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Photocleavage of Lysozyme by Cobalt(III) Complexes

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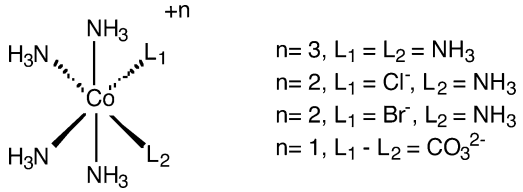
Photochemical reagents that cleave proteins at specific sites (photoproteases) are useful for studying protein structure and protein–ligand interactions. PolyammineCo(III) complexes are tested here as photochemical probes to cleave proteins. Irradiation of a mixture of lysozyme, a model protein, and polyammineCo(III) complexes resulted in the facile cleavage of the peptide backbone. Photocleavage yielded two fragments of molecular weights 10.6 and 3.7 kDa, and these masses sum to the molecular mass of lysozyme (14.3 kDa). No cleavage was detected in the absence of the metal complex, in the dark, or upon irradiation at wavelengths of >420 nm. The photocleavage yield increased with irradiation time and with the concentrations of the metal complex and the protein. N-terminal sequencing of the 10.6 kDa fragment indicated residues that are identical to the N-terminus of lysozyme, and sequencing of the 3.7 kDa fragment indicated Val-Ala-Trp-Arg, an internal sequence of lysozyme. From the known primary sequence of lysozyme and the sequencing data, the cleavage site was assigned to Trp108-Val109. Molecular modeling indicates that the observed cleavage site is within few angstroms from the proposed metal binding site at Glu35-Asp52. This is the first report of the successful photocleavage of proteins, with high selectivity, by transition metal complexes. This novel observation can facilitate the rational design of transition metal complexes for the photochemical footprinting of metal binding sites on proteins.

Photochemical reagents that cleave proteins at specific sites (photopeptidases) are useful for investigating protein−ligand interactions, protein−protein contact regions, and protein structure.[1] The photochemical approaches complement oxidative and hydrolytic methods of protein cleavage reported earlier.[2] Here, it is demonstrated that Co(III) complexes (Chart 1) cleave lysozyme under photochemical conditions with high selectivity. Such approaches are promising for the footprinting of metal binding sites on proteins.

Chart 1. Structures of Co(III) Complexes Used in This Study



Lysozyme is a well-characterized, small (molecular weight $\approx$ 14.3 kDa), water-soluble enzyme, and it is capable of hydrolyzing bacterial cell walls.[3] Metal ions such as Mn(II), Co(II), and Ni(III) bind weakly to lysozyme with binding constants in the range of 50−100 M^{-1}.[4] Metal binding to lysozyme was proposed to occur at specific residues of the protein. For example, Glu35, which is present in the active site of lysozyme, and Asp52, which is located within a few angstroms from Glu35, as well as Trp62,[5] have been proposed to be the metal binding sites.[6] Binding of lanthanide ions to Asp52 was suggested to inhibit aggregation of lysozyme.[7] The crystal structure of lysozyme indicates that these residues are exposed to the solvent, and they are accessible to small molecules or metal complexes. These metal binding sites on lysozyme presented interesting targets, and here, we describe the first report of the photocleavage of lysozyme by polyammineCo(III) complexes.

The chemical[8] and photochemical[9] properties of Co(III) complexes (Chart 1) are well-known. The ligand-to-metal

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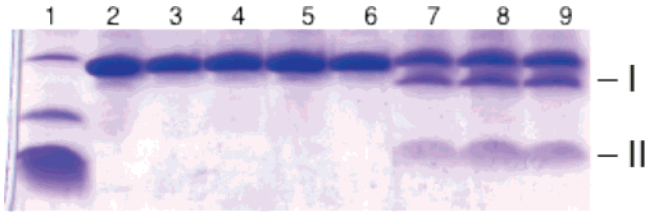

Figure 1. Photocleavage of lysozyme (75 μM) by $[Co(NH^3)_5Br]^{2+}$ (0.6 mM) by irradiation at different wavelengths (0.5 h). Lane 1 contained molecular weight markers, indicated in kilodaltons. Lane 2 contained lysozyme that was not irradiated, and lanes 6−9 contained lysozyme and $[Co(NH^3)_5Br]^{2+}$, not irradiated and irradiated at 310, 340, and 370 nm, respectively. Lanes 3−5 contained lysozyme irradiated at 310, 340, and 370 nm, respectively, for 30 minutes.

charge transfer (LMCT) states of these complexes are readily accessible, and population of these states results in products that are consistent with ligand oxidation. The solutions of these metal complexes are stable at pH 7 when protected from light over a period of >10 h.

Spectral studies show that these cobalt(III) complexes have weak affinities for lysozyme. NMR spectra of lysozyme (2.6 mM) recorded in the presence of $[Co(NH_3)_6]^{3+}$ (18 mM), for example, indicated reproducible shifts, up to 0.03 ppm, in the resonances of the β hydrogens of several residues, including Asp-101. In addition, the amide hydrogens of Asp18, Asp119, and Gly102 also indicated measurable shifts. Fluorescence spectra of lysozyme recorded in the presence of increasing concentrations of the Co(III) complexes (295-nm excitation, 350-nm monitoring, Supporting Information 1−5) indicated rapid quenching of lysozyme fluorescence. The Stern−Volmer quenching constants (K_{sv}) estimated from these data are 73, 192, and 488 M^{-1} for $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_4CO_3]^{+}$, and $[Co(NH_3)_5Br]^{2+}$, respectively. These are 2 orders of magnitude greater than values estimated for diffusion-limited quenching.[10] These large quenching constants are consistent with binding of the Co(III) complexes to the protein, and they provide strong support for the photochemical studies described below.

Photocleavage of lysozyme was achieved by irradiating (at 310, 340, or 370 nm) a mixture of lysozyme (75 μM) and $[Co(NH_3)_5Br]^{2+}$ (0.6 mM) in Tris buffer (10 mM, pH 7.0). The reaction mixture was analyzed for protein fragmentation, using SDS PAGE[11] (Figure 1), and the photoreaction resulted in two new bands of approximate masses 10 and 4 kDa (lanes 7−9). The molecular masses of the two fragments (I and II) nearly sum to that of lysozyme (14.3 kDa), suggesting that the products might arise from a single

Figure 2. Photocleavage of lysozyme (75 μM) by $[Co(NH_3)_4CO_3]^{+}$ (5 mM). Lane 1 contained molecular weight markers, indicated in kilodaltons. Lanes 2 and 3 contained lysozyme only, whereas lanes 4 and 5 contained a mixture of lysozyme and $[Co(NH_3)_4CO_3]^{+}$. Samples in lanes 3 and 5 were irradiated at 310 nm for 60 min, whereas samples in lanes 2 and 4 were left in the dark. A similar cleavage pattern was also observed when lysozyme/$[Co(NH_3)_4CO_3]^{+}$ samples were irradiated at 370 nm (data not shown).

Table 1. Yields (%) of Fragment I Observed with Co(III) Metal Complexes as a Function of Reaction Time[a]

time (min)	0.6 mM $[Co(NH_3)_5Br]^{2+}$	2.5 mM $[Co(NH_3)_4CO_3]^{+}$	5 mM $[Co(NH_3)_6]^{3+}$
10	27	16	7
20	27	22	10
30	30	22	12
40	29	25	13
50	29	26	15

[a] 310-nm irradiation, averages of several measurements are given, and the errors are within ±5%.

cleavage site. No photocleavage was observed when the samples were kept in the dark (lane 2) or when lysozyme was irradiated in the absence of $[Co(NH_3)_5Br]^{2+}$ (lanes 3−5). The appearance of clean, clear, sharp product bands in lanes 7−9 (fragment I yields of 28%, 32%, and 32%, respectively) indicate that protein cleavage occurs at a single site, or at sites that are within a few residues apart, and indiscriminate, random cleavage would have resulted in smears in the reaction lanes.

Irradiation at 340 or 370 nm, far from the protein absorption bands, also resulted in the facile photocleavage of the protein (lanes 8 and 9). Thus, any light absorption by aromatic residues of the protein is not responsible for the observed photocleavage. Direct population of the LMCT states is most likely responsible for the observed photocleavage.

Encouraged by these results, protein cleavage properties of $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_5Cl]^{2+}$, and $[Co(NH_3)_4CO_3]^{+}$ (Chart 1) were also tested. These metal complexes also indicated facile photocleavage of lysozyme. Irradiation of a mixture of lysozyme (75 μM) and $[Co(NH_3)_4CO_3]^{+}$ (5 mM) resulted in two fragments of lower mass (Figure 2, fragment I, 27% yield), and no reaction occurred when lysozyme was irradiated in the absence of the metal complex (lane 3) or when the reaction mixture was kept in the dark (lane 4). Similar cleavage patterns were also observed with $[Co(NH_3)_6]^{3+}$ (Supporting Information 6). The gel electrophoretic mobilities of these product bands matched with those from the $[Co(NH_3)_5Br]^{2+}$ photoreaction, and all metal complexes resulted in a single pair of product bands.

Product yields increased with increased irradiation time (0−50 min, 310-nm irradiation, Table 1), concentration of the metal complex, and concentration of lysozyme. The highest yields were obtained with $[Co(NH_3)_5Br]^{2+}$, and the yields increased in the order $[Co(NH_3)_6]^{3+}$ < $[Co(NH_3)_4$-

(10) The fluorescence lifetime of lysozyme is taken as ~2.8 ns (Formoso, C.; Forster, L. S. *J. Biol. Chem.* **1975**, *250*, 3738), and the mean diffusion rate of the metal complex and the protein is estimated to be 5×10^7 M^{-1} s^{-1} (Palamakumbura, A. H.; Foshay, M. C.; Vitello, L. B.; Erman, J. E. *Biochemistry* **1999**, *38*, 15647).

(11) The reaction mixture was dried under reduced pressure, and the residue was redissolved in loading buffer [7% SDS, 12% (v/v) glycerol, 0.1% bromophenol blue, 2% 2-mercaptoethanol]. Protein solutions were denatured at 100 °C for 6 min before being loaded on the gel. The gels were run by applying 60 V until the dye passed through the stacking gel, and the voltage was then increased to 110 V (2 h), as described in Schagger, H.; Von Jagow, G. J. *Anal. Biochem.* **1987**, 166, 368. Intensities of the product bands were quantified using NIH Image software, and the intensities reported here, for each lane, sum to 100%.

$CO_3]^+$ < $[Co(NH_3)_5Br]^{2+}$ (Table 1). These various observations clearly demonstrate the facile photocleavage of lysozyme by Co(III) complexes and suggest that the reaction most likely proceeds via the production of radicals generated from the corresponding LMCT states.[12]

To investigate the role of oxygen in the photoreaction, the photocleavage was carried out after the reaction mixture had been purged with nitrogen gas,[13] and this did not alter the yields of photoproducts with $[Co(NH_3)_6]^{3+}$. On the other hand, when the reaction was carried out in the presence of sodium azide (10 mM)[14] the yield increased from 13% to 23% (fragment I, 5 mM $[Co(NH_3)_6]^{3+}$, 75 μM lysozyme, 1-h irradiation at 310 nm), and a new fragment band (6 kDa) appeared. Thus, azide presumably participates in the photoreaction and alters the reaction path, and this aspect will be pursued in future studies. The generation of radicals during the photoreaction was also tested by using specific quenchers.[15] Irradiation (310 nm) of a reaction mixture of lysozyme (75 μM) and $[Co(NH_3)_4CO_3]^+$ (1 mM) in the presence of 2-propanol (2 M), for example, quenched the yield of fragment I from 18% to 2%.[16] Radical intermediates, generated from the LMCT state of Co(III) complexes, therefore, are most likely involved in the protein cleavage reaction.

To identify the cleavage site, the protein fragments obtained by photocleavage were isolated, purified, and analyzed by amino acid sequencing. N-terminal sequencing of the product I from the $[Co(NH_3)_5Br]^{2+}$ reaction mixture indicated the sequence Lys-Val-Phe-Gly-Arg, which corresponds to the native N-terminal sequence of lysozyme. The N-terminal sequencing of the corresponding product II revealed Val-Ala-Trp-Arg, a sequence that is internal to lysozyme. By comparing the observed N-terminal sequences of fragments I and II with the known sequence of lysozyme, the identity of the major cleavage site was established (Chart 2). $[Co(NH_3)_5Br]^{2+}$, therefore, cleaves lysozyme at Trp108-Val109.

Chart 2. Lysozyme Cleavage Site

NH_2 Lys-Val-Phe-Ala-…..Trp(108) _Val(109)-Ala-Trp-Arg……..Arg-Leu-COOH

Sequencing analysis further indicated that the reaction mixture also contained a small amount of an additional product (mol wt $\approx$ 4 kDa) that is not distinguishable in the gels. This product had residues that are identical to the N-terminal sequence of lysozyme, but the corresponding C-terminal fragment was not detectable. Perhaps, its yield is too small or is not amenable to sequencing. Therefore, a minor, secondary cleavage site was also observed, in addition to the major site, Trp108-Val109.

Molecular modeling studies indicate that binding of the metal complexes to Glu35-Asp52 site will position them across a cleft, within 5 Å from Trp108-Val109. It is plausible that the production of ligand radicals at this metal binding site can readily access the observed cleavage site or that the metal complex binds at another unidentified site.

In any event, the above Co(III) complexes cleave lysozyme, with high selectivity, under photochemical conditions. Even though the yields are <30%, for a photochemical reaction involving a macromolecule, these are significant. The wavelengths of irradiation used here are in the near-UV region, and irradiation far from the protein absorption bands (<300 nm) also resulted in protein cleavage. Studies are in progress to test whether these transition metal complexes can be used for the footprinting of metal binding sites on specific proteins.

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Supporting Information Available: Fluorescence spectra of lysozyme recorded in the presence of increasing concentrations of the Co(III) complexes and protein cleavage pattern observed with $[Co(NH_3)_6]^{3+}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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