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Mild and Chemoselective Peptide-Bond Cleavage of Peptides and Proteins at Azido Homoalanine**

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The chemical cleavage of amide (peptide) bonds usually requires harsh conditions.[1] As a result, side reactions and the lack of specificity of chemical amide-bond hydrolysis limits its scope in chemical biology and synthetic applications. Herein, we disclose our results on the selective cleavage of amide bonds in peptides and proteins that is milder than any previously reported chemical method.

The azide functional group is well suited for the in vivo labeling of biomolecules.[2] Azides combine a high chemical stability under physiological conditions with a unique reactivity that enables mild and selective organic transformations, such as the Staudinger ligation[3] and the azide–alkyne [3+2] cycloaddition.[4] Azido-functionalized amino acids[5] and azido sugars[4e] have been introduced into biomacromolecules and have subsequently been derivatized with specific labels to enable their detection, thereby demonstrating full bioorthogonality.[2] It has been shown that azido homoalanine (**1**) is effectively incorporated into proteins by the native methionyl tRNA synthetase of *E. coli*, thus allowing specific modification reactions.[5a] Azides, however, are susceptible to reduc-

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tion to amines with, for example, phosphines[3,6] and (di)thiols,[7] which are amongst the most widespread reagents to promote reduction in samples of biological origin, or that are present intracellularly (e.g., in the form of glutathione).

In our investigations into Cu^{I}-catalyzed [3+2] "click" cycloadditions of several different alkynes to peptides containing **1**,[4a–c] with the in situ reduction of Cu^{II} to Cu^{I}, we discovered that aside from the expected products smaller fragments were produced. Control experiments in which Cu^{II} or the alkyne moiety, or both, were omitted and a reducing agent was added showed a marked increase in peptidolysis, and so were chosen to investigate the underlying mechanism.

Two products were formed when the model octadecapeptide **P** (sequence: PPHHHHHHPPRGFG**1**GFR, synthesized by standard Fmoc chemistry) was incubated in buffers containing either tris(carboxyethyl)phosphine (TCEP), 2-mercaptoethanol (2ME), or dithiothreitol (DTT). As expected, a peak corresponding to the reduction of **1** to 2,4-diaminobutyrate (DAB) at *m*/*z* 2108.0 was observed under these conditions. Surprisingly, an additional product at *m*/*z* 1729.8 was observed. Low-energy collision-induced dissociation (CID) of this product in an ESI-Q-FT mass spectrometer revealed an N-terminal fragment of **P** that resulted from cleavage of the peptide bond C-terminal to element **1** together with the loss of the azide group.

To test the general occurrence of this cleavage reaction in proteins that contain **1** instead of methionine,[5a] we have produced His-tagged recombinant photoactive yellow protein (PYP) from *Ectothiorhodospira halophila* in methionine-auxotrophic *E. coli* grown on media containing **1**. A pure preparation of azido PYP (AzPYP), in which all six methionine residues have been replaced by **1**, was obtained, as detected by ESI mass-spectrometric analysis of the intact protein (see the Supporting Information) and MALDI-TOF mass-spectrometric analysis of a tryptic digest (data not shown). Reduction and cleavage of purified AzPYP by TCEP, 2ME, or DTT was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (Figure 1 a, b). It was found that TCEP was able to cleave the protein under all the conditions investigated, whereas both the rate and yield of the cleavage with DTT and 2ME increased drastically under denaturing conditions (4 M urea). Fragments N- or C-terminal to all positions of **1** in reductively treated AzPYP were detected (Figure 1 c).

All fragments, except for the C-terminal peptide that ends in valine, have a C-terminal residue with a mass of 83 Da which corresponds to **1** minus a HN_3 moiety, as confirmed by CID ESI Fourier Transform tandem mass spectroscopy (FTMSMS). Peptides with "missed cleavages" have masses that are in accordance with a reduction of the internal azidohomoalanine residues. The fact that reduction-induced cleavage occurs C terminal to all positions of element **1** in AzPYP indicates that the cleavage reaction apparently poses no special prerequisites to the residue C terminal to the scissile bond. This result underscores the high specificity and general applicability of this mild chemical cleavage reaction of peptide bonds C terminal to **1**.

Next, we carried out cleavage of peptide **P** by tris(carboxy ethyl)phosphine (TCEP) in 50% ^{18}O-enriched water, which

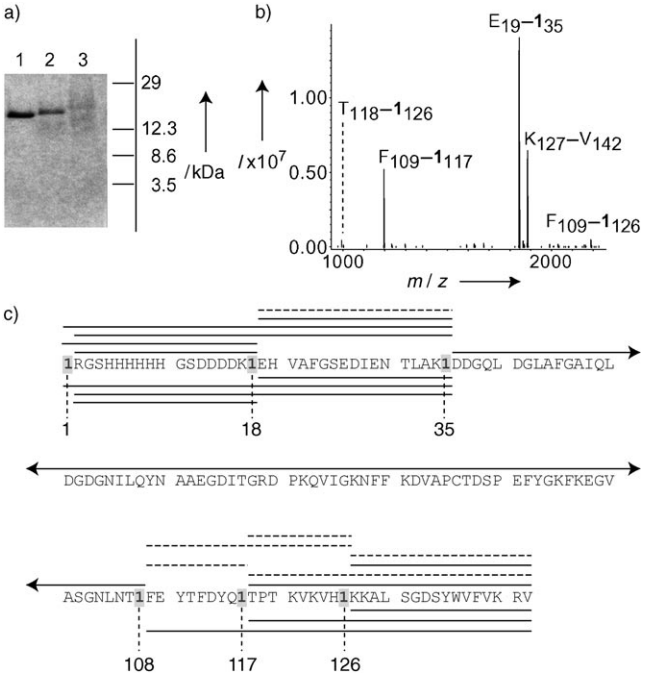


Figure 1. Recombinant AzPYP is cut by TCEP. a) Coomassie Blue stained gel containing equal amounts (23 μg) of AzPYP incubated without (lane 1) and with 10 mM (lane 2) and 100 mM (lane 3) TCEP at pH 5; the intact protein disappears and is cut into smaller fragments that do not resolve on the polyacrylamide gel. b) Deconvoluted ESI FTMS spectrum of TCEP-cleaved AzPYP. c) Sequence of His-tagged PYP from *E. halophila*. Intact molecular-mass and tryptic-peptide mapping confirmed >95% incorporation of **1** at methionine-coded residues. A coverage map obtained with TCEP (above the sequence) and DTT (below the sequence) is presented. Solid lines indicate peptides that result from cleavage that were detected with MALDI-TOF MS; dashed lines indicate peptides that were detected with ESI FTMS.

confirmed that the peptide bond C terminal to **1** is cleaved by hydrolysis, and indicated that only one ^{18}O atom is incorporated into the resulting C-terminal residue of the N-terminal peptide fragment (see the Supporting Information).

The C-terminal residue is considered to have a homoserine lactone structure based on the following observations: 1) When the cleavage product of peptide **P** was incubated under basic conditions, the mass of the product increased by 18 Da, which was added to the C-terminal residue as confirmed by CID; this addition could be reversed by incubation for one hour in anhydrous TFA.[8] 2) Incubation of the cleaved product of peptide **P**, or the cleaved products of AzPYP in butylamine, led to full conversion with an irreversible increase in the mass of the cleavage products by 73 Da, which is consistent with the addition of a C-terminal butylamine group,[8] as verified by CID. 3) It was found that both RP-HPLC retention times and CID MSMS analysis (see Figure 2 and the Supporting Information) of the presumed homoserine lactone ending peptides from TCEP-cleaved AzPYP and the corresponding homoserine lactone ending peptides from CNBr cleaved PYP were identical.

With TCEP, the reaction runs to completion in 100 min at 50 °C and pH 5, based on the disappearance of starting

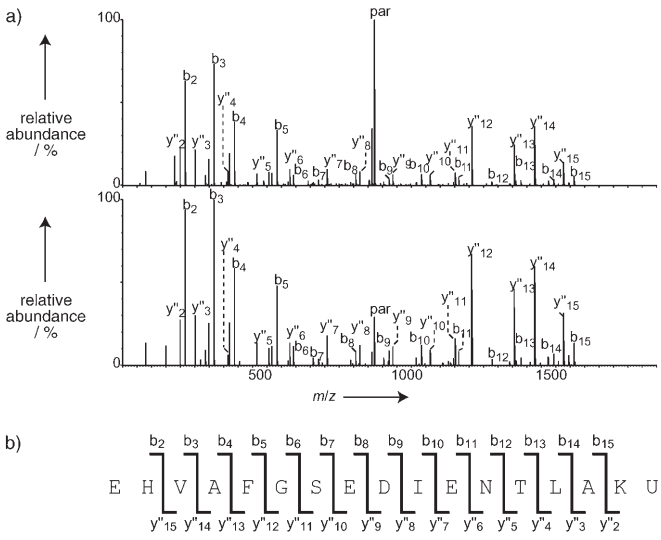


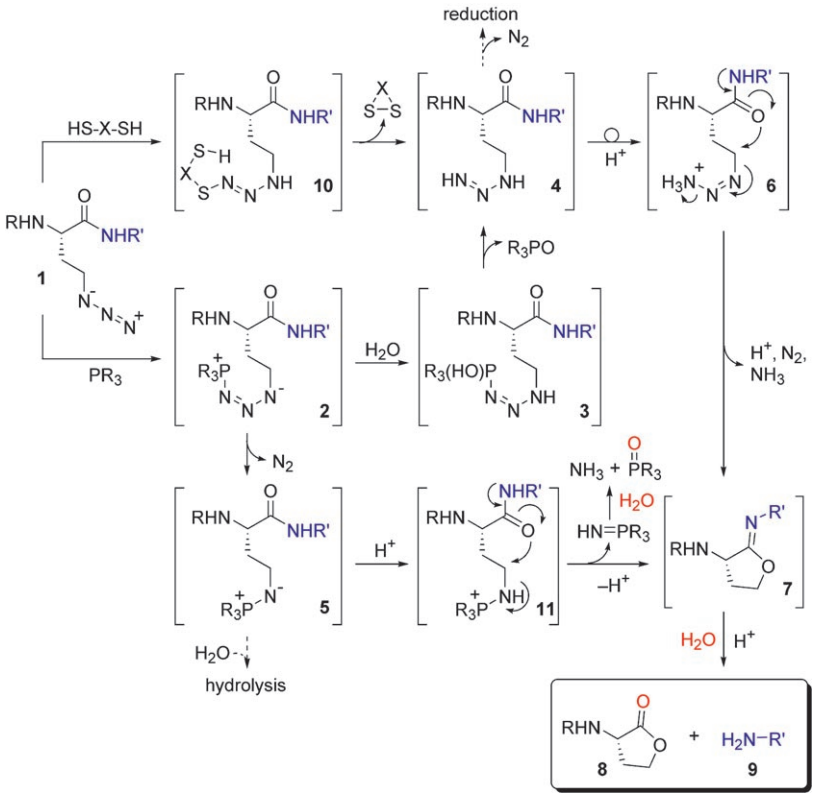
Figure 2. Comparison of the cleavage of AzPYP with TCEP and methionine-containing PYP with cyanogen bromide. Digests were loaded onto an LC–MSMS system (see Supporting Information). a) Charge-deconvoluted MSMS fragmentation spectra of the doubly charged ion of m/z 921.9 that eluted at identical retention times with annotation of fragment ions and parent ion (par). Identical fragment spectra verify the equivalence of both peptides. b) Sequence of the peptide and annotation of the retrieved fragment ions. U = homoserine lactone.

material. The ratio of cleaved/reduced product varied little as a function of pH value (the range of pH 3–11 was tested) or temperature, as quantified from RP-HPLC followed by MALDI-TOF-MS identification of the collected peak fractions. A maximized ratio at room temperature was accomplished at pH 5, thus resulting in 55% (±5%) cleavage, whereas at pH 9 approximately 40% (±5%) was cleaved (sum total of homoserine and homoserine lactone cleavage products).

With thiol reagents, the reaction only became significant at pH > 7, which is indicative of the requirement for the more nucleophilic thiolate anion. The peptide was significantly cleaved under these conditions, and a 30% (±8%) cleaved product was obtained with DTT within 16 hours with full conversion, whereas 84% of the azide compounds were still intact after 48 hours with 2ME. These results are consistent with the previously reported efficiencies of dithiols versus monothiols for azide reduction.[7a,9] In the case of 2ME, reduction into DAB (14%) predominated over cleavage (1%) as well. Cleaved peptides were mainly recovered with homoserine C termini, as could be expected at pH 9.2. Cleavage of AzPYP with thiols required unfolding of the protein with 4 M urea, which was not necessary for TCEP.

Peptide-bond hydrolysis that resulted in lactone formation can be rationalized by the mechanism depicted in Scheme 1. Phosphines attack at the electrophilic terminal nitrogen atom of azide **1**, initially forming intermediate **2**.[10] Water may add to **2** to give the postulated intermediate **3**, which fragments into the triazene **4** or lose dinitrogen to yield aza-ylide **5**.[10] It has been shown previously that triazenes may be substituted by suitable nucleophiles of either intra- or intermolecular origin.[9] It is worth mentioning that at elevated pH values conversion of **1** into homoserine without peptide cleavage occurs, which is indicative of direct hydroxide attack. The protonated triazene **6** may cyclize to imido ester **7** via an energetically favorable five-membered transition state, a pathway analogous to the cyanogen bromide induced cleavage of the methionine peptide bond.[11] Finally, hydrolysis of **7** liberates the homoserine lactone **8** and amine **9**.[1,8,11] This mechanism is supported by the above-discussed ^{18}O-labeling experimental results, which allow the carbonyl oxygen atom to be conserved in the lactone.

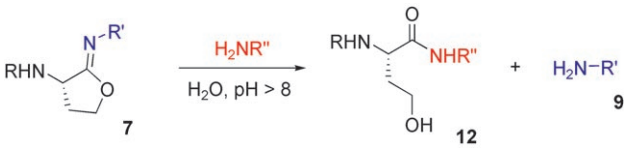
Alternatively, **5** can be hydrolyzed to an amine or may become protonated to generate intermediate **11**, which, through intramolecular S_N2 displacement involving the amide oxygen atom, yields the common imido ester intermediate **7**, which will then again hydrolyze to give **8** and **9**. The cleavage induced by (di)sulfides is presumed to be



Scheme 1. Mechanism for peptide cleavage C terminal to **1**. Initiation of the reaction is by reduction of the azide **1** into triazene **4**. Intramolecular S_N2 reaction of the carbonyl oxygen atom displaces the reduced azide to yield imido ester **6**, which is hydrolyzed and liberates a homoserine lactone ending peptide **8** and a peptide with an N-terminal free amine **9**. X denotes a three- or four-carbon spacer that can be substituted (e.g., X = -CH_2-CHOH-CHOH-CH_2- for DTT).

initiated by attack of the thiolate anion at either the α- or, as depicted, the γ-azide nitrogen atom (intermediate **10**).[7a] Loss of a cyclic disulfide from **10** gives the triazene **4**, which follows the pathway to **7** or fragments into DAB.

Lactones are amenable to further derivatization, for example, with amines in near quantitative yields,[8] as we have shown above for the cleaved peptide **P** and butylamine. As the cleavage reaction is not significantly affected by pH value, we were able to perform the cleavage reaction at elevated pH values (8–9) in the presence of amines. Under these conditions, we obtained products that were verified to be **P** cleaved C terminal to **1**, followed by addition of the selected amines (see the Supporting Information) and propose that the reaction proceeds as depicted in Scheme 2.



Scheme 2. Transamidation by exogeneous amines added to the reaction. Nucleophilic attack of an amine at the carbon atom of imido ester **7** may directly provide amide **12** while liberating amine **9**.

Other desired functionalities may similarly be installed with selected amines in a "one-pot" procedure. It is noteworthy that the stereocenter at C_α remains conserved, as is evident from the protein semisynthesis experiments with cyanogen bromide cleaved protein fragments.[12]

For optimal yield, however, the two-step alternative, in which first lactones are harvested and homoserine residues are closed by acid incubation,[8] may be preferred, as the initial yield of the TCEP-induced cleavage is higher at pH 5.

To our knowledge, this new cleavage reaction is milder than any chemical proteolysis described so far and, in contrast to I_2-induced allyl glycyl cleavage,[13] is directly applicable to overexpressed recombinant proteins. This report is also the first description of the use of an aza ylide as a leaving group amenable to intramolecular displacement by an amide group followed by hydrolysis to obtain an acid and amine. Until now, activated carboxylic acids were converted into amides using aza ylides and amidines were formed from amides,[3,14] the latter mainly in cyclization reactions. Performing this reaction in water has opened up a new pathway with far-reaching potential.

Direct application may be found in the preparation of isotope-labeled peptides by recombinant techniques, similar to the method described by Lindhout et al.[15] or protein semisynthesis from homoserine lactone,[8,12] but then after mild reduction-induced cleavage instead of CNBr. The yield of the cleavage reaction can be up to 60%; furthermore, a C-terminal function may be installed in a single step: an amine of choice elongates the cleaved polypeptide chain, thus providing the desired functionality.

Another powerful possibility lies in the targeted degradation of biopolymers.[16] However, as it is evident from earlier studies on azidothymidine (AZT) metabolism[9,17] and our findings that thiols reduce azides into highly reactive intermediates, one must remain conscious that the long-term intracellular presence of azides can lead to unintentional decomposition into unexpected products. On the other hand, methionines are often buried inside the protein core, and we have shown that degradation by thiols is accelerated under denaturing conditions, so at least for short-term experiments the azide function may be considered bio-orthogonal in vivo.

However, when phosphines or other reducing agents are added in subsequent reactions for the purpose of Staudinger ligations or to promote the in situ reduction of Cu^{II} to Cu^{I} for the catalysis of [3+2] azide–alkyne cycloadditions, it is conceivable that competitive nucleophilic substitutions at the (formerly) azide functionalized carbon atom may take place. The fate of intermediates **2** and **5** in Scheme 1, for example, will depend on the availability of suitable nucleophiles towards the phosphorane or the carbon nucleophilic center.[18] The proposal of using ultrapure Cu^{I} salts instead of the in situ reduction of Cu^{II} to catalyze the cycloadditions of azides and alkynes in biological samples[5c] may suppress reductive cleavage when this is not desired.

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