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PROSPECTS

Lysyl Oxidase: Properties, Specificity, and Biological Roles Inside and Outside of the Cell

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Abstract Lysyl oxidase (LO) plays a critical role in the formation and repair of the extracellular matrix (ECM) by oxidizing lysine residues in elastin and collagen, thereby initiating the formation of covalent crosslinkages which stabilize these fibrous proteins. Its catalytic activity depends upon both its copper cofactor and a unique carbonyl cofactor and has been shown to extend to a variety of basic globular proteins, including histone H1. Although the three-dimensional structure of LO has yet to be determined, the present treatise offers hypotheses based upon its primary sequence, which may underlie the prominent electrostatic component of its unusual substrate specificity as well as the catalysis-suppressing function of the propeptide domain of prolysyl oxidase. Recent studies have demonstrated that LO appears to function within the cell in a manner, which strongly modifies cellular activity. Newly discovered LO-like proteins also likely play unique roles in biology. J. Cell. Biochem. 88: 660–672, 2003. © 2003 Wiley-Liss, Inc.

Key words: lysyl oxidase; lysyl oxidase-like; specificity of lysyl oxidase; chromatin; prolysyl oxidase

Lysyl oxidase (LO; EC 1.4.3.13), an amine oxidase expressed and secreted by fibrogenic cells, initiates the covalent crosslinking of collagen and elastin in the extracellular space by oxidizing specific lysine residues in these proteins to peptidyl α-aminoadipic-δ-semialdehyde (AAS). These aldehyde residues can spontaneously condense with vicinal peptidyl aldehydes or with ε-amino groups of peptidyl lysine to generate the covalent crosslinkages which stabilize and insolubilize polymeric collagen or elastin fibers in the extracellular matrix (ECM) [Smith-Mungo and Kagan, 1998]. Thereby, LO plays a central role in the morphogenesis and repair of connective tissues of the cardiovascular, respiratory, skeletal, and other systems of the body. It had long been assumed that the biological role of this enzyme was restricted to its ability to oxidize peptidyl lysine exclusively in its collagen and elastin substrates. While these considerations were both complex and interesting, recent reports have revealed that LO and, possibly, a number of closely related "lysyl oxidase-like" (LOXL) proteins, exhibit biological effects and/or cell and tissue distributions suggesting that these proteins play exceptionally important roles in biology beyond the induction of crosslinkage formation in collagen and elastin. This review will consider aspects of the structure, enzymology, and biology of LO and of selected LOXL proteins. The reader is referred to a recent review, which considers the LOXL proteins in greater detail [Csiszar, 2001].

BIOSYNTHESIS OF LO

Initial insight into the molecular structure of LO derived from the cloning of full-length rat aorta LO cDNA sequence [Trackman et al., 1990, 1991]. Although the molecular weight of the functional catalyst isolated from connective tissues varied between 28 and 32 kDa, depending upon the vertebrate species of origin, a 46 kDa protein was the gene product predicted from the cDNA sequence obtained (Fig. 1). Subsequent studies [Trackman et al., 1992; Panchenko et al., 1996] confirmed that the enzyme of rat vascular smooth muscle cells (VSMCs) is synthesized as a 46 kDa preproprotein (preproLO) containing an N-terminal signal peptide sequence contiguous with a propeptide domain followed by the catalytic

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LOXL-1 MALARGSRQLGALVWGACLCVLVHGQQAQPGQGSDPARWRQLIQWENNGQ
       VYSLLNSGSEYVPAGPQRSESSSRVLLAGAPQAQQRRSHGSPRRRQAPSL
       PLPGRVGSDTVRGQARHPFGFGQVPDNWREVAVGDSTGMALARTSVSQQR
       HGGSASS                                          157
                                ↕
 RatLO MRFAWTVLFLGQLQFCPLLRCAPQA------PREPPAAPGAWRQTIQWEN   44
 HumLO ********L*****L*A*VH***P*AGQQQP************Q*****   50
LOXL-1 VSASAFASTYR*QPSY*QQFPY***PFVSQYENYD**SRTYDQGFVYYRP  207

 RatLO NGQVFSLLSLGAQYQPQRRRDSSATAPRADGNAAAQPRTPILLLRDNRTA   94
 HumLO ***********S*********PG*AV*G*ANAS*Q***************  100
LOXL-1 A*GGVGAGAAAVASAGVIYPYQPRARYEEYGGGEELPEYPPQGFYPAPER  257

 RatLO SARARTPSPSGVAAGRPRPAARHWFQVGFSPSGAGDGASRRAANRTASPQ  144
 HumLO A**T**AGS***T******T******A*Y*T*R*REAGAS**E*Q**PGE  150
LOXL-1 PYVPPP*P*PDGLDR*YSHSLYSEGTP**EQAYPDP*PEAAQ*HGGDPRL  307
                          ▼
 RatLO PPQLSNLRPPSHVDRMVGDDPYNPYKYSDDNPYYNYYDTYERPRSGSRHR  194
 HumLO V*A********R**G*****************************P*G**R  200
LOXL-1 GWYPPYAN**PEAYGPPRALEPPYLPVRSSDTPPPGGERNGAQQGRLSVG  357

 RatLO PGYGTGYFQYGLPDLVPDPYYIQASTYVQKMSMYNLRCAAEENCLASSAY  244
 HumLO ************************************************** 250
LOXL-1 SV*RPNQNGR*********N*V*******RAHL*S*******K****T**  407

 RatLO RADVRDYDHRVLLRFPQRVKNQGTSDFLPSRPRYSWEWHSCHQHYHSMDE  294
 HumLO ************************************************** 300
LOXL-1 APEAT***V***************A****N***HT***************  457
                          ■
 RatLO FSHYDLLDASTQRRVAEGHKASFCLEDTSCDYGYHRRFACTAHTQGLSPG  344
 HumLO *********N****************************************  350
LOXL-1 *********A*GKK*************ST**F*NLK*Y***S********  507
                ■
 RatLO CYDTYAADIDCQWIDITDVQPGNYILKVSVNPSYLVPESDYSNNVVRCEI  394
 HumLO *****G*************K**********************T******D*  400
LOXL-1 *****N**********************H***K*I*L***FT******D*  557

 RatLO RYTGHHAYASGCTISPY                                  411
 HumLO *****************                                  417
LOXL-1 *****************                                  574
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Fig. 1. Amino acid sequences of rat and human lysyl oxidase (LO) and lysyl oxidase-like gene (LOXL-1). The sequence of LOXL-1 was aligned by proceeding from the C-terminal to the N-terminal direction. The sequence of the first 156 residues of N-terminal region of this protein is shown separately at the top. Symbols:*, residue identical with the corresponding site in the rat sequence; ↕, signal peptide cleavage site; ▼, proLO proteolytic processing site; ■, Lys314 and Tyr349 (rat sequence) progenitors of the lysyl tyrosyl quinone (LTQ) cofactor. Two-NRT-consensus sequences for *N*-glycosylation are underlined within the propeptide domain sequences.

domain. The signal peptide is removed by cleavage at the Cys21-Ala22 bond and the propeptide domain is N-glycosylated during passage through the Golgi yielding a 50 kDa proenzyme (proLO), which is then secreted into the medium as a catalytically inactive protein [Trackman et al., 1992]. The 32-kDa active enzyme is released by proteolytic cleavage of proLO at Gly162–Asp163 (rat LO sequence; Fig. 1) by a metalloendoprotease indicated [Cronshaw et al., 1995; Panchenko et al., 1996] and recently confirmed [Uzell et al., 2001] to be procollagen C-proteinase (bone morphogenetic protein I). The N- and C-propeptides of fibrillar procollagens must be proteolytically removed in the extracellular space by procollagen N- and C-proteinases, respectively, to allow spontaneous assembly of individual collagen molecules into quarter-staggered fibrillar aggregates. Most interestingly, the LO-catalyzed oxidation of lysine in fibrillar collagens is restricted to these fibrillar aggregates [Siegel, 1974]. Clearly, nature has chosen a highly efficient mechanism to generate this crosslinking enzyme and its collagen substrate. The restriction of LO activity toward collagen in fibrillar aggregates appears to reflect the need for neutralization of unfavorable anionic charge vicinal to the peptidyl lysine substrate by neighboring collagen molecules within the collagen fibril [Nagan and Kagan, 1994]. The common role of procollagen C-proteinase in the extracellular activation of proLO and in the processing of procollagen to the substrate form of collagen required for its oxidation by LO suggests that this proteinase can act as a central point of control of the deposition of collagen fibers, which might be exploited in the search for agents that limit fibrotic diseases.

CATALYTIC AND STRUCTURAL PROPERTIES OF LO

Current understanding of the molecular and catalytic properties of the active enzyme have largely derived from kinetic, chemical, and spectroscopic studies on the processed LO species purified from bovine aorta. Comparison of the amino acid sequence of rat LO predicted from the rat cDNA sequence with those of several peptides isolated from digests of bovine LO revealed that there is nearly complete homology between these two enzymes [Trackman et al., 1990, 1991]. Moreover, the properties predicted and/or shown for rat LO and experimentally assessed with purified bovine LO are in agreement and are also likely to be fully relevant to the catalytically active forms of the cloned enzymes of human, mouse, and chick, the catalytic domains of which are $\geq$95% homologous to that of the rat. Initial attempts at the purification of LO from connective tissues were thwarted by the insolubility of the enzyme in neutral saline extracts of these tissues. The finding that the enzyme was readily solubilized by these buffers supplemented with 4 to 6 M urea [Narayanan et al., 1974] paved the way for its successful purification to apparent homogeneity. The insolubility of LO likely reflects its tight association with its substrates in the ECM [Cronlund et al., 1985; Kagan et al., 1986], consistent with the freely soluble behavior of LO secreted from fibrogenic cells in culture prior to the deposition of significant quantities of ECM proteins.

The mature catalyst isolated from bovine aorta migrates as a 32 kDa, apparently single protein band by SDS–PAGE [Kagan et al., 1979]. This apparently homogeneous enzyme resolved into four 32 kDa isoforms upon chromatography through DEAE cellulose, the origins of which are yet unknown. Each of these isoforms displayed the same catalytic mechanism, specificity and inhibitor profile [Sullivan and Kagan, 1982]. The activity of LO requires the presence of one tightly bound Cu(II) atom at its active site [Gacheru et al., 1990]. The presence of lysyl tyrosyl quinone (LTQ; Fig. 2), a unique peptidyl quinone functioning as a carbonyl cofactor in LO, is also essential to catalysis [Wang et al., 1996]. The LO carbonyl cofactor is presumed to arise by autocatalytic hydroxylation and oxidation of Tyr349 (rat LO sequence) occurring with the catalytic assistance of the copper atom at the nascent active site [Dove and Klinman, 2001]. Once in the form of peptidyl DOPA quinone, the developing cofactor can then be attacked by the ε-amino group of Lys314 (rat LO sequence) followed by the reoxidation of the resulting derivatized quinol to peptidyl LTQ [Dove and Klinman, 2001]. It is notable that this crosslinking enzyme is crosslinked within itself by the stable covalent bond within LTQ linking the peptidyl environment of Lys314 to that of Tyr349 (Figs. 1 and 2). This intramolecular crosslink likely contributes importantly to the remarkable physical-chemical hardiness of LO evidenced by its optimum assay temperature against

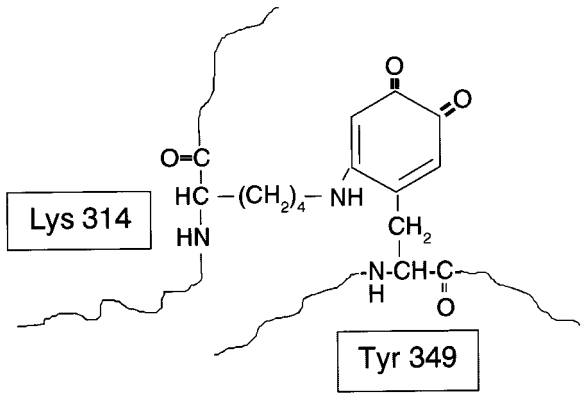

Fig. 2. Lysyl tyrosyl quinone (LTQ) cofactor of lysyl oxidase (LO).

synthetic amine substrates of 55°C, by its irreversible denaturation temperature of 92°C, and by the resistance of its catalytic potential to high urea concentrations [Trackman et al., 1981]. Clearly, the stability of the mature enzyme also depends upon the 5 disulfide bonds derived from the 10 cysteines in mature LO [Williams and Kagan, 1985]. As noted, the molecular weights found by SDS–PAGE for LO purified from different vertebrate species varied between 28 kDa (human and chick) and 32 kDa (bovine and rat). Sequence comparisons among these enzymes predicts that the processed, functional enzyme of each will be approximately 28,500 Da. These discrepancies may in part reflect the few amino acid substitutions occurring within the full catalytic domains and also may reflect the presence of the crosslinked cofactor which would prevent the complete unfolding of the mature protein under the conditions of SDS–PAGE.

The stoichiometry of the reaction catalyzed by LO is: $RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$. A continuous fluorometric assay for LO activity measuring the stoichiometric release of the H_2O_2 product of enzyme action upon oxidation of amines was developed several years ago [Trackman et al., 1981] and has recently been modified [Palamakumbura et al., 2002]. The enzyme operates by a ping-pong kinetic mechanism involving two half-reactions to complete the catalytic cycle. In the first half-reaction, the amine substrate binds at the active site through a Schiff base linkage with one of the two *ortho*-carbonyl moieties of the LTQ cofactor. Following general base-assisted removal of the α-proton of the bound substrate [Williamson and Kagan, 1986, 1987], migration of two electrons from the resulting α-carbanion into LTQ reduces the cofactor to the quinol state while oxidizing the substrate to the aldehyde, which remains linked as an aldimine to the reduced cofactor. The bound aldimine form of the substrate is then removed from the cofactor by hydrolysis liberating the free aldehyde product while leaving the amino group attached to the reduced quinone in the form of the enzyme-bound aminoquinol. In the second half of this two-stage reaction, molecular oxygen binds to the enzyme and oxidizes the reduced aminoquinol to the enzyme-bound quinoneimine. The resulting two-electron reduction of oxygen forms the hydrogen peroxide product, which is released from the active site. Finally, the ammonia product is released by hydrolysis of the quinoneimine form of the cofactor restoring the LTQ to its original state and completing the catalytic cycle [Williamson and Kagan, 1986, 1987]. This mechanism can be briefly summarized as follows:

$$LO_{ox} + RCH_2NH_2 \rightarrow LO_{red}(\text{-}NH_2) + RHC{=}O \quad (1)$$

$$LO_{red}(\text{-}NH_2) + O_2 + H_2O \rightarrow LO_{ox} + H_2O_2 + NH_3 \quad (2)$$

Histidine-containing sequences consistent with known copper-binding motifs bracket the center of the active site at the carbonyl residue. Evidence has been obtained that copper is incorporated into the nascent proenzyme in advance of the *N*-glycosylation that occurs within the propeptide domain of LO within the Golgi [Kosonen et al., 1997]. The precise role of copper in the mechanism of action of LO remains to be understood. It is of interest that LO expression is downregulated in Menkes disease [Gacheru et al., 1993], a disorder of copper transport reflecting a mutation in a P-type ATPase associated with the Golgi. Since LO expression can be upregulated by copper [Li et al., 1995], it is possible that a Menkes-related deficiency in this Golgi copper transporter may result in deficient insertion of copper in nascent LO and in the lowered LO expression seen in this genetic disease. Copper is an efficient mediator of single electrons from reduced quinones (quinols) to oxygen, forming, in turn, superoxide and then peroxide, and might participate, therefore, in the reoxidation of the substrate-reduced form of LO in the second half-reaction. However, the

formation of the aldehyde occurring in the first half-reaction requires the presence of copper, even though electrons are not transferred to oxygen in this half-reaction [Shah et al., 1993]. Nonetheless, it has been shown that the copper-free enzyme can catalyze a limited number of turnovers as it oxidizes a synthetic amine substrate [Tang and Klinman, 2001]. It is conceivable that copper may play a conformational role in the initial half-reaction, possibly by orientation of the amine substrate with the quinone cofactor.

SUBSTRATE SPECIFICITY OF LO

The significant difference in sequence surrounding the susceptible lysines in the collagen and elastin substrates of LO stimulated studies of factors critical to the substrate specificity of this enzyme. The enzyme proved to oxidize lysine-containing oligopeptides, showing particular sensitivity, both positive and negative, to peptide length and to the specific positions of dicarboxylic amino acids vicinal to lysine within these peptides [Nagan and Kagan, 1994]. The catalytic efficiency (k_{cat}/K_M) increased with increasing lengths of peptides containing one lysine residue symmetrically placed in a background of increasing numbers of glycine residues. Moreover, the k_{cat}/K_M for the oxidation of lysine within the *N*-acetyl-$(Gly)_4$–Glu-Lys-$(Gly)_5$-amide sequence was significantly increased over that for the *N*-acetyl-$(Gly)_5$–Lys-Glu-$(Gly)_4$-amide sequence. These results indicate that LO likely carries an extended active site in which substrate interactions distal to the susceptible lysine can modulate catalytic efficiency. Further, specific ionic charges of substrate residues vicinal to the susceptible lysine residue can also strongly influence catalytic potential.

While LO is sensitive to anionic residues at specific residue distances from peptidyl lysine, it also displays a broader sensitivity to electrostatic field effects between the enzyme and its protein substrates. Initial indications of the role of charge in LO specificity arose from the findings that elastin-bound anionic detergents markedly inhibited the oxidation of elastin by LO, whereas elastin-bound cationic detergents strongly stimulated the expression of LO activity toward this protein substrate [Kagan et al., 1981a]. Of further interest, purified LO readily oxidized a number of basic, globular proteins with pI values >8, notably including histone H1, but did not oxidize neutral or acidic proteins with pI values <8 [Kagan et al., 1984]. In addition to supporting the importance of net substrate charge in LO substrates, these results also demonstrated that the specificity of LO was not necessarily restricted to elastin and collagen as had been thought. Among its protein substrates, it was of particular interest that purified LO generated considerable quantities of peptidyl AAS as well as the aldol and lysinonorleucine crosslinkages derived from AAS and unmodified lysines within purified histone H1. Moreover, lesser amounts of these crosslinkages were identified within the H1 samples, which had not been incubated with LO [Kagan et al., 1983]. The resulting inference that LO might play critical roles in biology in addition to its role in stabilizing the ECM has been supported by a variety of recent reports as indicated herein.

STRUCTURAL IMPLICATIONS OF THE PRIMARY SEQUENCE

Significant information has been obtained about key physical–chemical and catalytic properties of lysyl oxidase during the recent decades, although the crystallization of LO and solution of its three-dimensional structure remains to be achieved. Nevertheless, certain inferences can be drawn from the amino acid sequence of the enzyme that seem likely to underlie key features of the specificity of this catalyst. The sequence of proLO is highly conserved among vertebrate species as illustrated for the rat and human preproenzymes in Figure 1. Moreover, there is nearly complete homology within the catalytic domains, which are released from the rat, human, chick, and mouse proenzymes by cleavage at the conserved -G-DD- sites in these enzymes. The sequence of LOXL-1 is also included in Figure 1, although that sequence is not intended to be representative of each of the LOXL-2, -3, and -4 species. Although, there is little homology seen in the first 300 residues of LOXL-1 with the sequences of the proLO species, significant sequence conservation is found within most of the catalytic domain, assumed to be contained within the remainder of the LOXL-1 sequence. LOXL-1 has recently been purified from bovine aorta as a 56 kDa protein. This protein was cleaved in vitro by procollagen C-proteinase resulting in the generation of BAPN-inhibitable

enzyme activity [Borel et al., 2001]. Clearly, this LOXL protein shares key catalytic and processing properties with LO.

The distributions of ionizable residues within rat and human preproLO as well as LOXL-1 are shown in Figure 3. The data are presented as the total cationic and total anionic residues within consecutive, non-overlapping 20 residue segments of the full sequences, numbering segments from the N- to the C-termini. Clearly, the profiles of the two LO enzymes are extremely similar. The charge profile of LOXL-1 displays significant differences from the profiles of rat and human proLO. These differences reflect in part the significantly greater residue length of LOXL-1, noting that the first 157

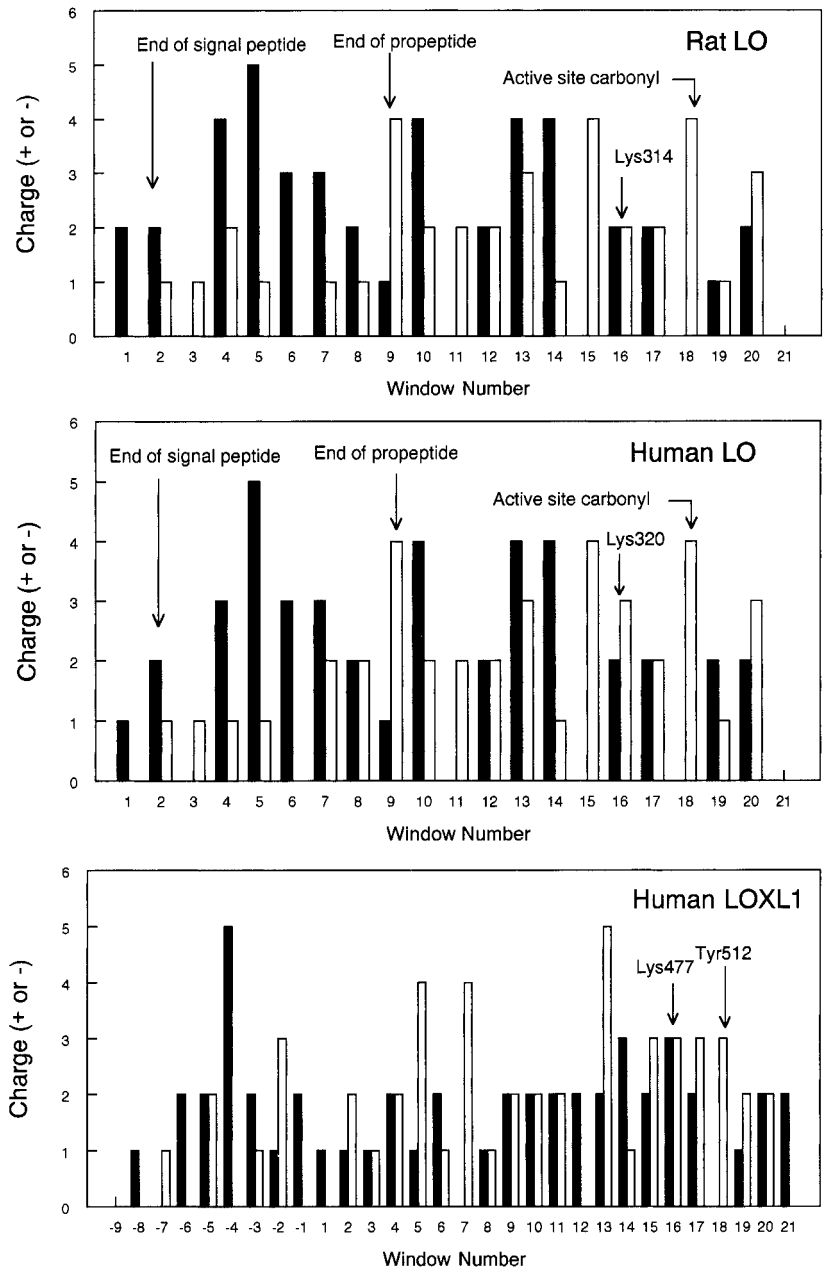

Fig. 3. Profiles of ionic residues in rat and human preprolo and in human lysyl oxidase-like gene (LOXL-1). Solid bars, cationic residue content; open bars, anionic residue content. The total anionic and cationic residue content of non-overlapping, consecutive 20 residue segments were summed and are presented in the bar charts. The N-terminal segments of the two preproLO enzymes are presented as Segments 1. Segment 1 of LOXL-1 initiates with the −VSAS-sequence (residues 157–160), consistent with the alignment of Figure 1. Segments −1 to −9 of LOXL-1 represent consecutive 20 residue segments proceeding toward the N-terminal residue from residue 156 and ending at residue 1.

residues of this protein precede the initiation of the two LO sequences in the arrangement of Figure 1. Considering the profile of rat preproLO, it is evident that segments which compose the propeptide (Segments 2 through 9) are preponderantly positively charged with 20 cationic and 11 anionic residues in these segments. The -G-DD- cleavage site occupies the second, third, and fourth residues of Segment 9, noting that the propeptide terminates with the Gly of this tripeptide sequence after cleavage. Taking this cleavage site into account, the propeptide sequence contains 20 cationic and only 7 anionic residues. Summing the potential charges of the catalytic domain beginning with the -DD- residues of the G-DD- cleavage site in Segment 9 reveals a total of 22 cationic and 30 anionic residues within the full catalytic domain. The calculated preponderance of negative charges in the mature enzyme is consistent with the estimated pI of 5.5 to 6 determined by isoelectric focusing of the 32 kDa bovine LO in the author's laboratory (unpublished data). It is striking that Segment 18 containing the active site carbonyl residue derived from Tyr349 (rat sequence) in both rat and human LO contains only anionic residues (aspartates 347, 352, 354, and 358 of rat LO; see Fig. 1). Similarly, Segment 15, terminating in Leu310 in the rat enzyme, also contains only anionic residues and immediately precedes Segment 16, the segment which contains Lys314 four residues downstream from Leu310. Since Lys314 is the residue which becomes covalently attached as a component of the carbonyl cofactor within Segment 18, it is expected that this covalent linkage would bring Segment 15 to the vicinity of the active site. One can reasonably infer that Segments 15 and 18 within the two LO enzymes shown, containing a net charge of minus 8, cooperatively generate strong anionic character at and in the broader vicinity of the active site. Such grouping of negative charges could then be responsible for the electrostatic field effects strongly inferred by the complete preference of LO for basic rather than acidic proteins as its substrates [Kagan et al., 1984]. It is also of interest that the thirteen residue sequence immediately C-terminal to the carbonyl cofactor derived from Tyr349 contains four aspartic acid residues within an otherwise neutral sequence while there is only one residue likely to be ionized at neutral pH (aspartic acid) in the thirteen residue sequence immediately preceding the N-terminal end of the carbonyl cofactor. This asymmetric distribution of anionic residues may well underlie the marked preference of LO for the -Glu–Lys- rather than the -Lys-Glu- sequence in oligopeptide substrates [Nagan and Kagan, 1994] because of the likelihood of greater charge–charge repulsion between aspartates and the substrate glutamate residue in one N to C orientation of the bound substrate over the other. The conservation of the chick and mouse LO sequences with those of the rat and human indicate that these possibilities will extend to those enzymes as well. Segment 18 of LOXL-1 also exhibits only anionic residues, although Segment 15 of that protein contains equal numbers of cationic and anionic residues. The presence of the conserved lysine and tyrosine constituents of the LTQ cofactor in LOXL-1 is consistent with the likelihood that LTQ forms at its active site and with the catalytic potential of this species. The difference in anionic charge density within the region of the active site of LOXL-1 with those of the LO enzymes suggests that there may be distinct differences in specificity for charges and/or sequences surrounding susceptible lysine residues in the putative protein substrates of LOXL-1.

Continuing along this speculative path, one might also question whether the distribution of charges within the 50-kDa proenzyme can account for the catalytic quiescence of the proenzyme. As noted in the profiles of the rat and human LO enzymes (Fig. 3), there is an abundance of positive charge within the propeptide moiety (Segments 2 through 9; 20 positive and 11 negative charges), with particularly notable cationic character in Segments 4–6. Since fully functional, recombinant rat LO was expressed and secreted by CHO cells which had been transfected with a construct encoding the LO signal peptide fused to the N-terminal of the catalytic domain but lacking the propeptide domain [Kagan et al., 1995a], it is very unlikely that the propeptide critically influences the proper folding of the catalytic domain during the synthesis of the enzyme. In view of the strong charge complimentarity between the propeptide and the active site region, it appears reasonable to suggest that the cationic propeptide within native proLO may interact electrostatically with the anionic active site region, thus blocking substrate access and preventing the uncontrolled oxidation by proLO of

intracellular proteins during the intracellular processing and secretion of the proenzyme. The abundance of cationic residues in Segments −7 to −1 of LOXL-1, and the resemblance of the charge profile generated by these segments to those of the propeptide segments of the LO species raises the additional possibility that these segments in LOXL-1, represent a propeptide domain.

Considering the amino acid composition of LO typified by the rat and human species shown in Figure 1, it is notable that these proteins contain remarkably few lysine residues. In contrast to a representative group of proteins, the compositions of which contain an average of 7 mol percent lysine [Van Holde, 1996], the rat and human LO proenzyme species contain only 1.2 and 1.4 mol percent lysine, respectively. The positions of those few lysines that do occur in these proteins are even more interesting. Thus, the rat enzyme contains five lysines out of 411 residues located at positions 170, 224, 264, 314, and 371, while the human enzyme has only 6 lysines out of 417 residues with lysines 176, 230, 270, 320, and 371 aligning with the five in rat LO with an additional lysine at 387 in the human enzyme. In view of the marked preference of LO for lysine within cationic protein sequences, it seems unlikely to be coincidental that the cationic propeptide domains of each of the proLO sequences available thus far (rat, chick, human, and mouse) lack lysine residues. The paucity of lysine in the full length of the proLO sequences and the complete lack of lysine within the propeptide domains would limit or prevent, respectively, the possibility of autocatalytic oxidation of lysine within the proLO and LO sequences. Certainly, if present and appropriately aligned with the active site, the availability of lysine within the cationic propeptide domain could lead to autocatalytic lysine oxidation and the generation of covalent crosslinkages between the propeptide and the catalytic domains, preventing dissociation of the propeptide and, therefore, the activation of the enzyme. Intra-and/or intermolecular autocatalytic oxidation of lysine within the fully processed catalytic domain might similarly lead to the formation of crosslinked polymeric forms of the enzyme with accompanying loss of activity. The tendency of purified LO to spontaneously form amorphous aggregates in the absence of modest concentrations of urea [Kagan et al., 1979] increases the possibility that intermolecular crosslinking between monomers could occur within such aggregates.

OTHER STRUCTURAL THEMES IN LYSYL OXIDASE

LO has been characterized at the protein and/or gene levels in tissues derived from human, rat, bovine, chicken, and mouse and has been localized in the human to heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Conserved elements found in these enzymes include a copper binding domain, other putative metal binding domains, a cytokine receptor domain, signal peptide, propeptide, and catalytic domains. Each of these enzymes contain a tyrosine and a lysine residue positioned appropriately for the formation of the LTQ cofactor, which has thus far been chemically identified only in the bovine enzyme. The most significant homology (>95%) among these species occurs in the C-terminal catalytic domain, with minor (mouse LO) or greater (chick LO) variability seen within the sequences of the propeptide domains relative to that of the rat enzyme [Kagan et al., 1995b]. The absence of lysine in the propeptide domains, the paucity of lysine (5 to 6 residues) in the full proenzyme sequences, and the distributions of cationic and anionic residues are strongly and sufficiently similar features within this group of enzymes to support the hypotheses proposed here regarding possible bases to the activation and specificity proposed in this treatise.

A region with close similarity to the consensus sequence of the cytokine receptor domain [C-x_9-C-x-W-x_{26-32}-C-x_{10-13}-C; where "x" refers to any amino acid and the four cysteines are C245 and C355, C392 and C406 in the rat LO sequence, (Fig. 1)] occurs in the C-terminal region of the catalytic domain and encompasses the active site region of LO. It is of interest in this regard that LO readily oxidizes basic fibroblast growth factor [bFGF; Li et al., 2002b] and selected additional growth factors (Li W and Kagan H, unpublished data). The mitogenic activity of bFGF is lost upon the oxidation of lysine residues accessible to LO within the native structure of this growth factor. Certainly, the cationic nature of bFGF is likely critical to its susceptibility to LO. Of further interest, the growth of tumorigenic NIH 3T3 IgBNM 6-1 cells is inherently stimulated by their overexpression of bFGF but is markedly reduced in the presence of nanomolar concentrations of

bovine aorta LO. The inhibition of the growth of these cells is accompanied by the LO-dependent oxidation and crosslinking of the bFGF within these cells. Moreover, the basal level of growth of these cells is strongly stimulated (2.7-fold over controls) by β-aminopropionitrile (BAPN) consistent with the limitation of the cell growth by endogenous LO [Li et al., 2002a]. The expression of LO has been correlated with the inhibition of cell proliferation, as seen, for example, by its suppression of the proliferation of Ras-transformed fibroblasts [Kenyon et al., 1995]. Conceivably, the cytokine receptor-like sequence in LO may provide a substrate recognition site for cationic growth factors the mitogenic functions of which can then be eliminated by LO-catalyzed oxidation of key lysine residues. Such a mechanism could then account for the anti-proliferative effect of this catalyst in selected cases.

NEW FUNCTIONS OF LO

As noted, recent evidence points to an anti-oncogenic effect of LO. Thus, expression of transfected, sense LO cDNA in cells transformed by p21 Ha-Ras suppresses Ha-Ras-induced tumorigenesis, indicating a Ras-suppressor effect of LO [Contente et al., 1990; Kenyon et al., 1991; Hamalainen et al., 1995; Contente et al., 1999; Giampuzzi et al., 2001]. LO mRNA expression is negligible in these cells before transfection as well as in a series of malignantly transformed human cells [Hamalainen et al., 1995]. Consistent with an anti-oncogenic function of LO, normal rat kidney fibroblasts became transformed by expression of antisense LO, demonstrating anchorage independent growth and elevation of the expression of p21-Ras [Giampuzzi et al., 2001]. In addition to its anti-oncogenic effects, the 32 kDa, purified LO is strongly chemotactic for monocytes and VSMC [Lazarus et al., 1995; Li et al., 2000]. The chemotactic property of LO was eliminated by inclusion of BAPN or of catalase in the chemotaxis chamber, indicating that catalytic expression is essential to the response and that the H_2O_2 product of the oxidation mediates the chemotactic response between LO and the cell. The medium conditioned by VSMC in the presence of added LO did not induce chemotaxis. This and related results indicated that the substrate oxidized by LO, resulting in the catalytic formation of the H_2O_2 mediator of the chemotactic response, appeared to be tightly associated with the cell layer [Li et al., 2000]. The identity of this substrate is not yet known. In view of this chemotactic effect of LO, it is of interest that LO activity is significantly increased in early arterial lesions in a rabbit model of atherosclerosis [Kagan et al., 1981b] and that the migration of VSMC from the medial portion of arteries to the sub-endothelial intimal region is a critical component of the response of these cells to atherogenic stimuli [Ross, 1999]. One might question whether the chemotactic activity of LO contributes to the chronic nature of this disease by maintaining the migration of medial VSMC to the sites of the intimal lesions and/or the migration of inflammatory blood monocytes through the vascular endothelium. The potential participation of LO in arterial disease is also inferred by the recent report that low density lipoproteins downregulate the expression of LO in vascular endothelial cells [Rodriguez et al., 2002].

In view of evidence that LO can affect cell function, it is of considerable interest that this catalyst as well as traces of lysine-derived crosslinkages have been identified within nuclei of cultured VSMC and 3T3 fibroblasts [Li et al., 1997]. The return of secreted and processed LO from the extracellular space into the cell is a feasible pathway for the nuclear localization of LO since fluorescein-labeled LO which was incubated with cultured VSMC at 37°C readily entered the cytosolic compartment and was then rapidly concentrated into the cell nucleus [Nellaiappan et al., 2000]. The uptake of LO is also observable at the ultrastructural level as shown in the electron micrograph of a fixed section of bovine aorta, which had been incubated with anti-LO and then with gold-labeled second antibody (Fig. 4). Gold particles representing the loci of LO are seen at the periphery of the smooth muscle cell, which occupies the microscopic field; within the cytosol in apparent aggregates of 5 to 10 particles each; at what appears to be periodically distributed sites on the nuclear membrane consistent with the distribution of nuclear pores; and, most interestingly, in apparent association with the condensed chromatin within the nucleus. In view of the nuclear localization seen here, it is of particular interest that transfection of LO sense cDNA into p21 Ha-Ras transformed cells alters chromatin packing [Mello et al., 1995]. The possibility that such cellular interactions of

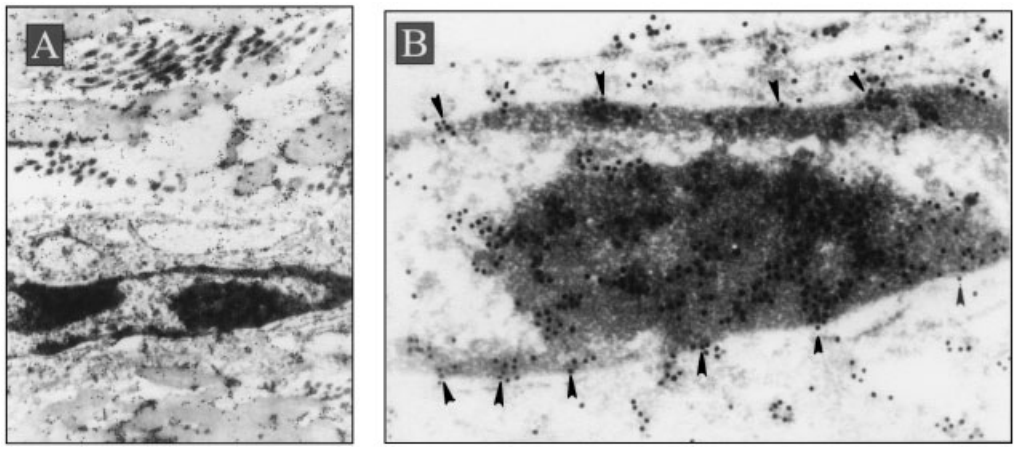

Fig. 4. Localization of lysyl oxidase (LO) in a section of bovine aortic tissue. Electron microscopy. Fixed specimens of the medial layer of bovine aorta were incubated with anti-LO and gold-labeled second antibody. Electron-dense gold deposits represent sites of LO. **A**: This section of the arterial media layer encompasses extracellular matrix (ECM) (upper one-half and lower one-quarter of the field) and a portion of a vascular smooth muscle cell aligned horizontally between the two ECM regions (60,000 ×). The nucleus of the smooth muscle cell containing prominent deposits of condensed chromatin is seen in this section. Gold deposits in the ECM are seen associated with amorphous elastin fibers (light gray areas) and bundles of tangentially cut collagen fibers. Gold deposits are also evident within the cell, within the nucleus, and at the cell periphery. **B**: Enlargement of an intracellular region of the cell seen in (A) emphasizing a nuclear region, containing a prominent deposit of condensed chromatin (1,43,000×). Gold deposits are seen at the site of the nuclear membrane (arrowheads) and in groups within the nucleus between the nuclear envelope and the condensed chromatin. Prominent deposits of gold occur in association with the condensed chromatin and appear to co-localize with sinuous, denser regions of the chromatin.

LO may affect gene expression is supported by reports that the addition of BAPN, an irreversible inhibitor of LO, to VSMC cultures reduces elastin mRNA expression [Jackson et al., 1991] and that overexpression of LO in COS-7 cells increased the promoter activity of the human collagen type III gene approximately 12-fold, an effect that was also abolished by BAPN. The latter effect was related to the LO-induced binding of Ku antigen to the type III collagen promoter [Giampuzzi et al., 2000]. Similarly, microinjection of recombinant LO antagonized p21-Ha-Ras-induced and progesterone-dependent *Xenopus laevis* oocyte maturation [Di Donato et al., 1997b]. Clearly, our view of the functions of LO must now consider its participation in the control of intracellular activities. It is an intriguing possibility that such effects may be due to the oxidation of lysine in nuclear proteins affecting gene transcription. Studies in progress in the author's laboratory indicate that histone H1, previously demonstrated to be a substrate of LO in assays in vitro [Kagan et al., 1983], becomes oxidized within VSMC nuclei in a BAPN-inhibitable manner [Li et al., 2002a], consistent with the role of nuclear LO catalysis in this process. Histone H1 binds internucleosomal DNA and also interacts with core histones in adjacent nucleosomes. The LO-catalyzed oxidation of lysine in histone may well alter DNA–histone and histone–histone interactions with consequent effects on DNA transcription in analogy to the effects resulting from the acetylation of histone lysines [El-Osta and Wolffe, 2000]. Although the oxidative deamination of the ε-amino group of peptidyl lysine to produce AAS is a reaction which is chemically distinct from that of *N*-acetylation of the ε-amino groups of lysine in histones, both reactions eliminate the positive charge of peptidyl lysine. The loss of this charge would then be expected to disrupt electrostatic interactions normally occurring between histone cationic ε-amino groups and DNA phospooxyanions within nucleosomes. In toto, these newly found activities and localizations of LO, coupled with its role in ECM production, suggest that this enzyme can serve as a critical messenger shuttling information between the intra- and extracellular compartments. The partitioning stimuli and mechanisms accounting for the distribution of LO between these two compartments remain to be solved.

LO-LIKE PROTEINS

Additional cDNA species have recently been described which encode a variety of LO-like

proteins, including LOXL [Kim et al., 1999], LOXL-2 [Jourdan-Le Saux et al., 2000], LOXL-3 [Jourdan-Le Saux et al., 2001; Maki and Kivirikko, 2001], and LOXL-4 [Asuncion et al., 2001], whose residue lengths are 574, 638, 753, and 756 kDa, respectively. By comparision, human preproLO is 417 residues in length. While there is little homology in the N-terminal regions of these proteins with the LO propeptide domain, there is significant homology within the C-terminal domains with the sequence of mature human LO. Consensus sites for cleavage by procollagen C-proteinases are absent in LOXL-2, -3, and -4, but are present in all proLO enzymes and in LOXL [Csiszar, 2001]. Csiszar [2001] has reported that LOXL expresses BAPN-inhibitable catalytic activity toward an elastin substrate and has noted that the sequences flanking the lysine and tyrosine progenitors of the LTQ cofactor in LOXL are highly conserved with those in LO. These similarities can be seen in Figure 1. Although not yet demonstrated, it is likely that LOXL contains the LTQ cofactor. The corresponding sequences in LOXL-2, -3, and -4 also exhibit similarities with those of LO but are not as highly conserved. The C-terminal domains of both LO and LO-like proteins contain 10 conserved cysteine residues and conserved copper binding domains. The overall sequences within the putative active site regions of LOXL-2, -3, and -4 suggest that they may also prove to be catalytically functional although with substrate specificities that may differ from that of LO and with differing biological roles yet to be determined. The possibility that each of these LOXL species can be proteolytically processed to functional catalysts has yet to be demonstrated.

FUTURE PROSPECTS

The development of research on LO has brought investigators to a new stage of awareness of the roles and importance of this enzyme in biology. Nevertheless, the function of this enzyme in the oxidation and crosslinking of its elastin and collagen substrates in the extracellular space remains inviolate. A number of observations have been made that relate the varied means of biological control of the expression of LO to those of these structural macromolecules that are its extracellular substrates, including, for example, effects of TGF-β, prostaglandins, copper availability, and other effectors that modulate the expression of both LO and elastin and collagen [Smith-Mungo and Kagan, 1998]. Clearly, both LO and its substrates are intimately related on several levels in the production of a structurally sound ECM.

The discovery that LO exists within cell nuclei has refocused much investigative effort on its possible functions within the intracellular space, although much remains to be accomplished in this arena of research. The available data suggest that LO can profoundly affect intranuclear dynamics, possibly impacting upon the control of mitosis, on the regulation of gene expression, and on the uncontrolled proliferation of transformed cells. Since the positive charge of peptidyl lysine can be critical to the interactions of proteins with anionic sites in DNA and RNA, it is not difficult to envision intranuclear, LO-dependent mechanisms, which could affect fundamental cellular activities. The possibility that there is a spectrum of nuclear protein targets of LO remains to be tested and this certainly will be a subject of considerable interest to many in the field. As noted, the loss of charge is a common consequence of the LO-catalyzed oxidation and the histone transacetylase-catalyzed acetylation of peptidyl lysine in histone substrates. However, histone acetylation is reversible by the action of histone deacetylases, whereas there is no evidence for the conversion of peptidyl AAS to peptidyl lysine by enzyme-catalyzed reductive amination. The persistent lack of information about the three-dimensional structure of LO remains a major obstacle to be overcome if we are to gain detailed insights into structure–function relationships of this catalyst. Clearly, hypothesis developed in this review about intramolecular interactions which could maintain the catalytic quiescence of the proenzyme and which may explain the specificity of LO for basic protein substrates can and doubtlessly will be experimentally tested. Critical analyses of these hypotheses will require the availability of the three dimensional structure of both proLO and LO as well as selective mutation of suspect residues of each. It is expected that this valuable structural information will become available in the near future.

The recent recognition that several LO-like proteins exist in nature adds additional exciting dimensions to this area of research. Should catalytic potential be demonstrated in each of these proteins, the variation in sequence,

structure, and charged residue content of these species in comparison to LO suggests that the substrate specificities of these putative enzymes may be distinct from that of LO. Since specific members of this group of LOXL proteins have been observed within neuronal and other tissues in which LO does not appear to be expressed, the possibilities that these enzymes have a variety of non-matrix substrates and may strongly influence the development and homeostatic condition of these tissues are intriguing.

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