A Five-coordinate Metal Center in Co(II)-substituted VanX

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A Five-coordinate Metal Center in Co(II)-substituted VanX*

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In an effort to structurally probe the metal binding site in VanX, electronic absorption, EPR, and extended x-ray absorption fine structure (EXAFS) spectroscopic studies were conducted on Co(II)-substituted VanX. Electronic spectroscopy revealed the presence of Co(II) ligand field transitions that had molar absorptivities of ~100 M⁻¹cm⁻¹, which suggests that Co(II) is five-coordinate in Co(II)-substituted VanX. Low temperature EPR spectra of Co(II)-substituted VanX were simulated using spin Hamiltonian parameters of $M_s = (|\pm 1/2|, E/D = 0.14, g_{\text{real}(xy)} = 2.37$, and $g_{\text{real}(z)} = 2.03$. These parameters lead to the prediction that Co(II) in the enzyme is five-coordinate and that there may be at least one solvent-derived ligand. Single scattering fits of EXAFS data indicate that the metal ions in both native Zn(II)-containing and Co(II)-substituted VanX have the same coordination number and that the metal ions are coordinated by 5 nitrogen/oxygen ligands at ~2.0 Å. These data demonstrate that Co(II) (and Zn(II) from EXAFS studies) is five-coordinate in VanX in contrast to previous crystallographic studies (Bussiere, D. E., Pratt, S. D., Katz, L., Severin, J. M., Holzman, T., and Park, C. H. (1998) Mol. Cell 2, 75–84). These spectroscopic studies also demonstrate that the metal ion in Co(II)-substituted VanX when complexed with a phosphate analog of substrate d-Ala-d-Ala is also five-coordinate.

Vancomycin is a cup-shaped glycopeptide that has been called the “antibiotic of last resort” (1, 2) because it is often used for antibiotic-resistant bacterial infections and in patients who are allergic to penicillins. Vancomycin inhibits bacterial cell wall synthesis by binding to the d-Ala-d-Ala end of the peptidoglycan pentapeptide via five hydroxy bonds, thereby preventing cross-linking of the cell wall precursors (3–6). However, bacterial resistance to vancomycin in human pathogens has emerged, particularly in Enterococcus and Staphylococcus strains (3–6). The genes responsible for inducible, high level resistance: VanR, VanS, VanH, VanA, and VanX (5). VanR and VanS make up a two-component, signal transduction system that regulates the expression of the other three genes and appears to be stimulated by the presence of vancomycin (8–10). VanH encodes for a d-specific α-ketoacid reductase, which catalyzes the reduction of pyruvate into D-lactate using NADPH (11, 12). VanA is a ligase that uses the D-lactate produced by VanH and generates a D-Ala-D-lactate depsipeptide (11, 13). This depsipeptide is added to a sugar-linked tripeptide by MurF, producing a modified bacterial cell wall precursor that can be incorporated into the growing peptidoglycan layer by the normal bacterial enzymes (3, 5, 12). Vancomycin binds to this modified cell wall pentapeptide 10³ less tightly than to the normal pentapeptide due to the loss of one hydrogen bond and to a key electrostatic repulsion (11, 14, 15). In other words, bacteria modify their peptidoglycan layers to reduce the binding affinity of vancomycin, thereby making the bacterium resistant to vancomycin.

The final gene product, VanX, is a d-Ala-d-Ala peptidase (16). Walsh and co-workers (17–21) have reported the cloning, over-expression, and purification of VanX. VanX is a Zn(II)-binding protein with a monomeric molecular mass of 23,365 Da that hydrolyzes a very narrow range of substrates and does not hydrolyze D-Ala-L-lactate or D-Ala-D-L-alanine (16, 17). Walsh and co-workers (18) used site-directed mutagenesis studies to predict the metal binding amino acids, and these predictions were confirmed when Bussiere and co-workers (1) published the crystal structure of VanX. The mutagenesis study also implicated Arg-71 and Glu-181 as having roles in catalysis, and these residues were shown in the crystal structure to be close to the Zn(II) ion. The crystal structure also revealed that VanX crystallized as a homohexamer, and each subunit bound one Zn(II). The Zn(II) ion was coordinated in a distorted tetrahedral arrangement with the side chains of His-116, His-184, and Asp-123 and a solvent water as ligands (Fig. 1). The crystal structure also demonstrated a narrow cavity leading to the active site (1), which nicely explained the very narrow substrate specificity of VanX.

Since VanX is essential for vancomycin resistance in bacteria, the enzyme has been targeted for drug/inhibitor design studies in hopes that an inhibitor can be given in combination with vancomycin as a treatment for vancomycin-resistant bacterial infections. Phosphinate and phosphonate analogs of d-Ala-d-Ala have been shown to be inhibitors of VanX, suggesting that the enzyme utilizes a nucleophile-driven hydrolysis reaction that may proceed via a tetrahedral transition state intermediate (17, 22, 23). However, these structurally similar compounds exhibit $K_I$ values varying over several orders of magnitude, and the crystal structure of VanX complexed with a phosphonate and a phosphinate demonstrated two distinct binding interactions (1). Wu and Walsh (24) reported several

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11074
dithiol compounds that exhibited potent, time-dependent inhibition of VanX, and Araoz et al. (25) reported that a modified D-Ala-D-Gly dipeptide is a mechanism-based inactivator of VanX. The former compounds apparently inhibit VanX by chelating Zn(II) and blocking access of the substrate. The latter compound, upon hydrolysis by VanX, releases a reactive 4-thiogalactopyranoside that reacts with a group on the surface of the enzyme. Unfortunately none of the reported inhibitors bind tightly enough or are specific enough to be useful as clinical inhibitors.

One of the major obstacles in studying VanX is the lack of a direct activity assay. The inability to find chromogenic substrates for VanX is presumably due to the narrow binding cavity in the enzyme that leads up to the active site. The lack of a direct activity assay complicates mechanistic studies, results from which could drive rational inhibitor design or redesign efforts. A ninhydrin-based assay, a capillary electrophoresis procedure, and several coupled assays have been reported (17, 25–27); however, all of these have experimental problems that limit their use in mechanistic studies. As an alternative, we propose to follow the mechanism by preparing the Co(II)-substituted form of VanX and probing the spectroscopic properties of the metal center during the catalytic cycle with use of stopped-flow and rapid freeze quench coupled with spectroscopic studies. This work describes the preparation of Co(II)-substituted VanX and the biochemical and spectroscopic characterization of the resulting enzyme.

**MATERIALS AND METHODS**

**Preparation of Co(II)-substituted Maltoose-binding Protein-VanX and VanX—Co(II)-substituted maltose-binding protein (MBP)1-VanX and VanX were prepared in minimal medium. The minimal medium contained 2.5 g/liter D(+)-glucose, 5 g/liter casamino acids, 10.6 g/liter K2HPO4, 5.5 g/liter KH2PO4, 10 g/liter NaCl, 1 g/liter (NH4)2SO4, 2 mg/liter thiamine, 1 mg/liter biotin, and a 1 ml/liter concentration of a compound, upon hydrolysis by VanX, releases a reactive 4-thiogalactopyranoside. Three 1-liter flasks, each containing 333 ml of minimal medium and 25 mg/ml kanamycin, were inoculated with 10 ml of the overnight preculture and were allowed to grow to an A600 of 1.6–1.8 at 30 °C. Protein production was induced by making the cultures 1 mM in isopropyl β-D-thiogalactopyranoside. At induction, the cultures were also made 100 μM in CoCl2. The cells were then collected by centrifugation for 15 min at 8,275 × g, flash frozen in liquid N2, and stored at −80 °C overnight.

The cell pellets were thawed on ice and were resuspended in 30 mM Tris-HCl, pH 7.6, containing 200 mM NaCl. Cells were lysed by passing the resuspension two times through a French press at 16,000 p.s.i., and the cellular debris were collected by centrifugation for 15 min at 32,583 × g. The cell lysate was then loaded on a 60-ml amylose column (26 mm × 20 cm), and protein was eluted with a 0–10 mM maltose gradient over 300 ml in 30 mM Tris-HCl, pH 7.6, containing 200 mM NaCl. Fractions containing MBP-VanX were identified by SDS-polyacrylamide gels, and pure fractions were pooled and concentrated to ~10 ml with an Amicon ultrafiltration concentrator equipped with a YM-10 membrane. This overexpression/purification protocol resulted in 410 mg of MBP-VanX fusion protein/liter of growth culture. The maltose-binding protein from the fusion MBP-VanX protein was removed by thrombin digestion. Three units of restriction grade thrombin were added for every 1 mg of uncleaved MBP-VanX, and the cleavage was allowed to incubate for 10 h in a water bath at 25 °C. The cleaved proteins were then separated by two methods: a 120-ml Sephacryl S-200 HR size exclusion column (16 mm × 60 cm) or a 25-ml Q-Sephrose anion exchange column (18 mm × 20 cm).

Fractions containing purified, cleaved VanX, as judged by SDS-PAGE, were pooled and concentrated using an Amicon ultrafiltration concentrator equipped with a YM-10 membrane. VanX concentrations were determined by using the sample’s absorbance at 280 nm, the extinction coefficient (ε280 nm), and Beer’s Law. The extinction coefficients of MBP-VanX (ε280 nm = 115,700 M−1 cm−1) and VanX (ε280 nm = 51,200 M−1 cm−1) were determined with amino acid analyses performed at the Protein Separation and Analysis Laboratory in the Department of Biochemistry at Purdue University.

**Steady-state Kinetic Studies**—Steady-state kinetics were performed using a coupled assay developed for VanX by Badet and co-workers (25). This assay involves the use of other enzymes, such as D-lactate dehydrogenase (Sigma), catalase (Sigma), and D-amino-acid oxidase (Calzyme Laboratories). All enzymes other than VanX were kept in excess so the rate-limiting process in the coupled reactions is the formation of D-Ala by VanX. All reactions were conducted at 37 °C on an Agilent 8453 UV-visible spectrophotometer. Concentrations of reagents used in the coupled assay were as follows: VanX at 1–5 nM, D-lactate dehydrogenase at 57 units/ml, catalase at 520 units/ml, D-amino-acid oxidase at 1.2 units/ml, NADH at 10 mM, and D-Ala-D-Ala was varied from 50 μM

Walsh at Harvard Medical School (18). The plasmid was transformed into BL21(DE3) Escherichia coli cells, and colonies containing the plasmid were selected on Luria-Bertani (LB) agar plates containing 25 μg/ml kanamycin. A single colony from this plate was used to make an overnight preculture in minimal medium containing 25 μg/ml kanamycin. Three 1-liter flasks, each containing 333 ml of minimal medium and 25 μg/ml kanamycin, were inoculated with 10 ml of the overnight preculture and were allowed to grow to an A600 of 1.6–1.8 at 30 °C.

**Spectroscopic studies**. This work describes the preparation of Co(II)-substituted VanX and the biochemical and spectroscopic characterization of the resulting enzyme.

**Fig. 1. Active site of VanX (1).** The figure was rendered by Rasmol version 2.6.
Metal Analyses—Metal analyses were performed using a Varian Liberty 150 ICP-AES spectrometer. All purified enzyme samples were diluted to 1 mM, and enzyme samples were loaded in a 0.5-ml quartz cuvette. The standard proteins were blue dextran and the supernatant was decanted. A small volume of 30 mM Tris, pH 7.6, was added, and the solid was resuspended. The resulting slurry was frozen in liquid nitrogen. Precipitated VanX samples were centrifuged, protein location was conducted using UV-visible spectroscopy measuring the absorbance at 280 nm of every 2 ml fraction.

RESULTS
Preparation and Characterization of Co(II)-substituted VanX—Our initial efforts to prepare Co(II)-substituted VanX involved using the direct addition method as described by Auld (34, 35). In our case, this method involved the removal of naturally occurring Zn(II) by dialysis versus chelators and the addition of Co(II) to apoVanX. Despite considerable efforts, we could not use this method to prepare Co(II)-substituted VanX in concentrations sufficient for spectroscopic studies as the protein precipitated during dialysis and concentration steps, and frequently Co(II) oxidized to Co(III) during the steps.

Therefore, a biosynthetic method to prepare Co(II)-substituted VanX was used (28, 35). By using a previously published method for the preparation of VanX as a fusion protein with MBP (18), Co(II)-substituted VanX was overexpressed in minimal medium supplemented with 100 µM CoCl₂ and purified using an amylose affinity column. The MBP-VanX fusion protein was cleaved with thrombin, and the cleaved proteins were separated by using size exclusion or ion-exchange chromatography. VanX eluted from the size exclusion column earlier than MBP despite VanX having a monomeric molecular mass of 23 kDa and MBP having a molecular mass of 42 kDa. This result suggests that VanX aggregates in solution into a species with a molecular mass greater than 42 kDa. To further test this hypothesis, size exclusion chromatography was used to determine the quaternary structures of VanX and MBP-VanX. Both proteins eluted from the column right after blue dextran and had elution times consistent with molecular masses of 500 and 630
kDa, respectively. The elution profile was unaffected by the inclusion of dithiothreitol or higher concentrations of salt in the buffer. The result with the reductant was not surprising since the crystal structure of hexameric VanX showed that disulfides are not involved in the aggregation of VanX. This result suggests that MBP-VanX, in the buffers and at the concentrations used in our studies, has a decameric structure, while VanX is composed of over 20 monomers. Efforts to concentrate these samples show that MBP-VanX can be concentrated to 1.6 mM, while VanX can only be concentrated to 0.3 mM.

The steady-state kinetic properties of Co(II)-substituted VanX and MBP-VanX were determined using the optimized coupled assay of Badet and co-workers (25) (Table I). Co(II)-substituted VanX exhibited \( k_{cat} \) and \( K_m \) values of 94 ± 6 \( s^{-1} \) and 140 ± 40 \( \mu M \), respectively, and these values were similar to those of the fusion protein Co(II)-MBP-VanX (\( k_{cat} = 110 \pm 20 s^{-1} \) and \( K_m = 100 \pm 40 \mu M \)). It is clear that the presence of MBP does not affect the steady-state kinetic constants exhibited by VanX in agreement with previous reports (18). The Co(II)-substituted analogs of VanX exhibited \( K_m \) values identical to Zn(II)-VanX and a 70% reduction in \( k_{cat} \) values, which is common for Co(II)-substituted enzymes (36). In our experiments, the \( k_{cat} \) value for Zn(II)-VanX was 3-fold higher than that previously reported (25). It is possible, in the previous report (25), the rate of \( \alpha \)-amino-acid oxidase contributed to the overall rate of reaction attributed to VanX.

Metal analyses on VanX produced from the MBP fusion construct showed that the isolated enzymes bind roughly 0.70 ± 0.02 equivalents of only cobalt or zinc. No other metal ions were found in the VanX samples. The inclusion of higher concentrations of Zn(II)/Co(II) in the growth medium used for overexpression did not result in purified VanX samples with more equivalents of metal ions. If excess Zn(II) or Co(II) was added to the as-isolated VanX (or MBP-VanX) and the enzymes were exhaustively dialyzed versus metal-free buffer, the resulting enzymes contained 1.0 ± 0.1 metal ion per monomer. We were unable to isolate Co(II)-substituted VanX that contained less than 0.06 equivalents of Zn(II), most likely due to Zn(II) from buffers, growth medium, glassware, etc.

**Electronic Spectra of Co(II)-substituted VanX**—The UV-visible spectrum of Co(II)-substituted MBP-VanX shows three peaks at 520, 550, and 580 nm and no other features above 300 nm (Fig. 2A). The peak at 580 nm often appears as a shoulder of the 550 nm peak. These peaks have molar extinction coefficients of ~100 \( M^{-1} \ cm^{-1} \), suggesting these transitions are ligand field transitions of high-spin Co(II) and that the Co(II) ion is five-coordinate (37). There were no peaks at ~320 nm in any of the UV-visible spectra of Co(II)-substituted VanX, suggesting that Co(II) does not bind to either of the solvent-accessible cysteines in VanX (38–40).

When Co(II)-substituted MBP-VanX was prepared in minimal medium, pH 7.5, the resulting enzyme exhibited similar ligand field transitions as in Fig. 2A; however, an additional feature at 425 nm that had an extinction coefficient of ~100 \( M^{-1} \ cm^{-1} \) was observed (Fig. 2B). The intensity of this peak implies that it is due to a ligand field transition rather than from a ligand to metal charge transfer band (expected \( \epsilon \) of >500 \( M^{-1} \ cm^{-1} \)) (40). The position of this peak suggests that it arises from Co(III) in the sample, indicating that Co(II) is air-oxidizing to Co(III) during protein preparation at pH 7.5. The preparation of Co(II)-substituted VanX in minimal medium, pH 6.8, reduces the amount of air oxidation (Fig. 2A).

In an effort to extend the use of UV-visible spectroscopy to

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**Table I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{cat} )</th>
<th>( K_m )</th>
<th>Metal</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(II)-VanX</td>
<td>41 ± 2</td>
<td>110 ± 10</td>
<td>NR</td>
<td>Ref. 25</td>
</tr>
<tr>
<td>Zn(II)-VanX</td>
<td>156 ± 16</td>
<td>109 ± 40</td>
<td>0.70 ± 0.02 Zn(II)</td>
<td>Our data</td>
</tr>
<tr>
<td>Co(II)-VanX</td>
<td>94 ± 6</td>
<td>140 ± 40</td>
<td>0.66 ± 0.09 Co(II)</td>
<td>Our data</td>
</tr>
<tr>
<td>MBP-Co(II)-VanX</td>
<td>110 ± 20</td>
<td>100 ± 40</td>
<td>0.66 ± 0.09 Co(II)</td>
<td>Our data</td>
</tr>
</tbody>
</table>

\( a \) Not reported.  
\( b \) Reported in Ref. 18.

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2 C. H. Park, personal communication.
probe inhibitor binding to VanX, UV-visible spectra were collected on samples of Co(II)-substituted MBP-VanX complexed with the phosphinate analog of d-Ala-d-Ala (Fig. 3). Previously Walsh and co-workers (23) reported that this phosphinate is a slow binding inhibitor of VanX with a $K_i$ value of 1.26 $\mu M$. Therefore, a 0.5 $mM$ Co(II)-MBP-VanX sample containing equimolar equivalents of phosphinate inhibitor was expected to result in $\sim 100\%$ enzyme-inhibitor complex. In addition, Bussei and co-workers (1) published a crystal structure of the Zn(II)-VanX-phosphinate complex; this structure showed that both phosphinate oxygens coordinate Zn(II) and that Zn(II) is five-coordinate. Based on this crystal structure and our data on uncomplexed Co(II)-MBP-VanX, we hypothesized that the intensities of the Co(II) ligand field transitions would be the same as those of the uncomplexed enzyme. In addition, we hypothesized that the peak positions of the ligand field transitions would shift to slightly longer wavelengths since the phosphinate oxygens were expected to be weaker field ligands than water. The UV-visible spectrum of Co(II)-MBP-VanX complexed with the phosphinate inhibitor is shown in Fig. 2C. As hypothesized, the intensities of the ligand field transitions in uncomplexed and complexed VanX are the same, suggesting that Co(II) is five-coordinate in both spectra (37). The simplest explanation for this observation is that the phosphinate oxygens replace two solvent molecules in the complexed enzyme. However, we cannot rule out the possibility that binding of the phosphinate results in the loss of one solvent molecule and the "shifting" of a chelating Asp-123 to a monodentate binding mode. This behavior has been reported previously for iron enzymes and has been called the "carboxylate shift" (41). Efforts to obtain the crystal coordinates of the VanX-phosphate complex were unsuccessful as these data are no longer available.2 EPR Spectra of Co(II)-substituted VanX—Initially we attempted to utilize unfused VanX in EPR studies; however, when we concentrated these samples, VanX precipitated. The precipitated protein could be diluted with buffer and resolubilized, but the final concentration of soluble VanX was never greater than 0.3 $mM$. We, therefore, collected EPR spectra on precipitated VanX and soluble MBP-VanX samples at 8 K. The 1.6 $mM$ Co(II)-MBP VanX sample exhibited an EPR signal with turning points at $g_{\text{eff}(x,y,z)} = 1.92, 3.71, \text{ and } 5.64 \text{ (Fig. 4C).}$ Computer simulation (Fig. 4D) returned spin Hamiltonian parameters of $M_N = \left| \frac{1}{2} \right|$, $E/D = 0.14$, $g_{\text{real} (x,y)} = 2.37$, and $g_{\text{real} (x,z)} = 2.03 \text{ (Fig. 4D).}$ These parameters are not dissimilar to those from other Co(II)-containing proteins (29, 42) and are indicative of either a five- or six-coordinate Co(II) center (43). Six-coordinate Co(II) ions typically exhibit low $E/D$ due to the high degree of axial symmetry, particularly when unconstrained ligands such as water allow for geometric relaxation, and examples of this include Co(H$_2$O)$_6^{2+}$ and the methionyl aminopeptidase from E. coli (44). The $E/D$ value for VanX, however, is intermediate for Co(II) centers that are axially symmetric ($E/D = 0$) and for those with the lowest possible degree of axial symmetry ($E/D = \frac{1}{3}$) (30). Thus, the EPR data, especially when considered along with the extinction coefficients of the electronic absorption ligand field transitions, suggest that the coordination number of Co(II) in Co(II)-MBP-VanX is five (29). For five-coordinate systems with no solvent-derived ligands, such as the leucine phosphonic acid complex of the aminopeptidase from Vibrio proteolyticus, $E/D$ is significantly higher (0.26) than for VanX, and the moderate $E/D$ (0.14) for VanX may signify the presence of at least one solvent-derived ligand.

The EPR spectrum of precipitated Co(II)-VanX contains a component that exhibits markedly higher rhombic distortion of the axial zero field splitting than that of soluble Co(II)-substituted MBP-VanX and is similar to spectra reported for Co(II)-substituted carboxypeptidase A (Fig. 4A) (42). The spectrum of precipitated Co(II)-VanX appears to result from two components (Fig. 4E, see difference spectrum). The minor component is indistinguishable from soluble Co(II)-substituted MBP-VanX (Fig. 4, C and E) and is presumably due to the soluble fraction of Co(II)-VanX. The major component has $g_{\text{eff}}$ values of around 1.69, 2.57, and 5.92 (Fig. 4E). The simulation of spectrum $E$ (Fig. 4) indicates values of $E/D = 0.277$, $g_{\text{real} (x,y)} = 2.235$, and $g_{\text{real} (x,z)} = 2.112$ (Fig. 4F). The similar values of $g_{\text{eff} (x,y)} - g_{\text{eff} (x,z)}$ for both the soluble MBP-VanX and the precipitated VanX suggest that there is no marked pseudotetragonal elongation or compression in going from the solution phase to the precipitate, but the higher $E/D$ value for the precipitated VanX indicates a decrease in axial electronic symmetry, and the narrower lines suggest a loss of conformational flexibility of the Co(II)-coordinating ligands. These phenomena are consistent with a solvent ligand being replaced by a rigid ligand and therefore may be due to Asp-123 providing a bidentate ligand to the metal center in the precipitated form with the second carboxylate oxygen atom replacing a coordinated water. The chelate binding mode of an Asp/Glu residue is preceded in carboxypeptidase A (45).

The EPR spectrum of the precipitated Co(II)-VanX with bound phosphinate was essentially due to a single species. The spectrum exhibited turning points at $g_{\text{eff}}$ values of 1.86, 2.87, and 5.78, and computer simulation returned values of $E/D =$
with the EPR data.

rigid ligand. The crystal structure of VanX with bound phosphinate revealed a high degree of rigidity compared with the uncomplexed soluble enzyme, possibly due to a bidentate ligand, and low conformational flexibility compared with the uncomplexed soluble enzyme. This mode of binding is entirely consistent with the EPR data.

The EPR spectrum of soluble Co(II)-MBP-VanX treated with phosphinate is markedly different from that of the untreated enzyme and appears to be made up of two components (Fig. 5A). Simulations of the major component yielded very similar values very similar to those for phosphinate-treated precipitated VanX: $g_{\text{eff}} = 1.83, 2.78,$ and $5.82; E/D = 0.245; g_{x,y} = 2.252; g_{z} = 1.77; M_S = \pm \frac{1}{2}$. Based on these values, major species is likely due to the bidentate binding mode of phosphinate to Co(II), similar to that predicted for precipitated Co(II)-VanX with bound inhibitor. The minor component could not be sufficiently well resolved to extract spin Hamiltonian parameters, but the presence of an additional sharp feature at low field suggests that it may be very similar to the major component and that only subtle geometrical differences exist between the two species.

**EXAFS Spectra of Zn- and Co(II)-substituted VanX—**

Fourier-transformed EXAFS spectra for Co(II) and Zn(II)-MBP-VanX are shown in (Fig. 6). Both Fourier-transformed EXAFS spectra show a narrowly distributed first shell at $\sim 2.0$ Å and outer shell scattering consistent with histidine (imidazole) coordination. Single scattering fits indicate that both cobalt and zinc are coordinated by five nitrogen/oxygen ligands at $\sim 2.0$ Å (Table II). The size of $\sigma^2$ is suggestive of a heterogeneous first coordination sphere, such as the His$_2$Asp(OH)$_2$$_2$ coordination indicated above. However, the data lack sufficient resolution to define two independent distances. The average bond lengths are consistent with those expected for five coordination with mixed nitrogen and oxygen ligands (46). The outer shell scattering for the Zn(II) and Co(II) enzymes is remarkably similar, indicating the presence of a pair of His ligands in both forms. Together with the curve fitting results, the similarity of the Zn(II)- and Co(II)-MBP-VanX Fourier-transformed EXAFS spectra demonstrate that zinc and cobalt adopt similar pentacoordinate geometries in the active site of VanX in solution. The similarity of the data further supports the transferability of spectroscopic studies on Co(II)-substituted MBP-VanX to direct investigation of native Zn(II)-VanX.

**Comparison of the EXAFS spectra for Co(II)-substituted VanX and Co(II)-MBP-VanX in the presence and absence of phosphinate.** First shell fits (dotted lines) are reported in Table II.

MBP-VanX with and without the phosphinate analog of d-Ala-d-Ala (Fig. 6) shows extremely similar first shell coordination with a slight decrease in average bond length on inhibitor binding (Table II). Although the two spectra differ dramatically in signal-to-noise ratio, the low $R$ features are sufficiently determined to conclude that the overall coordination number of the Co(II) ion has not changed. The absorption edge energies for Co(II)-substituted MBP-VanX with and without the inhibitor were 7,718.3 eV, strongly suggesting that the coordination numbers about Co(II) in both samples are the same and that the ligand types are similar. Qualitatively the only notable difference in the Fourier-transformed EXAFS spectra below $R + \alpha = 4$ Å is the apparent loss of the outer shell scattering peak at $2.1$ Å, a distance consistent with metal-carbon scattering from a coordinated carboxylate (47). Its absence in the inhibitor complex may indicate a shift in, or the loss of, a carboxylate ligand. However, the inhibitor bound data are not of sufficient quality to address this difference quantitatively.

**CONCLUSIONS**

Based on spectroscopic studies, the metal ion in Co(II)-substituted VanX is five-coordinate. An alternative explanation for the EPR data would require that the $M_S = \pm \frac{1}{2}$ spectra observed were, in fact, excited state spectra and that a $M_S = \pm \frac{3}{2}$ ground state existed due to tetrahedral Co(II). The observation of only $M_S = \pm \frac{1}{2}$ at 8 K would require that such a species exhibited an anomalously low zero field splitting, $D$. EXAFS analyses returned markedly better fits for 5-fold coordination than for 4-fold coordination, although in the absence of other evidence the assignment could not be taken as unambiguous. Electronic absorption spectroscopy does, however, appear to unambiguously preclude tetrahedral coordination. Taken together then, EXAFS, EPR, and electronic absorption spectroscopy are all consistent only with five-coordinate Co(II) in Co(II)-MBP-VanX.

This result was surprising since the crystal structure of Zn(II)-VanX showed a four-coordinate Zn(II) in the active site of VanX (1). There are at least three possible explanations for this contradictory result. 1) Co(II) in Co(II)-substituted VanX could have a different coordination number than Zn(II). 2) Zn(II) could be coordinated by both carboxylate oxygens of Asp-123, which would be similar to the coordination of Zn(II) by Glu-72 in carboxypeptidase A (45). 3) There could be an additional solvent (or buffer) molecule in VanX that was not reported in the crystal structure (1). Addressing the first expla-

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3 A. Costello and D. L. Tierney, unpublished results.
nation, EXAFS studies on Zn(II)-containing and Co(II)-
containing VanX demonstrate very clearly that these samples
have metal centers with the same coordination number even
though there is slight ambiguity as to what precisely this
number is. In the literature, there are only two examples of
Co(II)-substituted enzymes having a different coordination
number than Zn(II) in the native enzyme (48, 49). The likeli-
hood of a substituted Co(II) ion having a higher coordination
number than the native Zn(II) is not high when the physio-
chemical properties of these ions are considered. The ionic
radius of Co(II) is 0.02 Å smaller, and the Co(II)-ligand dis-
tances are typically shorter than those for native Zn(II) centers
(36); these characteristics would argue for Co(II) having a lower
coordination number rather than a higher one. The spectro-
scopic data combined indicate that Co(II) must be five-coor-
dinate in VanX. It is clear on experimental grounds and sup-
ported by both theoretical considerations and the extensive
literature on Co(II)-substituted Zn(II)-enzymes that Co(II) and
Zn(II) in VanX have the same coordination number. Therefore,
the evidence indicates that Zn(II) in VanX is five-coordinate.

The crystallographic data reported by Park and co-workers
(1) clearly indicate ligands due to His-116 (1 × nitrogen), His-
184 (1 × nitrogen), and Asp-123 (1 × oxygen at 2.55 Å). An oxygen atom presumed to be due to water was identified as a
fourth ligand. A second oxygen ligand from Asp-123 was mod-
elled at 3.01 Å from Zn(II). Significant differences between the
EPR spectra of soluble VanX and precipitated VanX clearly
indicate ligands due to His-116 (1
1
1
1
i
N
and
1
i
calc)
= \frac{\sum_{i=1}^{N} \left[ \text{Re}(x_{i}) \right]^{2} + \left| \text{Im}(x_{i}) \right|^{2}}{\sum_{i=1}^{N} \left[ \text{Re}(x_{i}) \right]^{2} + \left| \text{Im}(x_{i}) \right|^{2}}

where $N$ is the number of data points.

A Five-coordinate Metal Center in Co(II)-substituted VanX

<table>
<thead>
<tr>
<th>Sample</th>
<th>$N$/$O$</th>
<th>$R_{actual}$</th>
<th>$\sigma_{abs}^{2o}$</th>
<th>$\Delta E_{R}^{a}$</th>
<th>$R^{c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(II)-MBP-VanX</td>
<td>5 N/O</td>
<td>2.03</td>
<td>4.8</td>
<td>-21</td>
<td>109.0</td>
</tr>
<tr>
<td>Co(II)-MBP-VanX</td>
<td>5 N/O</td>
<td>2.00</td>
<td>3.4</td>
<td>-26</td>
<td>12.4</td>
</tr>
<tr>
<td>Co(II)-MBP-VanX + phosphinate</td>
<td>5 N/O</td>
<td>1.97</td>
<td>4.1</td>
<td>-18</td>
<td>22.6</td>
</tr>
</tbody>
</table>

$^a$ Integer coordination number giving the best fit, N/O, nitrogen/oxygen.
$^b$ Mean square deviation in absorber-scatterer bond length in $10^{-3}$ Å$^2$.
$^c$ The value of $\Delta E_{R}$ was allowed to vary for Co(II)-MBP-VanX data sets.

In summary, there are rational possible explanations why
the crystal structure and the spectroscopic data may initially
appear at odds. However, the inescapable conclusion from the
spectroscopic work is that Zn(II) in soluble VanX is five-coor-
dinate as is Co(II) in the cobalt-substituted form of the enzyme.
The presence of a five-coordinate Zn(II) or Co(II) in the active
site of VanX may have important catalytic consequences. The
most stable coordination numbers of Zn(II) or Co(II) in proteins
are $4 \rightarrow 6 > 5$ (36). It is likely then that 5-fold coordination
of the metal ion represents a state, the entatic state, that is stable
in the resting enzyme but is highly reactive toward a hydro-
lizable substrate (50). Mechanistic studies on VanX to probe
the metal center during catalytic turnover are currently in
progress.

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MBP-VanX, pBADL14.

REFERENCES
Biol. 3, 21–28
1172–1193
7. Arthur, M., Reynolds, P. E., Depardieu, F., Evers, S., Dutka-Malen, S., Quin-
33, 4625–4631
9. Haldimann, A., Fisher, S. L., Daniels, L. L., Walsh, C. T., and Wanner, B. L.
11417–11424
439–443
9336–9340
125, 9314–9315
10498–10505
19. Lessard, I. A. D., Pratt, S. D., McCafferty, D. G., Bussiere, D. E., Hutchins, C.,

The third possible structural scenario consistent with the
crystallographic data is that there is a second solvent ligand
to Zn(II) in soluble VanX. It is possible, although unlikely, that
such a ligand is present in the soluble form but not in the
crystalline form. A more likely explanation is that a second
solvent ligand may be present in the crystalline form but is
undetected. The crystal coordinates were kindly made avail-
able for inspection by Dr. Chang H. Park, Abbott Laboratories,
and the quality of the data were insufficient to rule out the
possibility of an additional solvent ligand, particularly if it
was disordered.
A Five-coordinate Metal Center in Co(II)-substituted VanX
