Studies with Mechanism-Based Inactivators of Lysine ε-Transaminase from *Achromobacter liquidum*†

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ABSTRACT: Analogues of lysine containing a 4,5-acetylenic linkage (lysyne) or a cis- or trans-4,5-olefinic linkage (lysenes) function as substrates for a homogeneous L-lysine ε-transaminase from *Achromobacter liquidum* but partition between transamination and time-dependent inactivation. The partition ratio is lowest for lysyne (40 per inactivation event) and higher for *trans*-lysene (160 per inactivation event), and the *cis*-lysene transaminates 1600 times per inactivation event. *cis*-Lysene yields α-picolinate as a detectable accumulating product, presumably from cyclization of initial 6-aldehyde to dihydropicolinate and spontaneous autooxidation. The trans isomer also yields some picolinate as an identifiable product. The product from the few lysyne turnovers is as yet unknown but has strong absorbance at 318 nm. The inactive enzyme species from all three lysine analogues slowly (overnight) regain full activity after gel filtration chromatography and dialysis, suggesting reversal of the initial adduct-forming reaction. Initial studies with partially purified pseudomonad lysine α-racemase show α-^3H incorporation from ^3H_2O but no inactivation, consistent with the expectation that these lysine analogues could act readily as mechanism-based inactivators for pyridoxal P enzymes which act at the ε- but not the α-carbon of lysine.

Two varieties of pyridoxal P (PLP) dependent amino acid transaminases can be catalogued: (a) those enzymes that oxidize the α-carbon of an L-α-amino acid (or a D-α-amino acid) and (b) those enzymes that act at ω-amino group of such ω-amino acids as lysine, ornithine, and γ-aminobutyrate (GABA). Studies in this and other laboratories in the past few years have indicated that certain natural and synthetic olefinic and acetylenic amino acids partition between turnover and suicidal inactivation of the processing transaminase (Rando, 1975a,b; Abeles & Maycock, 1976; Walsh, 1977). We (and others) have been attempting to devise analogues which will have sufficient structural specificity so as not to be adventitiously reactive with all transaminases or with other broad classes of PLP enzymes. In this regard the Merrell group has had dramatic success with γ-acetylenic and γ-vinylic GABA as GABA transaminase inactivators (although the 2,3-acetylenic analogue of GABA causes no inactivation) of apparent utility (Seiler et al., 1978; Jung, 1978). Also, investigators at Merck have reported the inactivation of DOPA decarboxylase, a key enzyme in neurotransmitter metabolism, by α-vinyl-DOPA and α-ethynyl-DOPA (Maycock et al., 1978).

In this paper we report studies with lysine analogues, the 4,5-acetylenic analogue (2,6-diamino-4-hexynoate ≡ lysyne) and the cis and trans 4-olefins (2,6-diamino-*cis*-4-hexenoate ≡ *cis*-lysene and 2,6-diamino-*trans*-4-hexenoate ≡ *trans*-lysene) as specific latently reactive enzyme substrates. The acetylenic or olefinic linkage ought to be activatable by enzymatic carbanion chemistry at the ε-amino but not at the α-amino group (the C_3 methylene should provide "insulation"). In fact, a homogeneous L-lysine ε-transaminase from *Achromobacter liquidum* (Soda & Misono, 1968, 1971) does undergo time-dependent inactivation during processing of these lysine analogues, while a partially purified pseudomonad lysine α-racemase (Yorifugi et al., 1971) shows no inactivation during α-hydrogen exchange. These three lysine analogues have been prepared previously and tested as bacterial growth inhibitors, but no molecular interpretations were offered for their modes of action (Jansen et al., 1979; Davis et al., 1964).

Materials and Methods

DL-2,6-Diamino-4-hexynoate (DL-lysyne) was synthesized according to the procedure of Jansen et al. (1970). DL-*cis*-2,6-Diamino-4-hexenoate (DL-*cis*-lysene) was prepared by starting from 2-butene-1,4-diol (91% cis isomer; Aldrich) which was converted to the cis dichloride by using PCl_3–pyridine as described by Mislow & Hellman (1951). Following fractional distillation of the dichloride under reduced pressure to yield the pure cis isomer (>99% pure by gas chromatography), *cis*-4-lysene was prepared in essentially the same fashion as 4-lysyne. Similarly, *trans*-4-lysene was prepared from *trans*-2-butene 1,4-dichloride (Eastman) after fractional distillation. Both *cis*- and *trans*-4-lysene had been prepared previously by Davis et al. (1964).

Specifically labeled [2-^{14}C]lysyne (250 μCi/mmol) was synthesized by incorporating diethyl acetamido[2-^{14}C]malonate (New England Nuclear) into the above scheme. A specific activity of 500 cpm/nmol of [2-^{14}C]lysyne was achieved.

The enzymic products from incubation of α-ketoglutarate and *cis*- or *trans*-lysene were separated and analyzed for comparison with authentic picolinic acid by thin-layer chromatography on 0.1-mm cellulose plates using 1-butanol–acetic acid–water (12:3:5) or 1-butanol–methyl ethyl ketone–17 N NH_4OH–H_2O (5:3:1:1).

Partially purified lysine racemase isolated from *Pseudomonas graveolens* (Yorifugi et al., 1971) was a generous gift of F. Jacobson (this laboratory).

Purification of Lysine ε-Aminotransaminase. Lysine ε-aminotransaminase for labeling studies was purified to >90%

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Table I: Interaction of Lysine ε-Transaminase with Lysine and Unsaturated Lysine Analogues[a]

compd	rel V_{max}	K_m (mM)	K_I (mM)	limiting k_{inact} (s^{-1})	partition ratio[b]	stability of inactivated enzyme	nature of accumulating transamination product
L-lysine	100	2.5					Δ^1-piperidine-6-carboxylate
DL-4-lysyne	1	5.0	6.0	0.07	35-40	labile	unidentified 318-nm chromophore
DL-4-*trans*-lysene	0.3	1.7	2.0	0.13	160	labile	α-picolinate and polymeric material
DL-4-*cis*-lysene	1	2.3	6.2	0.20	1700	labile	α-picolinate and polymeric material

[a] Incubations were carried out at 30 °C in 0.1 M Hepes buffer, 8.0. The cosubstrate α-ketoglutarate was present at 5 mM concentration. The V_{max} for lysine turnover under the conditions was 18–20 (μmol/min)/mg of enzyme. [b] The partition ratio represents the number of catalytic turnovers per inactivation event.

($A_{280}/A_{340}/A_{415}$ = 100:11:10) homogeneity (by disc gel electrophoresis) from *A. liquidum* essentially as described by Soda & Misono (1971) with the following modifications. For many kinetic studies, a Sephacryl S-200 Superfine (Pharmacia) column (1.5 × 60 cm) was substituted for the reported hydroxylapatite column. This resulted in enzyme of ca. 50% purity by specific activity and spectral ratio ($A_{280}/A_{340}/A_{415}$) criteria. This enzyme was found to be spectrally pure at wavelengths >300 nm (i.e., had literature values for A_{340}/A_{415}).

Assay Methods. Two methods for the assay of lysine ε-aminotransaminase were commonly used, depending on experimental constraints.

Assay Method A. Reaction in an assay mixture consisting of 10 mM L-lysine, 5 mM [^{14}C]-α-ketoglutarate (0.02 μCi/μmol; uniformly labeled) in 1 mL of 0.1 M Hepes, potassium phosphate, or potassium pyrophosphate buffer, pH 8.0, at 30 °C, was initiated by the addition of enzyme at time zero. At suitable time points, 100 μL is removed and placed on a 1-mL Dowex 50 cation-exchange column (200–400 mesh; H^+ form) and washed with 10 mL of water. The column is then washed with 3 mL of 3 N aqueous ammonia, and the radioactivity in this effluent, i.e., [^{14}C]glutamate produced in the transamination reaction, is measured.

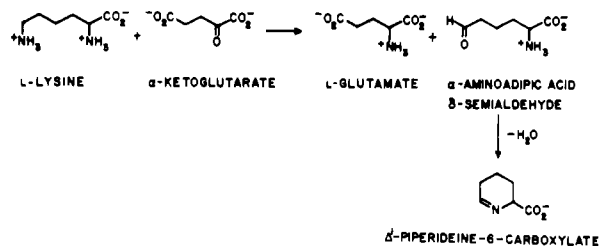
Assay Method B. The cyclic product, Δ^1-piperidine-6-carboxylate, from transamination of lysine can be trapped initially in the presence of *o*-aminobenzaldehyde (Sigma) to form the dihydroquinazolinium salt (which is in slow equilibrium with the piperidine-6-carboxylate at pH 8.0). Use of >5 mM *o*-aminobenzaldehyde and sufficiently small amounts of enzyme to maintain a pseudo-first-order rate for the formation of the dihydroquinazolinium salt [λ_{max} 460 nm (ε 925 M^{-1} cm^{-1}) by assay A] provides a sensitive continuous assay of enzymic activity.

Results

Kinetic Characterization of Substrate and Inactivator Behavior. The *A. liquidum* L-lysine ε-transaminase was homogeneous by gel electrophoresis, $A_{280}/A_{340}/A_{415}$ ratio, and specific activity measurements. In some experiments, 50% pure enzyme was used but it was the major (probably sole) PLP enzyme present in solution. The reaction catalyzed by this ε-transaminase is shown in Scheme I. The enzyme oxidizes lysine at the C_6(ε)-amino group, presumably to the α-aminoadipic semialdehyde, with the cyclized, dehydrated Δ^1-piperidine-6-carboxylate as the major accumulating product. The cosubstrate, α-ketoglutarate, is reductively aminated to give the α-amino acid L-glutamate (Soda & Misono, 1968, 1971).

When the 4,5-acetylenic (lysyne) and cis- and trans-4,5-olefinic (lysenes) lysine analogues were evaluated as substrates and irreversible inactivators, the data of Table I were collected; complete inactivation can be achieved by each of the analogues. Each shows saturation kinetics in the initial linear turnover

Scheme I



phase (allowing a K_m measurement) and behaves as a reversible inhibitor (K_I) against lysine under initial velocity conditions before significant enzyme inactivation ensues. From plots of reciprocals of first-order rate constants for kinetic inactivation vs. reciprocals of lysyne or lysene concentrations, limiting values for k_{inact}, the first-order rate constant for inactivation when all enzyme is complexed with inactivator, were obtained (Walsh, 1977).

Supporting the expectation of "suicide" substrate behavior are the observations of partitioning between normal transaminative turnover and inactivation (Walsh et al., 1978a,b). The partition ratio of 35–40 for lysyne, for example, was measured by the amount of lysyne-dependent [^{14}C]glutamate generated from [^{14}C]-α-ketoglutarate by the enzyme on its way to full inactivation, shown in Figure 1. One inactivation occurs per 35–40 turnovers in any given catalytic cycle. By this criterion, the olefinic lysenes, in particular *cis*-lysene, are less effective inhibitors. The latter compound undergoes 1700 turnovers, on the average, before an inactivation event occurs and is 10-fold less efficient than the trans olefin (160 turnovers).

Stoichiometric Labeling Studies. Subsequent to the observation of kinetic inactivation during catalytic turnover, we attempted to determine two additional parameters relevant to the mechanism of processing of the analogues: (a) stoichiometry of enzyme labeling and (b) the nature of the products which accumulate during the turnovers preceding inactivation. Only partial success has been obtained for both categories a and b.

When enzyme (0.31 mg; 2.7 nmol of active sites) of approximately 90% homogeneity was inactivated (>90%) with 2-[^{14}C]lysyne (500 cpm/nmol) in the presence of excess keto acid and subjected to Sephadex G-25 gel filtration chromatography, only fractional amounts of radioactivity (0.25–0.5 molar equiv/mol of active site)[1] eluted with the enzyme, and the eluting enzyme was no longer completely inactive. One interpretation might be that the covalent adduct between the lysyne ^{14}C-derived inactivator and enzyme was kinetically

[1] The dimeric holoenzyme (115 000 M_r) has two PLP groups but only one is catalytically active; the other PLP exhibits a λ_{max} at 340 nm and not at 435 nm, a finding of Soda et al. (1968) and reproducible by us.

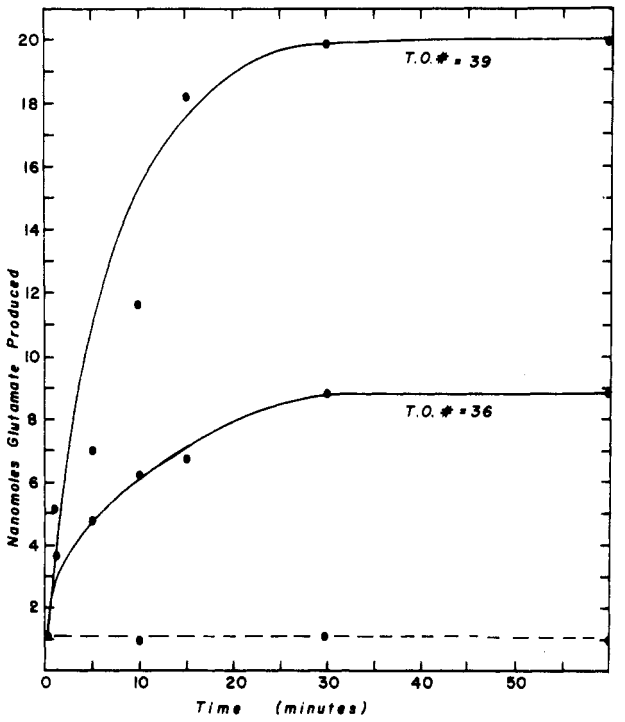
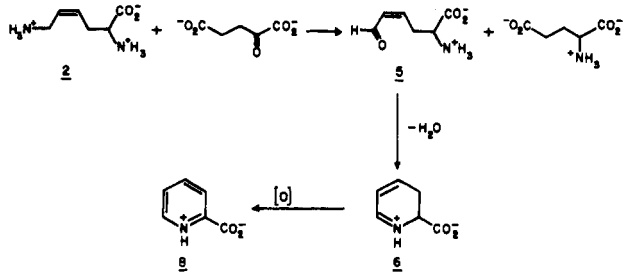


FIGURE 1: Determination of turnover numbers per inactivation event with DL-4-lysyne and lysine ε-transaminase. Enzyme, either 0.26 (lower curve) or 0.52 nmol (upper curve), was incubated in 1 mL of 100 mM Hepes buffer, pH 8.0, at 30 °C with 5 mM [U-^{14}C]ketoglutarate and 25 mM DL-lysyne. Activity was measured at the indicated intervals by using Dowex 50 H^+ column chromatography as noted in assay method A for [^{14}C]glutamate production. The dotted line is a control incubation lacking enzyme. For the lower curve the partition ratio may be calculated by (nmoles of product)/(nmoles of enzyme) = 9.2/0.26 = 36.

labile. This was verified by recovery of 100% of catalytic activity on overnight dialysis with or without 10^{-5} M PLP in the dialysis buffer. When enzyme inactivated in a cuvette assay was immediately electrophoresed in an analytical gel, a single radioactive, inactive band which displayed faster mobility to the + pole than the native enzyme was detected, bearing 0.85–0.95 mol of ^{14}C label per mol of enzyme active site. Elution of enzyme from the gel, followed by overnight dialysis, yielded unlabeled, fully active enzyme. The lability of the presumed covalent inhibitor–enzyme adduct has so far precluded other stoichiometric and structural study. Nor were studies with cis and trans olefin more promising. Prior to syntheses of radioactive *cis*- or *trans*-4-lysene, we investigated the stability of olefin-inactivated enzyme. With either isomer of olefinic lysene, essentially 100% of catalytic activity returned sequentially on prolonged dialysis (>10 h; pH 8.5; $\pm 10^{-5}$ M PLP).

Product Identification. It was anticipated that successful transaminase processing of lysyne and lysenes should yield the corresponding 6-aldehydes. The low turnover number (35–40/inactivation) has as yet thwarted identification of the acetylenic transamination product, but analysis of the UV–visible spectrum revealed an intense absorbance, λ_{max} at 318 nm, developing concomitant with inactivation. On the basis of 35–40 turnovers, the extinction coefficient of the low molecular weight product, separated from enzyme on the Sephadex G-25 column, approximates 14000 M^{-1} cm^{-1}. This cannot be the simple 2-amino-4-acetylenic 6-aldehyde since hexa-2-ynal has ϵ = 25 M^{-1} cm^{-1} at 318 nm (λ_{max}), but it could be a substituted picolinate.[2]

Scheme II



The cis-olefinic lysene does indeed yield picolinate (pyridine-2-carboxylate) as the accumulating product [λ_{max} 268 nm (ϵ 4000 M^{-1} cm^{-1})] (Table I) after a short (<1-min) lag. This pyridine derivative could arise from the initial cis-4-olefinic 6-aldehyde, prone to cyclization to the dihydropyridine and dihydropicolinate and then to spontaneous autoxidation to picolinate (Lyle, 1974; Charman & Rowe, 1971; Eisener & Kuthan, 1972). Likewise, the *trans*-4-lysene also generates some picolinate (quantitation is more difficult given ca. 10-fold fewer turnovers) as the product. Cyclization of an initial trans-olefinic aldehyde product would seem to require a geometrical isomerization step at some stage (Scheme II). From both the cis- and trans-olefinic substrates there was preliminary evidence for product polymerization, in keeping with reaction of simple unsaturated aldehydes such as crotonaldehyde and acrolein (Fischer, 1962) at pH 8, since gel filtration of large-scale (0.5 mg of enzyme) incubations after inactivation yielded a heterogeneous mixture of UV-absorbing material (λ_{max} ranging from 300 to 630 nm) in the small molecule fraction.

Other Enzymes. In preliminary studies to evaluate specificity of the 4-lysyne and *cis*- and *trans*-4-lysenes, partially purified lysine racemase (Yorifugi et al., 1971) was incubated with the analogues in ^3H_2O. No loss of activity was detected (ca. 2 h) during which time 2-^{3}H-labeled species were generated, and the racemase has been used preparatively to produce [2-^{3}H]-4-lysyne and [2-^{3}H]-*trans*-4-lysene. When rat liver ornithine δ-transaminase[3] was examined for susceptibility, the acetylenic and the cis-olefinic lysine analogues caused no inactivation. In contrast, *trans*-4-lysene shows the kinetic characteristics of a saturable, time-dependent, irreversible inactivator, and this inactivation is under active investigation.[3]

Discussion

The 4,5-acetylene and cis- and trans-4,5-olefinic analogues of lysine are processed by the ε-specific transaminase with k_{cat}/K_m values about 1% that of lysine itself, ~1 vs. 90 min^{-1} mM^{-1} (for lysine), with the reduction in catalytic efficiency totally in the k_{cat} term. With the varying frequencies noted in Table I, these unsaturated analogues induce time-dependent alkylation. The olefinic analogues presumably react after transamination but before hydrolytic release of the now conjugated imine product from the active site; the acetylene could react in this mode or, before transamination, the C_6 anion equivalent could rearrange to an electrophilic conjugated

[2] The expected initial product, 2-amino-4-hexyn-6-al, can probably not cyclize without prior addition of a nucleophile at C_4 in a Michael reaction to yield a 4-substituted 4-olefinic 6-aldeyde. Cyclization and dehydration and then aromatization could produce the picolinate. The product cannot be 4-hydroxypicolinate since its synthesis [according to Clark-Lewis & Mortimer (1961)] yields λ_{max} 270 ($\epsilon \approx 10000$ M^{-1} cm^{-1}). A 4-aminopicolinate structure, e.g., from addition of a molecule of unreacted lysyne, is under investigation.

[3] D. Valle, P. Shannon, M. Chang, and C. Walsh, unpublished experiments.

allene as often postulated for such acetylenic inactivators (Morisaki & Bloch, 1971, 1972; Abeles & Walsh, 1973; Walsh, 1977). Such allenic imines are qualitatively much more electrophilic than the olefinic imines (Marcotte & Walsh, 1978), so the lowest partition ratio for the acetylene is explicable by this mechanism. A possible rationale for the 10-fold difference in the partition ratio between cis and trans olefins could be a geometrically favored cyclization of the cis imine to dihydropicolinate as the actual release step; this could accelerate product release selectively from the cis isomer (and raise the turnover/inactivation ratio) since the trans-olefinic imine cannot cyclize directly.

The kinetic lability of enzyme inactivated with any of the three lysine analogues precludes direct structural analysis of the active-site residue participating in inactivation.[4] This lability is not unprecedented with mechanism-based inactivator adducts; for instance, the adducts from cyanoglycine processing by tryptophan synthetase (Miles, 1975), from α-vinyl-DOPA and α-ethynyl-DOPA by DOPA decarboxylase (Maycock et al., 1978), and from difluoroalanine processing by alanine racemase are all labile (Walsh et al., 1978a,b), reflecting equilibria in these covalent addition reactions which are readily reversed. Such lability may be the key determinant in potential in vivo specificity (e.g., bacteriocidal activity) even if the suicide substrate is targeted against only one specific enzyme. In contrast, the adducts from γ-acetylenic GABA and γ-vinylic GABA with GABA transaminase appear stable and so permit in vivo testing (Jung, 1978).

The distribution of L-lysine ϵ-transaminase in procaryotes (or eucaryotes) has not been reported, and it is unclear if this enzyme is the in vivo target for these lysine analogues (Jansen & Kerling, 1970; Davis et al., 1964). Few other enzymes process lysine at carbon 6. Clostridial species possess coenzyme B_{12} and PLP-dependent lysine ϵ-aminomutases (Stadtman, 1972) (these are likely targets yet untested) while eucaryotes have saccharopine dehydrogenase activity, but this is a nicotinamide-dependent enzyme, does not produce a carbanionic species at C_6 during catalysis, and should be unaffected. The lysyl oxidase activity of connective tissue does not process lysine or lysine analogues at the free amino acid level (Siegel, 1976; Siegel & Fu, 1976).

One possible use of the lysine analogues is in studies on mammalian ornithine δ-transaminase, an enzyme found in high levels in brain and recently shown to be the defect in lymphocytes from a patient with the disease gyrate atrophy, associated with second-decade onset of chorioretinal degradation (Valle et al., 1977). Studies currently in progress indicate that the *trans*-4-lysene but not the *cis*-4-lysene or the acetylenic 4-lysyne cause time-dependent irreversible inactivation of purified rat liver ornithine δ-transaminase.[3] Quite recently, it has also been observed that γ-acetylenic GABA (but not γ-vinylic GABA), previously held to be a specific GABA transaminase inactivator, also inactivates ornithine δ-transaminase in vitro and in vivo (Jung & Seiler, 1978). Since *trans*-4-lysene does not inhibit GABA transaminase (G. Berard and C. Walsh, unpublished experiments), it may offer more promise for specific in vivo induction of and evaluation of sequellae from ornithine δ-transaminase inactivation.

[4] We have not yet tried to stabilize the inactivated enzyme complex, e.g., by borohydride reduction which should prevent retro-Michael reactions. This will be analyzed with a radioactive inactivator.

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