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Citation	Biochimica et Biophysica Acta , 1730 : 206 - 217
Issue Date	2005
DOI	10.1016/j.bbaexp.2005.07.002
Self DOI	
URL	https://ir.lib.hiroshima-u.ac.jp/00000018
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Relation	



VanabinP, a novel vanadium-binding protein in the blood plasma of an ascidian, *Ascidia sydneiensis samea*

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Keywords: Ascidian; Vanadium; Metal accumulation, Blood plasma

Abstract

Some ascidians accumulate high levels of the transition metal vanadium in their blood cells. The process of vanadium accumulation has not yet been elucidated. In this report, we describe the isolation and cDNA cloning of a novel vanadium-binding protein, designated as VanabinP, from the blood plasma of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. The predicted amino acid sequence of VanabinP was highly conserved and similar to those of other Vanabins. The N-terminus of the mature form of VanabinP was rich in basic amino acid residues. VanabinP cDNA was originally isolated from blood cells, as were the other four Vanabins. However, Western blot analysis revealed that the VanabinP protein was localized to the blood plasma and was not detectable in blood cells. RT-PCR analysis and *in situ* hybridization indicated that the VanabinP gene was transcribed in some cell types localized to peripheral connective tissues of the alimentary canal, muscle, blood cells, and a portion of the branchial sac. Recombinant VanabinP bound a maximum of 13 vanadium(IV) ions per molecule with a K_d of 2.8×10^{-5} M. These results suggest that VanabinP is produced in several types of cell, including blood cells, and is immediately secreted into the blood plasma where it functions as a vanadium(IV) carrier.

1. Introduction

Some ascidians, the so-called sea squirts, of the suborder Phlebobranchia accumulate high levels of the transition metal, vanadium [1-3]. The blood cells of *Ascidia gemmata*, which is one of the vanadium accumulation ascidians, contain up to 350 mM vanadium [4], which corresponds to about 10^7 -times the concentration in seawater [5-6]. This unusual phenomenon observed in some ascidians has been studied by chemists, physiologists, and biochemists since its discovery about ninety years ago. The only other living organism known to accumulate such high levels of vanadium in such a selective manner is the Polychaete *Pseudopotamilla ocellata* [7]. The mechanism underlying this surprisingly efficient metal accumulation system requires clarification. It is known that vanadium is eventually accumulated in the vacuoles of blood cells, which are called vanadocytes. Most of the vanadium ions accumulated in vanadocytes are reduced to the vanadium(IV) form and are stored in the vacuoles in the vanadium(III) state as free ions.

We have isolated several proteins from the cytoplasm of the vanadocytes of *A. sydneiensis samea* that are likely to be involved in vanadium accumulation [8]. Of these proteins, Vanabin1 and Vanabin2, vanadium-binding proteins isolated from the cytoplasm fraction of vanadium-containing blood cells (vanadocytes) of the vanadium-rich ascidian *A. sydneiensis samea*, which are rich in lysine and cysteine residues, and the intervals between the cysteine residues are highly regular [9]. Furthermore, we have revealed that Vanabin1 and Vanabin2 can bind to multiples of 10 and 20 vanadium(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M,

respectively [9]. Neither magnesium(II) nor molybdate(VI) ions inhibit the binding of vanadium(IV) ions to either Vanabin type, which suggests specific interactions between Vanabins and vanadium(IV) ions [9]. In addition, EST analysis of vanadocytes has revealed that two novel Vanabin-like proteins, designated as Vanabin3 and Vanabin4, have primary structures that are similar to those of Vanabin1 and Vanabin2, bind qualitatively to vanadium(IV) ions, and are expressed in vanadocytes [10]. Moreover, we have recently reported the first 3D structure of Vanabin2 in an aqueous solution [11]. The structural analysis revealed a novel bow-shaped conformation, with four α -helices connected by nine disulfide bonds. No structural homologues have been reported to date. The ^{15}N -HSQC perturbation experiments of Vanabin2 indicate that vanadyl cations, which are exclusively localized on the same face of the molecule, are coordinated by amine nitrogens derived from amino acid residues, such as lysines, arginines and histidines, as suggested by the EPR results [11].

In ascidians, vanadium ions are supposedly taken up from seawater through the branchial sac or alimentary canal, transferred to the coelom, and concentrated in vanadocytes. It is not known if carrier proteins are involved in the transport of vanadium from the coelomic fluid (blood plasma) into the vanadocytes, although we have isolated at least two types of Vanabin from the cytoplasm of vanadocytes, as described above. In the present study, we use immobilized metal affinity column chromatography to identify several vanadium-associated proteins in the coelomic fluid (blood plasma) of *A. sydneiensis samea*. We cloned the cDNA for the major protein. Sequence analysis indicates that this protein is a novel Vanabin, which we have designated as VanabinP (Vanabin in plasma). We have characterized the tissue localization, gene expression pattern, and metal-binding ability of VanabinP.

2. Materials and Methods

2.1 Extraction of ascidian plasma proteins

Adults of the ascidian *Ascidia sydneiensis samea* were collected at the International Coastal Research Center, Ocean Research Institute, the University of Tokyo, at Iwate, Japan.

After washing the surface of each individual with Ca^{2+} - and Mg^{2+} -free artificial seawater [460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4 , 5 mM HEPES (pH 7.0), 5 mM EDTA-2Na, and 6 mM NaHCO_3], the coelomic fluid (blood plasma) was extracted. The coelomic fluids from individual ascidians were collected separately, as it has been reported for other ascidian species that the mixing of blood cells from different individuals causes destruction of the blood cells. Blood plasma was obtained after removing the blood cells from the coelomic fluid by two rounds of centrifugation: at $120 \times g$ for 10 min at 4°C and then at $1,000 \times g$ for 10 min at 4°C . After the addition of one-ninth volume of 500 mM Tris-HCl (pH 8.0), the proteins in the blood plasma were precipitated by 80% saturation with ammonium sulfate for 20 h at 4°C . The blood plasma proteins were obtained by centrifugation at $20,000 \times g$ for 30 min at 4°C and were resuspended in 20 mM NaH_2PO_4 (pH 7.2) plus 500 mM NaCl.

2.2 Extraction of vanadium-associated proteins from plasma

After dialysis with binding buffer [20 mM NaH₂PO₄ (pH 7.2), 500 mM NaCl], the soluble blood plasma proteins were obtained by centrifugation at 20,000 × g for 30 min at 4°C. The extraction of vanadium-associated proteins was performed using a Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) column that was charged with vanadium(IV) [VO²⁺]. After the soluble blood plasma proteins were loaded, the column was washed with binding buffer, and the proteins absorbed to the column were eluted using an elution buffer [20 mM NaH₂PO₄ (pH 7.2), 500 mM NaCl, 50 mM EDTA-2Na]. The protein concentration in each fraction was determined by the Bradford method (Bio-Rad).

2.3 Purification of the major vanadium-associated protein in plasma

The elution buffer was substituted with 0.1% TFA in 10% acetonitrile, and the proteins were concentrated by centrifugal filtration with a Centriplus YM-3 (Amicon). The concentrated proteins were applied sequentially to two different reverse-phase HPLC columns. In the first step, the proteins were applied to a COSMOSIL 5Ph-AR-300 packed column (4.6 × 150 mm; Nacalai Tesque Inc.) under the following conditions: flow rate, 1 ml/min; solvents, 0.1% TFA (solution A) and 0.1% TFA in acetonitrile (solution B); and linear gradient from 90% solution A and 10% solution B at 0 min to 30% solution A and 70% solution B at 60 min. In the next step, the purified proteins were loaded onto a COSMOSIL 5C18-AR-300 column (2.0 × 150 mm; Nacalai Tesque Inc.) under the following conditions: flow rate, 0.22 ml/min; solvents, solution A and solution B; and linear gradient from 80% solution A and 20% solution B at 0 min to 50% solution A and

50% solution B at 60 min. In both experiments, the amount of protein in the eluted solution was monitored using a UV detector (UV-8010 TOSOH) at 220 nm absorption. The protein eluted in each peak was examined by SDS-PAGE. The major bands for vanadium-associated proteins were digested using the Edman degradation method for sequence determination by N-terminal analysis. The molecular weights of the proteins were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

2.4 Cloning of the cDNA for the major vanadium-associated protein in plasma

A cDNA library has been constructed in the UniZap XR vector using the blood cells, which are signet ring cell (vanadocyte) rich of the ascidian *A. sydneiensis samea* [10]. To obtain a clone that encodes the major vanadium-associated protein in blood plasma, a degenerate primer (5'-(A/C)G(A/C/G/T) AA(A/G) AA(A/G) AA(A/G) AA(A/G) AA(A/G) ATG-3') was designed. The 3'-RACE method was then carried out using DNA obtained from the cDNA library of a signet ring cell (vanadocyte) as the template. The PCR mixture contained phage DNA, degenerate primer (250 pmol), T7 primer (50 pmol), dNTPs (20 pmol), 1× PCR buffer, and LA Taq DNA polymerase (TaKaRa Inc.) in a reaction volume of 50 µl. The following PCR conditions were used: 95°C for 2 min; 30 cycles at 95°C for 30 s, 45°C for 30 s and 72°C for 60 s; and a final extension at 72°C for 5 min. The 3'-RACE products were purified and cloned into the pBluescript vector. The DNA sequences of the 3'-RACE products were determined by the dideoxy method using the ALF express II automated DNA sequencer (Amersham Pharmacia Biotech). To determine the full-length cDNA sequence, two primers (R1,

5'-ATC CCT TTT TGC AAG CCT TC-3'; and R2, 5'-AGG TTT CAT GCA TGC TTT GG-3') were designed based on the DNA sequences of the 3'-RACE products. Phage DNA extracted from the cDNA library was used as the template for the 5'-RACE reaction. The PCR mixture contained phage DNA, R1 primer (100 pmol), T3 22-mer primer (10 pmol), dNTPs (10 pmol), 1× PCR buffer, and Pfu Turbo DNA polymerase (TaKaRa) in a reaction volume of 50 µl. The PCR conditions were as follows: 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. A portion of the 5'-RACE product was treated with exonuclease and alkaline phosphatase, and 1 µl of the treated product was used as the template for the nested PCR. The PCR mixture contained 5'-RACE product, R2 primer (10 pmol), T3 22-mer primer (10 pmol), dNTPs (10 pmol), 1× PCR buffer, and Pfu Turbo DNA polymerase (TaKaRa) in a reaction volume of 50 µl. The PCR conditions were as follows: 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR product was purified and cloned into the pBluescript vector. The DNA sequences of the 5'-RACE and nested PCR products were determined as described above.

2.5 Generation of antibody and Western blotting of ascidian tissues

To generate antibody against the vanadium-associated protein, the 15-aa peptide CLSTRNRPIPGEKPN, which corresponds to aa 84-98 of the mature protein, was synthesized and conjugated with keyhole limpet hemocyanin (KLH). Female BALB/c mice were immunized three times with the conjugate protein, and antiserum was obtained. The blood cell homogenate was prepared as described previously [12], and Western blot

analysis was performed [12]. For other tissues, homogenate was prepared from *A. sydneiensis samea* in 200 mM Tris-HCl (pH 8.0) that contained leupeptin, PMSF, and pepstatin A (5 µg/ml, respectively).

2.6 RT-PCR detection of VanabinP mRNA

Total RNA samples were extracted from the ascidian tissues using the cesium trifluoroacetic acid method. To remove proteins and genomic DNA, the extracted total RNA samples were treated with proteinase K for 1 h at 37°C and DNase I for 40 min at 37°C, respectively. Reverse transcription was performed using the SuperScript II reverse transcriptase (Invitrogen Life Technology) and dT15 primer. PCR with the F1 and R3 primers was used to detect the expression of the VanabinP gene in the ascidian tissues. The PCR mixture contained reverse-transcribed DNA from the total RNA samples of ascidian tissues, F1 primer (50 pmol), R3 primer (50 pmol), dNTPs (20 pmol), 1× PCR buffer, and rTaq DNA polymerase (TaKaRa) in a reaction volume of 100 µl. The PCR conditions used were as follows: 94°C for 4 min; 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. PCR with the As-actin fwd primer (5'-GAC CTA TGC TGC TCT TGA-3') and As-actin rev primer (5'-CAG GAT GGA TCC TCC AAT-3') was used to detect the expression of the actin gene in the ascidian tissues, as the positive control. The PCR mixture contained reverse-transcribed DNA from the total RNA samples of ascidian tissues, As-actin fwd primer (50 pmol), As-actin rev primer (50 pmol), dNTPs (20 pmol), 1× PCR buffer, and rTaq DNA polymerase (TaKaRa) in a reaction volume of 50 µl. The PCR conditions used were as

follows: 94°C for 2 min; 30 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The amplified fragment was examined by agarose gel electrophoresis.

2.7 *In situ* hybridization

Adult ascidians, 1.5-2 cm in length, were fixed in hs-PBS [450 mM NaCl, 2.7 mM KCl, 8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄] that contained 4% paraformaldehyde for 3 h at 4°C. The fixed samples were dehydrated by ethanol and embedded in Paraplast (Sigma Chemical Co.). These samples were serially sectioned at 8-µm thickness from the anterior to the posterior end in transverse sections, and the sectioned samples were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co.). After the removal of the Paraplast with xylene, the slides were hydrated and washed with PBS. The sectioned samples on the slides were permeabilized by treatment for 30 min at 37°C with a solution of 0.1 M Tris (pH 8.0) plus 0.05 M EDTA that contained 1 µg/ml proteinase K, and the samples were postfixated for 20 min at RT in PBS that contained 4% paraformaldehyde. After washing with PBS, the slides were incubated in pre-hybridization buffer [50% deionized formamide, 5× SSC, 1% SDS, 50 µg/ml yeast tRNA] for 2 h at 60°C. Digoxigenin (DIG)-labeled RNA probes were prepared from a plasmid that contained the cDNA fragment (nucleotides 82-462) of VanabinP using T3 or T7 RNA polymerase and digoxigenin-11-UTP (Roche). The slides were incubated in hybridization buffer, which consisted of pre-hybridization buffer plus 0.25 µg/ml DIG-labeled RNA probes, for 16 h at 60°C. The slides were washed twice in buffer A

[50% formamide, 3× SSC, 1% SDS] for 30 min at 60°C and three times in buffer B [50% formamide, 2× SSC] for 30 min at 60°C. After washing three times with 2× SSC, the slides were treated for 30 min at 37°C with TNE [0.01 M Tris (pH 7.0), 0.5 M NaCl, 0.001 M EDTA] that contained 20 µg/ml RNase A. The slides were then washed three times with 4× SSC for 5 min at RT, once in 2× SSC for 30 min at RT, once in 0.5× SSC for 30 min at RT, and three times in PBST [140 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3), 0.1% Tween]. The slides were incubated in PBST with 0.5% blocking reagent (Roche) for 60 min at RT and then in a 1:1500 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche) for 16 h at 4°C. The slides were washed three times in PBST for 5 min at RT, three times in PBST for 30 min at RT, and three times in a solution of 100 mM Tris (pH 9.5), 150 mM NaCl, and 50 mM MgCl₂ for 5 min at RT. The slides were incubated in BCIP/NBT substrate (DAKO) that contained 1 mM levamisole for 16 h at RT and were observed by Nomarski microscopy.

2.8 Recombinant protein expression

The cDNA region corresponding to the putative mature protein was amplified by the direct PCR method using a specific primer set with restriction enzyme sites as follows: F1, 5'-GAA TTC CGA AAG AAA AAG AAG-3'; R3, 5'-GTC GAC TCA ACC TTC AAA CAA-3'. One of the 3'-RACE clones was used as the template. The PCR mixture contained one of the 3'-RACE clones, F1 primer (250 pmol), R3 primer (250 pmol), dNTPs (10 pmol), 1× PCR buffer, and rTaq DNA polymerase (TaKaRa) in a reaction volume of 50 µl. The PCR conditions were as follows: 94°C for 2 min; 30 cycles

of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR product was purified and cloned into the pBluescript vector. The amplified fragment was digested with EcoRI and Sall and ligated into the multiple cloning site of the pGEX-6P-1 expression vector (Amersham Pharmacia Biotech), which has a lac promoter and the coding region for glutathione S-transferase (GST). The plasmid was introduced into the *E. coli* BL21 (codon plus) strain. *E. coli* cells that carried VanabinP-expressing plasmids were pre-cultured, and the expression of the fusion protein was induced by IPTG (0.5 mM) at 37°C for 2 h. The fusion protein was purified by glutathione Sepharose 4B column chromatography (Amersham Bioscience). The junction region between GST and VanabinP was cut using the PreScission protease (Amersham Bioscience). VanabinP was purified by removing GST using glutathione Sepharose 4B column chromatography and Ultrafree-MC centrifugal filters (Millipore). GST was eluted from the glutathione Sepharose 4B column after the purification of VanabinP and was used as a control protein. The resulting recombinant protein had eight additional amino acids (G-P-L-G-S-P-Q-F), which were derived from the junction region at its N-terminus.

2.9 Metal-binding assay

The metal-binding ability of the recombinant VanabinP was determined by means of the Hummel-Dreyer method [13], as described previously [9]. The metal concentration in each fraction was determined by atomic absorption spectrophotometry (AAS; SpectrAA-220Z; Varian Inc.), and the protein concentration was determined by the Bradford method (Bio-Rad).

3. Results

3.1 Identification of vanadium-associated proteins in ascidian plasma

By chelating a column charged with vanadium(IV), vanadium-associated proteins were first obtained from the blood plasma of the vanadium-rich ascidian *Ascidia sydneiensis samea* (Fig. 1). The ammonium sulfate-precipitated blood plasma proteins were loaded onto the vanadium-chelating column. After washing out the proteins that did not bind to the charged vanadium, vanadium-associated proteins were obtained by eluting with a buffer solution that contained 50 mM EDTA (Fig. 1A). The proteins were separated sequentially using two different types of HPLC column (Fig. 1B-C). The major peak obtained in the second HPLC contained a protein with an apparent molecular weight of 20 kDa according to SDS-PAGE analysis (Fig. 1D).

3.2 Cloning of the VanabinP cDNA

Edman degradation analysis revealed the N-terminal amino acid sequence of the 20-kDa protein in the major peak as: R-K-K-K-K-K-M-X-(R/G/X)-V-A-X-K-X, i.e., a sequence rich in basic amino acid residues. Using PCR with a degenerate primer, the cDNA for the 20-kDa protein was isolated from the vanadocyte cDNA library. The cDNA encoding the 20-kDa protein consisted of 581 bp, which corresponded to 146 amino acids (Fig. 2). The deduced amino acid sequence had a hydrophobic region consisting of 20 amino acid residues in the N-terminus, which may function as a signal

peptide and appears to be processed after translation. The region excluding the signal peptide was designated as the mature protein. The predicted amino acid sequence of the mature protein shows that its N-terminus is very rich in basic amino acids compared with the other protein regions. The molecular weight of the mature protein, as determined by MALDI-TOF MS, is 14 kDa (data not shown), which corresponds well to the value deduced from the cDNA sequence.

The deduced amino acid sequence of the mature protein is highly homologous to the Vanabin1 and Vanabin2 sequences [9], which have been identified in cytoplasmic extracts of vanadocytes, and to the Vanabin 3 and Vanabin 4 sequences, which have been identified by expressed sequence tag (EST) analysis of vanadocytes [10]. We conclude that the 20-kDa protein is a novel member of the Vanabin family, which we name as VanabinP (Vanabin in plasma). All five of the Vanabins contain a conserved motif of 18 cysteine residues, which can be described as the consensus sequence {C}- $\{X_{2-5}\}$ -{C} (Fig. 2). Homology searches using the BLASTP program to align the VanabinP sequence with the public protein databases did not reveal any remarkable similarities with proteins other than the Vanabins.

3.3 Localization of VanabinP

The anti-VanabinP polyclonal antibody was raised against the 15-aa peptide CLSTRNRPIPGKPN, which corresponds to aa 84-98 of the mature VanabinP protein. The obtained antibody recognized both the native and recombinant forms of VanabinP in a Western blot (Fig. 3). The difference of band size recognized between the native and recombinant forms of VanabinP was derived from additional eight amino acid residues in

the junction region at its N-terminus. The 20-kDa band for VanabinP was detected in the blood plasma, but not in any of the other tissues tested. In contrast, it is clear that Vanabin1 and Vanabin2 are localized to the vanadocyte cytoplasm [14]. Therefore, it seems likely that VanabinP plays a role different from that of Vanabin1 or Vanabin2.

3.4 VanabinP gene expression patterns in adult ascidian tissues

Although we isolated the cDNA for VanabinP from a blood cell cDNA library, it is not clear whether VanabinP is transcribed exclusively in blood cells. Therefore, we investigated the expression patterns of VanabinP mRNA in adult ascidian tissues.

RT-PCR analysis revealed that VanabinP mRNA was expressed in the blood cells, alimentary canal, muscle, and branchial sac (Fig. 4). To determine the cell types in these tissues that expressed VanabinP, we performed *in situ* hybridization on sectioned tissues. VanabinP-expressing cells were found in the peripheral connective tissues of the alimentary canal (Fig. 5, C-D), muscle (Fig. 5, G-H), and a portion of the branchial sac (Fig. 5, K-L). In particular, numerous VanabinP-expressing cells of two types, long and narrow-shaped cells (Fig. 5, D “Ex1”) and round-shaped cells (Fig. 5, D “Ex2”), were observed in the peripheral connective tissues of the alimentary canal. Although the former cell type was not identified, the latter cells morphologically resemble blood cells. The VanabinP-expressing cells in the peripheral connective tissue of the muscle (Fig. 5, H “Ex3”) were similar to the long and narrow-shaped VanabinP-expressing cells in the peripheral connective tissue of the alimentary canal (Fig. 5, D “Ex1”). Consequently, the results of the *in situ* hybridization were consistent with those of the RT-PCR analysis.

3.5 Metal-binding ability of VanabinP

To determine in a quantitative manner the metal-binding ability of VanabinP, the recombinant protein was overexpressed in *E. coli*. By means of the Hummel-Dreyer method [13], the ratios of bound vanadium ions per VanabinP protein molecule at various concentrations of free vanadium(IV) ion were determined (Fig. 6). For these experiments, we used vanadium(IV) ions coordinated by iminodiacetic acid (IDA), i.e., V(IV)-IDA, to avoid the precipitation of vanadium(IV) ions in the neutral pH range, as described previously [9]. The metal-binding assay was carried out using the metal free recombinant VanabinP. VanabinP was loaded onto a column, which was equilibrated with buffers that contained vanadium(IV)-IDA. VanabinP that bound to vanadium on the column was collected. The results show that VanabinP binds a maximum of 13 vanadium(IV) ions per mole protein with a dissociation constant of 2.8×10^{-5} M, which is similar to the values obtained previously for Vanabin1 and Vanabin2 [9]. We also attempted to determine the binding ability of VanabinP to vanadium(V) ions. However, we could not determine the binding constant or the number of binding sites (data not shown) owing to the weak binding ability.

4. Discussion

We report the isolation and cDNA cloning of a novel vanadium-binding protein, designated as VanabinP, from the blood plasma of the vanadium-rich ascidian *A. sydneiensis samea*. VanabinP differs in various characteristics from Vanabin1, Vanabin2 [9], Vanabin3, and Vanabin4 [10], which have been reported to be localized in

vanadocytes. However, the predicted amino acid sequence of VanabinP deduced from the cDNA is highly conserved and is similar to those of the other Vanabins, as shown in Fig. 2. Thus, the Vanabin family members share the characteristics of being cysteine-rich and having regular intervals between the cysteine residues. Furthermore, Vanabin1, Vanabin2, and VanabinP have in common a conservative N-terminal hydrophobic region that consists of about 20 amino acids (Fig. 2), which may function as a signal sequence for protein translocation [15]. Recently, an NMR study revealed the 3D structure of Vanabin2, which indicates that it has a novel bow-shaped conformation, with four α -helices connected by nine disulfide bonds [11]. It is possible that the Vanabin family members, including VanabinP, share a common structure, although this has not been confirmed.

Western blot analysis revealed that VanabinP was localized in the blood plasma (Fig. 3). In addition, the RT-PCR and *in situ* hybridization analyses indicated that the VanabinP gene was expressed in cells localized in the peripheral connective tissues of the alimentary canal, muscle, blood cells, and a portion of the branchial sac, although VanabinP-expressing cells have been not identified with any type of cells (Figs. 4 and 5). The *in situ* hybridization experiment revealed that most of the VanabinP-expressing cells were located in the peripheral connective tissues of the alimentary canal (Fig. 5, C-D). Signet ring cells and small signet ring cells, which react with the vanadocyte-specific monoclonal antibody, were reported to be localized in the connective tissues around the alimentary canal [16]. Further studies are needed to clarify which cell types, including signet ring cells and small signet ring cells, express VanabinP.

Using the recombinant protein, we found that VanabinP could bind to 13 vanadium(IV) ions with a dissociation constant of 2.8×10^{-5} M (Fig. 6). These values

were similar to those obtained for Vanabin1 and Vanabin2, which bind multiples of 10 and 20 vanadium(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively [9]. The similar values observed for the vanadium(IV)-binding abilities and dissociation constants of Vanabins may reflect a common structure in these proteins, as shown in Fig. 2. A recent NMR study [11] has indicated that vanadium(IV) ions, which are exclusively localized on the same face of Vanabin2, are coordinated by amine nitrogens that are derived from amino acid residues, such as lysines, arginines, and histidines, as suggested by the EPR study [17]. Analyses of the 3D structures using homology modeling of the proteins should elucidate the reasons for the similarities in binding abilities and dissociation constants among the Vanabins. On the other hand, we could not determine the binding constant or the number of binding sites of VanabinP for vanadium(V) ions. The reason for this may be that vanadium(V) species are unstable in neutral aqueous solutions as a result of protonation, oligomerization, and interaction with buffers, which produces weak binding of VanabinP to vanadium(V) [18, 19].

We have isolated at least five types of Vanabin, including the present VanabinP, which are likely to be involved in the vanadium accumulation processes that take place in *A. sydneiensis samea*. Vanabin1 and Vanabin2 proteins [9] are localized in vanadocytes, whereas VanabinP is found in the blood plasma (present study). However, the functional correlations between these Vanabins have not yet been revealed. Vanadium dissolved in seawater is taken up into the interior of the ascidian body via the branchial sac or alimentary canal, transferred into the coelomic fluid (blood plasma), and stored in the vacuoles of vanadocytes at concentrations in excess of 350 mM, which is about 10^7 -fold the concentration in seawater. However, the pathway of vanadium accumulation in ascidians remains unclear. In the process of accumulation, Vanabins may function as

metal carriers, so-called metallochaperones, rather than as proteins for metal storage or detoxification. The mechanisms by which VanabinP captures vanadium in the coelomic fluid and subsequently transfers the vanadium are the next problems to be resolved.

The accumulation of vanadium in ascidians is an unusual physiological phenomenon in one class of marine organisms. Although the unusual phenomenon whereby some ascidians accumulate vanadium to levels more than ten million times higher than those in seawater has attracted researchers in various fields, the physiological roles of vanadium remain to be explained. Endean and Smith proposed that the cellulose of the tunic might be produced by vanadocytes [20-23]. Carlisle suggested that vanadium-containing vanadocytes might reversibly trap oxygen under conditions of low oxygen tension [24]. The hypothesis has also been proposed that the vanadium in ascidians acts to protect them against fouling or as an antimicrobial agent [25, 26]. However, most of the proposals do not seem to be supported by sufficient evidence. Therefore, we convinced that Vanabins are an important clue toward resolving the physiological roles of vanadium in ascidians. In this study, we have isolated and analyzed VanabinP, which is a vanadium binding protein in blood plasma of the ascidian, *A. sydneiensis samea*. This is the extracellular vanadium binding protein that was identified for the first time, and may have a possibility to be the key substance in the process of vanadium accumulation from extracellular fluid to vanadocytes. The elucidation of the function of VanabinP must contribute to our understanding of the entire process of vanadium accumulation in ascidians as well as the processes of accumulation of other essential metals in other organisms and the physiological role of vanadium, which is an essential metal for various organisms, including mammals.

Acknowledgments

We would like to thank Prof. K. Yasui at Marine Biological Laboratory, Graduate School of Science, Hiroshima University, Onomichi, Hiroshima, Japan, for critical suggestion in performing *in situ* hybridization, and Mr. T. Morita and the staff at the International Coastal Research Center, Ocean Research Institute, the University of Tokyo, Iwate, Japan, for help in collecting adult ascidians. This work was supported, in part, by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#14340264 to H. M. and #14596005 to T. U.) and a Grant from the Toray Science Foundation (03-4402 to T. U.).

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Figure Legends

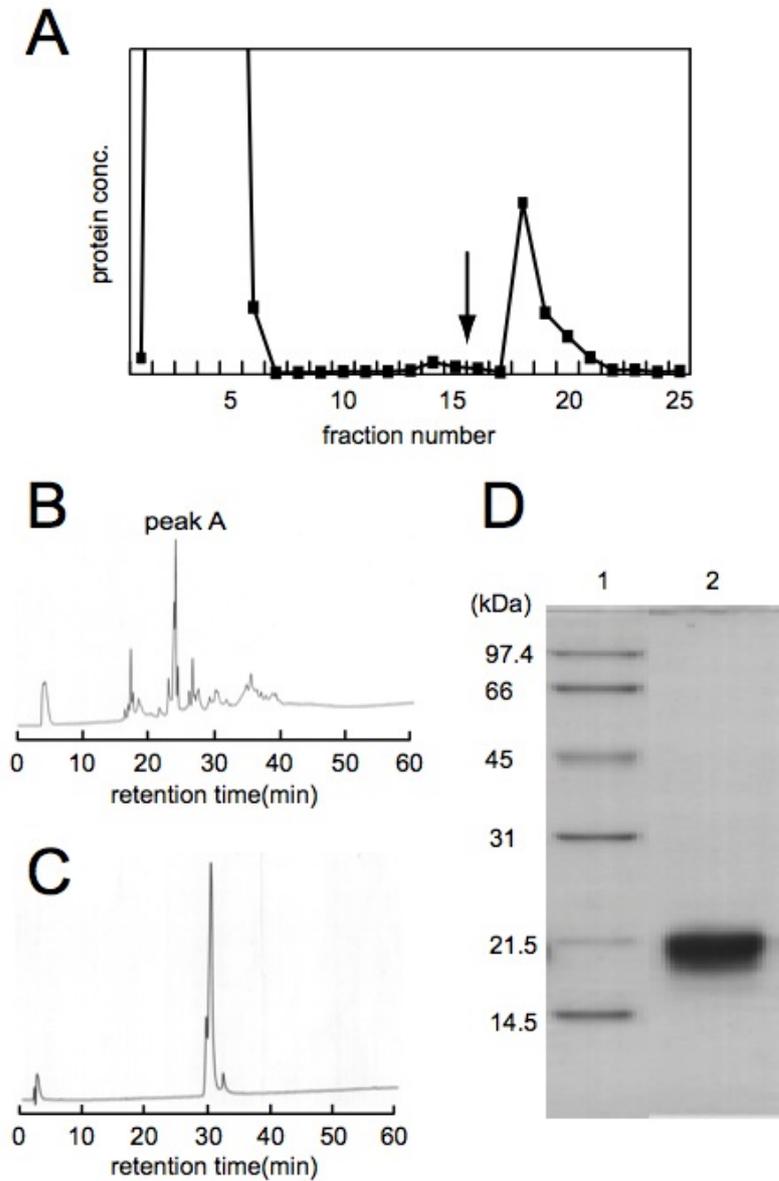


Fig. 1. Isolation of vanadium-associated proteins from ascidian blood plasma.

(A) Extraction of vanadium-associated proteins from blood plasma using immobilized metal affinity chromatography (IMAC). Vanadium-associated proteins were eluted by EDTA containing buffer (an arrow indicated) and eluted proteins were collected from

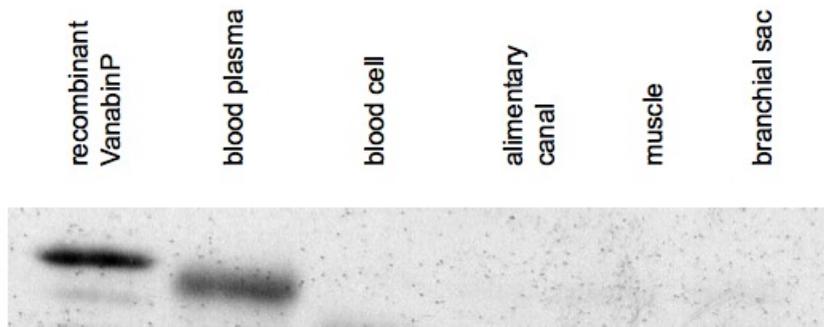


Fig. 3. Localization of VanabinP protein in *A. sydneiensis samea* tissues.

Western blot analysis was performed with the anti-VanabinP polyclonal antibody. The blood plasma proteins, blood cell homogenate, alimentary canal, muscle, and branchial sac, as well as recombinant VanabinP (1 μg recombinant VanabinP, 5 μg of blood plasma proteins, and 10 μg of the other proteins) were separated by 14% SDS-PAGE and reacted with anti-VanabinP antibody, followed by chemiluminescent detection. Recombinant VanabinP was used as the positive control.

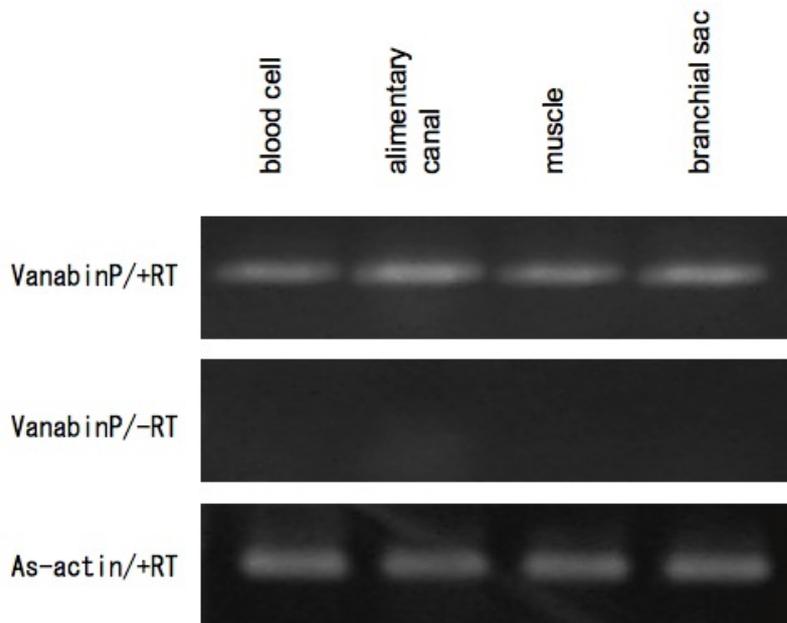
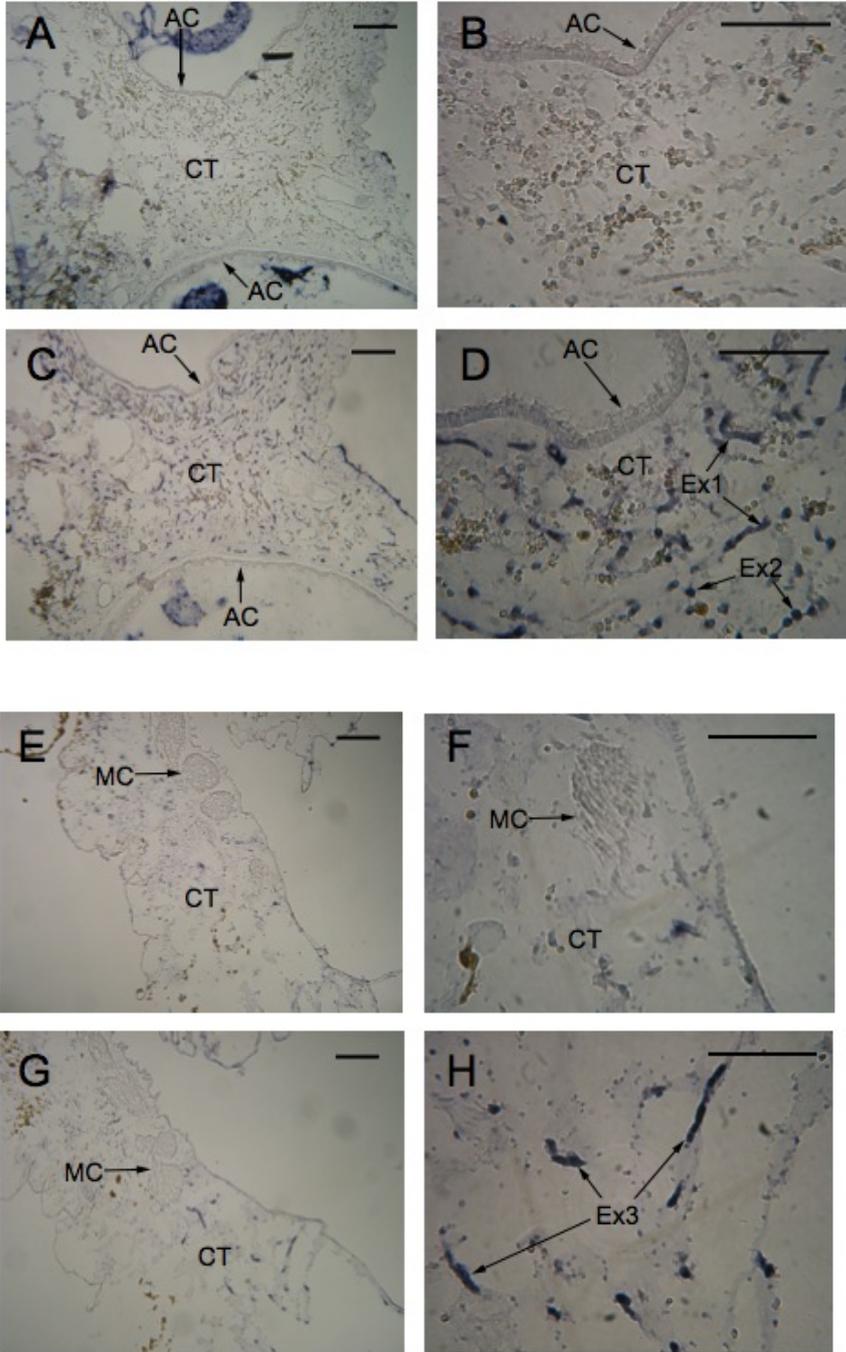


Fig. 4. RT-PCR analysis of VanabinP gene expression *A. sydneiensis samea* tissues.

Top panel: PCR was performed using a VanabinP-specific primer set and reverse-transcribed cDNA from the total RNA of each tissue as the template. Middle panel: PCR was performed using the VanabinP-specific primer set and the total RNA of ascidian tissues as the template (negative control). Bottom panel: PCR was performed using the *A. sydneiensis samea* actin-specific primer set and reverse-transcribed cDNA from the total RNA of each tissue as the template (positive control).



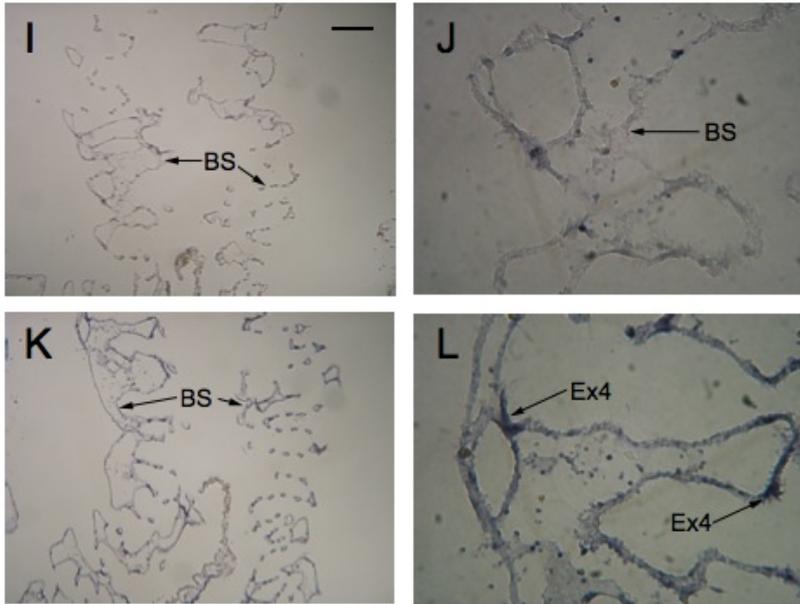


Fig. 5. *In situ* hybridization analysis of VanabinP gene expression in ascidian tissues. Panels A, B, C, and D, alimentary canal and peripheral connective tissues thereof; panels E, F, G, and H, muscle and peripheral connective tissues thereof; panels I, J, K, and L, branchial sac. The sense probe was used in panels A, B, E, F, I, and J. The antisense probe was used in panels C, D, G, H, K, and L. The left panels are relatively low magnification micrographs, and the right panels are relatively high magnification micrographs. The scale bars on the left and right panels indicate 200 μm and 50 μm , respectively. AC, alimentary canal; CT, connective tissue; MC, muscle cells; BS, branchial sac; Ex1-4, VanabinP gene-expressing cells.

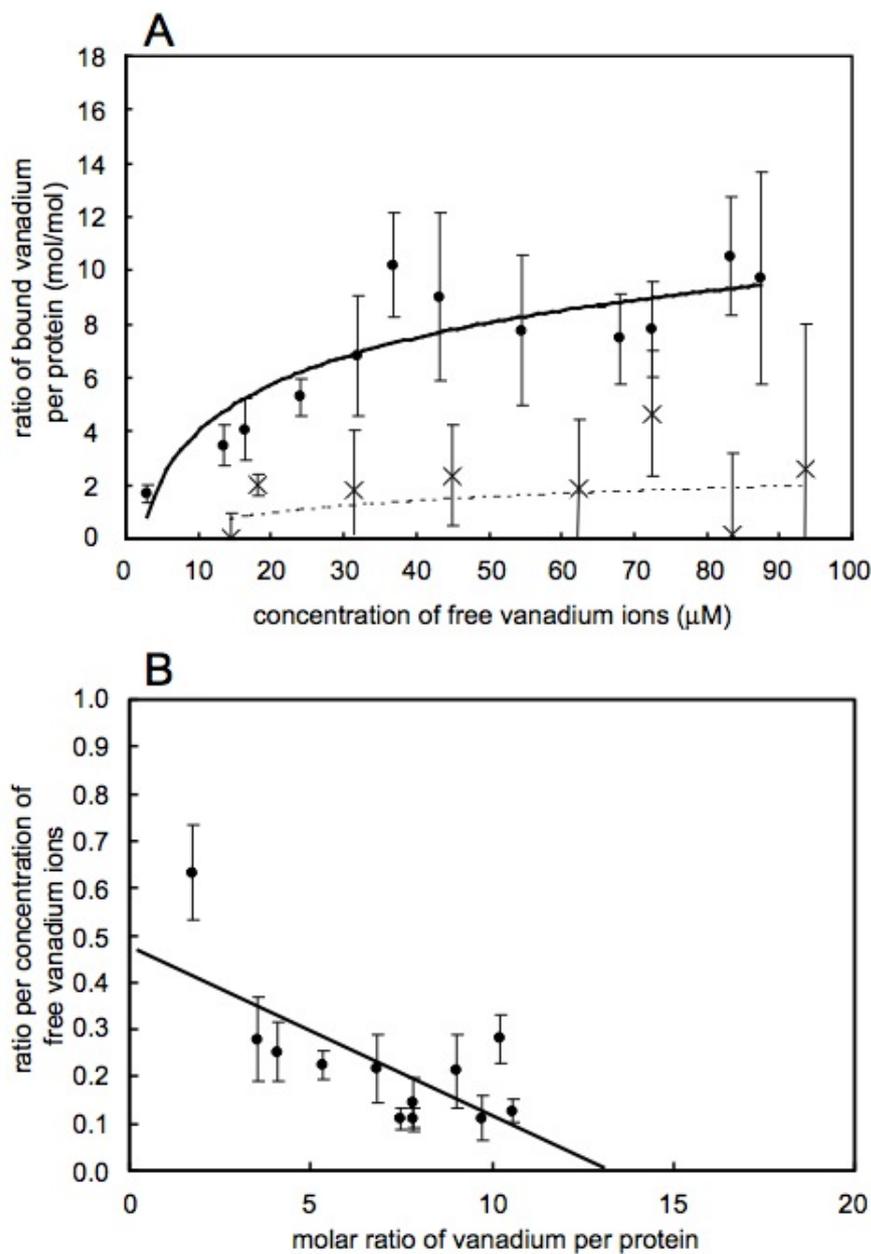


Fig. 6. Binding of VanabinP to vanadium(IV) ions as determined by the Hummel-Dreyer method. (A) Relationship between the concentration of free vanadium ions and the ratio of bound vanadium per protein. The proteins used in this experiment were recombinant VanabinP (filled circles) and GST (cross). GST was used as the negative control. Logarithmic fitting of the data is shown for VanabinP (solid line) and GST (dotted line). Data points are the means of three or six replicates, and the error bars represent \pm S.D.

(B) Scatchard plot analysis of the results shown in (A). Linear fitting of the data is shown for VanabinP (solid line).