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Human Copper-Dependent Amine Oxidases

Joel Finney, **Hee-Jung Moon**, **Trey Ronnebaum**, **Mason Lantz**, and **Minae Mure***

Department of Chemistry, The University of Kansas, Lawrence, KS 66045, USA

Abstract

Copper amine oxidases (CAOs) are a class of enzymes that contain Cu^{2+} and a tyrosine-derived quinone cofactor, catalyze the conversion of a primary amine functional group to an aldehyde, and generate hydrogen peroxide and ammonia as byproducts. These enzymes can be classified into two non-homologous families: 2,4,5-trihydroxyphenylalanine quinone (TPQ)-dependent CAOs and the lysine tyrosylquinone (LTQ)-dependent lysyl oxidase (LOX) family of proteins. In this review, we will focus on recent developments in the field of research concerning human CAOs and the LOX family of proteins. The aberrant expression of these enzymes is linked to inflammation, fibrosis, tumor metastasis/invasion and other diseases. Consequently, there is a critical need to understand the functions of these proteins at the molecular level, so that strategies targeting these enzymes can be developed to combat human diseases.

Keywords

copper amine oxidase; lysyl oxidase; quinoprotein

INTRODUCTION AND REACTION MECHANISM OF TYROSINE-DERIVED QUINONE COFACTORS

Copper amine oxidases (CAOs) are copper- and quinone-dependent enzymes that catalyze the oxidative deamination of primary amine functional groups to aldehydes, concomitantly producing hydrogen peroxide and ammonia. Currently, they are grouped into two nonhomologous subgroups based on the nature of their organic cofactors, namely 2,4,5-trihydroxyphenylalanine quinone (TPQ)-dependent CAOs and the lysine tyrosylquinone (LTQ)-dependent lysyl oxidase (LOX) family of proteins (1). A number of reviews of CAOs and LOXs are available (1-11), including an excellent recent review by Klema and Wilmot that focuses on structural biology studies of the mechanisms of TPQ biogenesis and catalysis of amine oxidation in the TPQ-containing bacterial and yeast CAOs (12). In the present review, we will first briefly summarize the current understandings of the mechanisms of 1) TPQ and LTQ biogenesis and 2) amine oxidation by CAOs and LOX. We will also discuss their commonly used *in vitro* inhibitors. We will then highlight recent research developments concerning human CAOs and the human LOX family of proteins, with an emphasis on their proposed roles in disease and health defects.

*To whom correspondence should be addressed: Minae Mure, Department of Chemistry, The University of Kansas, 1251 Wescoe Hall Drive, 2054 Malott Hall, Lawrence, KS 66045, USA, Tel.: (785) 864-2901, Fax: (785) 864-5396; mmure@ku.edu.

Tyrosine-derived Quinone Cofactors: TPQ and LTQ

TPQ and LTQ (Figure 1) were discovered by Klinman and coworkers as the respective organic cofactors of a CAO isolated from bovine plasma and a LOX isolated from bovine calf aorta (13,14). Both cofactors are post-translationally derived from a conserved active-site tyrosine residue via an autocatalytic mechanism requiring only Cu^{2+} and O_2 (15,16). Dopaquinone (DPQ) is proposed to be the common intermediate during the biogenesis of TPQ and LTQ, where the 1,4-addition of either water or the ε-amino side chain of a peptidyl lysine residue to DPQ yields TPQ or LTQ, respectively (13,14) (Figure 2). A careful inspection of the reaction product of TPQ biogenesis in the presence of $H_2{}^{18}O$ and $^{18}O_2$ by resonance Raman spectroscopy revealed that the C2 oxygen of TPQ is from solvent water, rather than O_2 (17). In the same study, substantial electron delocalization between the C2 and C4 oxygens of the TPQ cofactor was observed, whereas the C5=O bond had more carbonyl character. These results support a solution study demonstrating that the delocalization directs the addition of substrate amine at the C5 carbonyl group (18).

X-ray snapshot analysis of TPQ biogenesis revealed that the precursor tyrosine and the biogenesis intermediates (i.e. DPQ and the trihydroxybenzene form, i.e. TPQ_{red}) are all ligated to Cu^{2+} (i.e. “on-copper” forms) at their O4 oxygen atoms (19). In the last O_2-oxidation step of TPQ_{red} to TPQ, the TPQ ring finally moves away from the Cu^{2+} binding site and becomes trapped in a hydrophobic wedge-like cavity in the active site; this is the “off-copper” conformation (Figure 3)(described in greater detail under ***Reaction Mechanism***). The conformational change of TPQ is critical for optimal catalytic activity of CAOs, since the on-copper form of TPQ is unable to interact with substrate amines (7,20,21). The factor that drives TPQ to move off Cu^{2+} in the final step of biogenesis remains to be elucidated.

In contrast to TPQ, the details of the LTQ biogenesis mechanism (Figure 2) have not been explored, mainly due to the unavailability of diffracting crystals suitable for X-ray crystallography. However, to gain some insight in the intermediacy of DPQ in the biogenesis of TPQ and LTQ, a lysine residue was incorporated into the active site of a bacterial CAO by site-directed mutagenesis, replacing the conserved Asp residue located at the far end of the wedge (22). In this mutant, an LTQ-like quinone was produced instead of TPQ, where the covalent bond between the lysine side chain and DPQ was confirmed by X-ray crystallography (Figure 4). These results not only support the hypothesized common intermediacy of DPQ in the biogenesis of TPQ or LTQ (7,23), but also suggest that at room temperature the DPQ intermediate has sufficient motional flexibility to swing out of the Cu^{2+} site and interact with the ε-amino group of the lysine side chain in the wedge (Figure 4).

Reaction Mechanism of CAOs and LOX in Amine Oxidation

The reaction mechanism of CAOs in the oxidation of primary amines follows a classical ping-pong mechanism involving covalent intermediates formed between TPQ and amines, as well as oxidoreduction reactions of the TPQ cofactor (Figure 5) (7,8,12). A conserved Asp residue acts as an active site base to remove an α-proton from the first covalent intermediate between TPQ and the substrate amine (i.e. a substrate Schiff base), and also serves as a

proton sink to regulate the protonation state of the substrate and the TPQ-derived reaction intermediates, which are essential for optimal catalytic activity (24-26) (Figure 6). The protonation state of the reaction intermediates is also carefully controlled by the two conserved Asn and Asp residues (flanking the conserved precursor Tyr residue in the Asn-Tyr-Asp/Glu consensus sequence)(27,28) and water molecules in the active site (29). The reaction mechanism of amine oxidation by LOX is expected to be similar to CAOs, where an unidentified active site residue with pK_a ~ 7.6 is thought to be the catalytic base for a LOX isolated from bovine aorta (30).

TPQ is expected to have some motional flexibility in the active site, since it is connected to the peptide backbone by a single covalent bond. In the on-copper conformation, the O4 of TPQ ligates to the active site Cu^{2+} and the active carbonyl group at C5 of TPQ faces away from the substrate entry channel and the active site base (Asp)(Figure 3). Therefore, the on-copper TPQ form of CAOs is catalytically inactive. To prevent this, the mobility of the TPQ cofactor and TPQ-derived intermediates is carefully modulated in the active sites of CAOs by hydrogen bonding interactions among the O4 of TPQ, a conserved Tyr in the active site, a conserved Asp (the active site base), and the surrounding hydrophobic wedge-like cavity (Figure 3). These interactions maintain optimal activity by preventing the O4 of the TPQ ring from directly ligating to copper (i.e. by retaining TPQ in the off-copper conformation) (7,26,31-33). In contrast to TPQ, the LTQ cofactor of LOX is covalently linked to the peptide backbone at two positions, and is consequently fixed in one conformation.

Known Inhibitors of CAOs and LOX

CAOs and LOX can be inhibited irreversibly by hydrazine derivatives that form a hydrazone adduct with the active carbonyl group of TPQ and LTQ, mimicking the Schiff base reaction intermediates in the catalytic cycle (Figure 7). The most commonly used *in vitro* inhibitors for these proteins are phenylhydrazine and its derivatives, such as 4-phenylhydrazine, 2,4-phenylhydrazine and 2-hydrazinopyridine.

CAOs can also be inhibited by semicarbazide, which forms a semicarbazone adduct with the TPQ cofactor; therefore, CAOs are often classified as semicarbazide-sensitive amine oxidases (SSAOs) to distinguish them from other amine oxidases, such as monoamine oxidases A and B (maoA and maoB). However, it should be noted that semicarbazide also inhibits LOX from bovine aorta (IC_{50} = 30 μM), which is similar to CAOs from bovine and human plasma (IC_{50} = 50, 100 μM, respectively) (34). Additionally, semicarbazide-induced inactivation of LOX has been shown to induce abnormality in arterial structure and function in mice (35). These experiments indicate that the members of the LOX family of proteins are also likely to be SSAOs.

For the LOX family of proteins, β-aminopropionitrile (BAPN) is one of the most commonly used small molecule inhibitors for *in vitro* and *in vivo* experiments. BAPN is considered a LOX-specific inhibitor (K_i = 6 μM)(36) because it does not inhibit CAOs or flavin-dependent maoA or maoB (34). The IC_{50} for LOX isolated from chick embryo and bovine aorta were reported to be 10 mM and 25 μM, respectively (37,38), while the IC_{50} for LOX-like 2 (LOXL2, a member of the LOX family of proteins) produced in murine myeloma cells was reported to be 3-5 μM (39). For both LOX and LOXL2, the mode of inhibition is

competitive. However, there has been some controversy over the specificity of BAPN toward the LOX family of proteins, as a few groups have reported that BAPN does not inhibit LOXL2 in cell culture (39-42).

Sequences of Human CAOs and the LOX Family of Proteins

Humans have four genes encoding CAOs: AOC1 (diamine oxidase), AOC2 (retina-specific amine oxidase), AOC3 (vascular adhesion protein-1, VAP-1), and AOC4 (a pseudo-gene, truncated in the active site). The translated sequences of AOC2 and AOC3 share 65% identity, but AOC1 only shares ~38% identity with either AOC2 or AOC3 (43). The Tyr precursor for TPQ, the active-site base (Asp), three His for the copper-binding site, and a Tyr residue that has a hydrogen bond interaction with the TPQ cofactor are all conserved (44-46). AOC1 and AOC2 contain predicted secretion signals at their N-termini, while AOC3 does not contain a secretion signal, but has a helical transmembrane (type II) domain.

Humans also possess five genes encoding the LOX family of proteins: *lox* (LOX), *loxl*1 (lysyl oxidase-like 1, LOXL1), *loxl*2 (lysyl oxidase-like 2, LOXL2), *loxl*3 (lysyl oxidase-like 3, LOXL3), and *loxl*4 (lysyl oxidase-like 4, LOXL4). The LOX family of proteins can be grouped into two subgroups based on the nature of their N-terminal domains: LOX and LOXL1 contain a highly basic peptide at their N-termini (termed the propeptide), whereas LOXL2, LOXL3 and LOXL4 each contain four scavenger receptor cysteine-rich (SRCR) domains (Figure 8)(2). There is a conserved bone morphogenetic protein-1 cleavage site between the propeptide and the LOX catalytic domain of LOX and LOXL1 (47), but this site is not conserved in LOXL2, LOXL3 and LOXL4. Moreover, the C-terminal LOX catalytic domains of LOX and LOXL1 share 77% identity and 88% homology, while the C-terminal LOX catalytic domains of LOXL2, LOXL3 and LOXL4 share 71-72% identity and 84-88% homology. The LOX catalytic domains of the two subgroups share 51-54% identity and 64-68% homology. The precursor residues of the LTQ cofactor (Lys and Tyr) and the predicted Cu^{2+}-binding site (His-X-His-X-His) are conserved in all five family members. Additionally, all LOX family members possess an N-terminal secretion signal, but lack predicted transmembrane domains; therefore, they are generally considered to be secreted proteins. Whereas CAOs are known to be homodimers (reviewed in (12)), the oligomeric status of the LOX-family of proteins has not been characterized.

AMINE OXIDASE (COPPER-CONTAINING) FAMILY

Amine oxidase, copper-containing 1, AOC1 (EC1.4.3.22)

AOC1 was first described as a histaminase in 1929 (48), and is synonymous with diamine oxidase (DAO1), kidney amine oxidase (KAO), amiloride-sensitive amine oxidase precursor, and amiloride-binding protein (ABP1). AOC1 is mainly expressed in the kidney, placenta, intestine, thymus, and seminal vesicles (49), and is proposed to be released from the kidney and intestinal epithelial cells through basolateral vesicles at the plasma membrane in response to an external stimulus, such as heparin (50). AOC1 is the main enzyme responsible for metabolism of ingested histamine, and is implicated in histamine intolerance (51). Additionally, AOC1 is highly expressed in the placenta during a healthy pregnancy (1000-fold higher than in other organs), and low AOC1 activity has been linked to high-risk

pregnancies (52). A recent mice study indicated that AOC1 plays a critical role in homeostasis of histamine and putrescine levels (preferred substrates of AOC1, see below), which is essential for decidualization (i.e. remodeling of the endometrium in preparation for embryo implantation) and embryo implantation itself (53). In that study, the expression of AOC1 was shown to be under the control of estrogen via CCAAT/enhancer-binding protein.

The biochemistry of AOC1 has been studied mostly using recombinant protein produced in insect cells (49). The preferred substrates for AOC1 are histamine (K_m = 2.8 ± 0.07 μM), 1-methylhistamine (K_m = 3.4 ± 0.3 μM), and putrescine (K_m = 20 ± 1 μM) (Table 1). Longer polyamines, such as benzylamine (a common *in vitro* substrate for serum CAOs and LOX) and spermidine, are poor substrates for AOC1 (Table 1).

The X-ray crystal structure of AOC1 was solved at 1.8 Å resolution (43,54), using a template model (AOC3, PDB entry 2c10 (55)) and a sequence alignment of AOC1 and AOC3 using CHAINSAW (56). AOC1 is a homodimer of two 85-kDa subunits, and the crystal structure revealed the presence of an intermolecular disulfide bridge linking Cys736 of the A and B subunits. This intermolecular disulfide bridge has been detected in the crystal structures of AOC3 and a plant CAO, but is absent in bacterial and yeast CAOs.

By modeling histamine as the off-copper TPQ-Schiff base intermediate, it was discovered that Asp186 might be within hydrogen bonding distance (3.2 Å) of the imidazole nitrogen of histamine (43). Therefore, it was postulated that Asp186 might play an important role in binding diamine substrates in the active site of AOC1, though further studies are necessary to evaluate this hypothesis. The crystal structure also confirmed that AOC1 is *N*-glycosylated at Asn110, Asn538 and Asn745 (three of the four predicted *N*-glycosylation sites), and the electron density suggests that Asn168 (the remaining predicted site) is not *N*-glycosylated (43). The importance of the *N*-linked glycans for the biochemical and physiological functions of AOC1 has not been examined.

Crystal structures of AOC1 complexed with berenil (K_i = 13 ± 1 nM) or pentamidine (K_i = 290 ± 19 nM) have also been solved (43). Berenil and pentamidine are two antiprotozoal aromatic diamidine pharmaceutical compounds that noncovalently inhibit AOC1 in mixed fashions (43). In the active sites of the two inhibited forms of AOC1, the TPQ cofactor was detected in the inactive, on-copper conformation. Whether the binding of these inhibitors induces the conformational change of TPQ from off-copper (active) to on-copper (inactive) was not discussed.

Amine oxidase, copper-containing 2, AOC2 (EC 1.4.3.21)

AOC2 was originally cloned from the retina in 1997 (44). The mRNA of AOC2 has also been detected in adipose tissue and was found to be upregulated during *in vitro* adipocyte differentiation (57,58). Additionally, AOC2 has also been detected at the mRNA level in many tissues (lung, brain, kidney, cartilage, tonsil, and heart); however, AOC2 amine oxidase activity (using tyramine as a substrate) has only been detected in the retina (59). Therefore, AOC2 is alternatively known as retina-specific amine oxidase (RAO).

A recombinant form of AOC2 (rAOC2) produced in human embryonic kidney (HEK293) cells was detected at the cellular surface (59). In that study, crude cell lysates were used to conduct kinetic studies. The preferred *in vitro* substrates for AOC2 were 2-phenylethylamine, tryptamine and *p*-tyramine, instead of methylamine and benzylamine (the preferred substrates of AOC3, see below). AOC2 does not oxidize histamine (the preferred substrate for AOC1) or spermidine (Table 1).

Since AOC2 shares 65% sequence homology with AOC3, homology modeling based on the structure of AOC3 (60) was performed to create a structure model for AOC2 (depicted in Figure 4 of (59)). The monomers of AOC2 and AOC3 superimpose with a root mean square deviation (RMSD) of 0.91 Å. The active site of AOC2 appears to be much larger than AOC3, most likely because Val205 and Asn388 in the active site of AOC2 are smaller than the corresponding residues, Met211 and Tyr394, in that of AOC3 (Figure 4B and 4C in (59)). Not surprisingly, the differences in active site size and structure of AOC2 and AOC3 help explain their substrate preferences. For example, docking experiments revealed that the aromatic ring of benzylamine is sandwiched between Tyr384 and Leu469 in AOC3 (Figure 4D in (59)). However, in the AOC2 model, benzylamine is stabilized only by Tyr378, due to the replacement of Leu469 by Gly463 (Figure 4E in (59)). Additionally, 2-phenylethylamine, a good *in vitro* substrate for AOC2 (but not AOC3), fits generously into the modeled active site cavity of AOC2, due to the extra space generated from the Leu469 to Gly463 substitution (Figure 4G in (59)). However, 2-phenylethylamine (Figure 4F in (59)), *p*-tyramine, and tryptamine cannot be docked in the same position in the AOC3 active site; the additional -CH_2- groups makes their hydrocarbon chains longer, so that the aromatic ring collides with the surroundings.

Amine oxidase, copper-containing 3, AOC3 (EC 1.4.3.21)

AOC3 is the most studied of the three human CAOs, and has been reviewed previously (5,11,61-63). Alternative names for AOC3 are semicarbazide-sensitive amine oxidase (SSAO), vascular adhesion protein-1 (VAP-1), plasma amine oxidase (PAO) and primary amine oxidase. AOC3 is found in adipocytes, smooth muscle cells and endothelial cells, and is highly expressed in the lung, aorta, liver and ileum. AOC3 is a type II membrane-bound protein; soluble AOC3 is released upon proteolytic cleavage of the C-terminus by a metalloprotease (64). Healthy humans have a low level of soluble AOC3 activity in their sera, while an elevated level of AOC3 activity has been observed in the sera of patients suffering from diabetes, congestive heart failure, and liver disorders. The affected organs are thought to be the source of the soluble AOC3 (65,66).

Recombinant forms of AOC3 (rAOC3) have been produced in Chinese hamster ovary (CHO) cells (46,60), Ax endothelial cells (67), HEK293 cells (68,69), and *Drosophila S*2 cells (70). Recombinant AOC3s produced in HEK and S2 cells were expressed without the transmembrane domain (i.e. residues 29-763 were expressed). In addition to the currently known endogenous substrates for AOC3 (i.e. methylamine and aminoacetone (71,72)), rAOC3 oxidizes benzylamine *in vitro*, but does not oxidize the diamines histamine or putrescine, unlike rAOC1 (Table 1)(49,70).

The AOC3 monomer has six predicted *N*-linked and three putative *O*-linked glycosylation sites. A series of rAOC3s with single mutations at each of the 9 glycosylation sites were transiently-expressed in Ax cells (a rat high endothelial venule-derived cell line)(67). rAOC3 was shown to be glycosylated at all six putative *N*-linked glycosylation sites, while no *O*-linked glycosylation was detected. Among the six *N*-linked glycans, three *N*-linked carbohydrates are located on the top of the "cap" of AOC3, and could modulate AOC3-mediated lymphocyte adherence to the endothelium: when two or all three apical *N*-linked glycans were omitted from AOC3, the consequent lymphocyte adhesion to the endothelium was reduced by 25–35% under non-static assay conditions. Further, the glycosylation was shown to affect the catalytic activity of rAOC3. It was hypothesized that removal of the apical highly sialylated carbohydrates would effect changes in the charge of the rAOC3 molecule, thereby affecting the structural flexibility of rAOC3 and altering its enzymatic activity.

Recently, two independent detailed biochemical studies were conducted on a rAOC3 produced in HEK293-EBNA1 cells and insect cells (68,70). These rAOC3s were purified to >95% homogeneity from serum-free media and were detected as a single band at ~ 100 kDa (68,70). The stoichiometric amount of titratable TPQ cofactor was ~19% and ~6%, respectively, for the rAOC3s produced in HEK cells and insect cells. Incubation of the partially biogenized rAOC3 from HEK cells in buffer containing excess Cu^{2+} or O_2 did not change the amount of titratable TPQ. It was concluded that either 1) Cu^{2+} was replaced by another metal (most likely Zn^{2+}, which does not support TPQ biogenesis (73)) in a large fraction of the purified rAOC3, or that 2) the TPQ cofactor was somehow not able to react fully with phenylhydrazine.

Recombinant AOC3 produced in insect cells accepted a variety of primary amines with different chemical properties (i.e. nonphysiological branched-chain and aliphatic amines), with apparent (k_{cat}/K_m) values on the order of 10^2 to 10^4 $M^{-1}s^{-1}$ (70). The $K_m(O_2)$ approximated the partial pressure of oxygen found in the interstitial space. The apparent (k_{cat}/K_m) values for most of the screened amines only differed 3- to 4-fold between purified murine and human rAOC3; however, human rAOC3 was ~10-fold more active towards methylamine and aminoacetone (70).

The pH-dependency curve of the steady-state kinetic parameters of rAOC3 produced in HEK cells was fit using nonlinear regression (68). A bell-shaped curve was fit to the apparent k_{cat} versus pH plot, with two macroscopic pK_a values (7.0 ± 0.2 and 10.0 ± 0.4) representing ionizable groups in the rAOC3-substrate complex. The pH-dependency of the apparent (k_{cat}/K_m) revealed a single pK_a value (9.0 ± 0.1) that was assigned to the primary amino group of benzylamine.

A kinetic isotope effect (KIE) of 6 to 7.6 was obtained on apparent (k_{cat}/K_m) over the pH range of 6 to 10 using d_2-benzylamine. The KIE on apparent k_{cat} was found to be close to unity over the same pH range. The unusual KIE values on (k_{cat}/K_m) were explained by a mechanistic scheme including multiple isotopically sensitive steps (typical of CAOs). Analysis of quantitative structure-activity relationships (QSAR) using *para*-substituted protiated and deuterated phenylethylamines was also conducted. With phenylethylamines, a

large KIE on apparent k_{cat} (8.01 ± 0.28 with phenylethylamine) was observed, indicating that C–H bond breakage is limiting for TPQ reduction. Poor correlations were observed between steady-state rate constants and QSAR parameters.

The X-ray crystal structure of rAOC3 expressed in CHO cells was solved and refined to 2.9 Å (60), and the structures of two forms of rAOC3 expressed in HEK293 cells (i.e. the wild-type (WT) and 2-hydrazinopyridine (2-HP)-inhibited forms) were solved and refined to 2.5 Å and 2.9 Å, respectively (55). The major difference between the WT-rAOC3s produced in CHO and HEK cells is the conformation of the TPQ cofactor, i.e. on-copper (inactive) versus off-copper (active) TPQ. TPQ cofactor is known to have some mobility in the active site, and depending on the crystallization conditions, these two forms have been detected routinely (7,12,21). An additional disparity is that the structure of WT-rAOC3 produced in HEK293 cells contains an intermolecular disulfide bridge between Cys41 and Cys748; however, the authors acknowledged the possibility that this was an artifact from the crystallization procedure (55).

Overall, the crystal structures of the rAOC3s expressed in CHO and HEK cells are very similar to each other while differing from other CAOs in some important ways. As mentioned above, rAOC3 possesses an active site cavity that is markedly smaller than that of AOC2, owing to the presence of three active site amino acids with much bulkier side chains than those found in AOC2 (59). Additionally, the much narrower substrate entry channel of rAOC3 distinguishes it from human rAOC1 and CAOs from lower organisms. In both rAOC3 structures, Leu469 is proposed to function as a gate, controlling substrate access to the active site cavity (55,60). Leu468 and Leu469 are located at the bottom part of the substrate entry channel ‘funnel,’ which might sterically hinder larger substrates from entering the active site cavity. This is likely to contribute to the preference of AOC3 for small amine substrates (e.g. methylamine and aminoacetone) over larger amines (e.g. benzylamine and phenylethylamine) (Table1).

In addition to Leu469, Met211 and Tyr394 reside at the bottleneck of the substrate entry channel (Figure 7A in (74)), and a triple mutant form (M211V/Y394N/L469G) of rAOC3 exhibited substrate specificity similar to that reported for rAOC2 (59). In order to understand which of the three residues is critical for defining the substrate specificity of AOC3, single mutants (M211V, Y394N, or L469G) were transiently expressed in CHO cells, and crude cell lysates were used to obtain kinetic parameters (74). Leu469 and Met211 (but not Tyr394) were found to be critical for substrate recognition, and mutation of either of Leu469 or Met211 to the corresponding amino acids in AOC2 (i.e. L469G or M211V) changed the substrate specificity of AOC3. It was proposed that the larger active site of the M211V and L469G mutants and the absence of large hydrophobic side chains make the correct positioning of methylamine (a small substrate) difficult.

Despite these important differences between AOC3 and other CAOs, the active site configuration of the 2-HP-inhibited form of rAOC3 is very similar to a previously characterized 2-HP-inhibited form of a CAO from *E. coli* (31,32), and confirmed that Asp386 is the active site base for AOC3 and that the pyridine ring of the 2-HP is involved in

π-stacking interactions with Tyr (Tyr384). This Tyr residue is also conserved in AOC1 (Tyr371) and AOC2 (Tyr378) (43,59).

More recently, X-ray crystal structures of two imidazole-bound forms (on-copper and off-copper TPQ) of the soluble, proteolytically cleaved form of native AOC3 isolated from human serum were solved to 2.6-2.95 Å resolution (74). The overall structures of the imidazole-bound forms are largely similar to those of rAOC3s, except that Cys748 is reduced in the structures of the native AOC3, whereas Cys748 is involved in either an intermolecular or intramolecular disulfide bridge in the rAOC3 structures (55,60,75). It was found that at high concentration (100 mM), imidazole could covalently bind to the active carbonyl group of TPQ at C5. The saturation state of the bond between the N1 nitrogen of imidazole and TPQ was not clear at 2.95 Å resolution; however, imidazole most likely forms a substrate Schiff base-like adduct. The N3 of imidazole was within the necessary distance to hydrogen bond with Asp386, the active site base. The imidazole-bound (TPQ off-copper) form of AOC3 could not be derivatized with *p*-nitrophenylhydrazine and was inactive toward oxidation of substrate amines. Subsequently, it was determined that imidazole inhibits competitively, with an IC_{50} of 1.28 – 8.6 mM. A second molecule of imidazole was also seen in the AOC3 active site, away from TPQ and was involved in hydrogen bonding interactions with Tyr394 and the main chain nitrogen of Thr212 (through a water molecule), and hydrophobic interactions with Leu469 and Tyr176. Based on these observations, the authors noted the potential for inhibitor design based on secondary amine inhibition and/or the selectivity of inhibitors bridging the active site and the secondary imidazole binding site, which appears to be unique to AOC3.

In addition to the CAO catalytic domain, AOC3 has an adhesion domain that targets leukocytes for transmigration (76). Both sites and the amine oxidase activity of AOC3 are critical for AOC3-mediated induction of leukocyte rolling, adhesion and transmigration in response to inflammatory stimuli (77). Inhibition of AOC3 has been shown to be effective in mice models of inflammation (in the eyes, carrageenan-injected air pouch, and lungs), rheumatoid arthritis, liver fibrosis, and stroke (78,79). These results indicate that AOC3 has potential as a therapeutic target for inflammation and fibrosis. Consequently, several pharmaceutical companies have developed alkylhydrazino-, guanidine-, and imidazole-derivatives as AOC3 inhibitors with therapeutic potential (reviewed in (63)). An alternative strategy is to use monoclonal antibodies against AOC3 to disrupt its role in leukocyte trafficking (reviewed in (61)). *In vitro* and *in vivo* experiments show that genetically engineered chimeric monoclonal mouse-human antibodies can block sites used by AOC3 to promote leukocyte transmigration in humans without leading to side effects caused by immunogenecity or activation of effector functions (80). BTT-1023 (81), a fully human monoclonal antibody that specifically binds to AOC3, has been developed and has shown promising efficacy and safety in early clinical studies in rheumatoid arthritis and psoriasis patients, and in a range of preclinical models of inflammatory diseases, including chronic obstructive pulmonary disease (COPD), certain neurological conditions, and certain niche liver inflammatory fibrotic diseases. Currently, it is undergoing phase 2 clinical trials.

LYSYL OXIDASE FAMILY

Protein-lysine 6-Oxidase, LOX (EC.1.4.3.13)

Protein-lysine 6-oxidase, which is more commonly referred to as lysyl oxidase, is expressed highly in the heart, placenta, skeletal muscle, kidney, lung and pancreas (82). LOX is initially translated as pre-pro-LOX containing an N-terminal secretion signal (pre), a highly acidic propeptide (pro) and the C-terminal catalytic domain (LOX) (Figure 8). LOX is proposed to be *N*-glycosylated at the predicted *N*-glycosylation sites (Asn81, Asn97, and Asn144) in the propeptide domain (83). There are no *N*- or *O*-glycosylation sites predicted in the LOX catalytic domain. After being secreted from cells, the propeptide is proteolytically cleaved by bone morphogenetic protein-1 (BMP-1), releasing mature LOX (47,84).

Recombinant forms of secreted LOX have been prepared from CHO and RFL cell growth media (83,85), and biochemical characterization of LOX has been conducted using crude cell lysate and/or crude medium, with the cell lysate or medium from mock-transfected cells serving as negative controls (83,85). For LOX activity assays conducted using crude lysate/media, the activity is generally expressed in terms of BAPN-inhibitable amine oxidase activity, since BAPN is specific for the LOX-family of proteins, and does not inhibit CAOs or maoA or maoB (34). Studies have implicated that pro-LOX is catalytically latent, so processing by BMP-1 has been proposed to be essential for the LOX amine oxidase activity (47). However, no biochemical study using purified proteins has yet compared the relative activities of pro-LOX and mature LOX.

In order to assess the importance of *N*-glycosylation of the propeptide for secretion and protein maturation, a triple mutant form (N81Q/N97Q/N144Q) of pro-LOX was expressed in CHO cells (83). The triple mutant was secreted into the medium and underwent BMP-1 cleavage, suggesting that *N*-glycosylation of the propeptide is not essential for secretion or proteolytic activation. Intriguingly, the catalytic activity of the triple mutant in the crude medium was ~ 40% of that of WT-LOX. Because the propeptide and the associated *N*-linked glycans are not retained by mature LOX, these results suggest that the *N*-linked glycans in the propeptide may play an important role in LTQ biogenesis prior to secretion.

When the propeptide domain was omitted altogether by fusing the catalytic domain of LOX to the signal peptide, LOX was not secreted; instead, it was rapidly degraded in the cells via endoplasmic reticulum-associated protein degradation (ERAD)(83). These data indicate that the propeptide is essential for proper folding and secretion of LOX. Interestingly, the propeptide may also play a role in the recognition of LOX substrates in the extracellular milieu. To support this, the propeptide domain of pro-LOX was shown to be essential for deposition of pro-LOX onto elastic fibers produced in cultures of rat lung fibroblast cells (RFL-6) (85). Additionally, when the pre-propeptide without the C-terminal LOX catalytic domain was expressed in RFL-6 cells, it was secreted into the medium and still co-localized with elastic fibers.

Aberrant expression of LOX has been linked to many diseases. Downregulation or decreased activity of LOX is associated with connective tissue disorders, such as cutis laxa (86) or

occipital horn syndrome (87), Menkes' syndrome (87), myocardial ischaemia (88), and pelvic organ prolapse (89,90). Upregulation of LOX has been associated with liver cirrhosis (91), Alzheimer's and non-Alzheimer's dementia (92), Wilson's disease and primary biliary cirrhosis (41), and metastatic/invasive colorectal, breast, head and neck, prostate, and renal clear cell cancers (Table 2). Much of the recent study of LOX in disease has focused on elucidating its role in promoting the invasion/metastasis of breast cancer cells. Because LOX catalyzes the crosslinking of extracellular matrix (ECM) proteins such as collagen and elastin, LOX thus promotes stiffening of the ECM, leading to alteration of cellular mechanotransduction and activation of oncogenic signaling pathways such as the FAK/Src and Akt/PI3K pathways (93,94). Alternatively, LOX is proposed to regulate breast cancer cell migration and adhesion via activation of FAK/Src signaling pathways through H_2O_2 produced as a byproduct of ECM substrate oxidation (95).

In an apparent paradox to the metastasis/invasion-promoting function of LOX in breast cancer cells, *lox* has been identified as a tumor suppressor gene in *ras*-transformed murine fibroblast cells (96). Additionally, in human gastric cancers, *lox* is inactivated by methylation and loss of heterozygosity (97). The silencing of *lox* has also been observed in colon, lung and ovarian cancer cell lines (97), and LOX is absent in basal and squamous cell carcinomas (98). The mechanism by which LOX suppresses tumors at the molecular level is not completely understood. For *ras*-related tumors, it was originally proposed that the amine oxidase activity of LOX is essential for tumor suppression, based on the observation that BAPN can block tumor suppression (96). In recent years, the propeptide rather than the catalytic domain has been proposed to play critical roles in tumor suppression by inhibiting Ras signaling. Ectopic expression of the propeptide has been shown to inhibit the transformed phenotype of breast, pancreatic, lung, and prostate cancer cells *in vitro* (99-102), and also inhibits the formation of tumors by human epidermal growth factor receptor 2 (Her-2/neu)-driven breast cancer cells *in vitro* (103,104). Most recently, it was proposed that the propeptide functions as a tumor suppressor via interaction with Hsp70 and c-Raf to inhibit the *ras*-induced MEK signaling pathway (104). *In vitro* results demonstrate that the propeptide of LOX, but not the catalytic domain, can function as a tumor suppressor (104). An immunofluorescent study demonstrated that when exogenous propeptide is added to the culture medium of fibroblast cells, it translocates into the cytosol within 20 min and localizes to the perinucleus (105). The mechanism whereby the propeptide domain (~25 kDa) enters the cells has not been defined. Alternatively, pro-LOX is proposed to function in tumor suppression in the cytosol prior to secretion (106). In any case, the substrate in tumor suppression is currently unknown. Therefore, intracellular functions of LOX at the molecular level remain undefined.

Lysyl Oxidase Homolog 1, LOXL1 (EC.1.4.3.-)

LOXL1 is strikingly similar to LOX: it contains a secretion signal peptide, a propeptide, and a highly conserved LOX catalytic domain (77% identity and 88% homology) (Figure 8). Like LOX, LOXL1 is catalytically activated upon propeptide cleavage by extracellular BMP-1 (107). Recombinant LOXL1 has been produced in RFL-6 cells, and similar to LOX, its propeptide was shown to be essential for depositing the LOX catalytic domain onto elastic fibers, as the catalytic domains of LOX and LOXL1 were unable to interact with

elastic fibers when the propeptides were absent (85). However, unlike LOX, the catalytic domain of LOXL1 could be secreted into the medium even when the propeptide was absent. Given the high degree of homology between LOX and LOXL1, this is an intriguing and unexpected finding, and merits further investigation. Importantly, no biochemical studies have been conducted yet to compare the catalytic activities and substrate specificities of LOX and LOXL1.

LOXL1 is expressed in ocular tissues, including the ciliary body, lens, optic nerve, retina, and especially in the iris. LOXL1 has gained some attention due to the fact that SNPs of *loxl1* are associated with 99% susceptibility to exfoliation syndrome (XFS) in Scandinavian males over 60 years old (108). XFS is a disorder characterized by accumulation of abnormal fibrillar deposits in the anterior segment of the eye. A risk haplotype includes two LOXL1 coding non-synonymous SNPs (R141L and G153D) and one intronic SNP (108).

Recently, aberrant expression of LOXL1 has been associated with diseases involving female reproductive tissues. A microarray study revealed that LOXL1 was significantly upregulated among the ~15,000 genes and expressed sequence tags (ESTs) in peripheral blood lymphocytes isolated from patients with endometriosis (109). In a different study, a role for LOXL1 in elastic fiber renewal in adult tissues was proposed, based on high incidence of pelvic organ prolapse (POP) and permanent damage to the pelvic floor in post partum LOXL1-null mice (110). Subsequent studies of human populations have produced conflicting data regarding the role of LOXL1 in POP: one group has reported an *increase* in LOXL1 mRNA and protein in the uterosacral ligaments of patients with POP (111), while others report that expression of LOXL1 mRNA (89,112,113) and protein (114,115) are significantly *down-regulated* in the pelvic connective tissues of POP patients. While the majority of the data support the hypothesis that LOXL1 plays a critical role in elastin maturation, additional investigation is needed to resolve this discrepancy.

It has been also suggested that LOXL1 has a tumor suppressor function in bladder cancer cells, where it was discovered that LOXL1 was epigenetically silenced, predominantly by promoter methylation (116). Reintroduction of LOXL1 in bladder cancer cells was shown to inhibit colony formation and antagonize Ras-mediated activation of the extracellular signal-regulated kinase (ERK) signaling pathway (116).

Lysyl Oxidase Homolog 2, LOXL2 (EC.1.4.3.13)

Lysyl oxidase homolog 2, also known as lysyl oxidase-like protein 2 (LOXL2), lysyl oxidase-related protein 2, or lysyl oxidase-related protein WS9-14, contains a secretion signal, four SRCR domains and a LOX catalytic domain (Figure 8). LOXL2 contains no *O*-linked glycosylation sites and three potential *N*-linked glycosylation sites (Asn-X-Ser/Thr): Asn288, Asn455 and Asn644. LOXL2 is expressed in many tissues, with the highest expression observed in reproductive tissues, e.g. the placenta, uterus and prostate (117). LOXL2 is also upregulated in many cancer cells and tissues (Table 2). LOXL2 is a direct transcriptional target of HIF1A and its expression is induced by hypoxia (118,119).

LOXL2 is generally expected to function similarly to LOX in regard to ECM crosslinking and stiffening. LOXL2 expression is linked to upregulation of tissue inhibitor of

metalloproteinase-1 (TIMP-1) and matrix metalloproteinase-9 (as also proposed for LOX), thereby promoting ECM degradation and dissemination of metastatic breast cancer cells (95,120). However, there has been no *in vitro* biochemical study to directly compare the respective activities of LOX and LOXL2 in ECM stiffening.

An allosteric inhibitor of LOXL2, AB0023 (an antibody specific for the 4th SRCR domain of LOXL2), has been developed to target secreted LOXL2 (39). AB0023 has been shown to be effective in preventing tumor and fibrotic microenvironment formation, and reduces the metastatic potential of tumor cells in mice (40). Use of AB0023 as a research tool has also led to the discovery that inhibition of the enzymatic activity of secreted LOXL2 may not be sufficient as a therapeutic strategy in every pathological context in which LOXL2 is implicated. This realization originated from data demonstrating that mutant LOXL2 with abrogated enzymatic activity was still capable of preventing keratinocyte differentiation and promoting the development of squamous cell carcinomas (121). The capacity of LOXL2 to inhibit keratinocyte differentiation was subsequently traced to the 4th SRCR domain, and treatment with AB0023 was effective in relieving repression of involucrin, a marker of differentiation.

Recombinant LOXL2s lacking either the first three or all four SRCR domains (i.e. Δ1-3SRCR-LOXL2 or Δ1-4SRCR-LOXL2) have been expressed in the culture medium of *Drosophila* Schneider 2 (S2) cells stably-transfected with the corresponding expression constructs. Using these truncated secreted LOXL2s, *N*-linked glycosylation at Asn455 and Asn644 was recently confirmed; additionally, the *N*-linked glycans at these sites were shown to be independently important for proper protein folding and secretion from S2 cells (122,123). The *N*-glycosylation site at Asn644 in the LOX catalytic domain is conserved in the SRCR domain-containing LOXLs (LOXL2, LOXL3 and LOXL4), but is not conserved in LOX or LOXL1 (Figure 8). The LOX catalytic domains of LOX and LOXL1 (comparable to Δ1-4SRCR-LOXL2) were also expressed in S2 cells; however, the proteins were only produced as inclusion bodies, suggesting that there are some important differences between the structures of the LOX catalytic domains of LOXL2 and LOX/LOXL1, even though they share 50% sequence identity. The LTQ cofactor and its precursor residues (Lys653 and Tyr689) in LOXL2 were also identified by mass spectrometry (122,123).

The two truncated rLOXL2s were catalytically competent toward amine oxidation *in vitro*, and could oxidize LOX substrates such as tropoelastin ($K_m \approx 0.6$ μM; $k_{cat} \approx 0.7$-2.0 min^{-1} at pH 8.0, 37 °C)(123). The K_m values are very similar with or without the 4th SRCR domain, but the k_{cat} value is ~3-fold higher when the 4th SRCR domain is present, supporting the proposal that the 4th SRCR domain positively regulates the catalytic activity of LOXL2 (39). The parameters are mostly comparable to those determined for bovine LOX (124,125), where the k_{cat} was reported in min^{-1}, confirming that the LOX-family of proteins comprises intrinsically “slow” enzymes.

In addition to the proposed extracellular roles of LOXL2, some intracellular functions have also been postulated, since a perinuclear expression pattern of LOXL2 has been observed in basal-like breast and larynx squamous carcinomas (126-128). Snail1 transcription factor and trimethylated Lys4 of histone H3 (H3K4(me3)) have been proposed as intracellular

substrates (129,130). When LOXL2 and Snail1 were transiently co-overexpressed in HEK cells, LOXL2 was shown to interact with and stabilize Snail1 protein (130). LOXL2 was proposed to oxidize Lys98 and/or Lys137 of Snail1 to induce some conformational change that protects Snail1 from GSK3β-catalyzed phosphorylation, subsequent ubiquitinylation, and proteasomic degradation Similarly, the interaction of LOXL2 and H3K4(me3) was detected in HEK cells, and the downregulation of methylated H3K4 was observed upon ectopic expression of LOXL2 in MCF-7 cells (129). However, in that study, two unprecedented roles (i.e. demethylation and alcohol oxidation) were proposed for LOXL2. Finally, it has also been suggested that LOXL2 regulates cell polarity in basal breast cancer cells by transcriptionally downregulating tight junction proteins independently of E-cadherin (127).

Recently, secreted full-length LOXL2 (~100-kDa) was shown to be *N*-glycosylated at Asn455 and Asn644, whereas intracellular LOXL2 (~75-kDa) was nonglycosylated, N-terminally processed, and primarily associated with the cell nucleus. Particularly in cells expressing nuclear-associated nonglycosylated (~75-kDa) LOXL2, Snail1 protein was stabilized in a LOX amine oxidase-dependent fashion. This Snail1 stabilization induced EMT by downregulation of epithelial markers and upregulation of mesenchymal markers, and additionally promoted *in vitro* invasion via upregulation of vimentin, fibronectin and MT1-MMP. In contrast, cells expressing secreted *N*-glycosylated LOXL2 exhibited an epithelial phenotype and relatively low invasiveness under the reported *in vitro* experimental conditions (131).

Lysyl Oxidase Homolog 3, LOXL3 (EC.1.4.3.-)

Commonly known as lysyl oxidase-like protein 3, LOXL3 is the least studied member of the LOX family of proteins. Like LOXL2 and LOXL4, LOXL3 contains a signal peptide, four SRCR domains and a C-terminal LOX catalytic domain (Figure 8). There are five potential *N*-glycosylation sites in LOXL3: Asn111, Asn266, Asn390, and Asn481 in the SRCR domains, and Asn625 in the LOX catalytic domain. The actual extent of *N*-glycosylation of LOXL3 has not yet been examined. LOXL3 expression has been detected in many tissues and is most highly expressed in the placenta, heart, ovary, testis, small intestine and spleen (132,133). Decreased expression of LOXL3 has been observed in POP and breast cancer effusions (89,134).

Lysyl Oxidase Homolog 4, LOXL4 (EC.1.4.3.-)

LOXL4 is commonly called lysyl oxidase-like protein 4 or lysyl oxidase-related protein C (LOXC). LOXL4 is expressed in many tissues, with the highest levels being in the skeletal muscle, testis, pancreas, and cartilage (135,136). The protein consists of a secretion signal, four SRCR domains and a C-terminal LOX catalytic domain (Figure 8). The four SRCR domains may be essential for LOXL4 secretion, since the catalytic domain of LOXL4 by itself could not be secreted from endothelial cells (137). There are two potential *N*-glycosylation sites in LOXL4: Asn198 in the second SRCR domain and Asn629 in the LOX catalytic domain. However, the actual extent of *N*-glycosylation of LOXL4 has not been examined.

LOXL4 has a variety of recognized roles in human disease. Like LOXL1, LOXL4 is epigenetically silenced in bladder cancer cells, and overexpression of either protein in bladder cancer cells has been shown to inhibit Ras/ERK signaling pathways (116). In PLC/PRF/5 cells (liver cancer), *LOXL4* was identified as the only gene in the LOX family whose expression was induced by transforming growth factor-β1 (TGF-β1)(138). TGF-β1-induced overexpression of recombinant LOXL4 in PLC/PRF/5 cells restricted cell migration through Matrigel, and suppressed the expression of laminins and α3 integrin and the activity of matrix metalloproteinase 2 (MMP2). Thus, the authors suggested that LOXL4 might have a role as a negative feedback regulator of TGF-β1 in cell invasion by inhibiting the metabolism of ECM components. Recently, LOXL4 was also found to be under the control of TGF-β1 in aortic endothelial cells, where an activator protein 1 (AP1) site and a Smad binding element were essential for TGF-β1-induced expression of LOXL4 (137). In this study, TGF-β1-induced LOXL4 was shown to be secreted into the growth medium, where it contributed to ECM deposition and construction. Thus, the authors concluded that TGF-β1-induced LOXL4 plays a role in maintenance of the endothelial ECM, contributing to vascular processes associated with ECM remodeling. Most recently, the ectopic overexpression of full-length LOXL4 in metastatic MDA-MB-231 breast cancer cells was shown to reduce the metastatic potential partially by downregulation of Snail1 transcription factor and MMP-2 (139). In that study, LOXL4 was detected as a ~95 kDa protein in the media, but no further characterization was conducted. In any case, the data suggest that full-length LOXL4 may function as a tumor suppressor.

The upregulation of LOXL4 has been seen in head and neck squamous cell carcinoma (HNSCC), where the overexpression of LOXL4 transcripts was detected in 74% of invasive HNSCC tumors and 90% of both primary and metastatic HNSCC cell lines (140). LOXL4 was detected as a single ~93 kDa band in the cell lysates of UTSCC-19A cells (derived from primary tumors) and HCFMK1 cells (derived from metastatic tumors), but absent in normal non-neoplastic squamous epithelial cells. The molecular mass of the ~93 kDa protein detected in the cell lysate is larger than the predicted mass (84.5 kDa) of LOXL4, but is similar to the recombinant LOXL4s (97-100 kDa) produced in and secreted from HT-1080 and CHO cells (141). The differences in molecular mass are assumed to be due to the differences in glycosylation. Importantly, neither the native nor the recombinant LOXL4s seem to undergo proteolytic processing in the cytosol or in the ECM (140,141). In these cell lines, LOXL4 was detected predominantly at the perinucleus, as well as in cytosol as a diffused pattern. The high expression level of LOXL4 was associated with local lymph node metastases at progressed tumor stages but not with primary tumor types. The expression level of LOXL4 at the protein level correlated with the increased mRNA transcription in HNSCC cells.

CONCLUSION

In the previous 60 years, scientists have made great strides in the field of copper-dependent amine oxidases, particularly with the discovery of novel tyrosine-derived cofactors, namely TPQ and LTQ. Within the AOC subfamily, crystal structures have been solved for AOC1 and AOC3. This structural knowledge, coupled with data from several detailed studies of the substrate preferences of the AOCs, has facilitated the development of a variety of highly

selective small molecule inhibitors with some promise as therapeutic agents for AOC3-mediated inflammation. Proliferation and refinement of AOC3-specific inhibitors, in addition to the development of AOC3-targeting antibody therapies, will likely continue throughout the coming decade.

In contrast to the AOCs, much less is known about the structures and molecular functions of LOX and the LOXLs. Approximately 40 years after the discovery and isolation of LOX from bovine aorta, no crystal structure has been solved for any member of the LOX family, and very few biochemical studies have been conducted, aside from those on LOX. Consequently, while numerous associations between LOX family members and various diseases have been identified (and novel pathological roles are discovered yearly), the molecular functions of the lysyl oxidases and the degree to which their functions overlap remain unsatisfactorily understood. A few antibodies are being evaluated for their therapeutic value in treating fibrotic disease and cancers; however, a number of intracellular functions for different LOX family members have been proposed, highlighting the need to discover and optimize cell-permeable treatment options, such as small molecule inhibitors. Such discovery, as well as the capacity to distinguish the functions of the LOX family members, is likely to be severely hampered until the deficiencies of currently reported systems for recombinant LOX/L expression are addressed. To remedy this, many groups are currently expending great effort in the pursuit of more optimal expression systems for the LOX family. If their persistence bears fruit in the near future, the following 20 years are likely to be as exciting for the LOX family as the past two decades were for the AOCs.

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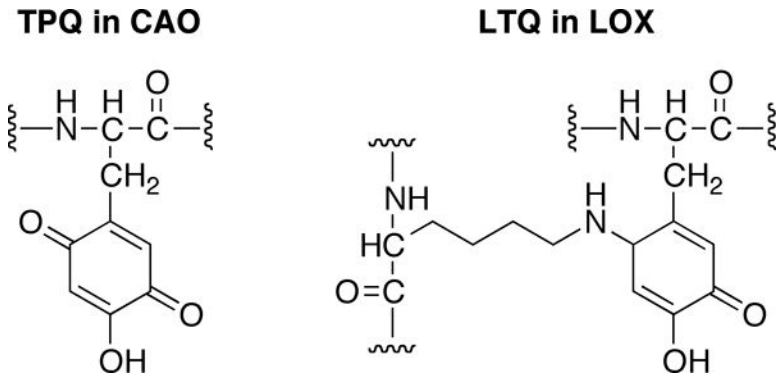

Figure 1. Structures of TPQ and LTQ

Tyrosine-derived quinone cofactors of human CAOs. TPQ (*left*) is derived from a conserved Tyr residue in CAOs, while LTQ (*right*) is derived from conserved Tyr and Lys residues in LOX. From (13,14).

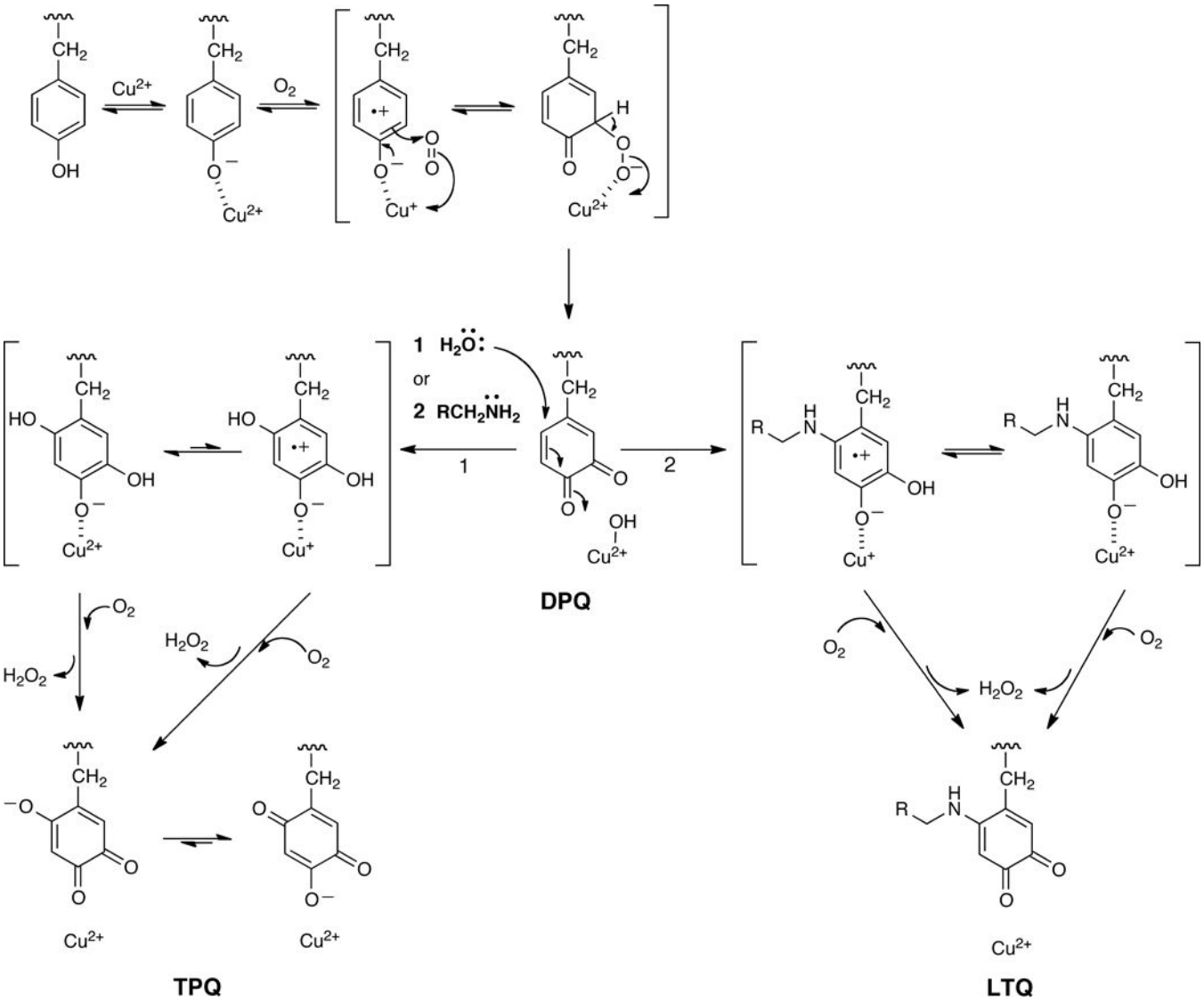

Figure 2. DPQ as a common intermediate in the biogenesis of TPQ and LTQ

The proposed mechanisms for the biogenesis of the TPQ and LTQ cofactors share a dopaquinone (DPQ) intermediate. The 1,4-addition of either water (*Pathway 1*) or the ε-amino side chain of a peptidyl lysine residue (*Pathway 2*) to DPQ yields TPQ or LTQ, respectively. RCH_2NH_2 represents the side chain of a peptidyl lysine residue (-$CH_2CH_2CH_2CH_2NH_2$) within the polypeptide of a LOX family member. Adapted from (13,14).

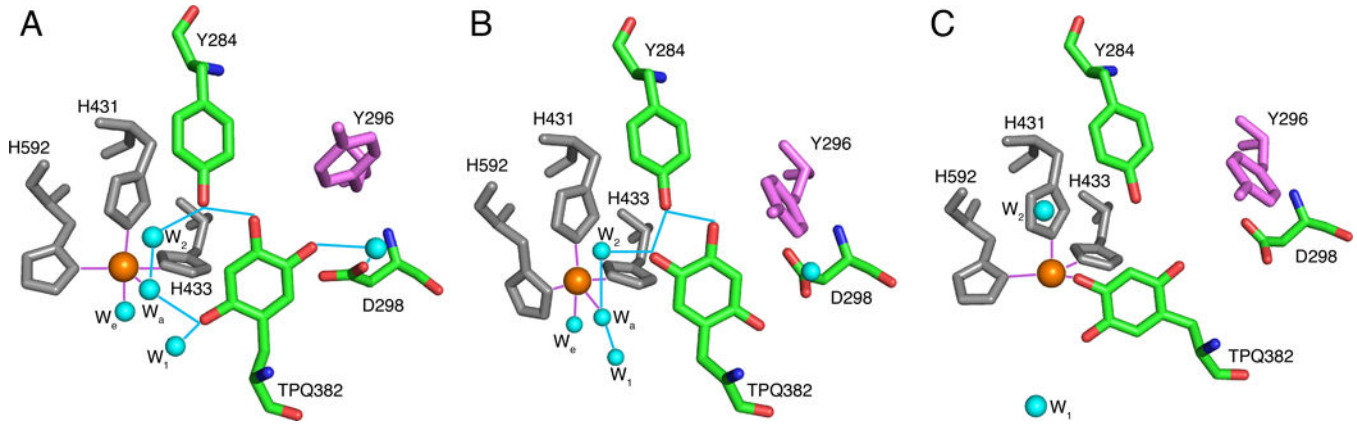

Figure 3. Three different conformations of the TPQ ring detected in crystals of *Arthrobacter globiformis* amine oxidase (AGAO)

(a) In the active "off-copper" conformation, O4 of TPQ_{ox} is hydrogen-bonded to Tyr284. The reactive carbonyl group at C5 faces the substrate entry channel (Tyr296 in *purple* is located at the base of the proposed substrate channel) and the active site base (Asp298)(PDB accession number: 1IU7). (b) In the inactive "flipped" conformation of TPQ_{ox}, the TPQ ring has flipped 180° from the active conformation at the C2 axis. Consequently, the reactive carbonyl at C5 faces away from the substrate entry channel and Asp298 (PDB: 1AV4). (c) In the inactive "on-copper" conformation, O4 of TPQ_{ox} is directly ligated to Cu^{2+}, where the C5 carbonyl group resides away from Asp298 and the substrate channel (PDB: 1AVL). Cu^{2+} is represented by an orange sphere, water molecules are represented by small blue spheres, the three His residues constituting the copper-binding site are in gray, hydrogen bonding interactions are represented by blue lines, and ligand interactions are represented by purple lines. Adapted from (7).

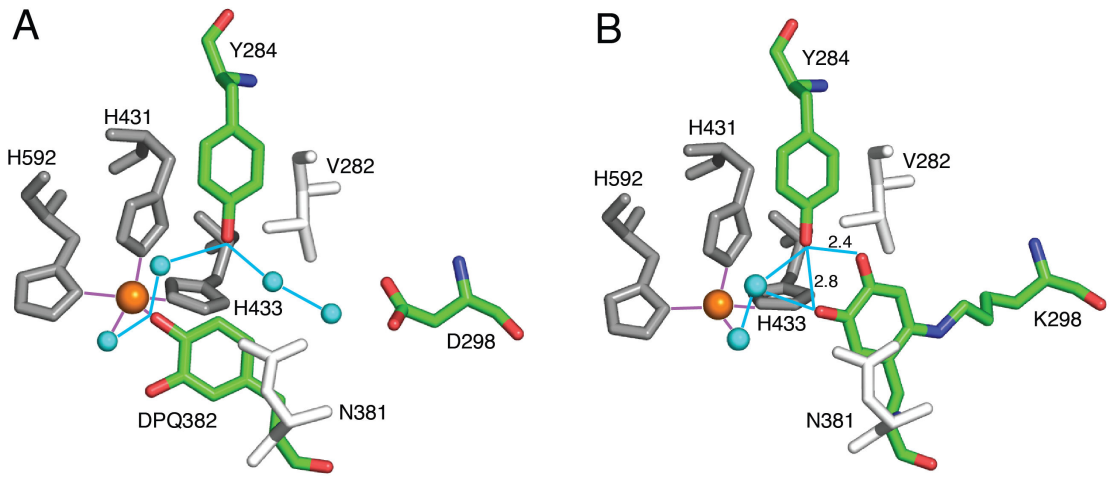

Figure 4. Active-site structures of mature D298K-AGAO and the putative DPQ intermediate detected during snapshot analysis of TPQ biogenesis in WT-AGAO

(A) DPQ intermediate (PDB: 1IVV), (B) D298K (PDB: 2YX9). Cu^{2+} is shown as an orange sphere, water molecules are shown as light-blue spheres, hydrogen bonding interactions are represented by blue lines, and ligand interactions are represented by purple lines. Val282 and Asn381 (*white*) form the edges of a wedge-shaped pocket. Hydrogen bonding distances are denoted in angstroms. From (22).

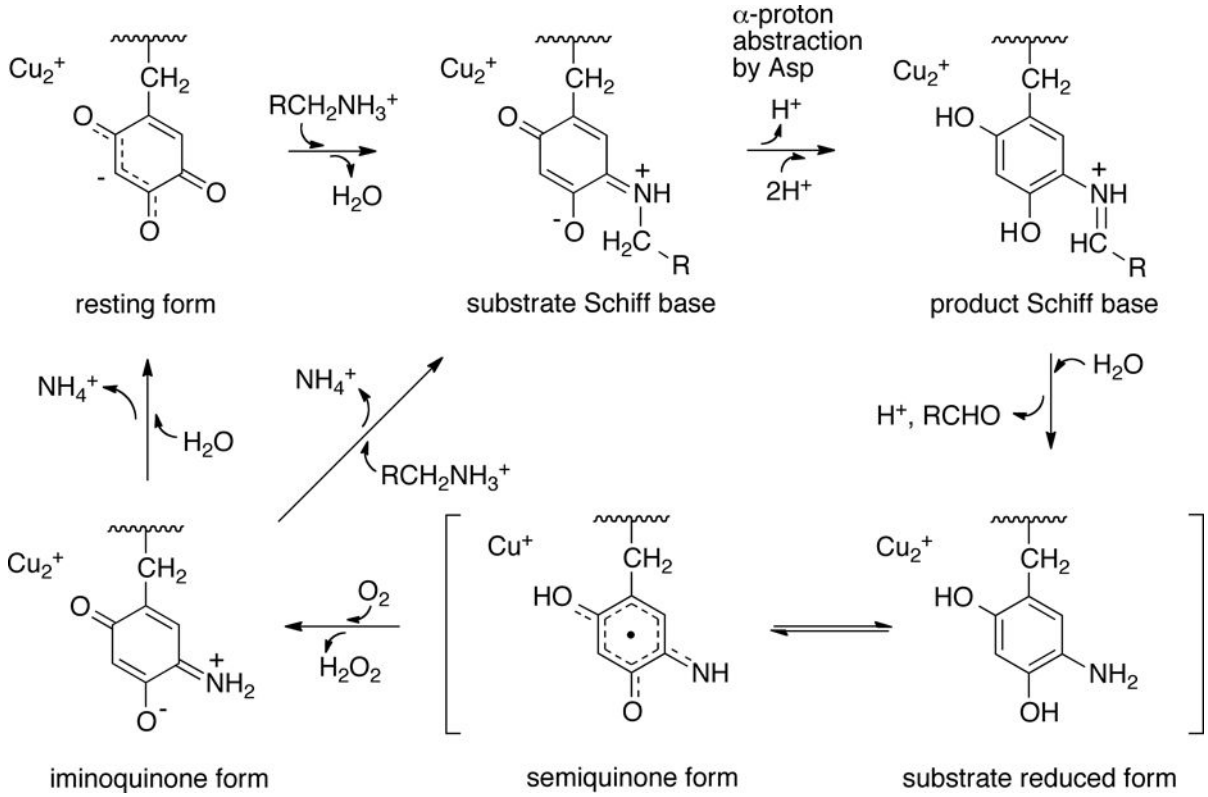

Figure 5. A proposed mechanism for the oxidation of an amine substrate by CAO

Scheme showing the classical ping-pong mechanism by which a copper amine oxidase oxidizes primary amines. Covalent intermediates are formed between TPQ and amines, in addition to oxidoreduction reactions of the TPQ cofactor. Adapted from (7).

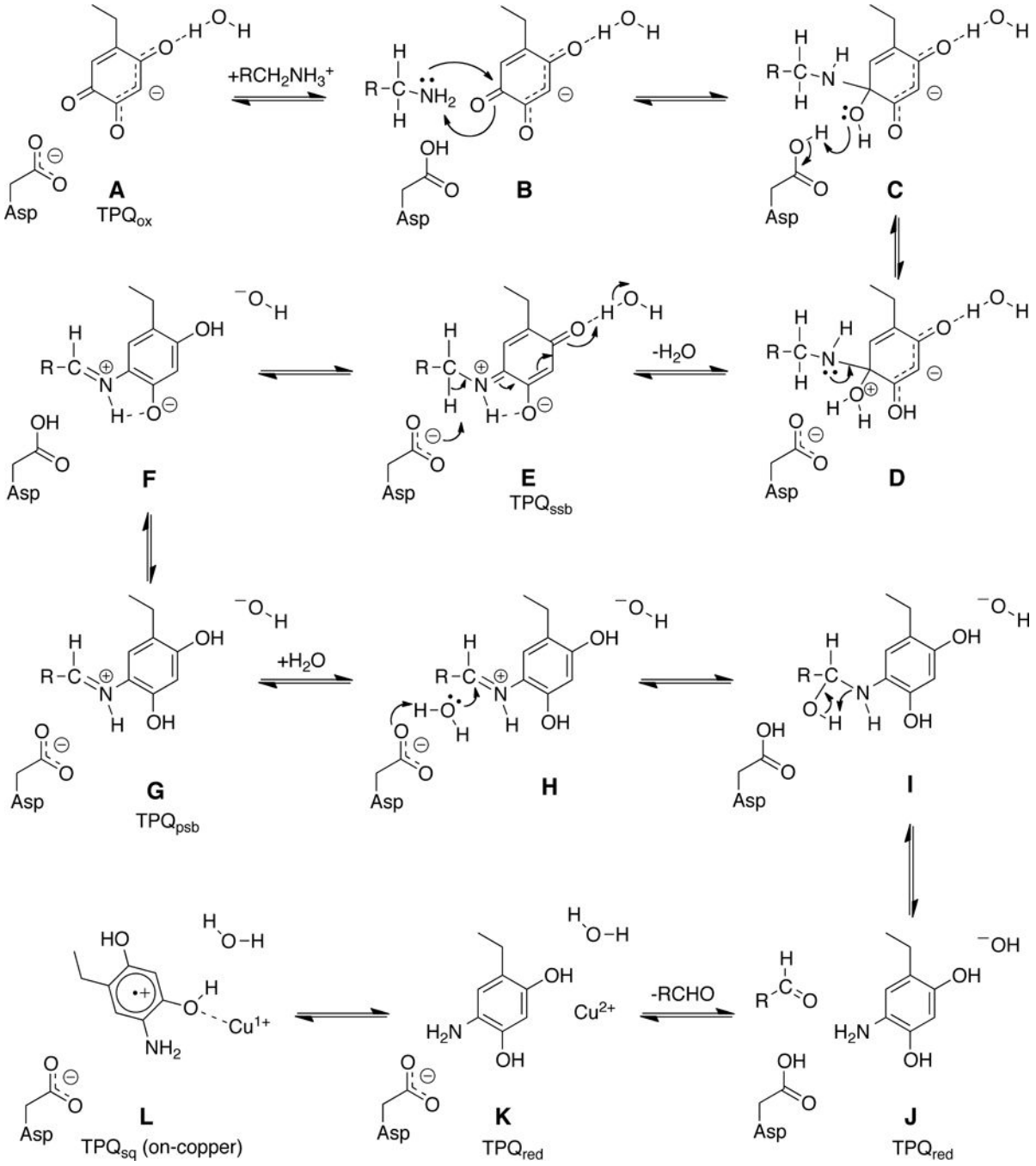

Figure 6. Detailed proposed mechanism of the reductive half-reaction

The protonation states of the substrate and reaction intermediates are carefully controlled to achieve the optimal activity. The conserved Asp in the active site serves as a proton sink. The protonated substrate amine ($RCH_2NH_3^+$) binds to the active site and is deprotonated by the Asp residue (which is deprotonated in the resting state). The reaction proceeds through two Schiff bases, i.e. a substrate Schiff base (TPQ_{ssb}, E) and a product Schiff base (TPQ_{psb}, G). TPQ_{psb} is mono-protonated to undergo facile hydrolysis to yield TPQ-aminoresourcinol

(TPQ_{red}, J and K). In some amine oxidases, disproportionation reactions between TPQ_{red} and Cu^{2+} yield a semiquinone form of TPQ (TPQ_{sq}, L). From (8,24).

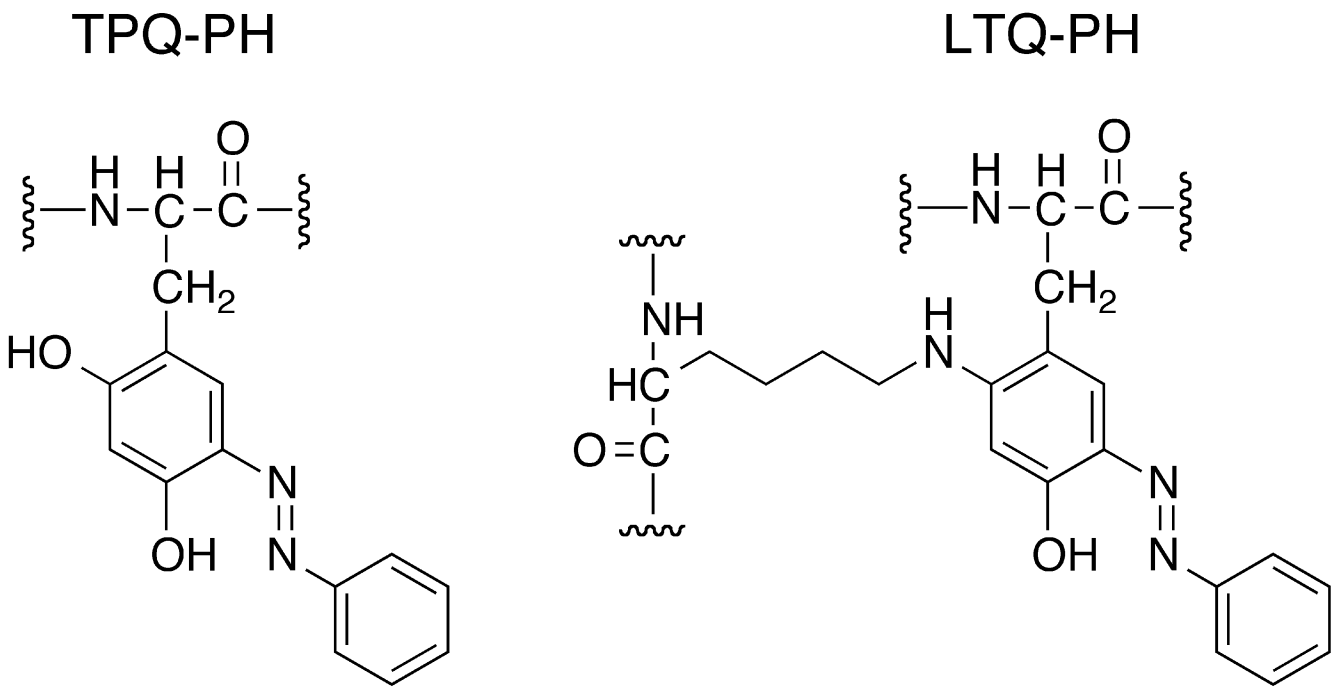

Figure 7.
Phenylhydrazine (PH) adducts of TPQ and LTQ

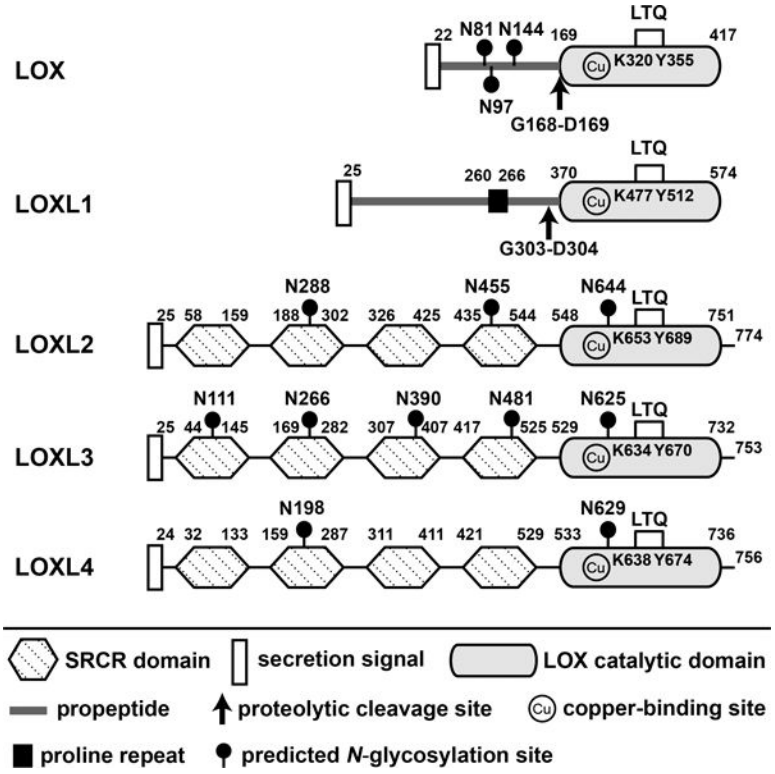

Figure 8. LOX family of proteins

Cartoon summarizing the features of the LOX family of proteins. Each member retains a conserved C-terminal lysyl oxidase-like catalytic domain, which encompasses a copper-binding site composed of a His-*X*-His-*X*-His sequence, as well as a covalently bound LTQ cofactor formed from the linkage of conserved Tyr and Lys residues in the catalytic core. Each member also possesses an N-terminal secretion signal, and LOX and LOXL2-4 also contain predicted *N*-linked glycosylation sites (i.e. Asn-*X*-Ser/Thr). LOX and LOXL1 constitute one subfamily, possessing an N-terminal propeptide sequence, which is proteolytically removed by procollagen c-proteinase (BMP-1) at a conserved site (*black arrow*). LOXL2-4 constitute the other subfamily, with four SRCR domains instead of a propeptide.

Table 1

Steady-state kinetic parameters and substrate specificities of human AOCs.

AOC Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	V_{max} (nmol H_2O_2 mg^{-1} hr^{-1})[a]	V_{max}/K_m	Ref.
AOC1						
Putrescine[b]	20 ± 1	475 ± 11	24 ± 1			(49)
Benzylamine	+[c]	+	+			(49)
Histamine[b]	2.8 ± 0.07	139 ± 0.6	50 ± 1			(49)
1-Methylhistamine	3.4 ± 0.3	103 ± 1.5	30 ± 3			(49)
Spermidine	1100 ± 480	187 ± 0.4	0.17 ± 0.07			(49)
AOC2						
Methylamine	1700 ± 710			2.4 ± 0.4	0.001	(59)
Benzylamine	167 ± 132			11 ± 2.2	0.06	(59)
Histamine[d]	–			–	–	(59)
2-Phenylethylamine	77 ± 15			44 ± 2.5	0.6	(59)
p-Tyramine	178 ± 51			43 ± 2.4	110	(59)
Tryptamine	56 ± 34			28 ± 3.2	72	(59)
Spermidine[d]	–			–	–	(59)
AOC3						
Methylamine	652 ± 77	336 ± 12	0.515 ± 0.007			(70)
Aminoacetone	66 ± 18	87.6 ± 6.0	1.33 ± 0.17			(70)
Benzylamine	84.5 ± 9.8	206 ± 6	2.44 ± 0.072			(70)
2-Phenylethylamine	2050 ± 620	67.2 ± 4.8	0.033 ± 0.002			(70)

[a] nmol H_2O_2 produced per mg of total protein (clarified soluble cell lysate) per hour

[b] Partial substrate inhibition was observed only with histamine and putrescine.

[c] The positive symbol (+) denotes substrate oxidation could be detected, but at rates too low to determine kinetic parameters.

[d] Tested substrates lacking observable level of oxidation.

Table 2

LOX family members in cancer

Type of Cancer	Family member	Expression	Reference
Basal and squamous skin cell carcinoma	LOX	P, I; ↓	(98)
Breast	LOX	R, P, A, I, C; ↑	(95,142-151)
	LOXL1	R, I; ↑	(152)
	LOXL2	R, P, C, I; ↑	(128,130,145,147,150,153-156)
	LOXL3	R; ↑	(152)
Bladder	LOXL1	R ↓	(116)
	LOXL4	R ↓	(116)
Choriocarcinoma	LOX	R, A ↓	(157,158)
Colon	LOX	R ↓	(97,159,160)
Colorectal	LOX	P ↑	(161-163)
	LOXL2	P ↑	(164)
Endometrial	LOXL2	P ↑	(40)
Esophageal	LOXL2	R ↑	(165)
Fibrosarcoma	LOX	R, A ↓	(157,158)
Gastric	LOX	R, I ↓	(97,166,167)
	LOXL2	P ↑	(40,120)
Head and neck squamous cell carcinoma	LOX	P, C ↑	(143,168)
	LOXL2	R, C ↑	(169)
	LOXL4	R ↑	(140,170,171)
Hepatocellular	LOXL2	P ↑	(40)
Laryngeal	LOXL2	P, I ↑	(40,128)
Lung	LOX	R, P, I ↑	(172-174)
	LOXL1	R ↑	(175)
	LOXL2	R ↓	(176)
Melanoma	LOX	R ↑	(145)
	LOXL3	R ↑	(130)
Oesophageal	LOXL2	P ↑	(40,177)
Oral cavity	LOX	I ↑	(178)
Oropharynx	LOX	I ↑	(178)
Osteosarcoma	LOX	R, P, A ↑	(105,179)

Type of Cancer	Family member	Expression	Reference
Ovarian cancer	LOXL1	R ↑	(134)
	LOXL2	R ↑	(134)
	LOXL3	R ↑	(134)
	LOXL4	R ↑	(134)
Pancreatic	LOX	R ↓	(97)
	LOXL2	P ↑	(180)
Prostate	LOX	R, C ↑	(145,181)
	LOXL2	P ↑	(180)
Renal clear cell	LOX	R, C ↑	(151,182-184)
	LOXL2	P ↑	(40)
Rhabdomyosarcoma	LOX	A ↓	(158)
Testicular seminoma	LOXL2	P ↑	(40)

R, RNA; P, protein; A, enzymatic activity; I, *in vivo* model validation; C, clinical validation; ↑, upregulation; ↓, downregulation.