Structure−Activity Studies of L-Canaline-Mediated Inhibition of Porcine Alanine Aminotransferase

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Received February 5, 1996[⊗]

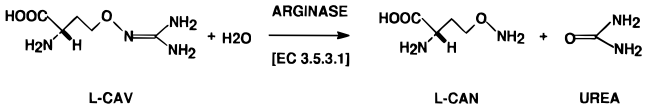
L-Canaline [L-2-amino-4-(aminooxy)butanoic acid] (L-CAN) and a family of eleven structurally related analogs were synthesized and evaluated for their inhibitory effect on PLP-dependent alanine aminotransferase (AlaAT) (EC 2.6.1.2) obtained from porcine heart. These congeners were selected to determine the stereochemical, aliphatic chain length, and aminooxy substitutional effects on L-CAN-mediated inhibition of AlaAT activity. L-CAN was the most effective inhibitor of the tested compounds; 10^{-7} M L-CAN elicited a 55% reduction in AlaAT activity after a 5 min exposure. This deleterious effect results from the ability of L-CAN to react avidly with the PLP moiety of the enzyme to form a stable, L-CAN-PLP oxime. In contrast, the methyl and ethyl esters of L-CAN reduced AlaAT activity by only 8% and 6%, respectively. While all of the L-enantiomeric forms of the tested compound were more potent AlaAT inhibitors than their corresponding D-stereoisomers, the D-enantiomers, particularly D-canaline, were active. Chain shortening or lengthening dramatically curtailed L-CAN-mediated loss in AlaAT activity, but the replacement of the α-amino group with a hydrogen was of little consequence in this regard. AlaAT was treated with L-CAN in the presence of free PLP to assess PLP capacity to protect AlaAT against 10^{-7} M L-CAN-dependent inactivation. L-CAN retained approximately two-thirds of its inhibitory ability in the presence of equimolar PLP, but AlaAT inhibition was reduced 90% by a 10-fold excess of PLP over L-CAN.

Introduction

L-Canavanine, L-2-amino-4-(guanidinooxy)butanoic acid (L-CAV),[1] is a naturally occurring L-arginine analog stored by a variety of leguminous plants (*1*). L-CAV is a potent L-arginine antagonist that exhibits antimetabolic activity in many living systems (*2*). CAV's biological effects include nitric oxide synthase inhibition (*3*), antimicrobial activity (*4*), leukocyte chemotaxis modulation (*5*, *6*), autoimmune reactions (*7*), and insecticidal activity (*8*). L-CAV may also give rise to other toxic compounds, including vinylglyoxylate, produced after L-CAV undergoes oxidation and subsequent *β*,*γ*-elimination (*9*). The chemotherapeutic potential of L-CAV and its derivatives has also been demonstrated in several *in vitro* and *in vivo* models; they possess great promise as novel antineoplastic agents (*10*−*12*).

L-CAV undergoes arginase (EC 3.5.3.1)-catalyzed hydrolytic deguanidination, producing L-canaline (L-CAN) and urea (Scheme 1) (*13*). L-CAN, L-2-amino-4-(aminooxy)butanoic acid, a structural analog of L-ornithine, reacts avidly with the PLP moiety of vitamin B_6-containing enzymes to form a stable oxime that rapidly inactivates the enzyme (*14*, *15*). L-CAN is a potent inhibitor of ornithine aminotransferase (EC 2.6.1.13) by virtue of its reaction with the PLP moiety of the enzyme to form an oxime that destroys the catalytic activity of the enzyme (*16*, *17*). L-CAN may also act as an antimetabolite by forming stable oximes with 2-oxo acid-containing substrates of PLP-dependent enzymes (*18*).

Scheme 1. Arginase-Mediated Hydrolytic Deguanidination of L-Canavanine to L-CAN and Urea



Since the utility of L-CAV as a chemotherapeutic agent may be compromised by the inherent toxicity of its L-CAN derivative, a more detailed understanding of the toxicity of L-CAN and its congeners may be useful in furthering the design of less toxic L-CAV analogs. To better understand the structure−activity relationship of L-CAN in the inactivation of PLP-containing enzymes, we synthesized a series of L-CAN analogs and evaluated their inhibitory effect on the PLP-dependent activity of alanine aminotransferase (EC 2.6.1.2) (AlaAT) isolated from porcine heart. These compounds were designed to permit an examination of the effects of changing the stereochemistry about the chiral center, varying aliphatic chain length, removing the α-amino group, and modification of the aminooxy moiety of L-CAN and related compounds on PLP-dependent AlaAT activity. The capacity of free PLP to counteract L-CAN-mediated inactivation of AlaAT was also evaluated.

Materials and Methods

Chemicals. L-CAV was isolated from an acidic methanolic extract of Jack bean (*Canavalia ensiformis)* seed and purified

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[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.
[1] Abbreviations: L-CAN, L-canaline; L-CAV, L-canavanine; PLP, pyridoxal phosphate; AlaAT, alanine aminotransferase; D-CAN, D-canaline; Tris, tris(hydroxymethyl)aminomethane; mU, milliunits.

S0893-228x(96)00019-7 CCC: $12.00

by recrystallization (*19*). L-CAN, prepared by arginase-mediated hydrolysis of L-CAV, was isolated as the dipicrate salt and converted to the free base via L-CAN sulfate (*19*). All of the remaining L-CAN derivatives were synthesized as described elsewhere (*19*).[2] All biochemicals used in this study, including a suspension of porcine heart alanine aminotransferase in 1.8 M ammonium sulfate [120 units (mg of protein)$^{-1}$] and rabbit muscle lactic dehydrogenase [Type II, 960 units (mg of protein)$^{-1}$] were purchased from Aldrich/Sigma Chemical Co. (St. Louis, MO).

Enzyme Assay. Alanine aminotransferase activity was assayed by reacting 100 μmol of L-alanine and 5 μmol of 2-oxoglutarate in 0.3 mL of 125 mM Tris buffer (pH 7.6) with 0.4 mL of 125 mM Tris buffer (pH 7.6) containing 26.25 mU of AlaAT/0.4 mL for 10 min at 37 °C. After 10 min, the reaction was terminated by immersing the assay tubes in a boiling water bath for 1 min. After cooling on ice to 23 °C, 0.7 mL of a 440 μM solution of reduced NAD (NADH) in 250 mM Tris buffer (pH 7.6) containing 52.8 units of rabbit muscle lactate dehydrogenase per 0.7 mL was added, and the mixture allowed to stand at 23 °C for 5 min. The oxidation of NADH was monitored at 340 nm using a Gilford Response spectrophotometer. This coupled enzyme assay was employed to quantify AlaAT activity by comparing the reduced coenzyme content of the samples containing AlaAT to comparable samples containing all reagents except AlaAT. All assays were conducted in quadruplicate employing two independent experiments.

Inhibition Studies. AlaAT inhibition studies were conducted in an analogous manner. Here, the enzyme was incubated in 125 mM Tris buffer (pH 7.6, 26.25 mU of AlaAT/0.4 mL) which contained the tested inhibitor at the specified concentration. After the indicated AlaAT/inhibitor incubation time, 0.4 mL of the enzyme−inhibitor mixture, containing 26.25 mU of AlaAT, was added to 0.3 mL of the substrate solution and assayed for enzymatic activity as described above. Enzymatic activity of AlaAT exposed to the various inhibitors at each concentration and after each exposure time was compared to that of similarly processed AlaAT preparations that did not contain an inhibitor.

Pyridoxal Phosphate Protection. In order to determine the capacity of free PLP to protect AlaAT from L-CAN-directed inhibition, free PLP at the indicated concentration was added to the enzyme and control assay tubes and incubated for 5 min. The specified concentration of L-CAN was then added, and the assays were conducted as described above. These assays were conducted in order to quantify the inhibitory effect of L-CAN over time in the presence of various ratios of L-CAN:free PLP.

Determination of the IC_{50} Value. The concentration of drug required to reduce AlaAT activity by 50% after 10 min (IC_{50} value) was estimated from the *x*-intercept of a log-linear plot of % AlaAT activity remaining vs $\log_{10}$ of the inhibitor concentration.

Results

Figure 1 illustrates the typical concentration and time-dependent inhibition of AlaAT by L-CAN. At an L-CAN concentration greater than 10^{-7} M, L-CAN-mediated inhibition of AlaAT is rapid and significant after 5 min (Figure 1). At lower concentrations, loss of AlaAT activity is decreased (Figure 1). The potency of L-CAN as an AlaAT inhibitor is demonstrated by a 55% reduction in AlaAT after a 5 min exposure to 10^{-7} M drug. In contrast, the esterified L-CAN derivatives, namely, 1-methyl-L-canaline and 1-ethyl-L-canaline, reduce AlaAT activity only 8% and 6%, respectively. Most interesting is our finding that D-CAN was significantly less active

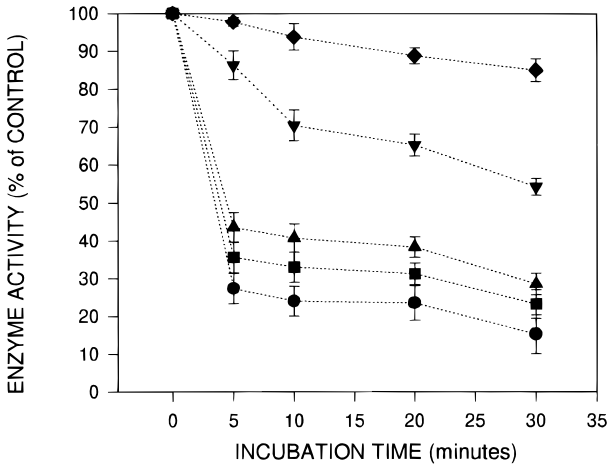


Figure 1. L-Canaline-mediated inhibition of alanine aminotransferase activity. AlaAT, at a concentration of 26.25 mU/0.4 mL, was incubated in 125 mM Tris buffer (pH 7.6) containing L-CAN at the specified concentration at 37 °C. After the specified incubation time, a 0.4 mL aliquot of the enzyme−inhibitor mixture, containing 26.25 mU of AlaAT, was added to 0.3 mL of the substrate solution and assayed for enzymatic activity as described in the text. L-CAN: 10^{-9} (◆), 10^{-8} (▼), 10^{-7} (▲), 10^{-6} (■), and 10^{-5} M (●).

than the naturally occurring stereoisomer in curtailing AlaAT activity. The estimated IC_{50} of D-CAN was approximately one-seventh that of L-CAN.

The lower homologues of D- and L-CAN, namely, D- and L-2-amino-3-(aminooxy)propanoic acid, show minimal (ca. 20%) inhibitory activity and do not significantly differ in their respective ability to inhibit AlaAT. The conformationally restrained, five-membered aminooxy heterocycle (2*S*,4*S*)-(aminooxy)proline, assayed at 10^{-7} M, similarly attenuates AlaAT activity by 15% after 10 min. At this concentration, the five-carbon linear congener L-2-amino-5-(aminooxy)pentanoic acid, the longest analog tested, decreases AlaAT activity by 6% after 10 min and by 16% after 30 min.

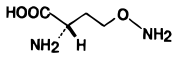
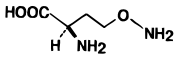
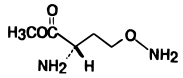
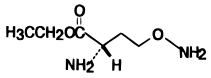
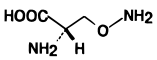
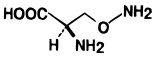
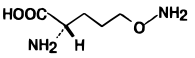
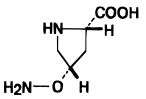
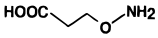
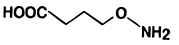
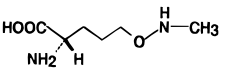
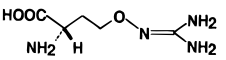
Carboxylic acids bearing a terminal aminooxy moiety and lacking an α-amino group are among the most active compounds examined. Both ω-(aminooxy)propanoic acid and ω-(aminooxy)butanoic acid display time- and concentration-dependent AlaAT inhibition that is comparable to L-CAN. At 10^{-7} M, ω-(aminooxy)propanoic acid reduces AlaAT activity 42% after 10 min and 52% after 30 min; the comparable values for ω-(aminooxy)butanoic acid were 49% and 55%, respectively.

N-Substituted ω-(aminooxy) α-amino acids have no effect on AlaAT activity. In contrast to L-2-amino-5-(aminooxy)pentanoic acid, which contains a free terminal aminooxy group, the *N*-methylaminooxy compound, L-2-amino 5-(*N*-methylaminooxy)pentanoic acid, is inactive at all tested concentrations over the entire time course of the experiment. A similar finding is noted for L-CAV, the guanidinooxy-containing precursor of L-CAN. A summary of the chemical structures of the tested drugs and their AlaAT inhibition activity constitutes Table 1. Table 2 provides a comparison of AlaAT inhibition by all of the tested drugs used in our study.

When L-CAN is added to analogous enzyme solutions containing various concentrations of free PLP, L-CAN-mediated inactivation of AlaAT is reduced in a PLP concentration-dependent manner (Figure 2). Equimolar PLP, in the presence of 10^{-7} M L-CAN for 10 min, reduces AlaAT inhibition from 57% to 38%. At 10:1 and higher ratios of PLP to L-CAN, AlaAT inhibition is reduced by

[2] Na Phuket, S. R., Trifonov, L. S., Crooks, P. A., Rosenthal, G. A., Freeman, J., and Strodel, W. E. (1996) Synthesis and structure−activity studies of some antitumor congeners of L-canavanine. *Drug Dev. Res.* (submitted).

Table 1. Summary of Tested Compounds and Their Estimated IC_{50}

compound	structure	IC_{50}
L-2-amino-4-(aminooxy) butanoic acid (L-CAN)		2.5×10^{-7} M
D-2-amino-4-(aminooxy) butanoic acid (D-CAN)		1.8×10^{-6} M
Methyl L-2-amino-4-(amino-oxy)butanoate		$> 10^{-5}$ M
Ethyl L-2-amino-4-(aminooxy) butanoate		$> 10^{-5}$ M
L-2-amino-3-(aminooxy) propanoic acid		5.0×10^{-6} M
D-2-amino-3-(aminooxy) propanoic acid		8.0×10^{-6} M
L-2-amino-5-(aminooxy) pentanoic acid		$> 10^{-5}$ M
2*S*,4*S*-aminooxy proline		$> 10^{-5}$ M
3-aminooxypropanoic acid		6.3×10^{-7} M
4-aminooxybutanoic acid		4.0×10^{-7} M
L-2-amino-5-(*N*-methyl amino-oxy)pentanoic acid		$> 10^{-5}$ M
L-2-amino-4-(guanidinooxy) butanoic acid		$> 10^{-5}$ M

more than 90%. Interestingly, detectable inhibition of AlaAT activity by L-CAN was observed when the PLP to L-CAN ratio was as high as 10 000:1. AlaAT control assays revealed that free PP in the reaction mixture did not affect base-line AlaAT activity.

Discussion

The nonprotein amino acid L-CAN is a potent inhibitor of AlaAT activity; it can affect profoundly PLP-containing enzymes whose substrates bear little structural relationship to L-CAN. Our experimental efforts confirm that, regardless of substrate, stereochemical, or functional analogy to L-CAN, a free aminooxy group is essential for the expression of L-CAN's antimetabolic effects. When compared to the activity of their L-stereoisomers, the significant loss of AlaAT inhibitory activity noted with D-CAN and D-2-amino-3-(aminooxy)propanoic acid suggests that the intact aminooxy moiety may be more important than the stereochemistry of the chiral center. This finding is also supported by the lack of inhibitory activity for L-CAN derivatives with an *N*-substituted aminooxy group. L-2-Amino-5-(aminooxy)pentanoic acid, which contains a free aminooxy group, is an effective AlaAT inhibitor. In contrast, its aminooxy *N*-methylated derivative is completely inactive, regardless of drug concentration or exposure time assayed. This methylated compound may be devoid of inhibitory activity, or it may simply react much more slowly with the enzyme-bound

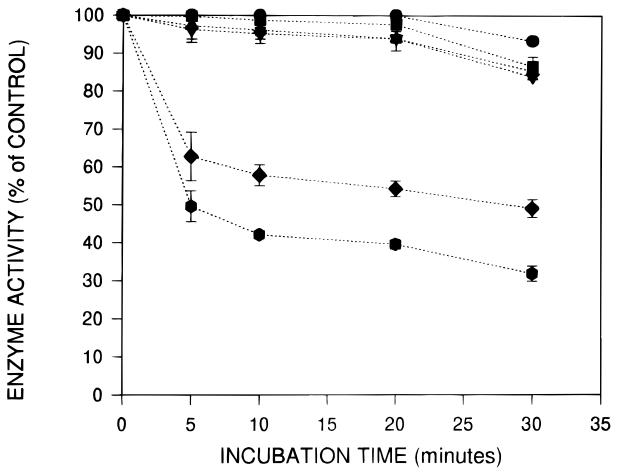


Figure 2. The ability of free PLP to protect against L-CAN-dependent inhibition of alanine aminotransferase. AlaAT was dissolved in 125 mM Tris buffer (pH 7.6) containing various concentrations of free PLP and incubated for 5 min at 37 °C. L-CAN was then added to give a final concentration of 10^{-7} M L-CAN, and the mixture was incubated for 10 min at 37 °C. After a 10 min incubation, a 0.4 mL aliquot of the mixture, containing 26.25 mU of AlaAT, was added to 0.3 mL of the substrate solution and assayed for activity as described in the text. The presence of free PLP did not affect AlaAT activity in the control samples. The PLP:L-CAN ratio: 10 000:1 (●), 1000:1 (■), 100:1 (▲), 10:1 (▼), 1:1 (◆), and 0.1:1 (⬣).

cofactor than its free aminooxy-containing analog. Chain lengthening of L-CAN to generate L-2-amino-5-(aminooxy)pentanoic acid significantly curtails inhibition of AlaAT activity.

The presence of a carboxyl group attached to the chiral center is important for inhibitory activity while an attached α-amino group is not. Inhibition of AlaAT activity is reduced when the aliphatic chain length, relative to L-CAN, is shortened or lengthened. A bulky cyclic congener such as (2*S*,4*S*)-(aminooxy)proline is a poor inhibitor.

Strecker and Eliasson (*21*) had reported that L-CAV is an effective aminotransferase inhibitor. Our study reveals that L-CAV is devoid of inhibitory activity after exposing AlaAT to a 10^{-5} M concentration of this compound for 30 min. These researchers (*21*) also reported that terminal amino carboxylates analogous to native enzyme substrates are effective aminotransferase inhibitors. Though hydroxylamine itself is an effective aminotransferase inhibitor (*16*, *17*), our experimental findings suggest that an unsubstituted or terminal carboxyl group may be a key structural feature of this series of substituted hydroxylamine AlaAT inhibitors. The effectiveness of (2*S*,4*S*)-(aminooxy)proline at reducing AlaAT activity at 10^{-7} M and lower drug concentrations supports this belief. Despite its bulky, rigid five-membered heterocyclic structure, which bears little structural resemblance to the substrates for this enzyme, its free aminooxy and carboxyl groups conferred significant capacity to inhibit aminotransferase activity, even more than had been attributed to the parent compound, L-proline, in rat liver (*22*). In addition, our intention was to utilize an analog of (2*S*,4*S*)-(aminooxy)proline having defined or fixed stereochemistry, i.e., transoid arrangement of the $-ONH_2$ and $-COOH$ groups, in order to obtain information on the conformation required for inhibitor binding to the enzyme.

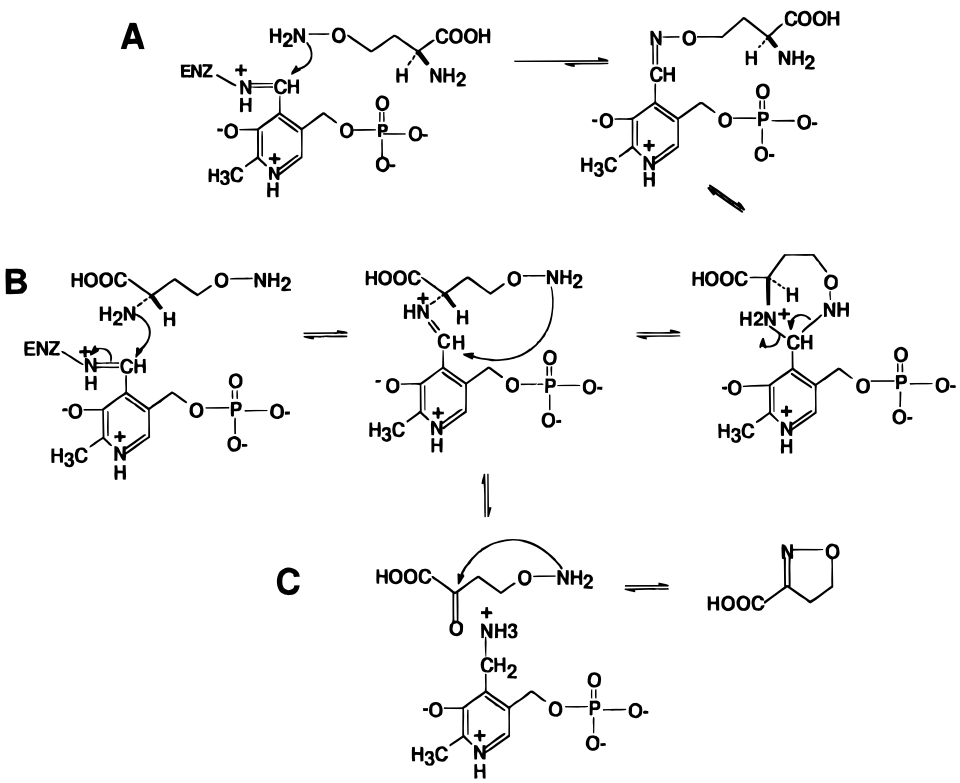
Despite their capacity to present a freely-rotating aminooxy group for nucleophilic attack on the enzyme-

Table 2. Percent of AlaAT Activity Remaining after Incubation with 10^{-7} M of the Indicated Compound[a]

compound	incubation time (min)			
	5	10	20	30
Group 1. Canaline and Canaline Esters				
L-canaline	43.5 ± 3.9	40.7 ± 3.7	38.3 ± 2.7	28.5 ± 2.8
D-canaline	77.5 ± 3.0	73.8 ± 3.4	72.1 ± 2.4	67.7 ± 3.8
1-methyl-L-canaline	92.2 ± 2.0	91.3 ± 2.2	89.8 ± 2.0	81.8 ± 2.4
1-ethyl-L-canaline	94.7 ± 2.1	94.0 ± 1.8	91.1 ± 2.1	83.7 ± 3.0
Group 2. ω-(Aminooxy) Amino Acids				
L-2-amino-3-(aminooxy)propanoic acid	91.0 ± 2.8	85.9 ± 1.1	79.5 ± 1.2	73.4 ± 1.5
D-2-amino-3-(aminooxy)propanoic acid	96.7 ± 3.8	89.5 ± 3.7	86.3 ± 3.6	92.2 ± 3.4
L-2-amino-5-(aminooxy)pentanoic acid	93.0 ± 2.1	93.6 ± 1.6	84.6 ± 3.6	82.2 ± 3.4
(2*S*,4*S*)-(aminooxy)proline	89.0 ± 2.9	84.7 ± 2.2	83.4 ± 2.2	82.0 ± 2.1
Group 3. ω-(Aminooxy) Carboxylic Acids				
ω-(aminooxy)propanoic acid	66.1 ± 4.0	58.4 ± 3.2	53.3 ± 3.1	48.6 ± 3.2
ω-(aminooxy)butanoic acid	51.6 ± 2.5	50.9 ± 3.2	50.0 ± 3.1	46.5 ± 2.8
Group 4. Substituted ω-(Aminooxy) Amino Acids				
L-2-amino-5-(*N*-methylaminooxy)pentanoic acid	99.8 ± 2.6	100 ± 2.4	100 ± 2.6	100 ± 2.0
L-canavanine	100 ± 3.2	98.2 ± 2.8	98.0 ± 2.1	97.9 ± 3.3

[a] Each value represents the AlaAT activity remaining after exposure to 10^{-7} M drug for the stipulated time period. AlaAT activity was assayed as described in the text. Value represents the mean ± SEM of 2 independent determinations conducted in quadruplicate.

Scheme 2. Proposed Mechanisms for L-CAN-Directed Inhibition of a PLP-Containing Enzyme[a]



[a] Reaction A represents the direct nucleophilic attack of the terminal aminooxy group on the enzyme-bound imine form of PLP, leading to the production of an aminooxy aminal, hydrolysis, and formation of the stable oxime without substantial functional or stereochemical influence on the reaction. Reaction B represents the classical trans-Schiffization reaction, where an initial attack by the α-amino group of the amino acid on the protonated imine is immediately followed by intramolecular nucleophilic attack by the terminal aminooxy group on the resulting protonated imine. This cyclic aminooxy aminal is rapidly hydrolyzed, giving the ultimate chemically stable oxime and preventing further active site catalysis. This oxime does not undergo facile acid-mediated hydrolysis to the parent amino acid and PLP due to the expected low pK_a value of the imino function. Some L-CAN may undergo transamination to give 2-oxo-L-CAN and pyridoxamine phosphate (path C). The pyridoxamine phosphate form of AlaAT would be resistant to the further action of L-CAN, and 2-oxo-L-CAN has been shown to undergo cyclization (*18*) to an inactive product.

bound PLP moiety, L-2-amino-5-(aminooxy)pentanoic acid, L-2-amino-3-(aminooxy)propanoic acid, and (2S,4S)-(aminooxy)proline were significantly less active than L-CAN. These observations raise an important mechanistic question regarding the precise mechanism of enzyme inhibition by L-CAN. L-CAN and its congeners may bind enzyme-bound PLP directly with their ω-aminooxy groups, as does (aminooxy)acetate (*17*). The strong nucleophilic character of the aminooxy nitrogen, enhanced by an α-effect from lone pair electrons on the adjacent oxygen, may favor this mechanism (Scheme 2, path A). It is important to note that the $-ONH_2$ group of L-CAN is strongly nucleophilic, yet has a relatively low pK_a value (ca. pH 3). Thus, L-CAN is not protonated at physiological pH and therefore is much more nucleophilic than the corresponding $-CH_2NH_2$ moiety of L-ornithine

under these conditions. We propose that the oxime resulting from the reaction of PLP and L-CAN will be very stable due to resistance to the protonation of the −ON=C moiety (Scheme 2). It may be less likely that L-CAN and its congeners first combine via the enzyme-bound PLP imine with their α-amino group in the first step of the classical PLP-dependent enzyme trans-Schiffization reaction, followed by intramolecular attack by the terminal aminooxy group on the imine carbon to form the ultimate terminal aminooxy-PLP oxime (Scheme 2, path B).

Beeler and Churchich (*15*) demonstrated that L-CAN attacks the Schiff base linkage of the enzyme-bound PLP moiety of cystathionase much faster than it reacts with free aldehydic PLP, an effect also observed in other enzyme systems (*16*). This finding suggests that cooperative binding at the active site is an important component of the enzyme inhibitory activity of L-CAN. The carboxyl group may be particularly important for this cooperative effect. As was observed with (aminooxy)-acetate (*16*), we demonstrate that three- and four-carbon terminal aminooxy carboxylic acids are also highly efficacious aminotransferase inhibitors. The potent AlaAT inhibition exerted by ω-(aminooxy)propanoic acid and ω-(aminooxy)butanoic acid obviates the necessity of an α-amino group for such activity in the three- and four-carbon compounds. Indeed, such compounds with an α-amino group in the D-configuration (L-2-amino-3-(aminooxy)propanoic acid and D-CAN) are less active than their parent terminal aminooxy carboxylic acids.

An intriguing finding of our investigation is that ω-(aminooxy)butanoic acid, containing the same number of carbons as L-CAN, is nearly as inhibitory as L-CAN to AlaAT activity, while chain shortening to yield L-2-amino-3-(aminooxy)propanoic acid, the lower homologue of L-CAN, dramatically curtails AlaAT inactivation.

Acknowledgment. This work was supported by National Science Foundation Grant IBN-9302875 and a Graduate Fellowship from the Graduate School of the University of Kentucky to D.R.W.

Registry Numbers Supplied by Author. L-Canavanine, 543-38-4; L-canaline, 496-93-5; arginase, 9000-96-8.

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