Hydrolysis of Unactivated Peptide Bonds by a Macrocyclic Copper(II) Complex: $Cu([9]aneN_3)Cl_2$ Hydrolyzes Both Dipeptides and Proteins

Eric L. Hegg and Judith N. Burstyn*

Department of Chemistry, University of Wisconsin Madison, Wisconsin 53706

Received June 14, 1994
Revised Manuscript Received February 3, 1995

There is great interest in designing synthetic metallopeptidases,[1−7] as such complexes could be useful in elucidating the solution structure of proteins as well as in the sequencing of large proteins. Substitutionally inert metal complexes of Co(III)[1,3] and complexes of Cu(II),[2,3a,4] Zn(II),[2c,3a] and Ni(II)[2c,3a] were used to study the mechanism and reaction intermediates of metal-promoted peptide hydrolysis. However, because of the extreme stability of peptide bonds,[8] their hydrolysis is often studied using activated amides[2] or metal ions tethered to an amide affording an intramolecular reaction[1,3] or by enforcing a favorable stereochemical relationship between the nucleophile and the amide bond.[3] Metal-promoted hydrolysis of proteins is not well studied, and there are few examples.[5c,7] In this Communication, we report the discovery that $Cu([9]aneN_3)Cl_2$[9] (**1**) hydrolyzes not only the unactivated dipeptide glycylglycine but also the protein bovine serum albumin at near-physiological pH. This is only the second example of protein hydrolysis by a small metal complex not tethered to the protein.[5c] Furthermore, **1** is the first metal complex to demonstrate the ability to hydrolyze both proteins and phosphodiester bonds.[10]

We discovered that glycylglycine (Gly-Gly) is hydrolyzed to glycine when incubated with varying concentrations of **1**.[11]

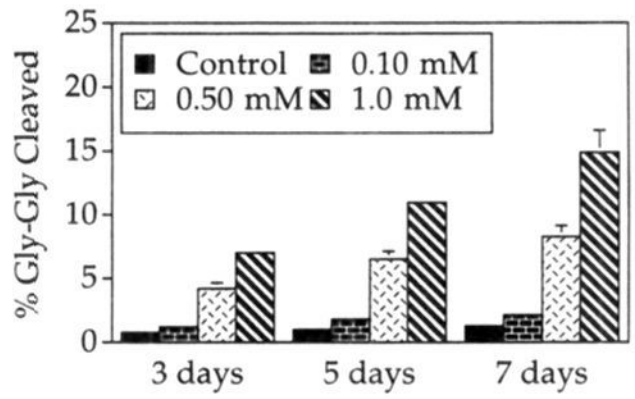

Figure 1. Extent of hydrolysis of glycylglycine (1.3 mM) with varying concentrations of **1** at 50 °C. The pH was maintained at 8.1 ± 0.1 with 50 mM $NaHCO_3$. All reactions were performed in triplicate, and standard deviations are shown in cases were the deviation is >0.4%.

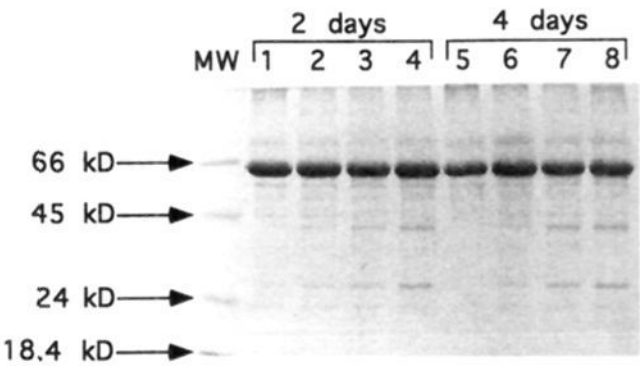

Figure 2. BSA (0.21 mM) incubated with **1** at pH 7.8 (50 mM HEPES) and 50 °C. Lanes 1, 5: control, no metal complex. Lanes 2, 6: 0.50 mM **1**. Lanes 3, 7: 1.0 mM **1**. Lanes 4, 8: 1.5 mM **1**. Each lane was loaded with 4.0 μg of total protein. BSA incubated at pH 8.1 ± 0.1 (50 mM HEPES) yielded identical results.

Significantly, glycine is the only product observed, confirming that **1** cleaves Gly-Gly hydrolytically. As shown in Figure 1, the extent of hydrolysis is dependent on both metal concentration and reaction time. Furthermore, $Cu([9]aneN_3)Cl_2$ is a catalyst; after extended periods of incubation, we obtained greater than stoichiometric yields of glycine with no loss in rate of hydrolysis.[12] Interestingly, the reaction shows a large buffer effect; Gly-Gly is cleaved much more rapidly in $NaHCO_3$ than in HEPES. Groves and Baron previously reported a similar effect on amide hydrolysis in HEPES vs phosphate buffers.[3b] They hypothesized that bifunctional buffers such as phosphate and bicarbonate are able to aid in proton transfer and facilitate the breakdown of the tetrahedral intermediate.

Bovine serum albumin (BSA), a 66 000 Da protein, is also a substrate for hydrolysis by **1**. BSA was incubated with varying concentrations of **1**, and the products were separated by denaturing polyacrylamide gel electrophoresis (SDS−PAGE).[13] Remarkably, when the reaction is performed in HEPES buffer, BSA is hydrolyzed into two discrete polypeptides of approximately 27 and 40 kDa (Figure 2). When the incubation time is extended to 7 and 13 days, nonspecific cleavage is

(1) (a) Buckingham, D. A.; Binney, G. S.; Clark, C. R.; Garnham, B.; Simpson, J. *Inorg. Chem.* **1985**, *24*, 135−141. (b) Sutton, P. A.; Buckingham, D. A. *Acc. Chem. Res.* **1987**, *20*, 357−364. (c) Takasaki, B. K.; Kim, J. H.; Rubin, E.; Chin, J. *J. Am. Chem. Soc.* **1993**, *115*, 1157−1159.

(2) (a) Reddy, K. V.; Jacobson, A. R.; Kung, J. I.; Sayre, L. M. *Inorg. Chem.* **1991**, *30*, 3520−3525. (b) Sayre, L. M.; Reddy, K. V.; Jacobson, A. R.; Tang, W. *Inorg. Chem.* **1992**, *31*, 935−937. (c) Fife, T. H.; Przystas, T. J. *J. Am. Chem. Soc.* **1986**, *108*, 4631−4636.

(3) (a) Groves, J. T.; Chambers, R. R. *J. Am. Chem. Soc.* **1984**, *106*, 630−638. (b) Groves, J. T.; Baron, L. A. *J. Am. Chem. Soc.* **1989**, *111*, 5442−5448.

(4) (a) Duerr, B. F.; Czarnik, A. W. *J. Chem. Soc., Chem. Commun.* **1990**, 1707−1709. (b) Chin, J.; Jubian, V.; Mrejen, K. *J. Chem. Soc., Chem. Commun.* **1990**, 1326−1328.

(5) (a) Burgeson, I. E.; Kostić, N. M. *Inorg. Chem.* **1991**, *30*, 4299−4305. (b) Zhu, L.; Kostić, N. M. *J. Am. Chem. Soc.* **1993**, *115*, 4566−4570. (c) Zhu, L.; Qin, L.; Parac, T. N.; Kostić, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218−5224.

(6) (a) Yashiro, M.; Takarada, T.; Miyama, S.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1994**, 1757−1758. (b) Singh, L.; Ram, R. N. *J. Org. Chem.* **1994**, *59*, 710−711.

(7) (a) Ermácora, M. R.; Delfino, J. M.; Cuenoud, B.; Schepartz, A.; Fox, R. O. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6383−6387. (b) Rana, T. M.; Meares, C. F. *J. Am. Chem. Soc.* **1990**, *112*, 2457−2458. (c) Rana, T. M.; Meares, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10578−10582.

(8) Kahne, D.; Still, W. C. *J. Am. Chem. Soc.* **1988**, *110*, 7529−7534.

(9) Copper(II) 1,4,7-triazacyclononane dichloride ($Cu([9]aneN_3)Cl_2$, **1**), was synthesized according to the procedure of Schwindinger et al.: Schwindinger, W. F.; Fawcett, T. G.; Lalancette, R. A.; Potenza, J. A.; Schugar, H. J. *Inorg. Chem.* **1980**, *19*, 1379−1381.

(10) Burstyn, J. N.; Deal, K. A. *Inorg. Chem.* **1993**, *32*, 3585−3586.

(11) Glycylglycine was incubated with **1** at pH 8.1 ± 0.1 (50 mM HEPES or $NaHCO_3$). Reactions using $NaHCO_3$ as a buffer were performed in a CO_2 incubator, and the pH was monitored daily. The reactants and products were derivatized with 5× (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl), followed by addition of a 50% ethanol/50% 50 mM phosphate buffer solution. Samples were analyzed on a Shimadzu-6A HPLC equipped with an Alltech Spherisorb ODS-2 reverse-phase column. The products were eluted with an 18%−28% acetonitrile/buffer A gradient over 30 min at 1 mL/min, where buffer A was 4% DMF in 25 mM sodium acetate at pH 6.5, and detected by UV absorption at 275 nm. The mass balance of products and reactants was confirmed to ±7%. Possible metal-catalyzed formation of 2,5-piperazinedione was ignored (Long, D. A.; Truscott, T. G.; Cronin, J. R.; Lee, R. G. *Trans. Faraday Soc.* **1971**, *67*, 1094−1103).

(12) Gly-Gly (5.0 mM) was incubated with **1** (0.50 mM) at pH 8.1 ± 0.1 (50 mM $NaHCO_3$) and 50 °C in a CO_2 incubator. Product analysis was performed as described in footnote 11; incubation for 28 days resulted in 1.3 turnovers.

(13) BSA was incubated with **1** at 50 °C and pH 7.8 (50 mM HEPES) or 8.1 ± 0.1 (50 mM HEPES or $NaHCO_3$). Reactions using $NaHCO_3$ as a buffer were performed in a CO_2 incubator. The polypeptide fragments were separated via SDS−PAGE by the method of Laemmli (Laemmli, U. K. *Nature* **1970**, *227*, 680−685) on a 10% polyacrylamide gel, and the resulting bands were stained with Coomassie Brilliant Blue.

(14) The protein fragments were electroblotted onto nitrocellulose using a Bio-Rad Mini Trans-Blot electrophoretic transfer cell. Immunoblotting was accomplished using the ECL western blotting system from Amersham according to the protocol provided. Densitometric analysis was performed using a Model 300A computing densitometer from Molecular Dynamics. Because only the site-specific cleavage was quantitated, the extent of total cleavage is somewhat higher. Densitometric analysis was also performed on Coomassie-stained gels, and these experiments yielded identical results within the error of the method (±5%).

0002-7863/95/1517-7015$09.00/0

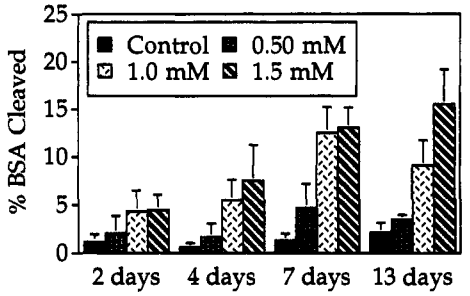

Figure 3. Extent of hydrolysis of BSA determined by densitometric analysis. BSA (0.21 mM) incubated at pH 7.8 (50 mM HEPES) and 50 °C with varying concentrations of **1**. These data are the average of six trials, and the standard deviation is shown.

evident at higher metal concentrations. The extent of site-specific hydrolysis was quantitated by densitometric analysis of western blotted protein,[14] and cleavage is again dependent on both metal concentration and reaction time (Figure 3). Evidence for a hydrolytic mechanism is afforded by performing the reactions anaerobically.[15] BSA incubated with **1** under argon showed no change in either the extent of cleavage or the products observed via SDS–PAGE.

The BSA fragments were isolated and subjected to N-terminal sequencing by the Edman degradation method.[16] The fact that the termini are susceptible to Edman degradation is notable because only free amino termini are expected to be sequenced successfully; this result is therefore further evidence for the hydrolytic cleavage of peptide bonds in BSA by **1**. The 27 kDa fragment contains the N-terminus of BSA, while the sequence map of the 40 kDa fragment is consistent with multiple internal cleavage sites. Cu([9]aneN$_3$)Cl$_2$ hydrolyzes BSA primarily between residues C243–C244, although hydrolysis between residues Q219–K220 is also important; evidence for minor hydrolysis between residues S270–S271 and E250–C251 is less certain.[17] Other fragments too minor to be successfully sequenced were also present. Thus, **1** is not selective for hydrolysis of a specific peptide bond, but rather for a specific region of the protein.

The hydrolysis of BSA exhibits a large buffer effect, consistent with the results observed with Gly-Gly. When BSA is incubated with **1** in sodium bicarbonate, the protein is cleaved at numerous sites, and the rate increases dramatically. While the 27 and 40 kDa fragments are observed initially, the BSA is subsequently cleaved into a myriad of fragments, resulting in a significant loss in intensity of the BSA band and a concomitant smear on the polyacrylamide gel. One hypothesis compatible with the observed data is that **1** hydrolyzes BSA in HEPES at sites that are accessible to the metal complex and where the departing alkyl amine can be protonated by an adjacent amino acid. The bifunctional nature of sodium bicarbonate may allow the buffer to act as a proton transfer agent and aid in the rate-determining breakdown of the tetrahedral intermediate. Thus, BSA is hydrolyzed more rapidly and at more sites in bicarbonate buffer.

In summary, Cu([9]aneN$_3$)Cl$_2$ hydrolyzes Gly-Gly and BSA at near-physiological pH. Gly-Gly contains no special metal binding sites, and it is in no way activated or oriented so as to promote hydrolysis. In contrast, many other metal complexes that hydrolyze peptide bonds require harsh conditions[4b,5,6b] or substrates activated toward hydrolysis.[2,3] The large buffer effect observed is consistent with a mechanism in which the bifunctional buffer aids in proton transfer. Although the rate of hydrolysis is slow, the reaction with Gly-Gly is nevertheless catalytic. Furthermore, Cu([9]aneN$_3$)Cl$_2$ is only the second nontethered small metal complex shown to hydrolyze an intact protein.[5c]

Acknowledgment. The authors thank Dr. Jane Wallant and Dr. Ronald Niece at the Protein Sequence and Peptide Synthesis Service in the NAP Facility of the University of Wisconsin Biotechnology Center, Madison, WI, for the N-terminal sequence analysis. Funding was provided by NIH 5T32GMO8349 (Biotechnology Training Grant for E.L.H.) and the University of Wisconsin Graduate School Research Committee.

JA941874M

(15) Anaerobic reaction mixtures were prepared in a glovebag under N_2 using rigorously degassed water. The samples were transferred to an argon-filled desiccator before being placed in an incubator at 50 °C. Under identical conditions, the oxidative cleavage of single-stranded DNA by Fe(EDTA)$^{2-}$/DTT was completely inhibited, confirming the absence of oxygen.

(16) The BSA fragments were electroblotted onto a sheet of poly(vinylidene difluoride) (PVDF) and stored under Ar prior to sequencing (Matsudaira, P. *J. Biol. Chem.* **1987**, *262*, 10035–10038. LeGendre, N.; Matsudaira, P. *Biotechniques* **1988**, *6*, 154–159). N-Terminal sequence analysis was conducted for a minimum of 10 residues using an automated Model 477A liquid pulse sequencer and Model 475A gas phase sequencer with on-line Model 120A PTH analyzer and Model 610A data analysis system (Applied Biosystems, Foster City, CA). All reagents were sequencing grade (Applied Biosystems).

(17) An OPA isolation experiment afforded partial verification of our interpretation of the sequence map (Wadsworth, C. L; Knuth, M. W.; Burrus, L. W.; Olwin, B. B.; Niece, R. L. In *Techniques in Protein Chemistry III*; Angeletti, R. H., Ed.; Academic: San Diego, 1992; pp 61–68). Chemical isolation by OPA in cycle 3 confirmed the presence of the minor peptide fragment resulting from hydrolysis after residue Q219.