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TETRAHYDROPTEROYLPOLYGLUTAMATE DERIVATIVES AS SUBSTRATES OF TWO MULTIFUNCTIONAL PROTEINS WITH FOLATE-DEPENDENT ENZYME ACTIVITIES

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Summary

Several of the folate-mediated reactions in eucaryotic cells are carried out by multifunctional proteins using the naturally occurring pteroylpolyglutamate derivatives. The compounds tetrahydropteroyl(glutamate)$_n$ where n = 1, 3, 5, or 7 were used to determine whether the additional glutamyl residues on the substrates provide kinetic advantages with two folate-dependent multifunctional protein. Methylenetetrahydrofolate dehydrogenase(EC 1.5.1.5)-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)-formyltetrahydrofolate synthetase (EC 6.3.4.3) activities comprise a trifunctional protein, and formiminoglutamate:tetrahydrofolate formiminotransferase(EC 2.1.2.5)-formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) for a bifunctional one. The dehydrogenase, transferase and synthetase were found to have 10—40-fold lower K_m values for the tetrahydropteroylpolyglutamate derivatives with essentially unchanged values of V. Specificities with cyclodeaminase and cyclohydrolase were determined by using pteroylglutamates as inhibitors of the activities; pteroylpentaglutamate is a 70-fold better inhibitor than folate with cyclodeaminase, but is only10-fold better with cyclohydrolase. Because of the sequential nature of the enzymic activities in these multifunctional proteins,

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Abbreviation: DTNB, dithiobis(2-nitrobenzoic acid). Enzymes: formiminoglutamate:tetrahydrofolate formiminotransferase (5-formiminotetrahydrofolate:L-glutamate *N*-formiminotransferase, EC 2.1.2.5), formiminotetrahydrofolate cyclodeaminase (5-formiminotetrahydrofolate ammonia-lyase (cyclizing), EC 4.3.1.4), methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.5), methenyltetrahydrofolate cyclohydrolase (5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing), EC 3.5.4.9), formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3).

the tetrahydropteroylpolyglutamate substrates were examined to see if they provide a kinetic advantage by promoting transfer of folate intermediates between active sites on a single enzyme molecule. With the sequential transferase-deaminase activities, it was observed that the product of the transferase accumulates in the medium with tetrahydropteroylmonoglutamate as the substrate, but does not when the pentaglutamate is used. Chemical modification to selectively inactivate the transferase and deaminase, followed by recombination, demonstrated that this kinetic property is observed because the intermediate formiminotetrahydropteroylpentaglutamate is transferred preferentially to the deaminase site rather than equilibrating with the medium.

Introduction

Most of the folate derivatives in cells exist as polyglutamate forms [1—6], with additional glutamate residues attached in γ linkage to the folate molecule. Their significance in metabolism is not fully understood, although numerous examples are known where the pteroylpolyglutamate derivatives have been demonstrated to be the preferred substrates for various enzymes [7—10]. Whether these forms exist simply to provide substrates with greater affinities, and thus are able to function at low intracellular concentrations, or in addition provide means for compartmentalization or regulation is not yet resolved. The existence of some folate-dependent enzyme activities as multifunctional proteins raises the additional question as to whether the polyglutamates have any special significance with respect to these structures. Formiminoglutamate: tetrahydrofolate formiminotransferase(EC 2.1.2.5)-formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) a bifunctional protein [11,12] and methylenetetrahydrofolate dehydrogenase(EC 1.5.1.5)-methenyltetrahydrofolate cyclohydrolase(EC 3.5.4.9)-formyltetrahydrofolate synthetase (EC 6.3.4.3), a trifunctional protein [13—15] are two multifunctional proteins that have reported in the last few years. In this paper, we have assessed the specificity of three of these activities for the number of glutamates in the tetrahydrofolate substrate, and observed the inhibition by pteroylpolyglutamates on two other enzyme activities. We have also examined the two multifunctional proteins to see if tetrahydropteroylpolyglutamates are beneficial in 'channeling' intermediates between two sequential folate-dependent activities in a single polypeptide chain.

Materials and Methods

$NADP^+$, ATP, folic acid, formiminoglutamate, 5,5′-dithiobis(2-nitrobenzoic acid), diethylpyrocarbonate, bovine serum albumin, and glucose-6-phosphate dehydrogenase were from Sigma; formaldehyde was obtained from British Drug House. For use in standard assays, (±)-tetrahydrofolic acid was prepared by reduction of an aqueous, neutral solution of folic acid at atmospheric pressure with hydrogen and platinum [16] and purified by chromatography on DEAE-cellulose [17] using triethanolamine-HCl (pH 7.2) instead of Tris-HCl and stored at 4°C in 10-ml ampoules. Formiminotetrahydrofolate was prepared as

described previously [18] and purified by the method of Rabinowitz [19]. *Lactobacillus casei* dihydrofolate reductase was a gift of Dr. Roy Kisliuk, Tufts University. Pteroylpolyglutamates were synthesized and purified as described previously [20]. Common chemicals were reagent grade from Fisher Scientific Co.

Preparation of (—)-tetrahydropteroylglutamates. (—)-Tetrahydropteroylglutamates were prepared by reduction of 5 μmol of the folate to the dihydro derivative with sodium dithionite at pH 6.0 [21] followed by conversion to the tetrahydro compounds using dihydrofolate reductase. This incubation mix contained approx. 5 μmol unpurified dihydropteroylglutamate, 50 μmol glucose-6-phosphate, 1 μmol NADP, 19 μg glucose-6-phosphate dehydrogenase and 1 mg dihydrofolate reductase in 3 ml of 0.05 M Tris-HCl, 0.5 M 2-mercaptoethanol, pH 7.5. After 15 min at room temperature, the sample was diluted with an equal volume of 0.5 M 2-mercaptoethanol and applied to a 0.7 × 5 cm column of DEAE-cellulose (Whatman) in the chloride form, washed with 15 ml of 0.075 triethanolamine-HCl and eluted with an exponential gradient formed with 60 ml of 0.075 M triethanolamine-HCl, 0.5 M 2-mercaptoethanol, pH 7.0, in the closed vessel, and 50 ml of the same buffer containing 0.6 M NaCl in the upper reservoir. The purified tetrahydropteroylglutamates were assayed using the synthetase activity.

Preparation and assay of enzymes. The dehydrogenase-cyclohydrolase-synthetase multifunctional protein was purified from pig liver as described previously [14] through chromatography on DEAE-Sephadex A25 in 20% Me_2SO and had a dehydrogenase specific activity of 2.5—3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Formiminotransferase-cyclodeaminase was also purifed from frozen pig liver [18] and had a transferase-specific activity of 36—40 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Standard assays were as used previously [11,12,14] and the concentrations of the tetrahydropteroylglutamates were varied to obtain K_m values of the various tetrahydrofolates with dehydrogenase, synthetase and transferase activities. In these assays, tetrahydrofolate is added directly to the assay mixture.

Measurement of the cyclohydrolase and cyclodeaminase activities requires preparation of the corresponding substrates, methenyl- and formiminotetrahydrofolate, both of which are unstable under the assay conditions. It was not possible to prepare and purify these derivatives from each of the tetrahydropteroylpolyglutamates because of the limited quantities available. Instead, the pteroylglutamates were employed as inhibitors. The cyclohydrolase and cyclodeaminase were assayed as previously described [12,14] in the presence of increasing concentrations of the respective pteroylglutamates.

The time course of appearance of products of the dehydrogenase and cyclohydrolase activities used to monitor 'channeling' of the methenyl intermediate was modified as previously described [15]. The time course for the appearance of products of the transferase-deaminase was determined in the standard assay but with $5 \cdot 10^{-5}$ M tetrahydropteroylglutamates. Transferase activity was measured after acidification and heating which converts the formimino intermediate to the final product methenyltetrahydropteroylglutamate. Appearance of only the final product, methenyltetrahydropteroylglutamate, was monitored on a Gilford 2000 recording spectrophotometer at 355 nm. All assays were conducted at 30°C.

Chemical modification of transferase-deaminase. Enzyme (250 μg/ml) was modified with 50 μM DTNB at pH 8.0 for 15 min at room temperature as described earlier [12]. Aliquots were diluted 80 fold into 0.1 M potassium phosphate (pH 7.3) containing 400 μg/ml bovine serum albumin. Diethylpyrocarbonate (10^{-3} M) was used to modify the enzyme (125 μg/ml) by incubation in 0.1 M potassium phosphate (pH 6.2) at 0°C for 15 min. Aliquots were diluted 40 fold into 0.1 M potassium phosphate (pH 7.3) containing 400 μg/ml bovine serum albumin.

Results

The activities of the multifunctional proteins methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase and formiminoglutamate:tetrahydrofolate formiminotransferase-formiminotetrahydrofolate cyclodeaminase can be determined separately using the proper tetrahydrofolate derivatives as substrates. The first objective was to determine whether these activities show specificity for the number of glutamates in the folate substrates. Three of these activities, dehydrogenase, synthetase, and transferase, simply require the addition of tetrahydrofolate to the respective assay mixes and thus were readily examined for substrate specificity with the tetrahydropteroylpolyglutamates. The relative values of V were not changed significantly, although the tetrahydropteroylpolyglutamates have much lower values of K_m with each activity than does tetrahydrofolate (Table I). The cyclohydrolase and cyclodeaminase assays require synthesis of one-carbon tetrahydrofolate derivaties for use as substrates which was not practical with limited amounts of the tetrahydropteroylpolyglutamates available. Instead, the relative effectiveness of the pteroylpoly-γ-glutamates as inhibitors of these reactions was determined. The concentration of each compound required for 50% inhibition was obtained using the standard assay conditions and are shown in Table II. These results indicate the relative affinities of the pteroylpolyglutamates and illustrate that the penta and hepta compounds are approximately equivalent, and are much better inhibitors than

TABLE I

(—)-TETRAHYDROPTEROYLGLUTAMATES AS SUBSTRATES FOR TRANSFERASE, DEHYDROGENASE AND SYNTHETASE ACTIVITIES

Appropriate concentrations of (—)-tetrahydropteroylglutamate were added to otherwise standard assay media and values of K_m and V were obtained from double-reciprocal plots. Values represent the averages of 3—5 determinations.

(—)-Tetrahydropteroyl(glutamate)$_n$ (n)	Enzyme activity					
	Transferase		Dehydrogenase		Synthetase	
	K_m	% V	K_m	% V	K_m	% V
1	48	100	25	100	89	100
3	31	87	6.8	83	2.0	98
5	3.5	96	3.0	104	—	—
7	4.6	140	2.7	80	—	—

TABLE II

PTEROYLGLUTAMATES AS INHIBITORS OF CYCLODEAMINASE AND CYCLOHYDROLASE

Increasing amounts of pteroylglutamates were added to standard assay mixtures and the concentration for 50% inhibition determined under these conditions. Values represent the averages of 2—3 determinations.

Pteroyl(glutamate)$_n$ (n)	Concentration (μM) for 50% inhibition	
	Cyclodeaminase	Cyclohydrolase
1	70	195
3	13	60
5	1.0	18
7	2.3	18

the pteroylmono- and triglutamates. These values can be used for comparison of relative affinities, but the calculation of actual K_i is difficult due both to the instability of the substrates for these two activities as well as the limited range of the spectrophotometric assays, requiring substrate concentrations below the apparent K_m.

The primary interest with these particular enzymes and the tetrahydropteroylpolyglutamate derivatives involves the pairs of sequential actvities, dehydrogenase-cyclohydrolase and transferase-deaminase, which were examined for 'channeling' of the corresponding tetrahydropteroylglutamate intermediate in each case. The objective was to observe whether, in the reaction A → B → C, the intermediate compound B equilibrates with the medium or is preferentially transferred through the second enzyme activity to form C. In this latter case, the intermediate B would not accumulate to high enough concentrations to account for the rate of production of C, which would appear without a time lag. If B should equilibrate with the medium, however, one would expect a lag in the appearance of C. The dehydrogenase-cyclohydrolase was shown earlier to partially channel the methenyltetrahydrofolate intermediate. This conclusion was reached from studies on the time course of appearance of products where it was demonstrated that the rate of formation of formyltetrahydrofolate, the final product, could not be accounted for by the accumulation of methenyltetrahydrofolate, the intermediate [15]. While preferential transfer between sites was observed, there was still significant release of the intermediate to the solution [15]. Similarly, in this study, the rates of production of these compounds with time were observed using substrates where n = 1, 3, 5, and 7. As shown in Fig. 1, the number of glutamates on the substrate has little effect on reducing the amount of methenyl intermediate released to the bulk solution.

A similar approach when applied to the transferase-deaminase showed, with (—)-tetrahydrofolate, a significant lag in the appearance of the final methenyl product, and a large accumulation of formiminotetrahydrofolate (Fig. 2). The lag is somewhat reduced with tetrahydropteroyltriglutamate and is not seen with the penta and hepta compounds. The channeling is essentially complete with tetrahydropteroylpentaglutamate, i.e. there is no significant accumulation of formiminotetrahydropteroylpentaglutamate during the reaction.

Two explanations for this kinetic observation were considered: a situation

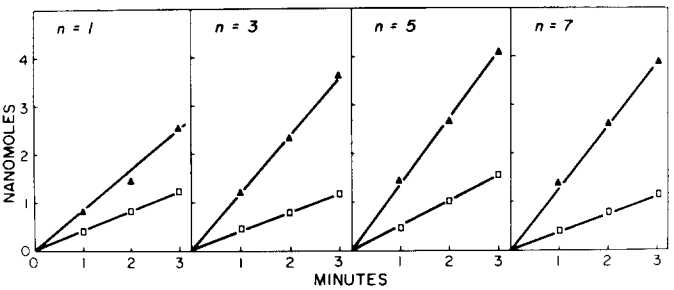


Fig. 1. Rate of appearance of methenyltetrahydropteroylglutamate (□) and formyltetrahydropteroylglutamate (▲) using 22 μM methylenetetrahydropteroyl(glutamate)$_n$ (n = 1, 3, 5, 7) and 36 μM $NADP^+$ as substrates.

where the formiminotetrahydropteroylpentaglutamate is preferentially transferred between sites on the same enzyme molecule and the alternative where the intermediate dissociates but rebinds to any deaminase site with a very high affinity. Because preparation of formiminotetrahydropteroylpentaglutamate was not feasible, this latter possibility could not be tested directly by kinetic approaches. Instead, aliquots of enzyme were chemically modified to retain either transferase or deaminase activity as shown in Table III, and then recombined to give transferase-deaminase but where the activities were not on separate molecules. Modification of the transferase activity with diethylpyrocarbonate does not alter the remaining deaminase which retains both its activity and its sensitivity to inhibition by pteroylpentaglutamate. The modified enzymes showed the same time course of appearance of products as native enzyme with the tetrahydropteroylmonoglutamate substrate (Fig. 3), but exhibited a pronounced lag in the appearance of the methenyl derivative when the substrate was tetrahydropteroylpentaglutamate (Fig. 4). Under these conditions formiminotetrahydropteroylpentaglutamate accumulated in the assay mixture.

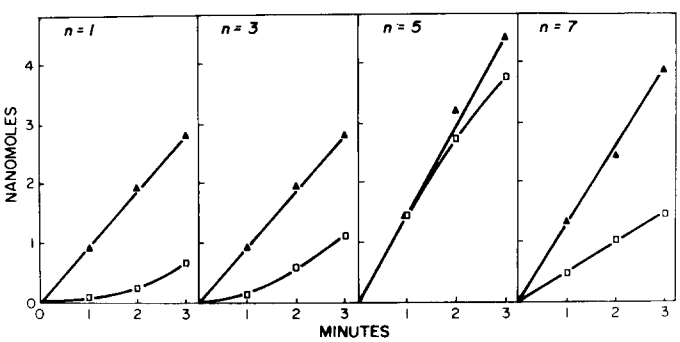


Fig. 2. Rate of appearance of the sum of formimino- and methenyltetrahydropteroylglutamate (▲) and the methenyl derivative alone (□) using 50 μM tetrahydropteroyl(glutamate)$_n$ (n = 1, 3, 5, 7) and 5 mM formiminoglutamate as substrates.

TABLE III

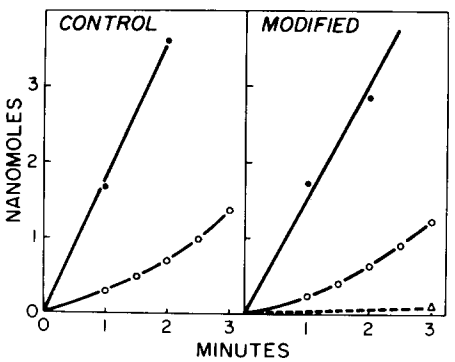
ACTIVITIES OF CHEMICALLY MODIFIED ENZYME

Separate aliquots of enzyme were treated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) or diethylpyrocarbonate (DEPC). Aliquots were diluted into 0.1 M potassium phosphate buffer (pH 7.3) containing 400 μg bovine serum albumin to stop the modification reactions. These diluted samples were subsequently used for assay.

Enzyme	% original activity	
	Transferase	Deaminase
Control	100	100
DTNB modified	93	6
DEPC modified	6	96

Discussion

The observation that the polyglutamate forms of the substrates of the dehydrogenase, synthetase and transferase have lower K_m values than the tetrahydropteroylmonoglutamates is in general agreement with reports on other folate-dependent enzymes [7—10]. Several studies [22—24] have indicated that the pentaglutamate is probably the predominant form of naturally occurring folates in liver. The primary concern in this investigation was to compare the monoglutamates, the usual substrates for in vitro assays, with the pentaglutamate derivatives, the most likely in vivo substrates. We could detect no significant differences in the values of V indicating that the role of the additional γ-glutamyl residues with these three enzyme activities is entirely to enhance affinity. The K_m values of the transferase and dehydrogenase with the appropriate tetrahydropteroylpentaglutamate substrate are about ten fold lower than with the monoglutamate. The synthetase, however, has a K_m with



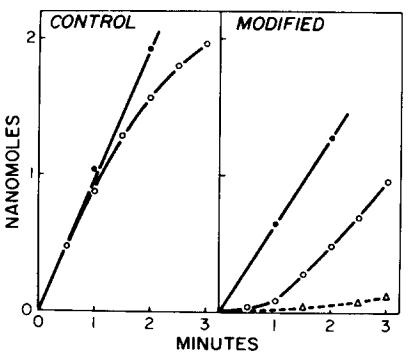


Fig. 3. Rate of appearance of products as described in Fig. 2 with tetrahydropteroylglutamate (n = 1) as substrate using unmodified (control) or a mixture of equal amounts of DTNB and diethylpyrocarbonate-modified enzyme (modified). •, the sum of formimino- and methenyltetrahydropteroylglutamate; ○, accumulation of methenyltetrahydropteroylglutamate, △, and the accumulation of the methenyl derivative when either one of the modified enzymes is used alone.

Fig. 4. As for Fig. 3 with tetrahydropteroylpentaglutamate as substrate.

tetrahydropteroyltriglutamate about 45 fold lower than with monoglutamate; substrates with additional γ-glutamyl residues showed very strong substrate inhibition within the limits of the assay, and reliable values of K_m could not be determined for the substrates where $n = 5$ and 7. Similar specificies were observed when the pteroylpolyglutamates were used as inhibitors of the cyclodeaminase and cyclohydrolase activities. The cyclodeaminase is particularly sensitive to inhibition by the pteroylpentaglutamate.

The potential of the folate substrates containing additional γ-glutamyl residues for allowing preferential transfer of intermediates between enzymes organized within cells, or, in the case of the multifunctional proteins, between active sites covalently linked in the single molecule, is an intriguing one. The long anionic polyglutamate chain could provide not only tighter binding to active sites but also serve as a type of anchor to allow the tetrahydropteroyl moiety to release and preferentially bind to the next active site. This possibility does not seem to hold with 'channeling' through the dehydrogenase-cyclohydrolase activities. While tetrahydrofolate itself shows preferential channeling [15], the higher affinity of the dehydrogenase and cyclohydrolase sites for the tetrahydropteroylpolyglutamates is not reflected in an increased efficiency of this process. The preferential transfer of the methenyltetrahydropteroylglutamate intermediate through the cyclohydrolase could be beneficial to produce formyltetrahydropteroylglutamate for both purine synthesis as well as for the overflow pathway suggested by Krebs et al. [25]. It is not clear why the channeling is not complete; perhaps complete channeling could reduce the production of the methenyl derivative required for purine synthesis to unacceptably low levels. Whether the efficiency of channeling can be modulated by some regulatory mechanism other than length of the polyglutamate chain is not known.

The efficiency of channeling seen with the transferase-deaminase enzyme, however, is clearly a function of the number of γ-glutamyl residues on the substrate. Pentaglutamate is the optimum length, and this specificity is reflected also in the large difference in K_m between the tri-and pentaglutamate for the transferase activity. The functional aspect is very clear: the tetrahydropteroylpentaglutamate substrate is converted efficiently to the methenyl product without accumulation of the formimino intermediate, for which there is no other metabolic function known. The mechanism of this process is true channeling in the sense that the intermediate formiminotetrahydropteroylpentaglutamate does not normally dissociate from the enzyme molecule as is shown by the time course studies using modified enzyme. Under experimental conditions where the intermediate is required to dissociate from one enzyme molecule and bind to another to complete the two reactions, formiminotetrahydropteroylpentaglutamate accumulated, as demonstrated by the time lag in appearance of the final methenyl product. This result is not explained by an altered deaminase activity since its inhibition by pteroylpentaglutamate was shown not to be reduced after the transferase was inactivated. Since the lag observed with tetrahydrofolate was not increased under the same conditions, the intermediate in this case normally equilibrates completely with the medium and rebinds randomly at deaminase sites on the same or other molecules. The transferase-deaminase has apparently evolved to utilize the polyglutamate sub-

strates to enhance the transfer of the intermediate between sites. The 'channeling' is essentially complete with tetrahydropteroylpentaglutamate but not with the hepta substate, although there is no lag in the time course of appearance of final product in either case. Apparently the number of glutamates on the substrate determines the fate of the formimino intermediates such that with $n = 1$ and 3 this intermediate preferentially dissociates from the enzyme. With a substrate where $n = 5$, the glutamates are the optimum length to assist transfer of the formimino intermediate from the transferase to the deaminase site; however, with $n = 7$, the 'anchor' is too long, the efficiency of transfer is reduced, and the intermediate has similar probabilities for dissociation as for transfer.

The structure of this enzyme is unusual; it is an octomer of identical subunits arranged in a ring [11]. Because both activities must be the responsibility of each of the identical polypeptides, it cannot be decided from the current experiment whether the channeling with tetrahydropteroylpentaglutamate occurs between sites on a single polypeptide, or between polypeptides, although this latter possibility is less likely.

The tetrahydropteroylpolyglutamate substrates thus provide a means to operate at low intracellular concentrations due to their greater affinities for the enzymes, and, in the case of the transferase-deaminase, combine with the bifunctional nature of this protein to increase the efficiency of the pathway and to avoid the unnecessary accumulation of another labile intermediate and potential inhibitor of other folate-mediate reactions.

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