Peptide Structures

DOI: 10.1002/anie.201205308

Analysis of Peptide Secondary Structures by Photoactivatable Amino Acid Analogues**

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Di- and trifunctional diazirines have been used extensively for studies of protein–ligand and protein–membrane interactions[1] (see also Ref. [2]). More recently, the diazirine-containing amino acid analogues photo-leucine (see Table S1 in the Supporting Information) and photo-methionine have been incorporated in vivo into proteins to detect protein–protein interactions by cross-linking in living cells.[3] Follow-up studies, in which photoamino acids were incorporated into peptides or proteins, focused solely on the detection of intermolecular interactions.[4] High-resolution mass spectrometry (MS and MS/MS) can provide further information, such as low-resolution structures of proteins and protein complexes.[5] Here, we combine these methods for the first time to characterize reverse turns in diazirine-labeled peptides by photochemical cross-linking followed by an in-depth mass spectrometric analysis of the reaction products.

Based on experiments with various substituted diazirines and solvents, biradicalic and zwitterionic excited states were predicted to form upon photoactivation, which produce rather inert linear diazo isomers and reactive carbenes.[6,7] The latter are known to react intramolecularly to form alkenes[8] and to insert into C–H and heteroatom–H bonds.[6,9] When we subjected a solution of photo-leucine[10] to weak UV-A irradiation (1 J cm^{-2}, ca. 5.5 min) and LC/ESI-MS analysis, we observed three peaks representing the intact protonated photoamino acid and species with specific losses (Figure 1a). Their m/z ratios hint an insertion into water creating an alcohol (−10 amu) and an intramolecular elimination reaction forming alkenes (−28 amu). Photo-leucine was also found to insert into methanol (+4 amu) and phosphoric acid (+70 amu) upon photo-activation—at least, when it was incorporated into peptides (Table S1 in the Supporting Information). The peak comprising a species at m/z 144.0744 might either correspond to the diazirine substrate or to linear diazo isomers, which are isobaric and thus indistinguishable by mass. This species, however, diminished upon extended UV-A irradiation, accompanied by an increased formation of the alcohol and the alkene.

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[**] K.K. is funded by the BMBF (ProNet-T^3), A.S. acknowledges financial support by the DFG, the EU, and the Land Sachsen-Anhalt.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201205308.

The relative chromatographic peak areas of all three species were plotted versus the applied UV-A dose (Figure 1b), which reflects the time domain in good approximation and thus allows for kinetic analysis. All species displayed apparent first-order kinetics with nearly identical rate constants ($8.0–8.6 \times 10^{-7}$ % J^{-1} cm^2). Apparently the disappearance of the species at m/z 144.0744 kinetically limits product formation, indicating the absence of inert linear diazo isomers.

UV-A-irradiated photo-methionine formed products similar to those of photo-leucine, albeit with different relative product yields and slightly higher rate constants ($1.6–1.7 \times 10^{-6}$ % J^{-1} cm^2, Figure 1c). Conceivably as a result of multiple isomerization and association states, the alkenes eluted as five distinct peaks (Figure S1 in the Supporting Information). Importantly, alkenes and alcohols of both photoamino acids occurred exclusively in irradiated samples and eluted as separate peaks. Thus, we exclude that the photo-reaction took place during HPLC analysis or the ESI process, but was indeed induced by UV-A irradiation. However, the mere presence of the alkenes had major consequences on subsequent MS peptide analysis: Since elimination and cross-linking reactions result in identical constant neutral losses (−28 amu), alkenes and intramolecularly cross-linked peptides are indistinguishable by mass. Therefore, a proteolytic cleavage step had to be included to create two new termini to allow for an unambiguous identification by MS/MS analysis.

The first peptide studied herein is derived from the sequence KKYTVSINGKKITVSI, which forms a stable β-hairpin in aqueous solution.[11] This type of secondary structure is characterized by a reverse turn connecting two antiparallel β-strands. The formation of multiple H-bonds between peptide bonds results in a rigid structure. An intrapeptide cross-link should therefore specifically connect the incorporated photo-amino acid to its opposing amino acid within the hairpin. In our experiments we used a modified version of this β-hairpin-forming peptide (GLGGKKYTV-SINGGKKITVSIGLLG), which is henceforward designated as peptide H, while the peptide containing photo-leucine (Λ) at position 2 is referred to as H* (Figure 2b). When peptide H was subjected to an energy-based structure prediction algorithm [http://www.scfbio-iitd.res.in/bhageerath/index.jsp],[12] one of the five obtained structure proposals showed indeed a β-hairpin-like secondary structure with both termini being in close proximity to each other (Figure 2c). According to our far UV–CD measurements (data not shown), peptides H and H* behave exactly as their progenitor showing pH- and methanol-dependent secondary structure.[11]

Peptide H* was irradiated by UV-A light and digested with trypsin (cleaving C-terminally of Arg and Lys). The

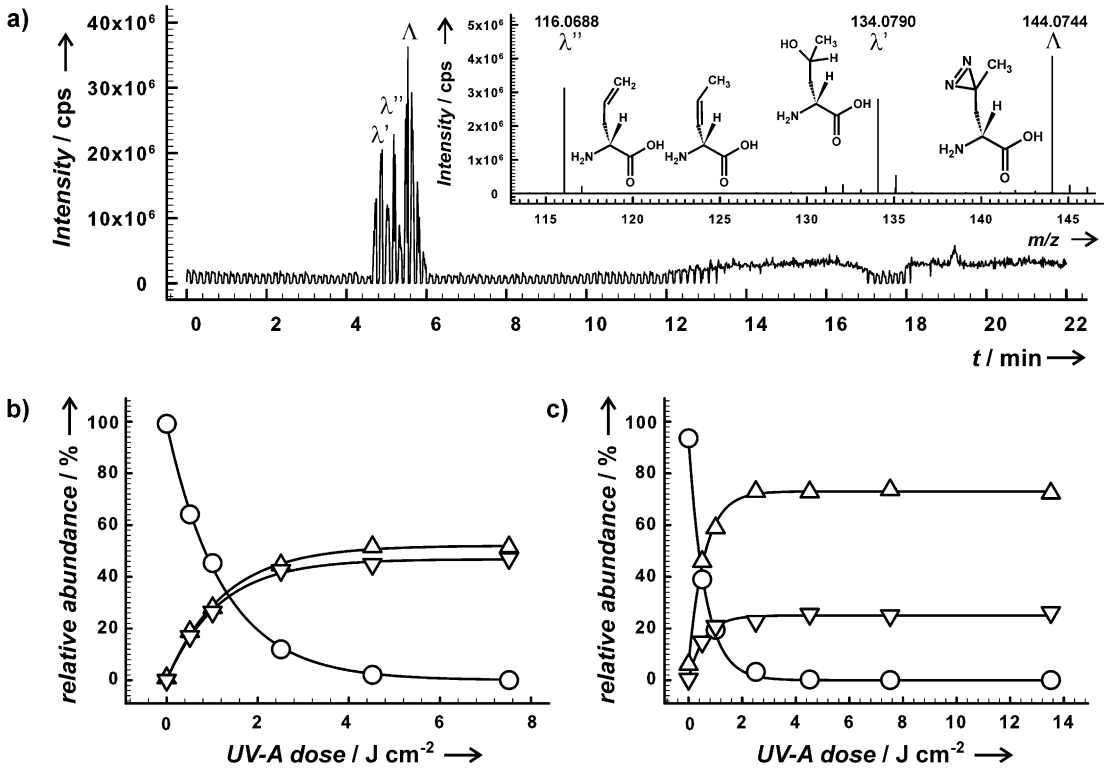

Figure 1. LC/ESI-MS analysis of photoamino acid reaction products. a) Baseline-corrected reconstructed total ion current chromatogram of UV-A-irradiated photo-Leu. Inset: Averaged spectra of precursor ions accumulated at 4.65–5.9 min. b) Reaction progress of photo-Leu. c) Reaction progress of photo-Met. Relative peak areas of intact photoamino acids (○) and alkene (△), alcohol (▽) reaction products were obtained from the chromatograms and plotted versus the applied UV-A dose.

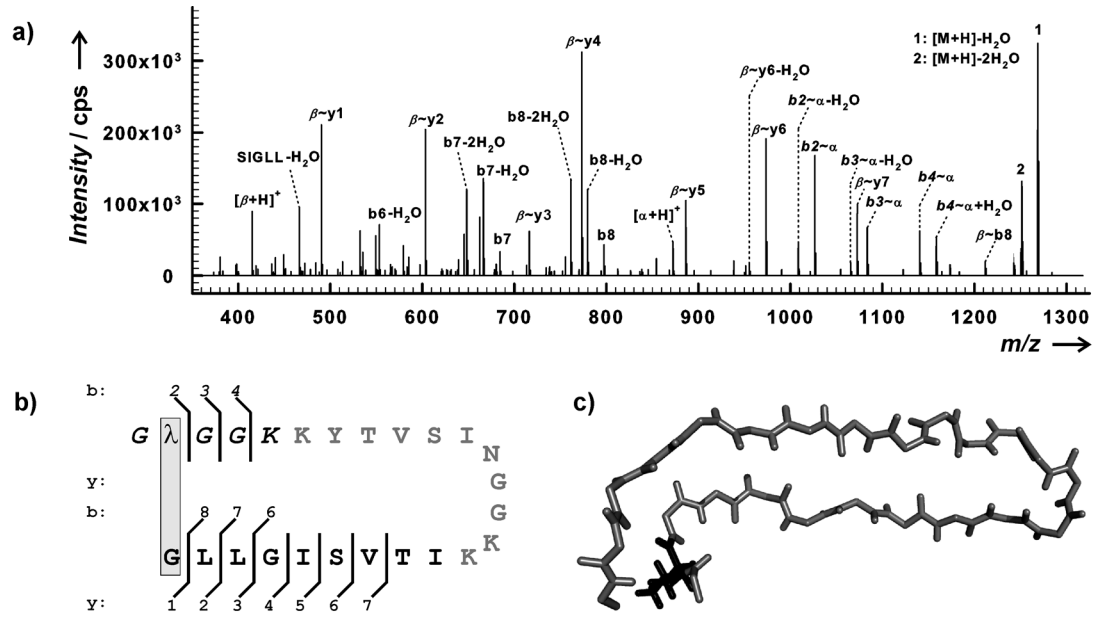

Figure 2. LC/ESI-MS/MS analysis of peptide H*. A peptide solution (400 μM) was exposed to UV-A light, digested with trypsin, and analyzed by LC/ESI-MS and LC/ESI-MS/MS. a) CID fragment-ion mass spectrum of the singly charged precursor ion of cross-linked tryptic peptides [1–5]~[17–25]. b) Schematic view of identified fragment ions; α- and β- (italics) peptide as well as their b- and y-type fragment ions are indicated (for nomenclature see Ref. [13]). Cross-linked amino acids are shaded; amino acids that were removed by tryptic digestion are indicated with gray letters. c) Structure model of peptide H. The peptide backbone is colored gray, Leu[2] is colored black. Coordinates were obtained by an energy-based structure-prediction algorithm [http://www.scfbio-iitd.res.in/bhageerath/index.jsp].[12]

mixture was separated by reversed-phase HPLC and analyzed online by high-resolution nano-ESI-MS and nano-MS/MS (Figure 2a and Figure S2 in the Supporting Information). Unmodified tryptic fragments dominated the mass spectra, while signals of the proposed cross-link between λ^2 and Gly^{25} were expectedly less abundant (Figure S2). Fragmentation of ions corresponding to cross-linked peptides [1–5]~[17–25] yielded expedient fragment-ion spectra[13] allowing for unambiguous identification of a cross-link between λ^2 and Gly^{25} (Figure 2a, Figure S2). We also identified an additional cross-linked product between redundant tryptic peptides, albeit with very low signal intensity, pointing to an intermolecular cross-link between two separate peptides forming an H* dimer. This agrees well with the proposed β-strand structure of both peptide "flanks" (Figure S3).

The second peptide, which is designated as peptide T ($GLGGPRGNGLLGY\text{-}NH_2$) and peptide T* ($G\Lambda GGPRGNGLLGY\text{-}NH_2$) respectively, comprises a PRGN sequence. According to previous studies,[14] this motif adopts a β-turn-like conformation. Secondary structures of this type are much less stabilized by noncovalent interactions than hairpins. Indeed, all five structure proposals predicted by the above-mentioned algorithm indicate a reverse-turn structure in the region of Pro^5 and no defined secondary structure in the N- and C-terminal regions of the peptide (Figure 3). Far-UV–CD analysis of peptide T proved difficult, probably because of the simultaneous presence of more than one conformation. At least one of these non-random conformations was stabilized by addition of up to 50% 2,2,2-trifluoroethanol (TFE) (Figure S4a).

Peptide T* yielded comparable CD spectra (data not shown), indicating that the secondary structure of the peptide remained unperturbed by the incorporated photo-amino acid. UV-A irradiation of peptide T*, tryptic digestion, and LC/ESI-MS/MS analysis revealed the formation of a cross-link between λ^2 and Leu^{10} or Leu^{11} as well as between λ^2 and Gly^{13} or Tyr^{14} (Figure S5 in the Supporting Information). The addition of 50% TFE resulted in increased relative signal intensities of the cross-linked products. In agreement with the proposed reverse-turn structure surrounding Pro^5, the cross-links were unambiguously assigned to occur between λ^2 and Leu^{10} as well as between λ^2 and Tyr^{14} (Figure 3a,b).

Peptide P (GLGGPPPPPGLLGY) was designed as a negative control to assure the specificity of the cross-linking

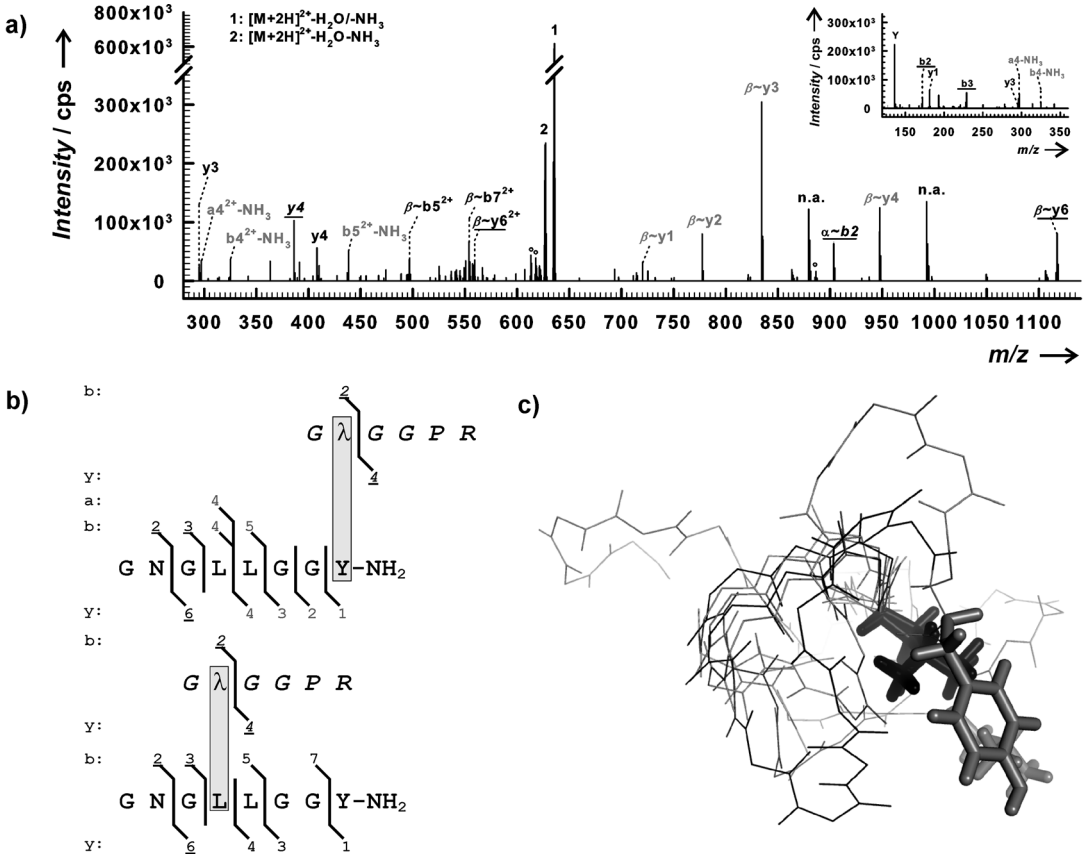

Figure 3. LC/ESI-MS/MS analysis of peptide T*. A peptide solution (160 μM) in 50% TFE was exposed to UV-A light, tryptically digested, and analyzed by LC/ESI-MS and LC/ESI-MS/MS. a) CID fragment-ion mass spectrum of the doubly charged precursor ion of cross-linked tryptic peptides [1–6]~[7–14]. Inset: Enlarged region of HCD fragment-ion mass spectrum. Black numbers and letters denote fragment ions of the C-terminally cross-linked product shown schematically in (b) (upper panel); gray numbers denote these from the alternative cross-linked product (lower panel). Underlined numbers and letters indicate an assignment to both species. b) Schematic view of identified fragment ions. Letters and numbers in italics denote the β-peptide and its fragment ions (see also (a)), cross-linked amino acids are shaded. c) Structure model of peptide T. Coordinates were obtained as described above. All five models are aligned. The peptide backbone and cross-linked amino acids (Leu^{10}, Tyr^{14}) are colored gray, Leu^2 is shown in black.

reactions in peptides H* and T*, as its pentaproline core should prevent any contacts of the peptide's termini owing to its inherent rigidity.[15] Especially in the presence of TFE, peptide P displayed polyproline II helix like properties as indicated by far-UV–CD spectra of acidic aqueous solutions (Figure S4b in the Supporting Information). Concomitantly, no intrapeptide cross-linked products of peptide P* (GΛGGPPPPPGLLGY) were detected after UV-A irradiation, pepsin digestion, and LC/ESI-MS or LC/ESI-MS/MS analysis. Obviously, cross-linked products are not randomly created, but indeed depend on the presence of reverse turns with the photoamino acid and the cross-linked amino acid being in close spatial proximity.

In conclusion, diazirine-containing analogues of leucine and methionine were used for in-depth studies of the reactions these amino acids can undergo upon photoactivation. Elimination reaction products, adducts with solvent components, and cross-linked products of diazirines with polar as well as with nonpolar groups were identified. No structural changes were observed in the peptides upon incorporation of the diazirine ring. The rather prolonged photo-activation, not the cross-linking reaction itself, is the rate-limiting step. These essential results prove the suitability of incorporating photoamino acids into peptides or proteins to specifically catch spatially close interactions, both stable and transient. This, combined with MS analysis, lays the foundation for further peptide and protein conformational studies as well as for monitoring various kinds of protein–ligand interactions. Owing to the broad occurrence of leucine in proteins, large numbers of cross-linking products can be expected. The inherent sensitivity of modern mass spectrometers will allow the identification of low-abundance species that otherwise escape detection by conventional methods like NMR spectroscopy and X-ray crystallography.

Experimental Section

Photo-leucine, photo-methionine, peptides H, H*, T, T*, P, and P* (> 95% purity) were obtained from ThermoFisher Scientific, analytical grade methanol (> 99.9%) and TFE (> 99%) from VWR and Sigma. Deionized water was used for HPLC solvents and buffers. Photo-leucine and photo-methionine were dissolved in 10 mM K_2HPO_4/KH_2PO_4 (pH 7.5) to final concentrations of 26 and 8 mM, respectively. Solutions were kept on ice and placed into a homemade UV-irradiation device[16] equipped with a UV-A filter and a photo cell. After application of specified UV-A doses, samples were taken and analyzed by LC/ESI-MS using an analytical HPLC system (Agilent1200) coupled to an ESI-LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific). Peptides H* and T* were dissolved in 100 mM K_2HPO_4/KH_2PO_4 pH 7.5, while peptide P* was dissolved in water. Concentrations were determined photometrically at 280 nm based on the theoretical absorption coefficient. Final concentrations varied (2.5 to 1600 μM). Cross-linking reactions were conducted as described above, except for the constant UV-A dose (8 J cm^{-2}). After the cross-linking reaction, trypsin (peptides H*, T*) or pepsin (peptide P*) digestions were performed followed by LC/ESI-MS and MS/MS analysis using an UltiMate plus nano-HPLC system (LC Packings) coupled to an LTQ-Orbitrap XL mass spectrometer (equipped with nano-electrospray ionization source (Proxeon)). Details of the LC/MS methods are given in Tables S2 and 3 in the Supporting Information. TFE, if present (peptide T*), was removed in a vacuum concentrator prior to tryptic digestion. Spectra representing potential cross-linked products were identified with the StavroX software[17] and validated by manual inspection.

Received: July 5, 2012
Published online: October 26, 2012

Keywords: analytical methods · circular dichroism · mass spectrometry · photoaffinity labeling · secondary structure

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