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Serine Hydroxymethyltransferase: Mechanism of the Racemization and Transamination of D- and L-Alanine†

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ABSTRACT: The reaction specificity and stereochemical control of *Escherichia coli* serine hydroxymethyltransferase were investigated with D- and L-alanine as substrates. An active-site H228N mutant enzyme binds both D- and L-alanine with K_d values of 5 mM as compared to 30 and 10 mM, respectively, for the wild-type enzyme. Both wild-type and H228N enzymes form quinonoid complexes absorbing at 505 nm by catalyzing the loss of the α-proton from both D- and L-alanine. Racemization and transamination reactions were observed to occur with both alanine isomers as substrates. The relative rates of these reactions are quinonoid formation > α-proton solvent exchange > racemization > transamination. The observation that the rate of quinonoid formation with either alanine isomer is an order of magnitude faster than solvent exchange suggests that the α-protons from both D- and L-alanine are transferred to base(s) on the enzyme. The rate of racemization is 2 orders of magnitude slower than the formation of the quinonoid complexes. This latter difference in rate suggests that the quinonoid complexes formed from D- and L-alanine are not identical. The difference in structure of the two quinonoid complexes is proposed to be the active-site location of the α-protons lost from the two alanine isomers, rather than two orientations of the pyridoxal phosphate ring. The results are consistent with a two-base mechanism for racemization.

Serine hydroxymethyltransferase (SHMT)[1] (EC 2.1.2.1) catalyzes the reversible interconversion of serine and glycine with tetrahydrofolate serving as the one-carbon carrier (Schirch, 1982). In addition to this physiological reaction, SHMT catalyzes several other reactions characteristic of pyridoxal-P enzymes:

$$\text{allothreonine} \rightleftharpoons \text{glycine} + \text{acetaldehyde} \quad (1)$$

$$\text{aminomalonate} \rightarrow \text{glycine} + CO_2 \quad (2)$$

$$\text{glycine} + \text{lipoate} \rightarrow NH_2CH_2\text{-lipoate} + CO_2 \quad (3)$$

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D-alanine + pyridoxal-P ⇌ pyruvate + pyridoxamine-P (4)

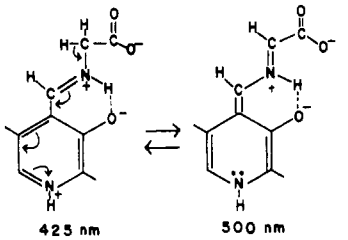
Reaction 1 is a H_4folate-independent aldol cleavage of a 3-hydroxy amino acid. The enzyme catalyzes this reaction with a wide variety of 3-hydroxy amino acids including L-threonine and 3-phenylserine (Schirch & Gross, 1968; Ulevitch & Kallen, 1977a). Reaction 2 is a decarboxylation reaction (Palekar et al., 1973) but differs from reaction 3 which resembles glycine decarboxylase in the glycine cleavage system (Zieske & Davis, 1983). Reaction 4 is a half-transamination reaction and was one of the first examples of a pyridoxal-P enzyme being able to catalyze alternative reactions (Schirch & Jenkins, 1964a).

The above reactions are proposed to occur by the conversion of an enzyme-bound external aldimine (structure I, Scheme I) to an intermediate where a bond at C_α of the amino acid is cleaved, leaving a resonance-stabilized structure referred to as a quinonoid complex (structure II) (Davis & Metzler, 1972). Dunathan proposed that in the mechanism of pyridoxal-P enzymes that the C_α bond of the amino acid cleaved by the enzyme is perpendicular to the plane of the coenzyme ring in the external aldimine (structure I) (Dunathan, 1966). In partial proof of this theory was the example that SHMT catalyzes the formation of structure II from D-alanine but not L-alanine (Schirch & Jenkins, 1964b). This is consistent with the observation that SHMT catalyzes solvent exchange of only the *pro*-2*S* proton of glycine, which has the same stereochemical configuration as the α-proton in D-alanine (Jordan & Akhtar, 1970; Besmer & Arigone, 1968). According to Dunathan's theory, when substrates bind to SHMT only the bond corresponding to the *pro-S* position of glycine is perpendicular to the plane of the pyridoxal-P ring and will therefore be cleaved.

The apparent stereochemical consistency of the reactions catalyzed by SHMT was first brought into question by the observation that the rapid decarboxylation of aminomalonate by SHMT (reaction 2) went without any selectivity for the two carboxyl groups (Palekar et al., 1973). In theory, the enzyme should be able to distinguish between the two carboxyl groups if only the *pro-S* proton is lost from glycine. It was next shown that several other L-amino acids also slowly form a quinonoid complex with SHMT (Ulevitch & Kallen, 1977b; Hansen & Davis, 1979). This observation was studied in detail with L-phenylalanine which forms a significant amount of stable quinonoid complex (Ulevitch & Kallen, 1977b). The reactions discussed in this paragraph can occur only if the bond at C_α of the amino acid substrate, corresponding to the *pro-R* proton of glycine, is broken by the enzyme. The cleavage of groups at either stereochemical position at C_α of the amino acid substrate by SHMT appears to violate Dunathan's hypothesis.

The broad reaction specificity of pyridoxal-P enzymes has been attributed to the reactivity of the Schiff base formed between the coenzyme and the amino acid substrate (structure I) (Metzler, 1957; Miles et al., 1986). This may be particularly true with substrate analogues which distort the geometry of active-site residues (Snell, 1985). A key feature of understanding the mechanism of a pyridoxal-P enzyme is the ability to determine how the external aldimine (structure I) is converted to the quinonoid complex (structure II) and how

Scheme I



I II

the enzyme further processes this very reactive intermediate. A unique property of several pyridoxal-P enzymes is that the quinonoid complex accumulates in the presence of substrates and substrate analogues, giving a unique spectral band near 500 nm (Davis & Metzler, 1972). This spectral property of the quinonoid complex permits the determination of rates of interconversion of these putative intermediates.

In this study, we further probe the broad reaction and substrate specificity of SHMT as a tool to understand the mechanism of pyridoxal-P enzymes. We have purified and studied the mechanism of three different SHMT's: rabbit liver cytosolic, rabbit liver mitochondrial, and *Escherichia coli*. All of these enzymes have a common amino acid sequence near the active-site lysine, which binds pyridoxal-P (Barra et al., 1983). In the *E. coli* enzyme, we have changed a conserved histidine residue adjacent to the lysyl residue to an asparagine by site-directed mutagenesis. The mutant enzyme retains 25% of the catalytic activity of the native enzyme for the cleavage of both L-serine and allothreonine (Hopkins & Schirch, 1986). Previous studies have provided evidence that the H228N mutation has not altered either the structure or the mechanism of this enzyme. In this paper, we report that this mutant enzyme, H228N, has a higher affinity for both D-alanine and L-alanine than the wild-type enzyme and forms quinonoid complexes with both isomers at similar rates. The enzyme transaminates both alanine isomers and also catalyzes an alanine racemase reaction. This is the first reported example of SHMT catalyzing a racemase reaction. Although the wild-type *E. coli* enzyme also catalyzes these nonphysiological reactions, the mutant enzyme appears to have lost the ability to distinguish between D- and L-alanine and offers a much better system for determining how SHMT can remove the α-proton from either D- or L-alanine. A critical question is whether the symmetrical quinonoid complexes, formed from either D- or L-alanine, are identical in structure. These studies contribute to our understanding of the mechanism of both SHMT and pyridoxal-P racemases.

Experimental Procedures

Materials. Rabbit liver cytosolic and *E. coli* SHMT's were purified to homogeneity as previously described (Schirch & Peterson, 1980; Schirch et al., 1985). Enzyme concentrations were determined by calculating the amount of bound pyridoxal-P released from the enzyme in 0.1 N NaOH (Harruff & Jenkins, 1976). All enzyme concentrations given in this work refer to the concentration of active sites. Most amino acids, folate derivatives, buffers, and coupling enzymes were purchased from Sigma. D,L-Allothreonine was obtained from ICN, and D,L-[2-^{2}H]alanine was purchased from Merck.

pro-S-Deuteriated glycine was synthesized by incubating cytosolic SHMT from rabbit liver with 20 mM D,L-*threo*-3-phenylserine in D_2O until the absorbance at 279 nm, corresponding to benzaldehyde production, stopped increasing. The sample was boiled and centrifuged to remove the enzyme. The [2(*S*)-^{2}H]glycine was purified from the mix through extrac-

[1] Abbreviations: SHMT, serine hydroxymethyltransferase; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; H_4folate, tetrahydrofolate; NMR, nuclear magnetic resonance; H228N, a site-directed mutant of *E. coli* serine hydroxymethyltransferase where the histidine at position 228 was substituted for an asparagine.

tions with decolorizing charcoal to remove benzaldehyde and 3-phenylserine.

L-[2-^{2}H]Alanine was prepared by reacting 4 mmol of D,L-[2-^{2}H]alanine with 15 units of D-amino acid oxidase and 500 units of catalase in 4 mL of 0.02% azide, pH 8.3. After 30 h of incubation at 37 °C, the reaction solution was loaded onto a 2.5 × 4.5 cm Dowex AG 50W-X8 ion-exchange column which had been equilibrated with 15 mM HCl. After the column was washed with several milliliters of H_2O, the residual amino acid was eluted with 3 column volumes of 0.4 N NH_4OH. The solution was lyophilized and the amino acid redissolved in 0.5 mL of 10 mM sodium BES, pH 7.0. D-[2-^{2}H]Alanine was prepared by the same procedure except the racemic compound was incubated with 6 units of L-alanine dehydrogenase, 1 mmol of NAD^+, and 10 units of lactate dehydrogenase at pH 10. The optical purity of L-[2-^{2}H]alanine was determined by assaying for the D isomer with the D-amino acid oxidase system. The purity of D-[2-^{2}H]alanine was determined by assaying for the L-isomer with the L-alanine dehydrogenase system. Both assays are described below. For each isomer, the optical purity was greater than 90%.

Spectral Characterization of Enzyme–Substrate Complexes. All UV–visible spectra were obtained on a Cary 210 spectrophotometer. Circular dichroism measurements were made on a Jasco J-500 spectropolarimeter. K_d[2] values were obtained for L-phenylalanine and alanine isomers by titration of enzyme with increasing concentrations of each amino acid and noting the increase in absorbance at 505 nm. Double-reciprocal plots of the absorbance at 505 nm versus the amino acid concentration were linear and yielded K_d values from the negative abscissa intercept. Alanine K_d values were confirmed by stopped-flow titration where increasing concentrations of alanine were flowed against 44 μM enzyme. All determinations were performed in 20 mM NaBES, pH 7.0.

Racemization of D- and L-Alanine. The racemization reaction contained 25 mM either D- or L-alanine, 22 μM SHMT, 0.03 mM pyridoxal-P, and 1 mM dithiothreitol in 50 mM Na-BES, pH 7.6 at 37 °C. A control reaction contained no enzyme. At various times, 0.2-mL aliquots were removed and loaded onto a Dowex AG 50W-X8 cation-exchange column in a Pasteur pipet. The column was washed first with the equilibration solution of 0.15 mM HCl and then by 2 column volumes of H_2O. The amino acid was eluted with 3 column volumes of 0.4 N NH_4OH. The eluate was lyophilized and redissolved in 50 μL of H_2O and assayed for either D-alanine or L-alanine. The assay for L-alanine consisted of 0.2 mM NAD^+, sample, and 2.5 units of L-alanine dehydrogenase in 100 mM sodium borate, pH 9.5. The change in absorption at 340 nm due to NAD^+ reduction was used to calculate the micromoles of L-alanine produced. The assay for D-alanine consisted of 0.2 mM NADH, sample, 2 units of D-amino acid oxidase, 1 unit of catalase, and 5 units of lactate dehydrogenase in 20 mM NaBES, pH 7.0. The concentration of D-alanine in the sample was determined from the decrease in absorbance at 340 nm.

Transamination of Alanine. The transamination of alanine isomers to pyruvate by SHMT was followed spectrophotometrically by observing the decrease in the absorption of the enzyme-bound pyridoxal-P absorbing at 422 nm and the appearance of free pyridoxamine-P absorbing at 325 nm. Each reaction contained 22 μM SHMT and 100 mM D- or L-alanine in 50 mM NaBES, pH 7.6 at 37 °C. The stoichiometric production of pyruvate and pyridoxamine-P was determined as previously described (Schirch & Jenkins, 1964a). The rate constant for transamination was determined from first-order log plots of the disappearance of absorbance at 422 nm.

Rate of Formation of the Quinonoid Complex. The rate of formation of the quinonoid complex absorbing near 500 nm, E·Q, was determined in a stopped-flow absorbance spectrophotometer from Kinetic Instruments, Inc. The cell has a 2-cm path length, and the dead time of the instrument is 1.5 ms. Absorbance versus time data were stored on an IBM XT computer and curve fitted with either a single-exponential or a double-exponential curve-fitting program written in ASYST from McMillan software. An average of three traces were used to obtain each rate constant. The final concentrations of enzyme and substrate were 22 μM and 100 μM, respectively. The buffer was NaBES, pH 7.0, except where the effect of pH was being studied. At pH values other than 7.0, the enzyme was loaded in one syringe in a 1 mM potassium phosphate solution, pH 7.0. The other syringe contained the amino acid, 200 mM, in 100 mM NaBES of the desired final pH. The pH of the reaction was verified by measuring the pH of the mixed solutions.

At pH values below 7.5, the rate of transamination was sufficiently slow that the formation of E·Q reached a maximum without any decrease in absorbance due to the conversion of the pyridoxal-P to pyridoxamine-P. However, at higher pH values, this was not true. To determine the true rate constant and the equilibrium concentration of E·Q, the absorbance data were curve fitted to the two-exponential equation:

$$Y = A_0 + A_1e^{-k_1t} + A_2e^{-k_2t}$$

where Y is the absorbance at time t, A_0 is the final absorbance, and A_1 and A_2 are the amplitudes for the first and second exponential terms. The value of A_1 was used for the true equilibrium concentration of E·Q and k_1 as the rate constant for its formation.

Rate of Amino Acid Proton Exchange. The exchange of the α-protons of D- and L-alanine and *pro-S*-deuteriated glycine was determined by solvent exchange experiments in D_2O using proton NMR to follow the reaction. These studies were performed on a 270-MHz IBM/Bruker AF270 instrument maintained at 37 °C. The enzyme was solvent exchanged in 99.8% D_2O in 20 mM potassium phosphate buffer, pD 7.6. The reaction was performed with 22 μM enzyme, 0.03 mM pyridoxal-P, and 5 mM amino acid. With a 2-s presaturation pulse to remove the residual water peak, spectra were collected every 10 min over a 2.5–5-h period with 20 transients being collected per spectrum. The exchange of [2-^{3}H]glycine protons with the solvent was performed as previously described (Jordan & Akhtar, 1970).

RESULTS

Amino Acid Dissociation Constants. We have previously shown that the mutation H228N in *E. coli* SHMT resulted in a 2–4-fold increase in the K_m values for the substrates serine, glycine, and allothreonine (Hopkins & Schirch, 1986). We have extended this study to look at the dissociation constants of several other amino acids (Table I). We found that in the mutant enzyme both D- and L-alanine have smaller K_d values for the mutant enzyme than the wild-type enzyme. Another difference is that the wild-type enzyme binds L-alanine more tightly than D-alanine but the mutant enzyme binds them with equal affinity. Apparently, the active site of the mutant enzyme can accommodate small side chains of either D- or L-

[2] This is not a true dissociation constant since several intermediates are known to exist in addition to the quinononid complex. These include the geminal diamine and the external aldimine. Therefore, the bound form of the amino acid is the sum of these complexes and not just the quinonoid complex.

Table I: Dissociation Constants for Amino Acids with Wild-Type and H228N Serine Hydroxymethyltransferase

amino acid[a]	K_d (mM) wild type	H228N
L-serine	1	2
glycine	1	10
L-alanine	10	5
D-alanine	30	5
L-phenylalanine	67	>100

[a] Values for glycine and L-serine are from Hopkins and Schirch (1986). Values for D-alanine, L-alanine, and L-phenylalanine were determined by observing changes in quinonoid absorbance with increasing substrate as described under Experimental Procedures.

amino acids but not large side chains since L-phenylalanine has a very high K_d value (Table I).

Transamination of D- *and* L-*Alanine.* We have previously shown that D-alanine reacts with SHMT from rabbit liver and *E. coli* to undergo a slow half-transamination reaction resulting in the formation of apoenzyme, pyridoxamine-P, and pyruvate (Schirch & Jenkins, 1964a; Hopkins & Schirch, 1986). We have now found that the H228N form of SHMT undergoes this reaction with L-alanine (Figure 1). The addition of L-alanine to the mutant enzyme results in the appearance of a species absorbing at 505 nm (Scheme I, structure II). The absorption bands at 505 and 422 nm slowly disappear with the concomitant appearance of a new absorption band at 325 nm. We have shown that the compound absorbing at 325 nm is pyridoxamine-P and that an equivalent amount of pyruvate is also formed during the reaction. The wild-type enzyme also transaminates L-alanine but at a much slower rate than D-alanine. With the mutant enzyme, L-alanine also transaminates faster than D-alanine (Table II). On reinvestigation of the rabbit cytosolic and mitochondrial isoenzymes, we found that mitochondrial SHMT also forms a quinonoid complex with L-alanine and undergoes the half-transamination reaction. These effects with L-alanine do not occur with the cytosolic isoenzyme.

The rate of transamination with either D- or L-alanine (k_D and k_L) was proportional to the concentration of the quinonoid complex as determined from the absorbance at 505 nm. This relationship of $k_D/k_L = [E{\cdot}Q_D]/[E{\cdot}Q_L]$ held for both forms of the *E. coli* enzyme at several pH values.

We have looked at a variety of L-amino acids to see if they form a quinonoid complex and are transaminated by the mutant enzyme. L-Phenylalanine, L-leucine, L-valine, L-tryptophan, and L-isoleucine all form a small amount of complex absorbing near 500 nm, and all slowly give spectral changes characteristic of the half-transamination reaction. No evidence for either slow transamination reactions or formation of quinonoid complexes was observed with L-serine, glycine, D-serine, D-threonine, aminomethyl phosphonate, or D-phenylalanine. The transamination reactions are reversible since incubation of apoenzyme with pyridoxamine-P and keto acids such as glyoxalate, pyruvate, and fluoropyruvate results in the formation of active enzyme and an absorption band at 422 nm characteristic of holoenzyme.

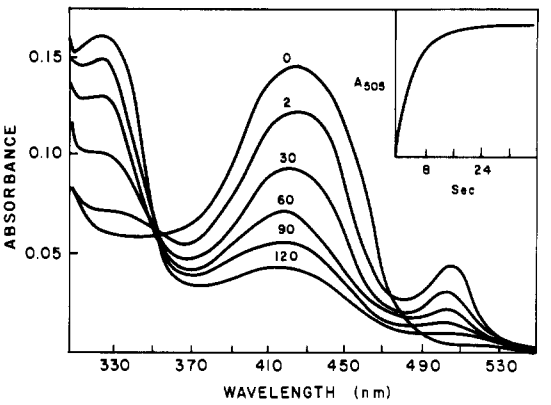


FIGURE 1: Spectra of H228N SHMT with L-alanine as a function of time. The solution was 22 μM enzyme and 100 mM L-alanine in 50 mM NaBES, pH 7.6 at 37 °C. The values above each curve represent incubation time in minutes. The curve at zero time contained no L-alanine. (Inset) Rate of formation of the enzyme–L-alanine quinonoid complex as determined by stopped-flow spectroscopy.

Rate of Formation of the Quinonoid Complex. A key intermediate in all of the reactions catalyzed by SHMT is the formation of the resonance-stabilized quinonoid complex (Scheme I, structure II). All forms of SHMT yield this complex with D-alanine by catalyzing the loss of the C_α-proton. Since rabbit cytosolic SHMT had been shown to catalyze the solvent exchange of only the *pro-2S* proton of glycine, the stereochemical integrity of the two substrates was maintained. However, the observation that L-amino acids are also capable of forming the quinonoid complex is unexplained since the enzyme must catalyze the removal of the proton equivalent to the *pro-R* proton of glycine. The mutant *E. coli* enzyme H228N forms quinonoid complexes with both D- and L-alanine. Since the enzyme binds these two isomers with equal affinity and tighter than the wild-type enzyme, it provides us with a model to explore the mechanism of this apparent lack of stereochemical specificity.

Figure 1 shows the spectrum of H228N SHMT with L-alanine. The optical activity of the quinonoid complex absorbing at 505 nm formed from either D- or L-alanine was examined. After correction for the different absorbances at 505 nm, positive optical rotations were observed for both D- and L-alanine, and the values were identical from 490 to 530 nm with a value of 7 mdeg per absorbance unit at 505 nm.

The equilibrium concentration of quinonoid complex was determined by following the increase in absorbance at 505 nm in a stopped-flow spectrophotometer (inset, Figure 1). The

Table II: Rate Constants for the Rates of Transamination, Racemization, Formation of $E{\cdot}Q_{505}$, and Proton Solvent Exchange of D-Alanine and L-Alanine with Wild-Type and H228N Serine Hydroxymethyltransferase

	k_{obsd} ($s^{-1} \times 10^3$)[a]							
	transamination		racemization		$E{\cdot}Q_{505}$		solvent exchange	
reaction enzyme form[b]	H	N	H	N	H	N	H	N
D-alanine + H_4folate	0.36	0.19	6	4	420	900	27	19
			15	5	19500	20000	>500	>500
L-alanine + H_4folate	0.10	0.28	4	13	720	1500	102	38
			7	14	800	1700	57	19
glycine + H_4folate[c]							378	150
					15200	16000	>500	>500

[a] K_{obsd} values were corrected for incomplete saturation of the enzyme with substrate using the equation $k_{obsd} = k\{K_d + [S]\}/[S][E_t]$. Reactions were performed at 37 °C, pH 7.6. [b] H is the wild-type enzyme with a histidine at position 228, and N is the mutant enzyme with asparagine at position 228. [c] Values from Hopkins and Schirch (1986).

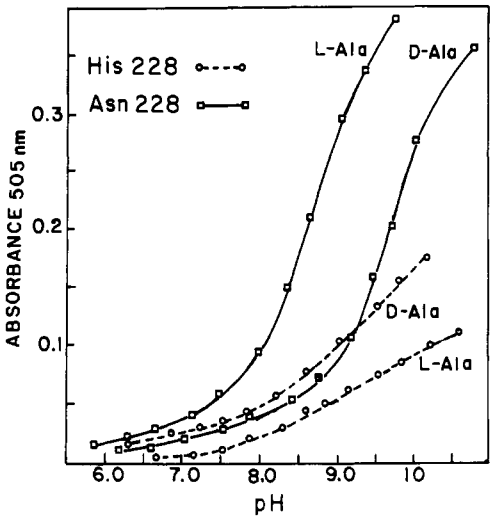


FIGURE 2: Dependence of the concentration of quinonoid complexes formed with D- and L-alanine as a function of pH. Solid lines are for H228N SHMT, and dashed lines are for wild-type SHMT.

variation of the maximum absorbance at 505 nm as a function of pH for each amino acid isomer and both wild-type and mutant enzymes is recorded in Figure 2. A plot of the reciprocal absorbance versus the hydronium ion concentration is linear, suggesting it fits the model for the ionization of a monoprotic acid (Sizer & Jenkins, 1963). From this plot, apparent pK values of 8.6 and 9.6 were determined for L- and D-alanine, respectively, with the mutant enzyme. The wild-type enzyme produces lower equilibrium concentrations of the quinonoid complexes with both amino acid isomers compared to H228N SHMT, so accurate pK values could not be determined for this enzyme. However, qualitative inspection of the variation of the absorbance at 505 nm of the wild-type enzyme with D- and L-alanine shows that the quinonoid complex formed from D-alanine has a lower pK than the one observed with L-alanine, which is the reverse of that found with the mutant enzyme (Figure 2).

The rates of formation of the quinonoid complexes absorbing at 505 nm were also determined as a function of pH for both wild-type and H228N SHMT's. The variation of the first-order rate constant with pH was bell-shaped with a maximum value at pH 8.4. The shapes of the curves were essentially identical with both D- and L-alanine as substrates. The decrease in the rate of formation of the quinonoid complexes with pH values above 8.4 was unexpected. At pH 7.6, the rate of formation of E·Q is faster with H228N than with the wild-type enzyme for both isomers (Table II). Also, L-alanine reacts more rapidly than D-alanine to form this complex. Previous studies with rabbit cytosolic SHMT showed that both NH_4^+ and H_4folate accelerated the rate of formation of the quinonoid complex with D-alanine (Schirch & Jenkins, 1964b). The addition of 50 mM NH_4^+ ion had no effect on the rate of formation of the quinonoid complex with the *E. coli* enzyme in the presence of either amino acid isomer. However, when the rate of formation of E·Q was determined in the presence of H_4folate, 0.5 mM, the reaction with D-alanine occurs at least 2 orders of magnitude faster with both *E. coli* enzyme forms with a final equilibrium concentration about 2-fold higher than in the absence of this ligand (Table II). These results are similar to those observed with the rabbit enzyme. With L-alanine as substrate, H_4folate slightly decreased the equilibrium concentration of E·Q for both enzyme forms and had no effect on the rate of formation of the complex.

To determine if the rate-determining step for E·Q formation was breaking the C_α–H bond, we used both D-[2-^{2}H]alanine and L-[2-^{2}H]alanine as substrates. The deuterium isotope effects for quinonoid formation were found to be 3.0 and 2.5 for L- and D-alanine, respectively, with the mutant enzyme.

Racemase Activity. The quinonoid complex (Scheme I, structure II) is a symmetrical compound with respect to the amino acid and pyridoxal-P components. Since the symmetrical E·Q complex can be formed readily from either D-alanine or L-alanine suggested that *E. coli* SHMT would be able to catalyze the racemization of D- and L-alanine. Initial rate studies show that indeed the enzyme does catalyze racemization of the alanine isomers starting with either D- or L-alanine (Table II). Evidence that the quinonoid complex is an intermediate in racemization is suggested by the 4-fold increased rate of conversion of L-alanine to D-alanine by H228N as compared to the wild-type enzyme. We assume this reflects the greater concentration of E·Q formed with H228N and L-alanine. Of particular interest is that the rate of racemization is 2 orders of magnitude slower than the rate of formation of E·Q in the absence of H_4folate (Table II).

Solvent Exchange of D-[*2-H*]- *and* L-[*2-H*]*Alanine and Glycine.* The enzyme-catalyzed exchange of the α-proton of the two alanine isomers was studied by following the disappearance of the C_α-proton signal with NMR when enzyme and amino acid were incubated in D_2O. Reactions with no enzyme showed no loss of the α-proton over a time period of 24 h. With D- and L-alanine, the loss of the α-proton signal at 3.8 ppm was not the result of transamination as no ^{1}H resonance for the methyl group of pyruvate appeared during the time of the study. Further evidence that the decrease in the proton signal was due to solvent exchange with D_2O was seen from a collapse of the alanine methyl doublet to a singlet centered at 1.5 ppm. The rate constant for the exchange of the α-proton with solvent was calculated from initial rates of disappearance of the α-proton signal. D-Alanine exchanges very slowly with solvent while L-alanine exchanges more rapidly (Table II). Upon the addition of 0.2 mM H_4folate, the rate of exchange with D-alanine was too rapid to determine by this method, but the rate with L-alanine was decreased by 50%.

Previous experiments have suggested that SHMT catalyzes the solvent exchange of only the *pro-S* proton of glycine (Jordan & Akhtar, 1970). We reinvestigated this using rabbit cytosolic, *E. coli*, and H228N SHMT's. In both the presence and absence of H_4folate, only 50% of racemic [2-^{3}H]glycine exchanged its tritium with the solvent, in agreement that only the *pro-S* proton of glycine exchanges with the solvent. This was confirmed by showing that none of the enzymes catalyzed the proton solvent exchange of [2(*R*)-^{2}H]glycine.

DISCUSSION

The goal of this study was to try and understand some of the stereochemical factors in the formation of the quinonoid complex with serine hydroxymethyltransferase and its amino acid substrates. The study was greatly aided by having the mutant *E. coli* enzyme H228N in which an active-site histidine was converted to an asparagine. The enzyme is important in this study because it binds both D- and L-alanine with equal affinity, has a greater affinity for these amino acids than the wild-type enzyme, transaminates both amino acids, forms a quinonoid complex with both isomers, and catalyzes the interconversion of the two isomers. The wild-type enzyme also catalyzes these reactions but forms much less quinonoid intermediate than the mutant enzyme so that a comparison of the reactions of D- and L-alanine was much more difficult to follow. We have focused our study on determining the rates of four reactions catalyzed with D- and L-alanine. These reactions are the transamination of the amino acids to pyruvate

and pyridoxamine-P, the racemization of D- and L-alanine, the formation of the quinonoid complex, and the solvent exchange of the α-proton. The transamination of L-alanine and the racemization reactions were not previously known to be catalyzed by SHMT.

We have shown that the removal of α-protons from either D- or L-alanine in going to the quinonoid intermediate is enzyme catalyzed. The fact that the rate of formation of the quinonoid complex is more than an order of magnitude faster than exchange of the α-proton with solvent protons under identical experimental conditions means that base(s) on the enzyme, not readily accessible to solvent, is(are) accepting the proton in the conversion of the external aldimine to the quinonoid complex. The observation that ammonia does not accelerate the rate of formation of the quinonoid complex further supports this interpretation that quinonoid formation is not dependent on solvent buffer catalysis. If either of the protons were transferred to the buffer, the rates of E·Q formation and proton solvent exchange would be identical. Buffer catalysis by ammonia had previously been shown to occur with cytosolic SHMT (Schirch & Jenkins, 1964b).

Previous investigators have proposed that racemization of amino acids occurs by proton removal and addition to opposite sides of the pyridoxal-P ring in the quinonoid complex (Floss & Vederas, 1982). According to the Dunathan hypothesis, the removal and addition of the α-proton during the interconversion of the external aldimine and the quinonoid complex occurs with the proton perpendicular to the plane of the pyridoxal-P ring. This suggests that SHMT must catalyze the removal of the protons of D- and L-alanine from opposite faces of the ring. An alternative is that L-alanine binds to the active site with the methyl and carboxyl groups reversed with respect to the binding of D-alanine. This would permit the removal of both protons from the same face of the pyridoxal-P ring, but this mechanism seems highly unlikely.

We have found that the rate of formation of the quinonoid complex is about 2 orders of magnitude faster than the racemization reaction and about 50-fold faster then C_α-proton solvent exchange under identical experimental conditions (Table II). These observations can be explained by the enzyme retaining the proton, removed in the conversion of the external aldimine to the quinonoid complex, so that it almost always replaces this same proton to the same side of the planar quinonoid complex. These results suggest that the quinonoid complexes formed from D- and L-alanine are not identical. The quinonoid complexes from D- and L-alanine are refered to as $H{\cdot}E{\cdot}Q_D$ and $H{\cdot}E{\cdot}Q_L$, respectively (Figure 3). The H in this notation (H·E·Q) represents the α-proton from the amino acid which is now present on some base on the enzyme. A schematic of the formation of the quinonoid complexes from D- and L-alanine is shown in Figure 3. In this figure, we are viewing the plane of the pyridoxal-P ring on an edge, and it is represented as a rectangle.

The amino acid and pyridoxal-P components of the quinonoid complex are completely symmetrical and would have the same structure whether the complex was formed from either L-alanine or D-alanine. Since the quinonoid complex of pyridoxal-P and an amino acid is symmetrical, the asymmetry of $H{\cdot}E{\cdot}Q_D$ and $H{\cdot}E{\cdot}Q_L$ must be imposed by the protein in one of two ways. This asymmetry can be due either to a different orientation of the coenzyme in the active-site pocket or to the placement of protons with respect to the face of the quinonoid complex. In the study of amino acid racemases, the mechanism proposed for a single base removing H_R and H_S of the substrates occurs with two quinonoid orientations in the active

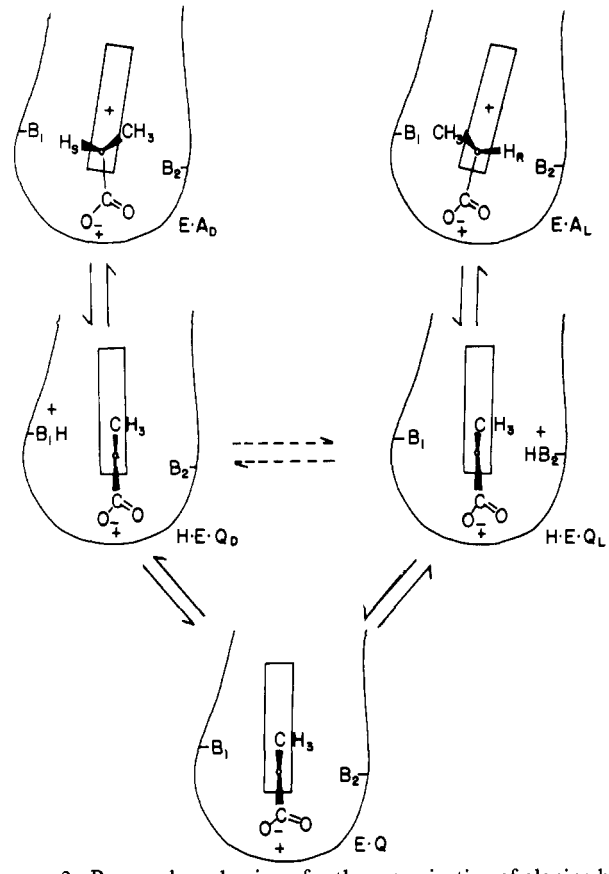


FIGURE 3: Proposed mechanisms for the racemization of alanine by SHMT. $E{\cdot}A_D$ and $E{\cdot}A_L$ represent external aldimines formed with D- and L-alanine, respectively. The respective quinonoid complexes are denoted as $H{\cdot}E{\cdot}Q_D$ and $H{\cdot}E{\cdot}Q_L$ where the proton removed from the α-carbon is at the active site on one of two bases, shown here as B_1 and B_2. E·Q represents a symmetrical intermediate where neither base is protonated. Another symmetrical intermediate not shown is where both B_1 and B_2 are protonated.

site and is referred to as the swinging-door mechanism (Henderson & Johnston, 1976). In a two-base mechanism, the quinonoid complex has a single orientation but differs by placement of the removed α-proton in the active site.

Our results with SHMT suggest that the asymmetry with respect to $H{\cdot}E{\cdot}Q_D$ and $H{\cdot}E{\cdot}Q_L$ is the location of the proton, and not different orientations of the ring. We propose that there are two bases at the active site which lie on opposite sides of the pyridoxal-P ring. We refer to these bases as B_1 and B_2 in Figure 3. In our study, there are three observations which argue against the single-base–swinging-door mechanism for *E. coli* SHMT. First, the relative rates of transamination for D- and L-alanine are directly proportional to the absorbance at 505 nm (the concentration of E·Q). In other words, the relationship $k_D/k_L = [E{\cdot}Q_D]/[E{\cdot}Q_L]$ holds true for both enzyme forms at several different temperatures and pH values. This suggests that the rate-determining step in transamination is the rate of proton addition to the 4′-carbon of the quinonoid complex to yield the product pyridoxamine-P. The similarity in the rate of addition of a proton to $H{\cdot}E{\cdot}Q_D$ and $H{\cdot}E{\cdot}Q_L$ suggests that the orientation of the quinonoid complex with respect to solvent is the same regardless of whether the complex was formed from D- or L-alanine. Second, the positions of λ_{max} for the quinonoids $H{\cdot}E{\cdot}Q_D$ and $H{\cdot}E{\cdot}Q_L$ are the same. There is evidence that the value of λ_{max} is sensitive to both amino acid structure and the protein component of the quinonoid complex (Kallen et al., 1985). Third, we have carefully determined the optical activity of the quinonoid complex and normalized the optical activity to the absorbance of the com-

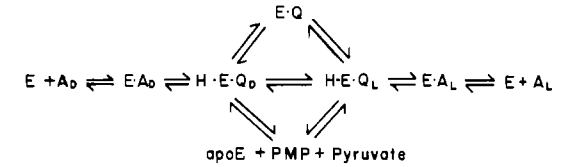


FIGURE 4: Proposed interrelationship of the reactions catalyzed by SHMT with D- and L-alanine. The abbreviations are the same as those used in Figure 3.

plex. At 505 nm, the optical activities of H·E·Q_D and H·E·Q_L are identical. The optical activity is conferred by the orientation of the quinonoid complex in the active site, and the identical optical activity for the two quinonoids further suggests that the orientation with respect to the protein is the same for both complexes. The swinging-door–one-base mechanism for racemization would be expected to elicit different optical properties for the two quinonoid complexes.

Figure 4 shows a reaction pathway incorporating the four reactions catalyzed by SHMT. There are two possible pathways for the enzyme to interconvert D- and L-alanine. First, H·E·Q_D could directly be converted to H·E·Q_L. In our mechanism shown in Figure 3, this could be accomplished by a transfer of the proton between B_1 and B_2 by some proton relay system at the active site. The alternative is that the ring flips over or swings as proposed in the swinging-door mechanism which seems highly unlikely for SHMT. A second method for racemization is that in the quinonoid complex either H·E·Q_D or H·E·Q_L loses a proton to the solvent to form E·Q which would be a truly symmetrical intermediate. (Another symmetrical intermediate not shown is where both B_1 and B_2 are protonated.) In this mechanism, the rate of solvent exchange of the α-proton would be expected to reflect the rate of racemization. Our results show that this is not true. H_4-folate increases the rate of solvent exchange of the D-alanine α-proton by at least 2 orders of magnitude but has essentially no effect on racemization rates with either D- or L-alanine. This suggests that in the rapid equilibration of the D-alanine proton with solvent the enzyme still remembers from which face of E·Q the proton was removed. If the rate of racemization was controlled by the formation of E·Q (Figure 3), then the rate-determining step would have to be from the direction of L-alanine. However, the rate of solvent exchange for L-alanine also exceeds the rate of racemization. The mechanism for racemization of D- and L-alanine by SHMT must be more complex than the model shown in Figure 3.

In a two-base mechanism, as proposed in Figure 3, the argument has been made that there would be no internal return of the H_S proton to the H_R position during racemization. With amino acid racemase from *Pseudomonas striata*, 10.2% internal return of the H_S to the H_R position was observed (Shen et al., 1983). Furthermore, internal return values of 11–18% with α-amino-ϵ-caprolactam racemase have been reported under single-turnover conditions (Ahmed et al., 1986). This internal return has been used as an argument for a one-base mechanism for pyridoxal-P racemases. However, if SHMT had a proton shuttle system between the putative bases B_1 and B_2 (Figure 3, dashed arrows), the enzyme would be an effective racemase and also catalyze internal return of protons between the two alanine isomers. This possibility of a proton shuttle with a pool of interchangeable protons at the active site seems more plausible from the recent studies with aconitase. This enzyme has a network of at least five interchangeable protons at the active site which exchange slowly with the solvent (Kuo & Rose, 1987).

The existence of multiple quinonoid complexes, which differ only by the location of a proton on the enzyme, has also been suggested for aspartate aminotransferase (Chen et al., 1987). These authors argue that to explain their kinetic observations, they need at least three quinonoid complexes which differ by the degree of protonation of two bases at the active site. Our observation that the rates of formation of H·E·Q_D and H·E·Q_L decrease above pH 8.4 could also be explained by invoking the same sequence of multiple quinonoid complexes differing by the degree of protonation as described for aspartate aminotransferase. The recent results with aspartate aminotransferase also suggest it is important that the enzyme maintain a constant net charge at the active site during the interconversion of intermediates. The advantage of having a pool of interchangeable protons which do not exchange with the solvent is that protons can be added or removed from reaction intermediates without changing the net charge at the active site.

We have not identified the nature of the putative bases B_1 and B_2 in our mechanism. We cannot rule out that they are activated bound water molecules. One possible binding site for water is in the hydroxymethyl binding site for the substrate L-serine. The structure of either B_1 or B_2 could also be Lys-228 which is expelled from being bound to pyridoxal-P during the formation of the external aldimine. This is the base which has been proposed for the removal of the α-proton of the amino acid substrate in aspartate aminotransferase (Jansonius et al., 1985; Arnone et al., 1985). However, it is clear that neither B_1 nor B_2 is the active-site histidine at position 228.

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Substrate Flux through Methylenetetrahydrofolate Dehydrogenase: Predicted Effects of the Concentration of Methylenetetrahydrofolate on Its Partitioning into Pathways Leading to Nucleotide Biosynthesis or Methionine Regeneration†

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ABSTRACT: Folic acid exists in mammalian cells with a poly-γ-glutamate tail that may regulate the flux of folates through the various cellular pathways. The substrate polyglutamate specificity of methylenetetrahydrofolate dehydrogenase from pig liver has been examined by using a competitive method and measuring apparent tritium kinetic isotope effects on V_{max}/K_m for methylenetetrahydrofolate. This competitive method yields very accurate ratios of K_m values for alternate substrates of an enzyme and may also be applied to reactions with no isotope effect. In combination with published data from our own and other laboratories, the kinetic parameters of methylenetetrahydrofolate dehydrogenase were used to calculate the initial velocities of pig liver methylenetetrahydrofolate dehydrogenase, thymidylate synthase, and methylenetetrahydrofolate reductase, at physiological concentrations of substrates and enzymes. These calculations suggest that the cellular concentration of methylenetetrahydrofolate may regulate the flux of this metabolite into the pathways leading to nucleotide biosynthesis and methionine regeneration. An increase in the cellular level of methylenetetrahydrofolate would permit more one-carbon units to be directed toward nucleotide biosynthesis.

Most cellular folates in mammals possess four to seven glutamyl residues linked in amide bonds involving the γ-carboxyl group. Several research groups have examined the polyglutamate specificites of folate-dependent enzymes from a variety of sources [e.g., MacKenzie and Baugh (1980), Matthews et al. (1985, 1987), and Allegra et al. (1985a); reviewed by McGuire and Coward (1984)]. While a regulatory role for the polyglutamate chain in determining the flux of folates in the cell has been proposed (Baggot & Krumdieck, 1979), no concrete model has been described. In particular, if the polyglutamate chain distribution changes only slowly (Eto & Krumdieck, 1982), alterations in chain length are unlikely to play a significant role in cellular regulation.

We present a model that predicts how changes in the cellular concentration of methylenetetrahydrofolate will alter the flux of this substrate into the pathways leading to thymidylate/purine biosynthesis or methionine regeneration (Figure 1). Our calculations indicate that increasing levels of methylenetetrahydrofolate result in an increased flux of this metabolite into pathways leading both to thymidylate synthesis and to de novo purine biosynthesis, as compared to the flux into the pathway for methionine regeneration.

This model is based on calculations of the initial velocities of pig liver methylenetetrahydrofolate reductase, pig liver methylenetetrahydrofolate dehydrogenase, and fetal pig liver thymidylate synthase and takes into account the polyglutamate specificities of these three enzymes for their polyglutamate substrates. The cellular concentrations of the pertinent enzymes and cosubstrates in pig liver were also considered. While most of the kinetic parameters needed for this calculation have been published, the reported K_m values for methylenetetrahydrofolate dehydrogenase were inconsistent. MacKenzie and Baugh (1980) observed changes in K_m for

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