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AMINOALKYLAZIRIDINES AS SUBSTRATES AND INHIBITORS OF LYSYL OXIDASE: SPECIFIC INACTIVATION OF THE ENZYME BY N-(5-AMINOPENTYL)AZIRIDINE

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1. ABSTRACT

The reaction of lysyl oxidase was assessed with members of a series of aminoalkylaziridines in which the primary amino group and the aziridinyl nitrogen were separated by 3-7 methylene carbons. Among these, N-(5-aminopentyl)aziridine proved to be the poorest substrate by far and to inhibit the enzyme activity. Aminoalkylaziridines with chain lengths shorter or longer than five carbons did not inhibit the enzyme. The resulting inhibition was competitive with productive substrates and became irreversible with time, following pseudo first order kinetics with a K_I of 0.22 mM. N-(5-aminopentyl)aziridine appears to act as a bifunctional affinity label covalently interacting with the active site of this enzyme.

2. INTRODUCTION

Lysyl oxidase is a copper-dependent amine oxidase which initiates the biosynthesis of lysine-derived crosslinkages by oxidizing peptidyl lysine residues to alpha-aminoadipic-delta-semialdehyde in elastin and collagen. This peptidyl aldehyde is the precursor of the variety of crosslinkages found in these proteins (1,2). In addition to copper, lysyl oxidase also contains a covalently bound carbonyl prosthetic group which is essential for catalytic function. This cofactor has recently been identified in rat lysyl oxidase as lysyl topaquinone (LTQ) deriving from tyrosine 349 and lysine 314 (3). The irreversible inactivation of this enzyme by 1,2-alkyldiamines (4) is consistent with the reactivity of this orthoquinone moiety.

The possibility that the increased accumulation of insoluble, crosslinked collagen in fibrotic disease states may be restricted by the specific suppression of lysyl oxidase activity has stimulated the search for selective and potent inhibitors of this enzyme. Thus, this catalyst is subject to mechanism-based inhibition by beta-aminopropionitrile (5) and beta-haloethylamines (6) and is significantly more sensitive to these agents than other mechanistically similar amine oxidases acting on non-peptidyl amine substrates (7). Certain para-substituted benzylamines (8) and 1,2-alkyldiamines (4) have also been found to be active site-directed inhibitors of lysyl oxidase. Aminoalkylaziridines (figure 1) have been shown to be effective inhibitors of diamine oxidase (9), which, like lysyl oxidase, contains both a copper(II) and a tyrosine-derived quinone cofactor (10,11). N-(4-Aminobutyl)aziridine, a potent inhibitor of cellular polyamine uptake, irreversibly inactivates diamine oxidase (9). These compounds are potentially covalent inactivators in view of their ability to alkylate an enzyme nucleophile at the aziridinyl moiety and to form Schiff base adducts with the carbonyl cofactor at the primary amino group (12-15). Thus, a series of aminoalkylaziridines have been assessed as novel active site directed inhibitors of lysyl oxidase in the present report.

3. MATERIALS AND METHODS

3.1 Enzyme purification and assay

Lysyl oxidase was purified to apparent homogeneity from calf aortas as previously described (16). The product migrated as a single band equivalent to a molecular mass of 32,000 Da when analyzed by sodium dodecyl sulfate gel electrophoresis (17). The purified enzyme preparation consists of four, 32 kDa ionic variants exhibiting a high degree of structural similarity, essentially the same substrate specificities and which appear to operate by the same catalytic mechanism (18,19). Aminoalkylaziridines were synthesized and purified as described (20,21).

The specific activity of lysyl oxidase was determined by a tritium release assay against 125,000 cpm of a chick aortic elastin substrate labeled in organ culture with L-(4, 5- ^{3}H)lysine (22). The specific activities of purified enzyme preparations ranged from 400,000 to 800,000 cpm (^{3}H)H_2O released per mg^{-1} protein per 2 h of assay. All activities were corrected for background rates of tritium release of enzyme-free controls, and all activities were fully

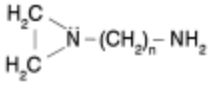


Figure 1. Generic structure of alkylaminoaziridines; "n" = 3 through 7.

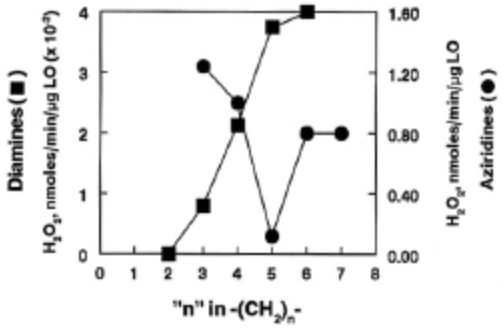


Figure 2. Chain length dependency of the substrate potential of diamines and aziridines.

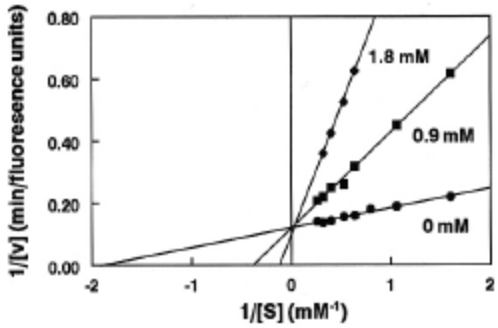
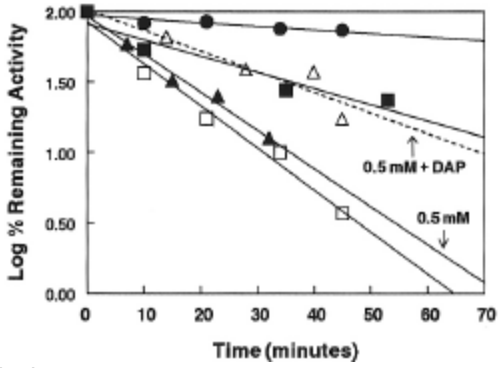


Figure 3. Lineweaver-Burk plot: inhibition of lysyl oxidase



by APAZ.

Figure 4. Inactivation of lysyl oxidase by APAZ: semi-log plot. Lysyl oxidase was preincubated for the indicated times in the presence of: 0 mM APAZ (control), ●; 0.2 mM APAZ, ■; 0.5 mM APAZ + 2.5 mM 1,5-diaminopentane (DAP), Δ; 0.5 mM APAZ, Δ; 2 mM APAZ, □. Remaining enzyme activity was assayed after dilution (1:50) of aliquots into the peroxidase-coupled assay mixture.

inhibited by 50 μM β-aminopropionitrile. Enzyme activities were also determined using a peroxidase-coupled fluorometric assay to obtain initial rates of lysyl oxidase-catalyzed H_2O_2 formation using 2.5 mM 1,5-diaminopentane, or other alkylamines, as specified, in assay mixtures containing 0.25 mg of homovanillic acid, 40 μg of horseradish peroxidase, and 1.2 M urea (23). Assays were initiated by the addition of lysyl oxidase to otherwise complete assay mixtures pre-equilibrated at the optimal assay temperature of 55°C.

4. RESULTS

4.1 Substrate potential of aminoalkylaziridines

The substrate or inhibitory potential of a series of aminoalkylaziridines of varying chain length (figure 2) was assessed in the peroxidase-coupled fluorescence assay. Aziridinyl compounds of different alkyl carbon chain lengths (Figure 1) were each tested at 3 mM in the assay mixtures. Among these, as shown in Figure 2, N-(5-aminopentyl)aziridine (n = 5; figure 1) was the poorest substrate of lysyl oxidase. The similar, marked increase in substrate potential as the chain length was shortened or increased by 1 methylene group (n = 4 or 6, respectively) indicated a uniquely unfavorable substrate effect at n = 5. This variation in the substrate potential of aminoalkylaziridines differed markedly from that obtained with a series of diamine substrates of lysyl oxidase, in that 1,5-diaminopentane and 1,6-diaminohexane (with 5 or 6 methylene groups, respectively, between the two primary amino groups) proved to be most rapidly oxidized at the concentrations tested in the assay system (5 mM). The concentration of diamines (5 mM) used in these assays yielded rates of oxidation which approximated the V_{max} for these compounds (6).

The possibility that N-(5-aminopentyl)aziridine (APAZ) was not only a poor substrate but also an inhibitor of lysyl oxidase was assessed. Indeed, this compound inhibited the activity of the enzyme, using either 1,5-diaminopentane or n-hexylamine as substrate in the peroxidase-coupled fluorescence assay, yielding an IC_{50} value of 0.5 mM with either substrate. Aminoalkylaziridines with chain lengths shorter or longer than five carbons did not inhibit the enzyme (not shown). Free aziridine did not inhibit the enzyme, indicating both that the inhibition required the primary amino and aziridine moieties and that inhibition was not due to traces of free aziridine possibly present in the preparations of the aminoalkylazirdine.

4.2 Competitive nature of inhibition

A Lineweaver-Burk plot (figure 3) of the data from an initial rate analysis of the mode of inhibition of 1,5-diaminopentane oxidation by APAZ resulted in a series of lines intersecting at the 1/v axis, indicating that the inhibitor competes with the amine substrate for interaction at the active site (figure 3). The K_I value calculated from the slopes of these plots was found to be 0.22 mM.

4.3 Irreversibility of inhibition

Lysyl oxidase was preincubated at 37°C in the presence or absence of increasing concentrations of APAZ followed by dilution of aliquots of these mixtures into the peroxidase-coupled assays for 1,5-diaminopentane oxidation, thus reducing the concentration of the aziridinyl compound to non-inhibitory levels in the lysyl oxidase assays. The rates of loss of enzyme activity increased with increasing inhibitor concentrations with inactivation following apparent first order kinetics. As shown (figure 4), the presence of the substrate, 2.5 mM 1,5-diaminopentane, in the preincubation mixture together with the inhibitor partially protected against

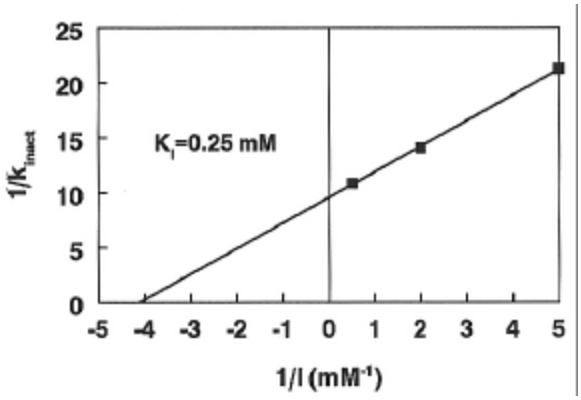


Figure 5. Kitz and Wilson plot. k_{inact} values were calculated from the $t_{1/2}$ values at each concentration of APAZ (k_{inact} = $0.693/t_{1/2}$; see Figure 4).

the loss of enzyme activity caused by the aminoalkylaziridine. The apparent first order rate constant for inactivation (k_{inact}) determined from the intercept of a secondary reciprocal plot (24) of the data of figure 5 was 0.1 min^{-1}. The linearity of this plot is characteristic of an inhibitor exhibiting saturation kinetics. The intercept of the plot at the 1/(I) axis, equal to $-1/K_I$, yielded a K_I value of 0.25 mM, closely similar to the value of 0.22 mM determined from the steady-state kinetic experiments described.

Consistent with the pseudo first order rate of loss of enzyme activity (figure 4), the inhibition of lysyl oxidase by APAZ was also determined to be irreversible by dialysis. Thus, the enzyme which had been preincubated with 5 mM of the inhibitor at 37°C for 1.5 hours and then dialyzed thoroughly to remove the compound was catalytically inactive, whereas enzyme treated in parallel in the absence of APAZ retained approximately 90% of its original activity.

5. DISCUSSION

In summary, the inhibition of lysyl oxidase by APAZ is competitive, develops irreversibly with time, and is selective for the five-carbon chain length separating the amino and aziridinyl moieties. Since the compound is also productively and catalytically, although minimally, processed, *in toto,* the data indicate its interaction both as an inhibitor and as a substrate with the active site. Thus, it seems reasonable to speculate that APAZ initially interacts through its primary amino group with the LTQ cofactor, forming a Schiff base adduct with one of the *ortho*-carbonyls of this prosthetic group, following the usual course of amine oxidation (25). Since shorter and longer aminoalkylaziridines are substrates but do not inhibit the enzyme, this suggests that APAZ exists in its fully extended configuration upon such interaction with the active site, situating its electrophilic aziridine moiety proximal to an electron donating site of the enzyme. Moreover, since the pK_a (8.1) of free aziridine in aqueous solution approximates the pH (8.2) at which lysyl oxidase is assayed, it would be expected that approximately one-half of the available aziridinyl moieties would be protonated, thus increasing electrophilicity and generating a positive charge at that end of the molecule. Notably, oxyanions of dicarboxylic amino acid residues of other enzymes have been shown to covalently attack aziridine rings (12,14,15). Thus, it is of some interest that the sequence surrounding the carbonyl cofactor in rat lysyl oxidase is GCYDT***Y***AADID, where the progenitor of the LTQ cofactor, tyr349, is represented in bold and italicized. Anionic charge stemming from the local abundance of aspartate residues may underlie the apparently exclusive preference of lysyl oxidase for a series of basic protein substrates whereas a variety of acidic proteins were not substrates for the enzyme (26). These aspartate residues are potential sources of nucleophilic oxyanions one of which may covalently react with the aziridine moiety of APAZ to inactivate lysyl oxidase. Indeed, molecular models illustrate the feasibility of a fully extended APAZ unit covalently bridging the β-carboxyl function of asp352 with the carbonyl cofactor, the latter linked through a Schiff base involving the primary amino function of APAZ. The future availability of isotopically labeled APAZ should permit the assessment of this hypothesis.

The K_I and IC_{50} values for the inhibition by APAZ indicate that this reagent is not an unusually potent inhibitor of lysyl oxidase. Nevertheless, the results obtained with APAZ in the present study uniquely point to the potential of bifunctional reagents as covalent probes of the active site of this enzyme and should serve as a paradigm for the development of other inhibitors which might find use as anti-fibrotic agents.

6. ACKNOWLEDGEMENTS

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