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Copper–Peptide Complex Structure and Reactivity When Found in Conserved His-X_{aa}-His Sequences

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Supporting Information

ABSTRACT: Oxygen-activating copper proteins may possess His-X_{aa}-His chelating sequences at their active sites and additionally exhibit imidiazole group δN vs εN tautomeric preferences. As shown here, such variations strongly affect copper ion's coordination geometry, redox behavior, and oxidative reactivity. Copper(I) complexes bound to either δ-HGH or ε-HGH tripeptides were synthesized and characterized. Structural investigations using X-ray absorption spectroscopy, density functional theory calculations, and solution conductivity measurements reveal that δ-HGH forms the Cu^I dimer complex $[\{Cu^I(\delta\text{-HGH})\}_2]^{2+}$ (**1**) while ε-HGH binds Cu^I to give the monomeric complex $[Cu^I(\varepsilon\text{-HGH})]^+$ (**2**). Only **2** exhibits any reactivity, forming a strong CO adduct, $[Cu^I(\varepsilon\text{-HGH})(CO)]^+$, with properties closely matching those of the copper monooxygenase PHM. Also, **2** is reactive toward O_2 or H_2O_2, giving a new type of O_2-adduct or Cu^{II}–OOH complex, respectively.

The study of peptide complexation to copper ions has been of great interest to (bio)chemists since the most common ligands at copper active sites in proteins are amino acids, most often histidine.[1] A survey of His imidazole group binding to copper proteins involved in redox chemistry, including O_2 reactivity, indicates that the His-X_{aa}-His (X_{aa} = amino acid) tripeptide motif is a frequently observed sequence, including, for example, His-Thr-His in PHM[2,3] and DβM,[4] His-Val-His in SOD5[5] and *p*MMO,[6] and His-Gln-His in APLP2 and LYOX.[7] Four highly conserved His-X_{aa}-His sequences exist in a bridging fashion in the trinuclear copper ion cluster of MCOs (Figure 1).[8] Also, a similar motif appears in pentapeptide domains (HLHWH) present in the amyloid precursor protein (APP) associated with the development of Alzheimer's disease.[7,9]

The imidazole group of histidine ligands can bind to Cu ion through either the δN or εN site, and tautomeric preferences occur in different classes of copper proteins.[10] The variations most certainly are critical in determining the functions and properties of the enzymes because of differences in decisive steric/electronic effects imparted to the copper ion center, for example controlling the exact nature of O_2 binding and consequent structure–reactivity and the specificity of substrate approach. For example, in PHM, O_2 binds at the Cu_M site, which is close to where the substrate docks; Cu_M is ligated by two εN sites of His's in an HTH sequence. Cu_H, which is ~11 Å away,

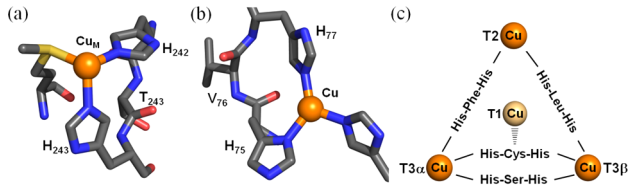


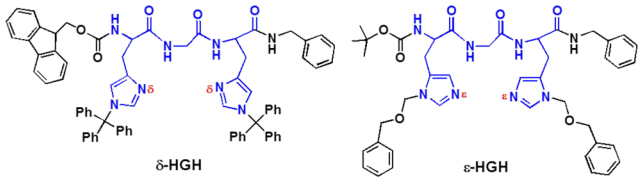
Figure 1. His-X_{aa}-His sequences present in the copper active site of (a) PHM (PDB entry 1SDW),[3] (b) SOD5 (4N3T),[5] and (c) MCO (1ZPU).[8]

facilitates electron transfer, but it binds to three δN_{His} sites (in fact where two of the His residues are adjacent in the overall peptide sequence).[3] These observations raise basic questions relevant to PHM active-site structure and function: how do these specific tautomeric imidazole N atom configurations imposed by nature control (i) copper coordination number and geometry, (ii) Cu^{II}/Cu^I redox potential, (iii) electronic structure/bonding and associated spectroscopic properties, and (iv) exogenous ligand preferences?

We have previously reported studies of Cu^I complexes of modified histidylhistidine (HisHis) peptides[11] where imidazole N atoms were specifically blocked, allowing study of δ-HH (δN of both H's available for metal coordination) or ε-HH (εN of both H's available for metal coordination). Significantly, both dipeptides adopt a linear two-coordinate N_{His}–Cu^I–N_{His} environment. In the present work, we aimed to understand why the unique His-X_{aa}-His sequence is particularly "selected" in nature by generating Cu^I complexes of His-Gly-His tripeptides[1h] with varying δN versus εN atom availability and investigating their structural features and chemical properties.

The tripeptides δ-HGH and ε-HGH (Chart 1) were synthesized by modifications of literature procedures and standard solution-phase peptide synthesis techniques.[12] The

Chart 1



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N-/C-tripeptide terminal groups were also "protected" using either fluorenylmethyloxycarbonyl (Fmoc), *tert*-butyloxycarbonyl (Boc), or benzyl groups to avoid any likelihood of terminal-group Cu coordination.[13] δ-HGH and ε-HGH were metalated with $[Cu^{I}(CH_3CN)_4]ClO_4$ in CH_2Cl_2. Solid complexes were isolated by precipitation and purified by recrystallization from CH_2Cl_2/Et_2O; their elemental analysis and electrospray ionization mass spectrometry envelope isotope patterns were consistent with the $[ligand{-}Cu^{I}]^+$ cation formulations.[14]

Extended X-ray absorption fine structure (EXAFS) spectroscopy (Figure 2)[14] of LCu^{I} complex solids and accompanying

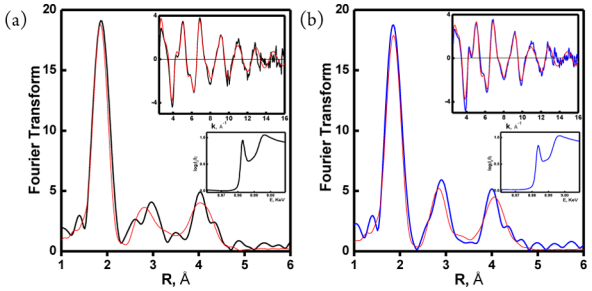

Figure 2. EXAFS and XANES spectroscopic data for (a) $[\{Cu^{I}(\delta\text{-HGH})\}_2]^{2+}$ (**1**) [data (black), fit (red)] and (b) $[Cu^{I}(\varepsilon\text{-HGH})]^+$ (**2**) [data (blue), fit (red)]. Overlapped spectra for comparison are shown in the Supporting Information.

computational analyses (Figure 3)[14] provide strong evidence that Cu^{I} complexes of both δ-HGH and ε-HGH possess two-coordinate $N_{His}{-}Cu^{I}{-}N_{His}$ geometries. Multiple scattering definitively reveals the patterns known for $N_{His}{-}Cu$ coordination. For the Cu^{I} complex of δ-HGH, the data given in Figure 2a display the best fit to two δN_{His}-ligand scatterers with Cu−N = 1.867 Å, indicative of linear two-coordinate Cu^{I}, as observed earlier with the HisHis peptides.[11] These very short $Cu^{I}{-}N$ bonds are characteristic of this very low coordination, being significantly shorter than those found in three-coordinate $Cu^{I}{-}N_3$ compounds.[15]

The EXAFS data for the solid complex of Cu^{I} with ε-HGH are extremely similar. The best and only fit was found with two-His ligation (Figure 2b)[14] and a $Cu^{I}{-}N_{His}$ bond length of 1.878 Å, also indicating two-coordinate Cu^{I}. The only significant difference is a small decrease in the intensity of the 8983 eV pre-edge transition found in the X-ray absorption near-edge structure (XANES) spectroscopic data, which may suggest some deviation from a strictly linear geometry.[11,16] As described below, this deviation seems to directly relate to this Cu^{I} compound's remarkably different (compared with the δ-HGH Cu^{I} complex) electrochemical and CO-binding behavior and its reactivity toward O_2 and H_2O_2.

Density functional theory (DFT) structural analyses and supporting solution conductivity measurements lead to differing formulations for δ-HGH and ε-HGH in comparison with our previous findings for HisHis dipeptides. The EXAFS data indicate near-perfect linear two-coordination for Cu^{I} in the δ-HGH complex. Solution conductivity data in dimethylformamide (DMF) provide an Onsager plot for a $Cu^{I}{-}\delta$-HGH complex with a slope in the range expected for 2:1 electrolyte behavior, thus indicating a dimer formulation, $[\{Cu^{I}(\delta\text{-HGH})\}_2]^{2+}$ (**1**). We note that Figure 3a is a geometry optimization assuming a dimer formulation. In fact, higher-level computations and energy comparisons (in vacuum) reveal that a monomeric formulation and structure are slightly favored

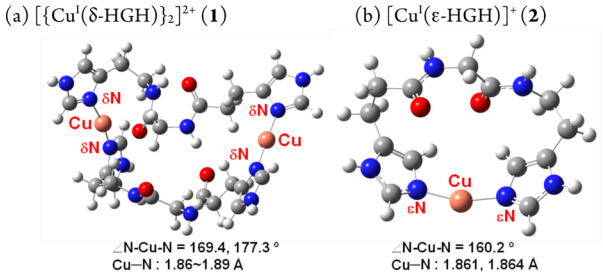

Figure 3. DFT-optimized geometries (RB3LYP/6-311G**) for (a) dimer **1** and (b) mononuclear **2** ($\angle\varepsilon N{-}Cu{-}\varepsilon N$ = 160.2°). The calculations were carried out with the protecting groups on the non-copper-coordinating N_{His} atom and N-/C-termini replaced with H atoms.[14]

(by 11.5 kJ/mol).[14] The stronger solution experimental evidence thus points to the dimer formulation; apparently, intramolecular two-coordination leads to an excessively strained structure. By contrast, structural energy minimization for a $Cu^{I}{-}\varepsilon$-HGH complex leads to a preferred mononuclear formulation, $[Cu^{I}(\varepsilon\text{-HGH})]^+$ (**2**) (Figure 3b), on the basis of electronic energies corrected for zero-point energy; a dimer structure as in **1** is thermodynamically disfavored by 44.0 kJ/mol. Also, a dimer is ruled out by solution conductivity measurements showing that this complex behaves as a 1:1 electrolyte.[14,17] Notably, the DFT-derived structure for complex **2** reveals a significant bending in the two-coordinate Cu^{I} coordination, with $\angle N{-}Cu^{I}{-}N$ = 160.2°, as suggested by the XANES data and the unexpected oxidative reactivity (vide infra); nevertheless, short $Cu^{I}{-}N_{Im}$ bond distances are present that are typical of this coordination number for Cu^{I} and much shorter than those observed in three-coordinate $Cu^{I}{-}N_3$ compounds (vide supra).

The features observed here for Cu^{I} binding to His-X_{aa}-His peptides contrast greatly with those observed for the previously studied HisHis peptides,[11] where the $[Cu^{I}(\delta\text{-HH})]^+$ complex showed monomeric behavior (DFT and solution conductivity) while $[Cu^{I}(\varepsilon\text{-HH})]^+$ is a 2:1 solution electrolyte with a dimeric structure. Just inserting a Gly amino acid between two His residues leads to significant changes in the Cu coordination environment. Do these alterations affect other physical/spectroscopic properties or reactivity patterns?

To address such questions, we first examined the CO binding behavior of the new Cu^{I}–peptide complexes, as CO is a Cu^{I}-specific ligand (and more generally an O_2 surrogate) and can provide insights into coordination number and ligand donation ability. CO adducts of acetone solutions (under Ar) of **1** and **2** were generated by direct CO bubbling. As previously established for near-linear two-coordinate $[Cu^{I}(HisHis)]^+$ complexes, CO binding is very weak, and high-frequency stretching vibrations (ν_{CO} = 2110−2112 cm^{-1}) of low intensity are observed.[11b,15c,18] This is also the case here, as the IR spectrum of **1**−CO exhibits ν_{CO} = 2103 cm^{-1} (Table 1). By contrast, $[Cu^{I}(\varepsilon\text{-HGH})(CO)]^+$ (**2**−CO) displays a high-intensity absorption at lower frequency (ν_{CO} = 2092 cm^{-1}).[14] This observation suggests that there is a significant geometric-coordinative effect leading to stronger ligation of CO to Cu^{I} and better back-donation from Cu^{I} when it is bound to the ε-HGH ligand rather than to either the δ-HGH or HisHis system (Table 1). This ν_{CO} of 2092 cm^{-1} for **2**−CO in fact compares very well with that observed for the enzyme Cu_M sites in PHM (2093 cm^{-1})[19] and DβM (2089 cm^{-1}),[20] which are ligated by two histidyl εN atoms of the His-Thr-His active-site tripeptide sequence (Figure 1a). Thus, **2**−CO possesses a

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Table 1. Comparison of Properties of Cu^I–Peptide Complexes

complex[a]	Cu–N_{His} (Å)[b]	ν_{CO} (cm^{-1})[c]	redox behavior	O_2 /H_2O_2 reactivity
$[\{Cu^I(\delta\text{-HGH})\}_2]^{2+}$	1.867	2103	irreversible	no
$[Cu^I(\varepsilon\text{-HGH})]^+$	1.878	2092	quasi-reversible	yes
$[Cu^I(\delta\text{-HH})]^+$	1.876	2110	irreversible	no
$[\{Cu^I(\varepsilon\text{-HH})\}_2]^{2+}$	1.863	2112	irreversible	no

[a]Determined by solution conductivity. [b]Measured by XAS. [c]IR stretching frequency.

chemical environment reasonably mimicking that of the protein active sites.

Cyclic voltammetry measurements were performed on **1** and **2** in DMF under argon to probe their electrochemical properties. Complex **1** displays irreversible redox behavior (Table 1),[14] as expected for a two-coordinate Cu^I species; the same was shown previously for analogous complexes formed from HisHis ligand systems.[11b] On the other hand, **2** shows quasi-reversible redox behavior ($E_{1/2} \approx -390$ mV vs Fc^+/Fc).[14] Such chemical effects derived from tautomeric preferences for binding of Cu^I to these HGH tripeptides were further clarified by studies of oxidative reactivity (vide infra).

Complex **1** is unreactive toward O_2 as a solid or in solution below 0 °C [with only extremely slow oxidation and color change occurring at room temperature (RT)].[21] This behavior is analogous to that observed for linear two-coordinate Cu^I complexes studied previously.[11b,15a−c] In the context of other works, as an exception (i.e., the only case where we observe facile reactivity with O_2), the reaction of **2** with O_2 in acetone results in the formation of a metastable complex at −80 °C that exhibits absorptions at 336 nm (ε = 1110 M^{-1} cm^{-1}) and 606 nm (ε = 110 M^{-1} cm^{-1}).[14] Frozen-solution electron paramagnetic resonance (EPR) measurements were silent, indicating that whatever species is present is diamagnetic but that warming to RT results in the formation of a paramagnetic mononuclear species. The low-temperature UV–vis absorptions are not characteristic of any well-known O_2–Cu^I adduct, such as a superoxo–Cu^{II}, peroxo–dicopper(II) (μ-1,2 or μ-η^2:η^2), or bis(μ-oxo)dicopper(III) complex.[22] Also, warming the intermediate to RT and testing for peroxide using iodometric titrations gave a 100% yield of H_2O_2 based on a stoichiometry of two molar equiv of **2** per H_2O_2.[14] This suggests that the reaction of **2** with O_2 leads to a peroxide-level species (i.e., two-electron reduction of O_2) but with unusual UV–vis features (see above). Further detailed studies are warranted. Following Na_2EDTA/H_2O/CH_2Cl_2 demetalation procedures,[23] the organic product was identified as unreacted starting ligand. Therefore, whatever Cu^I_n–O_2 (n = 1, 2, ...) adduct forms at low temperature does not affect any ligand oxidation/oxygenation chemistry, as is sometimes observed.

As Cu^{II}–hydroperoxo complexes are of interest as basic entities derived from Cu^I/O_2 reactions and have been discussed in the realm of possible enzyme active intermediates capable of substrate oxidative behavior, we sought to study the present copper–peptide complexes. **1** is in fact unreactive toward H_2O_2, a remarkable observation that further demonstrates the extreme stability of Cu^I in a linear two-coordinate nitrogen ligand environment. However, we could generate a green-colored species (presumed to possess a $[Cu^{II}(\varepsilon\text{-HGH})(OOH)]^+$ formulation) in acetone at −80 °C by (i) addition of H_2O_2 (10 equiv)/Et_3N to a mononuclear Cu^{II} derivative of ε-HGH, the newly synthesized cupric complex $[Cu^{II}(\varepsilon\text{-HGH})(H_2O)](ClO_4)_2$,[14] or (ii) the reaction of **2** with 1.5 equiv of H_2O_2 (Figure 4a), a recently reported procedure for generating Cu^{II}–

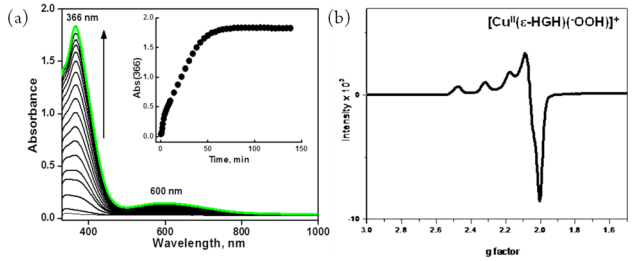
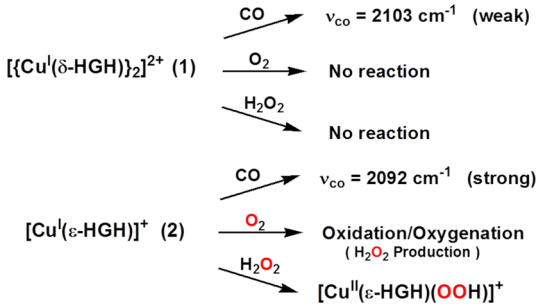


Figure 4. (a) UV–vis spectra of $[Cu^{II}(\varepsilon\text{-HGH})(OOH)]^+$ generated by addition of 1.5 equiv of H_2O_2 to a 3.5 mM solution of **2** in acetone at 193 K. (b) EPR spectrum of $[Cu^{II}(\varepsilon\text{-HGH})(OOH)]^+$ at 77 K ($g_\parallel$ = 2.25, $g_\perp$ = 2.05, $A_\parallel$ = 192 G, $A_\perp$ = 15 G).

OOH species.[24] $[Cu^{II}(\varepsilon\text{-HGH})(OOH)](ClO_4)$ is presently characterized by (i) its UV–vis features [λ_{max} = 366 nm (ε = 2600 M^{-1} cm^{-1}), assignable to a −OOH → Cu^{II} ligand-to-metal charge transfer absorption on the basis of the correspondence with a number of literature examples,[24,25] and λ_{max} = 600 nm (ε = 200 M^{-1} cm^{-1}), a d–d transition band] and (ii) its distinctive mononuclear-type axial EPR spectrum at 77 K ($g_\parallel$ = 2.25, $g_\perp$ = 2.05, $A_\parallel$ = 192 G, $A_\perp$ = 15 G; Figure 4b).

In conclusion, we have generated new Cu^I complexes with His-Gly-His tripeptides to probe fundamental aspects of Cu^I chemistry with this particular histidine-containing sequence; we have also probed the presence of synthetically imposed tautomeric preferences (δN_{Im} vs εN_{Im} availability) for Cu^I. The dimer $[\{Cu^I(\delta\text{-HGH})\}_2]^{2+}$ (**1**) exhibits favorable near-linear twofold coordination via intermolecular Cu–δN_{His} binding. This complex is not redox-active and only weakly binds CO. Furthermore, it does not react with either O_2 or H_2O_2 (Scheme 1). However, $[Cu^I(\varepsilon\text{-HGH})]^+$ (**2**) shows two-His ligation with

Scheme 1



deviation from linearity. The similarity of our synthetic construct to the protein is notable: the IR spectrum of the carbonyl adduct **2**–CO matches that observed for the enzyme PHM Cu_M–CO adduct with its active-site εH-X_{aa}-εH chelating moiety. Also, **2** displays redox activity and readily reacts with O_2 and H_2O_2 to afford the first oxygen-intermediate species to be noted with Cu^I ligated to biologically relevant His-containing peptides.

It is striking that a switch in the imidazole tautomer can radically influence the reactivity of a Cu^I center. These results, in conjunction with our previous work with Cu^I-HisHis complexes,[11] highlight the manner in which nature exerts its control of function. Even slight changes (dipeptide vs tripeptide; δN_{Im} vs εN_{Im} availability) can significantly affect an enzyme metal

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center's structure and reactivity. Thus, our continuing research will add to an understanding of structure−function relationships in copper enzymes and the role of His binding motifs in facilitating $Cu-O_2$ (and even reactive oxygen species) intermediate formation.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic and analytical details; UV−vis, IR, and EPR spectra; cyclic voltammograms; and Onsager plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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(2) Abbreviations: PHM = peptidylglycine α-hydroxylating monooxygenase; DβM = dopamine β-monooxygenase; SOD5 = superoxide dismutase; *p*MMO = particulate methane monooxygenase; APLP2 = Aβ precursor-like protein 2; LYOX = lysyl oxidase; MCO = multicopper oxidase.

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(13) (a) Isidro-Llobet, A.; Alvarez, M.; Albericio, F. *Chem. Rev.* **2009**, *109*, 2455. (b) A reviewer asked about our choice of blocking groups, e.g., the use of Fmoc and trityl for blocking εN imidazole positions in the δ peptide. Fmoc was preferred over Boc and trityl was preferred over benzyl because Boc and benzyl protection was more expensive, gave lower yields in synthesis, and afforded final peptides exhibiting poor solubility in organic solvents. Examination of molecular models indicated that the nature of the protecting group should not influence Cu^I binding.

(14) See the Supporting Information.

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