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A Novel Intermediate in the Interaction of Thiosemicarbazide with Sheep Liver Serine Hydroxymethyltransferase*

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An unusual intermediate bound to the enzyme was detected in the interaction of thiosemicarbazide with sheep liver serine hydroxymethyltransferase. This intermediate had absorbance maxima at 464 and 440 nm. Such spectra are characteristic of resonance stabilized intermediates detected in the interaction of substrates and quasisubstrates with pyridoxal phosphate enzymes. An intermediate of this kind has not been detected in the interaction of thiosemicarbazide with other pyridoxal phosphate enzymes. This intermediate was generated slowly ($t_{1/2}$ = 4 min) following the addition of thiosemicarbazide (200 μM) to sheep liver serine hydroxymethyltransferase (5 μM). It was bound to the enzyme as evidenced by circular dichroic bands at 464 and 440 nm and the inability to be removed upon Centricon filtration. The kinetics of interaction revealed that thiosemicarbazide was a slow binding reversible inhibitor in this phase with a k_{on} of 11 M^{-1} s^{-1} and a k_{off} of 5 × 10^{-4} s^{-1}. The intermediate was converted very slowly (k = 4 × 10^{-5} s^{-1}) to the final products, namely the apoenzyme and the thiosemicarbazone of pyridoxal phosphate. A minimal kinetic mechanism involving the initial conversion to the intermediate absorbing at longer wavelengths and the conversion of this intermediate to the final product, as well as, the formation of pyridoxal phosphate-thiosemicarbazone directly by an alternate pathway is proposed.

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1)[1] a pyridoxal 5′-phosphate (PLP) protein, catalyzes the first step in the pathway for the interconversion of folate coenzymes. SHMT facilitates the formation of 5,10-methylenetetrahydrofolate from serine and tetrahydrofolate (H_4-folate) as well as the aldolytic cleavage of a number of β-hydroxyamino acids, decarboxylation of aminomalonate, and transamination of D-alanine in addition to other reactions (Schirch, 1984; Ulevitch and Kallen, 1977; Palekar *et al.*, 1973; Mathews and Drummond, 1990). It is also a component of the thymidylate synthesis cycle (Snell, 1984) along with dihydrofolate reductase and thymidylate synthase which have been favorite target enzymes for the design of chemotherapeutic agents. Attempts at developing inhibitors specific to SHMT have not been successful (Tendler *et al.*, 1989). The systematic examination of the interaction of various carbonyl-directed reagents with SHMT appeared promising for the design of specific inhibitors for this enzyme. This has been our approach in the study of interaction of aminooxy compounds with SHMT (Manohar *et al.*, 1984; Baskaran *et al.*, 1989a, 1989b; Appaji Rao, 1991; Acharya *et al.*, 1991). We had demonstrated that *O*-amino-D-serine and aminooxyacetic acid, which could be considered as aminooxy analogues of serine and glycine, were potent inhibitors of the enzyme and generated a unique intermediate in their reaction with SHMT.

Hydrazides are extensively used as inhibitors of PLP enzymes and to probe into the interactions at the active site of these enzymes (Sizer and Jenkins, 1963; Torchinsky and Koreneva, 1964; Wood and Abrahams, 1971; Wood and Peesker, 1974; Fisher and Davies 1974; Klosterman, 1986). It was therefore of interest to examine the mechanism of reaction of hydrazide derivatives with sheep liver SHMT. In this paper we describe the slow binding of thiosemicarbazide with SHMT resulting in the formation of a hitherto undetected enzyme-bound intermediate, characterized by absorption maxima at 464 and 440 nm. This intermediate was converted to the PLP-thiosemicarbazone extremely slowly, indicating the potential of these reactions in the design of more effective and specific inhibitors for SHMT.

EXPERIMENTAL PROCEDURES

Materials

The following biochemicals were obtained from Sigma. 2-Mercaptoethanol, PLP, N^{α}-acetyl-L-lysine, EDTA. Centricon-30 were obtained from Amicon Division, W. R.Grace and Co., Danvers, MA. L-[3-^{14}C]Serine (53 mCi/mmol) was purchased from Amersham International, Bucks, England. H_4-folate was prepared by the method previously described (Hatefi *et al.*, 1959). Thiosemicarbazide was obtained from E. Merck Darmstadt, Germany.

Methods

Enzyme Purification and Assay—The enzyme was purified as described by Baskaran *et al.* (1989a). The enzyme (100 mg) was dialyzed for 24 h against 1 liter of 0.05 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A) with four changes to remove free PLP. The enzyme activity was assayed by the procedure of Taylor and Weissbach (1965) as modified by Manohar *et al.* (1982). The specific activity of the enzyme ranged from 7 to 9 μmol of HCHO formed per min per mg of protein.

Spectral Measurements—All the spectral measurements were carried out using buffer A at 25 ± 1 °C, unless otherwise mentioned. Absorption spectra were recorded in a Shimadzu UV 240 double-beam spectrophotometer. Circular dichroism (CD) measurements were carried out in a Jasco J 20C spectropolarimeter equipped with a DP-500 N data processor and monitor scope UP 3830 A. The instrument was continuously purged with pure nitrogen before and during

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[1] The abbreviations used are: SHMT, serine hydroxymethyltransferase; PLP, pyridoxal 5′-phosphate; H_4-folate, 5,6,7,8-tetrahydrofolate; CD, circular dichroism; TSC, thiosemicarbazide; E-TSC_1, enzyme-thiosemicarbazide intermediate; HPLC, high-performance liquid chromatography.

the experiments. Slits were programmed to 10-Å bandwidth. The CD spectra were plotted as molar ellipticity in the visible and as mean residue ellipticity in the far UV region (Greenfield and Fasman, 1969).

Preparation of PLP-Thiosemicarbazone—The enzyme (25 μM) was mixed with TSC (2 mM), incubated at 25 °C for 2 h, and heated at 90 °C for 2 min. The precipitated protein was removed by centrifugation at 20,000 × *g*. The supernatant (25 μl) was used for identifying the thiosemicarbazone. The PLP-thiosemicarbazone was prepared by reacting equal amounts of PLP and thiosemicarbazide (10 mM) at 50 °C for 1 h (Furst and Gustafson, 1967).

HPLC of PLP-Thiosemicarbazone—The thiosemicarbazone obtained upon the interaction of the enzyme with TSC and in nonenzymatic model systems were processed separately on a LKB Spherisorb octyl 3-μm (4.6 × 100 mm) column using 0.1% trifluoroacetic acid in water (solvent A) and 50% acetonitrile (solvent B) as the solvent system. The products were eluted with increasing concentrations of 0.1% trifluoroacetic acid in 50% acetonitrile (solvent B) as follows: 0–5 min, 0% solvent B; 5–15 min, 25% solvent B; 15–30 min, 50% solvent B; 30–45 min, 100% solvent B. The flow rate was 0.5 ml/min. The column effluents were monitored at 390 nm in a Shimadzu LC-6A HPLC system.

Centricon Filtration—The enzyme (5 μM) was incubated with TSC (200 μM) for 90 min at 25 °C in a 3-ml Centricon tube. An enzyme sample similarly incubated but in the absence of TSC served as a control. The control (1 ml) and one aliquot (1 ml) of the test enzyme were then centrifuged at 4,000 × *g* for 30 min. A duplicate set of the test sample was centrifuged in a standard tube. The contents of the upper chamber of the Centricon set up were reconstituted to the original volume (1 ml). The spectra of the reconstituted portions, the filtrates, and also of the centrifuged enzyme were recorded in a Shimadzu UV-240 spectrophotometer.

RESULTS

Inhibition of the Activity of Sheep Liver SHMT by TSC—The time course of the reaction, initiated by the addition of the enzyme (10 nM) to solutions containing saturating concentrations of L-serine (3.6 mM) and H_4-folate (2 mM) was linear up to 15 min under the conditions of the assay. However in the presence of varying concentrations of TSC (1–3 mM) it was not linear, and significantly higher inhibition was observed at later points in the time course of the reaction (Fig. 1). Such inhibition could occur, among other reasons, due to slow binding of the inhibitor to the enzyme or mechanism-based inactivation. The instability of the enzyme at low concentration (10 nM) precluded studying the reaction until the enzyme-inhibitor complex could reach steady state equilibrium. When SHMT (1 μM) was preincubated with increasing concentrations of TSC (0–300 μM) (Fig. 2), the overall activity decreased as a function of time and TSC concentrations. The loss of activity followed a biphasic mode of inhibition. The initial phase was linear up to 20 min and was dependent upon the concentration of the inhibitor used. This was followed by a slower phase which extended over long periods of time and terminated with almost total loss of enzyme activity. These results suggested that there could be more than one mode of interaction of the enzyme with the inhibitor.

TSC was a noncompetitive inhibitor of SHMT in the first phase of interaction when serine was the varied substrate (data not shown). A replot of the slope of the reciprocal plot *versus* concentration of TSC gave an apparent K_i of 50 μM. Although such intersecting plots are often indicative of reversible interactions between the inhibitor and an enzyme form, it could also arise by irreversible inactivation of the enzyme. Incubation of the enzyme (1 μM) for 10 h at 25 °C with TSC (300 μM) resulted in almost complete (95%) loss of activity, while the enzyme similarly treated but in the absence of TSC lost little activity. Incubation of the inhibited enzyme (1 μM) with PLP (500 μM) for 5 min, restored 48% of the activity compared to the control enzyme, which was also similarly incubated with PLP (500 μM) for 5 min. The enzyme (1 μM) incubated with TSC (300 μM) for 20 min had 14% of residual activity and upon preincubation of this enzyme (1 μM) with PLP (500 μM) for 5 min regained only 24% of the activity as the control (Table I). This paradoxical observation suggested that, upon incubation for longer periods, the enzyme-inhibitor complex changed to a form which was readily reconstituted by PLP to the active enzyme. These results implied that there were multiple steps in the interaction of SHMT with TSC. The presence of PLP at the active site with its characteristic spectral properties enabled further examination of the interaction between the enzyme and TSC.

Spectral Studies—The sheep liver SHMT has an absorbance maximum at 425 nm due to an internal aldimine (Manohar *et al.*, 1982). The addition of 200 μM of TSC to the enzyme (5 μM) resulted in a red shift of the absorbance maximum at 425 nm, an increased absorbance at 464, 440, 390, and 320 nm. After 15 min, the spectra had clear maxima

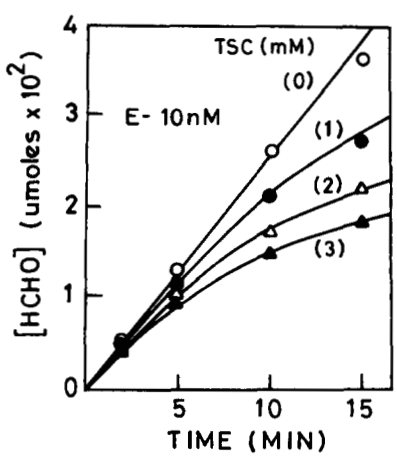

FIG. 1. **Progress curves for the reaction of sheep liver SHMT in the presence of TSC.** The activity of the enzyme (10 nM) was assayed in the presence of increasing concentration (0, 1, 2, and 3 mM) of TSC at 37 °C in reaction mixtures containing 0.4 M phosphate buffer (pH 7.4), 1 mM EDTA, 1.8 mM dithiothreitol, 2 mM H_4-folate, and 3.6 mM L-[3-^{14}C]serine (210,000 cpm/100 μl). Aliquots (100 μl) were withdrawn at various time intervals indicated, the reaction was arrested by the addition of an equal volume of dimedone, and the product formed was estimated (Taylor and Weissbach, 1965; Manohar *et al.*, 1982).

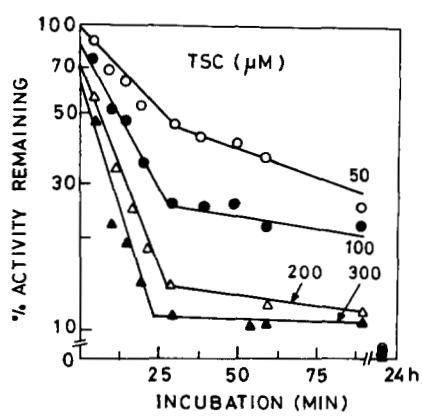

FIG. 2. **Time- and concentration-dependent inhibition of SHMT by TSC.** The enzyme (1 μM) in buffer A was separately preincubated with 50, 100, 200, and 300 μM of TSC. Aliquots (10 μl) were withdrawn at time intervals indicated into the assay mixture containing 0.4 M phosphate buffer (pH 7.4), 1 mM EDTA, 1.8 mM dithiothreitol, 2 mM H_4-folate, and 3.6 mM L-[3-^{14}C]serine (72,000 cpm) and incubated for 2 min at 37 °C. The amount of HCHO formed was determined (Taylor and Weissbach, 1965; Manohar *et al.*, 1982).

TABLE I

Time-dependent inhibition of sheep liver SHMT by TSC and reactivation by PLP

The enzyme (1 μM) in buffer A was incubated at 25 °C with TSC (300 μM). Aliquots were withdrawn at time points indicated and incubated for 5 min at 25 °C in buffer A either with 500 μM PLP or without it. Enzyme (1 μM), similarly treated but in the absence of TSC, served as control. A portion (10 μl) of this was then assayed for activity. The activity of the control enzyme (1 μM) at 10 h was more than 97% of its activity at 0 time.

Time	Activity remaining	
	Without PLP	With PLP
h	%	
0.3	14	24
1.5	14	32
3.0	11	34
6.0	6	47
10.0	5	48

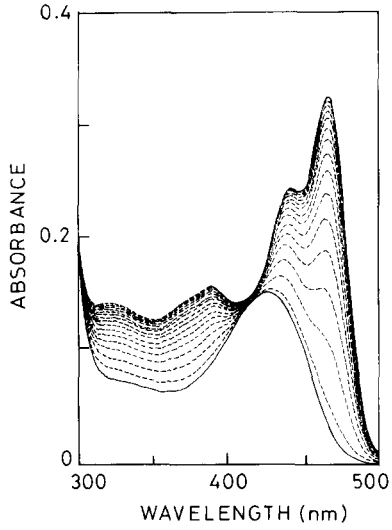

FIG. 3. **Visible absorbance spectra of sheep liver SHMT upon its interaction with TSC.** The enzyme (5 μM) (*continuous line*) in buffer A was mixed with TSC (200 μM), and the spectrum was recorded 15 s after the addition of the inhibitor and every minute thereafter for 15 min (*discontinuous lines*).

at 464, 440, 390, and 320 nm (Fig. 3). During the next 15 min, there was no appreciable change at 464- and 440-nm spectral peaks, whereas there was a slow increase in absorbance at 390- and 320-nm peaks. Thereafter, there was a slow decrease in absorbance at 464 and 440 nm and a continued increase in absorbance at 390 and 320 nm (Fig. 4). The spectral change in this instance had a clear isosbestic point at 411 nm. This phase of interaction was very slow, and the conversion was not complete even after 24 h. At these periods of time, there was no discernible change in the spectral properties of the native enzyme. Upon the addition of TSC (200 μM) to the enzyme (5 μM), there was no appreciable change in the absorbance between 250–300 nm except for a slight (10%) increase in absorbance at 278 nm (data not shown). It was, therefore, of interest to monitor whether the intermediate represented a form bound to the enzyme and, if so, the strength of its binding.

The characteristic CD spectrum of the holoenzyme with a maximum at 430 nm provided a convenient method to examine the possibility of the intermediate being bound to the active site of the enzyme. Upon the addition of TSC (400 μM) to sheep liver SHMT (5 μM), the CD spectrum was altered, forming a new spectrum with positive dichroic maxima at 464 and 440 nm (Fig. 5) corresponding to the absorbance peaks

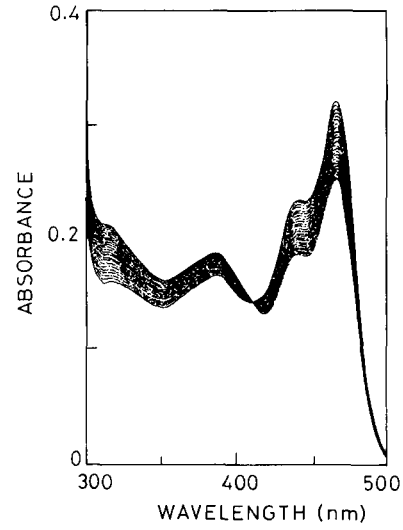

FIG. 4. **Visible absorbance spectrum of sheep liver SHMT during the second phase of its interaction with TSC.** The enzyme (5 μM) in buffer A was mixed with TSC (200 μM) as described in Fig. 3. The absorbance spectrum was recorded 30 min after the incubation and every 3 min for 90 min thereafter.

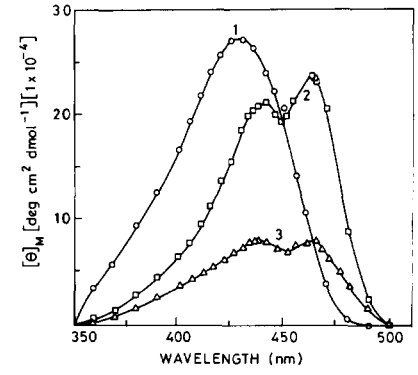

FIG. 5. **Changes in the visible circular dichroic spectrum of sheep liver SHMT upon its interaction with TSC.** The enzyme (5 μM) in buffer A (*1*) and TSC (200 μM) were mixed, and the spectrum was recorded 50 min (*2*) and 24 h (*3*) after the addition of TSC to the enzyme. The spectrum were reconstructed for molar ellipticity according to the method of Greenfield and Fasman (1969).

seen in Fig. 4. The CD spectrum recorded 45 min after the addition of TSC showed peaks at these wavelengths. Subsequently, the intensity of these bands decreased very slowly, and a spectrum recorded 24 h later retained about 25% of the maximal absorbance of the peaks (Fig. 5). These observations suggested that the intermediate was probably still bound to the enzyme. The far ultra-violet CD spectrum of the enzyme was not altered appreciably in the presence of TSC suggesting that TSC was not probably causing a gross change in the protein (data not shown).

Separation of Products by Centricon Filtration—As the intermediates formed on the addition of TSC to SHMT were stable, it was thought worthwhile to separate them from the enzyme and also determine if they were loosely or tightly bound to the enzyme. The enzyme (5 μM) was incubated at 25 °C for 90 min, transferred to a Centricon-30 tube, and centrifuged at 4000 × *g* for 30 min. The retentate was reconstituted to the original (1 ml) volume. The spectra of the filtrate and the retentate were recorded. The filtrate had no clear spectra (Fig. 6, *curve 1*), whereas the retentate gave the characteristic spectrum of SHMT absorbing maximally at 425 nm (*curve 2*). The enzyme (5 μM) was reacted with TSC (200

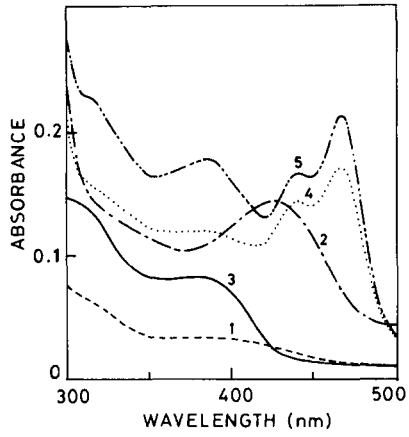

FIG. 6. **Evidence for the generation of an enzyme-bound intermediate when TSC was added to sheep liver SHMT.** The enzyme (5 μM) in buffer A was incubated at 25 °C for 90 min. It was then transferred onto a Centricon-30 tube and centrifuged at 4000 × *g* for 30 min. The spectrum of the filtrate (*1*) and of the retentate in the upper chamber after reconstitution to the original volume (*2*) were recorded. The enzyme (5 μM) treated with TSC (200 μM) was similarly processed, and the spectra of the filtrate (*3*) and the retentate (*4*) were recorded. The enzyme was treated exactly as described with TSC but only centrifugation instead of Centricon treatment was carried out and the spectrum was recorded (5).

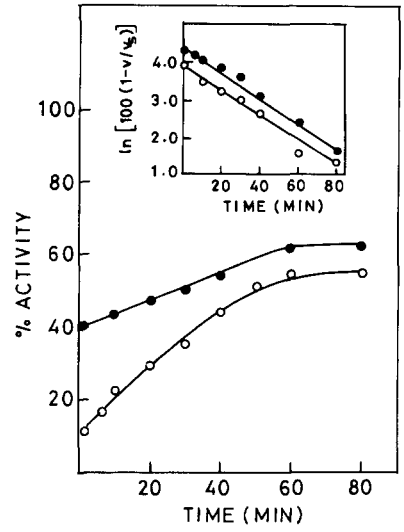

FIG. 7. **Reversal of the initial phase of inhibition of sheep liver SHMT by TSC.** The enzyme (25 (●) or 50 μM (○)) in 1 ml of buffer A was mixed with 100 (●) or 400 μM (○) of TSC. Spectra were recorded. Aliquots were diluted (when the spectral species showed maximal absorbance at 440 and 464 nm) 50 (●)- or 100 (○)-fold in buffer A. 10-μl aliquots of this were then assayed for activity at time points indicated, after dilution. *Inset*, ln [100(1-v/v_s)] plotted against the time of incubation after dilution. v = velocity at time t, v_s = final velocity regained. The slope is equal to $-k_{off}$.

μM) for 90 min and subjected to Centricon filtration as described above. The filtrate in this instance had spectrum with absorbance maxima at 390 and 320 nm (*curve 3*). The retentate had a spectrum with clear maxima at 464 and 440 nm, but the absorbance at 390 and 320 nm had decreased considerably (*curve 4*) compared to the spectrum of the enzyme (5 μM) treated with TSC (200 μM) but centrifuged instead of Centricon treatment (*curve 5*). These experiments suggested that the 464- and 440-nm absorbing species was probably a tightly bound enzyme intermediate, which was stable for considerable periods of time.

Reversibility and Dissociation of the Inhibitor from the Enzyme—The results described above suggested that TSC was probably a slow binding inhibitor forming an intermediate showing visible absorbance maxima at 464 and 440 nm and the k_{off} was probably very low. To examine this possibility, the enzyme (25 and 50 μM) was incubated with varying concentrations of the inhibitor for different periods of time. A time course of the spectral changes was continuously monitored. After the spectral species with maximal absorbance at 464 and 440 nm were formed, aliquots were withdrawn and diluted 50-, 100-, and 200-fold (data for the last concentration of the enzyme not given) in buffer A. These dilutions were adequate since the calculated second order rate constant for the association was small. Aliquots were assayed for activity in the absence of PLP (Fig. 7). A zero order regain of activity was noted, and a first order rate of 5.0×10^{-4} s^{-1} was calculated (Fig. 7, *inset*). The recovery of activity was not complete in the absence of PLP and was dependent on the concentration of the inhibitor used. This was due to the existence of the second step in the interaction of TSC with SHMT where the intermediate was converted very slowly to the apoenzyme.

Identification of the Product Formed in the Reaction of TSC with Sheep Liver SHMT—The spectral properties of the final product was compatible with the suggestion that it was probably a PLP-thiosemicarbazone. The following experiment confirmed the suggestion. The supernatant of the enzyme treated with TSC, obtained as described under "Methods" had a visible absorbance spectrum at 390 and 320 nm. When this was subjected to reversed-phase HPLC, a peak appeared with a retention time of 21.6 min which was identical to that obtained with an authentic sample of PLP·TSC (21.8 min). These results suggest that the final product of the reaction of enzyme with TSC was PLP-thiosemicarbazone.

Kinetics of Formation of Intermediates upon Interaction of the Enzyme with TSC—The analysis of the spectra in Figs. 3 and 4 suggested that the kinetics of the individual steps in the reaction could best be monitored by measuring the absorbance changes at 464, 440, 390, and 320 nm. The reaction was monitored at each of these wavelengths under pseudo-first order conditions. The increase in absorbance was biphasic at 390 and 320 nm. The calculated second order constants from the first order plots was 11 M^{-1} s^{-1} at 464 and 440 nm. The faster phase of increase in absorbance at 390 and 320 nm followed pseudo-first order kinetics with a second order rate constants of 8 M^{-1} s^{-1}. In addition to this step there was a slow phase of decrease in absorbance at 464 and 440 nm and the slower phase of increase in absorbance at 390 and 320 nm with the isosbestic point at 411 nm. An approximate first order rate constant for this reaction was calculated to be 4×10^{-5} s^{-1} by following the rate of decrease at 464 and 440 nm for time periods when the absorbance at these wavelengths were about 20% of its original amplitude. Measurements could not be taken beyond these time points because of the extremely slow nature of this transition, and the enzyme began to denature.

Interaction of TSC with PLP and N^{α}-Acetyl-L-lysine-PLP Schiff Base—Upon the addition of TSC (1 mM) to PLP (100 μM) there was an increase in absorbance at 390 and 320 nm indicating the formation of PLP-thiosemicarbazone (data not shown). Upon the addition of TSC (1 mM) to PLP-N^{α}-acetyl-L-lysine Schiff base, there was a decrease in absorbance at 410 nm associated with a blue shift slowly and a subsequent increase in absorbance at 390 and 320 nm. This indicated that TSC interacted with the Schiff base to form the thiosemicarbazone.

DISCUSSION

The data presented in this paper clearly demonstrates that the time-dependent inactivation of sheep liver SHMT by TSC

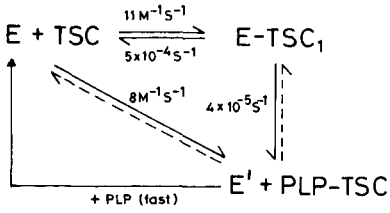

FIG. 8. **Minimal kinetic mechanism for the interaction of TSC with sheep liver SHMT.** *E*, holoenzyme; *E'*, apoenzyme; *PLP·TSC*, pyridoxal phosphate thiosemicarbazone.

probably represents slow binding inhibition (Williams and Morrison, 1979; Morrison, 1982; Morrison and Walsh, 1987). The onset of the reaction was very slow and could be monitored, as in the case of the inhibition of galactosidase by D-galactal (Wentworth and Wolfenden, 1974) using conventional spectrophotometry. The results described in this paper are compatible with the following minimal kinetic mechanism given for the interaction of TSC with the sheep liver SHMT in Fig. 8.

The first step in the interaction of TSC with sheep liver SHMT was slow and had a second order rate constant of 11 M^{-1} s^{-1} when absorbance change was monitored at 440 and 464 nm and 8 M^{-1} s^{-1} at 390 and 320 nm. A similar rate constant was obtained for the interaction of L-alanine phosphonate with alanine racemase from *Thermophillus stearothermophillus* (Badet *et al.*, 1986). The first step in the interaction was probably bifurcated into two pathways: one leading to the formation of E-TSC_1 characterized by the absorbance maxima at 464 and 440 nm and the other leading to the formation of apoenzyme and PLP-thiosemicarbazone absorbing at 390 and 320 nm. The catalytically active enzyme upon interaction with TSC under pseudo-first order reaction conditions for 15 min (the time taken to form E-TSC_1 as in Fig. 3) leads to a concentration-dependent inhibition of enzyme activity. From the double reciprocal plots at varying serine concentrations and different fixed concentrations of TSC, a K_i of 50 μM was calculated. PLP partially reversed the inhibition (Table I). This observation can be explained by the minimal kinetic mechanism as due to the inability of E-TSC_1 to be reversed by PLP. The apoenzyme and PLP-thiosemicarbazone formed by the alternative pathway could be converted to catalytically active enzyme upon the addition of PLP. As there was a mixture of the two forms at this point (15 min) partial reversal by PLP was in accordance with the mechanism. The increased reactivation with time upon addition of PLP was due to the slow conversion of E-TSC_1 to the apoenzyme. Morrison and Walsh (1987) classified reversible inhibitors as classical, tight binding, slow binding, and slow tight binding. Tight binding inhibitors are defined as those which produce substantial inhibition at concentrations that are comparable to the total enzyme concentration in the assay. Slow binding inhibitors are defined as those for which, at concentrations that cause moderate inhibition, equilibrium between enzyme, inhibitor, and enzyme-inhibitor complex is not reached before substantial depletion of the substrate has taken place. TSC could thus be regarded as a slow binding inhibitor of SHMT resulting in the generation of E-TSC_1 at the active site of the enzyme and was probably enzyme-bound. PLP bound to several enzymes have been shown to produce characteristic dichroic signals (Wilson and Meister, 1966; Martinez-Carrion *et al.*, 1970; Bayley and Harris, 1975; Miles and Moriguchi, 1977; Johnson and Graves, 1966). SHMT also has a characteristic CD band at 430 nm (Quashnock *et al.*, 1983; Manohar *et al.*, 1982). Free PLP and N^{α}-acetyl-L-lysine-PLP Schiff base exhibit little CD rotations in this region. It may be argued that characteristic visible CD spectra represent the presence of PLP at the active site of these enzymes. It may therefore be suggested that the E-TSC_1 which showed dichroic bands at 464 and 440 nm was present at the active site, and the subsequent very slow decrease in the CD spectrum at these wavelengths would indicate that this intermediate remained within the asymmetric milieu of the active site for long periods of time. Additional evidence that this intermediate was enzyme-bound was the observation that upon Centricon filtration or upon passage of E-TSC_1 through Sephadex G-25 gel-filtration column (data not shown), the protein fraction contained the 464- and 440-nm absorbing species. Upon denaturation of the enzyme by heating or with guanidinium hydrochloride (data not shown), the intermediate was rapidly converted to the PLP-thiosemicarbazone. All attempts to obtain the intermediate free of the enzyme were unsuccessful. Intermediates absorbing at longer wavelengths have been observed upon interaction of glycine and H_4-folate with the enzyme and have been assigned a resonance-stabilized quinonoid structure (Quashnock *et al.*, 1983). In the case of aspartate aminotransferase (Jenkins, 1961), tryptophan synthase (Miles, 1980), and SHMT (Schirch and Jenkins, 1964a, 1964b) and several other PLP enzymes, the interaction with a variety of substrates and substrate analogues yielded intermediates absorbing at longer wavelengths. These intermediates have been identified as resonance-stabilized semiquinones. In analogy with these observations, it can be suggested that E-TSC_1 could be a resonance-stabilized quinonoid form of PLP·TSC at the enzyme-active site. The presence of a thioketone group in the inhibitor seems to be essential for the formation of the presumed quinonoid intermediate as semicarbazide does not yield such an intermediate upon interaction with SHMT.[2] Such an unique intermediate was not reported in the reaction of TSC with aspartate aminotransferase (Jenkins and D'Ari, 1966), glutamate decarboxylase (Fisher and Davies, 1974), 3,4-dihydroxyphenylalanine hydroxylase, and 5-hydroxytryptophan decarboxylase (Slow and Dakshinamurti, 1990).

This intermediate dissociates to yield the holoenzyme with a calculated first-order rate constant (k_{off}) of 5×10^{-4} s^{-1} measured by the dilution of E-TSC_1 complex and monitoring the recovery of enzyme activity (Fig. 7, *inset*). The next step in the kinetic mechanism is the conversion of E-TSC_1 to the apoenzyme (E') and PLP-thiosemicarbazone. This step was measured by the decrease in absorbance at 464 and 440 nm and the slow phase of increase in absorbance at 390 and 320 nm. This reaction was extremely slow, and a 50% decrease in absorbance occurred in about 5–6 h. An approximate rate for this step was calculated to be about 4×10^{-5} s^{-1}. The absence of any circular dichroic bands at 390 nm suggested that the PLP-thiosemicarbazone was probably not at the active site. The PLP-thiosemicarbazone could be separated from the enzyme protein by Centricon filtration or gel filtration. The apoenzyme thus generated (E') could be partially reactivated with PLP.

In a detailed study Jenkins and D'Ari (1966) examined the interaction of TSC with aspartate aminotransferase in the presence and absence of glutarate. In addition, they also examined the effects of TSC on the interaction of aminooxy compounds with aspartate aminotransferase (Raunio *et al.*, 1984). These results indicated that the reaction of TSC with the enzyme was slow, with a second order rate constant of about 1 M^{-1} s^{-1} compared to about 10 M^{-1} s^{-1} observed in this study. It is pertinent to mention that in the case of aspartate aminotransferase the pH at which the rates were measured

[2] J. K. Acharya and N. Appaji Rao, unpublished observations.

was 6.9, and in the case of SHMT it was 7.4 (pH values at which the reactions were optimal). The k_{off} in the case of aspartate aminotransferase was about 8×10^{-4} s^{-1} compared to 5×10^{-4} s^{-1} for the initial phase of the reaction of TSC with SHMT (Fig. 7). One significant difference, in the interaction of TSC with the two enzymes, is the formation of longer wavelength absorbing species in the interaction of TSC with SHMT. Although it was indicated that the complex absorbing at 390 nm, formed when TSC interacted with aspartate aminotransferase, was a mixture of two species, detailed kinetic analysis of the formation of the complex was not reported (Sizer and Jenkins, 1962). The product of the reaction in both the cases was probably PLP-thiosemicarbazone as indicated by its characteristic absorbance.

Davies and Johnston (1974), in their study conducted to correlate the regional and developmental patterns of glycine levels in the postnatal rat brain reported the inhibition of SHMT from cerebellar extract by TSC. No other details of interaction of TSC with SHMT were discussed in that paper. Our results are compatible with the suggestion that TSC is a slow binding inhibitor of SHMT and the slow-binding of TSC with sheep liver SHMT results in the formation, at the active site of SHMT, of a hitherto undetected intermediate absorbing at 464 and 440 nm. This intermediate is converted to the PLP-thiosemicarbazone extremely slowly, highlighting the potential of these reactions in the design of effective and specific inhibitors for SHMT.

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