The evidence for a conformational change in the enzyme upon binding of tetrahydropteroylpolyglutamates (Received for publication, February 20, 1987)

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The 10-formyltetrahydrofolate synthetase domain of the trifunctional enzyme C1-tetrahydrofolate synthase appears to undergo a conformational change in the presence of tetrahydropteroylpolyglutamates, MgATP, and ammonium ion. The binding of these ligands increases the denaturation temperature of the enzyme by 12°C, abolishes the cold lability of the enzyme, and alters its susceptibility to digestion by chymotrypsin. The results suggest that a conformational change is dependent upon binding of the third glutamate residue of C1-tetrahydrofolate synthase and serine hydroxymethyltransferase was developed to determine the activity of 10-formyltetrahydrofolate synthetase. The assay gives linear rates with the tetrahydrofolate cyclohydrolase (E.C. 3.5.4.9) and 5,10-methenyltetrahydrofolate dehydrogenase (E.C. 1.5.1.5). We will refer to the three activities on this trifunctional enzyme as the synthetase, cyclohydrolase, and dehydrogenase activities. For most organisms, the physiologically active tetrahydropteroylmonoglutamate is tetrahydrofolate 10-formyltetrahydrofolate synthetase (1). The 10-formyl-H4PteGlu synthetase fragment is a homodimer with a subunit Mr of 72,000. The dehydrogenase and cyclohydrolase activities are catalyzed by a single site on a fragment of Mr = 36,000. Thermograms from a differential scanning calorimeter show that the native enzyme has two thermal transitions occurring at 49 and 63°C. The thermal transition occurring at 49°C is due to the dehydrogenase activity, and the transition occurring at 63°C is due to the protein domain containing the synthetase activity. Our current model for this trifunctional enzyme is that it consists of two independent protein domains that are connected by a polypeptide called the "hinge" region. Studies on the primary and tertiary structure of the yeast enzyme suggest that it has a similar structure and that the hinge region is rich in proline residues (12).

The substrate normally used in the study of folate-requiring enzymes is tetrahydropteroylmonoglutamate. However, in eukaryotic cells, polyglutamate forms of this compound serve as the substrate. For most organisms, the physiologically active tetrahydropteroylpolyglutamate pool is comprised of a mixture of compounds containing from 4 to 8 glutamate residues (13). Why the cells make these polyglutamate forms of H4PteGlu is not clear. One function may be to serve as a cellular trap since only the monoglutamate forms are transported through the cell membrane (14). Other proposed functions include the regulation of various pathways in one-carbon metabolism and the "channeling" of the coenzyme between different folate-requiring enzymes (14-16). The polyglutamate forms of this coenzyme exhibit lower Kₘ values with most folate enzymes in contrast to those values determined with H4PteGlu (15, 16). We recently observed that the addition of H4PteGlu to C1-tetrahydrofolate synthase significantly increased the temperature at which the 10-formyltetrahydrofolate synthetase domain denatures (17). The monoglutamate form H4PteGlu did not change the denaturation temperature of this enzyme. These results suggest that the binding of H4PteGlu to 10-formyltetrahydrofolate synthetase results in a conformational change resulting in a more thermally stable protein. To our knowledge this is the first reported example that the binding of the polyglutamate chain of H4PteGlu results in a change in the structure of an enzyme. On the assumption that a change in protein conformation also causes a change in enzyme function, we have extended our observation to other polyglutamate chain lengths of H4PteGlu. In this paper we verify the requirements...
for the observed conformational change and characterize the properties of the enzyme H$_2$PteGlu, complex by several structural and kinetic experiments. The results suggest that the conformational change observed upon binding of polyglutamate forms of H$_2$PteGlu, significantly alter the functional properties of 10-formyltetrahydrofolate synthetase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glycine, formic acid, and all buffers were reagent grade and used without purification. NADP*, NADPH, MgATP, Na$_2$ADO4, AMP, MgCl$_2$, and chymotrypsin were purchased from Sigma. Stock ammonium formate solutions were made by neutralizing formic acid with ammonium hydroxide.

Cytoxic serine hydroxymethyltransferase and C$_1$-tetrahydrofolate synthetase were purified from rabbit liver as previously described (11, 18). The dehydrogenase-cyclohydrolase fragment of C$_1$-tetrahydrofolate synthetase was purified from a tryptic digest of the enzyme in the presence of NADP* (11). Dihydrofolate reductase was purified from methotrexate-resistant Lactobacillus casei by the method of Lerry and Kishikui (19).

**Synthesis of Pteroylpolyglutamates**—The isolation of pteric acid, and its coupling to protected oligo-$\gamma$-glutamic acid peptides, was performed by the method of D’Ari and Rabinowitz (20). Purity and determination of the number of glutamate residues were checked after the coupling step by thin layer chromatography (20). Amino acid analysis was used to verify the rate chain length of the pteroylpolyglutamate compounds. Duplicate samples of PteGlu, (n = 1-5) were hydrolyzed for 24 h in 6 N HCl and the glutamate concentration determined on an amino acid analyzer. For each compound, the observed concentration of glutamic acid was within 10% of the concentration determined on an amino acid analyzer. For each compound, the observed concentration of glutamic acid was within 10% of the expected value. A further check on the purity of the compounds was done by the high performance liquid chromatography method of Cashmore et al. (21). This method gives base-line resolution of the monoglutamates and 85% pure.

**Reduction of Pteroylpolyglutamates**—Twelve to fifteen mg of the oxidized pteroylpolyglutamates were reduced to H$_2$PteGlu, by a modification of the diethionite method of Zaro et al. (22). After dithionite reduction at pH 11.2 and adjusting the pH to 5.8, the solution was reduced in volume to 1 ml and chromatographed on a 2 x 35 cm Bio-Gel P2 column equilibrated with 20 mM potassium N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonate, 100 mM 2-mercaptoethanol, pH 7.5. The H$_2$PteGlu, eluted first and was identified by its absorption spectrum. The solution, was reduced from about 12 to 100 SEM by the addition of 6 N HCl. The resulting solution was 85% pure.

**Uncoupled Assay**—The activity of 10-formyltetrahydrofolate synthetase with either H$_2$PteGlu, or H$_2$PteGlu, as substrate was determined by a modification of the method of Rabinowitz and Pricer (23). The reaction was performed in 0.9 ml of 50 mM potassium phosphate, pH 6.5, containing 10 mM 2-mercaptoethanol, 25 mM ammonium sulfate, 5 mM MgATP, 25 mM ammonium formate, 0.1-0.5 mM H$_2$PteGlu, and 2-3 $\mu$g of C$_1$-tetrahydrofolate synthetase. After 2 min the reaction is stopped by the addition of 100 $\mu$l of 3 M HCl. The solution was then incubated under an Argon blanket at 30 °C until the absorbance at 355 nm (due to formation of 5,10-methenyl-H$_2$PteGlu) reached its maximum. The concentration of the final product was determined by dividing the absorbance at 356 nm by the molar absorptivity coefficient of 25,000 $\text{m}^{-1}\text{cm}^{-1}$ (1). All reactions were corrected by subtracting the absorbance of a no-enzyme control. For those reactions which compared the uncoupled assay to the coupled assay, NADP* and serine hydroxymethyltransferase were also added to the reaction solution.

**Coupled Assay**—Synthetase activity was also determined by coupling the reaction to serine hydroxymethyltransferase (Scheme 1). The buffer in this assay was the same as in the uncoupled assay except for the addition of 25 mM glycine, 170 $\mu$g of rabbit liver cytosolic serine hydroxymethyltransferase, and 0.2 mM NADPH. The rate of the synthetase reaction was determined from the decrease in absorbance at 340 nm. This assay did not give accurate rates when H$_2$PteGlu, was used as substrate. With H$_2$PteGlu, to H$_2$PteGlu, as substrates the assay was linear with time and C$_1$-tetrahydrofolate synthetase concentration. Since the H$_2$PteGlu, is recycled in this assay, the polyglutamates could be used at 1-5 $\mu$M concentrations with no change in rate of NADPH oxidation.
**RESULTS**

**Differential Scanning Calorimeter Studies**—Ligand-induced conformational changes of proteins can be detected by a variety of methods. One of the most sensitive methods for observing different conformational states of a protein is to follow its thermal denaturation in a differential scanning calorimeter (24). This instrument determines the heat capacity of the protein solution as a function of increasing temperature, which is recorded as a thermogram. Fig. 1 shows a thermogram of C1-tetrahydrofolate synthase in the presence of MgATP and either H4PteGlu2 (curve A) or H4PteGlu5 (curve B). The maximum change in heat capacity is called the transition temperature ($T_m$), and the area under the curve is related to the enthalpy of denaturation, $\Delta H_d$ (25). As shown by curve A in Fig. 1, C1-tetrahydrofolate synthase exhibits two thermal transitions. The first occurs with a $T_m$ of 50 °C and as we have shown previously is sensitive to the presence of MgATP suggesting it is due to the denaturation of the dehydrogenase-cyclohydrolase domain. Studies on isolated dehydrogenase fragment supported this interpretation (11). When H4PteGlu2 is substituted for H4PteGlu5, there is a 12 °C increase in the $T_m$ of the synthetase domain. This shift is dependent on the presence of both MgATP and ammonium ion. The determination of the $T_m$ for the synthetase domain was repeated with the addition of H4PteGlu5 of varying glutamate chain lengths. The results, in Fig. 2A, show that the binding of H4PteGlu2 or H4PteGlu5 to the synthetase domain does not alter its $T_m$ value. However, the addition of a third glutamate residue to H4PteGlu5 does result in an 11 °C increase in $T_m$ for the synthetase-H4PteGlu5 complex. The increase in $T_m$ reaches a maximum of 12 °C with H4PteGlu5.

The requirements for the change in conformation of the synthetase domain, as observed by a change in $T_m$, were determined from differential scanning calorimeter experiments in which one component of the system was changed in each experiment. These experiments were done with every glutamate chain length of H4PteGlu5 from $n = 1$ to $n = 5$. The results of these studies show that the conformational change is dependent on the presence of either MgATP or MgADP (only the data for H4PteGlu5 is recorded in Table I).
AMP, a poor competitive inhibitor of MgATP (K_i = 3.8 mM), did not give an increased T_m value for the synthetase domain. The pteroyl portion of the coenzyme is also required since the peptide poly-γ-pentaglutamate did not increase the T_m value. This compound binds at the active site with a K_i of about 0.5 mM. The pteroyl portion of the coenzyme must also be reduced to the tetrahydro form since the oxidized forms did not increase the T_m values. The exception to this statement is that PteGlu did give a 6 °C increase in the presence of MgATP. The loss of the cold lability property was also dependent on the presence of either MgATP or MgADP and ammonium ion as evidenced by the T_m of only 51 °C when it is absent from the buffer containing MgATP and either the tri-, tetra-, or pentaglutamates. Anions, such as sulfate, chloride, and acetate, have no effect on the T_m of the large domain. Also, formate, in the presence of MgADP and the polyglutamates did not significantly change the T_m value in the presence of the triglutamate.

Cold Lability Studies—We have previously shown that the 10-formyltetrahydrofolate synthetase activity is more stable at 23 than at 0 °C (11). The effect of MgATP, ammonium ion, and the tetrahydropteroylpolyglutamates on this cold lability property was investigated by incubating aliquots of the enzyme for 2 h at 0 and 23 °C with MgATP and each of the polyglutamates. The aliquots incubated at 23 °C retained all of their synthetase activity. For the 0 °C samples, those aliquots containing either the tri-, tetra-, or pentaglutamates retained greater than 96% of their original synthetase activity. Those enzyme aliquots containing either no H,PteGlu or the mono-, or diglutamate lost greater than 80% activity during the 2-h incubation (Fig. 2B). The loss of the cold lability property was also dependent on the presence of either MgATP or MgADP and ammonium ion in addition to the presence of each of the polyglutamates.

Chymotrypsin Digestion—We have previously described conditions for chymotrypsin digestion of C1-tetrahydrofolate synthase into two fragments. One fragment of M_r = 126,000 contains the synthetase activity and the other fragment, M_r = 36,000, contains the dehydrogenase and cytochrome activities (11). The synthetase-containing fragment is further slowly digested by chymotrypsin to catalytically inactive polypeptides. This slow digestion results in only about a 40% yield of the active synthetase fragment compared to a greater than 95% yield of the proteolytically stable dehydrogenase-cytochrome fragment (11). These chymotrypsin digestion experiments were repeated on aliquots of the enzyme containing MgATP and the different tetrahydropteroylpolyglutamates. One hour following the addition of chymotrypsin, an aliquot was removed from each reaction and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results from the electrophoresis experiment, plotted as the percent of the parent enzyme undigested by chymotrypsin, (determined by densitometry scanning of the gel) versus the number of glutamate residues, are shown in Fig. 2C (right ordinate). These results suggest that in the presence of either the tri-, tetra-, or pentaglutamates there was an approximately 2-fold increase in the rate of digestion of the native enzyme at the hinge region, as evidenced by the increased disappearance of the M_r = 108,000 protein band. As we have previously shown, the digestion produces two new bands with M_r values of 72,000 and 36,000 which are the synthetase and dehydrogenase-cytochrome fragments, respectively (11). On the gels, the fragment with M_r = 72,000 is present as more intense bands in those samples containing the tri-, tetra-, and pentaglutamates, indicating that these polyglutamates stabilized the synthetase domain from further digestion by this protease. This was confirmed by observing the synthetase activity after 1 and 2 h of digestion by chymotrypsin. The remaining synthetase activity after 2 h of digestion as a function of polyglutamate chain length, is shown in Fig. 2C (left ordinate). The results show that the aliquots containing either the tri-, tetra-, or pentaglutamates retained greater than 90% of their synthetase activity compared to the other aliquots which retained less than 50% of their synthetase activity. These activity and gel studies show that the polyglutamates increase the rate of digestion by chymotrypsin at the hinge region, as well as stabilize the synthetase fragment from further proteolysis. These chymotrypsin digestion effects were dependent on the presence of either MgATP or MgADP and ammonium ion.

Kinetic Studies—The most common assay used to measure 10-formyltetrahydrofolate synthetase activity is to convert the product 10-formyl-H,PteGlu to 5,10-methenyl-H,PteGlu by lowering the pH to about 2 (23). Methenyl-H,PteGlu absorbs intensely at 355 nm with a molar extinction coefficient of 25,000 M^-1 cm^-1 (1). This single point uncoupled assay permits product determination at about the 2 μM level, and therefore, is limited to determining K_m values of substrates that are greater than 2 μM. To develop a more sensitive assay, we included in the normal assay mixture NADP+, glycine, and serine hydroxymethyltransferase. As shown by the reactions in Scheme I, this assay mixture enzymatically converts the synthetase product 10-formyl-H,PteGlu to H,PteGlu. This regeneration of the H,PteGlu substrate permits the use of catalytic levels of the expensive and difficult to obtain tetrahydropteroylpolyglutamates in the assay. The coupled reaction is followed by observing the decrease in absorbance at 340 nm due to the oxidation of NADP+. Since the conversion of 10-formyl-H,PteGlu to 5,10-methylenecarbethoxy-H,PteGlu is catalyzed by the cyclohydrolase and dehydrogenase activities on the small domain of C1-tetrahydrofolate synthase, it was necessary to determine that the 10-formyl-H,PteGlu synthetase reaction was rate determining. This was done by two experiments. First, the rate of NADP+ oxidation when 10-formyl-H,PteGlu was used as substrate was an order of magnitude faster than when H,PteGlu was used as a substrate. This suggests that the conversion of H,PteGlu to 10-formyl-H,PteGlu is rate limiting. The second experiment involved adding to the assay solution an excess of the dehydrogenase-cytochrome fragment, which had been purified from a tryptic digestion of C1-tetrahydrofolate synthase. The addition of these two enzyme activities did not change the rate of NADP+ oxidation suggesting that the two reactions catalyzed by these activities are not rate determining.

We also investigated the effect of the concentration of serine hydroxymethyltransferase in the assay. In the absence of...
of this enzyme, the rate of oxidation of NADPH drops rapidly as equilibrium conditions are reached. The addition of serine hydroxymethyltransferase and glycine does not change the initial rate but increases the linearity of the assay from a few seconds to several minutes. We were also concerned about adding serine hydroxymethyltransferase in the presence of very low concentrations of the tetrahydropteroylpolyglutamates. Matthews et al. (16) have shown that the $K_a$ for \(H_4PteGlu\), with this enzyme decreases sharply as the number of glutamate residues increase. In the presence of glycine, the $K_a$ values for the tri-, tetra-, and pentaglutamates are about 0.1 $\mu$M. Although these values are determined for the pig liver enzyme, we find similar values for the rabbit liver enzyme. As the polyglutamates are bound so tightly by serine hydroxymethyltransferase, the addition of this enzyme to the assay in concentrations equal to or greater than the concentration of the substrate \(H_4PteGlu\), would result in a significant lowering of the free \(H_4PteGlu\), concentrations available to the synthetase active site and would, therefore, inhibit the reaction. We tested this possibility by determining the effect of increasing concentrations of serine hydroxymethyltransferase at concentrations up to 20 $\mu$M did not inhibit the rate of NADPH oxidation in this assay when the concentration of \(H_4PteGlu\) was 2 $\mu$M.

We conclude that the coupled cyclic assay for the synthetase activity, as shown by the reactions in Scheme I, is a convenient and accurate assay for the determination of kinetic constants for 10-formyl-\(H_4PteGlu\) synthetase. The only limitation to this assay is that it does not work when the substrate is \(H_4PteGlu\). With the monoglutaamate there is an initial lag in the reaction which can last for several minutes. Even when a linear decrease in absorbance at 340 nm is attained the rate of the reaction is only about 10% of the rate determined by the uncoupled assay. This lag and apparent decrease in the rate is not observed with the di- and higher glutamate analogues. For the results reported in the following paragraph, we have used the uncoupled assay when determining $K_a$ and $V_{max}$ values for \(H_4PteGlu\) and \(H_4PteGlu_2\) and the cyclic coupled assay for the polyglutamates \(H_4PteGlu_4\) through \(H_4PteGlu_5\). The two assays gave the same values for \(H_4PteGlu_5\).

The $K_a$ values for MgATP and formate were determined for each polyglutamate form of \(H_4PteGlu\). The results are recorded in Fig. 3. The $K_a$ for MgATP decreases from 0.13 to 0.04 mM as the substrate is changed from the monoglutaamate to the pentaglutaamate. All of the decrease in $K_a$ occurs in going from the mono- to the triglutaamate. With formate the $K_a$ value decreases from 2.5 mM to 4 $\mu$M. This nearly three-order of magnitude decrease in $K_a$ also occurs in going from the mono- to the triglutaamate. Attempts were made to determine $K_a$ values for each polyglutaamate. The $K_a$ for the monoglutaamate is 15 $\mu$M but decreases markedly with the addition of 1 or more glutamate residues. The $K_a$ drops well below 1 $\mu$M for the polyglutaamates and cannot be determined by the coupled assay.

For most folate-requiring enzymes, the addition of glutamate residues to \(H_4PteGlu\) not only decreases the $K_a$ values but also $V_{max}$ values. We have not determined true $V_{max}$ values for the synthetase reaction with each tetrahydropteroylpolyglutamate. The experimental extrapolation to infinite concentration for the three substrates and activator ammonium ion was beyond the scope of this study. We did, however, determine the velocity with all substrates at saturating conditions. The results show that the velocity decreases 6.5-fold in going from the monoglutaamate to the pentaglutaamate. The value of $k_{cat}/V_{max}$ with respect to MgATP remains relatively constant with each polyglutaamate as substrate. However, with respect to formate the value of $k_{cat}/K_a$ increases 80-fold when \(H_4PteGlu_5\) is used as the cosubstrate in comparison to \(H_4PteGlu_1\).

**DISCUSSION**

The function of the polyglutaamate chain length of \(H_4PteGlu\), on the $K_a$ values of formate and MgATP with 10-formyl-\(H_4PteGlu\) synthetase. Solid line: log $K_a$ values for formate with \(H_4PteGlu_2\) through \(H_4PteGlu_3\) as cosubstrate. Dashed line: $K_a$ values for MgATP with \(H_4PteGlu_2\) through \(PteGlu_3\) as cosubstrate.

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The function of the polyglutaamate chain length of \(H_4PteGlu\), in cellular metabolism is as yet not understood. Most work with the reduced pteroylpolyglutamates and enzymes in one-carbon metabolism have focused on determining $K_a$ and $V_{max}$ values and looking for evidence of channeling (28). To our knowledge no one has observed a polyglutamate-induced conformational change of an enzyme. It was, therefore, of interest when we observed an increase in the denaturation temperature ($T_m$) when 10-formyltetrahydrofolate synthetase bound \(H_4PteGlu\). (17). Our aim was to characterize the ligand requirements for this conformational change, to prove the nature of the conformational change, and to determine if the conformational change altered the kinetic characteristics of the enzyme.

Three separate experiments, which rely on different properties of a protein, suggest that the addition of the third glutamate residue on \(H_4PteGlu\), results in a conformational change in the synthetase domain of C, tetrahydrofolate synthase. The synthetase domain denatures at a higher temperature, loses its property of cold lability, and has altered sensitivity to protease digestion (Fig. 2). The $T_m$ for thermal denaturation of the enzyme is increased 12°C upon binding of the tri-, tetra-, or pentaglutaamtes. However, this information only provides evidence that a protein structural change has occurred, and it gives little information as to the nature of the conformational change. The loss of cold lability is more informative about this structural change. We have shown previously that the cold lability of the synthetase domain was pH insensitive from pH 6.0 to 8.0 (11). This argues for cold lability being the result of the enzyme having hydrophobic residues exposed to the solvent (27, 28). The loss of cold lability of the synthetase domain on binding of the polyglutaamtes would then mean that the conformational change has "folded in" these hydrophobic areas so that they are no longer exposed to the solvent. This folded in structure is more resistant to digestion by chymotrypsin which is consistent with the removal of hydrophobic amino acid residues.
from the surface of the domain. The chymotrypsin digestion experiments also suggest that the hinge region of C'-tetrahydrofolate synthase is more exposed in the polyglutamate bound conformation and is hydrolyzed more rapidly by chymotrypsin.

Our studies suggest that the conformational change of the synthetase domain involves the β-phosphoryl of MgATP, ammonium ion, and the reduced pteridine ring, in addition to the glutamate side chain of H₄PteGlu₄. The results from the denaturation, cold liability, and chymotryptic digestion studies suggest that only the third glutamate residue plays a role in the conformational change. These three techniques show no difference in properties of the enzyme when complexed to the tri-, tetra-, or pentaglutamate forms of the coenzyme. The observation that MgADP gives the same $T_m$ for the enzyme as MgATP shows that the conformational change is not dependent on the γ-phosphoryl group of ATP. The failure of AMP to substitute for ADP shows that the β-phosphoryl group is required. Both potassium ion and ammonium ion are activators of 10-formyl-H₄-folate synthetase activity (11).

However, there appears to be a difference in the mechanism by which these two ions interact with the enzyme since the conformational change is dependent on the presence of ammonium ion (Table I). An alternative view in which the effects of binding of the tri- to pentaglutamates do not cause a conformational change but simply reflect the interaction of the coenzyme with several residues at the active site seems unlikely. Both the oxidized forms of the coenzyme (PteGlu₄) and poly γ-pentaglutamate when bound at the active site at saturating levels do not significantly alter the thermal denaturation, cold liability, and protease sensitivity of 10-formyltetrahydrofolate synthetase. If there was not a conformational change, these compounds would be expected to produce some of the same effects as the reduced pteroylpolyglutamates since they must interact with many of the same residues at the active site. This model could not easily explain the increased rate of digestion of the hinge region and the decreased rate of digestion of the synthetase domain by chymotrypsin.

Evidence that the tetrahydropteroylglutamate-induced conformational change directly affects catalytic properties of the enzyme is less clear. The 3-fold decrease in $K_m$ for MgATP with increased polyglutamate chain length could be due to reasons other than a conformational change. However, the nearly three-orders of magnitude decrease in the $K_m$ for formate suggests that some structural change has occurred at the active site (Fig. 3). The observation that much of this lag is due to some property of the synthetase domain. Another dehydrogenase-cyclohydrolase activity has been depressed in these assays to about 50 nM with no excess over H₄PteGlu₄. The concentration of free H₄PteGlu₅ upon using H₄PteGlu₆ as cosubstrate and the lowering of the $K_m$ value of formate to the μM range.

Previous studies by Wendland et al. (29) have suggested that the prokaryotic enzyme 10-formyltetrahydrofolate synthetase also undergoes a conformational change upon binding of substrates. Although this study used only the monogluta-

mate form of H₄PteGlu₄, they provide convincing evidence from nuclear magnetic resonance and electron paramagnetic resonance experiments that the binding of the H₄PteGlu₄ to the enzyme-ATP-formate complex causes a conformational effect (29). They demonstrate that the effect was dependent on ammonium ion, which was bound at the active site. The conformational change resulted in a decrease in the off-rate of formate from the enzyme which resulted in a 10-fold decrease in its $K_m$ value. Although the prokaryotic enzyme is not multifunctional, several studies suggest that it is related in terms of structure and function to the eukaryotic forms of the enzyme (3). We are presently trying to determine with our rabbit liver enzyme if the 500-fold decrease in the $K_m$ of formate in the presence of the polyglutamates is also due to a decrease in the off-rate.

Our new coupled assay involving serine hydroxymethyltransferase (Scheme I) also suggests that an important catalytic property has been altered when H₄PteGlu₄ and higher polyglutamates are used as substrates in place of H₂PteGlu₄. The lag in NADPH reduction, when H₄PteGlu₄ is the substrate, is not reduced by the addition of the catalytically active dehydrogenase-cyclohydrolase fragment suggesting that the lag is due to some property of the synthetase domain. Another unexplained observation is the failure of excess serine hydroxymethyltransferase to inhibit the reaction when in a large excess over H₄PteGlu₄. The concentration of free H₄PteGlu₅ has been depressed in these assays to about 50 nM with no concomitant decrease in the rate of NADPH reduction. This suggests that either the $K_m$ of the synthetase for H₄PteGlu₄ is less than nM or there is channelling of the H₄PteGlu₄ from serine hydroxymethyltransferase to 10-formyl-tetrahydrofo-
late synthetase. Kinetic evidence for direct transfer of a coenzyme between two enzymes has been well established for several dehydrogenases. Srivastava and Bernhardt (30, 31) have provided convincing arguments that NADH can be reversibly channeled between an A side dehydrogenase and a B side dehydrogenase. Evidence for a role of H₄pteroylglutamates in channelling has previously been observed with the bifunctional enzyme formimino glutarate: tetrahydrofolate formiminotransferase (E.C. 2.1.2.5)-formiminotetrahydrofolate cyclodeaminase (E.C. 4.3.1.4) (26). Whether the two unusual catalytic properties of the coupled synthetase reaction, in fact, support this possibility. We have found that AMP is a noncompetitive inhibitor of formate when H₂PteGlu₃ is used as substrate but an uncompetitive inhibitor when H₄PteGlu₄ is used as substrate. Our studies on determining the order of addition of substrates have been difficult and are incomplete because the $K_m$ values for the polyglutamates are so low, and we have been unable to find a competitive inhibitor of formate. These two problems rule out the method of dead-end inhibition and product inhibition patterns to determine the order of addition and release of substrates and products. A more in-depth study is presently underway using nonkinetic methods to determine the change in mechanism of substrate addition associated with the longer chain polyglutamate forms of the reduced coenzyme.

The results of this study demonstrate that 10-formyl-

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synthetase assay, as discussed above, are related to the polyglutamate-induced conformational change and constitute another example of substrate channeling is currently under investigation.

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