The Role of Sugar Residues in Molecular Recognition by Vancomycin†

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The sugar residues of the glycopeptide antibiotic vancomycin contribute to the cooperativity of ligand binding, thereby increasing ligand affinity and enhancing antimicrobial activity. To assess the structural basis for these effects, we determined a 0.98 Å X-ray crystal structure of the vancomycin aglycon and compared it to structures of several intact vancomycin:ligand complexes. The crystal structure reveals that the aglycon binds acetate anions and forms back-to-back dimeric complexes in a manner similar to that of intact vancomycin. However, the four independent copies of the aglycon in each asymmetric unit of the crystal exhibit a high degree of conformational heterogeneity. These results suggest that the sugar residues, in addition to enlarging and strengthening the dimer interface, provide steric constraints that limit the vancomycin molecule to a relatively small number of productive conformations.

Introduction

Vancomycin is used worldwide to treat infections by Gram-positive bacterial pathogens. Until recently, vancomycin has remained active against organisms resistant to other antibiotics, and has therefore been useful as an antibiotic of last resort. However, the past decade has seen the emergence and rapid spread of vancomycin-resistant enterococcal strains, as well as *S. aureus* strains with an intermediate level of susceptibility to vancomycin. The specter of widespread vancomycin resistance is now spurring investigations into the structure and function of vancomycin, with the rationale that a full molecular understanding of the drug's function should assist in the design of next generation compounds that circumvent resistance.

Vancomycin is the archetypal member of the family of glycopeptide antibiotics that includes such compounds as teicoplanin, ristocetin, and avoparcin.[1] These compounds act by binding to a D-Ala-D-Ala dipeptide found at the C-terminus of nascent cell-wall peptides. This binding and sequestration of the D-Ala-D-Ala dipeptide interferes with cross-linking of the cell-wall peptides, which is necessary for the formation of the bacterial peptidoglycan. Glycopeptide antibiotics are built around a heptapeptide core; the side chains of this heptapeptide are covalently joined to form macrocycles, and these are decorated with sugar substituents at various positions. Vancomycin has an L-vancosaminyl-α(1→2)-D-glucopyranosyl disaccharide attached to the phenolic hydroxyl of the residue 4 side chain (Figure 1).

Vancomycin forms asymmetric homodimers.[2−4] The polypeptide chains of each monomer in the dimer are arranged antiparallel, displaying 2-fold symmetry. The sugar residues are arranged in a parallel fashion that ignores the 2-fold symmetry and provides a large hydrophobic contact interface between the disaccharides of each monomer. This large interaction area explains, at least in part, how glycosylation enhances dimerization by 2 orders of magnitude.[5]

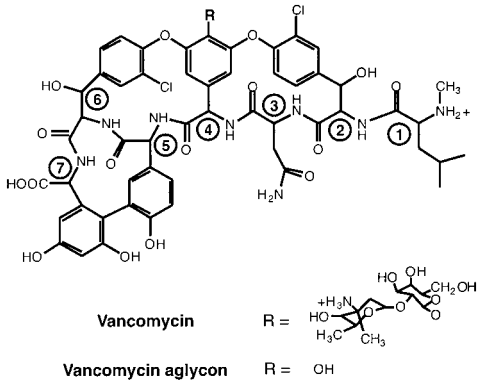

Figure 1. Chemical structure of vancomycin and its aglycon. Circled numerals indicate the seven amino acids of the heptapeptide backbone.

Because the sugars also form part of the ligand binding site, their asymmetric disposition gives rise to dimers with two nonequivalent binding sites. The two sites have different affinities for ligand[6,7] and are cooperative with respect to ligand binding. The disaccharide is clearly involved in the cooperativity, inasmuch as its removal leads to an anticooperative relationship between binding and dimerization in the aglycon.[8] Although the disaccharide is not essential for ligand recognition or antimicrobial activity, its removal decreases antibiotic potency and reduces water solubility.[9] The disaccharide unit has no antimicrobial activity of its own, but derivatives with antiglycosylase activity have been found.[10]

Prompted by the important and complex role played by the disaccharide group in modulating vancomycin activity, we have determined the X-ray crystal structure of vancomycin aglycon in complex with ligand. Comparison with the structure of intact vancomycin reveals similar dimeric relationships and ligand binding modes but considerably greater conformational freedom in the aglycon. This suggests that the disaccharide group in vancomycin gives rise to cooperative ligand binding and

† Abbreviations: Ac, acetate; AcDA, *N*-acetyl-D-alanine; AGV, aglycovancomycin; DMSO, dimethyl sulfoxide; rms, root-mean-square; TFA, trifluoroacetic acid; VMN, vancomycin.

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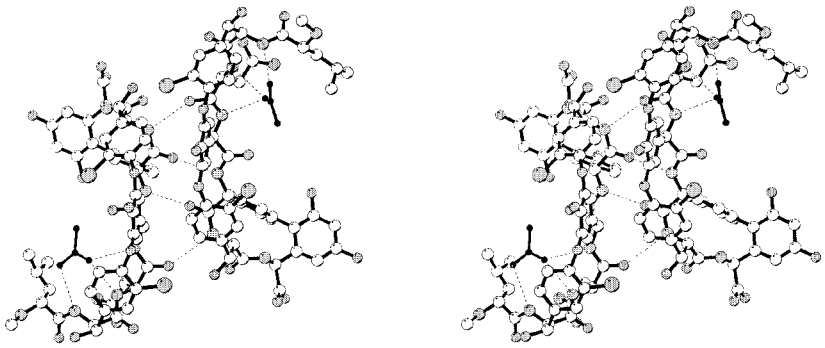

Figure 2. Stereo diagram of the back-to-back dimer formed by aglycon monomers 1 and 2. In the aglycon molecules, carbon atoms are colored white and heteroatoms are colored gray. Two acetate ligands are shown as well and are colored all black. Large circles denote chlorine atoms. Hydrogen bonds are shown as dashed lines.

enhanced ligand affinity by limiting the conformational freedom of the molecule, thereby preventing excursions into unproductive structural modes.

Results

Nomenclature. Four independent monomers of the vancomycin aglycon comprise the crystallographic asymmetric unit and have been arbitrarily numbered 1 through 4. Monomers 1 and 2 form a dimer, as do monomers 3 and 4. Each monomer contains seven amino acid residues, which are numbered as shown in Figure 1. The structure of the aglycon dimer with two bound acetate anions (AGV_2:Ac_2) will be compared with the crystal structures of intact vancomycin with one acetate ion (VMN_2:Ac) and with two *N*-acetyl-D-alanine molecules (VMN_2:$AcDA_2$).

Description of the Structure. Both of the AGV_2:Ac_2 complexes in the asymmetric unit adopt an antiparallel back-to-back configuration similar to that seen in the VMN_2:Ac complex [2−4,11] (Figure 2). Both also exhibit approximate 2-fold rotational symmetry, with the 2-fold axis passing between the two monomers, perpendicular to the plane formed by the intermonomer hydrogen bonds. Each monomer is cup-shaped; the backs of the cups form the dimer interface, leaving the concave surfaces of the monomers, which form the ligand-binding pockets, facing outward into solution (see Figure 2). By convention, this binding pocket is referred to as the "face" of the molecule, and the opposite side, which forms an antiparallel beta structure with another monomer, is referred to as the "back" of the molecule. The removal of sugar substituents has not altered the binding mode of the acetate ligand. The acetate methyl still packs against the face of the aromatic ring in the side chain of residue 4, and the distances and geometries of the three hydrogen bonds (between the ligand carboxylate and the amide nitrogens of residues 2, 3, and 4) are indistinguishable from those of other vancomycin−ligand complexes. However, it is possible that the sugars participate more fully in binding larger ligands, and in such cases the details of ligand binding may differ between the aglycon and intact molecule.

However, there are numerous and significant differences between the AGV_2:Ac_2 dimers and the VMN_2:Ac dimer. One difference is that both aglycon molecules in each AGV_2:Ac_2 dimer bind acetate anions, whereas there is only one acetate anion per VMN_2:Ac dimer. In monomers 1 and 2 in the aglycon asymmetric unit, the leucine side chain of residue 1 folds back over the binding pocket, shielding the ligand from solvent; this phenomenon is also observed in the VMN_2:$AcDA_2$ complex.[3,4] In monomers 3 and 4, the leucine side chain does not fold back as far as in monomers 1 and 2, but it still affords partial coverage of the acetate ion.

A second difference is that VMN_2:Ac exhibits no face-to-face interactions, instead binding a symmetry-related copy of itself and an intramolecular surrogate ligand.[3,4] Monomers 1 and 2 in AGV_2:Ac_2 also pack in such a way that the dimer interacts with a symmetry-related copy of itself. However, the interaction is limited to a salt bridge between the N-methylated amino terminus of monomer 2 and an acetate ion bound by a symmetry-related copy of monomer 1. Monomers 3 and 4, in contrast, form an infinite chain along the crystallographic *z*-axis with the face of monomer 3 interacting with the face of a symmetry-related copy of monomer 4. This face-to-face packing is mediated in part by the acetate ligands and closely resembles the face-to-face arrangement seen in crystals of the VMN_2:$AcDA_2$ complex.[11] Given that face-to-face dimerization could reflect a physiologically significant oligomerization mode for vancomycin in the presence of its natural ligand,[11] it is noteworthy that there exist alternative ways to assemble long chains of vancomycin monomers by alternating back-to-back and face-to-face packing (see Figure 3).

A third difference seen in the AGV_2:Ac_2 dimers is that only the 1−2 dimer contains the full set of intermonomer hydrogen bonds seen in VMN_2:Ac (these bonds connect the carbonyl oxygens of residue 3 with the amide nitrogens of residue 5, and the carbonyl oxygens of residue 5 with the amide nitrogens of residue 4). The other AGV_2:Ac_2 dimer (the 3−4 dimer) is missing a hydrogen bond between the carbonyl oxygen of residue 5 in monomer 3 and the residue 3 amide in monomer 4. Instead, both of these atoms form hydrogen bonds with water molecules. No crystal contacts are found anywhere near either of these atoms, and the crystal packing suggests no reasons that would explain why this fourth hydrogen bond is not formed. This slightly altered conformation in the dimer interface appears to reflect normal conformational variation, rather than a distortion imposed by the crystal lattice.

The four crystallographically independent aglycon monomers differ significantly from one another. These differences are particularly pronounced in the macrocycle formed by the cross-linked side chains of residues 5 and 7, and in the conformations of the side chains of

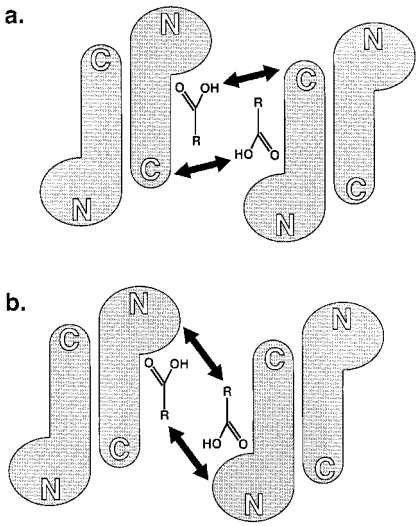

Figure 3. Schematic drawing of the two types of face-to-face dimers found in vancomycin crystals. (a) Face-to-face dimer found in the aglycon crystal structure. Contacts between dimers are made between the C-terminal of the ligand and the C-terminal of the vancomycin molecule. (b) Face-to-face dimer found in the VMN_2:$AcDA_2$ crystal structure. Interdimer contacts occur between the N-terminus of the ligand and the N-terminus of the antibiotic.

residues 1 and 3. The four monomers can be superimposed on one another with pairwise rms positional differences ranging from 0.53 to 0.96 Å. This range of structural variability is much larger than has been seen in previous vancomycin crystal structures. For example, four different crystal structures of vancomycin dimers complexed with low-affinity ligands can all be superimposed on each other with pairwise rms differences of less than 0.15 Å.[12] It is important to note that the variability seen among the aglycon monomers does not simply reflect uncertainty in the positions of the atoms; in the aglycon crystal structure, the mean estimated uncertainty in atomic positions is 0.013 Å, comparable to those of previous vancomycin structures. Hence, the differences between the four aglycon monomers are both significant and striking.

Discussion

Previous structural studies have identified two different vancoymcin conformational states. One form is seen in the presence of low-affinity ligands and another with high-affinity ligands. The rms difference between the high- and low-affinity forms is approximately 1 Å, whereas any two low-affinity complexes can be superimposed with rms differences of less than 0.15 Å.[12] Comparison of the aglycon structure with the high- and low-affinity forms of intact vancomycin reveals that the aglycon is not readily assigned to either conformational class. In fact, the different aglycon monomers are as different from one other as they are from either the low- or the high-affinity structures (rms differences between the aglycon and both the high- and low-affinity conformations range between 0.8 and 1.0 Å).

Thus, the aglycon conformations do not represent a distinct conformational family. Rather, if the various conformations available to the vancomycin molecule are considered as points lying along a continuum in conformational space, then the four different aglycon monomer structures are widely spread along this continuum. In contrast, the four monomers of the high-affinity VMN_2:$AcDA_2$ structure comprise a tight cluster of points along this same continuum, while the various low-affinity structures form a different, but equally tight, cluster. Hence, removal of the sugars allows for the expression of conformational heterogeneity otherwise not accessible to the vancomycin peptide backbone.

"Grafting" the disaccharide back onto the aglycon by computer modeling gives rise to numerous close contacts between the sugars and the aglycon, particularly between the glucose sugar and the aromatic ring of residue 4 and between the glucose and the phenolic oxygens connecting the side chains of residues 2, 4, and 6. Hence, structural heterogeneity is a consequence of the removal of the sugar residues, and the aglycon is able to explore conformations that are sterically prohibited in the intact vancomycin molecule.

An NMR structure has been reported for the vancomycin aglycon in neat DMSO.[13] Significant conformational differences exist between this structure and our crystal structure, most notably in the ligand binding site and in the aromatic rings of residues 2, 4, and 6. The NMR structure shows the peptide backbone bulging outward in the vicinity of residues 2 and 3 to form the so-called β-pleated sheet conformation, which is unfavorable for both ligand binding and dimerization.[14] This bulge is not seen in the crystal structure. The two structures also differ significantly in the torsion angles about the ether linkages connecting the side chains of residues 2, 4, and 6. The angle between the backbone and the ring plane of residue 4 differs by roughly 30° in the two structures.

The differences between the aglycon crystal structure and NMR structure are likely due to differences in the solvent environment. The aglycon does not bind ligand or dimerize in neat DMSO, evidently because of the rearrangement it undergoes in the absence of water. However, aglycon crystals are grown from an aqueous solution that supports dimerization.[8] The crystal structure demonstrates that, in an aqueous environment, the aglycon is indeed capable of adopting an active conformation much like that seen with intact vancomycin, and therefore the inactive conformation seen in the NMR structure does not inevitably result from removal of the sugar residues. This conclusion is supported by the one other available aglycon crystal structure, A-40926,[15] which is quite similar to intact vancomycin. Our crystal structure suggests that, having been freed of steric constraints contributed by the sugar residues, the aglycon is capable of exploring a greater conformational space than the intact antibiotic, explaining why the aglycon adopts the inactive conformation in DMSO, whereas the intact molecule does not.

The effect of vancomycin's sugars on cooperativity may be explained by their ability to limit dynamic fluctuations. Such fluctuations would compromise the geometry of the hydrogen bonds across the dimer interface, thereby weakening them. The aglycon conformation is clearly able to fluctuate in the dimer interface, as evidenced by the differences in this region seen between the two independent aglycon dimers. MacKay et al. suggest that cooperative ligand binding results from structural rigidity conferred by hydrogen

bond formation in the dimer interface that locks the ligand binding pocket into the correct conformation for dipeptide binding.[16] However, our crystallographic results show that dimerization alone is not sufficient to confer rigidity upon the heptapeptide backbone, since the aglycon forms dimers yet exhibits conformational heterogeneity. Williams et al. have suggested that the cooperativity between dimerization and ligand binding derives from cooperative "tightening" of the dimer interface and the drug:ligand interface.[17] The NMR evidence in support of this conclusion does not establish whether "tightening" means static conformational change or a change in conformational dynamics. The AGV_2:Ac_2 crystal structure demonstrates that the aglycon can form dimers with hydrogen bond geometries in the dimer interface and the ligand:antibiotic interface that are essentially identical to those seen with intact vancomycin. Thus, the dimer interface tightening associated with cooperativity corresponds to a reduction of conformational heterogeneity, not to a gross structural rearrangement.

Conclusions

The sugar residues of vancomycin, while not required for antimicrobial activity, increase the drug's affinity for ligands and enhance dimerization. An X-ray crystal structure for vancomycin aglycon at atomic resolution reveals that removal of the sugar residues causes no dramatic change in the conformation of the molecule. The aglycon forms back-to-back dimers and binds ligand in a manner similar to intact vancomycin. However, the aglycon exhibits a much higher degree of conformational heterogeneity, as reflected by substantial differences between the four independent molecules in the aglycon crystal structure. This suggests that an important role for the sugar substituents is to provide steric constraints that reduce the flexibility of the molecule and steer it toward a conformational state favorable for ligand binding and dimerization.

Experimental Section

Preparation and Crystallization. Vancomycin aglycon was prepared by acid hydrolysis[18] and purified by chromatography on a Vydac C-18 column (mobile phase 20−80% acetonitrile in 1% TFA). Crystals were prepared by vapor diffusion at 277 K, mixing 5.0 μL of 15 mg/mL aglycon in 20% (v/v) DMSO with 5.0 μL of reservoir buffer (1.5 M sodium acetate in 100 mM sodium cacodylate, pH 6.5). Rod-shaped crystals formed within 3 to 4 weeks, growing to maximum dimensions of 0.18 × 0.18 × 0.6 mm. Prior to data collection, crystals were transferred briefly to a cryprotectant solution containing 0.2 mL of DMSO, 0.6 mL of glycerol, and 1.8 mL of 1.5 M sodium acetate in cacodylate buffer. The crystals were mounted in nylon loops and flash-cooled in liquid N_2.

Data Collection and Processing. Diffraction data were collected from a single crystal at beamline X12-B at the National Synchrotron Light Source. The strategies for data collection and processing have been described previously.[12]

Structure Determination and Refinement. The structure was determined using anomalous scattering from the chlorine atoms; details will be given elsewhere (P. J. Loll, submitted). This structure was then refined against F^2 using the program SHELXL-97.[19] Molecular geometry and atomic displacement parameters were restrained throughout the refinement.[12] The final values of R and R_{free} for all data to 0.98 Å are 0.150 and 0.150, respectively. Coordinates have been deposited with the Protein Data Bank (accession number 1GHG).

Supporting Information Available: Details of data collection and refinement, values of selected torsion angles, hydrogen bond geometry in the dimer interface, and stereo figures demonstrating superposition of the different aglycon monomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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