Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroylpolyglutamate substrates and with dihydropteroylpolyglutamate inhibitors

ARTICLE *in* BIOCHEMISTRY · JUNE 1980

Impact Factor: 3.02 · DOI: 10.1021/bi00551a005 · Source: PubMed

2 AUTHORS, INCLUDING:

Rowena Green Matthews
University of Michigan
172 PUBLICATIONS **13,339** CITATIONS

Interactions of Pig Liver Methylenetetrahydrofolate Reductase with Methylenetetrahydropteroylpolyglutamate Substrates and with Dihydropteroylpolyglutamate Inhibitors†

Rowena G. Matthews* and Charles M. Baugh

ABSTRACT: Dihydrofolate and dihydropteroylpolyglutamates inhibit pig liver methylenetetrahydrofolate reductase. In all cases the inhibition is linearly competitive with respect to methylenetetrahydrofolate. The K_i values decrease with each additional glutamyl residue from one to six, from a value of 6.5 μM for dihydrofolate to 0.013 μM for dihydropteroylhexaglutamate. Dihydropteroylheptaglutamate has a K_i of 0.065 μM. These data indicate a free energy of binding of $\sim$0.75 kcal/mol for each of the five terminal glutamyl residues in dihydropteroylhexaglutamate. Methylenetetrahydropteroylpolyglutamates are substrates for the enzyme, and the increased free energy of binding is reflected in increased values for V_{max}/K_m with polyglutamate substrates. V_{max} is increased 1.76-fold on going from the mono- to the diglutamate substrate; additional glutamyl residues lead to decreases in K_m values for methylenetetrahydropteroylpolyglutamates. Our results suggest that the in vivo activity of methylenetetrahydrofolate reductase may also be sensitive to fluctuations in the ratio of methylenetetrahydropteroylpolyglutamates to dihydropteroylpolyglutamates and that this ratio may be important in determining the relative fluxes of methylenetetrahydropteroylpolyglutamates into the pathways leading to thymidylate biosynthesis and methionine regeneration.

Following the isolation from yeast of a polyglutamyl peptide linked to *p*-aminobenzoic acid (Ratner et al., 1946), studies from many laboratories have demonstrated that intracellular folate derivatives are present mainly as polyglutamyl derivatives with from two to seven glutamyl residues linked through their γ-carboxyl groups (Baugh & Krumdieck, 1971; Leslie & Baugh, 1974; Brown et al., 1974; McBurney & Whitmore, 1974; Taylor & Hanna, 1977). Generally, such pteroylpolyglutamyl derivatives have been shown to lead to increases in V_{max}/K_m relative to the corresponding monoglutamates. The following enzymes catalyzing folate-dependent reactions have been studied: mammalian dihydrofolate reductase (Coward et al., 1974), methionine synthetase from bovine brain (Coward et al., 1975), clostridial formyltetrahydrofolate synthetase (Curthoys & Rabinowitz, 1972), avian AICAR[1] transformylase (Baggott & Krumdieck, 1979), pig liver formiminotransferase/cyclodeaminase (Mackenzie, 1979), and both bacterial (Kisliuk et al., 1974) and human (Dolnick & Cheng, 1978) thymidylate synthetase [see, for example, Garrett et al. (1979)].

In general, polyglutamyl substrates are characterized by V_{max} values which are the same as or only slightly (two- to threefold) greater than those of the corresponding monoglutamate derivatives, and it is the K_m values which are decreased when polyglutamyl substrates are used.

Selhub et al. (1971) showed that cystathionine γ-synthase from *Neurospora* was activated by methyltetrahydrofolate and inactivated by *S*-adenosylmethionine. Methyltetrahydropteroylheptaglutamate was shown to be more effective than the diglutamate at preventing *S*-adenosylmethionine inactivation.

Kisliuk et al. (1974) showed that pteroyl- and dihydropteroylpolyglutamates were much more potent inhibitors of *Lactobacillus casei* thymidylate synthetase than the corresponding monoglutamates and suggested a regulatory role for cellular pteroylpolyglutamate derivatives in vivo as well as in vitro.

In this paper we present data on the K_i values of a series of dihydropteroylpolyglutamates which function as competitive inhibitors of methylenetetrahydrofolate reductase. Such studies can be used to estimate the free energy of binding associated with each additional glutamyl residue, yielding valuable information on the specificity of the binding site on the enzyme with respect to the polyglutamyl chain.

We have also examined the specificity of the enzyme with respect to methylenetetrahydropteroylpolyglutamate substrates. Our observations were designed to provide data which could be used to assess the possibility that regulation of the CH_2-$H_4PteGlu_n/H_2PteGlu_n$ ratio might be physiologically important in regulating the partitioning of CH_2-$H_4PteGlu_n$ into the competing pathways of thymidylate biosynthesis and the regeneration of methionine from homocysteine.

Experimental Procedure

Purification and Assay of Methylenetetrahydrofolate Reductase. Purification of pig liver methylenetetrahydrofolate reductase was performed as described by Matthews & Haywood (1979b). Enzyme preparations with specific activities of 0.5–1.0 μmol of CH_3-$H_4PteGlu_1$ oxidized per min per mg of protein were used for these experiments. NADPH–methylenetetrahydrofolate reductase activities were measured at 25 °C, under nitrogen, in 50 mM phosphate buffer, pH 6.7, 0.3 mM in EDTA, 2 μM in FAD, and 50 mM in β-mercaptoethanol. The buffer was bubbled with nitrogen in a cuvette and covered with parafilm. NADPH, any inhibitors, enzyme,

† From the Biophysics Research Division and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109, and the Department of Biochemistry, College of Medicine, University of South Alabama, Mobile, Alabama 36688. *Received November 29, 1979.* This work has been supported in part by U.S. Public Health Service Grant GM 24908.

* Correspondence should be addressed to this author at the University of Michigan. This work was performed during the tenure of an Established Investigatorship of the American Heart Association.

[1] Abbreviations used: $H_2PteGlu_n$, dihydropteroylpolyglutamate with *n* glutamyl residues; CH_2-$H_4PteGlu_n$, methylenetetrahydropteroylpolyglutamate with *n* glutamyl residues; $PteGlu_n$, pteroylpolyglutamate with *n* glutamyl residues; AICAR, 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide; EH_2, two electron reduced methylenetetrahydrofolate reductase.

0006-2960/80/0419-2040$01.00/0

and finally CH_2-$H_4PteGlu_n$ were introduced into the cuvette by using Hamilton gas-tight syringes. When V_{max} determinations of polyglutamate substrates were being performed, they were compared with a standard curve obtained by using 10–100 μM (±)-CH_2-$H_4PteGlu_1$.[2] This substrate was freshly prepared each day by dissolving Sigma Chemical Co. (±)-tetrahydrofolate in nitrogen-equilibrated 0.1 M ammonium carbonate buffer, pH 9.2, 50 mM in formaldehyde. The solution was stored at 25 °C for 1 h prior to use. Preincubation of the enzyme for 15 min in a solution containing 2 μM FAD and 100 μM NADPH was performed prior to each series of assays.

Preparation of Substrates and Inhibitors. Pteroylpolyglutamates were synthesized by the solid-phase method previously described (Krumdieck & Baugh, 1969, 1980).

Dihydropteroylpolyglutamates were prepared by dithionite reduction of pteroylpolyglutamates by a modification of the method described by Coward et al. (1975). $PteGlu_n$ (10–20 μmol) was dissolved in 2–5 mL of 0.05 M phosphate buffer, pH 7.6, 0.3 mM in EDTA, and placed in a Thunberg tube. Dithionite, in the amount indicated in Table I, was placed in the side arm. The Thunberg tube was alternately evacuated and equilibrated with oxygen-free nitrogen by using an anaerobic train previously described (Williams et al., 1979). The dithionite was then tipped into the solution, and the solution was incubated in the dark for 30 min. The $H_2PteGlu_n$ was purified by chromatography on DEAE-Sephadex A-25. A 0.9 × 25 cm column was equilibrated with 5 mM Tris buffer, pH 8.0, 0.2 M in NaCl and 50 mM in β-mercaptoethanol, and the $H_2PteGlu_n$ was eluted with a linear gradient of 500 mL of 0.2–0.7 M NaCl in the same buffer. The $H_2PteGlu_n$ concentration was determined by using an extinction coefficient of 28 400 M^{-1} cm^{-1} at 282 nm (Blakley, 1969).

Methylenetetrahydropteroylpolyglutamates were prepared starting from the corresponding $H_2PteGlu_n$ derivatives, formed by dithionite reduction as described above. The $H_2PteGlu_n$ derivatives were separated from residual dithionite and sulfite by chromatography on Sephadex G-10 equilibrated with 5 mM Tris buffer, pH 8.0, 50 mM in β-mercaptoethanol. Fractions containing $H_2PteGlu_n$ were pooled. Derivatives of chain length greater than two glutamyl residues eluted in a peak preceding the dithionite. The mono- and diglutamyl derivatives adsorb to the Sephadex under these conditions, and fractions eluting after the dithionite were pooled. Fractions containing $H_2PteGlu_n$ were brought to 10 mM in Tris buffer, pH 8.0, 100 mM in β-mercaptoethanol, and reduced in the presence of a 1.2-fold excess of NADPH by using purified dihydrofolate reductase from trimethoprim-resistant *Escherichia coli* (Poe et al., 1979). Formaldehyde was added to achieve a 10 mM concentration, and after 15 min the pH of the solution was raised above 8.6 by addition of 0.5 mL of 1 M ammonium carbonate, pH 9.2. The resultant (+)-CH_2-$H_4PteGlu_n$ was purified by chromatography on a 0.9 × 15 cm column of DEAE-52, equilibrated with 0.01 M ammonium carbonate, pH 9.2, 1 mM in formaldehyde, and eluted with 200 mL of a 0–0.5 M NaCl gradient in the same buffer. Fractions of 3 mL were collected in tubes containing 10 μL of 12.3 M formaldehyde. The CH_2-$H_4PteGlu_n$ content was determined in phosphate buffer, pH 7, by using an extinction coefficient of 32 000 M^{-1} cm^{-1}. The absorbance was maximal at 297 nm, rather than 294 nm as previously reported (Blakley, 1969). Purified CH_2-$H_4PteGlu_n$ was equilibrated with nitrogen and stored under nitrogen during the kinetic studies, which were performed within 3 h of preparation of substrates.

Table I: Preparation of Dihydropteroylpolyglutamates by Dithionite Reduction[a]

pteroylglutamate	yield (%)
$PteGlu_2$	48
$PteGlu_3$	69
$PteGlu_4$	74
$PteGlu_5$	55
$PteGlu_6$	80
$PteGlu_7$	70

[a] For reduction of $PteGlu_2$, a 25-fold excess of dithionite over $PteGlu_2$ was employed. For the longer chain pterolypolyglutamates, a 100-fold excess of dithionite was used.

Determination of V_{max} and K_m Values for Polyglutamate Substrates. All determinations of V_{max} and K_m for polyglutamate substrates were performed on aliquots of enzyme from the same preparation. In each case V_{max} for the polyglutamate substrate was compared with V_{max} for (±)-CH_2-$H_4PteGlu_1$ on the same day. Complete steady-state analyses, in which CH_2-$H_4PteGlu_n$ and NADPH were varied independently, were performed on the mono-, tri-, penta-, and hexaglutamate substrates. V_{max} for the diglutamate substrate was determined by extrapolation of data obtained at 130 μM NADPH and varied CH_2-$H_4PteGlu_n$, assuming a K_m for NADPH of 15 μM. V_{max} was determined for tetra- and heptaglutamate substrates by fixed ratio extrapolation (Segel, 1975) using an NADPH/CH_2-$H_4PteGlu_n$ ratio of 10:1. In all cases the concentration of (±)-CH_2-$H_4PteGlu_n$ was varied between 4 and 50 μM. For substrates with three or more glutamyl residues, the K_m for the polyglutamate substrate was too low to be measured directly from the slope of the double-reciprocal plot. For these substrates, the K_m was determined from the slope of double-reciprocal plots obtained at several concentrations of $H_2PteGlu_n$ of the same chain length as the substrate. Since the K_i values for these inhibitors have been measured, they can be used to calculate the K_m for the substrates by using the equation (Segel, 1975)

$$\text{slope}_{1/s} = (K_m/V_{max})(1 + I/K_i)$$

where the $\text{slope}_{1/s}$ is the slope of the Lineweaver–Burk plot at that concentration of I.

Measurements of Solvent Isotope Effects on CH_2-$H_4PteGlu_n$ Reduction. Values of pD were obtained by adding 0.4 to the pH meter readings. V_{max} was determined at each pH by fixed ratio extrapolation (Segel, 1975). The assay conditions were as described above. For the measurements in D_2O, enzyme stock in H_2O was diluted 10-fold with D_2O and incubated for 2 h prior to kinetic measurements. Dilution of this enzyme stock into an H_2O assay mixture established that enzyme activity was not lost during incubation in D_2O. The D_2O assays were performed at a final D_2O concentration of 97%.

Results

When $H_2PteGlu_n$ was produced by dithionite reduction under anaerobic conditions and the product rapidly separated from residual dithionite and sulfite, yields were considerably better than those reported by Coward et al. (1975), as shown in Table I. Presumably, side chain cleavage occurs during aerobic dithionite reductions. Amino acid analyses were

[2] The natural diastereoisomer of CH_2-$H_4PteGlu_n$ has the *R* configuration at C6 of the pteridine ring (Fontecilla-Camps et al., 1979) and the α-carbons of each glutamyl residue have *S* configurations. This isomer will be designated (+)-CH_2-$H_4PteGlu_n$ and the corresponding racemic mixture of isomers at C6 will be designated (±)-CH_2-$H_4PteGlu_n$. The natural diastereoisomers of $H_4PteGlu_n$ and CH_3-$H_4PteGlu_n$ have the *S* configuration at C6 and are designated (−)-$H_4PteGlu_n$ and (−)-CH_3-$H_4PteGlu_n$, respectively.

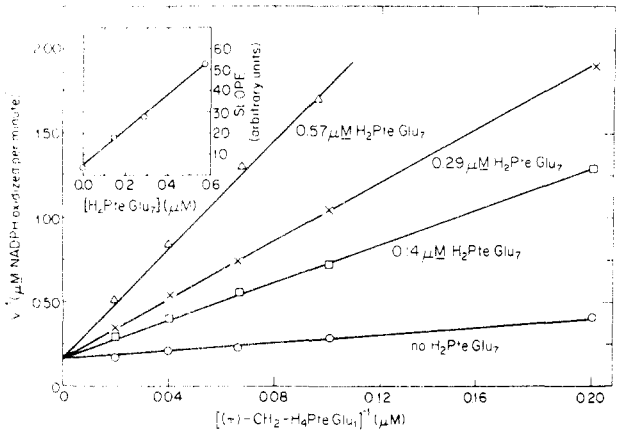


FIGURE 1: Inhibition of methylenetetrahydrofolate reductase activity by $H_2PteGlu_7$. Assay mixtures (2.0-mL volume) contained 125 μM NADPH and the indicated concentrations of (+)-CH_2-H_4PtGlu_1 [added as a (±) mixture] and $H_2PteGlu_7$ in 0.05 M phosphate buffer, pH 6.7, 0.3 mM in EDTA, 2 μM in FAD, and 50 mM in β-mercaptoethanol. The enzyme-bound FAD concentration was 4.9×10^{-9} M. Concentrations of $H_2PteGlu_7$ were (○) 0, (□) 0.14, (×) 0.29, and (△) 0.57 μM.

performed on hydrolyzed samples of $H_2PteGlu_3$ and $H_2PteGlu_5$, using the procedure described by Matthews et al. (1974). The results indicated 2.90 and 5.09 mol of glutamic acid per mol of dihydropteroylglutamate, respectively, suggesting that little if any side cleavage occurred under the described conditions of preparation.

Inhibition of the methylenetetrahydrofolate reductase activity was measured at three different inhibitor concentrations. The increment in NaCl concentration due to addition of inhibitor never exceeded 0.01 M. A typical experiment, utilizing $H_2PteGlu_7$ as the inhibitor, is shown in Figure 1. In each case the substrate utilized was (+)-CH_2-$H_4PteGlu_1$, added as a (±) racemic mixture. The K_i values for each dihydropteroylglutamyl derivative were determined from secondary plots of $slope_{1/s}$ vs. inhibitor concentration. Each derivative showed linear competitive inhibition with respect to methylenetetrahydrofolate. The variation of K_i with the number of glutamyl residues is shown in Figure 2.

The K_i value for $H_2PteGlu_1$ was also measured by using (+)-CH_2-$H_4PteGlu_3$, and the value obtained was 6.5 μM, in good agreement with data obtained by using (±)-CH_2-$H_4PteGlu_1$ as the substrate.

Preparations of (+)-CH_2-$H_4PteGlu_n$ prepared by the present method were free of detectable $H_2PteGlu_n$ contamination as judged by the symmetry of the 297-nm peak and the 340/297 nm absorbance ratio, which was 0.048. Amino acid analyses of CH_2-$H_4PteGlu_4$ and CH_2-$H_4PteGlu_7$ indicated 4.16 and 7.44 mol of glutamic acid per mol of methylenetetrahydropteroylglutamate. Spectroscopic examination of 10 and 50 μM (±)-CH_2-$H_4PteGlu_1$, diluted into assay cuvettes containing 0.05 M phosphate, pH 6.7, 2 μM in FAD and 50 mM in β-mercaptoethanol, and degassed as described under Experimental Procedure, showed no detectable formation of $H_2PteGlu_1$ during 30 min, although slow dissociation of formaldehyde took place as evidenced by decreases in the 297-nm absorbance.

When Lineweaver–Burk plots for (±)-CH_2-$H_4PteGlu_1$ prepared from Sigma tetrahydrofolate without further purification were compared with those for enzymatically prepared (+)-CH_2-$H_4PteGlu_1$, no differences were noted, provided that the results were plotted assuming that (−)-CH_2-$PteGlu_1$ is not a substrate. Thus, (−)-CH_2-$H_4PteGlu_1$ is not an inhibitor, in agreement with previous observations of Kutzbach & Stokstad

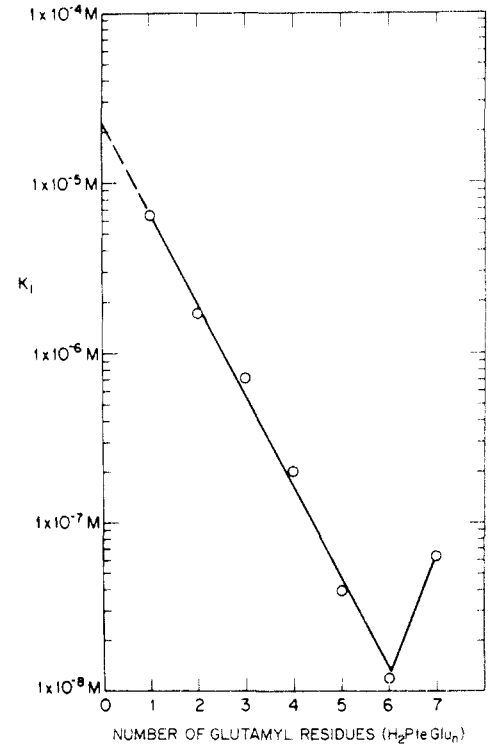


FIGURE 2: Variation of K_i values for dihydropteroylglutamate inhibitors with the number of glutamyl residues. Individual K_i values were determined in experiments like that shown in Figure 1. In no case was the concentration of enzyme-bound flavin more than 4% of the inhibitor concentration.

Table II: Kinetic Parameters for NADPH–CH_2-$H_4PteGlu_n$ Oxidoreduction

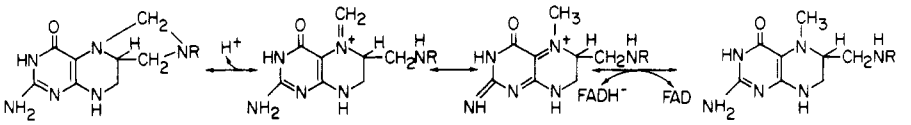
substrate	rel V_{max}	K_m(CH_2-$H_4PteGlu_n$) (μM)	K_m(NADPH) (μM)
CH_2-$H_4PteGlu_1$	1.00	7.1	16
CH_2-$H_4PteGlu_2$	1.76	5.2	
CH_2-$H_4PteGlu_3$	1.71	1.7	15
CH_2-$H_4PteGlu_4$	1.73	0.62	76
CH_2-$H_4PteGlu_5$	0.64	0.26	125
CH_2-$H_4PteGlu_6$	0.68	0.10	185
CH_2-$H_4PteGlu_7$	0.68	0.51	176

(1971). Values obtained for V_{max} and K_m for reactions involving polyglutamate substrates are shown in Table II. The increment in NaCl concentration due to addition of substrate to the assay mixture did not exceed 0.05 M. Parallel-line kinetics are observed for each of the substrates. For chain lengths of three glutamyl residues or more, inhibition of the NADPH–CH_2-$H_4PteGlu_n$ oxidoreductase reaction by $H_2PteGlu_n$ was examined, in order to determine the K_m for the polyglutamate substrate, and $H_2PteGlu_n$ was found to be linearly competitive with respect to the polyglutamate substrate.

Measurements of V_{max} for the NADPH–CH_2-$H_4PteGlu_1$ oxidoreductase reaction in D_2O and H_2O are shown in Figure 3A, and the analogous experiments for the NADPH–CH_2-$H_4PteGlu_5$ reaction are shown in Figure 3B. The results indicate a solvent isotope effect ($V_{max,H_2O}/V_{max,D_2O}$) of 3.4 for the reduction of the monoglutamate substrate and of 1.4 for the pentaglutamate substrate.

We have also examined the effect of $H_2PteGlu_n$ on the NADPH–CH_2-$H_4PteGlu_n$ oxidoreductase reaction when NADPH is the varied substrate. $H_2PteGlu_1$ and $H_2PteGlu_6$ are uncompetitive with respect to NADPH with CH_2-

Scheme I



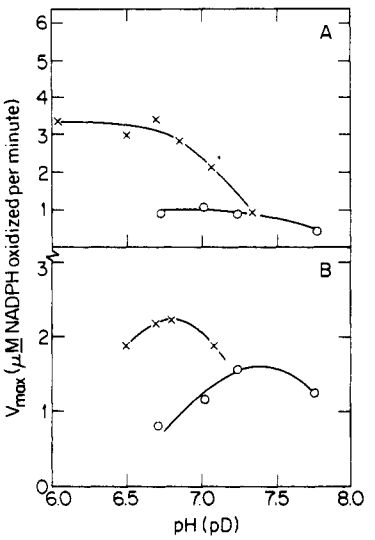
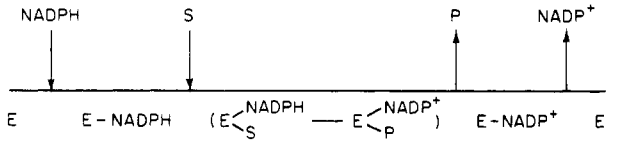


FIGURE 3: Measurements of the solvent isotope on V_{max} for reduction of CH_2-H_4PteGlu$_1$ (A) and CH_2-H_4PteGlu$_5$ (B). V_{max} values were determined by fixed ratio extrapolation. A ratio of NADPH/(+)-CH_2-H_4PteGlu$_1$ of 3:1 was used in (A), and in (B) a ratio of NADPH/(+)-CH_2-H_4PteGlu$_5$ of 21 was used. In both cases the enzyme-bound FAD concentration was 2.4×10^{-9} M. (×) V_{max} determined in H_2O; (○) V_{max} determined in D_2O.

H_4PteGlu$_1$ as the fixed substrate (Matthews & Haywood, 1979a,b), while H_2PteGlu$_5$ shows mixed-type inhibition with respect to NADPH when CH_2-H_4PteGlu$_5$ is the fixed substrate.

Discussion

Methylenetetrahydrofolate reductase exhibits parallel-line kinetics when the NADPH–methylenetetrahydropteroylmonoglutamate oxidoreductase activity is examined. While these results are consistent with a ping-pong bi-bi mechanism, an ordered bi-bi mechanism may also show such kinetic patterns if the rate constants are of appropriate magnitude. The inhibition pattern in the presence of either dihydropteroylmonoglutamate or dihydropteroylhexaglutamate, both of which are competitive with respect to methylenetetrahydrofolate and uncompetitive with respect to NADPH, eliminates many possible ternary mechanisms but fails to distinguish between a ping-pong bi-bi mechanism and an ordered bi-bi mechanism of the type



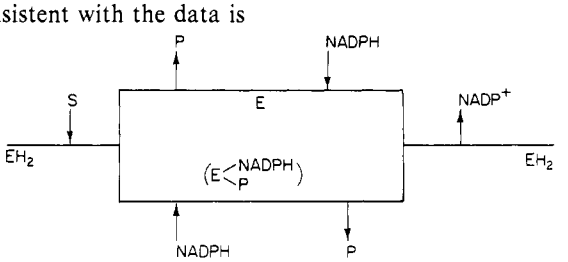
where inhibitor would combine with the E–NADPH binary complex to form a dead end ternary complex. However, in both these cases plots of slope$_{1/s}$ vs. inhibitor concentration, measured at saturating NADPH, can be used to measure the K_i of the dead end inhibitor, and this K_i represents a dissociation constant for the enzyme form which is binding the inhibitor (Segel, 1975).

We have observed a linear relationship between $-\log K_i$ and the number of glutamyl residues on H_2PteGlu$_n$ for up to six glutamyl residues. These results indicate that each of the five terminal glutamyl residues in dihydropteroylhexaglutamate contributes equally to the net free energy of binding, with a binding energy of about 0.75 kcal/mol. Since dihydropteroylglutamates are linearly competitive inhibitors with respect to methylenetetrahydrofolate and are structurally analogous compounds, we presume that they are binding at the substrate binding site on methylenetetrahydrofolate reductase. We may infer from these data that the enzyme active center contains a cleft which can accommodate up to six glutamyl residues. It should be noted that the observed binding energy of 0.75 kcal per glutamyl residue must represent a *difference* in binding energy between the glutamyl residue in aqueous solution and that on the enzyme surface.

While the affinity of the enzyme for its polyglutamate substrates may differ from its affinity for dihydropteroylpolyglutamates, the variation in affinity with chain length should be the same. The studies on the variation of V_{max} and the K_m for CH_2-H_4PteGlu$_n$ with substrate chain length thus tell us how the binding energy is expressed in the catalytic reaction. Substrate binding energy can be expressed as enhanced substrate affinity in the Michaelis complex (lower K_m) or in the transition state (higher V_{max}) as has been pointed out by Jencks (1975). In the present case, V_{max} is increased 1.76-fold on introduction of the second glutamyl residue, but thereafter the increased free energy of binding serves to lower the K_m for CH_2-H_4PteGlu$_n$.

With short-chain substrates (one to three glutamyl residues), the kinetic studies are consistent with simple ping-pong bi-bi kinetics in which the enzyme-bound flavin is alternately reduced by NADPH and reoxidized by CH_2-H_4PteGlu$_n$. In agreement with this, H_2PteGlu$_n$ is competitive with respect to CH_2-H_4PteGlu$_n$ and uncompetitive with respect to NADPH, and the K_m for NADPH is unaffected by the number of glutamyl residues. The overall reaction is characterized by a rather large (3.4) solvent isotope effect on V_{max}. Since the reactions involved in methylenetetrahydrofolate reduction (Matthews & Haywood, 1979b) (shown in Scheme I) could all show solvent isotope effects, whereas NADPH-linked reduction of FAD would not be expected to show a solvent isotope effect, these results are consistent with a rate-limiting step in the half-reaction in which CH_2-H_4PteGlu$_n$ is reduced.

With longer chain substrates (four or more glutamyl residues), we see an increase in the value for the K_m for NADPH, from 15 to 185 μM, and a small decrease in V_{max}. H_2PteGlu$_6$ is now a mixed-type inhibitor with respect to NADPH, indicating a transition to a mechanism involving ternary complexes. At the same time we see a reduction in the solvent isotope effect from 3.4 to 1.4. These results are consistent with a shift to a sequential mechanism. A mechanism which is consistent with the data is



The results suggest that the reduction of CH_2-H_4PteGlu$_n$ is

Table III: Values for I_{50} for $H_2PteGlu_n$ Inhibition of CH_2-$H_4PteGlu_n$ Reduction

inhibitor/substrate	I_{50} at 5 μM substrate[a] (μM)	I_{50} at 1 μM substrate[a] (μM)
$H_2PteGlu_1/CH_2$-$H_4PteGlu_1$	11.1	7.4
$H_2PteGlu_2/CH_2$-$H_4PteGlu_2$	3.3	2.0
$H_2PteGlu_3/CH_2$-$H_4PteGlu_3$	2.8	1.1
$H_2PteGlu_4/CH_2$-$H_4PteGlu_4$	1.8	0.52
$H_2PteGlu_5/CH_2$-$H_4PteGlu_5$	0.81	0.19
$H_2PteGlu_6/CH_2$-$H_4PteGlu_6$	0.61	0.13
$H_2PteGlu_7/CH_2$-$H_4PteGlu_7$	0.69	0.19

[a] Calcd from the formula (Segel, 1975) $I_{50} = K_i(1 + S/K_m)$.

no longer fully rate limiting and that either product release or flavin reduction by NADPH partially controls the rate of reaction.

It should be noted that our observations on the shift in the rate-limiting step(s) with longer chain length substrates and the appearance of a mixed-type pattern of inhibition with respect to NADPH can also be explained by a shift from an ordered bi-bi to a rapid equilibrium random sequential mechanism with longer chain length substrates.

The present work contributes to the growing body of evidence suggesting that folate polyglutamates, rather than monoglutamates, are the physiologically significant intracellular metabolites. However, the rationale for the energetically expensive polyglutamyl chain remains controversial. In general, polyglutamate substrates and inhibitors appear to bind folate-dependent enzymes more tightly than their monoglutamate analogues, but the contribution to V_{max} is generally small and in some cases V_{max} is decreased (Baggott & Krumdieck, 1979). Mackenzie (1979) has shown that substrate channeling occurs at the active center of formiminotransferase/cyclodeaminase, a multifunctional protein, and that channeling increases as the chain length is increased from one to five glutamyl residues. In the present case, the binding energy of the polyglutamyl side chain is used to lower the K_m for CH_2-$H_4PteGlu_5$ and CH_2-$H_4PteGlu_6$ well below the estimated concentration of these metabolites in cells. Intracellular CH_2-$H_4PteGlu_n$ is present mainly as the pentaglutamate and hexaglutamate in mouse liver, with an estimated concentration of 1–5 μM (D. G. Priest, personal communication). Little is known about polyglutamate chain length distribution of intracellular $H_2PteGlu_n$, but we may perhaps assume that this too is largely present as the pentaglutamate and hexaglutamate in mammalian cells, since it is generated primarily, or exclusively, from methylenetetrahydrofolate by the action of thymidylate synthetase. The cellular concentration of dihydropteroylglutamates is less than 1 μM and has been estimated to lie between 0.02 and 0.06 μM (Jackson & Harrap, 1973). Segel (1975) has pointed out that inhibition is quantitatively most significant when both inhibitor and substrate are present at concentrations greater than their K_i and K_m values, respectively. This is illustrated for methylenetetrahydrofolate reductase in Table III, where the I_{50} (the inhibitor concentration needed for a 50% inhibition of the reaction) is calculated for 1 and 5 μM substrate concentrations. It can be seen that increasing the polyglutamate chain length leads to a marked reduction in the inhibitor concentration necessary for 50% inhibition. With CH_2-$H_4PteGlu_6$ as the substrate and $H_2PteGlu_6$ as the inhibitor, the I_{50} is 0.13 μM when the substrate concentration is 1 μM. Thus, the methylenetetrahydrofolate reductase reaction velocity should be extremely sensitive to variations in the CH_2-$H_4PteGlu_6$/$H_2PteGlu_6$ ratio in cells. The K_m of dihydrofolate reductase from mammalian tumor cell lines for its dihydropteroylglutamate substrates lies between 0.6 and 10 μM and is rather independent of the number of glutamyl residues (Coward et al., 1974). Thus, increases in the rate at which dihydropteroylglutamates are produced (e.g., when thymidylate biosynthesis is activated) will result in increased steady-state levels of $H_2PteGlu_n$. We suggest that the consequent decrease in the CH_2-$H_4PteGlu_n/H_2PteGlu_n$ ratio will lead to inhibition of CH_2-$H_4PteGlu_n$ reduction, sparing CH_2-$H_4PteGlu_n$ for purine and pyrimidine biosynthesis.

Acknowledgments

We thank Dr. Martin Poe for helpful discussions during the course of this work and for the gift of dihydrofolate reductase from trimethoprim-resistant *Escherichia coli*. We are grateful to Dr. Charles H. Williams for performing the amino acid analyses. We also thank Dr. Roy L. Kisliuk for valuable discussions.

References

Baggott, J. E., & Krumdieck, C. L. (1979) *Biochemistry 18*, 1036–1041.

Baugh, C. M., & Krumdieck, C. L. (1971) *Ann. N.Y. Acad. Sci. 186*, 7–28.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland Publishing Co., Amsterdam.

Brown, J. P., Davidson, G. E., & Scott, J. M. (1974) *Biochim. Biophys. Acta 343*, 78–88.

Coward, J. K., Parameswaran, K. N., Cashmore, A. R., & Bertino, J. R. (1974) *Biochemistry 13*, 3899–3903.

Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., DeAngelis, L. M., & Bertino, J. R. (1975) *Biochemistry 14*, 1548–1552.

Curthoys, N. P., & Rabinowitz, J. C. (1972) *J. Biol. Chem. 247*, 1965–1971.

Dolnick, B. J., & Cheng, Y.-C. (1978) *J. Biol. Chem. 253*, 3563–3567.

Fontecilla-Camps, J. C., Bugg, C. E., Temple, C., Jr., Rose, J. P., Montgomery, J. A., & Kisliuk, R. L. (1979) *J. Am. Chem. Soc. 101*, 6114–6115.

Garrett, C., Wataya, Y., & Santi, D. V. (1979) *Biochemistry 18*, 2798–2804.

Jackson, R. C., & Harrap, K. R. (1973) *Arch. Biochem. Biophys. 158*, 827–841.

Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219–240.

Kisliuk, R. L., Gaumont, I., & Baugh, C. M. (1974) *J. Biol. Chem. 249*, 4100–4103.

Krumdieck, C. L., & Baugh, C. M. (1969) *Biochemistry 8*, 1568–1572.

Krumdieck, C. L., & Baugh, C. M. (1980) *Methods Enzymol.* (in press).

Kutzbach, C., & Stokstad, E. L. R. (1971) *Biochim. Biophys. Acta 250*, 459–477.

Leslie, G. I., & Baugh, C. M. (1974) *Biochemistry 13*, 4957–4961.

Mackenzie, R. E. (1979) in *The Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 443–446, Elsevier/North-Holland, New York.

Matthews, R. G., & Haywood, B. J. (1979a) in *The Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 459–464, Elsevier/North-Holland, New York.

Matthews, R. G., & Haywood, B. J. (1979b) *Biochemistry 18*, 4845–4851.

Matthews, R. G., Arscott, L. D., & Williams, C. H., Jr. (1974) *Biochim. Biophys. Acta 370*, 6–38.
McBurney, M. W., & Whitmore, G. F. (1974) *Cell 2*, 183–188.
Poe, M., Breeze, A. S., Wu, J. K., Short, C. R., Jr., & Hogsteen, K. (1979) *J. Biol. Chem. 254*, 1799–1805.
Ratner, S., Blanchard, M., & Green, D. E. (1946) *J. Biol. Chem. 164*, 691–701.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Selhub, J., Savin, M. A., Sakami, W., & Flavin, M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 312–314.
Taylor, R. T., & Hanna, M. L. (1977) *Arch. Biochem. Biophys. 181*, 331–344.
Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62D*, 185–198.

Kinetics of Inactivation of Erythrocyte Carbonic Anhydrase by Sodium 2,6-Pyridinedicarboxylate†

Y. Pocker* and Conrad T. O. Fong

ABSTRACT: The inactivation of bovine carbonic anhydrase by sodium 2,6-pyridinedicarboxylate (sodium dipicolinate) has been studied at pH 6.6, 25 °C. The catalytically essential zinc ion of the enzyme is removed with unprecedented speed by this chelating agent, producing inert apoenzyme and a zinc dipicolinate complex. This zinc complex rapidly reacts further with dipicolinate, forming the more stable $Zn(dipic)_2^{2-}$ species. The partitioning of zinc ion between enzyme and dipicolinate chelates was measured by separation of the two species using ultrafiltration and determination of their respective zinc concentrations by atomic absorption. The concentration of catalytically active enzyme in the presence of dipicolinate was measured by using either bicarbonate ion or *p*-nitrophenyl acetate as substrate. The rates of disappearance of catalytically active enzyme and appearance of zinc ions in the form of zinc dipicolinate chelates were measured in parallel runs and found to be identical. The exponential decay of enzymatic activity with dipicolinate in excess was analyzed by an integrated rate equation, and the resultant time dependence of the inactivation process was linear for nearly four half-lives. The apparent rate constant, k_{app}, was found to be directly proportional to the dipicolinate concentration. The second-order rate constant for inactivation, $k'' = k_{app}/[\text{dipicolinate}]$, with bicarbonate ion as the substrate was 1.1 M^{-1} s^{-1} at pH 6.6. Inactivation experiments employing *p*-nitrophenyl acetate as the substrate for determining enzymatic activity were performed in 10% v/v acetone. Under these conditions, the results were $k'' = 0.6$ M^{-1} s^{-1} at pH 6.5 and $k'' = 0.3$ M^{-1} s^{-1} at pH 6.9. The equilibrium constant at pH 6.6 for the reaction of enzyme and dipicolinate to produce apoenzyme and $Zn(dipic)_2^{2-}$ calculated from the residual enzymatic activity is $K_{eq} = 1 \times 10^2$ M^{-1}. The formal binding constant of sodium dipicolinate to the enzyme was measured as K_i with bicarbonate and CO_2 as substrates under initial conditions so that no inactivation could occur through loss of zinc. Competitive and noncompetitive behavior were observed, respectively, with $K_i = 0.1$ M for both substrates. The formal activation parameters for the extraction of zinc by dipicolinate at pH 6.6 and 25 °C are $\Delta H^{\ddagger} = 24$ kcal, $\Delta S^{\ddagger} = 22$ eu, and $\Delta G^{\ddagger} = 18$ kcal. By way of comparison, 1,10-phenanthroline, commonly used to produce the apoenzyme, is 5×10^3 slower than dipicolinate at pH 6.6 and 25 °C.

Salts of 2,6-pyridinedicarboxylic acid (dipicolinic acid) are present in large amounts in the spores of microorganisms (Leanz & Gilvarg, 1973; Woodruff et al., 1974). Dipicolinic acid has been studied as an inducer of sporulation (Fukuda et al., 1969), an enzymatic inhibitor (Mann & Byerrum, 1974; Tochikubo, 1974), and a herbicide (Naik et al., 1972). Recently it has been discovered that sodium dipicolinate is able to remove the catalytically essential zinc ion from the metalloenzyme carbonic anhydrase (Kidani et al., 1976).

The present paper reports on the kinetics and mechanism of the reaction between bovine erythrocyte carbonic anhydrase and sodium dipicolinate. Carbonic anhydrase catalyzes the interconversion of bicarbonate and carbon dioxide. Appropriate conditions for the monitoring of bicarbonate dehydration and CO_2 hydration have been delineated in some detail (Pocker & Bjorkquist, 1977; Pocker & Miksch, 1978). Accordingly, the inactivation of carbonic anhydrase by dipicolinate was studied by monitoring these physiological reactions at pH 6.6 and 25 °C. Additional data are presented, utilizing the hydrolysis of the synthetic substrate *p*-nitrophenyl acetate to measure the loss of enzymatic activity (Pocker & Stone, 1967, 1968). 1,10-Phenanthroline, a chelating agent commonly used with carbonic anhydrase for the removal of zinc at acid pH (Lindskog & Malmstrom, 1962), was also studied. We find that 2,6-pyridinedicarboxylate is much more efficient since it reacts 5×10^3 times faster at pH 6.6. This unprecedented speed of zinc removal allowed us to develop a facile procedure for the replacement of the zinc ion in carbonic anhydrase by other metal ions.

The inactivation of carbonic anhydrase by a chelating agent can be visualized as occurring through an initial binding equilibrium followed by a ligand exchange process, whereby the metal ion is transferred from the enzyme to the chelating agent through an intermediate which contains the zinc simultaneously bound to both. A comparison of $\Delta G^{\ddagger}$ for the dipicolinate-assisted removal of enzymatic zinc and the spontaneous dissociation [$(E)Zn \rightarrow E_{apo} + Zn^{2+}(aquo)$] reveals that $\Delta G^{\ddagger}$ is 6 kcal lower for dipicolinate. This substantial

† From the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received September 7, 1979.* Support of this work by grants from the National Institutes of Health of the U.S. Public Health Service, the the National Science Foundation, and the Muscular Dystrophy Association is gratefully acknowledged.

0006-2960/80/0419-2045$01.00/0