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Cleavage of Proteins by a Mixed-Ligand Copper(II) Phenolate Complex: Hydrophobicity of the Diimine Coligand Promotes Cleavage

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The mixed-ligand copper(II) complex [Cu(tdp)(tmp)](ClO_4), where H(tdp) is 2-[(2-(2-hydroxyethylamino)ethylimino)methyl]phenol and tmp is 3,4,7,8-tetramethyl-1,10-phenanthroline, exhibits cleavage of the proteins bovine serum albumin and lysozyme, producing approximately 5 and 4 kDa protein fragments respectively within a few minutes at micromolar concentrations. The hydrophobic tmp ligand recognizes the hydrophobic site and enhances protein binding and cleavage even at physiological pH and temperature.

The design of synthetic metalloproteases that cleave proteins at a specific site has elicited much interest very recently.[1] Because the peptide bond is one of the most stable chemical bonds in nature ($t_{1/2}$, 7−600 years at ambient temperature; pH 7),[2] the development of peptide bond cleaving reagents (artificial peptidases) is challenging.[3] Such chemical proteases can be utilized for breaking large proteins into smaller fragments that are more amenable to sequencing.[4] They will be useful in exploring the solution structures of proteins and in identifying the ligand and DNA binding sites on proteins. Small metal complexes may play the role of proteases and could be employed to generate reactive intermediates for peptide bond cleavage at selected sites on proteins. They can be directed to specific sites in proteins by using affinity ligands and peptide bond cleavage at the binding site achieved by mere incubation without the addition of external agents. Several metal complexes of cobalt(III),[5a] nickel(II),[5b] copper(II),[5c] and zinc(II)[5c] have been used to probe the mechanism of metal-promoted peptide cleavage. However, there are only a few examples of metal-promoted hydrolysis of peptide bonds in proteins.[3a,6−8] Burstyn and co-workers discovered[6] that [Cu(tacn)Cl_2] (tacn = 1,4,7-triazacyclononane) hydrolyzes the unactivated dipeptide glycylglycine and also the plasma protein bovine serum albumin (BSA) at 0.5−1.5 mM concentration under near physiological pH at 50 °C after 48−96 h of incubation. This is the first metal complex reported to cleave not only peptide bonds but also phosphodiester bonds including DNA.[9] Kostic and his co-worker reported a few palladium(II) complexes[7] that hydrolytically cleave BSA at millimolar concentrations at 50 °C after several days of the incubation period. Very recently, Neves and his co-workers reported a mononuclear copper(II) phenolate complex[8] that hydrolytically cleaves BSA ($t_{1/2}$, 30 min; pH 7.2) at 50 °C at micromolar concentrations.

In this Communication, we report our novel discovery that the mixed-ligand copper(II) complex $[Cu(tdp)(tmp)]^+$ (**4**), where H(tdp) is the tetradentate ligand 2-[(2-(2-hydroxyethylamino)ethylimino)methyl]phenol and tmp = 3,4,7,8-tetramethyl-1,10-phenanthroline (Chart 1), is remarkable in exhibiting rapid cleavage of the proteins BSA ($t_{1/2}$, 1.7 min) and lysozyme ($t_{1/2}$, 2.3 min) at micromolar concentrations under physiological pH and temperature. However, the simple copper(II) complex [Cu(tdp)(ClO_4)]·0.5H_2O (**1**) and the analogous ternary copper(II) complexes $[Cu(tdp)(diimine)]^+$, where diimine is 2,2′-bipyridine (bpy) (**2**), 1,10-phenanthroline (phen) (**3**), and dipyrido[3,2-*d*:2′,3′-*f*]quinoxaline (dpq) (**5**), fail to exhibit the cleavage. This is interesting because all of the complexes **1**−**5** have been shown[10] to bind to these proteins at ambient temperatures. Also, the dpq

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Chart 1. Structures of H(tdp) and Diimine (N−N) Coligands and Their Copper(II) Complexes

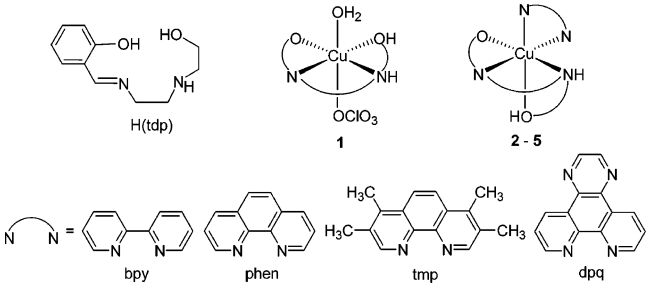
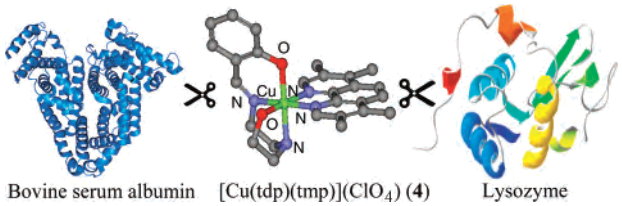


Chart 2. Schematic Diagram Showing the Energy-Optimized Molecular Structure of **4**, Which Cleaves the Proteins BSA and Lysozyme



complex **5** displays stronger DNA binding and better DNA cleavage[10] ability than all other complexes but, interestingly, only the tmp complex **4** exhibits the highest cytotoxicity against the ME180 cell line.[10] The tetradentate phenolate ligand is designed to provide donor elements such as amine nitrogen and phenolate and ethanolic oxygens to strongly bind to copper(II) and impose a square-planar geometry,[10] while the diimines are used as "affinity ligands" for recognizing and binding to proteins. Thus, the methyl groups on the 3, 4, 7, and 8 positions of the phen ring as in tmp would provide a hydrophobic recognition element.[11]

The ability of the complexes **1**−**5** to cleave protein peptide bonds was studied using the proteins BSA and lysozyme. When the proteins (15 μM) were incubated at 37 and 50 °C (Figure S8 in the Supporting Information) with **1**−**5** (300 μM) at pH 7.3 and 7.0 and then subjected to SDS-PAGE,[12] only the tmp complex **4** is found to exhibit cleavage of both of the proteins (Chart 2), producing approximately 5 kDa (BSA; Figures 1A and S9 in the Supporting Information) and 4 kDa (lysozyme; Figures 1B and S10 in the Supporting Information) protein fragments, while all other complexes, $Cu(ClO_4)_2{\cdot}6H_2O$ and the free ligands H(tdp) and tmp (Figure S11 in the Supporting Information), fail to cleave them. Also, we found that the corresponding mono- and bis(diimine)-copper(II) complexes (Figures 1 and S12 in the Supporting Information) fail to exhibit a similar efficient protein cleavage, revealing that only the mixed-ligand complex is involved in the cleavage. The observation of only one protein fragment for BSA is remarkable because it is not in agreement with the proposed solvent-accessible cleavage sites (50.2, 42.7, 41.6, 33.7, and 32.8 kDa) in BSA.[8] Also, this is in contrast to the observation of five (51.9, 44.1, 42.2, 32.6, and 31.1 kDa)[8] and two (27 and 40 kDa) protein fragments when respectively a copper phenolato complex[8] and [Cu(tacn)Cl_2][6] are incubated with BSA at 50 °C. However, Meares and his co-workers have reported the specific cleavage of BSA, a unique residue of which is attached to an iron(II) chelate, producing 5 kDa and additionally 45 and 17 kDa cleavage products but in the presence of 20 mM ascorbate and 5 mM H_2O_2.[3d] They have found that the N- and C-terminal sequences of the 5 kDa peptide fragments are produced by the cleavage of two peptide bonds, one between Ala150 and Pro151 and the other after the Ser190 amino acid. So, it is clear that the tmp complex **4** cleaves BSA to produce the 5 kDa fragment. Similarly, for lysozyme two cleavage sites, one major (Trp108−Val109) and the other minor (Glu35−Asp52), have been reported, producing approximately 10 and 4 kDa fragments, respectively.[13] It is evident that the tmp complex is also effective in cleaving lysozyme at the same minor cleavage site to produce the 4 kDa fragment. The more exposed protein secondary structure targets[14] of BSA and lysozyme are susceptible to binding and cleavage by the tmp complex at physiological (37 °C) and higher temperatures. The protein cleavage observed for **4** at physiological temperature is remarkable because metal complexes already known to hydrolyze peptide bonds at

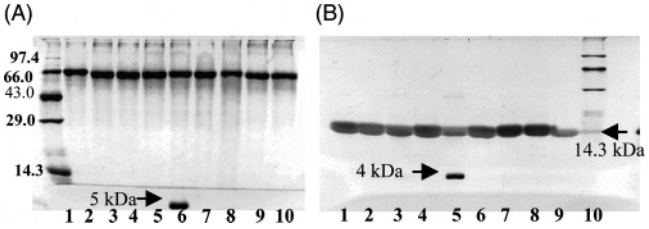


Figure 1. (A) SDS-PAGE of BSA incubated with complexes **1**−**5** (300 μM) in a 10% DMF/10 mM HEPES buffer (pH 7.3) and 37 °C for 4 h: lane 1, molecular weight standard for the mass values given on the left; lanes 2−10, BSA (15 μM); lane 2, control BSA; lane 3, BSA + **1**; lane 4, BSA + **2**; lane 5, BSA + **3**; lane 6, BSA + **4**; lane 7, BSA + **5**; lane 8, BSA + $[Cu(dpq)_2(H_2O)](ClO_4)_2$ (300 μM); lane 9, BSA + $[Cu(tmp)_2(Cl)]$-Cl (300 μM); lane 10, BSA + $Cu(ClO_4)_2{\cdot}6H_2O$ (300 μM). The main cleavage fragment is indicated by an arrow. (B) SDS-PAGE of lysozyme incubated with complexes **1**−**5** (300 μM): lanes 1−9, lysozyme (15 μM); lane 1, control lysozyme; lane 2, lysozyme + **1**; lane 3, lysozyme + **2**; lane 4, lysozyme + **3**; lane 5, lysozyme + **4**; lane 6, lysozyme + **5**; lane 7, lysozyme + $[Cu(dpq)_2(H_2O)](ClO_4)_2$ (300 μM); lane 8, lysozyme + $[Cu(tmp)_2(Cl)]Cl$ (300 μM); lane 9, lysozyme + $Cu(ClO_4)_2{\cdot}6H_2O$ (300 μM); lane 10, molecular weight standard for the mass values given on the right. The main cleavage fragments are indicated by arrows.

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(12) Protein cleavage experiments involve incubating BSA with **1**−**5** in 10% DMF/10 mM HEPES buffer at pH 7.0 and 7.3 for 4 h at 37 °C. At the end of the incubation period, an aliquot was mixed with a loading buffer two times (100 mM Tris-HCl; pH 6.8; 7% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.01% bromophenol blue) and kept in boiling water for 3 min. The samples were then analyzed by SDS-PAGE. For anaerobic reactions, samples were prepared according to a DNA cleavage procedure as previously described.[10] Protein samples were subjected to a discontinuous SDS-PAGE (5% acrylamide for the stacking gel and 10% acrylamide for the separation gel)[16] in a Genei vertical gel electrophoresis apparatus, carrying out the experiments at 50 V (stacking) and 100 V (separation). The gels were silver stained,[17] and their images were obtained using an Alpha Innotech Corp. Gel doc system and photographed using a CCD camera. Densitometry calculations were made using AlphaEase FC StandAlone software.

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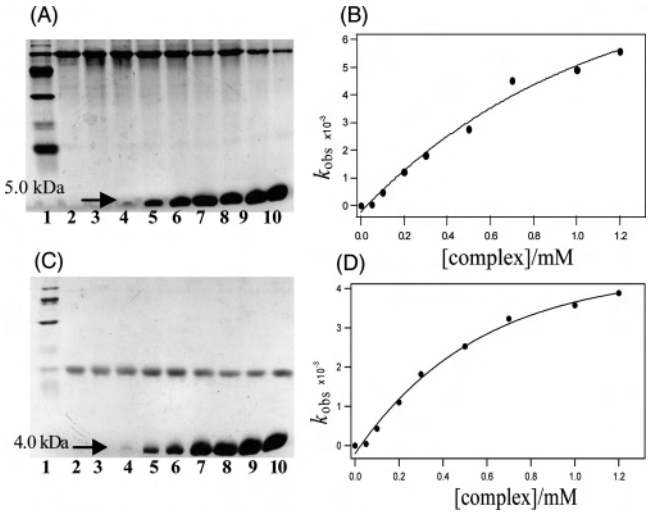

Figure 2. (A) SDS-PAGE of concentration-dependent BSA degradation by **4** in a 10% DMF/10 mM HEPES buffer (pH 7.3) and 37 °C for 4 h: lane 1, molecular weight standard for the mass values given on the left; lane 2, control BSA; lanes 3−10, BSA (15 μM) incubated with **4** (0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and 1.2 mM). The main cleavage fragment is indicated by an arrow. (B) Pseudo-Michaelis−Menten kinetics of the BSA degradation versus complex **4** concentrations. (C) SDS-PAGE of concentration-dependent lysozyme degradation by **4**: lane 1, molecular weight standard for the mass values given on the left; lanes 1−9, lysozyme (15 μM) incubated with **4** (0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and 1.2 mM). The main cleavage fragment is indicated by an arrow. (D) Pseudo-Michaelis−Menten kinetics of the lysozyme degradation versus complex **4** concentrations.

specific regions rather than specific peptide bonds require temperatures (50−70 °C) higher than physiological temperatures and longer incubation times[6] or substrates activated toward hydrolysis.[3d] Also, because the analogous mixed-ligand complexes of bpy (**2**) and other diimines (**3** and **5**) fail to cleave the proteins, it is evident that the coordinated tmp ligand with its hydrophobic methyl substituents in **4** acts as an affinity ligand to recognize the more exposed hydrophobic regions of the proteins (see below) and facilitate the selective binding and cleavage.

The effect of the concentration of complex **4** on rate of protein cleavage was studied using a constant concentration of proteins (15 μM) under "pseudo-Michaelis−Menten" kinetic conditions (Figure 2A,C). The calculated[15] rates of cleavage increase with an increase in the complex concentration (Figure 2B,D). The protein cleavage catalyzed by **4** was also studied as a function of time at 500 μM complex concentration (Figures S13A and S14A in the Supporting Information). The rates of protein degradation at 37 °C were calculated from the percentage of remaining BSA (66.0 kDa) and lysozyme (14.3 kDa) versus time plot (Figures S13B and S14B in the Supporting Information), and the $t_{1/2}$ values (BSA, 1.7; lysozyme, 2.3 min) derived from them reveal that BSA is cleaved faster than lysozyme. The BSA cleavage reaction occurs even in the presence of the hydroxyl radical scavenger manitol (Figure S15 in the Supporting Information), excluding the possibility of involvement of a freely diffusible hydroxyl radical in the cleavage. Also, it occurs even in the absence of dioxygen (argon atmosphere) with no change in the cleavage pattern or products, as observed in SDS-PAGE, supporting the hydrolytic pathway for the peptide bond cleavage. The effect of pH (Figure S16 in the Supporting Information) on the cleavage reaction was studied using BSA as the substrate, but no significant changes in the protein cleavage reaction are observed. It is expected that the ligand ethanolic oxygen in **4** is weakly bound to the axial site (two electrons in d_{z^2} orbital) of copper(II) as in the analogous phen complex **3** (Cu−O, 2.5120 Å).[10] We propose that the axially copper(II)-bound hydroxyl group might decoordinate in solution upon the addition of BSA and the now Lewis acidic copper(II) coordinates to the carbonyl oxygen atom, rendering the carbonyl group susceptible to nucleophilic attack by solvent water molecules. This is supported by absorption spectral studies (Figure S7 in the Supporting Information). It is obvious that the ligand ethanolic oxygen strongly bound at the equatorial site as in **1** cannot be easily dislodged from the equatorial site, so that it is involved in the peptide bond cleavage. Thus, the tmp complex exhibits a potential to cleave unactivated peptide bonds of BSA and lysozyme at micromolar concentrations under mild pH and temperature conditions. Also, using tryptophan emission quenching experiments,[10] we have already demonstrated the ability of **1**−**5** to bind to BSA at ambient temperatures, with the tmp complex displaying stronger protein binding.

In conclusion, we have discovered that the complex [Cu(tdp)(tmp)](ClO_4) is the only one among the present mixed-ligand complexes to exhibit very rapid peptide bond cleavage of the natural substrates BSA and lysozyme at micromolar concentrations under mild physiological pH and temperature conditions. We suggest that the hydrophobic tmp coligand recognizes the hydrophobic region of the proteins, facilitating site-specific protein bond cleavage by the complex. Further work on amino acid sequencing of the cleaved proteolytic fragments to identify and confirm the cleavage sites is in progress. To our knowledge, the tmp complex is the first mixed-ligand copper(II) complex to exhibit selective and fast protein cleavage at physiological conditions apart from displaying DNA cleavage. Thus, the complex has the potential to be exploited as a new synthetic protease in protein chemistry.

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Supporting Information Available: Synthesis and characterization (Figures S1−S6 and Tables S1−S6), UV−vis spectra (Figure S7), and SDS-PAGE (Figures S8−S16). This material is available free of charge via the Internet at http://pubs.acs.org.

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