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Peroxide Activation Regulated by Hydrogen Bonds within Artificial Cu Proteins

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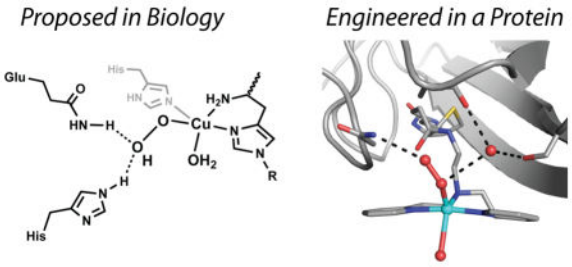
Abstract

Cu-hydroperoxido species (Cu^{II}–OOH) have been proposed to be key intermediates in biological and synthetic oxidations. Using biotin-streptavidin (Sav) technology, artificial copper proteins have been developed to stabilize a Cu^{II}–OOH complex in solution and *in crystallo*. Stability is achieved because the Sav host provides a local environment around the Cu–OOH that includes a network of hydrogen bonds to the hydroperoxido ligand. Systematic deletions of individual hydrogen bonds to the Cu–OOH complex were accomplished using different Sav variants and demonstrated that stability is achieved with a single hydrogen bond to the proximal O-atom of the hydroperoxido ligand: changing this interaction to only include the distal O-atom produced a reactive variant that oxidized an external substrate.

Graphical Abstract



The utilization of dioxygen in biology is critical for most forms of life and is often achieved using metalloproteins. A subset of these enzymes contain a monomeric Cu center as exemplified by the copper-dependent lytic polysaccharide monooxygenases (LPMOs). These fungal and bacterial enzymes assist in the extracellular degradation of polysaccharides such as cellulose into oligosaccharides via initial oxidation, and are linked in biomass

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Notes

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conversion to biofuels. The mechanistic details are still uncertain with questions persisting on the identity of the key dioxygen adducts that lead to oxidation. Possible candidates include Cu^{II}–hydroperoxido species (Figure 1A), whose structures are often only inferred as they are inherently unstable at room temperature.[1]

A persistent challenge in studying these systems is finding suitable model systems whose structure can simulate protein active sites. Because protein function is regulated largely by the structural properties of the protein matrix, it has been difficult to design model systems that closely mimic the local protein architecture that regulates O_2 activation.[2–6] Although site architectures are diverse in monooxygenases, they share the common characteristic that non-covalent interactions, especially hydrogen bonds (H-bonds), control metal-mediated O_2 activation.[7–9] For instance, dioxygen affinities to hemoglobins are correlated to H-bonding networks involving the Fe–O_2 unit (Figure 1B) where at least one H-bond to the proximal O-atom (O_p) is needed to form stable species.[10,11] In contrast, O–O bond cleavage in cytochrome P450s and peroxidases require specific H-bonds involving only the distal O-atom (O_d) of the Fe–OOH unit (Figure 1C).[12–14] Related proposals have been advanced for Cu–OOH species in LPMOs, in which H-bonds to the O_d-atom of the hydroperoxido ligand leads to productive C–H bond cleavage of substrates[15,16] (Figure 1A).

To investigate the relationship between H-bonding networks and regulation of Cu-hydroperoxido complexes, we present herein artificial copper proteins that were designed to stabilize a Cu^{II}–OOH complex in aqueous solution and *in crystallo* using biotin-streptavidin (Sav) technology. This approach relies on the strong binding of biotinylated metal complexes to the Sav host, leading to the precise localization of the synthetic metal cofactors within a protein environment.[17] Structure-function relationships between the local environment and the stability of the Cu–OOH complex were evaluated using a series of Sav variants in which individual H-bonds were systematically deleted.

Within this context, we initially examined the biotinylated Cu^{II} complex, $[Cu^{II}(\text{biot-et-dpea})(H_2O)_2]^{2+}$ (Figure 2A) and found that it can react with H_2O_2 in buffer A (50 mM MES at pH 6) at room temperature to form a new complex with spectroscopic properties that are similar to those of known Cu^{II}–OOH species (λ_{max} = 375 nm, ε_M = 1800 (Figures 2C & S1, Table S1).[15,18–25] However, the complex is unstable and decays with a half-life of 6 min (Figure S1). The decay of $[Cu^{II}(\text{biot-et-dpea})(H_2O)(OOH)]^{2+}$ is similar to that observed in other synthetic systems and illustrates the inherent instability of Cu^{II}–OOH under ambient conditions.[21]

Confinement of the Cu complex within Sav WT produced a stable Cu^{II}-OOH species. These studies utilized $[Cu^{II}(\text{biot-et-dpea})(H_2O)_2]^{2+}$ ⊂Sav WT (**1**) which is an ArM that contains a five-coordinate Cu^{II} center with two coordinated water molecules (Figure 2B). Its molecular structure (PDB 5WBC) revealed an H-bonding network involving the aqua ligand containing O3, the structural water molecule (O1), the side chain O-atom of residue S112, and the backbone carbonyl O-atom from A86.[26] The placement of O1 is noteworthy as it is present in many structures of Sav WT that we have studied and is positioned to H-bond to ligands coordinated to $[Cu^{II}(\text{biot-et-dpea})]^{2+}$. Treating **1** with excess H_2O_2 in buffer A (Figure 2C & 3) produced changes in the absorbance spectrum that are, again, indicative of formation of a

Cu^{II}–OOH species, that is $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)(OOH)]^+ \subset$Sav WT (**2**). Unlike in our control experiment with $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)_2]^{2+}$ without Sav, the band at λ_{max} = 375 nm for **2** persisted for over a day at room temperature, indicating that confinement within Sav increased the stability of the Cu^{II}–OOH adduct in solution (Figure S2).[27] In addition, the electron paramagnetic resonance spectrum of **2** showed small changes in the g- and A-values that are consistent with the binding of a hydroperoxido ligand to the copper center (Figure S2, Table S1).[20]

The formation of **2** was also achieved *in crystallo* by incubating single crystals of **1** in a cryoprotectant solution containing 1.0 mM H_2O_2 at pH = 6 for 10 minutes (see SI). The 1.62 Å resolution structure of **2** collected at short exposure times (Table S3) showed that each subunit contained a 5-coordinate Cu complex with a distorted trigonal bipyramidal structure (Figures 4 & S3): three of the coordination sites are occupied by the N-atoms from the dpea ligand and the fourth site is occupied by an aqua ligand (O2).[28] The remaining coordination site contains a ligand that was modeled to be a 2-atom fragment that is assigned to the end-on hydroperoxido ligand with Cu–O3 and O3–O4 bond lengths of 1.94(2) and 1.52(2) Å and a Cu-O3-O4 bond angle of 138(2)°(Figures 4A&B, S3, Table S2). Comparing the structures of **1** and **2**, the Cu(dpea) portion of the complexes occupies the same position, but replacing the aqua ligand (O3) in **1** with the peroxido ligand in **2** results in a shortening of the Cu–O3 bond distance by nearly 0.6 Å (Figure S3 & Table S2). Note that the metrical parameters for the Cu complex in **2** are similar to those reported for the only synthetic monomeric Cu–OOH complex: Cu-N bond distances are statistically the same, whereas the Cu–O3 and O3–O4 bond lengths are slightly longer in **2**.[18] The O–O bond length in **2** is also the same as that found in the structures of LPMO whose Cu^{II} active sites are proposed to also contain a peroxide molecule.[29,30] In addition, the O3–O4 and Cu–O3 bond distances are the same as those found for a proposed mononuclear Cu–OOH species in ascorbate oxidase trapped *in crystallo.*[31]

An important structural outcome was the H-bonding network that surrounds the Cu–OOH unit that we suggest contributes to the stability of this species. The Cu complex is anchored within Sav in such a way that the hydroperoxido ligand is canted toward residue N49 which forms an H-bond with the O_d-atom (O4). A second H-bond is formed between O1 and the O_p-atom (O3). This type of H-bonding network around a hydroperoxido ligand that involves both O-atoms is, to our knowledge, unknown in other Cu systems and highlights the natural cleft provided by Sav for this metallocofactor (Figures 4A & S3). In addition, the side chain of N49 and the water molecule containing O1 participate in extended H-bonding networks that assist in positioning the H-bonds to the Cu–OOH complex (Figure 4C). The position of the N49 side chain is fixed via a H-bonding network that includes the carboxylic acid sidechain of E51 and the guanidinium sidechain of R84. This H-bonding network suggests that the HN4 of the N49 amide side chain donates an H-bond to the Cu-OOH unit. The side chain in S112 has an additional H-bonding contact with another water molecule, which is part of a larger network that includes the carbonyl O-atom of K121. This orientation of H-bonds is consistent with the hydroxyl group of S112 serving as an H-bond donor to O1.

The premise that the stability of the Cu^{II}–OOH complexes in **2** is correlated to the H-bonding network within the local environment was evaluated using two different variants of

Sav. Mutations to alanine were engineered at either N49 or S112 that would alter the local environment to possibly modulate the stability of the Cu–OOH unit. In one variant, $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)_2]^{2+}\subset Sav\ S_{112}A$ (**3**) was prepared in order to remove the structural water molecule containing O1 because it can no longer form an H-bond with the engineered alanine residue at position $S_{112}A$. The 1.4 Å structure of **3** showed that the occupancy of the water molecule containing O1 is reduced substantially by approximately 50% and no H-bonds are formed with the confined Cu complex (Figure S4). This structural analysis showed that the Cu complex is no longer locked into a single position as in **1** and **2**, but is disordered over two positions that we attribute to the lack of a H-bond to fix the position of the complex within Sav (Figure S4). Treating a solution of **3** with H_2O_2 at room temperature in buffer A produced $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)(OOH)]^{+}\subset Sav\ S_{112}A$ (**4**) that had an initial absorbance spectrum nearly identical to that of **2**. However, **4** was not stable and decayed with a half-life of 20 min (Figure S5) that prevented formation *in crystallo*. The structure of the other variant $[Cu^{II}(biot\text{-}et\text{-}dpea)\ (H_2O)]^{2+} \subset Sav\ N_{49}A$ (**5**, 1.6 Å, Fig. S6) revealed a similar Cu^{II} complex to that in **1**. Moreover, the key water molecule (O1) is present in the same position and forms a H-bond to the coordinate aqua ligand O3. Variant **5** was reacted with H_2O_2 in buffer A to produce $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)(OOH)]^{+} \subset Sav\ N_{49}A$ (**6**) that had similar solution stability as **2** (Figure S5). Despite the stability of **6** attempts to prepare it *in crystallo* were unsuccessful: XRD analyses of all crystals showed a Cu^{II}–aqua complex that was similar to that observed in **5**.

The effects of the intramolecular H-bonds were further explored with reactivity studies involving external substrates. No reactivity was observed when **2** was treated with substrates having C–H bonds in solution at room temperature (buffer A). For example, treating **2** with 4-chlorobenzylamine showed no change in the absorbance spectrum and no detectable products after 1 h. A similar lack of reactivity was observed for **6**, the ArM that has $N_{49}A$ mutation. Gratifyingly, we observed oxidation products with **4**: in a typical reaction, ArM **4** was treated with 4-chlorobenzylamine at room temperature and the absorbance band at λ_{max} = 375 nm disappeared within 10 min (Figure S7). Analysis of the organic products revealed that *N*-(4-chorobenzyl)-1-(4-chlorophenyl)meth-animine (**7**) was produced (65% yield relative to Cu, Figure S8). Formation of this product is consistent with an initial oxidation of the amine and hydrolysis to afford the oxidized species 4-chlorobenzaldehyde that further reacts with the remaining amine to produce the Schiff base **7** (Scheme 1). In control experiments, $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)(OOH)]^{+}$ was treated with 4-chlorobenzylamine under the same conditions used with **4**: analysis of the reaction mixture by ESI-MS revealed a small molecular ion peak for **7**, but attempts to quantify the amount produced by NMR spectroscopy were below the detection limits.

Using biotin-Sav technology we demonstrate that ArMs can be designed and prepared that stabilize Cu^{II}–OOH species by precisely engineering the local H-bonding environment. The fusion of synthetic and protein chemistries offers the promise for capturing unattainable reactive species under ambient conditions in aqueous solutions— akin to what occurs in biology. Correlating the changes in reactivity of the Cu^{II}-OOH complex with modulation of the H-bonds highlights their critical role in regulating the function of metal-hydroperoxido intermediates (Figure 5). A single H-bond to the proximal O-atom of the hydroperoxido

ligand was essential to form a stable Cu–OOH within the protein host. Varying the H-bonding network to include only the distal O-atom produced a reactive variant that oxidized a substrate. For LPMOs, our experimental findings suggest a dioxygen binding site in which H-bonds participate in the activation of a Cu^{II}–OOH intermediate. Moreover, our correlations support the premise that H-bonds to the O_d-atom are important in the activation of a hydroperoxido ligand within the active site of proteins.[1,15,16] These findings highlight that the positioning of a single H-bond to a metal-hydroperoxido complex can be the difference between functional or dysfunctional metalloproteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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27. Attempts to measure the resonance Raman spectra of all ArMs and free Cu complexes were unsuccessful due to strong background fluorescence that obscured the signals.
28. We solved the structures of several crystals of 2 collected a different X-ray exposure times to evaluate the effects of possible photoreduction on the crystals. For the shorter times (0.35 s/0.5 deg) we observed the structure shown in Figure 4 with a 5-coordinate Cu^{II}–OOH species. For the longer times (1 s/0.5 deg) the Cu^{II}–OOH complex is 4-coordinate with aqua ligand containing O2 not observed (PDB 5WBA, Figure S2) that is attributed to partial photoreduction. However, all other metrical parameters are the same within experimental error (Table S2) and the structures support the assignment of a Cu^{II}–OOH.
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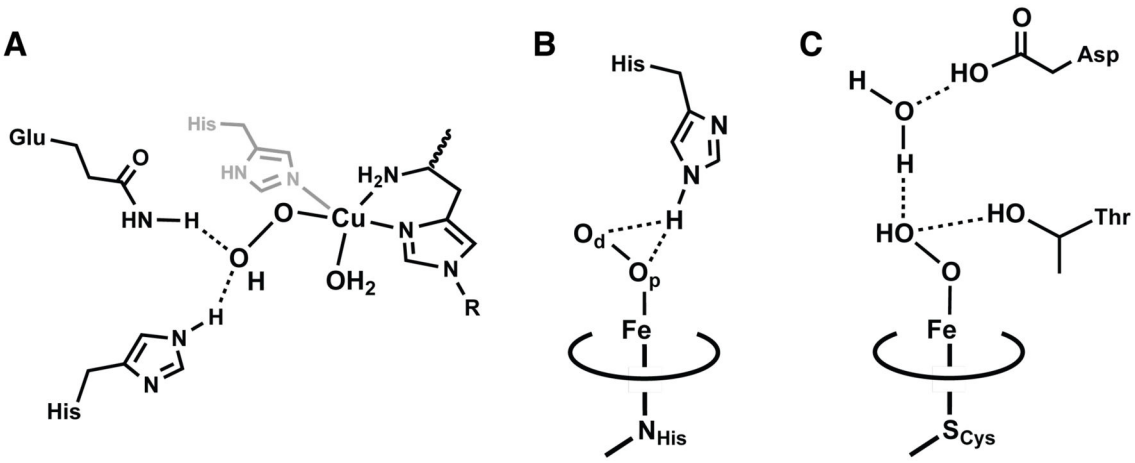


Figure 1.

Representations of the H-bonds involved in the proposed Cu^{II}-OOH intermediate formed during catalytic turnover for Cu-LPMOs (A),[1] in stabilizing the Fe-O_2 species in the active sites of hemoglobins (B, PDB 1GZX),[11] and in activating the Fe-OOH species in P450s (C).[14] The dashed lines indicate H-bonds, the circular lines represent protoporphyrin IX, and O_p and O_d denote the proximal and distal positions.

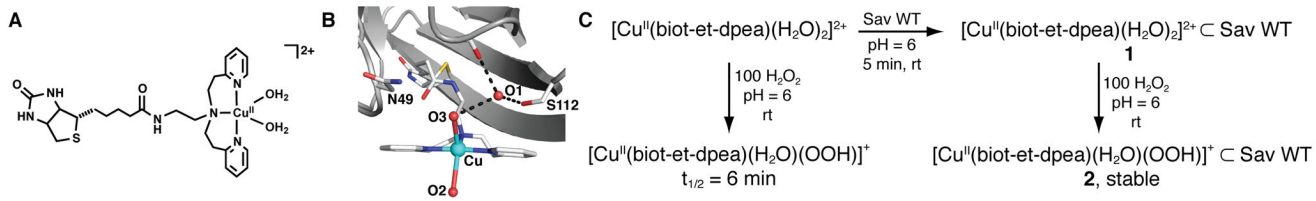


Figure 2.

Representation of biotinylated Cu complex $[Cu^{II}(biot\text{-}et\text{-}dpea)(H_2O)_2]^{2+}$ (A), the molecular structure of 1 (B, PDB 5K49),[26] and the reaction conditions to form 2 (C). The dashed lines indicate H-bonds.

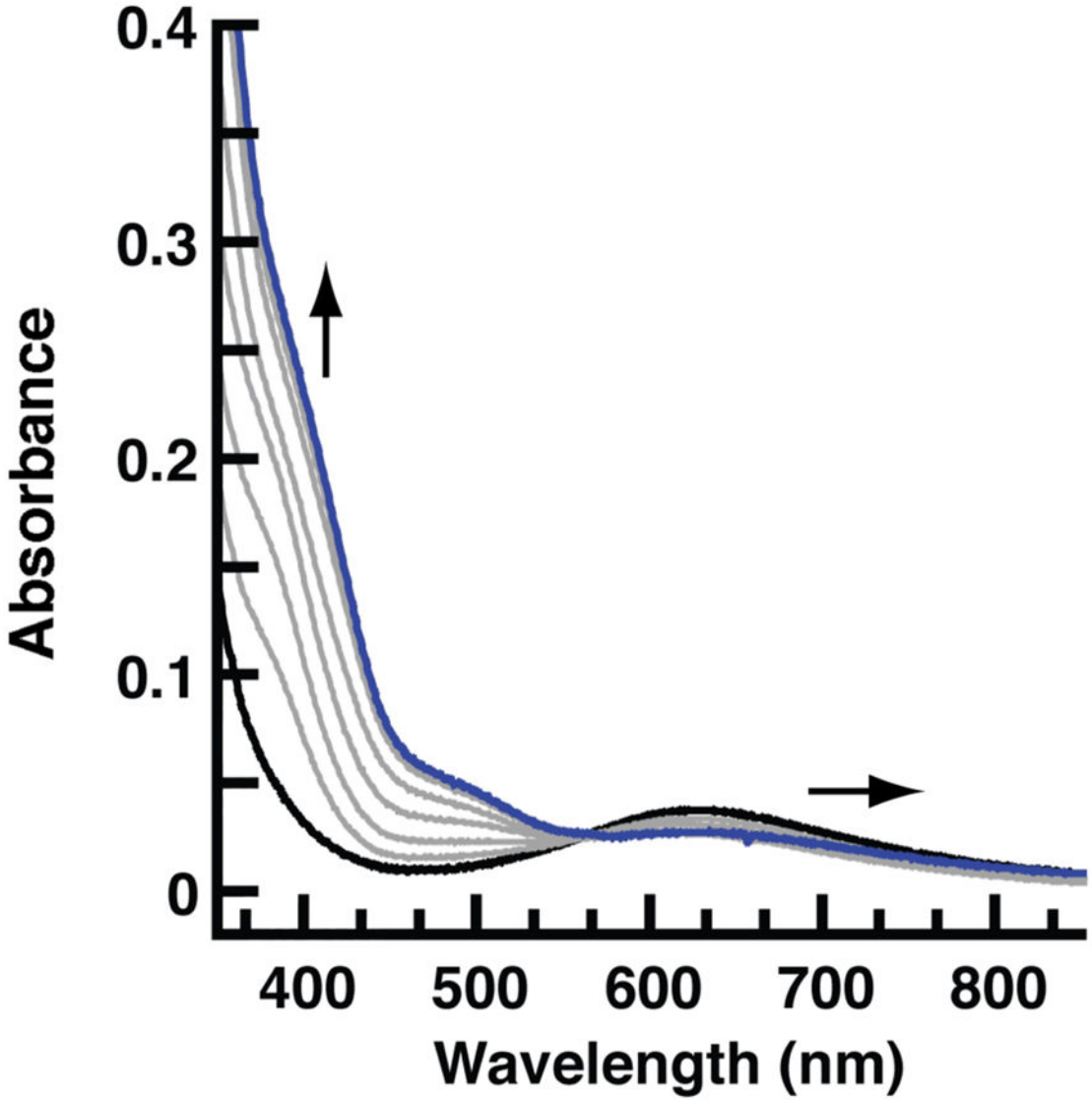


Figure 3.
Absorbance spectral changes for the conversion of 1 (black) to 2 (blue) at room temperature in buffer A.

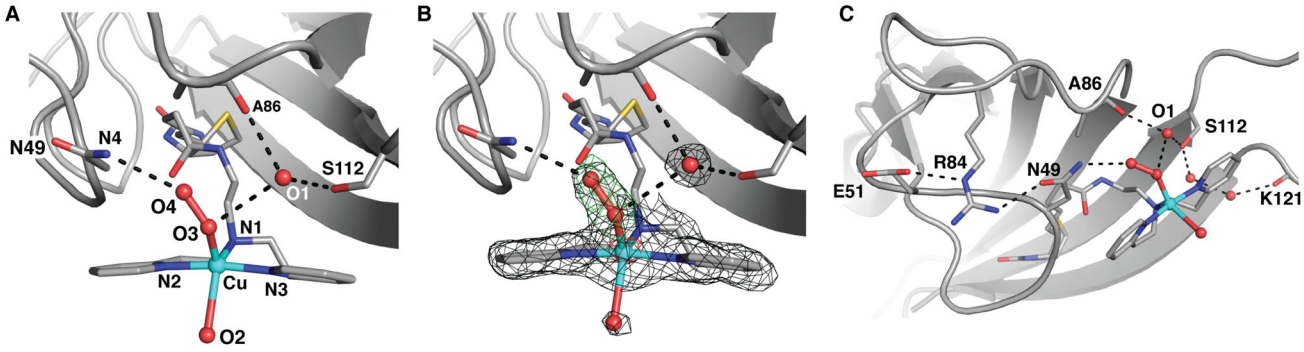


Figure 4.

The molecular structure of 2 (A & B, PDB: 6ANX) and the extended H-bonding network surrounding the Cu–OOH complex (C). The $2F_O–F_C$ electron density map (grey, contoured at 1σ), $F_O–F_C$ omit map (green, contoured at 3σ) and anomalous difference density (red, contoured at 10 σ) are highlighted in B. Copper ions are colored in cyan, O-atoms are colored in red, N-atoms are colored in blue, and C-atoms are colored in gray. H-bonds are indicated as dashed lines. Selected metrical data (Å or deg) for 2: Cu–O3, 1.94(2); Cu–O2, 2.60(2); Cu–N1, 2.05(2); Cu–N2, 1.96(2); Cu–N3, 2.05(2); O3–O4, 1.52(2); O1···O3, 3.18(2); O4···N4, 3.04(2); Cu-O3-O4, 138(2)°; N1-Cu-O2, 109(2)°. Note that some photo-reduction is possible during the collection as we have found diminished electron density for O2 at higher dose of X-ray.[28] See SI and reference 28 for details.

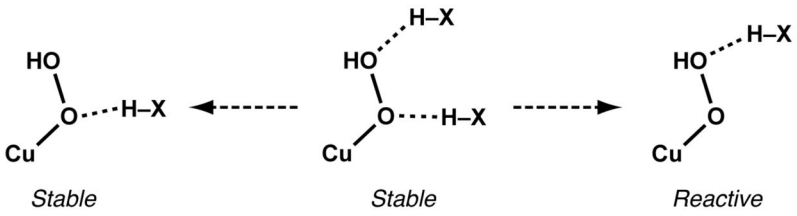
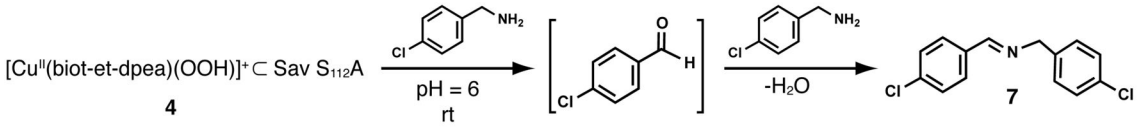


Figure 5.
Proposed effects of H-bonds on Cu–OOH species.



Scheme 1.