Unusually stable helical kink in the antimicrobial peptide – A derivative of gaegurin

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Abstract The structure of an active analog of the antibacterial peptide gaegurin was investigated by CD and NMR spectroscopy. The NOE connectivities showed that 21 out of 24 residues formed an α-helix despite the presence of a central proline. CD and NMR analysis indicates that the helix is in fast equilibrium with random coil. From chemical shift analysis of the amide protons, the distances of hydrogen bonding in the helix were calculated, and manifested obvious periodicity which implied a kink in the middle of the helix. 1D amide proton exchange experiments provided further evidence of an exceptionally stable kink. It is inferred that this kink is important not only to the function of the peptide but also to the early stage of the folding as a nucleation site.

Key words: Gaegurin; NMR; α-Helix; Kink

1. Introduction

Antimicrobial peptides have been found in a broad spectrum of species from insects to human beings [1–6]. A few well-known examples are mellitin [3,4] in bees, cecropin [5] in moths and magainin [6] in frogs. These peptides respond to the infection of bacteria or fungi by destroying the foreign bodies to protect the host. Structural studies of the antimicrobial peptides have revealed two main structural motifs. One, the α-helical motif, functions as an ion channel to perturb the membrane of the microorganism [7]. Mellitins and cecropins are included in this class. The other is the motif generally found in the defensin series. The structures of defensins are divided into two groups. Human and other mammalian defensins were found to have a triple β-sheet structure [8], while insect defensins were found to form two-stranded β-sheets with a flanking α-helix [9]. They all contain three disulfide bonds, but the topology of the disulfide bonds differs. Other categories are yet to be described.

Recently, gaegurin, an antimicrobial peptide which exhibits a broad spectrum of activity was isolated from a species of Korean frog [10]. From the six peptides in this series, we chose to study gaegurin-6 which is a 24 amino acid peptide. A derivative (GO 1-1), in which two cysteines in the C-terminal segment were replaced by serines, was shown to be as active as the wild type. We used this analog in our studies because the disulfide did not seem to play an important role in activity. The peptide has two prolines, one in the N-terminal segment and the other in the middle of the peptide. Among the antimicrobial peptides containing central prolines, mellitin is known to form an amphiphilic helix. However, mellitin has high hemolytic activity as well as antimicrobial activity. Gaegurin is reported to have a high and broad spectrum of antimicrobial activity with little hemolytic activity, which makes it a promising antibiotic agent.

2. Materials and methods

2.1. Peptide synthesis

The peptide (F-L-P-L-L-A-G-L-A-A-N-F-L-P-T-I-I-S-K-I-S-Y-K-S) was chemically synthesized using standard procedures for solid-phase peptide synthesis on an Applied Biosystems peptide synthesizer. All amino acids were protected at their α-amino groups with the Fmoc group The side-chain protecting groups were the *t*-butyl group (Ser, Thr, Tyr), and *t*-butyloxycarbonyl group (Lys). The fully synthesized peptide was applied to a TFA cleavage step, and after ether extraction, was purified on the RP-HPLC system.

2.2. CD spectroscopy

CD spectra were obtained on a Jasco J-600 spectropolarimeter using a 1 mm cell. The concentration was 200 μm and the spectra were recorded at ambient temperature. The solvent was a mixture of water and TFE (1:1, v/v). Preliminary tests showed that helix prevailed and all spectroscopy was performed in the water/TFE system.

2.3. NMR spectroscopy

All spectra were obtained on a Bruker AMX 500 MHz spectrometer at 295 K and the data were processed on a Silicon Graphics computer using the program FELIX 2.30. The peptide concentration was 2.2 mM. The solvent was a binary system of unbuffered water and TFE-d_3. pH was adjusted to 6. For sequential assignment, TOCSY and NOESY spectra were obtained. The TOCSY spectrum was recorded with a mixing time of 75 ms with MLEV-17 composite pulses used for the spin lock. The trim pulse was not used to reduce phase distortion in the water suppression process. NOESY spectra were recorded with mixing times of 100 and 250 ms. All spectra were acquired with 2K data points in the t_2 dimension and 512 points in the t_1 dimension. The time-domain data were zero-filled in both directions and $\pi/3$ or $\pi/2$ shifted squared sine bell functions were applied before Fourier transformation. A 1D amide proton exchange experiment was carried out in D_2O/TFE-d_3 solvent. The peptide was dissolved in water and lyophilized a few times. Then the freshly prepared sample was dissolved in D_2O/TFE-d_3 and 1D spectra were recorded in the pre-shimmed probe.

3. Results and discussion

CD indicated that the peptide was 50% helical while the secondary structure prediction program predicted 54.2% helix [11,12]. NOE connectivities, however, showed a remarkably

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Abbreviations: 1D, one-dimensional; CD, circular dichroism; Fmoc, 9-fluorenylmethyloxycarbonyl; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; RP-HPLC, reverse phase-high performance liquid chromatography; TOCSY, total correlation spectroscopy.

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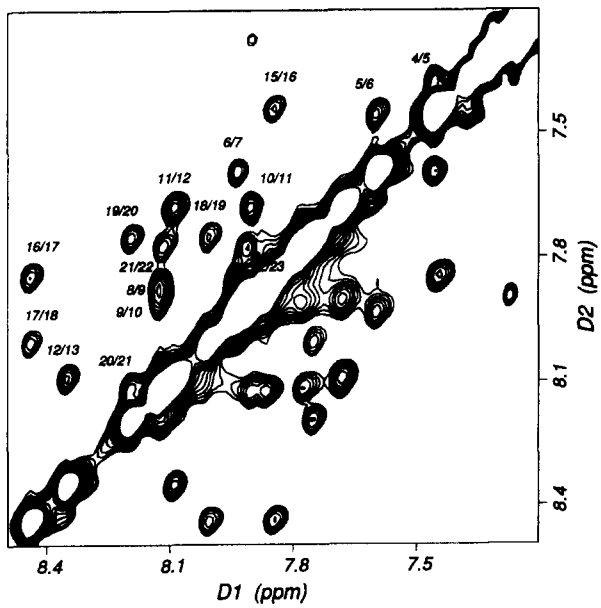

Fig. 1. Amide proton region of ^{1}H 2D NOESY spectrum of GO 1-1 in H_2O/TFE-d_3 (1:1) at pH 6 and 22°C. All cross-peaks between amide protons are annotated.

higher helical content of 87.5%. Sequential assignment was used to assign all the cross-peaks in the NMR spectra [13]. The chemical shifts of the proton resonances are given in Table 1. NH-NH connectivities, which are evidence of a helical structure, were found from residue 4 to 24, except for the central proline which has no amide proton (Fig. 1). Instead, NOEs between the δ protons of Pro-14 and the amide protons of adjacent residues were observed. Normally, proline does not permit helix formation because of its inability to hydrogen bond, but the NOEs between Pro-14 and its flanking residues imply that there is largely helical structure, if not regular α-helix, near Pro-14. A summary of all characteristic NOE connectivities is shown in Fig. 2. The characteristic amide to amide (*i*,*i*+1) and α proton to amide (*i*,*i*+3 and *i*+4) NOEs are observed. Considering both the CD and NMR data, we infer that a 21-residue-long helix is formed in the peptide and the helix is somewhat flexible so that there is a populational ensemble between helix and random coil.

To determine which part of the helix is stable, we investigated the chemical shift of each amide proton. ^{1}H-NMR chemical shifts have been shown to be strongly dependent on the character and nature of protein secondary structure [14]. In general, it was found that the α proton of all 20 naturally occurring amino acids experiences an upfield shift (with respect to the random coil values) when in a helical structure, and a comparable downfield shift when in a β strand or extended structure. We used reference values previously reported [15]. Chemical shifts of the α protons in gaegurin showed a typical upfield shift tendency, which is shown in Fig. 3A. The residues next to Pro-3 and Pro-14 showed the largest upfield shift of their amide protons. Most of the peptide showed the upfield shift expected of α-helix. Residues with a downfield shift or no shift were L2, L4, L5, L8, F12, L13, and I20 (see Table 2). The trends in the chemical shifts, shown in Fig. 3A, indicate helix in the same regions as the assignments 4–24.

We calculated the hydrogen bonding distance from the difference of the chemical shifts of amide protons between gaegurin and random coil. First, we obtained the difference of the

Table 1
Proton chemical shifts of GO 1-1 peptide[a]

	NH	αH	βH	γH	δH	Others
Phe-1		4.20	3.16,2.97			
Leu-2	7.78	4.63	1.51	1.51	0.88	
Pro-3		4.34	2.24,1.98	1.97	3.71,3.43	
Leu-4	7.36	4.13	1.59	1.58	0.93,0.86	
Leu-5	7.45	4.15	1.63	1.62	0.91,0.83	
Ala-6	7.59	4.08	1.42			
Gly-7	7.93	3.84,3.78				
Leu-8	7.86	4.17	1.82,1.74	1.61	0.86	
Ala-9	8.12	4.02	1.44			
Ala-10	7.90	4.08	1.44			
Asn-11	7.67	4.58	2.56,2.66			side chain NH 7.15,6.54
Phe-12	8.08	4.63	3.14			ring H 7.26,6.95
Leu-13	8.35	4.25	1.82,.167	1.45	0.92	
Pro-14		4.19	2.28,1.83	2.16	3.68,3.47	
Thr-15	7.44	3.93	4.32	1.21		
Ile-16	7.84	3.75	1.94	γCH_3 0.86, γCH_2 1.51,1.10	0.77	
Ile-17	8.44	3.69	1.83	γCH_3 0.86, γCH_2 1.59,1.21	0.77	
Ser-18	8.00	4.14	4.01			
Lys-19	7.74	4.21	2.03,1.93	1.50	1.64	εCH_2 2.92, NH_3^+ 7.53
Ile-20	8.19	3.94	1.95	γCH_3 0.89, γCH_2 1.63,1.23	0.79	
Ser-21	8.11	4.26	3.93,3.83			
Tyr-22	7.77	4.40	3.14			ring H 7.12,6.89
Lys-23	7.90	4.18	1.85,1.80	1.40	1.67	εCH_2 2.98, NH_3^+ 7.54
Ser-24	7.89	4.31	3.87,3.80			

[a]The chemical shifts are given in ppm and referenced to trifluoroethanol methylene resonance (3.88 ppm). The estimated error is ±0.02 ppm.

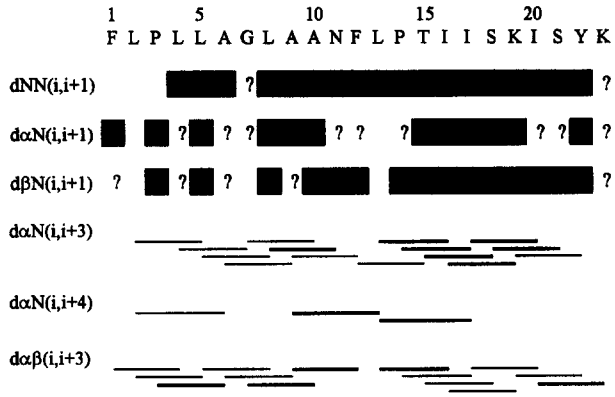

Fig. 2. Summary of proton-proton NOE connectivities of GO 1-1 in H_2O/TFE-d_3 (1:1). The intensities of NOE cross-peaks are indicated by the thickness of the lines. NOEs including the δ proton of proline are shown in gray. Ambiguities arising from overlapping of cross-peaks are indicated by question marks.

chemical shifts between amide protons in the α-helix and the random coil. Then the hydrogen bonding distances were calculated according to the equation $\Delta\delta_{HN}=19.2d^{-3}-2.3$ in which $\Delta\delta_{HN}$ is the difference of chemical shifts in ppm and d is the hydrogen bonding distance in Å [16]. The equation has been reported to be valid in water/TFE solution by comparison between chemical shift calculation and computer-modeling experiments of a curved α-helix [17]. In α-helix, each amide proton i makes a hydrogen bond to the carbonyl oxygen of the $i-4$ residue in the chain. The strength of hydrogen bonding is reported to be proportional to the distance between the donor and the acceptor. There was a remarkable periodicity in the hydrogen bonding distances. That is, the hydrogen bonding was strengthened and weakened in the center of the helix – near Pro-14. The strong hydrogen bonding forming amide protons were those of Leu-13, Ile-17, and Ile-20 (Fig. 3B). Ala-9 also showed a reinforced hydrogen bonding pattern in its local region. This peptide exhibits some amphiphilicity, with the strong hydrogen bonding forming residues roughly on the hydrophobic side.

We measured the exchange rates of amide protons to see how much they are shielded from solvent molecules. Residues N11, L13, I17, S18, and I20 showed protection, indicating some hydrogen bonding. The amide proton of Ile-17 showed unusual stability – it persists for over 48 h (Fig. 4). This is uncommon for short peptides. Furthermore, the amide proton of Ile-20 remained for about 3 h, with the amide proton of Leu-13 remaining for about 30 min. Other amide protons exchanged in about 10–15 min except for Ser-18 which persisted for 20 min. Even though Pro-14 presumably distorts the geometry of the peptide, there is very strong hydrogen bonding near this position. One possible explanation is that there is a kink made by Pro-14 and the amide proton of Ile-17 is in the concave face of the kink strengthening the hydrogen bonding. In this model, the amide proton is buried in the kink and surrounded by the side chains of other residues which screen it from solvent molecules. The stable amide protons of Leu-13 and Ile-20 indicate that the lack of upfield shift by Leu-13 and Ile-20 was not due to random coil population, but due to some distortion by Pro-14. They are also in the concave face of the kink, but are more accessible to the solvent molecules than Ile-17. It had been reported that there is a periodicity in hydrogen bonding distances in a designed amphiphilic α-helical peptides [17]. The amphiphilic peptide was shown to have a curved helical structure with hydrophilic face on the convex side and the hydrophobic face on the concave side. We see a similar periodicity in our peptide. These data suggest that Pro-14 kinks the helix and causes a similar characteristic periodicity in the hydrogen bonding length as that in the amphiphilic peptide. Furthermore, Pro-14 strengthened the kink with unusual stability as shown in the 1D exchange

Table 2
Differences of chemical shifts of amide protons, distances between hydrogen bonding partners (NH_{i+4} and CO_i groups), and the αH chemical shift pattern (see text)

	ΔδNH[a]	Distances (CO,NH)[b]	δαH pattern
Phe-1			−1
Leu-2	−0.21		+1
Pro-3			0
Leu-4	−0.63	2.26	0
Leu-5	−0.54	2.22	0
Ala-6	−0.53	2.21	−1
Gly-7	−0.43	2.17	−1
Leu-8	−0.13	2.07	0
Ala-9	0.00	2.03	−1
Ala-10	−0.22	2.10	−1
Asn-11	−0.66	2.27	−1
Phe-12	0.15	1.99	0
Leu-13	0.36	1.93	0
Pro-14			−1
Thr-15	−0.73	2.30	−1
Ile-16	−0.15	2.07	−1
Ile-17	0.45	1.91	−1
Ser-18	−0.30	2.13	−1
Lys-19	−0.55	2.22	−1
Ile-20	0.20	1.97	0
Ser-21	−0.19	2.09	−1
Tyr-22	−0.33	2.14	−1
Lys-23	−0.39	2.16	−1
Ser-24	−0.41	2.17	−1

[a]The chemical shift differences (ΔδNH) are between shifts of GO 1-1 and those of random coil, given in ppm.
[b]Distances are calculated and given in Å.

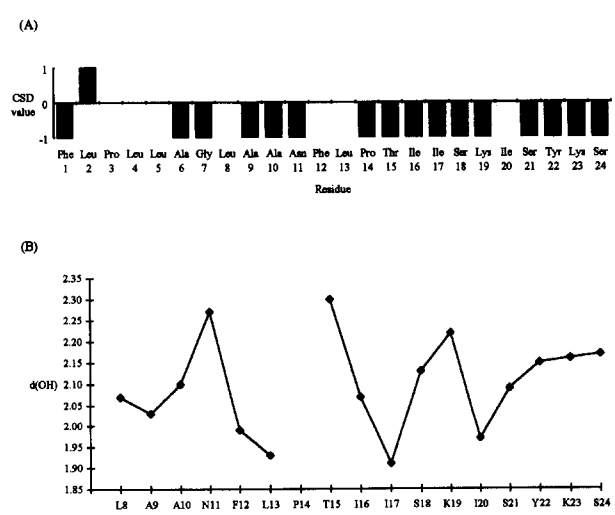

Fig. 3. (A) Diagram of α proton chemical shift pattern (see text). (B) Hydrogen bonding distances between NH and CO in the helix given in Å.

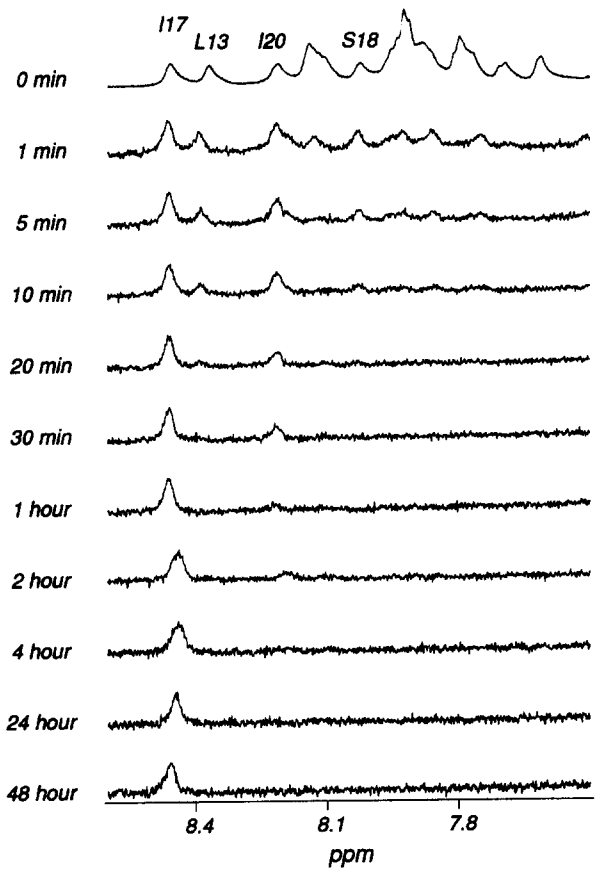

Fig. 4. Spectra of 1D proton exchange experiments. The spectrum of 0 min was recorded in H_2O/TFE-d_3 (1:1) and the others were recorded after dissolving the freshly prepared peptide in D_2O/TFE-d_3 (1:1).

data above. This stiffened kink is proposed to play an important role in the antimicrobial activity.

Prolines have been observed in bilayer-spanning regions [18] and it has been proposed that a kinked helix could function as a funnel in an ion-channel [19]. Our attractive explanation for the data in this study is that the helix in gaegurin not only kinks, but that the kink is quite stable. If this is the case, this may suggest that helical-kink motif plays an important role in ion-channel formation or in cell lysis. The peptide could be inserted into a membrane while maintaining the helical kink. At first, the hydrophilic patch on the convex face of the kink will interact with the head group of the bilayer to perturb membrane structure and cause penetration. Next, the hydrophobic patch will come in contact with the lipid chain. The mechanism of insertion is still unknown, but the kink will not be broken easily. Two possible mechanisms are as follows. One is a monomeric insertion mechanism in which the hydrophilic face interacts with membrane first and rotates to be inserted. The other is an oligomeric insertion mechanism in which the peptides oligomerize and are inserted into the membrane. These pathways will make the helical kink less distorted. Finally, the kink can be a nucleation site in protein folding. Generally, the rate limiting step of the folding of a helix is the nucleation step [20]. Once nucleation is achieved, the helical chain propagates in a fast and cooperative manner. The stable kink may serve as a stable nucleation core in solution at the initial stage of folding.

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