Invited Paper

Metallopeptides — from Drug Discovery to Catalysis‡

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Oligopeptides are involved in diverse biological activities, including neurotransmission and antibiotic. Many natural-occurring peptides and peptide-ketide hybrids exhibit specific biological activities and chemical reactivities upon binding with certain metal ions, such as divalent metal-binding antibiotic bacitracin and anticancer Fe/Cu-bleomycin. There are also numerous synthetic peptides designed to bind metal ions to exhibit wide range of physical properties and chemical and biological activities. In this review we summarize the background and discuss our research on metal binding properties, structures, and chemical reactivities of three metallopeptides, the bacterial antibiotic bacitracin, the Alzheimer's disease-related β-amyloid, and the salivary antimicrobial histatin. Despite their different structures and biological functions, the Cu^{2+} complexes of these peptides exhibit significant activities toward catechol oxidation and phenol hydroxylation. However, the mechanisms of the oxidative reactions among these three Cu-peptides seem to be distinctively different. Our current understanding about the structure-bioactivity relationship and chemical reactivities as well as implications in drug discovery of these peptides are summarized herein.

Keywords: Alzheimer's disease; Antibiotics; Antimicrobial; Bacitracin; β-Amyloid; Catechol oxidase; Copper; Histatin; Metalloantibiotics; Metallopeptide; NMR; Oxidation; Paramagnetic.

INTRODUCTION

The peptide –(C=O)–NH– and ketide –(C=O)–CH_2– moieties are two fundamental building blocks in numerous natural products which require multi-domain nonribosomal synthetases to bring together.[1] Oligopeptides are involved in diverse biological activities,[2] including neurotransmitters (e.g., N-acetylaspartylglutamate NAAG and β-endorphin) and other roles in the nervous systems (such as the neuropeptides dynorphin, enkephalin, galanin, and ghrelin), hormones (such as calcitonin and adrenocorticotrophic hormone ACTH), neurotoxins (such as α-latrotoxin from black widow spider and conotoxin from cone snails), antibiotics (such as mammalian defensins, human histatins, amphibian dermaseptins, insect melittins, fungal alamethicin, and bacterial bacitracin, actinomycin, polymyxins, and gramicidin), and the neurodegeneration-related β-amyloid in Alzheimer's disease. Likewise, there are many ketide-peptide-containing natural products which exhibit specific biological activities,[3] including antibiotics (such as erythromycin and rifamycin), anticancer reagents (such as bleomycin and epothilones), the potent immunosuppressant rapamycin, and many compounds of diverse biological activities.

There are a number of small natural products that can bind metal ions and exhibit specific biological activities, such as molecular recognition and redox activities.[4,5] Similarly, some natural-occurring peptides and peptide-ketide hybrids exhibit specific biological activities and chemical reactivities upon binding with certain metal ions, such as the antibiotics bleomycin (Blm),[6] histatin,[7] and bacitracin,[8,9] and the Alzheimer's disease-related β-amyloid (Aβ) peptide.[10] Blm exhibits Cu-dependent oxidative

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DNA cleavage[11] and forms a superoxide O_2^--Fe^{3+}-Blm complex by introducing dioxygen to Fe^{2+}-Blm[12] which is able to cleave DNA.[13] Metallo-bacitracin binds sugar-carrying lipid pyrophosphate which prevents bacterial cell wall synthesis.[8] The metallopeptides Cu-Aβs have been recently demonstrated to exhibit catechol oxidase and phenolase-like activities and also an activity toward oxidative DNA cleavage.[14] These peptide and ketide nature products represent a family of "Nature's minimalistic systems" that exhibit specific metal-dependent biological recognition and catalyses. Moreover, a number of peptides and peptide conjugates have recently been discovered or designed to bind metal ions to form various metallopeptides, which exhibit interesting physical properties and chemical and biological activities, such as heavy metal binding,[15] DNA recognition and cleavage,[16] spectroscopic detector,[17] assembling of mineral nanoclusters,[18] antifungal and antibacterial activities,[19] specific enzyme inhibition,[20] artificial receptors,[21] fluorescent sensing,[22] stabilization of secondary structure,[23] thio-ligand binding,[24] catalysis,[25] and combinatorial drug discovery.[26] The various properties and activities of these metallopeptides can be partially attributed to the versatility of amino acids as the building blocks. The highly versatile nature of these natural-occurring functional metallopeptides can serve as inspiring scaffolds for further exploration of various types of metal-centered chemistry. In this review, three metallopeptides, bacterial antibiotic metallobacitracin, Alzheimer's disease-related metallo-β-amyloid, and human salivary antimicrobial metallohistatin are discussed, which can serve as prototypical metallopeptides to show a wide range of biological, chemical, and physical properties. Better understanding of the structure, activities, and reactions of these metallopeptides can provide insights into design of peptides as chemical model systems, catalysts, drugs, or materials.

ANTIBIOTIC PEPTIDYL BACITRACIN

Bacitracin (Bc) is a cyclic dodecapeptide antibiotic excreted by *Bacillus* species, including *B. subtilis* and *B. licheniformis*, as a mixture of over 30 confirmed congeners.[27] The structural variation is mainly due to the change between Ile and Val at positions 1, 5, and 8 (R^1, X^5, and Y^8; Fig. 1A). It has widely been used in animal feed as a preventive drug for livestock[28,29] and in human medicinal "triple antibiotic" ointments (bacitracin, neomycin, and polymyxin B) such as Neosporin® and "double antibiotic" (bacitracin and polymyxin B) such as Polysporin®.[30] Bacitracin A_1 is the major component with the most potent antibiotic activity among all congeners,[31,32] which contains a thiazoline ring originated from Ile-1 carboxylate and the NH_2 and the SH groups of Cys-2 (R^1 and Cys^2 in Fig. 1).[33,34] In addition, it contains four D-amino acids and a unique cyclic heptapeptide structure formed via an amide linkage between the side chain of Lys-6 and the C-terminus of Asn-12, which may protect this peptide from easy degradation by proteases.

Bacitracin requires a divalent metal ion for its antimicrobial activity[35] and is prepared as the Zn^{2+} complex for medicinal use. Bc forms 1:1 complexes with several divalent metal ions,[36-38] with affinity constants following the trend of $Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+}$ based on microcalorimetric study.[38] Cu^{2+} binding to Bc was verified with electron paramagnetic resonance (EPR) spectroscopy,[37] revealing a typical tetragonally distorted coordination sphere ($g_x = 2.0575$, $g_y = 2.0469$, and $g_z = 2.2610$) and two superhyperfine-coupled coordinated N presumably from the imidazole ring of His-10 and the thiazoline ring. The binding of Mn^{2+} to Bc was shown to exhibit a significant superoxide dismutase (SOD) activity.[39] Since Mn^{2+} phosphate exhibits a significant SOD activity[40] and Mn^{2+} binds Bc weakly,[38] how these observations were taken into consideration was not discussed in the previous study of the SOD activity of Mn-Bc.

We have utilized paramagnetic metal ions extensively as NMR probes for structural and mechanistic studies of a variety of metal complexes,[41,42] metalloproteins,[43-53] and metallodrugs.[4,33,54-56] Owing to the paramagnetism, NMR signals of protons in close proximity of the metal are hyperfine-shifted outside the normal spectral window of less than 15 ppm in a large range which may reach more than

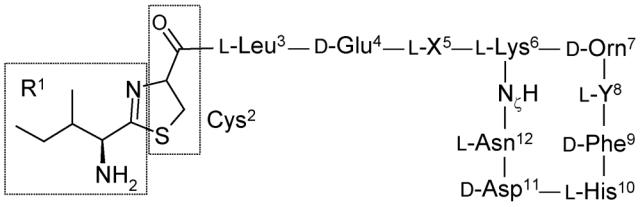

Fig. 1. Primary structure of bacitracin. The positions R^1, X^5, and Y^8 vary between Ile and Val to afford numerous congeners, with A1 ($R^1 = X^5 = Y^8$ = Ile) the major congener.

100 ppm in many cases.[57-60] Co^{2+} was used as a paramagnetic probe for structural study of metallobacitracin. A full analysis of the hyperfine-shifted ^1H NMR signals of Co^{2+} complexes of several bacitracin congeners in aqueous[33] and DMSO[61] solutions were done by means of 1D and 2D NMR techniques (Fig. 2). The NMR studies unambiguously revealed the structure of the metal binding environment, in which the metal binding ligands have been revealed to be the thiazoline ring nitrogen, the Nε nitrogen of His-10, and the carboxylate side chain of D-Glu-4. A structure was suggested based on the NMR study along with molecular mechanics calculations (Fig. 3), showing a hydrophobic pocket comprised of Ile-5, Ile-8, and D-Phe-9 (Black residues, Fig. 3). D-Glu-4 was found not to be a coordinated ligand in those few inactive congeners, including Bc-A_2 and Bc-F, suggesting that its coordination chemistry may play a significant role in the action of Bc. Our recent NMR study revealed that D-Glu-4 is detached from the metal in the Asn12-desamido derivative of low activity.[61] A relationship between the structure and the activity of this antibiotic has thus been suggested, wherein the congeners of high antibiotic activities have similar coordination sphere, including the bound D-Glu-4, and show similar hyperfine-shifted features different from those of low activities.

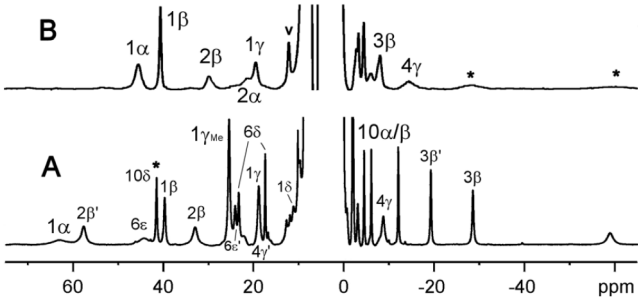

Fig. 2. Hyperfine-shifted ^1H NMR features of Co^{2+}-Bc in DMSO[61] (A) and in water at pH 5.0 (B).[33] The signals have been fully assigned as shown by means of 1D and 2D NMR techniques as labeled. The asterisked signals are solvent exchangeable signals.

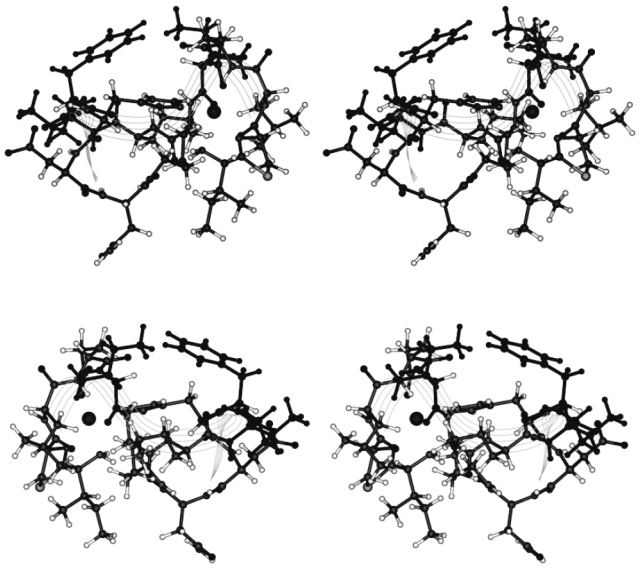

Fig. 3. Two different stereo relaxed-eye views of the structure of Co^{2+}-Bc obtained from NMR studies and molecular dynamic calculations from the direction of the metal-binding site (top) and the hydrophobic pocket (bottom). The residues comprised of the hydrophobic pocket are labeled in black.

Metallobacitracin binds tightly to C_{55}-isoprenyl pyrophosphate,[62] which prevents the lipid pyrophosphate from being hydrolyzed by a membrane pyrophosphatase and subsequently inhibits N-glycosylation of nascent proteins in the lumen of the endoplasmic reticulum. This inhibition thus serves as the key step in the inhibition of cell wall synthesis by this antibiotic.[63] Recent solid-state total synthesis of the enantiomer of Bc showed a similar activity as Bc in vitro, suggesting non-stereo-specific interaction for the antibiotic activity of this antibiotic which was suggested to be the achiral long-chain bactoprenyl-pyrophosphate.[64] Although a pyrophosphate-metal-bacitracin ternary complex was proposed[65] detailed binding and structural information about this ternary complex was lacking. The binding mode of D-Glu-4 side chain in bacitracin was found to reflect the activity of this antibiotic;[33] however, whether or not it is involved in the interaction with lipid pyrophosphate and its role in this interaction, if any, was not concluded. Our recent NMR study on the binding of pyrophosphate and derivatives shows direct binding of the pyrophosphate moiety to the metal in metallobacitracin.[61] The coordinated D-Glu-4 is suggested to detach from the metal upon binding of the pyrophosphate moiety based on the disappearance of its hyperfine-shifted signals upon trimetaphosphate binding, once again suggesting an important role of D-Glu-4 in the action of this antibiotic. When farnesyl pyrophosphate is docked onto metallobacitracin with the pyrophosphate moiety bound to the metal, the hydrophobic moiety of lipid pyrophosphate is found to fit into the "hydrophobic pocket"

of Bc (Fig. 3) which results in a significant expansion of the pocket from 3.91 to 7.55 Å for the Cδ-C4 distance between Ile-5 and Phe-9.[61]

Bc was reported to affect cleavage of DNA by the restriction enzymes HindIII and SmaI; however, the role of metal ions in such interaction was not investigated.[66] We found that the agrose gel pattern of plasmid DNA is not affected by apo-Bc; whereas the presence of Mn^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+} complex of Bc results in retardation in DNA migration.[61] This observation indicates that metal ions play an important role in the interaction of bacitracin with DNA. DNA is damaged by Bc in the presence of an iron or copper salt according to the formation of thiobarbituric acid-reactive material and observation of oxidative modification on the bases.[67,68] We also observed that plasmid DNA was rapidly cleaved by Cu^{2+}-Bc aerobically in the presence of 5.0 mM ascorbic acid which is readily visible at 5 min and nearly completely cleaved in 60 min, predominantly following single-stranded cleavage (Fig. 4). Therein, Cu^{2+}-Bc is reduced by ascorbic acid to yield Cu^{+}-Bc, which can bind O_2 and may eventually produce H_2O_2 and generate $^{\bullet}OH$ radical that can harm DNA similar to the well established Cu^{2+}-1,10-phenanthroline/O_2 chemistry under reduction conditions.[69]

Cu^{2+} complexes are widely known to catalyze oxidation of various organic compounds, such as epoxidation of alkenes, oxygenation of alkanes and aromatics, oxidation of alcohols, and sulfoxidation.[70] In addition to the oxidative activity toward DNA cleavage, Cu^{2+}-Bc can also oxidize catechol in the air to yield quinone which follows enzyme-like pre-equilibrium kinetics with $k_{cat} = 0.0070\ s^{-1}$, $K_m = 3.3$ mM, and a second-order rate constant $k_{cat}/K_m = 2.1\ M^{-1}\ s^{-1}$ and significant increase in k_{cat} ($0.38\ s^{-1}$) and k_{cat}/K_m ($14.7\ M^{-1}\ s^{-1}$) in the presence of 32 mM H_2O_2.[61] Quite interestingly, although the enzyme catechol oxidase contains a di-Cu active site and performs dinuclear catalysis,[71] the oxidation of catechol by Cu^{2+}-Bc seems to follow a mononuclear pathway based on "mechanistic Job plot" by the use of activity as the output which reveals that the substrate-bound intermediate [Cu^{2+}-Bc]-S has 1:1 stoichiometry. Moreover, titration of the inert substrate 4,5-dichlorocatechol (DCC) to Cu^{2+}-Bc monitored by the change of the charge transfer band at 304 nm reveals direct binding of this substrate to the metal center with an affinity constant of $2.40 \times 10^4\ M^{-1}$ (Fig. 5) to form a 1:1 DCC-to-[Cu^{2+}-Bc] complex, also reflecting a mononuclear catalytic pathway for catechol oxidation by Cu^{2+}-Bc. This 1:1 binding stoichiometry is also confirmed with Job plot.

Accordingly, a mechanism is proposed (Fig. 6) wherein the binding of the catechol substrate to Cu^{2+}-Bc (**A**, **B**) is followed by one-electron transfer from the substrate to Cu^{2+} to yield [Cu^{+}-Bc]-semiquinone intermediate (**C**). It then binds O_2 and is followed by electron transfer from Cu^{+} to O_2 to afford a Cu^{2+}-superoxo center (**D**), which then oxidizes the bound semiquinone to produce the final quinone product and a Cu^{2+}-peroxo intermediate (**E**). The intermediate can bind and oxidize another substrate (**F**). A number of Cu^{+} complexes can bind O_2 at low temperatures to form dinuclear Cu-O_2-Cu ternary complexes.[72] Whether or not such a dinuclear complex can be formed in the oxidative action of Cu-Bc under similar conditions awaits future in-

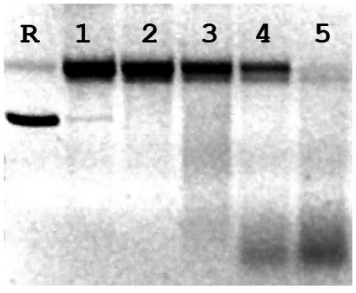

Fig. 4. Cleavage of 170-ng plasmid (R) by 75 μM Cu^{2+}-Bc in the presence of 5.0 mM ascorbic acid (lanes 1-5) at 5, 10, 20, 40, and 60 min from left to right in 100.0 mM HEPES at pH 7.0 and 37 °C.

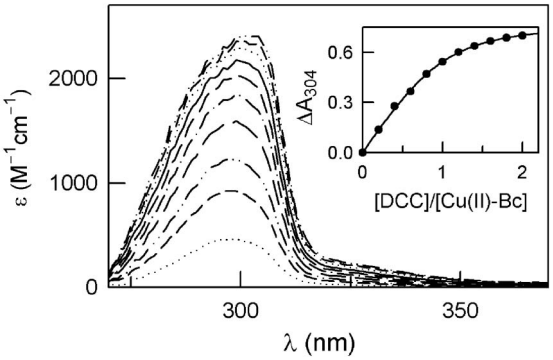

Fig. 5. DCC binding to Cu^{2+}-Bc in DMF to afford the [Cu^{2+}-Bc]-DCC complex (with 0.2 equivalent increment). The inset shows the change in absorbance at 304 nm with respect to the equivalent of DCC and fitted to a 1:1 [Cu^{2+}-Bc]-to-DCC stoichiometry.

vestigation.

ALZHEIMER'S DISEASE-RELATED β-AMYLOID PEPTIDES

Alzheimer's disease (AD) is a progressive brain-degenerative disease and is one leading health issue among elderly people. It is the most common cause of dementia which eventually leads to the loss of ability to perform daily routines, thus can cause significant burdens to the families of the patients. An estimate of about 2.4-4.5 million Americans have this disease, in most cases showing first symptom after age 60.[73] With a gradual increase in the average age of the population, AD has become a significant social and political issue. Although the etiology has not been fully revealed for sporadic AD, evidence for several different possible causes has been hypothesized and/or demonstrated. Coagulations of amyloid β-peptide (Aβ) in the brains as plaques and fibrils have been hypothesized to be associated with the pathogenesis of AD,[74] which has gained further support by some observations that Aβ plaques are toxic to neurons and produce toxic effects in some rat models.[74-76] Nevertheless, whether or not Aβ is a cause or a consequence of AD still cannot be concluded at this stage. Thus, the linkage between Aβ and AD must be further investigated and clarified in order to provide possible prevention and treatments of this disease. One missing link seems to include the lack of thorough understanding of the chemical and physical properties of Aβ and metallo-Aβ at the molecular level.

The Aβ peptides are generated from the cleavage of the ubiquitous amyloid precursor protein (APP) by α, β, and γ secretases, wherein Aβ(1-40/42) fragments are generated by secretases β and γ while Aβ(1-16) matches secretases α and β cutting sites.[77] Aβ(1-42) has a sequence of DAEFR **H**DSGY[10] EV**HH**Q KLVFF[20] AEDVG SNKGA[30] IIGLM VGGVV[40] IA. Aβ has several potential metal-binding side chains, including Asp, His, and Glu that are frequently found in the metal-binding sites of metalloproteins. Growing evidence has been accumulated in recent years which points the involvement of metal ions in the formation of fibril amyloid plaques and the role of metallo-Aβ in causing oxidative stress in the brains of AD patients.

Because full reduction of O_2 to water is a 4-electron process, any imbalance in redox reactions may result in the formation of intermediate reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet}$) from one-electron reduction and peroxide O_2^{2-} from two-electron reduction of O_2. Particularly, the latter can lead to further production of free radicals in the presence of a redox-active metal center under reduction conditions via Fenton-type chemistry.[78] The ROS are the culprits for initiating biological oxidative stress, wherein biomolecules can be oxidatively damaged. Since the concentrations of redox-active metal ions in the biological systems are negligibly low, how is oxidative stress initiated? One possible mechanism can be due to denaturation of metal-bound biomolecules which results in exposure of their metal center(s). Another mechanism may be misregulation of reactions, such as the loss of the oxygen transport capability of hemoglobin due to one-electron oxidation of the active Fe^{2+} center to yield methemoglobin and superoxide and excessive oxidation of catecholamine neurotransmitters. The activation of O_2 by biomimetic metal complexes and the structure of intermediate O_2 complexes of mononuclear and dinuclear metal centers have been extensively investigated.[72,79] Moreover, Fe and Cu centers can interact with ROS which may result in degradation of the ROS, such as catalase acting on peroxide and superoxide dismutase on superoxide, and/or may also be accompanied by catalytic oxidation of substrates, such as activation of H_2O_2 by peroxidases for oxygenation/peroxidation of organic substrates. If the production and activation of the ROS are not regulated, damages on biomolecules can occur.

Aβ and some of its fragments have been demonstrated to bind metal ions, including $Fe^{2+/3+}$, Cu^{2+}, and Zn^{2+}, which may result in the formation of fibrils.[80] The use of variants with His-13 replaced by a Gln revealed the importance of this His for metal binding and formation of Aβ plaques.[80g] This result is consistent with the observation that mouse Aβ with His-13 → Arg substitution is much less prone to form aggregates with metal ions.[80a] We have demonstrated by the use of Co^{2+} as a paramagnetic NMR probe that the metal binding site is comprised of three histidine residues, His-6, His-13, and His-14.[14a] The redox-active Cu- and Fe-Aβ have been shown to cause neurotoxicity via the generation of the ROS, which potentially can cause damages on cell membrane, proteins, DNA, and other biomolecules and lead to cell death.[81] However, those diseases where free Cu or Fe ions are accumulated such as Wilson's disease (Cu) and Hallervorden-Spatz disease (Fe) do not

show excessive Aβ plaque formation.[82] Moreover, significance of the soluble forms of Aβ in the pathogenesis of AD has recently been proposed and verified.[83] Further research is needed to clarify the roles of metal ions and metallo-Aβ in AD. Regardless, since metallo-Aβ forms aggregates and is a pathological feature of AD, it is thus important to reveal details about the interactions between metal ions and Aβ and to understand the mechanisms of the reactions associated with metallo-Aβ. Despite the immense knowledge about AD and massive publications about Aβ and its metal complexes, the chemistry of the redox-active Fe- and Cu-Aβ is largely unexplored. Gaining further insight into the structure and chemistry of metallo-Aβ is expected to provide the molecular basis for possible blockage of the formation of Aβ aggregates and inhibition of the oxidative activities of metallo-Aβ, which may lead to possible prevention and therapeutic strategies toward AD in the future.

Some information about the structure and metal binding of Aβ and the chemistry associated with metallo-Aβ has emerged by the use of various physical methods. For example, the morphology of Aβ fibrils was revealed with electron microscope,[84] X-ray fiber diffraction revealed β-sheet structure in Aβ,[85] the presence of α-helical intermediate in Aβ fibrillogenesis was suggested,[86] Raman spectroscopic[80i,k] and potentiometric[80l] studies have generated results about metal binding, the β-sheet structure of Aβ fibrils has been revealed with solid-state NMR techniques,[87] solution NMR techniques have been utilized for the study of Aβ in micelles to reveal helical structures,[88] fluorescence correlation spectroscopy has been applied to the investigation of aggregations of Aβ,[89] spin-trap electron paramagnetic resonance (EPR) method has been utilized for the investigation of free-radicals generation by metallo-Aβ,[81] the damages of cellular components by ROS have been determined from the reaction products,[81] and the interactions of lipid membranes with and damages by Aβ and metallo-Aβ have been investigated.[90] Despite the extensive studies, structural information about Aβ and metallo-Aβ under different conditions is still not complete and their chemistry not well established. Moreover, the chemical reactions associated with the insoluble Aβ fibrils are heterogeneous in nature which require different approaches by means of spectroscopic and kinetic methods, and can be more technically challenging relative to the investigation of homogeneous systems.

The mechanism for the oxidative stress in AD has not been completely revealed, other than that H_2O_2 is produced by redox-active metallo-β-amyloid.[91] We have demonstrated that Cu^{2+}-Aβ exhibits metal-centered oxidative chemistry toward the polyphenol trihydroxylbezene, catechol, and phenol to yield the corresponding *o*-quinones with and without H_2O_2 (Fig. 7).[14] In summary, *i*) the oxidation reactions follow enzyme-like kinetics with much higher activities by Cu-Aβ in the presence of H_2O_2 than by H_2O_2 alone, suggesting significant role of "metallo-ROS" in oxidative stress (herein ROS = H_2O_2); *ii*) a plot of k_{cat} as a function of [H_2O_2] gives a sigmoid curve, reflecting possible presence of cooperativity in this catalysis; *iii*) catechol oxidation by (Cu-Aβ)-H_2O_2 gives a significant catalytic efficiency k_{cat}/K' = 1.5 mM^{-1} s^{-1}, compared to 32 mM^{-1} s^{-1} for catechol oxidase;[92] *iv*) the slow substrate 4,5-dichloro-

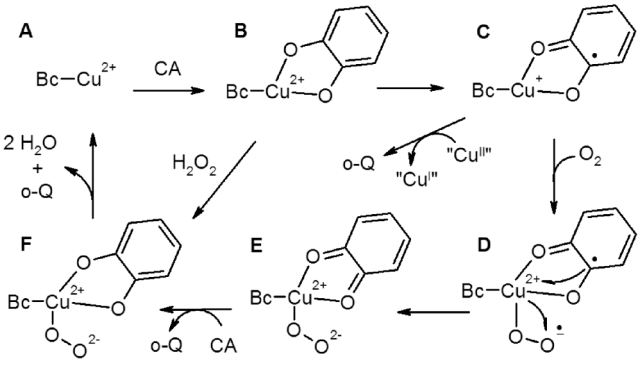

Fig. 6. Proposed mononuclear mechanism for catechol oxidation by Cu^{2+}-Bc in the presence (A, B, and F) and absence (A through F) of H_2O_2.

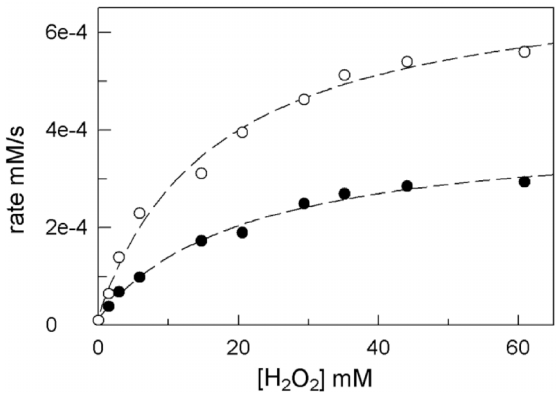

Fig. 7. H_2O_2 activation of Cu-$A\beta_{1\text{-}16}$ (o) and Cu-$A\beta_{1\text{-}20}$ (•) at a fixed concentration of trihydroxylbezene of 6.0 mM in 100-mM HEPES buffer at pH 7.0 and 25.0 °C. The saturation profiles indicate that H_2O_2 binds to the metal center to oxidize the substrate trihydroxylbezene.

catechol (DCC) shows direct binding to the metal center of Cu-A$\beta_{1\text{-}20}$ with the stoichiometry (DCC)-(Cu-Aβ)$_2$, resulting in an intense catecholate-to-Cu charge transfer transition at 437 nm which suggests that a dimeric (CuAβ)$_2$ center may be the active species for the oxidation catalysis; *v*) plasmid supercoiled DNA is noticeably cleaved in 10 minutes by 5.0-μM Cu-A$\beta_{1\text{-}20}$ with only 0.2% of H_2O_2 and is completely cleaved after 30 minutes, wherein the appearance of the linearized form in the early stage of cleavage indicates possible double-stranded DNA cleavage, whereas no cleavage was observed by H_2O_2 alone within the same time span (Fig. 8); *vi*) the oxidation chemistry of Cu-Aβ is inhibited by the reducing agents ascorbic acid and glutathione with different inhibition patterns,[93] competitive and noncompetitive, respectively; *vii*) the oxidation chemistry is activated by Met, which binds directly to the Cu^{2+} site via its thioether-S atom yet is not oxidized within a day by the metal as revealed by electronic, EPR, and ESEEM spectroscopic methods, suggesting Met35 in Aβ may not play a redox role.[93]

The oxidation of a prototypical neurotransmitter dopamine by Cu-A$\beta_{1\text{-}20}$ also showed enzyme-like pre-equilibrium kinetics as catechol with turn over k_{cat} of 0.0116 and 0.099 s^{-1} and catalytic efficiency k_{cat}/K_m of 22.3 and 190 M^{-1} s^{-1}, respectively, in the absence and presence of 0.17% H_2O_2 at pH 7.0,[94] which represents a significant enhancement in dopamine oxidation by 1,230 and 5,530 folds, respectively, with respect to the auto-oxidation of dopamine without Cu-Aβ. Likewise, oxidation of Levodopa (i.e., L-DOPA), the drug for Parkinson's disease, by Cu-A$\beta_{1\text{-}20}$ with and without H_2O_2 at pH 7.0 affords rates ~300-1,400 times higher than auto-oxidation of the drug; and the hydroxylation and oxidation of 5-hydroxy-Trp, the precursor of serotonin, affords dramatic (0.83-5.64) × 10^4-fold rate enhancements under the same conditions. Other neurotransmitters epinephrine, norepinephrine, and serotonin are also oxidized effectively, particularly with rate enhancement relative to the oxidation by H_2O_2 alone is in the range of 1,300-9,400-fold which demonstrates the potency of [Cu-Aβ]-H_2O_2 as a metallo-ROS for oxidative catalyses. The oxidation of dopamine by Cu-A$\beta_{1\text{-}16,20,40}$ is significantly affected by the detergent SDS, wherein k_{cat} is enhanced by 2 times before the critical micelle concentration of ~8 mM and 8 times by micellar SDS. These results indicate that metallo-Aβ can cause problem on normal neurotransmission by excessive oxidation of neurotransmitters, which becomes more significant when the reactions are taking place on/in the cell membrane. Since catecholamine neurotransmitters are involved in many physiological, psychological, and neuronal processes, including mode change, hormonal regulation, and sleep cycle, disturbance of neurotransmitter metabolism thus may render health disorders and abnormality in daily activities. Consequently, whether or not metallo-β-amyloid is a cause or a result of AD, it can always exacerbate the disease owing to the significant oxidative activities toward important biomolecules.

These oxidation reactions catalyzed by Cu-A$\beta_{1\text{-}20}$ with and without H_2O_2 match the mechanism of catechol oxidase,[95] which contains a di-Cu center and catalyzes

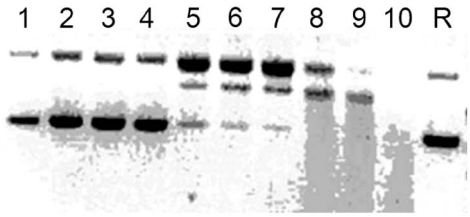

Fig. 8. Time course toward the cleavage of 150-ng plasmid DNA (R: pQE30Xa from Qiagen) by 5.0 μM Cu-A$\beta_{1\text{-}20}$ in the presence of 0.2% H_2O_2 at 10, 15, 20, 30, 40, and 60 min (lanes 5-10, respectively) and absence of H_2O_2 at 10, 20, 40, and 60 min (lanes 1-4).

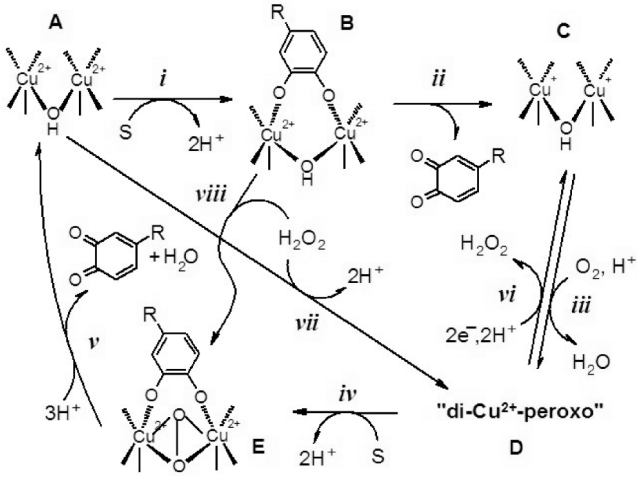

Fig. 9. Dinuclear reaction mechanism for aerobic oxidation of catechol and analogues by Cu^{2+}-Aβ in the absence (***i-v***) and presence (***vii***, ***viii***, ***iv***, and ***v***) of H_2O_2. Under reduction conditions in the air, H_2O_2 can be generated by going through the cycle of ***iii*** and ***vi***.

two-electron oxidation of catechol.[95] In this mechanism, a catechol substrate binds to a dinuclear center (Fig. 9, ***i***), followed by two-electron electron transfer from the substrate to the di-Cu^{2+} center to yield the oxidized product and a di-Cu^{+} center (**C**) which can bind O_2 to form a "di-Cu^{2+}-peroxo" intermediate (**D**), analogous to a highly active isoelectronic copper-dioxygen species (i.e. Cu_2^{2+}-μ-η^1:η^1-peroxo, Cu_2^{2+}-μ-η^2:η^2-peroxo, and Cu_2^{3+}-bis-μ-oxo)[72] responsible for Cu-dependent oxidation and hydroxylation reactions demonstrated in chemical model systems.[96-99] This intermediate can presumably also formed by binding of peroxide to the di-Cu^{2+} site (***vii***), which can bind and oxidize another substrate (***iv, v***). The substrate-bound di-Cu^{2+}-peroxo intermediate (***E***) can also be formed by binding of peroxide to the substrate-bound complex ***B***. In this mechanism, H_2O_2 is produced under reduction conditions (***vi***). Our preliminary results revealed the formation of a greenish intermediate upon addition of H_2O_2 to Cu^{2+}-$A\beta_{1\text{-}20}$ in DMF at -50 °C, presumably a di-Cu-hydroperoxo intermediate which changes color upon introduction of a substrate, suggesting the formation of a ternary complex as in ***E***. Many biologically significant molecules contain a phenol and/or catechol moiety, such as the neurotransmitters discussed herein. Abnormal metabolic pathways of these molecules, such as the unregulated oxidation reactions catalyzed by redox-active metallo-Aβ, can thus be expected to cause metabolic catastrophe. Better understanding of the reaction mechanism and prohibiting of the oxidation chemistry of metallo-Aβ should be a focus in drug discovery and prevention of AD.

METALLOPEPTIDYL ANTIMICROBIAL HISTATINS

Histatins are a family of His-rich cationic salivary peptides,[100,101] mainly expressed as histatin-1 and 3 in higher primates.[102,103] They exhibit antimicrobial activity against *Streptococcus mutans*,[104] *Saccharomyces cerevisiae*,[105] *Cryptococcus neoformans*,[106] *Porphyromonas gengivalis*, and *S. mitis*.[107] Moreover, they have fungistatic and fungicidal properties toward the prevalent and opportunistic pathogenic yeast *Candida albicans*.[108] There are up to 50 proteolytically cleaved fragments of Hn-related peptides isolated from the whole saliva.[109] Among them, histatin-5 (Hn5) has the highest concentration in the saliva which is consisted of the first 24 amino acids (DS**H**AK R**HH**GY[10] KRKF**H** EK**HH**S[20] **H**RGY) of Hn3 and displays the highest activity against *C. albicans* under the physiological concentrations (15-30 μM).[101]

There are different mechanisms for the actions of antimicrobial peptides, including pore formation on the membrane and specific recognition of some target molecules/receptors.[110] Unlike many cationic antibiotic peptides, Hn5 lacks the ability to form pores in the bacterial cell membranes.[111] One proposed antifungal mechanism suggests internalization of Hn5 by binding to the heat shock protein Ssa1/2 on the cell wall,[112] followed by interaction with the K^+ transporter TRK1[113] which leads to the release of K^+, ATP, and other cell components and the eventual loss of cell integrity.[104,108] Extracellular ATP, in turn, binds and activates purinergic-like receptors, which can also lead to apoptosis.[114] In another proposed mechanism,[115] Hn5 is internalized into the mitochondria possibly by translocation down the membrane-potential gradient followed by interference with the electron transfer processes, which leads to the generation of ROS and oxidative damage to biological molecules which leads to cell death.

Hn5 has several potential metal-binding residues, including seven His residues, one Asp, one Glu, and two Tyr residues, and has been shown to bind different metal ions by the use of CD, mass spectrometry, isothermal calorimetry, EPR, and NMR spectroscopy.[116] Both Hn3 and Hn5 have been shown to bind up to 5-equivalent metal ions.[116b,d] Calorimetric study suggests that Hn5 has three metal binding sites for Zn^{2+} and Cu^{2+}. Hn5 has a high-affinity Cu^{2+} and Ni^{2+} binding site at the amino-terminus (i.e., the N-terminal Asp-Ser-His site analogous to the Asp-Ala-His site in bovine serum albumin known as the ATCUN site)[117] and a preferential HEXXH metal binding site found in many metalloproteins. Moreover, there are also two His-His sequence fragments analogous to the His-His site in Aβ for metal binding. Hn5 was reported to bind several divalent metal ions in the order of $Cu^{2+} > Ni^{2+} > Zn^{2+} >> Ca^{2+} \sim Fe^{2+}$.[116b] Metal-binding plays an important role in histatin bioactivities. For example, (a) Hn5 can fuse negatively charged vesicles only in the presence of Zn^{2+} ions,[116a] probably through electrostatic interactions to destabilize cell membranes and (b) synthetic variants of Hn5 exhibit oxidative nuclease activity in the presence of Cu^{2+}.[118] However, how metal ions are involved in antimicrobial activity of Hn5 and the correlation between metal binding and anti-

microbial activities have not been extensively investigated.

We have recently investigated metal binding property of Hn5 and chemical activities of the Cu(II) complex of Hn5.[119] Binding of one equivalent of Cu^{2+} to Hn5 shows an electronic transition at 520 nm, characteristic of the square planar Cu^{2+} coordination sphere at the amino terminus analogous to the 525 nm transition in the Cu^{2+} complex of bovine serum albumin. The binding of the second equivalent of Cu^{2+} to Hn5 exhibits absorption at 620 nm, typical of a tetragonally distorted Cu^{2+} center. The EPR spectrum of the 1:1 Cu^{2+}-Hn5 complex (Fig. 10A) shows $g_{//} = 2.188$, $g_{\perp} = 2.053$, and $A_{//} = 612$ MHz, in good agreement with the square planar Cu^{2+}-ATCUN site of bovine serum albumin ($g_{//} = 2.177$, $g_{\perp} = 2.055$, and $A_{//} = 603$ MHz).[117] The EPR spectrum of the second site in the 2:1 Cu^{2+}-Hn5 complex can be clearly revealed in the difference spectrum with the first Cu^{2+}-binding site numerically subtracted out, showing $g_{//} = 2.261$, $g_{\perp} = 2.056$, and $A_{//} = 540$ MHz (Fig. 10B) which are consistent with the parameters of a Cu^{2+} center of N-rich ligands such as histidine.[120]

Co^{2+} is again used as a paramagnetic NMR probe[57-60] for the study of metal binding to Hn. The 1:1 Co^{2+}-Hn5 complex in DMSO shows five hyperfine-shifted ^{1}H NMR signals a-e (Fig. 11A) in the typical chemical shift range for imidazole ring of a Co^{2+}-bound histidine residue.[59,60] Co_2^{2+}-Hn shows increasing in the intensity of signals c and e (Fig. 11B). The signals a-c disappeared in the presence of 5% D_2O while the signal e significantly decreases in intensity, consistent with solvent exchangeable imidazole NH signals.[59,60] The results indicate the presence of two Co^{2+}-binding sites, with 3 His residues (showing the NH signals a-c) in the first site and 2 His residues (showing the NH signals c and e) in the second site. The first site may not be due to Co(II) binding to the N-terminus since there is only one coordinated His in this site shown in the Cu(II) binding. Analogous to metal binding preference of serum albumin, Hn5 seems also to exhibit a different Co^{2+}-binding sequence relative to Cu^{2+} binding (which binds the N-terminus first).[121]

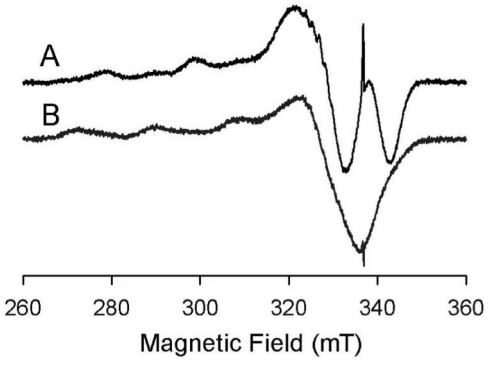

Fig. 10. X-band (9.4 GHz) EPR spectra at 4-5 K of (A) Cu^{2+}-Hn5 and (B) the second site of Cu_2^{2+}-Hn5 with the first site in (A) numerically subtracted out (100 mM HEPES buffer at pH 7.0).

Since Cu^{2+} has significant redox chemistry, we have investigated the oxidative activity of the complex Cu_2^{2+}-Hn5 toward catechol (CA).[119] The oxidation of CA by Cu_2^{2+}-Hn5 in the air follows enzyme-like pre-equilibrium kinetics (○, Fig. 12), with $k_{cat} = 0.011$ s^{-1} and $k_{cat}/K_m = 19$ M^{-1} s^{-1}. This kinetics indicates direct CA binding to the complex to form an intermediate (Cu_2^{2+}-Hn5)-CA, followed by electron transfer to afford *o*-quinone product and Cu_2^{+}-Hn5. The latter then re-oxidizes in the air and starts a new catalytic cycle again. CA oxidation by Cu_2^{2+}-Hn5 has an efficient 2.3×10^4-fold rate acceleration in terms of k_{cat}/k_o with the auto-oxidation rate constant $k_o = 4.7 \times 10^{-7}$ s^{-1}. In the presence of H_2O_2, which is a common ingredient

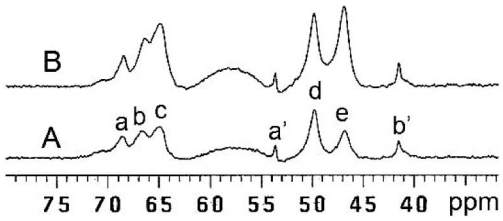

Fig. 11. The ^{1}H NMR spectra (500 MHz) of Co^{2+}-Hn5 (2 mM) in d_6-DMSO at (A) 1:1 and (B) 2:1 Co^{2+} : Hn5 ratios. Signals a-c, e, and b′ are solvent exchangeable which disappear upon addition of 5% D_2O to the samples.

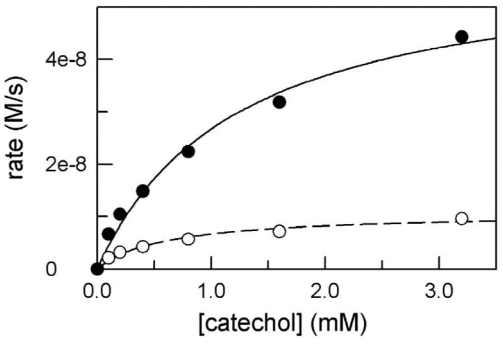

Fig. 12. Oxidation of CA by 1.0-μM Cu_2^{2+}-Hn5 in the absence (o) and presence (•) of 12.8-mM H_2O_2 in 100 mM HEPES buffer at pH 7.0 and 25 °C.

in some hygienic products, including toothpaste and thus may interact with salivary metallohistatins, the rate of CA oxidation significantly increases, with $k_{cat} = 0.060\ s^{-1}$ and $k_{cat}/K_m = 50\ M^{-1}\ s^{-1}$ at $[H_2O_2] = 12.8$ mM (•, Fig. 12), significantly higher than those in the absence of H_2O_2. However, whether or not both metal centers are involved in the oxidative catalysis could not be concluded in this study. Since metal binding is an intrinsic property of Hn5, the oxidative activity of Cu(II)-Hn we observed may play a role in creating oxidative stress in microorganisms and serve as an alternative route for the antimicrobial activity of this peptide. Our preliminary studies of Hn5 fragments reveal that there are indeed four Cu^{2+}-binding sites in Hn5, two on the N-terminal half and two on the C-terminal half.

Better understanding of the structure-reactivity relationship and the correlation of the structure and antimicrobial activity of this family of metallopeptides may hopefully lead to design of new antimicrobial metallopeptides with more effective activities and higher selectivity. Moreover, the highly versatile nature of the natural-occurring functional metallopeptides discussed herein can serve as inspiring "Nature's minimalistic model systems" for further exploration of various types of metal-centered chemistry.

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