



A plausible role of salivary copper in antimicrobial activity of histatin-5—Metal binding and oxidative activity of its copper complex

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ABSTRACT

Histatin-5 (Hn5) is an antimicrobial salivary peptide of 24 amino acids. Two specific metal-binding sites were revealed with electronic, NMR, and EPR spectroscopy. The complex Cu^{II}–Hn5 effectively oxidizes catechol, exhibiting enzyme-like kinetics ($k_{\text{cat}} = 0.011$ and 0.060 s^{-1} and $k_{\text{cat}}/K_{\text{m}} = 19$ and $50 \text{ M}^{-1} \text{ s}^{-1}$ without and with $12.8 \text{ mM H}_2\text{O}_2$, respectively). The significant oxidative activity may contribute to the biological activity of this antibiotic metallopeptide.

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The histatins (Hns) are antimicrobial cationic peptides, secreted by parotid and submandibular glands,¹ exhibiting activities against *Streptococcus mutans*, *Streptococcus mitis*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, and *Porphyromonas gingivalis*.^{2–5} They are also fungistatic and fungicidal against the prevalent and opportunistic pathogenic yeast *Candida albicans*,⁶ inhibit experimental plaque formation and gingivitis,⁷ and are suggested to be major wound-closure stimulating factors in human saliva.⁸ Hns are mainly expressed as Hn1 and Hn3 in higher primates,^{9,10} of which Hn5 is the most abundant in the saliva. It consists of the first 24 amino acids (DSHAK RHHGY KRKFH EKHHS HRGY) of Hn3 and displays the highest activity against *C. albicans* under physiological concentrations ($15\text{--}30 \mu\text{M}$).¹ Unlike many other antibiotic peptides, Hn5 cannot form pores in the bacterial cell membranes.¹¹ One antifungal mechanism suggests internalization of Hn5 by binding to the heat shock protein Ssa1/2 on the cell wall,¹² followed by interaction with the K⁺ transporter TRK1¹³ which leads to the release of K⁺, ATP, and other cell components and subsequently results in apoptosis.¹⁴ Alternatively, Hn5 is internalized into the mitochondria and interferes with the electron transfer processes, which leads to the generation of reactive oxygen species

and oxidative damage.¹⁵ Moreover, synthetic variants of Hn5 exhibit oxidative nuclease activity in the presence of Cu^{II}, suggesting a possible role of metal ions in the action of Hns.¹⁶

Hn3 and Hn5 have previously been suggested to bind up to 5 equiv of different metal ions, including Cu^{II}, Ni^{II}, Zn^{II}, Fe^{II}, and Ca^{II},¹⁷ and can fuse negatively charged vesicles in the presence of Zn^{II}.^{17a} Hn5 has a high-affinity Cu^{II} and Ni^{II} binding site at the N-terminus (analogous to the N-terminal metal-binding ATCUN site of Asp-Ala-His in serum albumin¹⁸) and a presumed Zn^{II} binding site (HEXXH). Herein, we report metal binding of Hn5 and a unique oxidative activity of its Cu^{II} complex by means of magnetic resonance and kinetic methods.

Upon addition of 0.5-equiv Cu^{II} to Hn5 in buffer at pH 7.0, an absorption at 520 nm is detected (Fig. 1). This absorption has a higher energy than that of a regular tetragonally distorted octahedral Cu^{II} center, characteristic of the d–d transition in a square planar Cu^{II} geometry attributable to Cu^{II} binding to the N-terminus of Hn5 (Asp-Ser-His). It also corroborates with the Cu^{II} in the N-terminal ATCUN site (Asp-Ala-His) of serum albumin at 525 nm^{18,19} and the N-terminal fragment of Hn5.^{16a} A plot of the absorption at 525 nm versus [Cu^{II}] yields a dissociation constant of 0.27 mM for Cu^{II} binding to the N-terminus (inset, Fig. 1). Absorptions at 250, 300, and 620 nm become visible with more Cu^{II} added and become distinct at >2.0-equiv Cu^{II}. The 620-nm absorption may be

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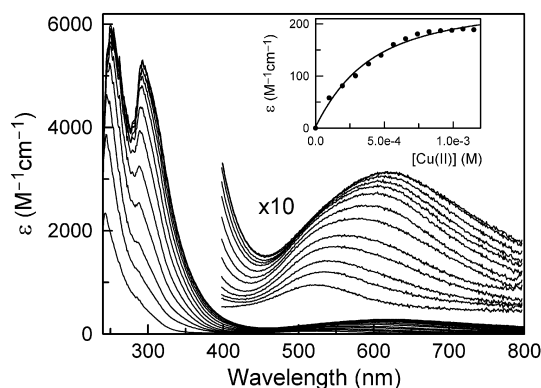


Figure 1. An optical titration of Cu^{II} to Hn5 (200 μM) in 100 mM HEPES buffer at pH 7.0 (with 0.5-equiv increment from bottom to top).

assigned to Cu^{II} in the HEXXH site and/or the di-His H7–H8 site as that in β -amyloid peptide (i.e., H13–H14).^{20a} The detection of the two distinct d–d transitions suggests the presence of at least two specific Cu^{II} -binding sites in Hn5.

Electron paramagnetic resonance (EPR) spectroscopy is a very useful tool for the detection of compounds and materials with unpaired electrons, such as Cu^{II} centers.²¹ The EPR spectrum²² of the 1:1 Cu^{II} –Hn5 complex (Fig. 2A) is axial with $g_{\parallel} = 2.188$, $g_{\perp} = 2.053$, and $A_{\parallel} = 612$ MHz and clear superhyperfine coupling in the g_{\perp} region best fitted to three N-14 nuclei ($I = 1$) with two $A_N = 39.5$ MHz and one $A_N = 36.4$ MHz (Fig. 2A'). The spectral features are in good agreement with those of the square planar Cu^{II} –ATCUN site of bovine serum albumin ($g_{\parallel} = 2.177$, $g_{\perp} = 2.055$, and $A_{\parallel} = 603$ MHz and N-14 superhyperfine couplings)¹⁹ and consistent with the conclusion from the electronic spectra. The superhyperfine couplings are possibly attributed to the two deprotonated amide-N atoms, the N_{δ} atom of His-3 residue, and/or the amino terminus which interact with the magnetic $d_{x^2-y^2}$ orbital of the square planar Cu^{II} . Particularly, the above amide and His N atoms are expected to bind to Cu^{II} better due to their higher Lewis basicity than the amino group and are expected to exhibit the three superhyperfine couplings with $\text{Cu}(\text{II})$.

The addition of 2-equiv Cu^{II} to Hn5 affords a spectrum which contains a mixture of two Cu^{II} -binding sites (Fig. 2B). The differ-

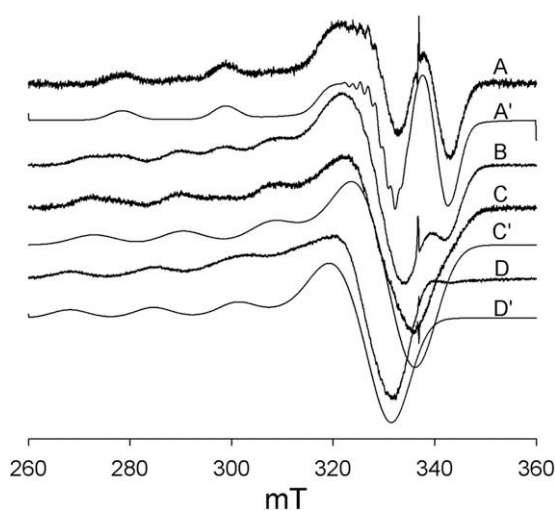


Figure 2. The EPR spectra of the Cu^{II} complexes of Hn5 with different Cu^{II} :Hn5 ratios in DMF: (A) 1:1, with a small population of the second species in (B) subtracted out and (A') is its fitted spectrum; (B) 2:1; (C) difference spectrum of (B–A) and the fitted spectrum (C'); and (D) 4:1, with the first two sites in B subtracted out and (D') is the fitted spectrum.

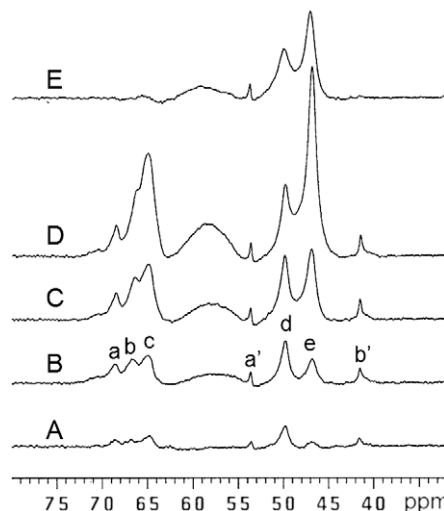


Figure 3. The ^1H NMR spectra of Co^{II} –Hn5 (2 mM) at (A) 0.5:1, (B) 1:1, (C) 2:1, and (D) 6:1 Co:Hn5 ratios in $\text{DMSO}-d_6$. (E) Addition of 5% D_2O to (D) revealed five solvent exchangeable signals a–c, e, and b'.

ence spectrum with spectrum A subtracted out (Fig. 2C) is due to the second Cu^{II} -binding site, which can be fitted with $g_{\parallel} = 2.261$, $g_{\perp} = 2.056$, and $A_{\parallel} = 540$ MHz (Fig. 2C'). These values are consistent with those observed for Cu^{II} centers with predominantly N-containing ligands,²³ such as the H7–H8 site. Addition of up to 4 equiv of Cu^{II} to the peptide results in another spectrum which is revealed in the difference spectrum with spectrum B subtracted out (Fig. 2D). It can be simulated with $g_{\parallel} = 2.312$, $g_{\perp} = 2.078$, and $A_{\parallel} = 520$ MHz (Fig. 2D'), consistent with predominantly oxygen-containing ligands,²³ such as an open coordination sphere with only a small number of coordinated His side chains in the equatorial position(s) interacting with the magnetic $d_{x^2-y^2}$ orbital of Cu^{II} . Possible candidates are the presumed Zn^{II} binding HEXXH, the di-His H18–H19, and a non-specific His binding.

Mononuclear Cu^{II} centers can broaden the NMR signals of nearby protons beyond detection, rendering it difficult to study the complexes with NMR. Conversely, Co^{II} has been utilized as a paramagnetic NMR probe for the study of the metal-coordination environment of various systems,²⁴ including serum albumin,²⁵ via the hyperfine-shifted signals. Upon addition of up to 1.0 equiv of Co^{II} to Hn5 in DMSO, five hyperfine-shifted signals a–e (and two weaker signals a' and b') were detected²⁶ (Fig. 3A and B) in the typical range for the imidazole ring CH and/or NH protons of a Co^{II} -bound histidine residue.²⁴ Further addition of Co^{II} increased the intensity of signals c and e (Fig. 3C and D). The spectral pattern indicates there are at least two Co^{II} binding sites, signals a–d from one site and the overlapped c and e signals from the second site. The signals a–c (and b') disappeared in the presence of 5% D_2O while the signal e (overlapped) significantly decreases in intensity (Fig. 3E), indicating the presence of five (c is due to two overlapped signals) solvent exchangeable imidazole NH protons of Co^{II} -bound histidine residues.²⁴ Apparently, three of them (a, b, and c) are in the first Co^{II} -binding site which thus cannot be the N-terminus ATCUN site with only one His, while two (the overlapped c and e) are in the second site. Since there are two solvent non-exchangeable signals (d and the overlapped e), two of the coordinated His residue must adopt N_{δ} -coordination mode; d in the first site and e in the second site. Such binding mode renders both C_{β}H and $\text{N}_{\delta}\text{H}$ protons farther away from the paramagnetic Co^{II} center than the $\text{C}_{\alpha}\text{H}$ proton and show relatively sharper hyperfine-shifted ^1H NMR signals comparable to each other, of which C_{β}H is not solvent exchangeable. In this case, the $\text{C}_{\alpha}\text{H}$ proton is *ortho* to Co^{II} , thus shows a much broader signal than the other two farther ring protons.

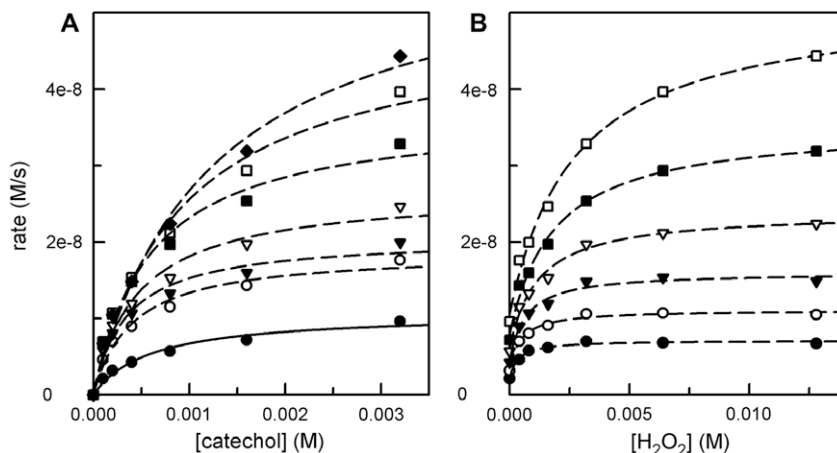


Figure 4. (A) Oxidation of catechol by 1.0 μM Cu^{II}-Hn5 in the presence of 0.0, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mM H₂O₂ (from bottom). (B) Oxidation of various amount of catechol (0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mM from bottom) by 1.0 μM Cu^{II}-Hn5 as a function of [H₂O₂]. Conditions: 100 mM HEPES buffer at pH 7.0 and 25 °C.

In a recent metal binding study of serum albumin,²⁵ Co^{II} was found not to bind to the N-terminal ATCUN site. Owing to the similarity of the N-terminal metal-binding site between Hn5 and serum albumin (Fig. 3 and Ref. 19), Co^{II} probably also does not bind to the N-terminus of Hn5. In stead, Co^{II} may bind to HEKHH or HH· ·H via two N_E and one N_δ atoms of His residues in the first site and two His residues via one N_E and one N_δ atom in the second site. The di-His metal-binding site in β-amyloid adopt N_E coordination mode,²⁰ which is likely to be the case for Co^{II} binding in Hn5. The results thus suggest that Co^{II} binds to Hn5 in an opposite order relative to Cu^{II}, wherein Cu^{II} binds the N-terminus first whereas Co^{II} does not seem to bind the N-terminus.

Since Cu^{II} may be involved in the bioactivity of Hn5¹⁶ and is well known to exhibit redox chemistry by the use of O₂ or H₂O₂ as an oxidant, potential oxidative activity and mechanism of the Cu^{II}-Hn5 complex (with Cu^{II} bound to the two sites in Fig. 2B) was investigated by the use of catechol (CA) as a substrate.^{20,27} The rate of aerobic CA oxidation by Cu^{II}-Hn5 reaches a plateau at a high [CA] (●, Fig. 4A), indicating direct CA binding to the complex to form (Cu^{II}-Hn5)-CA intermediate, similar to the ES intermediate in enzymatic catalysis. Fitting the data to the Michaelis-Menten equation affords $k_{\text{cat}} = 0.011 \text{ s}^{-1}$ and $K_m = 0.59 \text{ mM}$, which represents a 2.3×10^4 -fold rate acceleration in terms of k_{cat}/k_0 with respect to the auto-oxidation rate constant^{20b} $k_0 = 4.74 \times 10^{-7} \text{ s}^{-1}$.

The oxidation reagent H₂O₂ is a common ingredient in some hygienic products, including toothpaste. Thus, its influence on the oxidative activity of salivary Cu^{II}-Hn5 was investigated. The rate of aerobic CA oxidation by Cu^{II}-Hn5 increases with [H₂O₂], affording $k_{\text{cat}} = 0.060 \text{ s}^{-1}$, $K_m = 1.2 \text{ mM}$, and $k_{\text{cat}}/K_m = 50 \text{ M}^{-1} \text{ s}^{-1}$ at [H₂O₂] = 12.8 mM (◆, Fig. 4A), which is significantly higher than the rate without H₂O₂ (●, Fig. 4A). The rate of CA oxidative as a function of [H₂O₂] also follows the Michaelis-Menten kinetics at different [CA]'s (Fig. 4B), for example, $k_{\text{cat}}(\text{H}_2\text{O}_2) = 0.041 \text{ s}^{-1}$ and $K_m(\text{H}_2\text{O}_2) = 2.7 \text{ mM}$ at [CA] = 3.2 mM (□, Fig. 4B; affording a 4.7×10^5 -fold rate acceleration relative to $k_0(\text{H}_2\text{O}_2) = 8.7 \times 10^{-8} \text{ s}^{-1}$ determined for oxidation of 3.2 mM CA by H₂O₂). The kinetics indicates that H₂O₂ can bind to (Cu₂-Hn5)-CA to form a ternary H₂O₂-(Cu₂-Hn5)-CA complex.

The catalytic mechanism may follow (a) a dinuclear or (b) a mononuclear pathway. The dinuclear pathway is analogous to that in catechol oxidase,²⁸ wherein the binding of CA to the di-Cu^{II} center (which can be formed from the two bound Cu^{II} ions or from two Cu^{II} ions in different complexes) in the absence of H₂O₂ is followed by CA-to-Cu^{II}₂ two-electron transfer to afford the *o*-quinone product and a di-Cu^I center. Oxygen then binds to the di-Cu^I center, fol-

lowed by two-electron transfer to produce a di-Cu^{II}-peroxo intermediate as found in catechol oxidase²⁸ and model complexes,²⁹ which further binds and oxidizes CA. In the presence of H₂O₂, the active di-Cu^{II}-peroxo intermediate is formed directly, followed by CA binding and oxidation.

In aerobic mononuclear pathway in the absence of H₂O₂, CA binding to Cu^{II} is followed by CA-to-Cu^{II} electron transfer to generate *o*-semiquinone-Cu^I intermediate. Oxygen then binds to Cu^I, followed by two-electron transfer from the *o*-semiquinone-Cu^I moiety to the bound O₂ to yield *o*-quinone product and a Cu^{II}-peroxo active intermediate which can bind and oxidize CA. In the presence of H₂O₂, the Cu^{II}-peroxo intermediate is formed directly which is followed by CA binding and oxidation. Several mononuclear Cu^{II}-peroxo complexes have been characterized³⁰ to support mononuclear Cu-centered oxidation reactions. The confirmation of the mechanism for the oxidative catalysis by Cu-Hn awaits further characterization of the Cu₂-peroxo intermediate.

Copper in the whole saliva can reach low-μM concentration.³¹ Thus, it is essential to understand the interaction of Cu^{II} with those salivary components capable of metal binding such as Hn5. The Cu^{II}-Hn5 complexes have been implied to play a vital role in the defense against microorganisms in the oral flora.¹⁶ Herein, we conclude the presence of at least two specific Cu^{II}-binding sites in Hn5 and two sites (but excluding the ATCUN site) for Co^{II} binding and that the Cu^{II}₂-Hn5 complex exhibits significant oxidative activity. The results suggest possible involvement of metal ions in the antimicrobial activity of Hn5 via metal binding and oxidative chemistry. Moreover, this peptide may serve as a template for the design of antibiotic metallopeptides against bacterial and fungal infections and metallopeptides for oxidative catalysis.

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