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Studies on the Polyglutamate Specificity of Methylenetetrahydrofolate Dehydrogenase from Pig Liver†

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ABSTRACT: Methylenetetrahydrofolate dehydrogenase, which is one of the activities of a trifunctional folate-dependent enzyme isolated from pig liver, displays an ordered bi-bi kinetic mechanism when methylenetetrahydropteroylmonoglutamate is used as the folate substrate [Cohen, L., & MacKenzie, R. E. (1978) *Biochim. Biophys. Acta 522*, 311–317]. We have studied the inhibition of this activity by a series of pteroylglutamates containing one to seven glutamyl residues. Inhibitors with one to four glutamyl residues exhibit a kinetically determined K_D of about 56 μM for binding at the folate site of the enzyme, while inhibitors with five to seven glutamyl residues exhibit a K_D of about 16 μM. These results suggest that folylpolyglutamates are bound to the trifunctional enzyme relatively weakly, with the major interaction involving the fifth glutamyl residue of the polyglutamate "tail". A free energy decrease of about 0.74 kcal (3.1 kJ) is associated with this interaction. The possibility of a swinging arm mechanism for the trifunctional enzyme is discussed. We have also measured the kinetic parameters V_{max} and the K_m values for $NADP^+$ and the folate substrate associated with catalysis using a series of methylenetetrahydropteroylpolyglutamate substrates. The variation in these parameters with the length of the polyglutamate tail is small.

The majority of intracellular folate derivatives in mammalian cells appear to contain 5–8 glutamyl residues (Brown et al., 1974; Eto & Krumdieck, 1981; Foo & Shane, 1982). There is evidence to suggest that the distribution of folylpolyglutamates may differ from one species to another and also that the distribution may vary with the nature of the folate derivative and with metabolic conditions inside the cell (Eto & Krumdieck, 1981; Foo & Shane, 1982; Priest et al., 1981).

A number of suggestions have been advanced for the physiological roles of the polyglutamate "tails" on folate derivatives. They have been shown to assist in the intracellular retention of folate compounds (McBurney & Whitmore, 1974). The length of the polyglutamate tail has been postulated to play a role in the regulation of flux through competing pathways of one-carbon metabolism (Baggott & Krumdieck, 1979). According to this suggestion, enzymes that commit one-carbon units to the various pathways of folate-dependent one-carbon metabolism should show different specificities for polyglutamate chain length.

The free energy decrease associated with binding of the polyglutamate tail to the enzyme may be used to facilitate individual enzymatic reactions—either by increasing the affinity of the enzyme for its folate cosubstrate or by using the free energy decrease associated with binding of the folate cosubstrate to enforce a conformational change in the enzyme–folate complex that leads to enhanced binding of the non-folate substrate (enhanced ligand synergism) or enhanced binding at the transition state (an increase in V_{max}).

Polyglutamate substrates, but not the monoglutamate, are channeled between the active centers of the bifunctional enzyme formiminotransferase–formiminotetrahydrofolate cyclodeaminase (MacKenzie & Baugh, 1980; MacKenzie & Baugh, 1983), leading these authors to propose that the polyglutamate chain acts as an anchor to allow transfer of the pteroyl portion of the molecule from one active site to another without dissociation from the enzyme. Since a number of folate-dependent enzymes are isolated in association with one another, this may prove to be a very important role for the polyglutamate tail. The possible role of the polyglutamate tail of folate substrates in channeling between sites of the trifunctional enzyme methylenetetrahydrofolate dehydrogenase–methenyltetrahydrofolate cyclohydrolase–formyltetrahydrofolate synthetase has also been examined. MacKenzie & Baugh (1980) observed that no lag phase was associated with the production of CHO–H_4folate[1] from CH_2–H_4folate whether mono-, tri-, penta-, or heptaglutamyl derivatives were used as the substrates for the trifunctional enzyme from pig liver. They found that the number of glutamates on the substrate had little effect on the amount of methenyl intermediate released to the bulk solution during the course of the reaction. However, Wasserman et al. (1983) have compared channeling between the dehydrogenase and cyclohydrolase sites of trifunctional enzyme from chicken liver using pulse–chase experiments. They conclude that channeling of the triglutamate substrate proceeds to a significantly greater degree (85%) than channeling of the monoglutamate substrate (46%).

In order to assess the possible physiological roles of the polyglutamate tails of folate derivatives, we have been examining the polyglutamate specificities of a series of enzymes that are involved in the metabolism of CH_2–H_4folate. The metabolic pathways under consideration are shown in Figure 1.

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[1] Abbreviations: H_4folate, tetrahydrofolate; CH_2–H_4folate, 5,10-methylenetetrahydrofolate; CH=H_4folate, 5,10-methenyltetrahydrofolate; CH_3–H_4folate, 5-methyltetrahydrofolate; CHO–H_4folate, 10-formyltetrahydrofolate; DTT, dithiothreitol; $H_4PteGlu_n$, tetrahydropteroylpolyglutamate with n glutamyl residues; $PteGlu_n$, pteroylpolyglutamate with n glutamyl residues; CH_2–$H_4PteGlu_n$, methylenetetrahydropteroylpolyglutamate with n glutamyl residues; $H_2PteGlu_n$, dihydropteroylpolyglutamate with n glutamyl residues; GAR, glycinamide ribonucleotide; f-GAR, *N*-formylglycinamide ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide; f-AICAR, 5-(formylamino)imidazolecarboxamide ribonucleotide.

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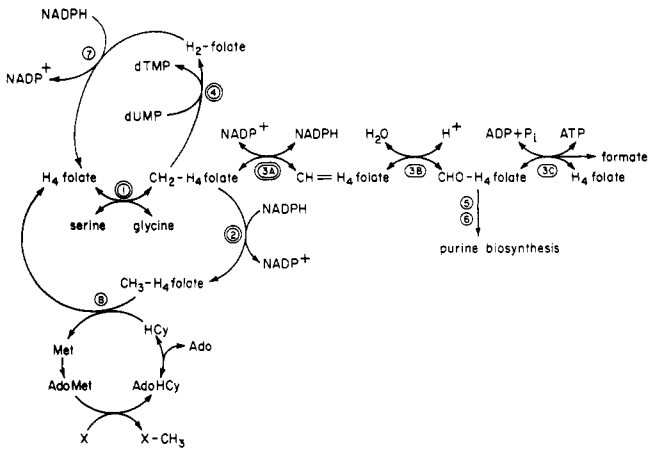


FIGURE 1: Outline of the major folate-dependent pathways in mammalian cells. The enzymes involved are (1) serine hydroxymethyltransferase, (2) methylenetetrahydrofolate reductase, (3) the trifunctional enzyme with methylenetetrahydrofolate dehydrogenase (3A), methenyltetrahydrofolate cyclohydrolase (3B), and formyltetrahydrofolate synthetase (3C) activities, (4) thymidylate synthase, (5) GAR transformylase, (6) AICAR transformylase, (7) dihydrofolate reductase, and (8) methyltetrahydrofolate–homocysteine methyltransferase. Enzyme activities designated by double circles have been or are being characterized with respect to their polyglutamate specificity by using the enzymes isolated from pig liver.

We have chosen to examine each of these enzymes from the same species and organ, viz., pig liver, so that interspecies differences in polyglutamate distribution will not be a factor in our comparisons. Studies on the polyglutamate specificities of serine hydroxymethyltransferase (Matthews et al., 1982) and methylenetetrahydrofolate reductase (Matthews & Baugh, 1980) have already been published, and studies on thymidylate synthase are currently in progress (Lu et al., 1984). This communication reports the results of our studies on the polyglutamate specificity of methylenetetrahydrofolate dehydrogenase from pig liver. This activity is one of three catalytic activities associated with a trifunctional enzyme, methylenetetrahydrofolate dehydrogenase–methenyltetrahydrofolate cyclohydrolase–formyltetrahydrofolate synthetase, which has now been isolated from pig liver (Tan et al., 1977), sheep liver (Paukert et al., 1976), rabbit liver (Schirch, 1978), yeast (Paukert et al., 1977), and chicken liver (Caparelli et al., 1978, 1980).

Experimental Procedures

Preparation of the Trifunctional Enzyme from Pig Liver. Enzyme was purified as described by Tan et al. (1977) through the first six steps of the preparation. After chromatography on phosphocellulose (step 6), $NADP^+$ was added to a concentration of 0.5 mM and ammonium sulfate to a concentration of 55% (w/v) and the enzyme was then stored at 0 °C. Prior to experiments, enzyme was further purified by chromatography on 2′,5′-ADP–Sepharose. Trifunctional enzyme was dialyzed against 60 mM potassium phosphate buffer, pH 7.2, 20% (v/v) glycerol, and 0.4 mM DTT and applied to the 2′,5′-ADP–Sepharose column (4-mL bed volume) that had been equilibrated with the same buffer. The column was washed with 180 mL of buffer. The enzyme was generally eluted from the 2′,5′-ADP–Sepharose affinity column with 1.5 mM $NADP^+$–20% glycerol–0.4 mM DTT in 60 mM phosphate buffer, pH 7.2. However, where steady-state parameters were being determined with CH_2–$H_4PteGlu_n$ substrates, the enzyme was eluted from the 2′,5′-ADP–Sepharose column with 0.5 M phosphate buffer (pH 7.2)–20% glycerol–0.4 mM DTT and was then dialyzed against 60 mM phosphate buffer–20% glycerol–0.4 mM DTT. Because the enzyme is moderately unstable after purification on 2′,5′-ADP–Sepharose columns, the final purification step was performed with small batches of enzyme, and the purified enzyme was then used for kinetic studies within 1 week. During 1 week, the enzyme activity decreased no more than 10–15%. Kinetic experiments were always performed on enzyme that had been assayed on the same day under standard conditions [100 μM (6*S*)-$H_4PteGlu_1$ added as 200 μM (6*RS*)-$H_4PteGlu_1$, 180 μM $NADP^+$], and all V_{max} values have been normalized. The specific activity of the freshly prepared purified enzyme in the standard assay was about 7.5 μmol min^{-1} mg^{-1}, in good agreement with previously reported values for homogeneous enzyme (MacKenzie & Tan, 1980; Tan et al., 1977).

Preparation of Polyglutamate Inhibitors and Substrates. Pteroylpolyglutamates were synthesized by a solid-phase method previously described (Krumdieck & Baugh, 1969, 1980). Tetrahydropteroylpolyglutamates were prepared by reduction of the corresponding pteroylpolyglutamates with NADPH using dihydrofolate reductase from *Lactobacillus casei* and were purified by chromatography on DEAE-52. Details of the procedure are given in Matthews et al. (1981). Methylenetetrahydropteroylpolyglutamates were formed nonenzymatically in the assay buffer by condensation of $H_4PteGlu_n$ with formaldehyde.

For standard assays, (6*RS*)-CH_2–H_4folate was used as the folate substrate. (6*RS*)-H_4folate was prepared by catalytic hydrogenation of a neutral aqueous solution of folate (Blakley, 1957) and was purified on DEAE-cellulose by elution with 0.25 M triethanolamine–chloride buffer, pH 7, and 0.5 M 2-mercaptoethanol (Tan et al., 1977). Stock solutions of $H_4PteGlu_n$ (0.5–10 mM) were stored under nitrogen at −20 °C.

Measurements of Inhibition of Methylenetetrahydrofolate Dehydrogenase Activity by Pteroylpolyglutamates. Pteroylpolyglutamates ($PteGlu_n$), at a final concentration of 30–200 μM, were added to assay mixtures containing 180 μM $NADP^+$, 0.15 M 2-mercaptoethanol, 2.3 mM formaldehyde, and 40–200 μM (6*S*)-$H_4PteGlu_1$ [added as (6*RS*)-$H_4PteGlu_1$] in 0.1 M phosphate buffer, pH 7.2, in a total volume of 0.5 mL. After preincubation at 30 °C for 5 min, enzyme was added to initiate the assay, and the reaction mixture was incubated for 5 min and then quenched by addition of 0.5 mL of 0.36 N HCl. After an additional 5 min at 25 °C, the absorbance at 350 nm was measured and corrected for the absorbance of a control mixture from which enzyme had been omitted. Each assay was performed in triplicate and the results were averaged. The averaged data points were displayed in double-reciprocal plots, and best fits were determined by linear regression analysis.

Determination of Steady-State Parameters with CH_2–$H_4PteGlu_n$ Derivatives. Assays were conducted at 30 °C in 0.1 M phosphate buffer, pH 7.2, at constant levels of formaldehyde (2.3 mM) and 2-mercaptoethanol (0.15 M). The $NADP^+$ concentration was varied between 20 and 200 μM. Incubations were quenched with HCl and the absorbance at 350 nm was measured as described above. Each assay was performed in triplicate or quadruplicate, the individual data points were displayed in double-reciprocal plots, and best fits were determined by linear regression analysis.

Results

We have examined the effects of a series of folypolyglutamates, $PteGlu_n$, on methylenetetrahydrofolate dehydrogenase activity. In each case, the folate cosubstrate was (6*R*)-CH_2–H_4folate, added as a racemic (6*RS*)-CH_2–H_4folate mixture. A typical experiment is shown in Figure 2. In each

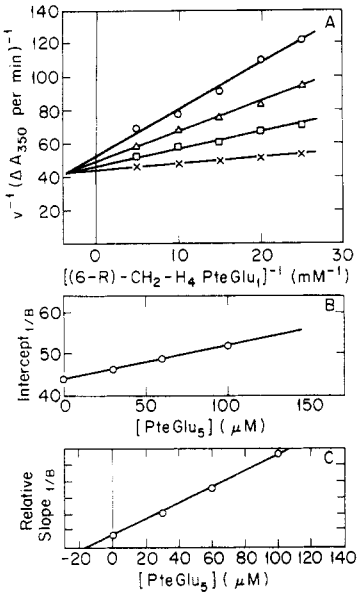
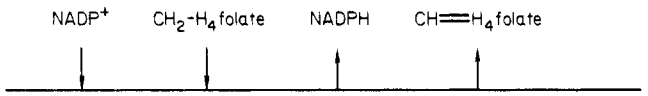


FIGURE 2: Effect of $PteGlu_5$ on methylenetetrahydrofolate dehydrogenase activity. (A) A double-reciprocal plot of velocity vs. (6*R*)-CH_2-$H_4PteGlu_1$ concentration in the presence of 0 (×), 30 (□), 60 (△), and 100 μM (○) $PteGlu_5$. (6*R*)-CH_2-$H_4PteGlu_1$ was added as the racemic (6*RS*)-CH_2-$H_4PteGlu_1$ mixture and the $NADP^+$ concentration was held fixed at 180 μM. Other conditions are as described under Experimental Procedures. (B) A replot of the *y*-axis intercepts of the lines in (A) as a function of $PteGlu_5$ concentration. (C) A replot of the slopes of the lines in (A) as a function of $PteGlu_5$ concentration.

case we observed a marked effect of the $PteGlu_n$ inhibitor on the slopes of double-reciprocal plots of velocity vs. the concentration of CH_2-H_4folate and a smaller effect of the inhibitor on the intercepts of these plots. Replots of both slopes and intercepts as a function of inhibitor concentration are linear, as illustrated in Figure 2B,C.

Studies of Cohen & MacKenzie (1978) on the steady-state kinetics of methylenetetrahydrofolate dehydrogenase from pig liver, using racemic CH_2-$H_4PteGlu_1$ as the substrate, have established that catalysis proceeds via an ordered bi-bi mechanism



and suggested that $PteGlu_1$ was competitive with respect to CH_2-H_4folate and uncompetitive with respect to $NADP^+$. While we observe generally similar inhibition patterns, in all cases the $PteGlu_n$ inhibitors show small, linear intercept effects in double-reciprocal plots of velocity vs. [CH_2-H_4folate]. These observations could readily be explained if $PteGlu_n$ inhibitors bind to the $NADP^+$ site on the enzyme as well as the CH_2-H_4folate site. The steady-state equation for v/V_{max} in a reaction governed by an ordered bi-bi kinetic mechanism, in the presence of an inhibitor that is purely competitive with respect to substrate *B*, is given by

$$v/V_{max} = [A][B]/\{K_{iA}K_{mB} + K_{mB}[A] \times (1 + [I]/K_i') + K_{mA}[B] + [A][B]\} \quad (1)$$

(Segel, 1975) where K_i' is the dissociation constant for binding of I at the site of substrate B on the enzyme. If inhibitor also can bind at the site of substrate A on the enzyme, with dissociation constant K_i, this equation must be modified as shown in eq 2.

$$v/V_{max} = [A][B]/\{K_{iA}K_{mB}(1 + [I]/K_i) + K_{mB}[A] \times (1 + [I]/K_i') + K_{mA}[B](1 + [I]/K_i) + [A][B]\} \quad (2)$$

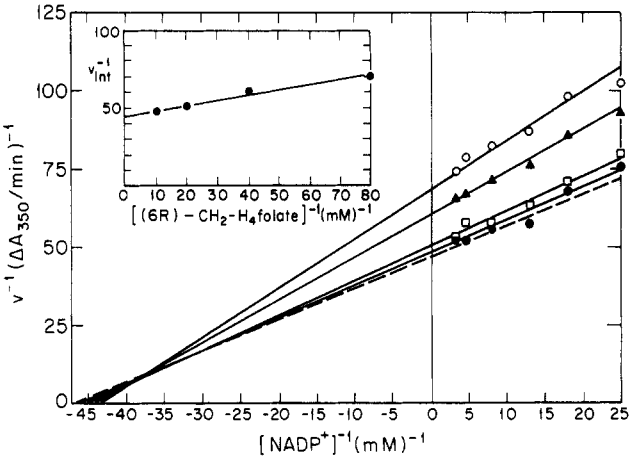


FIGURE 3: Measurement of the steady-state kinetic parameters associated with methylenetetrahydrofolate dehydrogenase activity when (6*RS*)-CH_2-$H_4PteGlu_1$ is used as the substrate. Double-reciprocal plots of velocity vs. $NADP^+$ concentration are shown for (6*R*)-CH_2-$H_4PteGlu_1$ concentrations of 12.5 (○), 25 (▲), 50 (□), and 100 μM (●). Assays were performed under the conditions described under Experimental Procedures. Each point shown is the average of three to four individual assays. The dashed line represents the extrapolated double-reciprocal plot when (6*R*)-CH_2-$H_4PteGlu_1$ is present at saturating concentrations. The inset shows a replot of the *y*-axis intercepts of the double-reciprocal plots vs. (6*R*)-CH_2-$H_4PteGlu_1$ concentration and was used for the estimation of the position of the dashed line at saturating CH_2-$H_4PteGlu_1$.

At any given inhibitor concentration [I] the intercept value for a double-reciprocal plot of velocity vs. [B] will be given by eq 3.

$$\text{intercept}_{1/B} = (K_{mA}/[A])(1 + [I]/K_i) + 1 \quad (3)$$

A replot of intercept$_{1/B}$ vs. [I] will have a slope given by $K_{mA}/([A]K_i)$ and an intercept of $(K_{mA}/[A]) + 1$. If K_{mA} and [A] are known, K_i can then be calculated. The slope value for a double-reciprocal plot of velocity vs. [B] at a given inhibitor concentration, [I], is given by eq 4.

$$\text{slope}_{1/B} = \frac{K_{iA}K_{mB}}{[A]}(1 + [I]/K_i) + K_{mB}(1 + [I]/K_i') \quad (4)$$

A replot of slope$_{1/B}$ vs. [I] will have a slope given by $K_{mB}[K_{iA}/([A]K_i) + 1/K_i']$ and an intercept given by $K_{mB}(1 + K_{1A}/[A])$. If K_{iA}, K_i, and [A] are known, K_i' can then be calculated.

We have measured the steady-state parameters K_{mA} and K_{iA} associated with methylenetetrahydrofolate dehydrogenase activity when (6*RS*)-CH_2-H_4folate is used as the substrate. The results of this experiment are shown in Figure 3. The values obtained for the kinetic parameters are K_{iA} = 27 μM, K_{mA} = 22 μM, K_{mB} = 6.5 μM, and $V_{max}/v_{standard\ assay}$ = 1.13. Using these values for K_{mA} and K_{iA} we can now calculate values for K_i and K_i' for each $PteGlu_n$ inhibitor. The replots of intercept$_{1/B}$ vs. [I] and slope$_{1/B}$ vs. [I] are shown for $PteGlu_5$ in parts B and C of Figure 2. Table I summarizes the values for K_i and K_i' obtained for each $PteGlu_n$ inhibitor. The values obtained for K_i' can be used to construct a linear free energy plot relating the decrease in free energy associated with binding of glutamyl residues in the polyglutamate tail of $PteGlu_n$ to the number of glutamyl residues, *n*. This plot is shown in Figure 4.

The kinetic parameters associated with the methylenetetrahydrofolate dehydrogenase activity have also been determined by using enzymatically prepared CH_2-$H_4PteGlu_1$, CH_2-$H_4PteGlu_4$, and CH_2-$H_4PteGlu_5$. These values are shown in Table II. Since the differences in kinetic parameters were small and might lie within the range of experimental error

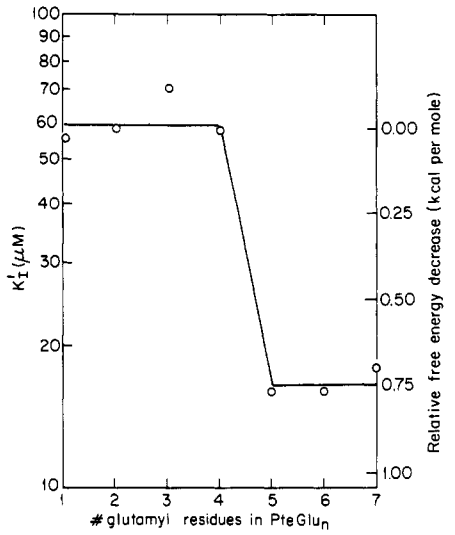


FIGURE 4: Free energy diagram of the binding of PteGlu$_n$ derivatives at the active center of the trifunctional enzyme that is responsible for methylenetetrahydrofolate dehydrogenase activity. A semilogarithmic plot of K_i' vs. the number of glutamyl residues, n, in PteGlu$_n$ is shown. On the right-hand margin are indicated the magnitudes of $\Delta(\Delta G)$, the incremental free energy decrease seen on binding of polyglutamate, as compared to monoglutamate, inhibitors.

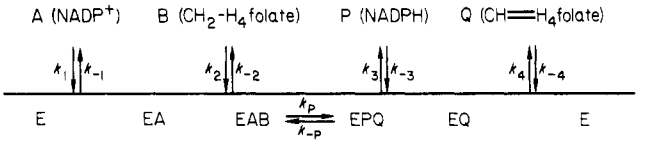
Table I: Inhibition Constants for PteGlu$_n$

inhibitor	K_i (at NADP$^+$ site) (μM)	K_i' (at CH_2–H_4folate site) (μM)
PteGlu$_1$	72	55
PteGlu$_2$	64	58
PteGlu$_3$	57	70
PteGlu$_4$	47	57
PteGlu$_5$	62	16
PteGlu$_6$	44	16
PteGlu$_7$	42	18

of the measurements, preparations of CH_2–H_4PteGlu$_4$ and CH_2–H_4PteGlu$_5$ were also compared directly with the same enzyme on the same day. At two concentrations of the folate substrate, 12.5 and 100 μM, kinetic measurements were made over a range of NADP$^+$ concentrations from 40 to 200 μM. No significant differences in velocity at saturating NADP$^+$ concentrations were observed at either high or low concentrations of the folate substrate, indicating that V_{max} and the K_m for the folate substrate do not change with polyglutamate chain length. Nor were significant differences in slope observed at either high or low folate substrate concentration. We conclude that the 3.5-fold difference observed when the K_i values of PteGlu$_4$ and PteGlu$_5$ are compared is not detectable in the kinetic parameters measured when the corresponding CH_2–H_4folate substrates are used.

Experiments have also been performed to ascertain that the order of substrate addition and product release does not change as a function of the number of glutamyl residues on the polyglutamate tail of the folate substrate. NADPH was found to be a mixed-type inhibitor with respect to NADP$^+$ whether CH_2–H_4PteGlu$_1$, CH_2–H_4PteGlu$_4$, or CH_2–H_4PteGlu$_5$ was

Scheme I[a]



[a] For an ordered bi-bi mechanism of this type, the following kinetic parameters are obtained (Segel, 1975): $K_{mA} = k_3k_4k_P/[k_1(k_3k_4 + k_3k_P + k_4k_P + k_4k_{-P})]$; $K_{mB} = k_4(k_{-2}k_3 + k_{-2}k_{-P} + k_3k_P)/[k_2(k_3k_4 + k_3k_P + k_4k_P + k_4k_{-P})]$; $V_{max}/[E]_T = k_3k_4k_P/(k_3k_4 + k_3k_P + k_4k_P + k_4k_{-P})$.

used as the substrate, and PteGlu$_5$ was found to show a "nearly competitive" pattern of inhibition with respect to CH_2–H_4-folate, similar to the pattern observed in Figure 2A, and to show a "nearly uncompetitive" pattern of inhibition with respect to NADP$^+$.

Discussion

It can be seen in Figure 4 that methylenetetrahydrofolate dehydrogenase exhibits relatively little discrimination between folate inhibitors on the basis of the length of their polyglutamate tail. The total free energy decrease associated with binding of the six terminal residues of PteGlu$_7$ is only 0.75 kcal, and this free energy decrease is apparently associated with a binding site for the fifth glutamyl residue in the polyglutamate tail. In contrast, the binding of H_2PteGlu$_6$ to methylenetetrahydrofolate reductase is associated with a free energy decrease of 3.75 kcal, and the enzyme has binding sites for the five terminal glutamyl residues of H_2PteGlu$_6$, each of which contributes about 0.75 kcal to the total free energy change (Matthews & Baugh, 1980). The binding of H_4PteGlu$_7$ to the enzyme–glycine complex of serine hydroxymethyltransferase is associated with a free energy decrease of 2.7 kcal, with 2 kcal of this free energy decrease associated with binding of the second and third glutamyl residues of H_4PteGlu$_n$ analogues and the fourth through seventh residues each contributing about 0.17 kcal to the observed free energy decrease (Matthews et al., 1982). What is unexpected in the free energy profile for methylenetetrahydrofolate dehydrogenase is the apparent absence of interactions between the enzyme and the second through fourth and sixth and seventh glutamyl residues, with retention of a considerable interaction between the enzyme and the fifth glutamyl residue in the chain.

As shown in Table II, we see no effect of the polyglutamate chain length of substrates on the kinetic parameters associated with methylenetetrahydrofolate dehydrogenase activity. Similar results were reported by Rabinowitz (1983) in studies on the dehydrogenase activity of the trifunctional enzyme from yeast. In view of our failure to observe alterations in the kinetic parameters K_{mA}, K_{mB}, and V_{max} when the polyglutamate chain length of substrates was varied, one may question whether our observations of a variation in K_i with polyglutamate chain length of PteGlu$_n$ inhibitors could be significant. The definition of these kinetic parameters in terms of the rate constants associated with binding of substrates and dissociation of products and with the interconversion of the central complexes

Table II: Determination of Kinetic Parameters Associated with Methylenetetrahydrofolate Dehydrogenase Activity

substrate	$V_{max}/v_{std\ assay}$	K_m(NADP$^+$) (μM)	K_m(CH_2–H_4folate) (μM)
(6*RS*)-CH_2–H_4PteGlu$_1$	1.13	22	6.5
(6*R*)-CH_2–H_4PteGlu$_1$	1.03	8	3.8
	1.11	13	5.9
(6*R*)-CH_2–H_4PteGlu$_4$	1.07	9	4.2
(6*R*)-CH_2–H_4PteGlu$_5$	1.02	13	2.4

is shown for an ordered bi-bi mechanism in Scheme I.[2] Given that a change in K_i values as the structure of a competitive inhibitor is varied reflects a change in the dissociation constants for the inhibitors, it is difficult to predict what effects are expected when the same structural variations are imposed on substrates. As discussed in the introduction, binding changes may be expressed in the binding of substrate or product to the enzyme to form binary complexes (in which case k_{-2}/k_2 and k_4/k_{-4} might be affected) or they may be expressed primarily at the transition state for the covalent catalysis (in which case they will affect only k_P and k_{-P}) or they may be expressed as enhanced ligand synergism (affecting k_3/k_{-3}). Whether or not such changes are apparent in K_{mA}, K_{mB}, and V_{max} depends both on where in the catalytic cycle the binding energy is utilized and on whether the affected terms are dominant in the expressions for these kinetic parameters. For example, changes in k_4 will not affect K_{mA}, K_{mB}, or V_{max} if $k_4 \gg k_P$. Conversely, it is difficult to infer binding properties from variations in kinetic parameters with substrate structure.

However, interpretation of the variation in K_i values for competitive inhibitors is not without its own difficulties. In this case, one should be wary of the possibility that a weakly bound substrate analogue might bind to a region that is not involved in substrate binding because the inhibitor is not subject to as many constraints as the substrate. Thus, additional evidence will be required to prove that our observation of a "distal binding site" for the fifth glutamyl residue of $PteGlu_n$ is germane to the binding of the folate substrates of the trifunctional enzyme from pig liver. Should such a distal site be verified, it may suggest the use of the polyglutamate tail as a swinging arm, weakly anchored by the fifth glutamyl residue. Such an arm could serve to move the folate substrate from one active site to another on the trifunctional enzyme, by analogy with its proposed function in formiminotransferase–formiminotetrahydrofolate deaminase (MacKenzie & Baugh, 1980; MacKenzie & Baugh, 1983).

Further experiments will be required to delineate these possibilities. It will be important to determine whether interconversions of the central complexes or product dissociation limits V_{max} for the dehydrogenase activity of the pig liver enzyme and whether the folate substrates are "sticky" or not. Evidence for or against a distal binding site for folylpolyglutamates could be sought by looking for evidence of overlap between the binding regions for polyglutamate substrates at the dehydrogenase, cyclohydrolase, and synthetase sites of the pig liver enzyme. Considerable evidence suggests that the dehydrogenase and cyclohydrolase sites are spatially separated from the synthetase site on trifunctional enzymes from eukaryotes, while in prokaryotes they are located on separate polypeptide chains. Paukert et al. (1977) showed that tryptic digestion of the yeast trifunctional enzyme led to coordinate loss of dehydrogenase–cyclohydrolase activities while the synthetase activity was retained on a fragment of 76 000 daltons. Tan & MacKenzie (1977) formed a bifunctional dehydrogenase–cyclohydrolase fragment of 30 000 daltons by tryptic digestion of trifunctional enzyme from pig liver and observed complete loss of synthetase activity. Cohen & MacKenzie (1978) observed that 60% of the $CH{=}H_4$folate produced by dehydrogenase activity appeared to be channeled into the cyclohydrolase site and converted to $CHO{-}H_4$folate. Later studies (MacKenzie & Baugh, 1980) indicated that the number of glutamyl residues on the $CH_2{-}H_4PteGlu_n$ substrate had little effect on the extent of channeling between dehydrogenase and cyclohydrolase sites. Schirch (1978) showed that dehydrogenase and cyclohydrolase activities from the rabbit enzyme showed the same heat inactivation profile and that $NADP^+$ protected both activities to the same extent. He also noted the failure of $CH{=}H_4$folate to accumulate during conversion of $CH_2{-}H_4$folate to $CHO{-}H_4$folate. Wasserman et al. (1983) have made an extensive study of the kinetic relationships between the various activities of the trifunctional enzyme from chicken liver. They observe 85% channeling of $CH{=}H_4PteGlu_3$, formed by dehydrogenase activity, into the cyclohydrolase site. However, using $H_4PteGlu_3$ as the substrate, they saw no evidence for channeling between synthetase and cyclohydrolase sites. Unfortunately, the polyglutamate specificity of the chicken liver enzyme is not known. It will be of interest to look for channeling between synthetase and cyclohydrolase sites of pig liver trifunctional enzyme by using $H_4PteGlu_5$, and we plan to perform these experiments.

Registry No. $PteGlu_1$, 59-30-3; $PteGlu_2$, 19360-00-0; $PteGlu_3$, 89-38-3; $PteGlu_4$, 29701-38-0; $PteGlu_5$, 33611-85-7; $PteGlu_6$, 35409-55-3; $PteGlu_7$, 6484-74-8; (6*RS*)-CH_2-H_4Glu_1, 3432-99-3; (6*R*)-CH_2-$H_4PteGlu_1$, 31690-11-6; (6*R*)-CH_2-$H_4PteGlu_4$, 65391-22-2; (6*R*)-CH_2-$H_4PteGlu_5$, 83679-35-0; NADP, 53-59-8; methylenetetrahydrofolate dehydrogenase, 9029-14-5.

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[2] We *cannot* assume that interconversion of the central complexes is rapid relative to product dissociation. The relative rates of these processes is unknown for the pig liver enzyme. For the enzyme from yeast, a 1.7-fold kinetic isotope effect was seen when the oxidation of $CH_2{-}H_4$folate was compared with that of $CD_2{-}H_4$folate, indicating that interconversion of the central complexes is at least partially rate limiting for the yeast enzyme (Farina et al., 1973).

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Purification and Characterization of a Human Platelet Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: A cyclic nucleotide phosphodiesterase was extensively purified from the 100000*g* supernatant fraction of human platelets. The purification was 2500–3000-fold with 30% recovery of activity. The enzyme was isolated by DEAE-cellulose chromatography followed by adsorption to blue dextran–Sepharose and elution with cAMP. The protein has a molecular weight of 140 000 as determined by gel filtration. On $NaDodSO_4$-containing polyacrylamide gels the major band is at 61 000 daltons, suggesting that the enzyme may exist as a dimer in solution under nondenaturing conditions. The enzyme requires Mg^{2+} or Mn^{2+} for activity. The calcium binding protein calmodulin does not stimulate hydrolysis of cAMP by this enzyme. The purified enzyme hydrolyzes both cAMP and cGMP with normal Michaelis–Menten kinetics with K_m values of 0.18 μM and 0.02 μM, respectively. The hydrolysis of cGMP, however, is only one-tenth as rapid as the hydrolysis of cAMP. Cyclic GMP does not stimulate cAMP hydrolysis but instead is a potent competitive inhibitor of cAMP hydrolysis. The enzyme is also competitively inhibited by the phosphodiesterase inhibitors papaverine, 3-isobutyl-1-methylxanthine, and dipyridamole. The enzyme did not cross-react with an antibody raised to a cAMP phosphodiesterase isolated from dog kidney, indicating that the enzymes are not immunologically related. The inhibition of cAMP hydrolysis by cGMP suggests a possible regulatory link between these two cyclic nucleotides. One of the roles of cGMP in platelets may be to potentiate increases in intracellular cAMP by inhibiting the hydrolysis of cAMP by this enzyme.

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17), the catabolic enzyme for the important regulatory nucleotides cAMP and cGMP, has been reported to exist in multiple forms in a wide variety of tissues and cell types [for recent reviews, see Wells & Hardman (1977), Strada & Thompson (1978), Vaughn et al. (1981), Appleman et al. (1982), and Beavo et al. (1982)]. These varieties of the enzyme differ in their substrate specificities, kinetic characteristics, and physical properties and in their response to natural and pharmacologic regulators. The physiological significance and function of these various enzymatic species are not well understood. Whether this diversity is due to the existence of unique enzymes, interconvertible molecules, or common subunits is still unclear. Several cyclic nucleotide phosphodiesterases from different species and sources have been purified and characterized (Miki et al., 1975; Ho et al., 1977; Morrill et al., 1979; Thompson et al., 1979a; Martins et al., 1982). In addition, antibodies have been raised against some of these purified phosphodiesterases (Tucker et al., 1981; Hansen & Beavo, 1982; Mumby et al., 1982; Sarada et al., 1982). Biochemical and immunological characterization of purified cyclic nucleotide phosphodiesterases should facilitate our understanding of the specific functions, interrelationships, and regulation of the multiple forms of cyclic nucleotide phosphodiesterase within cells.

In human platelets an increase in intracellular levels of cAMP is associated with the inhibition of platelet responses such as shape change, aggregation, adhesion, and release of granule contents [for reviews, see Salzman & Weisenberger (1972), Haslam (1973), and Mills (1982)] and thus may regulate platelet participation in physiological hemostasis and pathological thrombosis. Phosphodiesterase inhibitors such as the methylxanthines, papaverine, and dipyridamole have been shown to inhibit platelet activation (Ardlie et al., 1967; Markwardt et al., 1967; Vigdahl et al., 1971). Furthermore, such inhibitors potentiate the inhibition of platelets by such adenylate cyclase agonists as PGE_1, PGI_2, and adenosine (Markwardt et al., 1967; Mills et al., 1970; Mills & Smith, 1971; Jorgensen et al., 1979).

Hidaka and co-workers (Hidaka & Asano, 1976; Asano et al., 1977) reported the separation of three forms of cyclic nucleotide phosphodiesterase from human platelets by DEAE-cellulose chromatography. One enzyme appeared to be specific for cGMP, the second relatively nonspecific, and the third relatively specific for cAMP. In this study we report

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