A New Structure for the Substrate-Binding Antibiotic Ramoplanin

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Ramoplanin (Figure 1) is a cyclic lipoglycodepsipeptide antibiotic that inhibits the biosynthesis of peptidoglycan.[1] Like bacitracin and vancomycin, ramoplanin is believed to be a substrate binder, a molecule that inhibits an enzymatic transformation by docking to a required substrate. Ramoplanin binds selectively to Lipid II, a membrane-anchored β-(1,4)-linked GlcNAc-MurNAc disaccharide that is polymerized by the transglycosylases located on the outer surface of the bacterial membrane.[2] Both the disaccharide and the diphosphate linkage of Lipid II play a role in recognition by ramoplanin;[2b] however, there is no detailed information on the structure of the complex because it aggregates in water upon binding substrate.[2] Herein we describe the solution structure of a ramoplanin dimer that may provide insight into how this antibiotic assembles with itself and Lipid II.

The structure of a ramoplanin analogue in water was reported in 1991 and showed a bent antiparallel β-stranded conformation.[3] It was proposed that the ligand binds in the cleft created by the bend; however, a higher-resolution aqueous structure of ramoplanin shows no space in the purported cleft for a ligand.[4] Thus, the aqueous structure of ramoplanin does not shed much light on how Lipid II might bind. We began to wonder whether water was the best solvent in which to study ramoplanin given that the molecule binds to Lipid II at a membrane interface where the physical properties differ from those in bulk water.[5] Therefore, we examined the solution structure of ramoplanin under nonaqueous conditions to determine if it is capable of adopting another conformation that might be germane to Lipid II binding.

Figure 2 shows a pair of 1D NMR spectra of ramoplanin in D_2O and CD_3OD at a concentration of 0.5 mM. Two differences between the D_2O and CD_3OD spectra are immediately apparent. First, the resonance lines in D_2O are sharp, whereas those in CD_3OD are relatively broad. Second, there are more signals in CD_3OD than in D_2O. These observations indicate that ramoplanin in CD_3OD is in slow exchange between different states. Analysis of 2D NMR spectra in CD_3OD of ramoplanin reveals two sets of proton resonances, indicating two distinct states. The relative intensities of each set of resonances change with concentration (Figure 3). Therefore, we have concluded that each set of resonances corresponds to a different association state of ramoplanin.

The state predominating at low concentrations was identified as a monomer because both the chemical shifts and NOEs are similar to those for ramoplanin in aqueous solution. The other state involves more than one ramoplanin molecule, but it has only one set of signals for each ramoplanin proton. The simplest model consistent with this observation is a symmetric dimer. NMR diffusion measurements[6] show that the ratio of the diffusion constants for the two species is 0.84, which strongly supports the conclusion that the second species is a dimer.[7,8]

Full assignments for the proton resonances of the dimer were made from a combination of DQ-COSY, TOCSY, and NOESY spectra in CD_3OD and CD_3OH. Each subunit of the dimer in

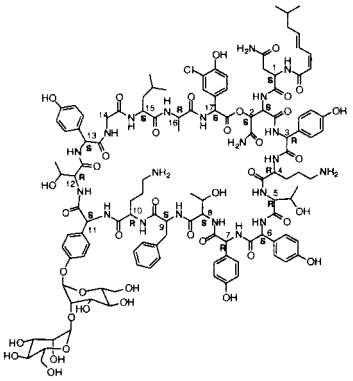

Figure 1. Structure of ramoplanin A2.

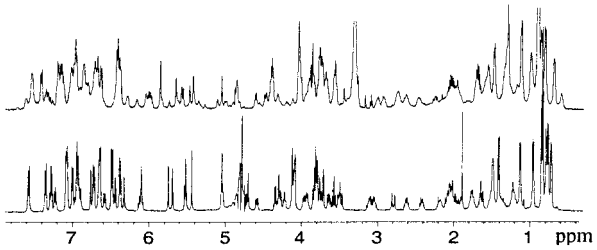

Figure 2. Ramoplanin A2 in D_2O (bottom), and CD_3OD (top) at 25 °C.

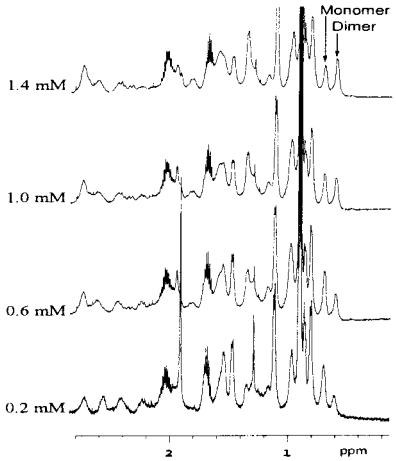

Figure 3. Upfield region of ramoplanin in CD_3OD at different concentrations (5 °C). Note the change in relative intensities of the upfield methyl resonances.

(1) (a) Somner, E. A.; Reynolds, P. E. *Antimicrob. Agents Chemother.* **1990**, *34*, 413. (b) Reynolds, P. E.; Somner, E. A. *Drugs Exp. Clin. Res.* **1990**, *16*, 385.

(2) (a) Lo, M.-C.; Men, H.; Branstrom, A.; Helm, J.; Yao, N.; Goldman, R.; Walker, S. *J. Am. Chem. Soc.* **2000**, *122*, 3540. (b) Lo, M.-C. Ph.D. Thesis, Princeton University, 2000.

(3) (a) Skelton, N. J.; Harding, M. M.; Mortishire-Smith, R. J.; Rahman, S. K.; Williams, D. H.; Rance, M. J.; Ruddock, J. C. *J. Am. Chem. Soc.* **1991**, *113*, 7522. (b) Maplestone, R. A.; Cox, J. P. L.; Williams, D. H. *FEBS Lett.* **1993**, *326*, 95.

(4) Kurz, M.; Guba, W. *Biochemistry* **1996**, *35*, 12570.

(5) The dielectric constant near the membrane−water interface is estimated to be ∼30, which is comparable to that of methanol. See: Zhou, F., Schulten, K. *J. Phys. Chem.* **1995**, *99*, 2194.

(6) Altieri, A. S.; Hinton, D. P.; Byrd, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 7566.

(7) The theoretical dimer/monomer ratio is ∼0.75. Deviations from theoretical values are common and can be significant. However, models involving higher order associations would be expected to give lower rather than higher ratios. See ref 6 and Krishnan, V. V. *J. Magn. Reson.*, **1997**, *124*, 468.

(8) The concentration dependent intensities can be fit to a monomer−dimer equilibrium better than to another model.

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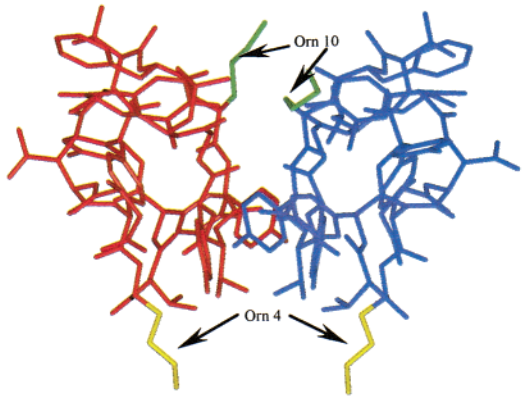

Figure 4. Ramoplanin aglycon dimer. 308 NOEs for each monomer, including 83 long-range NOEs ($\geq i, i+3$), and 28 NOEs across the dimer interface were used in the calculations. Key ornithine (Orn) residues flanking the potential binding clefts are indicated. The disaccharide on the side chain of residue 11 would project from the front and back of the structure shown.

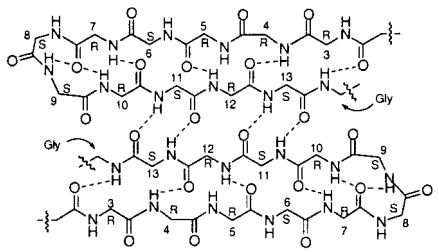

Figure 5. Partial schematic of the ramoplanin dimer backbone showing key inter- and intramolecular hydrogen bonds. Side chains were removed for clarity.

methanol has NOEs similar to those for the monomer, suggesting that the ramoplanin backbone is relatively rigid.[9] NOEs incompatible with those characteristic of the monomer were assigned as intermolecular.

Structures were calculated for the dimer using a simulated annealing procedure with intra- and intermolecular distance constraints obtained from NOESY spectra.[10,11] An average structure is shown in Figure 4.[12] Ramoplanin forms a symmetric dimer with the interface comprised of two bent antiparallel β-strands spanning residues 10−14 of each monomer. Four hydrogen bonds stabilize the interface (Figure 5). A chemical shift comparison of key proton resonances in the ramoplanin monomer and the dimer supports the calculated structure. For example, in the dimer both the amide and Cα proton resonances for residues 11−13 are shifted significantly downfield ($\Delta_{\text{dimer}-\text{monomer}} = 0.4-1.4$ ppm), consistent with the formation of intermolecular hydrogen bonds in a β-sheetlike arrangement.[13] Additional support for the hypothesis that dimerization involves stabilizing hydrogen bonds is the observation that ramoplanin exists almost exclusively in the dimer form in the less polar solvent CD_3CD_2OD, even at very low NMR concentrations.

The manner in which ramoplanin associates is reminiscent of the cyclic D,L-peptides studied by Ghadiri and others.[14] At the dimer interface, ramoplanin displays the same pattern of alternating stereochemistry as these peptides (Figure 5), with the result that successive side chains all project from the same face, perpendicular to the hydrogen bonded backbone. In organic solvents, cyclic D,L-peptides tend to form infinite hydrogen-bonded stacks unless one face of the ring is sterically blocked.[15] In hydrogen-bonding solvents, synthetic D,L-peptides typically do not associate into stable structures except at high concentrations that allow crystallization into ordered "nanotubes".[16] Ramoplanin is remarkable because it can self-assemble to form stable dimers in solvents that are capable of competitive hydrogen-bonding interactions.[17] Furthermore, it can exist as a monomer, dimer, or polymer, depending on solvent and the presence of ligand.[18,19] Unlike most of the synthetic D,L-peptides that have been studied previously, ramoplanin contains interruptions in the alternating D,L-pattern of amino acids. These interruptions influence the conformation of the macrocycle and may help control the delicate balance between aggregation states.

The ramoplanin dimer observed in CD_3OD suggests a mechanism of action in which the antibiotic exists as a monomer in water, but self-associates to form a dimer as it approaches the membrane−water interface. Although the monomer itself does not contain an apparent binding pocket, dimerization creates two possible clefts that could accommodate the disaccharide of Lipid II.[20] The positively charged amines of the Orn4 residues flank one cleft, while those of the Orn10 residues flank the other cleft, and either pair could interact with the negatively charged pyrophosphate that has been shown to be important for binding to Lipid II.[2b] The biological relevance of the dimer can be tested by chemically modifying ramoplanin and determining whether there is a correlation between biological activity, dimer formation, and the ability to bind Lipid I/II. In the meantime, we note that the structure of this ramoplanin dimer provides clues to new ways to engineer cyclic D,L-peptides to control their conformational properties and aggregation states.[15,21]

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Supporting Information Available: Assignments for proton resonances in the dimer, chemical shift changes for the Hα and NH resonances upon dimer formation, distribution of intra- and intermolecular NOEs used in the calculations, overlay of dimer structures and RMSD tables (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Intramolecular NOEs include long-range NOEs between residues 9 and 17, which are located at the turns on opposite ends of the molecule. See ref 4. The backbone conformation is enforced by cyclization and the large number of sterically hindered, β-branched amino acids, including the six hydroxyphenylglycine (Hpg) residues.

(10) Brunger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J.-S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr.* **1998**, *D54*, 905.

(11) For leading references on how to treat symmetric dimers, see: (a) Brunger, A.; Nilges, M. *Protein Eng.* **1993**, *17*, 297. (b) O'Donoghue, S.; King, G.; Nilges, M. *J. Biomol. NMR* **1996**, *8*, 193.

(12) The average structure is a composite of the six (of 30) lowest-energy structures that show no NOE violations greater than 0.3 Å. The average RMSDs for the backbone and all heavy atoms are 0.441 and 0.866 Å, respectively.

(13) (a) Wishart, D.; Sykes, B.; Richards, F. *J. Mol. Biol.* **1991**, *222*, 311. (b) Wishart, D.; Sykes, B.; Richards, F. *Biochemistry* **1992**, *31*, 1647.

(14) (a) Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. *Nature* **1993**, *366*, 324. (b) Clark, T. D.; Buriak, J. M.; Kobayashi, K.; Isler, M. P.; McRee, D. E.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1998**, *120*, 8949. (c) Ranganathan, D.; Haridas, V.; Gilardi, M. R.; Karle, I. L. *J. Am. Chem. Soc.* **1998**, *120*, 10793.

(15) Bong, D. T.; Clark, T. D.; Granja, J. R.; Ghadiri, M. R. *Angew. Chem., Int. Ed. Engl.* **2001**, *40*, 988.

(16) Karlstrom, A.; Undén, A. *Biopolymers* **1997**, *41*, 1.

(17) The hydrogen-bonded interface may be stabilized by side-chain aromatic ring interactions as well as additional hydrogen bonds between threonine 12 hydroxyls.

(18) For an example of a designed peptidomimetic that self-assembles into polymorphic β-sheet quaternary structures, see: Lashuel, H. A.; LaBrenz, S. R.; Woo, L.; Serpell, L. C.; Kelly, J. W. *J. Am. Chem. Soc.* **2000**, *122*, 5262

(19) Ramoplanin in methanol shows the same behavior with Lipid II as in water—*i.e.*, it polymerizes. See ref 2.

(20) (a) For a review of biological carbohydrate binders, see: Quiocho, F. *Curr. Top. Micro. Imm.* **1988**, *139*, 135; Quiocho, F. *Pure Appl. Chem.* **1989**, *61*, 1293. (b) For a recent review of designed carbohydrate binders, see: Davis, A. P.; Wareham R. S. *Angew. Chem., Int. Ed.* **1999**, *38*, 2979.

(21) For additional work on peptides or peptidomimetics engineered to control association, see *inter alia*: (a) Ranganathan, D.; Lakshmi, C.; Karle, I. L. *J. Am. Chem. Soc.* **1999**, *121*, 6103. (b) Nowick, J. S.; Chung, D. M.; Maitra, K.; Maitra, S.; Stigers, K. D.; Sun, Y. *J. Am. Chem. Soc.* **2000**, *122*, 7654. (c) Fisk, J. D.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, *123*, 343.