Purification and Characterization of Pyridoxal 5′-Phosphate Dependent Serine Hydroxymethylase from Lamb Liver and Its Action upon β-Phenylserines†

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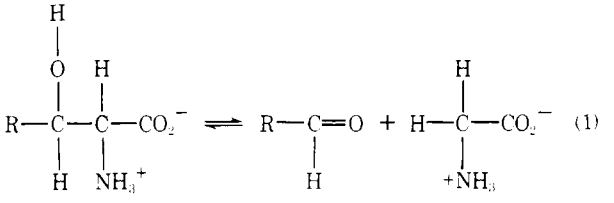
ABSTRACT: A serine hydroxymethylase (EC 2.1.2.1) has been isolated from lamb liver in an ultracentrifugally and electrophoretically homogeneous form and shown to have 4 pyridoxal 5′-phosphates bound per molecular weight of $227 \pm 20 \times 10^3$. This serine hydroxymethylase catalyzes the dealdolization of the L isomers of both *erythro*- and *threo*-β-phenylserine to benzaldehyde and glycine in the absence of tetrahydrofolic acid. No detectable decarboxylation, transamination, or deamination of β-phenylserines occurs. The enzyme forms spectrophotometrically detectable complexes at about 500 nm with L-β-phenylserines, glycine, D-alanine, aminomalonate, and L-phenylalanine attributed to the formation of enzyme bound quinonoid intermediates involving pyridoxal 5′-phosphate. The methyl ester of L-*erythro*-β-phenylserine is cleaved to benzaldehyde and glycine methyl ester. pH vs. K_m and V_m profiles suggest apparent pK_a values of 7.4 and 8.4, respectively. The data on competitive inhibitors indicate that the presence of a β-OH group assists while N-methylation hinders the formation of enzyme–inhibitor complexes. The equilibrium constants for the cleavage of *erythro*- and *threo*-β-phenylserine to glycine and benzaldehyde at 25 °C, pH 7.50, ionic strength 0.1 M, are 0.75 ± 0.25 M and 0.25 ± 0.05 M, respectively, indicating that the zwitterionic threo isomer is about 0.6 kcal/mol more stable than the erythro isomer under these conditions. There is a 1:1 ratio of the integrated areas of the signals of the ^1H nuclear magnetic resonance signals of the aldehydic and glycine protons in the products of the serine hydroxymethylase catalyzed cleavage of *erythro*-β-phenylserine in D_2O, which indicates that there is no exchange with solvent of the α-H of *erythro*-β-phenylserine before or during the cleavage and no exchange with solvent of the α-H remaining in glycine following the cleavage reaction.

The pyridoxal 5′-phosphate dependent liver aldolases catalyze the reversible cleavage of β-OH, α-NH_2 acids to glycine and aldehydes (eq 1) (Braunstein and Vilenkina, 1949; Gilbert, 1954; Bruns and Fiedler, 1958a,b).



(1)

Schirch and Gross (1968) have demonstrated that a monodisperse preparation of rabbit liver serine hydroxymethylase (EC 2.1.2.1), which catalyzes the cleavages of serine or α-methylserine (Schirch and Mason, 1963; Schirch and Diller, 1971; Wilson and Snell, 1962) in the presence of tetrahydrofolic acid (THF[1]), also catalyzes the dealdolization of threonine and allothreonine, although in the latter cases the presence of THF is not required. Thus, threonine aldolase and serine hydroxymethylase activities were inseparable.

Despite an earlier suggestion to the contrary (Malkin and Greenberg, 1964) and while this manuscript was in preparation, Schirch and Diller (1971) reported that rabbit liver serine hydroxymethylase catalyzes the cleavage of β-phenylserine. The present paper reports the purification, characterization, and properties of serine hydroxymethylase from lamb liver and its action upon D,L-*erythro*- and *threo*-β-phenylserine to form glycine and benzaldehyde. The accompanying papers detail aspects of the kinetic mechanism of the action of this serine hydroxymethylase upon these and related substrates (Ulevitch and Kallen, 1977a,b).

Materials and Methods

D,L-*erythro*-β-Phenylserine and D,L-*threo*-β-phenylserine were synthesized according to the methods of Shaw and Fox (1953) and were chromatographically homogeneous with R_f values of 0.29 and 0.44 in l-butanol/acetone/NH_4OH/water 50:6.25:6.25:37.5 (v/v) on silica gel.

The buffers utilized in this work were the following: (buffer I) 0.01 M potassium phosphate, 0.001 M EDTA, 0.014 M D,L-serine, 0.0001 M PLP, 0.1 M NaF, pH 7.50; (buffer II) 0.01 M potassium phosphate, 0.001 M EDTA, 0.001 M D,L-serine, 0.0001 M PLP, pH 7.50; (buffer III) 0.01 M potassium phosphate, 0.001 M EDTA, 0.001 M D,L-serine, pH 7.50; and (buffer IV) 0.05 M Hepes, 0.001 M EDTA, 0.025 M Na_2SO_4, pH 7.50.

Hepes, Bicine, Mes (Good et al., 1966), PLP, disodium NADH (type III), crystalline yeast alcohol dehydrogenase (ammonium sulfate suspension), BSA, and Tris were all pur-

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[1] Abbreviations: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; E_t, total tetrameric enzyme; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; PLP, pyridoxal 5′-phosphate; THF, tetrahydrofolic acid; Me_4Si, tetramethylsilane; TN, turnover number; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; NMR, nuclear magnetic resonance.

chased from Sigma; β-phenylethanolamine (Aldrich), L-phenylalanine, enzyme grade ammonium sulfate (Mann), and other materials of reagent grade were used without further purification. Phosphocellulose P11 was washed with acid and base according to instructions supplied by Reeve Angel, equilibrated with buffer III and stored at 4 °C. G-25, G-200, and CM-Sephadex (Pharmacia) were swelled in deionized water at room temperature, the fines decanted, and the gel was equilibrated with buffer II for 1 week at 4 °C. Dialysis tubing (A. H. Thomas) was boiled in a solution of 10^{-2} M D,L-serine and/or 10^{-2} to 10^{-3} M EDTA, pH 7.50, for 15 min and rinsed in deionized water before use.

Deionized water of greater than 5×10^5 ohms cm specific resistance was used throughout. Unless noted otherwise, the experiments were performed in buffer IV at pH 7.50 and 25 °C.

The *p*-nitrophenylhydrazone of benzaldehyde was synthesized according to Cheronis et al. (1965).

Assay of Enzymatic Activity. Measurements of initial velocity were made from absorbance determinations with a Gilford Model 2000 Multiple Sample Absorbance Recorder equipped with a Beckman DU monochromator and a thermostated cell compartment maintained at 25 ± 0.1 °C. After thermal equilibrium was attained, reactions were initiated by the addition of a small volume of enzyme (less than 25 μL) unless otherwise noted. Benzaldehyde production from either D,L-*erythro*- or D,L-*threo*-β-phenylserine was measured at 279 nm employing a molar absorptivity value of 1400 M^{-1} cm^{-1} (Forbes et al., 1958) for benzaldehyde.

Specific activity of enzyme preparations is expressed as μmol of benzaldehyde min^{-1} (mg of protein)$^{-1}$ and is measured with a 0.1 M solution of D,L-*threo*-β-phenylserine in buffer IV at pH 7.50 and 25 °C. An enzyme unit (U) is defined as the amount of enzyme that produces 1 μmol of benzaldehyde in 1 min under the above conditions. For the determination of the ratio of the specific activities during the purification, measurements with 0.1 M D,L-*erythro*-β-phenylserine were made as described above for D,L-*threo*-β-phenylserine.

The cleavage of L-threonine or D,L-allothreonine to form glycine and acetaldehyde was measured spectrophotometrically with yeast alcohol dehydrogenase and NADH as described by Malkin and Greenberg (1964) in buffer IV at pH 7.50 and 25 °C.

Protein was determined colorimetrically (Lowry et al., 1951) with a bovine serum albumin standard.

Kinetic Measurements. Initial rates of benzaldehyde production were calculated from linear absorbance vs. time traces and fit to a rectangular hyperbola with respect to substrate concentration (Viale and Kallen, 1971). The error bars reported denote the 95% confidence levels of the kinetic parameters. In some instances the kinetic data were analyzed graphically by alternate methods (Hanes, 1932; Lineweaver and Burk, 1934; and Dixon and Webb, 1964).

For the pH dependence of the steady-state kinetics, which were conducted at about 0.06 μM serine hydroxymethylase, the pH values were measured on a Radiometer 25 or 26 SE pH meter equipped with combined glass electrodes GK2301B or C and adjusted to a desired pH with HCl or KOH. At the conclusion of the kinetic runs, the pH values of the reaction mixtures were within ±0.03 of the desired value.

Acrylamide Gel Electrophoresis. Acrylamide gel electrophoresis at 4 °C, pH 9.5, with Tris–glycine buffer, in 7% gels was conducted at 3 mA/gel. Gels were either stained for protein (with 0.5% aniline blue black in 7% acetic acid at room temperature for 4 h) or for enzymatic activity (by equilibration at 37 °C for 4 or 10 min in 5.0 mL of 0.1 M D,L-*erythro*- or D,L-*threo*-β-phenylserine at pH 7.50 (buffer IV) on a Metabolyte rotary shaker at 100 rpm followed by incubation for 10 min at 37 °C in 10 mL of a 2 N solution of phenylhydrazine, the latter brought into solution with minimal amounts of glacial acetic acid). Destaining of aniline blue black treated gels was accomplished in a Canalco gel destainer with 7% acetic acid.

Ultracentrifugal Measurements. Equilibrium sedimentation and equilibrium velocity measurements were performed in a Spinco Model E ultracentrifuge equipped with Schlieren and Rayleigh interference optics. Fringe displacements were measured on a Beckman two-dimensional micro-comparator. Molecular weights were determined by the high speed sedimentation equilibrium method of Yphantis (1961) and a value of 0.256 was used for $(1 - \bar{V}\rho)$. Sedimentation coefficients were corrected to $s_{20,w}$. Enzyme used in these determinations was dialyzed overnight against 5×10^{-4} M Hepes, pH 7.5, 10^{-3} M EDTA, 0.032 M Na_2SO_4 and was fully active at the initiation of each run.

Determination of the Number of Moles of Pyridoxal 5′-Phosphate Bound per Mole of Enzyme. Stock solutions of L-cysteine (0.2 M) and PLP (0.01 M) in buffer IV were prepared daily; the cysteine solution was deaerated by bubbling with argon and both solutions were maintained at 2–4 °C protected from the light. The molar absorptivity at 335 nm determined for the thiazolidine generated from cysteine (0.2 M) and PLP ($5–20 \times 10^{-5}$ M) at pH 7.50 was unaltered by a 2-min equilibration at 100 °C.

The measurement of the number of moles of PLP bound per mole of enzyme was performed with 1.4 and 7.0 mg of purified serine hydroxymethylase (2.3 U/mg). Following a 5-min equilibration of the enzyme with L-cysteine in stoppered conical centrifuge tubes (protected from the light), a 2-min immersion in boiling water, and rapid cooling in ice, the denatured protein was removed by 15-min centrifugation in an International PR-2 refrigerated centrifuge (2000 rpm). The number of moles of PLP was determined from the absorbance at 335 nm of the thiazolidine present in the supernatant.

Characterization of the Enzymatic Reaction with D,L-threo-β-Phenylserine and D,L-erythro-β-Phenylserine. Solutions of serine hydroxymethylase and D,L-*erythro*- or D,L-*threo*-β-phenylserine were analyzed by paper chromatographic and spectrophotometric techniques for β-phenylethanolamine (Shaw and Fox, 1953), phenylpyruvate (Juni and Heym, 1962), and benzaldehyde through its conversion to the *p*-nitrophenylhydrazone (Juni and Heym, 1962); the latter material had behavior identical with synthetic material in several solvent systems.

Optical rotation measurements at 589.1 nm were made with a Bendix Automatic Polarimeter Series 1100 thermostated at 25 ± 0.1 °C of acidified solutions of D,L-*erythro*- and D,L-*threo*-β-phenylserines previously equilibrated with serine hydroxymethylase. The identification of the β-phenylserine isomer remaining was based on molar rotation (M_D^{25}) values for L-*threo*- and L-*erythro*-β-phenylserine of +147.3° and −91.1° in 5 N HCl, respectively (Greenstein and Winitz, 1961).

Spectral Measurements. Spectral measurements were performed in a Cary Model 14 with a thermostated cell compartment maintained at 25 ± 0.1 °C and a 0–0.1 absorbance slide wire. Difference spectra for enzyme–substrate intermediates employed tandem compartment mixing cells (Pyrocell) with a 0.44-cm lightpath per compartment.

Equilibrium Constants for the Cleavages of D,L-erythro- and D,L-threo-β-Phenylserine to Glycine and Benzaldehyde. The production of benzaldehyde from solutions of serine hy-

droxymethylase either D,L-*erythro*- or D,L-*threo*-β-phenylserines (pH 7.50) was measured with horse liver alcohol dehydrogenase (Boehringer). Solutions of substrate were equilibrated with serine hydroxymethylase at 25 °C in tightly stoppered serum vials and samples (5–25 μL) were removed at different times and assayed immediately for benzaldehyde at 25 °C and pH 7.50 (buffer IV with 0.1 M D,L-serine) with horse liver alcohol dehydrogenase, 0.1 mg/mL, and NADH (2.5×10^{-4} M). When the concentration of benzaldehyde became independent of time (90 min), 40 μL of 8 N HCl was added to the serum vial and benzaldehyde concentration was measured as described above.

Equilibrium mixtures were assayed for L-*erythro*-β-phenylserine with L-amino acid oxidase (isolated from *Crotalus adamanteus* venom and a generous gift from Dr. David Porter) using an oxygen electrode (Yellow Springs Instrument Co.). The concentration of the L-*erythro*-β-phenylserine present in the added sample was given by the amount of O_2 consumed. Reactions measured with the oxygen electrode were complete within 1–3 min and maintained a stable endpoint.

NMR Measurements of the Incorporation of Deuterium into Glycine and Benzaldehyde. Proton magnetic resonance measurements were performed with a Varian HR-220 Superconn NMR spectrometer with a probe temperature of 18 ± 1 °C. The pD of solutions in D_2O was measured and adjusted as described elsewhere (Ulevitch and Kallen, 1977b) with 10 N NaOD. The exchangeable (–OH and –NH) protons of D,L-*erythro*-β-phenylserine were deuterated by repeated dissolution in D_2O and lyophilization. A solution of 0.01 M potassium phosphate, 0.001 M EDTA, pD 7.50, containing 0.1 M D,L-*erythro*-β-phenylserine and 5 μM serine hydroxymethylase was utilized for NMR experiments in which the time dependence of the signals of the glycine and benzaldehyde protons was measured.

Chemical shifts are reported as ppm downfield from Me_4Si in D_2O.

Results

Enzyme Purification. Fresh (kosher) lamb livers were obtained from the abbatoir and transported on ice. Operations were carried out at 2–4 °C in buffer I except where noted otherwise.

Step 1: Preparation of Crude Extract. Cubes (2 to 3 cm) cut from four livers, and debrided of fat and connective tissue, were added to 1 L of buffer I and homogenized in a Waring commercial blender at high speed for 1 min, 23–25 °C. This material was diluted 1:1 with buffer I (to a protein concentration ~70 mg/mL) and was centrifuged for 30 min at 10 000*g* and the supernatant decanted through several layers of cheesecloth.

Step 2: Heat Steps, 54 °C. Fractions (~600 mL) in 2-L flasks were maintained at 54 °C for 15 min (Metabolyte rotary shaker 100 rpm) and rapidly cooled in an ice–water bath. The cooled material was centrifuged at 10 000*g* for 30 min and the precipitate was discarded.

Step 3: 50% Ammonium Sulfate Precipitation. Solid ammonium sulfate was added to a final concentration of 50% saturation (31 g/100 mL) (DiJeso, 1968) and the solution (pH 6.0–6.5) was stirred mechanically overnight. This solution was centrifuged for 30 min at 10 000*g*, the supernatant discarded, and the precipitate dissolved in buffer I. The redissolved precipitate was dialyzed against 15 L of buffer II for 36 h with a single change of buffer, centrifuged at 10 000*g* for 30 min and the precipitate was discarded.

Step 4: 0–35%, 35–50% Ammonium Sulfate Precipitation. Solid ammonium sulfate was added to the dialyzed material from step 3 to obtain a final concentration of 35% saturation (20 g/100 mL) and, after mechanical mixing for 3 h, the solution was centrifuged at 10 000*g* for 30 min. Solid ammonium sulfate was added to the supernatant to obtain a final concentration of 50% saturation, stirred mechanically for 3 h, and centrifuged. The supernatant was discarded and precipitates from the 0–35% and 35–50% ammonium sulfate cuts were separately dissolved in buffer II and dialyzed against 15 L of buffer for 24 h with a single change of buffer.

Step 5: Phosphocellulose Column Chromatography. Two columns (diameter 4.0 cm) were filled to a height of 38 cm, with phosphocellulose P11 in buffer III. The dialyzed material from step 4 (35–50% ammonium sulfate cut) was divided into two 365-mL fractions and applied to the columns. The columns were washed with 0.04 M potassium phosphate, 0.001 M EDTA, 0.001 M D,L-serine (pH 7.5) until the absorbance at 280 nm of the effluent was less than 0.3. The enzyme was eluted from the column as a discernibly yellow band with 0.3 M potassium phosphate, 0.001 M EDTA, 0.001 M D,L-serine (pH 7.5), concentrated by 50% ammonium sulfate precipitation resuspended in buffer II and dialyzed for 18 h with a single change of buffer.

Step 6: G-200 Sephadex Column Chromatography. The material from step 5 was applied in 45-mL fractions to a G-200 Sephadex column (6 × 82 cm, buffer III) and tubes containing enzyme of the highest specific activity were pooled, concentrated by 50% ammonium sulfate precipitation, dissolved in buffer II, and dialyzed against 3 L of buffer II overnight.

Step 7: CM-Sephadex (C-50) Column Chromatography–Gradient Elution. Dialyzed material from step 6 was applied to a CM-Sephadex column (2.5 × 26 cm, buffer III) and the column washed with 0.04 M potassium phosphate, 0.001 M EDTA, 0.001 M D,L-serine, pH 7.5 (700 mL). The enzyme was eluted at about 0.1 M potassium phosphate (or 100 mL eluent) with a linear gradient between 0.04 M potassium phosphate and 0.3 M potassium phosphate (250 mL each); fractions of the highest specific activity were pooled, concentrated by 50% ammonium sulfate precipitation, and dialyzed as described for step 6.

Step 8: Phosphocellulose Column Chromatography–Gradient Elution. The material from step 7 was applied to a phosphocellulose P11 column (2.5 × 12 cm, buffer III) and washed with 200 mL of 0.075 M potassium phosphate followed by elution of the enzyme with a linear gradient between 0.04 (or 0.075 M) and 0.3 (or 0.2 M) potassium phosphate, pH 8.0 (250 mL each). Fractions of the highest specific activity were pooled and concentrated by 50% ammonium sulfate precipitation.

Step 9: Storage of the Purified Enzyme. The enzyme from step 8 was concentrated by 50% ammonium sulfate precipitation, redissolved in buffer IV containing 0.0001 M PLP, and dialyzed overnight against 1 L of the same buffer. Enzyme solutions (5–20 mg/mL) could be stored indefinitely at 4 °C without appreciable loss of activity. Before kinetic or spectral experiments utilizing enzyme, excess PLP was removed by dialysis with buffer IV or by passage through a G-25 Sephadex column.

The results of the purification are summarized in Table I.

Acrylamide Gel Electrophoresis. Acrylamide gel electrophoresis with samples of purified enzyme (2.3 U/mg) was run in triplicate at pH 9.5 in the standard Tris-glycine buffer. When stained with aniline blue black, only a single protein component was noted. Phenylhydrazine treatment of the remaining two gels following exposure to D,L-*erythro*- or D,L-*threo*-β-phenylserine led to the development of an opaque white band, presumably the phenylhydrazone of benzaldehyde,

TABLE I: Purification of Serine Hydroxymethylase from Lamb Liver.

Step	Vol (mL)	Total units	Protein (mg/mL)	Spec. act. (U/mg)	Spec act. ratio[b]
(1) Crude extract	10000	6400	70.6	0.0091	10.7
(2) Heat step	7170	3872	45	0.012	7.6
(3) 50% ammonium sulfate ppt	1600	4704	50.1	0.060	7.3
(4) 35–50% ammonium sulfate ppt	730	3183	71.2	0.061	7.3
(5) Phosphocellulose P11	730	2190[a]	4.9	0.61	7.2
(6) Sephadex G-200	650	1460	2.65	0.85	6.7
(7) CM-Sephadex	166	900	3.53	1.54	7.4
(8) Phosphocellulose P11 gradient elution	218	570	1.13	2.3	7.1

[a] Approximately 1000 units of activity was found in the effluent which did not adsorb to phosphocellulose P11 in 0.04 M potassium phosphate and presumably represents the mitochondrial serine hydroxymethylase (see Discussion section). [b] *erythro*-β-Phenylserine/*threo*-β-phenylserine as described in Materials and Methods section.

which corresponded to the position of the protein staining.

Sedimentation Coefficient. A single symmetrical peak with sedimentation coefficients, $s_{20,w}$, of 9.1, 9.3, and 8.7 was obtained at 1.4, 5.0, and 10 mg/mL of protein (2.3 U/mg), respectively.

Equilibrium Sedimentation.. The molecular weight 2.27 ± 0.20 × 10^5, determined by the high speed method of Yphantis (1961), was used in calculations involving enzyme concentration.

Number of Moles of PLP Bound per Mole of Enzyme. The molar absorptivity of the thiazolidine of PLP and L-cysteine is 5580 M^{-1} cm^{-1} at 335 nm, 25 °C, and pH 7.50. After exposure of 6.1 and 30 μM serine hydroxymethylase (2.3 U/mg) to L-cysteine for 5 min and heat denaturation of the enzyme, the concentrations of the thiazolidine in the supernatant determined spectrophotometrically were 23 and 120 μM, respectively. These measurements indicate an average of 3.9 ± 0.1 mol of PLP bound per mol of serine hydroxymethylase.

Characterization of the β-Phenylserine Reaction. The products of the serine hydroxymethylase catalyzed cleavage of D,L-*threo*- and D,L-*erythro*-β-phenylserine are glycine and benzaldehyde. The presence of the latter compound was confirmed by UV and NMR spectroscopy, formation and isolation of the *p*-nitrophenylhydrazone from appropriately treated reaction mixtures, and, not unreliably, olfactory sensation. The presence of glycine was established by NMR spectroscopy and by the appearance of a ninhydrin positive spot (R_f = 0.12) which corresponded to a glycine standard (Shaw and Fox, 1953). With D,L-*erythro*-β-phenylserine as substrate a ninhydrin positive spot corresponding to *threo*-β-phenylserine appeared, and with D,L-*threo*-β-phenylserine as substrate a ninhydrin positive spot corresponding to *erythro*-β-phenylserine appeared. If glycine and benzaldehyde were equilibrated with enzyme and samples examined by paper chromatography, ninhydrin positive spots corresponding to both *erythro*- and *threo*-β-phenylserine could be identified. Reaction mixtures of enzyme and D,L-*threo*- or D,L-*erythro*-β-phenylserine were also treated with *p*-nitrophenylhydrazine and the resulting precipitates were indistinguishable from the *p*-nitrophenylhydrazone of benzaldehyde on Silica Gel G in two solvent systems: (v/v) (a) benzene/*n*-hexane (60/40) R_f = 0.025 and (b) benzene/ethyl acetate (20/1), R_f = 0.40. Thus, the enzyme purified from lamb liver catalyzes the reversible cleavage of both diastereoisomers of β-phenylserine (see below).

The occurrence of several instances of relaxation of reaction specificity of PLP-dependent enzymes (Huntley and Metzler, 1968; Bailey et al., 1970; Schirch and Jenkins, 1964a,b; Ulevitch and Kallen, 1977a) requires that consideration be given to other possible fates of β-phenylserine in accord with the general mechanisms for PLP-mediated reactions (Metzler et al., 1954; Braunstein and Shemakin, 1953; cf. Dunathan, 1966): (a) decarboxylation to form β-phenylethanolamine and CO_2, (b) deamination and dehydration to form phenylpyruvate, NH_3, and water, and (c) transamination to form β-hydroxyphenylpyruvate and the pyridoxamine form of the enzyme.

β-Phenylethanolamine (R_f = 0.72) is separable from D,L-*threo*- (R_f = 0.45), D,L-*erythro*-β-phenylserine (R_f = 0.30), and glycine (R_f = 0.12) by paper chromatography (Shaw and Fox, 1953). No β-phenylethanolamine was detected in 150-min incubation mixtures of D,L-*erythro*- or D,L-*threo*-β-phenylserine and serine hydroxymethylase by paper chromatography (Shaw and Fox, 1953). Furthermore, spectrophotometric measurements at 279 nm indicate that the enzyme does not catalyze the cleavage of β-phenylethanolamine to benzaldehyde. Thus it may be concluded that serine hydroxymethylase does not catalyze decarboxylation of β-phenylserine. Both lamb[2-4] and rat liver serine hydroxymethylases catalyze the decarboxylation of aminomalonate to CO_2 and glycine (Palekar et al., 1973).

Using the method of Juni and Heym (1962), the *p*-nitrophenylhydrazones of charged and neutral carbonyl compounds could be quantitatively separated and measured with a sensitivity to 0.1 μmol of phenylpyruvate in the presence of up to a 50-fold excess of benzaldehyde. Within the detectability limits of this assay (<8% conversion), no charged carbonyl compounds, for example, phenylpyruvate, β-hydroxyphenylpyruvate or glyoxylate were formed in a 27-h equilibration of enzyme with either D,L-*erythro*- or D,L-*threo*-β-phenylserine at which time specific activity of the enzyme had dropped by only 50%. These findings indicate that no significant deamination or transamination of β-phenylserines occurs.

Measurements of optical rotation of acidified aliquots of a solution of β-phenylserine and enzyme removed at different times after the initiation of the enzymatic reaction indicate positive changes in optical rotation with the erythro isomer (upper graph, Figure 1) and negative changes with the threo isomer (lower graph, Figure 1). Acidified solutions of the racemic mixtures of β-phenylserines show no measurable optical rotation. The nature of the changes in optical rotation, which corresponds to the accumulation of the D isomers, is evidence that the L isomers are the substrates for the enzyme.

The purified enzyme also catalyzes the cleavage of L-allothreonine and L-threonine to glycine and acetaldehyde

[2] R. J. Ulevitch and R. G. Kallen, unpublished observations.
[3] P. S. Tobias and R. G. Kallen, unpublished observations.
[4] S. Sato and R. G. Kallen, unpublished experiments.

TABLE II: Steady-State Kinetic Constants for Serine Hydroxymethylase Catalyzed Dealdolization of β-Phenylserines and Threonines.

Enzyme source	Substrate	pH	T (°C)	K_m (M)[c]	Catalytic center act. (s^{-1})
Lamb liver[a]	D,L-Allothreonine	7.50	25	0.0013	0.56
	L-Threonine	7.50	25	0.032	0.09
	D,L-*erythro*-β-Phenylserine	7.50	25	0.0095	21.0
	D,L-*threo*-β-Phenylserine	7.50	25	0.084	7.0
	D,L-*erythro*-β-Phenylserine methyl ester	7.50	25	0.070	29.0
Rabbit liver	D,L-Allothreonine	7.30, 7.20	37, 30	0.0013,[b] 0.0015[c]	2.21×10^{-3}[b,d,c]
	L-Threonine	7.30, 7.20	37, 30	0.050,[b] 0.04[c]	0.76×10^{-3}[b,d,c]
	D,L-*erythro*-β-Phenylserine	7.20	30	>0.062[c]	
	D,L-*threo*-β-Phenylserine	7.20	30	>0.062[c]	

[a] Catalytic center activity based upon mol wt 2.27×10^5 (4 mol of PLP per mol of enzyme). [b] Catalytic center activity based upon mol wt 3.31×10^5 (6 mol of PLP per mol of enzyme, Schirch and Gross, 1968). [c] K_m expressed as the concentration of the L isomer. [d] Catalytic center activities, based upon mol wt 2.15×10^5 (4 mol of PLP per mol of enzyme, Martinez-Carrion et al., 1972), are 1.91×10^{-3} and 0.66×10^{-3} s^{-1} for D,L-allothreonine and L-threonine, respectively. [e] Schirch and Diller (1971).

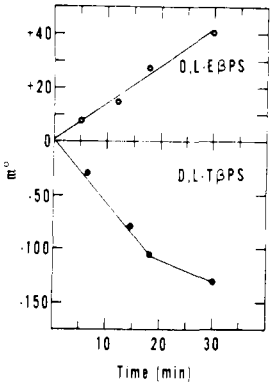

FIGURE 1: Time dependence of optical rotation in millidegrees (m°) at 589.1 nm during the serine hydroxymethylase catalyzed cleavage of D,L-*erythro*- or D,L-*threo*-β-phenylserine. Aliquots diluted 3.6-fold with HCl (final concentration 5 N). Pathlength: 2.0 cm. Incubation conditions: buffer IV, pH 7.50, 25 °C. (Top) With 0.1 M D,L-*erythro*-β-phenylserine, serine hydroxymethylase, 0.6 mg/mL, 2.3 U/mg; (bottom) 0.2 M D,L-*threo*-β-phenylserine, serine hydroxymethylase, 0.24 mg/mL, 2.3 U/mg.

TABLE III: Inhibition Constants for Serine Hydroxymethylase with D,L-*erythro*-β-Phenylserine as Substrate.

Inhibitor	K_i (M) Lamb[d]	K_i (M) Rabbit
Glycine	0.011	0.007[a]
		0.006[c]
Glycine methyl ester	0.030	*b*
Glycine ethyl ester	0.060	*b*
Sarcosine (*N*-methylglycine)	No inhibition	*b*
L-Serine	0.0007	0.0009[a]
		0.0008[c]
D,L-α-Methylserine	0.006[c]	0.007[c]
D,L-*O*-Methylserine	0.012[c]	*b*
L-Alanine	0.0076	0.014[c]
α-Aminoisobutyrate		0.013[c]

[a] Reported by Schirch and Gross (1968) for the reaction of rabbit liver serine hydroxymethylase with D,L-allothreonine, 37 °C, pH 7.3. [b] Not reported. [c] Calculated assuming the L isomer is the inhibiting species. [d] This work. [e] Reported by Schirch and Diller (1971) for the reaction of rabbit liver serine hydroxymethylase with D,L-allothreonine, 30 °C, pH 7.2.

(Table II) and the cleavage of L-*threo*-β-thienylserine to glycine and 2-thiophenaldehyde.[2]

Competitive Inhibitors. Several glycine and serine derivatives with the notable exception of sarcosine show simple competitive inhibition patterns with respect to D,L-*erythro*-β-phenylserine and the inhibitor constants, K_i, were obtained from plots of the apparent Michaelis constant ($K_{m_{app}}$) vs. inhibitor concentration (eq 2)

$$K_{m_{app}} = K_m(1 + [I]/K_i) \quad (2)$$

where $K_i = [E][I]/[EI]$ and I is the inhibitor. The results of these inhibition studies are summarized in Table III. While we and others (Schirch and Diller, 1971) have observed significant inhibition of serine hydroxymethylase activity at the lower pH values by anions (Cl^-, $H_2PO_4^-$, etc.), at the concentration of SO_4^{2-} utilized in the present experiments in order to maintain ionic strength constant at 0.1 M, no significant inhibition of catalytic activity was observed.

Steady-State Parameters: pH Dependence of the D,L-threo-β-Phenylserine and D,L-erythro-β-Phenylserine Dealdolization Reactions. The solubility maximum of the zwitterionic form of D,L-*threo*-β-phenylserine in water at 25 °C, ionic strength 0.1 M is on the order of the value of the K_m determined from steady-state kinetics (Figure 2); thus the values of the kinetic parameters for D,L-*threo*-β-phenylserine have larger 95% confidence levels than data obtained with D,L-*erythro*-β-phenylserine for which the K_m value is significantly lower.

The pH dependencies of the steady-state parameters K_m and catalytic center activity, where catalytic center activity is $V_{max}/([E_t] \times 4) = TN$, for the enzyme catalyzed cleavage of D,L-*threo*- and D,L-*erythro*-β-phenylserine from pH 6.0 to 9.3 are shown in Figures 3A and 3B. Although the pH dependencies of these parameters are small, inflection points appear to be present at about pH 7.4 for K_m and 8.4 for catalytic center activity. Instability of the enzyme made studies over a wider pH range difficult. Addition of 10^{-4} M PLP to enzymatic assays at pH 6.6, 7.5, and 8.5 had no effect on the kinetic parameters determined for D,L-*erythro*-β-phenylserine.

Visible Absorbance Spectral Changes Observed with Serine Hydroxymethylase. The spectrum of the purified enzyme (1.0 mg/mL, 2.3 U/mg) is pH independent between pH 6.6 and 8.6 with a 278 nm/425 nm ratio of 9.3. The 425-nm peak disappeared and a new peak at 335 nm appeared (most probably the thiazolidine of PLP and L-cysteine) upon mixing serine hydroxymethylase from lamb liver with 0.2 M L-cysteine as noted earlier with the rabbit liver enzyme (Schirch and Mason, 1962).

After mixing purified enzyme (0.7 mg/mL, 2.3 U/mg) with 0.4 M D-alanine, the appearance of a new absorbance band at

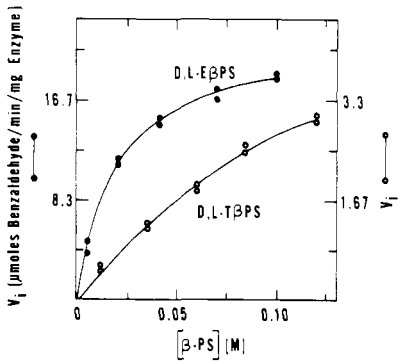

FIGURE 2: The substrate concentration dependence of the initial rate of benzaldehyde appearance in solutions of β-phenylserine and serine hydroxymethylase (0.6 mg/mL, 2.30 U/mg). Initial velocity, v_i, expressed as μmol of benzaldehyde min^{-1} mg of $enzyme^{-1}$. D,L-*erythro*- (●—●) and D,L-*threo*-β-phenylserine (○—○), pH 7.50 (buffer IV), 25 °C. The solid line is a calculated curve based upon the kinetic parameters (Table II) obtained by a least-squares method (Viale and Kallen, 1971). Concentrations are expressed in terms of total D,L-β-phenylserine concentration.

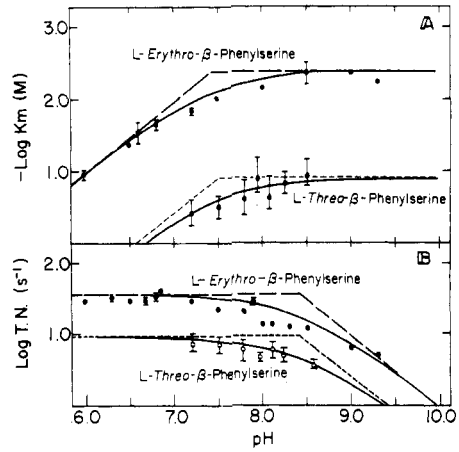

FIGURE 3: Dependence of K_m and turnover number (catalytic center activity: s^{-1}) values for the reaction of D,L-*erythro*- and D,L-*threo*-β-phenylserine with serine hydroxymethylase (2.3 U/mg) upon pH at 25 °C. The pH was maintained with 0.05 M Mes, Hepes, and Bicine buffers with 0.001 M EDTA and ionic strength adjusted to 0.1 M with Na_2SO_4. The K_m is expressed with respect to the L isomers of β-phenylserine and the error bars represent the 95% confidence levels from least-squares analysis of kinetic data (Viale and Kallen, 1971). Catalytic center activity is expressed as $V_{max}/([E_t] \times 4)$ based upon mol wt = 2.27×10^5 and 4 PLP bound per mol of enzyme.

505 nm was observed.

A 500-nm absorbance peak also appears within the time required to obtain a spectrum when serine hydroxymethylase is mixed with D,L-*threo*- or D,L-*erythro*-β-phenylserine (Figure 4). Similar spectral changes are observed when 0.1 M L-phenylalanine is mixed with serine hydroxymethylase; the appearance of the 500-nm absorbance band with L-phenylalanine is quite slow ($t_{1/2} \sim 5$ min) and, once formed, remains unchanged for at least 24 h.

NMR Studies: Incorporation of Deuterium into Glycine during the Serine Hydroxymethylase Catalyzed Dealdolization of D,L-erythro-β-Phenylserine. Following the addition of serine hydroxymethylase to a solution of D,L-*erythro*-β-phenylserine in D_2O, new absorbance signals appear at 2 and 10 ppm due to the α hydrogen of glycine and the aldehydic proton of benzaldehyde, respectively. The integrated areas under these signals increased over the course of 75 min and the ratio of the integrated areas under the respective peaks was 1.0, within experimental error, as equilibrium was attained (Ulevitch, 1971). The absorbance signals corresponding to the C_α (3 ppm) and the C_β (5.7 ppm) protons of *erythro*-β-phenylserine show no evidence of incorporation of deuterium into these positions in the presence of serine hydroxymethylase during the time-dependent cleavage to form glycine and benzaldehyde.

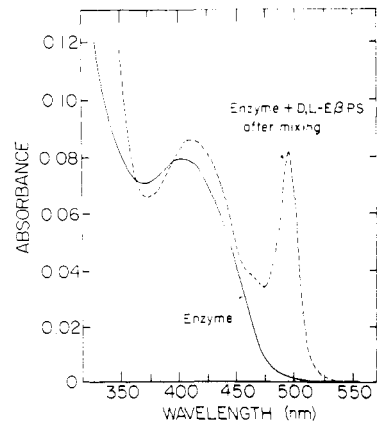

FIGURE 4: Visible absorbance spectrum of serine hydroxymethylase before and after mixing with D,L-*erythro*-β-phenylserine in split compartment cuvettes (pathlength 0.88 cm after mixing). Buffer IV, pH 7.50 25 °C. After mixing: 0.05 M D,L-*erythro*-β-phenylserine and serine hydroxymethylase, 0.7 mg/mL (2.3 U/mg). Spectrum taken 1 min after mixing (- - -).

Discussion

Identification of the Purified Enzyme as Serine Hydroxymethylase. The results of sedimentation velocity, sedimentation equilibrium, and acrylamide gel electrophoresis experiments indicate that the purification procedure (Table I) yields a preparation of serine hydroxymethylase which is homogeneous. The constant ratio of the specific activity with D,L-*erythro*- and D,L-*threo*-β-phenylserine during the purification (Table I) is evidence that a single enzyme catalyzes the reversible cleavage of both *erythro*- and *threo*-β-phenylserine to glycine and benzaldehyde. Since the same preparation also catalyzes the cleavage of L-threonine and L-allothreonine to glycine and acetaldehyde (Table II) and the THF-dependent cleavage of L-serine to glycine and N^5,N^{10}-methylene-THF,[3] the purified enzyme is almost certainly serine hydroxymethylase which has been shown to possess the above activities (Schirch and Gross, 1968; Biellmann and Schuber, 1970; Jordon and Akhtar, 1970; Schirch and Diller, 1971). Further evidence to support this conclusion derives from the following similarities between lamb liver and rabbit liver serine hydroxymethylases (Schirch and Gross, 1968): (a) the spectral changes at 505 nm observed with D-alanine and both enzymes are identical (Schirch and Mason, 1963; Schirch and Jenkins, 1964a,b; this work) and are at present unique to the interaction of serine hydroxymethylase and D-alanine; and (b) the lamb serine hydroxymethylase catalyzes the decarboxylation of aminomalonate to carbon dioxide and glycine[3,4] as does the rat liver cytoplasmic serine hydroxymethylase (Palekar et al., 1973).

In view of these data, serine hydroxymethylase almost certainly accounts for previous reports of PLP dependent aldol cleavage reactions of β-OH, α-amino acids (Braunstein and Vilenkina, 1949; Gilbert, 1954; Bruns and Fiedler, 1958a,b; Malkin and Greenberg, 1964; Riario-Sforza and Marinello, 1967; Riario-Sforza et al., 1969).

Mitochondrial and soluble serine hydroxymethylase dif-

TABLE IV: Comparison of Physical Properties and Steady-State Kinetic Constants of Serine Hydroxymethylase[c] Isolated from Various Mammalian Sources.[d]

Enzyme source[d]	Intracellular location	mol wt ($\times 10^{-5}$)	$s_{20,w}$	Moles of bound PLP	K_m L-Serine	K_m Glycine	A_{278}/A_{430}
Rat[g]	Soluble	*b*	*b*	*b*	0.00054	0.0012	*b*
Rat[g]	Mitochondrial	*b*	*b*	*b*	0.00054	0.0018	*b*
Rabbit[h]	Soluble	1.85	8.8	4	0.0013	*b*	8.9
Rabbit[h]	Mitochondrial	1.7	8.5	4	0.001	*b*	6.3
Rabbit	*b*	2.15[i]	*b*	4	0.0007[e]	0.0059[e]	10[e]
Lamb	*b*	2.27	9.0	4	0.0009[a]	0.011[a]	9.3[f]

[a] Determined as K_i for the reaction of D,L-*erythro*-β-phenylserine with serine hydroxymethylase; this work. [b] Not reported. [c] Serine hydroxymethylase from rabbit liver has threonine aldolase activity (Schirch and Gross, 1968; Biellmann and Schuber, 1967, 1970; Jordan and Akhtar, 1970) and the lamb liver serine hydroxymethylase has been shown to cleave other β-substituted serines (this work). [d] Enzymes isolated from liver. [e] Reported as A_{280}/A_{430} (Schirch and Gross, 1968) and revised as $A_{280}/A_{428} = 5.5$ (Schirch and Diller, 1971). [f] Reported as A_{279}/A_{425} (this work). [g] Nakano et al. (1968). [h] Fujioka (1968). [i] Martinez-Carrion et al. (1972); cf. Schirch and Gross (1968) and Schirch and Mason (1963).

fering in chromatographic and spectral characteristics have been isolated from *S. cerevisiae* (Zelikson and Luzzati, 1976), rat (Nakano et al., 1968), and rabbit liver (Fujioka, 1968; Akhtar et al., 1975). Although only one form of serine hydroxymethylase has been purified from lamb liver, two forms may be easily distinguished by enzyme activity measurements on acrylamide gels following electrophoresis in preparations prior to the phosphocellulose P11 chromatography step (Table I). The reported 278/430 nm absorbance ratios of 6.3 and 8.9 for the mitochondrial and soluble serine hydroxymethylase preparations from rabbit liver (Fujioka, 1968) suggest that our homogeneous preparation from lamb liver (278 nm/425 nm = 9.3) may be the "soluble" form of serine hydroxymethylase. There are no significant differences in the steady-state parameters for the L-serine reaction with the soluble and mitochondrial forms of serine hydroxymethylase (Nakano et al., 1968; Fujioka, 1968), although differences in inactivation by inhibitors have been detected (Fujioka, 1968; Akhtar et al., 1975). Physical and kinetic properties of serine hydroxymethylase isolated from different sources are summarized in Table IV. From the fact that there are 4 mol of PLP per molecular weight of 227 000 a monomer weight of 56 750 may be calculated for the lamb liver enzyme and is similar to the values of the monomer molecular weight reported for the rabbit liver enzyme (Martinez-Carrion et al., 1972).

Characterization of the Reaction of D,L-erythro-β-Phenylserine and D,L-threo-β-Phenylserine with Serine Hydroxymethylase. Measurement of optical rotation changes during the serine hydroxymethylase catalyzed dealdolization of D,L-*erythro*- or D,L-*threo*-β-phenylserine (Figure 1) indicates that the D isomers accumulate and is consistent with the observation of Gilbert (1954) and Bruns and Fiedler (1958a,b) that only the L isomers of *threo*- and *erythro*-β-phenylserine were utilized by a partially purified fraction from rat liver. Although these results do not eliminate interaction of the D isomers with the enzyme which might involve either deamination or transamination in accord with the hypothesis of Dunathan (1966) regarding the stereochemical control of reactions catalyzed by PLP enzymes, none of the expected products of deamination or transamination could be detected in long term reaction mixtures of D,L-*erythro*- or D,L-*threo*-β-phenylserine and enzyme. Most compellingly, the production of benzaldehyde and glycine in a 1:1 molar ratio stoichiometric with the disappearance of β-phenylserine has been unequivocally demonstrated. Thus interaction of D isomers of phenylserine with serine hydroxymethylase, if present at all, appears limited to nonproductive complexes.

While the enzyme can differentiate between the D and L isomers of D,L-*threo*- and D,L-*erythro*-β-phenylserine, the finding that both diastereoisomers of threonine and β-phenylserine are substrates demonstrates a lack of absolute stereoselectivity by the enzyme at the β carbon asymmetric center. Thus, the stereochemical control upon the carbonyl compounds in the formation of the second asymmetric center is not absolute and, although such observations may be relatively uncommon, they are not unique (Neuhaus and Lynch, 1964; Nishimura and Greenberg, 1961; Barker et al., 1959; Kagan and Meister, 1966). The pH dependencies of the steady-state kinetic parameters K_m and V_M in a pH region in which the ionic states of the substrates, D,L-*erythro*- and D,L-*threo*-β-phenylserines, are not changing significantly (Figure 4) suggest that groups on the enzyme with pK_a values of 7.4 and 8.4 are controlling the two parameters, respectively. Since the steady-state parameters are known to be composite constants at several pH values (Ulevitch and Kallen, 1977b), further discussion of their pH dependencies will be deferred.

Competitive Inhibitors of the D,L-erythro-β-Phenylserine Reaction. Studies of glycine analogues as inhibitors indicate a requirement for a primary amino group for effective EI complex formation since sarcosine (0.1 M) is not a competitive inhibitor. Glycine methyl and glycine ethyl esters are competitive inhibitors which suggests that the carboxylate anion may not be important in directing EI formation and that the carboxylic acid may be the significant enzyme bound species for natural substrates as suggested by Ivanov and Karpeisky (1961) (cf. Schirch and Diller, 1971). Further, D,L-*erythro*-β-phenylserine methyl ester is cleaved to benzaldehyde and glycine methyl ester by serine hydroxymethylase (Table II). The data on competitive inhibitors also suggest that the presence of a β-OH group assists in the formation of EI complexes.

Spectral Changes. The spectral changes at 505 nm observed with D-alanine and serine hydroxymethylase from lamb liver are identical with those reported by Schirch and Jenkins (1964a,b) for serine hydroxymethylase from rabbit liver. The 505-nm intermediate has been interpreted to be the quinonoid intermediate formed by the loss of the α-H from the enzyme bound Schiff base of PLP and D-alanine (Schirch and Slotter, 1966) and similar intermediates have been observed in both other PLP-mediated reactions (Jenkins, 1961, 1964; Morino and Snell, 1967; Martinez-Carrion et al., 1970) and in model studies (Maley and Bruice, 1968; Matsumoto and Matsushima, 1972; Thanassi and Fruton, 1962).

The characterization of the spectral changes observed when D,L-*erythro*-β-phenylserine (Figure 4) or L-phenylalanine (not shown) is mixed with serine hydroxymethylase will be dis-

cussed in the following papers (Ulevitch and Kallen, 1977a,b).

NMR Experiments. The maintenance of the 1:1 ratio of the α-H and aldehydic protons of glycine and benzaldehyde, respectively, during the serine hydroxymethylase catalyzed cleavage of *erythro*-β-phenylserine in deuterium oxide confirms simultaneously the nature of the products, their stoichiometry, and the lack of exchange of the α-H in the reactant β-phenylserine with solvent. This last observation is consistent with the similar results reported for the reactions of L-serine and glycine with serine hydroxymethylase (Jordan and Akhtar, 1970; Wellner, 1970; Besmer and Arigoni, 1968) and the ability of α-alkyl substituted serines to be cleaved by the action of the enzyme (Wilson and Snell, 1962; Schirch and Mason, 1963; Fujioka, 1969; Schirch and Diller, 1971).

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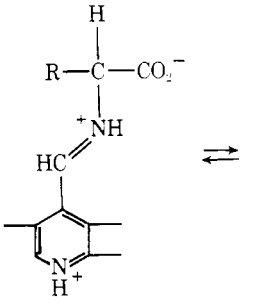
Studies of the Reactions of Lamb Liver Serine Hydroxymethylase with L-Phenylalanine: Kinetic Isotope Effects upon Quinonoid Intermediate Formation†

Richard J. Ulevitch‡ and Roland G. Kallen*

ABSTRACT: Serine hydroxymethylase (EC 2.1.2.1) reacts with L-phenylalanine in the absence of tetrahydrofolic acid to form an enzyme-bound amino acid–pyridoxal 5′-phosphate quinonoid intermediate (EQ_{503}) which is associated with the appearance of a new absorbance band at 503 nm. The enzyme also catalyzes the labilization of tritium from L-[α-^{3}H]phenylalanine. From the dependence upon the concentration of L-phenylalanine of the kinetic parameters for the approach to equilibrium and of the absorbance of the equilibrium concentration of EQ_{503}, the rate and/or equilibrium constants for the following minimal kinetic scheme were evaluated: E + S $\rightleftharpoons$ ES $\rightleftharpoons$ EQ_{503}, where ES is the enzyme-bound amino acid–pyridoxal 5′-phosphate Schiff base. The molar absorptivity of EQ_{503} at pH 8.00 is 4.1×10^4 M^{-1} cm^{-1} (per pyridoxal 5′-phosphate). Experiments with L-[α-^{1}H]- and L-[α-^{2}H]phenylalanine in H_2O and D_2O have established that there are kinetic deuterium isotope effects of 5–6-fold on the rate constant for the formation of EQ_{503} from ES and on the rate constant for the reverse reaction. These results are direct evidence that a hydrogen (proton) is transferred in the transition state of the ES $\rightleftharpoons$ EQ_{503} interconversion and, thus, for a general base catalytic contribution by the enzyme. The observed stereochemistry in which reactivity of serine hydroxymethylase is with L- and not D-phenylalanine is unexpected in terms of current formulations regarding pyridoxal 5′-phosphate mediated reactions.

Resonance stabilized quinonoid intermediates (II, eq 1) have been postulated to occur after the loss of the leaving group (α-hydrogen, CO_2, >C=O, etc.) from the Schiff bases formed from amino acids and pyridoxal 5′-phosphate (PLP)[1] during racemization, transamination, β elimination, decarboxylation, and dealdolization reactions catalyzed by PLP enzymes (Braunstein, 1947; Metzler et al., 1954; Morino and Snell, 1967; Jencks, 1969). In studies of several PLP model systems (Thanassi and Fruton, 1962; Matsumoto and Matsushima, 1972; Schirch and Slotter, 1966; Maley and Bruice, 1968) and enzymes (Morino and Snell, 1967; Jenkins, 1961; Schirch and Jenkins, 1964; Fonda and Johnson, 1970; Martinez-Carrion et al., 1970; Ulevitch and Kallen, 1977a) including serine hydroxymethylase (EC 2.1.2.1), an absorbance band at about 500 nm has been observed and assigned as a quinonoid intermediate.



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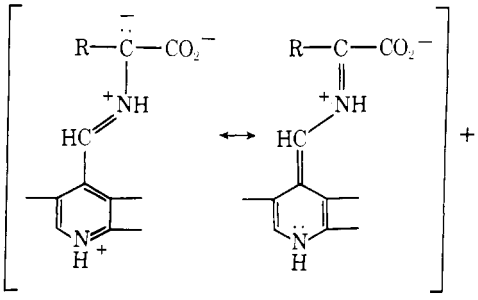

II (1)

During studies of the mechanism of the serine hydroxymethylase catalyzed cleavage of β-phenylserine to glycine and benzaldehyde (Ulevitch and Kallen, 1977a), the reaction of L-phenylalanine with the enzyme was investigated. A new

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[1] Abbreviations: buffer A, 0.05 M Hepes, 0.001 M EDTA, 0.025 M Na_2SO_4, pH 8.00; EDTA, ethylenediaminetetraacetic acid; E_t, total tetrameric enzyme (unless noted otherwise); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PLP, pyridoxal-5′-phosphate; THF, tetrahydrofolic acid.