Mechanism-Based Inhibition of L-Pipecolate Oxidase by 4,5-Dehydro-L-Pipecolic Acid

T. Mark Zabriskie

College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

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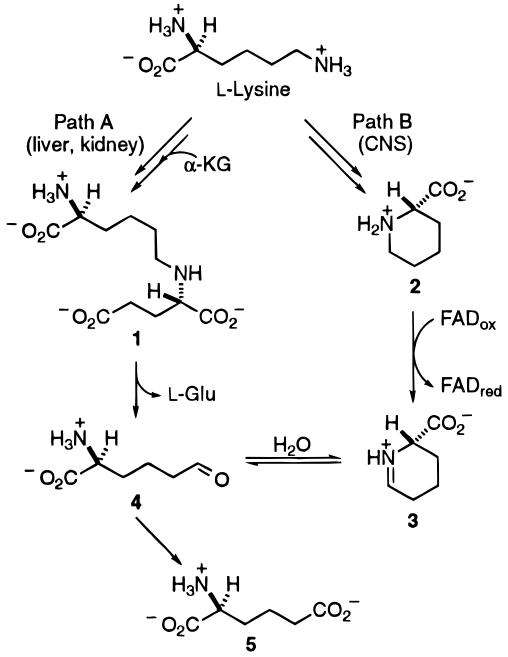
L-Lysine is an essential amino acid required in the mammalian diet for normal growth and development. An unusual aspect of lysine metabolism in mammals is that two distinct and tissue-specific degradative pathways have evolved. In most tissues, the predominant degradative route occurs via the intermediacy of saccharopine (**1**) in a process formally equivalent to the final steps of the lysine biosynthetic pathway in lower eukaryotes (Scheme 1, path A).[1−3] The notable exception is the central nervous system (CNS) where L-lysine is metabolized primarily through the pipecolate pathway (Scheme 1, path B).[4,5] In tissues where the saccharopine pathway is functional, especially liver and kidney, L-pipecolate (L-PA, **2**) formation is a secondary process and D-lysine serves as the precursor.[6−8] Scheme 1 also outlines the pathway for L-PA degradation that functions in all tissues studied. The first step is the flavin-dependent oxidation of **2** to the imine Δ^1-piperideine-6-carboxylate (Δ^1-P6C, **3**). This is a species-dependent process catalyzed by a peroxisomal oxidase in primates and by a mitochondrial dehydrogenase in lower mammals.[9] Hydrolysis of **3** produces L-α-aminoadipate-δ-semialdehyde (**4**), which upon further oxidation yields L-α-aminoadipate (**5**), the first chemically stable product of lysine degradation common to both the pipecolate and saccharopine pathways.[10,11]

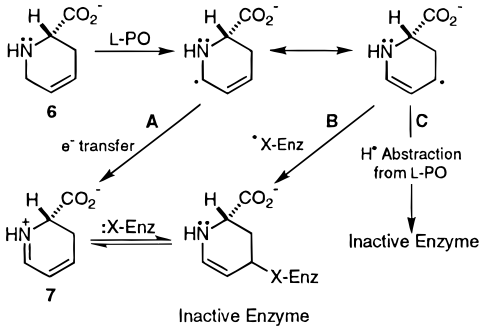
The specific conversion of L-lysine to L-PA in the brain,[4,5] and the existence of a specific degradative pathway,[12] suggests that L-PA may occupy a role in the CNS. Several reports have indicated that L-PA may function as a neuromodulator by interacting with γ-aminobutyric acid (GABA) receptor complexes to potentiate GABAergic inhibitory neurotransmission.[13] In contrast, L-α-aminoadipate (**5**) has excitotoxic properties[14] and also lowers the levels of the neuroprotective tryptophan metabolite kynurenic acid.[15] Recently, we began a program to develop specific inhibitors and mechanistic probes of L-pipecolate oxidase (L-PO), the primate flavoenzyme catalyzing the oxidation of **2** to **3**. L-PO has been purified from Rhesus monkey liver and characterized as a membrane-associated, 46 kDa monomer possessing a covalent flavin and exhibiting a K_m for L-PA of 3.7 mM and a V_{max} of 2.1 μmol·min^{-1}·mg^{-1}.[16] Specific inhibitors of L-PO should produce a concomitant increase in the synaptic concentration and neurological effects of **2** while lowering the levels and effects of **5**. These inhibitors may serve as useful pharmacological tools for studying the role of L-PA and other lysine metabolites in the CNS and provide new leads for treating convulsive disorders. Here we report that 4,5-dehydro-L-pipecolic acid ($\Delta^{4,5}$-L-PA, **6**) is the first example of a potent inhibitor of pipecolate oxidase.

Compound **6** was viewed as a potential mechanism-based inactivator of L-pipecolate oxidase based on the assumption that L-PO would share mechanistic similarities with other flavin-dependent amine oxidases such as monoamine oxidase (MAO). Substantial mechanistic data support the intermediacy of an α-aminyl radical in MAO-catalyzed oxidations, either by a mechanism involving single electron transfer from the amine to the flavin followed by proton transfer[17] or by a direct hydrogen abstraction mechanism.[18] Scheme 2 illustrates possible mechanisms for the inactivation of L-PO by **6**. After generation of an α-aminyl radical, loss of a second electron (path A) would result in an electrophilic conjugated iminium ion (**7**) able to react with an active site nucleophile. Alternatively, a delocalized allylic α-aminyl radical could inactivate L-PO by combining with the flavin semiquinone or other active site radical (•X-Enz in path B) or by abstracting a hydrogen atom from the surface of the enzyme (path C).

Scheme 1



Scheme 2



Racemic **6** was prepared according to a reported method[19] and converted to the homochiral $\Delta^{4,5}$-L-PA by a combined enzymatic and chemical procedure.[20] Pipecolate oxidase was isolated from Rhesus monkey liver as described by Mihalik *et al.*[16] Initial evaluation of **6** as an inhibitor of L-PO resulted in nonlinear progress curves characteristic of time-dependent inhibition (Figure 1).[21] A double-reciprocal plot of the dependence of the observed rate constants obtained from these curves on the concentration of **6** provided estimates for the kinetic parameters K_I and k_{inact} of 130 μM and 2.0 min^{-1},

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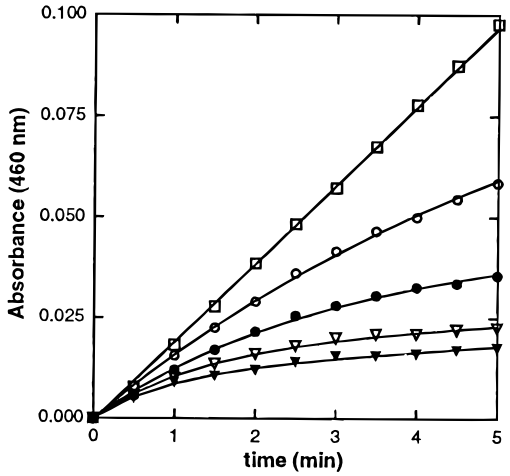

Figure 1. Progress curves showing the effect of the concentration of **6** on the rate of inactivation of pipecolate oxidase. Assays were carried out in a total volume of 0.5 mL at 37 °C in 40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5, buffer containing 10 mM L-PA, 320 μM *o*-dianisidine, 1.8 units of horseradish peroxidase, and **6** at the following final concentrations: (□) 0, (○) 12.5 μM, (●) 25 μM, (▽) 50 μM, and (▼) 125 μM. Reactions were initiated with enzyme.

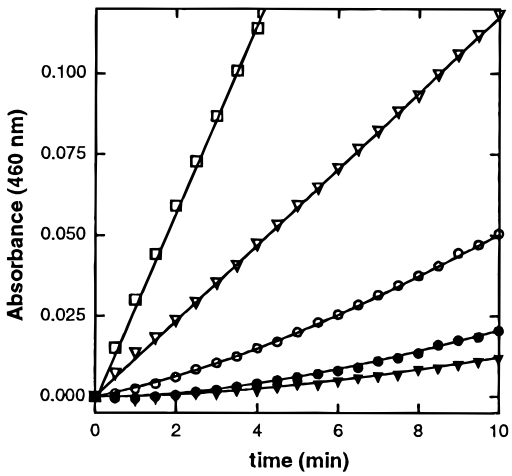

Figure 2. Recovery of pipecolate oxidase activity after preincubating with **6**. L-PO was incubated with various concentrations of **6** in 5 mM HEPES, 150 mM KCl, 0.1 mM EGTA, pH 7.8, buffer, in a total volume of 40 μL, at 25 °C for 5 min. Aliquots (20 μL) were removed and diluted into 480 μL of 37 °C assay cocktail (40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5; 10 mM L-PA, 320 μM *o*-dianisidine, and 1.8 units of horseradish peroxidase), and the enzyme activity was measured. Concentrations of **6**: (□) 0, (▽) 12.5 μM, (○) 25 μM, (●) 125 μM, and (▼) 250 μM.

respectively.[22] Interestingly, commercially available 3,4-dehydro-L-proline serves as an excellent substrate for pipecolate oxidase, and weak, time-dependent inhibition is only detected at millimolar concentrations in assays without L-PA.[24]

To determine if the progressive loss of activity resulted from irreversible inactivation or a reversible, slow-binding interaction, a sample of L-PO was incubated with 100 μM **6** under standard conditions for 30 min, after which no activity remained. The sample was dialyzed for 24 h and found to have regained 80% of the lost activity, relative to a similarly treated control. To further test the reversibility of the interaction, samples of L-PO were preincubated with various concentrations of **6**, diluted 25-fold into assay solutions containing a high concentration of substrate, and the activity was monitored for up to 30 min. The progress curves from these studies (Figure 2) revealed a slow return of activity, the initial reaction rate being a function of the concentration of **6** in the preincubation mixture. Similar data were obtained when the inactive complex was separated from unbound **6** by rapid gel filtration chromatography (data not shown).

Figure 3 illustrates the results from extended preincubation experiments where aliquots were assayed periodically for recovered activity. The initial inactivation is followed by a slow return of activity to a plateau level; the rate of recovery and the degree of permanent inactivation are dependent on the concentration of **6** in the preincubation mixture. Thus, it appears that a minor pathway exists leading to the irreversible inactivation of L-PO by $\Delta^{4,5}$-L-PA.

Incubating L-PO and various levels of L-pipecolate in the presence of a fixed concentration of **6** (25 μM) afforded protection of the enzyme in proportion to substrate concentration, demonstrating that $\Delta^{4,5}$-L-PA acts at the enzyme active site. When **6** was incubated with L-PO under normal horseradish peroxidase-coupled assay conditions in which L-PA was omitted, a burst of H_2O_2 was detected prior to a plateau phase. This evidence of flavin reoxidation confirms that **6** serves as

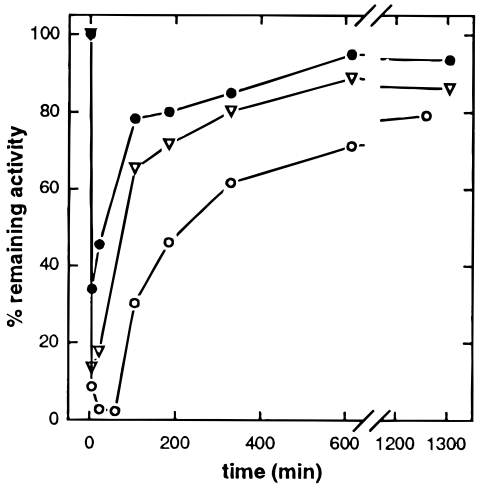

Figure 3. Pipecolate oxidase activity vs preincubation time at different concentrations of **6**. L-PO was incubated with various concentrations of **6** in 5 mM HEPES, 150 mM KCl, 0.1 mM EGTA, pH 7.8, buffer, in a total volume of 150 μL, at 25 °C in the dark. At the times indicated, 15 μL aliquots were removed and diluted into 485 μL of 37 °C assay cocktail (40 mM Tris, 80 mM KCl, 0.8 mM EGTA, pH 8.5; 10 mM L-PA, 320 μM *o*-dianisidine, and 1.8 units of horseradish peroxidase), and the enzyme activity was measured. Concentrations of **6**: (●) 25 μM, (▽) 50 μM, and (○) 125 μM.

an alternate substrate of L-PO and that the catalytic step occurs prior to inactivation. Supporting data that the turnover product is the imine **7** came from reacting the enzyme with **6** followed by treatment with NaB^3H_4. This yielded a radiolabeled species that comigrated with authentic **6** using both thin-layer and paper chromatography analyses and confirmed $\Delta^{4,5}$-L-PA had been oxidized by L-PO. The protein from this experiment was also analyzed for tritium incorporation, but no radioactivity was detected. If reversible inactivation is due to an unstable covalent complex, this species is not susceptible to borohydride reduction.

In order to demonstrate that released turnover product **7** does not indiscriminately alkylate L-PO or the

coupling enzyme, a second aliquot of L-PO was added to an assay mixture inactivated with 80 μM $\Delta^{4,5}$-L-PA. An immediate increase in H_2O_2 formation was detected followed by a loss of activity with the same apparent rate constant as that of the first aliquot of enzyme. Addition of a third aliquot of L-PO produced the same effect, confirming that progressive inhibition by $\Delta^{4,5}$-L-PA does not result from increasing levels of the turnover product **7**, nor is it due to nonselective inactivation by a highly reactive species released from the active site.

In summary, $\Delta^{4,5}$-L-PA (**6**) is the first example of a potent mechanism-based inhibitor of L-pipecolate oxidase. The time-dependent loss of activity is largely reversible, and the slow dissociation of the enzyme−inhibitor complex dominates the overall kinetics. Incubation of L-PO with high concentrations of **6**, or for extended times, results in a partial loss of activity and implies that a minor pathway leading to irreversible inactivation exists. Specific inhibitors of central lysine metabolism represent a new class of pharmacological tools for studying the role of pipecolic acid and other lysine metabolites in the CNS and may ultimately provide leads to new anticonvulsants. Demonstrating that **6** is a potent and selective inhibitor of Rhesus liver L-PO is the first step toward developing such compounds. Further studies examining the nature of the enzyme−inhibitor complex and the mechanism leading to permanent inactivation of L-PO are underway.

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Supporting Information Available: Information on the preparation of **6** including spectral and chromatographic data (4 pages). Ordering information is given on any current masthead page.

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(21) L-PO was assayed according to ref 16. Hydrogen peroxide produced during the oxidase reaction is coupled to the horseradish peroxidase-catalyzed oxidation of *o*-dianisidine. The reaction is followed spectrophotometrically by monitoring the increase in absorbance at 460 nm. Assays were carried out as described in the figure captions.
(22) The progress curve data were analyzed by direct fits to the integrated rate equation $P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k$, which describes inhibition mechanisms where the steady state is reached by a first-order process. This analysis provides estimates of the initial and steady-state velocities (v_0 and v_s, respectively) and the observed rate constants for the approach to steady state (k).[23]
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