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INHIBITION OF LYSYL OXIDASE BY DISULFHYDRYLS, DIAMINES AND SULFHYDRYL-AMINES[1]

Ronald L. Misiorowski and Mark J. Werner

Department of Surgery
Division of Surgical Biology
University of Arizona
Arizona Health Sciences Center
Tucson, Arizona 85724

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SUMMARY: Lysyl oxidase is the enzyme responsible for the oxidative deamination of lysine and hydroxylysine residues in collagen and elastin. Lysyl oxidase activity is irreversibly inhibited by disulfhydryls and diamines while the sulfhydryl-amine, penicillamine, inhibits reversibly. Monosulfhydryls or monoamines do not inhibit significantly. All inhibitors tested react directly with the enzyme. The disulfhydryls do not inhibit through thiolytic cleavage as an equivalent amount of β-mercaptoethanol does not produce significant inhibition (1). The possibility of adduct formation between these bifunctional inhibitors and aldehyde or pyridoxine derived cofactors within the enzyme is discussed.

INTRODUCTION: Page and Benditt postulated that the mechanism of β-aminopropionitrile induced lathyrism resides in its irreversible inhibition of lysyl oxidase (2,3), the enzyme responsible for the oxidative deamination of lysine residues in collagen and elastin. In addition to β-aminopropionitrile (4,5, 6), lysyl oxidase can be inhibited by carbonyl reagents (1,7), isoniazide (7,8,9), iproniazid (7,9), and, unexpectedly, dithiothreitol but not β-mercaptoethanol (1). Harris *et al.* (1) have suggested that dithiothreitol does not inhibit by thiolytic cleavage because equivalent amounts of β-mercaptoethanol show no inhibitory effect.

Although β-aminopropionitrile is the most effective lathyrogen, it has undesirable side effects *in vivo*. It would be advantageous to identify a highly specific inhibitor of lysyl oxidase to be used both for *in vivo* studies and to aid in enzyme purification. This research was undertaken to identify the structural requirements and characteristics of the peculiar inhibition of lysyl oxidase by DTT.

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MATERIALS AND METHODS: Inhibitors: The following were purchased from Sigma Chemical Company: dithiothreitol, dithioerythritol, monothioglycerol, 1,2-dithio-3-propanol (British Anti-Lewisite), L-cysteine, N-ethylmaleimide and oxidized lipoic acid. The following were obtained from the Aldrich Chemical Company: 1,3-dithio-2-propanol, dithioethane, dithiopropane, dithiobutane, 5,5'-dithiobis(2-nitrobenzoic acid), 1,3-diaminopropane, and n-propylamine. β-mercaptoethanol was from Eastman Kodak Company; glycerol from J.T. Baker Chemical Company; N,N-dimethyl-β-mercaptoethylamine from Pfaltz and Bauer, Inc. and D-penicillamine from Merck, Sharpe and Dohme. Inhibitor solutions were made in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.7.

Substrate: Collagenous substrate was prepared from chick calvarial organ culture as reported by Narayanan, Siegel and Martin (10) using 4,5-^{3}H lysine.

Assay: Lysyl oxidase activity was measured using prefibrilized collagen substrate in assay as described by Siegel (11) and modified by Misiorowski et al. (12). Inhibitors were added to the preincubated substrate prior to addition of enzyme preparations.

Oxidation of Dithiothreitol: 21 mM dithiothreitol was oxidized by shaking at 37° with continuous bubbling with oxygen for approximately 4 hours. Using Ellman's test for sulfhydryl (13), 0.05 mM reduced dithiothreitol remained. The resulting solution was used as stock 21 mM oxidized dithiothreitol. Solutions of 1,3-thio-2-propanol were partially oxidized by shaking for 1 hour at 37°.

Enzyme Purification: Lysyl oxidase was partially purified by the following procedures. The extraction was usually performed in the presence of the protease inhibitors N-ethylmaleimide and phenylmethylsulfonylfluoride, both at 1 mM. Whole leg (tibia/femur) or calvarial bones from 20 dozen 17 day embryonic white leghorn chicks were pre-extracted twice at 4°C in PBS. The 20,000 x g supernatants were found to have little lysyl oxidase activity and were discarded. The resulting insoluble residue was extracted twice with 5 M urea in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.7. These 20,000 x g supernatants were dialyzed extensively against 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.7, then incubated at 37°C in a shaking water bath for 60 minutes. The gelatinous material formed was pelleted at 20,000 x g, then extracted with 50 ml 0.05 M Tris, 5 M urea pH 7.7 for 30 minutes at 4°C. Following centrifugation at 20,000 x g, the supernatant was shaken for 30 minutes at room temperature with 30 ml preswollen DEAE ion exchange resin (DE52, Whatman) which has been pre-equilibrated in the same buffer. The DEAE resin slurry was filtered on a coarse scintered glass funnel and washed 5 times with 5 ml aliquots of 0.05 M Tris, 5 M urea, 1 M NaCl pH 7.7. The combined Tris-urea-salt effluents were dialyzed extensively against PBS and used as the enzyme source.

RESULTS: Extending the observation of Harris et al that dithiothreitol, but not β-mercaptoethanol, inhibited lysyl oxidase, we report that several disulfhydryls are effective inhibitors (Table 1A). The limited solubility of dithioethane, dithiopropane, and dithiobutane in aqueous buffers results in inexact quantitative comparison. Monothioglycerol and glycerol have little inhibitory effect while β-mercaptoethanol inhibits significantly less than disulfhydryl reagents (Table 1A).

TABLE 1

EFFECTS OF VARIOUS COMPOUNDS ON LYSYL OXIDASE ACTIVITY

Inhibitor	% Activity Remaining†						
	Concentration mM						
	0.1	0.2	0.3	0.5	1.0	2.0	3.0
A. Dithiothreitol	88.5			44.3	12.2		11.4
Dithioerythritol	90.0			43.9	17.8		8.1
1,3-dithio-2-propanol	88.1		74.8	44.5	22.0	14.0	
1,2-dithio-3-propanol	93.2		68.3	47.9	25.7	20.3	
Dithioethane	83.8				49.7		25.5
Dithiopropane	90.9	85.9			68.1		27.5
Dithiobutane	92.8	85.3			87.3		58.6
ß-mercaptoethanol	98.0			84.6	79.5		66.3
Monothioglycerol	98.6			102.7	88.1		80.0
Glycerol	113.7			92.5	104.6		110.5
	Concentration mM						
	0.1	0.75	1.0	3.0	5.0		10.0
B. Penicillamine	77.3	54.5	46.2	5.6			
Cysteine	96.1	70.8		15.7			
N,N-dimethyl-ß-mercapto-ethylamine			91.3		89.3		82.6
	Concentration mM						
	0.1	0.5	1.0	3.0	5.0		
C. Dithiothreitol-oxidized	106.6	103.2	101.2	96.7			
Oxidized Lipoic Acid	94.1	105.1	100.4	96.5			
1,3-dithio-2-propanol - partially oxidized	101.4	96.4	66.2				
N-ethyl maleimide	102.5		128.9	121.7			
5,5'-dithiobis(2-nitro-benzoic acid)					102.0		
	Concentration mM						
	1.0		3.0	5.0		10.0	
D. 1,3-diaminopropane	98.7		102.6	72.1		66.4	
Putrescine	86.7		68.9			54.9	
Cadaverine	113.1		96.0			75.7	
n-propylamine	105.8			102.3		112.4	

† Values expressed as percent of a control with no additions.

Since the disulfhydryls tested undergo rapid auto-oxidation with free molecular oxygen in aqueous solutions, the reduced or oxidized form could be the active form of the inhibitor. However, neither oxidized dithiothreitol (see methods) or oxidized lipoic acid (used as the sodium salt at pH 7.7), exhibit inhibition (Table 1C). 1,3-dithio-2-propanol, partially oxidized prior to assay, is a less effective inhibitor (Table 1C).

The sulfhydryl reagents N-ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoic acid) have no inhibitory effects (Table 1C).

Penicillamine and cysteine, sulfhydryl-amino compounds, are both effective inhibitors, whereas the sulfhydryl-tertiary amine N,N-dimethyl-β-mercaptoethyl amine produced less inhibition (Table 1B). Diamines are also inhibitory, although to a lesser extent than the disulfhydryls, whereas the monoamine, n-propylamine, is not (Table 1D).

To test the reversibility of lysyl oxidase inhibition by disulfhydryls or diamines, the enzyme was incubated in the presence of various inhibitor concentrations for 90 minutes at 37°C. Following extensive dialysis against PBS, they were assayed in the usual manner. Inhibition by dithiothreitol, 1,3-dithio-2-propanol and 1,3-diaminopropane is irreversible (Table 2), whereas penicillamine inhibition is readily reversible. Inhibition cannot be reversed by further dialysis of the inhibited enzyme with 10^{-4} M copper, 0.1 M acetic acid, or 5 x 10^{-5} M pyridoxal phosphate.

Lysyl oxidase prepared from either tibia-femur or calvaria, extracted in the presence or absence of phenylmethylsulfonylfluoride and N-ethylmaleimide yields similar results.

DISCUSSION: Lysyl oxidase inhibition requires a bifunctional sulfhydryl, bifunctional amine or a sulfhydryl-amine. Because oxidized disulfhydryls do not show inhibitory effects (Table 1C), it is the reduced form that is inhibitory. Although oxygen levels available to lysyl oxidase decrease in the assay medium during auto-oxidation of disulfhydryls, the observed inhibition is not due to significant oxygen competition but to irreversible interaction of the inhibitor with enzyme.

Dialysis against copper has no effect on inhibited enzyme. Both Harris *et al.* (1) and Siegel and Pinnell (5) have shown that lysyl oxidase activity is retained following removal and subsequent addition of copper. Harris *et al.* (1) also postulated that dithiothreitol may inhibit by either binding to or removing an essential copper ion. However, this mechanism is unlikely since dialysis against copper does not restore activity.

TABLE 2

EFFECT OF DIALYSIS ON INHIBITED ENZYME

Inhibitor	% Activity Remaining† After Dialysis
None	100
Dithiothreitol (3 mM)††	22
1,3-dithio-2-propanol (3 mM)	3
1,2-dithio-3-propanol (3 mM)	1
Penicillamine (5 mM)	96
1,3-diaminopropane (14 mM)	56

† Value expressed as percent of dialyzed control.

†† Incubated at room temperature for 60 minutes.

The inhibition of collagen cross-linking by penicillamine has been reported to be a function of thiazolidine formation with the allysine enzymatic product as well as direct interaction with the enzyme itself (14). Since our assay procedure measures tritium release from the tautomerization of newly formed allysine, the observed inhibition with penicillamine must be occurring at the enzyme level. Nimni (14) has shown that penicillamine and other sulfhydryl-amines react reversibly with aldehydes and pyridoxal phosphate to yield thiazolidine adducts. Similarly, one could speculate that a pencillamine adduct of an enzyme aldehydic group would be reversible. Our experimental results indicate that pencillamine is a reversible inhibitor of lysyl oxidase (Table 2). Furthermore, disulfhydryls react irreversibly with aldehydes to give quantitative yields to their cyclic products (15). The inhibition of lysyl oxidase with disulfhydryls is irreversible (Table 2). Although primary diamines do not readily form cyclic adducts with aldehydes (18), we suggest that cyclic adducts with an enzyme aldehydic group are, in fact, occurring as primary monoamines show no inhibitory effect. The primary diamines are the least effective bifunctional inhibitors tested. We

hypothesize that the enzyme aldehydic group is associated with the recently reported pyridoxine derived cofactor of lysyl oxidase (17,18).

We are currently pursuing this line of investigation.

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