Overexpression, Purification, and Characterization of VanX, a D-, D-Dipeptidase which Is Essential for Vancomycin Resistance in *Enterococcus faecium* BM4147†

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ABSTRACT: Vancomycin resistance in *Enterococcus faecium* requires five genes: *vanR, vanS, vanH, vanA,* and *vanX*. The functions and mechanism of four gene products have been known, with VanR/S for signal transduction and transcriptional regulation and VanH/A to synthesize D-Ala-D-lactate. But the function of the fifth gene product, VanX, has been unknown until very recently, when Reynolds and colleagues discovered D-, D-dipeptidase activity in crude extracts of a VanX overproducer [Reynolds, P. E., et al. (1994) *Mol. Microbiol. 13,* 1065−1070]. We report here the expression of VanX in *Escherichia coli* and its purification to homogeneity. VanX has been characterized as a metal-activated D-, D-dipeptidase with an optimal pH range of 7−9. The k_{cat} and K_m of D-Ala-D-Ala in the absence of divalent metal are determined to be 4.7 s^{-1} and 1 mM, respectively. However, in the presence of metal cations, k_{cat} can be as high as 788 s^{-1}. VanX is unable to hydrolyze D-Ala-D-lactate, the substituted moiety in the peptidoglycan that leads to vancomycin resistance, not only because of low binding affinity (K_i estimated at 242 mM) but also due to a k_{cat} less than 0.005 s^{-1}. The more than 10^5-fold differential in catalytic efficiency of VanX for hydrolysis of D-Ala-D-Ala vs D-Ala-D-lactate leaves D-Ala-D-lactate intact for subsequent incorporation into peptidoglycan. Phosphinate analogues of the proposed tetrahedral adduct for hydrolysis of D-Ala-D-Ala show mixed-type noncompetitive inhibition of VanX at K_{is} of approximately 0.4 μM, for a K_m/K_{is} ratio of 2500:1. The dipeptidase activity of VanX may be a target for drug design to reverse clinical vancomycin resistance.

The glycopeptide antibiotic vancomycin acts against Gram-positive bacteria by gaining access to the extracellular face of the cytoplasmic membrane. It binds to the D-Ala-D-Ala terminus of disaccharyl pentapeptide units which are to be incorporated into the peptidoglycan (PG)[1] layer. These vancomycin-*N*-acyl D-Ala-D-Ala complexes block the subsequent transglycosylation and transpeptidation reactions which attach and cross-link the incoming disaccharyl peptide to the growing PG layer. The net result of the formation of the drug−PG complex is that the PG layer has less tensile strength and the bacteria are susceptible to osmotic lysis [for reviews, see Barna and Williams (1984) and Reynolds (1989)].

In the past decade, clinically significant vancomycin resistance has arisen and been traced to a set of five *van* genes, *vanR, vanS, vanH, vanA,* and *vanX* on transposable elements (Arthur et al., 1993). Each of these genes is required, and the whole set is sufficient to confer the resistance phenotype (Arthur et al., 1992b). Protein sequence analysis has suggested the possible functions for four of the Van proteins (Arthur et al., 1991, 1993; Dutka-Malen et al., 1990), and we have previously reported the overproduction in *Escherichia coli* and purification of VanA, VanH, VanR, and VanS (Bugg et al., 1991a,b; Wright et al., 1993). VanH is a D-hydroxyacid dehydrogenase, and VanA is a D-Ala-D-lactate depsipeptide ligase. Together, VanH and VanA synthesize D-Ala-D-lactate rather than D-Ala-D-Ala. D-Ala-D-lactate is incorporated into PG to yield a sugar−tetrapeptide ester terminating in D-Ala-D-lactate rather than the normal cell wall intermediate sugar−pentapeptide ending in D-Ala-D-Ala. Vancomycin binds to *N*-acyl-D-Ala-D-lactate with 1000-fold lower affinity compared to *N*-acyl-D-Ala-D-Ala (Bugg et al., 1991a; Wright & Walsh, 1992; Messer & Reynolds, 1992), which explains the molecular strategy of VanH and VanA function. VanS and VanR encode a prototypical two-component regulatory system. VanS is a transmembrane sensor with a cytoplasmic histidine kinase domain (Wright et al., 1993), while VanR is a cytoplasmic response regulator that binds to *vanH* and *vanR* promoter regions (Arthur et al., 1992b; Holman et al., 1994). The binding affinity of VanR is enhanced by phosphorylation at its Asp53 residue from phospho-VanS, allowing activation of transcription of the *vanH, vanA,* and *vanX* structural genes and possibly regulation of transcription of the *vanS* and *vanR* genes (Holman et al., 1994).

Until recently, the function of the fifth gene, *vanX,* has been enigmatic. DNA sequence analysis revealed a 202 aa ORF (open reading frame) with no obvious homology to any known proteins (Arthur et al., 1993). Reynolds and the Institut Pasteur group (Reynolds et al., 1994a) have recently

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[1] Abbreviations: BSA, bovine serum albumin; ddl, D-Ala-D-Ala ligase; DTT, dithiothreitol; EC_{50}, effective concentration for 50% response; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl thiogalactopyranoside; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PG, peptidoglycan; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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observed that overproducing constructs of VanX lead to depletion of D-Ala-D-Ala but not D-Ala-D-lactate, suggesting that VanX is a D-Ala-D-Ala dipeptidase. In this work, we have confirmed those observations by purification of VanX to homogeneity and characterization of it as a divalent metal-activated D-, D-dipeptidase that does not catalyze the hydrolysis of D-Ala-D-lactate. We also describe the potent inhibition of VanX's dipeptidase activity by phosphinate analogues that may mimic tetrahedral intermediate in the hydrolysis of D-Ala-D-Ala.

MATERIALS AND METHODS

Materials. Plasmid pAT399, which carries the *vanX* gene, was a generous gift from Dr. M. Arthur, Institut Pasteur, Paris, France. D-3-[(1-Aminoethyl)phosphinyl]-2-heptylpropionic acid was a gift from Dr. A. A. Patchett, Merck Research, Rahway, NJ. 3-[(1-Aminoethyl)phosphinyl]-2-methylpropionic acid was a gift from Drs. P. Bartlett and B. Ellsworth, Department of Chemistry, University of California, Berkeley, CA. D-3-[(1-Aminoethyl)phosphonyl]-D-2-methylpropionic acid was a gift from Dr. J. Griffin, Department of Chemistry, Stanford University. CHELEX 100 resin was purchased from Bio-Rad. Sodium D-lactate was purchased from Fluka. Ninhydrin, amino acids, dipeptides, and tripeptides were purchased from Sigma, except D-Ser-D-Ala, D-Ala-L-Ala, L-Ala-D-Ala, and D-Ala-D-Phe, which were purchased from Chemical Dynamics Corp., South Plainfield, NJ. All other chemicals were reagent-grade and were purchased from Sigma or Aldrich.

Preparation of VanX Overproducing Construct. A plasmid suitable for overproduction of VanX was prepared by the expression cassette polymerase chain reaction (PCR) method (MacFerrin et al., 1990). PCR primers were designed to incorporate the ribosome binding site of the bacteriophage T7 gene 10 and a unique *Xba*I site 5′ to the initiation codon of *vanX* (GW1: 5′-GCATGCTCTAGAAGGAGATATACATATGGAAA TAGGATTTACTTT-3′) and a unique *Hin*dIII site 3′ to the gene after the stop codon (GW2: 5′-RGACATAAGCTTAGTTTATTTAACGGGGAAAT-3′). These primers were used to amplify the *vanX* gene from plasmid pAT399. The isolated DNA fragment was treated with *Xba*I and *Hin*dIII, ligated into M13mp19, cut with the same restriction enzymes, and sequenced to ensure that no mutations had occurred during the PCR. The *vanX* gene was then excised and ligated into vector pKK223-3* (Zawadzke et al., 1991) to place the *vanX* gene under control of the IPTG-inducible *tac* promoter. This new plasmid, pGW1, was transformed into *Escherichia coli* W3110.

Purification of VanX. E. coli W3110/pGW1 (1 L) was grown at 37 °C in LB medium containing ampicillin (100 μg/mL) to an absorbance of 1.3−1.5 at 595 nm. IPTG was then added to a final concentration of 0.8 mM, and cells were grown for an additional 1.5 h. Cells were harvested by centrifugation at 6000 rpm for 10 min, and the pellet was resuspended in 20 mL of 50 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, and 1 mM DTT. Cells were disrupted by two passages through a French press at 16000 psi, and cell debris was removed by centrifugation at 10000*g* for 30 min. The supernatant was dialyzed overnight in 50 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM DTT and then loaded onto a Q-Sepharose column (9 × 2.3 cm). The column was washed with 40 mL of 100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM DTT, followed by a salt gradient of 150 to 500 mM NaCl. VanX eluted at about 200 mM NaCl.

VanX was judged to be greater than 90% pure and showed a molecular mass of 24 200 Da by SDS−PAGE. Gel filtration on an FPLC Sephadex 75 column (Bio-Rad, 30/10) gave an apparent molecular mass of 38 000 Da. The purified VanX was electrophoresed on a 10% SDS−PAGE minigel, blotted onto a PVDF membrane, and submitted for N-terminal sequencing. The N-terminal sequence MEIGFTFLDE is identical to the sequence predicted from the DNA sequence.

Protein Concentration. Protein concentration was determined by the method of Bradford (Bradford, 1976) with BSA as standard. Amino acid analysis of purified VanX at the Microchemistry Facility at Harvard University showed that the concentration determined by Bradford assay was overestimated by 2.6-fold. All protein concentrations in this report use BSA as standard except where otherwise noted.

Enzyme Assays. Hydrolysis of peptides was qualitatively detected by silica gel thin-layer chromatography (TLC) with a 7:3 *n*-propanol /H_2O solvent system, followed by staining with 0.2% ninhydrin in ethanol. Hydrolysis of dipeptides and depsipeptide was also determined quantitatively by measuring the release of free amino acid with the modified cadmium−ninhydrin method (Doi et al., 1981). Cd−ninhydrin stock solution was prepared by dissolving 1 g of ninhydrin in 100 mL of ethanol, followed by addition of 12.5 mL of acetic acid and 1.25 g of $CdCl_2$ dissolved in 1.25 mL of H_2O. The color was developed by incubating the diluted hydrolyzed product (100 μL) with 0.75 mL of Cd−ninhydrin stock solution at 85 °C for 5 min. The optical density was measured at 505 nm (Perkin Elmer Lambda 6 UV/vis spectrophotometer) and quantified with free amino acid as standard.

All peptides were dissolved in H_2O, treated with CHELEX 100 resin to remove trace amounts of metal, and adjusted to pH 8.0 before use. All buffers were treated with CHELEX 100 resin and determined to be metal-free by plasma emission analysis. All enzyme assays were carried out in 50−100-μL reaction volumes at 37 °C with 50 mM HEPES (pH 8.0) as buffer except where otherwise noted.

For NMR studies, 2 mg of D-Ala-D-Ala was incubated with 1.2 μM VanX in 400 μL of D_2O pyrophosphate buffer (pH = 8.7) at 37 °C for 4 h. Then the reaction mixture was analyzed by ^{1}H-NMR (Varian VXR-500S NMR machine) to establish that D-Ala was the sole product.

Effect of Divalent Cations and Other Chemical Reagants on the Dipeptidase Activity. Purified enzyme (0.2 μM) was preincubated with metal cation for 10 min in 50 mM phosphate buffer (pH 6.8) before addition of D-Ala-D-Ala (10 mM). Ten microliters of incubation mixture was withdrawn every 5 min, and enzyme activity was determined by the Cd−ninhydrin method. Some precipitation was observed for 10 mM $ZnCl_2$ or $CoCl_2$. Purified VanX (0.2 μM) was also preincubated at room temperature with other chemical reagents, EDTA (10 mM, overnight), *o*-phenanthroline (1 mM, 45 h), DTT (2 mM, 20 min), spermidine (10 mM, 20 min), PMSF (2 mM, 10 min), pepstatin A (18 μM, 10 min), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) (1 mM, 10 min), and ATP (10 mM, 20 min), respectively. Consequently, the effect on enzyme activity was determined for each reagent.

Metal Content Analysis of VanX. A purified VanX sample (1.2 μM by amino acid analysis) was prepared by dialysis overnight in 50 mM HEPES, pH 7.5, and 10 mM EDTA at 4 °C, and submitted for plasma emission analysis of metal content (Analytic Chemistry Lab, University of Georgia, Athens, GA). The absorption spectrum (220−700 nm) of this sample displayed a maximal absorbance at 279 nm. The dialysis buffer was treated by CHELEX 100 resin three times and was metal-free by plasma emission analysis.

Effect of pH on the Dipeptidase Activity. The effect of pH in the presence or the absence of 7.5 mM $ZnCl_2$ was determined in the range of pH 4−10 by using a buffer consisting of 20 mM each of malic acid, 2-(*N*-morpholino)-ethanesulfonic acid (MES), HEPES, and boric acid adjusted to the appropriate pH values. Two microliters of crude VanX was preincubated in the buffer for 10 min at room temperature before addition of D-Ala-D-Ala (10 mM). Enzyme activity was determined by the Cd−ninhydrin method.

Determination of Substrate Specificity of VanX. Dipeptides and tripeptide (10 mM) were incubated with purified VanX (1 μM) in the presence of 7.5 mM $ZnCl_2$ at 37 °C for 8 h. The hydrolyzed product was analyzed by both the modified Cd−ninhydrin assay and the TLC method with free amino acids as standards.

Kinetic Analysis of VanX. K_m values were determined by varying substrate concentration from 0.1K_m (minimum 0.1 mM) to 5K_m (maximum 50 mM) in assays being carried out in duplicate. K_m and k_{cat} were determined by least-squares fit to the Michaelis−Menten equation on the KinetAsyst software package (IntelliKinetics, State College, PA) or KaleidaGraph (Synergy Software, Reading, PA). K_m and k_{cat} determinations in the absence of metal cation with D-Ala-D-Ala as substrate were carried out with 0.3 μM VanX. K_m and k_{cat} determinations in the presence of metal cation were carried out in the optimum concentration of Zn^{2+} (0.5 mM), Co^{2+} (2 mM), Ni^{2+} (1 mM), or Fe^{2+} (3 mM) with VanX concentrations of 200, 20, 12, and 50 nM, respectively. VanX was preincubated with metal cation in 50 mM HEPES (pH 8.0) at room temperature for 10 min before addition of substrate. Kinetic analysis of VanX with DL-Ala-DL-Ser or D-Ser-D-Ala as substrates was carried out with 0.8 and 1.2 μM VanX respectively. As optically pure D-Ala-D-Ser was not available, a racemic mixture of DL-Ala-DL-Ser was used with the assumptions that a quarter of the compound was D-Ala-D-Ser and the other three isomers did not interfere with assay, on the basis of the observation that D-Ala-L-Ala and L-Ala-D-Ala did not potently inhibit VanX activity.

Inhibition of VanX by Phosphorus-containing Substrate Analogues of D-Ala-D-Ala. The inhibition of VanX by aminoalkyl phosphonate analogue **II** was assessed with 2 μM VanX at inhibitor concentrations ranging from 0−17K_i and substrate concentration ranging from 0.5−20 K_m. The Lineweaver−Burk plots at different concentrations of inhibitor fitted simple competitive inhibition, and the K_i was determined by use of the KinetAsyst software package. The inhibition studies of VanX by aminoalkyl phosphinate analogues **III** and **IV** were performed in the presence of 2 mM Co^{2+}, in order to keep concentrations of VanX below 10% of inhibitor concentration. VanX (40 and 80 nM, respectively) was preincubated with inhibitor whose concentration ranged from 0−3K_{is} and was then combined with 0.5−20K_m of D-Ala-D-Ala. The kinetics followed mixed-type noncompetitive inhibition, and the K_{ii} and K_{is} were determined by use of the KinetAsyst software package.

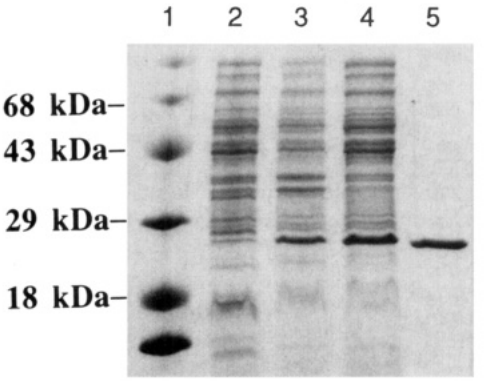


FIGURE 1: SDS−PAGE analysis of purification of VanX. Lane 1, molecular mass standards (in kilodaltons); lane 2, before IPTG induction; lane 3, whole cellular proteins after IPTG induction; lane 4, supernatant after French press; lane 5, Q-Sepharose pools.

Chemical Synthesis of D-Ala-D-lactate and Kinetic Analysis of its Hydrolysis Reaction. Chemical synthesis of D-Ala-D-lactate was carried out by following the reported procedure (Bugg et al., 1991b). The debenzylation was carried out in ethanol/water/acetic acid, 7:2:1, under an atmosphere of H_2 with 10% Pd on activated carbon overnight. The crude product was purified by RP-HPLC (Bio-Rad HI Pore 318, 250 × 21.5 mm) using 25 mM NH_4HCO_3 as solvent. The purified fractions were repeatedly lyophilized until they reached a constant weight.

Hydrolysis of D-Ala-D-lactate was also quantitatively determined by measuring the release of free N-terminal D-Ala with the modified cadmium−ninhydrin method. D-Ala-D-lactate was reasonably stable when stored as a powder at −20 °C, but once dissolved in aqueous solution, it hydrolyzed with a $t_{1/2}$ about 7.4 h. Therefore, D-Ala-D-lactate was always prepared with buffer just before use. pH effects on nonenzymatic hydrolysis rates of D-Ala-D-lactate (10 mM) were determined in 50 mM HEPES adjusted to pH 6−9 at 37 °C by measuring the amount of D-Ala produced in a 10-μL reaction mixture at 20-min intervals. The reaction order at pH 8.0 was determined by varying the concentration of D-Ala-D-lactate from 3−25 mM and fitted by least-squares analysis on KaleidaGraph. Hydrolysis rates of D-Ala-D-lactate (10 mM) were determined in the presence of metal cation (1 mM). The reaction rates in the presence of purified VanX ranging from 1 to 25 μM were also measured with up to 250 mM D-Ala-D-lactate.

The inhibition constant of D-Ala-D-lactate for VanX was estimated from a simple plot of 1/v versus [I] at 1 mM D-Ala-D-Ala (=K_m), at inhibitor concentrations of 70 and 140 mM, respectively, and a VanX concentration at 0.57 μM with the assumption that the kinetics followed simple competition inhibition.

RESULTS

Overproduction and Purification of VanX in E. coli. The *vanX* gene from plasmid pAT399 was subcloned into an overproducing construct pGW1 in *E. coli* , expressed and purified to homogeneity as described in Materials and Methods and as indicated by the protein gel in Figure 1. The production level is moderate, leads to soluble VanX protein, and gives a yield of about 5 mg of purified protein per liter under the indicated growth and induction conditions. The predicted molecular mass of the 202-aa VanX is 23 382

Table 1: Kinetic Data of VanX as Dipeptidase with Dipeptides and Depsipeptide as Substrate

metal ion or substrate	optimum concn (mM)	V_{max} (nmol min^{-1} μg)	K_m or EC_{50} (mM)	K_i (mM)	k_{cat} (s^{-1})	k_{cat}/K_m or k_{cat}/EC_{50} (mM^{-1} s^{-1})
D-Ala-D-Ala						
		12.3 ± 0.4	1.0 ± 0.1		4.7 ± 0.2	4.5 ± 0.2
Ni^{2+}	1	2064 ± 207	21 ± 5 (EC_{50})		788 ± 79	38 ± 10
Fe^{2+},	3	409 ± 18	11 ± 1 (EC_{50})		156 ± 7	14 ± 1
Co^{2+}	2	180 ± 6	1.2 ± 0.1		69 ± 2	58 ± 0.4
Zn^{2+}	0.5	78 ± 4	14 ± 2 (EC_{50})		30 ± 2	2.1 ± 0.3
D-Ala-D-Ser[a]		4.7 ± 0.5	2.8 ± 0.8		1.8 ± 0.2	0.64 ± 0.20
D-Ser-D-Ala		1.3 ± .03	1.7 ± 0.1		0.35 ± 0.01	0.20 ± 0.01
D-Ala-D-lactate		not detectable		242	<0.005	$<2 \times 10^{-5}$

[a] V_{max} and k_{cat} were estimated by using DL-Ala-DL-Ser as substrate with the assumptions that a quarter of the racemic mixture is D-Ala-D-Ser and the other three isomers have no inhibition effect on VanX.

Table 2: Substrate Specificity of VanX as Dipeptidase and Tripeptidase[a]

Substrate	Activity
D-Ala-D-Ala	+
D-Ala-L-Ala	−
L-Ala-D-Ala	−
L-Ala-L-Ala	−
NAc-D-Ala-D-Ala	−
D-Ala-D-Ala-D-Ala	−
D-Ala-D-Phe	+
D-Ala-Gly	+
DL-Ala-DL-Ser	+
DL-Ala-DL-Val	+
DL-Ala-DL-Asn	+
D-Ser-D-Ala	+
Gly-Gly	−
Gly-Gly-Gly	−
D-Ala-L-Leu	−
D-Ala-D-Lactate	−

[a] Each substrate (10 mM) was incubated with 1 μM VanX and 7.5 mM $ZnCl_2$ at 37 °C for 8 h, and the reaction activity was determined visually by the Cd−nindydrin assay and the TLC method.

Da, consistent with the molecular weight estimated by SDS gel analysis. Gel permeation chromatography of native VanX indicated a mobility consistent with a molecular mass of 38 000 Da: thus we provisionally assign a dimeric structure to native VanX.

Characterization of VanX as a D-, D-*Dipeptidase.* In confirmation of the recent studies on dipeptidase activity in crude extract of VanX overproducers (Reynolds et al., 1994a), pure VanX is active as a D-, D-dipeptidase and D-Ala was determined to be the sole product by NMR spectroscopy. As shown in Table 1, the enzyme as isolated has a catalytic constant k_{cat} of 4.7 s^{-1}, a Michaelis constant K_m of 1 mM for D-Ala-D-Ala, and a specificity constant k_{cat}/K_m of 4500 M^{-1} s^{-1}. Consistent with the observations of Reynolds et al. (Reynolds et al., 1994a), N-blocked D-Ala-D-Ala species, either *N*-acetyl or as tripeptide D-Ala-D-Ala-D-Ala, were not substrates by either ninhydrin or TLC assays, establishing VanX as a dipeptidase (Table 2). Similarly neither L-Ala-L-Ala, D-Ala-L-Ala, nor L-Ala-D-Ala shows detectable activity, indicating VanX is a D-, D-dipeptidase. No D-, D-carboxypeptidase activity of VanX was observed toward either UDP-MurNAc-pentapeptide terminating in either D-[^{14}C]Ala or D-[^{14}C]lactate (data not shown). The enzyme shows a broad pH optimum with a plateau between pH 7 and 9 (Figure 2). In the presence of 7.5 mM $ZnCl_2$, the plateau of pH optimum narrows. The enzyme activity cannot be inhibited by common protease inhibitors, such as PMSF, E-64, pepstatin, *o*-phenanthroline, EDTA, and DTT or be stimulated by 10 mM ATP (data not shown).

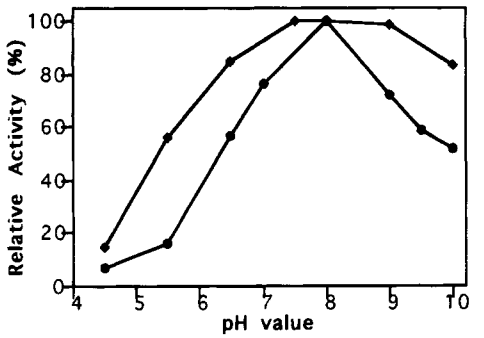


FIGURE 2: pH profile of VanX dipeptidase activity at 37 °C: (◆) without any metal cofactor; (●) in the presence of 7.5 mM $ZnCl_2$. The relative activities with and without $ZnCl_2$ on the *y* axis have not been normalized.

Table 3: Metal Ion Effect on VanX Dipeptidase Activity with D-Ala-D-Ala as Substrate

metal ion/reagent	concn (mM)	app rate (nmol/min)	app k_{cat} (s^{-1})	rel act.
control		3.2	2.7	1
KCl	10	1.8	1.5	0.56
KCN	10	1.3	1.1	0.41
$MgSO_4$	10	3.0	2.5	0.94
$NiSO_4$	7.5	96	80	30
$ZnCl_2$	7.5	13	11	4.0
$CoCl_2$	10	27	23	8.8
$MnCl_2$	10	2.2	1.8	0.69
$CuCl_2$	10	0.2	0.53	0.06
$FeCl_2$	10	24	19	7.3
$FeCl_3$	10	1.7	1.4	0.53
$CaCl_2$	2	7.6	2.5	1.2

A dipeptidase can be viewed either as an aminopeptidase or a carboxypeptidase; almost all aminopeptidases and many carboxypeptidases are activated by divalent cations. Table 3 indicates that Zn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} can clearly stimulate the dipeptidase activity of VanX, with Ni^{2+} having the largest effect under the indicated set of conditions. In contrast, 10 mM Cu^{2+} was found to inhibit the enzyme activity. No metal-catalyzed nonenzymatic hydrolysis of dipeptide was observed in the time scale of the assay, and the enzyme activity cannot be stimulated by 10 mM of the polycation spermidine. Further analysis of the effect of M^{2+} concentration on enzymatic activity indicates (Figure 3) that inhibition is developed at higher concentrations of all four divalent cations. Kinetic analysis of VanX in the presence of metal cation was then conducted at the optimal M^{2+} concentration for maximal stimulation (0.5 mM for Zn^{2+}, 1

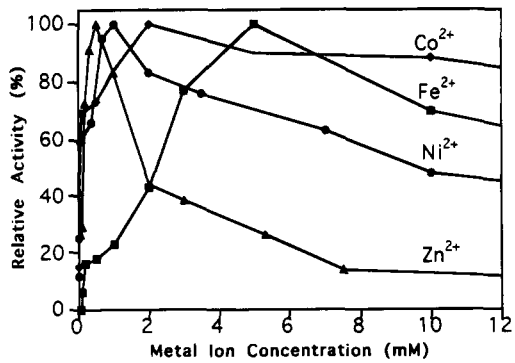


FIGURE 3: Effect of divalent cation on the VanX dipeptidase activity at 37 °C. For every metal cation, the VanX concentration varies with 25, 50, 60, and 200 nM for the assays in the presence of Ni^{2+}, Co^{2+}, Fe^{2+}, and Zn^{2+}, respectively, but with constant D-Ala-D-Ala concentration (10 mM) (See Materials and Methods). The relative activities have not been normalized with respect to enzyme concentration.

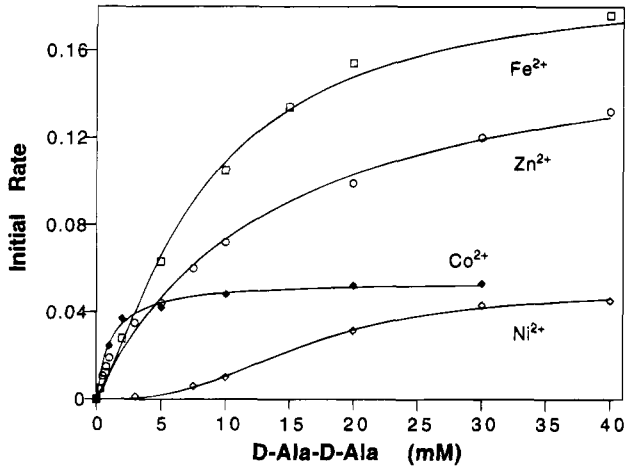


FIGURE 4: Initial velocity vs substrate plots of VanX in presence of 1 mM $NiSO_4$, 0.5 mM $ZnCl_2$, 3 mM $FeCl_2$, and 2 mM $CoCl_2$ as marked with VanX concentrations of 12, 200, 50, and 20 nM, respectively. The curves are fitted well by the Michaelis–Menten equation (Co^{2+}), the Hill equation (Ni^{2+}), or either of the equations (Zn^{2+}, Fe^{2+}). The initial rates on the y axis for each metal ion have not been normalized.

mM for Ni^{2+}, 3 mM for Fe^{2+}, and 2 mM for Co^{2+}) to obtain effects on k_{cat} and K_m. As seen in Table 1, 0.5 mM Zn^{2+} gave a 6.4-fold increase in k_{cat} over basal activity; Fe^{2+} gave a 33-fold increase; Co^{2+} ,a 15-fold increase; and Ni^{2+} was clearly the most effective at 168-fold at 1 mM Ni^{2+}. But in the presence of Zn^{2+}, Ni^{2+}, and Fe^{2+}, the velocity vs substrate profiles deviate from the rectangular hyperbolic relationships seen in the absence of these divalent cations and follow sigmoid kinetics, although Co^{2+} still showed standard Michaelis–Menten behavior (Figure 4). It is also clear from the curves in Figure 4 that the K_m for D-Ala-D-Ala remains in the 1 mM range with Co^{2+} as activator (K_m = 1.2 mM, Table 1), but clearly rises to the 10–20 mM range with Zn^{2+}, Ni^{2+}, and Fe^{2+} along with deviation from Michaelis–Menten behavior. Using K_m values for D-Ala-D-Ala in metal-free and Co^{2+} states, and EC_{50} (effective concentration for 50% response) values of 11, 14 and 21 mM for Fe^{2+}, Zn^{2+} and Ni^{2+}, the catalytic efficiency ratios of k_{cat}/K_m or k_{cat}/EC_{50} can be compared in Table 1. Co^{2+} produces a 13-fold enhancement in catalytic efficiency, essentially all on k_{cat}. The 6.4-fold increase in k_{cat} induced by Zn^{2+} is offset by a 14-fold drop in EC_{50} for D-Ala-D-Ala, so there is a slight net decrease in catalytic efficiency. Fe^{2+} gives a 3-fold, and Ni^{2+}, a net 8-fold, increase in k_{cat}/EC_{50}.

Transition-state metal ions can form tight complexes with dipeptides (Smith & Martell, 1975). Thus, deviation from simple saturation kinetics of VanX in the presence of metal cations could be caused by a change of free metal ion concentration at different concentrations of substrate. For example, Zn^{2+} and Gly-Gly can form ML and ML_2 complex with association constants of 2.8×10^3 and 2.0×10^6 M^{-1}, respectively (Smith & Martell, 1975). Assuming Zn^{2+} complexation with D-Ala-D-Ala in a similar manner, a (D-Ala-D-Ala)$_2$-Zn^{2+} complex was prepared and used as substrate for VanX. An inhibition curve similar to one of Zn^{2+} in Figure 3 was observed (data not shown), indicating that the function of metal cation in catalysis is not simply complexation with substrate.

To assess whether VanX as isolated is a metalloenzyme, two approaches were taken. First the enzyme was exposed to 10 mM EDTA overnight or to 1 mM *o*-phenanthroline for up to 2 days at 37 °C before its enzymatic activity was measured. There was no diminution in dipeptidase activity, arguing either for no chelatable metal cation or a metal kinetically inaccessible to the two exogenous chelating agents. Second, an enzyme sample (1.2 μM by amino acid analysis) was prepared by dialysis overnight in 10 mM EDTA and submitted for plasma emission analysis. There was no detectable Fe, Co, Ni, Cu, Ca, Mg, or Mn in the enzyme, and the net concentration of Zn in the isolated 1.2 μM enzyme was 0.08 μM. The VanX/Zn mole ratio of 15 suggests that the enzyme does not purify with stoichiometric levels of tightly bound divalent cation. Furthermore, the electronic absorption spectra of VanX also fail to reveal any absorption higher than 280 nm.

Further analysis of substrate selectivity indicates that the enzyme can accept side chains other than the methyl of D-Ala in either position (Table 2), although full kinetic characterization of k_{cat}/K_m ratios remains to be explored in order to rank the order of catalytic efficiency. The K_m and k_{cat} with DL-Ala-DL-Ser and D-Ser-D-Ala as substrate were determined for VanX (Table 1) with specificity constants k_{cat}/K_m of 0.64 and 0.2 mM^{-1} s^{-1}, respectively. It seems that replacement of either D-Ala by D-Ser will decrease both binding affinity and catalytic constant, although systematic studies of substrate specificity are necessary.

Characterization of Spontaneous Hydrolysis Reaction of D-Ala-D-lactate and D-Ala-D-lactate as Substrate for VanX. Most notable is the behavior of VanX toward the depsipeptide D-Ala-D-lactate. As suggested by the initial observations by Reynolds et al. in the crude extracts of overexpressed VanX (Reynolds et al., 1994a), D-Ala-D-lactate is not readily broken down by purified VanX. In order to confirm the observation, D-Ala-D-lactate was chemically synthesized and purified. N- and C-terminal protected Cbz-D-Ala-benzyl-D-lactate is very stable. But once debenzylated, it tends to undergo hydrolysis of the ester bond. The deprotected D-Ala-D-lactate can be stored as a solid at −20 °C when it is reasonably stable. When it is dissolved in solution, it undergoes spontaneous hydrolysis. The pH profile showed an increasing hydrolysis rate with an increasing pH (Table 4). The rate vs D-Ala-D-lactate concentration plot shows a pseudo-first-order reaction with a reaction constant of 2.4×10^{-5} s^{-1} and a half-life of 7.4 h at pH 8.0 (data not shown). The pseudo-first-order reaction with D-Ala-D-lactate argues

Table 4: Effects of pH, Divalent Metal Ion, and VanX on Hydrolysis Rates and Half-Life Time of D-Ala-D-lactate (10 mM)[a]

pH value/metal ion /VanX	rate constant (s^{-1})	rel act.	half-life $t_{1/2}$ (min)
pH 6.0	6.2×10^{-6}	0.3	1480
pH 7.0	1.0×10^{-5}	0.4	1110
pH 8.0	2.4×10^{-5}	1	444
pH 9.0	3.1×10^{-5}	1.3	342
1 mM Mg^{2+}	3.5×10^{-5}	1.5	304
1 mM Cu^{2+}	1.7×10^{-4}	7.1	63
1 mM Co^{2+}	2.0×10^{-4}	8.3	53
1 mM Ni^{2+}	2.3×10^{-4}	9.6	46
1 mM Zn^{2+}	1.2×10^{-3}	50	9
1 mM Co^{2+}; 25 μM VanX	1.5×10^{-4}	6.3	71
1 mM Zn^{2+}; 25 μM VanX	1.1×10^{-3}	46	10
VanX-catalyzed hydrolysis	less than 0.005		

[a] The pH effects of spontaneous hydrolysis of D-Ala-D-lactate were determined in 50 mM HEPES adjusted to different pH, while metal ion effects were determined in 50 mM HEPES, pH 8.0.

against an intermolecular hydrolysis mechanism catalyzed by the N-terminal amino group or the C-terminal carboxy group. The spontaneous hydrolysis reaction probably involves a general base mechanism or a direct nucleophilic attack of the C-terminus carboxyl group via an anhydride intermediate (Fife et al., 1979). The hydrolysis rate can be accelerated by as much as 50-fold in the presence of a metal ion, such as 1 mM Zn^{2+} with a reaction constant of 1.2×10^{-3} s^{-1} and a half-life of merely 9 min (Table 4).

The catalytic hydrolysis of D-Ala-D-lactate by VanX was then investigated. In the presence of 25 μM VanX and concentrations of D-Ala-D-lactate up to 250 mM, no higher than background hydrolysis rate was observed. Given the limits of the assay, the catalytic hydrolysis of VanX will be lower than 0.005 s^{-1} (vs 4.7 s^{-1} for D-Ala-D-Ala). Because D-Ala-D-lactate is not a good substrate of VanX, it was added to D-Ala-D-Ala with VanX to determine whether it can inhibit VanX activity. When the concentration of D-Ala-D-lactate was 70 or 139 mM, respectively, about 12% and 23% of activity was inhibited at 1 mM D-Ala-D-Ala. A Dixon plot using the K_m for D-Ala-D-Ala of 1 mM yielded an extrapolated inhibition constant K_i of 242 mM.

Test of Phosphorus-Containing Substrate Analogues as Inhibitors of VanX. Many Zn^{2+}-activated aminopeptidases and carboxypeptidases are potently inhibited by phosphorus-containing analogues of substrate peptides (Philips et al., 1992). The tetrahedral phosphorus atom mimics the tetrahedral geometry of the reaction intermediate in which H_2O can be added to the carbonyl carbon of the peptide bond under attack. In the case of VanX, such an intermediate would be the adduct **I** in Scheme 1.

Phosphonate and phosphinate analogues **II**−**IV** have been prepared previously (Hanson et al., 1989; Bartlett & Kezer, 1984; Parsons et al., 1988) and tested as inhibitors of VanA and bacterial D-Ala-D-Ala ligases (Duncan & Walsh, 1988), and have now been tested with VanX.

As shown in Figure 5 and Table 5, phosphonate analogue **II** shows competitive inhibition with a K_i of 0.3 mM, a 3-fold increase in affinity compared to substrate D-Ala-D-Ala. Both phosphinates **III** and **IV** give mixed-type noncompetitive inhibition (Figure 6). They are at least 2 orders of magnitude more potent than the phosphonate analogue with K_{is} of 1.26 and 0.81 μM, respectively. The sample of **III** was a mixture of all four diastereomers. Assuming only the D-, D-isomer is recognized by VanX, the K_{is} for that compound should be 315 nM, about 3170-fold tighter binding to VanX than D-Ala-D-Ala. Compound **IV** is a mixture of two diastereomers. Assuming only its D-, D-isomer is the inhibitor, then K_{is} for **IV** will be 405 nM, a 2469-fold tighter binding to VanX than D-Ala-D-Ala.

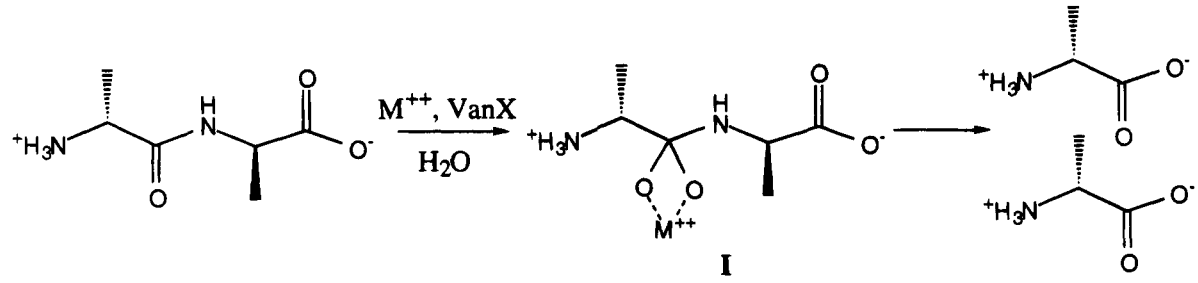
DISCUSSION

In this work we have reported the purification of the fifth and last protein for high-level vancomycin resistance, VanX. Together with the VanR, -S, -H, and -A proteins (Arthur et al., 1992b), VanX is required to confer inducible, high-level clinical resistance to vancomycin in opportunistic human pathogenic Gram-positive bacteria such as *Enterococci*. The discovery by Reynolds et al. (Reynolds et al., 1994a) that VanX has D-Ala-D-Ala dipeptidase activity has been confirmed by the studies here with the purified protein.

As a divalent metal-activated peptidase, VanX probably shares mechanistic and active site structural features with other hydrolytic enzymes. While Zn^{2+}-requiring aminopeptidases and carboxypeptidases are well known (Vallee & Auld, 1993b), very little is known about the structure and function of bacterial dipeptidases and oligopeptidases. Several di- and tripeptidase activities have been reported in *E. coli* and *Salmonella typhimurium* over the years [for a review, see Lazdunski (1989)]; only one tripeptidase from *Lactococcus lactis* (Bosman et al., 1990) and a dipeptidase from *Streptococcus cremoris* (van Boven et al., 1988) have been purified to homogeneity. No D-, D-dipeptidase activity has been well characterized previously. Interestingly, a D-aminopeptidase isolated and purified from the bacterium *Ochrobactrum anthropi* was reported to have some hydrolytic activity with D-Ala-D-Ala. It differs from VanX not only by its size (59 000 Da) but also by its activity being inhibited by metal cations (Asano et al., 1989 and Asano et al., 1992). Furthermore, it does not appear that VanX bears any obvious resemblance to the penicillin-sensitive D-, D-transpeptidases or the penicillin-sensitive D-, D-carboxypeptidases which were initially characterized as penicillin binding proteins (Frere et al., 1992). Nor does VanX have any of the previously recognized Zn^{2+}-binding motifs in Zn-enzyme families (Vallee & Auld, 1993a,b).

Isolated VanX contains little tightly bound M^{2+}. While several cations activate VanX, especially Ni^{2+}, Co^{2+}, and Fe^{2+}, it is most likely that Zn^{2+} would be the physiological activator given ambient concentrations of cations in cells. On the other hand there is a Co^{2+}-dependent methionine aminopeptidase (Roderick & Matthews, 1993) and a nickel-activated hydrogenase known in *E. coli* (Ballantine & Boxer, 1985). Use of $(D\text{-Ala-D-Ala})_2$-Zn as substrate or increase of the metal cation concentration does not show a simple saturation kinetics; the role of the M^{2+} could be both in catalysis and coordination of substrate dipeptides. Interestingly, it was found that Cu^{2+} can inhibit the enzyme activity, which was also observed in carboxypeptidase A and may be due to a shift of mechanistically important groups upon binding of Cu^{2+} (Rosenberg et al., 1975). In the presence of Zn^{2+}, Ni^{2+}, or Fe^{2+}, VanX kinetics follows sigmoid kinetics, i.e., co-operative kinetics (Tipton, 1992), which might be due to the fact that VanX is a dimer, and binding of substrate on one active site could affect affinity of the

Scheme 1



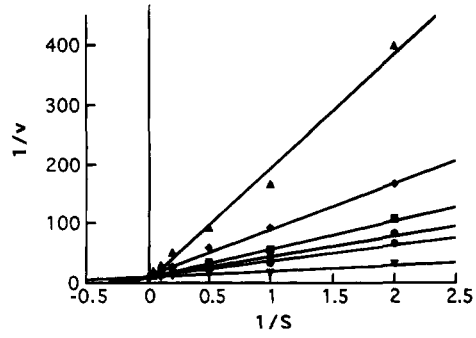


FIGURE 5: Lineweaver–Burk plot of simple competitive inhibition of VanX by D-3-[(1-aminoethyl)phosphonyl]-D-2-methylpropionic acid **II** with D-Ala-D-Ala as substrate. The concentrations of **II** added to the assay were 0, 0.2, 0.5, 1, 2, and 5 mM (from bottom line to top) with VanX concentration of 2 μM at 37 °C.

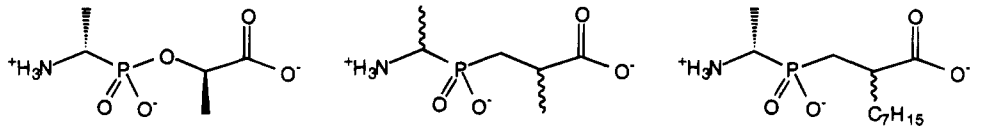
another site. These kinetic patterns can be fitted very well to the Hill equation (Segel, 1975) with the Hill coefficients of 0.7, 1, 1.3, and 1.6 for Zn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+}, respectively. There is a rich literature on the coordination chemistry of dipeptides with M^{2+}, including binding constant rankings, and the actual substrate for VanX is probably a M^{2+}-dipeptide conformer. The M^{2+} may also help coordinate and activate an attacking water molecule in the hydrolysis step. In order to ascertain the functions of different metal cations in VanX catalysis, the structure of VanX with a transition-state analogue in the active site should ultimately be solved.

With the VanS/R pair for signal transduction and transcriptional regulation and the VanH/A pair to synthesize D-Ala-D-lactate, it appears that the picture of vancomycin resistance in *Enterococcus* would be complete. But a detailed kinetic analysis of this picture reveals a problem: D-Ala-D-Ala synthesized by both D-Ala-D-Ala ligase (ddl) and VanA will compete with D-Ala-D-lactate produced by VanH/A to be incorporated into peptidoglycan in the cell wall as indicated in Scheme 2. In order to completely change the peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-lactate, the organism could either repress the normal D-Ala-D-Ala ligase or hydrolyze any D-Ala-D-Ala produced, as suggested by Reynolds (Messer & Reynolds, 1992). We do observe that VanX has a DNA-binding affinity to the *vanH* promoter or the *vanR* promoter with a relatively poor EC_{50} of 10 μM (Z. Wu and C. T. Walsh, unpublished results). As VanA has comparable ligase activity for both D-Ala D-Ala and D-Ala D-lactate (Scheme 2), it is impossible to repress the synthesis of D-Ala-D-Ala solely by repression of the transcription of the *ddl* gene. The only way to get rid of D-Ala-D-Ala is to hydrolyze it, and VanX is a D-, D-dipeptidase.

The penicillin-sensitive D-, D-transpeptidases, penicillin-sensitive D-, D-carboxypeptidases, and D-aminopeptidase use both peptide and depsipeptide/esters as substrates (Rasmusen & Strominger, 1978; Nguyen-Disteche et al., 1986). The efficiency of utilization of depsipeptide/ester vs peptides can be enhanced by up to 147-fold. Carboxypeptidase A also has a k_{cat} about 10^2 s^{-1} for peptide and 10^3 s^{-1} for esters (Christianson & Lipscomb, 1989). It is therefore surprising that VanX differentiates so clearly between the depsipeptide D-Ala-D-lactate and the dipeptide D-Ala-D-Ala as substrate. The K_m values of VanX is 1 mM for D-Ala-D-Ala vs a K_i of 242 mM for D-Ala-D-lactate. The k_{cat} of VanX for D-Ala-D-Ala and D-Ala-D-lactate are 4.7 and less than 0.005 s^{-1}, respectively. The more than 10^3-fold differential in k_{cat} and approximately 250-fold in affinity of VanX for D-Ala-D-Ala vs D-Ala-D-lactate leaves the enzyme efficiently equipped to function in vancomycin resistance. VanX action leaves D-Ala-D-lactate intact and eliminates the competition from D-Ala-D-Ala for the UDP muramyl-L-Ala-D-isoGlu-L-Lys elongation reaction via D-Ala-D-X addition catalyzed by D-Ala-D-Ala adding enzyme (MurF for *E. coli*). This will ensure, after translocation, that there are no D-Ala-D-Ala peptidyl termini to be recognized by vancomycin. As an ester bond is more activated than an amide bond, the muramyl-L-Ala-D-isoGlu-L-Lys-D-Ala-D-lactate will be easily cross-linked. It can be predicted that the D-lactate in the C-terminus of UDP-MurNAC-pentapeptide will undergo spontaneous hydrolysis if it is not cross-linked after a period of time, which was consistent with the observation that there was only UDP-MurNAC-tetrapeptide accumulated in high-level resistant *Enterococcus faecium* (Messer & Reynolds, 1992). The unstability of depsipeptide could also make VanY, a D-, D-carboxypeptidase in the *van* gene cluster which may contribute to vancomycin resistance by removing D-Ala residue from C-terminus of peptidoglycan (Arthur et al., 1992a), functionally unnecessary for high-level vancomycin resistance in *E. faecium*, although it was reported to contribute moderately in the VanB type *E. faecalis* when the concentration of free D-Ala is high (Arthur et al, 1994). If all the peptidoglycan chains end in D-Ala-D-lactate, vancomycin binds with 1000-fold lower affinity (Bugg et al., 1991a). The subsequent transpeptidation can still occur, and the bacterial cells are both viable and vancomycin resistant. The molecular discrimination of VanX between D-Ala-D-Ala and D-Ala-D-lactate is also mirrored by the 10^3-fold discrimination of vancomycin for the same moieties.

The clinically vancomycin resistant *Enterococcus* strains are divided into VanA, VanB, and VanC type based on their phenotype (Dutka-Malen & Courvalin, 1990). The cell wall analysis of VanC type in *Enterococcus gallinarum* showed that instead of D-Ala-D-Ala and D-Ala-D-lactate in the normal peptidoglycan and the VanA type, respectively, D-Ala-D-Ser was found in the C-terminus of peptidoglycan (Billot-Klein

Table 5: Phosphonate and Phosphinate Inhibitors and Their Inhibition Constants



II III IV

Simple Competitive Inhibition	Mixed-type Noncompetitive Inhibition	
K_i = 0.3 ± 0.06 mM	K_{is} = 1.26 ± 0.50 μM	0.81 ± 0.28 μM
	K_{ii} = 1.75 ± 0.22 μM	3.47 ± 0.61 μM
	For D-, D-isomer	
	K_{is} ≈ 0.32 μM	0.40 μM

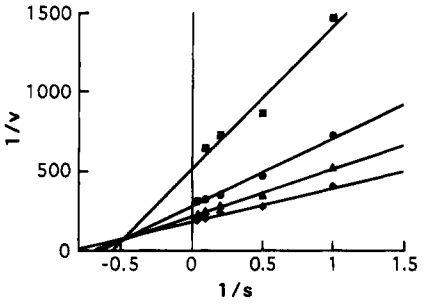


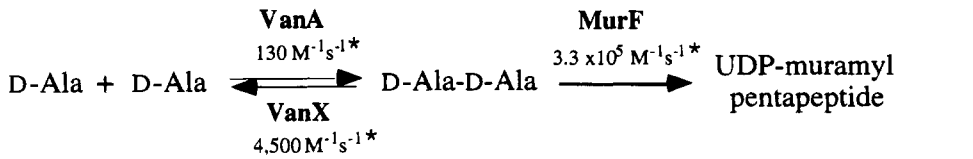
FIGURE 6: Lineweaver–Burk plots of mixed-type noncompetitive inhibitions of VanX by phosphinate compound 3-[(1-aminoethyl)-phosphinyl]-2-methylpropionic acid **III** and D-3-[(1-aminoethyl)-phosphinyl]-2-heptylpropionic acid **IV** with D-Ala-D-Ala as substrate. Only data of **III** is shown here. The concentrations of **III** added to the assay were 0, 0.4, 1, and 5 μM (from bottom line to top) with 2 mM $CoCl_2$ and VanX concentration of 80 nM at 37 °C.

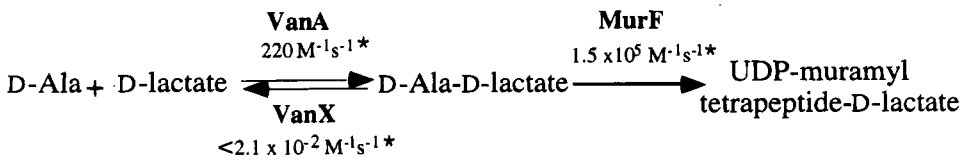
et al., 1994; Reynolds et al., 1994b). VanX activity has also been detected in the crude extract of *E. gallinarum* (Reynolds et al., 1994b). VanX is found to hydrolyze D-Ala-D-Ser with an estimated catalytic efficiency of 0.64 mM^{-1} s^{-1} when DL-Ala-DL-Ser is used as substrate. The merely 7-fold substrate discrimination of VanX between D-Ala-D-Ala and D-Ala-D-Ser could be the cause of the low-level resistance in VanC type *Enterococcus gallinarum*, comparing with more than 10^5-fold catalytic efficiency difference between D-Ala-D-Ala and D-Ala-D-lactate in the high-level resistance in VanA type *Enterococcus faecium*.

Knowledge of the catalytic role and specificity of VanX advances it as a target whose inhibition *in vivo* should restore vancomycin sensitivity to a Van^R pathogen. As an initial *in vitro* test of inhibition, we turned to tetrahedral phosphonate and phosphinate analogues of D-Ala-D-Ala. These molecules have previously been synthesized and evaluated for inhibition of the ATP-dependent D-Ala-D-Ala forming enzymes D-Ala-D-Ala ligase (Parsons et al., 1988; Duncan & Walsh, 1988) and VanA (Bugg et al., 1991a). In those ligases the phosphinates **III** and **IV** become potent slow-binding inhibitors in the presence of the cosubstrate ATP as they undergo ligase-mediated phosphorylation to phosphinophosphate species bound in the ligase active sites (Duncan & Walsh, 1988; Fan et al., 1994).

Typically phosphinate analogues of peptide substrates are potent inhibitors of Zn^{2+}-dependent aminopeptidases and carboxypeptidases with more than 1000-fold increased affinity compared to substrate. This difference is interpreted as mimicking of tetrahedral adduct geometry of bound reaction intermediates (Philips et al; 1992). In the VanX case, compounds **HI** and **IV** bind with K_{is} values 2500–3500-fold tighter than the D-Ala-D-Ala K_m of 1 mM; while phosphonate compound (**II**) binds VanX with a K_i of only 0.3 mM, a 1000-fold difference from **III**, even though they have similar structure (Table 5). One possible explanation is that the bridging O in phosphonate **II** decreases the nucleophilicity of the P–O group and thus affects its affinity, or perhaps induces a subtle geometric change that reduces

Scheme 2[a]





[a] *Specificity constants (*) k_{cat}/K_m of VanA and MurF are from Bugg et al. (1991b).

affinity of D-Ala^P-O-D-Ala vs D-Ala^P-CH_2-D-Ala. A crystallographic analysis of VanX with tetrahedral transition state analogue, as in the case of ddl (Fan et al., 1994), is necessary to clarify this issue. These results may lead to more potent inhibitors, but the *in vitro* efficiency for **III** and **IV** as ddl inhibitors was not borne out by *in vivo* studies as antibacterial agents, largely because of poor penetration into the bacterial cytoplasm (Parsons et al., 1988). Perhaps due to similar lack of uptake, initial analysis of phosphinate **III** against a vancomycin-resistant *Enterococcus* strain failed to show a significant effect (H. Gold, Z. Wu, and C. Walsh, unpublished results). The mixed-type noncompetitive inhibition of phosphinate analogues may be due to the slow-binding inhibition (Morrison & Walsh, 1987).

In summary, we have successfully overexpressed, purified, and characterized VanX, the last protein in the *van* cluster of *Enterococcus faecium,* whose mechanism had not been clear. Mechanistic analysis of VanX presents an unusual example of a peptidase which is able to hydrolyze an amide substrate but not the related kinetically and thermodynamically favorable ester substrate. Further work will be directed to structural studies of the VanX active site which is responsible for substrate selectivity, and to find out whether VanX is a Zn-peptidase. Effort will also be made to design better inhibitors in order to try to reverse clinical vancomycin resistance.

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