Kremzner, L. T., and Wilson, I. B. (1964), *Biochemistry 3,* 1902–1905.
Lee, C. Y. (1970), *Clin. Toxicol. 3,* 457–472.
Lenard, J. (1970), *Biochemistry 9,* 1129–1132.
Leuzinger, W., and Baker, A. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 446–451.
Leuzinger, W., Goldberg, M., and Cauvin, E. (1969), *J. Mol. Biol. 40,* 217–225.
Mann, K. G., Fish, W. W., Cox, A. C., and Tanford, C. (1970), *Biochemistry 9,* 1348–1354.
Massoulié, J., and Rieger, F. (1969), *Eur. J. Biochem. 11,* 441–455.
Massoulié, J., Rieger, F., and Tsuji, S. (1970), *Eur. J. Biochem. 14,* 430–439.
Millar, D. B., and Grafius, M. A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12,* 61–64.
Powell, J. T., Bon, S., Rieger, F., and Massoulié, J. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 36,* 17–22.
Pringle, J. R. (1970), *Biochem. Biophys. Res. Commun. 39,* 46–52.
Richards, F. M., and Vithayathil (1959), *J. Biol. Chem. 234,* 1459–1465.
Rieger, F., Bon, S., and Massoulié, J. (1972a), *C. R. Acad. Sci., Ser. D 274,* 1753–1756.
Rieger, F., Bon, S., and Massoulié, J. (1973), *Eur. J. Biochem. 34,* 539–547.
Rieger, F., Tsuji, S., and Massoulié, J. (1972b), *Eur. J. Biochem. 30,* 73–80.
Rosenberry, T. L., and Bernhard, S. A. (1971), *Biochemistry 10,* 4114–4120.
Rosenberry, T. L., Chang, H. W., and Chen, Y. T. (1972), *J. Biol. Chem. 247,* 1555–1565.
Schaffer, N. K., Michel, H. O., and Bridges, A. F. (1973), *Biochemistry 12,* 2946–2950.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1970), *Biochemistry 9,* 3591–3598.
Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971), *Biochem. Biophys. Res. Commun. 44,* 390–395.
Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815–820.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39,* 462–471.
Tanford, C. (1968), *Advan. Protein Chem. 23,* 121–282.
Ui, N. (1971), *Biochim. Biophys. Acta 229,* 267–581.
Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1130–1134.
Waxdal, M. J., Konigsberg, W. H., Henley, W. L., and Edelman, G. M. (1968), *Biochemistry 7,* 1959–1966.
Waxdal, M. J., Wang, J. L., Pflumm, M. N., and Edelman, G. M. (1971), *Biochemistry 10,* 3343–3347.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.
Weinberg, R. A., Loening, U., Willems, M., and Penman, S. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1088–1095.
Williams, R. C., and Rajagopalan, T. G. (1966), *J. Biol. Chem. 241,* 4951–4954.

A Kinetic Investigation of the Interaction of Serine Transhydroxymethylase with Glycine and Serine†

Sheng-Hsiung Liu and John L. Haslam*

ABSTRACT: A temperature-jump kinetic study indicates five reactions occur in the serine–serine transhydroxymethylase interaction. The number of reactions observed with glycine (Cheng, C. F., and Haslam, J. L. (1972), *Biochemistry 11,* 3512) is the same; however, the glycine reactions show a 495-nm-absorbing intermediate, whereas no similar species is observed with serine. The results of a temperature and pH study are also reported for the interaction of glycine with serine transhydroxymethylase. Both the enthalpy and entropy changes for the first two steps in the reaction are positive at 25° and pH 7.3. A possible reaction mechanism for the interaction of serine and glycine with the enzyme is discussed in terms of the present experimental information.

A previous paper, Cheng and Haslam (1972), described the interaction of glycine with the enzyme, serine transhydroxymethylase. The results given in this paper describe the interaction of that enzyme with the substrate serine. The effects of temperature and pH on the reactions of glycine with the enzyme are also reported.

The spectral changes observed in the absorption spectrum of the enzyme when substrates are added facilitate both equilibrium and kinetic studies. Specifically: the spectral changes occurring with glycine or serine have been used to monitor the reactions taking place with serine transhydroxymethylase. On the basis of such changes several intermediates, a Schiff base (Metzler, 1957), *gem*-diamine (O'Leary, 1971), and a quinoid- or carbanion-type structure (Jenkins, 1964), have been postulated.

Schirch and Mason (1963) have shown that addition of glycine to a solution of the enzyme results in a decrease in the absorption at 425 nm, an increase at 343 nm, and a new peak at 495 nm. With the substrate serine, the enzyme shows no 495-nm absorption: only a slight decrease in the absorption at 343 nm, and a 4-nm shift toward shorter wavelengths in the 425-

† From the Department of Chemistry, University of Kansas, Lawrence, Kansas 66044. *Received July 30, 1973.* Supported by grants from the Research Corporation and the University of Kansas General Research Fund.

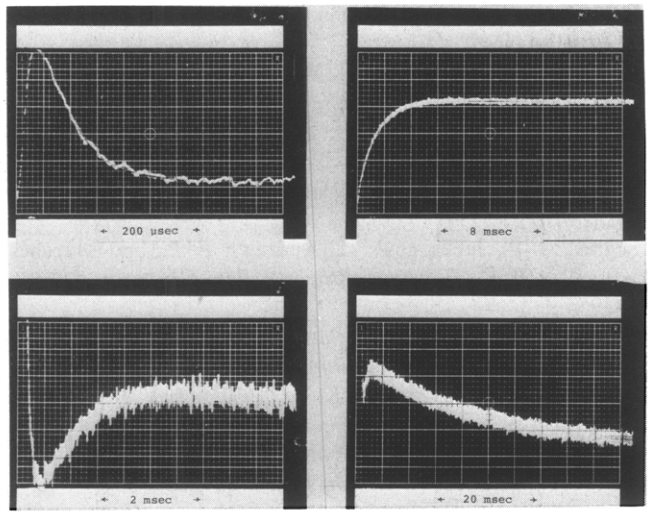

FIGURE 1: Oscilloscope traces of relaxation effects in the serine transhydroxymethylase–serine interaction: 0.05 M potassium phosphate buffer (pH 7.3), 8°, serine transhydroxymethylase concentration 5×10^{-5} M, vertical scale is in arbitrary units of decreasing absorbancy. Top left: (τ_1), horizontal scale is 50 μsec/major division, serine concentration is 20 mM, and wavelength 425 nm. Bottom left: (τ_2), horizontal scale is 0.5 msec/major division, serine concentration is 15 mM and 425 nm. Upper right: (combination of τ_3,τ_5) horizontal scale is 2 msec/major division, serine concentration is 6 mM and 460 nm. Bottom right: (τ_4) horizontal scale is 5 msec/major division, serine concentration is 20 mM and 425 nm.

nm peak with a slight increase in absorption. Schirch and Diller (1971) have observed temperature and pH effects on the spectrum of the enzyme–glycine complexes but no effects on the free enzyme.

Experimental Section

Serine transhydroxymethylase was prepared by the procedure of Schirch and Gross (1968) with the modifications described by Cheng and Haslam (1971). Enzyme concentrations were estimated using an extinction coefficient of 0.95 ml/(mg cm) at 280 nm (Fujioka, 1969). Activity measurements were made by the procedure of Schirch and Gross (1968), using DL-*allo*-threonine as substrate.

The enzyme concentration in the kinetic experiments ranged from 1×10^{-2} to 8×10^{-2} mM. The specific activity based on the serine assay was 8–11. All solutions were 0.05 M in potassium phosphate buffer and were adjusted to the desired pH with potassium hydroxide.

The temperature-jump apparatus is similar to that described by Erman and Hammes (1966) and Faeder (1970) except that it is not set up for the stopped-flow work.

A Beckman DU and a Cary 14 spectrophotometer were used in the determination of the enzyme–substrate binding constants and the enzyme assay.

DL-*allo*-Threonine was purchased from Nutritional Biochemical Co.; pyridoxal-P, DL-serine, L-serine, yeast alcohol dehydrogenase, and NADH grade III were purchased from Sigma Chemical Co. All other chemicals were standard reagent grade.

TABLE I: Wavelength Dependence of the Reciprocal Relaxation Times in the Serine Transhydroxymethylase–Serine Interaction[a]

λ (nm)	$1/\tau_1$	$1/\tau_2$	$1/\tau_3$	$1/\tau_4$	$1/\tau_5$
425	I	D	I	I	I
343	D	N	D	I	D
400	I	N	I	N	I
460	N	N	D	N	D

[a] I and D represent an increase and decrease in absorption and N means not observed at this wavelength.

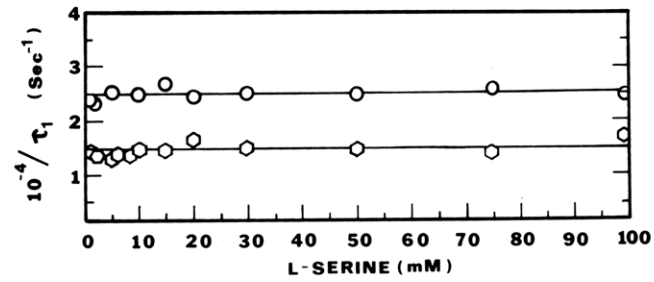

FIGURE 2: Variation of $1/\tau_1$ with L-serine concentration. Circles are data at 25° and hexagons for 8°. The solid lines have no theoretical significance.

Results and Treatment of Data

Reactions of Serine Transhydroxymethylase with L-*Serine.* Examples of the relaxation curves observed in the serine–enzyme interaction are presented in Figure 1. The experimental reciprocal relaxation times ($1/\tau$) were obtained from these oscilloscope traces by plotting on semilog paper the difference between the relaxation curve and the equilibrium value (arbitrary units) against time; the slope of the plot is $-1/\tau$. Table I indicates the wavelength dependence of each of the relaxation processes observed. Although large spectral differences were not observed between the free enzyme and the enzyme–serine complexes (Schirch and Mason, 1963), most of the relaxations could be observed reasonably well at one or more wavelengths.

Figure 2 is a plot of $1/\tau_1$, the fastest step observed, *vs.* L-serine concentration. In these experiments, the serine concentration was always larger than the enzyme concentration. Hence, the sum of the equilibrium concentration of the enzyme and the equilibrium concentration of the substrate (which sum normally would be the abscissa in Figure 2) was set equal to the total substrate concentration. The concentration independence of this reciprocal relaxation time is indicative of a first order reaction of an enzyme–serine complex.

Figures 3 and 4 represent relaxation times observed at 25 and 8°, respectively. At the higher temperature, two relaxation times could be separated. At low serine concentration (below 20 mM) all of the wavelengths at which the reactions were followed (343, 425, 460) showed two reactions. At serine concentrations of 20–80 mM, the values indicated by the circles were observed only at 343 nm, while the remaining wavelengths gave the values indicated by the hexagons. At higher serine concentrations, 80–300 mM (data not given), only a single relaxation was observed, which had a reciprocal relaxation time ($1/\tau$) of (6–7) $\times 10^3$ sec^{-1}. The data represented by the circles indicated a first-order reaction.

One explanation for the data designated by the hexagons is that two relaxations are involved, one occurring in the low concentration range and the other observed at high concentrations, and that there is a nearly linear transition between the two. Using this interpretation the relaxation represented by the circles has been designated as τ_3 and the relaxation represented by the hexagons in the low concentration region as τ_5.

Figure 5 shows another relaxation observed at 25 and 8°. This is the slowest step observed and is designated as τ_4.

None of the relaxation times at 25° indicate a bimolecular

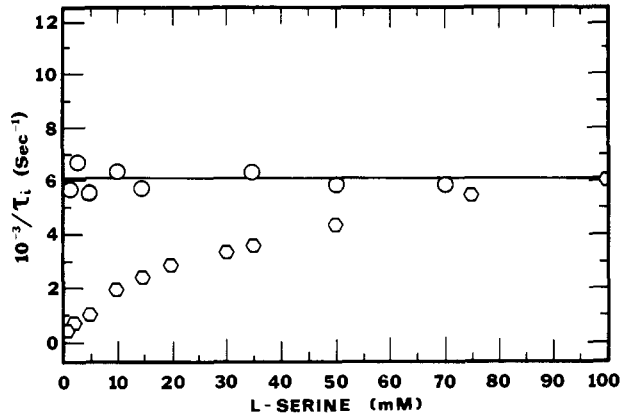

FIGURE 3: Variation of $1/\tau_3$ and $1/\tau_5$ with L-serine concentration at 25°. Circles are for $1/\tau_3$ and hexagons for both $1/\tau_3$ and $1/\tau_5$. See text for details.

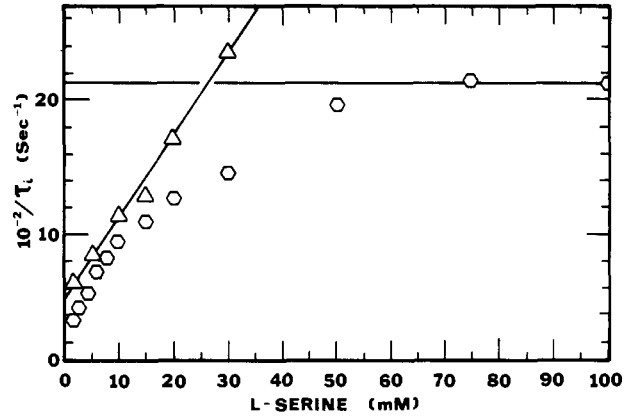

FIGURE 4: Variation of $1/\tau_2$, $1/\tau_3$, and $1/\tau_5$ with L-serine concentration at 8°. Triangles designate data for τ_2 and 425 nm. Hexagons are for data mainly at 460 and 343 nm and represent a combination of $1/\tau_2$, $1/\tau_3$, and $/1\tau_5$. See text for details.

step. However, since it was known that with the substrate glycine the amplitude of the bimolecular step increases as the temperature is lowered, experiments at lower temperatures were run. At about 17° the relaxation at 425 nm previously observed as an increase in absorption changed to a decrease in absorption while the amplitude of the relaxation continued to increase as the temperature was further lowered. The triangles of Figure 4 are the values observed for this relaxation time ($1/\tau_2$). This relaxation may be the bimolecular step since the concentration dependence appears to be linear, the amplitude decreases with increase in serine concentration (constant enzyme concentration), and a similar decrease in absorption for the bimolecular step was observed with the substrate glycine.

Figure 4 also shows the effect of the lower temperature on τ_3 and τ_5. At low concentration a single relaxation time is observed which appears to be a combination of τ_5 and τ_2. This relaxation process then makes a nearly linear transition to the value of τ_3 at higher concentration.

Similar difficulties separating experimental relaxation times have been observed with aspartate transaminase, another pyridoxal-P enzyme.The interactions of this enzyme with α-methylaspartic acid (Hammes and Haslam, 1968) had relaxation constants which were close to average values between the calculated bimolecular step and a unimolecular step. In the reactions of *erythro*-β-hydroxyaspartic acid with the same enzyme (Hammes and Haslam, 1969) a linear transition was also observed between two first-order steps.

The results at low temperature (Figures 2, 4, and 5) indicate that five reactions are involved in the interaction of serine with serine transhydroxymethylase. Since τ_3 and τ_1 do not show any concentration dependence, they must be isolated from the bimolecular step (τ_2) by one of the two slow steps, either τ_4 or τ_5. This also means that an ordering of the reactions beyond the slow step even in a linear mechanism is not possible with the present data. Another problem is that it is difficult to determine an acceptable value of τ_5 at high concentration.

The results are similar to the glycine studies in that five reactions—a slow bimolecular step and four unimolecular steps—are observed. One obvious difference is that with serine no reactions could be observed at 495 nm. Therefore, at least one intermediate must be different in the case of the serine interaction with the enzyme. Because of the problem of determining a value for τ_5 and insufficient information to order the reactions beyond the slow step, values for the individual rate constants were not calculated.

Temperature and pH Dependence of the Interaction of Glycine with Serine Transhydroxymethylase. The temperature dependence and pH dependence of the five reactions observed in the glycine–enzyme interaction were investigated. Experiments were done at four temperatures: 35, 25, 17, and 8°. All of the relaxation times were observed at these temperatures except for the relaxation time designated as τ_5. The relaxation amplitude for τ_5 decreased as the temperature was lowered and was not observed at 8°. The plots of $1/\tau$ *vs.* glycine concentration as a function of pH along with a table listing the values of $1/\tau$ for the individual experiments are given in Liu (1973). Previously reported results at pH 7.3 and 25° have been published (Cheng and Haslam, 1972).

The rate constants given in Tables II and IV (pH 7.3) were calculated from these data using a linear reaction mechanism (Haslam, 1972). The theoretical values consistent with these rate constants were also calculated and compared with the experimental data. The results are consistent with the data only when the slowest step (τ_4) follows the bimolecular step (τ_2). A unique ordering of the remaining three reactions is not possible since all permutations of the reciprocal relaxation times can be made to fit the data. However, one particular ordering (τ_1, τ_3, τ_5) for the last three reactions has been chosen to represent the data. The ordering does not affect the values of k_1 or k_{-1} but does have some effect on k_2 and k_{-2}.

The error in the individual rate constant is difficult to estimate since each is determined using all of the experimental relaxation times in the calculation. The average deviation observed in the 3–10 runs for each data point ranged from 10 to 30%.

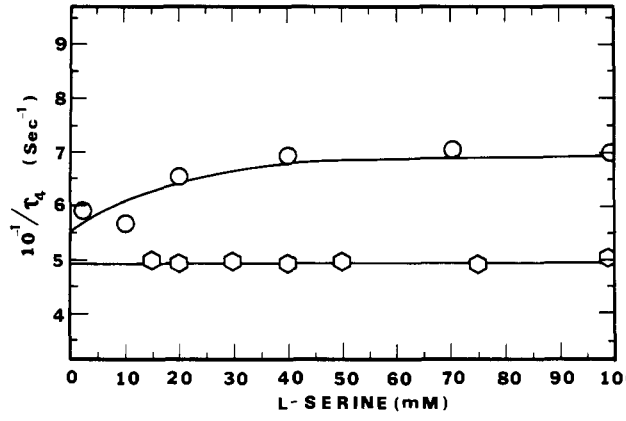

FIGURE 5: Variation of $1/\tau_4$ with L-serine concentration. Circles are for data at 25° and hexagons for 8°. Solid lines have no theoretical significance.

TABLE II: Summary of Rate and Equilibrium Constants in the Serine Transhydroxymethylase–Glycine Interaction at pH 7.3

Temp (°C)	i	k_i (sec^{-1})	k_{-i} (sec^{-1})	K_i	X_i (%)
35	1	1.20×10^5 M^{-1}	6.15×10^2	195 M^{-1}	84.73
	2	6.00×10^1	7.16×10^2	0.084	7.12
	3	5.92×10^3	6.07×10^3	0.976	6.94
	4	6.35×10^2	3.96×10^3	0.160	1.12
	5	1.10×10^2	1.39×10^3	0.079	0.09
	1–5			230 M^{-1}	100.0
17	1	4.95×10^4 M^{-1}	4.63×10^2	107 M^{-1}	88.49
	2	2.45×10^1	6.17×10^2	0.0397	3.51
	3	4.00×10^3	2.35×10^3	1.70	5.97
	4	6.50×10^2	2.55×10^3	0.255	1.53
	5	1.93×10^2	6.05×10^2	0.319	0.5
	1–5			120 M^{-1}	100.0
8	1	2.76×10^4 M^{-1}	3.60×10^2	76.6 M^{-1}	89.75
	2	1.35×10^1	4.08×10^2	0.033	2.96
	3	4.15×10^3	2.30×10^3	1.80	5.33
	4	5.10×10^2	1.85×10^3	0.275	1.46
	5	1.93×10^2	5.75×10^2	0.336	0.5
	1–5			85 M^{-1}	100.0

When plots of the logarithm of the ten rate constants *vs.* $1/T$ were made, only the results for the first two steps showed a reasonably good linear dependence. The first two steps are expected to give the best results because these rate constants are more sensitive to the experimental relaxation times than the other rate constants. The data at 25° were taken from previous work (Cheng and Haslam, 1972) except for the values of k_1 and k_{-1} which were redetermined in this work giving the larger values shown in Table IV. The data for the first two steps are plotted in Figures 6 and 7.

The activation energies and other thermodynamic information calculated from the data are presented in Table III. The uncertainties in the activation energies were estimated from the experimental error in the relaxation times neglecting the possible error introduced in the calculation of the rate constants. Even with the large uncertainty in the rate constants the values of ΔH and ΔS for the first two steps in the reaction are positive. The positive entropy indicates that the binding of glycine to the enzyme results in the breaking or loosening of other bonds associated with the enzyme or substrate. Schirch and Diller (1971) determined the energy changes by studying the effect of temperature on the absorption at 425 and 343 nm. Their results also indicated a large positive entropy change. However, the value determined here is a reflection of all the reactions of the system. The overall equilibrium constant $K = [\Sigma X_i]/[E][Gly]$, in which X_i represents all the enzyme–substrate complexes, was determined for these studies by titrating the enzyme with substrate and plotting the data as described by Fasella *et al.* (1966). The overall equilibrium constants are given in Tables II and IV as $K_{1\text{-}5}$. As can be seen from these

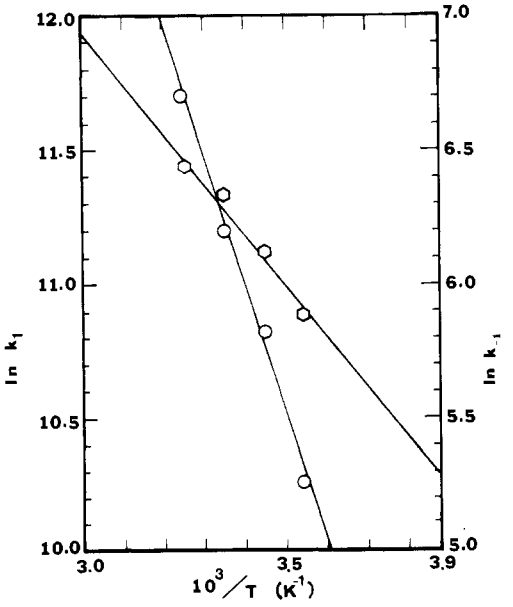

FIGURE 6: Plot of ln k_1 and k_{-1} *vs.* $1/T$ for the reaction of glycine with serine transhydroxymethylase at pH 7.3. Circles are for k_1 and hexagons for k_{-1}.

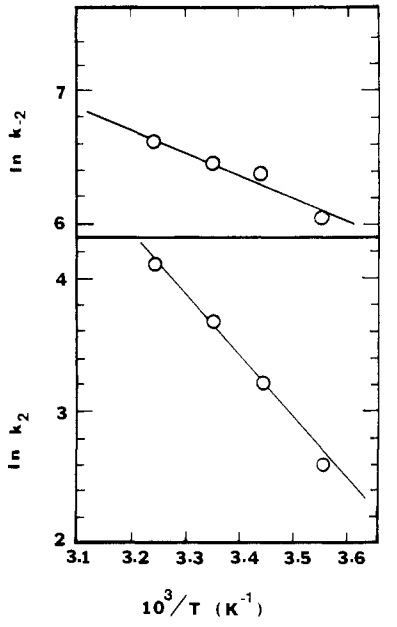

FIGURE 7: A plot of ln k_2 and ln k_{-2} with $1/T$ for the reaction of glycine with serine transhydroxymethylase at pH 7.3.

TABLE III: Thermodynamic Parameters for the First Two Steps in the Serine Transhydroxymethylase–Glycine Interaction at 25° and pH 7.3

	$E_{a(f)}$ (kcal/mol)	$E_{a(r)}$ (kcal/mol)	ΔH (kcal/mol)	ΔG^a (kcal/mol)	ΔS (cal/mol deg)
First step	9.8 ± 2.3	3.9 ± 1.8	5.9 ± 4.1	−2.9	29.5
Second step	9.2 ± 2.6	3.1 ± 2.7	6.1 ± 5.3	1.7	14.8

[a] Calculated from the equilibrium constant.

tables, a major part of the overall equilibrium constant is determined by the first reaction. The remaining reactions contribute only about 15% to the observed value.

Experiments were also performed at pH values of 7.8, 7.3, and 6.3 at 25° to determine the pH effect on the five reactions. A plot of $1/\tau_2$ (the bimolecular step) as a function of glycine concentration and several pH values is shown in Figure 8. The remaining four relaxation times show little concentration dependence. The individual reciprocal relaxation times and the plots of the data at each pH are given in Liu (1973). The only relaxation time which was not observed at all of the pH values was τ_5 which was not observed at pH 7.8. A few experiments were also tried at pH 8.2 but τ_5 was not observed and the amplitude of the τ_2 relaxation was small and decreased rapidly as the substrate concentration was increased.

Schirch and Diller (1971) also observed that the enzyme is unstable above pH 8.5 and that at pH's below 7 phosphate inhibition occurs with this enzyme. An inhibition pK for phosphate was found to be 6.2 in the reaction of *allo*-threonine.

The rate constants given in Table IV were calculated (as described earlier) for each pH assigning an arbitrary value of 1.65×10^3 sec^{-1} to $1/\tau_5$ at pH 7.8. A change in this value has little effect on the rate constants for the first two steps.

Figures 9 and 10 indicate the pH dependence of the rate constants for the first two steps in the glycine interaction. The pH range is so limited that a detailed analysis is not justified. However, the results show that k_1 and k_{-1} have a strong pH dependence above pH 7. This indicates that an ionizable group with a pK greater than 8 is affecting the rate. It also indicates that an unprotonated form of the enzyme or substrate reacts faster than the protonated species. The second pK of glycine (9.6) could account for the results. The solid lines of Figure 9 were constructed using the pK value of 9.6 and values shown at pH 5 on the graphs for the rate constants for the reaction of the protonated form of enzyme or substrate.

If only the anionic form of glycine reacts rapidly with the enzyme this would explain the very low bimolecular rate constant observed at pH 7.3. A possible charge repulsion in the approach of glycine to the binding site may account for the decreased rate at low pH.

The reverse action is also slower at low pH. Tighter binding of glycine is indicated, but, since k_1 increases faster than k_{-1}, the net effect is greater binding at higher pH.

The rate constant k_{-2} increases only slightly with pH but k_2 may have a bell-shaped pH dependence. Fujioka (1969) observed that the activity of the enzyme follows a bell-shaped curve reaching a maximum at pH 7.3. If k_2 is the slowest step in the enzyme reaction, the effect of pH on k_2 may explain the observed pH maximum in the enzyme activity. There are not sufficient data to determine the pK's for this step. However, a pK value of 6.9 has been observed by Schirch and Diller (1971).

A mechanism for the interaction of glycine and L-serine with serine transhydroxymethylase is presented in Figure 11. It is

TABLE IV: Summary of Rate and Equilibrium Constants in the Serine Transhydroxymethylase–Glycine Interaction at 25°

pH	i	k_i (sec^{-1})	k_{-i} (sec^{-1})	K_i	X_i (%)
7.80	1	1.56×10^5 M^{-1}	6.80×10^2	229 M^{-1}	89.9
	2	1.50×10^1	8.33×10^2	0.018	1.62
	3	4.31×10^3	2.97×10^3	1.45	2.35
	4	3.27×10^3	1.42×10^3	2.30	5.40
	5	1.39×10^2	1.16×10^3	0.12	0.64
	1–5			255 M^{-1}	100.0
7.30[a]	1	7.3×10^4 M^{-1}	5.8×10^2	126 M^{-1}	86.31
	2	4.0×10	6.5×10^2	0.06	5.31
	3	3.9×10^3	2.9×10^3	1.30	7.15
	4	2.2×10^2	1.4×10^3	0.16	1.12
	5	8.7×10	8.7×10^2	0.10	0.11
	1–5			141 M^{-1}	100.0
6.30	1	2.15×10^4 M^{-1}	2.20×10^2	97.7 M^{-1}	87.16
	2	1.80×10^1	4.0×10^2	0.045	3.92
	3	3.68×10^3	3.12×10^3	1.18	4.64
	4	1.15×10^3	1.35×10^3	0.85	3.94
	5	4.31×10^1	5.07×10^2	0.085	0.334
	1–5			112 M^{-1}	100.0

[a] Values from Cheng and Haslam (1972) except for k_1 and k_{-1} which are from the current work (see Figure 18).

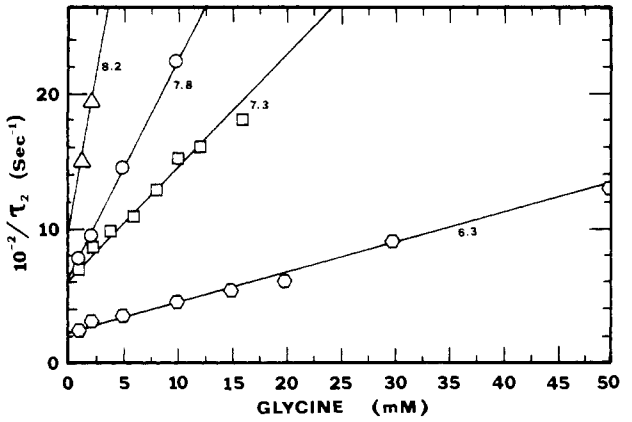

FIGURE 8: Variation of $1/\tau_2$ with glycine concentration at 25° and the pH values indicated in the figure.

based on studies of this enzyme and another pyridoxal-P enzyme, aspartate transaminase (Hammes and Haslam, 1968, 1969; Ivanov and Karpeisky, 1969). The structure E in Figure 11 is the free enzyme. The pyridoxal-P coenzyme is known to form a Schiff base with the ϵ-amino group of a lysine residue on the enzyme (Schirch and Mason, 1963). From model systems (Johnson and Metzler, 1970), the absorption of the enzyme at 425 nm was shown to result from the protonation of this Schiff base. The dissociation of this proton does not occur until the pH is raised above 9 at which point the spectrum begins to change. However, the activity at this pH has decreased more than the spectral change would indicate (Schirch and Mason, 1962).

A two-step binding process indicated by species 1 and 2 is proposed. The results of the temperature study with glycine show that as the temperature increases the equilibrium is shifted to the right forming more of species 1. Since the second reaction step is slower, the absorption change observed for the first reaction requires species 1 to have less absorption than the enzyme at 425 nm. The kinetic results show that the species 1 represents better than 85% of the total complex formed. Since the equilibrium spectrum of a glycine-saturated enzyme solution shows a decrease greater than 25% at this wavelength, species 1 must absorb less than the free enzyme at 425 nm. The bimolecular reaction with glycine or serine is observed only at 425 nm indicating that there is either no change at 343 nm or the change is too small to detect at this wavelength.

The first reaction is postulated to involve a binding interaction between the α-amino group of the substrate and possibly

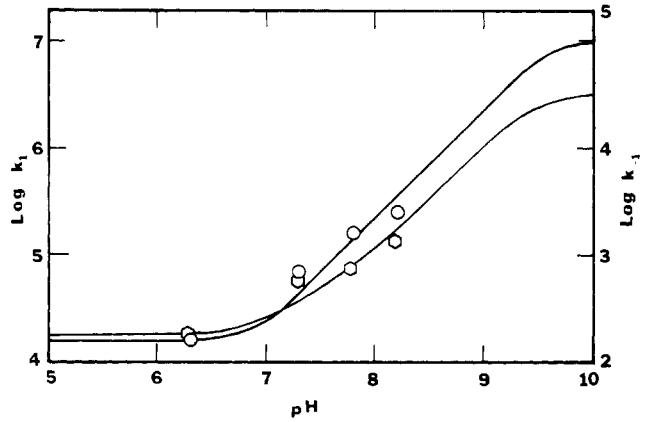

FIGURE 9: A plot of the logarithm of k_1 and k_{-1} *vs.* pH at 25° for the reaction of glycine with serine transhydroxymethylase. Circles are for k_1 and hexagons for k_{-1}. The solid line is a theoretical line. See text for details.

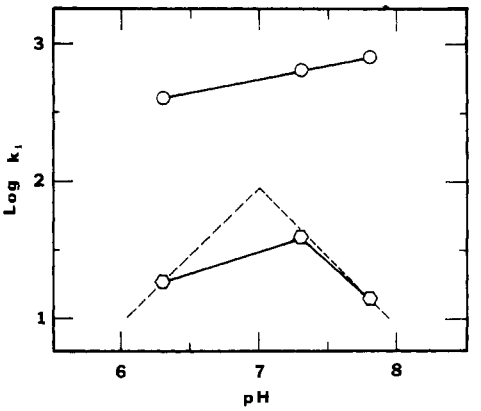

FIGURE 10: A plot of the logarithm of k_2 (hexagons) and k_{-2} (circles) *vs.* pH at 25° for the reaction of glycine with serine transhydroxymethylase. Solid lines are drawn through the data. Dashed line for a slope of +1 and −1 is drawn through the data.

the 3-hydroxy group of pyridoxal-P on the enzyme. The pH dependence of k_1 and k_{-1} can be explained by the electrostatic effects involved in the formation of species 1. If the protonated Schiff base and the protonated α-amino group of the substrate interact during the approach of the substrate, the reaction with the bipolar form of the substrate would be slower than with the anionic form, which indeed is observed for k_1. The reverse reaction rate would also be expected to increase with pH since binding would be weaker with the unprotonated substrate.

Schirch and Diller (1971) conclude from a study of the binding of substrate analogs and anions that both the amino and carboxyl groups of the substrate are necessary for strong binding and that the carboxyl-binding site is not exposed in the free enzyme but is generated after the initial binding step.

The second step in the reaction, the slowest step in the glycine reaction, is also one of the two slowest steps in the serine reaction. In the mechanism of Figure 11, the second step is postulated to involve a change in the protein structure such that the anion site which will bind the carboxyl group is exposed. During the process of carboxyl binding the amino group or the pyridine ring probably moves to a position such that nucleophilic attack by the α-amino group on the formyl carbon of pyridoxal-P can occur (Ivanov and Karpeisky, 1969). The kinetic results give no indication of the absorption maxima associated with species 2 since, being the slowest step, it should be

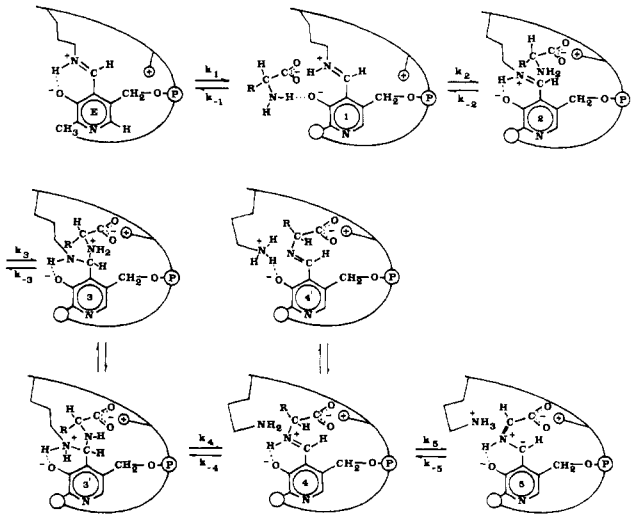

FIGURE 11: Schematic representation of a possible reaction mechanism for the interaction of glycine and L-serine with serine transhydroxymethylase.

observed at all wavelengths. The pH dependence of the second reaction implicates the involvement of two ionizable groups. When the hydrogen bonds associated with these groups are broken, a conformational change could occur with a simultaneous binding of the substrate carboxyl group to the anion site.

The formation of the *gem*-diamine, structure **3,** proposed as an intermediate in the interaction, may produce the observed absorption at 343 nm (O'Leary, 1971). A rapid proton transfer, structure **3** to structure **3'**, would allow the formation of the substrate Schiff base, species 4. This species is expected to have a spectrum similar to that of the free enzyme. If equilibration with the bulk solution is prevented, another species, structure **4'**, might be formed which might also absorb at 343 nm. The spectrum of L-leucine with 5-deoxypyridoxal in basic solution contains a peak at 343 nm which has been attributed to this type of structure (Johnson and Metzler, 1970).

The final step in the glycine reaction is shown as the formation of a carbanion, species 5, which represents the long-wavelength-absorbing species observed 495 nm. Jordan and Akthar (1970) have shown that tritium is lost specifically from [2S-^{3}H]glycine to the solvent. The nature of the remaining intermediate formed in the serine reaction with the enzyme is not known. Without tetrahydrolic acid, the enzyme apparently is unable to break the carbon–carbon bond with serine. The last step with serine could be the interaction of a basic group with the serine hydroxy group which could then help in the breaking of the carbon–carbon bond in the presence of tetrahydrofolic acid.

References

Cheng, C. F., and Haslam, J. L. (1972), *Biochemistry 11,* 3512.

Erman, J. E., and Hammes, G. G. (1966), *Rev. Sci. Instrum. 37,* 746.

Faeder, E. J. (1970), Ph.D. Thesis, Cornell University.

Fasella, P., Giartosio, A., and Hammes, G. G. (1966), *Biochemistry 5,* 197.

Fujioka, M. (1969), *Biochim. Biophys. Acta 185,* 338.

Hammes, G. G., and Haslam, J. L. (1968), *Biochemistry 7,* 1519.

Hammes, G. G. and Haslam, J. L. (1969), *Biochemistry 8,* 1591.

Haslam, J. L. (1972), *J. Phys. Chem. 76,* 366.

Ivanov, V. I., and Karpeisky, M. Ya (1969), *Advan. Enzymol. 32,* 21–53.

Jenkins, W. T. (1964), *J. Biol Chem. 239,* 1742.

Johnson, R. J., and Metzler, D. E. (1970), *Methods Enzymol. 18,* 433–471.

Jordan, P. M., and Akthar, M. (1970), *Biochem. J. 166,* 277.

Liu, S. H. (1973), Ph.D. Thesis, University of Kansas.

Metzler, D. E. (1957), *J. Amer. Chem. Soc. 79,* 485.

O'Leary, M. (1971), *Biochim. Biophys. Acta 242,* 484.

Schirch, L., and Diller, A. (1971), *J. Biol. Chem. 246,* 3961.

Schirch, L., and Gross, T. (1968), *J. Biol. Chem. 243,* 5651.

Schirch, L., and Mason, M. (1962), *J. Biol. Chem. 237,* 2578.

Schirch, L., and Mason, M. (1963), *J. Biol. Chem. 238,* 1032.

Modification of Fructose-1,6-diphosphatase with Pyridoxal 5′-Phosphate. Evidence for the Participation of Lysyl Residues at the Active Site†

Giovanna Colombo and Frank Marcus*

ABSTRACT: Treatment of purified pig kidney fructose-1,6-diphosphatase with pyridoxal 5′-phosphate (in the presence of substrate) followed by reduction with sodium borohydride leads to the selective alteration of the regulatory properties of fructose-1,6-diphosphatase due to the modification of up to four lysyl residues/mole of enzyme (G. Colombo *et al.* (1972), *Biochemistry 11,* 1798). Now, we have studied the inactivation of fructose-1,6-diphosphatase which occurs when modification with pyridoxal phosphate is carried out in the presence of the allosteric inhibitor AMP. Under these conditions up to four lysyl residues/mole of enzyme were modified, and the activity loss was linearly related with the formation of N^{ϵ}-(P-pyridoxyl)lysine. Enzyme inactivation was protected by the substrate fructose 1,6-diphosphate or the inhibitor fructose 6-phosphate, but not by the analog substrate fructose 1-phosphate. These results, as well as those obtained in kinetic studies of partially inactivated enzyme with the substrates fructose 1,6-diphosphate and fructose 1-phosphate, strongly suggest that the site of reaction of pyridoxal phosphate with fructose-1,6-diphosphatase (in the presence of AMP) is a lysyl residue at (or near) the 6-phosphate substrate binding site. These experiments provide another example of the usefulness of pyridoxal phosphate as a means of modifying lysyl residues in or near phosphate binding sites of enzymes, a subject which is herein shortly discussed.

Fructose-1,6-diphosphatase (EC 3.1.3.11, D-fructose-1,6-diphosphate 1-phosphohydrolase) is a regulatory enzyme which plays a key role in the control of gluconeogenesis (for a review, see Pontremoli and Horecker, 1971). The enzyme isolated from mammalian liver and kidney in its native form is composed of four presumably identical subunits with molecular weights of approximately 35,000 (Mendicino *et al.,* 1972; Tashima *et al.,* 1972; Traniello *et al.,* 1972). The existence of four substrate binding sites and four allosteric sites for the inhibitor AMP per enzyme molecule has been demonstrated (Pontremoli *et al.,* 1968a,b; Sarngadharan *et al.,* 1969). Amino acid analysis reveals a relatively large content of lysine (Byrne

† From the Instituto de Bioquimica, Universidad Austral de Chile, Casilla 567, Valdivia, Chile. *Received February 1, 1974.* This investigation was supported by a grant from the Research Fund of the Universidad Austral (Project 72-71).

* Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706.