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Conformational Shift of a β -Hairpin Peptide upon Complex Formation with an Oligo–proline Peptide Studied by Mass Spectrometry

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Abstract

So-called super-secondary structures such as the β -hairpin, studied here, form an intermediate hierarchy between secondary and tertiary structures of proteins. Their sequence-derived ‘pure’ peptide backbone conformation is combined with ‘remote’ interstrand or interresidue contacts reminiscent of the 3D-structure of full-length proteins. This renders them ideally suited for studying potential nucleation sites of protein folding reactions as well as intermolecular interactions. But β -hairpins do not merely serve as model systems; their unique structure characteristics warrant a central role in structural studies on their own. In this study we applied photo cross-linking in combination with high-resolution mass spectrometry and computational modeling as well as with ion mobility-mass spectrometry to elucidate these structural properties. Using variants of a known β -hairpin representative, the so-called trpzip peptide and its ligands, we found evidence for a conformational transition of the β -hairpin and its impact on ligand binding.

Keywords

cross-linking; ion mobility; mass spectrometry; peptide secondary structure; Rosetta modelling workflow

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Introduction

During the past decade, chemical cross-linking combined with mass spectrometry (XL-MS) and computational modeling has emerged as an alternative technique to elucidate three-dimensional structures of proteins. In addition to introducing external cross-linkers as ‘molecular rulers’ to derive distance information of reactive side chains in proteins, the use of artificial amino acids has proven to be beneficial.^[1] When incorporated directly into the polypeptide of interest, amino acid analogues carrying benzophenone or diazirine groups form ‘zero-length’ cross-links to adjacent residues upon photo-activation.^[2] In a proof-of-principle study, we had previously monitored the formation of reverse turns in synthetic peptides comprising the diazirine-containing amino acid homologue photo-leucine (Λ) using high-resolution mass spectrometry (MS).^[3] These initial results were promising and encouraged us to extend our cross-linking strategy with respect to spatial resolution, peptide flexibility, and, in particular, specificity of intermolecular interactions. Therefore, a simple and well-studied system was essential to test our approach. Both requirements are fulfilled by the so-called trpzip peptide that adopts a stable β -hairpin conformation and binds to oligo-proline containing peptides.^[4]

Results and Discussion

In order to meet the demands for cross-linking, three slightly different variants of the trpzip peptide (Table 1) were designed as follows. To a C-terminally extended trpzip variant^[4b] the amino acid sequence Ac-G(L/ Λ)G was N-terminally added (peptides W4/W4*). Also, we deleted (W3/W3*) or substituted (W3 L/W3 L*) Trp⁷. The flexible termini of W4* and W3* were designed to monitor unimpeded cross-linking of an intact (W4*) or destabilized (W3*) hairpin. In addition, peptide W3 L* allowed us to test photo-cross-linking within the rigid environment a β -sheet.

First, near- and far-UV circular dichroism (CD) spectra of W4 were recorded, which indicate an interaction of Trp residues across the β -sheets. Also, thermal denaturation profiles closely resemble those of the original peptides.^[4b, 5] This suggests that the newly created termini do not affect β -hairpin formation. In agreement with our objectives, the β -hairpin structure is largely maintained after substitution but is drastically destabilized upon deletion of Trp⁷ (Figures S1-2, Supporting Information).

UV-A irradiation of peptides W4*, W3*, and W3 L*, trypsin digestion, and nanoHPLC/nanoESI-MS/MS analysis lead to distinct cross-linked products. The first product of W4* (Λ^2 ~Lys¹⁷) reflects a tight contact between the termini of the peptide (Figure 1a) and is rather abundant as judged by the extracted ion chromatograms (EICs). On the other hand, the corresponding product of W3* (Λ^2 ~Lys¹⁶) was almost completely absent (data not shown). For W3 L*, a cross-linked product (Λ^7 ~Trp¹²) was observed, which is indicative of contacts between both β -strands (Figure S3). Additional cross-linked products of W4* and W3* (Λ^2 ~Trp¹²/Lys¹⁵ and Λ^2 ~Trp¹¹/Lys¹⁴) suggest less well-defined contacts between Λ^2 and all residues in the putative second β -strand of the peptides. These ‘internal’ and apparently low-abundant products are isobaric to linear alcoholic side products (Λ') with

missed tryptic cleavage sites, but are unequivocally assigned based on fragment ion mass spectra (Figure S3, Supporting Information).

Incorporation of the W4*-derived distance constraints into a Rosetta computational modeling workflow yielded two slightly different model structures. Although a compact β -hairpin is evident in both models, the first model (I) agrees much better with the distance information provided by the λ^2 -Lys¹⁷-cross-link than the λ^2 -Trp¹²/Lys¹⁵ cross-linked product (Figure 1b). The second model (II) displays the opposite behavior (Figure 1d). For W3 L*, both conformations agree with the formation of the λ^7 -Trp¹² product (Figure 1c, e). Considering the short length of W4, the finding of more than one folded and stable conformation is surprising and raises the question whether the cross-links of λ^2 with residues in close spatial proximity (Lys¹⁷ vs. Trp¹²-Lys¹⁵) indeed reflect distinct conformations.

To test the coexistence of two conformations, ion mobility–mass spectrometry (IM–MS) experiments using a drift-tube instrument^[6] were performed. In IM–MS, the time is measured for ions of a particular m/z to traverse a buffer-gas filled drift cell under the influence of a weak electric field. The resulting arrival time distributions (ATDs) are used to obtain absolute, angular-averaged collision cross sections (CCSs) and thus yield valuable information about size and shape of the species of interest.^[7] The ATDs of the doubly charged peptide W4 reveal the presence of two partly resolved peaks (Figure 2a), which inter-convert into each other depending on collisional activation before ion mobility separation. Similar observations were made also for the triply and quadruply charged ions of W4, albeit with different relative populations.

Absolute collision cross sections for helium as the drift gas ($^{DT}CCS_{He}$) were determined for all separable species of W4, W4*, W3, and W3 L (Table S1, Supporting Information). To obtain a clearer picture on gas-phase structure of the peptides, molecular modeling was performed. First, we generated a linear model of neutral W4 and attached protons to the lysine residues^[8] to obtain initial structures of doubly and quadruply charged W4. Subsequently, molecular dynamics (MD) simulations were performed for each protonated candidate structure,^[9] and theoretical CCSs were computed using different established methods^[10] (Figure S5, Table S2 and S3, Supporting Information). While doubly charged W4 species are more ordered than higher charged ions (Fig 2b-c), compact, reverse turn structures are maintained invariantly. In case both protons are located on two of the three C-terminal lysine residues in doubly charged W4, a less-ordered, more compact structure is obtained. On the other hand, proton attachment on both sides of the β -strand leads to a more hairpin-like, extended structure (Fig 2b). Apparently, a salt bridge between the functional groups of Glu⁸ and protonated Lys¹¹ is advantageous in overcoming Coulomb repulsion and maintaining the reverse turn in the gas phase (Figure 2d).

As W4 contains four lysine residues, there are six potential protonation isoforms of doubly charged peptide. According to our MD simulations, three out of the six isoforms appear to be energetically favorable for doubly charged W4 with, however, only two of them forming the more hairpin-line structure with the above-mentioned salt bridge. Like in comparable systems,^[11] the absolute values of computed CCSs and measured $^{DT}CCS_{He}$ differ

depending on the method, but the coexistence of distinct species is obvious from MD simulations and experimental ATDs alike (Figure S5) and has also been observed with related, singly charged peptides.^[12]

The question remains if and how solution and gas phase conformations are connected. In the LC elution profiles of W4 peptides, we observed multiple peaks for each charge state, which cannot be explained by net charge or proton uptake during ionization. The differently eluting quadruply charged W4 species even revealed different CID fragmentation patterns. This multitude of peaks was drastically reduced for W3, possessing less stable solution conformations than W4 (Figure S6, Supporting Information). Thus there is indirect evidence for a charge-structure relation also in solution. Although we cannot correlate with certainty p. e. the more hairpin-like solution conformation (model I, Figure 1b) with the extended protonation isoforms of doubly charged W4 (B, Figure 2b), we can conclude that several conformations coexist, both in gas phase and solution.

Heterogeneous solution conformations are also visible in the ligand-bound form of the trpzip peptides. We measured tryptophan fluorescence emission spectra of W4 and W3 in the presence of increasing concentrations of the ligand, peptide P5 (Table 1). The obtained binding isotherms are clearly indicative of a multistate equilibrium (Figure S7, Supporting Information).

Upon UV-A irradiation of W4*/P5 mixtures, we identified two- to fivefold charged intermolecular photo-reaction products (W4*~P5) by nanoHPLC/nanoESI-MS/MS analysis. In particular, the fragment ion mass spectra of highly charged W4*~P5 prove a close contact between Λ^2 (W4*) and the C-terminus of P5 with only sparse minor signals indicating other contacts (Figure S8a, Supporting Information).

Next, we varied the P5 concentration and used EICs to relatively quantify product formation. Contrary to diazirine-mediated cross-linking in the gas phase,^[13] photo-activation in solution yields an excess of linear alkene (λ'') and alcohol (λ') products, which don't provide any structural information (Figure S9, Supporting Information). Because of their predominant formation, the abundance of these linear products can be regarded as constant. Therefore, their EIC-peak areas were used as normalization standards for quantification of the cross-linked products.

Plotting the normalized peak areas of all W4~P5 species *versus* the P5 concentration yielded a sigmoidal quasi-binding isotherm (Figure 3a). Sigmoidal curves might have resulted from cooperative binding to at least two interdependent sites.^[14] However, the formation of ternary (or higher) P5-W4 complexes is certainly not feasible. With respect to W4* itself, we never observed unambiguous intermolecular cross-linked products agreeing with the original peptides.^[4] Also, the reverse experiment (varied [W4*] and constant [P5]) yielded a hyperbolic quasi-isotherm (data not shown). For these reasons and based on our complex fluorescence curves, we consider a conformational change of P5-bound W4 as a more plausible cause of the sigmoidal binding curves.

Consequently, we monitored both intramolecular cross-linked products in parallel to the formation of the W4~P5 complex. Whereas λ^2 ~K¹⁷ sharply diminishes at P5 concentrations

below the apparent half saturation point, the abundance of $\lambda^2\sim\text{Trp}^{12}/\text{Lys}^{15}$ clearly increases in parallel to complex formation (Figure 3b). This agrees with the results of our *in-silico* docking studies: The distance constraints derived from the major W4*~P5 cross-linked product (Figure S8a, Supporting Information) were included into a version of Rosetta docking workflow and the obtained structure model showed P5 binding to W4 to be most favorable in the $\lambda^2\sim\text{Trp}^{12}/\text{Lys}^{15}$ -derived conformation.

Apparently, the peculiar arrangement of *N*-terminal amino acids of W4 seen in model II is instrumental in forming a close contact to Tyr¹⁴ (Figure 3c). With respect to the peptide backbone, model I and the original peptide closely resemble an ideal β -hairpin (Figure 3d). In model II, representing the binding competent conformation, the β -strands are twisted (Figure 3d), concomitantly with a rearrangement of the tryptophan residues. Only this rearranged indole cage, which agrees with our fluorescence data, can accommodate P5 without steric clashes (Figure 3e).

There are two important aspects in this finding. First, one out of two coexisting conformations is incapable of P5 binding for steric reasons. Second, the alternative conformation is not only present in the absence of P5 as well, but is promoted upon ligand binding. This agrees with the data of peptide W3*. The formation of the $\lambda^2\sim\text{Lys}^{16}$ intramolecular cross-linked product of W3 reflecting the hairpin-like conformation, is evidentially impaired, in contrast to the $\lambda^2\sim\text{Trp}^{11}/\text{Lys}^{14}$ product. The W3*~P5 cross-linked product is formed nonetheless and gives rise to a small-amplitude hyperbolic quasi-binding isotherm (Fig 3a), which in turn implies that no conformational shift of W3 is required for ligand binding. Therefore the induced-fit mechanism, which had been shown for the original peptide^[4] and which would have explained the sigmoidal quasi-binding isotherms of W4*/P5, cannot be assumed for our peptides. As an alternative interpretation, a preferential binding mechanism fits much better to our results. Considering the crucial role of the *N*-terminal amino acids being arranged as in model II for complex formation, the different termini of the original and our peptides are obvious reasons for this divergence.

Additionally and as mentioned above, some minor signals in the fragment ion mass spectra of highly charged precursors indicated an alternative orientation of P5 in the complex. These became more dominant in number and intensity in the spectra obtained from doubly charged precursor ions (Figure S8b, Supporting Information). Likewise, cross-linked products of peptides W3 L* and P5 were observed that do not fit the structural model (Figure S10, Supporting Information). For these reasons we performed the reverse experiments, using either *N*- (P5*) or *C*-terminally (P5b*) labelled P5 variants (Table 1). Both yielded intermolecular cross-linked products (Figure S11, Supporting Information), with P5*~W3 agreeing well with structural model II (Figure 3c) while P5b*~W3 does not. Thus, trpzip-bound P5 adopts different orientations. This finding of alternative P5 orientations in the complex impedes the determination of apparent thermodynamic constants like those shown previously.^[15]

Conclusions

We have employed a low-resolution method, XL–MS backed by CD and fluorescence spectroscopy, to study the solution structure of a β -hairpin forming peptide and its behavior upon ligand binding. While the finding of W4 forming two β -strands connected by a reverse turn is more or less accepted knowledge, the observation of two coexisting conformations was surprising. As these conformations result in the differential cross-linking of spatially close residues within a small peptide, there is a considerable amount of subtlety involved. In order to shed more light on this, IM–MS experiments were performed and also here, several distinct species were observed. Molecular modeling suggested that these different gas phase conformations are characterized by, or are due to different charge locations. The question, whether this different protonation site occupation is caused directly by distinct solution conformations or just a result of gas phase rearrangement, is difficult to answer at the current stage but provides ample opportunity for our ongoing research.

The principal finding of the cross-linking experiments, the coexistence of several conformations of our trpzip peptide variants, however, was confirmed by the IM–MS results and also by computational solution modeling as well as by our semi-quantitative monitoring of ligand-induced changes. Based on this latter approach, we also report an alternative binding mode.

Taken together, cross-linking of dedicatedly placed photo-active labels, combined with a thorough product characterization, and sophisticated computational modeling enabled us to establish a near-atomic picture for the investigated trpzip peptide.

Experimental Section

All peptides were obtained from Thermo Fisher Scientific. For experimental details of cross-linking procedures, IM–MS measurements, gas phase modeling, CCS calculations, and Rosetta modeling see Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. β -hairpin formation of trpzp peptide W4

a) CID fragment ion mass spectrum of the cross-linked product of peptide W4*, Ac GAGSWTWENGK~K-NH₂ at m/z 703.346 (doubly charged species). Nomenclature: α (red) and β (blue) refer to the heavier and lighter peptide, respectively^[16] (see inset). When backbone fragment ions of the α -peptide include the cross-linking site, this is denoted as “ β ~”; neutral losses to already assigned fragment ions are indicated by “*” and internal fragment ions by “*”. b-e) Structural models I (b-c, grey) and II (d-e, cyan) from computational modeling aligned to peptide trpzp2 (pdb 1LE1,^[4a]yellow). The C $_{\alpha}$ atoms of λ^2 (W4*, b, d) and of λ^7 (W3 L*, c, d) are shown as red spheres; blue spheres indicate C $_{\alpha}$ atoms of interacting residues. C $_{\alpha}$ -C $_{\alpha}$ distances (—) are given in Å.

Figure 2. Compact conformations of peptide W4 in the gas phase

a) ATD of the doubly charged precursor ion of peptide W4 revealing at least two partly resolved features. Capital letters above the peaks indicate a tentative assignment to conformations as shown in b). b-d) Candidate gas phase structures of W4 as obtained from *in-silico* modeling and Molecular Dynamics (MD) simulations. Primary amine groups of lysine residues are shown as blue spheres (N) and grey sticks (H). b) Comparison of candidate structures of doubly charged W4. Upper panel (A): positive charges located at residues Lys^{16, 17} (yellow) and Lys^{15, 16} (grey). Lower panel (B): Positive charges located at residues Lys^{11, 15} (yellow) and Lys^{11, 17} (red). c) Candidate gas phase structures of quadruply charged W4 as obtained from MD simulations at 350 K (grey), 500 K (yellow), and 650 K (red). C_α atoms (spheres) of all lysine residues are shown to indicate charge locations. d) Comparison of candidate gas phase structures of doubly charged W4 with the positive charges (two and three grey sticks, respectively) located at Lys^{15, 16} (grey) and Lys^{11, 17} (red backbone). Distances (—) between the primary amine groups of Lys¹¹ (blue) and the oxygen atom of Glu⁸ (black spheres) are given in Å.

Figure 3. Complexes of trpzp and P5peptides

a) Semiquantitative binding isotherms of intermolecular cross-linked species $W4 \sim P5$ (*) and $W3 \sim P5$ (*). Peak areas of cross-linked products ($Wx \sim P5$) in extracted ion chromatograms (EICs) were normalized to peak areas of linear photoproducts and plotted *versus* the total concentration of P5. *Inset:* Zoomed-in presentation of $W3 \sim P5$ formation. *Note:* The P5 samples used in this study contained a minor component, GLLGPPPPGGLL, the relative amount of which varied between different preparations. Complex formation was exactly as for the major component and the corresponding data are therefore included in the curve. b) Abundance of the $\lambda^2 \sim \text{Lys}^{17}$ (*) and of the $\lambda^2 \sim \text{Trp}^{12} / \text{Lys}^{15}$ (~, inset) intramolecular cross-linked products in the presence of P5. Peak areas of cross-linked products in EICs were normalized to those of linear alkene and alcohol photoproducts and plotted *versus* the total concentration of P5. c) Structure model II in complex with P5 as obtained from Rosetta modeling workflows. Peptide W4 is shown in cartoon presentation and P5 as sticks. The C_{α} atoms of residues of λ^2 in W4 and P5* are indicated by red, those of their cross-linked partners by blue spheres. Distances (—) are given in Å d) Comparison of overall conformations. Peptide backbones of structure models I (grey) and II (cyan) were structurally aligned to that of trpzp2 peptide (yellow, pdb ID 1LE1,^[4a]). e) Conformational differences between models I and II. The peptide backbone of the trpzp peptides is shown in cartoon presentation with the Trp side chains as in model I (black) and II (blue/cyan) indicated as sticks; P5 is depicted as ball-and-sticks. Arrows indicate major steric clashes between P5 and indole moieties in W4-model I.

Table 1
Amino acid sequences of the peptides used

The amino acid sequence of the original trpzip2 peptide^[4a] is shown on top. Modifications of the trpzip2 sequence are shown underlined; bold letters denote photo-leucine (**Λ**) in diazirine-labeled variants (*).

	designation
H-SWTWENGKWTWK-NH ₂	trpzip2
<u>Ac-G(L/Λ)GSWTWENGKWTWKK-NH₂</u>	W4/W4*
<u>Ac-G(L/Λ)GSWT_ENGKWTWKK-NH₂</u>	W3/W3*
<u>Ac-G(L/Λ)GSWTLENGKWTWKK-NH₂</u>	W3 L/W3 L*
H-G(L/ Λ)GGPPPPGLLGY-OH	P5/P5*
H-GYGGPPPPGLLG A -OH	P5b*