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Serine Transhydroxymethylase: Evidence for a Sequential Random Mechanism†

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ABSTRACT: Initial velocity patterns in the presence of product and dead-end inhibitors suggest that in reaction 1 the addition of substrates and release of products occur by a sequential random mechanism: L-serine + tetrahydrofolate ⇌ glycine + 5,10-methylenetetrahydrofolate. This interpretation is supported by equilibrium isotope-exchange studies. The relative maximum rates of exchange of L-serine ⇌ glycine and L-serine ⇌ 5,10-methylenetetrahydrofolate in reaction 1 were not inhibited by high levels of substrates. The relative rates of these two exchange reactions were similar but were not identical. These results suggest that the catalytic interconversion and dissociation of substrates are of the same order of magnitude. Reaction 1 represents the transfer of a one-carbon group from the third carbon of L-serine to tetrahydrofolate. Inhibition studies showed that abortive enzyme ternary complexes are formed with L-serine and tetrahydrofolate compounds, which also contain a one-carbon group, e.g., 5-methyltetrahydrofolate and 5,10-methylenetetrahydrofolate. This suggests that the one-carbon binding site can accommodate two one-carbon groups simultaneously without serious steric hindrance.

Serine transhydroxymethylase (EC 2.1.2.1) catalyzes the following reactions at rates which are of physiological importance (Wilson and Snell, 1962; Schirch and Mason, 1963; Schirch and Gross, 1968; Palekar, et al., 1973; Ulevitch and Kallen, 1973). H_4-folate[1] is required only for reactions 1 and 2.

L-serine + H_4-folate ⇌ glycine + 5,10-CH_2-H_4-folate (1)

α-methyl-L-serine + H_4-folate ⇌ D-alanine + 5,10-CH_2-H_4-folate (2)

allothreonine ⇌ glycine + acetaldehyde (3)

L-threonine ⇌ glycine + acetaldehyde (4)

β-phenylserine ⇌ glycine + benzaldehyde (5)

aminomalonate ⇌ glycine + CO_2 (6)

In our previous work, we have been primarily concerned with substrate and reaction specificity and with identifying enzyme–substrate complexes (Schirch and Gross, 1968; Schirch and Jenkins, 1964a,b; Schirch and Diller, 1971; Schirch, 1975; Chen and Schirch, 1973b). In this paper, we explore further the properties of reaction 1 with emphasis on the role of H_4-folate.

Enzyme-bound pyridoxal phosphate is a required cofactor for all of the reactions listed. Considerable evidence has been accumulated to show that pyridoxal phosphate in these reac-

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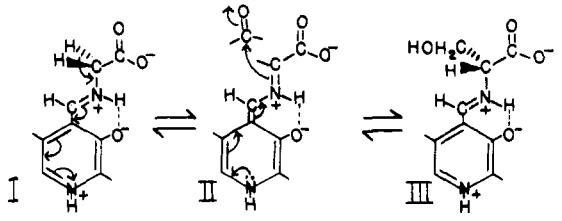
[1] Abbreviations used are: H_4-folate, *dl*-tetrahydrofolate; 5,10-CH_2-H_4-folate, 5,10-methylenetetrahydrofolate; 5-CH_3-H_4-folate, 5-methyltetrahydrofolate; 5-CHO-H_4-folate, 5-formyltetrahydrofolate; 10-CHO-H_4-folate, 10-formyltetrahydrofolate; 5,10-CH^+-H_4-folate, 5,10-methenyltetrahydrofolate; H_2-folate, dihydrofolate; E, serine transhydroxymethylase; NADH, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

tions forms a Schiff base with the amino acid substrates (structure I of Scheme I). Starting with glycine, the mecha-

Scheme I



nism involves the loss of the pro-2-*S* proton from the α carbon of glycine to form a glycine anion which exhibits an intense absorption band near 500 nm (structure II of Scheme I) (Schirch and Jenkins, 1964a,b; Schirch and Slotter, 1966; Besmer and Arigone, 1968; Jordan and Akhtar, 1970). Structure II then makes a nucleophilic attack on an aldehyde to form the enzyme-bound Schiff base of the β-hydroxyamino acid (structure III of Scheme I). When the aldehyde is formaldehyde the enzyme is a transferase as it accepts the one-carbon aldehyde from 5,10-CH_2-H_4-folate and transfers it to glycine to form serine. When the aldehyde is acetaldehyde or benzaldehyde, H_4-folate is not required and the aldehyde apparently reacts directly with the glycine anion (structure II). However, no information is presently available which helps establish the nature of the structure of the enzyme-bound aldehyde.

In the absence of an aldehyde, the absorbance at 500 nm by structure II can be used as a sensitive probe of the formation of the enzyme–glycine complex. The addition of either 5-CH_3-H_4-folate, 5-CHO-H_4-folate, or H_4-folate to a solution of enzyme and glycine results in about a 100-fold increase in the absorbance at 500 nm. This change in absorbance at 500 nm as a function of glycine and H_4-folate compounds has been used to determine dissociation constants for these compounds (Schirch and Ropp, 1967). These experiments revealed that there is cooperative binding to the enzyme by glycine and the three H_4-folate compounds studied.

H_4-folate is not an absolute requirement for reaction 1 (Chen and Schirch, 1973a). In the absence of H_4-folate there is a very slow reversible interconversion of glycine and serine. The addition and release of glycine and formaldehyde is ordered with glycine adding first and being released last. From these experiments, it was proposed that one function of H_4-folate was to catalyze the addition and release of formaldehyde at the active site. The substrate for the reverse of reaction 1 has been shown to be 5,10-CH_2-H_4-folate. 5-Hydroxymethyl-H_4-folate and H_4-folate plus formaldehyde do not serve as effective substrates for the conversion of glycine to serine (Chen and Schirch, 1973b).

In the present study, we have tried to establish whether reaction 1 goes by a ping-pong or sequential mechanism and whether substrates and products add in a random or ordered sequence. The primary tools for these studies have been steady-state kinetic experiments for the forward and reverse directions of reaction 1 in the presence of product and dead-end inhibitors and equilibrium isotope-exchange studies. The investigations also reveal some properties of the one-carbon binding site.

Experimental Procedure

Materials and Methods. Serine transhydroxymethylase was purified from rabbit liver and crystallized as previously described (Schirch and Diller, 1971). 5,10-CH_2-H_4-folate dehydrogenase was purified 1000-fold from rabbit liver.[2] The enzyme is free of serine transhydroxymethylase activity but does contain a highly active 5,10-methenyltetrahydrofolate cyclohydrolase activity (EC 3.5.4.9).

L-Threonine, L-serine, glycine, folic acid, 5-CH_3-H_4-folate, H_2-folate, NADH, NADP, and yeast alcohol dehydrogenase were purchased from Sigma. DL-Allothreonine was purchased from ICN Pharmaceuticals. α-Aminomethylphosphonic acid was obtained from Calbiochem. 5-CHO-H_4-folate was a gift from Lederle Laboratories. [^{14}C]Serine (10 mCi/mmol, Amersham Searle), [^{14}C]glycine (106 mCi/mol, Schwarz/Mann), and [^{3}H]$NaBH_4$ (50 mCi/mmol, New England Nuclear) were used without further purification.

For the initial velocity studies, stock solutions of H_4-folate were prepared as previously described (Chen and Schirch, 1973a). For these studies, 5,10-CH_2-H_4-folate was prepared by the addition of 15% excess of formaldehyde to a stock solution of H_4-folate. The concentration of 5,10-CH_2-H_4-folate was determined by adding an aliquot of the solution to a reaction mixture containing NADP and 5,10-CH_2-H_4-folate dehydrogenase in 0.02 M potassium phosphate–0.02 M 2-mercaptoethanol (Blakley, 1969)

$$5,10\text{-}CH_2\text{-}H_4\text{-folate} + NADP \rightleftharpoons 10\text{-}CHO\text{-}H_4\text{-folate} + NADPH \quad (7)$$

The 5,10-CH^+-H_4-folate is rapidly converted to 10-CHO-H_4-folate by the cyclohydrolase activity in the 5,10-CH_2-H_4-folate dehydrogenase preparation. The concentration of NADPH, which is equal to the original concentration of 5,10-CH_2-H_4-folate, was determined from the absorbance at 340 nm. This method gives the concentration for the L-(+)-diastereomer of 5,10-CH_2-H_4-folate.

For the initial velocity studies, the concentrations of stock solutions of H_4-folate were determined by coupling reactions 1 and 7. The H_4-folate was converted to 5,10-CH_2-H_4-folate by adding to the reaction mixture, described in the previous paragraph, 10 mM L-serine and 10 μg of serine transhydroxymethylase. This method gives the concentration of L-(−)-H_4-folate. The enzymatic assay showed that the addition of 15% excess formaldehyde to the stock solution of H_4-folate gave a 100% conversion to 5,10-CH_2-H_4-folate. Stock solutions of these two H_4-folate compounds were 6–8 mM and were found to be stable at −15 °C for several days.

For the isotope exchange studies, H_4-folate was first purified as follows. In a dry box under N_2, 250 mg of the cofactor was washed two times with 50 ml of methanol and two times with 50 ml of ether, and the resulting material was stored at −4 °C under vacuum. Standard solutions of 5–10 mM were made up just before use in buffer in the dry box under N_2, and assayed spectrophotometrically using $\epsilon_{298} = 28.4 \times 10^3$ (Blakley, 1960).

For the isotope exchange studies, 5,10-CH_2-H_4-folate was prepared by reduction of 5,10-CH^+-H_4-folate with $NaBH_4$ in Me_2SO/pyridine and purified via reprecipitation according to Tatum et al. (1977). The material was stored under vacuum at 4 °C. Standard solutions (8–10 mM) were made up just before use and assayed spectrophotometrically using $\epsilon_{294} = 32 \times 10^3$ (Blakley, 1960). The synthesis and purification of 5,10-CH_2-H_4-[^{3}H]folate were identical to the above, except that [^{3}H]$NaBH_4$ was used in place of $NaBH_4$. The specific activity of the product was approximately 8 mCi/mmol. It was stored and used the same as the unlabeled material.

Stock solutions of 5-CH_3-H_4-folate, H_2-folate, and folate

[2] Unpublished procedure by Schirch, Miller, and Donely.

were prepared by dissolving the compounds in 20 mM potassium phosphate–30 mM 2-mercaptoethanol solutions adjusting the pH to 7.3 with 1 M NaOH. The concentrations of the solutions were determined from their respective absorbance peaks (Blakley, 1969). For each of the folate compounds, it was assumed that only one of the diastereomers effectively binds to the enzyme and all concentrations reported in this study, which were determined from absorbance measurements, have been divided by 2. All stock solutions of these folate compounds were 6–8 mM and were prepared fresh immediately before use.

For the initial velocity studies, absorbance measurements were followed on a Cary, Model 15, recording spectrophotometer fitted with an expanded scale and thermostated at 30 °C. Radioactive counting for the isotope-exchange studies was carried out on a Packard TriCarb, Model 3320, and a Packard Radiochromatogram Scanner, Model 7201.

Assays. Three different assays were used to measure the steady-state velocities for reactions catalyzed by serine transhydroxymethylase. Assay 1 was used to determine the rate of reaction 1 as written. The reaction was coupled to reaction 7 by adding an excess of NADP and 5,10-CH_2-H_4-folate dehydrogenase. The initial rate of reaction 1 was determined from the formation of NADPH, as measured from the increase in absorbance at 340 nm. A typical 1-ml reaction mixture contained: potassium phosphate, 50 mM, pH 7.3; NADP, 0.24 mM; 2-mercaptoethanol, 30 mM; 5,10-CH_2-H_4-folate dehydrogenase, 8 μg; serine transhydroxymethylase, 10 μg; H_4-folate, 17–150 μM; and L-serine, 0.5–5 mM. The reaction was started by the addition of L-serine. At low concentrations of H_4-folate the initial velocity was often difficult to determine because the approach of the system to equilibrium gave small absorbance changes. For these reactions, the rate was followed on a tenfold expanded absorbance scale.

Assay 2 was used to follow reaction 1 in the reverse direction. The decrease in absorbance at 290 nm upon the conversion of 5,10-CH_2-H_4-folate to H_4-folate was used to follow the progress of this reaction (Kallen and Jencks, 1966; Chen and Schirch, 1973b). This assay gives initial velocities which are linear with enzyme concentration only at pH values above pH 8.1. For this reason, this assay was performed at pH 8.3. The intense absorption of 5,10-CH_2-H_4-folate at 290 nm limited the concentration range over which this substrate could be varied. To partially correct for this absorbance, reactions at the higher concentrations of 5,10-CH_2-H_4-folate were performed in 1-ml cuvettes with a 5-mm path length. Since the absorbance changes are small, all reactions were performed on a tenfold expanded absorbance scale. A typical 1-ml reaction mixture contained: sodium borate, 50 mM, pH 8.3; 2-mercaptoethanol, 30 mM; serine transhydroxymethylase, 20 μg; 5,10-CH_2-H_4-folate, 6–36 mM; and glycine, 1–10 mM. The reaction was started by the addition of glycine. The reference cell contained all of the reactants, except glycine. The small amount of HCHO in the 5,10-CH_2-H_4-folate solutions did not interfere in these assays.

Assay 3 was used to determine the rates for reactions 3 and 4. These reactions are coupled to the reduction of acetaldehyde by alcohol dehydrogenase and NADH (Schirch and Gross, 1968). The velocity of reactions 3 and 4 were followed by the rate of decrease in absorbance at 340 nm.

Dissociation constants for substrates and some inhibitors can also be determined by a nonkinetic method based upon the absorbance at 500 nm of structure II (Schirch and Ropp, 1967). As long as no aldehyde or aldehyde donor is added to the system, the absorbance at 500 nm is proportional to the equilibrium concentration of the enzyme–glycine complex. Since several folate compounds caused a large enhancement of the concentration of structure II, the absorbance at 500 nm can also be used to measure the concentration of the enzyme–glycine–folate complexes as a function of glycine and folate concentrations. This method has been used in this study to determine the K_d for 5-CH_3-H_4-folate in the presence of varying concentrations of L-serine, which behaves as an inhibitor since enzyme–serine complexes do not absorb at 500 nm. A typical composition in 1 ml is as follows: potassium phosphate, 50 mM, pH 7.3; 2-mercaptoethanol, 10 mM; glycine, 5 mM; serine transhydroxymethylase, 2 mg; 5-CH_3-H_4-folate, 0.036–0.66 μM; and L-serine, 2.5–20 mM.

The rate of dissociation of glycine from the enzyme–glycine–H_4-folate complex was determined by observing the rate of disappearance of absorbance at 500 nm when an excess of the glycine analogue α-aminomethylphosphonate was rapidly added to the solution. The compound α-aminomethylphosphonate is a competitive inhibitor of glycine which does not form an intermediate like structure II (Schirch and Diller, 1971). These reactions were performed in a Durrum, Model D-100, stopped-flow spectrophotometer thermostated at 14 °C. The signal was stored in a Physical Data, Model 514, transient recorder which was played back on an x–y recorder. The observed rate constants were determined by first-order plots of the decay of absorbance at 500 nm. In a typical experiment, one syringe contained: potassium phosphate, 50 mM, pH 7.3; 2-mercaptoethanol, 30 mM; serine transhydroxymethylase, 0.3 mg/ml; glycine, 2 mM; and either H_4-folate, 0.6 mM; or 5-CH_3-H_4-folate, 0.7 mM. The second syringe contained in the same buffer: α-aminomethylphosphonate, 200 mM; and either H_4-folate, 0.6 mM; or 5-CH_3-H_4-folate, 0.7 mM.

Determination of Equilibrium Constant. Two methods were employed. The first method determined the relative concentrations of H_4-folate and 5,10-CH_2-H_4-folate at equilibrium in the presence of an excess and fixed ratio of serine and glycine. The methods of determining the concentration of 5,10-CH_2-H_4-folate and H_4-folate employed NADP and 5,10-CH_2-H_4-folate dehydrogenase as discussed above. The combined concentrations of H_4-folate and 5,10-CH_2-H_4-folate are referred to as $[H_4\text{-folate}]_{tot}$. The experiment was performed as follows. Reaction 1 was allowed to come to equilibrium at 30 °C in a 0.1 ml volume containing: potassium phosphate, 20 mM, pH 7.3; 2-mercaptoethanol, 10 mM; serine transhydroxymethylase, 8 μg; H_4-folate, 64 mM; L-serine; and glycine. For determinations of $[H_4\text{-folate}]_{tot}$, the reaction was diluted to 1 ml with a solution containing: sodium borate, 50 mM, pH 8.3; 2-mercaptoethanol, 30 mM; and 5,10-CH_2-H_4-folate dehydrogenase, 20 μg. After adjusting to zero absorbance, NADP was added and the total increase in absorbance at 340 nm was determined (reaction 7). Under these conditions, both H_4-folate and 5,10-CH_2-H_4-folate will be converted to 10-CHO-H_4-folate. To determine only the concentration of 5,10-CH_2-H_4-folate in the equilibrium reaction mixture, the exact procedure was repeated, except the diluting buffer contained 10 mM L-cysteine which rapidly inactivates serine transhydroxymethylase (Schirch and Mason, 1962). Since reaction 1 is now blocked, the diluting buffer determines only the concentration of 5,10-CH_2-H_4-folate. The concentrations of H_4-folate in the equilibrium experiments were determined as the difference in concentration between $[H_4\text{-folate}]_{tot}$ and 5,10-CH_2-H_4-folate.

The second method is based on determining the distribution of [^{14}C]serine and [^{14}C]glycine in a reaction mixture at equilibrium. These reactions were carried out in 0.5-ml vol-

umes under N_2 at 35 °C in the following solution: L-serine, 2.2 mM; H_4-folate, 1.4 mM; potassium phosphate, 50 mM, pH 7.5; EDTA, 1 mM; 2-mercaptoethanol, 20 mM. After all the reagents had been combined, 0.22 unit of serine transhydroxymethylase, specific activity 9.6 (Schirch and Gross, 1968), was added to initiate the reaction. Reactions were allowed to incubate for 40–60 min to attain equilibrium before the addition of labeled substrates, as noted under exchange reactions. At various time intervals, 50-μl aliquots were removed and quenched with 5 μl of 12% Cl_3CCOOH until isotopic equilibrium was attained. After centrifugation, 15-μl samples were spotted on Whatman no. 1 paper, and serine and glycine were separated by ascending paper chromatography (1-butanol–acetone–diethylamine–water, 10:10:2:5, glycine R_f 0.22, serine R_f 0.35). The ratio of [^{14}C]serine/[^{14}C]glycine at each time point was determined directly employing the Radiochromatogram Scanner. Equilibrium concentrations of serine and glycine were calculated from the relative ratio of [^{14}C]serine/[^{14}C]glycine based on the initial concentration of serine. In all cases, equilibrium concentrations of H_4-folate and 5,10-CH_2-H_4-folate were calculated from the serine and glycine concentrations based on the initial concentration of H_4-folate. For all reactions employing [^{14}C]amino acids, the concentration of H_4-folate was taken as one half of the spectrophotometrically determined concentration of L-(±)-H_4-folate (Matthews and Huennekens, 1960).

Exchange Reactions. Reaction conditions and the determination of [^{14}C]serine and [^{14}C]glycine were identical to that described above. In those reactions, which were pulsed with 5,10-CH_2-H_4-[^{3}H]folate, the serine spot from the chromatograph was excised and counted in a liquid scintillation cocktail containing 2 ml of Soluene (Packard) and 10 ml of toluene scintillator solution (7 g, 2,5-diphenyloxazole, 0.6 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene to 1 l. with toluene). In all cases, equilibrium concentrations of the folates were calculated from the serine and glycine concentrations based on the initial concentration of L-(−)-H_4-folate.

The equilibrium exchange rates, R, were calculated according to eq 8 (Boyer, 1959).

$$R = \frac{-2.3\,[A][C]}{t([A] + [C])} \log (1 - F) \qquad (8)$$

where [A] and [C] are the molarities of the reactants involved in the exchange reaction, t is the time in minutes, and F is the fraction of isotopic equilibrium which has been attained in time t. F was calculated based on the observed counts per minute in the pertinent reactant after isotopic equilibrium had been attained.

The conditions for measuring serine ⇌ glycine exchange at variable glycine concentrations were as follows: each reaction initially contained L-serine, 1.1 mM; H_4-folate, 2.18 mM; and 0.22 unit of serine transhydroxymethylase. Varying amounts of a stock solution, 12.0 mM in serine and 42.0 in glycine, were added along with buffer to give a total volume of 0.500 ml and total final concentrations of substrates as noted under Results. The reaction mixture was incubated for 60 min at 35 °C to allow the attainment of equilibrium, and was then pulsed with 10 μl of [^{14}C]glycine (<0.5% of the equilibrium glycine concentration), containing 5.5×10^5 cpm. Aliquots (50 μl) were withdrawn at appropriate time intervals from the reaction mixture and the separation of isotope pools accomplished as described above.

The conditions and methods for monitoring serine ⇌ 5,10-CH_2-H_4-folate exchange were identical to the above, except that the pulse solution consisted of 10 μl of 5,10-CH_2-H_4-[^{3}H]folate containing 7.5×10^4 cpm (<1.5% of the equilibrium concentration of 5,10-CH_2-H_4-folate).

TABLE I: Determination of K_{eq} for Reaction 1.

[Gly] (mM)	[Ser] (mM)	[5,10-CH_2-H_4-folate] (mM)	[H_4-folate] (mM)	k_{eq}[a]
40	20	0.51	0.13	7.8
50	10	0.39	0.25	7.8
75	5	0.23	0.42	8.2
75	2	0.115	0.52	8.3

[a] K_{eq} = ([Gly][CH_2-H_4-folate]/[Ser][H_4-folate]).

The conditions for determining serine ⇌ glycine exchange at variable 5,10-CH_2-H_4-folate were as follows: each reaction initially contained L-serine, 2.07 mM; glycine, 10.23 mM; H_4-folate, 0.46 mM; 5,10-CH_2-H_4-folate, 0.63 mM; and 0.22 unit of serine transhydroxymethylase. Varying amounts of H_4-folate from a stock solution 10.6 mM in H_4-folate and varying amounts of 5,10-CH_2-H_4-folate from a stock solution 8.86 mM in 5,10-CH_2-H_4-folate were added in constant ratio along with buffer to give a final volume of 0.500 ml. All reactions were incubated for 40 min at 35 °C to allow them to come to equilibrium. Each one was then pulsed with 10 μl of [^{14}C]serine containing 5.5×10^5 cpm (<5% of equilibrium concentration of serine), aliquots of the reaction mixtures were withdrawn, and substrate pools were separated as described above.

The kinetic nomenclature and the interpretation of the experimental results are taken from the published methods of Cleland (1970) and Segel (1975).

Results

Equilibrium Constant. The equilibrium constant for reaction 1 had not previously been determined with a homogeneous enzyme preparation. Since many of the experiments described in this paper require a knowledge of the equilibrium constant, we decided to redetermine the previously reported value of 10 obtained by Blakley (1960).

The first method used to determine K_{eq} measured the concentrations of H_4-folate and 5,10-CH_2-H_4-folate in solutions which had a known ratio of serine and glycine. Table I records the results of this study. A value of 8 was found for K_{eq}.

The second method involved adding [^{14}C]serine or [^{14}C]glycine to a reaction mixture at equilibrium. The radioactivity was then allowed to reach an equilibrium distribution between glycine and serine. The ratios of concentrations of H_4-folate and 5,10-CH_2-H_4-folate were calculated from the known initial concentrations of L-serine, H_4-folate, and the final [^{14}C]serine/[^{14}C]glycine ratio. An average of seven determinations gave a value of 12 for K_{eq}.

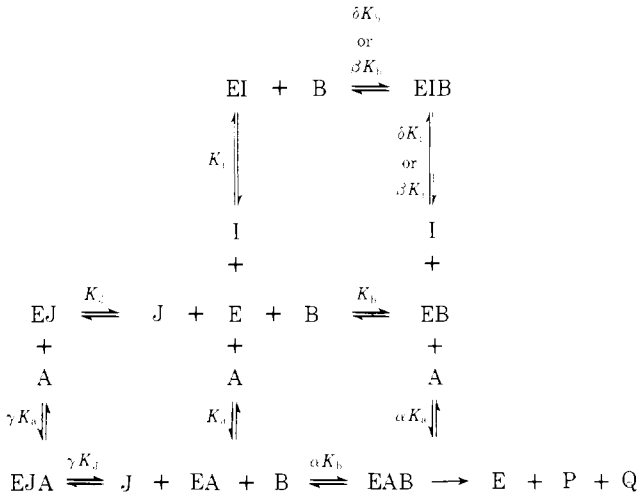
Initial Velocity Studies for Serine → Glycine. Initial velocity studies were performed using "assay 1" with serine and H_4-folate as substrates over the range of concentrations, as described under Methods. Double-reciprocal plots of the initial rates gave a series of straight lines which crossed above the horizontal axis when either serine or H_4-folate was the variable substrate. Secondary plots of slopes and intercepts were linear. No evidence for substrate inhibition or activation was observed. The values for kinetic constants obtained from these studies are recorded in Table II. The symbols for the constants are explained in Scheme II. The Greek letter prefixes represent the factors by which the dissociation constant of one substrate

TABLE II: Initial Velocity Data for Reaction 1.

Variable Substrate	Fixed Substrate	Double-Reciprocal Plot Pattern	K (mM)
Serine	H_4-folate	Intersecting	$K_a = 1.3^a$ $\alpha K_a = 0.36$
H_4-folate	Serine	Intersecting	$K_b = 0.06$ $\alpha K_b = 0.02$
Glycine	5,10-CH_2-H_4-folate	Intersecting	$K_q = 7.7$ $\alpha K_q = 1.7$
5,10-CH_2-H_4-folate	Glycine	Intersecting	$K_p = 0.08$ $\alpha K_p = 0.017$

[a] The nomenclature is based on Scheme I. The Greek letter prefixes represent the factors by which the dissociation constant for one substrate is changed by the presence of the second substrate (Segel, 1975).

Scheme II[a]



[a] Serine is designated A and H_4-folate is designated B. For the reverse of reaction 1, glycine is designated Q and 5,10-CH_2-H_4-folate is designated P. Competitive inhibitors of amino acids are designated I and competitive inhibitors of H_4-folate substrates are designated J.

is changed by the binding of the second substrate or inhibitor (Segel, 1975).

Glycine in the concentration range of 4–37.5 mM was found to behave as a product inhibitor. A competitive inhibition pattern was obtained when serine was the variable substrate and a noncompetitive pattern was observed when H_4-folate was the variable substrate. Secondary slope and intercept plots were linear. The values for kinetic constants obtained from these studies are recorded in Table III.

5-CH_3-H_4-folate, 7–200 μM, behaves as a dead-end inhibitor showing competitive inhibition with H_4-folate as variable substrate and noncompetitive inhibition with serine as variable substrate (Table III). Secondary slope plots when H_4-folate was the variable substrate were linear. However, both slope and intercept replots with serine as the variable substrate deviated slightly from linearity. It is not clear whether the small deviation from linearity is due to experimental error or some mechanistic feature of the reaction. The kinetic constants obtained from these studies are recorded in Table III.

TABLE III: Inhibition Data for Product and Dead-End Inhibitors of Reaction 1.

Variable Substrate	Inhibitor	Inhibition Pattern	K (mM)
Serine	Glycine	Competitive	$K_i = 8.0^a$ $\beta K_i = 2.9$
H_4-folate	Glycine	Noncompetitive	$\beta K_b = 0.027$
Serine	5-CH_3-H_4-folate	Noncompetitive	$\gamma K_a = 1.7$
H_4-folate	5-CH_3-H_4-folate	Competitive	$\gamma K_j = 0.03$
Serine	α-Aminomethylphosphonate	Competitive	$\delta K_i = 0.8$ $\delta K_i = 0.4$
H_4-folate	α-Aminomethylphosphonate	Noncompetitive	$\delta K_b = 0.025$
Glycine	Serine	Competitive	$K_i = 0.5$ $\beta K_i = 0.36$
5,10-CH_2-H_4-folate	Serine	Noncompetitive	$\beta K_p = 0.013$
Glycine	5-CH_3-H_4-folate	Noncompetitive	$\gamma K_q = 5.3$
Glycine	α-Aminomethylphosphonate	Competitive	$K_i = 3.9$ $\delta K_i = 2.9$
5,10-CH_2-H_4-folate	α-Aminomethylphosphonate	Noncompetitive	$\delta K_p = 0.03$

[a] The prefixes represent the factor by which the dissociation constant of the substrate is changed by the presence of the inhibitor. It also represents the factor for the same change in the dissociation constant of the inhibitor in the presence of the substrate (Segel, 1975).

α-Aminomethylphosphonate, an analogue of glycine, in the concentration range of 0.5–2 mM, behaves as a dead-end inhibitor giving a competitive inhibition pattern when serine is the variable substrate and a noncompetitive inhibition pattern when H_4-folate is the variable substrate (Table III). All secondary plots of slopes and intercepts were linear. The kinetic constants derived from these primary and secondary plots are recorded in Table III.

Initial Velocity Studies for Glycine → Serine. These studies were performed using "assay 2". Initial velocity double-reciprocal plots with 5,10-CH_2-H_4-folate and glycine as substrates gave intersecting patterns. The slope and intercept replots for these studies were linear and kinetic constants obtained from these graphs are recorded in Table II.

Serine was found to be a product inhibitor at concentrations of 0.3–1.2 mM. The range of serine concentrations which could be used was limited by the unfavorable equilibrium of reaction 1. Serine was found to be a competitive inhibitor of glycine and a noncompetitive inhibitor of 5,10-CH_2-H_4-folate. Slope and intercept replots of the data were all linear. Kinetic constants obtained from these studies are recorded in Table III.

As previously stated, 5-CH_3-H_4-folate behaves as a dead-end inhibitor. At a concentration of 19 μM it gave a noncompetitive inhibition pattern with glycine as the variable substrate (Table III). Inhibition studies with different concentrations of 5-CH_3-H_4-folate could not be done because of the limitation of the concentration of folate compounds which can be used in assay 2. When the absorbance at 290 nm approaches 2.0, the drift in the blank severely limits the accuracy of the assay. This limitation in the assay also precluded inhibition studies

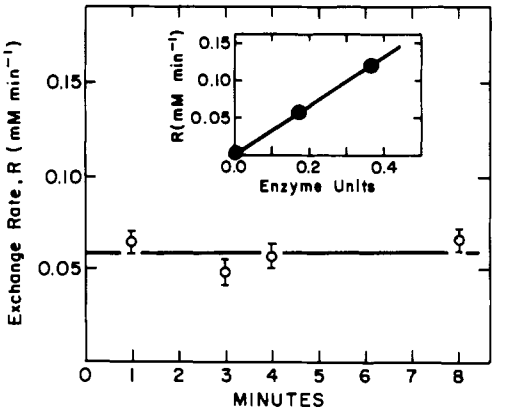


FIGURE 1: Equilibrium exchange rate of serine and glycine as a function of time. Reaction mixture initially contained 1.10 mM L-serine, 2.83 mM H_4-folate, 1 mM EDTA, 20 mM β-mercaptoethanol, and 0.17 unit of serine transhydroxymethylase in 0.05 M potassium phosphate at pH 7.50. After incubation for 60 min at 35 °C, the equilibrium concentrations were as follows: L-serine, 0.15 mM; glycine, 0.95 mM; L-(−)-H_4-folate, 0.46 mM; L-(+)-5,10-CH_2-H_4-folate, 0.95 mM. [^{14}C]glycine (4.7 nmol, 5.5 × 10^5 cpm) in 10 μl was then added. The appearance of [^{14}C]serine was followed and the exchange rate, R, was determined as described under Experimental Procedure. Insert shows the variation under the above conditions of the exchange rate with enzyme activity.

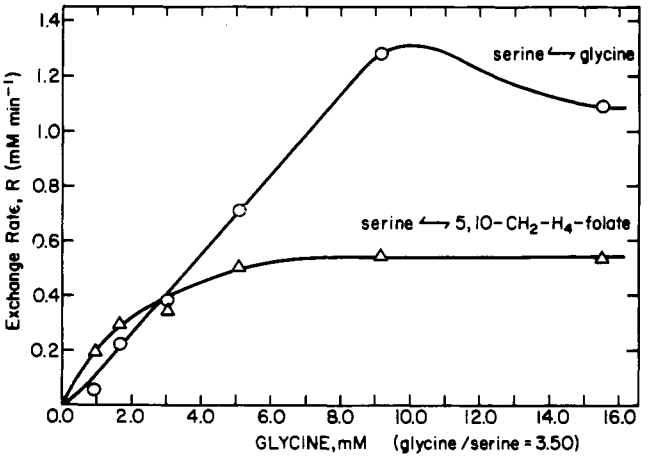


FIGURE 2: Effect of glycine and serine on equilibrium exchange rates. Initial concentrations of reaction components, excluding variable substrate pair, were the following: 0.22 unit of serine transhydroxymethylase; potassium phosphate, 0.05 M, pH 7.50; EDTA, 1 mM; β-mercaptoethanol, 20 mM; *dl*-H_4-folate, 2.2 mM. Aliquots of a concentrated solution 12 mM in L-serine and 42 mM in glycine were added in various amounts, and the reaction was allowed to incubate at 35 °C. When equilibrium was attained (after 60 min), the concentrations of substrates were the following: L-(−)H_4-folate, 0.24 mM, L-(+)-5,10-CH_2-H_4-folate, 0.86 mM; serine and glycine as shown in the figure. [^{14}C]glycine or [^{3}H]5,10-CH_2-H_4-folate in the following amounts was then added: [^{14}C]glycine (4.7 nmol, 5.5 × 10^5 cpm in 10 μl), 5,10-CH_2-H_4-[^{3}H]folate (14 nmol, 7.5 × 10^4 cpm in 10 μl). The appearance of labeled serine was followed and the equilibrium exchange rates determined as described under Experimental Procedure.

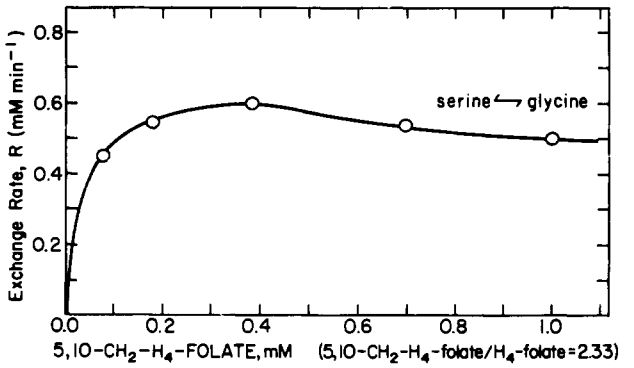


FIGURE 3: Effect of 5,10-CH_2-H_4-folate and H_4-folate on equilibrium exchange rates. Initial concentrations of reaction components, excluding variable substrate pair, were the following: 0.22 unit of serine transhydroxymethylase; potassium phosphate, 0.05 M, pH 7.50; EDTA, 1 mM; β-mercaptoethanol, 20 mM; L-serine, 2.07 mM; glycine 10.23 mM. Aliquots of concentrated solutions 10.6 mM in *dl*-H_4-folate and 8.86 mM in 5,10-CH_2-H_4-folate were added in various amounts and the reaction allowed to incubate at 35 °C. When equilibrium was attained (after 40 min), the concentrations of substrates were the following: L-serine, 2.0 mM; glycine, 10.3 mM; L-(+)-5,10-CH_2-H_4-folate and L-(−)-H_4-folate as shown in the figure. [^{14}C]glycine (4.7 nmol, 5.5 × 10^5 cpm) in 10 μl was then added. The appearance of [^{14}C]serine was followed and the exchange rates were determined as described under Experimental Procedure.

with 5-CH_3-H_4-folate as the dead-end inhibitor and 5,10-CH_2-H_4-folate as the variable substrate.

The glycine analogue α-aminomethylphosphonate, 1–4 mM, gave a competitive inhibition pattern with glycine as the variable substrate and noncompetitive inhibition pattern with 5,10-CH_2-H_4-folate as the variable substrate (Table III). Intercept and slope replots of these were linear. The kinetic constants associated with these experiments are recorded in Table III. It should be noted that the experiments using assay 2 were performed at pH 8.3, rather than 7.3.

Exchange Reactions. Under the equilibrium reaction conditions (0.05 M potassium phosphate, 1 mM EDTA, 20 mM β-mercaptoethanol, pH 7.5, 35 °C), all substrates appeared to be stable for at least 120 min, evidenced by the fact that the equilibrium concentrations of serine and glycine were invariant over that time period. Assay of the enzyme after incubation showed that it did not lose activity during that time.

As a test of the conditions that no net reaction occurs during the measurement of the isotopic-exchange rates and that the latter are time independent, three sets of exchange rates at each of the various concentrations of substrates were followed for periods up to 10 min, at 2- or 3-min intervals. The exchange rates at equilibrium were found to be constant for this time period, showing that no net reaction was occurring. Figure 1 shows typical rates of serine–glycine exchange for a reaction at saturating levels of 5,10-CH_2-H_4-folate and H_4-folate at chemical equilibrium measured at four time points. The insert to Figure 1, showing the variation in the same exchange rate as a function of the enzyme activity, illustrates that the rate increases linearly with enzyme concentration.

When glycine and serine were varied in constant ratio while holding the 5,10-CH_2-H_4-folate and H_4-folate concentrations constant, the exchange rates varied, as shown in Figure 2. As the glycine concentration was raised, the serine–glycine exchange rate increased to a maximum and then decreased slightly at high substrate levels. The serine and glycine concentrations were 12- and 9-fold above their respective αK_a and αK_q values (Schirch and Ropp, 1967). 5,10-CH_2-H_4-folate $\rightleftharpoons$ serine exchange also rose to a maximum as the glycine concentration was increased. However, the maximum exchange rate for this pair was reached at a lower glycine concentration than for the glycine–serine pair, and it remained constant at high glycine concentration. The maximal rate of glycine $\rightleftharpoons$ serine exchange is 2.4 times greater than 5,10-CH_2-H_4-folate $\rightleftharpoons$ serine exchange.

In Figure 3 is shown the variation in the rate of serine $\rightleftharpoons$ glycine exchange as the concentrations of 5,10-CH_2-H_4-folate and H_4-folate were varied in constant ratio. As can be seen, the rate reaches a maximum, and then shows a slight decrease at

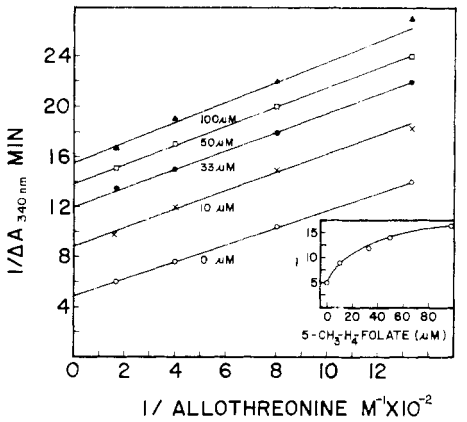


FIGURE 4: Double-reciprocal plot of the initial velocity of reaction 3 in the presence of 5-CH_3-H_4-folate. The concentrations of the inhibitor 5-CH_3-H_4-folate for each experiment are recorded on the data lines. Inset: intercept replot.

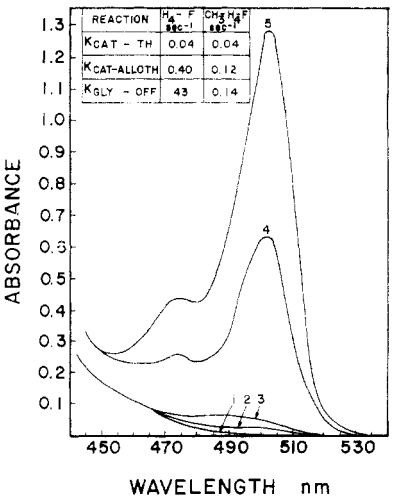


FIGURE 5: Spectra of reactions 3 and 4 under steady-state conditions in the absence and presence of threonine and allothreonine, H_4-folate, and 5-CH_3-H_4-folate. The reaction solution contained in 1 ml of 20 mM potassium phosphate: 2-mercaptoethanol, 20 mM; serine transhydroxymethylase, 3 mg; L-threonine, 50 mM; or DL-allothreonine, 50 mM; H_4-folate, 0.1 mM, or 5-CH_3-H_4-folate, 0.18 mM. Curve 1, E; curve 2, E + threonine; curve 3, E + threonine + H_4-folate; curve 4, E + threonine + 5-CH_3-H_4-folate; curve 5, E + allothreonine + 5-CH_3-H_4-folate. Inset: catalytic rate constants for reaction 4, $k_{cat\text{-}th}$, and reaction 3, $k_{cat\text{-}alloth}$, and the rate of dissociation of glycine from the enzyme, $k_{gly\text{-}off}$, in the presence of H_4-folate or 5-CH_3-H_4-folate.

high 5,10-CH_2-H_4-folate concentrations. The concentrations of 5,10-CH_2-H_4-folate and H_4-folate were 50- and 1-fold higher than their respective αK_b and αK_p values. The maximum exchange under these conditions was essentially identical to the serine $\rightleftharpoons$ 5,10-CH_2-H_4-folate exchange.

Nature of the One-Carbon Binding Site. The size of the one-carbon binding site was explored by observing the inhibition patterns of 5-CH_3-H_4-folate with various amino acids. We first used the previous observation that the E-Gly-5-CH_3-H_4-folate complex exhibits an intense absorption band at 502 nm, whereas the E-Ser complexes do not absorb at this wavelength (Schirch and Ropp, 1967). This permits us to determine from the absorbance at 502 nm the concentration of the E-Gly-5-CH_3-H_4-folate complex with serine acting as an inhibitor according to reaction 9.

$$\text{E + glycine + 5-CH}_3\text{-H}_4\text{-folate} \rightleftharpoons \text{E-Gly-5-CH}_3\text{-H}_4\text{-folate } (A_{max} = 502 \text{ nm}) \quad (9)$$

With 5-CH_3-H_4-folate as the variable substrate, 0.036–0.66 mM, serine behaved as a noncompetitive inhibitor over the concentration range of 2.5–20 mM. The intercept and slope secondary plots were linear. The kinetic constants derived from this data show that the dissociation constant for serine from the E-Ser complex, 3.6 mM, is only slightly smaller than the 4.6 mM value for the dissociation constant of serine from the E-Ser-5-CH_3-H_4-folate complex. This suggests that there is little hindrance in the binding of serine by the presence of a 5-methyl group. The data from this experiment also suggests that serine does not significantly alter the affinity of the enzyme for 5-CH_3-H_4-folate.The dissociation constant for 5-CH_3-H_4-folate from the E-Ser-5-CH_3-H_4-folate complex is 0.25 mM. This compares to a dissociation constant of 0.20 mM previously calculated for the E-5-CH_3-H_4-folate complex. This experiment was repeated with 5-CHO-H_4-folate in place of 5-CH_3-H_4-folate. Essentially identical results were obtained.

To further explore the amount of crowding at the one-carbon binding site, we tested the inhibition patterns obtained for 5-CH_3-H_4-folate with threonine and allothreonine as substrates (reactions 3 and 4). Although it is not clear how threonine and allothreonine are bound at the active site, we assume that the asymmetry at carbon 3 results either in a different orientation for the terminal methyl or hydroxyl group for the two substrates.

We found that at low threonine concentrations the addition of 5-CH_3-H_4-folate increased the rate of product formation by about 20%. The activation disappeared at higher concentrations of L-threonine.

With allothreonine as substrate, 5-CH_3-H_4-folate is a hyperbolic uncompetitive inhibitor, as shown in Figure 4. This suggests that 5-CH_3-H_4-folate interacts differently at the active site with threonine and allothreonine. However, further experiments did not support this interpretation. The effect of 5-CH_3-H_4-folate appears to be only on the rate of dissociation of the product glycine from the enzyme. This interpretation is supported by two experiments. First, 5-CH_3-H_4-folate greatly increases the steady-state concentration of the intermediate absorbing at 500 nm (structure II) in the degradation of both threonine and allothreonine (curves 4 and 5 in Figure 5). This in in contrast to the very low level of this intermediate in the presence or absence of H_4-folate (curves 2 and 3 in Figure 5). Second, the rate of dissociation of glycine from the E-Gly-5-CH_3-H_4-folate complex is 0.14 s^{-1}, which is 300 times slower than the rate of dissociation of glycine from the E-Gly-H_4-folate complex (data are recorded as $k_{gly\text{-}off}$ in the inset of Figure 5).

Also shown in the inset of Figure 5 are the catalytic rate constants for reactions 3 and 4 under the same conditions used to determine the rate of dissociation of glycine. (These are designated as $k_{cat\text{-}th}$ and $k_{cat\text{-}alloth}$ for threonine and allothreonine, respectively.) The data show that the reaction of allothreonine is slowed down in the presence of 5-CH_3-H_4-folate to a rate essentially equal to the rate of dissociation of glycine from the inhibited complex. The apparent effect of 5-CH_3-H_4-folate on reaction 3 then is to change the rate-determining step from one occurring before the formation of structure II to the dissociation of glycine.

Inhibition by Other Folate Compounds. We have tested several other folate compounds for their ability to bind at the active site of serine transhydroxymethylase. This was determined by either observing inhibition of reaction 1 or effects on the 500-nm absorbing E-Gly complexes.

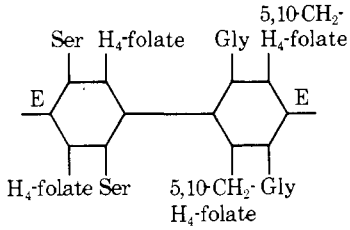
Folate was found to inhibit reaction 1 and slightly enhance the absorbance at 500 nm of the E–Gly complex. From these experiments, a dissociation constant of 0.2 mM was determined for folate for the enzyme saturated with glycine. Several folate compounds, that do not inhibit reaction 1 and have dissociation constants greater than at least 0.5 mM include H_2-folate, 5,10-CH^+-H_4-folate, and 10-CHO-H_4-folate.

Discussion

A variety of mechanisms for the addition of substrates and release of products are possible for transferases of which serine transhydroxymethylase is an example. These mechanisms can be subdivided into two groups. The first is based on whether all substrates must bind before release of any products (sequential mechanism) or whether some products are released prior to the addition of all substrates (ping-pong mechanism). The second subdivision of mechanisms relates to whether substrates (or products) can bind at the active site in any order (random mechanism) or whether they can add to the active site in only a certain sequence (ordered mechanism). Although the distinction between ordered and random addition has been criticized on kinetic grounds (Himoe, 1976), limiting cases can be recognized by inhibition and isotope-exchange studies. The rules for the interpretation of kinetic and exchange studies to differentiate among the various possible mechanisms have been discussed by Cleland (1970), Segel (1975), Boyer (1959), Silverstein and Boyer (1964), and Wedler (1974) and will not be repeated here.

The experimental results in this paper clearly indicate that serine transhydroxymethylase operates by a sequential random mechanism which can be summarized by the notation used by Cleland (1975), as shown in Scheme III.

Scheme III



The evidence that the mechanism is sequential rather than ping-pong is provided by the results of initial-velocity studies recorded in Table II. The intersecting patterns observed for the double-reciprocal plots are consistent with both substrates adding to the active site before release of any products. A ping-pong mechanism would have given a series of parallel lines in these studies.

The evidence that the mechanism is random rather than ordered comes mostly from the results of the equilibrium isotope-exchange studies recorded in Figures 2 and 3. The observation that the serine $\rightleftharpoons$ 5,10-CH_2-H_4-folate exchange is not inhibited at high glycine concentrations indicates that the reaction cannot involve an ordered sequence of dissociation of products with glycine dissociating before 5,10-CH_2-H_4-folate (Figure 2). In such a situation, a high glycine concentration would draw all of the enzyme into an E–Gly–5,10-CH_2-H_4-folate complex, thus, decreasing the level of binary complexes responsible for the exchange. Likewise, the fact that the serine $\rightleftharpoons$ glycine exchange is essentially constant at high levels of 5,10-CH_2-H_4-folate and H_4-folate shows that the reaction does not have a compulsory binding order with 5,10-CH_2-H_4-folate dissociating before glycine (Figure 3). These latter results also rule out a compulsory binding order with serine binding before H_4-folate on the reactant side. The high levels of H_4-folate would draw the enzyme into central complexes decreasing the concentration of free enzyme required for serine binding, resulting in inhibition of the exchange rate. The results in Figures 2 and 3 therefore rule out a compulsory release of the products glycine and 5,10-CH_2-H_4-folate and the compulsory addition of serine before H_4-folate on the reactant side. The data do not rule out the possible ordered sequence where H_4-folate binds before serine. The isotope-exchange experiments which would have eliminated this possibility are the exchange of H_4-folate and 5,10-CH_2-H_4-folate in the presence of increasing concentrations of glycine and serine. This exchange reaction was not attempted due to the difficulty of separating and assaying the two H_4-folate compounds. Studies of the exchange rates in the presence of increasing concentrations of glycine and H_4-folate also were not pursued owing to the probability of inhibition caused by the formation of the dead-end E–Gly–H_4-folate complex. The formation of this complex has previously been demonstrated (Schirch and Ropp, 1967).

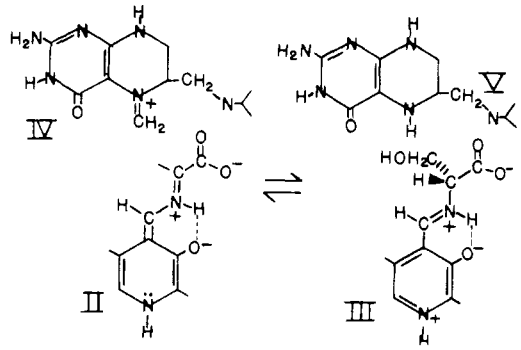
However, there are two experiments which show that serine can bind to the free enzyme, thus ruling out the one remaining possible ordered mechanism. The first of these is the observation that serine is a competitive inhibitor of allothreonine (reaction 3) which does not require H_4-folate. The second experiment is that reaction 1 does not show an absolute requirement for H_4-folate. Serine is converted slowly to glycine and formaldehyde in the absence of H_4-folate (Chen and Schirch, 1973a). The dissociation constants determined for serine in these experiments are essentially the same as the K_a values reported for serine in Table II.

Initial velocity patterns also give information concerning ordered vs. random mechanisms. The inhibition patterns in Table III are consistent with a random mechanism. Of particular importance are the inhibition patterns observed with the dead-end inhibitors α-aminomethylphosphonate and 5-CH_3-H_4-folate. The observation that α-aminomethylphosphonate is a noncompetitive inhibitor of H_4-folate and 5,10-CH_2-H_4-folate argues against an ordered Bi-Bi mechanism in which the H_4-folate compounds add first and are released last. Such a mechanism may have given a competitive inhibition pattern (Cleland, 1970; Segel, 1975). It can also be argued that the noncompetitive inhibition patterns observed for 5-CH_3-H_4-folate vs. serine and glycine (Table III) rule out an ordered Bi-Bi mechanism with the amino acids adding first and being released last. However, the rules for interpreting inhibition patterns are complicated when the dead-end inhibitors can bind to more than one enzyme form. Because of this, the data in Table III should be viewed as only being consistent with the proposed sequential random mechanism.

The initial-velocity studies show that the inhibitors 5-CH_3-H_4-folate and 5-CHO-H_4-folate can effectively bind to E, the E–Gly complex, and the E–Ser complex. These complexes would be the three forms of serine transhydroxymethylase in the cell to which folate compounds could bind. This study clearly establishes that these compounds can be important physiological inhibitors. However, the failure of 5,10-CH^+-H_4-folate and 10-CHO-H_4-folate to show any appreciable affinity for the active site suggests that these compounds may not play a role in the regulation of reaction 1. Also, the failure of these two compounds to show appreciable affinity for the active site suggests that substitution at the 10 position of H_4-folate may block the binding of these compounds. This is supported by our previous observation that 10-CH_3-H_4-folate is not an inhibitor of reaction 1 (Schirch and Ropp, 1967).

The immediate one-carbon donor in reaction 1 may be the 5-iminium cation (structure IV of Scheme IV) rather than

Scheme IV



5,10-CH_2-H_4-folate (Kallen and Jencks, 1966; Barrows et al., 1976). If this is true, then 5-CH_3-H_4-folate is an excellent substrate analogue of the one-carbon donor and, therefore, it is not surprising that it is a very effective inhibitor. What is not known is how the one-carbon group is transferred from structure IV to glycine to form serine (structure III). Several previous observations which have to be considered in this respect are: the lack of a H_4-folate requirement for reactions 2–6; the lack of stereospecificity at the β carbon for the cleavage of threonine and β-phenylserine; the nonstereospecific decarboxylation of aminomalonate (Palekar et al., 1973); the partial racemization of the C-3 hydrogens of serine on forming 5,10-CH_2-H_4-folate (Tatum et al., 1977); the conservation of oxygen in the cleavage of threonine to acetaldehyde (Jordan et al., 1976).

The data in this paper adds to this list of observations. We conclude that the one-carbon binding site on the enzyme is either very large or there are two one-carbon binding sites, i.e., one for C-3 of serine and one for 5,10-CH_2-H_4-folate. The first experiment which suggested that there is not a single locus for the one-carbon group was the observation that serine is a noncompetitive inhibitor of 5,10-CH_2-H_4-folate (Table III). If there is a single well defined site for the one-carbon group, high concentrations of 5,10-CH_2-H_4-folate would have totally excluded the inhibitor serine and a competitive inhibition pattern would have been observed. This observation is supported by the noncompetitive inhibition pattern observed for 5-CH_3-H_4-folate when serine is the variable substrate (Table III). This inhibition pattern shows that an E–Ser–5-CH_3-H_4-folate complex is formed. The formation of this ternary complex is also demonstrated in the experiment where serine behaves as a noncompetitive inhibitor of the formation of the 502 nm absorbing E–Gly–5-CH_3-H_4-folate complex. The dissociation constants calculated from this latter experiment show that there is little, if any, steric hindrance in the binding of the C-3 carbon of serine and the 5-methyl group of 5-CH_3-H_4-folate. This interpretation is further supported by the failure of 5-CH_3-H_4-folate and 5-CHO-H_4-folate to inhibit the binding and breakdown of threonine (reaction 4).

The inhibition of the breakdown of allothreonine by 5-CH_3-H_4-folate (Figure 4) does not appear to result from the 5-methyl group blocking the binding of allothreonine. The rate data in the inset of Figure 5 suggests that the effect of 5-CH_3-H_4-folate is to change the rate-determining step from one occurring before the formation of structure II to dissociation of glycine. The hyperbolic uncompetitive inhibition pattern in Figure 4 is explained then by 5-CH_3-H_4-folate exerting its effect at a step after the binding of allothreonine and changing the rate-limiting step[3] (Cleland, 1970). A similar situation has been described by Kallen and Blanck (1973) with the inhibition of reaction 5 by 5-CHO-H_4-folate. The small activation of reaction 4 by low levels of 5-CH_3-H_4-folate was not pursued further but may reflect the same type of mechanism observed by Jones and Priest (1976) for the folate activation of reaction 5.

In those cases where catalytic interconversion of the ternary complexes is the rate-limiting step—a truly rapid equilibrium mechanism—the maximum exchange rates for all substrate pairs are identical. If dissociation or binding of one of the substrates is completely or partially rate limiting, then the exchange rates are different. It can be seen from Figure 2 that the exchange rates of serine $\rightleftharpoons$ glycine and serine $\rightleftharpoons$ 5,10-CH_2-H_4-folate differ by a factor of about 2.4. This probably is a reflection of differences in the rates of dissociation of glycine vs. 5,10-CH_2-H_4-folate, suggesting that catalytic interconversion is not solely rate limiting. Figure 2 also shows the interesting feature that at high concentrations of serine and glycine, the serine $\rightleftharpoons$ glycine exchange rate is decreased slightly. Such a decrease may be due to nonproductive binding of substrate or cofactors (possibly L-(+)-H_4-folate) at or near the active site, thus decreasing the rate of exchange (Silverstein and Boyer, 1964). The present data are insufficient to determine the cause of this depression in rate. The slight depression seen in Figure 3 at high concentrations of cofactor may also be due to this phenomenon, or it may be a manifestation of the substrate synergism (Wedler, 1974) which decreases exchange rates at high substrate concentrations (Table II).

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[3] Uncompetitive inhibition patterns occur when the inhibitor can combine only with the E–substrate complex. However, in this case, the inhibitor, 5-CH_3-H_4-folate probably combines before the addition of the substrate but the point of inhibition occurs only after the formation of the *E-allothreonine* complex.

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Enzymatic Oxidation of Cobalt Protoporphyrin IX: Observations on the Mechanism of Heme Oxygenase Action†

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ABSTRACT: Studies on the enzymatic mechanism of microsomal heme oxygenase were made utilizing various porphyrins and metalloporphyrins of different ring substituents and central metal ions. Co-heme (cobalt protoporphyrin IX) was shown to be a substrate for the enzyme and the product of its oxidative metabolism was identified as the natural bile pigment, biliverdin IXα isomer. Metalloporphyrins, which do not bind molecular oxygen (Ni, Mn, and Sn protoporphyrin IX), were not substrates for heme oxygenase, although they could competitively inhibit oxidation of reactive substrates for the enzyme. The presence of lipophilic substituents on pyrrole rings I and II, as well as a central metal atom, were required for the heme oxidation reaction to occur. The oxidative cleavage of Co-heme displayed typical characteristics of an enzyme-mediated reaction, and the oxidation of this substrate, as well as that of Fe-heme (iron protoporphyrin IX), could be supported with either reduced nicotinamide adenine dinucleotide phosphate or reduced nicotinamide adenine dinucleotide. A hypothesis is proposed on the mode of action of heme oxygenase in which the enzyme and its substrate are considered to form a "transitory" hemoprotein which can activate molecular oxygen for cleavage of the heme tetrapyrrole ring. In this formulation, heme as substrate for heme oxygenase is synonymous with heme as prosthetic group for the enzyme.

Heme oxygenase is an enzyme which catalyzes the oxidative degradation of heme to biliverdin; the latter is subsequently reduced in the cytosol to bilirubin. The microsomal site of heme breakdown was established by Tenhunen et al. (1969) but the mechanism of heme oxidation to biliverdin has remained unclear. Certain analogies between heme degradation and the "mixed function" oxidation of drugs led to the belief that the heme oxygenase system contains cytochrome P 450 as the terminal oxidase (Schmid, 1972), that it has an absolute requirement for NADPH[1] (Tenhunen et al., 1969; Schmid, 1972), and that the central iron atom of heme is indispensable for heme oxidative activity (Tenhunen et al., 1969). Subsequently, studies from this laboratory conclusively dissociated cytochrome P 450 from heme oxidation in liver (Maines and Kappas, 1974; 1975a,b), an organ rich in P-450 content, and, in concurrent studies, the activity of heme oxygenase in spleen was also shown not to be cytochrome P 450 dependent (Yoshida et al., 1974).

In further studies on the mechanism of hepatic heme oxygenase, we have explored the substrate specificity and cofactor requirements of the enzyme. The results indicate that microsomal heme oxygenase can catalyze the oxidative cleavage of a non-iron heme substrate—specifically cobalt protoporphyrin IX (Co-heme), and that NADH, as well as NADPH, can serve as electron donor for the reaction. These and other findings reported here provide a basis for interpreting the role of chelated metal in the oxidative metabolism of heme and for defining the possible role of heme as substrate in relation to the catalytic activity of heme oxygenase.

Materials and Methods

Materials. Crystalline cobalt heme (cobalt protoporphyrin IX, Co-heme), Co-heme dimethyl ester, cobalt coproporphyrin III, and cobalt uroporphyrin I were purchased from Porphyrin Products (Salt Lake City, Utah). Iron-heme (iron protopor-

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[1] Abbreviations used are: NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP; NADH, reduced nicotinamide adenine dinucleotide.