Selective Metal Binding by Vanabin2 from the Vanadium-rich Ascidian, *Ascidia sydneiensis samea*

Norifumi Kawakami[a], Tatsuya Ueki[a], Koichi Matsuo[b], Kunihiko Gekko[b] and Hitoshi Michibata [a]*

[a] *Molecular Physiology Laboratory, Department of Biological Science and* [b] *Molecular Biophysics Laboratory, Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8526, Japan*

*Corresponding author. Tel/Fax: +81 82 424 7438

E-mail address: hmichi@hiroshima-u.ac.jp (H. Michibata)

Keywords: Ascidian; Vanadium; Copper; Metal selectivity; Metal-binding protein

Abstract

Vanadium-binding proteins, or Vanabins, have recently been isolated from the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Recent reports indicate that Vanabin2 binds twenty V(IV) ions at pH 7.5, and that it has a novel bow-shaped conformation. However, the role of Vanabin2 in vanadium accumulation by the ascidian has not yet been determined. In the present study, the effects of acidic pH on selective metal binding to Vanabin2 and on the secondary structure of Vanabin2 were examined. Vanabin2 selectively bound to V(IV), Fe(III), and Cu(II) ions under acidic conditions. In contrast, Co(II), Ni(II), and Zn(II) ions were bound at pH 6.5 but not at pH 4.5. Changes in pH had no detectable effect on the secondary structure of Vanabin2 under acidic conditions, as determined by circular dichroism spectroscopy, and little variation in the dissociation constant for V(IV) ions was observed in the pH range 4.5-7.5, suggesting that the binding state of the ligands is not affected by acidification. Taken together, these results suggest that the reason for metal ion dissociation upon acidification is attributable not to a change in secondary structure but, rather, that it is caused by protonation of the amino acid ligands that complex with V(IV) ions.

1. Introduction

Several species of ascidians (sea squirts) accumulate high levels of vanadium in blood cells known as vanadocytes [1, 2]. The intracellular vanadium concentration can be as high as 350 mM, which is 10^7 times the concentration in seawater [3]. From the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, we recently

isolated a number of proteins likely to be involved in vanadium accumulation [4-8]. Among them, the vanadium-binding proteins known as Vanabins should play an important role in the accumulation and they include homologues of Vanabin1-4 and VanabinP. These Vanabins are rich in lysine and arginine residues, and have conserved motifs described by the consensus sequence (C)-(X_{2-5})-(C). Three-dimensional structural analysis using nuclear magnetic resonance (NMR) [9] has shown that Vanabin2 has a novel, bow-shaped conformation consisting of four helices connected by nine disulfide bonds.

Electron paramagnetic resonance (EPR) studies have suggested that vanadium (IV) [V(IV)] ions, which are exclusively localized on a single face of the Vanabin2 molecule, are mostly coordinated by amine nitrogens and carboxyl oxygens derived from basic and acidic amino acid residues, respectively [10]. Furthermore, each molecule of Vanabin1 can bind ten V(IV) ions, with K_d of 2.1×10^{-5} M, and each molecule of Vanabin2 can bind twenty V(IV) ions, with K_d of 2.3×10^{-5} M. This binding is significantly inhibited by a 10-fold molar excess of Cu(II) ions, but not Mn(II) or Mo(V) ions [4].

The mechanism by which ascidians specifically accumulate metal ions is not yet well understood, but the inherent metal selectivity of Vanabins may play a role. In the present study, we examined selective metal binding to Vanabin2 to better understand this issue. Generally, metal ions compete with protons for binding to proteins under acidic conditions. Therefore, we evaluated selective metal binding to Vanabin2 at low pH, and we also examined the effect of low pH on the secondary structure of Vanabin2.

2. Materials and Methods

2.1. Cloning, expression, and purification of Vanabin2

Recombinant Vanabin2 was prepared as described previously [4]. To prepare a fusion protein of maltose binding protein (MBP) and Vanabin2, pMAL-c plasmid DNA containing the Vanabin2 coding region was introduced into *E. coli* strain TB1. Cells bearing the fusion protein-expressing plasmids were incubated at 37 °C for 16 h in LB medium containing 50 μg/ml ampicillin. To express the fusion protein, the culture was diluted with nine volumes of fresh LB medium, and the LB medium added 0.5 mM IPTG was grown at 37 °C for 6 h in LB medium. Cells were then harvested, suspended in lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 10 mM EDTA, 10 mM EGTA, 0.25% Tween 20, 10 mM 2-mercaptoethanol, pH7.0) containing 4 M urea, and sonicated using a UH-150 ultrasonic homogenizer (SMT Company). The insoluble fraction was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C.

The fusion protein was then purified from the soluble cellular fraction by amylose resin-column chromatography, according to the manufacturer's protocol (New England Biolabs). The protein was cleaved at the MBP junction region by incubation with Factor Xa at 4 °C for 16 h, and the released Vanabin2 was purified by anion-exchange column chromatography on DEAE-Sephacel. The resulting protein was dialyzed against 50 mM Tris buffer (pH 7.4) containing 25 mM EDTA to remove any metal contaminants. The protein was then dialyzed against 20 mM phosphate buffer containing NaCl to remove EDTA. The dialyzed Vanabin2 was dissolved in sodium phosphate buffer solutions of various pH values (4.5, 5.5, 6.5, and 7.5) and then filtered through a 0.45-μm syringe

filter unit (DISMIC-25cs; Advantec MFS, Inc.). Protein concentrations were measured at each step of the purification using the Bradford assay with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc.) and bovine serum albumin (Pierce Biotechnology, Inc.) as a standard.

2.2. Immobilized metal-ion affinity chromatography (IMAC)

IMAC on a polypropylene column (bed size, 1.5 cm × 3 cm) packed with Chelating Sepharose Fast Flow (Amersham Biosciences) was used qualitatively assess metal binding to Vanabin2. All buffer solutions were prepared using deionized water and ultrapure-grade reagents, and they were degassed for 10 min under vacuum before use.

Selected ions of the first-row transition elements [V(IV), Fe(III), Co(II), Ni(II), Cu(II), and Zn(II)] were examined for binding to Vanabin2. $VOSO_4 \bullet nH_2O$ (n = 3-4), $FeCl_3$, $CoSO_4 \bullet 7H_2O$, $NiCl_2 \bullet 6H_2O$, $CuCl_2 \bullet 2H_2O$, and $ZnCl_2$ were purchased from Wako Pure Chemical Industries, Ltd. Metal ion solutions were prepared by dissolving the metal reagents in DW at 200 mM just before use, and the metal ions were immobilized on the IMAC by passage of 5 ml of metal ion solution through the column. The column was then equilibrated with 20 mM sodium phosphate buffer adjusted to pH 4.5, 5.5, or 6.5. To prevent non-specific binding between protein and Sepharose, the pH 5.5 and 6.5 phosphate buffers included 100 mM NaCl, and the pH 4.5 buffer contained 200 mM NaCl.

Three ml of Vanabin2 solution (80-100 μg protein/ml) was applied onto the column, which was then washed thoroughly with equilibration buffer at a flow rate of 0.7 ml/min.

Protein fractions eluted from the column with equilibration buffer were designated as non-binding fractions. Elution buffers of pH 4.5, 5.5, or 6.5 consisting of 20 mM Na_2HPO_4, 500 mM NaCl, and 50 mM EDTA were used to release adsorbed proteins, which were designated as binding fractions. Each binding or non-binding fraction was concentrated by ultrafiltration to 3-ml, respectively. The concentrated fractions were subjected to SDS-PAGE on a 14% polyacrylamide gel.

2.3. Hummel-Dreyer method

The V(IV) and Cu(II) ion-binding ability of Vanabin2 was determined using the Hummel-Dreyer method [11]. Metal ions were mixed with iminodiacetic acid (IDA) at a 1:1 molar ratio to prevent metal ion precipitation in the neutral pH range. A gel-filtration column (bed size, 0.7 × 19 cm) packed with Bio-Gel P-6 DG resin (Bio-Rad Laboratories, Inc.) was equilibrated with 20 mM sodium phosphate buffer (pH 4.5 or 7.5) containing 100 mM NaCl and various concentrations of V(IV)-IDA or Cu(II)-IDA. Purified Vanabin2 was then loaded onto the column and fractionated at a flow rate of 0.3 ml/min. Protein concentrations of each fraction were determined as described above, and metal concentrations were measured using atomic absorption spectrometry (AAS; SpectrAA-220Z; Varian Inc.). Dissociation constants and the maximum numbers of metal ions bound were determined from Scatchard plots of the data [12].

2.4. Circular dichroism spectroscopy

Effects of V(IV) and Cu(II) ions and acidification on the secondary structure of

Vanabin2 were examined using CD spectroscopy [13]. Prior to CD spectroscopy, the purity of Vanabin2 was confirmed with SDS-PAGE, and the protein concentration was adjusted to 85 μg/ml. V(IV)-IDA and Cu(II)-IDA complexes were added to the Vanabin2 solution at various molar ratios (5:1, 1:1, 1:5, 1:10, 1:20, or 1:40), and the pH of the solution was adjusted to 7.5. To examine the effects of acidification in the absence of metal ions, vanabin2 solutions of pH 4.5, 5.5, and 6.5 were prepared. CD spectra were measured with a Jasco J720 spectropolarimeter at 20 °C for 9-16 accumulations (1.0-mm path length, 1.0-mm slit, 4-s time constant, 50-nm/min scan speed)

3. Results

3.1. Metal-binding selectivity of Vanabin2

Selective metal binding to Vanabin2 was qualitatively examined using IMAC and SDS-PAGE. Vanabin2 was applied to an IMAC column chelated with V(IV), Fe(III), Co(II), Ni(II), Cu(II), or Zn(II) ions at various pHs. Non-binding fractions were eluted from the column with the equilibration buffer, and binding fractions were subsequently eluted with elution buffers at each pH. Binding and non-binding proteins were analyzed by SDS-PAGE.

At pH 6.5, Vanabin2 was detected in all of the binding fractions (Fig. 1), as well as in the non-binding fractions from the Co(II), Ni(II), and Zn(II) ion-chelated columns. In contrast, no protein was detected in the non-binding fractions from the V(IV) or Fe(III) columns. A weak band of Vanabin2 was observed in the non-binding fractions from the Cu(II) column.

At pH 5.5, obvious bands were observed in the binding fractions from the V(IV), Fe(III), and Cu(II) columns. Weak bands, due to non-specific binding between protein and Sepharose were observed in the binding fractions from the Ni(II) and Zn(II) columns, and no band was observed for the binding fractions from the Co(II) column. This result suggests that, at acidic pH, Vanabin2 binds weakly to Ni(II) and Zn(II) ions but loses virtually all ability to bind Co(II) ions. At pH 4.5, all binding to the Ni(II) and Zn(II) column were lost, but binding to the V(IV), Fe(III), and Cu(II) columns was the same as it was at pH 6.5

3.2. Effect of acid pH on the V(IV) and Cu(II) ion-binding ability of Vanabin2

The Hummel-Dreyer method was used quantitatively to evaluate the affinities of Vanabin2 for the V(IV) and Cu(II) ions, which exhibited tight binding to Vanabin2 during IMAC. The maximum number of V(IV) or Cu(II) ions bound to each protein molecule was determined at various concentrations of free metal ions at pH 7.5 and 4.5. Figures 2 and 3 showed the plot for analysis of V(IV) and Cu(II) binding abilities, respectively. The values of K_d and maximum binding numbers were summarized in Table 1. These values were obtained from a Scatchard plot of the binding data (Figs. 2 and 3). . As shown in Table 1, under more acidic conditions, K_d values for both V(IV) and Cu(II) increased, although that for Cu(II) increased only slightly. Furthermore, the number of V(IV) ions, but not the number of Cu(II) ions, bound to Vanabin2 decreased (Table 1).

3.3. Secondary structure of Vanabin2

Changes in the secondary structures of proteins are generally detectable as changes in CD spectra in the far-ultraviolet region. CD spectra of Vanabin2 in the 260-195 nm region are shown in Fig. 4. The two negative peaks at 208 and 222 nm are typical of an α-helix-rich protein. No remarkable structural change was detected upon addition of a 40-fold molar excess of V(IV) or Cu(II) ions (Fig. 4A), or at other molar ratios (data not shown). The CD spectra exhibited no significant pH dependence in the pH region 4.5-7.5 (Fig. 4B), indicating that acidic conditions had no effect on secondary structure.

4. Discussion

The metal ions used in the present study were selected from the first-row transition metals because these metals are essential for many living organisms and have revealed chemical properties at some length. For iron, the trivalent ion Fe(III) was used instead of Fe(II) because the latter is easily oxidized to Fe(III) by dissolved oxygen. Of the metal ions examined, V(IV), Fe(III), and Cu(II) ions bound most tightly to Vanabin2 at low pH (Fig. 1) and, at all pH values, Vanabin2 was found only in the binding fractions from V(IV), Fe(III), and Cu(II) IMAC columns. In contrast, the amount of Vanabin2 detected in the binding fractions from the Ni(II), Zn(II), and Co(II) ion columns decreased as pH decreased. At pH 4.5, binding of Vanabin2 to these columns was virtually undetectable.

The binding data for the metal ions indicated that the ions could be categorized into two groups, a tight-binding group consisting of V(IV), Fe(III), and Cu(II), and a weak-binding group consisting of Co(II), Ni(II), and Zn(II). Certain chemical properties of these metal ions are summarized in Table 2 [14-17]. EDTA stability constants indicate

that, of the Irving-Williams metal ion series, which includes Mn(II), Fe(II), Co(II), Ni(II), Cu(II), and Zn(II), Cu(II) forms the most stable complexes with EDTA. V(IV) and Fe(III) ions also form stable complexes with EDTA.

In general, complexation of metal ions and ligands can be understood in terms of the Irving-Williams series, the Hard and Soft (Lewis) Acids and Bases (HSAB) principle, and ionic radii. However, neither the Irving-Williams series nor the ionic radii of the ions satisfactorily explains the present results. Rather, they are best explained by ionic bond interactions based on the HSAB principle, since Vanabin2 bound most tightly to “hard” metal ions, such as V(IV) and Fe(III). In addition, EPR analysis has revealed that V(IV) ions are bound to Vanabin2 by coordination to amine nitrogens and carboxyl oxygens, which are classified as “hard” bases [10]. Therefore, metal ion binding to Vanabin2 may be explained as an interaction between hard acids and hard bases.

A Scatchard plot of the binding data revealed that each Vanabin2 molecule bound an average of 20.2 V(IV) ions with a K_d of 2.3×10^{-5} M at pH 7.5, but, at pH 4.5, only 4.9 V(IV) ions were bound, and the K_d increased somewhat to 9.3×10^{-5} M (Fig. 2). On the other hand, the analysis disclosed that Vanabin2 binding of Cu(II) ions was not affected significantly by a change in pH from 7.5 to 4.5. At the higher pH, 3.2 Cu(II) ions were bound, with a K_d of 6.4×10^{-5} M at pH 7.5, and at the lower pH, 4.5 Cu(II) ions were bound, with a K_d of 7.0×10^{-5} M (Fig. 2). Thus, under acidic conditions, most V(IV) ions dissociated from Vanabin2, whereas Cu(II) ions remained bound.

We next assessed the effects of metal ion binding and acidic pH on the secondary structure of Vanabin2, using CD spectroscopy. No spectral changes were noted upon

changes in pH or in the molar ratio of protein to metal-IDA complex (Fig. 4), indicating that the dissociation of V(IV) ions at low pH is not attributable to gross secondary structural changes. The lack of spectral changes is consistent with previously reported NMR evidence that Vanabin2 has a stable conformation consisting of a bow-shaped structure with four α-helices connected by nine disulfide bonds [9].

Iron ions are readily released from transferrin upon a decrease in pH [18, 19], and a structural change accompanies this process [19, 20]. The release of metal ions from Vanabin2, however, may be a result of localized conformational changes or of protonation of amino acid residues in the metal binding sites. The fact that similar values of K_d were obtained at pH 7.5 and 4.5 suggests that the binding ligands were not affected by acidification. However, basic amino acids are almost certainly completely protonated at pH 4.5, which is significantly lower than the usual pK_a value range of lysine and arginine in protein. Accordingly, we surmise that binding of V(IV) ions by Vanabin2 at pH 4.5 occurs primarily through coordination by acidic amino acids, and that these amino acids play a key role in metal ion selection at neutral pH. We are currently conducting site-directed mutagenesis experiments of Vanabin2 to better understand its metal binding sites.

Binding of V(IV) to Vanabin1 and Vanabin2 is known to be inhibited by an excess of Cu(II) ions *in vitro* [4]. *E. coli* cells with periplasmic expression of MBP fusions, with Vanabin1 or Vanabin2, accumulated twenty-fold as much Cu(II) as control cells expressing MBP, whereas no accumulation of V(IV) was observed. Cell lines expressing MBP-Vanabin1 or MBP-Vanabin2 absorbed 80 or 70% of the Cu(II) ions in the medium,

respectively [21]. A homologue of mammalian Cu-ATPase has been found in ascidian vanadocytes; the function of this protein is currently under investigation (in preparation). EPR and NMR analyses have already revealed that the most of the EPR-active V(IV) ions are coordinated by amine nitrogens [10], and that V(IV) ions bind to a particular region of Vanabin2 where lysine and arginine are localized [9]. Whether Cu(II) ions behave similarly remains to be determined. On the other hand, ascidians are known to exclusively accumulate higher levels of V(IV) ions, not Cu(II) ions. Thus, they may select V(IV) ions during the accumulation process *via* selective metal ion transporters, ion-pumps, or selective membrane mechanisms that exclude Cu(II) ions.

Acknowledgments

We express our thanks to Mr. K. Morita and the staff of the International Coastal Research Center, Ocean Research Institute, of the University of Tokyo, for their cooperation in materials collection. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#17370026 and #17651048 to H. M.) and a grant from the Toray Science Foundation (03-4402 to T. U.).

References

[1] M. Henze, Untersuchungen über das Blut der Ascidien. I. Mitteilung. Die Vanadiumverbindung der Blutkörperchen, Hoppe-Seyler's, Z. Physiol. Chem. 72 (1911) 494-501.

[2] H. Michibata, N. Yamaguchi, T. Uyama, T. Ueki, Molecular biological approaches to the accumulation and reduction of vanadium by ascidians, Coord. Chem. Rev. 237 (2003) 41-51.

[3] H. Michibata, Y. Iwata, J. Hirata, Isolation of highly acidic and vanadium-containing blood cells from among several types of blood cell from *Ascidiidae* species by density-gradient centrifugation. J. Exp. Zool. 257 (1991) 306-313.

[4] T. Ueki, T. Adachi, S. Kawano, M. Aoshima, N. Yamaguchi, K. Kanamori, H. Michibata, Vanadium-binding proteins (vanabins) from a vanadium-rich ascidian *Ascidia sydneiensis samea*, Biochim. Biophys. Acta 1626 (2003) 43-50.

[5] N. Yamaguchi, K. Kamino, T. Ueki, H. Michibata, Expressed sequence tag analysis of vanadocytes in a vanadium-rich ascidian, *Ascidia sydneiensis samea*. Mar. Biotechnol. 6 (2004) 165-174.

[6] M. Yoshihara, T. Ueki, T. Watanabe, N. Yamaguchi, K. Kamino, H. Michibata, VanabinP, a novel vanadium-binding protein in the blood plasma of an ascidian, *Ascidia sydneiensis samea*. Biochim. Biophys. Acta 1730 (2005) 206-214.

[7] T. Ueki, T. Uyama, K. Yamamoto, K. Kanamori, H. Michibata, Exclusive expression of transketolase in the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, Biochim. Biophys. Acta 1494 (2000) 83-90.

[8] T. Uyama, Y. Moriyama, M. Futai, H. Michibata, Immunological detection of a vacuolar-type H^+-ATPase in vanadocytes of the ascidian *Ascidia sydneiensis samea* J. Exp. Zool. 270 (1994) 148-154.

[9] T. Hamada, M. Asanuma, T. Ueki, F. Hayashi, N. Kobayashi, S. Yokoyama, H.

Michibata, H. Hirota, Solution structure of Vanabin2, a vanadium(IV)-binding protein from the vanadium-rich ascidian *Ascidia sydneiensis samea*. J. Am. Chem. Soc. 127 (2005) 4216-4222.

[10] K. Fukui, T. Ueki, H. Ohya, H. Michibata, Vanadium-binding protein in a vanadium-rich ascidian *Ascidia sydneiensis samea*: CW and pulsed EPR studies. J. Am. Chem. Soc. 125 (2003) 6352-6353.

[11] J. P. Hummel, W. J. Dreyer, Measurement of protein-binding phenomena by gel filtration. Biochim. Biophys. Acta 63 (1962) 530– 532.

[12] G. Scatchard, The attractions of proteins for molecules and ions. Ann. N. Y. Acad. Sci. 51 (1949) 660–672.

[13] K. Matsuo, R. Yonehara, K. Gekko, Improved estimation of the secondary structures of proteins by vacuum-ultraviolet circular dichroism spectroscopy, J. Biochem. 138 (2005) 79-88.

[14] R. D. Shannon, Revised effective ionic radii and systematic studies of interatomic distances in halides and chalcogenides. Acta Cryst. A32 (1976) 751-767.

[15] R. G. Pearson, Hard and soft acids and bases. J. Am. Chem. Soc. 85 (1963) 3533-3539.

[16] A. P. Brunetti, G. H. Nancollas, P. N. Smith, Thermodynamics of ion association. XIX. Complexes of divalent metal ions with monoprotonated ethylenediaminetetraacetate. J. Am. Chem. Soc. 91 (1969) 4680-4683.

[17] G. Schwarzenbach, J. Sandera, Komplexone XXIV. Die Vanadiumkomplexe der äthylendiamin-tetraessigsäure (= H4Y), Helv. Chim. Acta 36 (1953) 1089-1101.

[18] L. Messori, G. D. Poggetto, R. Monnanni, J. Hirose, The pH dependent properties of metallotransferrins: a comparative study. BioMetals 10 (1997) 303-313.

[19] P. G. Thakurta, D. Choudhury, R. Dasgupta, J. K. Dattagupta, Tertiary structural changes associated with iron binding and release in hen serum transferrin: a crystallographic and spectroscopic study. Biochem. Biophys. Res. Commun. 316 (2004) 1124–1131.

[20] F. Kilar, I. Simon, The effect of iron binding on the conformation of transferrin. A small angle X-ray scattering study. Biophys. J. 48 (1985) 799-802.

[21] T. Ueki, Y. Sakamoto, N. Yamaguchi, H. Michibata, Bioaccumulation of copper ions by *Escherichia coli* expressing Vanabin genes from the vanadium-rich ascidian *Ascidia sydneiensis samea*. Appl. Environ. Microbiol. 69 (2003) 6442–6446.

Figure Legends

Figure 1. Selective binding of metal ions to recombinant Vanabin2 evaluated by IMAC. Vanabin2 was applied to V(IV), Fe(III), Co(II), Ni(II), Cu(II), and Zn(II) ion-chelated IMAC columns in 20mM sodium phosphate buffer at the indicated pH values. Non-binding fractions were eluted with the equilibration buffer, and binding fractions were subsequently eluted with elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, 50 mM EDTA) at each pH. Proteins in the non-binding and binding fractions were analyzed by SDS-PAGE and CBB staining.

Figure 2. Vanadium ion-binding ability of Vanabin2 at pH 4.5 evaluated by Hummel-Dreyer method. V(IV) ions were mixed with iminodiacetic acid (IDA) at a 1:1 ratio to prevent precipitation. Vanabin2 was applied to a Bio-Gel P-6 DG resin as described in Section 2.3. Protein and metal concentrations of each fraction were measured using the Bradford assay and AAS, respectively. **(A)** Raw data showing the relationship between the concentration of free V(IV) ions and the number of bound V(IV) ions per protein molecule. **(B)** Scatchard plots of the same data.

Figure 3. Copper ion-binding by Vanabin2 at pH 4.5 and 7.5 evaluated by Hummel-Dreyer method. To compare binding of Cu(II) ions to Vanabin2 under acidic and neutral conditions, the Hummel-Dreyer method was applied. All experiments were done under the same conditions as those used for the V(IV) ion-binding experiments. **(A)**

and **(B)** are Scatchard plots of Cu(II) binding at pH 7.5 and pH 4.5, respectively.

Figure 4. Far ultraviolet CD spectra of Vanabin2. (A) Effects of metal ions on Vanabin2 were evaluated at a Vanabin2:metal-IDA complex molar ratio of 1:40. Spectra were measured in the absence of metal ions (solid line), in the presence of V(IV)-IDA complex (dotted-dashed line), and in the presence of Cu(II)-IDA complex (dotted line). **(B)** The secondary structure of Vanabin2 was examined at pH 4.5 (dotted-dashed line), 5.5 (broken line), 6.5 (dotted line), and 7.5 (solid line).

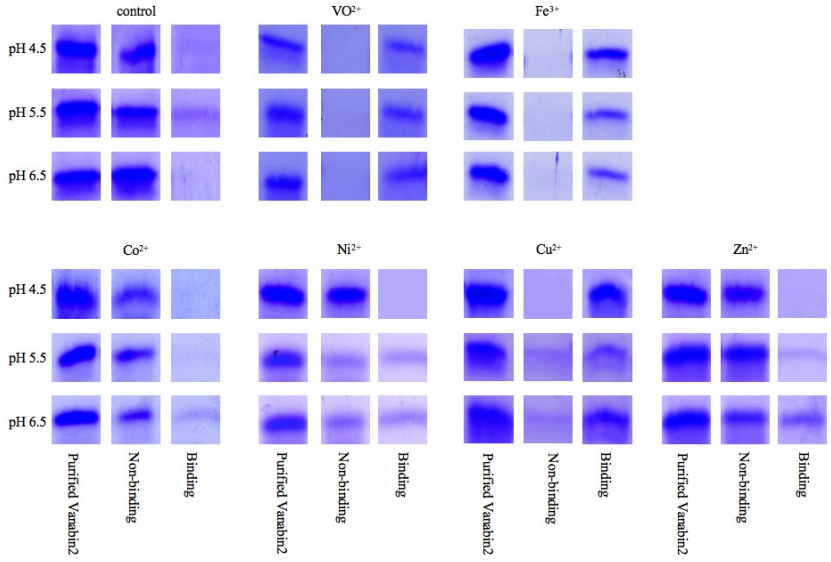

control
VO2+
Fe3+
pH 4.5
pH 5.5
pH 6.5
Co2+
Ni2+
Cu2+
Zn2+
Purified Vanabin2
Non-binding
Binding

Fig. 1

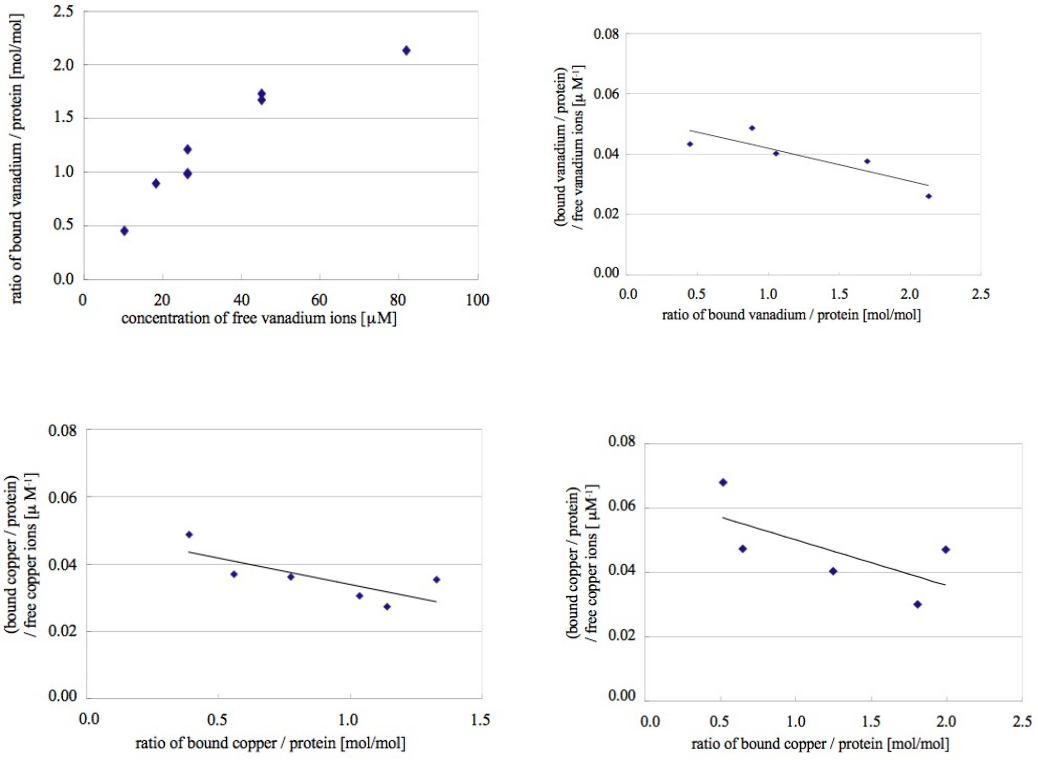
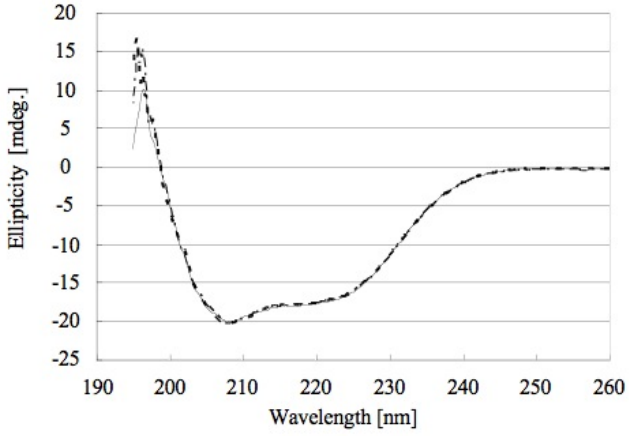

ratio of bound vanadium / protein [mol/mol]
concentration of free vanadium ions [μM]
(bound vanadium / protein) / free vanadium ions [μ M-1]
ratio of bound vanadium / protein [mol/mol]
(bound copper / protein) / free copper ions [μ M-1]
ratio of bound copper / protein [mol/mol]
(bound copper / protein) / free copper ions [μM-1]
ratio of bound copper / protein [mol/mol]

Fig. 2


20
15
10
5
0
-5
-10
-15
-20
-25
Ellipticity [mdeg.]
190
200
210
220
230
240
250
260
Wavelength [nm]

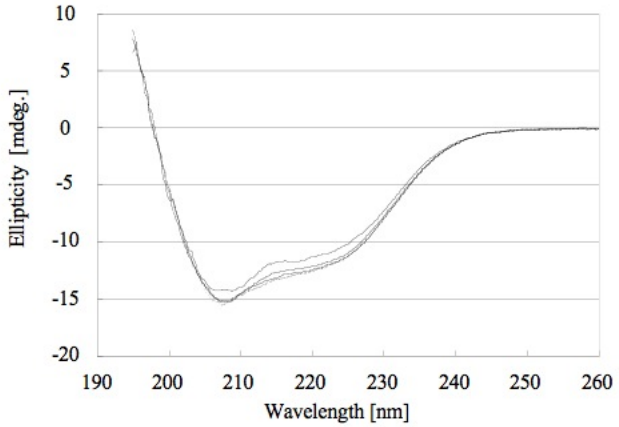

10
5
0
-5
-10
-15
-20
Ellipticity [mdeg.]
190
200
210
220
230
240
250
260
Wavelength [nm]

Fig. 3

Table 1 Metal ion binding abilities of Vanabin2

	Muximum binding number	Kd[μM]
pH4.5 VO^{2+}	4.9	9.3×10^{-5}
pH7.5 VO^{2+}	20.2	2.3×10^{-5}
pH4.5 Cu^{2+}	4.5	7.0×10^{-5}
pH7.5 Cu^{2+}	3.2	6.4×10^{-5}

Table 1

Table 2 Chemical properties of first transition metal ions

Metal ions	Stability constants with EDTA	HSAB principle	Ionic radii [Å]
VO^{2+}	18.0	Hard	0.72
Fe^{3+}	25.1	Hard	0.79
Co^{2+}	16.3	Medium	0.89
Ni^{2+}	18.6	Medium	0.83
Cu^{2+}	18.8	Medium	0.87
Zn^{2+}	16.5	Medium	0.88

Table 2