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Peptide Structures

Crystal Structure of a Spin-Labeled, Channel-Forming Alamethicin Analogue**

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Peptaibol antibiotics are membrane-active linear peptides of fungal origin that are characterized by a high population of the C^{α}-tetrasubstituted α-amino acid α-aminoisobutyric acid (Aib), an N-terminal acetyl group, and a C-terminal 1,2-amino alcohol.[1] Alamethicins, the longest peptaibols, are a group of closely sequence-related peptides composed of 19 amino acid residues.[2] They are able to form voltage-dependent pores in biological membranes and are the most extensively investigated among simple model compounds of large pore-forming proteins.[3] The formation of pores is known to be based on the transmembrane assembly of 6–8 helical alamethicin molecules, but many details of this mechanism are still under debate.

In this context, alamethicin analogues that are labeled at a single specific sequence position by the incorporation of a stable nitroxide free radical are expected to contribute to a better understanding of numerous details of pore formation in model membranes and intact cells by exploitation of the electron paramagnetic resonance (EPR) spectroscopic technique. To this aim, we have synthesized a spin-labeled analogue of alamethicin F50/5 bearing a 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) residue in place of the Aib residue at position 16, and three γ-methyl glutamate (Glu(OMe)) residues in place of Gln residues at positions 7, 18, and 19 of the natural sequence.[4] The primary structure of the analogue (**1**) is: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Glu(OMe)-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-TOAC-Aib-Glu(OMe)-Glu(OMe)-Phol (Ac = acetyl; Phol = phenylalaninol).

The C^{α}-tetrasubstituted α-amino acid TOAC has conformational properties similar to those of Aib.[5] Therefore, it is not expected to significantly affect the overall conformation of the alamethicin helical structure. In addition, the TOAC nitroxide moiety, being part of a piperidine ring which includes the C^{α} atom, is tightly connected to the peptide backbone. This property is quite favorable, compared to previously used spin probes which were characterized by flexible linkers,[6] for a study of the precise location of the peptaibol molecules in a membrane. However, a full conformational characterization of such an alamethicin analogue cannot be provided by NMR spectroscopic techniques, owing to the line-broadening effects exerted by the free radical.

The crystal structure of alamethicin F30 (a mixture of the F30 I and II components), solved by the heavy-atom isomorphous derivative procedure and refined at a resolution of 1.5 Å, was reported by Fox and Richards in 1982.[7] The major component, F30 I, differs from alamethicin F50/5 in that the sequence position 18 is occupied by a Glu residue in the former, while by Gln in the latter. Three peptide molecules, which are largely α-helical, but bent at Pro^{14}, are present in the asymmetric unit.

Although the X-ray diffraction structures of a few additional peptaibols of up to 17 amino acids in length have been reported,[8] that of alamethicin has not been determined at a higher resolution, nor has that of any analogue been described. Herewith, we report the X-ray diffraction structure of the [$TOAC^{16}$, $Glu(OMe)^{7,18,19}$] alamethicin F50/5 analogue **1**, which was solved ab initio and refined at a resolution of 0.95 Å.[9]

The two crystallographically independent peptide molecules **A** and **B** of **1** are shown in Figure 1. The residues are numbered from 1 to 20 (including the C-terminal 1,2-amino alcohol Phol) in molecule **A**, and from 21 to 40 in molecule **B**. Both molecules are bent helices, but they differ in significant details of their intramolecular hydrogen-bonding schemes.

Molecule **A** is almost fully α-helical from the N terminus up to N13. Indeed, the acetyl carbonyl oxygen atom acts as the acceptor of two intramolecular hydrogen bonds, from the N3–H and N4–H groups, thus, generating a ten-atom pseudocycle closed by a hydrogen bond (C_{10} structure or β bend) encompassed within a C_{13} structure (α bend).[10] Then, from N5 to N13, nine consecutive C_{13} forms are found. The regularity of the α helix is interrupted by Pro^{14}, as this residue lacks a hydrogen-bond donor. A shorter helix (one C_{10} structure, followed by four C_{13} structures, and an oxy-analogue of the C_{13} form,[10] which is formed by both of the

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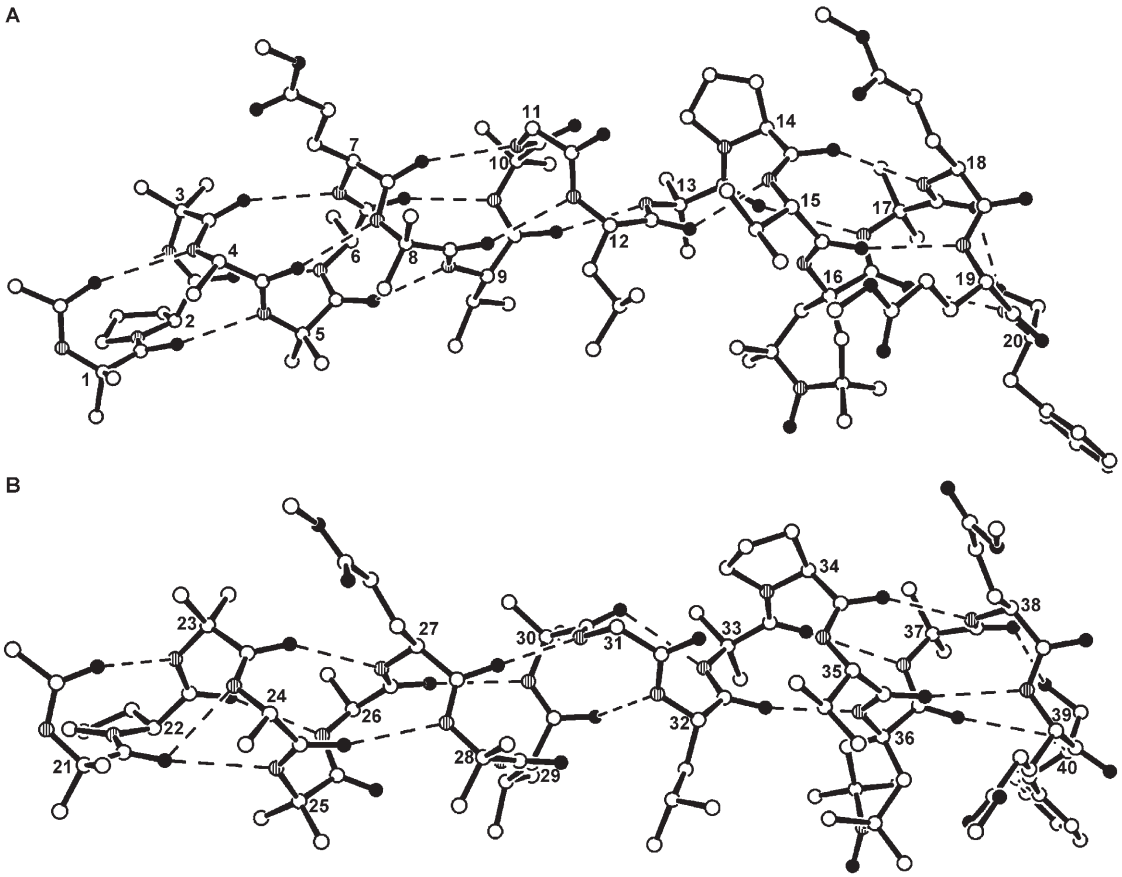

Figure 1. The two independent peptide molecules **A** and **B** of the [TOAC[16], Glu(OMe)[7,18,19]] alamethicin F50/5 analogue **1** with C^{α}-atom numbering. The minor site of the disordered C-terminal hydroxyl oxygen atom of Phol[20] in molecule **A** is omitted for clarity. Intramolecular hydrogen bonds are indicated by dashed lines. C white, N hatched, O black. Hydrogen atoms not shown.

partially occupied oxygen sites of the disordered Phol[20] hydroxyl group) characterizes the C-terminal stretch. Among the backbone carbonyl oxygen atoms of molecule **A**, O18, O19, and O20, which are located near the C terminus, as well as O10 and O11 do not take part in intramolecular hydrogen bonding.

In molecule **B** the long N-terminal helix is more irregular, as it shows a mixed $\alpha/3_{10}$ character. It features six C_{13} structures in its central part (with an interruption, as N29 and O25 are not hydrogen-bonded (N29···O25 3.73 Å), owing to the involvement of O25 in a hydrogen bond with a co-crystallized water molecule), and two consecutive C_{10} structures at each end. Again, the hydrogen bonding is discontinued by the lack of a donor at Pro[34], but the interruption extends also to N35. Then, from N36 to the C terminus, a fully α-helical fold is restored, terminating with an oxy-analogue of the C_{13} structure, in which the hydroxyl group of Phol[40] acts as the donor. Among the backbone carbonyl atoms of molecule **B**, O38, O39, and O40, as well as O25, O28, and O31 are not engaged in intramolecular hydrogen bonding.

Figure 2 shows the α-carbon traces of molecules **A** and **B**. Molecule **A** is more sharply bent near the Pro[14] residue than molecule **B** is near Pro[34]. In molecule **B**, the smaller kink at Pro[34] is accompanied by a slight bending in the opposite direction near the middle of the long, irregular N-terminal helix, which is determined by the interruption of hydrogen bonding between N29 and O25. As a result, molecule **B** is more linear overall than molecule **A** and the three independent molecules of alamethicin F30.[7] The bending of helices, although to a largely variable extent, is a common feature of peptaibol structures, even in the absence of Pro residues in their sequences,[7,8] but its exact functional role is not yet fully understood. Changes in the bending angle of alamethicin have been hypothesized to be related to its insertion into bilayers, channel self-assembly, and voltage gating, or to be

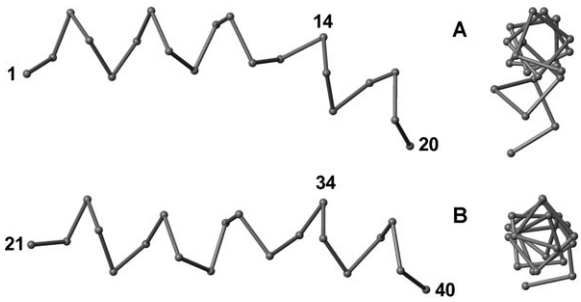

Figure 2. C^{α}-atom tracing of molecules **A** (top) and **B** (bottom), viewed perpendicular (left) and parallel (right) to their N-terminal helix axis.

required to accommodate the relative motions of the two leaflets of the bilayer.[3,11]

In both independent molecules **A** and **B**, all three Glu(OMe) side chains point outwards of the helical envelope. The carbonyl oxygen atoms of the side chains, which are neither intra- nor intermolecularly hydrogen-bonded, are located on the same side of the helical structure where the few free backbone C=O groups are found, thus, giving a hydrophilic character to the convex face of each molecule. The opposite, concave, face accommodates the most hydrophobic side chains and the bulky TOAC residue.

In the crystal, molecules **A** and **B** run antiparallel to each other along the *c* direction. In this packing mode, molecules of the same kind are head-to-tail hydrogen-bonded to their translational equivalents along the *c* direction, either directly (molecule **B**), or through two inserted water molecules (molecule **A**). Additional intermolecular hydrogen bonds involve the three water molecules as the donors.

In summary, the structure of the [TOAC^{16}, Glu(OMe)^{7,18,19}] analogue of alamethicin F50/5 has been determined by X-ray diffraction analysis at a resolution of 0.95 Å, thus, allowing its detailed conformational characterization. Such an achievement would have not been possible by NMR spectroscopic techniques, owing to the presence of the TOAC free radical. The overall folding of molecules **A** and **B** is similar to that reported for the three independent molecules in the structure of alamethicin F30 at 1.5-Å resolution.[7] The high resolution of the present structural analysis, which allows a precise discrimination between intramolecularly hydrogen-bonded C_{10} and C_{13} structures, supports the conclusions that alamethicin is largely α-helical, bent at the level of the internal Pro residue, and characterized by a significant degree of plasticity in terms of the pattern of intramolecular hydrogen bonding and the extent of bending. With respect to the structure of alamethicin F30,[7] the replacements of Aib^{16} by TOAC, and of Gln^{7}, Glu^{18} and Gln^{18} by three Glu(OMe) residues do not dramatically affect the backbone conformation, thus, validating our design of the analogue. In addition, preliminary results of patch-clamp experiments show that both the [TOAC^{16}, Glu(OMe)^{7,18,19}] and the [Glu(OMe)^{7,18,19}] alamethicin F50/5 analogues retain the capability to form ion channels into cell membranes, although the current produced by the TOAC-containing analogue is lower (Figure 3). On these bases, it may be concluded that the [TOAC^{16}, Glu(OMe)^{7,18,19}] alamethicin F50/5 analogue described herein is not only a conformationally, but also a functionally reliable model of its natural counterpart. Finally, as the TOAC spin label is tightly connected to the peptide backbone, the geometrical and conformational information gathered from the present structural analysis will be of help in the interpretation of the results of ongoing investigations by EPR spectroscopy and biophysical techniques.

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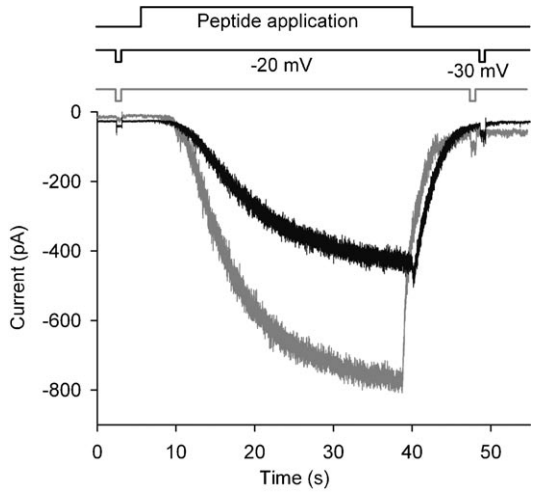

Figure 3. Permeabilization of isolated retinal-rod outer segments (OS) of frog photoreceptor induced by the [TOAC^{16}, Glu(OMe)^{7,18,19}] (**1**) and the [Glu(OMe)^{7,18,19}] (**2**) alamethicin F50/5 analogues at 1-μM concentration. The smooth traces show, from top to bottom, the timing of the peptide application (34 s for both peptides), and the timing of the holding voltage amplitude during application and withdrawal of **1** (black) and **2** (gray). The noisy traces are the voltage-clamp whole-cell current recordings from an OS perfused with **1** (black) and from another OS perfused with **2** (gray). Experimental details are given in the Supporting Information.

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[9] Crystallographic data: $2C_{101}H_{164}N_{21}O_{28}\cdot 3H_2O$, $M_r = 4295.1$, $0.40 \times 0.06 \times 0.02\ mm^3$, monoclinic, space group $C2$, $a = 39.767(4)$, $b = 20.192(3)$, $c = 32.655(3)$ Å, $\beta = 116.091(2)°$, $V = 23549(5)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.211\ Mg\,m^{-3}$, $\mu = 0.090\ mm^{-1}$, $\lambda = 0.6868$ Å, $T = 150$ K, $2\theta_{max} = 42.4°$. In total, 68928 reflections were collected, of which 28257 were independent ($R_{int} = 0.041$) and employed for refinement, except for a 5% fraction, which was reserved for R_{free} calculation. Data/restraints/parameters: 26845/851/2725, $R_1 = 0.117$ ($F \geq 4\sigma(F)$), $wR_2 = 0.305$ (all data), min/max residual electron density: $-0.38/0.95\ e\,Å^{-3}$. Data were collected at the microcrystal diffraction facility on Station 9.8 of the Synchrotron Radiation Source, CCLRC Daresbury Laboratory. The structure was solved ab initio using SHELXD,[12] and refined by least-squares procedures using SHELXL-97.[13] Details of the structure solution and refinement, along with tables of torsion angles and hydrogen-bond parameters, may be found in the Supporting Information. CCDC-604861 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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