

Solution Structure and Acid-Base Properties of Reduced α -Conotoxin MI

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The reduced derivative of α -conotoxin MI, a 14 amino acid peptide is characterized by NMR-pH titrations and molecular dynamics simulations to determine the protonation constants of the nine basic moieties, including four cysteine thiolates, and the charge-dependent structural properties. The peptide conformation at various protonation states was determined. The results show that the disulfide motifs in the native globular α -conotoxin MI occur between those cysteine moieties that exhibit the most similar thiolate basicities. Since the basicity of thiolates correlates to its redox potential, this phenomenon can be explained by the higher reactivity of the two thiolates with higher basicities. The folding of the oxidized peptide is further facilitated by the loop-like structure of the reduced form, which brings the thiolate groups into sufficient proximity. The 9 group-specific protonation constants and the related, charge-dependent, species-specific peptide structures are presented.

Keywords: thiol, oxidizability, disulfide, pKa.

Introduction

The co-dependent thiolate-disulfide redox and thiolate-thiol acid-base equilibria are crucial components to maintain redox homeostasis and to form the solution structure of cysteine-containing peptides. These issues have recently attracted significant scientific interest.^[1,2] While the related works mainly study molecules of one single thiol group and the concomitant heteronuclear disulfide formation,^[3,4] no thorough analysis appeared on the parameters and mechanism of basicity-influenced multiple intramolecular disulfide formations, despite the fact that natural compounds of appropriate properties for such studies do exist, as follows.

Marine cone snails from the genus *Conus* produce a plethora of cysteine-enriched peptides that act as neurotoxins.^[5] These conotoxins, approximately 50.000 in total, each have very specific pharmacological activities,^[6] becoming thus the subject of intensive

biochemical research. The A-conotoxin superfamily contains the α -conotoxins, which bind to nicotinic acetylcholine receptors (nAChR).^[7] One member of this family is the relatively small α -conotoxin MI of *Conus magus* (GRCCHPACGKNYSC, with disulfide linkages between C3–C8 and C4–C14).^[8–10] This relatively small peptide is important in the field of drug research on nAChRs.^[11] Concerning also the intriguing mechanism of multiple-choice disulfide bond formations, this peptide is also a suitable candidate for the investigation of the effect of thiolate protonation constants on disulfide bond formation patterns. While the native disulfide pattern in oxidized α -conotoxin MI was determined by Gray *et al.*^[9] to be uniform, it is known that these peptides can exhibit various disulfide patterns after reduction and guided oxidation under certain circumstances;^[12] i.e., ribbon (C3–C14/C4–C8) and bead (C3–C4/C8–C14) patterns besides the native globular pattern (C3–C8/C4–C14).

The solution structure of α -conotoxin MI was determined by Gouda *et al.* with $^1\text{H-NMR}$ spectroscopy (at pH 4.1),^[13] and refined with molecular dynamics simulation.^[14] In their work the oxidized peptide showed a 3_{10} helix and type I β -turn motif and significant structural similarity with other α -conotoxins. In their proposed mean structure, the tyrosine residue is placed roughly in the center of the structure, the N-terminal looped back in close proximity to the C-terminal, while the lysine and arginine side-chains point outward.

The objective of this study was to explore possible relationship(s) between the thiolate basicities and the pattern of disulfide bond formation, and the concomitant peptide structure. Therefore a reduced derivative of α -conotoxin MI (Figure 1) was used to determine

the group-specific protonation constants of the thiolates using $^1\text{H-NMR-pH}$ titrations. Note that in aqueous media, and pH below 13 the arginine side-chain is protonated, therefore, the positive charge on the guanidinium site is permanent throughout the pH scale. The remaining basic moieties can be characterized in terms of group-specific protonation constants, as described earlier in detail for other peptides.^[15] Due to the sufficiently large number of covalent bonds between any two sites, the protonation of every basic site takes place largely independently of the protonation state of the other sites. Note that for the C-terminal cysteine, the protonation of the thiolate and carboxylate occur at distant pH regions; therefore, they can also be dealt separately, treating them as

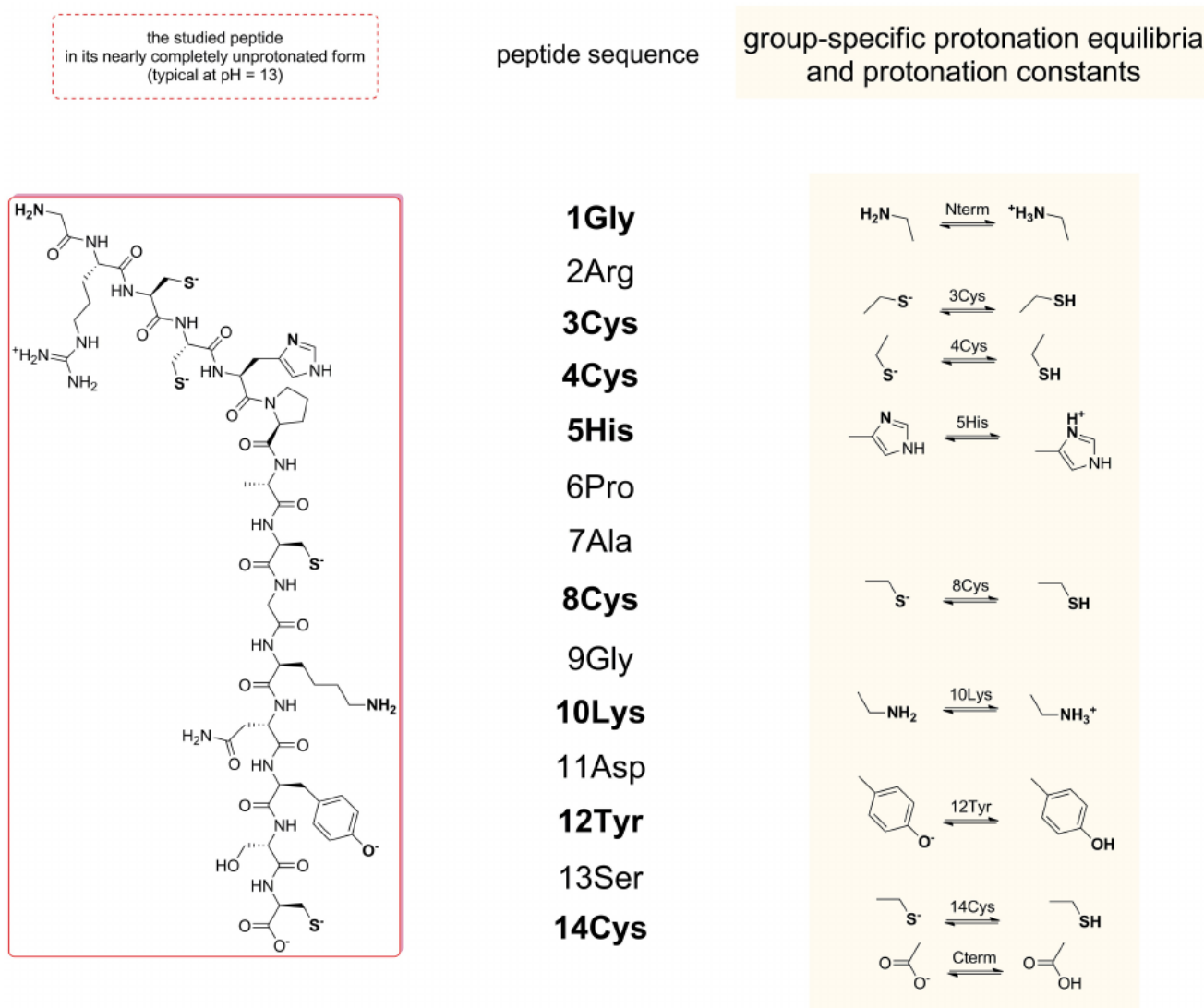


Figure 1. The group-specific protonation steps of the peptide with the symbols of groups undergoing protonation on the equilibrium lines.

site-specific constants, belonging to the major protonation pathway.

The hypothesis of our study is that since acid-base and redox characteristics of thiolates correlate, the thiolate groups with the highest protonation constants and therefore, highest oxidation propensities will form disulfide bridges first, unless a strong alternate conformational preference exists. In our work, the acid-base characteristics of the peptide are presented along with the structural factors that influence the native disulfide bond formation in α -conotoxin MI.

Results and Discussion

COSY, TOCSY, NOESY Assignment at pH=4.5

A 0.5 mmol/L aqueous solution (containing 5 V/V% D₂O) of the peptide was prepared at pH=4.5 (277 K and 0.15 mol/L ionic strength). The average charge of the peptide at this pH is +3, with a plateau, shown by

preliminary pH-titration experiments. The complete assignment of the peptide was performed using the COSY and NOESY spectra (Figure 2) and confirmed using the TOCSY spectrum of the peptide under the same conditions as above. The assignment table is in Table 1.

¹H-NMR-pH Titration

Following the assignment of the ¹H-NMR signals a D₂O solution of the peptide was titrated at 298 K and 0.15 mol/L ionic strength (adjusted with NaCl) with DCl and NaOD. A D₂O solution was used to perform the titration since the essential α H ¹H signals for the evaluation are close to the water signal and are often suppressed in ¹H measurements with solvent suppression. Titration curves corresponding to the basic moieties were fitted to the titration data for each select signal in order to obtain the group-specific protonation constant of the basic moieties. The fitted

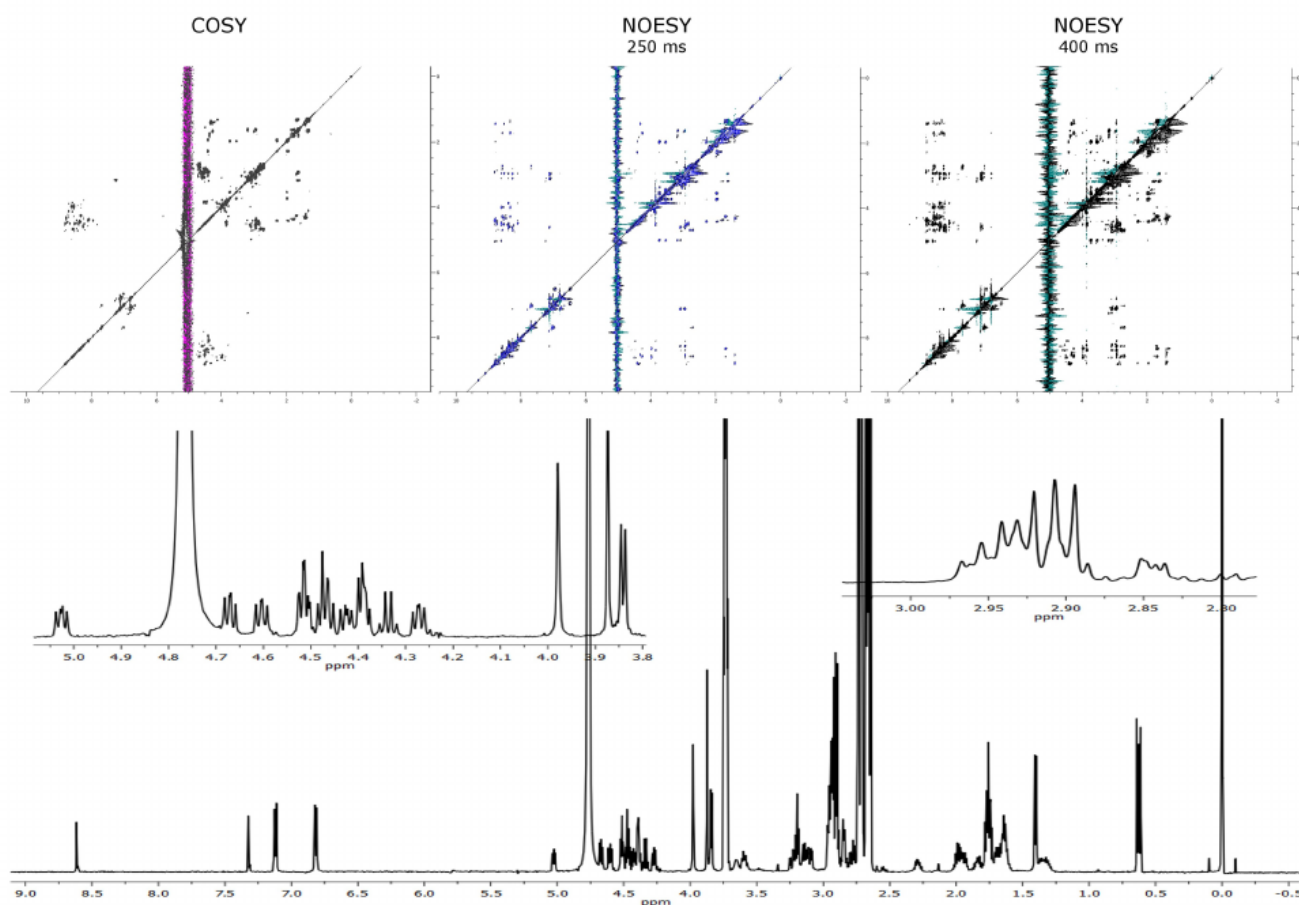


Figure 2. The COSY and two NOESY spectra (latters with two different mixing times) of the peptide in a pH=4.5 aqueous solution (containing 5 V/V% D₂O) – above. The ¹H spectrum of the peptide in a pH=4.3 solution with the α H region and Lys ϵ region enlarged – above.

Table 1. The ^1H signal assignment table of the peptide at $\text{pH}=4.5$. Chemical shift values are referenced to the methyl signal of internal DSS in aqueous solution containing 5 V/V% D_2O .

| | NH | αH | βHa | βHb | | | | |
|-------|------|------------------|------------------|------------------|--------------------------|--------------------------|--------------------------|--------------------|
| 1Gly | | 3.85 | | | | | | |
| 2Arg | 8.78 | 4.37 | 1.76 | 1.83 | 1.63(γ) | 3.18(δ) | 7.24(ω) | |
| 3Cys | 8.80 | 4.50 | 2.89 | | | | | |
| 4Cys | 8.73 | 4.46 | 2.84 | | | | | |
| 5His | 8.61 | 4.99 | 3.09 | 3.18 | 7.63(2H) | 6.96(5H) | | |
| 6Pro | | 4.42 | 1.94 | 2.29 | 1.97(γ) | 3.54(δa) | 3.75(δb) | |
| 7Ala | 8.78 | 4.33 | 1.41 | | | | | |
| 8Cys | 8.52 | 4.51 | 2.94 | | | | | |
| 9Gly | 8.60 | 3.98 | | | | | | |
| 10Lys | 8.33 | 4.26 | 1.68 | 1.69 | 1.29(γa) | 1.34(γb) | 1.63(δ) | 2.94(ϵ) |
| 11Asn | 8.57 | 4.68 | 2.70 | 2.79 | | | | |
| 12Tyr | 8.26 | 4.59 | 2.94 | 3.09 | 7.12(2H,6H) | 6.80(3H,5H) | | |
| 13Ser | 8.38 | 4.47 | 3.85 | | | | | |
| 14Cys | 7.99 | 4.39 | 3.10 | 3.25 | | | | |

titration curves and the measured data points are in *Figure 3*. The proposed protonation scheme of the peptide is depicted in *Figure 1* indicating the protonating moiety on top of equilibrium lines. Due to the large covalent distance between basic groups in the peptide it can be well assumed that none of the basic moieties can influence the protonation of any of the other basic moieties. Therefore, essentially all basic moieties protonate as independent monoprotic sites. This assumption does not hold for the 14Cys thiolate and carboxylate, however, since these moieties protonate at vastly distant pH regions, their observed protonation constants can be regarded as two separate monoprotic group-specific protonation constants along the major protonation pathway as depicted in *Figure 1*; i.e., the carboxylate protonation is only considered after the 14Cys thiolate has been fully protonated. The fitting of titration curves is best-conditioned on ^1H signals that lie closest to the protonating site in the molecule and therefore have the highest selectivity for those protonation shifts. This was not always feasible when βH signals were not discernable; therefore, αH signals were used as acceptable alternatives. The protonation constants are compiled in *Table 2* and the average charge of the peptide as a function of pH is presented in *Figure 4*. Note, that due to the similar chemical shifts of 3Cys and 8Cys, the titration data of these two residues could not be assigned by high level of certainty. This has caused, however, no real ambiguity since the protonation constants of these moieties are practically identical (see *Table 2*).

Table 2. The group-specific protonation constants of the peptide in \log_{10} value \pm standard deviation of the fitted values from the regression analysis.

| | $\log K$ |
|--------------|------------------|
| 3Cys or 8Cys | 8.53 ± 0.02 |
| 8Cys or 3Cys | 8.52 ± 0.09 |
| 4Cys | 8.97 ± 0.1 |
| 14Cys | 8.91 ± 0.03 |
| Nterm | 7.87 ± 0.03 |
| 5His | 6.24 ± 0.02 |
| 10Lys | 10.41 ± 0.09 |
| 12Tyr | 10.16 ± 0.01 |
| Cterm | 3.11 ± 0.03 |

CD Measurements

Following the titration experiments, the peptide solution samples were used to measure circular dichroism spectra at select pH values encompassing the entire pH region of protonation in order to gain insight in the secondary structure motifs that may be present in the peptide. Unfortunately, the CD spectra revealed no characteristic structural elements or a tertiary structure throughout the protonation of the reduced peptide. Some CD spectra are shown in *Figure 5*.

Structure Determination

In order to be able to deduce structure-chemical property relationships the conformation of the peptide was studied in a $\text{pH}=4.5$ medium using the NOESY peaks recorded at 2 different mixing times as described in the Materials and Methods section. The

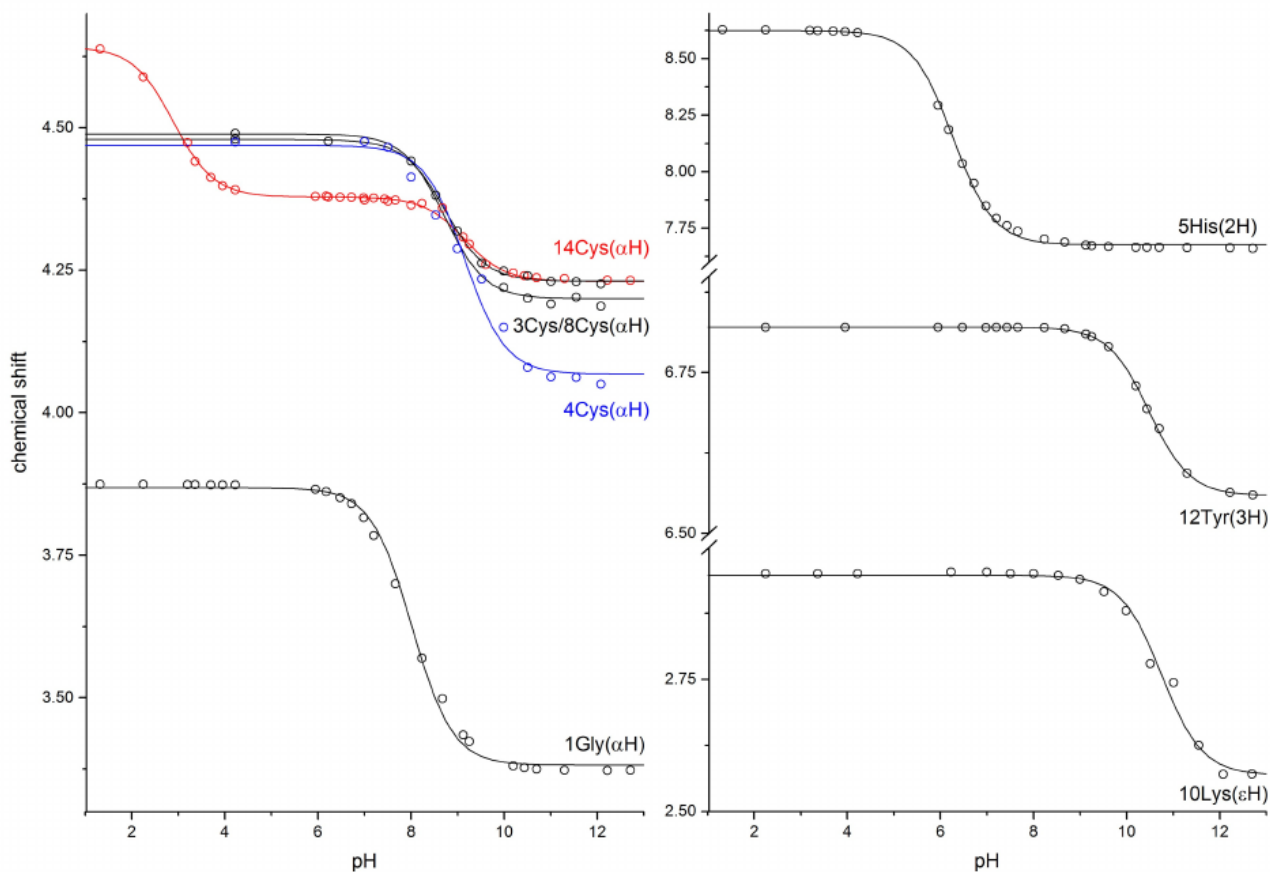


Figure 3. The ^1H -NMR-pH titration data and fitted curves of the peptide. The reading of the overlapping signals was aided with COSY and ^1H - ^{13}C HSQC measurements. The chemical shifts reported are referenced to the methyl signal of internal DSS in D_2O solution.

structure refinement produced 7 alternative structures; of which the best mean structure is presented here. We also attempted to determine the structure of the peptide under basic conditions where the average charge of the peptide is close to its minimum value of -5 and all basic moieties (except for the arginine guanidino) are unprotonated. These measurements, however, were hindered due to the appearance of a set of minor signals due to minor conformers and disappearance of the amide signals. The conformation search of the aforementioned protonation states (i.e., with +3 and -5 charges) was performed with molecular dynamics simulations. For each species the conformation search unambiguously identified one major conformation. The structures are presented in Figure 6.

Protonation constant values of the four cysteine residues in the reduced form of α -conotoxin MI, clearly indicate that the cysteine residues with similar $\log K$ values form disulfide linkages in the native form of the peptide. This supports the hypothesis that those cysteine residues tend to form disulfide bonds first that have the largest protonation constant values and therefore, the highest oxidizability propensities within the peptide. As it has been shown previously,^[16] thiolate basicities correlate with thiolate redox potentials; this correlation allows the calculation of the group-specific standard redox potential of the four cysteine residues. These values are as follows:

$$3\text{Cys and } 8\text{Cys } E^\circ = -0.350 \pm 0.01 \text{ V};$$

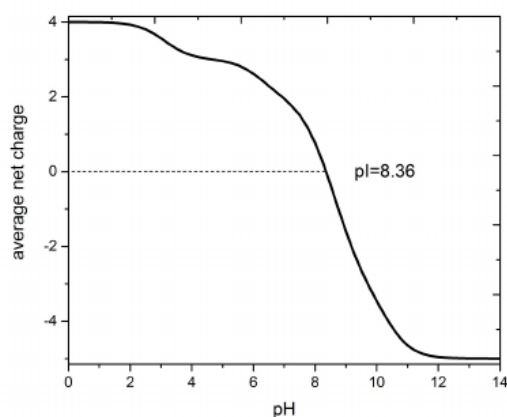


Figure 4. The calculated average charge of the peptide as a function of pH. For the structure determination experiments, pH=4.5 was chosen, as the peptide occurs predominantly in a +3 charged state at this plateau.

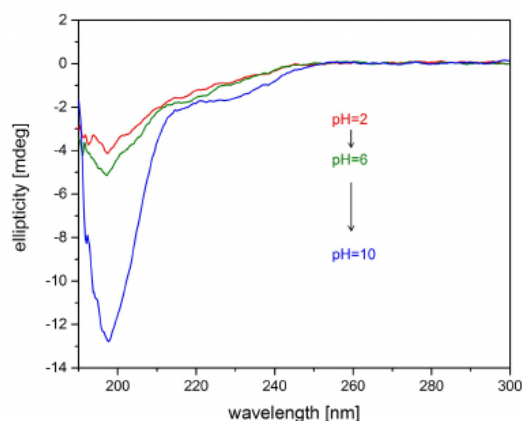


Figure 5. The accumulated CD spectra of the peptide at different pH values in aqueous solution.

$$4\text{Cys } E^\circ = -0.377 \pm 0.015 \text{ V};$$

$$14\text{Cys } E^\circ = -0.374 \pm 0.005 \text{ V}.$$

It was uniformly demonstrated by the CD and NMR measurements, and molecular dynamics simulations that the structure of the peptide at pH=4.5 exhibits no classical secondary structures. However, it is noteworthy that the reduced peptide forms a loop-like structure similar to that of oxidized α -conotoxin M1 with the same charge.^[13] In this conformation the termini come into close proximity with the tyrosine residue positioned in the center. This conformation of the reduced peptide enables the thiol groups to join

and form the disulfide bond via oxidation. Based on molecular dynamics simulations the completely deprotonated form of the reduced peptide at pH=12.5 demonstrates an untethered structure that locates the negatively charged thiolate groups in distant positions (Figure 5).

The peptide structure at intermediate pH values can be approximated by a structure that leads from the structure at pH=4.5 to the structure at pH=12.5. The oxidation of the completely deprotonated peptide therefore can result in a mixture of different oxidized products (globular, ribbon, bead) due to greater variabilities in the conformation. It is therefore reasonable to assess that the enzymatic oxidation of the peptide in the organism is essential for the correct disulfide linkages to form, as it has also been observed in our titration experiments that at pH values above 10 a mixture of oxidized products is formed due to the oxidation of the peptide by air, as indicated by the appearance of the NMR signals of the oxidized product.

Conclusions

In this work it was demonstrated that conformation and protonation changes in a peptide go hand-in-hand and are vital for the peptide functionality and correct folding. Since the pH-dependent conformational changes often change the proximal environment of the basic moieties, as can be seen in the determined structures, it can be stated that the protonation constants of the basic moieties are different for each conformation of the peptide, therefore, great care must be used when discussing the acid-base character of the residues.

Experimental Section

Materials

The reduced form of α -conotoxin M1 with free termini (sequence: GRCCHPACGKKNYSC) was purchased from ProteoGenix (Schiltigheim, France). All other reagents were purchased from Merck (Munich, Germany) and used without further purification.

NMR Measurements

For the pH-titration experiments NMR spectra were recorded on a 14.1 T Varian Unity Inova DDR spectrometer with a 5 mm $^1\text{H}\{^{13}\text{C}/^{31}\text{P},^{15}\text{N}\}$ pulse field

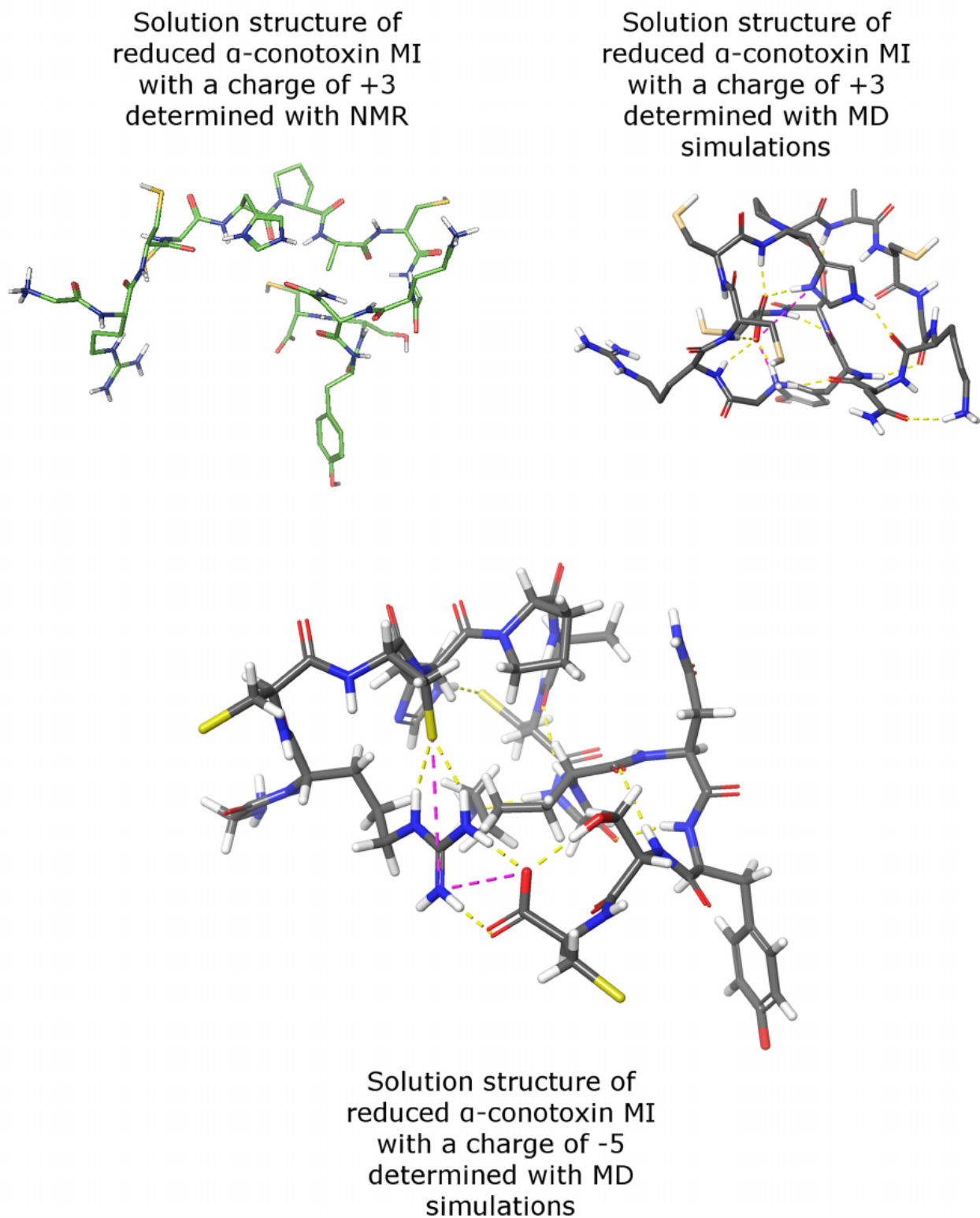


Figure 6. The solution structure of reduced α -conotoxin MI with charge +3 (top) and -5 (below) determined with-NMR measurements and MD simulations. In the structures determined with MD simulations pink dashed lines indicate ionic interactions and yellow dashed lines indicate H-bonds.

gradient triple resonance probehead at 298 K. The solvent was D₂O, ionic strength was adjusted to 0.15 mol/L and the spectra were referenced to the methyl signal of internal 3-(trimethylsilyl)-1-propane-sulfonate (DSS). The pH of the samples were determined by direct readings from a Metrohm 6.0204.100 combined pH glass electrode calibrated with aqueous NBS standard buffers, then, the direct readings were converted to corrected pH values using the work of Krézel et al.^[17] The solutions also contained dithiothreitol to preclude oxidation. Non-linear regression analysis on the titration data was carried out using Origin Pro 8 (OriginLab Corp., Northampton, MA, USA) with the following function:

$$\delta_{\text{obs}}(\text{pH}) = \frac{\delta_{\text{L}} + \delta_{\text{HL}} \times 10^{\log K - \text{pH}}}{1 + 10^{\log K - \text{pH}}} \quad (1)$$

where δ_{L} is the chemical shift of an unprotonated moiety, δ_{HL} is the chemical shift of the protonated moiety, and K is the group-specific protonation constant; log henceforth refers to the base 10 logarithm.

The structure determination experiments were performed with a 500 μL sample containing 1.49 mg peptide at pH=4.55 (277 K); under such acidic conditions oxidation by air is not significant. Datasets were collected with a 16.4 T Bruker Avance III spectrometer equipped with a 5 mm inverse TCI probe-head with z-gradient. Spinlock for ¹H-¹H TOCSY was 80 ms, while a mixing time of 150, 250 and 400 ms was taken for ¹H-¹H NOESY spectra. NMR structure calculations were performed and refined by cooperative use of CcpNmr Analysis 2.4.1, Aria 2.061 and CNS Solve 1.2.62.

CD Measurements

CD and UV spectroscopic measurements were performed using a Jasco J-815 spectropolarimeter (JASCO International Co., Ltd, Tokyo, Japan) equipped with a Jasco CDF-426 combined fluorescent/CD accessory attachment, a Peltier type thermostat, and sample stirrer functionality.

Molecular Dynamics Simulations

The peptide structures were introduced in the Schrödinger software using Maestro (Schrödinger Suite (Schrödinger Release 2020-4: Maestro, Schrödinger, LLC, New York, NY, 2019)). Maestro was also used for

generating the figures shown in the text. Conformation search was carried out with the OPLS3e force field and a mixed torsional/low-mode sampling method. Molecular dynamics simulations were carried out as implemented in the Desmond application (Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2021. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2021),^[18] using the NPT ensemble to achieve atmospheric pressure and 300 K temperature. The system was solvated with SPC water molecules in orthorhombic boxes with a size allowing 10 Å distances. The total charge was neutralized with Na⁺/Cl⁻ ions to a final salt concentration of 0.15 mol/L. Energy minimization of the starting structures was allowed for a total simulation time of 100 ps.

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Author Contribution Statement

Z. F., A. M., D. H., and P. H. performed the experiments, analyzed the data, and wrote the article. T. P. contributed to the samples/reagents/materials/analysis tools and analyzed the data. A. P. and B. N. conceived and designed the experiments.

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