Protein Hydrolysis

International Edition: DOI: 10.1002/anie.201502006
German Edition: DOI: 10.1002/ange.201502006

Highly Amino Acid Selective Hydrolysis of Myoglobin at Aspartate Residues as Promoted by Zirconium(IV)-Substituted Polyoxometalates**

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Abstract: *SDS-PAGE/Edman degradation and HPLC MS/MS showed that zirconium(IV)-substituted Lindqvist-, Keggin-, and Wells–Dawson-type polyoxometalates (POMs) selectively hydrolyze the protein myoglobin at Asp–X peptide bonds under mildly acidic and neutral conditions. This transformation is the first example of highly sequence selective protein hydrolysis by POMs, a novel class of protein-hydrolyzing agents. The selectivity is directed by Asp residues located on the surface of the protein and is further assisted by electrostatic interactions between the negatively charged POMs and positively charged surface patches in the vicinity of the cleavage site.*

Selective hydrolytic cleavage of the peptide bond in proteins is one of the most important procedures in analytical biochemistry and biotechnology applications. It is frequently used for protein structure/function/folding analysis, protein engineering, and the design of target-specific protein-cleaving drugs[1] but is still a challenging task. Commonly used natural proteases are expensive, operate only in a narrow temperature and pH-value range, often suffer from self-digestion, and often have limited selectivity. Therefore, new efficient and selective cleaving agents that are sufficiently active at nondenaturing pH values and temperatures are greatly needed.

The development of such reagents is not straightforward, as the peptide bond in peptides and proteins is extremely inert.[2] During the past decades it was shown that various metal ions, such as Pt^{II}, Pd^{II}, Cu^{II}, Co^{III}, Ni^{II}, Zn^{II}, Ce^{IV}, Hf^{IV}, Zr^{IV}, and their complexes, are able to hydrolyze peptide bonds in dipeptides, oligopeptides, and proteins.[1] Unfortunately, selectivity and reactivity under physiological conditions are difficult to achieve. For example, it was shown that despite being highly specific, Pd^{II} and Pt^{II} complexes are only hydrolytically active at very low pH values.[3] On the other hand, although they are active under physiological conditions, Co^{III} complexes only cleave N-terminal peptide bonds, thereby significantly limiting their use in protein applications.[4] Zr^{IV} complexes have higher reactivity, but tend to result in inhomogeneous reaction mixtures and do not show any specific selectivity.[5]

Interestingly, the incorporation of Zr^{IV} into polyoxometalate clusters results in metal-substituted polyoxometalates (MSPs), which have been shown to be effective homogeneous catalysts for a range of reactions, including the H_2O_2-based epoxidation of olefins,[6] oxygenation reactions of thioethers,[7] Mukaiyama aldol and Mannich-type addition reactions,[8] and the cyclization of citronellal.[9] MSPs are also a promising alternative in protein-hydrolysis experiments. We have recently shown that MSPs consisting of highly Lewis acidic metal ions, such as Ce^{IV} and Zr^{IV}, embedded into tungsten(VI) metal–oxygen frameworks act as catalysts for peptide-bond hydrolysis in oligopeptides and proteins, such as oxidized insulin chain B, human serum albumin (HSA), bovine serum albumin (BSA), and hen egg-white lysozyme (HEWL).[10] In the presence of a cerium(IV)-substituted Keggin-type MSP, HEWL was selectively hydrolyzed at only two sites, Trp28–Val29 and Asp44–Arg45.[10a] As both cleavage sites are characterized by a positively charged protein surface patch, it was postulated that electrostatic interactions with the negatively charged MSP skeleton led to the observed regioselectivity. Metal-directed binding between the embedded Ce^{IV} ion and the carboxylate side chain of negatively charged residues, such as Asp44, was also suggested, thereby opening the way to the development of MSPs as sequence-specific cleaving agents. Interestingly, the tendency of MSP to hydrolyze peptide bonds in the vicinity of amino acids with a carboxylate group in their side chain was further observed in HSA hydrolysis by a series of zirconium(IV)-substituted MSPs, as three out of four detected hydrolysis sites contained an Asp or Glu residue, all of which were located in the vicinity of a positively charged patch.[10b] From these studies it emerged that MSPs may possess remarkable selectivity towards the hydrolysis of peptide bonds linking amino acids with carboxylate groups in their side chain, but so far no direct proof for sequence selectivity has been presented.

Therefore, in this study, we set out to further examine the protease activity of a series of zirconium(IV)-substituted MSPs (Figure 1) towards a protein characterized by a high Asp and Glu content. Horse-heart myoglobin (HHM), a protein made up of 153 amino acids (16.951 kDa), including 8 Asp and 13 Glu residues (see Figure S1 in the Supporting Information), was selected as a suitable target in this study.

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[**] H.G.T.L. thanks the Vietnamese Government and KU Leuven for a doctoral fellowship. T.N.P.-V. and P.P. thank the FWO, KU Leuven and the Hercules Foundation for financial support. G.A. thanks the FWO for a postdoctoral fellowship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201502006.

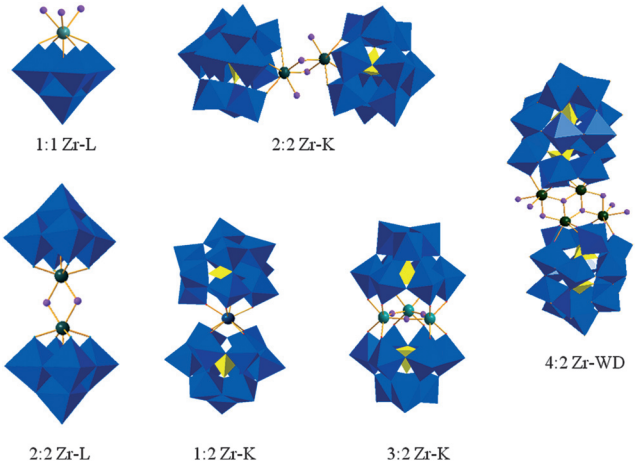

Figure 1. Chemical structure of 1:1 Zr^{IV}–Lindqvist $(Me_4N)_2[W_5O_{18}Zr(H_2O)_3]$ (1:1 Zr-L), 2:2 Zr^{IV}–Lindqvist $(nBu_4N)_6[\{W_5O_{18}Zr(\mu\text{-}OH)\}_2]\cdot 2H_2O$ (2:2 Zr-L), 1:2 Zr^{IV}–Keggin $(Et_2NH_2)_{10}[Zr(PW_{11}O_{39})_2]\cdot 7H_2O$ (1:2 Zr-K), 2:2 Zr^{IV}–Keggin $(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr\text{-}(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ (2:2 Zr-K), 3:2 Zr^{IV}–Keggin $(Et_2NH_2)_7H_2[Zr_3(\mu\text{-}OH)_3(A\text{-}\alpha\text{-}PW_9O_{34})_2]\cdot 12H_2O$ (3:2 Zr-K), and 4:2 Zr^{IV}–Wells–Dawson $Na_{14}[Zr_4(P_2W_{16}O_{59})_2(\mu_3\text{-}O)_2(OH)_2(H_2O)_4]\cdot 57H_2O$ (4:2 Zr-WD).

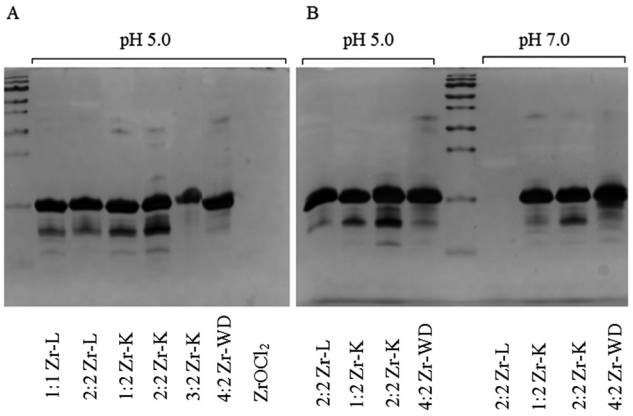

Figure 2. Silver-stained SDS-PAGE gels showing the results of HHM hydrolysis after 3 days at 60 °C. A) Hydrolysis at pH 5.0. B) The results of experiments performed at pH 7.0 are shown and compared in the same gel to those at pH 5.0. As a result of precipitation in the presence of the 2:2 Zr-L POM, no intact HHM was observed at pH 7.0.

Owing to its well-defined structure, this protein also enabled us to study the influence of the local environment on the selectivity of hydrolysis. HHM was previously hydrolyzed in the presence of $CuCl_2$ or a copper(II)–cyclen complex at the two peptide bonds Gln91–Ser92 and Ala94–Thr95;[11] in contrast, 13 cleavage sites adjacent to Met, Arg, and His residues were observed in the presence of *cis*-$[Pd(dtco\text{-}OH)(H_2O)_2]^{2+}$.[12] Whereas the first procedure required a lengthy synthetic route and the attachment of the Cu^{II} catalyst to cross-linked polystyrene, the second method resulted in a large number of cleavage sites at different residues.

In this study, a 0.02 mM solution of HHM was simply incubated with zirconium(IV)-substituted POMs (2.0 mM) in 10.0 mM acetate buffer (pH 5.0) at 60 °C. Subsequently, reaction aliquots were taken at different time intervals and analyzed by SDS-PAGE to evaluate the progressive hydrolysis of HHM in the presence of each POM. Figure 2 A shows the SDS-PAGE results after HHM hydrolysis for 3 days. The appearance of new bands during the course of the reaction indicates that HHM hydrolysis occurred, and quantitative analysis showed that the 2:2 Zr-K POM was the most active. Approximately 40 and 30 % of HHM was hydrolyzed by the 2:2 Zr-K and 1:2 Zr-K POM, respectively, whereas 25 % hydrolysis was observed in the presence of 1:1 Zr-L or 2:2 Zr-L. Only minor amounts of hydrolysis were observed when 4:2 Zr-WD (10 %) or 3:2 Zr-K (5 %) were present. However, despite a difference in reactivity, the bands appeared at the same position in the SDS-PAGE gel, thus indicating the presence of fragments with the same molecular weight. In all cases, no additional fragments were observed after longer reaction times of up to 9 days (see Figure S2). Interestingly, the same bands were observed in SDS-PAGE gels for samples with MSP/HHM ratios of 1:1 and 100:1, thus indicating that the selectivity does not depend on the POM concentration (see Figure S3). Control experiments, in which HHM was incubated with the salt $ZrOCl_2$ or $ZrCl_4$ (pH 5.0, 60 °C), were also performed (see Figure S4). In both reaction mixtures, gel formation was observed, thus resulting in heterogeneous reaction conditions and preventing the observation of the SDS-PAGE band of intact HHM. Moreover, in the absence of a POM or in the presence of the lacunary Keggin POM $Na_9[A\text{-}\alpha\text{-}PW_{11}O_{34}]$, which lacks Zr^{IV}, no hydrolysis of HHM was observed after 1 week (see Figure S4). These findings indicate that the POM framework stabilizes Zr^{IV} in aqueous solution and that both Zr^{IV} and the POM are necessary to facilitate HHM hydrolysis. The hydrolysis of HHM in the presence of the POM $(Me_2NH_2)_{10}[Ce^{IV}(\alpha\text{-}PW_{11}O_{39})_2]\cdot 14H_2O$ (1:2 Ce^{IV}-K) was also studied (see Figure S5). This POM was less active than the 1:2 Zr-K species, despite the similarity in structure and in the Lewis acidity of Zr^{IV} and Ce^{IV}. ^{31}P NMR spectra of 1:2 Zr-K and 1:2 Ce^{IV}-K in the absence and presence of HHM were recorded (see Figures S6 and S7). The results showed that whereas 1:2 Zr-K was stable in the presence of HHM, approximately 30 % of 1:2 Ce^{IV}-K was reduced to 1:2 Ce^{III}-K after 3 days, thus leading to the loss of its reactivity. The partial reduction of Ce^{IV} to Ce^{III} was previously also observed in the Ce^{IV}-K-promoted hydrolysis of HEWL.[10a]

To evaluate the reactivity of zirconium(IV)-substituted POMs towards HHM hydrolysis under physiological conditions, we also tested their hydrolytic activity at pH 7.0 (10.0 mM phosphate buffer). Despite somewhat lower reactivity as compared to that at pH 5.0, the POMs under study were also active near physiological pH conditions; again, 2:2 Zr-K was the most active POM (Figure 2 B). This result is in good agreement with our previous studies with dipeptides and oligopeptides, in which 2:2 Zr-K displayed superior reactivity as compared to other MSPs.[13] Experimental and theoretical studies indicate that 2:2 Zr-K partly dissociates into the catalytically active 1:1 species, in which Zr^{IV} has multiple free coordination sites at which to bind and activate the substrate.[14]

^{1}H NMR spectra of HHM indicated that HHM preserves its folded three-dimensional structure in the presence of the POM 2:2 Zr-K (see Figure S8). ^{31}P NMR spectra showed that under the hydrolysis conditions, slight conversion of 2:2 Zr-K into 1:2 Zr-K occurred after prolonged reaction times. This equilibrium was not caused by the presence of HHM, as similar spectra were observed in its absence (see Figure S9).

Owing to the superior reactivity of 2:2 Zr-K, HHM hydrolysis in the presence of this POM was studied further in detail. Reaction aliquots were taken after 2, 3, 4, 6, 7, and 9 days and analyzed by SDS-PAGE (see Figure S2). The rate constant for HHM hydrolysis (2.15×10^{-5} h^{-1}) was calculated by fitting the decrease in the intensity of the electrophoretic bands corresponding to HHM (see Figure S10). After 2 days at 60 °C, all hydrolysis fragments could be observed, and the band intensity further increased as the incubation time increased (see Figure S2). To identify the cleavage sites of HHM, we blotted the peptide fragments separated on an SDS-PAGE gel to a polyvinylidene fluoride (PVDF) membrane (see Figure S11) and determined the NH_2-terminal sequence of the individual Coomassie-stained fragments by Edman degradation. A combination of Edman degradation with LC–MS and LC–MS/MS techniques (see Table S1 and Figure S12) unambiguously confirmed that HHM was hydrolyzed at six peptide-bond positions: Asp4–Gly5, Asp20–Ile21, Asp44–Lys45, Asp60–Leu61, Asp126–Ala127, and Asp141–Ile142, thus resulting in fragments visible on the gel with molecular masses of 16579, 14738, 13625, 12049, 11785, and 10175 Da (see Figure S11).

Remarkably, all hydrolyzed peptide bonds in HHM were found to be upstream from the aspartate residue. The exclusive hydrolysis at Asp–X type peptide bonds indicates that 2:2 Zr-K acts as a site-selective hydrolysis agent. Interestingly, although HHM contains eight Asp residues, hydrolysis was only observed at six of these residues. This result can be explained by taking the protein-surface charge distribution at the cleavage sites into account (see Figure S13). Each of the hydrolyzed peptide bonds is located in the near vicinity of a positively charged surface patch that can electrostatically interact with the negatively charged POM surface (Figure 3), whereas the Asp109–Ala110 bond, which did not undergo hydrolysis, has no such surface characteristics that promote docking of the MSP (see Figure S14). The absence of hydrolysis at the Asp122–Phe123 bond can be explained by the fact that the width of the positively charged cavity between Asp122 and Asp126 is about 10 Å (see Figure S15), which would fit only one Keggin POM.[15] Therefore, the observed Asp126–Ala127 bond hydrolysis probably results from a correct orientation of Zr^{IV} towards this bond, and this interaction cannot simultaneously occur with the Asp122–Phe123 peptide bond.

Accelerated hydrolysis of peptides containing Asp residues, such as Val-Tyr-Pro-Asp-Gly-Ala, was also previously observed at Asp–X bonds and was attributed to the presence of a carboxyl group in the side chain that assists hydrolysis by intramolecular nucleophilic attack on the amide carbonyl carbon atom to form a cyclic anhydride intermediate under mildly acidic solutions.[16] Interestingly, although 13 Glu residues are present in HHM, no hydrolysis was observed at peptide bonds with these amino acids. The less preferential hydrolysis of the Glu–X bond as compared to an Asp–X bond has previously been observed in large peptide drugs and has been attributed to the formation of a less stable six-membered-ring intermediate as opposed to a more thermodynamically favorable five-membered ring in the case of Asp-X.[17]

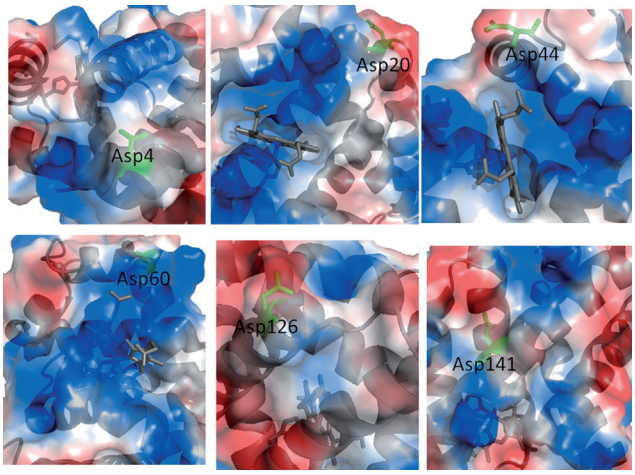

Figure 3. 3D structure and surface charge distribution. Negatively charged surfaces areas are shown in red, and positively charged surface areas are shown in blue. Asp residues in hydrolyzable Asp–X bonds are highlighted in green.

The different reactivity of Zr^{IV}-POMs towards HHM observed in this study is most likely not related to their charge distribution, as no clear correlation was observed between their charge and reactivity (see Table S2). Moreover, an increase in the number of hydrolytically active Zr^{IV} ions in the structure did not result in an increase in reactivity. Therefore, the most likely reason for different reactivity lies in different tendencies of Zr^{IV}-POMs to dissociate in solution and undergo monomer/dimer equilibration. The 3:2 Zr-K and 4:2 Zr-WD POMs are particularly resistant towards dissociation into monomers and preserve their structure over a broad range of pH values, concentrations, time, and temperatures (see Figures S16 and S17). We have previously shown that 1:2 Zr-K is also not very prone to dissociation, although the presence of a protein could influence the monomer/dimer equilibrium.[10a] The large dimeric structure of these POMs most likely hinders the effective interaction of Zr^{IV} with Asp–X peptide bonds, thus resulting in lower reactivity. However, we recently detected a monomer/dimer equilibrium in the case of 2:2 Zr-K, and have proven the existence of a monomeric 1:1 form both by experimental and theoretical approaches.[14] The monomeric 1:1 form is smaller and has more coordination sites available for interaction with HHM; these features could explain our observation of the highest reactivity for 2:2 Zr-K.

In conclusion, the simple incubation of HHM with 2:2 Zr-K resulted in remarkable selectivity towards the hydrolysis of Asp–X bonds in HHM. Previous studies on Zr^{IV}-POM-promoted protein hydrolysis indirectly indicated the importance of amino acid residues with carboxyl side chains for selectivity, and this study unambiguously showed that HHM

was selectively hydrolyzed only at Asp residues located in the vicinity of positively charged protein patches. The Asp residues may play a dual role in inducing the selectivity. The carboxyl group in the Asp side chain may help in the anchoring of the MSP catalyst to the protein through Zr^{IV} coordination, or the carboxyl group could assist hydrolysis by nucleophilic attack to form a tetrahedral intermediate. Given the current lack of efficient and selective cleaving agents for emerging biochemical applications, site-selective MSPs could be interesting alternatives as artificial proteases that are water-soluble, nonvolatile, and easy to handle. Moreover, their synthesis can be scaled up readily, and their use does not result in undesired side reactions. As the cleavage is purely hydrolytic and occurs under mildly acidic or physiological pH conditions, protein fragments are left in pristine condition for further biochemical condensation.

Keywords: homogeneous catalysis · horse-heart myoglobin · hydrolysis · metalloproteases · polyoxometalates

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 7391 – 7394
Angew. Chem. **2015**, *127*, 7499 – 7502

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Received: March 3, 2015
Revised: April 17, 2015
Published online: May 7, 2015

