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Solution Structure of Physiological $Cu(His)_2$: Novel Considerations into Imidazole Coordination

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A disagreement on the mode of histidine binding to copper and the structure of $[Cu^{2+}(His)_2]$ in solution still exists. Spectroscopic data in solution support a six-coordinate species with N4O2 donor atoms, while X-ray crystallography reveals five-coordinate N_3O_2 donor atoms. We modified $[Cu^{2+}(His)_2]$ in solution using diethyl pyrocarbonate (DEPC) and monitored the products spectrophotometrically and by mass spectrometry. Our spectrophotometric study indicates the presence of a free imidazole in the $[Cu^{2+}(His)_2]$ complex in solution. Mass spectral characterization of a DEPC-modified $[Cu^{2+}(His)_2]$ complex yielded a peak at 587.8 amu corresponding to three DEPC adducts. Taken together, our data indicate that the $[Cu^{2+}(His)_2]$ complex in solution exists as a neutral five-coordinate structure with N3O2 donor atoms.

L-Histidine (L-His, HisH) in the form of free amino acid acts as a tridentate ligand toward transition-metal ions.[1,2] Among divalent metal ions, the copper−histidine system has generated a great deal of interest since its discovery in human blood and its use for the treatment of Menkes disease.[3] Usually, histidine forms a bis-complex with divalent metal ions, where it coordinates through a combination of imidazole nitrogen, amino nitrogen, and carboxylate oxygen atoms.[1] The composition of the predominant complexes of copper and HisH in aqueous solution depends upon the metal ion to ligand ratio, temperature, and pH. The copper−histidine complexes detected progressively as the pH increases are $[Cu^{2+}(HisH)]^{2+}$, $[Cu^{2+}(His)]^{+}$, $[Cu^{2+}(HisH)_2]^{2+}$, $[Cu^{2+}(His)(HisH)]^{+}$, $[Cu^{2+}(His)_2]$, and $[Cu^{2+}(His)_2(OH)]^{-}$.[4] A detailed titration study indicates that, among all of these complexes, the major copper−histidine complexes are $[Cu^{2+}(HisH)]^{2+}$, $[Cu^{2+}(His)(HisH)]^{+}$, and $[Cu^{2+}(His)_2]$.[5] It is generally accepted that only the $[Cu^{2+}(His)_2]$ complex is present around physiological pH in aqueous solution (>99% at pH 7.4) and is a dominant species in solution within the pH range of 7.0−9.0.[2] Crystal structures for the copper−histidine system are available in the literature. Evertsson obtained the $[Cu^{2+}(HisH)_2]^{2+}$ complex at pH 3.7.[6] The structure of the cationic $[Cu^{2+}(HisH)_2]^{2+}$ complex showed that two amino nitrogen and two carboxylate oxygen atoms are coordinated to the copper ion. Both imidazole groups of the His ligand were protonated and pointed away from the copper center, giving a glycine-like four-coordinate complex with a N2O2 donor atom set. Camerman et al. published the structure of $[Cu^{II}(L\text{-His})(D\text{-His})(H_2O)_2]\cdot 4H_2O$ at pH 8.0.[7] The copper atom is octahedrally coordinated by amino and imidazole nitrogen atoms of L-His and D-His in a square-planar arrangement and by oxygen atoms of the water molecule at the axial positions. These structures provided insight into a possible arrangement between copper and L-His.

However, a structure for the physiological $[Cu^{2+}(His)_2]$ complex had eluded researchers for over 40 years.[2] Deschamps et al. solved the structure of this physiological $[Cu^{2+}(His)_2]$ complex.[8] The crystal structure yielded a neutral five-coordinate complex with one of the imidazole groups of the His ligand not involved in copper coordination (Scheme 1A). The structural features were quite unexpected, especially the presence of only one imidazole involved in coordination, because most of the spectroscopic data appeared to point to the presence of the imidazole in the coordination sphere (Scheme 1B). Myriads of techniques have been used to obtain the structure of $[Cu^{2+}(His)_2]$ in solution, ranging from spectroscopies, like UV−vis/near-IR, IR, Raman, ^1H and ^{13}C NMR, electron spin resonance, and extended X-ray absorption fine structure, to potentiometric experiments.[2,4,5,9] Despite the reporting of a five-coordinate

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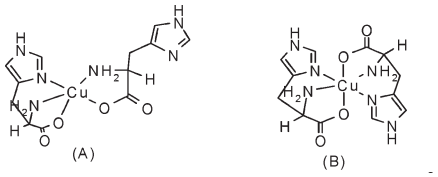
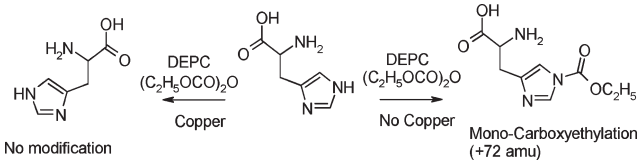
Scheme 1. Structures of a Physiological $[Cu^{2+}(His)_2]$ Complex: (A) Solid-State Structure Obtained by X-ray Crystallography;[8] (B) Proposed Solution Structure Obtained Using a Variety of Spectroscopic Techniques[9]



Scheme 2. Modification of Histidine Using DEPC in the Absence of Copper[a]



[a] The binding of copper protects histidine from covalent modification.

structure obtained by X-ray crystallography, a six-coordinate structure with N4O2 donor atoms for $[Cu^{2+}(His)_2]$ in solution was proposed in most of the above studies. The coordination geometry inferred from a crystal structure does not always agree with the spectroscopic data, as was famously observed in the case of the ribonucleotide reductase R2 subunit.[10] However, despite all of these studies, disagreement on the mode of histidine binding to copper and the structure of $[Cu^{2+}(His)_2]$ in solution still exists.[9d] It is likely that the exact nature of $[Cu^{2+}(His)_2]$ in solution was difficult to determine because of the fact that each technique allowed identification of some structural features but not all.[2] The conundrum surrounding the involvement of one or two imidazole group(s) in the copper coordination has become crucial in the understanding of the structure of $[Cu^{2+}(His)_2]$ in solution and the copper transport in human blood.

Diethyl pyrocarbonate (DEPC) modification coupled with mass spectrometry has been imperative in the study of the involvement of histidine residues in copper binding in human prion and COMMD1 proteins.[11,12] The fundamental concept involves covalent modification of the imidazole nitrogen atom of the histidine residue by DEPC (Scheme 2), while the binding of copper protects the imidazole group from this modification. A single carboxyethylation modification increases the molecular weight of histidine by 72 amu in the absence of copper, while copper binding protects histidine against modification. The number of histidines involved in copper binding thus can be calculated using the differences in their masses. Further, ms/ms technology can also be used to find out the exact position of the modified histidine in the protein sequence using peptide mapping.[11,12] It is known that, in addition to histidine residues, DEPC also shows reactivity toward lysine and the N-terminal NH_2 group.[13] Therefore, the number of adducts is always greater than the number of histidine residues. To overcome this limitation,

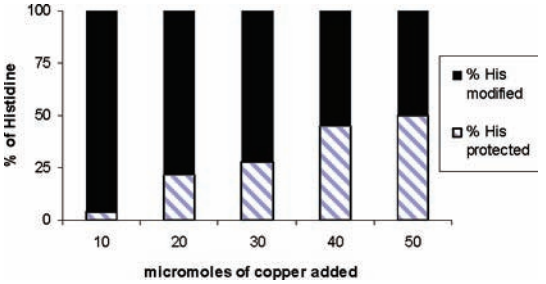


Figure 1. DEPC modification of 100 μM histidine in the presence of various concentrations of Cu^{2+} (10, 20, 30, 40, and 50 μM).

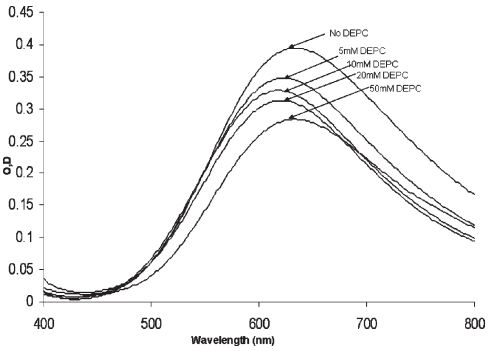


Figure 2. Effect of DEPC modification on the $[Cu^{2+}(His)_2]$ complex. A 5 mM $[Cu^{2+}(His)_2]$ solution was modified with various concentrations of DEPC. Values on the curves indicate the concentration of DEPC in mM.

DEPC modification is monitored spectrophotometrically prior to mass spectrometry. The DEPC adduct of imidazole absorbs at 240 nm with a ε value of 3200 M^{-1} cm^{-1}, which is quenched upon copper binding.[12,14] The combined results reveal precisely the number of histidine residues involved in copper binding. Here, we used this method for understanding imidazole coordination in the physiological $[Cu^{2+}(His)_2]$ complex.

In the first experiment, we carried out chemical modification of 100 μM histidine in the presence of various concentrations of Cu^{2+} (10, 20, 30, 40, and 50 μM).[15] Our result shows that copper protects the imidazole group of histidine against DEPC modification. The addition of 10 μM copper protects 10% of histidine. The degree of histidine protection was found to be proportional to the amount of copper added (Figure 1). At 50 μM copper concentration, which represents 1:2 stoichiometry for Cu/His, only 50% of the histidines were protected. With 1:2 stoichiometry for Cu/His in the physiological $[Cu^{2+}(His)_2]$ complex, our result indicates that only one imidazole is protected through copper binding. $[Cu^{2+}(His)_2]$ has been found to be a dominant species in solution in the pH range 7.0−9.0.[2,5] Therefore, we further studied the DEPC modification of His and $[Cu^{2+}(His)_2]$ at various pH levels in aqueous solution. Our results indicate minor variations in the degree of protection in the pH range

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(15) Two sets of 100 μM histidine solutions in phosphate-buffered saline were incubated with various concentrations of Cu^{2+} (prepared from $CuSO_4 \cdot 5H_2O$). One set was then treated with a 10 molar excess ethanolic solution of DEPC for 30 min at 27 °C, while the other set acting as the control was treated just with ethanol. The reaction was terminated using excess Tris-HCl buffer (pH 8.3). The optical density of the DEPC-containing tubes was measured at around 240 nm using respective control tubes containing ethanol on a Shimatzu 1601 spectrophotometer.

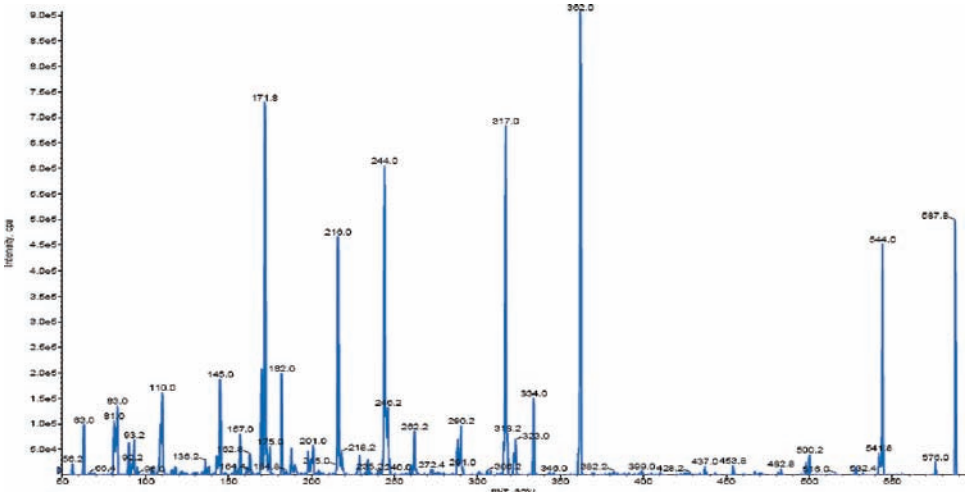


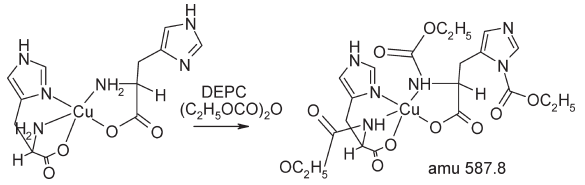
Figure 3. LCMS spectrum of DEPC-modified $[Cu^{2+}(His)_2]$ showing the highest mass peak at 587.8 amu corresponding to three DEPC adducts [371.8 + 3(72)].

from 7.0 to 9.0, indicating the involvement of only one histidine imidazole group in copper binding.

We further carried out experiments to evaluate the effect of DEPC modification on the structure of the $[Cu^{2+}(His)_2]$ complex.[16] The $[Cu^{2+}(His)_2]$ complex shows a broad peak at 640 nm (ε = 88 M^{-1} cm^{-1}).[8,14] For DEPC concentrations of 5−25 mM, we observed a small blue shift from 634 to 618 nm. At 50 mM DEPC concentration, the peak red shifts to 634 nm with a decrease in the ε value upon DEPC modification (Figure 2). The shift in the peak can be attributed to covalent modification of $[Cu^{2+}(His)_2]$ at more than one position. However, the modification does not disturb the original geometry and structure of the original complex. A similar blue shift was also observed in the Schiff base modified $[Cu^{2+}(His)_2]$ complex; however, a five-coordinate geometry around copper with a N3O2 donor atom set remains intact.[17]

We, therefore, monitored the DEPC modification of $[Cu^{2+}(His)_2]$ using liquid chromatography−mass spectrometry (LCMS).[18] DEPC-modified histidine alone showed a mass peak at 300 amu [156 + 2(72) amu] corresponding to two DEPC adducts at the imidazole and amino groups. DEPC is shown to react with N-terminal amino groups of prion and COMMD1 proteins.[12,13] DEPC-modified $[Cu^{2+}(His)_2]$, on the other hand, showed a mass peak at 587.8 amu [371.8 + 3(72)] corresponding to three DEPC adducts (Figure 3). Of the three adducts, two are formed at the amino position, while the third is formed at the free imidazole group that is not involved in copper binding (Scheme 3).

Scheme 3. DEPC Modification of $[Cu^{2+}(His)_2]$



In summary, we have for the first time demonstrated the presence of a free imidazole histidine group in the physiological $[Cu^{2+}(His)_2]$ complex in solution. DEPC modification assay using spectrophotometry and mass spectrometry reveals that only one imidazole is modified. This confirms a five-coordinate neutral structure for physiological $[Cu^{2+}(His)_2]$ in solution; however, a six-coordinate structure with weak coordination of the solvent molecule may also form. A small fraction of copper bound to HisH maintains an exchangeable pool of copper in equilibrium with albumin in human blood.[19] Subsequently, it was demonstrated that HisH enhances the cellular uptake of copper, and the exchange of copper between HisH and albumin modulates the availability of copper to the cell.[20] Perhaps, the pendant imidazole observed in the $[Cu^{2+}(His)_2]$ complex may be the key to its interaction on the cell membrane receptor, ultimately modulating the availability of copper to the cell.

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(16) Aqueous solutions of 5 mM $[Cu^{2+}(His)_2]$ (pH 7.4) were treated with various concentrations of ethanolic solutions of DEPC (0−50 mM) for 30 min at 27 °C. The reaction was terminated using excess Tris-HCl buffer (pH 8.3). Spectra were measured between 400 and 800 nm against water containing equal volumes of ethanol on a Jasco V630 spectrophotometer.

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