ON THE INHIBITION OF LYSYL OXIDASE BY β-AMINOPROPIONITRILE

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SUMMARY. β-Aminopropionitrile binds to and inactivates lysyl oxidase, the enzyme carrying out the first step in the cross-linking of collagen and elastin.

Administration of β-aminopropionitrile (BAPN) to growing animals results in the experimental disorder known as lathyrism. In this condition, the supporting tissues are markedly weakened (1). Connective tissue from lathyritic animals has collagen and elastin fibers with decreased tensile strength (2,3,4) and collagen that is abnormally soluble (2). The basic defect has been shown to be decreased cross-linking of collagen and elastin (5) due to the inhibition of lysyl oxidase by BAPN (6,7,8). Lysyl oxidase catalyzes the conversion of certain lysines in collagen and elastin to the corresponding δ-semialdehyde. Lysine derived aldehydes condense spontaneously to form the various cross-links in collagen and elastin (9-12). Little lysyl oxidase activity is found in animals receiving BAPN and BAPN is a potent inhibitor of this enzyme *in vitro* (6,8). In this report we present further studies on the inhibition of lysyl oxidase by BAPN including its binding to the enzyme.

MATERIALS AND METHODS

Preparation of Lysyl Oxidase and Assay of Activity: An extract with lysyl oxidase activity was prepared from the cartilagenous ends of tibiae and femora of 17 day old embryonic chicks. Lysyl oxidase was partially purified (5 fold) from the extract as described previously (8). For the

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present studies reported here a 490-fold purified enzyme, obtained by DEAE-cellulose chromatography, was used. Details of the procedures and properties of the purified enzyme are to be published elsewhere (A. S. Narayanan, J. Greene and G. R. Martin, in preparation). Enzyme activity was assayed by measuring the release of tritium from chick aortic elastin biosynthetically labelled with lysine-6-^{3}H after incubation with the enzyme preparation for 8 hours (6).

Inhibition and Binding Studies: β-Aminopropionitrile-1-^{14}C (Specific Activity 1.1 mCi/mM) was obtained from New England Nuclear. Radioactive BAPN was also prepared by a catalytic exchange procedure for labelling the compound with tritium (carried out for us by New England Nuclear). Prior to use BAPN was isolated from the radioactive samples by thin layer chromatography on silica gel followed by chromatography on CM-cellulose paper using the solvent system n-butanol:acetic acid:water in the proportion 5:1:4 (13). BAPN.HCl-^{14}C was found to be essentially free of contaminants while the tritiated sample was grossly contaminated.

To measure the rate of inactivation of lysyl oxidase by BAPN, 0.9 ml of enzyme (100 - 200 µg of protein) was incubated with 100 µg of BAPN in 0.1 ml of 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.5 at 37° for 2 hours. After this incubation, unreacted BAPN was removed by dialysis against 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.5 at 4° and the remaining enzyme activity was assayed. To measure the binding of labelled BAPN to lysyl oxidase, 250,000 - 500,000 cpm of the labelled compound in 0.2 ml of buffer was incubated with 0.8 ml of the enzyme at 37°. The controls were incubated at 0° at which temperature no loss of activity was observed (Table I). After two hours of incubation, unreacted BAPN was removed either by dialysis or by passage through a G-25 Sephadex column. An aliquot of the enzyme was counted to measure the binding.

Molecular Sieve Chromatography: Five ml of purified enzyme was incubated at 37° with BAPN-3H as indicated above, then dialyzed and concentrated to about 3 ml by pH 5 precipitation (8) and loaded on a 8% agarose

column (Bio-Gel A 1.5 M, 200-400 mesh, 112 x 1.5 cm) equilibrated with 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.5 at 4° and eluted with the same buffer at a flow rate of 9 ml per hour. The absorbance of the column effluent was measured at 280 mμ. Fractions of 4.5 ml were collected and the radioactivity in one ml portions was measured in Beckman Ready-solv VI counting solution using a Packard Model 3375 liquid scintillation spectrometer. A 5 ml portion of untreated enzyme was concentrated and chromatographed in the same way. One ml portions of the collected fractions were assayed for enzyme activity.

RESULTS AND DISCUSSION

Previous studies have demonstrated that lysyl oxidase is irreversibly inactivated after incubation with BAPN (6). We expected to find that BAPN became bound to the enzyme. Preliminary studies with our 5-fold purified preparation of lysyl oxidase indicated that added radioactive BAPN was metabolized to a variety of products. Indeed, the 5-fold purified preparation was found to contain significant amounts of amine oxidase activity (16 units/mg protein), an enzyme known to degrade BAPN. The DEAE-cellulose purified enzyme was virtually free of amine oxidase activity (0.5% of the original amine oxidase activity per unit of lysyl oxidase activity) and its use was more suitable for this study.

Using the DEAE-cellulose preparation of lysyl oxidase, we have determined the levels of BAPN which inhibit and inactivate the enzyme. For this study, lysyl oxidase was incubated with various concentrations of BAPN for 4 hours at 37°. To assess inactivation, we removed free BAPN from the enzyme by dialysis prior to enzyme assay in one experiment. In another experiment, free BAPN was not removed by dialysis. A similar decrease in enzyme activity was seen with increasing levels of BAPN in both cases with half the activity lost at approximately 0.25 μg BAPN/ml (Fig. 1). The DEAE-cellulose enzyme is about 4 times as sensitive to inhibition by BAPN as the 5-fold purified enzyme (6,8) which may be due to the removal of

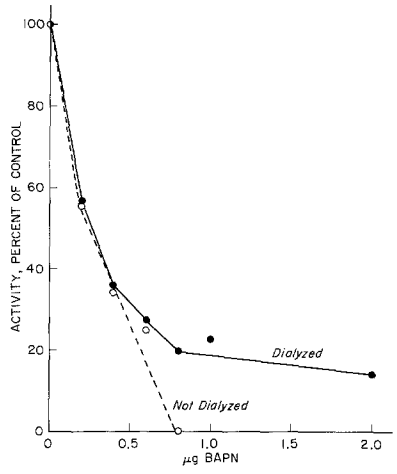

Fig. 1. Effect of BAPN concentration on inactivation of lysyl oxidase. o—o after reaction unreacted BAPN dialyzed; o- - -o BAPN present in the assay mixture.

enzymes such as amine oxidase that degrade BAPN. Enzyme activity was completely inhibited in those samples where BAPN was present throughout the enzyme assay while about 15% of the activity was present in those samples dialyzed prior to assay. Apparently a portion of the lysyl oxidase in our preparation was in a form resistant to inactivation during the preincubation with inhibitor.

To measure the rate of inactivation of lysyl oxidase when incubated with an excess of BAPN, the enzyme preparation was incubated with 50 μg/ml of BAPN for varying periods at 37° and then dialyzed against buffer at 4° overnight prior to assay. More than 60% of the activity was lost after the first 30 minutes of incubation with BAPN, although significant levels of activity persisted for up to six hours (Fig. 2). This is in contrast to the results obtained by adding BAPN to incubation mixtures of labelled elastin substrate and lysyl oxidase. Here enzyme activity is immediately and completely blocked (see ref. 8, Fig. 3). The controls, which were

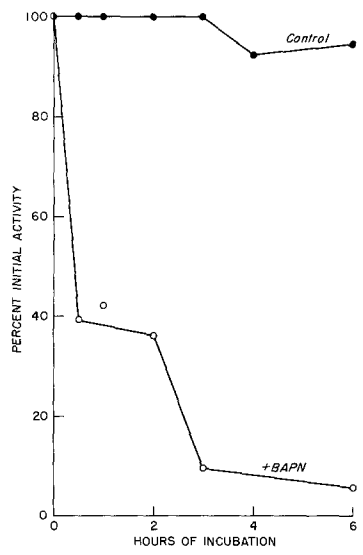

Fig. 2. Inactivation of lysyl oxidase by BAPN with time. Samples were incubated for the time indicated at 37° with BAPN (o——o) and dialyzed to remove free BAPN and then assayed for lysyl oxidase activity. Controls (●——●) were treated similarly but without BAPN.

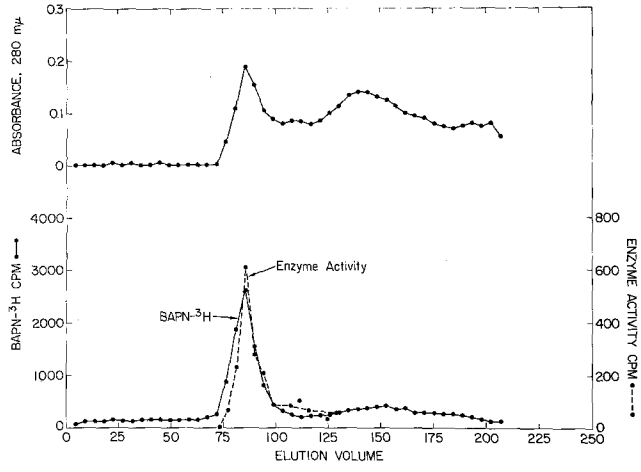

Fig. 3. Superimposed molecular sieve chromatograms on agarose (8%) of lysyl oxidase preincubated with tritiated BAPN and active lysyl oxidase. The column was equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5 and eluted at 9 ml/hr. Radioactivity (●——●), enzyme activity (●---●) and absorbance (●——●, top line) of aliquots of each 4.5 ml fraction are shown.

TABLE I. Binding of ^{14}C-BAPN to lysyl oxidase

Enzyme Sample	^{14}C-BAPN bound/ml (cpm)	Enzyme Activity (cpm tritium water)
1. Control	--	2714
2. Incubated with BAPN-^{14}C at 0°	3116	2593
3. Incubated with BAPN-^{14}C at 37°	17967	62

incubated at 37° for varying periods without BAPN, retained almost all of their activity.

The possibility that BAPN became bound to lysyl oxidase during the incubation period was investigated using labelled BAPN and the DEAE-cellulose purified enzyme. When labelled BAPN was incubated with lysyl oxidase, a portion of the radioactivity became nondialyzable. The amount of label that became nondialyzable was some 6-fold higher at 37° than at 4° (Table I) where enzyme activity was not lost. Preincubation of lysyl oxidase with unlabelled BAPN (50 μg/ml) at 37° for 4 hours followed by dialysis at 4° to remove free BAPN also reduced to a fourth the amount of unlabelled BAPN that would bind to the enzyme preparation upon subsequent incubation (not shown). These observations suggest that the binding of BAPN occurs under those conditions where the enzyme is active.

To demonstrate that the label was indeed bound to the enzyme, we have used chromatographic procedures. Lysyl oxidase was incubated with labelled BAPN under conditions leading to inactivation of the enzyme (37° for 4 hours). After the incubation unbound BAPN was removed by dialysis, the protein was concentrated as indicated in METHODS and the sample was placed on a molecular sieve of 8% agarose. Portions of fractions collected from the column effluent were assayed for radioactivity and absorbance. To identify the position at which lysyl oxidase emerged from the column, active lysyl oxidase was run on the same column and the fractions assayed for

enzyme activity. The bulk of the label emerged in the position that lysyl oxidase activity eluted from the molecular sieve (Fig. 3). Similar studies have been made using DEAE-cellulose to fractionate the BAPN tagged enzyme. Again label emerged with lysyl oxidase activity (not shown).

Our studies indicate that BAPN is bound to lysyl oxidase as the enzyme is inactivated by this compound. Binding of BAPN and the inactivation of the enzyme occur only under those conditions where the enzyme is also active on its macromolecular substrates. The chemical basis for this binding and the mechanism of action of lysyl oxidase are not known. However, it is possible that BAPN possessing an amino group like the natural substrate, forms a stable enzyme-inhibitor complex by reacting with the active center of lysyl oxidase. This would account for the conditions necessary to achieve binding of BAPN to lysyl oxidase and the inactivation of the enzyme. BAPN may prove useful in labelling the active site of this enzyme.

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