Sequence variation of Vanabin2-like vanadium-binding proteins in blood cells of the vanadium-accumulating ascidian *Ascidia sydneiensis samea*

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Abstract

The blood cells of ascidians accumulate extremely high levels of the transition metal vanadium. We previously isolated four vanadium-binding proteins (Vanabins 1–4) and a homologous protein (VanabinP) from the vanadium-rich ascidian *Ascidia sydneiensis samea*. In the present study, we identified cDNAs encoding five different Vanabin2-related proteins in *A. sydneiensis samea* blood cells. It was notable that the sequences of the encoded proteins vary from that of Vanabin2 at up to 14 specific positions, while both the polypeptide length and the 18 cysteine residues were completely conserved. The most divergent protein, named 14MT, differed from Vanabin2 at all 14 positions. Using immobilized metal-ion affinity chromatography, we found that Vanabin2 and 14MT have the same metal ion selectivity, but the overall affinity of 14MT for VO^{2+} is higher than that of Vanabin2. Binding number for VO^{2+} ions was same between Vanabin2 and 14MT as assessed by gel filtration. These results suggested that sequence variations were under strict evolutionary constraints and high affinity binding sites for VO^{2+} are conserved among Vanabin2 variants.

1. Introduction

The ascidians (also known as sea squirts), especially those of the class Ascidiidae, have long been known to accumulate extremely high levels of vanadium in specialized vanadium-accumulating blood cells called vanadocytes [1-3]. The concentration of vanadium depends on species and tissues. Species belonging to suborder Phlebobranchia are revealed to accumulate higher levels of vanadium in their blood cells, and the highest concentration of vanadium (350 mM) was found in the blood cells of *Ascidia gemmata*, a 10^7-fold increase over its concentration in seawater (35 nM) [4, 5]. This remarkable ability to concentrate vanadium is thought to result in the highest metal accumulation factor of any living organism. During the accumulation process, vanadium in the +5 oxidation state (HVO_4^{3-} or $H_2VO_4^{2-}$ at physiological condition; V^V) is reduced to +3 oxidation state (V^{3+}; V^{III}) via +4 oxidation state (VO^{2+}; V^{IV}) [6]. NADPH is a strong reductant of V^V to V^{IV} as shown by the fact that enzymes for the pentose phosphate pathway are exclusively expressed in the cytoplasm of vanadocytes [7–10], and detailed *in vitro* studies suggest that chelating agents are necessary for this reaction [11, 12].

Vanadium-binding proteins (Vanabins) must function as metallochaperones or chelating agents in the accumulation and reduction of vanadium. We have isolated several vanadium-binding proteins and related proteins from the vanadium-rich ascidian *Ascidia sydneiensis samea* [13–19], which accumulate 12.8 mM vanadium in blood cells [5]. Among them, Vanabin family is remarkable for several reasons. First, Vanabins share a conserved amino acid motif described by the consensus sequence {C}-{$X_{2–5}$}-{C} (C: cysteine, X: any amino acids except for cysteine). Second, they bind VO^{2+}. Third, nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) studies have

shown that Vanabin2 has a novel bow-shaped conformation consisting of four α-helices connected by nine disulfide bonds [21], and VO^{2+} is mostly coordinated to side-chain nitrogen atoms of amino acids such as lysines and arginines [22]. Fourth, our recent studies have shown that Vanabin2 selectively binds to VO^{2+}, Fe^{3+}, and Cu^{2+}, but not to Co^{2+}, Ni^{2+}, or Zn^{2+} [23].

During the search for novel vanadium-binding proteins, we identified several variants of Vanabin2 from blood cells of *A. sydneiensis samea*. We extensively examined the sequence variation and identified five variants of Vanabin2 in this study. The lengths of the open reading frames of these variants were identical to that of Vanabin2, although the amino acid sequences encoded by the cDNA clones differed from the Vanabin2 sequence at 1, 9, 10, or 14 positions. All of the 18 cysteine residues are conserved. We designated these cDNA clones as 1MT, 9MT, 10MT, and 14MT, respectively. In addition, 10MT is divided into two, 10MT-1 and 10MT-2, according to the 16th amino acid residue. By immobilized metal-ion affinity chromatography (IMAC), we found that that Vanabin2 and 14MT have the same metal selectivity. Regardless of the difference of number of lysine residues, 14MT and Vanabin2 bound a similar number of VO^{2+} ions by a gel filtration, suggesting that high affinity binding sites for VO^{2+} ions are conserved.

2. Materials and methods

2.1 Preparation of blood-cell proteins

Specimens of adult *A. sydneiensis samea* were collected near the International Coastal Research Center of the Marine Research Institute, University of Tokyo, Otsuchi, Iwate, Japan. Their blood cells were extracted, suspended in artificial seawater

(CMFASW; 460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 5 mM HEPES, 6 mM $NaHCO_3$, 5 mM EDTA, pH 7.0), and centrifuged at 300 × *g* for 10 min at 4°C. The supernatant fraction including the blood plasma was removed, and the cell pellet was resuspended in CMFASW containing 0.2 M sucrose and 20 mM MOPS (pH 7.0). After centrifugation at 150 × *g* for 20 min at 4°C, giant cells floating on the surface were removed. The cell pellet, which contained vanadocytes, was washed with CMFASW, collected by centrifugation at 300 × *g* for 10 min at 4°C, and homogenized in 9 volumes (vol/wt) of 200 mM Tris-HCl (pH 8.0). Soluble proteins were separated by successive 10-min centrifugations at 1,000, 10,000, and 100,000 × *g* at 4°C and then dialyzed against 20 mM NaH_2PO_4 (pH 7.4).

2.2 Purification of vanadium-binding proteins

All buffers were prepared from deionized water and ultrapure-grade reagents and degassed for 10 min under vacuum before use. Vanadyl sulfate (V^{IV}; $VOSO_4 \cdot nH_2O$, n=3–4) was purchased from Wako Pure Chemical Industries Inc. Vanadyl sulfate was dissolved at 0.1 M in distilled water immediately before use. Chelating Sepharose-FF resin (GE Healthcare) was packed in a column (18 × 19 mm), washed with water, and charged with VO^{2+} by loading 0.1 M $VOSO_4$ solution at 1.7 ml/min. The resin was washed with distilled water and then equilibrated with binding buffer (20 mM NaH_2PO_4, pH 7.4). After the soluble blood-cell proteins were loaded onto the column, the column was washed with the binding buffer, and absorbed proteins were eluted with a stepwise gradient of NaCl (100, 200, 400, and 500 mM) in binding buffer. Finally, all proteins, as well as the VO^{2+} ions, were eluted using 50 mM EDTA in binding buffer.

The eluted fractions were concentrated by ultrafiltration using Centriplus YM-3

devices (Millipore), and their constituent proteins were further separated by reverse-phase HPLC on a 5Ph-AR300 column (4.6 × 150 mm, Nakalai Tesque). The column was run at 1.0 ml/min with a linear gradient of 10–70% acetonitrile over 60 min. N-terminal amino-acid sequencing was performed using Edman degradation [24].

2.3 Isolation of cDNAs for vanadium-binding proteins

Of the partial N-terminal amino acid sequences derived from the eluted protein peaks, five protein peaks with strong homology to Vanabin2 were identified. Four types of degenerate primers were designed based on the N-terminal sequences of these five proteins. One of the four primers, P-V-D-C-K-G/f, had a sequence common to all five proteins [5′ -CCN GTN GAY TGY AAR GG- 3′ for Pro-Val-Asp-Cys-Lys-Gly]. The other primers were peak5/f (5′- TGY CAN CAN GAR TGY GG-3′ for Cys-Tyr-Tyr-Glu-Cys-Gly), peak6/f (5′- TGY AAR GGN CAR CAR GC-3′ for Cys-Lys-Gly-Gln-Gln-Ala), and peak8/f (5′- GGN CAR TGY CAN GCN AC-3′ for Gly-Gln-Cys-Tyr-Ala-Tyr).

Using a cDNA library of *A. sydneiensis samea* blood cells (with giant cells extracted) constructed with UniZap XP vector [8] as the template, 3′ RACE was performed with the degenerate primers and a T7 primer using rTaq DNA polymerase (TaKaRa, Inc.). As a control, the Vanabin2-specific primer 15F2 (5- GGA ATT CGC TCC GGT GGA TTG C-3′) and the T7 primer were used to perform PCR with Easy A High-Fidelity PCR Cloning Enzyme (Stratagene). The PCR mixture contained 1× PCR buffer, 0.2 mM dNTPs, 40 pmol of each degenerate primer, 10 pmol of T7 primer, 280 ng/ml of the phage DNA template prepared from the cDNA library, and 1 U DNA

polymerase. The PCR conditions were denaturation at 90°C for 2 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 90 sec; and a final 5-min extension at 72°C. The amplified fragments were TA-cloned into the pBluescript SK vector (Stratagene) according to the manufacturer's instructions. The constructed plasmids were sequenced using Thermosequenase with an ALF Express DNA sequencer (GE Healthcare) or 3130xl DNA sequencer (Applied Biosystems).

2.4 Expression and purification of recombinant 14MT

The cDNA region corresponding to the putative mature protein of 14MT was amplified by PCR using a specific primer set with restriction enzyme sites. The primers used were 15F2 (described above) and 15R2 (5′-CGT CGA CTC ACT TGC AGT TTG TC-3′), and one of the 3′ RACE clones was used as the template. The PCR mixture contained 1× PCR buffer, 0.2 mM dNTPs, 10 pmol each 15F2 and 15R2 primers, 0.5 µl template cDNA, and 1 U rTaq DNA polymerase (TaKaRa). The PCR conditions were: denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 45 sec, and extension at 72°C for 45 sec; and a final extension at 72°C for 5 min. The PCR products were TA-cloned into the pBluescript SK vector, and the amplified fragment was excised with *Eco*RI and *Sal*I. Ligation of this fragment into the multiple cloning site of the pMAL-p2X expression vector (New England BioLabs, Inc.) resulted in a vector encoding an MBP-14MT fusion protein. DNA sequence was confirmed by automated DNA sequencer as above.

The MBP-14MT plasmid was introduced into *E. coli* strain TB1. Positive transformants were pre-cultured in LB medium containing 100 µg/ml ampicillin at 37°C for 18 h, diluted 1:10 in fresh LB medium containing 50 µg/ml ampicillin, and cultured at

37°C for 8 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were collected by centrifugation at 10,000 × *g* for 10 min and suspended in lysis buffer (10 mM Na_2HPO_4•$12H_2O$, 30 mM NaCl, 0.25% Tween20, 10 mM 2-mercaptoethanol, 10 mM EDTA•2Na, pH 7.0) containing 4 M urea. The suspension was sonicated on ice until it became clear and then centrifuged at 10,000 × *g* for 10 min. The resulting supernatant fraction was removed and dialyzed against 100 vol low-salt column buffer (LSC buffer; 10 mM Na_2HPO_4•$12H_2O$, 30 mM NaCl, pH 7.0) using a dialysis membrane with a 6,000–8,000 Da cut-off). The 14MT-MBP fusion protein was purified by amylose resin column chromatography according to manufacturer's protocol. The eluted 14MT-MBP fusion protein was digested by Factor Xa, which recognizes and cleaves the junction region between MBP and 14MT, and the reaction solution was dialyzed against 100 vol of 50 mM Tris-HCl (pH 7.4) using a dialysis membrane with a 3,500-Da cut-off).

The 14MT was purified by anion-exchange column chromatography. The mixture of 14MT and MBP was loaded onto a DEAE-Sephacel anion-exchange column (GE Heathcare) equilibrated with 50 mM Tris-HCl (pH 7.4). The column was washed with the same buffer and then eluted with 400 mM NaCl in the same buffer. The concentration of 14MT protein in the flow-through elution fractions of the column was determined by the Bradford method (Bio-Rad Laboratories Inc.), and the molecular size and purity of the 14MT was analyzed by 14% SDS-PAGE and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using Axima CFRplus (Kratos).

2.5 Metal-binding assay

The metal selectivity of 14MT was analyzed using immobilized metal-affinity chromatography (IMAC) by the batch method. Chelating Sepharose Fast Flow resin (bed

volume 100 µl; GE Healthcare) in 1.5-ml micro test tubes (Eppendorf) was washed three times with distilled water, and Mg^{2+}, Ca^{2+}, VO^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+}, and Zn^{2+} were chelated to the resin by addition of 0.1 M $MgCl_2$, $CaCl_2$, $VOSO_4$, $MnCl_2$, $FeCl_3$, $CoSO_4$, $CuCl_2$, or $ZnCl_2$, respectively (all from Wako Pure Chemical Industries Inc), to the tubes. The resin was washed twice with distilled water and then equilibrated with binding buffer (20 mM Na_2HPO_4, 100 mM NaCl, pH 7.5). The 14MT protein (60 µg/ml) was loaded onto the resin and incubated for 30 min. The resin-containing tube was centrifuged, and the supernatant fraction was removed as the flow-through fraction. Finally, the resin was eluted with 50 mM EDTA (pH 7.5). Binding of 14MT to each metal was evaluated by SDS-PAGE on a 14% polyacrylamide gel.

The vanadium-binding assay of Vanabin2 and 14MT were also studied using IMAC. Chelating Sepharose Fast Flow Resin (200 µl) was added to an 1.5-ml micro test tube and washed three times with deionized water (DW). Vanadium solution (1ml of 100 mM $VOSO_4$ in DW) was add to the resin. The resin was washed three times with DW and then equilibrated with binding buffer (20 mM Na_2HPO_4, 100 mM NaCl, pH 7.5). Each of the Vanabin2 and 14MT (100 µg/ml, respectively) was loaded onto the resin and incubated for 30 min. The resin was sedimented by a centrifugation, and the supernatant was collected as the flow-through fraction. The following elution buffers were then used in successive steps in order to elute the protein bound to the vanadium ions immobilized on the resin: (1) 20 mM Na_2HPO_4, 200 mM NaCl, pH 7.5; (2) 20 mM Na_2HPO_4, 300 mM NaCl, pH 7.5; (3) 20 mM Na_2HPO_4, 400 mM NaCl, pH 7.5; (5) 20 mM Na_2HPO_4, 500 mM NaCl, pH 7.5; (6) 50 mM EDTA, pH 7.5. After adding each elution buffer onto the resin and incubating for 30 min, the resins were centrifuged and the supernatant were collected as the binding fraction, respectively. Binding of Vanabin2 or 14MT to the

vanadium was evaluated by SDS-PAGE on a 15% polyacrylamide gel.

The vanadium-binding abilities of Vanabin2 and14MT were also assayed by a gel filtration method. A gel filtration column (bed size, 7 × 190 mm) filled with Biogel P-6 DG resin (Bio-Rad Laboratories Inc.) was equilibrated with binding buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5). Proteins (100 μg/ml) were premixed with 2 mM vanadium-IDA complex, loaded onto the column and separated at a flow rate of 250 μl/min. A protein peak-containing fraction that eluted about 12 min after loading was collected, and the vanadium concentration in the fraction was determined using atomic absorption spectroscopy (AAS; Spectra AA-220Z, Varian Inc). The protein concentration was determined by the Bradford method using BSA as standard and used to calculate the molar ratio of metal bound to protein.

2.6 Homology modeling

The 14MT amino acid sequence was subjected to homology modeling based on the solution structure of Vanabin2 [21]. SWISS-MODEL (http://swissmodel.expasy.org/) was used in full-automatic mode to model the overall structure of 14MT, and SWISS-PDB-viewer was used to generate a pictorial representation of the structure [25, 26].

3. Results

3.1 Identification of novel vanadium-binding proteins from blood cells of A. sydneiensis samea

To identify novel vanadium-binding proteins from *A. sydneiensis samea*, we

prepared soluble protein fractions from blood cells of about 100 adult individuals. After removing the giant cells, which comprise most of the total cell volume and do not accumulate vanadium, we obtained about 60 mg protein. Further purification yielded ~9.3 mg of soluble protein, which was then dialyzed against 20 mM NaH_2PO_4 (pH 7.4) and loaded onto an IMAC column charged with VO^{2+} ions. Vanabin1 was found in the fraction eluted by 400 mM NaCl, and Vanabin2 was found in the fractions eluted by 500 mM NaCl and EDTA (Fig. 1A). The EDTA fraction also contained proteins that yielded 16-, 26-, and 40-kDa bands on an SDS-gel. We separated the proteins in the EDTA fraction by reverse-phase HPLC (Fig. 1B) with monitoring at 200 nm. Twelve peak fractions were detected, and the N-terminal amino-acid sequences for six of these peak fractions were determined (Table 1). Of the partial N-terminal amino acid sequences derived from the eluted protein peaks, five protein peaks with strong homology to Vanabin2 were identified. The other one was identified as Vanabin1. This result indicated that blood cells contain multiple types of Vanabin2-like vanadium-binding proteins.

3.2 PCR cloning of cDNAs for vanadium-binding proteins

Based on the N-terminal amino-acid sequences for Vanabin2-like proteins, we made four types of degenerate primers and performed 3′ RACE PCR using an *A. sydneiensis samea* blood-cell cDNA library as a template. As a result of 3′ RACE, we isolated many different cDNA clones that were highly homologous to Vanabin2. Their coding regions were identical in length to that of Vanabin2, and all 18 cysteine residues of Vanabin2 were completely conserved. However, the predicted amino acid sequences of the clones differed from that of Vanabin2 at up to 14 different positions. Among all the cDNA clones, five types of clones were most frequently observed (Fig. 2). The amino

acid sequences encoded by these cDNAs differed from that of Vanabin2 at 1, 9, 10, or 14 positions, although all were the same length as Vanabin2. The cDNA clones were designated 1MT, 9MT, 10MT, and 14MT, respectively, according to the number of differences. In addition, 10MT is divided into two, 10MT-1 and 10MT-2, according to the 16th amino acid residue. The sites of amino acid variation appeared to be specific, rather than random. The 1MT protein differs from Vanabin2 only at residue 70, and this difference also occurs in 10MT-1, 10MT-2, and 14MT. On the other hand, the lysine contents of 9MT, 10MT-1, 10MT-2, and 14MT are all greater than that of Vanabin2.

We modeled the structure of the most divergent protein, 14MT, using the solution structure of Vanabin2 [21] as a template. Although the resulting structure is almost identical to that of Vanabin2, its electrostatic surface is more positive, reflecting the increased number of lysine residues in 14MT (Fig. 3). The calculated isoelectric point (pI) of 14MT is 8.47, whereas that of Vanabin2 is 8.00. The amino acid differences between 14MT and Vanabin are distributed over the three-dimensional structure, as illustrated in Fig. 3B. The relationship between the VO^{2+}-binding sites and sites of variation are discussed below.

3.3 Metal-binding ability of a Vanabin2-like protein 14MT

We focused on 14MT because it was the most divergent among the Vanabin2-like cDNA clones. We overexpressed recombinant 14MT protein in *E. coli* and assayed its selectivity for Mg^{2+}, Ca^{2+},VO^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+}, and Zn^{2+} ions. As shown in Fig. 4A, IMAC analysis revealed that 14MT bound tightly to VO^{2+}, Fe^{3+} and Cu^{2+} and weakly to Co^{2+} and Zn^{2+}. In contrast, no detectable binding of 14MT to Mn^{2+}, Ca^{2+}, or Mg^{2+} was observed. These results indicate that the metal selectivity of 14MT is similar to that of

Vanabin2 [23]. The affinity of Vanabin2 and 14MT for VO^{2+} were assessed by stepwise elution on IMAC. Proteins were bound to the IMAC column with immobilized VO^{2+} in buffer containing 100 mM NaCl and then eluted by stepwise increases in the NaCl concentration. The results of this analysis indicated that overall affinity of 14MT for VO^{2+} is higher than that of Vanabin2 (Fig. 4B), although this is rather qualitative analysis.

Quantitative determination of the vanadium-binding abilities of Vanabin2 and 14MT was done according to the gel filtration method. 14MT protein was mixed with excess of VO^{2+} ions in a buffer at pH7.5, and free VO^{2+} ions were removed by gel filtration. This assay revealed that Vanabin2 bound to 3.46 ± 0.79 VO^{2+} ions and 14MT bound to 3.24 ± 0.74 VO^{2+} ions, respectively. Student's *t*-test value indicated that there were no significant differences in binding number of VO^{2+} between Vanabin2 and 14MT. This indicated that high affinity binding sites for 3–4 VO^{2+} ions were conserved between Vanabin2 and 14MT.

4. Discussion

The Vanabin family has at least five homologous members in *Ascidia sydneiensis samea*. Two of these homologues, Vanabin1 and Vanabin2, were first extracted from the cytoplasmic fraction of vanadocytes as major vanadium-binding proteins [13, 14]. Vanabin3 and Vanabin4 were identified by an EST database analysis of vanadocytes [15], and VanabinP was isolated from coelomic fluid [17]. In these five Vanabins, all 18 cysteine residues are conserved, and the intervals between these cysteine residues are conserved as well. Vanabin1, Vanabin2, and Vanabin3 are exclusively localized in the cytoplasm of signet ring cells, while Vanabin4 is loosely associated with the cytoplasmic

membrane of signet ring cells [20]. VanabinP is exclusively localized in the blood plasma, but its mRNA has been found in all examined tissues [17].

During the search for novel vanadium-binding proteins, we found that several variants of Vanabin2 are present in the blood cells of the vanadium-rich ascidian *A. sydneiensis samea*. Therefore, we extensively examined the sequence variation and identified five variants of Vanabin2 in this study. All of these variants have the conserved {C}-{X_{2-5}}-{C} motifs common to the Vanabin family. Their open reading frames also encode polypeptides of the same length as Vanabin2 (91 amino acid residues). In general, residues that function in maintaining structural stability and in metal binding sites are tightly conserved. In fact, as shown in Figure 2, we observed no replacements of any cysteine residues involved in disulfide bondings [21]. Among the five variants, amino acid replacements were commonly observed at positions 16, 18, 21, 22, 25, 32, 52, 64, 70, and 80, where substitutions of proline, threonine, alanine, glutamate, and histidine by lysine; proline or glutamine by glutamate; and glutamate by glycine or glutamine, were seen (Figs. 2 and 3). These substitutions increase the total number of lysine residues in the variants, compared to Vanabin2, especially in 14MT, in which the number of lysine residues is higher by 6, for a total of 20.

We examined the most divergent variant, 14MT, to determine whether its abundant substitutions affected its VO^{2+}-binding ability. Results of IMAC and gel filtration assays indicated that high affinity binding sites for 3–4 VO^{2+} ions were conserved between Vanabin2 and 14MT, while overall affinity seemed to increase by amino acid substitutions. We previously reported that Vanabin2 can bind 20 VO^{2+} ions at maximum [14]. The difference of binding number is due to the gel filtration step; in the buffer for gel filtration in this study contained no vanadium, but the previous study used

Hummel-Dreyer method that utilize ligand containing buffer [27]. The present result also suggested that the 14 substitution at maximum did not affect its physiological function as metallochaperone in the vanadium-accumulating cells. The sites of variation appeared to be specific, rather than random, suggesting that these variations occurred under some physiological constraints. The non-varying amino acid residues in the putative VO^{2+}-binding region are shown in yellow in Fig. 3C. Our parallel studies on several artificially mutated Vanabins (Ueki et al., unpublished data) have suggested that some of these conserved amino acids are responsible for high-affinity VO^{2+}-binding. Thus, these conserved VO^{2+}-binding residues supposed to be physiologically necessary and unchangeable.

Whether the 1MT, 9MT, 10MT-1, 10MT-2, and 14MT variants of Vanabin2 arose through gene duplication, allelic variation, or alternative splicing remains a riddle, as does their biological significance. Moreover, whether such variation also occurs for the other Vanabins remains to be determined. Our preliminary PCR-based analysis of Vanabin genes in genomic DNAs from individual *A. sydneiensis samea* adults suggested that one individual possesses four types of Vanabin2-like genes (Ueki et al., unpublished data); that is, the haploid genome has two genes. Because Vanabin2 itself was not always found in all individuals, it appeared to be one of the variants. SDS-PAGE analysis of proteins from each individual also suggested that the expression of Vanabin2 variants of 15- to 16-kDa differs from one individual to another. Therefore, the pattern of 15- to 16-kDa bands shown in Fig. 1A (lane 1) is a sum of Vanabin2 variants from many individuals. In contrast, the 12-kDa band (Vanabin1) always appears as a single, distinct band in each individual. Extensive analysis of a genomic DNA library is in progress.

Another ascidian species, *Ciona intestinalis*, which accumulates 0.6 mM vanadium

[5], possesses five Vanabin homologues, CiVanabins 1–5 [28]. Draft genome analysis has shown that the five Vanabins are encoded by five independent genes. However, there is no clear relationship between the five Vanabins in *A. sydneiensis samea* and the five Vanabins in *C. intestinalis*. Extensive EST analysis of *C. intestinalis* did not yield Vanabin variants such as those found in our present study of *A. sydneiensis samea*. One hypothesis is that duplication of Vanabins occurred independently in *Ascidia* and *Ciona*, and ancestral Vanabin2 in *Ascidia* duplicated and diversified to perform new physiological functions that allow accumulation of extremely high levels of vanadium ions. We are now conducting EST analysis of another vanadium-rich ascidian, *Ascidia gemmata*, which accumulates the highest known level of vanadium (350 mM); we have already identified several Vanabin homologues in this species (Ueki et al., unpublished data). A comparative analysis of gene structure and gene expression in the two *Ascidia* species will be useful in understanding the reasons for Vanabin diversification.

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Table 1

N-terminal amino-acid sequences of vanadium-binding proteins

HPLC chromatogram peak [a]	N-terminal amino acid sequence [b]
Peak 5	A-P-V-D-X-K-G-Q-X-T-T-**E**-X-**G**-P-L-**I**-A
Peak 6	A-P-V-D-X-K-G-Q-**Q**-**A**
Peak 7	A-P-V-D-X-K-G-Q-X-T-T-P
Peak 8	A-P-V-D-X-K-G-Q-X-T-**A**-**T**
Peak 9	A-P-V-D-X-K-G-Q-X-T-T-P
Peak 11	G-P-G-X-K-X-Q

[a] Peak numbers correspond to those shown in Fig. 1.

[b] Amino acid residues that differ from residues of Vanabin2 are shown in bold type.

Figure legends

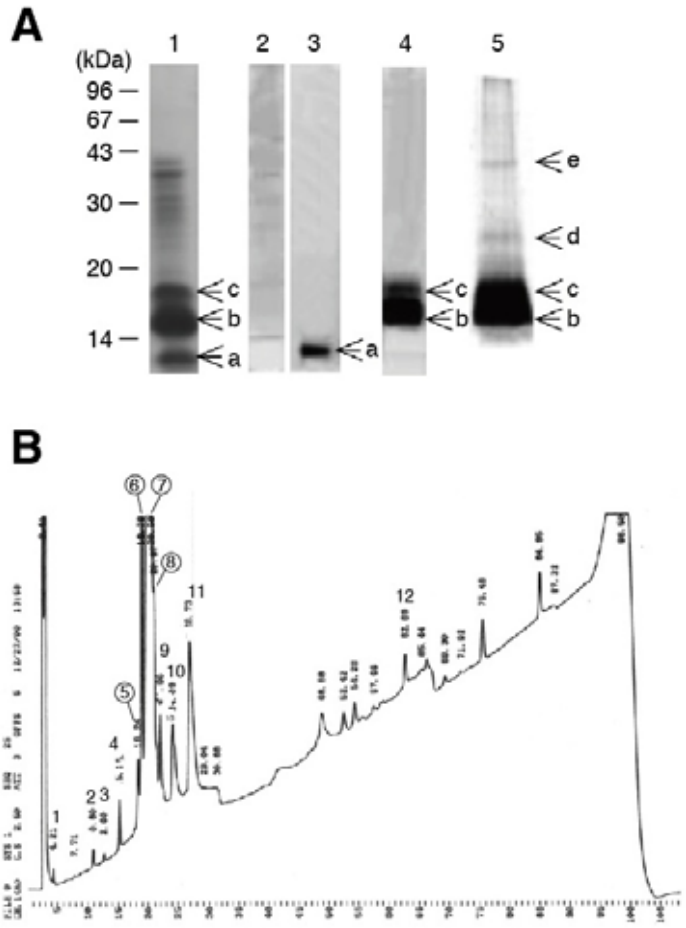

Fig. 1 (A) Isolation of vanadium-binding proteins from blood cells of *A. sydneiensis samea* by immobilized metal-ion affinity chromatography (IMAC) on resin charged with VO^{2+}. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, total soluble proteins from blood cells; lane 2, flow-through fraction; lane 3, proteins eluted by 400 mM NaCl; lane 4, proteins eluted by 500 mM NaCl; lane 5, proteins eluted by EDTA. Arrows *a* and *b* indicate Vanabin1 and Vanabin2, respectively. Arrows *c–e* indicate 16-, 26-, and 40-kDa vanadium-binding proteins. **(B)** Separation of the EDTA-eluted fraction by reverse-phase HPLC. Proteins were separated on an

5Ph-AR-300 column using a linear gradient of 10–70% acetonitrile over 60 min and detected by monitoring absorbance at 220 nm. The twelve numbered peaks indicated on the chromatogram were analyzed. Those peaks found to contain Vanabin2-like proteins are indicated by circles.

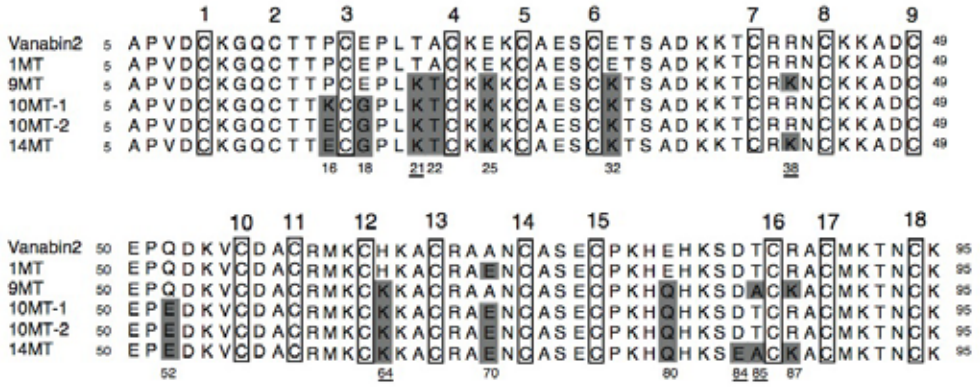

Fig. 2 Amino acid sequences of Vanabin2 and its variants identified in *Ascidia sydneiensis samea*. The amino acid sequence is numbered to include the four additional N-terminal amino acids encoding the vector junction region (I-S-E-F; not shown here). Numbers above the rows indicate the 18 conserved cysteine residues (boxed). Amino acid residues that differ from those at the same position in Vanabin2 are shaded and labeled (underneath the rows) by residue number. Underlining indicates residues that are within VO^{2+}-binding sites as revealed by NMR study [21].

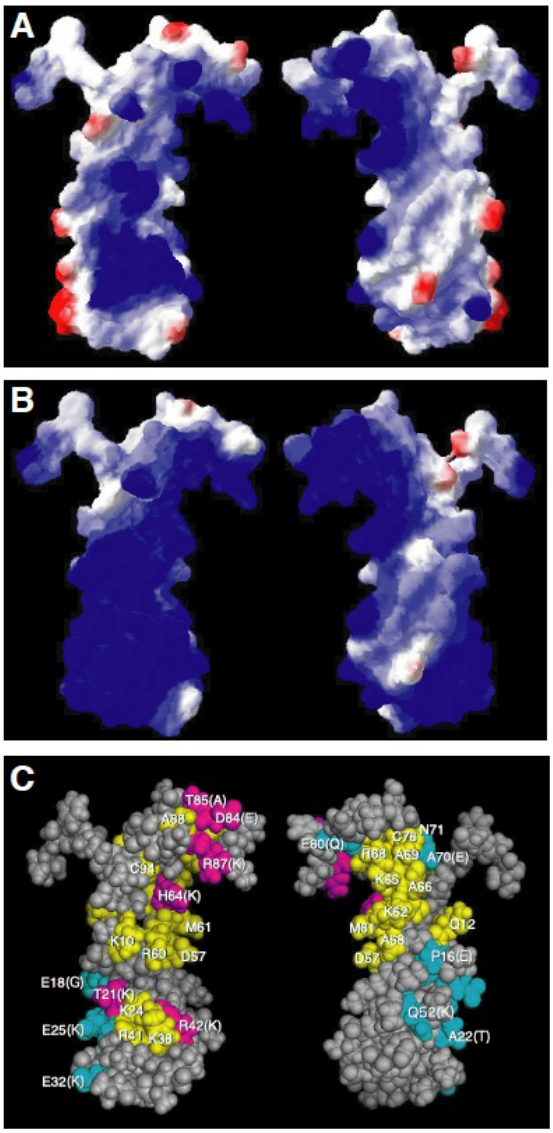

Fig. 3 Comparison of structural models of Vanabin2 and 14MT. **(A–C)** Each panel depicts two opposite faces of the same molecule. **(A)** Electrostatic surface potential of solution structure of Vanabin2 [21]. The image was produced by SWISS-PDB-viewer with the parameters: Red -2.2, White 0.0, Blue 4.0. Blue, positively charged; red, negatively charged. **(B)** The electrostatic surface potential of 14MT was predicted by homology modeling based on the solution structure of Vanabin2 [22] and is depicted as described in (A). **(C)** Positions of amino acid differences between Vanabin2 and 14MT. Yellow, unchanged residues in the VO^{2+}-binding site; purple, altered residues in the

VO^{2+}-binding site; blue, altered residues outside of the VO^{2+}-binding site. Residue names are shown, and substitutions present in 14MT are added in brackets. The amino acid sequence is numbered to include the four additional N-terminal amino acids encoded by the pMal-c vector junction region (I-S-E-F; not shown), which were also included in the structural modeling [21].

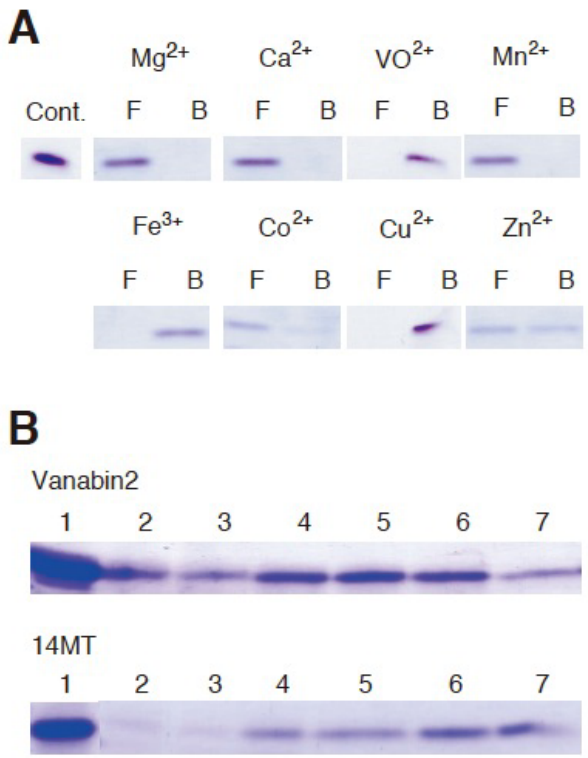

Fig. 4. Metal selectivity and affinity of a variant 14MT**(A)** Selective binding of metal ions to recombinant 14MT evaluated by IMAC with columns charged with various metal ions. 14MT protein was applied to IMAC columns equilibrated in 100 mM NaCl, 20 mM sodium phosphate buffer (pH 7.4). Non-binding fractions (F) were eluted with the equilibration buffer. Binding fractions (B) were subsequently eluted with elution buffer, and eluted proteins were analyzed by SDS-PAGE and staining with Coomassie brilliant

blue. **(B)** Affinity of Vanabin2 and 14MT for VO^{2+} as assessed by stepwise elution with NaCl. After binding the column in 20 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl, a stepwise gradient of NaCl was used to elute the bound proteins. lane 1, loaded protein; lane 2, flow-through fraction; lane 3–6, eluted by 200, 300, 400 and 500 mM NaCl, respectively; lane 7, eluted by 50 mM EDTA. Eluted proteins were analyzed by SDS-PAGE and staining with Coomassie brilliant blue.