Oligomerization of protegrin-1 in the presence of DPC micelles. A proton high-resolution NMR study

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Abstract Protegrins are members of a family of five Cys-rich naturally occurring cationic antimicrobial peptides. The NMR solution structure of protegrin-1 (PG-1) has been previously determined as a monomeric β-hairpin both in water and in dimethylsulfoxide solution. Protegrins are bactericidal peptides but their mechanism of action is still unknown. In order to investigate the structural basis of their cytotoxicity, we studied the effect of lipid micelles on the structure of PG-1. The NMR study reported in the present work indicates that PG-1 adopts a dimeric structure when it binds to dodecylphosphocholine micelles. Moreover, the amide proton exchange study suggests the possibility of an association between several dimers.

Key words: Protegrin; Antimicrobial peptide; Nuclear magnetic resonance structure; Micelle

1. Introduction

Small antimicrobial peptides secure the front lines of host defense through direct physicochemical attack on the surface membranes of invading microorganisms. Generally positively charged, they are perfect suited to interact with membranes and to cause disruptive changes in membrane permeability [1]. The majority of these peptides are thought to form amphiphilic helices that facilitate membrane incorporation and disruption. Protegrins (PG) are a family of peptides isolated from porcine leukocytes [2–4], that have a broad range of antimicrobial activities and that are thought to be important in defending various tissues from infection. PG are unusual because they have a β-sheet structure stabilized by two disulfide bonds [5,6]. Thus, they bear a striking resemblance to other families of antibiotic peptides such as tachyplesins [7] and, to a lesser extent, mammalian defensins [7–11].

Unlike amphiphilic α-helical peptides such as the magainins, a structural basis for membrane disruption is not so apparent. In a previous study, we showed that PG-1 is able to alter the cellular membrane permeability by forming ionic pores that present some specific properties in common with human defensin-induced channels [12]. But the different NMR structural studies made so far indicate that PG is a monomer both in aqueous solution [5,6] and in dimethylsulfoxide [5], a solvent supposed to mimic the membrane environment. In order to find evidence of a possible oligomerization state of PG in a lipid environment, we investigate in the present work its NMR solution structure in the presence of perdeuterated dodecylphosphocholine (DPC) micelles. DPC is a commonly used zwitterionic detergent for the solubilization of membrane peptides and proteins because the small uniform micelles reorient rapidly enough for solution NMR spectroscopy [13]. The use of perdeuterated DPC allowed us to observe all the peptide resonances without interference from the detergent and was essential for eliminating spin diffusion effects that obscure the analysis of NMR data [14,15].

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Abbreviations: NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy

2. Materials and methods

2.1. Sample preparation

Synthetic PG-1 was prepared as previously described [5]. The peptide (4 mg) was solubilized in an aqueous solution (H_2O or ^2H_2O, 500 μl) containing 20 mg perdeuterated DPC (molar ratio ∼1:12). Additionally, a 2 mg peptide sample in ^2H_2O was prepared for the titration experiment. Sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate (TSP) was added as an internal chemical shift standard. The pH (pD) was adjusted to an uncorrected glass electrode reading of 3.0. Perdeuterated [$^2H_{38}$]DPC (98% ^2H), ^2H_2O, ^2HCl, NaO^2H, and TSP were purchased from EURISOTOPE.

2.2. NMR spectroscopy

Proton NMR spectra were recorded at 600 MHz (Bruker AMX600) at a probe temperature of 25°C and the data processed on a Silicon Graphics computer using indifferently UXNMR or the program Gifa [16]. TOCSY and NOESY experiments were recorded in the phase-sensitive state-TPPI mode [17], with mixing times of 60 ms and 100 ms, respectively. Solvent suppression was carried out using the WATERGATE method in association with water-flip-back pulses [18,19]. A total of 32 (TOCSY) or 64 (NOESY) transients were acquired with a recycling delay of 1 s. All two-dimensional spectra were recorded with 512×4096 data points and with a spectral width of 7500 Hz. Shifted sine-bell functions were used for apodization.

An amide proton exchange experiment was carried out by recording a series of TOCSY experiments on the freshly lyophilized NMR sample (4 MG PG-1, 20 mg DPC) dissolved in ^2H_2O. The NH resonance detectable after 2 h was classified as slowly exchanging. For the titration experiment, minute amounts of a concentrated DPC solution (40 mg/ml) were added to a sample of PG-1 dissolved in ^2H_2O, and a 1D spectrum was recorded after each addition.

3. Results and discussion

3.1. Titration of PG-1 with DPC micelles

The proton resonances of PG-1 in aqueous solution were completely assigned in a previous work, and the refined 3D structure was determined [5]. Fig. 1 shows the ^1H NMR spectra of PG-1 at pH 3.5 and 298 K in H_2O solution and in the presence of DPC at a detergent/protein molar ratio of ∼12.

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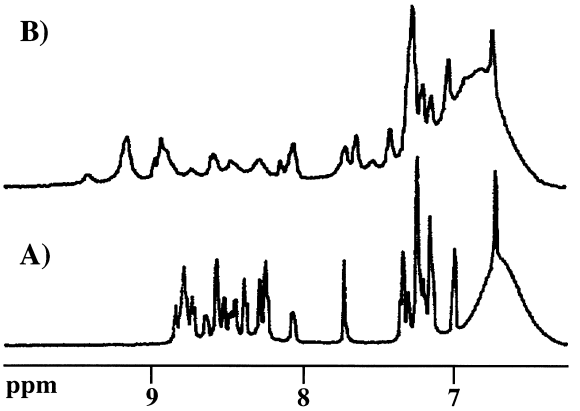

Fig. 1. One-dimensional ^{1}H NMR spectra (aromatic and amide regions) of PG-1 at 600 MHz, pH 3.5 and 298 K in H_2O (A) and in the presence of DPC at a detergent/peptide molar ratio of ~12 (B).

There is a significant broadening of all proton resonances, compared to unbound peptide, but they remain sharp enough to be compatible with high-resolution NMR techniques. In addition, numerous resonances undergo a significant shift in the presence of DPC. This observation, together with the line broadening, constitutes a strong indication in favor of the existence of an interaction between zwitterionic lipids and PG-1.

Fig. 2 shows the effect of varying the molar ratio of DPC on the 1D NMR spectrum of PG-1: additional broad resonances, assigned to the micelle-bound peptide, appear and grow up in the 1D spectra, whereas the sharp resonances belonging to the free peptide disappear concomitantly. This effect is particularly clear when looking at the HC^{α} resonances of the four cysteine residues, since the corresponding resonances in the bound peptide appear in an empty zone of the spectrum of the peptide. Consequently, the peptide-micelle complex exists in slow exchange with free PG-1, with regard to the NMR chemical shift time scale, which indicates a strong interaction between the two partners. This is a striking result since PG-1 is highly soluble in water – in contrast to nearly all membrane-associated peptides – and one would expect fast exchange conditions, corresponding to weak non-specific interactions. This might suggest that PG-1 undergoes strong structural change upon binding the lipid micelle.

The titration curve was established by integration of the well-resolved C15 HC^{α} resonance in the series of 1D spectra. The binding curve displays an initial lag at DPC concentrations below or around the CMC (1 mM [20]), indicating that there is no interaction between PG-1 and DPC monomers, but increases sharply upon crossing the threshold concentration. At a detergent/peptide ratio of about 6, the resonance reaches virtually a constant integral. Since there are about 40–50 molecules of DPC per micelle [20], this indicates that about eight molecules of PG-1 bind a single micelle of DPC. Nevertheless, this should be considered as a rough estimation, since we have at this step no indication of the number of DPC molecules in the peptide-micelle complex.

3.2. Effect of DPC micelles on the structure of PG-1

Sequence-specific resonance assignments of the ^{1}H resonance of PG-1 in DPC micelles were obtained using standard methods of protein NMR spectroscopy [21] (Table 1). Interestingly, the 2D spectra of PG-1 in the presence of DPC were found to be more spread out than those previously obtained in aqueous solution. When calculating their chemical shift

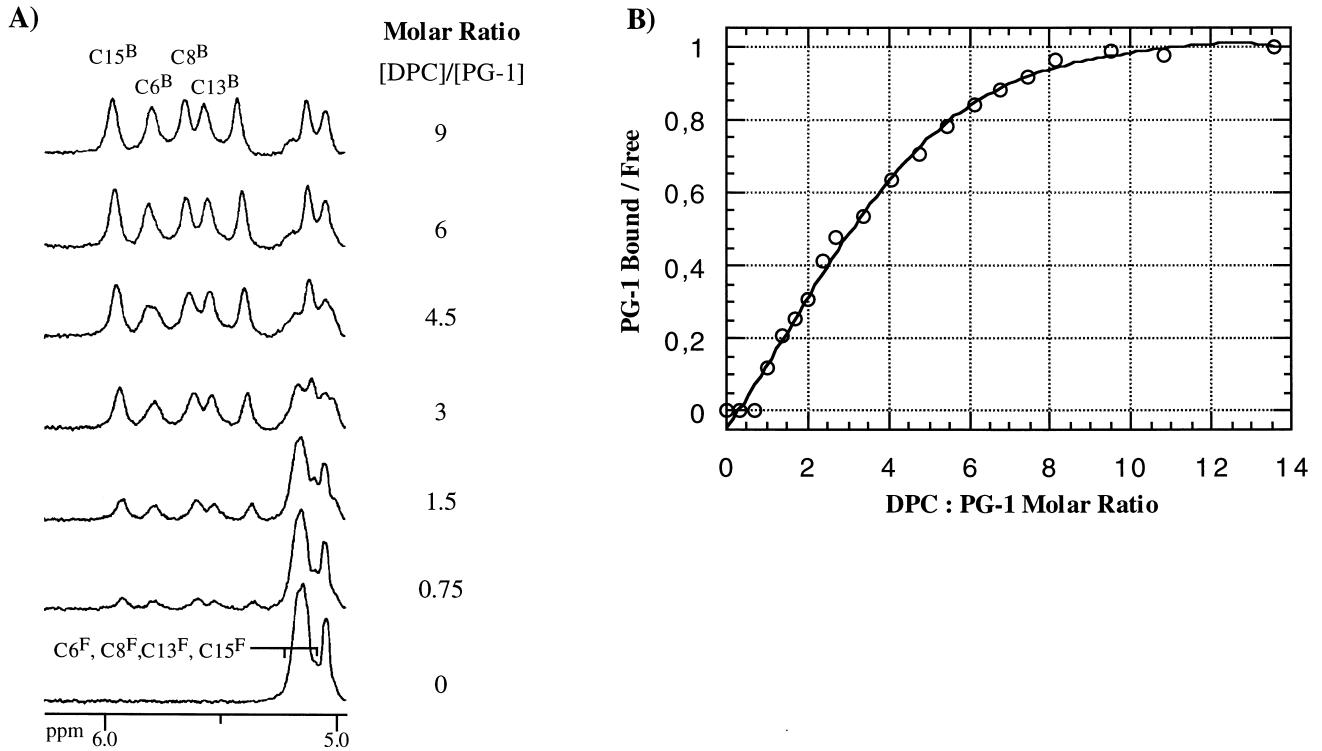

Fig. 2. Titration of PG-1 with perdeuterated DPC. A: Effect of the addition of DPC on the HC^{α} proton resonances of residues C6, C8, C13 and C15. The letters B and F are for the bound and free peptide, respectively. The corresponding detergent/peptide molar ratios are indicated. B: Titration curve deduced from the experiment.

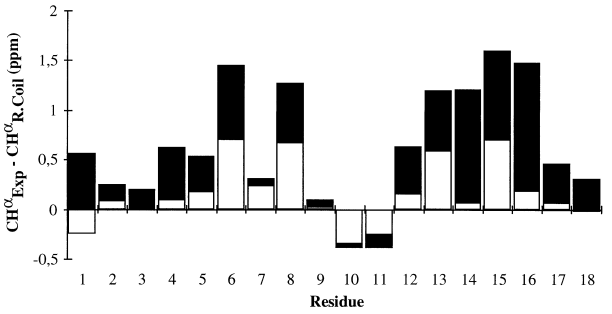

Fig. 3. Chemical shift deviations of α-proton resonances relative to random coil values in water [22]. Open bars are for PG-1 in water solution [5], filled bars are for PG-1 in the presence of DPC micelles (detergent/peptide molar ratio ∼12).

deviation relative to random coil values [22], most of the HC^{α} proton resonances exhibit an increased downfield shift as compared to our previous analysis in water solution (Fig. 3), and inspection of the NOESY maps allowed us to gather an increased number of NOE effects (see Fig. 5) indicating a better structuration, albeit apparently similar, of the β-hairpin in the presence of DPC. Similarly, the six amide protons potentially involved in H-bonds in the β-sheet were found as slowly exchanging protons, whereas they were exchanging in less than 5 min in ^2H_2O solution. Only two H-bonds were assigned in the previous study on the basis of the weak temperature coefficients measured on the corresponding amide protons.

Besides the β-sheet regular NOEs observed in the NOESY maps, additional NOEs were detected which appear inconsistent with the β-sheet structure. These are, for example, strong $d_{\alpha\alpha}$ NOE effects observed between residues R18 and F12 or V14 and V16 (Fig. 4, lower panel), which are far apart in the β-sheet structure. Second, amide protons of different residues which should be solvent exposed in the β-sheet structure were found to exchange slowly (Fig. 4, upper panel). Most of these effects can be readily interpreted if we assume that they are not intramolecular but intermolecular effects. As reported in Fig. 5, if we assume that two monomers adopt an antiparallel disposition, forming a large four-stranded antiparallel β-sheet, all the previously quoted 'inconsistent' NOEs can be explained by the formation of an additional antiparallel β-sheet between the two monomers. Similarly, the slow exchange exhibited by the amide protons of residues C15, C13 and G17 can be explained by the involvement of these protons in regular H-bonds stabilizing the protegrin dimer.

If these results demonstrate the dimerization of PG-1 in the presence of DPC micelles, a doubt remains about the possibility of a further association of several dimers. Nevertheless, different signs suggest this possibility. First, if we assume that the number of DPC monomers per micelle does not change in the presence of PG-1, the titration curve indicates that there are about eight peptide molecules (four dimers) per micelle. Second, the slow exchange exhibited by the amide protons of residues C6 and C8 suggests a possible association of these dimers: these residues are located on the outer strands of the large four-stranded β-sheet formed by the dimer, and their corresponding NH protons are not involved in the regular H-bond pattern of the dimer. Invoking a deep burying of the dimer on the micelle to explain the slow exchange seems hardly compatible with the dynamic equilibrium which is known to exist in solution between the DPC micelle and the unassociated DPC molecules. An explanation might be the formation of inter-dimer H-bonds. At this step of the study, no NOEs were found to support this hypothesis, but this could be due to a fortuitous symmetry in the oligomeric association.

Table 1
Proton chemical shifts measured in water for PG-1 in the presence of perdeuterated dodecylphosphocholine micelles (detergent/peptide molar ratio ∼12) at pH 3.5 and 298 K

Residue	HN	Hα	Hβ	Hγ	Others
Arg-1	9.07	4.91	2.07	1.81	Hδδ′ 3.33 Hε 7.44
Gly-2	9.05	4.21–4.15			
Gly-3	8.7	4.16–4.10			
Arg-4	8.13	4.97	1.81–1.87	1.69	Hδδ′ 3.16 Hε 7.27
Leu-5	8.99	4.85	1.89	1.76	CH_3δ 1.07
Cys-6	8.57	5.99	2.91–3.10		
Tyr-7	9.54	4.861	2.99		H2–6 6.84 H3–5 7.14
Cys-8	8.36	5.81	2.99–2.86		
Arg-9	8.97	4.45	1.95–1.75	1.57	Hδδ′ 3.45 Hε 7.815
Arg-10	9.26	3.96	2.14–2.24	1.82	Hδδ′ 3.36 Hε 7.755
Arg-11	8.37	3.96	2.00–1.90	1.79	Hδδ′ 3.23 Hε 7.305
Phe-12	8.16	5.26	3.17		H2–6 7.30 H3–5 7.38 H4 7.38
Cys-13	9.02	5.74	2.98–2.86		
Val-14	9.25	5.33	2.20	1.24–1.16	
Cys-15	9.24	6.15	2.83–2.91		
Val-16	9.24	5.60	2.22	1.23–1.16	
Gly-17	8.56	4.42–4.28			
Arg-18	8.84	4.66	1.77–1.53	1.66	Hδδ′ 3.21 Hε 7.27

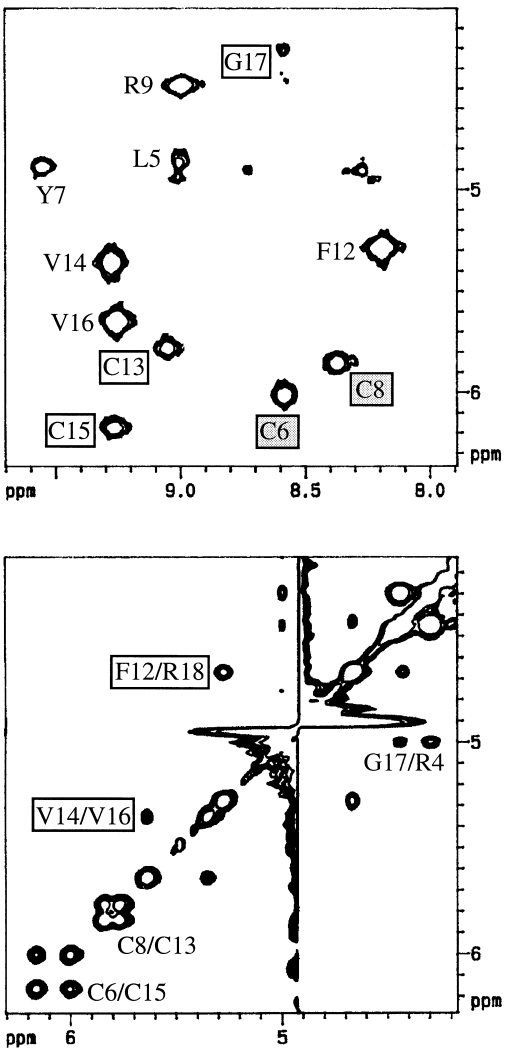

Fig. 4. Fingerprint region of the TOCSY spectrum (upper panel) recorded on a freshly dissolved sample of PG-1 in ^2H_2O in the presence of DPC micelles (detergent/peptide molar ratio ~12) showing the slowly exchangeable amide protons. The open rectangles are for amide protons involved in intermolecular H-bonds which stabilize the dimer (see Fig. 5), the dashed rectangles are for slowly exchangeable amide proton potentially involved in H-bonds which might stabilize further association. The remaining slowly exchangeable amide protons are involved in the regular pattern of H-bonds of the monomer. Lower panel: The corresponding NOESY spectrum showing the $d_{\alpha\alpha}$ NOE effects characteristic of the β-sheet structure. The open rectangles indicate intermolecular interactions characteristic of the dimer (see Fig. 5).
←

4. Conclusion

The results of the present work demonstrate that PG-1 forms a dimer in the presence of DPC micelles, and suggest that there is a possible association of these dimers. From the knowledge of the structure of PG-1 in solution, models have been built to extend the protegrin sheet [6]. The association turn-next-to-tail was one way for the self-association of PG-1 to form antiparallel β-sheets between monomers. But this model was found to be inappropriate since it placed positively charged arginine residues close to other arginines on the neighboring PG-1 molecules, creating large electrostatic repulsion [6]. Our present work demonstrates the validity of this model in the presence of DPC micelles. It has been suggested that the guanidinium ion of the arginine side chain is capable of interacting with two phosphate groups [23]. Conceivably, this occurs with PG-1 within DPC micelles in order to neutralize the electrostatic repulsion.

The dimeric structure adopted by PG-1 in a lipid environment is closely related to that of the human defensin HNP-3: the crystal structure of HNP-3 demonstrated dimers formed from monomers that come into close contact along the edges of their β-hairpins to form a local twofold rotation axis [11]. The result is a six-stranded β-sheet within the dimer stabilized by hydrogen bonds and hydrophobic contacts. Moreover, a speculative model in which an annular pore is formed by a

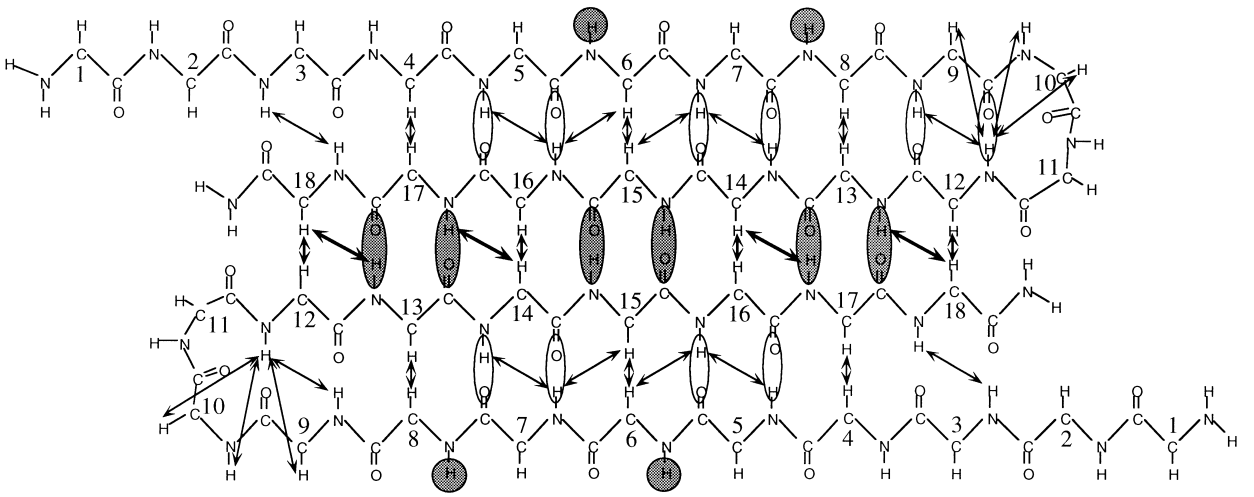

Fig. 5. The secondary structure of PG-1 in the presence of micelles. The double arrows indicate long- or medium-range NOEs effects characteristic of the β-sheet structure, the bold arrows stand for intermolecular effects. The ellipsoids indicate the H-bonds deduced from proton/deuteron exchange, the dashed ones are for intermolecular H-bonds which stabilize the dimer structure. The dashed circles indicate amide protons potentially involved in H-bonds which might stabilize further association.

hexamer of dimers has been proposed from studying the release of entrapped dextrans from large unilamellar vesicles in the presence of the related HNP-2 defensin [24]. In a previous work, we showed that PG-1 is able to alter the cellular membrane permeability by forming pores that present some specific properties in common with human defensin-induced channel. The present work suggests that these similar biological properties are based on similar structural proterties.

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