Vancomycin Binding to Low-Affinity Ligands: Delineating a Minimum Set of Interactions Necessary for High-Affinity Binding§

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Received July 14, 1999

Bacterial resistance to vancomycin has been attributed to the loss of an intermolecular hydrogen bond between vancomycin and its peptidoglycan target when cell wall biosynthesis proceeds via depsipeptide intermediates rather than the usual polypeptide intermediates. To investigate the relative importance of this hydrogen bond to vancomycin binding, we have determined crystal structures at 1.0 Å resolution for the vancomycin complexes with three ligands that mimic peptides and depsipeptides found in vancomycin-sensitive and vancomycin-resistant bacteria: *N*-acetylglycine, D-lactic acid, and 2-acetoxy-D-propanoic acid. These, in conjunction with structures that have been reported previously, indicate higher-affinity ligands elicit a structural change in the drug not seen with these low-affinity ligands. They also enable us to define a minimal set of drug−ligand interactions necessary to confer higher-affinity binding on a ligand. Most importantly, these structures point to factors in addition to the loss of an intermolecular hydrogen bond that must be invoked to explain the weaker affinity of vancomycin for depsipeptide ligands. These factors are important considerations for the design of vancomycin analogues to overcome vancomycin resistance.

Introduction

Vancomycin is a glycopeptide antibiotic derived from the soil microorganism *Nocardia orientalis*. While the class of glycopeptide antibiotics has many members, vancomycin is the only one in common clinical use, and it occupies an important medical niche in the treatment of infections due to broadly resistant Gram-positive organisms. However, its effectiveness is threatened by the emergence of resistant pathogens that are spreading rapidly and becoming a major public health menace. The need for new therapeutic agents to combat resistance is prompting detailed examinations of the structure and mechanism of action of vancomycin and related compounds.

Glycopeptide antibiotics act by inhibiting biosynthesis of the bacterial cell wall. The specific targets of vancomycin are peptides terminating with the sequence -L-Lys-D-Ala-D-Ala-COOH. The binding of vancomycin to these targets prevents the transpeptidation reaction necessary for the synthesis of peptidoglycan. The most common form of bacterial resistance substitutes D-lactate for the C-terminal D-alanine residue, producing a depsipeptide. This depsipeptide still functions as a substrate in the transpeptidation reaction, allowing resistant bacteria to synthesize normal peptidoglycan. However, the affinity of vancomycin for its target is reduced by 1000-fold. This reduction in affinity has been attributed chiefly to the loss of a hydrogen bond donor that would otherwise interact with an acceptor on the vancomycin backbone.[1,2]

X-ray crystal structures have been previously reported for complexes of vancomycin with the ligands acetate (Ac)[3,4] and *N*-acetyl-D-alanine (AcDA).[5] We now report three new crystal structures of vancomycin complexes with *N*-acetylglycine (AcG), D-lactate (DLac), and 2-acetoxy-D-propanoic acid (ACDLac), the latter being a mimic of the depsipeptide found in resistant bacteria (see Figure 1). These structures suggest that additional factors beyond the loss of an intermolecular hydrogen bond are required to explain the reduced affinity of vancomycin for depsipeptide ligands. Insights derived from the new structures have important implications for the rational development of novel agents active against resistant bacteria.

Results

Nomenclature. The asymmetric unit of this crystal form contains two vancomycin molecules, referred to herein as V1 and V2. The seven amino acid residues of the vancomycin monomer are denoted V1:1, V1:2, ...V1:7 and V2:1, V2:2, ...V2:7. The glucose and vancosamine sugars on each monomer are indicated as "G" or "V", instead of a residue number. The asymmetric unit also contains one ligand molecule, bound to V2.

Antibiotic Structures. The vancomycin:AcG, vancomycin:DLac, and vancomycin:ACDLac structures are isomorphous with the vancomycin:Ac complex previously described.[3,4] In each case, two antibiotic molecules form an unsymmetric homodimer in which the peptide portions of the dimer are related by an almost perfect 2-fold noncrystallographic symmetry axis. Hydrogen bonds between the two halves of the dimer follow the pattern of a two-stranded antiparallel β-sheet. The N- and C-terminal ends of the peptide bend outward away from the dimer interface, giving each monomer a concave shape and forming the ligand recognition

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§ Abbreviations: Ac, acetate; AcG, *N*-acetylglycine; AcDA, *N*-acetyl-D-alanine; DLac, D-lactate; ACDLac, 2-acetoxy-D-propanoic acid; NMR, nuclear magnetic resonance; rms, root-mean-square.

10.1021/jm990361t CCC: $18.00
Published on Web 10/07/1999

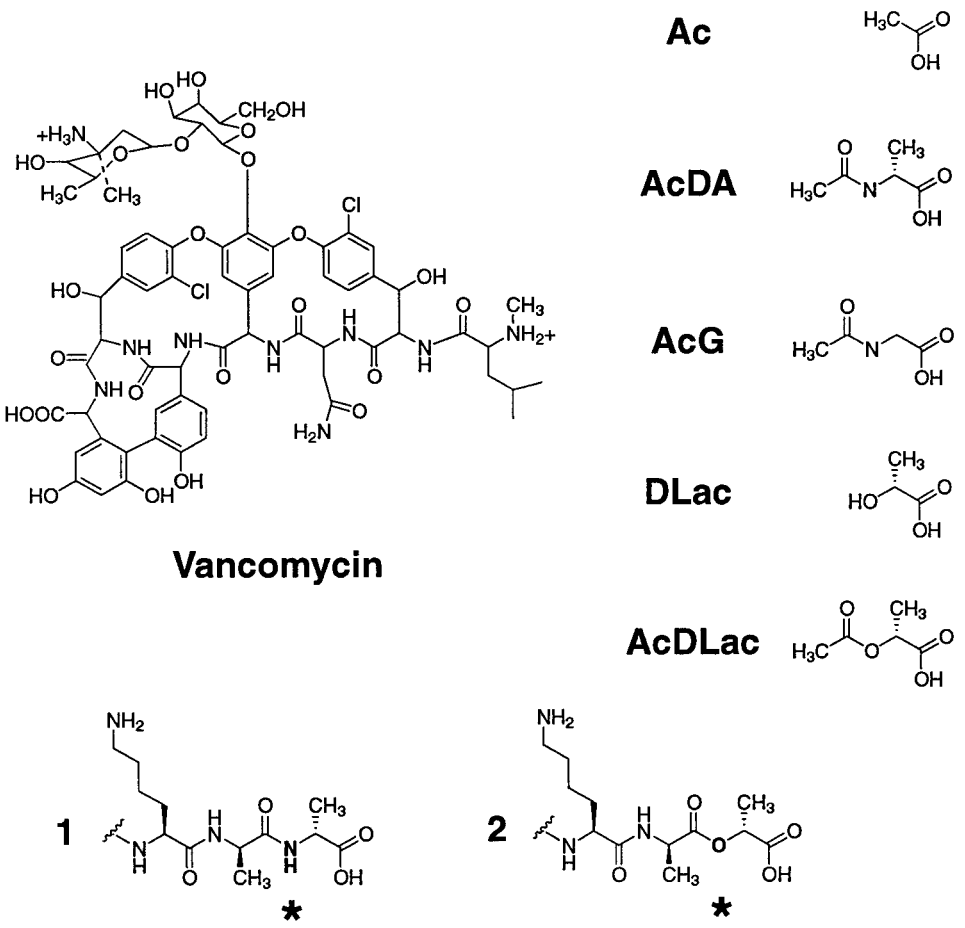

Figure 1. Structures of vancomycin and certain of its ligands. **1** and **2** represent the physiological ligands from vancomycin-sensitive and vancomycin-resistant bacteria, respectively; the site of the peptide → depsipeptide change is marked with a star. Structures of ligands for which vancomycin complex structures are available are shown at right.

Table 1. Rms Differences (in Å) in Atomic Positions between Various Vancomycin:Ligand Complexes[a]

	AcG	DLac	AcDLac	Ac	AcDA #1[b]	AcDA #2[b]
AcG		*0.089*	*0.124*	*0.091*	*0.711*	*0.737*
DLac	0.108		*0.067*	*0.035*	*0.699*	*0.724*
AcDLac	0.147	0.105		*0.064*	*0.714*	*0.730*
Ac	0.103	0.072	0.102		*0.692*	*0.717*
AcDA #1	0.996	0.972	0.987	0.986		*0.224*
AcDA #2	0.996	0.972	0.983	0.985	0.325	

[a] Superpositions were carried out with all 202 non-hydrogen atoms of the vancomycin dimer (bottom half of matrix, plain typeface) and with the 134 core atoms remaining after carbohydrate and side chains are removed (upper half of matrix, italics). [b] There are two crystallographically independent dimers in the asymmetric unit of the N-Ac-D-Ala crystals.

pocket. The carbohydrate portions of the dimer project outward above these concave surfaces and form part of the ligand recognition pockets. The carbohydrate does not obey the 2-fold symmetry of the rest of the dimer, rendering the two ligand recognition surfaces non-equivalent. The ligand binding site in V1 is occupied by a symmetry-related copy of V1 and the Asn side chain of V1:3, while V2 is occupied by ligand.

Rms differences in atomic positions after pairwise least-squares superposition of the non-hydrogen vancomycin atoms in these four complexes are approximately 0.1 Å (Table 1). Rms differences for atoms in the polypeptide 'backbone' are about one-half as large, approaching the level of uncertainty expected in the coordinate determination. Thus, these four independent refinements converged to essentially identical results for the antibiotic structure. In contrast, rms differences between these four structures and that of the previously described vancomycin:AcDA complex[5] are significantly greater, indicating that vancomycin adopts a somewhat different structure in the latter complex. This difference may be a consequence of the higher affinity that vancomycin has for AcDA and/or differences in crystal packing.

Ligand Binding. There are significant differences in the manner by which Ac, AcG, AcDA, DLac, and AcDLac bind to vancomycin (see Figure 2). Although the two carboxylate oxygens of each ligand form the same three hydrogen bonds to the vancomycin backbone, the positions of the other ligand atoms are different in each complex. The amide proton of AcG makes a hydrogen bond interaction with the carbonyl oxygen of V2:4. The carbonyl oxygen of the acetyl group points away from vancomycin into solution and does not participate in any hydrogen bonds with vancomycin or any symmetry-related complexes. Although the binding mode of AcG is similar to that of AcDA, AcG sits higher in the binding pocket (i.e., closer to V2:G) because there are no bulky substituents at the C_α position.

The crystals of the DLac complex were grown from a racemic mixture of D- and L-lactate, but only one isomer appears in the crystal. The bound ligand is identified as D-lactate from (a) the relative intensities of the hydroxyl oxygen and methyl carbon peaks in the

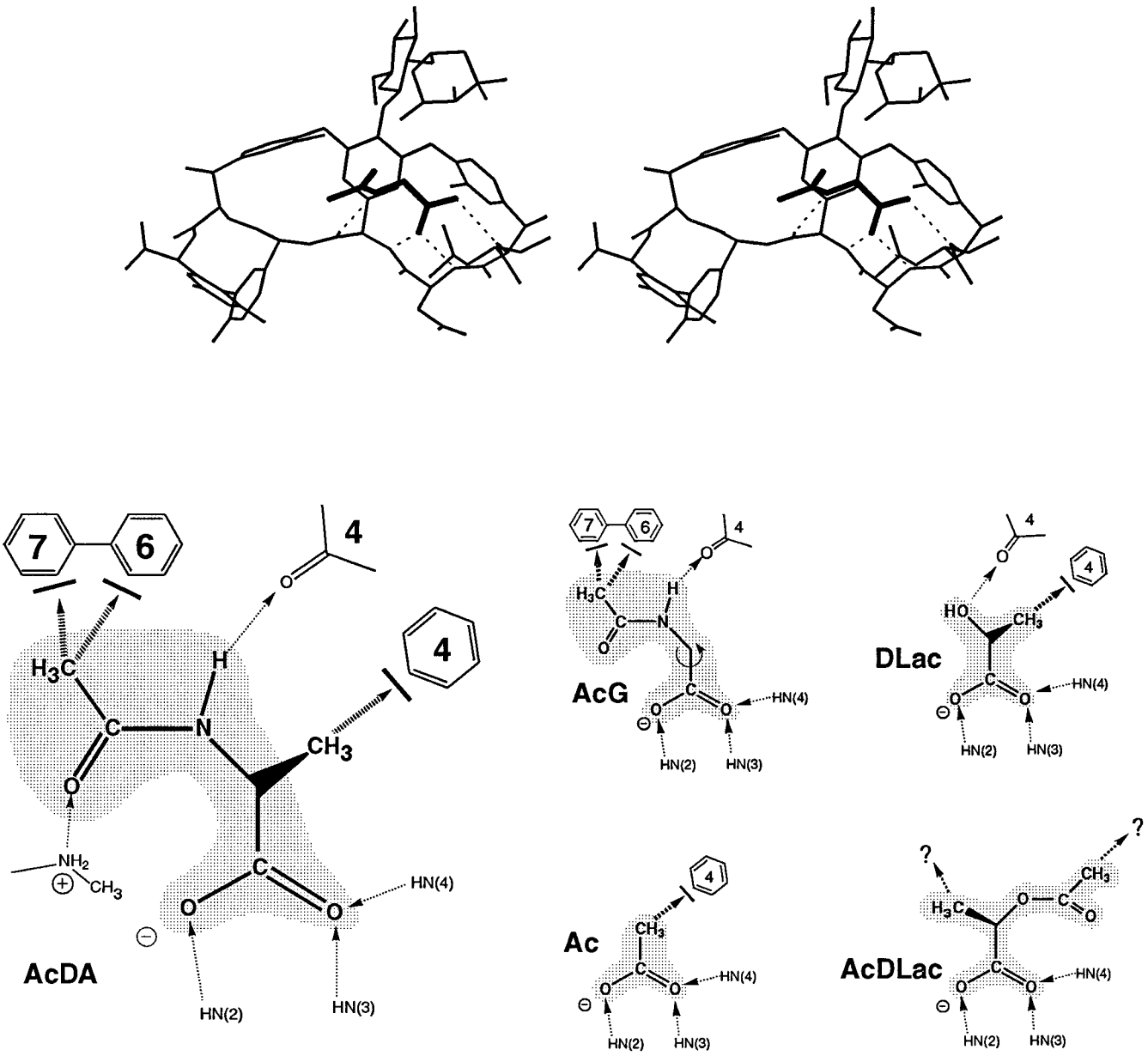

Figure 2. (a) Stereoillustration of ligand binding by vancomycin. Shown is the AcG ligand bound by the vancomycin monomer V2. AcG is shown in bold, and hydrogen bonds between the ligand and drug are shown as dashed lines. (b) Schematic representation of how ligand recognition by vancomycin varies for five different ligands.

electron density map and (b) the geometry of the hydrogen bond interactions available to the hydroxyl oxygen (see below). The methyl group of DLac points toward V2:G causing the C_α of DLac to be 0.6 Å further away from V2:G than the C_α of AcG. The hydroxyl group of DLac is well-positioned to donate a hydrogen bond to the carbonyl oxygen on residue V2:3 (oxygen−oxygen distance = 2.8 Å).

The most surprising result is seen in the AcDLac complex. Strong density is only seen for the lactate portion of the ligand molecule. Diffuse density can be seen near the ester oxygen of the ligand, but the acetyl group cannot be positioned with confidence into the electron density map, and we presume it to be present but disordered. The portion of the ligand that can be positioned shows the φ torsion angle of the ligand is rotated 180° relative to that in the DLac complex, with the ester oxygen pointing toward V2:G, rather than toward V2:3. It is unlikely that the bound ligand is hydrolyzed AcDLac because the products of hydrolysis would be acetate and DLac, and the electron density we observe in the vancomycin:AcDLac complex is clearly different from the density observed for the vancomycin: Ac and vancomycin:DLac complexes. In addition, after the DLac complex crystals were harvested, the reservoir buffer from the crystallization experiment was examined by reverse-phase HPLC, and no evidence for hydrolysis was observed.

DLac Binding Affinity. Binding affinities have been obtained previously for vancomycin and Ac, AcG, DLac, and AcDLac using a magnetic resonance.[6] We have repeated this measurement of the affinity of Ac for vancomycin, obtaining a value of 15 M^{-1}, in reasonable agreement with the value previously reported (see Table 2). When the method was applied to AcDLac, minimal changes in ^{13}C NMR chemical shifts were observed. Due to limited solubility of the AcDLac:vancomycin complex at high AcDLac concentrations, some uncertainty exists

Table 2. Affinities of Ligands for Vancomycin

ligand	abbreviation	affinity (M^{-1})
acetate	Ac	30[a]; 15[b]
N-acetyl-D-alanine	AcDA	300[a]
N-acetylglycine	AcG	80[a]
2-acetoxy-D-propanoic acid	AcDLac	40[b]
D-lactate	DLac	80[a]

[a] Reference 6. [b] This work.

in the limiting chemical shift values and hence in the calculated binding constant. Nonetheless, we can conclude that AcDLac binds relatively weakly to vancomycin and can place an upper estimate on the binding constant of 40 M^{-1}. Furthermore, the ^{13}C NMR chemical shifts indicate that AcDLac binds differently to vancomycin than other ligands. For ligands such as Ac or AcDA, the largest changes in ^{13}C NMR chemical shift (ca. 100 Hz) are observed for the carbonyl carbons of residues 3, 4, and 5.[6,7] However, when AcDLac is titrated into vancomycin, these same carbonyl carbons exhibit only small changes in chemical shift (less than 10 Hz). This suggests that AcDLac binds differently to vancomycin than do other ligands, in agreement with the crystal structure described in this report.

Discussion

The structural data reported in this paper in combination with previously reported structures reveal that a number of factors control high-affinity binding of ligand to vancomycin. This is best demonstrated by pairwise comparison of different complexes.

First, comparison of the vancomycin complexes with AcDA and AcG confirms that the lower affinity of AcG is not due to a drastically altered binding mode. An earlier computational study of ligands terminating in D-alanine and glycine identified three factors expected to contribute to lowered affinity for AcG: a larger entropic cost to the binding of AcG due to greater freedom of rotation for its C_α and carboxylate group (hindered by the α-methyl group in AcDA), favorable van der Waals interactions between the α-methyl group of AcDA and the aromatic ring of V2:4, and bond angle strain induced in the AcG ligand upon binding.[8] The resolution of these crystal structures does not permit unrestrained refinement, so we cannot address the question of bond angle strain, but by showing that AcG and AcDA bind in the same general way, the structural data help support the other two computational predictions. When bound to AcDA, vancomycin forms face−face and back−back dimers in the crystal;[5] however, the vancomycin:AcG complex forms only back−back dimers, even though AcG appears to be properly positioned to mediate face−face dimerization. This implies that higher ligand affinity helps promote dimer formation.

Second, comparison of the AcG and DLac complexes shows that AcG interacts with the aromatic rings of V2:6 and V2:7, yet the affinity of AcG for vancomycin is no greater than that of DLac. This indicates that any enhancement of AcG affinity attributable to its acetate group is either insignificant or negated by entropic, electrostatic, and angle strain factors.

Third, comparison of the AcDA and DLac complexes shows that the carboxylate groups in both ligands accept hydrogen bonds from the same donors, their α-methyl groups form the same van der Waals contacts with the V2:4 aromatic ring, and they both donate hydrogen bonds to the carbonyl of V2:4. Yet, the affinity of AcDA is almost 4-fold higher even despite the entropic cost of restricting rotation about the N−C (ψ) bond in AcDA upon binding. The acetate methyl group of AcDA does interact with the aromatic rings of V2:6 and V2:7, but as mentioned in the previous paragraph, these interactions are unlikely to be responsible for significantly higher affinity. It is more likely that the acetate carbonyl forms a hydrogen bond with the charged *N*-methylleucyl residue of another vancomycin monomer in a face−face dimer. This hydrogen bond is clearly formed in the crystal structure of AcDA. Inferences drawn here from the crystal structure of the DLac complex and ligand affinity measurements now support its energetic significance in solution.

Fourth, comparison of the Ac and AcDLac structures suggests that both of these ligands depend primarily on the binding of carboxylate for their affinity to vancomycin.

These comparisons reveal that a variety of different factors contribute to the recognition of ligands by vancomycin, including the ligand's flexibility and conformational preferences, its ability to participate in specific nonbonded interactions, and its ability to support formation of face−face vancomycin dimers. Identification of these factors has important implications for the design of vancomycin analogues with an affinity for depsipeptide ligands and the potential to overcome vancomycin resistance. For example, Stack et al.[9] have shown that vancomycin monomers, when covalently linked with a tether segment of sufficient length, are effective against bacteria exhibiting depsipeptide targets; Loll et al.[5] have argued that this antibacterial activity is likely due to the formation of face−face dimers and have shown that the requisite length of the tether corresponds precisely to that which is needed to span the linkage sites in a face−face dimer. In this context, the data reported herein suggest that the favorable free energy associated with formation of a face−face dimer more than compensates for the loss of an intermolecular bond associated with binding a depsipeptide ligand. The difference between the free energies of binding ($\Delta\Delta G$) of AcDA and DLac is roughly 3.3 $kJ \cdot mol^{-1}$, which can be attributed primarily to the electrostatic interaction across the dimer interface between the terminal acetate of the ligand and the *N*-methylleucine group of the drug. In contrast, the $\Delta\Delta G$ for Dlac versus AcDLac is only 2.4 $kJ \cdot mol^{-1}$, corresponding to the loss of the hydrogen bond between ligand and the carbonyl of V2:4.

The structure of vancomycin in the AcDA complex differs substantially from that seen in all of the other complexes considered here; the drug is in a more closed conformation, apparently gripping AcDA more tightly than other ligands. The affinity of AcDA for vancomycin is also higher than any other ligand considered here (Table 2). This suggests that tightly bound ligands can elicit a structural change not observed with lower-affinity ligands, and the threshold for this change corresponds to an affinity constant between 100 and 300 M^{-1}. This is reflected in the crystal packing seen in low-affinity complexes where one of the two ligand binding pockets in each dimer is occupied by a crystal contact.

Presumably, the ligands are so weakly bound that under crystallization conditions they are unable to displace the symmetry-related molecule. However, the addition of AcDA to any of the tetragonal crystals immediately causes them to crack and dissolve, indicating that the more tightly bound ligand can displace a symmetry-related vancomycin molecule from this binding pocket.

From the structures currently available, we can identify a minimum set of drug−ligand interactions necessary to trigger this structural change in vancomycin. The set includes: (1) three hydrogen bonds formed between the carboxylic acid of the ligand and amide protons of the antibiotic, (2) interactions between the α-methyl group of the ligand and the aromatic ring of residue 4, (3) the hydrogen bond donated by a nitrogen or oxygen of the ligand to the carbonyl oxygen of residue 4 of the antibiotic, and (4) various contacts between an acyl group attached to the C-terminal residue and both monomers in a face−face dimer. This acyl group is acetyl in AcG and AcDA but would be D-alanyl in the natural ligand. Each of the relatively low-affinity structures described herein exhibits only three of these four interactions, implying that all four interactions must be formed in order for vancomycin to adopt the high-affinity conformation.

Conclusions

Binding of high-affinity ligands by vancomycin is associated with a structural change in the drug and the formation of ligand-mediated face−face dimers. The structural change and dimerization may be coupled, since while low-affinity ligands bind in a manner compatible with face−face dimer formation, they elicit neither the structural change nor face−face dimers. We have identified a minimal set of interactions, all of which must occur between the drug and its ligand in order for high-affinity binding to be observed. These results show that the loss of an intermolecular hydrogen bond is only one of the factors needed to explain the failure of vancomycin to recognize depsipeptide ligands found in resistant bacteria, and it will be important to compensate for these other factors when designing agents targeted against vancomycin-resistant organisms. A question that remains is whether the high-affinity conformation of vancomycin seen with AcDA reflects the full extent of the structural change that is induced in vancomycin by ligands, or if tighter binding by longer and more physiologically relevant ligands will elicit further structural changes.

Experimental Section

Materials and Methods. Pharmaceutical grade vancomycin hydrochloride (Vancocin, Eli Lilly) was used without further purification. 2-Acetoxy-D-propanoic acid was synthesized as described below; *N*-acetylglycine was obtained from Sigma and lithium D-lactate from Aldrich. Other materials were obtained commercially and were of the highest purity available. TLC was carried out using silica-60 plates (EM Science) and a mobile phase of 1% acetic acid in ethyl acetate. HPLC analyses were conducted with an analytical Vydac C18 column, using 50 mM K_2HPO_4 as the mobile phase and identifying analytes by absorbance at 220 nm. NMR spectra were acquired on a Varian XL-300 spectrometer.

Synthesis of AcLac. The sodium salt of D-lactic acid (5.5 g, 49 mmol) was dissolved in 25 mL of a 65:35 (v/v) mixture of water and concentrated HCl. This solution was extracted repeatedly with diethyl ether. The organic phase was dried over $CaSO_4$ and filtered; its volume at this point was 150 mL. Pyridine was added to the solution (4.9 g, 62 mmol), after which acetyl chloride (3.9 g, 50 mmol) was added dropwise with stirring. A white precipitate appeared immediately. After 40 min excess pyridine was precipitated by the addition of 1 mL of concentrated HCl; the solution was then filtered and concentrated in a rotary evaporator. The remaining material was dissolved in water, frozen, and lyophilized; 1.35 g of a clear colorless oil remained after lyophilization (21%): ^{1}H NMR (300 MHz, D_2O) δ 5.04 (q, 1H, J = 7.0 Hz), 2.16 (s, 3H), 1.50 (d, 3H, J = 7.1 Hz); ^{13}C NMR (75 MHz, D_2O) δ 178.2, 176.2, 72.5, 22.8, 18.8. Anal. ($C_5H_8O_4$) C, H, O.

Measurement of Ligand Binding Constants via ^{13}C NMR Spectroscopy. Stock solutions of 400 mM ligand and 24 mM vancomycin were prepared in 50 mM potassium phosphate buffer, pH 6.0, containing 10% D_2O. The stock solutions were mixed with buffer to yield a final solution of 12 mM vancomycin and 0−200 mM ligand. A solution of 400 mM AcDLac, 12 mM vancomycin was prepared by the addition of solid vancomycin to the 400 mM AcDLac stock. NMR spectra were acquired immediately after sample preparation. ^{13}C NMR spectra were acquired on a Varian XL-300 spectrometer at a ^{13}C frequency of 75 MHz. Spectra were acquired with composite pulse proton decoupling, using a 90° (15-ms) excitation pulse and a 2-s interpulse delay. Spectra were acquired into 32K data points with a spectral width of 233 ppm. Approximately 12 000 acquisitions were averaged per spectrum. Spectra were acquired at room temperature (297 ± 2 K). Limiting chemical shift changes were estimated from plots of ^{13}C chemical shift versus concentration of AcDLac. Binding constants were calculated from plots of $\Delta\delta/\Delta\delta_{lim}$ versus $(\Delta\delta/\Delta\delta_{lim}) \times$ (concentration).

Crystallization. Crystals were prepared by the hanging drop vapor diffusion method; 5.0-μL drops containing 30 mg/mL vancomycin in water were mixed with equal volumes of reservoir buffer and suspended from a siliconized cover slip over 1.0 mL of reservoir buffer. The plates were sealed with plastic tape and maintained at 291 K. The reservoir buffers contained 100 mM ligand, which was used as a buffering agent at pH 4.6, and 2.0−2.4 M NaCl. Racemic lactic acid was used in the reservoir buffer for the DLac complex. Bipyramidal crystals formed within 1 week, growing to maximum dimensions of 0.2 × 0.3 × 0.5 mm. Shortly prior to data collection, the crystals were transferred to a solution of 30% v/v glycerol in reservoir buffer. After 1−2 min, the crystals were mounted in nylon loops and flash-cooled by plunging into liquid N_2.

Data Collection and Processing. Diffraction data were collected at beamline A1 at the Cornell High Energy Synchrotron Source and beamline X-12B at the National Synchrotron Light Source. Beamline A1 at CHESS was fitted with a binned 2K×2K CCD detector,[10] while beamline X-12B utilized a 30-cm MAR image plate detector. At CHESS, several data collection passes were made, using the unattenuated beam for high-resolution data and attenuation for the more intense low-resolution data. A similar strategy was used at NSLS, except that instead of attenuating the beam the exposure time was drastically reduced. In addition, the crystal used at X-12B was returned still frozen to the home laboratory, where additional low-resolution data were collected using a 30-cm MAR image plate detector mounted on a rotating anode source equipped with focusing mirrors and a Ni foil. Crystals were maintained at ca. 100 K in a stream of N_2 gas at all times during data collection. Images were processed using the programs DENZO and SCALEPACK.[11] Observations containing overloaded pixels were not included in the data set.

Refinement. Refinement was carried out against F^2 using the program SHELXL-93.[12] All three of the complexes are isomorphous with the vancomycin:Ac complex structure determined previously.[3,4] Thus, starting structures were obtained by removing the carbohydrate groups, ligand, and residue 1 and 3 side chains from the vancomycin:Ac structure and subjecting this fragment to rigid body refinement. R values after rigid body refinement were typically about 0.45. Missing

vancomycin atoms, as well as the ligands and solvent molecules, were positioned into difference maps during successive rounds of refinement and map inspection.

The molecular geometry and atomic displacement parameters were restrained throughout the refinement. The independent vancomycin monomers in the asymmetric unit were restrained to have similar 1,2- and 1,3-distances. Restraints were also imposed to limit deviations from planarity in rings and sp^2 systems. Distance and angle restraints were applied to the ligands. Along-bond components of anisotropic displacement parameters were made subject to hard restraints, and softer similarity restraints were applied to atoms closer than 1.7 Å. Solvent water atoms were restrained to be approximately isotropic and were also subjected to antibumping restraints to limit close contacts. Conjugate gradient refinement was used throughout, except for the end stage of the refinements, when a blocked least-squares method was used. Hydrogen atoms were included for the antibiotic and ligands and were refined with a riding model. The final values of R and R_{free} for all data to 1.0 Å are 0.110/0.129, 0.114/0.142, and 0.119/0.134 for AcG, DLac, and AcDLac, respectively. Coordinates have been deposited with the Protein Data Bank (accession numbers 1QD8, 1C0Q, and 1C0R).

Supporting Information Available: Data collection and refinement statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM990361T