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Review

Lysyl oxidase: an oxidative enzyme and effector of cell function

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Abstract. Lysyl oxidase (LOX) oxidizes the side chain of peptidyl lysine converting specific lysine residues to residues of α-aminoadipic-δ-semialdehyde. This posttranslational chemical change permits the covalent crosslinking of the component chains of collagen and those of elastin, thus stabilizing the fibrous deposits of these proteins in the extracellular matrix. Four LOX-like (LOXL) proteins with varying degrees of similarity to LOX have been described, constituting a family of related proteins. LOX is synthesized as a preproprotein which emerges from the cell as proLOX and then is processed to the active enzyme by proteolysis. In addition to elastin and collagen, LOX can oxidize lysine within a variety of cationic proteins, suggesting that its functions extend beyond its role in the stabilization of the extracellular matrix. Indeed, recent findings reveal that LOX and LOXL proteins markedly influence cell behavior including chemotactic responses, proliferation, and shifts between the normal and malignant phenotypes.

Keywords. Lysyl oxidase, protein oxidation, cofactor, chemotaxis, malignancy.

Introduction

The importance of posttranslational modification of proteins has become eminently clear in recent decades. While the primary sequence of proteins underlies the variations in secondary and higher-ordered levels of protein structure, the covalent, postranslational modification of specific residues within the primary sequence of proteins can exert profound effects on the biological function, cellular localization, and metabolic fate of these biopolymers. More than 100 different types of covalent modifications are known including such diverse enzyme-catalyzed reactions as phosphorylation, sulfation, thiol oxidation, methylation, N-acetylation, lipidation, proteolysis, ubiquitylation, glycosylation ADP-ribosylation, hydroxylation, carboxylation, and others. As many as 15 of the 20 amino acids which serve as protein building blocks are peptidyl targets of such modifications [1]. The specificity of these reactions is maintained by two key elements: (i) a consensus recognition sequence in the target protein and (ii) the compartmentalization of the converter enzymes. For example, complex glycosylation, prolyl hydroxylation, and disulfide isomerization take place only in the lumen of the secretory pathway, while others, including proteasome-dependent protein degradation, O-linked N-acetylglucosamine (O-GlcNAc) modification, and ubiquitination, occur in the cytoplasm and in the nuclei. In addition, some converter enzymes are localized in the extracellular space. A corollary of this compartmentalization is that the relocation of a converter enzyme to another subcellular compartment [e.g. lysyl oxidase (LOX) from the extracellular matrix (ECM) to the nuclei] may lead to unusual cellular responses mediated by unusually modified proteins. The movement of converter enzymes (physiologically induced, pathological and others) among subcellular compartments is a relatively unexplored area in cell biology.

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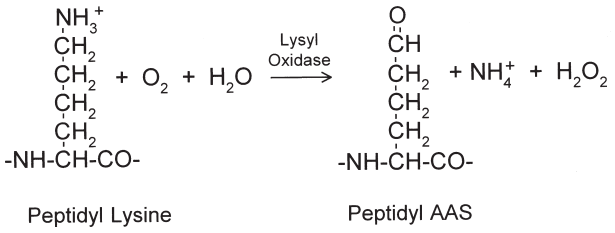

Figure 1. Stoichiometry of the LOX-catalyzed reaction.

As the catalyst of the posttranslational oxidation of peptidyl lysine to the peptidyl aldehyde, α-aminoadipic-δ-semialdehyde (AAS), LOX has long been the subject of considerable interest. Since the first demonstration of the activity of LOX *in vitro* in 1968 [2], this secreted enzyme has been recognized as the catalyst that accounts for the insolubilization and stabilization of its extracellular collagen and elastin substrates. The conversion of the soluble precursors of these ECM proteins to insoluble fibers results from the intra- and interpeptide chain crosslinkages that are spontaneously generated from the peptidyl AAS product of LOX action on these structural macromolecules. The stoichiometry of the LOX-catalyzed oxidative deamination reaction is shown in Figure 1.

The conversion of the Є-amino group of peptidyl lysine to the aldehyde residue significantly changes the chemical microenvironment at the sites of oxidation. Clearly, there is a loss of cationic charge of the substrate lysine residue. The generation of the aldehyde function also introduces a potentially electrophilic carbonyl in place of the normally unreactive methylene carbon of the original lysine residue. Thus, the aldehyde can condense with a neighboring Є-amino group of an unmodified lysine to form the anhydrolysinonorleucine (deLNL) crosslink. Similarly, two of these aldehydic residues can react to form the aldol condensation product (ACP; Fig. 2). Accordingly, both the deLNL and ACP crosslinkages are formed in collagen and elastin. Aldehydes can also participate in the reversible formation of hemiacetals or thiohemiacetals when condensing with oxygen or sulfur anions, respectively, although there is no evidence thus far that such condensations occur between peptidyl AAS and ionized thiolate, tyrosyl, or carboxylate functions in proteins and/or phosphate functions in nucleic acids. Thus, the generation of such peptidyl aldehydes can alter local macromolecular structure as well as the nature of protein-protein or protein-nucleic acid interactions. The oxidation of cellular proteins is predominantly viewed as a non-enzymatic process mediated by free radicals and non-radical oxidants, resulting in the oxidation of many side chain amino acids including lysyl residues. These uncontrolled, chemical oxidations often lead to the increased proteolytic degradation of oxidatively damaged proteins [3–5]. In contrast to non-enzymatic oxidants, the generation of peptidyl AAS by LOX occurs with specificity to enzyme-selected, accessible lysine residues. The

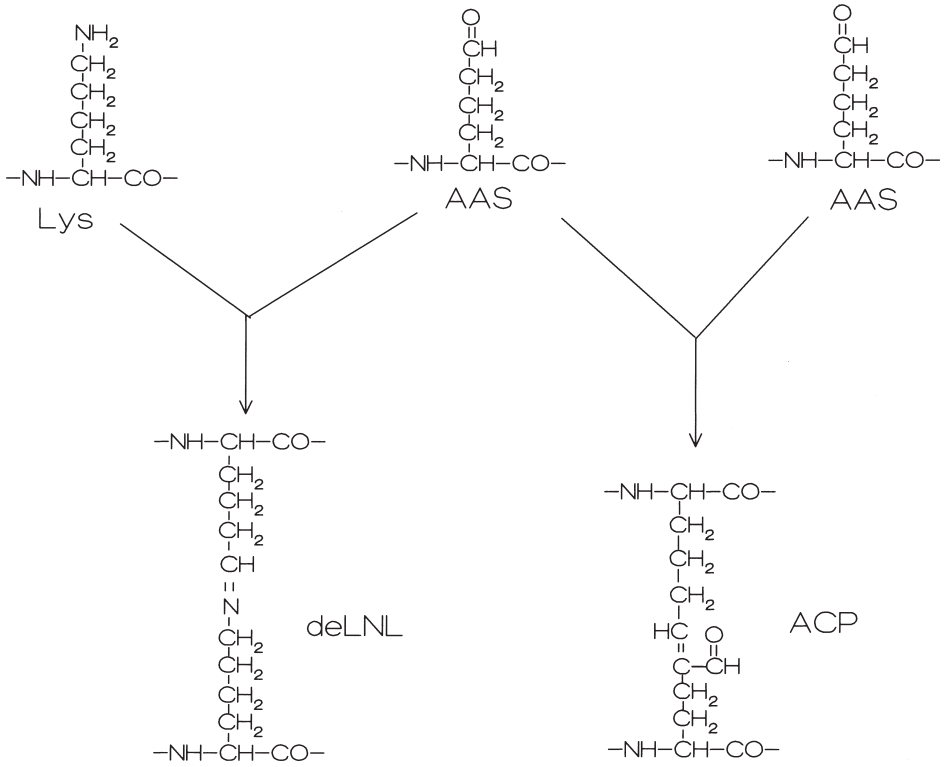

Figure 2. Spontaneous formation of the dehydrolysinonorleucine (deLNL) and aldol condensation product (ACP) crosslinkages from peptidyl AAS and lysine residues.

enzyme-catalyzed oxidation may also be regulated by effectors of the converter enzyme.

Recent investigations have illustrated that the biological role of LOX extends beyond the oxidation of structural proteins of the ECM. Indeed, several reports describe its influence on tissue development, cell proliferation, intracellular signal responses, and cell migration and reveal that it can act as an antagonist or a protagonist of malignant processes. It has also been shown that the secreted enzyme can reenter cells and then concentrate within cell nuclei [6]. In addition to LOX, at least four different LOX-like (LOXL) proteins, (LOXL1, LOXL2, LOXL3, and LOXL4) have now been described [7], thus establishing a gene family containing at least five members. The predicted sequences of the full-length gene products of each of these proteins contain an N-terminal signal peptide domain followed by a region of variable sequence and length followed by a C-terminal region which demonstrates significant similarity to the catalytic domain of LOX. Following intracellular signal peptide cleavage, LOX and LOXL1 are secreted as proproteins (proLOX and proLOXL1, respectively) which are proteolytically cleaved to release the free catalysts and the N-terminal propeptide regions which immediately precede the catalytic domains [6, 7]. Whether LOXL2, 3, and 4 are also secreted as proenzymes from which propeptides are subsequently proteolytically released remains to be established. The regions immediately following the signal peptide sequences of LOXL2, LOXL3 and LOXL4 each contain four scavenger receptor cysteine-rich (SRCR) domains and, as a result, likely exist as stable, distinctly folded protein structures. In contrast, the propeptides of LOX and LOXL1 do not contain cysteine residues, although 10 cysteine residues are found within the catalytic domains of each of these five enzymes. Based upon these differences, LOXL2, 3, and 4 appear to be related to each other as a LOX subfamily while the similarities between LOX and LOXL1 suggest that these exist as a separate LOX subfamily. The functional significance of the SRCR domains in the LOXL enzymes remains to be elucidated. The present review will emphasize the biosynthesis, physical-chemical and enzymatic properties as well as recently discovered biological activities of LOX with limited reference to specific LOXL enzymes in selected instances.

Biosynthesis and proteolytic processing

The cloning and sequencing of rat LOX cDNA revealed that the molecular weight of the full-length biosynthetic product was 46 kDa [8], thus differing from the 32-kDa mass of the mature, catalytically functional protein isolated from bovine aorta [9]. LOX cDNA encodes a consensus signal peptide sequence at the N-terminal region, consistent with the secreted fate of this enzyme. Further studies [10] revealed that this enzyme emerges from the cell as an N-glycosylated, 50-kDa pro-protein (proLOX). The propeptide domain contains two consensus sites for this posttranslational N-glycosylation. The mature enzyme retains the 10 cysteine residues of proLOX, all of which exist in disulfide linkage [11]. The mature enzyme also contains histidine-rich sequences which can function as copper-binding sites for this copper-dependent catalyst. The distributions of cysteines in the propeptide regions of LOXL2, 3, and 4 are consistent with SRCR domains found in cell membrane-associated proteins that function in cell adhesion [12]. For example, the WS914, 87-kDa protein predicted from a cDNA clone derived from senescent fibroblasts and now identified as LOXL2 has a 48% homology within its putative catalytic domain with the catalytic domains of both LOX and LOXL1. WS9-14 was abundantly expressed in tumor cell lines that attach to culture dishes but not in cell lines that grow in suspension, suggesting that this gene encodes an extracellular protein involved in cell adhesion [13]. Similarly, Giampuzzi et al. [14] have noted that downregulation of LOX induced anchorage-independent growth in NRK-49F cells.

The secreted proLOX molecule is catalytically quiescent but is activated by proteolytic cleavage between Gly162 and Asp163 (rat LOX sequence) by procollagen C-proteinase [15, 16]. It was subsequently found that other extracellular proteases, including mammalian tolloid and tolloid-like-1 and 2 proteases, also cleaved proLOX at the correct physiological site but at lower efficiency [17]. The redundancy of proteases involved in the maturation and activation of LOX underscores the importance of LOX activity in ECM homeostasis. Since purified LOX does not act upon the procollagen precursor of the collagen molecule, it is of interest that the fibrillar collagens are secreted as procollagen species containing N- and C-terminal propeptide domains on each of their three component α chain polypeptides. The form of type I collagen that is susceptible to oxidation by LOX requires both the prior removal of these N- and C-procollagen propeptides by N- and C-procollagen proteinases as well as the subsequent, spontaneous aggregation of the mature collagen molecules into microfibrils [18]. Thus, the same protease that processes procollagen to the collagen substrate of LOX is also the more efficient converter of the proLOX precursor of LOX to the catalytically functional, mature enzyme, representing a highly integrated mechanism for the formation of crosslinked collagen fibril production. Purified LOX also readily oxidizes purified tropoelastin, the secreted precursor of insoluble elastin [19], ultimately leading to insoluble elastin deposits, as are found in connective tissues.

Fogelgren et al. [20] have recently reported that the proteolytic activation of proLOX can occur at the surface of human fibroblasts in a bound complex with cellular fi-

bronectin (FN). The association between fibronectin and proLOX was implied by the results of a yeast two-hybrid system utilizing the mature 30-kDa LOX as the bait molecule to identify expressed cellular protein(s) which have binding affinity for this protein. Additional experiments in this study demonstrated that tight complexes formed *in vitro* between purified bovine LOX and FN that retained catalytic function against synthetic amine substrates. Moreover, the proteolytic processing of proLOX to LOX was largely reduced in mouse embryo fibroblasts in which FN biosynthesis was genetically knocked out. A matter that remains to be resolved with the findings of this study was the report that purified recombinant BMP-1 (procollagen C-proteinase), as well as the tolloid proteinases, mTLD, mTLL-1, and mTLL-2, each functioned as proteolytic activators of proLOX *in vitro* in the absence of other proteins [17], thus indicating that the cleavage site of proLOX was accessible under these simple conditions.

Catalytic mechanism

The LOX-catalyzed reaction falls into the oxidation-reduction category of enzymes. Redox catalysts must include a mechanism for the removal of electrons (oxidation) from the atomic target within the substrate and provide for a final acceptor of electrons (reduction) to restore the enzyme to its original state. Many redox enzymes bind cofactors such as nicotinamide adenine dinucleotide (NAD^+) or flavin adenine mononucleotides (FMNs) or dinucleotides (FADs) which act as electron sinks accepting the electrons removed from the substrate. These cofactors also serve as the final depository of the electrons removed in oxidation and emerge from their enzymes as reduced species, i.e. NADH and $FADH_2$. Oxidases, including LOX, have chosen a different path. LOX contains two cofactors essential to its catalytic function. This enzyme contains one tightly bound copper ion (Cu^{2+}) and a unique, covalently integrated organic cofactor identified as lysine tyrosylquinone (LTQ) [21]. The structure of LTQ is shown in Figure 3. LTQ is autocatalytically derived from a specific tyrosine and a specific lysine residue within the nascent enzyme. A pathway for the formation of LTQ has been proposed [21–23] according to which the enzyme-bound copper atom catalyzes the oxidation of the tyrosine residue (Tyr345 in the rat enzyme) to peptidyl dihydroxyphenylalanine quinone followed by covalent addition to the quinone ring of the Є-amino group of the attacking lysine residue (rat LOX, Lys314). Oxidation of the resulting peptidyl lysine tyrosylquinol to the functional LTQ cofactor would be accomplished by passage of electrons to molecular oxygen, the final acceptor of electrons in this enzyme. LTQ plays a critical cofactor role as a transient electron sink in the

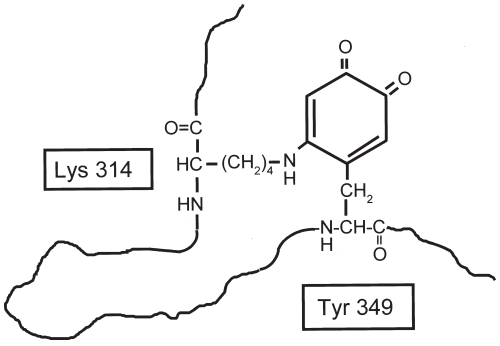

Figure 3. The lysyl tyrosine quinone (LTQ) cofactor of LOX.

catalytic mechanism of LOX and is readily reduced by acceptance of two electrons from the Є-carbon of the substrate lysine residue.

The tyrosine-derived trihydroxyphenylanine quinone (TPQ) cofactor of plasma amine oxidase and that of cellular semicarbazide amine oxidase are also autocatalytically generated from active-site tyrosine residues in these copper-dependent enzymes [22, 23]. The aromatic ring of the TPQ residue is not covalently attached to any other residue in these proteins. Moreover, the specificities of these catalysts appear to be restricted to simple, non-peptidyl amines. The covalently linked lysyl component of LTQ of LOX (Fig. 3) might then play an important role in the preference of LOX for peptidyl lysine substrates in cationic protein microenvironments both as a result of its donation of anionic charge to the active site and, possibly, by restricting the rotation of the LTQ ring in a manner that properly orients the carbonyl cofactor with respect to peptidyl lysine substrates. The quinone ring of TPQ is not covalently restricted from rotation, although non-covalent interactions of the TPQ cofactor with neighboring amino acid residues could help to restrict the orientation of this cofactor within the active sites of these other amine oxidases.

A variety of chemical, kinetic, and spectroscopic investigations have detailed a likely mechanism of action of LOX [24–27]. As shown in Figure 4, the Є-amino group of the substrate lysine residue condenses with one of the two carbonyls of LTQ ① in Fig. 4). The resulting Schiff base linkage coupled with general base-assisted abstraction of a hydrogen on carbon 6 of the substrate lysine residue permits the flow of two electrons into LTQ ② generating the reduced peptidyl lysine tyrosyl aminoquinol ③. Hydrolysis of the resulting imine linkage releases the peptidyl aldehyde (AAS) product from the reduced cofactor ③–④. Subsequent reoxidation of the peptidyl aminoquinol ④ occurs by transfer of two electrons to molecular oxygen, forming and releasing hydrogen peroxide ④–⑤. The quinoneimine so formed is then hydrolyzed to regenerate LTQ ① and to release the free ammonia product of the reaction.

Substrate specificity

An early supposition in LOX research was that the soluble precursors and immature fibrillar forms of elastin and collagen, respectively, were the unique biological targets of this enzyme The possibility that this view was too limited arose when *in vitro* assays demonstrated that the purified enzyme readily oxidized a number of basic globular proteins with pI values ≥8.0, among which H1 histone was included, but did not oxidize globular proteins with pI values less than 8 [28]. Although bovine serum albumin (BSA) is an acidic protein and, therefore, not oxidized by LOX, chemical conversion of its glutamate and aspartate residues to their corresponding amides (glutamine and asparagine) resulted in a highly basic protein readily oxidized by LOX [28]. These and related data led to the conclusion that the electrostatic potential between LOX, an acidic protein, and its basic protein substrates was essential to productive catalysis. Characterization of the substrate specificity of LOX and analyses of its catalytic mechanism were greatly simplified by the finding that LOX can also oxidize non-peptidyl amine substrates such as n-butylamine and 1,5-diaminopentane, thus facilitating the development of a fluorescence-based assay for LOX-dependent H_2O_2 production [29] which has been recently modified [30].

The effects of ionic charge on the potential of proteins as substrates seemed likely to reflect the distribution of anionic residues within LOX. Although information about the three-dimensional structure of mammalian LOX is not available, inspection of the amino acid sequence of rat and human LOX species revealed that anionic residues are distributed in a manner that likely provides localized density of anionic charge in the immediate vicinity of their active sites [6]. The sequence region within which the tyrosine progenitor of LTQ is found, as well as that in which the lysine residue (Lys314) that becomes part of LTQ occurs, are enriched in anionic residues. Since these two regions of LOX become covalently crosslinked to each other as the LTQ cofactor is generated, both of these regions would cooperatively provide an abundance of negatively charged sites in the microenvironment of the active site, as shown in Figure 5. It is likely that such an arrangement underlies the strong preference of LOX for cationic protein substrates.

It had been noticed some time ago that the sequences surrounding susceptible lysines in fibrillar collagens are quite different from those in the vicinity of susceptible lysines in tropoelastin [31]. Prior to the oxidation of its lysine residues, tropoelastin is a cationic molecule seemingly appropriate to the electrostatic features of the specificity of LOX. In contrast, the sequences surrounding the susceptible lysines in collagen are in hydrophilic sequences containing anionic residues. For example, the lysine residue within the Asp-Glu-Lys-Ser sequence which occurs at the N-terminal region of the α1(I) collagen chain within the mature type I collagen molecule is oxidized by LOX *in vivo*. In contrast, a collagen-like synthetic peptide which contains this sequence was inert as a LOX substrate in assays *in vitro* although oxidation occurred if the Asp residue within this sequence was replaced with a Gly residue [32]. Coupled with the observa-

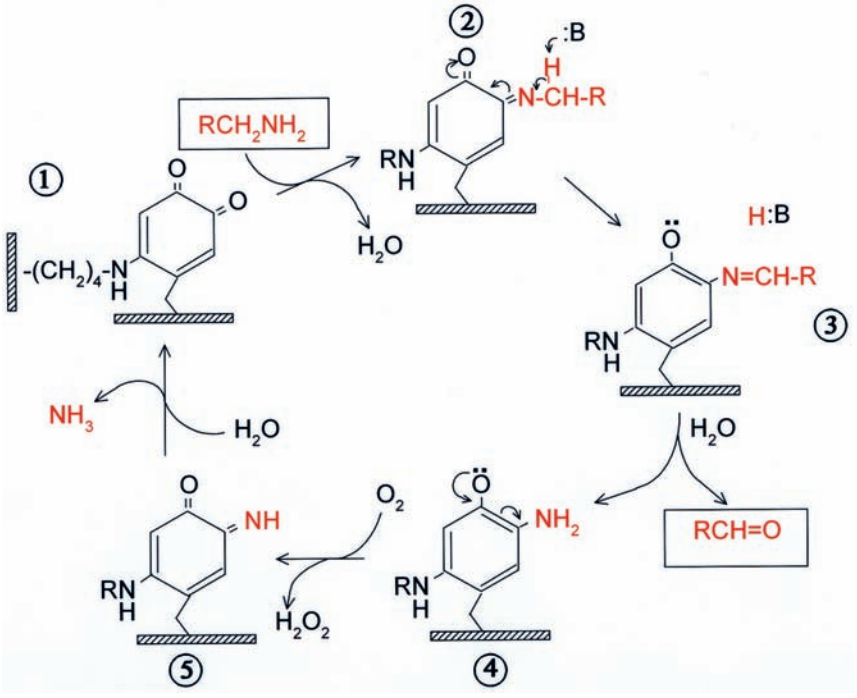

Figure 4. Mechanism of action of LOX.

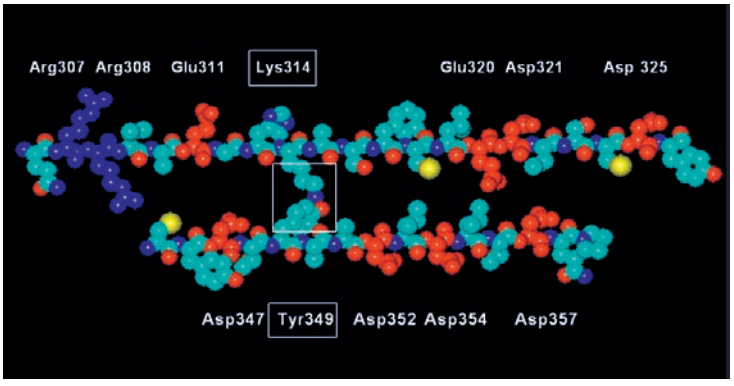

Figure 5. Space-filling models of the sequences adjacent to Lys314 and Tyr349 of rat LOX that become linked to each other as the LTQ cofactor is formed. The square between the two peptides defines the site of LTQ formation. Residues in red are anionic amino acids while two cationic arginine residues are shown in dark blue. The secondary structural features of these peptide chains are unknown.

tion that collagen molecules must condense into microfibrillar arrays prior to their oxidation by LOX [18], these results led to the hypothesis that the unfavorable negative charge contributed by this Asp residue can be neutralized by a specific cationic site in the neighboring collagen molecule within the quarter-staggered microfibril, thus allowing lysine oxidation by LOX. Molecular models of collagen molecules within the microfibrillar array supported this view of this enabling interaction [32].

Along with a report documenting that LOXL1 forms a complex with elastin fibers that appears to be essential for elastin fiber homeostasis [33], Thomassin et al. [34] have reported that the propeptide regions of recombinant proenzymes of LOX and LOXL1 mediate the binding of these enzymes to soluble precursor and fibrous forms of elastin in cultures of transfected RFL-6 fibroblasts. These studies lead to the conclusion that the binding of the LOX proenzymes to elastin substrates was essential for the oxidation of lysine in elastin by the activated LOX and LOXL1. Recombinant mature LOX encoded by a construct from which the propeptide region had been deleted was not secreted by the RFL-6 fibroblasts, thus precluding conclusions about its binding affinity for extracellular elastin. The cell culture results differ from those of cell-free studies which have demonstrated that the purified, mature LOX catalyst readily interacts with, oxidizes, and insolubilizes homogenous, recombinant tropoelastin in the absence of the LOX propeptide or any other proteins in assays *in vitro* [19]. As previously noted, it has been reported that proLOX must bind to FN to be proteolytically activated to the functional catalyst [20]. According to that requirement, the expected order of events would be (i) proteolytic activation of proLOX in a bound complex with FN followed by (ii) migration of the newly released mature catalyst to its elastin substrate, thus bypassing the requirement for the binding of the proenzyme to elastin. Noting the prominent cationic charge density of tropoelastin, especially at its C terminus [35], and that of the propeptide domain of LOX [6], one might also expect that the interaction of the LOX propeptide with tropoelastin, while certainly possible, would not be energetically highly favored. It clearly will be important to continue probing into the roles of FN and of the LOX propeptide in the processing and substrate specificity of LOX.

Expression of recombinant LOX

Recombinant LOX was first expressed at the analytical level from cDNA constructs encoding full-length or a truncated form of the murine enzyme [36]. The truncated cDNA construct encoded the expression of the last 30 residues of the propeptide domain through the C-terminal residue of the full-length rat preproenzyme sequence. Both the full-length and truncated proteins were expressed and secreted as a protein exhibiting normal LOX catalytic function and inhibitor profiles [36]. These experiments supported the view that the bulk of the propeptide is not required for the folding of the nascent product into the catalytically functional conformation. More recently, Sommer and colleagues [37] reported that secretion of catalytically functional, recombinant LOX specifically required the presence of the N-glycosylation site of the propeptide domain that had been retained in the truncated form expressed in the earlier study [36]. This requirement is consistent with the need for protein-linked carbohydrate units for the proper folding of nascent secretory proteins by the quality control system in the lumen of the endoplasmic reticulum [38].

Quantities (1–10 mg) of the 32-kDa mature enzyme have been purified from 300 to 1000 g of bovine aorta. This procedure adapts to the lack of solubility of connective tissue LOX in phosphate-buffered saline (PBS) by solubilizing the enzyme from the PBS-extracted aortic pellet with buffered 4–6 M urea [39, 40]. The relatively poor solubility of this catalyst in simple aqueous buffers is also made evident by the appearance of amorphous precipitate in moderately concentrated solutions of the protein

in urea-free buffer, reflecting the tendency of the enzyme to form multimers in the absence of urea [39]. These features of the enzyme have frustrated efforts to crystallize the protein for analysis of its structure. It is promising that Jung et al. [41] have successfully expressed and purified recombinant, catalytically active LOX and LOXL proteins from *Escherichia coli* transformants at levels of 10–50 mg per liter of bacterial culture. Althought oxidative activities of the expressed enzymes against protein substrates were not tested in this report, the recombinant enzymes were fullly inhibited by 10- to 100-fold molar excesses of the active site-directed agent, *β*-aminopropionitrile (BAPN), when assayed against benzylamine as substrate, consistent with the properties of LOX purified from connective tissues. The extraction of the functional recombinant LOX from the inclusion bodies did require the supplementation of buffers with 8 M urea, suggestive of solubility issues similar to that of the bovine aorta enzyme [41]. The availability of relatively large quantities of this recombinant enzyme should permit extensive studies on its solubility and its potential for crystallization, possibly leading to the determination of the three-dimensional structures of these proteins.

Modulation of cell phenotypes by LOX

The important role played by LOX at least in its function as the initiator of crosslinking of elastin and collagen and in the repair of these ECM molecules during aging and disease [42, 43] predicts that inactivation of the LOX gene would have debilitating consequences. Not surprisingly, $LOX^{-/-}$ mice came to term but died at the end of gestation or as neonates. Defects in the cardiovascular system, including aortic aneurisms and severe elastic fiber defects, characterized the developed pathology [44], consistent with the role of LOX in oxidation and crosslinking and possibly as an element of the scaffold to ensure spatially defined deposition of elastin.

LOX-dependent chemotaxis. Purified 32-kDa LOX was found to act as a potent chemoattractant for human peripheral blood mononuclear cells, with a 237% increase in migration over the enzyme-free or catalytically inhibited LOX controls seen at 10^{-9} M LOX [45]. Extension of these studies revealed that vascular smooth muscle cells (VSMCs) are also strongly chemotactically responsive to the catalytically active enzyme but not if the enzyme was inactivated nor if it was added to the chemotaxis chambers in the presence of catalase. Thus, the H_2O_2 product of the LOX-catalyzed reaction appeared to mediate the chemotactic response [46]. The lack of a chemotactic effect toward conditioned VSMC culture medium which had been preincubated with added LOX suggested that the chemotactic response was not due to the reaction of LOX with secreted proteins but more likely required direct access of the enzyme to cell-associated substrate(s). The addition of active but not catalytically inhibited LOX to VSMCs elevated levels of intracellular H_2O_2, enhanced stress fiber formation, and increased focal adhesion assembly, consistent with the induction of the chemotactic response. Each of these changes was prevented by the prior addition of catalase, again indicating the essential participation of the hydrogen peroxide product of LOX catalysis [46].

Kirschman and colleagues subsequently noted that LOX mRNA is upregulated in invasive breast cancer cells and that invasion of these cells in an *in vitro* system was facilitated by active but not inactive LOX [47]. A further report from this laboratory [48] demonstrated that this LOX-dependent chemotactic response of breast cancer cells was elicited by the H_2O_2 product of LOX acting on unidentified substrate(s). Thus, the LOX- and peroxide-dependent chemotactic mechanism initially detailed in VSMCs [46] appears to be critically involved in the invasive cellular response of breast cancer tissue as well. Most recently, Payne et al. [49] have shown that actin stress fiber formation and Rho activity in breast cancer cell lines are increased through the p130(Cas)/Crk/DOCK180 signaling complex by inhibition of LOX in these cells [49]. The upregulation of these parameters is typically associated with the non-motile phenotype. These results suggest that depression of LOX activity could limit the invasiveness of breast cancer cells. Other observations have noted that LOXL1 is also highly expressed in metastatic breast cancer cells and is significantly correlated with increased tumor malignancy and increased fibrotic foci [50]. The highly invasive nature of cells expressing LOXL1 [50] suggests that this member of the LOX family may also exert a chemotactic effect on the transformed cells. Similar considerations may apply to the observation that elevated levels of LOX occuring in head and neck tumor cells under hypoxic conditions appear to be essential for the hypoxia-induced metastatic response of these cells [51]. While the elevation of LOX under hypoxic conditions is consistent with an earlier report [52], the authors of the current study observed that all of the various human cancer cells assessed showed increased invasion under hypoxic or anoxic conditions and that this increased invasiveness was prevented by treatment of the cells with BAPN, LOX antisense oligonucleotides, LOX antibody or shRNA expression but not with LOX sense oligonucleotides [51]. The inhibitory effect of BAPN on the invasiveness of these cells suggests, once again, that the movement of these cells might be stimulated by the H_2O_2 produced by LOX catalysis, although this hypothesis was not tested in this study.

Oncogenic cellular transformation Interest in the ability of LOX to affect the cell phenotype began with the discovery of a newly found *ras* recission gene (*rrg*). The

rrg transcript was highly expressed in normal NIH 3T3 fibroblasts, markedly decreased in *ras*-transfected, tumorigenic derivatives (RS485) of these cells, and reexpressed at high levels in PR4, non-oncogenic revertants of the RS485 cells. Remarkably, cloning and sequencing of mouse *rrg* cDNA revealed its nearly complete (>96%) identity with the cDNA sequence of rat LOX [53, 54]. This inverse relationship between the oncogenic phenotype and LOX expression has been observed in other instances as well [55–58]. Investigation of the transcriptional regulation of LOX in transformed cells derived from gastric cancers revealed that the LOX gene becomes inactivated by methylation and by loss of heterozygosity. Cancer cell lines with complete methylation of LOX reexpressed elevated levels of LOX when treated with the demethylating reagent, 5-azadC [59]. Loss of heterozygosity as well as LOX gene mutations have also been identified in colorectal tumor cells [60]. Trackman and colleagues [61] reported that blocking of basic fibroblast growth factor (bFGF) in RS485 cells either by the use of anti-bFGF or by exposure of cells to suramin reversibly increased LOX expression tenfold in these transformed cells, suggesting that the typically reduced expression of LOX in transformed cells [62] involves autocrine growth factor pathways. The *ras*-LOX connection has proved relevant to the maturation of *Xenopus laevis* oocytes, since microinjected LOX blocked the inducing effects of oncogenic Ha-*ras* and progesterone on embryonic development [63]. The course of differentiation of developing *X. laevis* embryos did not appear to be altered by incubating embryos with BAPN, although collagen fiber formation, as expected, was compromised by this inhibitor of LOX activity [64]. LOX or LOX-like enzymes also occur in *Drosophila* in which both Dmloxl-1 and Dmloxl-2 have been identified [23, 65]. Contrasting with the effects noted in *Xenopus*, the development of *Drosophila* larvae was delayed by exposure to BAPN which also resulted in a shift in sex ratios. Moreover, overexpression of Dmloxl1 in the w^{m4} variegating strain resulted in increased production of diopterin [65]. This study thus implicates the catalytic activity of LOX in developmental and gene-regulating effects seen in this species.

Noting that *ras* mediates transformation partially by its activation of the NF-κB transcription factor, Jeay et al. [66] have reported that ectopic expression of LOX in *ras*-transformed NIH 3T3 cells resulted in decreased NF-κB transcriptional activity and decreased binding of this transcription factor to the upstream NF-κB-binding element of the *c-myc* promoter. Prior to its action as a transcription factor within the nucleus, NF-κB is sequestered in the cytosol as a complex with IκB inhibitory proteins. Phosphorylation of IκB by an IκB kinase complex initiates ubiquitination and then rapid degradation of the IκB component of the cytosolic NF-κB:IκB complex, thus allowing the translocation of NF-κB into the nucleus. Evidence was obtained that LOX expression decreased both the turnover rate of IκBα and the phosphorylating activities of the two IκB kinases, IKKα and IKKβ, thus inhibiting nuclear uptake of NF-κB. Exploration by these authors of additional signal transduction components through which *ras* induces NF-κB revealed that LOX expression strongly downregulated both PI3K and Akt kinases and partially inhibited MEK kinase activity. These studies led to the conclusion that the antioncogenic effects of LOX on *ras*-mediated transformation result from LOX-dependent inhibition of signaling pathways leading to activation of NF-κB [66]. In a continuation of these studies, it was reported that the induction of the reversion of *ras*-transformed NIH 3T3 cells to the non-oncogenic phenotype is due to the propeptide domain and not to the catalytic LOX domain [67, 68]. Thus, reversion was inhibited in cells grown on culture surfaces coated with the isolated LOX propeptide but was not inhibited by BAPN or in cells grown on surfaces coated with the catalytic domain, indicating that LOX catalysis was not involved in the change to the non-oncogenic phenotype. Since the propeptide fragment was insolubilized on well surfaces, one might surmise that the site of action of the propeptide was extracellular, possibly involving specific binding of this fragment to receptor-like sites on the cell membrane, although this remains to be determined. Corresponding results were obtained when quantifying the growth of these cells in soft agar to which these fragments were added. It was also observed that *ras*-induced NF-κB activity was inhibited in cells expressing the recombinant signal peptide-propeptide fragment of preproLOX. This study clearly points to a unique and important biological role for the LOX propeptide.

Recent evidence has also pointed to roles for LOXL2 and LOXL3, members of the lysyl oxidase gene family, in the progression of carcinoma. Snail is a transcription factor that controls epithelial-mesenchymal transition in the progression of carcinoma by repressing the expression of E-cadherin and other epithelial genes. Cadherin mediates cell-cell adhesion and has been proposed to influence actin polymerization through its interaction with β-catenin [14, 56].The stability and cellular localization of Snail are subject to control by phosphorylation catalyzed by glycogen synthase kinase-3 (GSK3) and by subsequent ubiquitination. Phosphorylation of Snail permits its nuclear export and its degradation. Yeast two-hybrid screening and selective inhibition of LOXL2 by RNA interference yielded evidence that Snail interacts with LOXL2 and LOXL3. The members of this protein complex cooperatively downregulated E-cadherin expression and attenuated GSK3-dependent Snail degradation. These results point to a unique mechanism of regulation of carcinoma progression involving LOXL proteins [69, 70]. Giampuzzi et al. [71] reported the oncogenic transformation of normal rat kidney fibroblasts (NRK-49F) by transfecting

with antisense LOX cDNA [71]. β-Catenin was upregulated as was cyclinD1 while the *ras* oncogene became activated in response to the downregulation of LOX in these cells. These studies point to alterations in cell-cell adhesion as an important mode of transformation of cells by repression of LOX and/or LOXL proteins. Both LOXL2 and LOX have also been seen to be down-regulated in squamous cell carcinoma of the head and neck [72]. In contrast, LOXL4 mRNA was overexpressed in head and neck squamous cell carcinomas [73].

As noted here, the reports documenting changes in cellular phenotypes in response to LOX or LOXL enzymes vary with respect to the requirement for LOX catalysis in the effects seen. In some instances, the effects are muted or prevented by exposure to the LOX inhibitor, BAPN, while no apparent effects of BAPN are seen in other cases. The data concerned with the effects of LOX on *ras*-mediated transformation are consistent with the enzyme inhibiting the initiation and/or progress of the carcinogenic phenotype in a manner that appears to be independent of the catalytic function of LOX. As noted above, LOX exerts a procarcinogenic influence in the case of breast cancer cells by stimulating their migration and thus their invasion of adjacent tissue. In this case, catalysis by LOX is necessary, since the chemotactic mediator is the hydrogen peroxide product of the LOX catalyzed reaction. As another example, bFGF (pI > 9) is readily oxidized by LOX *in vitro*, resulting in oxidization of lysine residues and formation of covalent crosslinkages between bFGF monomers [74]. Specific lysine residues of bFGF are involved in its function and in its binding to cell membranes [75]. NIH 3T3 IgBNM 6–1 cells overexpress bFGF thus transforming these cells to a tumorigenic state. The addition of nanomolar concentrations of purified LOX markedly reduced the proliferative rates of these cells in a BAPN-inhibitable manner. Marked inhibition of proliferation also occurred if BAPN was added to these cells in the absence of exogenous LOX, consistent with a role for endogenous LOX activity in the modulation of proliferation. Stimulation of phosphorylation of the MAP kinases, ERK1 and ERK2, induced by bFGF was significantly reduced by the addition of LOX [74]. This study illustrates a different mechanism of suppression of oncogenesis which relies upon LOX-catalyzed inactivation of a cationic growth factor. Simlarly, Giampuzzi et al. [76] have reported that the promoter activity of the type III collagen gene was increased 12-fold in COS-7 cells due to ectopic overexpression of the mature form of LOX and that this effect was abolished by incubation of the cells with BAPN. Evidence was also presented in this report that the overexpression of LOX increased the specific binding of Ku antigen to the collagen promoter.

Overall, the demonstrated preference of LOX for protein substrates that are cationic in nature and have accessible lysine residues raises the possibility that there may be several intra- and extracellular protein substrates for LOX, assuming the accessibility of this enzyme and its substrate in each case. An uncertainty that arises in instances in which phenotypic changes are attributed to LOX catalysis concerns the fact that alterations in the ECM have been shown to initiate such cellular responses [77, 78]. Thus, the inhibition of LOX activity at least results in deficient crosslinkage formation in its extracellular collagen and elastin substrates and, consequently, to altered cell-matrix interactions which could contribute to intracellular events which have been attributed to LOX. On the other hand, some cases which appear to be independent of LOX catalysis might involve catalytically productive interactions of LOX with pericellular and/or intracellular proteins which are sufficiently stable to prevent the access of BAPN to the active site of the enzyme.

Extracellular and intracellular localization of LOX

Immunogold electron microscopy, immunofluorescence microscopy and Northern blot analyses have localized lysyl oxidase in the extracellular compartment of several tissues, including skin, aorta, heart, lung, liver, and cartilage [8, 79, 80]. Li et al. [81] have documented the presence of mature (32-kDa) LOX within nuclei of VSMCs and fibroblasts by confocal immunomicroscopy and by Western blot analyses of nuclear extracts. The nuclear enzyme appeared to retain its catalytic activity. Adding purified, fluorescently labeled 32-kDa LOX to vascular smooth muscle cells or NIH 3T3 fibroblasts in culture revealed that the exogenous enzyme rapidly entered the cells and then concentrated within the nuclei within 4 h of the addition of the enzyme [82]. The passage of the enzyme into the cells and into nuclei was not inhibited by BAPN nor competed against by adding 29-kDa carbonic anhydrase together with the fluorescently tagged enzyme, although uptake of the labeled LOX was competed against by unlabeled 32-kDa LOX, pointing toward specificity in the mechanism of cellular uptake. LOX does not appear to contain a classical nuclear localization signal (NLS), although localized regions of cationic sequences exist within its primary structure. Ongoing studies in the authors' laboratory have explored for possible NLS loci in LOX by site-directed mutagenesis of selected arginines and the few lysine residues in the enzyme. None of several single or double mutations have prevented the nuclear localization of the enzyme, raising the possibility that nuclear uptake of LOX may require assistance of an intracellular chaperone. The observation by Hayashi et al. [80] that both LOX and LOXL are seen in the nuclei of cells in several different tissues emphasizes the yet unsolved but important issue of the putative function of nuclear LOX. It certainly is relevant to note that histone H1

has long been known to be a substrate for LOX *in vitro* [83], consistent with its abundance of lysine residues and its net cationic character. Moreover, amino acid analyses of H1 samples that had been incubated in the absence of LOX contained endogenous, low levels of crosslinkages derived from oxidized lysine suggestive of intracellular peptidyl lysine oxidation [83]. The potential of histone H1 to be oxidized by LOX has been recently confirmed while histone H2 has also been shown to be a substrate of this enzyme *in vitro* [84]. In this regard, histone H1 has been related to the degree of compaction of chromatin and appears to influence expression of selected genes [85, 86]. Since histone H1 electrostatically interacts both with the DNA bridge linking consecutive nucleosomes and with adjacent DNA wound about the nucleosome core, the loss of Є-amino groups resulting from oxidative deamination induced either by LOX or non-enzymatic oxidants would be expected to significantly alter DNA-histone interactions between cationic amino groups of histone lysine residues and anionic phosphoanions of DNA, in analogy to the effects of acetylation of lysine in histones. Disruption of these electrostatic bonds could change the availability of promoter elements in DNA to transcription factors and/or alter the degree of condensation of nuclear chromatin. Indeed, the introduction of antisense LOX cDNA into non-tumorigenic revertants of *ras*-transformed RS485 cells visibly altered chromatin texture to the more compacted state of the original RS485 cells [87]. Clearly, further investigation is warranted to assess the effects of nuclear LOX on the changes in cell phenotypes seen in the several instances cited here.

Prospects

As reviewed here, it is clear that LOX and LOXL proteins are proving to be multifunctional enzymes with important consequences for normal and disease-related cellular functions. The current literature provides new insights into intermolecular interactions and cellular sites and processes that involve these enzymes. The effects of LOX and LOXL enzymes in cellular adhesion, migration, proliferation and malignancy are striking and widen our understanding of these biological phenomena.

While significant progress has been made in this area of research, molecular details of the mechanisms involved in many of these cellular responses to LOX remain to be elucidated. In particular, the role(s) and specific substrate targets of LOX catalysis in the phenotypic responses are largely unsolved aspects of this area of research. As noted, the response to LOX appears to require catalysis in some instances while the response is resistant to BAPN in others. In either case, it will be important to identify cellular binding partners of LOX and to analyze for the presence or absence of product(s) of LOX catalysis. In cases where LOX catalysis may be involved, LOX-dependent cellular responses might reflect not only the enzymatic conversion of cationic lysine side chains to non-ionic aldehyde residues but also the resultant formation of intra- and intermolecular crosslinkages derived from peptidyl AAS. The latter possibility would be evidenced by the appearance of polymeric forms of its protein substrate(s).

Additional challenges include the resolution of the relative contributions of LOX and of the individual LOXL enzymes to the various cellular responses which have been observed. It seems reasonable to suggest that these oxidases will prove to have differing substrate specificities expressed both at the level of the individual protein substrates and at the level of the sequence regions within which susceptible lysine residues are found. Such differences in specificity could then segregate the specific cellular targets to specific LOX and/or LOXL enzymes.

The evidence cited here concerning the involvement of FN in the activation of LOX proenzyme species, the role of the propeptide in the recognition of the elastin substrate and in the suppression of the NFκB-dependent proliferation of transformed cells focuses increased attention on the function of the propeptide domains of the LOX family of enzymes. In view of the preponderance of cationic residues in the propeptide and of anionic residues at the active-site region, it has been hypothesized that electrostatic bonding could provide much of the force that would allow the propeptide to bind and restrict access to the active site within the proenzyme [6]. It had also been noted that none of the unusually small number of lysine residues within proLOX (5 of 411 residues) occur within the propeptide domain but are exclusively contained within the catalytic domain. This distribution would be advantageous to prevent the undesirable oxidation of a lysine residue within the cationic propeptide domain which might then crosslink the propeptide to the catalytic domain, thus preventing propeptide release and exposure of the active site to protein substrates [6]. These proposals for the role of the propeptide as well as the impacts of LOX and LOXL enzymes on the control of cell phenotypes will doubtlessly prove to be focal points in continued investigations on these enzymes.

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