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Mechanism of Serine Hydroxymethylase Catalyzed Cleavage of L-*erythro*-β-Phenylserine: pH Dependence of Elementary Kinetic Processes from Spectroscopic, Pre-Steady-State Kinetic, and Competitive Inhibition Studies†

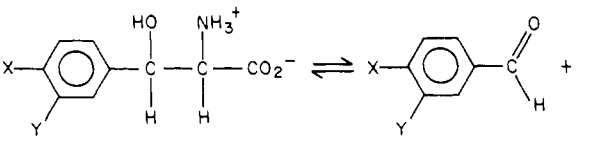
Wei-Mei Ching and Roland G. Kallen*

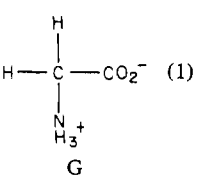
ABSTRACT: The serine hydroxymethylase catalyzed dealdolization reaction of L-*erythro*-β-phenylserine (S) to form benzaldehyde (B) and glycine (G) obeys the following minimal kinetic scheme

$$E + S \underset{K_1}{\rightleftharpoons} ES \overset{k_2}{\underset{\pm B}{\rightleftharpoons}} EQ \overset{k_3}{\rightleftharpoons} EG \overset{K_4}{\rightleftharpoons} E + G$$

in which ES and EG are the complexes formed by transimination sequences common to amino acid binding with pyridoxal 5′-phosphate (PLP) enzymes. The intermediate EQ (formed as a result of the C–C bond cleavage step) is an enzyme-bound quinonoid intermediate in which Q is $^{-}O_2CC^{-}H(N^{+}H{=}CHR)$ and R is the substituted pyridinium ring moiety of PLP. The apparent rate and equilibrium constants, K_1, k_2, and k_3, were evaluated from the dependence of k_{obsd} upon the concentration of S with the pre-steady-state rate equation, $k_{obsd} = k_2\{[S]/([S] + K_1)\} + k_3$ by monitoring EQ spectrophotometrically and were confirmed by appropriate steady-state kinetic and absorbance measurements for the pH range 6–10. The transimination sequence to form the ES complex, $1/K_1$, is controlled by an apparent pK_a value of 7.1. The rate constants for k_2 and k_3 exhibit sigmoid dependencies upon pH, increasing from 10 s^{-1} for both k_2 and k_3 to 450 s^{-1} and 130 s^{-1} in the acid limit with apparent pK_a values of 6.9 and 7.7 for k_2 and k_3, respectively. The agreement between pre-steady-state and steady-state parameters is quite good at pH >8 and is within threefold at the acid limit. The results are consistent with more highly protonated enzyme forms being more efficient catalysts due to (a) increases in the electron sink character of the PLP moiety; (b) microscopic ionization constant changes for neighboring groups which influence acid–base contributions by the enzyme; and/or (c) altered protein conformations which increase the reactivity of β-oxy anion and quinonoid intermediates.

Serine hydroxymethylase (EC 2.1.2.1) has been purified from bacteria and mammalian liver or kidney (Wilson & Snell, 1962; Schirch & Mason, 1963; Fujioka, 1968; Palekar et al., 1973; Jones & Priest, 1976; Kumar et al., 1976; Ulevitch & Kallen, 1977a). This pyridoxal 5′-phosphate (PLP)[1] requiring enzyme catalyzes the reversible cleavage of substituted β-phenylserines (S) to form substituted benzaldehydes (B) and glycine (G, eq 1). Recent pre-steady-state and steady-state kinetic and spectroscopic studies have provided evidence that the minimal kinetic mechanism of eq 2 is applicable to these





(1)

$$E + S \underset{K_1}{\overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}}} ES \overset{\pm B}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}} EQ_{500} \overset{k_3}{\rightarrow} G + E \qquad (2)$$

dealdolization reactions at pH 7.5 (Ulevitch & Kallen, 1977c) in which ES and EQ_{500} are enzyme–substrate intermediates,

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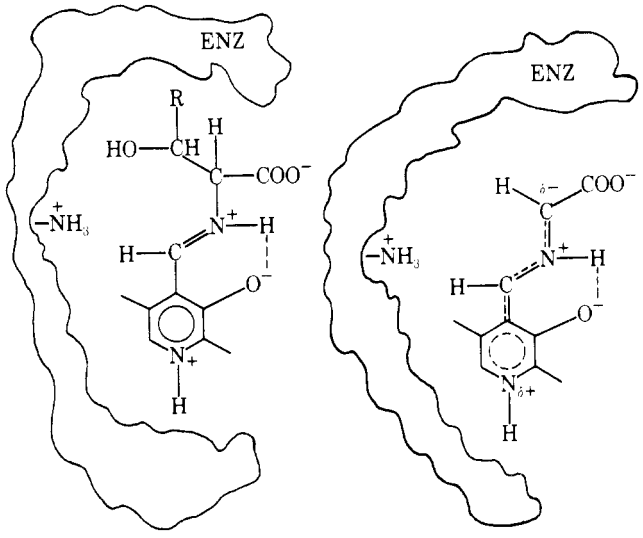
namely, the enzyme-bound Schiff base formed from PLP and amino acid and the enzyme-bound quinonoid conjugate base of the Schiff base formed from PLP and glycine, respectively. In eq 2 the immediate product of the carbon–carbon bond cleavage step, k_2, is a spectrophotometrically detectable enzyme-bound quinonoid intermediate (Q), and the protonation of the quinonoid intermediate is the process to which k_3 applies. The rate constants applicable to eq 2 have been evaluated for substituted L-*erythro*-β-phenylserines and correlations by linear free-energy relationships of the Hammett σ–ρ type are consistent with the chemical and kinetic mechanisms of eq 2.



ES (I) EQ_{500} (II)

In view of the capability for isolating elementary kinetic steps, this system appeared to provide an opportunity to study pH dependencies of such elementary steps to circumvent some of the ambiguities (Knowles, 1976) associated with the interpretation of pH dependencies in kinetic studies of less tractable systems. In this paper we report the results of these studies over the pH range 6 to 10 which are relevant to possible acid–base contributions of dealdolization reactions catalyzed by serine hydroxymethylase.

Experimental Section

Materials

(*1*) *Enzyme.* Serine hydroxymethylase (2.3 U/mg, A_{279}/A_{425} = 7.5 ± 0.5[2]) was purified from lamb liver by a slight modification of the procedure[2] previously described (Ulevitch & Kallen, 1977a) and exhibited similar stability characteristics. The gradient elution of the phosphocellulose column was performed prior to the CM-Sephadex (C-50) column chromatography and the step involving Sephadex G-200 column chromatography was omitted. The enzyme was stored at 4 °C in 0.05 M Hepes, pH 7.5, 10^{-3} EDTA, 0.025 M Na_2SO_4, 10^{-4} M PLP (Sigma), dialyzed for >12 h against the solution described for syringe A below, centrifuged at 10000 rpm for 20–30 min at 4 °C, and assayed for activity just prior to its utilization.

(*2*) *Chemicals.* D,L-*erythro*- and D,L-*threo*-β-phenylserine were synthesized according to the methods of Shaw & Fox (1953).

(*3*) *Buffers.* The buffers containing 10^{-3} M EDTA utilized in this work were the following: Mes (Sigma), pH <6.5; Hepes (Sigma), pH 6.5–8.5; and Dabco (Aldrich, purified by sublimation), pH >9.0. All other materials were of reagent grade and were utilized without further purification. Deionized water of greater than 5×10^5 ohms cm specific resistance was used throughout. Ionic strength was maintained at 0.1 M with Na_2SO_4.

Methods

(*1*) *Enzyme Activity.* Initial velocity measurements were obtained and analyzed as previously described (Ulevitch, 1971; Ulevitch & Kallen, 1977a).

(*2*) *Spectral Measurements.* Spectral measurements were recorded with a Cary Model 118 spectrophotometer at 23–25 °C. Difference spectra for enzyme–substrate intermediates employed stoppered tandem compartment mixing cells (Pyrocell) with a 0.44-cm light-path length per compartment.

(*3*) *Pre-Steady-State Kinetics.* Stopped-flow experiments were performed in a thermostated Durrum-Gibson stopped-flow spectrophotometer equipped with 2-cm light-path cell maintained at 25 ± 0.1 °C. Syringe A was filled with serine hydroxymethylase (12–36 μM active site concentration) in 5×10^{-4} M Hepes, pH 7.5, ionic strength 0.1 M. Syringe B was filled with substrate solution in 0.1 M buffer at ionic strength 0.1 M. These experiments constituted pH-jump experiments, and the enzyme was shown to be stable during the time period for which data are reported. Control experiments employing buffered (0.05 M) solutions at the same pH value of 6.8 in both syringes A and B yielded identical results. The pH values reported are those for final reaction mixtures as indicated in the above-mentioned pH-jump experimental protocol. Transmittance values at 500 nm from two superimpossible oscilloscope traces were converted to $\Delta A = A_\infty - A_t$, where A_∞ and A_t are the absorbance values at steady state and at a given time after mixing, respectively, and were analyzed as described elsewhere (Kallen & Jencks, 1966; Kallen, 1971a). The data are estimated to have ±15% errors, when standard deviations are not reported.

(*4*) *Steady-State Absorbance.* The absorbance at 500 nm upon mixing of serine hydroxymethylase and substrates were obtained either from oscilloscope traces of stopped-flow experiments (A_∞ values, see above) or from steady-state experiments with manual mixing of tandem compartment mixing cells and Gilford 2000 or Cary 118 recording spectrophotometers by extrapolation to time zero (in actuality, steady state).

(*5*) *Proton NMR Measurements.* The chemical shifts of the α hydrogen of serine or of the serine–PLP Schiff base generated in situ from a mixture of serine (0.025 M) and PLP (0.025 M) in D_2O were measured by 220-MHz NMR (HR-220 Varian) at 15 °C, using 2,2-dimethylsilapentane-5-sulfonic acid as internal reference.

Amino acid proton dissociation constants were determined titrimetrically as described elsewhere (Kallen, 1971b).

Measurements of pH were obtained with a Radiometer Model 26E pH meter or Model 63 digital pH meter equipped with combined electrodes (GK2302B) calibrated with standard buffers (Beckman) at pH 4, 7, and 10.

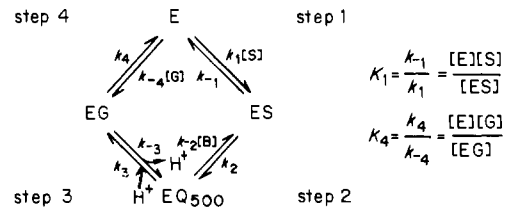
Results

Pre-Steady-State Kinetic and Steady-State Absorbance Studies. The appearance of a new absorbance peak at 500 nm with identical λ_{max} and width at half-height values upon mixing a variety of substituted L-*erythro*- and L-*threo*-β-phenylserines (H, 4-NO_2, and 3-CH_3O, 4-HO-) and glycine

[1] Abbreviations used: a_{H^+}, hydrogen ion activity; Dabco, 1,4-diazabicyclo[2.2.2]octane; Hepes, *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; Q, quinonoid intermediate; EDTA, ethylenediaminetetraacetic acid.

[2] See Ching (1977).

Scheme I



$$K_1 = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$

$$K_4 = \frac{k_4}{k_{-4}} = \frac{[E][G]}{[EG]}$$

with serine hydroxymethylase at pH 7.5 has been attributed to the formation of a common quinonoid intermediate, Q (Ulevitch & Kallen, 1977c), by the release of the carbonyl compounds.

The time course for the approach to steady state, obtained from stopped-flow experiments in which the absorbance at 500 nm is monitored, is described by a single exponential, k_{obsd} (Figure 1A). The minimal kinetic Scheme I has been shown to be applicable to this system at pH 7.5 where ES and EG are the enzyme-bound Schiff bases of PLP with substrate and glycine, respectively (Ulevitch & Kallen, 1977c). Steps 1 and 4 are transimination sequences involved in substrate binding and glycine release, respectively, step 2 involves the carbon–carbon bond cleavage step (carbon–carbon bond formation in the reverse reaction), and step 3 represents protonation of the quinonoid intermediate to form EG prior to glycine release (α-hydrogen removal from EG to form EQ in the reverse reaction). For this formulation, with the application of the rapid equilibrium assumptions to steps 1 and 4, in the absence of added glycine and benzaldehyde and with the irreversibility of step 3 under the experimental conditions employed, the substrate concentration dependence of k_{obsd} is given by eq 3 (Ulevitch & Kallen, 1977c) for the apparent parameters at any given pH value.

$$k_{obsd} = k_2\{[S]/([S] + K_1)\} + k_{-2}[B] + k_3 \qquad (3)$$

The dependence of k_{obsd} upon L-*erythro*-β-phenylserine concentration at a given pH value is described by a rectangular hyperbola with a finite ordinate intercept k_3^{app}, and an asymptote at $[S] \gg K_1^{app}$ such that $k_{obsd} = k_2^{app} + k_3^{app}$. The substrate concentration at the half-maximal substrate dependent increment in k_{obsd} is given by K_1^{app}. Experimental results at pH 9.0 and 10.0 (Figure 1B) indicate that the rectangular hyperbolas are shallow leading to less precise determinations of K_1^{app} values from this type of experiment. However, the steady-state absorbance values are also determined by the same kinetic parameters, K_1^{app}, k_2^{app}, and k_3^{app}, the concentrations of substrate and enzyme, and the molar absorptivity value of EQ, ϵ_{500}^{EQ}, as described by eq 4[3] where

$$A_{500} = [EQ]\epsilon_{500}^{EQ} = \frac{[E_T]\epsilon_{500}^{EQ}\left(\frac{k_2^{app}}{k_2^{app} + k_3^{app}}\right)[S]}{[S] + \frac{K_1^{app}k_3^{app}}{k_2^{app} + k_3^{app}}} = \frac{A_{500}^{max}[S]}{[S] + K_m^{app}} \qquad (4)$$

E_T is the enzyme active site concentration based upon a subunit molecular weight of 55 000 (Ulevitch & Kallen, 1977c). Thus, the rectangular hyperbolic dependence of A_{500} upon the substrate concentration provides additional experimental data for the determinations of K_1^{app}, k_2^{app}, k_3^{app}, and, as well, ϵ_{500}^{EQ}. The data for experiments at pH 9 and 10 (Figure 1C) indicate that K_m^{app} values are satisfactorily determined in this type of experiment.

[3] The extinction coefficient calculated from eq 4 is very sensitive to the variations in k_2 and k_3.

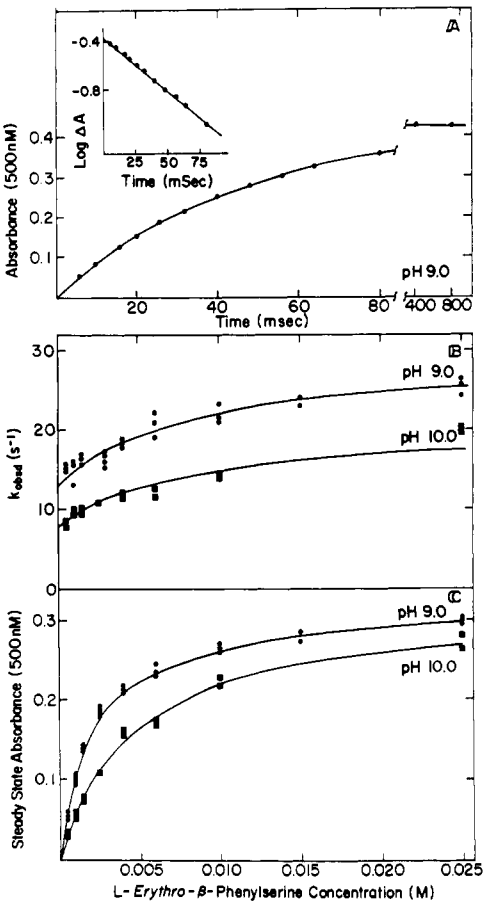


FIGURE 1: (A) The approach to steady state at 500 nm upon mixing of serine hydroxymethylase (10.3 μM active site concentration) and L-*erythro*-β-phenylserine (0.01 M). Reaction conditions after mixing: Dabco, 0.05 M, pH 9.0, ionic strength 0.1 M, 25 °C. Solid lines are calculated. $\Delta A = \Delta A_{max}(1 - e^{-k_{obsd}})$, where t = time, $\Delta A_{max} = 0.437$, and $k_{obsd} = 23.2\ s^{-1}$. (B) The dependence of the pseudo-first-order rate constant, k_{obsd}, for the appearance of EQ upon the concentration of L-*erythro*-β-phenylserine. For pH 9.0 (●) and 10.0 (■) after mixing. [Serine hydroxymethylase (2.3 U/mg)] = 10.3 and 16.5 μM active site concentration, respectively; [Dabco] = 0.05 M, ionic strength 0.1 M, 25 °C. Solid lines for pH 9.0 and 10.0 are calculated from eq 3, and $K_1 = 0.008$, 0.010 M; $k_2 = 15.9$, 13.0 s^{-1}; and $k_3 = 13.2$, 8.2 s^{-1}, respectively. (C) The steady-state absorbance at 500 nm upon mixing serine hydroxymethylase with various concentrations of L-*erythro*-β-phenylserine. The reaction conditions are those described in the legend of B.

The pH dependence of the constants for ES complex formation, $1/K_1^{app}$ (Figure 2A), for carbon–carbon bond cleavage, k_2^{app} (Figure 2B), and for protonation of the quinonoid intermediate, k_3^{app} (Figure 2C), based upon the data of Table I is fit to apparent pK_a values of 7.1, 6.9, and 7.7, respectively.

Complex Formation between Enzyme and L-*Serine or Glycine.* More precise data for ES or EG complex formation result from inhibition studies in which glycine or L-serine is employed as a competitive inhibitor (Figure 3) of the serine hydroxymethylase catalyzed dealdolization of L-*erythro*-β-phenylserine as described by eq 5 (Dixon, 1953), where E_T

$$\frac{[E_T]}{v_i} = \frac{K_m^{app}(1 + [I]/K_i^{app})}{TN^{app}[S]} + \frac{1}{TN^{app}} \qquad (5)$$

is the active site concentration, v_i is the initial velocity, I is the inhibitor, TN^{app} is the apparent turnover number (per active site), and K_m^{app} and K_i^{app} are the apparent (i.e., pH dependent) Michaelis constants for L-*erythro*-β-phenylserine

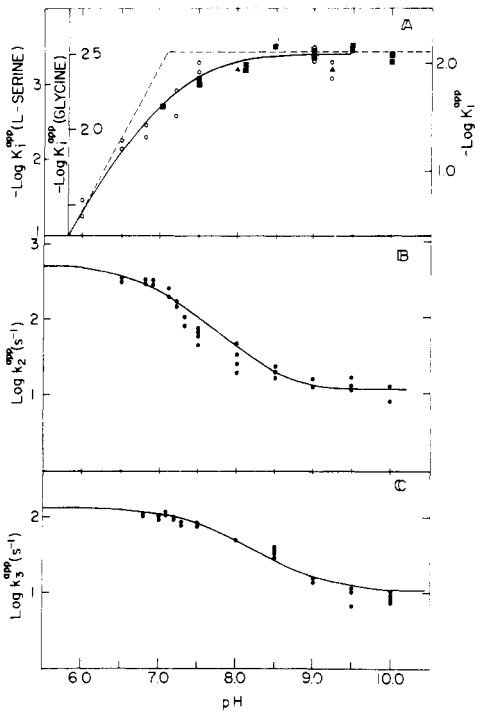


FIGURE 2: (A) The pH dependence of association constants for binary complex formation from serine hydroxymethylase and L-*erythro*-β-phenylserine, ■ ($1/K_1$ in Table I), glycine, ▲ ($1/K_4$ in Table I), and L-serine, ○ ($1/K_1$ in Table I). For the latter two compounds the association constants are from inhibition studies (see text). The apparent pK_a value is 7.10 ± 0.13. (B and C) The pH dependence of the C–C bond cleavage step (k_2) (Figure 2B) and the protonation of the enzyme bound quinonoid intermediate (k_3) (Figure 2C) in the serine hydroxymethylase catalyzed cleavage of L-*erythro*-β-phenylserine from pre-steady-state kinetic measurements at 500 nm, 25 °C, ionic strength, 0.1 M. The apparent pK_a values, acid, and alkaline limiting rate constants are: k_2, 6.9, 450 s^{-1}, 10 s^{-1}, and k_3, 7.7, 130 s^{-1}, 10 s^{-1} (Tables I and II).

and inhibition constants for glycine (or L-serine), respectively.

There is no detectable absorbance at 500 nm upon mixing L-serine with serine hydroxymethylase, in the absence of other ligands, which indicates that there is no detectable carbon–carbon cleavage occurring (cf. Chen & Schirch, 1973a–c). The K_i^{app} value then refers to a dissociation constant describing the equilibrium ES ⇌ E + S; i.e., $K_i^{app} = K_1^{app}$.

For glycine interactions with serine hydroxymethylase, the K_i^{app} value at a given pH is given by eq 6 (Ulevitch & Kallen, 1977c), but under the conditions of the present study the

$$K_i^{app} = K_4^{app}\{k_3^{app}/(k_3^{app} + k_{-3}^{app})\} \quad (6)$$

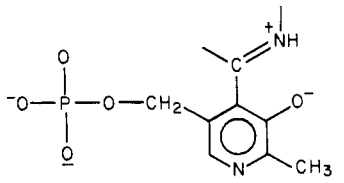
amount of absorbance at 500 nm attributable to α-hydrogen removal from EG, k_{-3}^{app}, to form EQ is negligible and $K_i^{app} \sim K_4^{app}$. The data for EG complex formation from glycine and serine hydroxymethylase appear to be superimposable on those for $1/K_1$ with L-*erythro*-β-phenylserine and L-serine (when normalized), and the apparent pK_a value of 7.1 (Figure 2A) applies to the data for the three compounds.

Estimation of the β-Alcoholic Proton Dissociation Constant for the Schiff Base Formed from Pyridoxal 5′-Phosphate and β-Phenylserine. An estimate of the inductive effect substituent constant value, σ^I, for W (Taft, 1953; Charton, 1964) was obtained from the correlation of the chemical shifts of the α hydrogen of zwitterionic (3.84 ppm) and anionic (3.34 ppm) serine, $HOCH_2CH(R)CO_2^-$, in D_2O at pD values of 7.2 and 12.0, where R = NH_3^+ ($\sigma^I = 0.58$) and R = NH_2 ($\sigma^I = 0.05$), respectively (Taft, 1953; Charton, 1964), which yields $\rho^I = (3.84 - 3.34)/(0.58 - 0.05) = 0.94$. The α hydrogen of the Schiff base formed from an equimolar solution of L-serine and

Table I: pH Dependence of Rate and Dissociation Constants for the Serine Hydroxymethylase Catalyzed Dealdolization of L-*erythro*-β-Phenylserine, 25 °C, Ionic Strength 0.1 M

constant[a]	pH 6.0	6.5	6.8	7.0	7.1	7.2	7.3	7.5	8.0	8.5	9.0	9.3	9.5	10.0
K_1[b] (M)								0.0164	0.011	0.0071	0.0084		0.0069	0.0087
				(0.024)				(0.013)[j]	(0.012)[j]		(0.0079)		(0.0070)	0.0100
K_1[c] (M)	0.0075	0.0062	0.0023			0.0015		0.0007[j]			0.00064	0.00082		
K_4[d] (M)									0.004[j]		0.0031	0.004		
k_2[e] (s^{-1})		320	300	290	206	157	94	60	32	19.4	14.0		13.5	10.2
k_{-2}[e] (s^{-1})								4.1×10^4[j]						2.6×10^3
K_3[f]						0.0118	0.0144	0.0123	0.0104	0.009	0.022		0.031	0.077
k_3[e] (s^{-1})		133	112	104	115	97	82	82	55	34	14.4		9.8	8.6
ϵ_{500}^{EQ}[g] (M^{-1} cm^{-1})				2.76×10^4	3.19×10^4	2.65×10^4	2.76×10^4	3.46×10^4	4.21×10^4	5.33×10^4	5.60×10^4		4.47×10^4	3.91×10^4
TN[h] (s^{-1})	28.6			33		28		22	13.5		6.2	4.55		
K_m[i] (M)	0.12	0.044	0.023			0.015		0.0095	0.007	0.0045	0.0043	0.0059		
				0.023				0.0082	0.0067		0.002		0.0044	0.0034
				0.006				0.0075	0.0076		0.0038		0.0031	0.0042

[a] See Scheme I and text for definition of constants. [b] For L-*erythro*-β-phenylserine. Calculated from $K_m = K_1 k_3/(k_2 + k_3)$ and the values of k_2, k_3, and K_m contained in this table, except for the values at pH 9.5 and 10, for which K_m was taken as 0.004 M, the asymptotic value from pH dependence of K_m at high pH (Figure 4A). The values in parentheses were obtained from the substrate dependence of k_{obsd} (eq 3). [c] For L-serine from inhibition studies (see text). [d] From glycine inhibition studies (see text). [e] From pre-steady-state experiments (see text). [f] From the equilibrium absorbance upon mixing enzyme with glycine and values of ϵ_{500}^{EQ} in this table (see text); $K_3 = k_{-3}/k_3$. [g] From steady-state absorbance measurements, eq 4. [h] From steady-state (turnover) experiments. [i] The values from steady-state (turnover) experiments and steady-state absorbance measurements, calculated from $K_m = K_1 k_1/(k_2 + k_3)$, are the upper middle and lower values, respectively. [j] Ulevitch & Kallen, 1977c.



W

PLP (0.025 M) in situ at pD 7.75–8.77 (Tobias & Kallen, 1978) exhibited a chemical shift of 3.81 ppm which yields a σ^I value for W of 0.55 from 3.81 − 3.34 = 0.94σ^I.

The pK_a values of primary alcohols have been correlated by a ρ_I value of −8.2 (Takahashi et al., 1971). Thus, starting with the pK_a value of 15.4 for benzyl alcohol (Takahashi et al., 1971), substituting for a benzylic hydrogen by CH_3 (σ^I = −0.05), the methyl hydrogens of $PhCH(CH_3)OH$ by $-CO_2^-$ (σ^I = 0.05) and W (σ^I = 0.55, see above), and employing the principle of additivity of substituents and a fall-off factor of 2.5 for the intervening carbon atom (Hammett, 1970), the pK_a value for the β-alcoholic group of the β-phenylserine–PLP Schiff base (not protonated at the pyridine nitrogen atom) is estimated at 13.8 in aqueous solution.

Discussion

pH Dependence of Binary Complex Formation. The three types of complexes that have been studied in the reactions of serine hydroxymethylase are those formed between enzyme and glycine, L-serine, and L-*erythro*-β-phenylserine. Each of these three molecules can in principle form additional enzyme amino acid complexes, most importantly the enzyme-bound quinonoid intermediate, EQ, but in the cases of glycine and L-serine the enzyme partition function is dominated by complexes EG and ES at relatively high concentrations of ligand (i.e., the fraction of enzyme that exists as EQ is negligible) enabling the approximation $K_i^{app} \sim K_4$ and K_1, respectively, from the inhibition by glycine and L-serine of the steady-state kinetics.

The pre-steady-state kinetic and steady-state absorbance studies outlined above for L-*erythro*-β-phenylserine enable the evaluation of the K_1^{app}, k_2^{app}, k_3^{app}, and apparent ϵ_{500}^{EQ} values. Due to technical reasons, these data for binary complex formation, K_1^{app}, are less precise than those obtained from K_i^{app} values for glycine and L-serine (see above) and the data have been combined (with normalization) in Figure 2A. The formation of enzyme–amino acid complexes is pH independent in the pH range 8.5–10.0 and is pH dependent from pH 6 to 8.5, characterized by an apparent pK_a value of about 7.1. The data in these two pH regions which are similar to data for the rabbit liver cytoplasmic serine hydroxymethylase (Liu & Haslam, 1974) will be discussed sequentially.

In order to observe a pH-independent binding function in a pH range in which ligands are undergoing a change in ionization state, the affinity of the enzyme for the conjugate acid and base forms of the ligand must be the same; i.e., $K_b = K_b'$ in Subscheme A, where X = glycine, L-serine, or L-*erythro*-β-phenylserine and the relevant $pK_{a_2}^X$ values are 9.6, 9.2, and 8.8, respectively, for these ligands (Table II).

The ionic form of the enzyme that binds to the two ionic states of ligand, $XH^{\pm}$ and X^-, is depicted as EH^+ throughout this pH range and is *not* meant to indicate that groups on the enzyme are not undergoing ionization state changes in this pH range, but rather that any groups that do ionize have no effect upon the affinity of the enzyme for ligand and are therefore undetected. The consequence of a thermodynamic square such as that presented in Subscheme A when $K_b = K_b'$ is that $K_{a_2}^X = K_{a_3}^{EX}$, in other words, that enzyme–ligand binary complex and ligand have the same proton dissociation constants. Note that, while the equality $K_{a_2}^X = K_{a_3}^{EX}$ must hold, the groups involved in the proton dissociations from the ligand and from the binary complex need not be and are almost certainly not

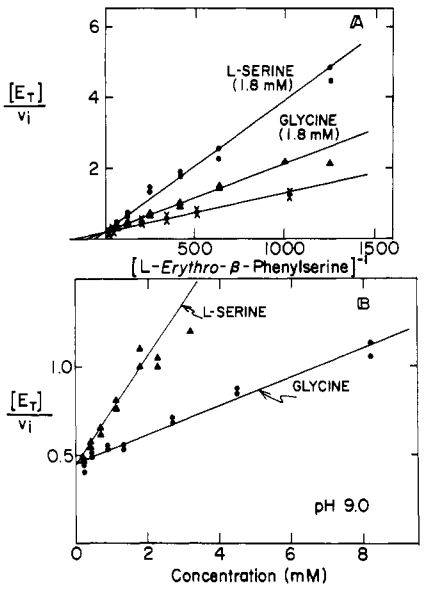


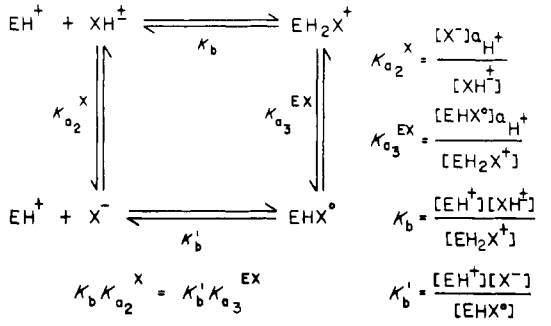
FIGURE 3: (A) Double-reciprocal plots of the serine hydroxymethylase (2.6–5.2 nM, active site concentration) catalyzed dealdolization of L-*erythro*-β-phenylserine (1.0–36 mM) at pH 9.30 (Dabco, 0.05 M), ionic strength 0.1 M, 25 °C. (X) without inhibitor; (▲) 1.82 mM glycine; (●) 1.82 mM L-serine. (B) Competitive inhibition of serine hydroxymethylase (3–6 nM active site concentration) catalyzed dealdolization of L-*erythro*-β-phenylserine (3.3 mM) by glycine (0.23–8.2 mM), and L-serine (0.25–3.2 mM) at pH 9.0 (Dabco, 0.05 M) ionic strength 0.1 M, 25 °C. Solid lines are calculated from $[E_T]/v_i = K_m(1 + [I]/K_i)/(TN[S]) + 1/TN$, where ordinate intercept = $K_m/(TN[S]) + 1/TN$, slope = $K_m/(K_iTN[S])$, ordinate intercept/slope = $K_i(1 + [S]/K_m)$, K_m = 4.4 mM, TN = 5.4 s^{-1}, K_i(glycine) = 3.1 mM, and K_i(L-serine) = 0.8 mM.

Table II: Summary of pH-Independent Constants (Scheme II) for Serine Hydroxymethylase and L-*erythro*-β-Phenylserine, Glycine, and L-Serine, Ionic Strength 0.1 M, 25 °C

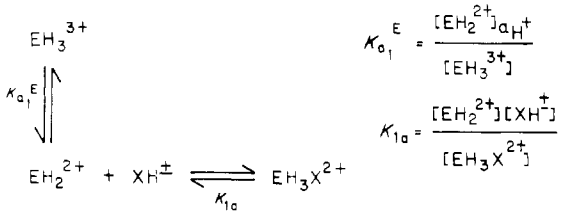
	ligand		
constant[a]	Gly	L-Ser	L-*erythro*-β-phenyl-Ser
$pK_{a_2}^S$	9.6[c]	9.2[c]	8.8[c]
$pK_{a_1}^E$	7.10 ± 0.13[d]	7.10 ± 0.13[d]	7.10 ± 0.13[d]
$pK_{a_2}^E$			6.90 ± 0.10[b]
K_{1a}, K_{1b}, K_{1b}' (mM)		0.64 ± 0.07[d]	7.7[d]
k_{2a} (s^{-1})			450 ± 22[e]
k_{2b}, k_{2b}' (s^{-1})			10 ± 3[e]
k_{3a} (s^{-1})			127 ± 6[f]
k_{3b}, k_{3b}' (s^{-1})			11.0 ± 3.2[f]
K_{4a}, K_{4b}, K_{4b}' (mM)	4.0[g]		
TN_a (s^{-1})			30 ± 6,[h] 96[i]
TN_b (s^{-1})			5.2[i]
K_{mb} (mM)[j]			4[k]
$pK_{a_2}^{ES}$ or $pK_{a_2}^{EG}$	6.9 ± 0.10[e]		6.9 ± 0.10[e]
$pK_{a_3}^{ES}$ or $pK_{a_3}^{EG}$	9.6[l]	9.2[l]	8.8[l]
$pK_{a_2}^{EQ}$			7.7 ± 0.11[m]

[a] Defined in Scheme II and text: $K_{a_2}^S = [X^-]a_{H^+}/[XH^{\pm}]$, where X = glycine, L-serine, or L-*erythro*-β-phenylserine. [b] See Scheme II, Figure 2B, and text. [c] At ionic strength 1.0 M. [d] From Figure 2A. [e] From Figure 2B. [f] From Figure 2C. [g] See Table I. [h] From Figure 4B. [i] Calculated from $TN = k_2k_3/(k_2 + k_3)$. [j] Calculated from $K_m = K_1k_3/(k_2 + k_3)$. [k] From Figure 4A. [l] See Scheme II and text. [m] See Scheme II, Figure 2C, and text.

Subscheme A



Subscheme B

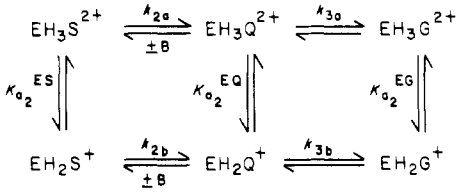


the same (see above). Furthermore, in the application of this Subscheme A to the specific amino acids glycine and serine, while $K_{a_2}^{G} = K_{a_3}^{EG}$ and $K_{a_2}^{S} = K_{a_3}^{ES}$, note that $K_{a_2}^{G} \neq K_{a_2}^{S}$ and, significantly, $K_{a_3}^{EG} \neq K_{a_3}^{ES}$ (Scheme II). This means that inasmuch as glycine and the serines are different molecules, and so too are the complexes EG and ES, their respective pK_a values may be different and are constrained only within the homologous system.

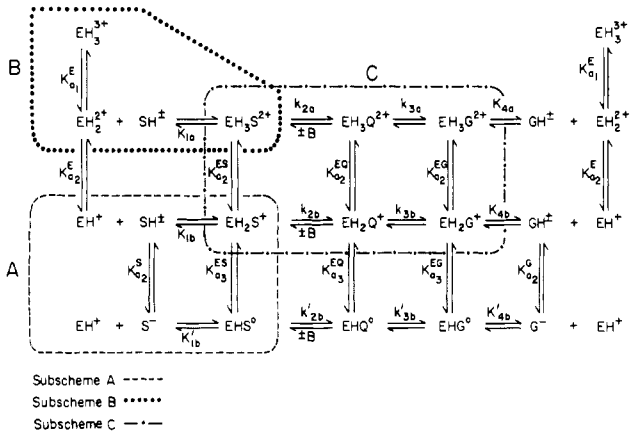
The pH dependencies for the apparent constants for the formation of the binary complexes from serine hydroxymethylase and glycine, L-serine, and L-*erythro*-β-phenylserine in the pH range 6–8.5 indicate that the affinity of the enzyme for ligands decreases with increasing acidity, characterized by an apparent pK_a value of about 7.1 (Figure 2A). Since the ligands undergo no change in ionization state in the pH range <7, the pH dependence of binary complex formation must be governed by an ionization of the enzyme itself, such that the more protonated form of the enzyme has undetectable affinity for these ligands. Subscheme B is the simplest formulation which accounts for these observations, where X = glycine, L-serine, or L-*erythro*-β-phenylserine. Although in formal terms one might introduce the species EH_4X^{3+}, with the formation of a thermodynamic square, the experimental observations are accounted for without the inclusion of such a species; i.e., the apparent association constants appear to approach zero with increasing acidity rather than to approach a plateau at a finite ordinate value (Figure 2A). The omission of EH_4X^{3+} from Subscheme B is tantamount to the statement that EH_4X^{3+} has negligible stability.

pH Dependencies of Rate Constants for Elementary Kinetic Processes. C–C Bond Cleavage and Protonation of the Enzyme-Bound Quinonoid Intermediate. The sigmoid-shaped pH dependencies of the apparent rate constants for both the C_β–C_α bond cleavage step, k_2^{app}, and the protonation of the enzyme-bound quinonoid intermediate (EQ), k_3^{app}, obtained from pre-steady-state kinetic studies are presented in Figures 2B and 2C, respectively. Both kinetic processes clearly exhibit *no* tendency for rate constants to approach zero, but rather they tend to level off at substantial ($\sim$10 s^{-1}) pH-independent rate constants at pH values >8.5 (Figures 2B and 2C). Such behavior is consistent with the minimal kinetic Subscheme C in which the acidic asymptotes for k_2^{app} and k_3^{app} are given by k_{2a} and k_{3a}, the basic asymptotes for k_2^{app} and k_3^{app} are

Subscheme C



Scheme II



given by k_{2b} and k_{3b}, and the apparent pK_a values are p$K_{a_2}^{ES}$, p$K_{a_2}^{EG}$, and p$K_{a_2}^{EQ}$ for the proton dissociation of the binary complexes formed from enzyme and L-*erythro*-β-phenylserine (ES), glycine (EG), and the quinonoid intermediate (EQ), respectively, with the values contained in Table II. In Subscheme C thermodynamic squares and reverse reactions have been introduced in order to accommodate the known reversibility of the entire reaction sequence. The value of p$K_{a_2}^{EG}$ of 6.9 for the EG complex from lamb liver is quite comparable to the value of 6.9 reported for the EG complex from rabbit liver serine hydroxymethylase (Schirch & Diller, 1971).

Fusion of Subschemes A, B, and *C to Form Scheme II.* A complete and symmetrical Scheme II, constructed from the fusion of Subschemes A–C, accounts for the experimental observations (Figures 2 and 3) and the known reversibility of the overall reaction. There are two aspects of this formulation that deserve comment.

First, the introduction of the ionization constant, $K_{a_2}^{E}$, for the proton dissociation reaction, $EH_2^{2+} \rightleftharpoons EH^+ + H^+$, in the formal linkage of Subschemes B and C, which have in common species EH_3S^{2+}, requires that $K_{a_2}^{E} = K_{a_2}^{ES}$, in order that K_{1a} and K_{1b} be equal in accord with the experimental observations (Figure 2A). Under these circumstances the pH dependence of the binding function is totally insensitive to $K_{a_2}^{ES}$ and remains governed by $K_{a_1}^{E}$ (as in Subscheme B, since $K_{a_2}^{E}$ and $K_{a_2}^{ES}$ completely offset each other, eq 7) in contrast to the

$$\frac{1}{K_1^{app}} = \frac{1}{K_{1a}} \frac{\left(1 + \frac{K_{a_2}^{ES}}{a_{H^+}} + \frac{K_{a_2}^{ES}K_{a_3}^{ES}}{a_{H^+}^2}\right)}{\left(1 + \frac{K_{a_2}^{E}}{a_{H^+}} + \frac{a_{H^+}}{K_{a_1}^{E}}\right)\left(\frac{K_{a_2}^{S}}{a_{H^+}} + 1\right)} \quad (7)$$

kinetic function, k_2^{app}, which is highly pH dependent, being governed by $K_{a_2}^{ES}$ (as in Subscheme C). The constellation of functional groups, the changing ionizaton state of which is responsible for the apparent ionization constant, $K_{a_2}^{E}$, may be (1) significantly involved with the kinetic events but not all with binding processes or (2) involved in both but with

whatever alterations in stability of EH_3S^{2+} caused by protonation of EH_2S^+ completely compensated for by equivalent alterations in the stability of EH_2^{2+} caused by protonation of EH^+ (perhaps even at totally different sites).

Second, the intermeshing of Subschemes A and C, which have in common species EH^+, $XH^{\pm}$, and EH_2X^+, requires the insertion of species EHQ° and the rate constants k_{2b}' and k_{3b}' leading to and away from EHQ° in order to maintain the pH independence of the k_2^{app} and k_3^{app} values in the pH region in which the ionizations $K_{a_2}^S$, $K_{a_2}^G$, $K_{a_3}^{ES}$, and $K_{a_3}^{EG}$ occur (see above). With the following equalities, $k_{2b} = k_{2b}'$ and $k_{3b} = k_{3b}'$, the k_2^{app} and k_3^{app} values remain pH independent at pH values greater than 8.5 (eq 8 and 9).

$$k_2^{app} = k_{2a}\alpha_{EH_3S^{2+}} + k_{2b}\alpha_{EH_2S^+} + k_{2b}'\alpha_{EHS^\circ} \quad (8)$$

$$k_3^{app} = k_{3a}\alpha_{EH_3Q^{2+}} + k_{3b}\alpha_{EH_2Q^+} + k_{3b}'\alpha_{EHQ^\circ} \quad (9)$$

where

$$\alpha_{EH_3X^{2+}} = \frac{1}{1 + \frac{K_{a_2}^{EX}}{a_{H^+}} + \frac{K_{a_2}^{EX}K_{a_3}^{EX}}{a_{H^+}^2}}$$

$$\alpha_{EH_2X^+} = \frac{1}{1 + \frac{a_{H^+}}{K_{a_2}^{EX}} + \frac{K_{a_3}^{EX}}{a_{H^+}}}$$

$$\alpha_{EHX^\circ} = \frac{1}{1 + \frac{a_{H^+}}{K_{a_3}^{EX}} + \frac{a_{H^+}^2}{K_{a_2}^{EX}K_{a_3}^{EX}}}$$

and X = S or Q for eq 8 and 9, respectively.

Concordance of Steady-State and Pre-Steady-State Experiments. The data (Figures 1–3) illustrate comparisons between observation and calculation (solid lines). That the pre-steady-state observations obtained at high enzyme concentrations correspond to measurements made at low enzyme concentrations under steady-state "turnover" conditions is evidenced by the satisfactory comparison between the measured turnover number (TN^{app}) and K_m^{app} (from steady-state experiments) with the values based upon the same parameters calculated from pre-steady-state experiments for which $TN^{app} = k_2^{app}k_3^{app}/(k_2^{app} + k_3^{app})$ and $K_m^{app} = K_1^{app}k_3^{app}/(k_2^{app} + k_3^{app})$ in the pH range 7.5–9.0 (Figures 4A and 4B) (Ulevitch, 1971; Ulevitch & Kallen, 1977c).

In the more acidic pH region, the behavior of the parameters is qualitatively similar but the quantitative agreement is not as good with the differences between steady-state and pre-steady-state experiments up to threefold. There are three possible explanations for such differences (since control experiments indicate that the pH-jump protocol employed had no influence on the results): (i) the pre-steady-state and steady-state kinetic experiments involve enormously different concentrations of enzymes. In the case of chymotrypsin one finds precedent for concentration- and pH-dependent polymerization processes (Rao & Kegeles, 1958) and, as well, pH-dependent partitioning between active and inactive forms (McConn et al., 1969). Many other such examples can be cited that might be associated with different forms and, thus, different activities at high and low enzyme concentration. (ii) Turnover experiments require that the enzyme release product and recycle to interact with another substrate molecule. Should product release or some step in the return of the enzyme to the form that accepts substrate become rate determining (conformation change or proton transfer), then correspondence between single and multiple turnover experiments are not to be expected (Schirch, 1975; Schirch et al., 1977; Akhtar et al., 1975). (iii) The kinetic Scheme I requires some modification for application to the most acidic range studied, a possibility that only further experiments can substantiate.

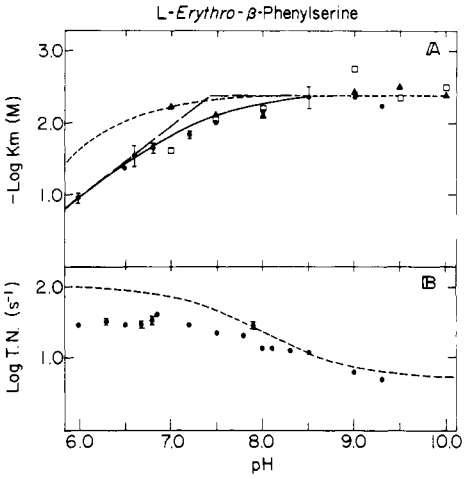


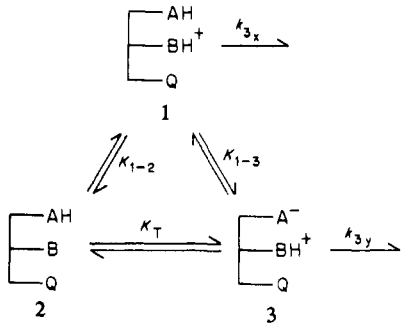
FIGURE 4: (A) Dependence of K_m for the reaction of L-*erythro*-β-phenylserine with serine hydroxymethylase (2.3 U/mg) upon pH from steady-state experiments. Dashed line is calculated from values of K_1, k_2, k_3 in Table II and $K_m^{app} = K_1^{app}k_3^{app}/(k_2^{app} + k_3^{app})$, where $K_1^{app} = K_{1a}/\alpha$, $k_2^{app} = k_{2a}(1 - \alpha) + k_{2b}\alpha$, $k_3^{app} = k_{3a}(1 - \alpha) + k_{3b}\alpha$, where $\alpha = 1/(1 + [H^+]/K_a)$ and $K_a = K_{a_1}^E$, $K_{a_2}^{ES}$, and $K_{a_2}^{EQ}$ for K_1^{app}, k_2^{app}, and k_3^{app}, respectively. (●) Steady-state kinetics; (□) steady-state absorbance; (▲) calculated from pre-steady-state kinetic studies. (B) Dependence of TN (per active site) for the reaction of L-*erythro*-β-phenylserine with serine hydroxymethylase (2.3 U/mg) upon pH from steady-state experiments. Dashed line is calculated from values of k_2 and k_3 in Table II and $TN = k_2^{app}k_3^{app}/(k_2^{app} + k_3^{app})$, where k_2^{app} and k_3^{app} are calculated in the same way as described in A.

Mechanism of Serine Hydroxymethylase Catalyzed Dealdolization. Step 3: Interconversion of EQ and EG. In terms of the chemical mechanism the most uncomplicated step would appear to be the protonation of the enzyme-bound quinonoid intermediate, EQ_{500}(II). It was anticipated that the rate constant for proton donation by a group on the enzyme might follow the titration curve of that group with the apparent rate constant, k_3^{app}, increasing from approximately zero as the fraction of protonated group increased with increasing acidity. Although a decrease in k_3^{app} occurs with increasing pH, it is surprising that at the alkaline extreme the k_3^{app} values are not asymptotic to zero but level off at about 10 s^{-1}, a very appreciable rate constant. Three possible explanations for this behavior are, first, that solvent water or the conjugate acid of the buffer has become the proton donor, protonating the enzyme bound quinonoid intermediate at 10 s^{-1} (Figure 2C); second, that the titration curve observed for k_3^{app} is not of the proton donor directly but of an adjacent perturbing group (see below); and third, that the state of ionization of the electron sink (PLP) portion of the quinonoid intermediate or an adjacent group is changing with changing pH.

The first possibility, assuming access of solvent to the active site,[4] might be expected to proceed at or close to diffusion-controlled rates as do the protonation rates for cyanide ion (Stuchr et al., 1963) and some other carbanions (Eigen, 1964). If this is the case, the pK_a value of the enzyme-bound qui-

[4] Solvent access at the active site has been discussed (Kallen, 1971c; Tatum et al., 1977) and appears likely in the present studies in view of the experiments demonstrating equilibration of protonated enzyme groups with solvent during serine hydroxymethylase catalyzed reactions (Ulevitch & Kallen, 1977a,b).

Scheme III



$K_{a_2}^{EQ} = ([2] + [3])a_{H^+}/[1] = K_{1-2} + K_{1-3}$
$K_T = [3]/[2]$
$K_{1-3} = [3]a_{H^+}/[1]$
$K_{1-2} = [2]a_{H^+}/[1]$

nonoid intermediate is estimated at 5 for protonation by water from $k_{3b} = 10\ s^{-1} \simeq 10^{10}(1/(1 + 10^{-\Delta pK_a}))\ s^{-1}$ where ΔpK_a = pK_a(acceptor) − pK_a(donor) and pK_a(donor) = 14 (Eigen, 1964). For protonation of EQ by the conjugate acid of buffer, the pK_a value estimated for EQ is <1. This possible explanation can be ruled out since the reverse reaction (the removal of the α hydrogen of glycine in the EG complex by hydroxide ion or conjugate base of the buffer) would be required by the principle of microscopic reversibility to proceed at about 10^9 to $10^{10}\ s^{-1}$ rather than about ~ 0.1–$1\ s^{-1}$, estimated from values of k_3 and K_3 (Table I; see also Schirch, 1975). Furthermore, pK_a values for EQ of less than 5 should enable accumulation of large fractions of total enzyme as EQ in neutral solutions of serine hydroxymethylase at saturating glycine concentration: this is not in accord with observation (see K_3 values, Table I).

The second possibility is illustrated in a minimal fashion by Scheme III in which species **1** might be EH_3Q^{2+} (Scheme II), while species **2** and **3** might represent inactive and active prototropic tautomeric forms of EH_2Q^+, respectively.[5] In terms of Scheme III in which the species are interconnected by *microscopic* ionization constants (Edsall & Wyman, 1958; Kallen, 1971b), species **1** and **3** accomplish the protonation of the quinonoid intermediate with the characteristic rate constants k_{3_x} and k_{3_y}, respectively. The observed rate constants for quinonoid intermediate protonation then are a function of the specific rate constants k_{3_x} and k_{3_y} and the pH-dependent partitioning of enzyme among the states **1**, **2**, and **3**. In this formulation, in the acid extreme the entire population of quinonoid intermediate exists as **1**, and k_3^{app} will be $k_{3a} = k_{3_x}$. In the more alkaline region k_3^{app} will be $k_{3b} = k_{3_y}/(1 + 1/K_T)$, where $K_T = [3]/[2] = K_{1-3}/K_{1-2}$. Without further data the numerical evaluation of K_T and k_{3_y} from the composite constant k_{3b} is not possible (Edsall & Wyman, 1958; Kallen, 1971). To the extent that the tautomerization constant, K_T, is $\ll 1$, the microscopic ionization constant governing the partitioning of the protonation pathways among **1** and **3** will differ markedly from macroscopic ionization constant, $K_{a_2}^{EQ}$, according to $K_{a_2}^{EQ} = K_{1-2} + K_{1-3}$. At the more alkaline pH values then, a pH-independent pathway persists based upon the existence of species **3**.[6] That the enzyme has other ionizable groups adjacent to the active site which might serve as the perturbing group, AH, seems likely based upon the occurrence of a histidine adjacent to the "active site" PLP-binding lysine of some PLP containing proteins including rabbit liver serine hydroxymethylase (Applebaum et al., 1975; Boeker et al., 1975; Bossa et al., 1976; Fluri et al., 1971; Kagamiyama et al., 1970; Maurer & Crawford, 1971; Sabo & Fischer, 1974; Strausbauch & Fischer, 1970). Both the active site histidine and lysine are candidates for assignment as groups AH and BH^+. Similar perturbations by neighboring groups appear to occur in kinetics of alkylation of lipoamide dehydrogenase (Matthews et al., 1977) and papain (Polgár, 1973).

The third possibility, that an ionization on the electron sink portion of the PLP system or of an adjacent group is altered, might be revealed by further studies of the various spectrally distinct species.[5] The trend to larger ϵ_{500}^{EQ} values (with the exception of the data for pH 7.1, Table I) with increasing pH to about 9 suggests that this explanation of such a contribution to the pH dependence of k_3 remains viable and, additionally, enables a rationalization for the pH dependence of k_2 (see below). We are, however, not aware of data regarding the effects of changing ionization states upon the spectra of such quinonoid intermediates.

Other less satisfying explanations involving pH-dependent conformation changes have been omitted from this discussion, although it should be noted that there is evidence that serine hydroxymethylases are conformationally mobile enzymes (Cheng & Haslam, 1972; Liu & Haslam, 1974; Schirch, 1975).

One possible method of choosing between the second and third possibilities is by the pH dependence of the k_{-3}^{app} values. An increase in k_{-3}^{app} values with increasing pH is consistent with a simple formulation for the microscopic reverse of the k_3 process in terms of a base catalytic contribution by the enzyme in the ionization of the α-hydrogen of EG to form EQ.[7] The third explanation offered above involving increasing electron sink character with increasing acidity would predict that k_{-3} values increase with increasing acidity. The data presently available on the pH dependence of k_{-3}^{app} are deemed too preliminary to be employed in this regard.

Step 2. Carbon–Carbon Bond Cleavage and Formation. The pH dependence of the carbon–carbon bond cleavage step, k_2, is at first glance paradoxical since β-alkoxide ion formation in ES, favored with increasing pH, would be expected to increase the magnitude of k_2^{app}. However, it has been argued previously on the basis of studies with substituted β-phenylserines that β-oxyanion formation is complete or almost so prior to the k_2 step at pH 7.5 (Ulevitch & Kallen, 1977c). If this were so, the present data suggest that the proton acceptor has not been protonated and therefore rendered inactive at pH values as low as 6. Increasing the electron deficiency of the electron sink character of PLP in ES by protonation on or adjacent to the PLP moiety could account for the increasing k_2^{app} values with decreasing pH and be consistent with one explanation offered with respect to the pH dependence of k_3^{app} (see above). The pK_a values for the iminium ion of

[5] The possibility of changes in the ionization of the phosphate moiety of PLP has been minimized in this discussion of pH dependencies of enzyme intermediates due to the probability that this group is primarily involved in cofactor binding and not catalysis.

[6] In terms of this explanation, the fact that the k_3^{app} values do not approach zero in the alkaline limit (Figure 2C) requires a more expanded Scheme III involving additional enzyme "active site" ionization and multiple microscopic forms of EHQ°, only some or one of which is active.

[7] This criterion is not unambiguous since slightly more complicated formulations permit a rationalization of an increase in k_{-3}^{app} values with decreasing pH, for example, by the placement of the AH group in the EG complex in a position that increases the acidity of the α hydrogen of glycine. If the AH group has changed its position in the EG complex relative to its position in the EQ complex, then a *hybrid* mechanism involving microscopic ionization *and* protein conformational influences upon an apparent rate constant is postulated.

the Schiff base linkage and the pyridinium ion of amino acid–PLP Schiff bases in aqueous solution are about 12.3 and 5.6, respectively (Tobias & Kallen, 1978; Metzler et al., 1977), almost certainly eliminating the former but *not* the latter from consideration as a candidate for the assignment of the apparent $pK_{a_2}^{ES}$ values.

Dependencies of Binary Complex Formation upon pH. The K_1 values in the present study refer to the complex transimination sequence involved in the conversion of the PLP–Schiff base linkage with the active site lysine to the substrate amino acid–PLP Schiff base (ES). Scheme II postulates that the constellation of ionizations assigned as $K_{a_1}^{E}$ governs the decreased formation of ES with decreasing pH according to eq 7. This formulation accounts for the experimental observations (Figure 2A) but there is little information currently on the identity of the group or groups assignable as $K_{a_1}^{E}$ or $K_{a_2}^{E}$.

Comparison of Model and Enzyme-Catalyzed Carbon–Carbon Cleavage Reactions. On the basis of phenyl ring substituent effects on carbon- carbon cleavage reactions (Ching & Kallen, 1978) and benzylic hydroxyl group ionizations (Stewart & Van der Linden, 1960), we have previously suggested that the formation of the ES binary complex proximal to the carbon–carbon cleavage step involves β-oxyanion formation during or immediately following the transimination sequence; $1/K_1$ then represents this overall process (Ulevitch & Kallen, 1977c). Conversely, transimination equilibrium constants are quite insensitive to substituents (Tobias & Kallen, unpublished observations) and, further, in the case of the substituted β-phenylserines, the substituents are relatively distant and are insulated by interposed methylene groups. Thus, the phenyl ring substituents are expected to exert only a small inductive effect at the α nitrogen. The observed substituent effects upon K_1 therefore support β-oxyanion formation during ES formation. Other systems in which enzymes may foster alkoxide ion formation upon binding substrates include liver alcohol dehydrogenase (McFarland & Chu, 1975) and glucose oxidase (Bright & Appleby, 1969). It should be noted that tautomerization equilibria, such as E–A^-....HOS $\rightleftharpoons$ E–AH....$^-$OS, are pH independent and cannot in of themselves account for pH-dependent binding or kinetic functions.

The pH dependence of $1/K_1$ is consistent with β-alkoxide ion formation since the binding function falls off with increasing acidity (Figure 2A) as expected were the proton acceptor to be converted to its conjugate acid and were this to be a dominant influence upon binding. As a consequence of this, it might be predicted that the ρ value for $1/K_1$ for ring-substituted β-phenylserines will change with increasing acidity (cf. Ulevitch & Kallen, 1977c). Although the pK_a value for the β-OH group in aqueous solution is estimated at 13.8 (see Results section), it would be expected to be more acidic if the ring nitrogen were maintained cationic in the amino acid–Schiff base bound to the enzyme. Such an effect is tantamount to an increase in pK_a value for this site on the enzyme of several units above that for the pyridinium ion proton dissociation of 5.6 for an amino acid–PLP Schiff base free in aqueous solution (Metzler et al., 1977). The observation of an enhanced affinity of β-OH rather than β-OCH_3 or β-H α-amino acids for the enzyme is consistent with β-oxyanion formation (Ulevitch & Kallen, 1977a–c), but clearly further experiments are desirable on this point.

It also should be noted that the hypothesis that the β-oxyanion exists in the ES binary complexes at all pH values studied appears to require that the pK_a value for the conjugate acid (β-OH) is depressed substantially (at least 8 pK_a units) upon binding compared with the pK_a value estimated for the β-phenylserine–PLP Schiff base free in aqueous solution ($pK \sim 13.8$, see Results section). Although such a depression in a pK_a value would be favored by pyridinium ion formation at the active site, the magnitude has no precedent of which we are aware for enzymatic reactions; to date shifts of up to 4 pK_a units have been reported (Schmidt & Westheimer, 1971).

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Investigation of the Pre-Steady-State Kinetics of Fructose Bisphosphatase by Employment of an Indicator Method†

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ABSTRACT: The pre-steady-state kinetics for the hydrolysis of fructose 1,6-bisphosphate by rabbit liver fructose bisphosphatase have been investigated by stopped-flow kinetics utilizing an acid–base indicator method that permits the continuous monitoring of the inorganic phosphate product. The reaction sequence is characterized by two successive first-order steps followed by establishment of the steady-state rate. The first exponential process results from a conformational change in the protein that is dye sensitive owing to a perturbation of an acidic residue on the protein. A second process reflects the rapid initial turnover of all four subunits of the enzyme with the concomitant release of inorganic phosphate followed by the rate-limiting step of the catalytic cycle. This latter step may involve a product release (fructose 6-phosphate) or a second conformational change. The catalytic cycle ends with decay of the enzyme to its initial unreactive resting state.

In an effort to elucidate additional mechanistic details of the hydrolysis reaction catalyzed by fructose bisphosphatase (FBPase),[1] our interest centered on the locus of the rate-determining step(s) as well as the possibility of covalently bound intermediates. An investigation of the pre-steady-state kinetics offered the potential of some insight into these questions. Initial experiments monitoring fructose-6-P formation with a coupled enzyme assay system that produces NADPH indicated that the lag phase inherent in this method interfered with the observation of the pre-steady-state kinetics. It appeared possible, however, to exploit the pH change arising from the hydrolysis of fructose-1,6-P_2 in order to develop a continuous assay based on a dye indicator couple for inorganic phosphate that would be responsive on a millisecond time scale. This paper describes the resulting method and findings relevant to the mechanism of action of FBPase.

Experimental Procedures

Materials

Fructose bisphosphatase (rabbit liver) was purified by the method of Ulm et al. (1975) as modified by Benkovic et al. (1974). Glucose-6-P dehydrogenase (yeast) and phosphoglucose isomerase (yeast) were obtained as crystalline suspensions in ammonium sulfate from Sigma. Tetrasodium fru-1,6-P_2, disodium fru-6-P, NADP, tetrasodium EDTA, and Tris were purchased from Sigma. Tris was recrystallized from 95% ethanol–water containing 0.0001% EDTA prior to use. The substrate analogue (α+β)-methyl D-fructofuranoside-1,6-P_2 was prepared as previously described (Benkovic et al., 1970). Phenol red (Fisher) was purified according to the method of Freas & Provine (1928). All other chemicals including $MgCl_2 \cdot 4H_2O$ were reagent grade. Double-distilled, deionized, and degassed water was used throughout.

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[1] Abbreviations used: FBPase, fru-1,6-P_2, fru-6-P, and P_i are used throughout this paper to symbolize fructose bisphosphatase, fructose 1,6-bisphosphate, fructose 6-phosphate, and inorganic phosphate, respectively.

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