The Formation of Lysine Tyrosylquinone (LTQ) Is a Self-Processing Reaction. Expression and Characterization of a *Drosophila* Lysyl Oxidase†

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ABSTRACT: Recent work in our laboratory has established methods for the expression and purification of a recombinant form of *Drosophila* lysyl oxdidase (rDMLOXL-1) [Molnar, J., Ujfaludi, Z., Fong, S. F. T., Bollinger, J. A., Waro, G., Fogelgren, B., Dooley, D. M., Mink, M., and Csiszar, K. (2005) *J. Biol. Chem. 280*, 22977−22985]. Previous investigations on the expression and purification of recombinant forms of lysyl oxidase [Kagan, H. M., Reddy, V. B., Panchenko, M. V., Nagan, N., Boak, A. M., Gacheru, S. N., and Thomas, K. (1995) *J. Cell. Biochem*. *59*, 329−338] and lysyl oxidase-like proteins [Jung, S. T., Kim, M. S., Seo, J. Y., Kim, H. C., and Kim, Y. (2003) *Protein Expression Purif. 31*, 240−246] [Molnar, J., Fong, K. S. K., He, Q. P., Hayashi, K., Kim, Y., Fong, S. F. T., Fogelgren, B., Szauter, K. M., Mink, M., and Csiszar, K. (2003) *Biochim. Biophys. Acta 1647*, 220−224] have been reported in the literature. However, this is the first time that an expression system has been developed yielding sufficient amounts of a recombinant lysyl oxidase for detailed characterization. rDmLOXL-1 is secreted into the medium from S2 cells, and the protein is readily purified by Cibacon blue affinity chromatography yielding 10 mg of protein per liter of medium. The protein, as initially purified, is inactive and has no detectable copper or cofactor present. Following aerobic dialysis against copper, the protein is active and displays an electronic absorption spectrum with λ_{max} at 504 nm, consistent with the presence of an organic cofactor. Addition of phenylhydrazine to the copper-loaded protein produced a high-affinity adduct with λ_{max} at 454 nm. Comparison of the resonance Raman spectra of this adduct and a phenylhydrazine-labeled model compound of lysine tyrosylquinone (LTQ) establishes that the cofactor in the active, copper-containing enzyme is LTQ. Collectively, the data demonstrate that LTQ biogenesis most likely occurs by self-processing chemistry, requiring only the precursor protein, copper, and oxygen. Electron paramagnetic resonance and circular dichroism spectroscopy were used to characterize the Cu(II) site in rDmLOXL-1. The data are consistent with a tetragonal Cu(II) site with nitrogen and oxygen ligands. Recombinant DmLOXL-1 displayed significant activity toward tropoelastin and a wide variety of amines including polyamines and diamines. β-aminoproprionitrile (βAPN), a well-known irreversible inhibitor of mammalian lysyl oxidases, is also a potent inhibitor of rDmLOXL-1. Results from this investigation have important implications for the lysyl oxidase family.

Lysyl oxidase (LOX; EC 1.4.3.13)[1] is a copper-containing amine oxidase well-known for its involvement in the posttranslational maturation of extracellular connective tissue (*1*, *2*). Lysyl oxidase catalyzes the oxidative deamination of specific lysine residues of extracellular matrix proteins to generate peptidyl α-aminodipic-δ-semialdehyde or allysine. These aldehyde residues then can spontaneously condense with other lysine residues and peptidyl aldehydes on neighboring polypeptides to generate the cross-linkages found in, for example, mammalian collagen and elastin (*1*, *3*). Furthermore, LOX has been implicated in a variety of cellular processes, including differentiation, development, motility, and reversion of *ras*-transformed cells (*1*, *4*−*6*).

The LOX family includes four lysyl oxidase-like proteins (LOXLs) identified as LOXL, LOXL2, LOXL3, and LOXL4 (*4*, *7*, *8*). Lysyl oxidase-like proteins contain the LOX catalytic domain and are frequently coexpressed with LOX (*4*). The physiological importance of LOX and the LOX-like (LOXLs) proteins has been established by multiple studies of the effects of β-aminopropionitrile (βAPN), which is an irreversible inhibitor of LOX and LOXLs (*9*−*11*). βAPN treatment has been shown to perturb connective tissue maturation, development, cell proliferation, morphogenesis, cell migration, and wound healing (*4*, *12*−*15*). At this time, it is difficult to deconvolute the effects of LOX and LOXLs inhibition. In principle, studies of knockouts and antisense interference could help distinguish the physiological roles of the LOX and LOXLs proteins. The phenotypes of LOX knockout mice are consistent with an essential role for LOX in elastin and collagen cross-linking, as expected, but did not reveal additional roles for LOX (*16*, *17*). Antisense interference experiments were consistent with a role for LOX in differentiation and transformation (*16*, *17*). Abnormal LOX activity has been associated in such diseases as athero-

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[1] Abbreviations: LOX, lysyl oxidase; rDmLOXL-1, recombinant lysyl oxidase from *Drosophila*; LOXLs, lysyl oxidase-like; LTQ, lysine tyrosylquinone; rR, resonance Raman; βAPN, β-aminopropionitrile fumarate; TBS, tris-buffered saline; CBA, cibacron blue agarose; HRP, horseradish peroxidase.

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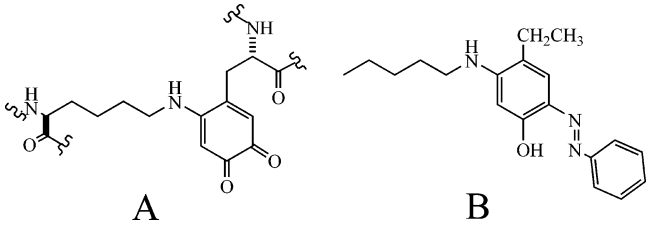

FIGURE 1: Structures of (A) LTQ cofactor and (B) phenylhydrazine-labeled LTQ model compound.

sclerosis, pulmonary and hepatic fibrosis, and disorders of the skin and joints (*1*, *9*, *16*). Consequently, the development of specific inhibitors of LOX would be of substantial interest. Designing specific agents that potentially control or limit the formation of connective tissue would be useful in the treatment of certain fibrotic diseases.

Purification of mammalian LOX is difficult due to aggregation and solubility problems, but the eventual discovery that LOX was soluble and active in buffers containing 6 M urea paved the way for the successful development of purification protocols. Investigations with bovine aorta lysyl oxidase provided the initial insight into its catalytic and structural properties (*18*−*20*). The active form of the enzyme migrates between 28 and 32 kDa, depending on species, as determined by SDS−PAGE, and contains one tightly bound Cu(II) and a covalently bound cofactor identified as lysine tyrosylquinone (LTQ) in its active site (Figure 1). The biogenesis of LTQ is thought to proceed in a similar manner as in the biogenesis of the copper-containing proteins containing 2,4,5-trihydroxyphenylalanine quinone (TPQ) as their cofactor, although there is no direct evidence available on the mechanism of LTQ biogenesis. Our hypothesis is that LTQ biogenesis is a self-processing reaction involving copper-mediated or -assisted oxidation of a specific peptidyl tyrosine residue and formation of a covalent cross-link with a conserved lysine residue. Tyr 349 and Lys 314 have been identified as the precursors for LTQ in bovine aorta lysyl oxidase (*20*).

The cloning and sequencing of the cDNA of LOX from rat aorta was first accomplished in the early 1990s, and the human, mouse, and chick cDNA sequences were subsequently determined (*1*). A significant degree of sequence homology exists among the species. The LOX family has three strictly conserved regions in the C-terminal end where the residues of the copper-binding domain, the carbonyl cofactor domain, and the cytokine receptor-like domain are present (*4*). The postulated copper-binding histidines, the lysine and tyrosine residues that compose LTQ, and the 10 C-terminal cysteines are conserved between the two *Drosophila* lysyl oxidases and the human enzyme (Figure 2). In contrast, the N-terminal regions of LOX family members show very little sequence homology (*4*) owing in part to four scavenger receptor cysteine-rich (SRCR) domains found in LOXL2, LOX3, and LOXL4, which are missing in LOX itself. The N-terminal domains are thought to modulate protein−protein interactions, substrate specificity, activity, and the processing of the LOX protein in its particular physiological context (*4*, *15*, *21*).

Despite the substantial advances in our understanding of the structure and function of LOX and LOXLs and the reports of success in expressing and purifying recombinant human LOX (*22*) and LOXLs proteins from *Escherichia coli* inclusion bodies, (*21*, *23*, *24*) detailed structural, spectroscopic, and mechanistic studies of these proteins have been hampered by insufficient amounts of homogeneous enzyme. We have characterized the genes, domain structures, and expression patterns of the two lysyl oxidases in *Drosophila*, *Dmloxl-1* and *Dmloxl-2* (Genbank Accession Nos. AJ295625 and AJ295626). Our initial results are consistent with a role for *Dmloxl-1* in heterochromatinization and gene expression regulation (*15*). To obtain LOX for structural, spectroscopic, and mechanistic experiments, we have expressed, purified, and characterized a recombinant form of a *Drosophila* lysyl oxidase (rDmLOXL-1).[2] This is the first time an expression system had been developed yielding sufficient amounts of recombinant protein for characterization. We also present the first direct evidence that LTQ biogenesis is a self-processing reaction.

MATERIALS AND METHODS

Expression and Purification of Recombinant Lysyl Oxidase (rDmLOXL-1). The expression and purification of recombinant lysyl oxidase (rDmLOXL-1) was first carried out using the methods developed in our laboratory. These results have recently been reported in the literature (*15*).

For copper loading, rDmLOXL-1 in TBS−6 M urea was dialyzed sequentially against TBS−2 M urea containing 10 mM 2,2′-dipyridine for 1 day, 0.5 mM $CuSO_4$ for 2−4 days, and 10 mM EDTA for 1 day. The sample was then dialyzed against TBS−2 M urea for 1 day to remove EDTA. Metal-free rDmLOXL-1 (apo-rDmLOXL-1) was prepared similarly except the dialysis step against $CuSO_4$ was omitted. Copper content of the samples was measured by atomic absorption (AA) spectroscopy (Buck Scientific 210 VGP). Standards were prepared using copper sulfate dissolved in TBS−2 M urea. Five readings of each sample were taken and averaged for comparison to the standard curve.

Protein concentrations were estimated spectrophotometrically by the absorbance at 280 nm using a predicted extinction coefficient (ExPASy); an OD of 1.56 is equivalent to 1.0 mg/mL. The purity and the molecular weight of rDmLOXL-1 were determined by SDS−PAGE analysis on a Pharmacia Phastsystem. SDS gels (8−25%) were stained with Coumassie blue. Concentration of samples was carried out using a Microcon-30 microcentrifugal filtering device (Millipore). LTQ content was calculated using the extinction coefficient of 15.4 mM^{-1} cm^{-1} as reported for the phenylhydrazine-labeled adduct from bovine aorta LOX (*20*).

Spectroscopy. Freshly prepared samples of the copper-loaded and apo forms of rDmLOXL-1 in TBS−2 M urea were concentrated to the desired volume using a Microcon-30 microcentrifugal filtering device (Millipore). All UV−vis data were collected on either a Hewlett-Packard HP8452 or HP8453A spectrophotometer equipped with a thermostated cell. EPR spectra were recorded using a Bruker EMX spectrometer interfaced to a PC computer. Either 150 μL of

[2] *Drosophila melanogaster* contains two genes designated *Dmloxl-1* and *Dmloxl-2* that code for two proteins containing the lysyl oxidase catalytic domain. We refer to the protein expressed and purified in this paper as rDmLOXL-1 to reflect its genetic origin. Consistent with the description of mammalian enzymes, we refer to both *Drosophila* proteins as belonging to the lysyl oxidase class and use this term generically to describe these proteins.

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                1                                                    50
Human           DLVADPYYIQ  ASTYVQKMSM  YNLRCAAEEN  CLASTAYRAD  ....VRDYDH
DmLOXL-1        DLEIGLVDIE  RTARLEAVPM  SRLTCAMEEH  CVSADAYEIR  RTNPH...AA
DmLOXL-2        DLVVDYLEIE  QTAHLEDRPM  LLMQCAMEEN  CVANEAYQIQ  RDDPHWRYRS
               51                                                   100
Human           RVLLRFPQRV  KNQGTSDFLP  SRPRYSWEWH  SCHQHYHSMD  EFSHYDLLDA
DmLOXL-1        RILLRFSVKA  SNVGTADVSP  YANYKEWVWH  QCHRHYHSMN  VFATFDVYDL
DmLOXL-2        RRLLKFTAAA  INAGNADFRP  FKEKSQWEWH  MCHMHFHSME  VFATFDIFNL
               101                                                  150
Human           NTQRRVAEGH  KASFCLEDTS  CDYGYHRRFA  CTAHT.QGLS  PGCYDTYGAD
DmLOXL-1        NY.RKVAQGH  KASFCLMDSE  CRPGVRQKYT  CGNTT.QGIS  VGCADTYTDV
DmLOXL-2        RG.IKVAQGH  KASFCLEDSN  CLPGVAKKYN  CANSGDQGIS  INCSDVYLYN
               151                                                  200
Human           IDCQWIDITD  VK.PGNYILK  VSVNPSYLVP  ESDYTNNVVR  CDIRYTGHHA
DmLOXL-1        LDCQWVDVTR  VPINRRYILR  VALNPEYKLG  EISFENNGAE  CLLDYTGVRQ
DmLOXL-2        LDCQWVDVTD  L.IPGTYVLK  IAINPEFKVA  EMNYDNNAAI  CDLIYTA..N
               201
Human           YASG..C
DmLOXL-1        TTRIFNC
DmLOXL-2        FARVQNC
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FIGURE 2: Lineup of the catalytic domains of *Drosophila* DmLOXL-1 and DmLOXL-2 with human lysyl oxidase. Conserved cysteines (yellow), copper-binding region (blue), and lysine and tyrosine that form the LTQ cofactor (red).

copper-loaded rDmLOXL-1 (9.8 mg/mL) or 140 μL of apo-rDmLOXL-1 (17.9 mg/mL) in TBS−2 M urea was placed in EPR tubes. The data were collected at 77 K. Circular dichroism (CD) spectra were recorded using a JASCO J-710 spectropolarimeter. A volume of 125 μL of either copper-loaded rDmLOXL-1 (9.8 mg/mL) or apo rDmLOXL-1 (17.9 mg/mL) was placed in a semi-micro-masked cuvette (1 cm path length). Spectra were collected at room temperature. Resonance Raman (rR) spectra were obtained using a Coherent Innova 400 Argon ion laser and a Spex Triplemate spectrophotometer equipped with a CCD detector. A sample of rDmLOXL-1 (0.5 mg/mL) was incubated with a 10-fold molar excess of phenylhydrazine in the dark for 60 min. The solution was then extensively dialyzed against TBS−2 M urea and then concentrated down to ~50 μL for data collection. A model compound of phenylhydrazine-labeled LTQ (kindly provided by Professor Judith Klinman, University of Californina, Berkeley, CA) (Figure 1) was prepared by dissolving a small amount (~5 mg) in 5 μL of acetonitrile. Two microliters of the dissolved compound were further diluted into 100 μL of TBS−2 M urea buffer. Data were collected between 3 and 60 min using an excitation wavelength at 457.9 nm at 40 mW. Raman frequencies were calibrated relative to an aspirin standard using Grams (Thermo Galactic Industries, NH) software. Background spectra of TBS−2 M urea were subtracted from each data collection.

Activity Assays. Rates of amine oxidation were measured via the rate of hydrogen peroxide formation as previously described (*15*). All substrates used in Table 1 were prepared in TBS and purchased from Sigma. Tropoelastin was prepared as described in ref *25*. A volume of 100 μL of TBS containing Amplex Red, HRP, and substrate was placed in the well of a microplate. Final substrate concentrations were 5 mM for small molecular weight amines and 0.5 mg/mL for tropoelastin. Under these conditions, the amount of tropoelastin was sufficient to saturate the enzyme. Reactions were started by adding 100 μL TBS containing 0.5 μg of rDmLOXL-1 and 40 mM urea with or without βAPN. Control reactions for the survey of substrates contained 0.25 mM βAPN, which served as a control for background. Reactions for the dose response study of βAPN contained 0−0.25 mM βAPN. Assays were run in quadruplicate and reported as mean ± 1 standard error. Data were fit by linear regression (Origin 7.0, Northampton, MA).

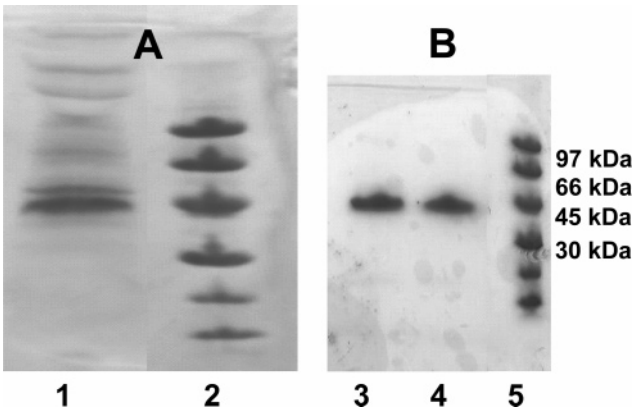


FIGURE 3: SDS/PAGE showing overexpressed recombinant DmLOXL-1. (A) Lane 1, *Drosophila* S2 media (concentrated 10×; lane 2, molecular weight standards. (B) Lanes 3 and 4, purified rDmLOXL-1, after CBA column, sample run in duplicate; lane 5, molecular weight standards.

RESULTS

Expression and Purification of rDmLOXL-1. Following induction by copper, the recombinant S2 cell line, created by the methods described in ref *15*, secretes lysyl oxidase into the medium. The recombinant enzyme was readily purified by Cibacron blue affinity chromatography yielding 10 mg of the protein per liter of medium. SDS/PAGE analysis of the recombinant DmLOXL-1 protein gives a single band with an apparent molecular weight of ~38 kDa (Figure 3), in good agreement with the predicted value of 38.5 kDa from the primary sequence (ExPASY). This "*as purified*" form of the protein has no detectable copper or cofactor present and displayed no amine oxidase activity. The addition of copper to the "as purified" form did not stimulate LTQ formation. We were surprised by this result and suspected that other metals may be bound in the active site. Therefore, prior to the biogenesis experiments the "as purified" form of the protein was dialyzed against 2,2′-dipyridine and EDTA to remove any adventitiously bound metal ions: this form of the protein is designated "apo". Neither the "as purified" form of the protein (Figure 5) nor

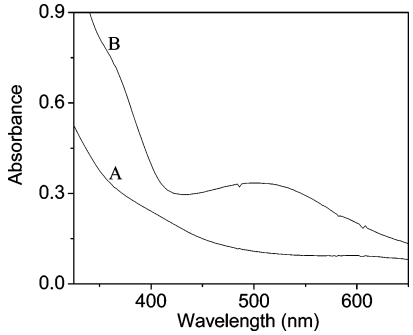


FIGURE 4: Absorption spectra of (A) apo-rDmLOXL-1 and (B) rDmLOXL-1 in TBS−2 M urea. Spectra were normalized for protein concentration (9.7 mg/mL).

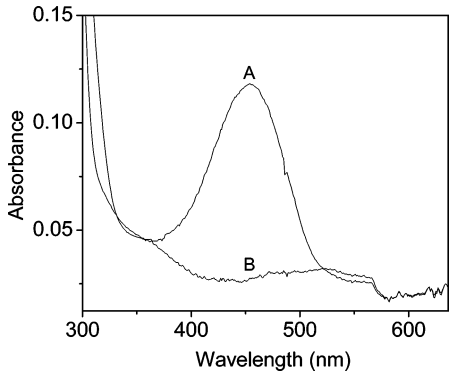


FIGURE 5: UV−vis spectra showing (A) rDmLOXL-1 (0.5 mg/mL) after the addition of a 10-fold excess of phenylhydrazine and (B) the "as purified" form of rDmLOXL-1 (0.5 mg/mL) in TBS−2 M urea.

the apo form of rDmLOXL-1 (Figure 4) displays any detectable electronic transitions in the visible region. The lack of any spectral features between 300 and 600 nm indicates that the LTQ cofactor, which has a characteristic absorption band near 500 nm, is absent. This inference is supported by the lack of activity and the observation that no spectral changes were observed following addition of phenylhydrazine to either the "as purified" or the apo forms of the protein (data not shown).

Activation and LTQ Biogenesis. Several protocols for loading the protein with copper were explored, and the most successful for incorporating copper into the protein proved to be a four-step dialysis treatment (see Materials and Methods). A copper content of 0.6 g atoms of copper per monomer of rDmLOXL-1 (as determined by atomic absorption spectroscopy) could be achieved with this methodology. The copper-containing protein was pink in color and displayed a visible absorption spectrum with λ_{max} at 504 nm (Figure 4). This electronic transition is consistent with the presence of an organic cofactor and strongly resembles both the visible absorbance spectrum attributed to the oxidized form of a lysyl oxidase isolated from bovine aorta and a synthetic model compound of LTQ, which has λ_{max} at 506 nm in urea buffer (*20*). Importantly, the pink, copper-containing form of the enzyme was also catalytically active against a variety of substrates (vide infra), which is strong evidence for the presence of LTQ. Because the homogeneous preparations of rDmLOXL-1 used in these studies are purified in 6 M urea and the biogenesis experiments are conducted in 2 M urea, it is highly unlikely that any enzymatic activity, or protein−protein interactions, are involved. Collectively, these data constitute the first direct evidence that the biogenesis of the cofactor in rDmLOXL-1 is a self- processing event.

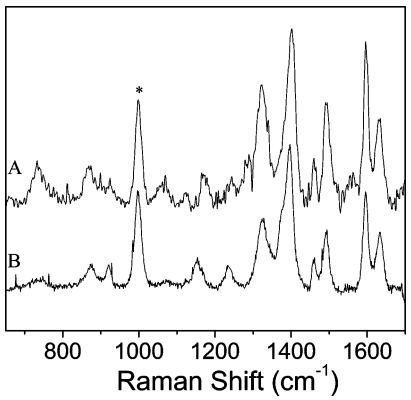


FIGURE 6: Resonance Raman spectra of (A) the phenylhydrazine derivative of rDmLOXL-1 and (B) the LTQ−phenylhydrazine model compound prepared in TBS−2 M urea. Excitation wavelength 457.9 nm at 40 mW power. * = solvent peak.

Derivatization of copper amine oxidases with phenylhydrazine is a commonly used technique to quantitate the amount of the cofactor present in the processed enzyme. Reaction of recombinant DmLOXL-1 with phenylhydrazine produced an intense yellow chromophore (λ_{max} = 454 nm, Figure 5). In this experiment, 57% of LTQ in the rDmLOXL-1 was titratable by phenylhydrazine using a calculated extinction coefficient previously reported for the phenylhydrazine-labeled LOX from bovine aorta (*20*). The results correlate well with the amount of copper reported for this sample (see below).

Confirmation of the Cofactor as LTQ. Some TPQ-containing amine oxidases, particularly the "lysyl oxidase" from *Pichia pastoris* (PPLO), display activities toward lysyl oxidase substrates; in the case of PPLO, the activities are comparable to that of the mammalian lysyl oxidase (*26*). Since the biogenesis of TPQ has been established to be a self-processing reaction requiring only Cu(II) and dioxygen (*27*, *28*), it is therefore essential to unambiguously identify LTQ as the product of the reaction of the precursor protein with Cu(II) and dioxygen. rR spectroscopy of native quinoproteins or their derivatives with phenylhydrazine reagents is a well-established method for the identification of the quinone cofactors (*20*, *29*, *30*). Accordingly, the rDmLOXL-1-phenylhydrazine derivative was prepared (Figure 5, λ_{max} = 454 nm). This spectrum is identical to the spectrum of the LTQ−phenylhydrazine adduct of bovine aorta LOX (*20*). Resonance Raman spectra were obtained on the phenylhydrazine-labeled, copper-loaded form of rDmLOXL-1 and on the corresponding LTQ model compound using an excitation wavelength at 457.9 nm in TBS−2 M urea (Figure 6). The rR spectra of the protein and of the model compound are essentially identical, particularly in the region between 1200 and 1640 cm^{-1}. Disregarding the differences in the signal-to-noise ratio, the spectra in Figure 6 are nearly superimposable with respect to frequencies and relative intensities, which establishes that the cofactor in rDmLOXL-1 is LTQ.

Spectroscopic Characterization of the Cu(II) Site. The availability of rDmLOXL-1 permits the characterization of the copper site. Figure 7 shows the EPR spectrum of the copper-loaded form of rDmLOXL-1 and the spectrum of apo-

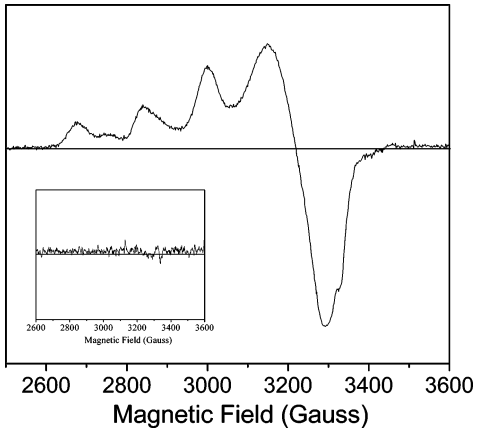


FIGURE 7: X-band EPR spectra of rDmLOXL-1 prepared in TBS−2 M urea. Inset: apo-rDmLOXL-1 in TBS−2 M urea. Protein concentrations were 9.7 and 17.9 mg/mL, respectively. Experimental conditions: 77 K, 9.33 GHz, 1.2 mW power, 10 accumulations. For apo: 77 K, 9.32 GHz, 6.32 mW power, 3 accumulations.

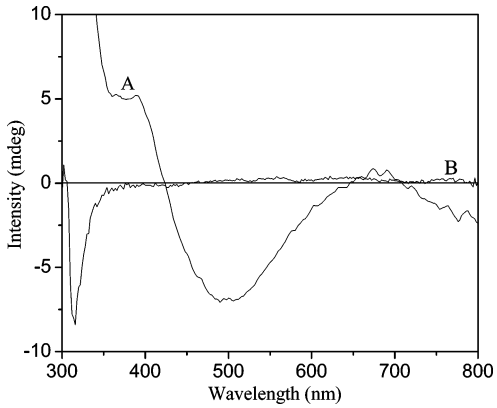


FIGURE 8: CD spectra of (A) rDmLOXL-1 and (B) apo-rDmLOXL-1 in TBS−2 M urea at 25 °C. Experimental conditions: resolution, 2 nm; response time, 1 s; bandwidth, manual; accumulations, 15. Spectra were normalized for protein concentration.

rDmLOXL-1 (inset). This latter spectrum is featureless, as expected. Inspection of the spectrum of the Cu(II)-containing enzyme indicates that $g_{\|} > g_{\perp} > 2.0$, which is consistent with a tetragonal Cu(II) site (*31*, *32*). Comparison to the $g_{\|}$, $A_{\|}$ correlation diagrams of Peisach and Blumberg (*33*) suggests a N_3O coordination in the equatorial plane. Double integration of the signal indicated 0.55 g atoms of copper per monomer, in good agreement with atomic absorption results. The CD data of the copper-loaded enzyme shows a strong negative band at 500 nm, corresponding to LTQ, and a less intense, broader band from 600 to 800 nm, which is assigned to Cu(II) d−d transitions (Figure 8). The energy of these ligand-field transitions are consistent with the tetragonal N_3O coordination inferred from the EPR parameters. The CD spectrum of the apo form of the enzyme (Figure 8) is featureless in the 400−800 nm region, indicating the absence of LTQ and copper in the active site.

Substrate Specificity and Inhibition of rDmLOXL-1. Measurements of the initial rates of oxidation of potential substrates are summarized in Table 1. It is well-known that mammalian LOX proteins oxidize collagen and elastin substrates to generate the necessary steps for cross-linking of these connective tissue proteins (*1*). *Drosophila* rDmLOXL-1 displays significant activity toward a tropoelastin substrate, even though tropoelastin is not considered a native substrate. However, it is evident from Table 1 that a variety of amines, including polyamines, are oxidized by rDmLOXL-1. The physiological substrates of rDmLOXL-1 are not known, but the ability of the enzyme to catalyze the deamination of other natural, nonpeptidyl substrate amines may be functionally important. βAPN is recognized as a selective inhibitor of LOX and LOXLs proteins. The rate of cadaverine oxidation after the addition of βAPN is reduced; catalytic activity was significantly reduced upon addition of micromolar amounts (<2 μM) of βAPN (data not shown), as has been observed for mammalian lysyl oxidases and lysyl oxidase-like proteins.

Table 1: Initial Rates of Amine Oxidation by rDmLOXL-1

monoamines		diamines	
histamine	37.4 ± 2.1[a]	putrescine	79.9 ± 4.6
agmatine	64.5 ± 5.2	cadaverine	115.7 ± 3.9
polyamines		lysines	
spermine	36.2 ± 10.9	L-lysine	49.9 ± 1.8
spermidine	57.2 ± 1.1	tropoelastin	28.6 ± 3.3

[a] Reported rates are picomoles of hydrogen peroxide formed per minute per micrograms of rDmLOXL-1.

DISCUSSION

The investigation of redox-active amino acids, and post-translationally introduced redox cofactors derived from amino acids, has emerged as an active and expanding area in enzymology. Figure 9 shows the structures of several amino acid-derived cofactors that are involved in catalysis. Some of these, such as TPQ and the Tyr-Cys radical cofactor of galactose oxidase, have been demonstrated to arise from remarkable self-processing reactions of the proteins themselves (*27*, *34*, *35*), while others, such as TTQ, require the action of separate processing enzymes (*36*). Despite its similarity to TPQ, the origins of LTQ were uncertain owing to the lack of a source of unprocessed lysyl oxidase where the conserved Tyr and Lys residues are unmodified. The data presented herein establish that LTQ is produced by a self-processing reaction requiring only the addition of Cu(II) and oxygen to the unprocessed protein. The rR spectra (Figure 6) definitively establish that LTQ is the product of the reaction with Cu(II) and oxygen. Plausible mechanisms for LTQ formation are outlined in Scheme 1. We have previously argued that pathway 1A should be preferred because nucleophilic attack at C2, as required by pathway 1B, is unprecedented in TPQ model chemistry and has not been observed in amine oxidase reactions (*20*). Consequently, both the initial oxidation of the tyrosine ring to a dopa intermediate (Scheme 1, pathway 1A), and the formation of the covalent cross-link between the lysine residue and the oxidized tyrosine may proceed without the assistance of other proteins or cofactors.

This result for DmLOXL-1 is also very likely to be the case for the mammalian enzymes as well. Although mammalian lysyl oxidases are synthesized as a proenzyme and are active following the cleavage of the propeptide, Kagan and co-workers have shown that the propeptide in rat lysyl oxidase is not essential to the folding, processing, and secretion of the mature, active lysyl oxidase (*22*). Hence, the capability for self-processing is an intrinsic property of the enzyme sequences in the *Drosophila* protein and in

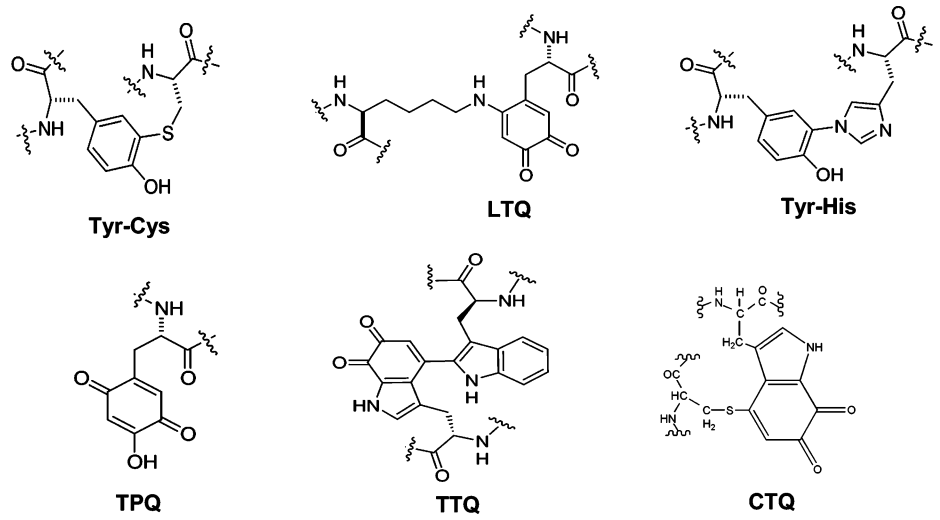
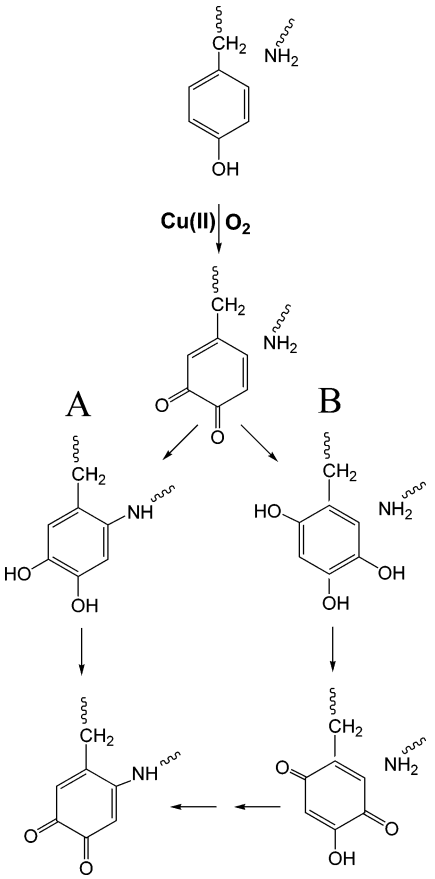


FIGURE 9: Structures of amino acid-derived cofactors and radicals involved in catalysis. (Adapted from ref *35*).

Scheme 1. Proposed Mechanism for LTQ Formation



mammalian lysyl oxidases. Furthermore, it is highly likely that LTQ formation in the "lysyl oxidase-like" proteins is also a self-processing reaction, given the sequence homologies in the putative catalytic domains of these proteins. Clearly, considerable work remains to be done to define the mechanism of LTQ biogenesis more completely, and experiments along these lines are currently underway in our laboratory.

The energies of the Cu(II) ligand-field transitions (Figure 8) and the EPR parameters indicate that the electronic structure of Cu(II) in rDmLOXL-1 is similar to that of Cu(II) in the TPQ-containing amine oxidases. This conclusion is consistent with the multiple histidine imidazole coordination inferred from sequence alignments. Further, the data are consistent with a structure for the Cu(II) site in rDmLOXL-1 similar to those of the Cu(II) sites in amine oxidases (Figure 10). If, as is the case for amine oxidases, the active-site copper is responsible for oxidation of the precursor tyrosine in lysyl oxidase, the copper and LTQ cofactors must be in close proximity in the active site.

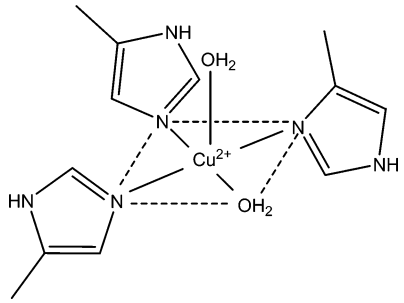


FIGURE 10: Cu(II) site in amine oxidases.

rDmLOXL-1 is very active against at least one of the important physiological substrates of the mammalian lysyl oxidases, tropoelastin. It is also active against a wide variety of primary amines (Table 1). The actual physiological substrates of DmLOXL-1 are not known and could include both protein lysine residues and primary amines. Additional experiments are warranted to define its biological role in the development and life of the organism.

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