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Use of a molybdenum(VI) complex as artificial protease in protein photocleavage

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Use of a molybdenum(VI) complex as artificial protease in protein photocleavage

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ABSTRACT

In this study, a molybdenum(VI) peroxo α-amino acid complex, $MoO(O_2)_2$(α-leucine) (H_2O), was prepared and used as an artificial protease for site-specific cleavage of porcine pepsin, a model protein. Cleavage of pepsin by $MoO(O_2)_2$(α-leucine) (H_2O) was achieved under photochemical conditions at room temperature and pH 7.0. The reaction was activated by irradiation of the $MoO(O_2)_2$(α-leucine) (H_2O)-protein mixture by UV light (320 and 340 nm) for up to 30 min. No cleavage was observed in the absence of $MoO(O_2)_2$(α-leucine) (H_2O) or the light. The photocleavage yield increased with irradiation time. The cleaved fragments were sequencable, and the cleavage site was assigned to Leu(112)–Tyr(113). The cleavage reaction was quenched by ethanol. Therefore, hydroxyl radicals may be involved in the reaction and responsible for the cleavage of the protein. This is the first demonstration of the successful photocleavage of proteins by a molybdenum complex. This observation can provide a new approach for the photochemical footprinting of metal binding sites on proteins.

1. Introduction

The development of chemical reagents to cleave proteins with a high specificity is of interest in this study since uncatalyzed hydrolysis of peptides is extremely slow. At room temperature and neutral pH, the peptide bond is unreactive towards hydrolysis, with the half-life of 350–600 years [1]. Reagents that can cleave the protein backbone as chemical proteases with a high specificity can be useful for converting large proteins into smaller fragments, understanding biomolecular recognition with small ligands [2,3], and design of new therapeutic agents [4,5]. Many protein cleaving reagents have been developed to induce protein cleavage at selected sites. The design of such reagents should include appropriate recognition elements for binding to the target site with high affinity and reactive groups which can be activated to produce the desired cleavage chemistry with high selectivity.

The reactions can be activated using heat (thermal reaction) or light (photoreaction). Attempts were made to design new reagents for protein scission [6–8]. However, the studies for protein photocleavage have not been widely investigated. Using light to induce protein cleavage is one way to activate the chemistry with many advantages. The reaction can be initiated, sustained, or terminated conveniently, and light can be a nontoxic, green component of the reaction mixture. In our laboratories, designed small organic molecules have been developed for photocleavage of proteins [9]. Pyrenyl chromophores linked to short peptides or to specific substrates have been developed and protein photocleavage at the probe binding site can be achieved. However, pyrenyl excited states itself cannot cleave proteins. An electron acceptor is required to quench the pyrenyl excited states to generate radical intermediates, which are responsible for the cleavage of the peptide backbone. Therefore, development of new cleaving reagents without the need of an electron acceptor is the aim of this study.

Metal complexes were directed to specific cleavage of proteins by using affinity ligands. The achievement of peptide bond cleavage was accomplished *via* oxidative or hydrolytic methods [10–21]. These studies could be helpful in elucidating the role of metal ions in natural hydrolases, even though, the precise role of the metal ion in the hydrolysis reactions is still not clear. To date, only a handful of transition metal complexes have been known to have the ability to photocleave proteins. Photocleavage of lysozyme, for example, by Co(III) complexes has been reported [22]. Two photocleaved fragments were achieved upon irradiation of lysozyme-pentammineaquocobalt(III) complex at 310, 340, or 370 nm, at room temperature and pH 7.0. Searching for new molecules to expand this repertoire is very challenging.

The use of molybdenum complexes as artificial proteases under thermal conditions has been recently reported [23,24]. Here, the ability of a molybdenum complex to selectively cleave a protein, under photochemical conditions, is reported for the first time. Molybdenum is one of the important metals found in metalloenzymes, and it is the only 4d element with a biological function [25]. However, photocleavage of proteins by molybdenum

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complexes has not been reported. A molybdenum(VI) peroxo α-amino acid complex were synthesized by attaching an amino acid (leucine) to molybdenum trioxide (MoO_3) (Scheme 1). Porcine pepsin, a zymogen-derived protein, was chosen for the current study as a model protein since the complete amino-acid sequence of porcine pepsin and its crystal structure are known [26–30]. This new observation may provide the rational design of transition metal complexes for the footprinting of metal binding sites on proteins and in addition facilitate in the development of more efficient artificial proteases.

2. Materials and methods

All materials were reagent grade and were used without further purification unless otherwise noted. Porcine pepsin (Mol. Wt. = 34,623) was purchased from Sigma Chemical Co. MoO_3, H_2O_2 and leucine were purchased from Sigma Chemical Co. All solutions were freshly prepared in 50 mM Tris–HCl buffer, pH 7.0 unless noted otherwise.

2.1. Synthesis of $MoO(O_2)_2$(α-amino acid) (H_2O)

$MoO(O_2)_2$(α-leucine) (H_2O) was synthesized by following the previously reported method [31]. MoO_3 (2.85 g) was dissolved in 30% H_2O_2 (10 mL) with stirring at 30 °C for 24 h. Leucine (2.70 g) was gradually added to the previous solution. The solution was stirred at 30 °C for 24 h. The yellowish precipitate was obtained (5.51 g; 85.03% yield). The product was identified using UV–Vis (λ_{max} 350 nM), IR (Nujol) 980.26 cm^{-1} (Mo═O), 917.25 cm^{-1} (O—O), 630.16 cm^{-1} (Mo—O(O_2)), 531.35 (Mo—O(O_2)), ^{1}H NMR (400 MHz, d6-DMSO): 3.82 ppm (1H), 2.67 ppm (2H), 1.98 ppm (1H), 0.98 ppm (6H).

2.2. Protein cleavage conditions

The protein cleavage was carried out at room temperature. The protein solution (15 μM) was treated with $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM) in 50 mM Tris–HCl buffer, pH 7.0 (total volume 200 μL), and the reaction mixtures were irradiated at 320 and 340 nm for 10–30 min with 150 W xenon lamp, using a grating monochromator to generate the light at selected wavelengths. Dark control sample was prepared under the same conditions, as described above, except that the solution was protected from light, and left at room temperature for the same reaction time as that of the irradiated reaction mixtures. All reaction mixtures were lyophilized (freeze-drying) until dryness.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein samples were electrophoresed following the literature procedure with minor modifications [32]. The dried protein samples were redissolved in loading buffer (24 μL) (containing SDS (7% w/v), glycerol (13% v/v), Tris–HCl (50 mM, pH 6.8), mercaptoethanol (2% v/v) and bromophenol blue (0.01% w/v)). Protein solutions in loading buffer were heated for 3 min, and then the samples (8 μL) were loaded on the gel. The gels (10%) were run by applying 60 V until the dye passed through the stacking gel. The voltage was then increased to 110 V, as described in the previous report [9]. The gels were run for 1.5 h, stained with Coomassie brilliant blue, and destained in acetic acid solution (10%). The migration distance of molecular weight markers showed a logarithmic relationship to molecular weight. Therefore, band centers were used to assign approximate molecular weights using the molecular weight markers for calibration of each gel.

The gels were scanned with a Hewlett–Packard scanner. The images of the fragment bands were quantitated using ImageJ software (v 1.46r), and the photoproduct yields were calculated with respect to the unreacted protein bands. The ratio of the cleaved product band intensities to the sum of the intensities of the products and the starting material in each lane was calculated for the yields. Light intensities at specific wavelengths (320 and 340 nm) were determined using a ferrioxalate actinometer [33] which was carried out in the dark room. The actinometer solution was prepared by dissolving potassium ferrioxalate [$K_3Fe(C_2O_4)_3.3H_2O$, (0.59 g)] in sulfuric acid (0.05 M, 100 mL). Two sets of the diluted solutions (2, 4, 6, 8 and 10 mM) were prepared. The first set of diluted actinometer solutions (3 mL) was irradiated at 320 nm for 10 min, while the other set was irradiated at 340 nm for 10 min. At the end, the irradiated actinometer solutions were mixed with 0.1% buffered phenanthroline (0.5 mL), and concentration of the ferrous ion was estimated from absorbance at 510 nm.

2.4. Peptide transfer and amino acid sequencing

The separated peptide fragments on SDS–polyacrylamide gel were transferred to PVDF membrane with a current of 140 mA for 1 h using the semi-dry system (BIORAD) with CAPS buffer, pH 10.5. The transferred protein fragments on PVDF membrane were stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue R-250 in 40% methanol and 1% acetic acid). The desired bands were cut and sent for N-terminal amino acid composition analysis (Midwest Analytical, Inc., MO, USA). Chemical sequencing was performed on an automated protein sequencer. Five cycles were performed to identify the N-terminus of the cleaved fragments.

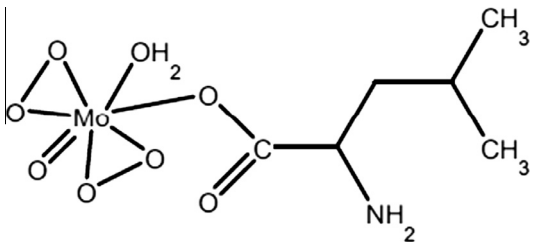
2.5. Quenching of protein cleavage reaction by ethanol

The participation of hydroxyl radical intermediate in the cleavage reaction is tested in quenching studies with ethanol. Ethanol (0.5 mM) was added to the reaction mixture (pepsin + $MoO(O_2)_2$(α-leucine) (H_2O)), and the solution was irradiated at 340 nm for 20 min. The reaction mixture was dried, and the protein sample was electrophoresed following the above procedure.

3. Results and discussion

3.1. Cleavage of pepsin

Pepsin was successfully cleaved by $MoO(O_2)_2$(α-leucine) (H_2O), under activation by light, at mild conditions (room temperature, pH 7.0). The protein cleavage was monitored in gel electrophoresis experiments under denaturing conditions. Irradiation of pepsin (15 μM) in the presence of $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM) at 320 nm and 340 nm for 10, 20 and 30 min resulted in cleavage of the protein as demonstrated in SDS–PAGE experiments (Fig. 1). The cleaved pepsin resulted in at least three fragments (I, II and III) with the molecular weights of approximately 25, 20



Scheme 1. The structure of molybdenum(VI) peroxo α-amino acid complex, ($MoO(O_2)_2$(α-leucine) (H_2O)).

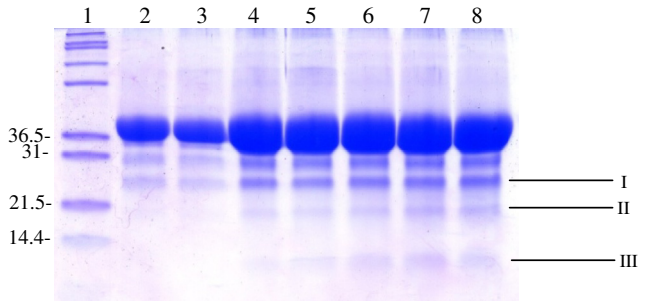


Fig. 1. SDS–PAGE of the photocleaved products of pepsin by $MoO(O_2)_2$(α-leucine) (H_2O). Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2–8 contained pepsin (15 μM) and $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM). Lane 2 was the dark control. Samples in lanes 3–5 were irradiated at 320 nm for 10, 20, and 30 min, respectively while samples in lanes 6–8 were irradiated at 340 nm for 10, 20, and 30 min, respectively.

and 12 kDa, respectively (lanes 4–8). However, irradiation of the protein-$MoO(O_2)_2$(α-leucine) (H_2O) mixture at 320 nm for 10–30 min gave faint bands of the cleaved fragments (lanes 3–5, respectively), compared to the results obtained at 340 nm (lanes 6–8). No cleavage was observed in the absence of $MoO(O_2)_2$(α-leucine) (H_2O) (data not shown), while the weak but detectable cleavage band (band I) was observed even the absence of the light (dark control, lane 2). Self cleavage of pepsin was not observed at room temperature, indicating that the weak band (band I) could be the reaction mediated by the metal complex. However, leaving the mixture (the dark control) for longer reaction times at room temperature (without shining the light) did not result bands II and III.

The backbone cleavage of pepsin by the $MoO(O_2)_2$(α-leucine) (H_2O) showed the expected dependence on reaction time. Analysis of the cleavage data with pepsin clearly indicated increased yields of the fragments with reaction time (Fig. 2). The highest yield (14.3%) was obtained when irradiating the samples at 340 nm for 30 min. The yields of the cleavage fragments from pepsin were followed as a function of molybdenum complex concentration (0.125–2.00 mM) (Fig. 3). The product yield increases with concentration as expect, suggesting that the metal complex is activated by light and responsible for the cleavage reaction. However, at concentration higher than 1.00 mM, the product yield increases only marginally.

Although the complex absorbs more light at 320 nm compared to 340 nm, the spectrum is not a sharp absorption band (see Supplementary Information). However, from the actinometry experiment, the light intensity at 340 nm is higher than that at 320 nm by ~31%. Therefore, the observed higher yields at 340 nm could be due to the higher light intensity of this wavelength or this might be due to the coordination sphere of the molybdenum atom in the

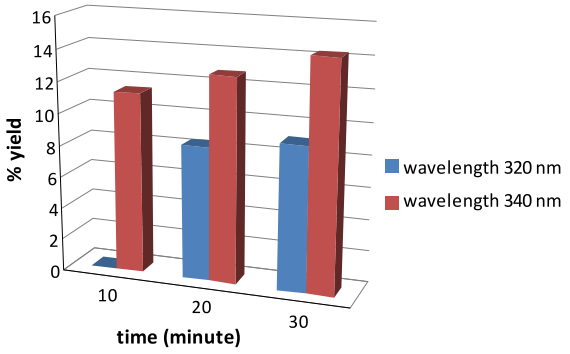


Fig. 2. Plot of % photoproduct yields vs. time of irradiation.

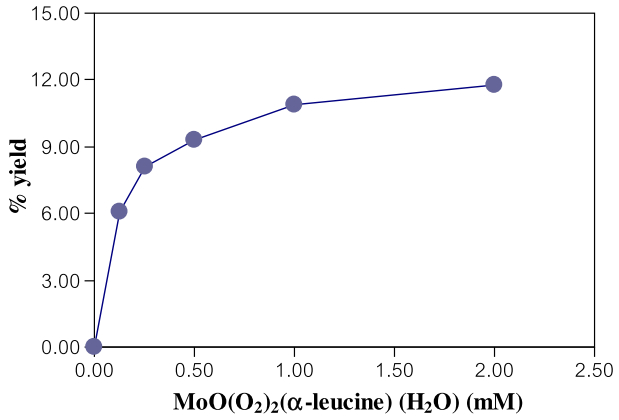


Fig. 3. Plot of cleavage yield vs. $MoO(O_2)_2$(α-leucine) (H_2O) concentration. The mixtures of pepsin (15 μM) and $MoO(O_2)_2$(α-leucine) (H_2O) (0.000, 0.125, 0.250, 0.500, 1.000 and 2.000 mM) were irradiated at 340 nm for 10 min.

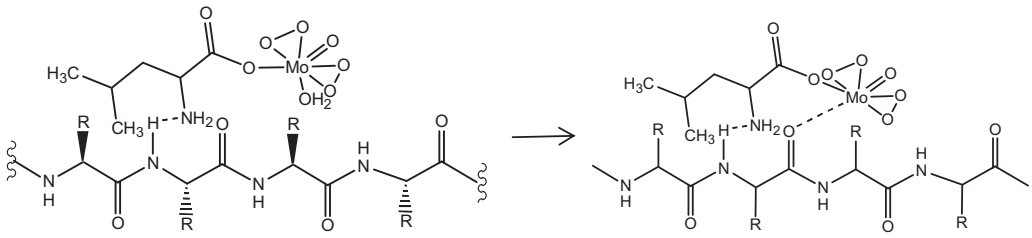
complex changed from water to oxygen atom in amino group or any atom in substituent of amino acid, during binding to the protein, resulting in red shift of the absorption band (Scheme 2). To elaborate this result, more investigation on the binding of this complex and pepsin are needed in the future studies.

3.2. Amino acid sequencing and cleavage sites determination

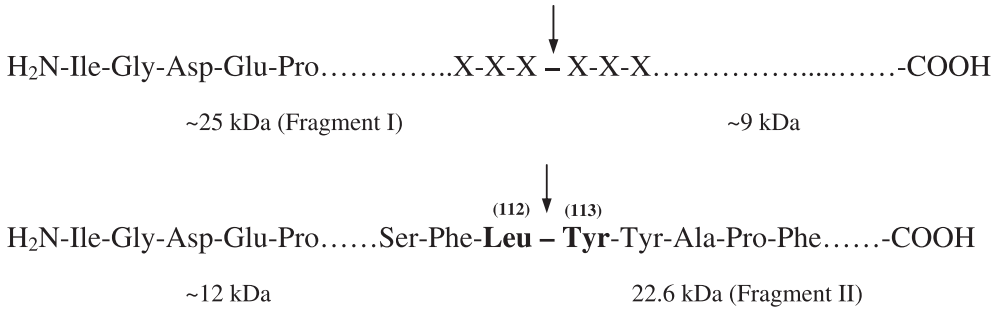
The peptide fragments from the gels were isolated and subjected to amino acid sequencing to examine the location of cleavage site on pepsin. N-terminal sequencing of fragment I (~25 kDa) indicated the sequence IGDEP which corresponds to the known N-terminal sequence of native pepsin. N-terminal sequencing of fragment II (~20 kDa) showed an amino acid sequence YYAPF. The sequence of YYAPF indicated the cleavage site at Leu 112–Tyr 113. The protein band of fragment III (~12 kDa) was very faint, and the observed N-terminal sequencing data were not clear. This might be due to the insufficient amount of the blotted protein for the sequence analysis, or the fragment may not be amenable to sequencing. The cleavage pattern can be concluded as shown in Scheme 3.

From the cleavage results, we can conclude that three observed cleaved fragments may arise from two cleavage sites, with a small amount of another fragment (~9 kDa) that is not distinguishable in the gels. However, only one cleavage site was clearly concluded. As seen from Scheme 3, the cleavage between Leu 112 and Tyr 113 gives another sequence (N-terminal sequence) with molecular weight of ~12 kDa, which could be the observed fragment III on the gels. The structure of the binding site residues on the protein is expected to account for specific binding of the metal complex to the protein. By looking down the three-dimensional structure of pepsin backbone, the major cleavage site of pepsin was quite expose to the aqueous media and, therefore, accessible to the ligand to bind at this active site. However, such binding is expected to be sensitive to the three-dimensional structure of the molybdenum complex as well. Substitute leucine in the probe structure with glycine did not result in the cleavage of pepsin in the same reaction conditions.

It is noteworthy in this study that $MoO(O_2)_2$(α-leucine) (H_2O) specifically cleaves pepsin under photochemical conditions. The used wavelengths are far away from the absorption bands of aromatic residues on the protein. Therefore, the molybdenum complex is directly responsible for the photocleavage of pepsin. The ligand-to-metal charge transfer (LMCT) process could be involved in the cleavage reaction. Transient species, such as radical



Scheme 2. Possible mechanism for the binding of $MoO(O_2)_2$(α-leucine) (H_2O) to pepsin. Hydrogen bonding between NH_2 in the metal complex with one residue of the protein may be the initial step in the binding reaction.



Scheme 3. Photocleavage sites of pepsin.

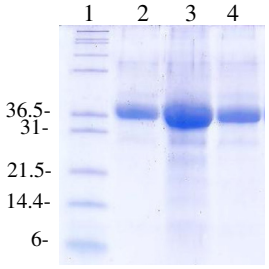


Fig. 4. Quenching of photocleavage of pepsin by ethanol. Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2–3 contained pepsin (15 μM) and $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM). Lane 4 contained pepsin (15 μM), $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM) and ethanol (0.5 mM). Lane 2 was the dark control, while samples in lanes 3–4 were irradiated at 340 nm for 20 min.

intermediates, generated from excitation of the metal complex, may be involved in the photocleavage reaction.

3.3. Quenching of protein cleavage reaction by ethanol

Exposure of peptides to hydroxyl radical is known to effect backbone cleavage, although the mechanism is still not fully elucidated. The participation of hydroxyl radical intermediate in the cleavage is tested in quenching studies with ethanol. Ethanol quenches hydroxyl radicals at diffusion controlled rates, and reacts with carbon centered radicals at much slower rates [34]. Ethanol (0.5 mM) was added to the pepsin/$MoO(O_2)_2$(α-leucine) (H_2O) mixture, and the mixture was irradiated at 340 nm for 20 min. The cleavage of pepsin was quenched by ethanol (lane 4), as shown in Fig. 4.

4. Conclusion

The data in the current studies clearly show that $MoO(O_2)_2$(α-leucine) (H_2O) successfully cleaves pepsin at specific sites under photochemical conditions. Cleavage specificity is expected to occur due to specific binding of the metal complex to the selective sites on the protein. The activation of the metal complex at 340 nm results in higher yield of protein cleavage compared to the yield obtained at 320 nm. From the above results, hydroxyl radicals may be responsible for the cleavage of the protein, as indicated by the decrease of cleavage yields in the presence of ethanol. Hydroxyl radicals may be generated at or near the cleavage sites. However, more details in the mechanism for photocleavage of pepsin need more investigation. Even though the yields are small (~14%), but the photocleavage reaction has shown high selectivity. Furthermore, this is the first demonstration of protein photocleavage by a molybdenum complex without the need of adding other chemical reagents. This study will be useful for the footprinting of metal binding sites on specific proteins in the future.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotobiol.2013.07.004.

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