy-5′-methylpteric acid (20).

It is well known that most commercial preparations of MTX contain considerable amounts of impurities (21, 22). These impurities could either inhibit FPGS, act as alternate substrates of FPGS, or be inert toward FPGS. In order to obtain valid kinetic values, we have purified both MTX and AM. In addition, we have tested the 7-hydroxy metabolites of both MTX and AM as substrates for mammalian FPGS. These compounds have been compared for reactivity, and for the extent of their conjugation, to 5-CHO-H4-folate and to tetrahydrofolate. High doses of MTX, with rescue by later administration of folic acid, are often used in antineoplastic chemotherapy (6). Possibly, folic acid exerts its rescue activity after it is converted to tetrahydrofolate or polyglutamates of tetrahydrofolate in vivo.
MATERIALS AND METHODS

AM, 5-CHO-H4-folate, L-MTX [also called (+)-MTX], and α-MTX [also called (−)-MTX] were purchased from Sigma Chemical Co. AM was purified by crystallization of its magnesium salt (23). MTX was purified by gradient elution from a DEAE-cellulose column with NH4HCO3 (0.1 to 0.4 M); MTX eluted at approximately 0.23 M NH4HCO3. This was followed by lyophilization and chromatography on Sephadex G-10. Salt was removed by washing of precipitated MTX with water at pH 3.5, redissolving of the MTX, and precipitation with glacial acetic acid. The precipitated material was washed with cold acetone. 7-OH-MTX was prepared by the enzymatic method of Johns and Loo (24), except that the mixture was allowed to stand at room temperature for 16 h before being cooled to 5 °C. 7-OH-AM was prepared from AM by the same procedure. These 2 compounds were identified by their absorption spectra (24, 25). The purity of each of these compounds was tested by chromatography on columns of DEAE-cellulose, with elution being effected by a linear gradient of 0.1 to 0.4 M NH4HCO3. 7-OH-AM was not eluted by this gradient, but by 0.1 N NaOH after the gradient. Standard spectra (24, 25). The purity of each of these compounds was tested by chromatography on columns of DEAE-cellulose, with elution being effected by a linear gradient of 0.1 to 0.4 M NH4HCO3. 7-OH-AM was not eluted by this gradient, but by 0.1 N NaOH after the gradient. Standard poly-γ-glutamyl derivatives of folate and MTX were obtained from Dr. C. M. Beaug, University of South Alabama. 1,2,3-[3H]Glutamic acid (NET-395) and [3H]MTX (NET-730) were purchased from New England Nuclear. The [3H]MTX was purified on a column of Sephadex G-15 by the method of Whitehead et al. (8), except that the concentration of 2-mercaptoethanol in the eluting buffer was 100 mM. All other chemicals were obtained from the previously reported sources (15). The concentrations of the folates were obtained by measuring their absorbances and using the published extinction coefficients (24–26).

FPGS was purified from beef liver as described previously (15), except that it was eluted from the blue dextranagarose by a linear gradient (0.2 to 1.2 M) of KCl. Its specific activity was 7,000 to 10,000 pmol/mg/h. FPGS was purified from rat liver by the same method. Frozen male Sprague-Dawley rat livers were purchased from Pel-Freez Biologicals. The specific activity of the rat liver preparations was 750 to 800 pmol/mg/h. Its activity was assayed by measuring the incorporation of [3H]glutamate into polyglutamates. We have recently described the assay conditions and the method used to separate the substrate from the labeled products using chromatography on DEAE-cellulose (15). Antifolates or folic acid have replaced tetrahydrofolate as the folate substrate, as noted in the text. The only changes in the methods have been to count 1 ml of product in 5 ml of Beckman HP/b liquid scintillation cocktail (this change has increased the counting efficiency slightly and decreased the amount of cocktail used in each assay by one-half) and to wash columns with 80 ml of buffer when [3H]glutamate was substrate. All experimental points were determined in at least duplicate. Kinetic results are reported as plots of initial velocity (cpm/2 h) versus concentration of substrate. Kinetic constants were calculated on a microcomputer, using the unweighted curve-fitting equations of the program KINFIT (27).

RESULTS AND DISCUSSION

Substrate Activity of Folate Monoglutamate Analogues. McGuire et al. (12) have demonstrated that, with mammalian FPGS, there is a requirement for the pteridine moiety of folates or their analogues, but there is a lack of specificity for this moiety. With rat liver FPGS (12), tetrahydrofolate gave the highest level of activity of the folate coenzymes tested, but the analogue MTX had greater activity than tetrahydrofolate. AM was nearly as good a substrate as MTX. A recent paper (28) shows that 7-OH-AM, DCM, and 7-OH-DCM also are substrates of the rat liver FPGS. These workers have not presented Km values for any substrates because of the potential formation of multiple products that can be competing substrates.

We have shown that, under our standard assay conditions (15), only tetrahydrofolyliglutamate is formed from tetrahydrofolate. Even at much lower concentrations of tetrahydrofolate (e.g., 5 μM), the diglutamate is the major product. We have calculated that small errors in determination of diglutamates at low concentrations of either tetrahydrofolate or folic acid would not appreciably change their Km values. Irrespective of their concentrations, MTX and AM form very little product (less than 1%) other than diglutamate in 2 h. In addition, incorporation of [3H]glutamate with all pteridine substrates is linear for longer than the standard incubation time of 2 h. Therefore, under these and similar conditions with other folate substrates, Michaelis-Menten kinetic calculations can correctly be used for quantitative comparisons of the activities of substrates. We had found that commercially available MTX was a much poorer substrate with the beef liver synthetase (15) than it was with the rat liver enzyme (12). AM appeared to have much higher activity with the beef enzyme than did MTX (15). Therefore, we have examined these compounds as substrates more thoroughly.

Because the presence of impurities could affect the apparent activities of various preparations of MTX or AM as substrates for FPGS, we have compared the commercially available materials with MTX and AM that we have purified. We have previously reported a Km value of about 90 μM for commercial MTX (15). We have repeated these experiments and obtained a Km value of 100 μM for the commercial MTX (96.5% purity by our column analysis) and a Km value of 100 μM for the commercial MTX (96.5% purity by our column analysis). Chart 1 shows the kinetic data for the MTX that we have purified. The Km value is 100 μM, the same as that for the
less pure material. Therefore, we may conclude that there is no major effect of the contaminants on the substrate activity of the commercial preparation of MTX which we have used. We may also conclude, in agreement with the recent results of McGuire et al. (28), that the high activity of MTX with rat liver FPGS and its lower activity with other mammalian synthetases are due to differences between the enzymes from different species.

The enantiomer of MTX which is used for therapy and which we have used as the substrate for FPGS is L-MTX. This isomer contains a residue of L-glutamic acid. When d-MTX was tested as a substrate, incorporation of [3H]glutamate could be observed only at a high concentration of the MTX. At 300 µM d-MTX, the incorporation of [3H]glutamate observed was less than 2% of that with a similar concentration of L-MTX. This low activity of the d-enantiomer might be due to the presence of small amounts of the l-enantiomer. d-MTX is known to be present as an impurity in preparations of L-MTX (29). d-MTX can inhibit the glutamyltaion of L-MTX. For example, with 187 µM L-MTX as substrate, there was a 21% inhibition of glutamate incorporation when an equal concentration of d-MTX was added. Thus, d-MTX can bind to FPGS, but less avidly than does L-MTX.

Having shown that the impurities in MTX preparations do not have an appreciable effect on FPGS and that d-MTX [present in small amounts in most L-MTX preparations (29)] binds more weakly to FPGS than does L-MTX, we may conclude that these impurities probably have no effect during clinical use on the reactivity of L-MTX with FPGS, even during high-dose therapy.

AM also has been examined as a substrate of FPGS. We had previously estimated (15) a Km value for commercially available AM as 100 µM using the ammonium sulfate fraction of FPGS from beef liver. Repetition of this estimation with more highly purified FPGS gave the lower values of 23 to 26 µM. In addition, we found that the Km value for purified AM is essentially the same, 25 µM (Chart 2). This value is significantly lower than that for MTX, suggesting that AM is a better substrate than is MTX. We shall show below that AM also has higher activity than does MTX by virtue of a higher Vmax. We had shown that the pH optimum for the reaction of MTX in the presence of mammalian FPGS was 8.4 (15), the same as that for tetrahydrofolate (12).

In addition, we have found that AM has an identical pH optimum of 8.4 (data not shown).

In mammals, both MTX and AM are metabolized to their 7-hydroxy derivatives, probably by the effect of aldehyde oxidase in liver (16). These metabolites are poorer inhibitors of dihydrofolate reductase (25) and are less soluble in water, possibly explaining the nephrotoxicity that occurs in high-dose MTX therapy. Whole cells incorporate 7-OH-MTX into polyglutamates (30), so the 7-hydroxy compounds should be substrates for FPGS. This activity was reported by McGuire et al. (28), who found 7-OH-MTX and 7-OH-DCM to be substrates of rat liver FPGS. We have tested 7-OH-MTX and 7-OH-AM as substrates of the beef liver FPGS. The effects of their concentrations on the activity of the synthetase are shown in Charts 3 and 4. The Km values determined from these results are 50 µM for 7-OH-MTX and 20 µM for 7-OH-AM. Both of these values are below those of the parent drugs. The Km for 7-OH-MTX with FPGS from beef liver is higher than the value for the rat liver enzyme, as described below. 7-OH-AM differs from the other antifolates by showing an inhibition of the reaction at higher concentrations (above about 50 µM).

Folinic acid is the folic acid derivative usually administered as
the rescue agent in high-dose MTX chemotherapy. It is therefore useful to compare its properties as a substrate of FPGS to those of the antifolate compounds. As can be seen from Chart 5, folinic acid can saturate the beef liver FPGS at a concentration close to that of tetrahydrofolate, which has a $K_m$ value of 9 $\mu$M (15). We have estimated the $K_m$ value for folinate as 7 $\mu$M. Thus, the beef liver FPGS has a much greater affinity for the rescue agent than it has for either MTX or AM. This affinity would allow folinate to preferentially form polyglutamates if it were at a concentration similar to that of MTX, for instance during the time when concentrations of MTX in plasma are 10 $\mu$M to 1 mM during the critical period of the rescue from high-dose therapy (17, 18).

Further evidence that folinic acid can compete with MTX as a substrate is shown in the following experiment (Chart 6). When the conversion of [3H]MTX to polyglutamate was measured in the presence and absence of 9.3 $\mu$M unlabeled folinic acid, it was found that folinic acid was a potent competitive inhibitor of the glutamylation of MTX. For example, when the concentration of MTX was 62 $\mu$M, 9.3 $\mu$M folinate caused a 28% inhibition. Therefore, we have demonstrated that a low concentration of a rescue agent can effectively inhibit the substrate activity of MTX by having a greater activity with the FPGS. In a recent abstract, Sato and Moran (31) reported that folinate and MTX are competitive with mouse liver FPGS.

When MTX is oxidized to 7-OH-MTX in vivo, the hydroxy metabolite should gradually replace MTX as a substrate of FPGS. We have shown that, as expected, 7-OH-MTX can inhibit the enzyme-catalyzed formation of polyglutamates of [3H]MTX. As the concentration of 7-OH-MTX is increased, the extent of inhibition is increased. When equimolar (104 $\mu$M) concentrations of [3H]MTX and 7-OH-MTX were present, we observed 51% inhibition.

After measuring the $K_m$ values with FPGS for the folate analogues, we have compared their velocities with that obtained using an optimal concentration of tetrahydrofolate (35 $\mu$M). In a single experiment, velocities were measured under near-saturating conditions, at 4 and 8 times the $K_m$ values. The determination of each $K_m$ could take several days. Therefore, it would be inaccurate to compare $V_{max}$ values from Michaelis-Menten curves because there might be differences in the activity of the FPGS. As may be seen from the data of Table 1, only AM has a velocity approaching that of tetrahydrofolate. All of the compounds tested have appreciable activity, although high concentrations are required for the antifolates. The velocities of the 7-hydroxy metabolites are slightly below those of their parent compounds. The $V_{max}$ for folinic acid is about two-thirds that for tetrahydrofolate.

The ratio of $V_{max}$ to $K_m$ can be used as a measure of kinetic preference of an enzyme for its substrates. We have calculated this ratio for 6 substrates of the beef liver FPGS using the data of Table 1 and the $K_m$ values. The ratios of $V_{max}$ to $K_m$ relative to that of the poorest substrate, MTX, are: tetrahydrofolate, 20; folinic acid, 16; AM, 6.8; 7-OH-AM, 3.5; 7-OH-MTX, 1.4; and MTX, 1.0. Clearly, AM and 7-OH-AM are better substrates than MTX and 7-OH-MTX, but not as active as the natural cofactors.

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (M)</th>
<th>Activity relative to tetrahydrofolate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dl-l-Tetrahydrofolate</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>28</td>
<td>52</td>
</tr>
<tr>
<td>Folinic acid</td>
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<td>64</td>
</tr>
<tr>
<td>MTX</td>
<td>400</td>
<td>49</td>
</tr>
<tr>
<td>MTX</td>
<td>800</td>
<td>57</td>
</tr>
<tr>
<td>7-OH-MTX</td>
<td>200</td>
<td>38</td>
</tr>
<tr>
<td>7-OH-MTX</td>
<td>400</td>
<td>40</td>
</tr>
<tr>
<td>AM</td>
<td>100</td>
<td>78</td>
</tr>
<tr>
<td>AM</td>
<td>200</td>
<td>97</td>
</tr>
<tr>
<td>7-OH-AM</td>
<td>80</td>
<td>36</td>
</tr>
<tr>
<td>7-OH-AM</td>
<td>160</td>
<td>25</td>
</tr>
</tbody>
</table>
Comparison of Mammalian FPGS. In order to compare the activities of folate analogues with several mammalian FPGS, we have tested tetrahydrofolate, folinic acid, and several analogues with rat liver FPGS and also calculated $K_m$ values from published results with rat liver and human leukemic cell synthetases (12, 28). These results are summarized in Table 2. With all the substrates tested, the $K_m$ values are lower with rat liver FPGS than with the other mammalian synthetases. None of the analogues or their metabolites has as low a $K_m$ as tetrahydrofolate or folinate. When the approximate maximal velocities, at 4 and 8 times the $K_m$ values, were measured for the rat enzyme (Table 3), we noted that all the substrates had high velocities. The velocities with AM, 7-OH-MTX, and MTX exceeded that of tetrahydrofolate. The range of ratios of $V_{max}$ to $K_m$ is much smaller than with the beef liver FPGS. The values, relative to MTX, are: tetrahydrofolate, 3.9; folinic acid, 3.1; AM, 2.1; MTX, 1.0; 7-OH-AM, 0.96; and 7-OH-MTX, 0.93. As noted previously (28), the relative substrate activities depend on the source of the enzyme. MTX is one of the poorest substrates tested with all of these mammalian tissues. In addition, the FPGS from mouse liver has been reported to have the high apparent $K_m$ value of 163 $\mu M$ for MTX (32). Similar to the beef and rat enzymes, mouse FPGS has a low $K_m$, 8 $\mu M$, for folinic acid (14).

**Table 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Beef liver</th>
<th>Rat liver</th>
<th>Human leukemic cells</th>
</tr>
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<tbody>
<tr>
<td>d-L-Tetrahydrofolate</td>
<td>9</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>7</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>MTX</td>
<td>90</td>
<td>22</td>
<td>55</td>
</tr>
<tr>
<td>MTX</td>
<td>100</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>7-OH-MTX</td>
<td>50</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>7-OH-MTX</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>25</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>7-OH-AM</td>
<td>20</td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td>DCM</td>
<td>ND</td>
<td>17</td>
<td>ND</td>
</tr>
<tr>
<td>7-OH-DCM</td>
<td>ND</td>
<td>12</td>
<td>ND</td>
</tr>
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</table>

* From Ref. 34.
* ND, not determined.
* Calculated from the data presented in Ref. 29.
* Calculated from the data presented in Ref. 28. Values without other reference are from work in this paper.

**Table 3**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration ($\mu M$)</th>
<th>Activity relative to tetrahydrofolate ( %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-L-Tetrahydrofolate</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>12</td>
<td>76</td>
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<tr>
<td>Folinic acid</td>
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<td>114</td>
</tr>
<tr>
<td>MTX</td>
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<td>127</td>
</tr>
<tr>
<td>7-OH-MTX</td>
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<td>125</td>
</tr>
<tr>
<td>7-OH-MTX</td>
<td>128</td>
<td>126</td>
</tr>
<tr>
<td>AM</td>
<td>32</td>
<td>142</td>
</tr>
<tr>
<td>AM</td>
<td>64</td>
<td>139</td>
</tr>
<tr>
<td>7-OH-AM</td>
<td>48</td>
<td>98</td>
</tr>
<tr>
<td>7-OH-AM</td>
<td>96</td>
<td>68</td>
</tr>
</tbody>
</table>

**Chart 7.** Incorporation of $[^{3}H]$glutamate into polyglutamates of MTX. The reactions were performed under standard assay conditions, except that 50 $\mu M$ MTX replaced the tetrahydrofolate, and the reaction volumes were 4 times the normal size. The reaction mixtures were analyzed by gradient elution from the DEAE-cellulose columns, as described in "Materials and Methods." --- , products after 2 h; ---- , products after 48 h. $M$, positions of elution of, from left to right, standard samples of MTXGIu$_3$, MTXGIu$_4$, and MTXGIu$_5$.
Chart 8. Incorporation of \(^{3}H\)glutamate into polyglutamates of folinic acid. The reactions were performed under standard assay conditions, except that 5 μM folinic acid replaced tetrahydrofolate, and the time of incubation was 72 h. The reaction mixture was 5 times the normal volume. The ammonium sulfate fraction from beef liver was used as FPGS. The assay was stopped by addition of sodium acetate buffer, pH 4.5. —, radioactive compounds formed; ——, radioactive compounds remaining after treatment of the products with beef kidney conjugase for 30 min.

Chart 9. Effect of the concentration of MTXGlu\(_2\) on the rate of the reaction catalyzed by FPGS from beef liver. Reactions were performed under standard assay conditions, except that the concentration of MTXGlu\(_2\) was varied as indicated, and that the total reaction volume was 0.27 ml. Abbreviations as in Chart 1.

(9) or in whole cells (10), with polyglutamates as long as MTXGlu\(_6\) being formed. However, when MTX is incubated with mammalian FPGS, the predominating product is the diglutamate (Chart 7). Therefore, individual polyglutamates of MTX were tested as substrates of the beef enzyme. Chart 9 shows the effect of the concentration of MTXGlu\(_2\) on the activity of FPGS from beef liver. The \(K_m\) for MTXGlu\(_2\) was found to be 110 μM. Within experimental error, this value is the same as that for MTXGlu\(_1\) (100 μM). However, the \(V_{max}\) for MTXGlu\(_2\) was only 19% that of MTXGlu\(_1\). MTXGlu\(_3\) was found to have 3 to 5% the activity of MTX as a substrate, and longer polyglutamates had no activity. Few studies have been reported on the effect of chain length of folypolyglutamates on their activity with FPGS. Foo and Shane (34) have found that the activity of folypolyglutamates as substrates of FPGS from Chinese hamster ovary cells decreases with increasing chain length. The hexa- and heptaglutamates of tetrahydrofolate had little or no activity. Glutamates of 10-formyltetrahydrofolate showed a pattern of reactivity that was very similar to that of MTX polyglutamates in our experiments. The pentaglutamate of tetrahydrofolate is a poor substrate for rat liver FPGS but a potent inhibitor of this enzyme (28). The decrease in the effectiveness of antifolate substrates as their chain lengths increases could partially explain why no polyglutamates larger than MTXGlu\(_2\) are formed from MTX in our enzymatic incubations (Chart 7). If appreciable amounts of MTXGlu\(_1\) and MTXGlu\(_2\) were present, they would act as substrates in preference to any small amounts of MTXGlu\(_3\) formed.

The inability to observe any of the longer polyglutamates of MTX and the lack of activity of conjugates longer than MTXGlu\(_3\) could be caused by any of several reasons. A hydrolytic enzyme might be present as a contaminant in the FPGS preparation. However, we have shown previously that, although conjugase-like activity can be detected in the beef liver FPGS fraction at pH 4.4 or pH 5.2, this activity is very low (15), and at more basic pH values, there appears to be little hydrolysis of folypolyglutamates. This hydrolytic activity appears to be too low to be the only reason for a lack of long-chain polyglutamates. It is possible that reaction conditions (e.g., pH of incubation, concentrations of substrates, or membrane binding of FPGS) necessary for elongation of oligoglutamates catalyzed by FPGS are different from those for their formation in monoglutamates. This possibility, to our knowledge, has not yet been fully tested. It is possible that additional protein, removed during purification, is required for efficient elongation of MTXGlu\(_2\). Cossins and Chan (35) have found more than one FPGS in Neurospora crassa. The cytosol requires 2 fractions for the formation of long-chain polyglutamates, and mitochondria contain an FPGS that uses tetrahydropteroyldiglutamate as substrate. Mitochondria prepared from beef liver possess apparent FPGS activity which is resistant to solubilization by digitonin. This or some other enzyme may be essential for the elongation reactions. Recently, Cowan and...
Jolivet (36) described an MTX-resistant cell line that, although possessing full FPGS activity, accumulates only short-chain MTX polyglutamates, similar to those observed in the FPGS-catalyzed reactions (Chart 7; Ref. 28). It is possible that the cells of their resistant subline lack a protein (possibly a synthetase activity or a protein cofactor for binding long-chain products) essential for the elongation of oligoglutamates. Further investigations are needed to find the enzymatic processes of elongation of polyglutamates of MTX.

We have tested polyglutamates of MTX as inhibitors of the glutamylation of MTXGIu7. The data of Chart 10 show that, when 8 μM [3H]MTX was the substrate for the reaction, addition of MTXGIu2 through MTXGIu6, at 130 μM (Curve C) caused an inhibition of glutamylation that increased with the length of the polyglutamate chain. When lower concentrations of the polyglutamates were used, results were similar, except that MTXGIu5 to MTXGIu gave approximately the same extent of inhibition (e.g., 50% inhibition at 65 μM). This leveling off of inhibition at MTXGIu5 could indicate that FPGS has binding sites for only 4 glutamate residues of a polyglutamate. In any case, MTXGIu4 to MTXGIu must bind to FPGS, even though they appear not to be substrates.

Several points are becoming clear as more information becomes available on enzymatic reactions involved in the metabolism of MTX and other analogues of folic acid in mammals. Large differences in reactivity of substrates can occur with enzymes from different species. For example, AM is not a substrate for hepatic aldehyde oxidase from mouse or rat, but is a substrate for the oxidases from rabbit and guinea pig liver (37). MTX and DCM are substrates of all of these enzymes. AM is more toxic than is MTX to those animals that can oxidize MTX but not AM (37). Differences have also been observed in efficiency of folate analogue substrates with FPGS from various species (28; this paper). For example, AM is a better substrate than MTX, although its relative activity differs between tissues. Although 7-OH-MTX has approximately the same efficiency as a substrate as MTX, 7-OH-AM is only one-half as efficient as AM with both beef and rat FPGS. If this pattern of better reactivity of AM than MTX with FPGS also occurs in other mammalian species, it could help to explain the greater toxicity of AM. Greater reactivity would cause greater accumulation of polyglutamates of AM within cells. In support of this proposal, Goldman et al. (38) have shown that, in Ehrlich ascites cells, the rate of formation of polyglutamates of AM is greater than those of MTX. Folinic acid (and any tetrahydrofolate formed from it) is known to exert a rescue effect by circumventing the block of dihydrofolate reductase and thus replenishing the supply of tetrahydrofolate coenzymes. However, based upon our results, it may be suggested that it also may assist the rescue phenomenon by inhibiting the conjugation of MTX catalyzed by FPGS. This would allow MTX to preferentially exit from host cells as short-chain glutamates. Galivan and Nimec (39) have shown with hepatoma cells that folinic acid prevents the uptake and glutamylation of MTX, and after a pulse of MTX, it prevents the conversion of MTXGIu3 to longer polyglutamates. In addition to continuing studies on mammalian FPGS, we are currently examining the numerous other mammalian enzymes affecting antifolates [conjugases, folate-hydrolyzing enzyme, and folate oligoglutamate:aminol acid transpeptidase (40)] with the aims of further understanding the controls of metabolism of polyglutamates and assessing their roles in therapy of neoplasms.

REFERENCES


Folate Analogues as Substrates of Mammalian Folylpolyglutamate Synthetase


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