Mode of Interaction of Aminooxy Compounds with Sheep Liver Serine Hydroxymethyltransferase†

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

ABSTRACT: The interaction of aminooxy compounds such as aminooxyacetate (AAA), L-canaline, and hydroxylamine with sheep liver serine hydroxymethyltransferase (EC 2.1.2.1) was studied by absorption spectra and stopped-flow spectrophotometry and compared with the unique feature of interaction of *O*-amino-D-serine (OADS) with the enzyme [Baskaran, N., Prakash, V., Appu Rao, A. G., Radhakrishnan, A. N., Savithri, H. S., & Appaji Rao, N. (1989) *Biochemistry* (preceding paper in this issue)]. The reaction of AAA (0.5 mM) with the Schiff base of the enzyme resulted in the formation of pyridoxal 5′-phosphate (PLP) and was biphasic with rate constants of 191 and 19 s^{-1}. The formation of the PLP–AAA oxime measured by decrease in absorbance at 388 nm on interaction of AAA with the enzyme had a rate constant of 5.2 M^{-1} s^{-1}. On the other hand, the reaction of L-canaline with the enzyme was slower as measured by the disruption of enzyme–Schiff base than the reaction of OADS and AAA. In contrast, the formation of PLP as an intermediate could not be detected upon the interaction of hydroxylamine with the enzyme. The reaction of D-cycloserine with the enzyme was much slower (1.6×10^2 M^{-1} s^{-1}) than the aminooxy compounds. These observations indicate that the aminooxy compounds that are structural analogues of serine (OADS, AAA, and canaline) formed PLP as an intermediate prior to the formation of oxime, whereas with hydroxylamine such an intermediate could not be detected.

Aminooxy compounds such as aminooxyacetate (AAA)[1] and L-canaline as well as the parent compound hydroxylamine inhibited pyridoxal phosphate (PLP)-dependent enzymes by breaking the enzyme-Schiff base to form oxime type complexes (Braunstein, 1973; Beeler & Churchich, 1976; John et al., 1978; Rosenthal, 1981; Raunio et al., 1984; Klosterman, 1986). This property of aminooxy compounds was exploited to examine the physiological role of PLP–enzymes (Baxter & Roberts, 1961; Hotta, 1968; Rognstad & Clark, 1974; Aniento et al., 1988). Recently, it was demonstrated that 1-(aminooxy)-3-aminopropane acted as an antineoplastic agent by inhibiting ornithine decarboxylase (EC 4.1.1.17; Hyvonen et al., 1988). Our studies described in the previous paper indicated that the interaction of *O*-amino-D-serine (OADS) with sheep liver serine hydroxymethyltransferase (EC 2.1.2.1; SHMT) resulted in the formation of PLP–OADS oxime with PLP as an intermediate (Baskaran et al., 1989). It was, therefore, of interest to examine whether this mechanism was unique to aminooxy compounds related to the substrates or a general feature of the interaction of aminooxy compounds with SHMT. This paper describes our studies on the mode of interaction of aminooxy compounds such as AAA, L-canaline, hydroxylamine, and other analogues of serine with sheep liver SHMT.

Experimental Procedures

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO: 2-mercaptoethanol (2-ME), ethylenediaminetetraacetic acid (EDTA), D-cycloserine (DCS), PLP, AAA, L-canaline, and hydroxylamine. DL-2,3-Diaminopropionic acid and DL-2,4-diamino-*n*-butyric acid were the kind gifts of Dr. D. Rajagopal Rao, Central Food Technological Research Institute, Mysore, India. OADS and β-isopropylidene-OADS were prepared as described by Stammer (1962).

Methods

Enzyme Purification and Estimation of Activity. The sheep liver SHMT was purified as described by Baskaran et al. (1989). The free PLP was removed by dialyzing the enzyme against 0.05 M potassium phosphate buffer, pH 7.4, containing 1 mM 2-ME and 1 mM EDTA (buffer A). The enzyme activity was assayed as described earlier (Manohar et al., 1982) by using L-[3-^{14}C]serine as the substrate.

Spectral Measurements. The absorption, fluorescence, and stopped-flow spectrophotometric measurements were carried out in buffer A at 25 ± 1 °C as described earlier (Baskaran et al., 1989).

Preparation of PLP Oximes. The enzyme (5 mg/mL) was mixed with AAA or hydroxylamine (0.2 mM) and incubated at 25 °C for 30 min, and the solutions were heated at 90 °C for 2 min. The precipitated proteins were removed by centrifugation at 10000*g* for 5 min. The supernatant (25 μL) of the enzyme treated with hydroxylamine (E–NH_2OH) or AAA (E–AAA) was used for identifying the oximes. The PLP oximes of AAA and NH_2OH were prepared by mixing equal amounts of PLP with AAA or NH_2OH (10 mM).

High-Performance Liquid Chromatography (HPLC) of PLP Oximes. The HPLC of the oximes formed with the enzyme and in nonenzymatic model systems was separated on a Spherisorb 5 ODS (C_{18}) column using 10% methanol in

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

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[1] Abbreviations: AAA, aminooxyacetic acid; PLP, pyridoxal 5′-phosphate; OADS, *O*-amino-D-serine; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid disodium salt; DCS, D-cycloserine; TFA, trifluoroacetic acid.

0006-2960/89/0428-9613$01.50/0

Table I: Inhibition of SHMT Activity by Aminooxy Compounds[a]

compound (μM)	inhibition (%)	compound (μM)	inhibition (%)
aminooxyacetic acid		L-canaline	
1	15	20	8
3	50	100	45
10	90	200	75
hydroxylamine			
1	18		
3	55		
10	82		

[a] The enzyme (1 μM) in buffer A was preincubated with different fixed concentrations of aminooxy compounds at 37 °C. Aliquots were withdrawn from this reaction mixture after 5 min, and the residual enzyme activity was determined (Manohar et al., 1982). The activity of the enzyme treated similarly but without the inhibitor was normalized to 100, and the inhibition (%) was calculated.

water containing 0.1% trifluoroacetic acid (TFA) as the solvent. The column effluents were monitored at 280 nm on an LKB HPLC system.

Results

Inhibition of the Enzyme Activity by Aminooxy Compounds. Increasing concentrations of aminooxy compounds progressively inhibited the activity of the enzyme (Table I). Incubation (5 min) of the enzyme with PLP (100 μM) prior to the addition of aminooxy compounds completely protected the activity of the enzyme against the inhibition caused by these compounds. On the other hand, β-isopropylidene-OADS had an IC_{50} value of 5 mM. DL-2,3-Diaminopropionic acid and DL-2,4-diaminobutyric acid (10 mM) inhibited the enzyme activity by 50 and 12%, respectively.

Changes in the Visible Absorption Spectrum of SHMT on Addition of Aminooxy Compounds. The addition of AAA (50 μM) to sheep liver SHMT (5 μM) resulted in the rapid disappearance of the 425-nm peak and the appearance of a peak at 390 nm (Figure 1). The peak at 390 nm disappeared slowly with time, concomitant with the appearance of a peak at 325 nm (Figure 1). The difference spectrum of the enzyme (5 μM) treated with AAA (50 μM) against an enzyme blank exhibited a peak at 375 nm and a trough at 430 nm at 1 min. The peak at 375 nm rearranged slowly to give a new peak at 335 nm at 10 min.[2] The higher homologue of OADS, L-canaline (100 μM), when added to the enzyme caused a slow disappearance of the 425-nm peak and the appearance of a broad peak in the region 385–395 nm with an isosbestic point at 395 nm (Figure 1, inset A). With increasing periods of reaction, a peak appeared at 330 nm (Figure 1, inset A) with an isosbestic point at 375 nm. Addition of hydroxylamine (50 μM) to SHMT (5 μM) resulted in the rapid disappearance of the peak at 425 nm and the appearance of a peak at 325 nm in 1 min,[2] and the difference spectrum showed a peak at 345 nm (Figure 1, inset B). The addition of β-isopropylidene-OADS (5 mM), DL-2,3-diaminopropionic acid, and DL-2,4-diaminobutyric acid (10 mM) did not cause any change in the spectrum of the enzyme (5 μM).

Identification of PLP Oximes Formed upon the Reaction of Aminooxy Compounds with SHMT. AAA and hydroxylamine treated enzyme supernatants (E–AAA and E–NH_2OH, respectively) prepared as described under Methods gave upon HPLC a peak with a retention time of 13.1 and 7.5 min, respectively.[2] Similar retention times were obtained for the oximes of PLP–AAA and PLP–NH_2OH.

[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.

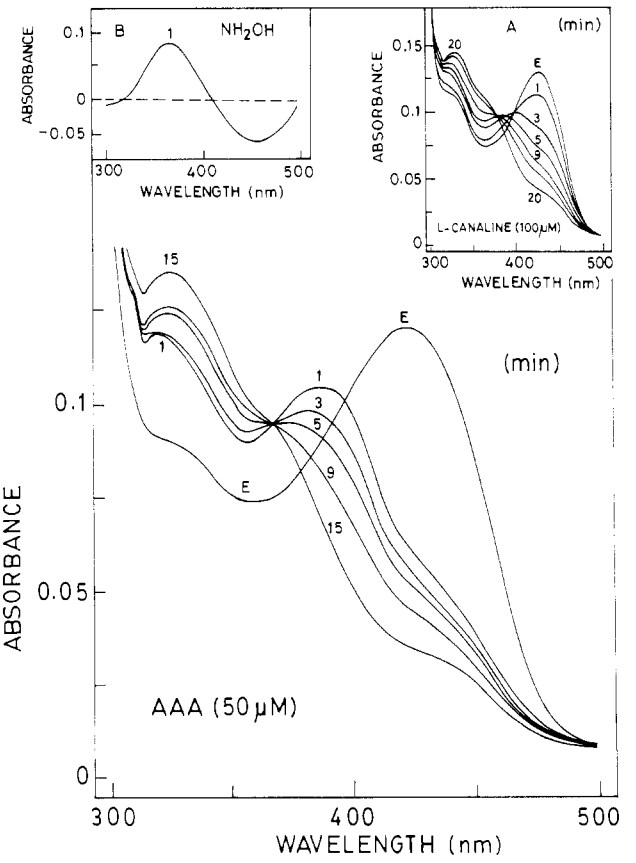

FIGURE 1: Changes in the visible absorption spectrum of sheep liver SHMT on addition of AAA. The enzyme (5 μM) in buffer A was mixed with AAA (50 μM), and the spectrum was recorded. Curve E represents the spectrum of the holoenzyme, and the numbers on the curves indicate the time in min. (Inset A) Changes in the spectrum of the enzyme (5 μM) on addition of L-canaline (100 μM). (Inset B) Difference spectrum of the enzyme treated with hydroxylamine (50 μM) recorded against enzyme blank (5 μM).

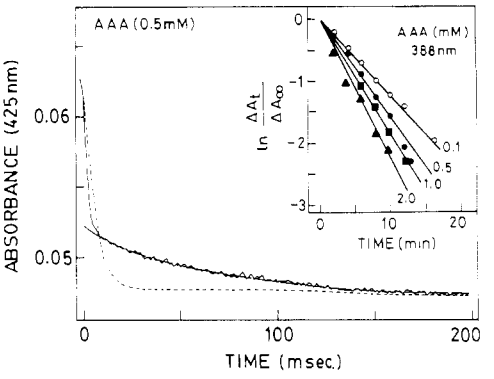

FIGURE 2: Time course of the reaction of AAA with SHMT monitored at 425 nm. The enzyme (10 μM, 5 mL) and AAA (1 mM, 5 mL) were taken in reservoirs A and B of an Union-Giken RA401 stopped-flow spectrophotometer, respectively. The base line was set by repeated sweeping of the flow cell by mixing the enzyme and AAA. The reaction curve (—) was curve-fitted (---, – – –). The curve represents the average of five sets of experiments with an average deviation in the range 5–10%. (Inset) The first-order plot for the disappearance of PLP formed on addition of AAA to SHMT. The enzyme (5 μM) in buffer A was mixed with AAA (0.1, 0.5, 1, and 2.0 mM), and the decrease in absorbance at 388 nm was monitored. ΔA_∞ represents the maximal absorbance change observed at the end of the reaction (1 h), and ΔA_t represents the ΔA_∞ minus the absorbance change at time t.

Kinetics of Interaction of AAA, L-Canaline, and Hydroxylamine with SHMT. The disappearance of 425-nm absorbance upon the interaction of AAA (0.5 mM) with sheep liver

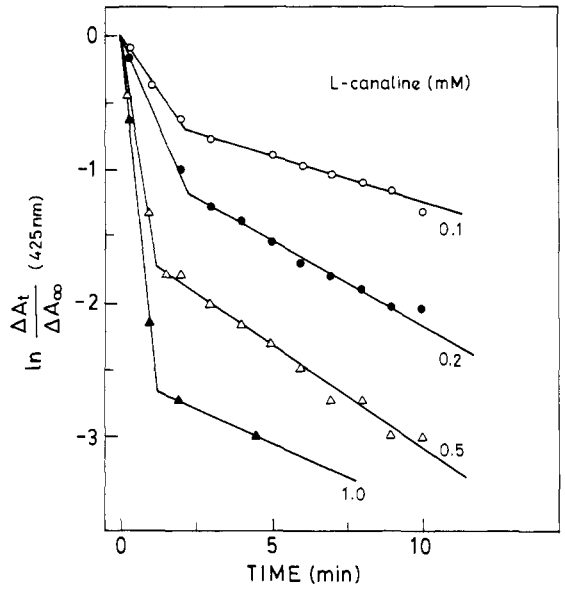

FIGURE 3: Time course of the disappearance of the absorbance at 425 nm on addition of L-canaline to SHMT. The enzyme (5 μM) in buffer A was mixed with L-canaline (0.1, 0.2, 0.5, and 1.0 mM), and the disappearance of the absorbance at 425 nm was monitored.

SHMT was complete within 200 ms (Figure 2). The first-order plot was biphasic with rate constants of 191 and 19 s^{-1} for the fast and slow phases of the reaction, respectively. The rate constants for the increase in absorbance at 388 nm were 100 and 32 s^{-1} (at 0.5 mM AAA) for the fast and slow phases of the reaction (data not given), respectively. At higher concentrations of AAA, the rates of the reaction were very fast, and the first fast phase occurred within the dead time (0.5 ms) of the instrument. For this reason, the rate constants could not be evaluated accurately at higher concentrations of AAA. The rates at lower concentrations were not determined as the pseudo-first-order conditions were not met. The formation of the oxime, which was monitored by the decrease in absorbance at 388 nm (Figure 2, inset) and the increase in absorbance at 335 nm, had a rate constant of 5.2 M^{-1} s^{-1} when AAA interacted with SHMT.

The reaction of L-canaline with sheep liver SHMT (5 μM) monitored at 425 nm (Figure 3) followed pseudo-first-order kinetics and was biphasic (Figure 3). The second-order rate constant calculated for the fast phase was 39 M^{-1} s^{-1}, and the second slow phase had a rate constant of 1.6 $\times$ 10^{-3} s^{-1} (at 0.1 mM).

The reaction of hydroxylamine with SHMT was complete within 8 s and followed pseudo-first-order kinetics (Figure 4). The second-order rate constants obtained at 425 and 335 nm were similar (2.1 $\times$ 10^3 M^{-1} s^{-1} at 425 and 335 nm). Similarly, the slow phase had first-order rate constants of 0.8 and 0.7 s^{-1} (at 1 mM) at 425 and 335 nm, respectively.

Interaction of DCS with SHMT. The DCS interaction with SHMT monitored at 425 nm was over within 30 s (Figure 5) and followed first-order kinetics with a fast and a slow phase. The first step was dependent on the concentration of DCS (2.5, 5, 15, and 20 mM) with a second-order rate constant of 1.6 $\times$ 10^2 M^{-1} s^{-1}, whereas the second slow step was independent of the concentration (1.2 $\times$ 10^{-1} s^{-1}). The increase in absorbance at 335 nm on interaction of DCS with the enzyme (Figure 5, inset) had a rate constant of 1.3 $\times$ 10^{-1} s^{-1} at all concentrations used (2.5, 5, and 20 mM).

Discussion

Hydroxylamine and AAA were as potent as OADS in inhibiting the activity of SHMT (IC_{50} = 2–3 μM), whereas

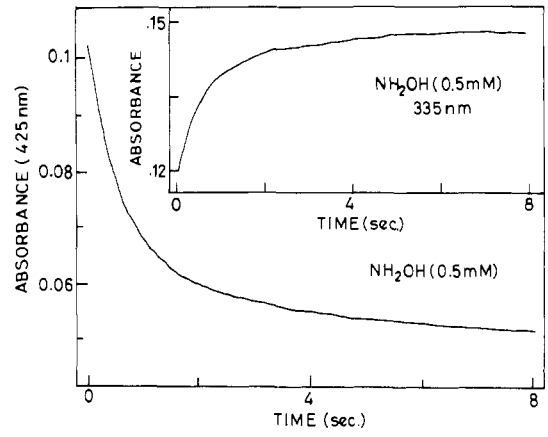

FIGURE 4: Time course of the disappearance of the absorbance at 425 nm on addition of hydroxylamine to SHMT. The rate of this reaction was determined as described for Figure 2 by using hydroxylamine instead of AAA. (Inset) Time course of the appearance of the absorbance at 335 nm on addition of hydroxylamine to SHMT.

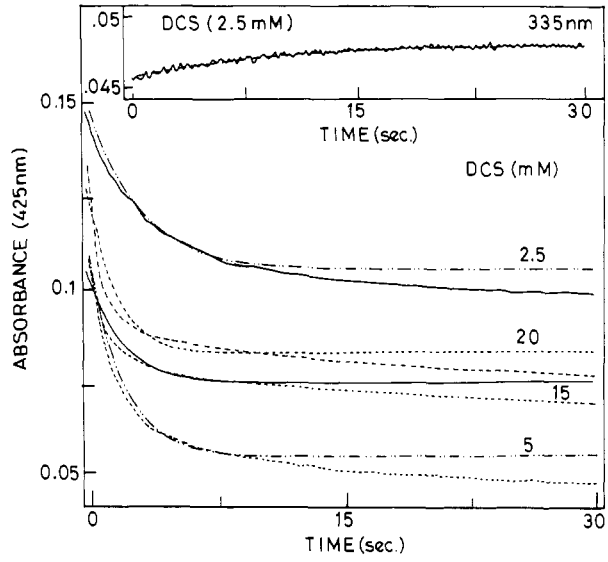

FIGURE 5: Time course of the disappearance of the absorbance at 425 nm on addition of DCS to SHMT. The rate of the reaction was performed as described in the legend for Figure 2. The experimental curve and the curve-fit lines are given [DCS (mM) 2.5, —, -··-; 5, ---, -··-; 15, ---, —; 20, – – –, ---]. (Inset) Reaction of DCS (2.5 mM) with SHMT monitored at 335 nm.

L-canaline was not as effective (IC_{50} = 100 μM, Table I), suggesting that the larger size of L-canaline probably hindered the effective interaction at the active site of SHMT. When the aminooxy group was blocked, as in β-isopropylidene-OADS, the IC_{50} value was increased to 5 mM, suggesting that the reactive aminooxy group was essential for potent inhibition. Moreover, when the aminooxy group was replaced by an amino group as in DL-2,3-diaminopropionic acid and DL-2,4-diamino-*n*-butyric acid, there was essentially no inhibition (IC_{50} $\geq$ 10 mM). The protection of the enzyme activity by PLP against the inhibition caused by these aminooxy compounds suggested that, like OADS, these compounds were probably inhibiting SHMT activity by interacting with enzyme-bound PLP. Although AAA and hydroxylamine were as potent as OADS, the closer structural similarity of OADS with substrate serine could introduce specificity in the interaction with SHMT.

As in the case of OADS (Baskaran et al., 1989), the disappearance of the absorbance at 425 nm (Figure 1) after the addition of AAA indicated that the Schiff base of PLP with

Table II: Summary of the Second-Order Rate Constants ($k \times 10^{-3}$ M^{-1} s^{-1}) for the Interaction of Aminooxy Compounds with PLP Enzymes

enzymes	OADS	AAA	L-canaline	NH_2OH	DCS
serine hydroxymethyltransferase[a]	6.5	191[f]	0.04	2.1	0.16
cystathionase[b,c]		0.060	0.80		0.0004
aspartate aminotransferase[d]		0.4			
GABA-aminotransferase[d]		1.3		1.4	
ornithine aminotransferase[e]			1		

[a] The rate constants determined in this study. [b] Beeler and Churchich (1976). [c] Churchich and Bieler (1971). [d] John et al. (1984). [e] Kito et al. (1978). [f] Pseudo-first-order rate constant at 0.5 mM AAA.

the ϵ-amino group of lysine was disrupted, and an absorption spectrum characteristic of PLP with a peak at 390 nm was formed. The pseudo-first-order rate constant, 191 s^{-1} at 0.5 mM AAA (Figure 2), was 50 times higher than that for OADS (3.5 s^{-1} at 0.5 mM; Baskaran et al., 1989), suggesting that the presence of the α-amino group in OADS might have reduced the reactivity of the aminooxy group with the enzyme–Schiff base. The second-order rate constants for the interaction of AAA and OADS with SHMT were 3.8×10^5 and 6.5×10^3 M^{-1} s^{-1} compared to 4.0×10^2 M^{-1} s^{-1} (John et al., 1978) observed with aspartate aminotransferase (EC 2.6.1.1), indicating that both OADS and AAA, which are substrate analogues, are more reactive with SHMT than with asparate aminotransferase. The biphasic nature of the first-order plot indicated that all the PLP molecules at the active site of the tetrameric SHMT were probably not equally reactive with AAA. A similar observation was also reported for the interaction of canaline with cystathionase (EC 4.2.1.15; Beeler & Churchich, 1976).

The second step in the interaction of AAA with the enzyme was the formation of an oxime, indicated by the presence of a peak at 325 nm (Figure 1). The formation of oxime was further confirmed by (a) the enzyme difference spectrum with a peak at 335 nm, which was similar to that of PLP–AAA oxime, and (b) the identical retention time on HPLC of PLP–AAA oxime and E–AAA obtained upon interaction of SHMT with AAA.[2] The formation of PLP–AAA oxime with the enzyme showed concentration dependency (K = 5.2 M^{-1} s^{-1}, Figure 2, inset), whereas the formation oxime with free PLP and AAA had a K value of 0.8 M^{-1} s^{-1} (Raunio et al., 1984). The higher rate constant observed with SHMT compared to that with free PLP probably reflects the facilitation of the reaction of PLP with AAA at the active site of SHMT. On the other hand, the formation of oxime on interaction of OADS with SHMT was unimolecular (Baskaran et al., 1989). This observation indicated that although OADS and AAA caused similar spectral changes, their mechanism of interaction was probably different, probably reflecting the effect of additional interactions of the α-NH_2 group of OADS with amino acid residues at the active site of SHMT.

The absence of a fast change in the 425-nm peak (Figure 1, inset A) suggested that L-canaline was not as effective as OADS in disrupting the Schiff base. The peak formed at 330 nm (Figure 1, inset A) was due to PLP–canaline oxime (Beeler & Churchich, 1976).

Hydroxylamine, which has only the reactive function, aminooxy group, interacted with the enzyme, forming PLP oxime as evidenced by spectral characteristics (Figure 1, inset B) as well as by the similar retention time on HPLC of the PLP–NH_2OH and E–NH_2OH supernatant. Unlike in the case of OADS, the formation of PLP as an intermediate could not be detected in the interaction of hydroxylamine, probably due to the absence of functional groups in hydroxylamine for interaction with amino acid residues at the active site. Hence, it is suggested that hydroxylamine reacted directly and exclusively with enzyme–PLP Schiff base. The rate constant for the formation of the oxime with free PLP and hydroxylamine was 2.7 M^{-1} s^{-1} compared to 2.1×10^3 M^{-1} s^{-1} with the enzyme, indicating that hydroxylamine attacks the Schiff base of the enzyme faster (3 orders of magnitude) than the aldehydic groups of PLP. The higher reactivity of the enzyme with hydroxylamine could be due to the enhanced reactivity of the internal aldimines compared to free aldehyde (Jencks & Cordes, 1963).

D-Cycloserine (DCS), a cyclic compound with a blocked $-ONH_2$ group, is a well-known inhibitor of PLP–enzymes including SHMT (Braunstein et al., 1961; Brown et al., 1969; Roze & Strominger, 1966; Wang & Walsh, 1978; Manohar et al., 1984). The interaction of DCS with the Schiff base of SHMT had a rate constant of 1.6×10^2 M^{-1} s^{-1} (Figure 5) compared to 6.5×10^3 and 2.1×10^3 M^{-1} s^{-1} for OADS and hydroxylamine, respectively. Glycine interacted with SHMT (7×10^4 M^{-1} s^{-1}) to form a geminal diamine (Schirch, 1975). These observations suggested that the aminooxy compounds and glycine interacted with the enzyme faster than DCS. The rigid structure and the absence of a carboxyl group might have decreased the reactivity of DCS with enzyme–PLP Schiff base.

A comparison of the rates of the reaction of these aminooxy compounds with SHMT and other PLP enzymes would shed light on the kinetic advantage gained by aminooxy compounds that are substrate analogues. Table II summarizes the rate constants obtained with SHMT as well as cystathionase (EC 4.2.1.15), aspartate aminotransferase, γ-aminobutyrate (GABA) aminotransferase (EC 2.6.1.19), and ornithine aminotransferase (EC 2.6.1.13). It is evident from the table that aminooxy compounds, which have a structural similarity with substrates of the enzyme, are at least 1 order of magnitude more reactive than other aminooxy analogues.

Interaction of aminooxy compounds with other PLP-dependent enzymes (John et al., 1978; Kito et al., 1978; Raunio et al., 1984) revealed the presence of a spectral absorbing at 380 nm that was ascribed to the formation of an oxime. However, evidence in support of this suggestion was not provided. Korpela et al. (1979) showed that the PLP oximes are very stable over a wide range of pH (4–11) with high affinity (K_{eq} = 10^7 M^{-1}). The higher stability of the oximes was explained by the electronegativity at the C_4' of PLP, i.e., the contact of electronegative oxygen with nitrogen, as well as by the ability of the oxygen to possess a partial positive charge such as $C^- - N = O^+$—which can be stabilized by resonance (Jencks, 1964). The higher stability of oximes, the reversible inhibition of aspartate aminotransferase with aminooxy compounds, and the observation of spectra at the 390-nm region (John et al., 1978; Kito et al., 1978; Raunio et al., 1984) all suggest that PLP was probably formed in these enzymes also. However, further studies are necessary to unravel the mode of interaction of these compounds with other PLP enzymes.

In conclusion, aminooxy compounds such as OADS, AAA, and L-canaline, which have a structural similarity to the substrate or product, interacted with SHMT by forming PLP as an intermediate before the formation of oxime complex, and

hydroxylamine, which did not have much structure similarity, formed PLP oxime without any intermediate.

Registry No. SHMT, 9029-83-8; OADS, 20311-84-6; AAA, 645-88-5; DCS, 68-41-7; PLP–AAA oxime, 17780-81-3; PLP–canaline oxime, 77111-63-8; PLP oxime, 634-25-3; PLP, 54-47-7; NH_2OH, 7803-49-8; L-canaline, 496-93-5.

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Inhibition of Restriction Endonuclease Cleavage via Triple Helix Formation by Homopyrimidine Oligonucleotides†

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Received August 31, 1989; Revised Manuscript Received October 6, 1989

ABSTRACT: A 17-mer homopyrimidine oligonucleotide was designed to bind to the major groove of SV40 DNA at a 17 base pair homopurine·homopyrimidine sequence via Hoogsteen base pairing. This sequence contains the recognition site for the class II-S restriction enzyme *Ksp* 632-I. The oligonucleotide was shown to inhibit enzymatic cleavage under conditions that allow for triple helix formation. Inhibition is sequence-specific and occurs in the micromolar concentration range. Triple helix formation by oligonucleotides opens new possibilities for sequence-specific regulation of gene expression.

The recognition of DNA sequences by regulatory proteins is central to the control of cellular processes at the level of gene expression. Techniques for selectively inhibiting protein–nucleic acid recognition would provide means for controlling gene expression at the level of DNA replication and transcription. Homopyrimidine oligonucleotides were recently shown to bind to the major groove of double-stranded DNA at homopurine·homopyrimidine sequences (Le Doan et al., 1987; Moser & Dervan, 1987; Praseuth et al., 1988; Lyamichev et al., 1988; François et al., 1988, 1989). A triple helix is locally formed where the homopyrimidine oligonucleotide is oriented parallel to the homopurine-containing strand of DNA. Thymine forms two hydrogen bonds with adenine in a Watson–Crick A·T base pair. The formation of two hydrogen bonds between cytosine and a Watson–Crick G·C base pair requires protonation of cytosine.

Hoogsteen base pairing of thymine and protonated cytosine to a homopurine sequence of duplex DNA might not be the only molecular code for recognition of Watson–Crick base pairs by oligonucleotides. A purine-rich oligonucleotide, 27 nucleotides in length, was recently shown to bind duplex DNA and to reduce the transcription of the human c-myc gene in

† This work was supported in part by Rhône-Poulenc-Santé.

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0006-2960/89/0428-9617$01.50/0