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Kinetic Relationships between the Various Activities of the Formyl–Methenyl–Methylenetetrahydrofolate Synthetase†

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ABSTRACT: The formyl–methenyl–methylenetetrahydrofolate synthetase from chicken liver catalyzes the formation of the 10-formyl- and 5,10-methenyltetrahydrofolate cofactors via three enzymatic activities. In this report we define the kinetic relationships between the activities of this trifunctional protein. An investigation of the time course for 10-formyl cofactor synthesis by computer modeling indicates that commencing with tetrahydropteroyltriglutamate, the activities of the synthetase/cyclohydrolase couple act as separate enzymic species. In contrast, 10-formyl cofactor formation from the 5,10-methylene cofactor utilizing the dehydrogenase/cyclohydrolase couple is described by a single or interactive site model that partitions the 5,10-methenyl intermediate primarily (85%) to the 10-formyl product. An unusual characteristic of the latter coupled activities is the negligible cyclohydrolase activity toward exogenous 5,10-methenyl cofactor, which serves as substrate in the individual activity assay. This is based on (1) competitive inhibition by 5,11-methenyltetrahydrohomofolate against the 5,10-methenyl derivative in the cyclohydrolase-catalyzed hydrolysis but the absence of such inhibition in the dehydrogenase/cyclohydrolase couple and (2) a pulse–chase experiment showing the failure of chase 5,10-methenyl cofactor to dilute the 10-formyl product derived from the coupled activities. The result of this coupling is to minimize the concentration of the 5,10-methenyl species, consistent with its noninvolvement in de novo purine biosynthesis.

The trifunctional protein (10-formyltetrahydrofolate synthetase/5,10-methenyltetrahydrofolate cyclohydrolase/5,10-methylenetetrahydrofolate dehydrogenase) from chicken liver catalyzes the formation of several reduced folate cofactors[1] via the reactions

$$\text{formate} + \text{ATP} + \text{H}_4\text{folate} \xrightarrow{\text{synthetase}} \text{10-formyl-H}_4\text{folate} + \text{ADP} + \text{P}_i$$

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$$\text{NADP}^+ + \text{5,10-methylene-H}_4\text{folate} \xrightarrow{\text{dehydrogenase}} \text{5,10-methenyl-H}_4\text{folate} + \text{NADPH}$$

$$\text{H}_2\text{O} + \text{5,10-methenyl-H}_4\text{folate} \xrightleftharpoons{\text{cyclohydrolase}} \text{10-formyl-H}_4\text{folate} + \text{H}^+$$

We have shown (Smith et al., 1980) that the purified protein consists of two identical subunits and has an overall molecular weight of 190 000.

This multifunctional protein is not unique to avian liver and has also been isolated from other eukaryotic sources including ovine liver, M_r 218 000 (Paukert et al., 1976), porcine liver, M_r 150 000 (Tan et al., 1977), and rabbit liver, M_r 215 000 (Schirch, 1978), as well as from yeast, M_r 201 000 (Paukert et al., 1977). The bacterial enzymes are discussed in detail elsewhere (Paukert et al., 1976), and from these sources the dehydrogenase and synthetase enzymes are purified as separate species. Uyeda & Rabinowitz (1967a,b) and Rabinowitz & Pricer (1962) have isolated the synthetase, dehydrogenase, and cyclohydrolase from *Clostridium cylindrosporum* as three separate proteins. In *Escherichia coli* the cyclohydrolase and dehydrogenase were purified as a M_r 100 000 protein containing five nonidentical subunits (Dev & Harvey, 1978).

Because of separation of the synthetase and dehydrogenase activities in bacterial systems several workers have investigated the relationship between the three activities of the trifunctional protein. Paukert et al. (1977) have reported that upon tryptic digestion of the yeast enzyme the dehydrogenase and cyclohydrolase activities were coordinately lost, whereas the synthetase activity was maintained on a M_r 76 000 protein. Schirch (1978) presented evidence for a combined dehydrogenase/cyclohydrolase site in the rabbit protein including the protection of both activities by $NADP^+$ against heat inactivation and the failure of 5,10-methenyl-H_4folate to accumulate during the coupled enzyme reaction. More extensive studies on the porcine trifunctional protein (Tan & MacKenzie, 1977; Cohen & MacKenzie, 1978; MacKenzie & Baugh, 1980) also suggest an association between the dehydrogenase and cyclohydrolase sites. In this case tryptic digestion of the native protein yields a M_r 30 000 bifunctional protein. Additionally, the dehydrogenase may channel 60% of the 5,10-methenyl product directly to the cyclohydrolase without prior equilibrium with solution.

In the present study we examined the relationship of the three activities of the chicken liver enzyme by computer simulation of the time course for formation of 5,10-methenyl- and 10-formyl-H_4folate, by radiochemical channeling experiments and by comparison of the inhibition of the cyclohydrolase activity by 5,11-methenyl-H_4homofolate against 5,10-methenyl-H_4folate which is provided either by the dehydrogenase activity or as added cofactor to the solution. Due to the increasing evidence that the reduced folate cofactors function as their poly(γ-glutamate) derivatives, the triglutamate forms of the required folate substrates were synthesized for these studies.

[1] Abbreviations: $NADP^+$, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate; GAR, glycinamide ribonucleotide; FGAR, *N*-formylglycinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribonucleotide; H_4folate, L(−)-tetrahydrofolate or tetrahydropteroylmonoglutamate; H_4pteroyl-$(Glu)_3$, L(−)-tetrahydropteroyltriglutamate; GAR TFase, GAR transformylase.

Experimental Procedures

Materials

Malate dehydrogenase, oxalacetate, ATP, $NADP^+$, and pteroyl-L-glutamic acid were purchased from Sigma Chemical Co., St. Louis, MO. QAE-Sephadex A-25 was purchased from Pharmacia Fine Chemical, Uppsala, Sweden. Liquiscint is a product of National Diagnostics, Somerville, NJ. [^{3}H]-Formaldehyde (85 Ci/mol) was purchased from New England Nuclear, Boston, MA. α,β-Glycinamide ribonucleotide was prepared by the method of Chettur & Benkovic (1977). Pteroyl-(γ-L-glutamyl)$_2$-L-glutamic acid was prepared by the method of Baugh et al. (1970). Pteroyl-L-glutamic acid and pteroyl-(γ-L-glutamyl)$_2$-L-glutamic acid were converted enzymatically to L(−)-H_4folate and L(−)-H_4pteroyl$(Glu)_3$, respectively, according to the methods of Blakely (1960) and Mathews & Huennekens (1960) and stored under vacuum at −4 °C in 50 mM Tris-HCl and 0.1 M 2-mercaptoethanol, pH 7.4. L(+)-5,10-Methenyl-H_4pteroyl$(Glu)_3$ was prepared as described by Rowe (1968), and 5,11-methenyl-H_4homofolate was prepared by the method of Caperelli et al. (1981).

Methods

Preparation of Trifunctional Enzyme. The trifunctional enzyme was purified from chicken liver according to the procedure of Smith et al. (1980) and concentrated to 0.8 mg/mL with an Amicon ultrafiltration unit utilizing a YM-10 membrane. Protein concentration was determined by a UV–biuret assay (Zamenhof, 1957) for which a standard curve was established with ribonuclease A.

Determination of Kinetic Parameters. Parameters for 10-formyl-H_4folate synthetase were measured by following the disappearance of L(−)-H_4pteroyl$(Glu)_3$ at 298 nm by using $\Delta\epsilon_{298} = 19\,700\ M^{-1}\ cm^{-1}$ (Black et al., 1978). Conditions for the assays were as described for the coupled enzyme assay except for the use of varying amounts of L(−)-H_4pteroyl$(Glu)_3$. One-milliliter reactions were run in sealed cuvettes under nitrogen, and reactions were initiated with 0.2 μg of trifunctional protein [6.9 μmol min^{-1} (mg of synthetase)$^{-1}$]. Kinetic constants were calculated from initial velocity data by using the hyperbolic computer program of Cleland (1967).

Parameters for 5,10-methenyl-H_4folate cyclohydrolase were calculated from data of initial velocities obtained by the spectrophotometric assay described by Caperelli et al. (1980) using L(+)-5,10-methenyl-H_4pteroyl$(Glu)_3$. Reactions were initiated with 0.08 μg of trifunctional protein [18.6 μmol min^{-1} (mg of cyclohydrolase)$^{-1}$] and kinetic constants calculated as previously indicated (Cleland, 1967). Initial velocity data from 90, 180, 450, and 950 μM $NADP^+$ and 3, 5, 7, and 10 μM L(+)-5,10-methenyl-H_4pteroyl$(Glu)_3$ were computer fit by Cleland's competitive inhibition program (1967). A K_i value for the inhibition of 5,10-methenyl-H_4folate cyclohydrolase by H_4pteroyl$(Glu)_3$ was determined from initial velocity data for 3, 7.5, and 10 μM H_4pteroyl$(Glu)_3$ and 6.25, 8.3, 12.5, and 25 μM 5,10-methenyl-H_4folate. An average value of 5 μM was determined.

A maximal velocity value for 5,10-methylene-H_4folate dehydrogenase was estimated by using the initial velocity data for 40 μM L(−)-H_4pteroyl$(Glu)_3$ and 325 μM formaldehyde, by using the measured K_m value of 5 μM for 5,10-methylene-H_4folate (L. Slieker, unpublished results), and by correcting for the nonsaturating level of the 5,10-methylene cofactor. The initial concentration of the 5,10-methylene cofactor was calculated by using the equilibrium constant of $3.2 \times 10^4\ M^{-1}$ (Kallen & Jencks, 1966) for the condensation of formaldehyde with H_4folate. The assay conditions were

Table I: Comparison of Kinetic Parameters for Monoglutamate and Triglutamate Substrates

	dehydrogenase of 5,10-methylene-H_4pteroyl-		cyclohydrolase of 5,10-methenyl-H_4pteroyl-		synthetase of H_4pteroyl-	
	Glu_1	$(Glu)_3$	Glu_1	$(Glu)_3$	Glu_1	$(Glu)_3$
K_m[a] (μM)	5.0		20	4.3	67	4.1
V_{max}[b] (μmol min^{-1} mg^{-1})	9.0	15.5	5.3	18.6[a]	5.1[a]	6.9[a]
turnover no.[c] [μmol min^{-1} (μmol of enzyme)$^{-1}$]	1720	2950	1000	3530	970	1300

[a] Values were calculated from double-reciprocal plots obtained from standard assays as described under Methods. Assay conditions were 50 mM sodium maleate, pH 6.8, and 25 °C. [b] Unless otherwise indicated, values were calculated from standard assays as described under Methods and corrected for folate cofactor saturation using $v = V[S]/K_m + [S]$ and the predetermined K_m value. [c] Concentration of enzyme was determined by using a molecular weight of 190 000.

identical with those of the acid quenched point assay at 350 nm as described elsewhere by using $\Delta\epsilon_{350} = 26000$ M^{-1} cm^{-1}.

10-Formyl-H_4folate Synthetase/5,10-Methenyl-H_4folate Cyclohydrolase Assay. The synthetase assays were performed at 25 °C in 50 mM sodium maleate, pH 6.8, using a 1-mL reaction mixture containing 2 mM formate, 0.1 M NH_4Cl, 4 mM $MgCl_2$, 40 μM L(−)-H_4pteroyl$(Glu)_3$, 2 mM ATP, 0.28 mM 2-mercaptoethanol, and 4×10^{-4} mg of trifunctional protein [6.9 μmol min^{-1} (mg of synthetase)$^{-1}$ and 18.6 μmol min^{-1} (mg of cyclohydrolase)$^{-1}$]. After initiation of the reaction with ATP the production of 5,10-methenyl-H_4pteroyl$(Glu)_3$ was followed spectrophotometrically by the change in absorbance at 355 nm by using $\Delta\epsilon = 24900$ M^{-1} cm^{-1} (Uyeda & Rabinowitz, 1967a). Spectrophotometric assays were performed in sealed cuvettes which were prepared in a glove bag under nitrogen. The total of 5,10-methenyl-H_4pteroyl$(Glu)_3$ plus 10-formyl-H_4pteroyl$(Glu)_3$ produced was determined by quenching 100-μL aliquots, removed at various times, in 300 μL of 6 N HCl and measuring the change in absorbance at 350 nm where $\Delta\epsilon = 26000$ M^{-1} cm^{-1} (Rowe, 1968). Acidification assays were performed in a glove bag under nitrogen in thermostated cells. Solutions for both assays were degassed by evacuating and saturating with argon for a total of 12 exchanges. The level of 10-formyl-H_4pteroyl$(Glu)_3$ was determined by the difference between the total of 10-formyl- plus 5,10-methenyl-H_4pteroyl$(Glu)_3$ and 5,10-methenyl-H_4pteroyl$(Glu)_3$ alone.

5,10-Methylene-H_4folate Dehydrogenase/5,10-Methenyl-H_4folate Cyclohydrolase Assay. The dehydrogenase/cyclohydrolase assays were performed at 25 °C in 50 mM sodium maleate, pH 6.8 buffer containing 325 μM CH_2O, 40 μM L(−)-H_4pteroyl$(Glu)_3$, 0.1 mM EDTA, 0.5 mM oxalacetate, 0.25 mM $NADP^+$, 0.28 mM 2-mercaptoethanol, 0.18 mg of malate dehydrogenase[2] (1000 μmol min^{-1} mg^{-1}), and 4×10^{-4} mg of trifunctional protein [15.5 μmol min^{-1} (mg of dehydrogenase)$^{-1}$ and 18.6 μmol min^{-1} (mg of cyclohydrolase)$^{-1}$] in a 1-mL reaction volume. In these assays H_4pteroyl$(Glu)_3$ was reacted with formaldehyde for 20 min in 50 mM maleate, pH 6.8, prior to the addition of the remaining components in order to prepare the 5,10-methylene-H_4pteroyl$(Glu)_3$ cofactor. Reactions were initiated with $NADP^+$, and the time course production of 5,10-methenyl- and 10-formyl-H_4pteroyl$(Glu)_3$ was measured as previously described.

Inhibition studies used 100 μM 5,11-methenyl-H_4homofolate, 100 μM L(−)-H_4folate, and 3 mM CH_2O in the assay mixture just described. These reactions were initiated with trifunctional enzyme, and the time course for production of folate cofactors was measured as before.

Measurement of k_{obsd} for Reaction of H_4pteroyl$(Glu)_3$ and Formaldehyde. The rate constant for the formation of 5,10-methylene-H_4pteroyl$(Glu)_3$ was measured by the procedure of Kallen & Jencks (1966) in 50 mM sodium maleate, pH 6.8, at 25 °C with 40 μM L(−)-H_4pteroyl$(Glu)_3$, 325 μM formaldehyde, and 0.28 mM 2-mercaptoethanol.

Measurement of the 5,10-Methenyl-H_4pteroyl$(Glu)_3$/10-Formyl Equilibration during the Dehydrogenase/Cyclohydrolase Assay. The assay solution contained all of the components of the dehydrogenase/cyclohydrolase reaction as before except for the following: (1) 325 μM [^{3}H]formaldehyde (44 Ci/mol), (2) 0.5 mM α,β-GAR, (3) 2.9×10^{-3} mg of GAR TFase (1.6 μmol min^{-1} mg^{-1}) and (4) 4.2×10^{-4} mg of trifunctional protein [15.5 μmol min^{-1} (mg of dehydrogenase)$^{-1}$ and 18.6 μmol min^{-1} (mg of cyclohydrolase)$^{-1}$]. After initiation of the reaction with $NADP^+$, 180-μL portions were removed at various times and quenched in 0.093 N NaOH. Each quenched sample was applied to a 0.55×12.5 cm column of QAE-Sephadex which had been previously equilibrated with 0.01 M $NaHCO_3$, pH 9.9. FGAR was eluted with 0.02 M $NaHCO_3$, pH 9.9. Fractions of 1.5 mL were collected into 20-mL scintillation vials, 15 mL of Liquiscint cocktail was added to each vial, and radioactivity measurements were done on a Beckman LS-8100 liquid scintillation counter.

The equilibration experiment was performed by adding 50 μM unlabeled 5,10-methenyl-H_4pteroyl$(Glu)_3$ to the running assay at 2 min and then quantitating the labeled FGAR produced at various times as just described.

Computer Simulation. All computation was done on an IBM 3033 processor complex running under OS/VS2 MVS. Simulations were performed by Chemical Reaction Analysis and Modeling System (CRAMS), an integrated system of Fortran and OS Assembler modules designed to simulate and predict data (Butler & DeMaine, 1975; DeMaine, 1980a,b). The simulator module of the system uses a predictor–corrector algorithm started by a Runge-Kutta method to solve the applicable system of differential equations.

Results

Kinetic Parameters. The kinetic parameters of the folate triglutamate substrates for the trifunctional protein are listed in Table I along with the corresponding values for their monoglutamate counterparts. The Michaelis constant for 5,10-methylene-H_4pteroyl$(Glu)_3$ was taken to be less than or equal to the value of the monoglutamate substrate since smaller values could not be accurately measured spectrophotometrically. Increasing the number of glutamyl residues results in 5- and 16-fold tighter binding constants for the 5,10-methenyl (cyclohydrolase) and tetrahydrofolate (synthetase) substrates, respectively.

[2] Malate dehydrogenase removes the NADPH ($\Delta\epsilon_{340} = 6220$ M^{-1} cm^{-1}) formed in the assay since it interferes with the measurement of 5,10-methenyl-H_4pteroyl$(Glu)_3$ at 355 nm.

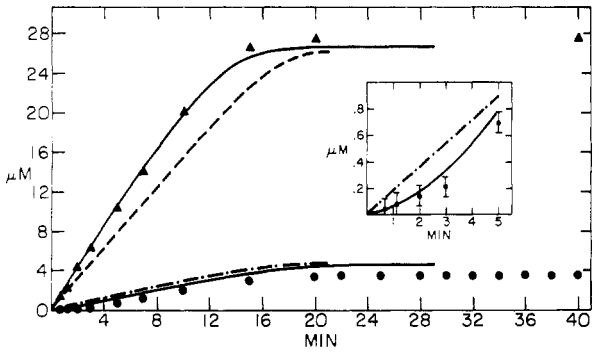


FIGURE 1: Experimental and simulated time courses for the synthetase/cyclohydrolase coupled enzyme reaction. Reactions were performed in 50 mM maleate, pH 6.8 at 25 °C, as described under Methods. The points represent the observed concentrations of (●) 5,10-methenyl-H_4pteroyl(Glu)$_3$ measured by ΔA_{355} and (▲) 10-formyl-H_4pteroyl(Glu)$_3$ measured by the difference between ΔA_{350} after acidification and ΔA_{355}. The solid curves represent the simulation using the two-site model. The dashed curves are the simulated concentrations of (---) 10-formyl-H_4pteroyl(Glu)$_3$ and (-·-) 5,10-methenyl-H_4pteroyl(Glu)$_3$ using the partitioning model.

Turnover numbers were calculated by using a molecular weight value of 190 000 for the trifunctional protein. Assays were performed at 25 °C in maleate buffer in order to stabilize the 5,10-methenyl cofactor by decreasing the rate of buffer-catalyzed hydrolysis. The number of glutamyl residues has a greater influence on the turnover numbers for the dehydrogenase and cyclohydrolase activities than for the synthetase activity. On average, the triglutamate substrates enhance the activity of the former activities approximately 2-fold whereas the synthetase activity is enhanced about 1.2-fold.

It has previously been found in our laboratory that the cyclohydrolase activity is competitively inhibited by the substrate of the dehydrogenase reaction L(−)-5,10-methylene-H_4folate, with a K_i of 13 μM (P. Domanico, unpublished results). In this paper we report the additional finding that $NADP^+$, also a substrate for the dehydrogenase, inhibits the cyclohydrolase competitively against 5,10-methenyl-H_4-pteroyl(Glu)$_3$ with a K_i of 171 μM. It was also found that ATP and H_4pteroyl(Glu)$_3$, both substrates for the synthetase, inhibit the cyclohydrolase with K_i values of 2 mM and 5 μM, respectively.

Synthetase/Cyclohydrolase Coupled Enzyme Reaction. Production of the 10-formyl and 5,10-methenyl cofactors by the synthetase and presumably the cyclohydrolase is shown in Figure 1. The final equilibrium ratio of the two cofactors from the enzymatic reaction is close to the 85:15 (10-formyl:5,10-methenyl) ratio expected in 50 mM maleate, pH 6.8, resulting from the simple buffer-catalyzed interconversion of the two folate forms. The observed rate constant for conversion of the 5,10-methenyl to the 10-formyl derivative was calculated from the hydrolysis reaction in 50 mM maleate, pH 6.8, and found to be 1.2×10^{-2} min^{-1}. By use of this value and the chemical equilibrium ratio, the observed rate constant for the nonenzymatic formation of the 5,10-methenyl cofactor from the 10-formyl species is given by

$$\text{5,10-methenyl} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{10-formyl}$$

where $k_{-1} = k_1/K_{eq} = (1.2 \times 10^{-2}\ \text{min}^{-1})/(85/15) = 2.1 \times 10^{-3}$ min^{-1}. Since the chemical rate is small relative to V_{max}, the production of the 5,10-methenyl cofactor at the observed rate in the coupled assay must primarily result from cyclohydrolase equilibration of the cofactors.

Table II: Computer Simulation of Synthetase/Cyclohydrolase Reaction Time Course: Model for Independent Sites

no.	reaction[a]	kinetic constant	determination of kinetic constant[b]
1	$S + H_4P \rightleftharpoons S(H_4P)$	2.4×10^5 M^{-1}	K_m^{-1}
2	$S(H_4P) \rightarrow CHO + S$	1.3×10^3 min^{-1}	k_{cat}
3	$CHO \rightarrow C^+H$	2.1×10^{-3} min^{-1}	*c*
4	$C^+H \rightarrow CHO$	1.2×10^{-2} min^{-1}	*d*
5	$C^+H + Cy \rightleftharpoons Cy(C^+H)$	2.3×10^5 M^{-1}	K_m^{-1}
6	$Cy(C^+H) \rightarrow CHO + Cy$	3.5×10^3 min^{-1}	k_{cat}
7	$CHO + Cy \rightleftharpoons Cy(CHO)$	2.4×10^5 M^{-1}	K_i^{-1} [e]
8	$Cy(CHO) \rightarrow C^+H + Cy$	5.9×10^2 min^{-1}	*f*
9	$H_4P + Cy \rightleftharpoons Cy(H_4P)$	2.0×10^5 M^{-1}	K_i^{-1} [g]
10	$ATP + Cy \rightleftharpoons Cy(ATP)$	5.0×10^2 M^{-1}	K_i^{-1} [h]

[a] Abbreviations: S = synthetase; Cy = cyclohydrolase; H_4P = tetrahydropteroyltriglutamate; CHO = 10-formyl-H_4P; C^+H = 5,10-methenyl-H_4P. [b] K_m and k_{cat} values from Table I for triglutamate substrates. [c] Rate constant was calculated from measured rate constant for reaction 4 and the observed equilibrium ratio at pH 6.8 and 25 °C, K_{eq} = 85:15 (10-formyl:5,10-methenyl). [d] Rate constant for hydrolysis of 5,10-methenyl-H_4pteroyl(Glu)$_3$ was measured at 25 °C in 50 mM sodium maleate buffer, pH 6.8. [e] K_i for 10-formyl-H_4pteroyl(Glu)$_3$ was approximated to be 4.1 μM by presuming the same K_m/K_i ratio as for the monoglutamate substrates where K_m(5,10-methenyl) = 20 μM and K_i(10-formyl) = 19 μM. [f] Rate constant calculated by using kinetic constants from reactions 5, 6, and 7, K_{eq}, and the Haldane Relationship for equilibrium reactions (Fersht, 1977). [g] K_i for H_4pteroyl(Glu)$_3$ was determined to be 5 μM. [h] K_i for ATP was determined to be 2 mM.

The production of 5,10-methenyl-H_4pteroyl(Glu)$_3$ by the cyclohydrolase shows a lag as would be expected for a multifunctional enzyme catalyzing sequential reactions at two distinct and noninteracting active sites. This necessitates that the 10-formyl intermediate produced by the synthetase equilibrates with solution prior to binding at the cyclohydrolase site. The computer simulation of the reaction time course using a two-site model (Table II) compares well to the observed data in Figure 1. Both the rates of formation of products and their equilibrium levels were closely fit by using a model in which the synthetase and cyclohydrolase sites act independently of one another. The experimental points cannot be fit by a one-site partitioning model (see below) or models assuming separate but interacting sites. In these cases the rate of formation of the 5,10-methenyl species would be faster than the observed rate.

Dehydrogenase/Cyclohydrolase Coupled Enzyme Reaction. The equilibrium ratio of products from the dehydrogenase/cyclohydrolase coupled assay is also equal to the value expected from the chemical equilibrium in solution. In contrast to the synthetase/cyclohydrolase assay 10-formyl-H_4pteroyl(Glu)$_3$, the product of the second enzyme in the couple, does not exhibit a lag phase during the initial portion of the reaction. A lag time, *t*, can be calculated from the Storer & Cornish-Bowden expression (1974) shown in eq 1 and the kinetic

$$t = \phi K_2/v_1 \qquad (1)$$

constants listed in Table III where K_2 is the binding constant of 5,10-methenyl-H_4pteroyl(Glu)$_3$ to the cyclohydrolase (4.3 μM) and v_1 is the initial velocity of the dehydrogenase reaction (5.3 μM min^{-1}) under our conditions. The constant ϕ was approximated by using a value of $v_2/v_1 = 0.9$ and $v_1/V_2 = 5.3$ μM min^{-1}/7.4 μM min^{-1} (Table III) where v_2 is the initial velocity and V_2 the maximal velocity of the cyclohydrolase. From Storer & Cornish-Bowden (1974) these values yield a minimum value of $\phi = 6.5$. Substitution of ϕ into eq 1 results in an expected lag time of at least 5.3 min which is significantly

Table III: Trifunctional Protein-Enzymatic Activities in Coupled Enzyme Assays

	dehydrogenase	cyclohydrolase	synthetase
maximal sp act.[a] (μmol min^{-1} mg^{-1})	15.5	18.6	6.9
V_{max}[b] (μM min^{-1})	6.2	7.4	2.8
$v_{initial}$[c] (μM min^{-1})	5.3		2.4

[a] Values from Table I for triglutamate forms of reduced folate substrates. [b] Calculated from maximal specific activity by using a 1-mL reaction volume containing 4×10^{-4} mg of trifunctional protein. Buffer and temperature conditions for coupled assays were identical with those used in assaying the individual enzymes. Details of the assays are described under Methods. [c] Initial velocities for the dehydrogenase and synthetase activities used in eq 1 were calculated from initial concentrations of reduced folate substrates by using $v = V_{max}[S]/K_m + [S]$ and K_m values listed in Table I.

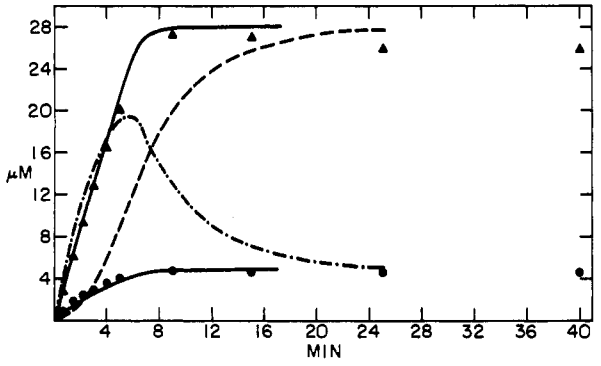


FIGURE 2: Experimental and simulated time courses for the dehydrogenase/cyclohydrolase coupled enzyme reaction. The details are identical with those described under Figure 1 except that the solid curves represent the simulation using the partitioning model. The dashed curves are the simulated concentrations of (---) 10-formyl-H_4pteroyl(Glu)$_3$ and (---) 5,10-methenyl-H_4pteroyl(Glu)$_3$ assuming two independent sites.

different from the experimental data in which no lag time was observed. This calculated lag time represents a minimal value relative to a value calculated by using $v_2/v_1 = 0.99$ and by considering the observed competitive inhibition of the cyclohydrolase activity against 5,10-methenyl-H_4pteroyl(Glu)$_3$ by both $NADP^+$ and 5,10-methylene-H_4pteroyl(Glu)$_3$.

The initial rate of formation of the 10-formyl species expected during the coupled assay is the sum of the chemical and enzymatic rates and is calculated by using eq 2, the kinetic

$$v_{10\text{-formyl}} = k_1[5,10\text{-methenyl}] + \frac{V_{max}[5,10\text{-methenyl}]}{K_m + [5,10\text{-methenyl}]} \quad (2)$$

parameters from Tables I and III, and the concentration of the 5,10-methenyl cofactor in the assay solution at any time (Figure 2). At 2 min the expected amount of the 10-formyl species from 2.2 μM 5,10-methenyl-H_4pteroyl(Glu)$_3$ is calculated to be 2.5 μM by using eq 2 where $k_1 = 1.2 \times 10^{-2}$ min^{-1}, $V_{max} = 7.4$ μM min^{-1}, and $K_m = 4.3$ μM. This is considerably lower than the observed concentration of 9.6 μM shown in Figure 2. Thus the initial amount of the 10-formyl cofactor in solution cannot stem from the observed levels of the 5,10-methenyl intermediate in solution during the initial portion of the reaction.

Both the absence of a lag phase during the formation of the 10-formyl cofactor and the enhanced rate of production of the 10-formyl species indicate that the dehydrogenase and cyclohydrolase sites are not independent of one another. As

Table IV: Computer Simulation of Dehydrogenase/Cyclohydrolase Reaction Time Course: Model for Interacting Sites or Combined Site

no.	reaction[a]	kinetic constant	determination of kinetic constant[b]
1	$CH_2O + H_4P \rightarrow CH_2$	2.3×10^3 M^{-1} min^{-1}	c
2	$CH_2 \rightarrow H_4P + CH_2O$	7.2×10^{-2} min^{-1}	d
3	$DCy + CH_2 \rightleftharpoons DCy(CH_2)$	1.0×10^6 M^{-1}	K_m^{-1}
4	$DCy(CH_2) \rightarrow DCy(C^+H)$	2.9×10^3 min^{-1}	k_{cat}
5	$DCy(C^+H) \rightarrow DCy + CHO$	1.7×10^4 min^{-1}	e
6	$DCy(C^+H) \rightarrow DCy + C^+H$	2.9×10^3 min^{-1}	e
7	$C^+H \rightarrow CHO$	1.2×10^{-2} min^{-1}	f
8	$CHO \rightarrow C^+H$	2.1×10^{-3} min^{-1}	f

[a] Abbreviations: DCy = dehydrogenase/cyclohydrolase; H_4P, C^+H, and CHO as described in Table II; CH_2 = 5,10-methylene-H_4P. [b] K_m and k_{cat} values from Table I for triglutamate substrates. [c] Rate constant for condensation of CH_2O and H_4P measured in 50 mM maleate buffer, pH 6.8 at 25 °C, as described under Methods. [d] Rate constant calculated from measured rate constant for reaction 1 and overall equilibrium constant of 3.2×10^4 M^{-1} (Kallen & Jencks, 1966). [e] Assumed rate constants to account for observed ratio of 85:15 (10-formyl:5,10-methenyl) produced by trifunctional protein where reaction 4 is rate limiting. [f] Described in Table II.

illustrated in Figure 2, the experimental data from the dehydrogenase/cyclohydrolase coupled assay could not be simulated with the two-site model used for the synthetase where the 5,10-methenyl product from the dehydrogenase freely equilibrates with solution prior to binding to the cyclohydrolase. The two-site model also includes equations to account for the inhibition of the cyclohydrolase activity by $NADP^+$ and 5,10-methylene-H_4pteroyl(Glu)$_3$ which was observed in the individual activity assay.

The observed folate levels were fit by a combined dehydrogenase/cyclohydrolase one-site model where the relative rates for formation of the free 5,10-methenyl and 10-formyl cofactors produce the observed equilibrium ratio of folates in solution (Table IV). This model assumes the trifunctional protein–5,10-methenyl cofactor complex partitions so that 15% of the product formed leaves the enzyme in the 5,10-methenyl form whereas 85% is directly hydrolyzed by the cyclohydrolase to 10-formyl-H_4pteroyl(Glu)$_3$. The fit is very sensitive to the partitioning ratio: changing the ratio from 85:15 to 75:25 leads to a 60% deviation from the original fit for the methenyl concentration.

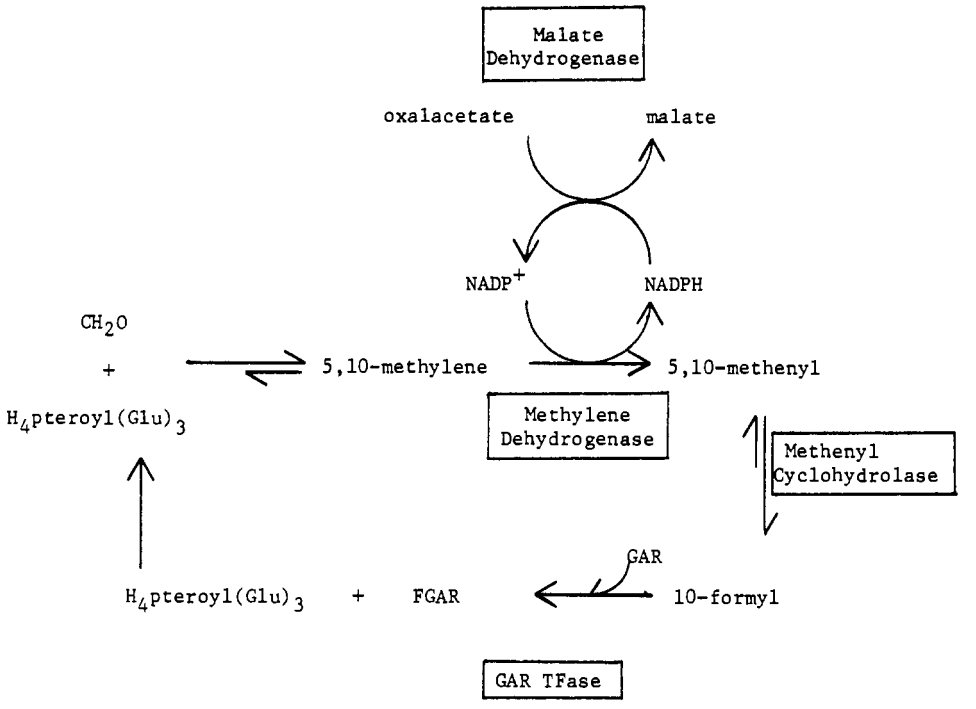
5,11-Methenyl-H_4homofolate Inhibition of the Dehydrogenase/Cyclohydrolase Coupled Assay. It was previously determined by Caperelli et al. (1981) that 5,11-methenyl-H_4homofolate inhibits the cyclohydrolase activity and is competitive with 5,10-methenyl-H_4folate ($K_i = 41$ μM) but does not inhibit the dehydrogenase activity. Since the inhibitor is the monoglutamate form, the coupled enzyme inhibition experiments were done with H_4folate monoglutamate to generate the initial pool of 5,10-methylene-H_4folate. Since the amount of the 5,10-methenyl cofactor produced during the coupled enzyme reaction initially increases with time, the expected inhibition of the cyclohydrolase should decrease with time. At any time the fractional inhibition can be calculated from the equation (Segal, 1975)

$$i = [I]/[[I] + K_i(1 + [5,10\text{-methenyl}]/K_m)] \quad (3)$$

where [I] = 100 μM, $K_i = 41$ μM, and $K_m = 20$ μM.

By use of eq 3, at 4 min the measured concentration of the 5,10-methenyl cofactor in the assay solution is 7.5 μM (Figure 3), and the expected inhibition is 64%. Therefore, given the

Scheme I



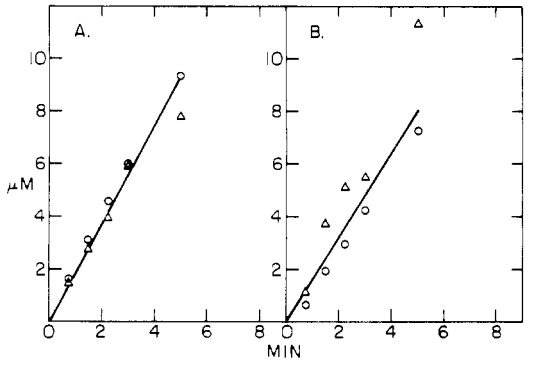


FIGURE 3: Inhibition of the dehydrogenase/cyclohydrolase coupled enzyme reaction by 5,11-methenyl-H_4homofolate. (A) Comparison of 5,10-methenyl-H_4folate produced in the absence (○) and presence (△) of 100 μM inhibitor. (B) Comparison of 10-formyl-H_4folate produced in the absence (○) and presence (△) of 100 μM inhibitor. Reactions were performed in 50 mM maleate, pH 6.8 at 25 °C, as described under Methods.

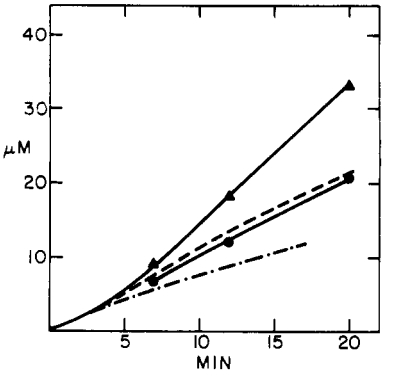


FIGURE 4: [^{3}H]FGAR produced in the pulse–chase experiment from the trifunctional protein/GAR TFase enzyme mixture. Reactions performed in 50 mM maleate, pH 6.8 at 25 °C, by using dehydrogenase/cyclohydrolase assay conditions as described under Methods. (▲) Control reaction starting with 30 μM [^{3}H]5,10-methylene-H_4pteroyl(Glu)$_3$; (●) pulse–chase reaction where 50 μM unlabeled 5,10-methenyl-H_4pteroyl(Glu)$_3$ was added to the running reaction at 2 min; (---) dilution in rate from enzymatic hydrolysis by total trifunctional protein of the unlabeled 5,10-methenyl cofactor chase; (---) dilution in rate from the nonenzymatic plus enzymatic hydrolysis by 14% of the total trifunctional protein of the unlabeled chase.

low concentrations of the 5,10-methenyl species observed and the 100 μM concentration of inhibitor present in the assay, a significant amount of inhibition in 10-formyl cofactor formation should have been observed during the time frame shown in Figure 3. The plots show that there is no difference in the distribution of the 5,10-methenyl and 10-formyl cofactors in solution in the absence or presence of 5,11-methenyl-H_4homofolate. Therefore, there is no inhibition of the cyclohydrolase reaction when the 5,10-methenyl cofactor is provided by the dehydrogenase reaction rather than from bulk solution.

Hydrolysis of the 5,10-Methenyl Intermediate by the Trifunctional Protein. A pulse–chase experiment was designed that efficiently removed the 10-formyl product by coupling it to GAR TFase (Scheme I) in order to determine if the observed solution of 5,10-methenyl-H_4pteroyl(Glu)$_3$ formed from enzymatic dehydrogenation of the 5,10-methylene species undergoes subsequent hydrolysis at the cyclohydrolase site. The difference in the amount of ^{3}H label incorporated from 5,10-[^{3}H]methylene-H_4pteroyl(Glu)$_3$ into FGAR with and without the addition of excess unlabeled 5,10-methenyl-H_4pteroyl(Glu)$_3$ is a measure of the utilization of the 5,10-methenyl cofactor pool. If the 5,10-[^{3}H]methenyl intermediate pool functions as a source of the 10-formyl species through interconversion at the cyclohydrolase site, a large dilution in the specific radioactivity of [^{3}H]FGAR should occur when an unlabeled chase of 5,10-methenyl-H_4pteroyl(Glu)$_3$ is added to the reaction. Dilution owing to the nonenzymatic hydrolysis of the 5,10-methenyl chase should be much less because the nonenzymatic rate is approximately 12-fold less than the enzymatic one.

The results in Figure 4 show that when 50 μM unlabeled 5,10-methenyl-H_4pteroyl(Glu)$_3$ is added to the running reaction at 2 min, [^{3}H]FGAR is initially produced at a linear rate of 1.1 μM/min relative to the control rate of 1.7 μM/min. Since the 5,10-methenyl derivative does not inhibit any of the

enzymatic activities, this decrease in rate is due to a decrease in the specific radioactivity of 10-[^{3}H]formyl-H_4pteroyl(Glu)$_3$, the substrate for GAR TFase. From the time course for production of labeled and unlabeled 10-formyl-H_4pteroyl-(Glu)$_3$, the calculated dilution in [^{3}H]FGAR synthesis caused by enzymatic hydrolysis of the unlabeled intermediate pool is far greater than the observed data (Figure 4).[3] Under the assumption (1) that only the free form of the trifunctional protein (14% on average) not involved in a dehydrogenase/cyclohydrolase couple is capable of catalyzing the hydrolysis of the 5,10-methenyl-H_4pteroyl(Glu)$_3$ pool and (2) that non-enzymatic hydrolysis of this pool to the 10-formyl species now is a significant contributor, a dilution curve (Figure 4) may be calculated that closely fits the observed data.[4]

Discussion

The differences in K_m values between the mono- and triglutamate substrates tabulated in Table I are similar to those reported by MacKenzie & Baugh (1980) for the porcine multifunctional protein. These authors observed K_m = 6.8 μM (tri) vs. 25 μM (mono) for the dehydrogenase and K_m = 2.0 μM (tri) vs. 89 μM (mono) for the synthetase. This enhancement in binding and frequently V_{max} of folate substrates by additional glutamyl residues is commonly observed for folate-dependent enzymes including 5-aminoimidazole-4-carboxamide–ribonucleotide transformylase (Mueller & Benkovic, 1981), methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), the bifunctional protein formiminoglutamate:tetrahydrofolate formiminotransferase and formiminotetrahydrofolate cyclodeaminase (MacKenzie & Baugh, 1980), thymidylate synthetase (Kisliuk et al., 1981), methionine synthetase (Coward et al., 1975), and dihydrofolate reductase (Coward et al., 1974). The turnover numbers for the trifunctional protein also compare well with those for the porcine enzyme whose values are 1700 min^{-1} dehydrogenase, 2100 min^{-1} synthetase, and 8700 min^{-1} cyclohydrolase (Tan et al., 1977). Because the assay conditions were different than those used in this study, a strict comparison cannot be made.

The computer-simulated data using our model for the synthetase/cyclohydrolase coupled enzyme reaction is in good agreement with the basic features of the observed data and well within the experimental errors of our techniques. The results constitute strong supportive evidence that the synthetase and cyclohydrolase activities of the trifunctional protein occur at two separate and independent active sites. This means that the 10-formyl intermediate fully equilibrates with the bulk solution prior to binding to the cyclohydrolase site. Independent sites are consistent with studies showing the selective removal of the synthetase vs. the cyclohydrolase activity by proteolysis of the yeast (Paukert et al., 1977) and porcine (Tan & MacKenzie, 1977) multifunctional proteins.

However, the dehydrogenase and cyclohydrolase sites are not independent of one another. Product formation from the 5,10-methylene species through the dehydrogenase could only be simulated by assuming a partitioning in which 85% of the bound 5,10-methenyl intermediate is converted to the bound 10-formyl form and 15% is released from the enzyme into solution. Hydrolysis of the 5,10-methenyl cofactor before its loss to solution eliminates the expected lag in the rate of formation of the 10-formyl cofactor and accounts for the enhanced rate of production of the 10-formyl product over that predicted from the measured level of the 5,10-methenyl intermediate in solution. These results support a model in which the products are partitioned from a single site in the observed ratio of 85:15 (10-formyl:5,10-methenyl). Alternatively a two-site model with 85% channeling of the 5,10-methenyl intermediate to form the 10-formyl species at a second site likewise is consistent with the data. Since direct channeling between two proximal and interacting sites on the enzyme and a combined one-site model are kinetically indistinguishable, the description of the enzymatic reactions used for the computer simulation analysis is valid for both cases. Cohen & MacKenzie (1978) reported an enhanced rate of production of the 10-formyl cofactor and 60% conversion of the 5,10-methenyl species to the 10-formyl derivative for the monoglutamate cofactor using the porcine enzyme. Although the evidence for interacting sites in the rabbit trifunctional protein is not conclusive, Schirch (1978) demonstrated that 5,10-methenyl-H_4folate does not accumulate in solution during the dehydrogenase/cyclohydrolase coupled enzyme reaction.

It is unusual that the trifunctional protein produces the two triglutamate cofactors in their equilibrium ratio at pH 6.8. This result may be coincidental, or the ratio of products released from the enzyme could be dependent on the pH of the assay solution. Although we have not measured the ratio of products as a function of pH, Cohen & MacKenzie (1978) assayed the dehydrogenase/cyclohydrolase activity of the porcine protein at pH 7.3. At this pH the equilibrium ratio of the 10-formyl and 5,10-methenyl cofactors is greater than 85:15, yet the observed product ratio is 60:40. This finding argues against a site or sites that release products at chemical equilibrium.

The problem of a combined site vs. separate sites may be examined by monitoring the response of the partitioning ratio to the polyglutamate length of the 5,10-methenyl intermediate. MacKenzie & Baugh (1980) proposed that the polyglutamate tail may serve as an "anchor" for folate-utilizing multifunctional proteins so that the folate intermediate can pivot from one active site to another. The partitioning to products would then be dependent on the number of glutamyl residues present on the intermediate relative to the distance between the two active sites. Since the initial product is the 5,10-methenyl species, its extent of conversion to the 10-formyl product should increase with increasing chain length. In the experiment using 5,10-methylene-H_4folate as substrate for the dehydrogenase/cyclohydrolase (Figure 3), the initial rates show that 46% of the product is the 10-formyl species as compared to 85% observed for the triglutamate case and favors this role for the glutamyl moiety. Because of the increasing evidence that substrates of folate-dependent reactions have an optimal polyglutamate length, perhaps complete conversion of the 5,10-methenyl intermediate to the 10-formyl product would occur by using the penta- or heptaglutamate substrates.

From several lines of evidence it appears that the operation of the dehydrogenase/cyclohydrolase pathway markedly affects the binding characteristics of the cyclohydrolase site. Since 5,10-methylene-H_4folate is a competitive inhibitor against 5,10-methenyl-H_4folate for the cyclohydrolase (K_i = 13 μM), one would expect to see inhibition of 10-formyl co-

[3] The dilution factor in the rate of [^{3}H]FGAR synthesis is the ratio of the amount of labeled 10-formyl cofactor divided by the total amount of 10-formyl cofactor produced in the assay at any time. The production of labeled cofactor is obtained directly from Figure 2. The rate of hydrolysis of unlabeled 5,10-methenyl cofactor is calculated from the parameters in Tables III and IV based on the initial concentration of 50 μM.

[4] An interaction between GAR transformylase and the trifunctional enzyme that prevented access by chase to the cyclohydrolase site also would be in accord with these results. However, this assumption is not necessary in view of the lack of inhibition by 5,11-methenyltetrahydrohomofolate in the coupled assay.

factor production. Assuming the equilibrium binding of the 5,10-methenyl and the 5,10-methylene cofactors to a single common site on the trifunctional protein, one can readily derive the relationships

$$[E{\cdot}S_1] = [E_T][K_2[S_1]/(K_1K_2) + K_2[S_1] + K_1[S_2]]$$

and

$$[E{\cdot}S_2] = [E_T][K_1[S_2]/(K_1K_2) + K_2[S_1] + K_1[S_2]]$$

where S_1 = 5,10-methylene-, S_2 = 5,10-methenyl-H_4-pteroyl(Glu)$_3$, $K_1 = K_i$,[5] and $K_2 = K_M$ (Table I). At 2 min $[E{\cdot}S_2] \simeq 0.16[E_T]$ so that the maximal estimate of 10-formyl cofactor produced in this interval is 2.4 μM (7.4 μM/min × 2 min × 0.16). The observed concentration is 9.4 μM (Figure 2), 4-fold greater than expected. The absence of 5,10-methylene inhibition of 10-formyl cofactor formation also was noted for the trifunctional protein from rabbit (Schirch, 1978) where the 5,10-methylene species did not inhibit the cyclohydrolase activity in the dehydrogenase/cyclohydrolase coupled assay but acted competitively when the single activity was assayed.

The results with the 5,11-methenyl-H_4homofolate inhibition study further substantiate those regarding the peculiar 5,10-methylene cofactor inhibition of the cyclohydrolase. Although 5,11-methenyl-H_4homofolate is an inhibitor of the cyclohydrolase in the separate activity assay, no inhibition is observed during the time course of the dehydrogenase/cyclohydrolase coupled assay. In the coupled reaction there should be three competitors for the cyclohydrolase site: the 5,10-methenyl and 5,10-methylene cofactors and 5,11-methenyl-H_4homofolate. The actual partitioning of the trifunctional protein between the three species can be readily calculated by using the above equilibrium assumption expanded to include an additional species so that

$$[E{\cdot}S_2] = [E_T][K_1K_3[S_2]/(K_1K_2K_3) + K_2K_3[S_1] + K_1K_2[S_3]]$$

where S_3 = 5,11-methenyl-H_4homofolate and $K_3 = K_i$. For the monoglutamate the V_{max} for the cyclohydrolase activity under these conditions is 2.1 μM/min.[6] At 4 min the value of [E·5,10-methenyl-H_4folate] = 0.04[E_T] so that the maximal value of 10-formyl-H_4folate expected is 0.3 μM contrasted to the observed value of 6.4 μM (Figure 3). Consequently we conclude that this inhibitor no longer blocks the cyclohydrolase activity in the coupled assay. Moreover, the actual substrate, 5,10-methenyl-H_4folate, is not hydrolyzed under coupled assay conditions since there is minimal dilution of [^{3}H]FGAR in the pulse–chase experiment. One cannot view this simply as a decreased binding of substrates by increasing their off rate at the cyclohydrolase site since this would be accompanied by an increased amount of the 5,10-methenyl species in solution, contrary to observation (Figures 1 and 2). It appears that operation of the dehydrogenase/cyclohydrolase couple reduces both the on/off rates for the 5,10-methenyl cofactor–enzyme combination, but the synthetase/cyclohydrolase couple does not.

Schirch (1978) made the interesting observation that $NADP^+$ activates the cyclohydrolase above pH 7 by lowering the K_m of 5,10-methenyl-H_4folate and inhibits the rabbit enzyme below pH 7, although here it is not competitive with the 5,10-methenyl species. $NADP^+$ is a competitive inhibitor against 5,10-methenyl-H_4folate for the porcine protein, with K_i = 500 μM (Tan et al., 1977), and is a competitive inhibitor (K_i = 171 μM) for the chicken liver cyclohydrolase. However, $NADP^+$ alone cannot be responsible for blocking hydrolysis of 5,10-methenyl-H_4folate in the chase experiment given the large value for K_i and the levels of $NADP^+$ employed in the assay.

The restricted formation of the 5,10-methenyl derivative by the trifunctional protein is physiologically very significant in view of our recent finding that 5,10-methenyl-H_4folate is not the cofactor for GAR TFase from chicken liver (Smith et al., 1981). Because there is now no known reaction in chicken liver where 5,10-methenyl-H_4folate functions as cofactor, it would be inefficient for the cell to tie up the limited supply of reduced folate cofactors as a nonreactive species. The cyclohydrolase activity is necessary to bridge the flow of 1-carbon units from serine to the de novo purine biosynthetic pathway. Therefore, by minimization of loss of the 5,10-methenyl intermediate on the trifunctional protein to solution the accumulation of this cofactor would be kept to a minimum.

Acknowledgments

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Registry No. 5,10-Methylene-H_4folate, 3432-99-3; L-5,10-methylene-H_4pteroyl(Glu)$_3$, 36034-70-5; L(+)-5,10-methenyl-H_4folate, 73611-11-7; L(+)-5,10-methenyl-H_4pteroyl(Glu)$_3$, 84254-12-6; H_4-folate, 71963-69-4; H_4pteroyl(Glu)$_3$, 4227-85-4; 5,11-methenyl-H_4-homofolate, 84254-13-7; formyl–methenyl–methylene-H_4folate synthetase, 73699-18-0; 10-formyl-H_4folate synthetase, 9023-66-9; 5,10-methenyl-H_4folate cyclohydrolase, 9027-97-8; 5,10-methylene-H_4folate dehydrogenase, 9029-14-5.

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[5] This K_i is based on data for the monoglutamate derivative. Since on average the K_m values for the triglutamate species are at least a factor of 5 smaller than the monoglutamate values (Table I), the concentration of E·S_2 is probably reduced by 3-fold.

[6] P. Benkovic, unpublished results.

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Reaction of Arsenite Ions with the Molybdenum Center of Milk Xanthine Oxidase†

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ABSTRACT: A study has been made of the reaction of arsenite ions with milk xanthine oxidase. In agreement with earlier work, the reaction was found to take place exclusively at the molybdenum site of the functional enzyme and is accompanied by loss of enzymic activity. The product of the reaction may be reduced and reoxidized without change of properties and can exist in Mo(VI), Mo(V), and Mo(IV) states. The Mo(VI) state of the arsenite complex is confirmed to have a characteristic absorption band at 380 nm, relative to the untreated enzyme, but this band is not present in the Mo(IV) state. Kinetic studies of the reaction of arsenite with the oxidized molybdenum center indicate a complex process in which initial reversible binding is followed by a slower irreversible process with $k = 0.020\ s^{-1}$ at pH 8.2 and 25 °C. The dithionite-reduced enzyme reacts much faster with arsenite than does the oxidized enzyme. The presence of competitive inhibitors or substrates slows the reaction with arsenite, both for the reduced and for the oxidized enzyme. The Mo(V) state of the arsenite complex gives a very complicated electron paramagnetic resonance (EPR) spectrum that has been successfully interpreted with the aid of computer simulations for spectra at 9 and 35 GHz. There is not only strong anisotropic hyperfine coupling but also strong quadrupole coupling of a single arsenic nucleus to molybdenum. Relevant parameters, including angular relationships, have been evaluated. Studies involving ^{2}H, ^{17}O, and ^{33}S substitution show no coupling to ^{1}H but weak coupling to ^{33}S and to ^{17}O. The nature of the reaction with arsenite and the structure of the arsenite complex are discussed. A structure of the form >Mo(=O)S—As= is proposed, in which the sulfur atom is the cyanide-labile sulfur originally present as Mo=S in the oxidized enzyme. In the arsenite complex, substrates and small inhibitor molecules have partial access to the active site, since they modify the EPR spectrum somewhat.

Studies of the reactions of various inhibitors with molybdenum centers in enzymes have been important in helping to elucidate their structures and mechanisms of action (Bray, 1980a). Arsenite ions have long been known to be inhibitory to xanthine oxidase [e.g., Barrey et al. (1928) and Peters & Sanadi (1961)] and to the other molybdenum-containing hydroxylases, xanthine dehydrogenase, and aldehyde oxidase (Coughlan et al., 1969). This inhibition is accompanied by changes in the absorption spectra of the enzymes with a substantial increase in absorption at 380 nm (Coughlan et al., 1969; Cleere & Coughlan, 1974). Johnson & Rajagopalan (1978) found that the molybdenum(V) EPR[1] spectrum of

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[1] Abbreviations: EPR, electron paramagnetic resonance; EXAFS, X-ray absorption edge extended fine structure; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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