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Control of lysyl oxidase activity through site-specific deuteration of lysine

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ABSTRACT

Lysyl oxidase (LOX) is implicated in several extracellular matrix related disorders, including fibrosis and cancer. Methods of inhibition of LOX in vivo include antibodies, copper sequestration and toxic small molecules such as β-aminopropionitrile. Here, we propose a novel approach to modulation of LOX activity based on the kinetic isotope effect (KIE). We show that 6,6-$_{D2}$-lysine is oxidised by LOX at substantially lower rate, with apparent deuterium effect on V_{max}/K_m as high as 4.35 ± 0.22. Lys is an essential nutrient, so dietary ingestion of D_2Lys and its incorporation via normal Lys turnover suggests new approaches to mitigating LOX-associated pathologies.

The lysyl oxidase family (LOX, EC 1.4.3.13) comprises five isoforms of homologous Cu-dependent amine oxidases that catalyze oxidative deamination of ε-amino groups of lysine residues in some proteins.[1] The resulting allysine can form Schiff bases, allysine-aldol and pyridinoline cross-links that stabilize some types of collagen and elastin; and modify other peptides and proteins such as an important cytokine TGF-β.[2] LOX modulates properties of extracellular matrix/stromal tissue, and as such has been implicated in a variety of pathologies related to connective tissue, including fibrotic processes, neurodegenerative, ophthalmological and cardiovascular diseases.[3] LOX expression is up-regulated in hypoxic tumors and affects cell motility.[4] For this reason, LOX is important for metastasis in many cancers including breast, colon, and esophagus cancers,[5] and recruitment of bone marrow-derived cells for premetastatic niche formation.[6] LOX is secreted by cells and processed in the extracellular space, but some LOX is transported to nuclei where it may have an effect on gene expression and cell cycle.[1,7] This LOX-mediated control of intracellular activities can be due to the oxidation of Lys in nuclear proteins, affecting gene transcription. Histone H1 can be oxidized within nuclei of vascular smooth muscle cells in a BAPN-inhibitable manner,[1] consistent with the role of nuclear LOX catalysis. Oxidation of Lys in H1 may have an epigenetic effect on DNA-histone and histone-histone interactions similar to that of histone acetylation (although histone acetylation, unlike LOX oxidation, is reversible).

Development of new methods for LOX inhibition in vivo is important for further elucidation of the role that LOX isoforms play in these pathologies. LOX may be inhibited by copper sequestration,[8] antibodies,[6,9] and small molecules[10] such as BAPN and derivatives.[11] However, it is desirable to modulate rather than inhibit LOX, in order to avoid side-effects such as increased elasticity of blood vessels that may lead to aneurisms.[12]

LOX requires the presence of a Cu (II) atom and a unique quinone carbonyl cofactor, lysyl tyrosyl quinone (LTQ[13]), which forms an initial Schiff base with the ε-amino group of Lys. This mechanism of amine oxidation consists of five steps[14] and can be summarised as a reductive amination of LOX, followed by its O_2-mediated oxidation, which yields ammonia and H_2O_2. The major rate-limiting step of the process is the base-assisted hydrogen abstraction from the ε-CH_2 group.[15,16]

An isotope effect is an influence of substitution of a heavy atom for a light one (e.g., deuterium for hydrogen) on the strength of a chemical bond. The bonds between heavier isotopes will have lower energy in the ground state, so the dissociation of these bonds will require more energy. The primary KIE arises when the bond is cleaved during or before the transition state (rate-limiting step). Since the discovery that deuterated substances may have significantly improved pharmacological properties due to the KIE,[17,18] various deuterated substrates for MAO family were prepared and tested. The in vivo effects of deuteration may be substantially larger than the in vitro measured KIE values. For example, a signif-

Abbreviations: BAPN, β-aminopropionitrile; LOX, lysyl oxidase; MAO, monoamine oxidase; KIE, kinetic isotope effect; ^{D}VK, deuterium isotope effect on V_{max}/K_m; ^{D}V, deuterium isotope effect on V_{max}; SSAO, semicarbazide-sensitive amine oxidase.

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icantly higher stability of dideuterotryptamine to deamination by MAO in the brain was demonstrated, the ratio of the remaining substance after pulse-chase being as high as six,[19] although the measured KIE for this substrate was substantially lower, about 1.7.[20,21] KIE may be much larger for copper-dependent AOs, which include LOX. For example, for tyramine, substituted benzylamines, and butylamine the measured ^{D}V values were 2–3, whereas for ^{D}KV much larger KIEs of 4–6 were observed.[16,21–23]

KIE for the physiological LOX substrates, including free Lys, and Lys residues in peptides and proteins were never measured before. Moreover, the potential of 'protection' against LOX should be evaluated as a means to obtain 'reinforced' versions of LOX substrates for pharmacological purposes. In this study, we have prepared deuterated substrates of LOX and compared their stability towards LOX oxidation. The results show that site-specific deuteration of Lys substantially slows down the LOX mediated oxidation, suggesting new approaches to modulating LOX activity.

Deuteration of Lys at position 6 (ε-CH_2) was carried out as shown in Figure 1.[24] The side chain amino group was first converted to a nitrile,[25] which was then deuterated[26] giving the title compound. Site-specific deuteration (position 6 only), rather than per-deuteration, is important because other positions in the Lys side chain may have functions that should not be compromised. For example, Lys residues in collagen hydroxylated at position 5 by lysyl hydroxylase have an important function[27,28] that can be affected by perdeuteration. LOX-produced hydroxylysine and hydroxyallysine can form Schiff bases which can undergo dehydration/Amadori rearrangement, eventually forming pyridinoline derivatives or, upon oxidation, adducts with Arg.[29] Moreover, 5-hydroxylysine can play other roles in collagen. We did not want to interfere with the formation of 5-hydroxylysine or other metabolic pathways, and so prepared the selectively 'reinforced' 6,6-D_2-Lys derivative. The identity and isotope purity (>95%) of this compound was confirmed by ^{1}H and ^{13}C NMR (Fig. 2). 6,6-D_2-Lys was converted into an appropriately protected Fmoc(Boc) derivative **5**, which was then used to synthesise a LOX peptide substrate.[29–31]

LOX used for our studies was prepared from aorta[32] by several methods and was tested using immunoblotting with a monoclonal antibody in parallel with a recombinant, bacterially expressed LOX that was purified by immobilized metal chelate chromatography (Fig. 3). Care was taken to prevent contamination by other amine oxidases, like SSAO (also referred to as VAP-1, encoded by AOC3 gene), which is abundant in aorta. Although SSAO cannot oxidize Lys,[37] it may still interfere with LOX assays, especially because BAPN, the standard inhibitor of LOX, is both a substrate and a competitive inhibitor of SSAO.[38] Methylamine is a specific substrate for SSAO, and berenil is a highly potent inhibitor of SSAO and its homologs,[39] whereas LOX is insensitive to this benzamidine derivative. Thus, berenil-sensitive oxidation of methylamine was also monitored and the purification process[34] was found to yield LOX free of contamination by SSAO (data not shown).

KIE measurements for 6,6-D_2-Lys are shown in Figure 4. To monitor the oxidation process, we used a fluorometric assay based on stoichiometric release of hydrogen peroxide.[40] The measured ^{D}VK effect (^{D}KIE on V_{max}/K_m) was 4.35 ± 0.22 (n = 4) at 37 °C, similar to that reported for butylamine (4.3)[16,21] and smaller than that for substituted benzylamines (5.1–6.4)[22] or tyramine (4.8).[23] This is consistent with the view that the chemical structure proximal to the $–CH_2NH_2$ moiety affects substrate properties; indeed, both Lys and butylamine are similar in this respect. More distant substituents have a negligible influence on ^{D}VK. Interestingly, much smaller ^{D}V effects (^{D}KIE on V_{max}) were reported for the various amines;[16,21–23] for Lys (Fig. 4) it is close to unity (1.34 ± 0.3). The reaction cycle of LOX and other copper-containing AOs obeys a ping-pong mechanism that involves hydrogen abstraction as one rate-limiting step of the reaction cycle,[16,21] the other being oxidation of quinonimine by dioxygen. Therefore, low DV values suggest a strong limitation by the oxidative half-reaction.

The KIE may be used to reduce the activity of LOX without complete blocking of its activity, by selectively 'reinforcing' Lys residues with deuterium thus creating stronger bonds that are less susceptible to oxidation. The effect on V_{max}/K_m is a better estimate of stability gain in vivo that may be achieved by site-specific deuteration of proteins and peptides, since their physiological concentration may be quite low. Also, in case of high molecular weight substrates, formation and dissociation of enzyme-substrate complexes may significantly limit reaction rate and mask isotope effects. For this reason, it is important to measure the KIE on a polypeptide substrate.

LOX is sensitive to anionic residues vicinal to peptidyl-Lys.[29] For instance, Glu (but not Gln or Asp) N-terminal to Lys substantially increased the catalytic efficiency of LOX oxidation as compared to Glu C-terminal to Lys.[41] LOX oxidizes basic globular proteins (pI >8) such as histone H1, but does not oxidize neutral or acidic proteins (pI <8).[42] The rate of autocatalytic oxidation of LOX is low as it has a small number of Lys residues (six Lys residues out of 417 in the human enzyme) in its pro-LOX sequence compared to an average of about 7% for other proteins.[1] We have prepared a typical peptide fragment of collagen containing a single deuterated Lys residue[31] and known to be a good substrate for LOX.[27–29] Rate of its oxidation was measured in the concentration range from 0.1 to 0.8 mM and it was found that the isotope effect on V_{max}/K_m of the 6,6-D_2-Lys-containing peptide is 3.1 at 37 °C (Fig. 5). This confirms that the KIE measurements for 6,6-D_2-Lys described above are consistent with the estimates that deuterated peptides and proteins should be approximately three times more resistant to oxidation by LOX in vivo.

Lys is an essential amino acid for many animals including primates. Thus, a mechanism is in place to take up Lys, as well as isotope

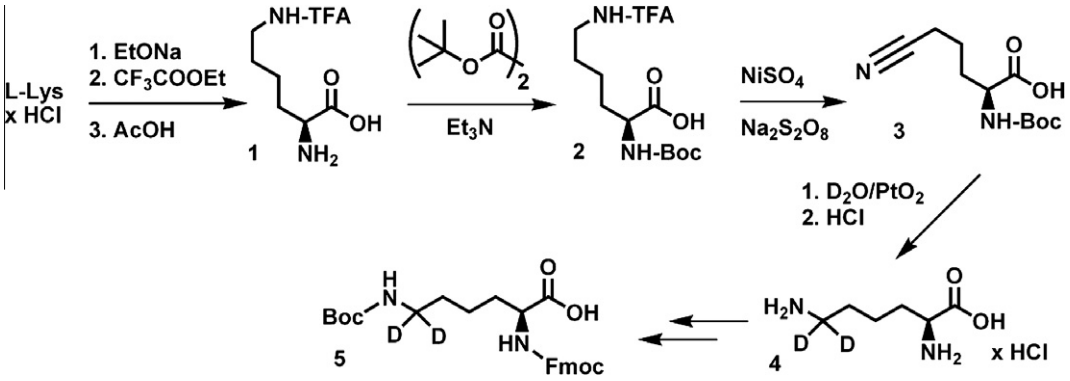

Figure 1. Synthesis of 6,6-dideutrolysine.[24,30]