Vanadium-binding proteins (Vanabins) from a vanadium-rich ascidian

Ascidia sydneiensis samea

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[1]The nucleotide and amino acid sequences reported in this paper have been entered in GenBank/DDBJ/EMBL under the accession number AB088203 and AB088204 for *vanabin1* and *vanabin2*, respectively.

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Abstract

Since the beginning of the last century, it has been known that ascidians accumulate high levels of a transition metal, vanadium, in their blood cells, although the mechanism for this curious biological function remains unknown. Recently, we identified three vanadium-binding proteins (vanabins), previously denoted as vanadium-associated proteins (Kanda et al., 1997), from the cytoplasm fraction of vanadium-containing blood cells (vanadocytes) of the vanadium-rich ascidian *Ascidia sydneiensis samea*. Here, we describe the cloning, expression, and analysis of the metal-binding ability of vanabins. Recombinant proteins of two independent but related vanabins, vanabin1 and vanabin2, bound to 10 and 20 vanadium(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively. The binding of vanadium(IV) to these vanabins was inhibited by the addition of copper(II) ions, but not by magnesium(II) or molybdate(VI) ions. Vanabins are the first proteins reported to show specific binding to vanadium ions; this should provide a clue to resolving the problem regarding the selective accumulation of vanadium in ascidians.

1. Introduction

Early last century, it was revealed that the blood cells of ascidians (tunicates, or sea squirts), especially those belonging to the class Ascidiacea in the suborder Phlebobranchia, accumulate extremely high levels of vanadium from seawater [1]. Simultaneously, it was discovered that a homogenate of their blood cells was extremely acidic [1-4]. These unusual phenomena have attracted the interdisciplinary attention of both biologists and chemists because of considerable interest in the possible role of vanadium in oxygen transport as a third possible prosthetic group in respiratory pigments in addition to iron and copper, and in part because of the strong interest in the extraordinarily high levels of vanadium never before reported in other

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organisms.

The other reason why much of the interest developed is because of ascidian phylogeny. Ascidians belong to the Chordata. Many researchers regard ascidians as a good "model" organism because molecular biology has revealed that ascidians have key chordate features in common with the vertebrates. Moreover, their genome size is relatively small (~160 million bases) and they possess fundamentally all of basic developmental genes. They are, therefore, one of model organisms for genome sciences [cf. 5]. Many scientists have been interested in the reason why ascidians have such unique nature to accumulate vanadium the more because ascidians are relatively close animal to the vertebrates (for review, see [6-8]).

Recently, we identified three vanadium-binding proteins (vanabins), originally called vanadium-associated proteins (VAPs), in the blood cells of *Ascidia sydneiensis samea* [9]. These vanabins were identified by a combination of anion exchange chromatography and atomic absorption spectrometry. They include at least two major proteins with apparent molecular weights of 12.5 and 15 kDa, and a minor 16-kDa protein. These proteins bound vanadium at a ratio of 16 vanadium ions per protein on average [9], suggesting that there is a specific interaction between these proteins and vanadium ions. By using a specific antibody, the proteins have been shown to be localized in the cytoplasm of vanadocytes [9, 10].

In this article, we report the cloning and metal binding analysis of 12.5- and 15-kDa vanabins. These proteins were rich in cysteine residues and the intervals between the cysteines were highly regular. We used recombinant proteins of these two independent but related vanabins, named as vanabin1 and vanabin2, for metal binding assay and revealed that vanabins can bind to multiple 10 or 20 vanadium(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively. Neither magnesium(II) nor molybdate(VI) ions inhibited the binding of vanadium(IV) ions to both vanabins, suggesting a specific interaction between vanabins and vanadium(IV) ions. Vanabins are the first proteins reported to show specific binding to vanadium ions; this should provide a clue to resolving the problem regarding the selective accumulation of

vanadium in ascidians.

2. Materials and methods

2.1. Cloning vanabin cDNA

Vanabins (12.5-, 15- and 16-kDa) were purified from blood cells of *Ascidia sydneiensis samea* as described [9]. The N-terminal partial amino acid sequences of the three vanabins were determined by the Edman degradation method [11]. The molecular weights of the vanabins were determined by the matrix assisted laser desorption/ionization (MALDI) method using Voyager DE (Perceptive Biosystems) with the help of Dr. Aimoto and Dr. Kawakami at the Institute for Protein Research, Osaka University.

cDNA library of blood cells – except for giant cells, which have an extremely acidic vacuole but do not contain vanadium – of the ascidian *A. sydneiensis samea* has been constructed in UniZap XR vector [12]. To clone cDNAs encoding vanabins, we first used an immunoscreening methods as previously described [12]. Briefly, a nitrocellulose membrane dipped in 10 mM IPTG was placed on the plaques for 3 hours at 37°C. After the membrane had been incubated in a buffer containing 1% bovine serum albumin, it was reacted with polyclonal antibodies against vanabins [9]. Positive plaques were re-screened until they were cloned. The DNA sequence was determined by dideoxy methods using ALFexpressII automated DNA sequencer (Amersham Pharmacia Biotech., Inc).

To clone the cDNAs encoding 15-kDa vanabin, we designed a set of nested degenerate primers (F1, 5'–GCN CCN GTN GAY NNN AAR GGN CA–3' for Ala–Pro–Val–Asp–Xxx–Lys–Gly–Gln; F2, 5'–AAR GGN CAR NNN GCN ACN CC–3' for

Lys–Gly–Gln–Xxx–Ala–Thr–Pro), since by immunoscreening we failed to identify cDNAs for

15- and 16-kDa vanabins. Phage DNA was extracted from the amplified cDNA library mentioned

above. One microgram of phage DNA was used as the template for a 3'-RACE reaction with 200

pmole F1 and 20 pmole M13 primers. One microliter of the 50-microliter reaction product was

used as the template for PCR with 200 pmole F2 and 20 pmole M13 primers. The PCR products

were purified and cloned into pBluescript vector. The cDNA fragment was used as a probe to

screen full-length cDNAs from the cDNA library.

2.2. Recombinant Protein Expression

The cDNA regions corresponding to putative mature vanabins were amplified by PCR

using specific primer sets with artificial restriction sites as follows: 12F2, 5'-GGA ATT CGG CCC

AGG CTG CAA A-3'; 12R2, 5'-CGT CGA CTC ACA CAC AAT TCA A-3'; 15F2, 5'-GGA ATT

CGC TCC GGT GGA TTG C-3'; 15R2, 5'-CGT CGA CTC ACT TGC AGT TTG TC-3'. 12F2

and 12R2 were used for the 12.5-kDa vanabin, while 15F2 and 15R2 were used for the 15-kDa

vanabin. The amplified fragments were digested with *Eco*RI and *Sal*I and ligated into the

corresponding site of pMAL-c expression vector (New England BioLabs Inc.). This vector has a

lac promoter and a coding region for *E. coli* maltose binding protein (MBP), to which the vanabin

coding region was ligated to produce a fusion protein. The plasmid was introduced into *E. coli*

TB1 strain. An overnight culture of non-induced *E. coli* cells bearing vanabin-expressing

plasmids was diluted 1:10 in LB medium containing 50 μg/ml ampicillin. IPTG (0.5 mM) was

added and the cells were grown at 37°C for 6 hours. The fusion protein was purified by amylose

resin column chromatography according to the manufacturer's protocol (New England BioLabs

Inc.). The junction region between MBP and vanabin was cut using Factor Xa, and the vanabin

was purified by DEAE–Sephacel anion exchange column chromatography (Amersham

Pharmacia Biotech Inc.). The resulting recombinant protein had four additional amino acids

(I-S-E-F) derived from the junction region at the N-terminal. The protein concentration was measured with a BioRad protein assay reagent (BioRad Laboratories Inc.) using bovine serum albumin (Pierce Inc.) as a standard.

2.3. Metal Binding Assay

All buffers were prepared from deionized water and ultrapure-grade reagents, and degassed for 10 min under vacuum before use. Vanadyl sulfate (vanadium(IV); $VOSO_4 \cdot nH_2O$, n=3–4), sodium orthovanadate (vanadium(V); Na_3VO_4), magnesium chloride (magnesium(II); $MgCl_2 \cdot 6H_2O$), cupric chloride (copper(II); $CuCl_2 \cdot 2H_2O$), and sodium molybdate (molybdate(VI); $Na_2MoO_4 \cdot 2H_2O$) were from Wako Chemical Inc. (Japan). Vanadyl sulfate was dissolved in water and mixed with an equal molar ratio of iminodiacetic acid. Sodium orthovanadate was dissolved in water at 10 mM, and the pH was adjusted to 7.0 by adding hydrogen chloride. The resulting yellow solution was stored at 37°C until it became colorless.

The Hummel-Dreyer method was used to determine the metal-binding ability of the recombinant vanabins [13]. A gel filtration column (bed size, 7 mmΦ × 190 mm) filled with Biogel P–6 DG resin (Bio-Rad Laboratories Inc.), which consists of polyacrylamide beads, was equilibrated with a binding buffer (100 mM NaCl, 10 mM Tris-HCl, pH7.4) containing the desired concentration of metal ions. Proteins were loaded onto the column and separated at a flow rate of 0.3 ml/min. Under these conditions, a protein peak appeared around 5 min after loading. The metal concentration in each fraction was determined by atomic absorption spectrophotometry (AAS, SAS–7500, Seiko Instruments Inc.), and the protein concentration was determined with BioRad reagent (Bio-Rad Laboratories Inc.). To analyze vanadium by AAS, 10 μl of each sample, diluted if necessary, was placed on a tungsten board, which was heated at 95°C for 30 seconds, then from 95°C to 900°C in 10 seconds, at 900°C for 10 seconds, at 1,600°C for 3 seconds, and at 2,700°C for 3 seconds. The molar ratio of metal per protein was calculated for the fraction with

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the protein peak. The results were analyzed by Scatchard plot [14] and statistical significance was assessed using Student's two-tailed *t*-test.

3. Results

3.1. Isolation of vanabin cDNAs

In this study, we first determined the N-terminal amino acid sequence of the three vanabins in order to clarify their molecular nature. The N-terminal amino acids were almost identical in the 15- and 16-kDa vanabins (15-kDa vanabin; A-P-V-D-X-K-G-Q-X-(A/T)-T-P-X-E-P-L-K-A-A-K-K-K-X-A-E-S, 16-kDa vanabin; A-P-V-D-X-K-G-Q-X-T-T-P-X-E-P-L-T-A-X-K), suggesting that they are encoded by the same gene or by very similar genes. The other vanabin, the 12.5-kDa vanabin, had a different N-terminal sequence (G-P-G-X-K-X-Q-S-V-X-G-E-V-K-K-X-G-V-K-K-F-R-S-X-N-X-D-R-D-X-T-K-D-X-A-K-A-K-X-G-K-V-P-N-A-G-D-X-X-H-X-M-L). We first tried to isolate cDNAs for the three vanabins by immunoscreening with polyclonal antibodies that recognize the three vanabins, and succeeded to clone cDNAs for 12.5-kDa vanabin. For the cloning of cDNAs for 15-kDa vanabins, we used polymerase chain reaction (PCR) method using degenerate primers corresponding to the N-terminal sequences of the vanabin. The amino acid sequences deduced from the cDNAs of both the 12.5- and 15-kDa vanabins had an N-terminal hydrophobic region, which may function as a signal sequence for protein translocation and seems to be processed after translation (Fig. 1). In both vanabins, the hydrophobic region is followed by amino acid sequence corresponding to that determined by Edman degradation method. Here, we call the region from the N-terminal of the vanabins purified from blood cells to C-terminals deduced from their cDNAs the "mature" protein region. The molecular weights of both the 12.5- and 15-kDa vanabins purified from

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vanadocytes determined by MALDI-TOF MASS was identical to that of the mature protein sequence deduced from their respective cDNAs. Therefore, we concluded that these cDNAs encode the 12.5- and 15-kDa vanabins, and we called the respective genes encoding the 12.5- and 15-kDa vanabins *vanabin1* and *vanabin2*. We have not yet identified the gene encoding the 16-kDa vanabin.

The deduced amino acid sequences of the vanabins were highly conserved (Fig. 1). Most strikingly, both were rich in cysteine residues (18/87 and 18/91 for mature vanabin1 and vanabin2, respectively) and the intervals between the cysteines were highly regular. Homology search using both sequences by a BLASTP program against public protein databases did not show any proteins with a striking similarity; the scores did not exceed 40, and the E values were no less than 10^{-4}. Although we know that metallothioneins are also rich in cysteines, and that they bind to divalent metal cations, the repetitive pattern of cysteine residues differs between metallothioneins and vanabins. The conserved motif of vanabins can be described as the consensus sequence {C}-{X$_{2-5}$}-{C}. Vanabins are rich in charged residues, such as arginine (3/87 and 5/91), aspartate (6/87 and 6/91), glutamate (2/87 and 7/91), and lysine (12/87 and 14/91), while metallothioneins are rich in serine and lysine. In addition, analysis of expression sequence tags (ESTs) in the *A. sydneiensis samea* blood cell cDNA library has already identified two cDNAs that encode proteins that are closely related to vanabins and one cDNA encoding metallothionein-like protein (unpublished data). These results suggest that vanabins compose a unique gene family.

3.2. Metal binding ability of vanabins

To examine the metal-binding ability of these vanabins, we produced recombinant proteins in *E. coli*. Using these proteins, we applied the Hummel-Dreyer method [13] to

Fig. 1

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determine the ratio of vanadium atoms per protein molecule at various concentrations of free

vanadium(IV)(VO^{2+}) and vanadium(V)($[VO_4]^{3-}$) ions. We used vanadium(IV) ions coordinated

by iminodiacetic acid (IDA), because free vanadium(IV) ions precipitate at neutral pH. IDA was

chosen because it does not bind too strongly to exclude the coordination of proteins [15]. Since

both vanadium(V) and vanadium(IV) ions were somehow absorbed by dextran- or agarose-based

column beads, we did not use these columns for Hummel-Dreyer's method (data not shown). This

does not mean vanadium ions bound to vanabins are taken off by the resin. The only resin suitable

for this method was Bio-Gel P resin (Bio-Rad Laboratories, USA), which is prepared by

polymerizing acrylamide and *N, N′*-methylene-bis-acrylamide. Neither vanadium(V) nor

vanadium(IV) ions are absorbed by this resin (data not shown).

A metal binding assay was done in Tris-HCl buffer at pH 7.4 containing 100 mM NaCl,

which prevents non-specific ionic interaction between proteins and vanadium ions. Consequently, Fig. 2

vanabin1 was revealed to bind to 10 vanadium(IV) ions per protein (Fig. 2). The apparent

dissociation constant was 2.1×10^{-5} M. By contrast, vanabin2 bound to 20 vanadium(IV) ions per

protein for a dissociation constant of 2.3×10^{-5} M. The dissociation constants of these vanabins

are quite similar, although vanabin2 has twice as many apparent binding sites as vanabin1. The

difference might be due to the difference in the amino acid composition of these two vanabins.

The most striking difference was found for glutamate, which can coordinate to vanadium.

Vanabin2 contains seven glutamate residues, while vanabin1 only contains two. Conversely,

vanabin1 contains eleven glycine residues, while vanabin2 contains only one.

We also analyzed the binding of vanadium(V) ions to these vanabins. Vanadium(V) ions

seem to interact with both vanabins, but we could not determine the binding constant or the

number of binding sites. The results were irreproducible, probably due to the weak binding of

vanadium(V) ions to vanabins or to the complex behavior of vanadium(V) ions in aqueous

solution, which includes the effects of protonation, oligomerization, and interaction with buffers

[16, 17]. We did not examine binding of vanadium(III) to vanabins because vanadium(III) ions

are soluble only under the strongly acidic conditions below pH 2.2 unless any ligands participate in the stabilization of vanadium(III) ions [18].

3.3. Metal selectivity of vanabins

We then performed competition experiments in order to determine metal selectivity. We analyzed three metal ions: magnesium(II), molybdate(VI), and copper(II). Magnesium(II) was chosen for its structural and functional similarity to vanadium(IV). Vanadium(IV) ions mimic the structural interactions of magnesium(II) with nucleotides [19, 20], and vanadium(IV) ions can substitute at the magnesium(II) binding site of pyruvate kinase and xylose isomerase [21, 22]. Molybdate(VI) was chosen because molybdate acts as a structural mimic of vanadium(V) ions, that is, as a potent inhibitor of phosphate-metabolizing enzymes. For example, both vanadium(V) and molybdate inhibit *E. coli* alkaline phosphatase [23]. Oligomeric vanadium(V), tungsten(VI), and molybdate(VI) ions all inhibit glycogen phosphorylase by competing against glucose-1-phosphate [24]. The third metal ion chosen was copper(II), since vanabins contain 18 cysteine residues and appear to bind to copper(II) ions, as do metallothioneins.

Neither magnesium(II) nor molybdate(VI) ions inhibited the binding of vanadium(IV)

Fig.

ions to both vanabins (Fig. 3), suggesting a specific interaction between vanabins and vanadium ions. On the contrary, the binding of vanadium(IV) ions was significantly inhibited by a 10 molar excess of copper(II) ions (Fig. 3). Our preliminary analysis indicated that vanabin2 bound to approximately four copper(II) ions. One possible reason for the inhibition by the copper(II) ion is that vanadium(IV) and copper(II) ions bind to the same binding sites and compete with each other. This is likely to occur, since the preferable coordination geometries of the vanadium(IV) and copper(II) ions are similar, while those of other metals differ. The vanadium(IV) ion usually adopts a square pyramidal structure with an oxo ligand at the apical position. Thus, square planar

coordination sites are available for the coordination of vanabins. The copper(II) ion prefers a square planar coordination, while other metal ions prefer other coordination geometries, such as an octahedron. In other words, vanabins afford coordination sites favorable for square planar coordination. Another possible reason is that copper(II) ions bind to different sites and may cause a structural change affecting the binding sites for vanadium(IV) ions. We intend to examine the three-dimensional structure of vanabins, as this may provide insight into the effect of copper(II) ions. Since vanadium nitrogenases have Fe-S clusters coordinated with vanadium(V) as an active center [25], it is possible that iron(III) ions bind to vanabins in cooperation with vanadium(V) ions. We tested whether iron(III) ions bound to vanabin2 alone, but they did not (data not shown). Combinatorial binding of vanadium and other ions, including iron, to vanabins needs to be analyzed.

4. Discussion

In this article, we first report the cloning of cDNAs encoding 12.5- and 15-kDa vanabins (vanabin1 and vanabin2, respectively) from an vanadium-accumulating ascidian *Ascidia sydneiensis samea*. The mature protein deduced from the cDNAs were rich in cysteine residues, whose repetitive patterns were highly conserved between the two vanabins and were distinct from known cysteine-rich proteins such as metallothioneins. As far as we know for the organisms other than *A. sydneiensis samea*, vanabin-like proteins have only been identified in other vanadium-accumulating ascidian species *A. ahodori* and *Ciona intestinalis* [10] and in a polycheate worm *Pseudopotamilla occelata* which accumulate high levels of vanadium in its fan epithelial cells [26]. It is possible that vanabins only exist in vanadium-accumulating organisms.

We then revealed that vanabin1 and vanabin2 can bind to multiple 10 and 20 vanadium(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively.

These values are comparable to those of a nickel chaperone protein UreE (1.0×10^{-5} M for Ni^{2+}) that assist the insertion of Ni^{2+} in the active site of urease [27], copper-binding site of Menkes protein (4.5×10^{-5} M for Cu^{2+}) [28], or periplasmic molybdate-binding protein ModA (3×10^{-6} M for molybdate and 5×10^{-6} M for tungstate) [29]. The number of binding sites of recombinant vanabins can account for the ratio of vanadium per vanabins purified from blood cells (16:1).

In competition experiments, neither magnesium(II) nor molybdate(VI) ions inhibited the binding of vanadium(IV) ions to both vanabins (Fig. 3), suggesting a specific interaction between vanabins and vanadium(IV) ions. While the overall amino acid sequences of vanabins remind us of the binding of divalent soft metal ions to sulfhydryl residues of cysteine-rich metal-binding proteins, such as metallothioneins, our preliminary studies suggest that vanabins do not use cysteine residues to bind vanadium ions. Electron spin resonance (ESR) studies indicate that vanadium(IV) ions do not interact with sulfur of cysteine residues but interact with nitrogen and oxygen (unpublished data). Supporting data have been obtained from mass spectrometry that indicate all the eighteen cysteine residues of vanabin2 make intramolecular disulfide bonding in vitro (unpublished data). In addition, no significant difference of circular dichroism measurement was observed between vanadium-free and vanadium-loaded vanabin2. Taken together, vanabins seem to make a new family of metal binding protein. Structural analysis of vanabin2 as well as vanabin1 is now in progress.

Since vanabins are localized in the cytoplasm of vanadocytes [10] and can bind to vanadium ions in the +4 oxidation state (vanadium(IV)) at binding constant of $\sim 2 \times 10^{-5}$ M, vanabins might function as metal chaperone proteins in the cytoplasm rather than proteins for metal storage or detoxification (Fig. 4). Vanadium is dissolved in the +5 oxidation state (vanadium(V)) in the natural environment, such as in seawater [30]. Vanadium(V) might be taken up by ascidians via the branchial sac, stomach or intestine, and transferred into the coelomic fluid (blood). In the coelomic fluid, vanadium ions might be captured by a carrier protein, such as a vanabin-like protein, or a transferrin-like protein that we have already identified (unpublished

data). Most of the vanadium ions in the vanadocytes are reduced to vanadium(IV) and transferred to vacuoles, where vanadium(IV) ions are further reduced to vanadium(III) and stored, although some ions remain as vanadium(IV) in the cytoplasm [31-38].

Previously, we showed that vanadocytes express several enzymes in the pentose phosphate pathway, which produces the reducing agent NADPH, and that NADPH can reduce vanadium(V) ions to vanadium(IV) ions [12, 39-42]. Therefore, we postulate that vanadium(V) ions are transferred across the cytoplasmic membrane by a metal transporter. Since the cytoplasm of vanadocytes is not acidic and free vanadium(IV) ions readily precipitate at neutral or higher pH, we propose that vanabins function as cytoplasmic carrier proteins for vanadium(IV) ions, like a copper chaperone for superoxide dismutase (SOD) [43]. Vanabins may also chelate vanadium(V) and help NADPH reduce vanadium(V) to vanadium(IV), which is to be examined in our future study. The reduction of vanadium(V) becomes more favorable when a ligand binds more strongly to vanadium(IV) than to vanadium(V) [42], and peptides and proteins bind more strongly to d^1 vanadium(IV) ions than to d^0 vanadium(V) ions [44]. Next, vanadium(IV) ions are probably transported into the vacuole by a metal transporter and reduced to vanadium(III) by an unknown mechanism. The vacuoles of vanadocytes are extremely acidic, and high concentrations of protons and sulfate ions coexist with vanadium(III) ions [38, 45, 46]. We have already identified and cloned several subunits of a vacuolar-type proton ATPase (V-ATPase) localized in vanadocytes, and have shown that V-ATPase functions to accumulate protons in the vacuoles of vanadocytes [47-49]. The identification of vanabins that bind vanadium(IV) ions specifically links the pathway of vanadium transfer from the cytoplasmic membrane to the vacuolar membrane, and provides insight into the reduction mechanism of vanadium(V) to vanadium(IV).

Ions of heavy metals such as iron, copper, zinc, cobalt, or nickel are essential micronutrients, required for function of a large number of proteins. However, neither the accumulation mechanism of the ions by living organisms nor the trafficking mechanisms to target proteins have been completely revealed because of their low concentrations in living organisms.

Fig. 4

Attempts to characterize the unusual phenomenon shown in ascidians can be, therefore, expected to promote more information not only about the accumulation mechanism of vanadium by one class of marine organisms but also about the accumulation and trafficking of metal ions by almost all living organisms, although the physiological roles of vanadium remain to be explained.

Acknowledgements

References

[1] M. Henze, Hoppe-Seyler's Z. Physiol. Chem. 72 (1911) 494-501.

[2] M. Henze, Hoppe-Seyler's Z. Physiol. Chem. 79 (1912) 215-228.

[3] M. Henze, Hoppe-Seyler's Z. Physiol. Chem. 88 (1913) 345-346.

[4] M. Henze, Hoppe-Seyler's Z. Physiol. Chem. 213 (1932) 125-135.

[5] E. Pennisi, Science 296 (2002) 1792-1795.

[6] H. Michibata, T. Uyama, T. Ueki and K. Kanaomori, The mechanism of accumulation and reduction of vanadium by ascidians, in H. Sawada, H. Yokosawa and C. C. Lambert (Eds.),

The Biology of Ascidian, Springer-Verlag, Tokyo, 2001, pp. 366-373.

[7] H. Michibata, T. Uyama, T. Ueki and K. Kanamori, Microscop. Res. Technol. 56 (2002) 421-434.

[8] H. Michibata, N. Yamaguchi, T. Uyama and T. Ueki, Coord. Chem. Rev. (2002) in press.

[9] T. Kanda, Y. Nose, J. Wuchiyama, T. Uyama, Y. Moriyama and H. Michibata, Zool. Sci. 14 (1997) 37-42.

[10] J. Wuchiyama, Y. Nose, T. Uyama and H. Michibata, Zool. Sci. 14 (1997) 409-414.

[11] P. Edman and G. Begg, Eur. J. Biochem. 1 (1967) 80-91.

[12] T. Uyama, T. Kinoshita, H. Takahashi, N. Satoh, K. Kanamori and H. Michibata, J. Biochem. 124 (1998) 377-382.

[13] J. P. Hummel and W. J. Dreyer, Biochim. Biophys. Acta 63 (1962) 530-532.

[14] G. Scatchard, Ann. N. Y. Acad. Sci. 51 (1949) 660-672.

[15] D. Sanna, I. Bodi, S. Bouhsina, G. Micera and T. Kiss, J. Chem. Soc. Dalton Trans. (1999) 3275-3282.

[16] A. S. Tracey and M. J. Gresser, Inorg. Chem. 27 (1988) 1269-1275.

[17] D. Crans, Comments Inorg. Chem. 16 (1994) 1-33.

[18] L. V. Boas and J. C. Pessoa, Vanadium, in G. Sir Wilkinson (Ed.), Comprehensive Coordination Chemistry, Pergamon Press, Oxford, 1987, pp. 453-583.

[19] D. Mustafi, J. Telser and M. W. Makinen, J. Am. Chem. Soc. 114 (1992) 6219-6226.

[20] F. S. Jiang and M. W. Makinen, Inorg. Chem. 34 (1995) 1736-1744.

[21] K. Lord, .A. and G. H. Reed, Arch. Biochem. Biophys. 281 (1990) 124-131.

[22] R. Bogumil, J. Hütterman, R. Kappel, R. Stabler, C. Sudfeldt and H. Witzel, Eur. J. Biochem. 196 (1991) 305-312.

[23] P. J. Stankiewicz and M. J. Gresser, Biochemistry 27 (1988) 206-212.

[24] G. Soman, Y. C. Chang and D. J. Graves, Biochemistry 22 (1983) 4994-5000.

[25] J. Chen, J. Christiansen, R. C. Tittsworth, B. J. Hales, S. J. George, D. Cocuvanis and S. P.

Cramer, J. Am. Chem. Soc. 115 (1993) 5509-5514.

[26] T. Uyama, Nose, Y., Wuchiyama, J., Moriyama, Y., and Michibata, H., Zool. Sci. 14 (1997) 43-47.

[27] M. Y. Lee, H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, D. W. Christianson and R. P. Hausinger, Protein Sci. 2 (1993) 1042-1052.

[28] P. Y. Jensen, N. Bonander, N. Horn, Z. Tümer and O. Farver, Eur. J. Biochem. 264 (1999) 890-896.

[29] S. Rech, C. Wolin and R. P. Gunsalus, J. Biol. Chem. 271 (1996) 2557-2562.

[30] G. C. McLeod, K. V. Ladd, K. Kustin. and D. L. Toppen, Limnol. Oceanogr. 20 (1975) 491-493.

[31] R. M. K. Carlson, Proc. Natl. Acad. Sci. USA 72 (1975) 2217-2221.

[32] T. D. Tullius, W. O. Gillum, R. M. K. Carlson and K. O. Hodgson, J. Am. Chem. Soc. 102 (1980) 5670-5676.

[33] A. L. Dingley, K. Kustin, I. G. Macara and G. C. McLeod, Biochim. Biophys. Acta 649 (1981) 493-502.

[34] P. Frank, R. M. K. Carson and K. O. Hodgson, Inorg. Chem. 25 (1986) 470-478.

[35] S. Lee, K. Kustin, W. E. Robinson, R. B. Frankel and K. Spartalian, J. Inorg. Biochem. 33 (1988) 183-92.

[36] S. G. Brand, N. Edelstein, C. J. Hawkins, G. Shalimoff, M. K. Snow and E. R. T. Tiekink, Inorg. Chem. 29 (1989) 434-438.

[37] J. Hirata and H. Michibata, J. Exp. Zool. 257 (1991) 160-165.

[38] T. Ueki, K. Takemoto, B. Fayard, M. Salomé, A. Yamamoto, H. Kihara, J. Susini, S. Scippa, T. Uyama and H. Michibata, Zool. Sci. 19 (2002) 27-35.

[39] T. Uyama, Yamamoto, K., Kanamori, K. and Michibata, H., Zool. Sci. 15 (1998) 441-446.

[40] T. Uyama, T. Ueki, Y. Suhama, K. Kanamori and H. Michibata, Zool. Sci. 15 (1998) 815-821.

[41] T. Ueki, T. Uyama, K. Yamamoto, K. Kanamori and H. Michibata, Biochim. Biophys. Acta

1494 (2000) 83-90.

[42] K. Kanamori, M. Sakurai, T. Kinoshita, T. Uyama, T. Ueki and H. Michibata, J. Inorg. Biochem. 77 (1999) 157-161.

[43] T. D. Rae, P. J. Schmidt, R. A. Pufahl, V. C. Culotta and T. V. O'Halloran, Science 284 (1999) 805-808.

[44] D. Shriver and P. Atkins, Inorganic Chemistry, 3rd ed., New York, 1999.

[45] H. Michibata, Y. Iwata and J. Hirata, J. Exp. Zool. 257 (1991) 306-313.

[46] K. Kanamori and H. Michibata, J. Mar. Biol. Ass. U. K. 74 (1994) 279-286.

[47] T. Uyama, Y. Moriyama, M. Futai and H. Michibata, J. Exp. Zool. 270 (1994) 148-154.

[48] T. Ueki, T. Uyama, K. Kanamori and H. Michibata, Zool. Sci. 15 (1998) 823-829.

[49] T. Ueki, T. Uyama, K. Kanamori and H. Michibata, Mar. Biotechnol. 3 (2001) 316-321.

Figure legends

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vanabin1  mvskftillgvvvlmalsvnayesefddetfekGPGCKCQSVCGEVKKCG
vanabin2  m-skvifalvlvvvlvacinatyvefeeayAPVDCKGQCTTPCEPLTACK
consensus m-sk----l--vv------na----------------C---C-----C-

vanabin1  VKCFRSCNGDRD---CTKDCAKAKCGKVPNAGDCGHCMLSCEGKCRADHC
vanabin2  EKCAESCETSADKKTCRRNCKKADCE--PQDKVCDACRMKCHKACRAANC
consensus -KC---C----D---C---C-KA-C---P----C--C---C---CRA--C

vanabin1  ASACPGKVSKAPGCLDCMKLNCV
vanabin2  ASECPKHEHKSDTCRACMKTNCK
consensus AS-CP----K---C--CMK-NC-
```

Fig. 1. Amino acid sequences of vanabin1 and vanabin2 deduced from their cDNA clones. The consensus sequence is shown below the sequences. Conserved cysteine residues are shown in bold. The N-terminal partial sequences determined by the Edman degradation method are shaded. The arrows indicate the N-terminal of putative mature vanabins.
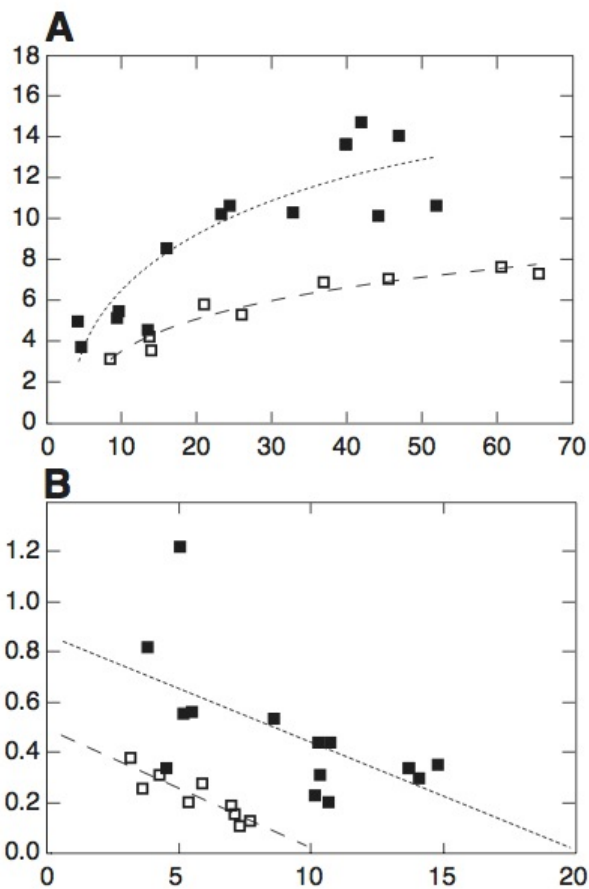

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Fig. 2. Binding of vanabins to vanadium(IV) ions determined by the Hummel-Dreyer method.

(A), Relationship between the concentration of free vanadium ions (horizontal axis, μM) and the

ratio of bound vanadium per protein (vertical axis, mol/mol). The proteins used were

recombinant vanabin1 (open rectangles) or vanabin2 (filled rectangles). Logarithmic fitting of

the data is shown for vanabin1 (dashed line) and vanabin2 (dotted line). (B), Scatchard plot of the

results shown in (A). The horizontal axis indicates the molar ratio of vanadium per protein, and

the vertical axis indicates the ratio per concentration of free vanadium ions. Linear fitting of the

data is shown for vanabin1 (dashed line) and vanabin2 (dotted line).
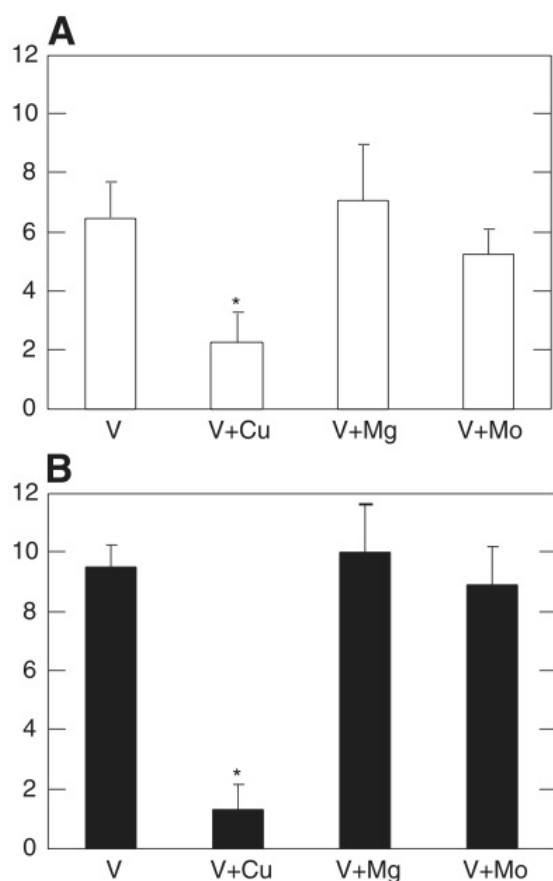

Fig. 3. Inhibition of binding of vanabins to vanadium(IV) ions by excess amounts of other

transition metals. Vanabin1 (A) or vanabin2 (B) was loaded on a gel equilibrated in buffer

containing 50 μM vanadium(IV)-IDA without other metal ions (V) or with 500 μM of $CuCl_2$

(V+Cu), $MgCl_2$ (V+Mg), or Na_2MoO_4 (V+Mo). The vertical axis indicates the ratio of bound

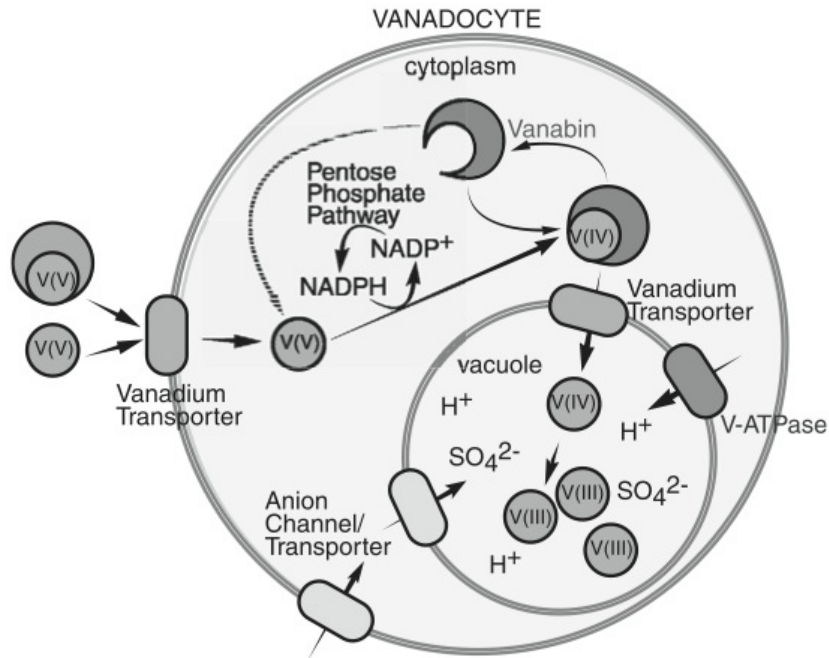vanadium per protein (mol/mol) as the mean $\pm$ S.E. (n=7-15). *P<0.05.

Fig. 4. A model of the pathway for the reduction and accumulation of vanadium in ascidian vanadocytes. The vanadium taken into the vanadocytes is bound by vanabins. The pentose phosphate pathway exists in vanadocytes and functions to generate NADPH, which reduces V(V) to V(IV) with the assistance of vanabins or other chelators. The V(IV) ions are transported into vacuoles and reduced to V(III) by unknown reductant(s). The vacuoles have extremely high levels of H^+ and SO_4^{2-}. Vacuolar-type H^+-ATPase (V-ATPase) accumulates protons in the vacuoles. Metal transporters and anion transporters localize on the plasma and vacuolar membranes.