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Artificial metallopeptidases: regioselective cleavage of lysozyme†

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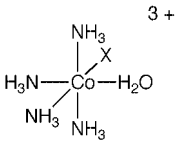
Received (in Columbia, MO, USA) 10th September 1999, Accepted 30th October 1999

Pentammineaquocobalt(III) chloride and tetramminediaquocobalt(III) chloride cleave chicken hen egg lysozyme between Ala 110 and Trp 111 at neutral pH and 37 °C.

Metal complexes that can cleave proteins with a high specificity under mild conditions will be useful for structure–activity studies of proteins, investigation of protein structural domains, and in converting large proteins into smaller fragments that are more amenable for sequencing.[1–8] Such reagents are also useful for the chemical modification of proteins, or for heavy atom labeling.[2–3] Metal complexes have been attached to specific sites on proteins and subsequent activation resulted in protein fragmentation *via* oxidative or hydrolytic chemistry. Hydrolytic chemistry is preferred over oxidative chemistry as the latter can potentially damage the protein side chains and the resulting fragments may not be amenable to sequencing. Exopeptidase activities of pentammineaquocobalt(III) (CoPA) and tetramminediaquocobalt(III) (CoTA) ions (Scheme 1), under mild conditions, is demonstrated here for the first time.



Scheme 1 Structure of the cobalt(III) metal complexes used to cleave lysozyme with high specificity. X = NH_3 for CoPA and H_2O for CoTA.

Metal complexes with labile ligands may bind to the side chains of amino acid residues such as Cys, His, Trp, Asp and Lys and such coordination may function as an anchor to position the reagent for the controlled hydrolysis of protein backbone.[2,3] In this context, Cu, Pt and Pd complexes showed significant artificial metallopeptidase activities.[3] Co(III) complexes are known to induce N-terminal hydrolysis of peptides (endopeptidase activity)[9,10] but the exopeptidase activity of Co(III) is not known. Hydrolysis of N-4(1-pyrenyl)butyroylphenylalanine by CoPA (hydrolysis occurs at the C-terminus) prompted us to investigate the catalytic hydrolysis of proteins by Co(III) complexes.[11] Chicken hen egg lysozyme was chosen for the current studies because lysozyme can bind a number of metal ions,[12] its three-dimensional structure is known[13] and catalytic activity of lysozyme provides a convenient handle to test if the protein cleavage results in the loss of activity.

Incubation of lysozyme (15 μM) with CoPA or CoTA (1 mM) at 37 °C for 21–26 h resulted in facile cleavage of the protein into two fragments as demonstrated in SDS PAGE experiments (6% acrylamide, 0.4% bisacrylamide, 13% glycerol; pH 8.4).[14] Two new bands with molecular weights of *ca.* 12 and 2 kDa are clearly visible in Fig. 1(a) (lanes 4 and 6). If the protein cleavage were to be random, one would have observed a smear in these lanes. Occasionally, the 2 kDa band was difficult to see in the gels (lane 2). The rate of protein cleavage increases with the concentration of CoTA (0.2, 0.5 and 1 mM) and similar activity was observed with CoPA [Fig. 1(b)].

† Electronic supplementary information (ESI) available: self cleavage of lysozyme at room temperature and 37 °C. See http://www.rsc.org/suppdata/cc/a9/a907477e/

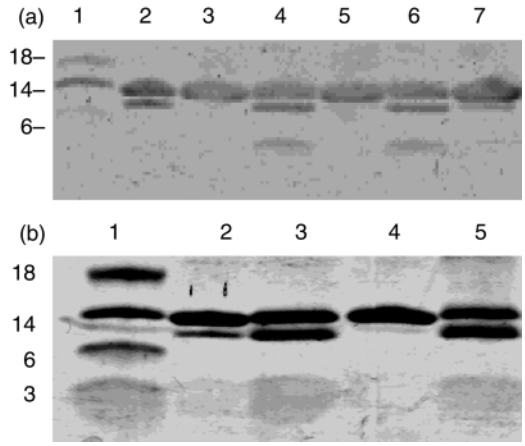


Fig. 1 (a) Lysozyme cleavage by CoTA at 37 °C. Lane 1 contains molecular weight markers at 18, 14 and 6 kDa. Lanes 2–7 contain lysozyme (15 μM) and increasing concentrations of CoTA (lanes 2, 3 contain 0.2 mM, lanes 4, 5 contain 0.5 mM and lanes 6, 7 contain, 1 mM CoTA, respectively). Lanes 2, 4 and 6 were kept at 37 °C (26 h) while lanes 3, 5, 7 were kept at room temperature (26 h). Two new bands corresponding to lower molecular weights than the native protein are indicated in lanes 4 and 6. (b) Lane 1 contains molecular weight markers at 18, 14, 6 and 3 kDa. Lanes 2–5 contain lysozyme (15 μM). Lane 2 contains protein treated with CoTA (1 mM, 22 °C); lane 3, CoTA (1 mM, 37 °C); lane 4, CoPA (1 mM, 22 °C) and lane 5, CoPA, 1 mM, 37 °C). Facile cleavage of lysozyme at 37 °C (26 h) by the aquo complexes is evident. In addition to the major new band in lanes 3, and 5, a faint band with a molecular weight around 2000 Da is also present.

No cleavage was observed when the protein was incubated with CoPA at room temperature (26 h, lane 4) while CoTA induced weak but detectable cleavage even at room temperaure (lane 2, 6%). The ratio of the major product band intensity to the sum of the intensities of the major product and the starting material in lanes 3 and 5 are 45 and 35%, respectively.

Even though the reaction does not proceed at room temperature with CoPA [Fig. 1(b), lane 4] and the reaction is very weak with CoTA [Fig. 1(b), lane 2] self cleavage of lysozyme at room temperature and at 37 °C was examined in the absence of the metal complexes (ESI†). No reaction occurs at 37 °C, 26 h (lane 3) or at room temperature (lane 2) indicating that the reaction is mediated by the metal complexes.

By contrast, no reaction was observed with the *cis* or *trans* isomers of bisethylenediaminediaquocobalt(III) bromide (Fig. 2, lanes 4, 5). Other cobalt complexes such as chloropentamminecobalt(III), chlorotetramminecobalt(III) or chlorotetramminecarbonatocobalt(III) also induced protein cleavage, although, at much slower rates (data not shown). These metal complexes can undergo hydrolysis under the reaction conditions to give rise to the corresponding aquo complexes which are active. Hexamminecobalt(III) chloride did not induce protein cleavage suggesting that the Co(III) center with one or more aquo ligands are needed for activity.

The reaction rate is accelerated with temperature and protein cleavage products could be detected within 5 h at 45 and 55 °C. The protein cleavage was optimal at pH 7 and was slowed down

DOI: 10.1039/a907477e

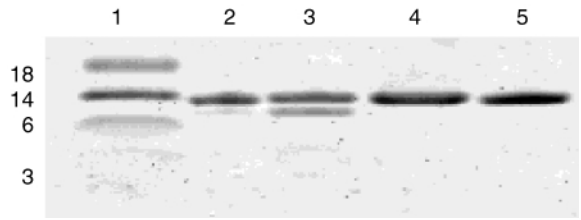
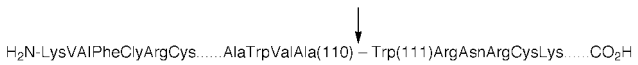


Fig. 2 Lane 1 contains molecular weight markers at 18, 14, 6 and 3 kDa. Lanes 2, 3, 4 and 5 contain lysozyme (15 μM) treated with the metal complexes (1 mM) under different conditions. Lane 2 contains CoTA at 25 °C; lane 3 contains CoTA at 37 °C; lane 4 contains *trans*-$[Co(en)_2Br_2]Br$ at 37 °C and lane 5 contains *cis*-$[Co(en)_2Br_2]Br$ (37 °C). An intense product band is evident in lane 3, and another faint band at 2000 Da is also present in this lane. No products were formed with the *cis*- or *trans*-ethylenediammine complexes treated at 37 °C or at 22 °C even after 21 h.

at pH 4.5 or 9. Protein cleavage proceeded smoothly when Tris–HCl buffer was replaced by acetate or phosphate.

The peptide fragments from the gels were isolated and their molecular weights have been determined from electrospray ionization mass spectrometry. The larger fragment had a molecular weight of 11998 Da while the smaller fragment had a molecular weight of 2343 Da. N-terminal sequencing by Edman degradation method revealed that the 12 kDa fragment had the sequence KVFGRCELAAAM, the known N-terminal sequence of lysozyme (Scheme 2). The N-terminus of the smaller fragment had the sequence, WRNRCK, a sequence internal to lysozyme. The cleavage thus occurs between Ala 110 and Trp 111, as inferred from the known sequence of lysozyme.[13] A minor product (< 3%) of molecular weight 3 kDa had the N-terminal sequence KVFGRCELAAAM, suggesting cleavage of lysozyme at a secondary site, in addition to the major cleavage site described above.



Scheme 2 Lysozyme sequence that has been targeted by the metal complex at neutral pH.

Catalytic activity of lysozyme was examined before and after the cleavage by CoTA, using glycol chitin as the substrate.[5b] The enzyme activity was unaffected by the protein cleavage reaction, and this observation is consistent with the location of the active site in the 12 kDa fragment. The major fragment thus retains the active site with little or no damage to the key residues involved in the enzyme catalysis. Preliminary ^{1}H NMR studies of lysozyme–CoPA solutions (2.9 mM lysozyme, 18 mM CoPA) indicate a number of shifts when compared to those of the protein (3 mM) in the absence of the metal complex. Further studies are needed to assign these changes but the resonances due to Trp 108 appears to have undergone significant changes. The known three-dimensional structure of lysozyme[13] indicates that Trp 108 is accessible to the aqueous medium and Trp108 could anchor the Co(III) reagent. Such coordination could position the metal complex proximate to the Ala 110 and Trp 111 peptide bond where the cleavage was observed.

The mechanism for protein cleavage by Co(III) is expected to be similar to that reported for amide hydrolysis by CoTA and CoPA.[15,16] The amide hydrolysis rates were higher for complexes with two open coordination sites.[15] Water molecules bound to the metal ion may participate in acid–base catalysis at the metal binding site, facilitating peptide bond cleavage. Size is also important as larger size may preclude the metal complex from reaching the peptide back bone (ethylenediamine complexes are not active). Current results demonstrate that Co(III) complexes can cleave internal peptide bonds, possibly *via* a hydrolytic mechanism. Chemical proteases derived from inorganic materials may prove to be useful as tools in molecular biology.

Financial support from the University of Connecticut Research Foundation, ACS Petroleum Research Fund and the Naional Science Foundation is gratefully acknowledged. We are grateful to Mary Moyer and William Burkhart of Glaxo Wellcome Inc., for the sequencing and mass spectral data.

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Communication a907477e