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Modulation of Lysyl Oxidase Substrate Specificity by the Oleate Anion[1] (42077)

THOMAS A. LOSTY, DANIEL S. MILLER, AND BOYD L. O'DELL[2]

Department of Biochemistry, University of Missouri, Columbia, Missouri 65211

Abstract. Extracts of bovine ligamentum nuchae have been assayed for lysyl oxidase activity using as substrates soluble elastin and soluble collagen labeled with tritiated lysine. The assays were performed in the presence and absence of sodium oleate. At 0.8 m*M*, oleate decreased activity with elastin more than 50% and enhanced activity with collagen to approximately 200% that of controls without oleate. The results show that this hydrophobic anion modulates lysyl oxidase specificity in crude extracts and suggests a mechanism for modifying activity in tissues. © 1985 Society for Experimental Biology and Medicine.

Lysyl oxidase is a copper-dependent enzyme which catalyzes the key reaction in the crosslinking of collagen and elastin (1). The crosslinks are formed from peptidyl allysine, α-aminoadipic-δ-semialdehyde, which arises from oxidative deamination of peptidyl lysine residues. The latter reaction, catalyzed by lysyl oxidase, is impaired by copper deficiency (2) and lathyrogens such as β-aminopropionitrile (BAPN) (1).

The enzyme has been purified from chick aorta (2) and cartilage (3, 4) and from bovine aorta (5), ligamentum (6), and cartilage (7) using insoluble chick aorta elastin as the assay substrate. Generally four catalytically active forms of the enzyme can be separated on DEAE-cellulose (4, 7, 8) and all show the same relative activity when assayed with soluble collagen and insoluble elastin substrates (4, 7). However, we have observed different relative activities in fractions separated on a Sephadex G-100 column (9).

The difference in relative activities noted in crude fractions may be due to modulation by other tissue components. Kagan *et al.* (10) have shown that anions, such as sodium dodecyl sulfate, fatty acid salts, and bile salts, inhibit lysyl oxidase activity when measured with an insoluble, elastin-rich substrate. Neutral detergents had no effect while cationic amphiphiles stimulated activity.

In this study the effect of an anion, oleate, on the catalytic oxidation of two soluble substrates has been investigated. In confirmation of the earlier observations, oxidation of elastin was inhibited; however, oxidative deamination of collagen lysine was stimulated by the same concentration of oleate.

Materials and Methods. *Assay.* Lysyl oxidase activity was measured by the rate of release of ^3H from soluble elastin and soluble collagen substrates according to the procedure of Pinnell and Martin (1) except that soluble substrates were used. All components of the 1.0 ml assay were in KR buffer (0.016 *M* $KHPO_4$, pH 7.7, and 0.125 *M* NaCl). The final NaCl concentration was 0.92 *M* (11) and each milliliter contained 0.05 mg each of streptomycin and penicillin G. Tubes, in triplicate, were incubated at 39°C for 4 hr, frozen, the water was distilled under vacuum and the radioactivity of the distillate counted. Blanks contained 1.0 m*M* BAPN. Sodium oleate (Sigma Chemical Co., St. Louis) was dissolved in KR buffer and the concentration calculated on the basis of formula weight, 304. This solution was pipetted directly into the assay tubes when appropriate.

Soluble elastin substrate. Aortas from 18-day chick embryos were incubated in 95% O_2–5% CO_2 with [^3H]lysine for 8 hr at 37°C. Fifty aortas were minced with scissors and placed in 25 ml of Waymouth's medium without lysine but with added glutamine HCl (7 mg), ascorbic acid (1.25 mg), BAPN (1.25

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[2] To whom correspondence should be addressed.

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mg), penicillin G (1.25 mg), streptomycin sulfate (1.25 mg), pepstatin A (5 μg), and [4,5-^{3}H]lysine (250 μCi). Following incubation the cultures were made 0.5 *M* with acetic acid and frozen or immediately homogenized and stirred overnight at 4°C. The homogenate was centrifuged at 12,000*g* and the pellet re-extracted. The combined supernates were treated with alcohols to isolate soluble elastin (12). The solution was adjusted to pH 5.2 and 1.5 volumes of *n*-propanol was added dropwise followed by 2.5 volumes of *n*-butanol. After centrifugation at 16,000*g* the supernate was evaporated to dryness under vacuum and the residue dissolved in KR buffer containing the proteolytic inhibitors, phenylmethylsulfonyl fluoride (10^{-5} *M*), *N*-ethylmaleimide (10^{-5} *M*), EDTA (10^{-4} *M*), and ϵ-aminocaproate (10^{-1} *M*). The solution was then dialyzed against 100 vol of KR buffer containing the inhibitors. This procedure eliminated unbound lysine.

Soluble collagen substrate. Fifty embryonic chick calvaria were incubated as above for 24 hr with 500 μCi of [4,5-^{3}H]lysine and soluble collagen was isolated by the method of Siegel and Martin (13). The soluble collagen was precipitated with 20% NaCl and the pellet taken up in KR buffer containing the proteolytic inhibitors described above. The solution was dialyzed as for the elastin substrate.

Enzyme preparation. Bovine ligamentum nuchae from 18-month animals served as the enzyme source. They were powdered in liquid nitrogen and extracted twice with 4 vol of KR buffer. The insoluble pellet was then homogenized in a buffer containing 0.016 *M* $KHPO_4$ (pH 7.7), 0.015 *M* NaCl, and 4 *M* urea and extracted twice with 5 vol of the buffer. The extract was concentrated to 5% of the original volume on a YM-10 ultrafilter (Amicon, Inc.). The retentate was then dialyzed against KR buffer. Frequently a translucent insoluble precipitate formed during dialysis. This fraction was removed by centrifugation at 44,000*g* and designated "KR insoluble"; the supernate was the "KR soluble" fraction although both fractions were originally solubilized with urea. The KR insoluble fraction was added to assay tubes as a suspension.

Results. These urea solubilized fractions prepared from bovine ligament showed lysyl oxidase activity with soluble collagen as well as soluble elastin substrates. In agreement with the results obtained earlier with insoluble elastin (10), sodium oleate inhibited the catalytic activity with soluble elastin as the substrate. When compared on the basis of tritium counts released, the activity was more than 10-fold greater for the elastin than for the collagen substrate. The KR soluble lysyl oxidase fraction released approximately 1.8% of the tritium in the elastin substrate compared to 0.11% of the collagen counts (Table I). The KR insoluble fraction, which was more active per unit volume, released 6.7% of the elastin counts compared to 0.29% of those in collagen.

TABLE I. INVERSE EFFECTS OF SODIUM OLEATE ON LYSYL OXIDASE ACTIVITY DEPENDING UPON SUBSTRATE

Lysyl oxidase preparation	Elastin substrate[a]		Collagen substrate[b]	
	No oleate	0.8 m*M* oleate	No oleate	0.8 mM oleate
	DPM released		DPM released	
KR soluble fraction	3740 ± 980 (4)[c]	1423 ± 376 (38)[d]	328 ± 59 (3)[c]	774* ± 73 (235)[d]
KR insoluble fraction	13,380 ± 4275 (3)[c]	4554 ± 2040 (34)[d]	870 ± 10 (2)[c]	1590 ± 350 (185)[d]

[a] The soluble elastin substrate contained approximately 200,000 dpm per assay tube.
[b] The soluble collagen substrate contained on average 300,000 dpm per assay tube.
[c] Number of trials involving different substrate and enzyme preparations. The values are means ± standard errors of the means.
[d] The percentage of the mean without oleate.
* As determined by the paired Student *t* test, this value is significantly ($P < 0.05$) different from the control.

Regardless of the potency of the enzyme preparations, 0.8 m*M* oleate inhibited tritium release from the soluble elastin substrate. The KR soluble fraction was inhibited to a level of 38% of that of the control tubes without oleate and the insoluble fraction, 34%. In contrast, with the soluble collagen substrate, oleate stimulated the soluble lysyl oxidase activity 235% and the insoluble activity 185%. It was necessary to show that lysyl oxidase was affected specifically because tissues contain amine oxidases which are not inhibited by BAPN and these could be either inhibited or stimulated by oleate. That this is not the case was demonstrated by the fact that oleate had little or no effect on the BAPN blanks. Furthermore, the oleate effect is not strictly that of a detergent since nonionic detergents, such as Triton X-100, had no effect (data not shown).

Discussion. Lysyl oxidase plays a key role in the crosslinking of two distinctly different connective tissue proteins collagen and elastin. There are at least four different species of the enzyme which can be separated on the basis of charge (4, 7, 8). Soluble collagen and soluble elastin have different isoelectric points and would carry a quite different charge at physiological pH, yet the relative activities of the lysyl oxidase species with elastin and collagen substrates are the same (4, 7). A priori one might predict that there exists a control mechanism which imparts substrate specificity. This paper presents a model of a possible mechanism for this type of control.

Lysyl oxidase appears to exist in a highly polymerized or membrane bound form which can be solubilized by urea or similar agents which break hydrophobic bonds. As shown by Kagan *et al.* (10) ionic detergents modulate lysyl oxidase activity when insoluble elastin serves as substrate. Anionic compounds inhibited the enzyme while cationic detergents enhanced activity. This anionic effect has been confirmed here with soluble elastin. In contrast, oleate stimulates the activity of lysyl oxidase when soluble collagen serves as substrate. Such compounds may act as modulating agents of the enzyme under natural conditions. Crude extracts of tissues, such as bovine aortas and ligaments, can be separated by gel filtration into fractions with different relative activities against collagen and elastin (9). This phenomenon could be explained by the presence of hydrophobic anions in the extracts.

Theoretically oleate could interact with lysyl oxidase and either or both of its substrates. Based on the respective charges at physiological pH and the relative hydrophobicity of these molecules, oleate would be expected to interact readily with elastin but much less so with collagen. Lysyl oxidase probably carries a negative charge and is a hydrophobic protein (10). If oleate reacts with lysyl oxidase at apolar sites, local concentrations of negative charge might well change its conformation and substrate specificity. In the presence of oleate the change in conformation might make the collagen binding site more accessible while excluding elastin from its active site. It is also possible that oleate affects the coacervation of soluble elastin and the formation of fibrils from soluble collagen. While interactions with the substrates could explain the modulating effect of oleate on lysyl oxidase activity, a more direct and simple explanation involves a conformational change which relates to the specificity of substrate binding.

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