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Folylpoly-γ-glutamates as Cosubstrates of 10-Formyltetrahydrofolate:5′-Phosphoribosyl-5-amino-4-imidazolecarboxamide Formyltransferase†

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ABSTRACT: N^{10}-Formyltetrahydropteroylpoly-γ-glutamates (N^{10}-formyl-H_4PteGlu$_n$) having n = 1, 3, 4, 5, 6, and 7 glutamyl residues have been tested as cosubstrates of the purine biosynthesis enzyme 10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase (AICAR transformylase) of chicken liver. The cosubstrates were synthesized by solid-phase synthesis, reduced catalytically, and formylated; a purified enzyme preparation was used and assayed spectrophotometrically following the ΔOD at 298 nm resulting from conversion of the formylated folate to the free tetrahydro form. K_m values and V_m values determined at saturating concentrations of AICAR and at 25 and 150 mM KCl were used to calculate the relative specificity constants V_m/K_m for the N^{10}-formyl-H_4PteGlu$_n$. At physiologic [K^+] (150 mM) they were 1.0, 52, 250, 93, 120, and 59 and at the lower (25 mM) [K^+] the relative specificity constants were 1.0, 64, 78, 34, 48, and 37 for n = 1, 3, 4, 5, 6, and 7, respectively. The poly-γ-glutamates are clearly the preferred cosubstrates, particularly when tested at physiologic [K^+]. The maximal relative specificity constant observed with N^{10}-formyl-H_4PteGlu$_4$ supports the hypothesis that regulation of certain pathways of one-carbon metabolism may operate via alterations of the poly-γ-glutamyl chain length. No inhibition by the unnatural (*d*) isomers of the N^{10}-formyl-H_4PteGlu$_n$ was observed.

The enzyme 10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase (EC 2.1.2.3), from here on referred to as AICAR transformylase, was originally purified by Flaks and co-workers and Warren and co-workers (Flaks et al., 1957a,b; Warren et al., 1957). This enzyme catalyzes the transfer of the formyl moiety from N^{10}-formyltetrahydropteroylglutamate (N^{10}-formyl-H_4PteGlu) to the 5-amino position 5′-phosphoribosyl-5-formamido-4-imidazolecarboxamide (AICAR) to yield 5′-phosphoribosyl-5-formamido-4-imidazolecarboxamide (formyl AICAR) and tetrahydropteroylglutamate (H_4PteGlu).

We have chosen to investigate AICAR transformylase in order to determine enzyme–cosubstrate specificity with respect to the poly-γ-glutamyl moiety of the folate cosubstrate (N^{10}-formyl-H_4PteGlu$_n$; n = 1, 3, 4, 5, 6, and 7). It is now well established that pteroylpoly-γ-glutamates are the naturally occurring forms of the folate cosubstrates (Baugh & Krumdieck, 1971). One of us (C.L.K.) has postulated that the poly-γ-glutamyl chain serves a role in the regulation of one-carbon metabolism (Krumdieck et al., 1977). An important mechanism which would fulfill this role would be the regulation of folate-dependent pathways via enzyme–cosubstrate specificity mediated by alterations in the poly-γ-glutamyl chain length. In this paper we report the kinetic parameters V_m and K_m and the ratio V_m/K_m which we use as a measure of the enzyme–cosubstrate specificity. The kinetic parameters and the specificity constants, V_m/K_m, were determined at low (25 mM KCl) and physiologic (150 mM KCl) potassium ion concentration. These studies were conducted by use of a partially purified chicken liver enzyme and a new spectrophotometric assay for AICAR transformylase.

Experimental Procedures

Materials

Substrates. Pteroylpoly-γ-glutamates were synthesized by the method of Krumdieck & Baugh (1969) and were reduced to their 5,6,7,8-tetrahydro forms (H_4PteGlu$_n$; n = 1, 3, 4, 5, 6, and 7) by the method of Hatefi et al. (1960). N^5,N^{10}-Methenyltetrahydropteroylpoly-γ-glutamates (N^5,N^{10}-

† From the Department of Nutrition Sciences, University of Alabama, Birmingham, Alabama. *Received August 11, 1978; revised manuscript received December 7, 1978.* Support for this research was provided in part by Grant IN 66Q from the American Cancer Society, U.S. Public Health Service Grant 1 T32 DE07020-02, Contract HD-4-2862 from the Contraceptive Evaluation Branch, Center for Population Research, NICHD, and Grant GM 23453.

0006-2960/79/0418-1036$01.00/0

CH=H_4PteGlu$_n$) were synthesized by the method of Rowe (1968). The N^5,N^{10}-methenyl derivatives were stored in evacuated ampules at −20 °C until needed and converted to the N^{10}-formyl-H_4PteGlu$_n$ when we dissolved the former in 50 mM Tris-HCl, 50 mM mercaptoethanol, pH 7.4. The solution remained at room temperature in the dark, for 2 h, resulting in equilibrium concentrations of the N^5,N^{10}-CH=H_4PteGlu$_n$ and N^{10}-formyl-H_4PteGlu$_n$. The equilibrium greatly favors the N^{10}-formyl form (97% of the total) at pH 7.4 (Tabor & Wyngarden, 1959).

The concentration of N^5,N^{10}-CH=H_4PteGlu$_n$ in the stock solution was determined spectrophotometrically after we acidified an aliquot in 1.0 N HCl and used an extinction coefficient of 2.65×10^4 M^{-1} cm^{-1} at 348 nm (Huennekens et al., 1963). The chemically reduced folates are racemic mixtures (*dl*) of the asymmetric carbon-6 of the pteridine ring, where the *d* and *l* forms are the unnatural and naturally occurring optical isomers, respectively.

Crystalline AICAR was a gift from Dr. Conrad Wagner. Stock solutions of AICAR were made in 50 mM Tris-HCl, 50 mM mercaptoethanol, pH 7.4. The concentration of AICAR was determined spectrophotometrically at 269 nm by use of an extinction coefficient of 1.26×10^4 M^{-1} cm^{-1} (Huang, 1965).

All other chemicals used were of reagent grade or higher quality. Optical density measurements were performed on a recording GCA/McPherson spectrophotometer (EU-700 series) by use of 1- or 0.2-cm path length quartz cuvettes equipped with air-tight screw tops.

Methods

Assay of AICAR Transformylase. The assay of AICAR transformylase utilizes the increase in optical density at 298 nm when N^{10}-formyl-H_4PteGlu is converted to H_4PteGlu. The assay solution contained 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 50 mM mercaptoethanol, 50 μM (*dl*) N^{10}-formyl-H_4PteGlu, and the enzyme preparation in a volume of 3.2 mL. This solution was incubated for 10 min in the cuvette to attain temperature equilibration; after this period the change in optical density at 298 nm was monitored for several minutes to establish a blank rate (minus AICAR). AICAR was then added to a final concentration of 50 μM (a negligible volume change occurred), and the increase in optical density was followed for 1–3 min. Initial rates (plus AICAR) and blank rates were calculated by linear regression. Blank rates were consistently 5% or less of the initial rates. The enzyme rates (initial rate minus blank rate) are reported. Assays for the determination of kinetic parameters were performed at 37 °C by use of the enzyme at stage V in the purification (see below).

The enzyme rate, ΔOD, units/min, was converted to (nmol of H_4PteGlu$_n$ formed/min)/mL, by use of an estimated ϵ_m of 1.7×10^4 for the reaction (see Experimental Results). The specific activity was obtained by dividing the above rate by the protein concentration (mg/mL) to give (nmol of H_4PteGlu$_n$ formed/min)/mg. One unit of activity is defined as the amount of enzyme which forms 1 nmol of H_4PteGlu$_n$/min.

Assay of Conjugase. Conjugase (pteroylpoly-γ-glutamyl hydrolase) was assayed by use of the radioactive method described by Krumdieck & Baugh (1970). The assay was performed in 50 mM Tris-HCl, 50 mM mercaptoethanol, 25 mM KCl, pH 7.4. The concentration of the synthetic radioactive substrate pteroyl-γ-glutamyl-γ-glutamyl[U-^{14}C]-glutamate was 67 μM, sp radioact. 0.3 μCi/μmol. The specific activity of conjugase is given in terms of (nanomoles of glutamic acid liberated/min)/mg of protein. One unit of activity is defined as that amount of enzyme which will liberate 1 nmol of glutamic acid/min.

Determination of Kinetic Parameters. The data sets (enzyme rate and substrate concentration) were fitted to the Michaelis–Menten equation by nonlinear regression. A computer program, furnished by Dr. K. M. Pruitt (Department of Biochemistry, University of Alabama), was used to calculate K_m values and V_m values and their standard errors. K_m values are reported as concentrations of the natural (*l*) optical isomer.

Purification of AICAR Transformylase. The procedure originally described by Flaks et al. (1957a) was modified as follows: 250 g of fresh chicken liver were homogenized (all operations were performed at 2 °C) in 500 mL of 0.9% NaCl, 10 mM Tris-HCl, pH 7.4, by use of a Polytron homogenizer operated at low speed. The homogenate was filtered through gauze (stage I).

The filtrate was centrifuged at 13000*g* for 30 min and the pellet discarded. The supernatant was then centrifuged for 4 h at 13000*g*, decanted, and dialyzed overnight against 4 L of 10 mM Tris-HCl, pH 7.4 (stage II).

Thirty-eight milliliters of 1 M Tris-HCl buffer, pH 8.0, was added to the dialysant (380 mL), followed by 101 g of ammonium sulfate. After we stirred the mixture for 30 min the precipitate was removed by centrifugation (10000*g* for 10 min), and 72 g of ammonium sulfate were slowly added to the supernatant with continuous stirring. The precipitate was collected, dissolved in 70 mL of 10 mM Tris-HCl buffer, pH 7.4, and dialyzed overnight against 4 L of the same buffer (stage III).

The following were added to the 124 mL of dialysant: 62 mL of 10 mM Tris-HCl buffer, pH 7.4, 1.24 mL of saturated ammonium sulfate solution, and 1.81 mL of 1 M zinc acetate solution (dropwise, over a 10-min period). The solution was centrifuged at 10000*g* for 10 min and the pellet discarded. The supernatant was dialyzed (with rapid stirring) against 3 × 1 L of 10 mM Tris-HCl, 10 mM EDTA, pH 7.4, for 90 min with buffer changes every 30 min. One-tenth volume of 1 M Tris-HCl, pH 8.0, was added to the 159 mL of amber-colored dialysant, followed by 51 g of ammonium sulfate. The solution was stirred for 15 min and centrifuged (10000*g* for 10 min), and the pellet was discarded. Fourteen grams of ammonium sulfate was then added to the supernatant, and the precipitate was collected by centrifugation, dissolved in 10 mL of 10 mM Tris-HCl, pH 7.4, and dialyzed overnight against 4 L of the same buffer (stage IV).

Four milliliters of the dialysant from stage IV were passed through a Millipore filter, loaded on a 2.5 × 35 cm Sephadex G-150 column, and eluted with 10 mM Tris-HCl, pH 7.4, buffer as shown in Figure 1. Fractions 23–42 were pooled and lyophilized. The resulting solids were dissolved in 3 mL of water and stored frozen at −20 °C (stage V).

The purification is summarized in Table I. Protein concentration was estimated by the Biuret method.

Experimental Results

Validity of the AICAR Transformylase Assay. The enzyme rates of AICAR transformylase determined by use of the monoglutamate cosubstrate as described above are proportional to the protein concentration when measured over the range of 1.0–22.0 μg of protein/mL at both low (25 mM) and high (150 mM) KCl concentrations.

The production of H_4PteGlu is indicated by the appearance of a peak at approximately 300 nm in the difference spectrum (Figure 2). The $\Delta\epsilon_{298}$ for the reaction was found to be 1.7×10^4 M^{-1} cm^{-1}. This is consistent with $\epsilon_{m_{298}} = 1.1 \times 10^4$ for N^{10}-formyl-H_4PteGlu (Blakley, 1960) and $\epsilon_{m_{298}} = 2.8 \times 10^4$ for H_4PteGlu (Blakley, 1969). The known requirement for

Table I: Purification of AICAR Transformylase and Conjugase Activity

purifcn stage	total protein (mg)	sp act.[a] of AICAR transformylase [(nmol/min)/ mg of protein]	total act. (units)	sp act.[a] of conjugase [(nmol/min)/ mg of protein]	total act. (units)
I	5.53×10^4	0.234	12900	0.026	1400
II	1.13×10^4	1.13	12800	0.025	280
III	5.58×10^3	2.46	13900	0.023	130
IV	285	8.05	2300	0.060	17
V	32.3	37.1	1200 (6900)[b]	0.31	10 (57)[b]

[a] Assay conditions are described under Methods. AICAR transformylase was assayed at 25 °C. [b] Only a fraction (50 mg of protein) of the stage IV preparation was used in stage V. Numbers in parentheses represent total activity had the entire stage IV preparation been used.

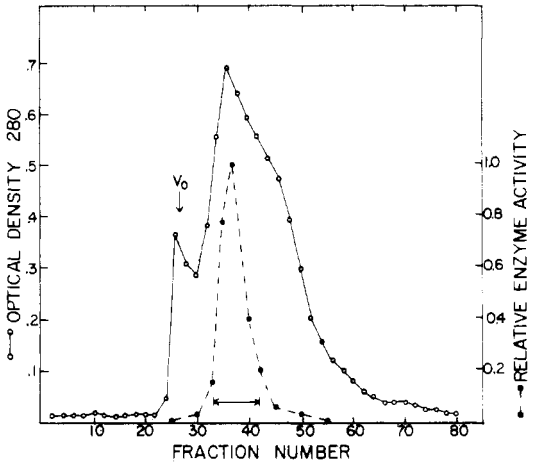

FIGURE 1: Elution profile of AICAR transformylase from Sephadex G-150. Protein was eluted at a flow rate of 6 mL/h (2.2-mL fractions). The double arrow indicates fractions which were pooled. V_0 is the elution position of blue dextran.

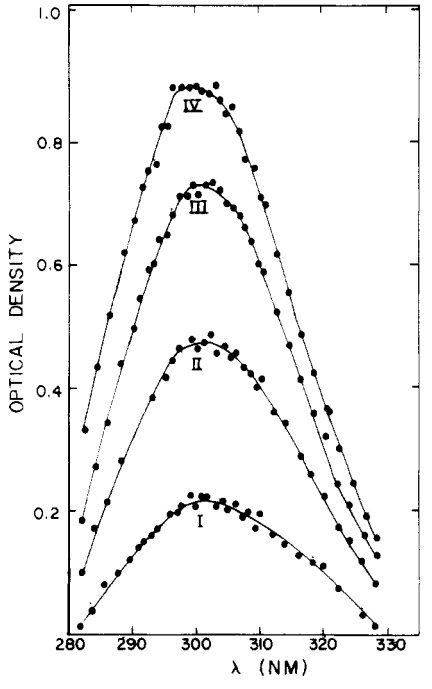

FIGURE 2: Difference spectra of the AICAR transformylase reaction. Difference spectra were taken at 10 (I), 20 (II), 68 (III), and 239 min (IV) during the reaction. Scanning time was approximately 1 min. Initial concentrations were 100 μM *dl*-N^{10}-formyl-H_4PteGlu, 120 μM AICAR, 50 mM Tris-HCl, 50 mM mercaptoethanol, 25 mM KCl, 0.01 mg of protein/mL (stage V in the purification), at pH 7.4, 37 °C, in a 1-cm cuvette.

potassium ions of AICAR transformylase (Flaks et al., 1957a) was demonstrated by use of the assay as shown in Figure 3.

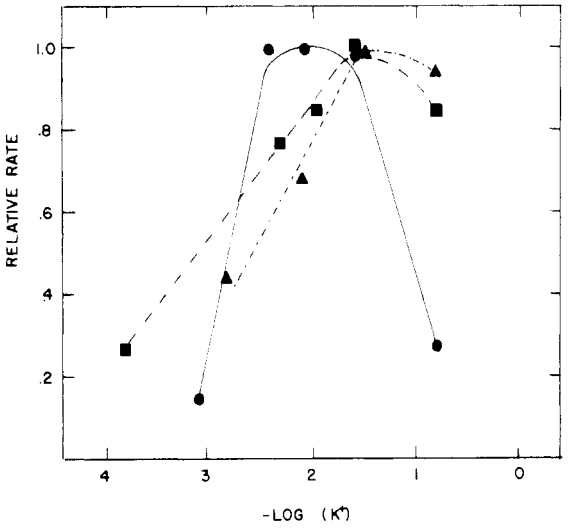

FIGURE 3: The effect of KCl concentration on the relative enzyme rate. Concentrations of *dl*-N^{10}-formyl-H_4PteGlu$_n$ were 80 (n = 1; ●), 30 (n = 4; ■), and 19 μM (n = 6; ▲). Assays were performed by use of 120 μM AICAR, 50 mM Tris-HCl, 50 mM mercaptoethanol, KCl as shown, pH 7.4, 37 °C. Enzyme at stage V in the purification was used.

In addition, the nucleoside and the free base of AICAR are inactive as substrates (Flaks et al., 1957b). These observations indicate that the assay is a valid measurement of AICAR transformylase activity. The $\Delta\epsilon_{298}$ of the AICAR transformylase reaction with the various poly-γ-glutamyl cosubstrates is assumed to be the same as that observed with the monoglutamate.

Purification of AICAR Transformylase. The enzyme was purified approximately 160-fold from a homogenate of chicken liver (Table I). Since the copurification of conjugase would have confounded the kinetics of AICAR transformylase with the poly-γ-glutamyl cosubstrates, the activity of the former was followed during the purification (Table I). Fortunately the purification of AICAR transformylase is not an effective purification of conjugase. The specific activity of AICAR transformylase in stage V was over 100-fold greater than that of conjugase. At a concentration of polyglutamyl cosubstrate of 1 μM, it is estimated that 1% would be degraded by the remaining conjugase during the assay period (including the incubation period prior to the assay). This amount of degradation would not alter the kinetic parameters. AICAR transformylase is very stable when stored frozen (−20 °C), with no loss of activity observed over a period of 6 months.

Kinetic Parameters of AICAR Transformylase. (a) Optical Isomer Enrichment Experiment. Enzyme rates of the AICAR transformylase reaction were measured at increasing concentrations of the unnatural (*d*) isomer of N^{10}-formyl-H_4PteGlu. This was accomplished by determining the rate

Table II: Effect of d-N^{10}-formyl-H_4PteGlu on AICAR Transformylase[a]

l-N^{10}-formyl-H_4PteGlu (μM)	d-N^{10}-formyl-H_4PteGlu (μM)	sp act. [(nmol of H_4PteGlu formed/min)/mg of protein]
2.0	2.0	5.57
2.0	4.0	5.90
2.0	6.0	5.10
2.0	8.0	5.53
2.0	10	5.38
	mean	5.50 (0.29)[b]

[a] Assay conditions: 120 μM AICAR, 50 mM Tris-HCl, 50 mM mercaptoethanol, 25 mM KCl, pH 7.4, 37 °C. AICAR transformylase at stage V in the purification was used. [b] Standard deviation.

at a 4 μM concentration of the (*dl*) racemic mixture and at 120 μM AICAR. The reaction was allowed to proceed to completion and then restarted by adding *dl*-N^{10}-formyl-H_4PteGlu such that the concentration of (*l*)-N^{10}-formyl-H_4PteGlu was again 2 μM. The enzyme rate was measured and the process repeated three more times with the resulting accumulation of the unnatural (*d*) isomer to a final concentration of 10 μM. The data are summarized in Table II. The possibility that the *d* isomer inhibits the enzyme was thus tested. No change in enzyme rates was observed with a fivefold enrichment in the *d* isomer.

(*b*) *Determination of K_m Values and V_m Values.* The kinetic parameters for the N^{10}-formyl-H_4PteGlu$_n$ cosubstrates were initially determined in the presence of 25 mM KCl. All of the poly-γ-glutamyl cosubstrates (n = 3, 4, 5, 6, and 7) displayed Michaelis–Menten kinetics. Their apparent K_m values varied between 1 and 3 μM, and there were small differences in V_m values (Table III). The apparent K_m and V_m for the monoglutamate were significantly higher than the corresponding parameters for the poly-γ-glutamates.

Since changing the number of glutamyl residues of the poly-γ-glutamyl cosubstrates could alter the K_m for AICAR and confound the determination of the kinetic parameters, the K_m for AICAR was determined by use of both N^{10}-formyl-H_4PteGlu and N^{10}-formyl-H_4PteGlu$_4$. It was found to be nearly independent of the length of the poly-γ-glutamyl chain and the concentration of the folate cosubstrate (Table IV). Thus the apparent K_m for AICAR is not strongly affected by the degree of saturation of the enzyme with the folate cosubstrate.

AICAR transformylase requires potassium ions for activity; when the monoglutamate was used as cosubstrate, maximal enzyme activity occurs in the range of 3–30 mM KCl (Flaks et al., 1957a). This range of concentrations lies far below the intracellular levels of [K^+] ($\simeq$150 mequiv/L) which led us to assay the enzyme at 150 mM KCl. Figure 3 shows that the enzyme is markedly inhibited when we increase the concentration of K^+ to physiological ranges only if the cosubstrate used is the monoglutamate. When we employed N^{10}-formyl-H_4PteGlu$_4$ or N^{10}-formyl-H_4PteGlu$_6$, this inhibitory effect of K^+ was greatly reduced.

It should be noted that in this experiment the poly-γ-glutamyl cosubstrates were tested at concentrations higher than their corresponding apparent K_m values determined at 25 mM KCl. Since it was conceivable that changes in the kinetic parameters could go undetected, a complete kinetic analysis at 150 mM KCl was performed. The results appear in Table III. Michaelis–Menten kinetics were observed with all cosubstrates (n = 1, 3, 4, 5, 6, and 7) up to a concentration of about 10 μM. At higher concentrations the longer chain cosubstrates ($n \geq 4$) deviated from the theoretical curve as shown in Figure 4. This is opposite to what was observed with the monoglutamate which deviated from Michaelis–Menten kinetics when tested at concentrations greater than 100 μM in the low (25 mM) but not in the high (150 mM) KCl medium. Figure 5 shows the deviation from Michaelis–Menten kinetics in the low-KCl media (curve a). The figure clearly shows that the Michaelis–Menten equation adequately describes the kinetics at concentrations below 100 μM (–log [N^{10}-formyl-H_4PteGlu] $\geq$ 4.0).

Two important changes in the kinetic parameters are observed at high KCl concentrations. At 150 mM KCl there

Table III: Kinetic Parameters: Folate Cosubstrates[a]

N^{10}-formyl-H_4PteGlu$_n$, n	K_m ± SE (μM)		V_m ± SE [(nmol of H_4PteGlu$_n$ formed/min)/mg of protein]	
	25 mM KCl	150 mM KCl	25 mM KCl	150 mM KCl
1	674[b] ± 209	353[b] ± 58	1750 ± 480	301 ± 33
3	1.65 ± 0.26	5.95[c] ± 0.82	274 ± 10	261 ± 15
4	0.99 ± 0.30	1.07 ± 0.12	199 ± 12	232 ± 7
5	2.56 ± 0.62	2.06 ± 0.27	227 ± 12	164 ± 6
6	1.64 ± 0.20	1.95 ± 0.49	204 ± 6	192 ± 16
7	1.83 ± 0.49	3.13 ± 0.59	173 ± 15	157 ± 14

[a] Assay conditions: 120 μM AICAR, 50 mM Tris-HCl, 50 mM mercaptoethanol, pH 7.4, 37 °C. [b] Significantly higher than n = 3, 4, 5, 6, and 7 ($p < 0.01$). [c] Significantly higher than n = 4, 5, 6, and 7 ($p < 0.01$).

Table IV: Kinetic Parameters: AICAR[a]

n	l-N^{10}-formyl-H_4PteGlu$_n$] (μM)	[l-N^{10}-formyl-H_4PteGlu$_n$]/K_{m_n}	KCl (mM)	K_m ± SE (μM)	V_m ± SE [(nmol/min)/mg]
1	15.2	0.023[b] (2.2)	25	8.67 ± 0.77	45.5 ± 1.3
1	30.4	0.045 (4.3)	25	9.27 ± 0.60	88.0 ± 1.6
4	3.00	3.03 (75)	25	9.28 ± 1.71	158 ± 10
4	15.7	15.9 (94)	25	12.0 ± 2.7	196 ± 13
1	35.0	0.099 (9.0)	150	22.0 ± 1.8	31.7 ± 0.9
1	70.0	0.198 (17)	150	20.3 ± 4.5	56.4 ± 4
4	3.58	3.35 (77)	150	24.1 ± 6.0	192 ± 22
4	10.8	10.1 (91)	150	32.3 ± 7.8	227 ± 32

[a] Conditions were the same as in Table III except that the AICAR concentration was varied. [b] Percent saturation by folate cosubstrate is given in parentheses.

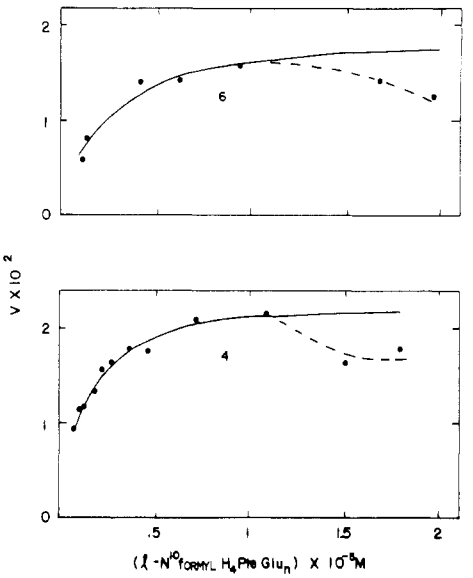

FIGURE 4: Specific activity (V) of AICAR transformylase vs. [N^{10}-formyl-H_4PteGlu$_6$] (top) and [N^{10}-formyl-H_4PteGlu$_4$] (bottom) at 150 mM KCl. Assays were performed by use of 120 μM AICAR, 50 mM Tris-HCl, 50 mM mercaptoethanol, pH 7.4, 37 °C. Solid lines were drawn by use of kinetic parameters given in Table III.

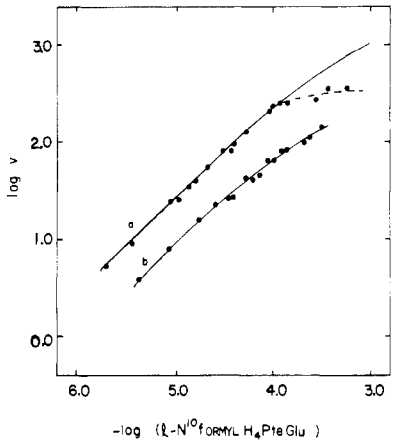

FIGURE 5: Log specific activity (log V) of AICAR transformylase vs. log concentration [-log (mol/L)] of N^{10}-formyl-H_4PteGlu. Assays were performed by use of 120 μM AICAR, 50 mM Tris-HCl, 50 mM mercaptoethanol, 25 mM (a) or 150 mM (b) KCl, pH 7.4, 37 °C. The solid lines were drawn from kinetic parameters given in Table III.

is approximately a sixfold decrease in the apparent V_m for the monoglutamate. In addition, the apparent K_m of N^{10}-formyl-H_4PteGlu$_3$ increases about fourfold at the higher K^+ concentration. The apparent K_m values and V_m values of the remaining cosubstrates are not greatly altered (Table III).

To exclude the possibility that the changes in the kinetic parameters observed at high KCl concentration resulted from changes in the K_m for AICAR, the apparent K_m was determined at 150 mM KCl by use of both the monoglutamate and the tetraglutamate cosubstrates. As shown in Table IV, the apparent K_m values for AICAR increased about twofold when determined at 150 mM KCl with either cosubstrate. This increase is too small to have any appreciable effect on the degree of saturation of the enzyme by AICAR at the concentration used in the assay (120 μM).

Use of a Specificity Constant. It was recognized by Hofstee that the ratio, V_m/K_m, is a better measure of the catalytic effectiveness of an enzyme than either V_m or K_m alone (Hofstee, 1952a,b, 1954). He pointed out that the constant

Table V: Relative Specificity Constants[a]

N^{10}-formyl-H_4PteGlu$_n$, n	V_m/K_m	
	25 mM KCl	150 mM KCl
1	1.0	1.0
3	64	52
4	78	250
5	34	93
6	48	120
7	37	59

[a] V_m/K_m for N^{10}-formyl-H_4PteGlu = 1.0

V_m/K_m is proportional to the enzyme rate when [S] << K_m. Thus, $v/[S] \simeq V_m/K_m$ when [S] << K_m (Hofstee, 1954). The condition, [S] << K_m, is most likely encountered in vivo with N^{10}-formyl-H_4PteGlu$_n$ cosubstrates.

We have elected to use the constant, V_m/K_m, to describe the specificity of chicken liver AICAR transformylase for the various N^{10}-formyl-H_4PteGlu$_n$ studied. Table V compares the relative specificity constants at high and low KCl concentration. As the specificity increases, the relative specificity constant becomes larger. At low KCl concentration there is a trend in the relative specificity constant which reaches a maximum at N^{10}-formyl-H_4PteGlu$_4$. At physiological K^+ concentration, the trend is more pronounced; again there is a maximum at the tetraglutamate.

Discussion

Inhibition of the enzyme by the unnatural optical isomer (d) of N^{10}-formyl-H_4PteGlu was not observed. Although the possibility remains that inhibition of AICAR transformylase by extremely high concentrations of the d isomers of N^{10}-formyl-H_4PteGlu$_n$ may be demonstrable, it is clear that they are not inhibitory at concentrations equimolar with the l isomers. This is in agreement with reports indicating no inhibition of thymidylate synthetase (Blakley, 1963), tetrahydropteroylglutamate methyltransferase, and 5,10-methylenetetrahydrofolate reductase (Cheng et al., 1974), formyltetrahydrofolate synthetase (Himes & Rabinowitz, 1962; Curthoys & Rabinowitz, 1972), and ketopantoate hydroxymethyltransferase (Powers & Snell, 1976) by the d isomer(s) of their corresponding folate cosubstrates.

Inhibition of thymidylate synthetase has been observed by the addition of d-tetrahydropteroylmono-, -tri-, and -hexaglutamates to an assay system containing dl-methylenetetrahydropteroylglutamate (Kisliuk et al., 1974). In this system the d-monoglutamate proved to be a weak competitive inhibitor while the tri- and hexaglutamate were stronger. The d isomers of the tri- and hexaglutamate apparently bind to thymidylate synthetase with greater affinity than the d isomer of the monoglutamate. In another study of thymidylate synthetase the K_m for l-methylenetetrahydropteroylglutamate and the K_i for d-methylenetetrahydropteroylglutamate were found to be 15 and 50 μM, respectively (Leary et al., 1974). The d isomer was found to be a competitive inhibitor. Thymidylate synthetase may be sensitive to inhibition by the d isomer because the enzyme mechanism involves generation of the asymmetric carbon of the pteridine ring.

The K_m for AICAR is nearly independent of the apparent degree of saturation of the enzyme by the folate cosubstrate (Table IV). At 25 mM KCl, the K_m for AICAR ranges from 8.7 to 12 μM when the apparent saturation of the enzyme by the folate cosubstrates was varied from 2 to 94%. Similarly, at physiological concentration of K^+, the K_m ranges from 20 to 32 μM while varying the apparent saturation by folate from 9 to 91% (Table IV, colums 5 and 3). These observations are

consistent with a quasi-equilibrium random mechanism in which both substrates bind independent of each other in rapid equilibria preceding the rate determining step (Wong, 1975).

The binding of AICAR and the folate cosubstrates is sensitive to the K^+ concentration. Potassium ions may cause a change in the conformation of the enzyme, bind to the active site, or both. The data suggest that K^+ does both. Potassium ions are clearly required for maximum enzymatic activity. At physiological concentrations, K^+ alters the K_m for AICAR and many of the kinetic parameters of the folate cosubstrates. The effect of physiological K^+ concentration is greatest with those substrates of low ionic density: AICAR and N^{10}-formyl-$H_4PteGlu_n$, n = 1 and 3. The kinetic parameters of N^{10}-formyl-$H_4PteGlu_n$, n = 4, 5, 6, and 7, are less sensitive to K^+. In addition, non-Michaelis–Menten behavior is either created (n = 4, 5, 6, and 7) or destroyed (n = 1) at high cosubstrate concentration by increasing the KCl concentration. These results suggest that the K^+ is bound to the enzyme producing a change to the active conformation and that an enzyme–(co)substrate–K^+ complex is formed at the active site.

An interpretation of the kinetics of AICAR transformylase is confounded by (without excluding others) the following considerations. All of the folate cosubstrates are present at equilibrium concentrations of the N^5,N^{10}-methenyl and N^{10}-formyl forms. Although the equilibrium lies in favor of the N^{10}-formyl forms (approximately 97% at pH 7.4; Tabor & Wyngarden, 1959), the effect of small but finite amounts of the N^5,N^{10}-methenyl forms is unknown. Aside from this, the number of possible ionic species of the poly-γ-glutamate cosubstrates at pH 7.4 is large, and the relative amount of each species is unknown. It is unreasonable to expect AICAR transformylase to bind each species with equal affinity or catalytic efficiency. Finally, the poly-γ-glutamyl cosubstrates most likely possess tertiary structure, and certain structural species may bind to the enzyme with different affinities or catalytic efficiencies.

Intracellular Folate Concentration and the Specificity Constant V_m/K_m. Based on differential microbiological assays before and after treatment with two different conjugases, Noronha & Silverman (1962) demonstrated that the majority of folates in chicken liver occurred as the N^5-methyltetrahydropteroylpolyglutamates. From their data we have calculated that methylfolates must account for at least 75% of the total. The remaining 25% must bear all of the other one-carbon substituents. It is thus reasonable to assume that the fraction present as N^{10}-formylfolates would not exceed 5–10% of the total pool. Since the concentration of folates in chicken liver is approximately 20 μM, the concentration of N^{10}-formyl forms (including all polyglutamyl chain lengths) would amount to 1 or 2 μM. This concentration is at or below the apparent K_m for any of the folate cosubstrates of AICAR transformylase tested. At this low concentration only the poly-γ-glutamyl cosubstrates can be physiologically important, and the specificity constant becomes a meaningful measurement. The monoglutamate should indeed be considered a "convenient analogue" (Rabinowitz, 1960) of the folate cosubstrates. Furthermore, the trend in the specificity constants of the poly-γ-glutamates with a maximum at N^{10}-formyl-$H_4PteGlu_4$ is consistent with the hypothesis that an in vivo regulatory mechanism of purine biosynthesis could operate via alterations in the poly-γ-glutamyl chain length of the folate cosubstrates.

Acknowledgments

The expert technical assistance of Barbara Hudson is gratefully acknowledged.

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