Structural and Functional Characterization of VanG \(\text{D-Ala}:\text{D-Ser}\) Ligase Associated with Vancomycin Resistance in \textit{Enterococcus faecalis}*

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**Background:** \(\text{D-Ala}:\text{D-Lac}\) and \(\text{D-Ala}:\text{D-Ser}\) ligases are key enzymes in vancomycin resistance of Gram-positive cocci. They catalyze a critical step in the synthesis of modified peptidoglycan precursors that are low binding affinity targets for vancomycin. The structure of the \(\text{D-Ala}:\text{D-Lac}\) ligase VanA led to the understanding of the molecular basis for its specificity, but that of \(\text{D-Ala}:\text{D-Ser}\) ligases had not been determined. We have investigated the enzymatic kinetics of the \(\text{D-Ala}:\text{D-Ser}\) ligase VanG from \textit{Enterococcus faecalis} and solved its crystal structure in complex with ADP. The overall structure of VanG is similar to that of VanA but has significant differences mainly in the N-terminal and central domains. Based on reported mutagenesis data and comparison of the VanG and VanA structures, we show that residues Asp-243, Phe-252, and Arg-324 are molecular determinants for D-Ser selectivity. These residues are conserved in both enzymes and explain why VanA also displays D-Ala:D-Ser ligase activity, albeit with low catalytic efficiency in comparison with VanG. These observations suggest that D-Ala:D-Lac and D-Ala:D-Ser enzymes have evolved from a common ancestral D-Ala:D-X ligase. The crystal structure of VanG showed an unusual interaction between two dimers involving residues of the omega loop that are deeply anchored in the active site. We constructed an octapeptide mimicking the omega loop and found that it selectively inhibits VanG and VanA but not \textit{Staphylococcus aureus} D-Ala:D-Ala ligase. This study provides additional insight into the molecular evolution of D-Ala:D-X ligases and could contribute to the development of new structure-based inhibitors of vancomycin resistance enzymes.

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The glycopeptide antibiotics vancomycin and teicoplanin are important for the treatment of serious infections caused by Gram-positive cocci. \textit{Enterococcus} spp. resistant to vancomycin are a serious public health threat in hospitals worldwide. Their increased prevalence and their ability to transfer vancomycin resistance to other bacterial species, including methicillin-resistant \textit{Staphylococcus aureus}, have made them a challenging nosocomial pathogen (1).

The mechanism of action of glycopeptides is the inhibition of extracellular steps during peptidoglycan synthesis (2). These antibiotics interact with the D-Ala-D-Ala terminus of the uncross-linked peptidoglycan pentapeptide (\(N\)-acetyl-muramyl-L-Ala-D-Glu-Lys-D-Ala-D-Ala). This interaction involves five hydrogen bonds that sequester the D-Ala-D-Ala dipeptide, thereby inhibiting the activity of the transpeptidases necessary for cell wall cross-linking (3). Resistance results from the production of modified peptidoglycan precursors ending in D-Ala-D-Lac\(^2\) (VanA-, VanB-, VanD-, and VanM-type) or D-Ala-D-Ser (VanC-, VanE-, VanG-, VanL, and VanN-type) (4–6). This modification results in the loss of a hydrogen bond in the complex between D-Ala-D-Lac and vancomycin and to hydroxyl-mediated steric hindrance with D-Ala-D-Ser. As a consequence, vancomycin exhibits, respectively, a 1000- and 6-fold decrease in binding affinity (7, 8). In VanA-, VanB-, VanD-, or VanM-type enterococci, synthesis of D-Ala-D-Lac requires the presence of an ATP-dependent ligase (VanA, VanB, VanD, or VanM) of altered specificity when compared with the host D-Ala-D-Ala ligase (Ddl) (4, 9). In VanC-, VanE-, VanG-, VanL, and VanN-type strains, the ligase genes encode proteins that catalyze the synthesis of D-Ala-D-Ser (8, 10). These enzymes act at a critical step in reprogramming the peptidoglycan biosynthetic pathway and are therefore important targets for developing new antibiotics. The enzymatic reaction is a two-step process, with the transfer of the \(\gamma\)-phosphoryl of ATP to the carboxyl group of the first D-Ala leading to an acylphosphate intermediate and ADP. The acyl carbon of the acylphosphate then reacts with the amino group of the second D-Ala to yield a tetrahedral intermediate that dissociates to produce

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* This article was selected as a Paper of the Week. The atomic coordinates and structure factors (code 4FU0) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: D-Lac, D-lactate; Ddl, D-Ala:D-Ala ligase; LmDdl2, Ddl2 from \textit{L. mesenteroides}; StaDdl, Ddl from \textit{S. aureus}. 

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phosphate and D-Ala-D-Ala. Kinetic studies have identified two subsites for D-Ala binding in the active site (11, 12). Structural (13–18) and mutagenesis (19) studies have shown that many of the residues involved in substrate binding and catalysis are conserved in the D-Ala:D-X ligase superfamily.

The D-Ala:D-X ligase superfamily can be divided into six families based on sequence homology (Fig. 1). Three are D-Ala:D-Ala ligases, two are D-Ala:D-Lac ligases, and one is a D-Ala:D-Ser ligase (20). VanG is a singular example because it is associated with a D-Ala-D-Ser mechanism of resistance, whereas it is phylogenetically closer to the VanA group. An intriguing question in vancomycin resistance is the molecular evolution of the D-Ala:D-Ala ligases that led to a switch in substrate specificity and thus to the reprogramming of peptidoglycan synthesis.

Because the first reaction step is common to all D-Ala:D-X ligases, specificity for the second substrate must be attributed to differences in the second binding site (subsite 2). D-Ala:D-Lac ligase specificity was previously studied by the structure determination of VanA (14) and of the naturally resistant LmDdl2 from Leuconostoc mesenteroides (16). Both structures were determined in complex with a phosphinophosphate inhibitor, which is a close analog of a tetrahedral intermediate in the catalytic reaction. The basis for D-Ala:D-Ser ligase specificity is not fully understood due, in part, to the absence of three-dimensional structures for this family of enzymes. Previous studies of the VanC2 D-Ala:D-Ser ligase suggested that residues Arg-322 and Phe-250 corresponding to, respectively, Arg-324 and Phe-252 in VanG could be responsible for the greater affinity of the second binding site for D-Ser (21). However, the role of these residues in controlling substrate specificity has not been elucidated. To gain insight into the molecular specificity of D-Ala:D-Ser ligases, we have studied the function and crystal structure of VanG and provide evidence that the triad Asp-243, Phe-252, and Arg-324 is the molecular signature for D-Ala-D-Ser specificity.

**EXPERIMENTAL PROCEDURES**

**Cloning, Protein Expression, and Purification**—Production and purification of VanG have been reported previously (22). Briefly, VanG engineered to have a C-terminal His tag was produced from Escherichia coli (23). Production and purification of VanA and the D-Ala:D-Ala ligase from Staphylococcus aureus (StaDdl) were produced and purified from E. coli (24). Substrate Specificity and Kinetic Analysis—Substrate specificity was assayed by thin-layer chromatographic (TLC) analysis of radioactive dipeptides produced by VanG (20) following incubation of D-[1-14C]Ala or D-[1-14C]Ser (5 mM) with 80 mM of additional amino acid or hydroxy acid (25). The D-Ala:D-Lac ligase activity was studied by incubation of D-Ala (5 mM) with 80 mM D-[1-14C]Lac. Kinetic parameters of D-Ala:D-X ligase activities were determined using the ADP release spectrophotometric assay previously described (26). The assay mixture contained 100 mM Hepes (pH 7.5), 10 mM KCl, 10 mM
MgCl₂, 10 mM ATP, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 50 units/ml pyruvate kinase/l-lactate dehydrogenase, and substrate at increasing concentrations. Kinetic constants (the mean values of at least three independent measurements) were obtained by fitting experimental data to the equations described by Neuhaus (11) using the program EnzFitter (BIOSOFT, Cambridge, UK). 

Inhibition by Peptide Omega-1—Peptide Omega-1 (formyl-DYTEKYTL-NH₂) was obtained from Peptide 2.0 Inc. (Chantilly, VA). Inhibition of VanG, VanA, and StaDdl was assayed against D-Ala-D-Ala formation by the ADP release spectrophotometric (VA). Inhibition of VanG, VanA, and StaDdl was assayed (BIOSOFT, Cambridge, UK). 

Described by Neuhaus (11) using the program EnzFitter were obtained by fitting experimental data to the equations (the mean values of at least three independent measurements) were obtained by nonlinear regression analysis using EnzFitter.

Structure Determination—Crystallization, data collection, and structure determination of the VanG-ADP complex were reported previously (22). Briefly, crystals were obtained with the hanging-drop vapor diffusion method by mixing 2 μl of protein solution at 14.5 mg/ml containing 10 mM ADP with 2 μl of crystallization solution containing 0.5 M ammonium sulfate, 0.9 M lithium sulfate, and 0.1 M sodium citrate, pH 5.6, and equilibrated against 1 ml of reservoir solution. The best crystal was flash-cooled in liquid nitrogen using the crystallization solution containing 25% (v/v) of glycerol as a cryoprotectant, and x-ray diffraction data were collected on beamline Proxima 1 at the SOLEIL synchrotron (St. Aubin, France). The crystallographic parameters and data statistics were reported (22); the protein crystallized in space group P3₁21 (a = b = 116.1 Å, c = 177.2 Å) with two independent molecules forming a dimer in the asymmetric unit, and diffraction data extended to 2.35 Å resolution. The structure was determined by molecular replacement with the program Phaser (27) using coordinates of the VanA D-Ala-D-Lac ligase from Enterococcus faecium (Protein Data Bank (PDB) code 1E4E) (14), which has 45% sequence identity with VanG. The structure was refined by alternate cycles of restrained maximum likelihood refinement using the program Refmac5 (28) as implemented in the CCP4 program suite (29). Manual adjustments to the model were made with Coot (30). The final refinement statistics and model parameters are shown in Table 1. All structural representations were generated with PyMOL (31).

RESULTS

Substrate Specificity of VanG—Thin layer chromatographic assay showed that similarly to VanA (25) and VanC2 (32), VanG was able to synthesize several mixed dipeptides (Fig. 2). Using D-[1-¹⁴C]Ala and D-X (amino acid or hydroxy acid), VanG synthesized simultaneously D-Ala-D-X and D-Ala-D-Ala. However, in the presence of D-Ser, D-Ala-D-Ala was not detected, suggesting that VanG exhibits a high specificity for D-Ser at subsite 2 (Fig. 2A). D-Threonine was also a good substrate as judged by the low amount of D-Ala-D-Ala formed. Alternatively, VanG could also accommodate a number of amino acids such as D-amino butyrate, D-norvaline, D-norleucine, D-methionine, and to a lesser extent, D-valine, D-leucine, and D-isoleucine. Aromatic amino acids such as D-phenylalanine and D-tryptophan were poor substrates, arguing that subsite 2 could be a relatively small cavity unable to accommodate bulky amino acid side chains. VanG did not synthesize D-Ala-D-Lac or D-Ala-D-hydroxyvalerate as confirmed by using D-[¹⁴C]lactate and D-Ala in the assay mixture (data not shown).

![FIGURE 2. Substrate specificity of VanG as D-Ala-D-X ligase (A) and D-Ser-D-X ligase (B) tested by TLC. Lane 1, no enzyme; lane 2, D-alanine, lane 3, D-serine; lane 4, D-lactate; lane 5, D,L-aminobutyrate; lane 6, D,L-hydroxyvalerate; lane 7, D-norvaline; lane 8, D-valine; lane 9, D-norleucine; lane 10, D-threonine; lane 11, D-leucine; lane 12, D-phenylalanine; lane 13, D-isoleucine; lane 14, D-methionine; lane 15, D-tryptophan.](image-url)
Interestingly, incubation of VanG in the presence of D-[1-14C]Ser and the same series of substrates showed that VanG was able to synthesize only a few D-Ser-D-Ser activity was detected (32).

### Table 2: Kinetic parameters of VanG, VanA, and VanC2 D-Ala:D-Ser ligases

| Product/Substrate | VanG enzyme | VanA enzyme | VanC2 enzyme |
|-------------------|-------------|-------------|--------------|
|                   | $K_m$ (ms)  | $K_m$ (ms)  | $K_m$ (ms)  |
|                   | $K_m$ (ms)  | $K_m$ (ms)  | $K_m$ (ms)  |
|                   | $s^{-1}$    | $s^{-1}$    | $s^{-1}$    |
| $s^{-1}$          | $10^3$      | $10^3$      | $10^3$      |
| D-Ala-D-Ala        | 0.4 ± 0.17  | 32.7 ± 9.5  | 7.8 ± 0.6   |
| D-Ala-D-Lac        | ----        | ----        | ----        |
| D-Ala-D-Ser        | ----        | 0.22 ± 0.01 | 5.5 ± 0.2   |
| D-Ser-D-Ser        | 1.8 ± 0.9   | 16.3 ± 2.6  | 6.1 ± 1.4   |
| D-Ala-D-Abu        | 4.5 ± 1.4   | 2.0 ± 0.2   | 0.44 ± 0.14 |
| D-Ser-D-Abu        | 3.0 ± 0.7   | 3.5 ± 0.6   | 1.16 ± 0.3  |
| D-Ala-D-Abu        | NA          | 0.05 ± 0.01 | NA          |
| D-Thr-D-Thr        | NA          | 0.1 ± 0.03  | NA          |
| ATP                | 3.6 ± 0.65  | ----        | ----        |

Data are from Refs. 21 and 32.

### Enzyme Kinetic Characterization

Kinetic parameters of VanG were determined for a number of mixed dipeptides (Table 2). As expected, high catalytic efficiency was obtained for D-Ala-D-Ala formation, which was ~5-fold higher than for VanC2 due to a 8 times lower $K_m$ value. This result is in contrast with that obtained for VanC2 where no D-Ser-D-Ser activity was detected (32).

### Overall Structure of VanG

VanG crystallized with two independent molecules forming a homodimer in the asymmetric unit (Fig. 3A). Difference electron density maps revealed clear density for the polypeptide chains from residues 2–348 of both monomers, except for a gap from residues 89–92 near a pseudo two-fold axis of the dimer. Each monomer comprised three domains (Fig. 3B) as reported for VanA (14) and DdB (13). The N-terminal domain (residues 2–130) was formed by three α-helices and a core of seven β-sheets, whereas the central domain (residues 131–223) and C-terminal domain (residues 224–348) were formed by four α-helices and four and five β-strands, respectively. The nucleotide and substrate binding sites were located between the central and the C-terminal domains. Interactions in the dimer interface were both hydrophobic and electrostatic, with a buried surface area of 1790 Å² as calculated from the European Bioinformatics Institute (EBI) PISA web-based server (33). The overall structures of the two monomers of VanG were very similar except for the omega loop regions (residues 248–262), which differed significantly in conformation and orientation (Fig. 4A).

Superposition of the VanG monomer with that of VanA (PDB code: 1E4E) showed that the nucleotide and substrate binding sites were structurally homologous except for significant variations in the conformation and position of the omega loop and in the serine-serine 150S loop (Gly-188–Phe-191) (Fig. 3B). The major secondary and tertiary structural variations occurred in the central domain, mostly in the number and position of the α-helices (Fig. 3B).

### Nucleotide Binding Site

As reported for other D-Ala:D-X ligases (13–15), the nucleotide binding site is formed by two antiparallel β-sheets from the central and C-terminal domains. The position and orientation of ADP in the binding site are highly conserved. Solvent accessibility calculations indicated that ADP is completely buried in the active site of the VanG ligase. The principal interactions involved in ADP binding are hydrophobic, ion pairs, and hydrogen bonds. The adenine ring of ADP is buried in a hydrophobic pocket formed by Phe-181, Ile-193, Ile-222, Phe-252, Tyr-258, and Phe-301. Access to the ADP binding site of monomer A in the dimer is partly blocked by residues from the omega loop of a symmetry-related molecule in the crystal (Fig. 4A). The nucleotide base of ADP makes a π-π stacking interaction with the aromatic side chain of Phe-181. The N6 atom of ADP forms hydrogen bonds with the side chain oxygen atom of Gln-219 and the carbonyl group of Glu-220, whereas the N1 and N7 atoms make hydrogen bonds with the main chain of Ile-222. In the ribose moiety, the O5’ atom forms a hydrogen bond with the side chain of Glu-226, and the O3’ atom interacts with a water molecule in the binding pocket. The interaction established by the ribose differentiates VanG from other D-Ala:D-X ligase structures where O3’ also interacts with Glu-226. The α- and β-phosphate atoms of ADP form...
ion-pair interactions/hydrogen bonds with the side chains of Lys-140, Lys-183, Asn-311, and Glu-312. The β-phosphate atom also makes hydrogen bonds with Ser-190. This residue is part of the 150S loop that is present in all d-Ala:d- X ligases and is responsible for refolding of the active site during catalysis (13).

Ligand Binding Site—Our attempts to determine the structure of VanG in complex with the phosphinate analog of d-Ala- d-Ser by co-crystallization or by soaking were unsuccessful. This could be due to the presence of a bound sulfate ion (from the crystallization solution) in the ligand binding site near the position of the phosphinate analog described in other Ddl structures (Fig. 3B). Nevertheless, the structure of VanG reported here provides a description of the topology of the active site and insight into its molecular specificity. The residues present at subsite 1 are conserved and are in similar orientations as in VanA, in particular, the strictly conserved residues Glu-16, Val-19, and His-106 involved in d-Ala recognition (14). At subsite 2, the corresponding residues forming the oxyanion hole that interacts with the phosphinate analog are Arg-297, Asn-314, and Gly-318, but their position is shifted when compared with VanA (Fig. 5). However, a conformational shift upon substrate binding could bring these residues into proximity of the ligand. Based on interactions of the phosphinophosphinate inhibitor in the active site of VanA, the carboxyl group of d-Ser in VanG could form hydrogen bonds with the side chain of conserved residue Ser-323 and with the amide group of Arg-324, and the hydroxyl group of d-Ser could be stabilized by the side chain of Arg-324, thus contributing to the specificity for serine at subsite 2 (Fig. 5).

FIGURE 3. Overall structure of VanG. A, structure of the VanG homodimer. The omega loops of the two monomers have significantly different conformations. ADP is shown in red. B, VanG monomer A (green) superimposed on VanA (yellow) (PDB code 1E4E). The phosphophosphinate inhibitor in the VanA structure is colored in magenta, and the magnesium ions are shown as gray spheres. A sulfate ion (yellow sphere) is located in the subsite 2 active site of VanG. The omega loops of VanG and VanA are colored in dark red and light blue, respectively. The serine-serine (150S) loops are indicated by arrows.
Structural and Functional Properties of the Omega Loop—Comparison of the two monomers of VanG revealed major structural differences in the omega loop region from residues 248–262, with differences of more than 12 Å in α-carbon positions at residues Tyr-258 and Thr-259 (Fig. 3A). The omega loop of monomer A of the dimer completely buries the ADP binding site, leaving partial access to the substrate binding site.

Monomer B’ from a neighboring dimer makes extensive hydrophobic and electrostatic interactions with monomer A in the crystal, with a total buried surface area of 1359 Å² (Fig. 4A). These interactions involve direct contacts of the omega loop with the ADP and substrate binding sites of an adjacent monomer A related by crystallographic symmetry (Fig. 4A). The interface contains a number of buried water molecules. Details of the electrostatic interactions in the binding interface are summarized in Table 3 and Fig. 4C. The buried surface of omega loop residues 248–262 in the interface is 814 Å². Residue Thr-255 of monomer B’ makes a hydrogen bond with Glu-16 of the adjacent symmetry-related monomer A (Glu-16 participates in the binding of D-Ala at subsite 1 in other Ddl ligases) (Fig. 4, B and C). Glu-256 of the omega loop forms a
Omega-1 (formyl-DYTEKYTL-NH₂), mimicking the omega loop sequence, to inhibit D-Ala:D-X ligase activity. Omega-1 was selected based on the contacts that the omega loop makes with the side chains of key active site residues involved in substrate binding or catalysis, i.e. Glu-16, Ser-190, and Asn-314 (Fig. 4C, Table 3). To ensure high similarity with the omega loop, Omega-1 was synthesized with C-amidated and N-formylated termini and was tested as a potential inhibitor of VanG, VanA, and StaDdl. Omega-1 was found to inhibit, in a dose-dependent manner, VanG and VanA but not StaDdl. At 2 mM final concentration, the rates of inhibition were, respectively, 32, 58, and 9%, corresponding to IC₅₀ values of, respectively, 2.6 ± 0.06, 1.7 ± 0.06, and 11.2 ± 2.8 mM. As a control, two unrelated peptides of nine residues (RYYPYGSL and GYNVTRYEV) were also tested and showed no inhibition at 5 mM final concentration, suggesting that inhibition by Omega-1 is specific.

**DISCUSSION**

Understanding the molecular basis of D-Ala:D-X ligase specificity is important for the development of antibiotics to overcome vancomycin resistance. The specificity of D-Ala:D-Lac ligases has been investigated by mutagenesis (21, 23, 34), three-dimensional structure determination (14, 16), and molecular docking of the LmDdl2 D-Ala:D-Lac ligase (35, 36). These studies provide evidence that the omega loop plays a central role in substrate selectivity at subsite 2. Selectivity of VanA for D-Lac was attributed to the positive charge of His-244, located in the omega loop, which could attract the negatively charged D-Lac substrate and reject the protonated form of the D-Ala amino group at the second subsite (14, 23), whereas in LmDdl2, Phe-261 could orient D-Lac favorably to achieve catalysis (36). The situation for D-Ala:D-Ser ligases is less clear. Based on the results of mutagenesis of VanA and VanC2 (21, 23), we have carried out the kinetic characterization and crystal structure determination of the VanG D-Ala:D-Ser ligase. This protein is phylogenetically close to the VanA D-Ala:D-Lac ligase (Fig. 1) despite the difference in resistance mechanisms mediated by these enzymes, an observation suggesting that they could share determinants for substrate specificity. However, kinetic analysis showed that VanG had high catalytic efficiency for D-Ala:D-Ser formation but no detectable depsipeptide ligase activity (Table 2).

The overall structure of VanG was found to be very similar to that of VanA, and the position of most active site residues was conserved in both nucleotide and ligand binding sites of the two enzymes (Figs. 3B and 5A). However, in the VanG ADP binding site, Glu-226 made a single hydrogen bond to the ribose moiety, whereas in VanA, both the O2' and the O3' atoms of the ribose ring established hydrogen bonds with the side chain of the structurally equivalent residue Glu-214. This difference may explain the lower affinity of VanG for ATP (Table 2). In addition, significant differences in the omega loop conformation were observed and could be due to the absence of the phosphophosphate inhibitor in the active site of VanG.

Interestingly, residues Arg-322 and Phe-250, which have a role in the D-Ser specificity of VanC2 (21), are also conserved in VanA (Arg-317 and Phe-241) and in VanG (Arg-324 and Phe-250).
Molecular Specificity of D-Ala-D-Ser Ligases

FIGURE 6. Alignment of the C-terminal domains of representative D-Ala-D-X ligases harboring the omega loop (box). Sequences are grouped as follows: group 1, VanA and VanC groups; group 2, D-Ala-D-Ala ligases; and group 3, D-Ala-D-Lac ligases from lactic bacteria. Conserved amino acids are highlighted in blue, and color intensity is dependent on the percentage of identity. Active site residues conserved in all D-Ala-D-X ligases are colored in gray. The residues of the DFR triad involved in D-Ala-D-Ser specificity are colored in red, and those stabilizing His-244 in VanA are shown in green. The phenylalanine determinant of D-Ala-D-Lac ligase in lactic bacteria is displayed in purple. Alignment was done by ClustalW (40), and the figure was generated using Jalview (41).

VanA was unable to synthesize D-Ala-D-Lac and D-Ala-D-hydroxyvalerate (Table 2), which is mainly due to the lack of the His-244 counterpart in the VanG omega loop. The sequence identity of VanG with the VanA group includes a number of residues (e.g. Lys-35, Ser-77, Asp-130, Phe-191, Glu-219, Asp-341 . . . ), which are distributed among all three structural domains and are thus unlikely to play a significant role in specificity. Although the omega loop containing His-244 appears to be a major determinant for D-Ala-D-Lac specificity, replacement of the VanC2 omega loop by that of VanA did not result in D-Ala-D-Lac or other depsipeptide synthesis (23). This indicates that other residues also play a role in specificity. In VanA, His-244 is part of a hydrogen-bond network involving Lys-22, Tyr-315, and Glu-250 (Fig. 5) (14), which are conserved in the VanA group; Tyr-315, which makes direct contact with His-244, is conserved only in the VanA group. The evolutionary relationship between these two Van groups is of practical significance because these enzymes determine, in part, the resistance level of pathogenic bacteria to glycopeptides. Unlike the Lmdll2 ligase where the mutation F261Y switches synthesis of D-Ala-D-Lac to D-Ala-D-Ala (34), the evolution of ligase activity from D-Ala-D-Ser to D-Ala-D-Lac involves more than one or two residues because it includes the omega loop and at least Tyr-315. As reported for Lmdll2 (36), the switch of specificity and thus emergence of resistance could be due to an appropriate substrate orientation at subsite 2. The question then arises: could D-Ala-D-Ser ligases evolve to acquire depsipeptide ligase activity by selecting the hydroxyl group of D-Ser rather than the amino group for reaction with the alanylphosphate intermediate? This could generate a new mechanism for ligase activity conferring high levels of vancomycin resistance. It may be of interest to study the orientation of D-Ser and to see whether any mutations could favor ester over peptide bond formation.
Interaction of the omega loop with the active site of an adjacent monomer has not previously been described in other D-Ala:D-Ala ligase structures, and it is not known whether this intermolecular binding has any functional role. The extensive interactions and complementarity of the binding surfaces (Fig. 4) suggest using the omega loop sequence to select a potential peptide inhibitor for this family of enzymes. Glu-16, Ser-190, and Asn-314 of monomer A, strictly conserved residues involved in substrate binding or catalysis (13, 19), interact with the neighboring omega loop (Fig. 4, Table 3). The Omega-1 peptide inhibits VanG and VanA, but not StaDdl, suggesting a different mode of binding at the active sites of these enzymes. Omega-1 probably binds to the active site of VanG in the same manner as the omega loop in the crystal structure. However, it is difficult to predict the interaction geometry in VanA. Apart from the conserved active site residues involved in the interaction with the omega loop (Table 3), residues Thr-15, Glu-18, Gly-255, and Phe-316. These differences could be responsible for the 2-fold difference in the inhibition rates of VanA and VanG and the very weak inhibition of StaDdl. To get more insight into the molecular recognition of Omega-1, we are currently trying to obtain the crystal structures of VanA and VanG in complex with the peptide inhibitor. Only a few peptide inhibitors of D-Ala:D-Ala ligases have been described (37), and all are analogs of the D-Ala:D-Ala product with $K_{i}$ or IC$_{50}$ values ranging from 0.5 to 6 mM. The IC$_{50}$ values of Omega-1 for VanG and VanA were of the same order of magnitude. Thus, Omega-1 could provide a valuable model for the structure-based design of new D-Ala:D-Ala ligase inhibitors.

In conclusion, we report the first structure of a member of the vancomycin resistance D-Ala-D-Ser ligases and provide new insight into the molecular specificity and evolution of this family of enzymes. The structure of VanG reported here enabled us to describe a new peptide inhibitor and provided clues for the search for more effective peptide derivatives and for new antibiotics active against glycopeptide-resistant pathogens.

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