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⁻¹ cm⁻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 $M_S = |\pm \frac{1}{2}|$, $E/D = 0.14$, $g_{\text{real(x,y)}} = 2.37$, 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 phosphinate analog of substrate $\beta$-Ala-$\beta$-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 $\alpha$-Ala-$\alpha$-Ala end of the peptidoglycan pentapeptide via five hydrogen bonds, thereby preventing cross-linking of the cell wall precursors (3–6). How-}
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-thioquinonefluoromethide 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 naphthyl-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)-VanX and the biochemical and spectroscopic characterization of the resulting enzyme.

**MATERIALS AND METHODS**

Preparation of Co(II)-substituted Maltose-binding Protein-VanX and VanX—Co(II)-substituted maltose-binding protein (MBP)-VanX and VanX were prepared in minimal medium. The minimal medium contained 2.5 g/liter d(-+)-glucose, 5 g/liter casamino acids, 10.8 g/liter K2HPO4, 0.5 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 plasma with atomic emission spectroscopy.

Extended x-ray absorption fine structure; ICP-AES, inductively coupled plasma mass spectrometry; EXAFS, extended x-ray absorption fine structure; ICP-AES, inductively coupled plasma with atomic emission spectroscopy.

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**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 that the 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 to 100 μM.
to 2 mM with all solutions being made in 50 mM Hepes, pH 8.0, containing 200 mM NaCl. The reactions were monitored at 340 nm, and the reactions were designed to ensure the loss of ~1 absorbance unit over the course of 10 min. Absorbance values for the linear portion of all curves were converted into velocities using the molar extinction coefficient for NADH (ε<sub>280 nm</sub> = 6,220 M<sup>-1</sup> cm<sup>-1</sup>) and the time interval of the absorbance change. These values were then plotted against the corresponding substrate concentrations, and the resulting plots were fitted to the Michaelis-Menten equation ε<sub>0</sub> = V<sub>max</sub>/(S/K<sub>m</sub> + [S]) using Igor Pro version 4.05A.

**Metal Analyses—**Metal analyses were performed using a Varian Liberty 150 ICP-AES spectrometer. All purified enzyme samples were diluted to ~10 μM with 30 mM Tris-HCl, pH 7.6. A calibration curve with four standards and a correlation coefficient of greater than 0.999 was generated using serial dilutions of Fisher metal standards. Three trials of each sample were averaged. The following wavelengths for the indicated metal ions were used to ensure the lowest detection limit possible: zinc, 213,856 nm; copper, 324,754 nm; nickel, 321,604 nm; cobalt, 238,892 nm; iron, 259,940 nm; and manganese, 257,610 nm.

**Size Exclusion Chromatography—**A 120-ml Sephacryl S-200 HR size exclusion column was used to determine the molecular masses of MBP-VanX and VanX. A calibration curve was used to relate molecular weight to the retention time. The standard proteins were blue dextran 2000 (11.7 M), thyroglobulin (20 S, 2 MDa), ferritin (molecular mass, 440 kDa), catalase (molecular mass, 222 kDa), aldolase (molecular mass, 158 kDa), and albumin (molecular mass, 67 kDa). The mobile phase buffer was 0.05 M Na<sub>2</sub>PO<sub>4</sub>, pH 7.6, containing 150 mM NaCl, and the flow rate was 0.5 mL/min. Analysis of protein location was conducted using UV-visible spectroscopy measuring the absorbance at 280 nm of every 2-ml fraction.

**UV-visible spectroscopy—**All UV-visible spectra were collected on an Agilent 8453 UV-visible spectrophotometer with photodiode array detection measuring the UV-visible absorbance at each wavelength between 200 and 1,100 nm. Enzyme concentrations ranged from 200 μM to 1 mM, and enzyme samples were loaded in a 0.5-mL quartz cuvette. The buffer used for dilutions and for the blanks was 30 mM Tris-HCl, pH 7.6. Spectra were recorded at 25 °C. The phosphate inhibitor (22, 23) of the enzyme was added in concentrations sufficient for spectroscopic studies as the inhibitor-scatterer (as) pair, φ<sub>as</sub>(k) is the backscattering amplitude of the absorber-scatterer (as) pair, φ<sub>as</sub>(k) is the phase shift experienced by the photoelectron as it encounters the electron clouds of the scattering and absorbing atoms, λ is the photoelectron mean free path, and the sum is taken over all shells of scattering atoms. Theoretical amplitude and phase functions, A<sub>k</sub>(exp(-2πφ<sub>as</sub>(k)), and φ<sub>as</sub>(k)), were calculated using FEFF version 8.00 (31). Similar results were obtained using a single shell of oxygen atoms to model the first shell. Markedly better fit residuals were obtained with mixed nitrogen/oxygen models; however, the data do not possess sufficient degrees of freedom to warrant the use of multiple atom types, and these fits are not reported.

## 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 CoCl<sub>2</sub> 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 μM, respectively, and these values were similar to those of the fusion protein Co(II)-MBP-VanX ($k_{cat} = 110 ± 20$ s$^{-1}$ and $K_m = 100 ± 40$ μM). 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 3-amino-acid oxidase contributed on 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).

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C. H. Park, personal communication.

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**Table I**

| Enzyme          | $k_{cat}$  | $K_m$  | Metal Content | Source  |
|-----------------|------------|--------|---------------|---------|
| Zn(II)-VanX     | 41 ± 2     | 110 ± 10 | NR$^a$        | Ref. 25 |
| Zn(II)-VanX     | 156 ± 16   | 109 ± 40 | 0.07 ± 0.02 Zn(II) | Our data |
| Co(II)-VanX     | 94 ± 6     | 140 ± 40 | 0.06 ± 0.03 Zn(II) | Our data |
| MBP-Co(II)-VanX | 110 ± 20   | 100 ± 40 | 0.06 ± 0.03 Zn(II) | Our data |

$^a$ Not reported.

$^b$ Reported in Ref. 18.

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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 $\varepsilon$ 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...
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, Bussiere 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 “shrinking” 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). 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 Co(II)-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 $g_{//} = 1.92, 3.71$, and 5.64 (Fig. 4C). Computer simulation (Fig. 4D) returned spin Hamiltonian parameters of $M_S = |z/z|, E/D = 0.14, g_{//} = 2.37$, and $g_{\perp} = 2.03$ (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 ($E/D = 1/3$) (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). 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 $g_{//}$ values of around 1.69, 2.57, and 5.92 (Fig. 4E). The simulation of spectrum E (Fig. 4) indicates values of $E/D = 0.277, g_{//} = 2.235$, and $g_{\perp} = 2.112$ (Fig. 4F). The similar values of $g_{//} - g_{\perp}$ 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 $g_{//}$ values of 1.86, 2.87, and 5.78, and computer simulation returned values of $E/D =$
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0.233, $g(x,y) = 2.245$, and $g(z) = 2.165$ with an $M_g$ value of $|+\frac{1}{2}|$ (Fig. 5B). While these values are certainly sufficiently different from those of untreated, precipitated VanX to distinguish the spectrum of the phosphinate complex as being due to a separate chemical species, they suggest a similar chemical structure, viz. a five-coordinate species with low axial electronic symmetry, possibly due to a bidentate ligand, and low conformational flexibility compared with the uncomplexed soluble enzyme, probably due to replacement of a water molecule by a rigid ligand. The crystal structure of VanX with bound phosphinate showed that the inhibitor binds through both phosphinate oxygens (1). 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 values very similar to those for phosphinate-treated precipitated VanX: $g_{\text{eff}} = 1.83, 2.78$, and $5.82$; $E/D = 0.245$; $g(x,y) = 2.252$; $g(z) = 2.177$; and $M_g = |+\frac{1}{2}|$. Based on these values, the 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 ~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 ~2.0 Å (Table II). The size of $\sigma^2$ is suggestive of a heterogeneous first coordination sphere, such as the $\text{His}_2\text{Asp(OH)}_2$ 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

3 A. Costello and D. L. Tierney, unpublished results.
EXAFS fitting results for Zn(II)- and Co(II)-MBP-VanX

| Sample                  | N°  | R_m | \(\sigma_{R_m}^{2}\) | \(\Delta E_{c}\) | R²   |
|------------------------|-----|-----|----------------------|------------------|------|
| Zn(II)-MBP-VanX        | 5 N/O | 2.03 | 4.8                  | −21              | 109.0|
| Co(II)-MBP-VanX        | 5 N/O | 2.00 | 3.4                  | −26              | 12.4 |
| Co(II)-MBP-VanX        | 5 N/O | 1.97 | 4.1                  | −18              | 22.6 |
| Co(II)-MBP-VanX        | 5 N/O | 1.97 | 4.1                  | −18              | 22.6 |

\(a\) Integer coordination number giving the best fit. N/O, nitrogen/oxygen.
\(b\) Mean square deviation in absorber-scatterer bond length in 10⁻³ Å².
\(c\) The value of \(\Delta E_{c}\) was allowed to vary for Co(II)-MBP-VanX data sets.

Goodness of fit (R) defined as:

\[
R = \sqrt{\frac{\sum_{i=1}^{N} [\text{Re}(X_{\text{fit}}) - \text{Re}(X_{\text{obs}})]^2 + [\text{Im}(X_{\text{fit}}) - \text{Im}(X_{\text{obs}})]^2}{\sum_{i=1}^{N} [\text{Re}(X_{\text{obs}})]^2 + [\text{Im}(X_{\text{obs}})]^2}}
\]

where \(N\) is the number of data points.

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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 modeled at 3.01 Å from Zn(II). Significant differences between the EPR spectra of soluble VanX and precipitated VanX clearly show that the metal center can adopt different geometries depending on the aggregation state of the protein. It must, therefore, be at least considered possible that the second oxygen atom of Asp-123 may adopt a slightly different orientation with respect to Zn(II) in the soluble form of VanX than in the crystalline state, affording a fifth ligand to Zn(II). The precise conformational changes required to lengthen the Zn(II)-oxygen bond in the crystalline state will presumably be specific and dependent on the nature of the global conformational change that is necessary to maximize the energetic favorability of the crystal contacts. In contrast, in the precipitated material, there is insufficient driving force to break the Zn–O bond, although the geometry of the site is altered. Although technically difficult, EPR and electronic absorption studies of the crystalline Co(II)-VanX may prove insightful.

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 available 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 were disordered.

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-coordinate 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 ≤ 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 hydrolyzable substrate (50). Mechanistic studies on VanX to probe the metal center during catalytic turnover are currently in progress.

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