Peptide Conformations

DOI: 10.1002/ange.200502300

Effect of C-Terminal Amidation on Folding and Disulfide-Pairing of α-Conotoxin ImI**

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α-Conotoxins are disulfide-rich neurotoxins isolated from the venom of the marine predatory cone snail. These structurally compact toxins form well-ordered three-dimensional structures through the formation of two crucial disulfide bridges. All currently identified members of the α-conotoxin family exhibit a highly conserved four-cysteine framework, with an absolute preference for folding into the globular form (C1–C3, C2–C4) as the native conformation.[1–3] They specifically bind to nicotinic acetylcholine receptors. Recently, a new family of χ/λ-conotoxin was identified and shown to have conserved the positions of the cysteine residues characteristic of α-conotoxins. However, χ/λ-conotoxins revealed an alternative disulfide-pairing pattern (C1–C4, C2–C3) that results in the ribbon conformation[4–6] and distinct biological activity such as inhibition of the noradrenaline transporter by MrIA conotoxin.[6] Functional assays with the non-native conformations of both α-conotoxin and χ/λ-conotoxin resulted in a tenfold and three-order decrease in activities, respectively.[4,7]

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[**] This work is supported by the Biomedical Research Council, Agency for Science, Technology, and Research, Singapore. T.S.K. acknowledges the Singapore Millennium Foundation for granting the research scholarship. The authors thank the Sophisticated Instrument Facility at the Indian Institute of Science and the Chemical and Molecular Analysis Center, Department of Chemistry at the National University of Singapore for allowing the use of their NMR spectrometers.

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These results indicate that the disulfide pairing and the consequent conformation play a crucial role in the biological potencies of native toxins. To identify the structural features that determine specific disulfide pairing, we analyzed the amino acid sequences of α-[8–19] and χ/λ-conotoxins.[4–6] The α-conotoxins, with the exception of GID conotoxin, have a conserved C-terminal amide, whereas all members of χ/λ-conotoxins bear a free carboxylic acid at the C terminus. Herein we report the influence of C-terminal amidation in the determination of the folding tendencies of ImI α-conotoxin in vitro.

Oxidation of the synthetic ImI conotoxin (termed ImI amide) and the corresponding deamidated analogue (termed ImI acid) was conducted in either folding buffer (100 mM Tris-HCl, 2 mM EDTA, pH 8.5) or denaturant buffer (100 mM Tris-HCl, 2 mM EDTA, 6 M GndCl, pH 8.5) by oxidation with air and a glutathione redox system (Gnd = guanidinium; for detailed protocols, see the Supporting Information). Upon oxidation, each of the synthetic variants folded into three monomeric isoforms that correspond to the three peaks observed in the HPLC chromatograms. Each band contained peptides that were completely oxidized as was verified by a decrease by four mass units, which corresponds to the formation of two disulfide bridges (1351.35 ± 0.47 Da and 1352.65 ± 0.04 Da for ImI amide and ImI acid, respectively). The folding experiments conducted in the presence of redox systems also generated isoforms in similar proportions (Table 1).

Table 1: Oxidation studies of peptide analogues under folding and denaturant conditions.[a]

	% Total oxidized peptides		
	Globular	Ribbon	Beaded
A) Folding buffer			
ImI amide	54.0 ± 0.4	43.0 ± 1.0	3.0 ± 0.6
	(52.1±0.1)	(43.8±0.4)	(4.0±0.2)
ImI acid	30.1 ± 0.6	67.2 ± 0.4	2.7 ± 0.5
	(29.4±0.3)	(67.9±0.1)	(2.8±0.2)
ImI Gly	40.3 ± 0.1	57.6 ± 0.1	2.1 ± 0.03
	(41.5±0.3)	(54.0±0.8)	(4.5±0.5)
B) Denaturant buffer			
ImI amide	48.9 ± 0.1	39.7 ± 0.0	11.4 ± 0.1
	(48.3±0.5)	(38.0±0.4)	(13.7±0.1)
ImI acid	37.6 ± 0.7	44.6 ± 1.8	17.8 ± 2.4
	(50.3±0.3)	(39.8±1.1)	(9.9±0.1)

[a] The results shown in parentheses represent data acquired when the study was performed in the presence of a redox system.

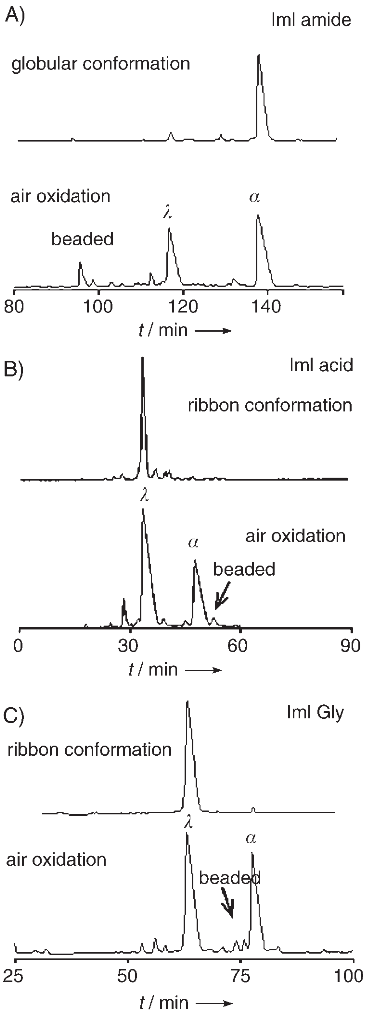

Figure 1. Chromatographic profiles of ImI conotoxin analogues. Comparison of the retention times for the dominant isoform of A) ImI amide B) ImI acid, and C) ImI Gly, with the corresponding regioselectively synthesized isoforms.

The various isoforms were initially identified by comparison of the retention times of the conotoxin isoforms with the corresponding regiospecifically synthesized globular and ribbon conformations (Figure 1). The dominant isoform of ImI amide coelutes with the globular form. In contrast, the major isoform of the deamidated ImI acid has the same retention time as its ribbon conformation but not its globular form. In folding buffer, ImI amide shows a tendency to fold into the globular conformation. Substitution of the C-terminal amide to free carboxylic acid, however, resulted in a switch in folding preference of ImI acid to that of the ribbon conformation. Thus, it appears that C-terminal amidation plays a pivotal role in the folding tendencies of ImI conotoxin.

In the presence of a denaturant, the overall folding tendencies remained the same as when the peptides were oxidized with air. However, the amounts of disfavored beaded conformation in both variants were found to have increased, and the presence of guanidine resulted in a "normalizing" effect on the lead of the predominant isoform in both variants. We therefore concluded that side-chain interaction has a significant, but non-absolute, role in the folding to the final conformation of ImI peptides.

To further confirm the disulfide pairings and the folding of ImI amide and ImI acid isoforms, we determined their three-dimensional structure by NMR spectroscopy. Figure 2A shows the amide region in the 1D ^{1}H NMR spectrum of the dominant forms of the two variants. The 1D ^{1}H NMR spectrum of the major isoform of ImI amide matches well with that of the force-folded globular conformation. Con-

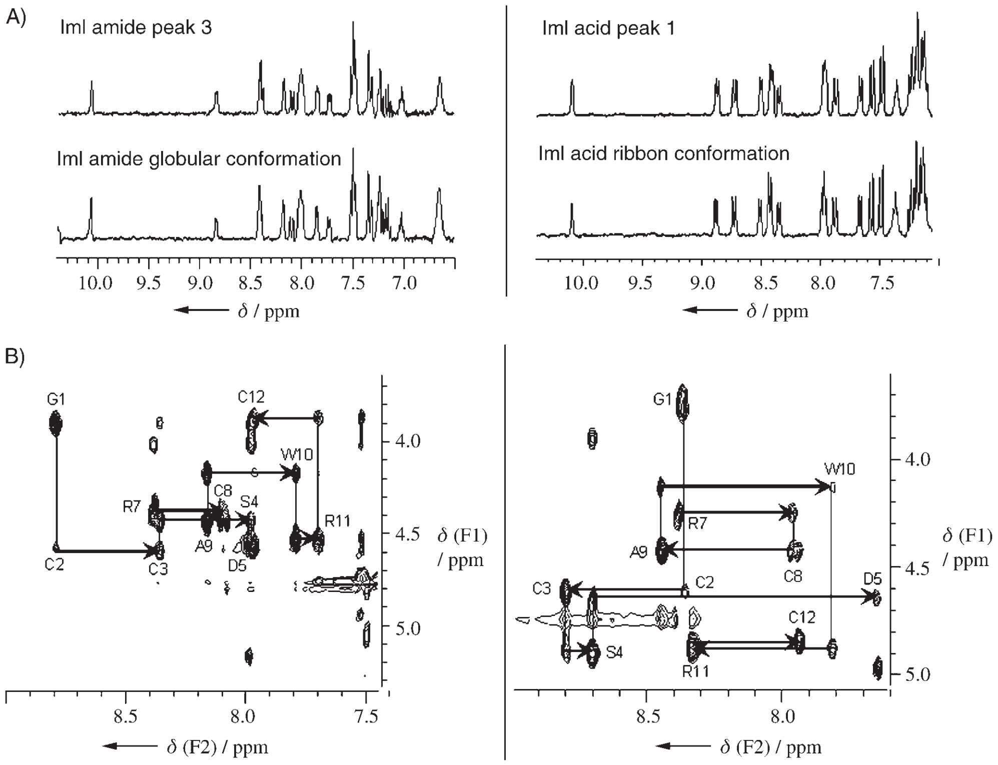

Figure 2. Structural differences between ImI amide and ImI acid. A) Comparison of 1D NMR spectra of the dominant isoforms of ImI amide and ImI acid with the corresponding globular or ribbon conformations. B) Sequential assignment of 2D NMR ROESY spectra of ImI amide (left) and ImI acid (right).

versely, the 1D ^{1}H NMR spectrum of the major isoform of ImI acid corresponds well to that of its ribbon form.

2D ^{1}H NMR spectroscopic experiments for ImI amide and ImI acid yielded good-quality spectra. The TOCSY spectrum obtained for ImI amide was similar to that reported earlier,[20] and the 88 distance constraints derived from its ROESY spectrum fitted well with the reported structure of ImI conotoxin. The 2D NMR ROESY spectrum for ImI acid yielded 110 distance constraints of which the presence of a strong αH–δH ROE between Asp5–Pro6 suggests a *trans* peptide bond between these two residues. Identification of the CβH–CβH ROEs between Cys3–Cys8 and Cys2–Cys12 is indicative of the disulfide-pairing characteristic of the ribbon conformation. The amide regions of the ROESY spectra revealed distinct chemical shift patterns between the globular form of ImI amide and the ribbon conformation that was seen with ImI acid (Figure 2B). Significant differences in chemical shift were observed despite the similarity in amino acid sequences between the analogues. These spectra further support the theory that ImI amide folds preferentially into the globular form and ImI acid into the ribbon conformation.

Figure 3A shows the three-dimensional structure of ImI acid calculated from 2D NMR constraints, the statistics of which are shown in Table 2. The solution structure clearly indicates that ImI acid folds into the ribbon conformation. As the ^{1}H NMR spectra for ImI amide were similar to those reported earlier, we used the structure previously published.[21] Figure 3B shows the superimposition of the ribbon conformation of ImI acid with that of the native globular conformation of ImI conotoxin (PBD accession code 1G2G). Based on ^{1}H NMR spectra, the solution structure of the isoforms, as well as coelution profiles, we concluded that ImI amide folds predominantly into the globular conformation with C1–C3, C2–C4 disulfide pairings, whereas ImI acid folds preferentially into the ribbon conformation with C1–C4, C2–C3 disulfide pairings. Thus the C-terminal amide has been shown to play a very important role in the determination of the disulfide pairing and resultant protein folding in these two classes of short conotoxins.

Amidation is caused by post-translational oxidative cleavage of a C-terminal glycine residue, and hence we also examined the folding of the precursor peptide with a further C-terminal glycine (termed ImI Gly). ImI Gly, with its free carboxylic acid, folded predominantly into a ribbon conformation (Table 1). Thus, the presence of a negatively charged group appears to favor the ribbon conformation even in the presence of a further C-terminal residue. Although the three sets of peptides fold into different predominant isoforms, the excess of one form over the other is not drastically different, which therefore suggests that the folding may occur through independent pathways. Figures 3C and D show the proposed hydrogen-bonding interactions of the C-terminal regions of ImI amide and ImI acid, respectively. These noncovalent interactions were derived from the averaged 3D structures calculated. The two variants exhibit distinct hydrogen-bonding patterns. It is not clear whether *cis–trans* isomerization of proline or the postulated hydrogen bonds contribute to these folding pathways.

In several earlier studies, a number of bioactive peptides in nature were shown to have undergone amidation at the C terminus. Such a modification has been shown to have a substantial impact on the biological activities of several of these peptides, presumably owing to the neutralization of negative charges conferred by the carboxylic acid at the C terminus.[22–26] In these cases, however, the C-terminal amide is not known to influence the conformation of the peptides. In earlier reports, post-translational modifications such as γ-carboxylation of glutamate residues have been shown to be important for the folding in P-superfamily

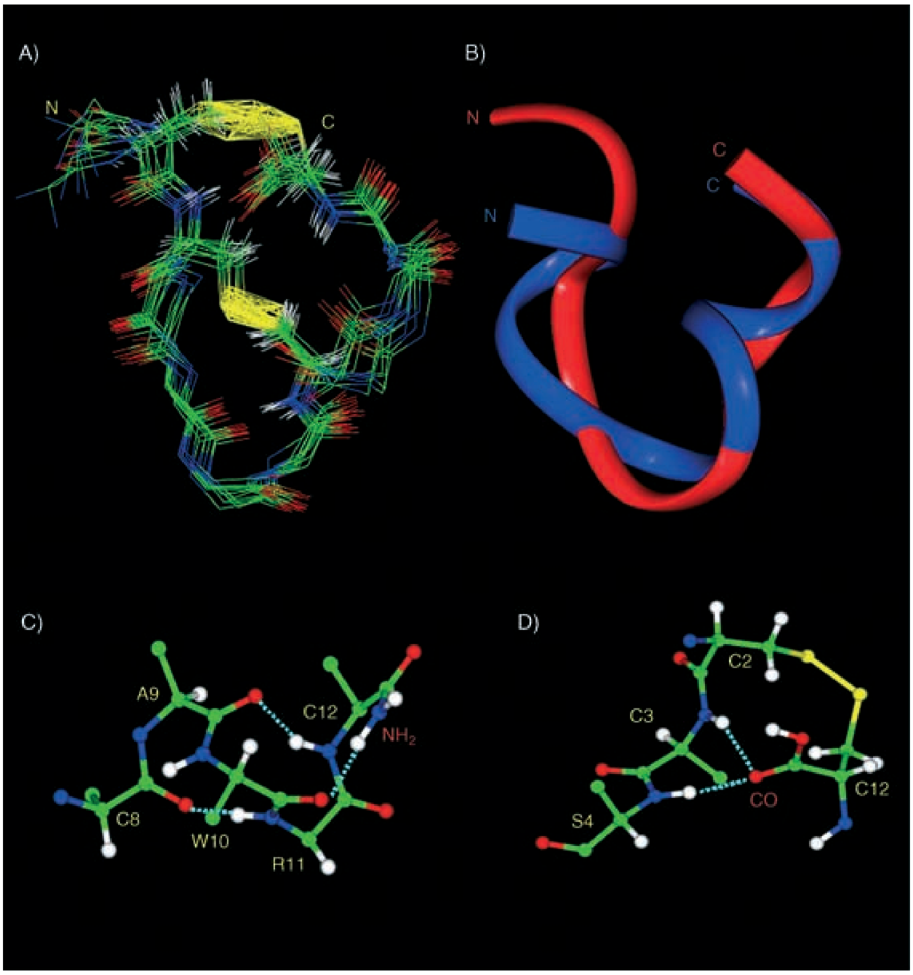

Figure 3. Comparison of solution structures of ImI amide and ImI acid. A) Backbone superimposition of 15 structures of ImI acid determined by NMR spectroscopy. B) Backbone overlay of ImI acid structure (red) with ImI amide (blue). Proposed hydrogen-bonding interactions in the C-terminal region of C) ImI amide and D) ImI acid. The hydrogen bonds are labeled with cyan broken lines, and the C-terminal amide and carbonyl groups are labeled in red.

Table 2: Summary of statistics for structure calculations.

Restraining NMR constraints	RMSD[a]
intra residue = 73	backbone atoms: 0.48 ± 0.10
short range (distance $\lvert i{-}j \rvert = 1$) = 23	heavy atoms: 0.70 ± 0.14
medium range (distance $1 < i \leq 2$) = 4	all atoms: 0.84 ± 0.13
long range (distance > 2) = 10	
total = 110	

[a] RMSD = root-mean-square deviation.

conotoxins.[27,28] This is the first example in which amidation was shown to influence the folding tendencies and hence the biological activity of two-disulfide-bonded conotoxins.

Received: July 1, 2005
Revised: July 26, 2005

Keywords: amidation · neurotoxins · NMR spectroscopy · peptides · protein folding

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