interest is the way in which the enzymic mechanism alters if the glutathione-binding pocket is deprived of this particular protonatable residue. In this context, it is relevant to note that the only homologous flavoprotein disulfide oxidoreductase to lack a histidine residue at this position is mercuric reductase, in which it is replaced by a tyrosine residue (Brown et al., 1983; Misra et al., 1985; Laddaga et al., 1987). This enzyme is unique in that the substrate is not an organic disulfide.

Registry No. GSSG, 27025-41-8; NADPH, 53-57-6; His, 71-00-1; Tyr, 60-18-4; glutathione reductase, 9001-48-3.

REFERENCES

Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 1264–1269.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Boggoram, V., & Mannervik, B. (1978) *Biochem. Biophys. Res. Commun. 83*, 558–564.

Brown, N. L., Ford, S. J., Pridmore, D., & Fritzinger, D. C. (1983) *Biochemistry 22*, 4089–4095.

Carter, P., Bedouelle, M., Waye, M. M., & Winter, G. (1985) in *Oligonucleotide Site-Directed Mutagenesis in M13. A Laboratory Manual*, Anglian Biotechnology, London.

Greer, S., & Perham, R. N. (1986) *Biochemistry 25*, 2736–2742.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.

Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729.

Karplus, P. A., Pai, E. F., & Schulz, G. E. (1989) *Eur. J. Biochem. 178*, 693–703.

Laddaga, R. A., Chu, L., Misra, T. K., & Silver, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5106–5110.

Lederberg, J., & Zinder, N. (1948) *J. Am. Chem. Soc. 70*, 4267–4268.

Lowe, C. R., Hans, M., Spibey, N., & Drabble, W. T. (1980) *Anal. Biochem. 104*, 23–28.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem. 252*, 3199–3207.

Misra, T. K., Brown, N. L., Haberstroh, L., Schmidt, A., Goddette, D., & Silver, S. (1985) *Gene 34*, 253–262.

Pai, E. F., & Schulz, G. E. (1983) *J. Mol. Biol. 258*, 1751–1757.

Pai, E. F., Karplus, P. A., & Schulz, G. E. (1988) *Biochemistry 27*, 4465–4474.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. M., & Roe, B. A. (1980) *J. Mol. Biol. 143*, 161–178.

Schirmer, R. H., Krauth-Siegel, R. L., & Schulz, G. E. (1989) in *Glutathione: Chemical, Biochemical and Medical Aspects. Part A* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) pp 553–596, Wiley, New York.

Scrutton, N. S., Berry, A., & Perham, R. N. (1987) *Biochem. J. 245*, 875–880.

Scrutton, N. S., Berry, A., & Perham, R. N. (1988) *FEBS Lett. 241*, 46–50.

Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8749–8764.

Williams, C. H., Jr. (1976) *Enzymes (3rd Ed.) 13*, 89–173.

Wong, K. K., Vanoni, M. A., & Blanchard, J. S. (1988) *Biochemistry 27*, 7091–7096.

Mechanism of Interaction of *O*-Amino-D-serine with Sheep Liver Serine Hydroxymethyltransferase†

N. Baskaran, V. Prakash, A. G. Appu Rao, A. N. Radhakrishnan, H. S. Savithri, and N. Appaji Rao*

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India, and Biophysical Chemistry Section, Department of Food Chemistry, Central Food Technological Research Institute, Mysore 570013, India

Received March 16, 1989; Revised Manuscript Received July 25, 1989

ABSTRACT: The mechanism of interaction of *O*-amino-D-serine (OADS) with sheep liver serine hydroxymethyltransferase (EC 2.1.2.1) (SHMT) was established by measuring changes in the enzyme activity, absorption spectra, circular dichroism (CD) spectra, and stopped-flow spectrophotometry. OADS was a reversible noncompetitive inhibitor (K_i = 1.8 μM) when serine was the varied substrate. The first step in the interaction of OADS with the enzyme was the disruption of enzyme–Schiff base, characterized by the rapid disappearance of absorbance at 425 nm (6.5×10^3 M^{-1} s^{-1}) and CD intensity at 430 nm. Concomitantly, there was a rapid increase in absorbance and CD intensity at 390 nm. The spectral properties of this intermediate enabled its identification as pyridoxal 5′-phosphate (PLP). These changes were followed by a slow unimolecular step (2×10^{-3} s^{-1}) leading to the formation of PLP–OADS oxime, which was confirmed by its absorbance and fluorescence spectra and retention time on high-performance liquid chromatography. The PLP–OADS oxime was displaced from the enzyme by the addition of PLP as evidenced by the restoration of complete enzyme activity as well as by the spectral properties. The unique feature of the mechanism proposed for the interaction of OADS with sheep liver SHMT was the formation of PLP as an intermediate.

Serine hydroxymethyltransferase [L-serine:tetrahydrofolate; 5,10-hydroxymethyltransferase (SHMT), EC 2.1.2.1] catalyzes the conversion of L-serine to glycine and tetrahydrofolate (H_4folate)[1] to 5,10-methylene-H_4folate. The role of SHMT in cellular proliferation was reviewed (Schirch, 1982; Appaji Rao et al., 1987). D-Cycloserine (DCS) (Figure 1) functioned

† This work was supported by the Departments of Science and Technology and Biotechnology, Government of India, New Delhi, India.

* To whom correspondence should be addressed.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; H_4folate, 5,6,7,8-tetrahydrofolate; DCS, D-cycloserine; OADS, *O*-amino-D-serine; EDTA, ethylenediaminetetraacetic acid disodium salt; 2-ME, 2-mercaptoethanol; CM, carboxymethyl; A_{280}, absorbance at 280 nm; A_{425}, absorbance at 425 nm; CD, circular dichroism; TFA, trifluoroacetic acid.

0006-2960/89/0428-9607$01.50/0

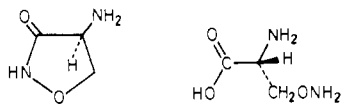

FIGURE 1: Structure of D-cycloserine and *O*-amino-D-serine.

as an antitumor drug in mice bearing Freund's virus leukemia by inhibiting SHMT (Bukin et al., 1970, 1979). In an earlier study from our laboratory (Manohar et al., 1984), it was shown that DCS binds rapidly and irreversibly to sheep liver SHMT to form an E–DCS–PLP Schiff base complex that slowly dissociated from the active site, leaving behind an apoenzyme. On the other hand, when DCS functioned as a suicide substrate (e.g., alanine racemase, EC 5.1.1.1), the cyclic structure of DCS was opened by a nucleophilic attack followed by acylation of an active-site residue (Karpeiskii et al., 1964; Roze & Strominger, 1966; Braunstein, 1973).

It was, therefore, of interest to examine the mechanism of inhibition of SHMT by *O*-amino-D-serine (OADS) (Figure 1), a hydrolytic product of DCS and also an aminooxy analogue of serine. While the mechanism of inhibition of a few aminotransferases and decarboxylases by aminooxy compounds has been studied (Free et al., 1967; Beeler & Churchich, 1976; John et al., 1978; Kito et al., 1978; Rosenthal, 1981; Raunio et al., 1984; Khomutov et al., 1985), no information is available on the interaction of aminooxy compounds with SHMT. In this paper we report the mechanism of interaction of SHMT by OADS monitored by spectral and kinetic methods.

Experimental Procedures

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO: 2-mercaptoethanol (2-ME), ethylenediaminetetraacetic acid (EDTA), DCS, pyridoxal 5′-phosphate (PLP), pyridoxamine 5′-phosphate hydrochloride (PMP), N^{α}-acetyl-L-lysine, carboxymethyl (CM)-Sephadex C-50, Sephacryl S-200, L-serine, and glycine. L-[3-^{14}C]Serine (53 mCi/mmol) was purchased from Amersham International, Bucks, England. OADS was prepared by hydrolyzing DCS as described by Stammer (1962). H_4folate was prepared by the method of Hatefi et al. (1959). All the other chemicals used were of analytical reagent grade.

Methods

Enzyme Purification and Assay. Large-scale purification of SHMT was achieved by a modification of the procedure of Manohar et al. (1982). The cytosolic fraction of sheep liver (1 kg) homogenate prepared in 0.05 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM 2-ME, and 0.05 mM PLP (buffer A) was adsorbed to CM-Sephadex in a separating funnel. The gel was washed extensively, and the enzyme was eluted with 0.4 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM 2-ME, and 0.05 mM PLP. The enzyme was precipitated at 50% ammonium sulfate saturation. This precipitate was dissolved in buffer A and fractionated by using a Sephacryl S-200 column (2 × 90 cm). The fractions (2 mL) containing enzyme activity (>7 units/mg of protein) and with an A_{280}:A_{425} ratio of >10 were pooled (Table I). The purified enzyme was identical in its physicochemical properties with that reported by Manohar et al. (1982). The enzyme was dialyzed against 0.05 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM 2-ME (buffer B) with four changes for 24 h to remove free PLP. The enzyme activity was assayed as described by Manohar et al. (1982).

Table I: Purification of Sheep Liver Serine Hydroxymethyltransferase

step	protein (g)	total act. (units)[a]	sp act. (units/mg of protein)
crude extract	150.4	5565	0.04
CM-Sephadex	1.12	2783	2.49
Sephacryl S-200	0.22	1606	7.30

[a] Micromoles of HCHO formed per minute at 37 °C and pH 7.4.

Spectral Measurements. All the spectral measurements were carried out by using buffer B at 25 ± 1 °C. 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 3830A. The instrument was continuously purged with pure nitrogen before and during the experiments. Slits were programmed to 10-Å bandwidth at each wavelength. The CD spectra of the enzyme were plotted as molar ellipticity (Greenfield & Fasman, 1969). The fluorescence spectra were obtained by using a Hitachi 650-60 fluorescence spectrophotometer attached to a 057 X–Y recorder.

Stopped-Flow Spectrometry. The absorption stopped-flow experiments were performed by using a Union-Gikon RA 401 stopped-flow spectrophotometer equipped with a 10-mm cell. The data were collected by using a NEC 9801 computer interfaced to the spectrophotometer. The solutions were mixed under N_2 pressure (6 kg/cm^2). The dead time of the instrument was 0.5 ms, and the slit width was set at 1.4 nm in all the experiments. All the reaction curves presented were the average of at least five sets of experiments. The k values that were determined by the built-in curve-fitting method agreed with the values calculated manually (Hiromi, 1979).

Preparation of PLP–OADS Oxime. The enzyme (5 mg/mL) in buffer B was incubated with OADS (1 mM) for 15 min at 37 °C. This mixture was heated at 95 °C for 2 min, and the denatured protein was removed by centrifugation at 10000g. The supernatant (E–OADS) was used for analysis. PLP–OADS oxime was prepared by mixing PLP (10 mM) and OADS (10 mM) in buffer B and incubated at 4 °C for 18 h.

High-Performance Liquid Chromatography (HPLC) of PLP–Oximes. Spherisorb 5 ODS (0.46 × 250 mm; 0.5-μm particle size) was used to separate the oxime formed upon the reaction of OADS with the enzyme. The separation of the oxime was carried out on an LKB 2152 HPLC system using water containing 0.1% trifluoroacetic acid (TFA) as the solvent.

Results

Inhibition of Sheep Liver SHMT Activity by OADS. The inhibition of the enzyme by OADS was time and concentration dependent (Figure 2). The activity of the enzyme was partially protected (57%) against OADS inhibition when the enzyme was incubated with serine (24 mM) prior to the addition of OADS (10 μM), whereas PLP (100 μM) completely protected the enzyme activity (Table II). H_4folate, on the other hand, did not have any effect on the inhibition by OADS. OADS was a noncompetitive inhibitor (K_i = 1.8 μM) when serine was the varied substrate (Figure 2, inset A). The plots of velocity in the absence (●) and in the presence (■) of OADS (5 μM) against enzyme concentration passed through the origin but with different slopes (Figure 2, inset B), indicating that OADS was a reversible noncompetitive inhibitor (Segal, 1975).

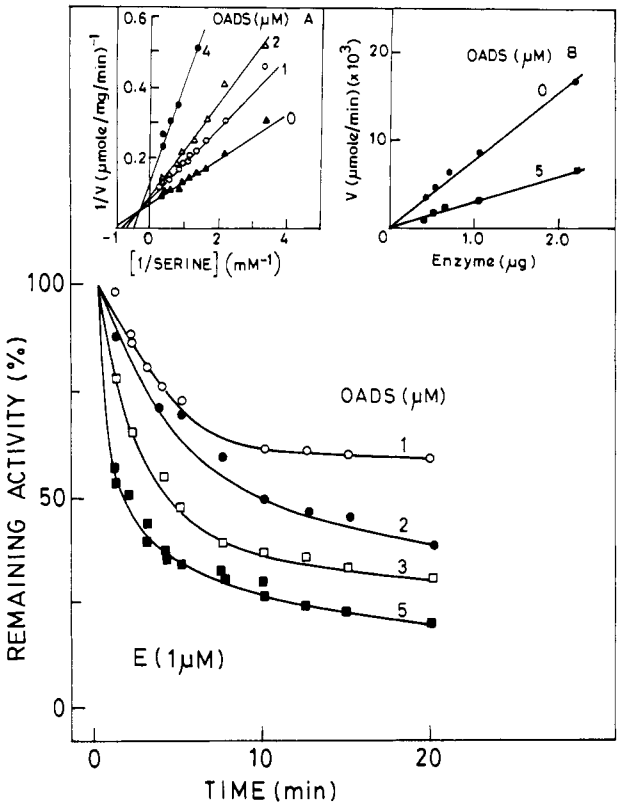

FIGURE 2: Time course of inhibition of sheep liver SHMT activity by OADS. The enzyme (1 μM) in buffer B was preincubated with 1, 2, 3, and 5 μM OADS, and aliquots (10 μL) were withdrawn at different time intervals into assay mixtures containing 0.4 M phosphate buffer (pH 7.4) containing 1 mM EDTA, 1.8 mM DTT, 1.8 mM H_4folate, and 3.6 mM L-[3-^{14}C]serine (70 000 cpm) and incubated for 2 min at 37 °C. The amount of formaldehyde formed was determined (Manohar et al., 1982). The OADS (0.5 μM) that was carried over into the assay mixture did not inhibit the enzyme activity significantly. (Inset A) Noncompetitive inhibition by OADS. The enzyme (1 μM) in buffer B was preincubated with serine (0.15–3.6 mM) for 5 min followed by a second incubation with OADS (0–4 μM) for 5 min. The residual enzyme activity was assayed. (Inset B) Reversible noncompetitive inhibition by OADS. The enzyme (0–2.5 μg) was incubated with either 5 μM OADS or buffer B for 5 min at 37 °C. The residual enzyme activity was assayed by withdrawing aliquots (10 μL) from the reaction mixture.

Table II: Protection of SHMT Activity by PLP against Inhibition by OADS[a]

compound	remaining act. (%)
OADS (10 μM)	4
OADS (10 μM)	
+5 μM PLP	25
+10 μM PLP	50
+20 μM PLP	78
+50 μM PLP	90
+100 μM PLP	100

[a] The enzyme (1 μM) in buffer B was preincubated with 10 μM OADS for 5 min at 37 °C, an aliquot (10 μL) was withdrawn, and the remaining activity was determined (Manohar et al., 1982). The enzyme (1 μM) was incubated with PLP (5, 10, 20, 50, and 100 μM) for 5 min followed by a second incubation with OADS (10 μM) for 5 min at 37 °C. Aliquots were withdrawn and assayed for the remaining activity. The activity of the enzyme that was processed identically but in the absence of OADS was normalized to 100.

Spectral Studies. The sheep liver SHMT had an absorbance maximum at 425 nm due to the PLP at the active site of the enzyme (Figure 3). Addition of an equimolar concentration of OADS (20 μM) to the enzyme resulted in a sudden decrease in the absorbance at 425 nm and appearance of a new peak at 388 nm (Figure 3). The peak at 388 nm

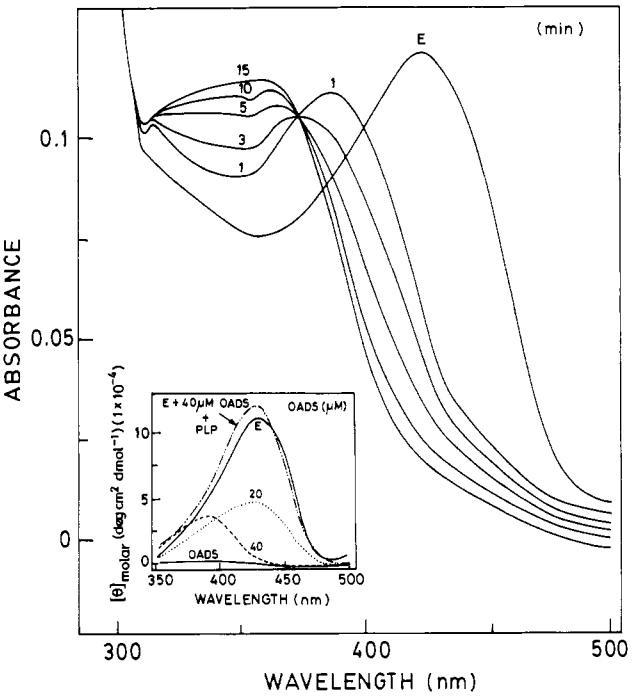

FIGURE 3: Changes in the visible absorption spectrum of SHMT on addition of OADS. The enzyme (5 μM, curve E) in buffer B was treated with OADS (20 μM), and the spectrum at 1, 3, 5, 10, and 15 min was recorded (numbers indicated on the curve). (Inset) Changes in the visible CD spectrum of SHMT on addition of OADS. Enzyme (10 μM, curve E) in buffer B and OADS was mixed, and the spectrum was recorded [(···) 20 μM; (- - -) 40 μM]. PLP (0.4 mM) was added to the enzyme treated with OADS (40 μM), and the spectrum was recorded (curve E + 40 μM OADS + PLP).

slowly disappeared (1–15 min) to form a broad peak centered around 340 nm (Figure 3) with an isosbestic point at 375 nm. The difference spectrum of the enzyme (5 μM) treated with OADS (20 μM) against an enzyme blank showed a peak at 380 nm and a trough at 435 nm at 1 min. The peak at 380 nm in the difference spectrum slowly disappeared with the appearance of a new peak at 360 nm at 10 min.[2] There was no change in the absorption spectrum of the enzyme between 250 and 300 nm after interaction with OADS (data not shown).

Upon addition of OADS (20 μM) to sheep liver SHMT (10 μM), the 430-nm CD band decreased rapidly (Figure 3, inset). At equal concentrations of OADS and enzyme active sites (40 μM), the CD band at 430 nm disappeared almost completely and concomitantly a positive CD band at 390 nm was observed. The addition of PLP (400 μM) to this enzyme treated with OADS resulted in the reappearance of a CD band at 430 nm (Figure 3, inset). Addition of OADS (40 μM) to the enzyme (10 μM) resulted in reduction of intensity of CD bands in the near-UV region (250–350 nm), and addition of PLP (400 μM) to this enzyme treated with OADS restored the original spectrum. On the other hand, the far-UV CD spectrum (200–250 nm) of the enzyme did not change significantly on addition of OADS.[2] OADS, PLP, and PLP–OADS oxime did not have any CD bands.

Identification of the Product Formed in the Reaction of OADS with Sheep Liver SHMT. The supernatant of the enzyme treated with OADS (E–OADS), obtained as described under Methods, had a visible absorption spectrum with a

[2] These figures were submitted to the scrutiny of the reviewers and will be furnished to the interested reader by writing directly to the authors.

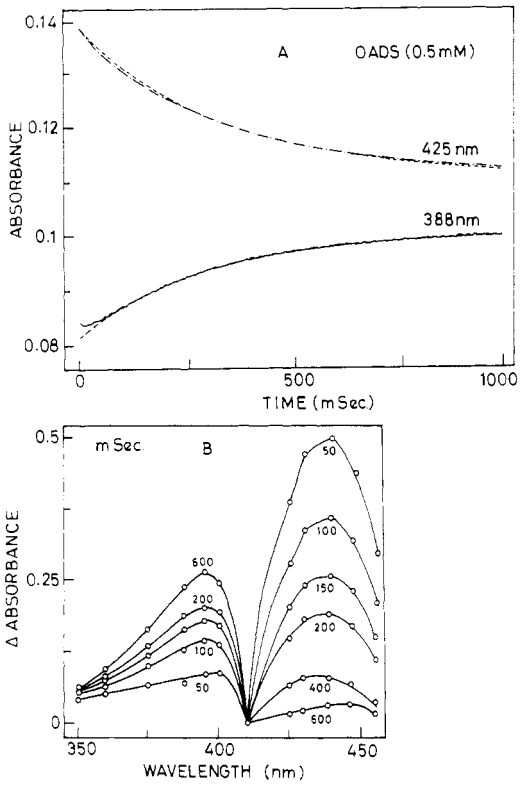

FIGURE 4: (A) Interaction of OADS with SHMT monitored by stopped-flow spectrophotometer at 425 and 388 nm. The enzyme (10 μM, 5 mL) in buffer B was taken in reservoir A and OADS (1 mM, 5 mL) in reservoir B. The flow cell was flushed with two or three sweepings (0.2 mL from each reservoir/stroke) with the reactants after mixing under nitrogen pressure (6 kg/cm^2), and the base line was fixed. The absorbance change was monitored at both 425 and 388 nm up to 1000 ms. The actual curve (---, —) and the curve fits are shown in the figure (-·-, ---). (B) Time difference spectra of the interaction of OADS with SHMT. The absorbance change at different wavelengths (350–460 nm) was determined as described above at 0.5 mM OADS for 600 ms. From the time course of this reaction, the spectra were constructed by plotting change in absorbance (ΔA) against wavelength at different time points (50, 100, 150, 200, 400, and 600 ms).

maximum at 360 nm, which was identical with PLP–OADS oxime. When the enzyme (0.1 μM) was treated with OADS (1 μM) for 15 min at 25 °C, a fluorescence spectrum with an emission maximum at 450 nm was obtained when excited at 360 nm. The E–OADS and PLP–OADS oxime had similar fluorescence spectra.[2] The holoenzyme, PLP or OADS, did not have any fluorescence when excited at 360 nm. When the E–OADS supernatant was subjected to HPLC, a sharp peak appeared with a retention time of 23 min, which was identical with that of PLP–OADS oxime. On the other hand, PLP and PMP had retention times of 7.3 and 4.8 min, respectively.[2]

Early Events in the Interaction of Sheep Liver SHMT with OADS (Stopped-Flow Studies). Since the rate of disappearance of absorbance at 425 nm was fast (Figure 3), we resorted to the use of stopped-flow spectrophotometry to evaluate the rate constants for the interaction of sheep liver SHMT with OADS. The reaction was almost complete within 600 ms and followed pseudo-first-order kinetics (Figure 4A) with a second-order rate constant (K) of 6.5×10^3 M^{-1} s^{-1}. The reaction monitored at 388 nm (Figure 4A) also had a similar rate constant. The time difference spectra, which were constructed (Figure 4B) as described by Hiromi (1979), showed a gradual decrease in the absorbance at 440 nm and coincided with an increase in the absorbance at 395 nm (Figure 4B).

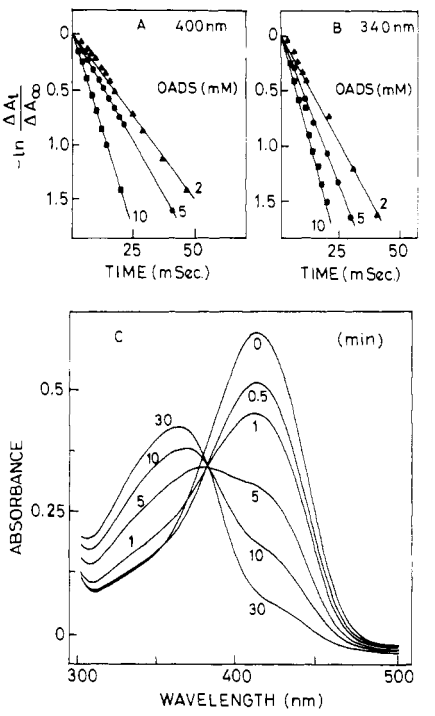

FIGURE 5: Rapid reaction of PLP and OADS. Free PLP (0.125 mM) was mixed with OADS (2, 5, and 10 mM), and the reaction was monitored for 100 ms as given in Figure 4. First-order plots are shown in for changes measured at 400 (A) and 340 nm (B), respectively. (C) Interaction of OADS with PLP–N^{α}-acetyl-L-lysine. PLP (0.2 mM) and N^{α}-acetyl-L-lysine (20 mM) in buffer B were mixed and incubated for 18 h at 0 °C. OADS (2 mM) was added, and the spectrum was recorded at different time intervals (0.5, 1, 5, 10, and 30 min). Curve 0 represents the spectrum of Schiff base before mixing with OADS.

Rate of Formation of PLP–OADS Oxime. The formation of oxime, monitored by the increase in absorbance at 340 nm upon interaction of OADS (0.5–3 mM) with the enzyme (5 μM), had a similar rate constant ($k = 2 \times 10^{-3}$ s^{-1}). The interaction of free PLP (0.2 mM) and OADS (2 mM) occurred with an initial rapid decrease in absorbance at 400 nm, and the rate was enhanced when the concentration of OADS was increased. Concomitantly, a rapid increase in absorbance at 340 nm was observed (figure not given). The rate constants measured at 2, 5, and 10 mM OADS at 400 and 340 nm were 29.4 and 38.0, 38.5 and 58.9, and 66.7 and 83.3 s^{-1}, respectively (Figure 5A,B). Following these rapid changes, there was a slow increase in absorbance at 360 nm with a rate constant of 2.8 M^{-1} s^{-1} when OADS (0.1–2 mM) was added to PLP (0.1 mM).

Interaction of OADS with N^{α}-Acetyl-L-lysine–PLP Schiff Base. The PLP–N^{α}-acetyl-L-lysine Schiff base had an absorption maximum at 410 nm (Figure 5C). On addition of OADS (2 mM) to this solution, the absorbance peak at 410 nm disappeared slowly, and a peak at 360 nm appeared corresponding to PLP–OADS oxime with an isosbestic point at 375 nm (Figure 5C). This result indicated that OADS interacted with the Schiff base to form PLP–OADS oxime without the formation of any discernible intermediate.

Discussion

OADS inhibited the enzyme activity very effectively (Figure 2). OADS (2 μM) produced about 50% inhibition of the enzyme activity, whereas 1 mM DCS was required to inhibit the enzyme activity to a similar extent (Manohar et al., 1984). OADS was a reversible noncompetitive (Figure 2, inset A) type of inhibitor with a K_i value of 1.8 μM, whereas the K_m

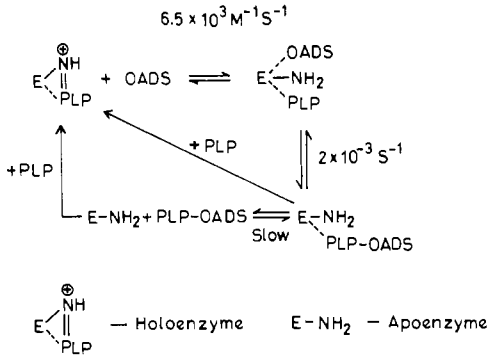

FIGURE 6: Minimal kinetic scheme for the interaction of OADS with sheep liver serine hydroxymethyltransferase.

for serine was 1 mM. This suggested that OADS had greater affinity (almost 3 orders of magnitude) for SHMT compared to L-serine. PLP completely protected the enzyme activity (Table II), indicating that the inhibition of OADS was by interaction with PLP. The protection by PLP may be due to its reaction with OADS, thereby making it unavailable for inhibition, or alternatively it could displace the PLP–OADS oxime from the active site. On the basis of the results presented (Figure 2; Table II) it is suggested that, like DCS, OADS was probably not forming a covalent adduct with SHMT.

On the basis of the results presented in this paper, we propose a minimal kinetic scheme for the interaction of OADS with sheep liver SHMT shown in Figure 6. This kinetic scheme suggests that OADS interacts with the enzyme rapidly ($K = 6.5 \times 10^3$ M^{-1} s^{-1}) and reversibly (Figure 2, insets A and B) by disrupting the enzyme–lysine–PLP Schiff base to form PLP (Figures 3 and 4A). In a second slow step PLP–OADS oxime was formed in a unimolecular reaction (2×10^{-3} s^{-1}). It could be postulated that in this step a local conformational change separated the PLP from OADS still bound with the enzyme by noncovalent interactions and thus prevented a rapid reaction between PLP and OADS as observed in nonenzymatic reactions (Figure 5A,B). In the third step, the PLP–OADS oxime could be released from the enzyme by slow dialysis, and the apoenzyme formed could be partially reactivated by PLP. PLP–OADS oxime could be displaced from the enzyme by the addition of PLP.

The first step in the kinetic scheme (Figure 6) was supported by the following observations. The absorbance at 425 nm rapidly disappeared due to the disruption of Schiff base, and a new peak appeared at 388 nm (Figure 3). The CD spectral band at 430 nm also disappeared rapidly, and a weak CD band appeared at 390 nm (Figure 3, inset). The spectral properties of the intermediate were in agreement with the postulate that this intermediate was probably PLP. The rate of this reaction monitored at both wavelengths was dependent on the concentration of OADS ($K = 6.5 \times 10^3$ M^{-1} s^{-1}) (Figure 4A). This result indicated that the disruption of enzyme–Schiff base by OADS probably resulted in the formation of PLP, unlike the transschiffization reaction that normally occurred with the substrate amino acid(s) in several PLP-dependent enzymes, including SHMT (Snell, 1958; Fasella, 1967; Schirch, 1975).

The spectral studies at 425 and 388 nm, however, did not rule out the possibility that there might be intermediary step(s) whose rate of reaction was similarly rapid. To test this possibility, time difference spectra were constructed (Figure 4B) which showed that with increasing time the spectrum characteristic of the Schiff base with a maximum at 440 nm decreased with a concomitant increase in the absorbance (λ_{max} = 395 nm) corresponding to PLP. This observation clearly indicated that the formation of PLP probably occurred without the occurrence of any other spectrally distinct intermediate. However, the initial peak in the difference spectra was at 440 nm (Figure 4B) and not at 425 nm, as observed in the static measurements (Figure 3). The static difference spectrum measured against an enzyme blank showed a trough at 435 nm. This difference could be due to the binding of OADS at the active site prior to the interaction with the Schiff base. Such red shifts were observed when cytosolic aspartate aminotransferase (EC 2.6.1.1) interacted with inhibitory dicarboxylic acids such as glutarate, maleate, and succinate, and a shift in the absorption spectrum from 430 to 440 nm was observed even though these inhibitors did not disrupt the Schiff base (Fonda & Johnson, 1970).

The second step in the kinetic scheme (Figure 6) postulates the formation of PLP–OADS oxime. The formation of the oxime was indicated by the following observations: (a) the difference spectrum obtained after the interaction of OADS showed a peak at 360 nm which was similar to that of PLP–OADS oxime; (b) the enzyme treated with OADS had a fluorescence emission spectrum at 450 nm when excited at 360 nm which was similar to that of oximes formed by aminooxyacetate and L-canaline after interaction with the cystathionase or PLP (Beeler & Churchich, 1976); and (c) the formation of PLP–OADS oxime was also confirmed by analyzing the E–OADS supernatant which had a retention time identical with that of PLP–OADS. All these observations clearly indicated that the peak at 340 nm (Figure 3) was probably due to the formation PLP–OADS oxime type of complex.

The rate of formation of PLP–OADS oxime with SHMT and OADS was concentration independent ($k = 2 \times 10^{-3}$ s^{-1}), suggesting that the reaction was unimolecular. The reaction of free PLP with OADS, on the other hand, showed a fast step and a slow step. The first fast step could be the formation of carbinolamine with the aminooxy group of OADS, which was characterized by the rapid decrease in absorbance at 400 nm and concomitant rapid increase in absorbance at 340 nm (Figure 5A,B). The slow step was probably due to the formation of the oxime as shown by the increase in absorbance at 360 nm. This step was also observed for the interactions of PLP with aminooxyacetate, L-canaline, and hydroxylamine (Beeler & Churchich, 1976; Raunio et al., 1984).

The final step in the kinetic scheme (Figure 6) visualizes the release of the oxime from the enzyme. Unlike the DCS–PLP complex, which readily dissociates from the active site of SHMT (Manohar et al., 1984), the PLP–OADS oxime complex does not dissociate as easily. The PLP–OADS complex was completely lost only after prolonged dialysis, suggesting that it binds strongly to the enzyme by the noncovalent interactions. Addition of PLP to the PLP–OADS oxime complex of the enzyme results in the formation of the active holoenzyme.

It was shown by Korpela et al. (1981) that OADS interacts with PLP at alkaline pH (pH >12) to form a cyclic geminal diamine which finally rearranges to form PLP–OADS oxime. Such a mechanism does not seem to be operative in our case as a peak due to geminal diamine (λ_{max} = 318 nm) was not observed with the enzyme. Under alkaline conditions (pH <12) the α-amino group is more reactive than the aminooxy group ($pK_a \simeq 4.0$). In neutral and slightly alkaline conditions (pH <10), however, the aminooxy group is more reactive, leading to the formation of oxime. This raises the question of whether the α-amino group of OADS plays any role in the mechanism of interaction of OADS with SHMT.

In conclusion, OADS interacted with SHMT with greater avidity than serine, and the mechanism proposed for the interaction of OADS with SHMT is novel in that PLP was formed as an intermediate prior to the formation of the oxime.

ACKNOWLEDGMENTS

We are thankful to Dr. K. Yagi, Director, Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Japan, and Dr. K. Hiromi, Kyoto University, Sakyoku, Kyoto, Japan, for helpful discussions on the stopped-flow studies.

Registry No. OADS, 20311-84-6; PLP, 54-47-7; PLP–OADS oxime, 123505-87-3; SHMT, 9029-83-8; N^{α}-acetyl-L-lysine–PLP Schiff base, 73715-42-1; L-serine, 56-45-1.

REFERENCES

Appaji Rao, N., Ramesh, N., Rao, D. N., Manohar, R., Vijayalakshmi, D., & Baskaran, N. (1987) *J. Sci. Ind. Res. 46*, 248.

Beeler, T., & Churchich, J. E. (1976) *J. Biol. Chem. 251*, 5267.

Braunstein, A. E. (1973) *Enzymes (3rd Ed.) 9*, 379.

Bukin, Y. V., Sergeev, A. V., & Raushenbackh, M. O. (1970) in *Chemistry and Biology of Pteridines, Proceedings of the International Symposium on Pteridines, the Chemistry and Biology (Folic Acid Included), 4th, July 1969* (Iwai, K., Ed.) p 445, International Academic Printing, Tokyo.

Bukin, Y. V., Draudin-Krylenko, V. A., & Korytnyk, W. (1979) *Biochem. Pharmacol. 28*, 1669.

Fasella, P. (1967) *Annu. Rev. Biochem. 36* (Part I), 185.

Fonda, M. L., & Johnson, R. J. (1970) *J. Biol. Chem. 245*, 2709.

Free, C. A., Julius, H., Arnow, P., & Barry, G. T. (1967) *Biochim. Biophys. Acta 146*, 608.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108.

Hatefi, Y., Talbert, P. T., Osborn, M. J., & Huennekens, F. M. (1959) *Biochem. Prep. 7*, 89.

Hiromi, K. (1979) in *Kinetics of Fast Enzyme Reactions, Theory and Practice*, p 189, Halsted Press–Wiley, New York.

John, R. A., Charteris, A., & Fowler, L. J. (1978) *Biochem. J. 171*, 771.

Karpeiskii, M. Y., Khomutov, R. M., Severin, E. S., & Brensov, Y. N. (1964) *Mekh. Kinet. Ferment. Katal.*, 123.

Khomutov, R. M., Hyvonen, T., Karvonen, E., Kauppienen, L., Paalanen, T., Paulin, L., Eloranta, T., Pajula, R. L., Andersson, L. C., & Poso, H. (1985) *Biochem. Biophys. Res. Commun. 130*, 596.

Kito, K., Sanada, Y., & Katunuma, N. (1978) *J. Biochem. 83*, 201.

Korpela, T., Makela, M., & Lonnberg, H. (1981) *Arch. Biochem. Biophys. 212*, 581.

Manohar, R., Ramesh, K. S., & Appaji Rao, N. (1982) *J. Biosci. 4*, 31.

Manohar, R., Appu Rao, A. G., & Appaji Rao, N. (1984) *Biochemistry 23*, 4116.

Raunio, R. P., Lindberg, R. K., & Jenkins, W. T. (1984) *Arch. Biochem. Biophys. 233*, 43.

Rosenthal, G. A. (1981) *Eur. J. Biochem. 114*, 301.

Roze, U., & Strominger, J. L. (1966) *Mol. Pharmacol. 2*, 92.

Schirch, L. (1975) *J. Biol. Chem. 250*, 1939.

Schirch, L. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 83.

Segal, I. H. (1975) in *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, p 128, Wiley-Interscience, New York.

Snell, E. E. (1958) *Vitam. Horm. 16*, 77.

Stammer, C. H. (1962) *J. Org. Chem. 27*, 2957.