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Relation	

Molecular mechanism of the transport and reduction pathway of vanadium in ascidians

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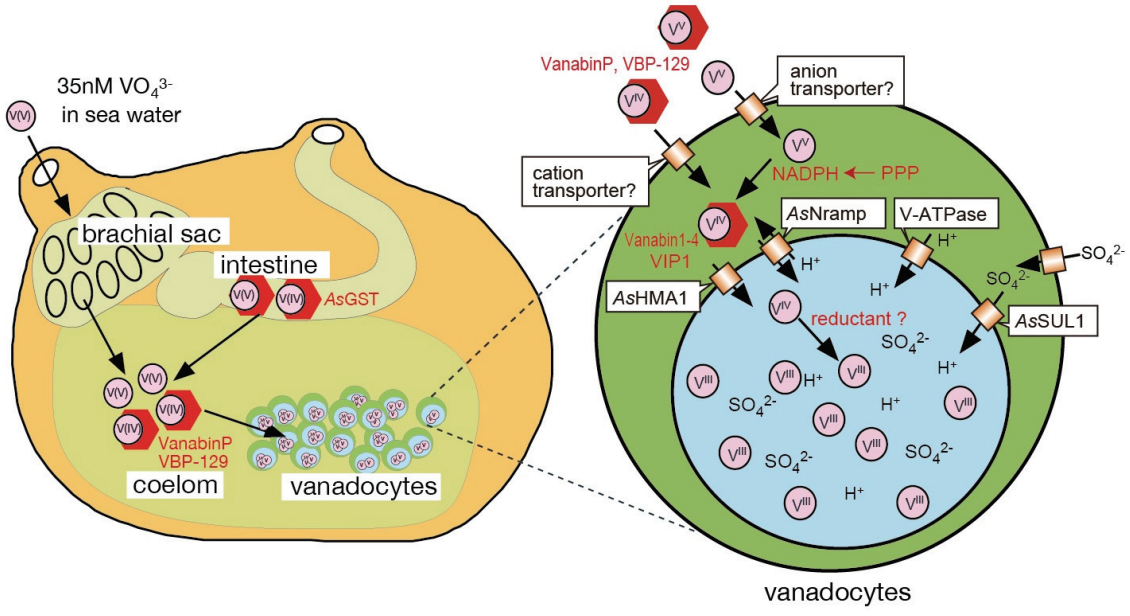
Contents

Abstract

Metal ions are required for physiologically essential functions such as metalloenzymatic reactions, redox reactions, electron transfer, regulation of transcription factor activity, and respiration in living cells. Generally, metal ions are homeostatically maintained at very low concentrations in the sub-micromolar to micromolar range in living cells. However, some organisms, called hyperaccumulators, collect extremely high levels of metal ions, thereby providing experimental systems in which to study the mechanisms underlying the selective accumulation of metal ions. Typical of such organisms are the ascidians, more commonly known as sea squirts or tunicates. They are sessile marine animals belonging to the chordates. Several species of ascidians are known to accumulate extremely high levels of vanadium ions in their blood cells. Vanadium is usually in the V^{V} state in the natural environment, but in ascidians, most of vanadium is reduced to V^{III} via V^{IV} during the assimilation process. In this review, we first summarize the history of the studies on vanadium accumulation in ascidians and then focus on the recent progress of molecular studies, especially on the transport and reduction of vanadium, using primarily two ascidian species, *Ascidia sydneiensis samea* and *Ciona intestinalis*. Several candidate genes for V^{IV} transporters and enzymes catalyzing the redox reactions of V^{V}/V^{IV} are addressed in detail. The function of accumulated V^{III} is discussed in relation to redox reactions.

Graphical Abstract



Keywords

Vanadium; Sulfate; Proton; Ascidian; Redox; Membrane Transporters

Abbreviations

CW, constant-wave; DCT, divalent cation transporter; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; GR, glutathione reductase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate. Nramp, natural resistance-associated macrophage protein, XAS; X-ray absorption spectrometry.

1. Introduction

Metal ions are required for physiologically essential functions such as metalloenzymatic reactions, redox reactions, electron transfer, regulation of transcription factor activity, and respiration in living cells. Inherent to these functions are the incorporation of metal ions from the external environment and their cellular delivery, which are processes dependent on the mechanistic properties, regulation, and chemical specificity of the respective metal ion transporters.

Metal ions in biological systems are generally found as a component of proteins, which are known by the general term "metal-related proteins" or "metalloproteins." Enzymes that contain one or more metal ions at an active site are called "metalloenzymes," which generally harbor one or a few transition metal ions that act as a catalytic center (e.g., dehydrogenase, reductase) [1-3]. Another function of metal ions is as a structural component at a coordination center (e.g., heme protein) [4] or as a structural ligand for amino acid residues in a protein (e.g., Zn finger) [5]. Some proteins carry a few or many metal ions or clusters and function as metal carrier proteins, metal storage proteins or "metallochaperones" (e.g., ferritin, transferrin) [6-8].

Generally, in living cells, metal ions are homeostatically maintained at very low concentrations in the sub-micromolar to micromolar range [9-12]. However, some organisms gather extremely high levels of metal ions and are called "hyperaccumulators"; these organisms provide experimental systems in which to study the mechanisms underlying the selective accumulation of metal ions [13-16]. Typical of such organisms are members of the ascidians, sessile marine animals more commonly known as sea squirts or tunicates, belonging to the phylum Chordata. The ascidians make good model systems for biological metal redox reactions because vanadium is usually in the +5 state (HVO_4^{2-} or $H_2VO_4^-$; V^V) in the natural environment but most of vanadium is reduced to the +3 state (V^{3+}; V^{III}) via the +4 state (VO^{2+}; V^{IV}) during assimilation and hyperaccumulation by the ascidians.

2. Vanadium accumulation in ascidians

2.1. Discovery of high levels of vanadium accumulation

About a hundred years ago, the German physiological chemist Martin Henze discovered high

levels of vanadium in the blood (coelomic) cells of the ascidian *Phallusia mammillata*, collected from the Bay of Naples [17]. His discovery attracted the interdisciplinary attention of chemists, physiologists, and biochemists, in part because of considerable interest in vanadium as a possible prosthetic group, in addition to iron and copper, in respiratory pigments. This would have implied a role for vanadium in oxygen transport, a hypothesis that later proved to be false [18]. After Henze's finding, many chemists looked for vanadium in other species of ascidians.

The concentration of vanadium within the tissues of many ascidians has been determined by neutron activation analysis, electron paramagnetic resonance (EPR), or atomic absorption spectrometry. Ascidians belonging to the suborder Phlebobranchia appear to contain higher levels of vanadium than those of the suborder Stolidobranchia [19]. Of the tissues examined, blood cells contain the highest amounts of vanadium. The highest amount was found in blood cells of the ascidian *Ascidia gemmata* (Fig. 1). The vanadium concentration in this species can reach 350 mM [19, 20], which is 10^7 times the concentration found in seawater (35 nM) [21, 22]. This is thought to be the highest degree of accumulation of a metal in any living organism.

The accumulation of vanadium is also found in the fan worms *Pseudopotamilla occelata* [23] and *Perkinsiana littoralis* [24]. In these fan worms, the concentration of vanadium is as high as 60 mM. Fan worms belong to the phylum Polychaeta, which is phylogenetically distant from Chordata. Unlike the chordates, in fan worms, the highest level of vanadium is found not in blood (coelomic) cells but in the epithelial cells of the branchial crown. We previously reported that the same antigenic signal as Vanabins (see Section 3.2) was found in *P. occelata* [25], but the molecular nature is unclear.

2.2. Identification of vanadocytes

Ascidian blood cells can be classified into 9–11 different types, which are grouped into six categories on the basis of their morphology: hemoblasts, lymphocytes, leukocytes, vacuolated cells, pigment cells, and nephrocytes [26]. The vacuolated cells can be further divided into at least four different types: morula cells, signet ring cells, compartment cells, and small compartment cells.

For many years, morula cells were thought to be the vanadium-containing cells, or vanadocytes [27-30]. These cells have a pale green color resembling that of an aqueous solution of vanadium, and their dense granules, which can be observed under an electron microscope after fixation with osmium tetroxide, were assumed to be vanadium deposits. At the end of the 1970s, scanning transmission electron microscopes with energy dispersive X-ray detectors became available, and the finding was made that the characteristic X-ray due to vanadium was detected not from morula cells, but from granular amoebocytes, signet ring cells, and type-II compartment cells [31-33]. Complementary to the X-ray microanalytical research, the chelating reagent 2,2’-bipyridine, which

is known to complex with vanadium in the +3 oxidation state [34], was successfully used as a V^{III}-specific staining agent to visualize V^{III} in the blood cells of *P. mammillata* and *Phallusia fumigata* [35, 36]. Another analytical approach used was the direct measurement of vanadium in each type of blood cell. Using density-gradient centrifugation to separate the specific types of blood cells, followed by thermal neutron-activation analysis to quantify vanadium in the isolated subpopulations of blood cells, vanadium was found to be accumulated in signet ring cells in *Ascidia ahodori* [37]. However, all of these approaches have limitations due to the freezing/drying steps, specificity, and purity, all of which affect the conclusiveness of the results.

In 2001, we used an X-ray microscope installed at beamline 21 of the European Synchrotron Radiation Facility to visualize vanadium in living blood cells of *P. mammillata* and *Ascidia sydneiensis samea* [38]. Without fixation, freezing or staining, we observed vanadium localized in the signet ring cells and vacuolated amoebocytes of two vanadium-rich ascidian species. A combination of transmission and fluorescence imaging of signet ring cells suggested that the vacuoles of both species contained vanadium. An X-ray beam with an energy of 5.500 keV was used to obtain good yields of vanadium transmission and fluorescence. At this energy, vanadium ions at the +3, +4, and +5 oxidation states were detected simultaneously.

2.3. Vanadium, protons, and sulfate in vanadocytes

Henze [17], in addition to discovering the very high levels of vanadium in the blood cells of ascidians, also reported that the homogenate of the blood cells was extremely acidic [17, 39-41]. This unusual phenomenon attracted the interest of investigators because of the possible role of the highly acidic environment in changing or maintaining redox potentials.

The reported pH values varied considerably. It is probable that one of the reasons for the variation in pH values is that the measurement of pH has been made without fractionation of the population of blood cells. Thus, we designed a study to isolate the acidic blood cells from among several types of blood cell and to examine whether the acidic blood cells were identical to the vanadocytes [20].

From microelectrode measurements of blood cell lysate, and non-invasive ESR measurements on intact cells under anaerobic conditions, we found that ~~A~~ a correlation exists between the concentration of V^{III} ions and the pH within the vacuoles of three *Ascidia* species. In *A. gemmata*, which contains the highest concentration of vanadium (350 mM), the vacuoles have the lowest pH of 1.86. Vacuoles of *A. ahodori*, containing 60 mM vanadium, have a pH of 2.67, and those of *A. sydneiensis samea*, containing 13 mM vanadium, have a pH of 4.20 [20] (Table 1).

In general, vacuolar-type H^+-ATPases (V-ATPases) play a central role in pH homeostasis in various intracellular organelles, including vacuoles, clathrin-coated vesicles, endosomes, lysosomes, Golgi-derived vesicles, multivesicular bodies, and chromaffin granules, which belong

to the central vacuolar system [42-44]. The proton-motive force generated by V-ATPases in these organelles is used to drive transport processes.

By immunocytological analysis, subunits A and B of V-ATPase were found to be localized on the vacuolar membrane of the vanadocytes in *A. sydneiensis samea*, and inhibition of the proton pump by bafilomycin A, which specifically inhibits V-ATPases, resulted in pH neutralization of the vacuoles [45]. The cDNAs for subunits A, B, and C were cloned from *A. sydneiensis samea*, and a functional study by a yeast complementation assay was performed [46, 47]. As a follow-up to these studies, a functional assay to examine whether the protons concentrated by V-ATPase are linked to the accumulation of vanadium should be conducted.

A considerable amount of sulfate has always been found in association with vanadium in ascidian blood cells [31-33, 48-52], suggesting that sulfate might be involved in the biological function and/or the accumulation and reduction of vanadium ions. Table 1 summarizes the results of measurements of sulfate ion concentrations in ascidian blood cells. The concentration of sulfate ions in the blood cells of *A. gemmata* is 500 mM, which suggests that V^{III} (350 mM) and sulfate ions coexist as counterions in the vacuole [53] (the ideal ratio of V^{III} ions to sulfate ions, existing as counterions, is 1:1.5). Frank *et al*. reported the concentration of sulfate ions to be 250 mM in the blood cells of *Ascidia ceratodes* [54]. We determined the sulfate and vanadium concentrations in blood cells (except for giant cells) in *A. sydneiensis samea* to be 38 mM and 86 mM, respectively; although the ratio is not exactly 1:1.5, the concentrations are still within an appropriate range for their existence as counterions [55]. Sulfate ions incorporated in ascidian blood cells exist as complex cations with V^{III} ions, such as $[V^{III}(H_2O)_5(HSO_4)]^{2+}$ [50, 51].

Sulfate ions are very important constituents in biological systems, and sulfate ion transporters are known from a wide range of organisms [56]. Recently, we isolated a homolog of sulfate-ion channels, *As*SUL1, from the blood cells of *A. sydneiensis samea* [55]. Functional analysis of *As*SUL1 revealed that it could act as a sodium-dependent sulfate transporter when expressed in *Xenopus laevis* oocytes (Fig. 2A). Because the sodium concentration in the blood plasma of *A. sydneiensis samea* is very high, one can reasonably suppose that *As*SUL1 is a plasma membrane sulfate transporter in blood cells of the ascidians. The K_m value for sulfate was 1.75 mM (Fig. 2B), which is sufficiently less than the 25 mM concentration of sulfate ions in the blood plasma of *A. sydneiensis samea* [55] so that *As*SUL1 should be functional as an active transporter. Considering these observations, we hypothesize the accumulation pathway of vanadium, sulfate, and protons in ascidians to be as depicted in Fig. 3.

In general, the cytoplasm is the major compartment in the cell where metal ions exert their physiological functions. The concentration of free metal ions in the cytoplasm is strictly regulated, primarily by membrane transporters involved in uptake and export. Metal-binding proteins can

absorb excess metal ions to control the free metal ion concentration, or to stabilize metal ions, in the cytoplasm. Compartmentalization of metal ions into organelles decreases the concentration of free metal ions in the cytoplasm and also acts as a reservoir of metal ions, without affecting the physiological function of the ions in the cytoplasm.

In vanadocytes, the concentration of vanadium is extremely high, and most of the vanadium is reduced to V^{III}. V^{III}-aqua ions are only stable at low pH, and the acidic vacuole is the most suitable compartment for storage of V^{III} ions. V^{V} ions outside the cell are transported into the cytoplasm and reduced to V^{IV}. V^{IV}-aqua ions are also stable at low pH, but the cytoplasm is not. Therefore, V^{IV}-binding proteins must exist in the cytoplasm to absorb and stabilize V^{IV} ions at physiological pH. Reducing agents must participate in the accumulation of vanadium in vanadocytes, since the reduction of V^{V} to V^{IV} and/or V^{IV} to V^{III} is presumed to accelerate this accumulation.

3. Vanadium transport and reduction

3.1. Transport of V^{V} into the cytoplasm

As Dingley et al. [57] pointed out, the phosphate anion transporter is a candidate for the specific transporter of V^{V} ions, since the chemical features of V^{V} ions (HVO^{2-} or $H_2VO_4^-$) resemble those of phosphate anions (HPO_4^{2-} or $H_2PO_4^-$). We are currently attempting to identify homologs of NaP_i- or P_iT-type phosphate transporters in blood cells and other tissues of *A. sydneiensis samea*. Such anion transporters may be involved in the first step(s) of vanadium uptake in the branchial sac or the intestine.

Another possible pathway of V^{V} ion transport is a transferrin (Tf)–transferrin receptor (TfR)-like pathway, as is known for iron homeostasis in mammals [11, 58]. Tf–TfR-mediated endocytosis is the major pathway by which all cells take up Fe^{III} ions, which is released from transferrin in the acidified endosome. The released Fe^{III} ions are reduced to Fe^{II} by a member of the STEAP metalloreductase family (STEAP3 in the erythroblast) and transported out of the endosome by Nramp/DCT/DMT [59].

In mammals, transferrin is reported to transport V^{IV} ions. The average vanadium concentration in human blood serum is 200 nM, with most of the vanadium associated with the plasma fraction, while that in tissues reaches 6 μM [61]. The first report of V^{IV} binding to human transferrin was by Cannon and Chasteen [62], and more recent work has extended the studies on V^{V}–Tf interactions [63]. Also, the distribution of insulin-mimicking vanadium compounds in blood plasma are extensively studied in relation to their interaction with Tf [64, 65].

Transferrin homologs have already been cloned from three ascidian species, *A. sydneiensis*

samea [60], *Halocynthia roiretzi* [66], and *Ciona intestinalis* [67], although TfR gene is not found in ascidians [68]. Functional analyses suggested that *H. roretzi* transferrin binds to Fe^{III} [66], while one of the two *C. intestinalis* transferrin binds to both Fe^{III} and V^{V} [69-71]. These transferrins are monolobal transferrins that are unique to ascidians.

In *A. sydneiensis samea*, two vanadium-binding proteins that are expressed in blood plasma have been identified. One is a member of the Vanabin family, VanabinP. Recombinant VanabinP binds a maximum of 13 V^{IV} ions per molecule, with a K_d of 2.8×10^{-5} M [72]. Another one is VBP-129, which binds to V^{IV} ions, as well as to Fe^{III}, Co^{II}, Cu^{II}, and Zn^{II} ions [73]. The mechanisms by which VanabinP and VBP-129 capture vanadium in the blood plasma and subsequently transfer the vanadium ions are the next problems to be resolved.

3.2. Reduction of V^{V} to V^{IV} in the cytoplasm

NADPH is a strong candidate for the reductant involved in the conversion of V^{V} to V^{IV} because the enzymes involved in the pentose phosphate pathway are expressed exclusively in the cytoplasm of vanadocytes in *A. sydneiensis samea* [74-77]. Detailed *in vitro* studies have suggested that the reduction of V^{V} to V^{IV} occurs with the assistance of several chelating substances [78, 79]. Biologically relevant thiols, such as glutathione (GSH) and cysteine, are also candidates for the direct reductant of V^{V} to V^{IV} [80].

Recently, we revealed that the vanadium-binding protein, Vanabin2, catalyzes the reduction of V^{V} to V^{IV} in the presence of NADPH, glutathione reductase (GR), and GSH [81]. Without Vanabin2, the reduction proceeds at a very slow rate. Rapid reduction was observed after the addition of 4 μM Vanabin2 to 0.1–10 mM V^{V} in the presence of 2 mM GSH, 200 μM NADPH and 0.25 U/ml GR (Fig. 4).

Vanabin2 was first isolated from *A. sydneiensis samea* as a novel vanadium-binding protein by anion exchange column chromatography [82]. In this species, the Vanabin family consists of at least five closely related proteins, Vanabins 1–4 and VanabinP [72, 83, 84]. All of these five Vanabins possess 18 cysteine residues, and the intervals between cysteines are very well conserved (Fig. 5A). *In vitro* studies indicate that nine disulfide bonds are partially cleaved by 1–4 mM GSH, forming intermediate structures [81]. This suggests that electrons may be transferred from the donor NADPH to the acceptor V^{V} ions via thiol–disulfide exchange reactions in Vanabin2. The resultant disulfides in Vanabin2 are converted to thiols by reduced GSH, and the oxidized GSH is re-reduced by GR. The disulfides of GR are reduced to thiols by NADPH [85], which is linked to the pentose phosphate pathway. Since the concentration of GSH is 1.83 mM in vanadocytes of *A. sydneiensis samea* [81], the existence of a redox cascade from NADPH to V^{V} ions in ascidians is likely.

In our model, V^{V} ions are readily reduced to V^{IV} in the cytoplasm, and V^{IV} ions are stabilized by Vanabins, which act as both vanadium reductases and vanadium chaperones. Two of the cytoplasmic Vanabins in *A. sydneiensis samea*, Vanabin1 and Vanabin2, bind up to 10 and 20 V^{IV} ions, with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively, in Tris buffer at pH 7.4 and 20°C [83]. NMR titration experiments showing the ^{1}H–^{15}N heteronuclear single-quantum coherence (^{15}N HSQC) spectra of Vanabin2 upon the gradual addition of V^{IV} ions revealed the putative vanadium-binding sites on Vanabin2 [86]. The resonances of 32 residues were quenched or disappeared, implying that these residues are located near the V^{IV} interaction sites (Fig. 5B).The locations of the residues involved in the V^{IV} interaction sites agree well with the positively charged areas in the electrostatic potential plot, where basic residues are gathered. X-band CW EPR studies revealed that the V^{IV}–Vanabin2 complex invariably exhibits a usual mononuclear-type V^{IV} signal up to a 20:1 molar ratio of V^{IV}:Vanabin2, and no allosteric effects were observed in the process of binding multiple vanadium ions [87]. A two-pulse ESEEM study suggested that most EPR-active V^{IV} ions have amine nitrogen ligands. This suggests that all or almost all amine nitrogens are used in the vanadium-saturated Vanabin2 because the EPR-active vanadium number is 20, while Vanabin2 has 15 amine nitrogens (14 lysines and one N-terminal amine).

The mechanism of metal selectivity in Vanabins has not been determined, although the effects of acidic pH on selective metal binding and on the secondary structure of Vanabin2 was studied [88]. Using immobilized metal ion affinity chromatography (IMAC), Vanabin2 was shown to selectively bind V^{IV}, Fe^{III}, and Cu^{II} ions under acidic conditions. In contrast, Co^{II}, Ni^{II}, and Zn^{II} ions were bound at pH 6.5 but not at pH 4.5. Changes in pH had no detectable effect on the secondary structure of Vanabin2 under acidic conditions, as determined by CD spectroscopy. Little variation in the dissociation constant for V^{IV} ions was observed in the pH range 4.5–7.5, suggesting that the binding state of the ligands is not affected by acidification. Taken together, these results suggest that the reason for metal ion dissociation upon acidification is not because of a change in secondary structure but rather because of protonation of the amino acid ligands that complex with the V^{IV} ions [88].

Site-directed mutagenesis of Vanabin2 has been performed to reveal the functional contribution of each binding site. We prepared several mutants of Vanabin2 by *in vitro* site-directed mutagenesis and analyzed their metal selectivity and affinity by IMAC and the Hummel–Dreyer method [89]. Mutation at K10/R60 (Site 1) markedly reduced the affinity for V^{IV} ions. Mutation at K24/K38/R41/R42 (Site 2) decreased the maximum binding number, but only slightly increased the overall affinity for V^{IV} ions. Mutation of disulfide bonds near Site 1 did not affect the protein's high-affinity binding capacity, while those near Site 2 decreased the overall affinity for V^{IV} ions. These results suggest that Site 1 is a high-affinity binding site for V^{IV} ions, while Site 2 is a moderate affinity site for multiple V^{IV} ions (Fig. 5B). Therefore, we propose that V^{V} is reduced to

V^{IV} by thiol–disulfide exchange reactions using cytoplasmic Vanabins as reductase, with the resultant V^{IV} ions bound to the Vanabins and stabilized in the cytoplasm.

Vanabin2 is a very abundant protein in the cytoplasm of vanadocytes in *A. sydneiensis samea*. Recently, we extensively examined the sequence variation of Vanabin2 and identified five variants of Vanabin2 [90]. 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 were conserved. Using IMAC, we found that Vanabin2 and the most divergent one of the five variants, named 14MT, which differs from Vanabin2 at all 14 positions, have the same metal ion selectivity [90]. These results suggest that sequence variations have been under strict evolutionary constraints, with the high-affinity binding sites for V^{IV} ions conserved among all the Vanabin2 variants. We speculate that gene duplication of Vanabin2 may account for the very high degree of vanadium accumulation.

A homology search of public DNA and protein databases, using both the Vanabin1 and Vanabin2 amino acid sequences, revealed no proteins with striking similarities, other than from two ascidian species, *Ciona intestinalis* and *Ciona savignyi*. We identified five Vanabins (*Ci*Vanabin1 to *Ci*Vanabin5) from *C. intestinalis* [91], the whole genome of which has been sequenced [92]; we have reported that these *Ci*Vanabins bind V^{IV} ions. Two of them, *Ci*Vanabin1 and *Ci*Vanabin2, have a long C-terminal domain, the function of which is still unclear, that is rich in aspartic and glutamic acids. We have also found five Vanabin homologs from the related species *C. savignyi*, although its genome assembly is still incomplete (http://www.broad.mit.edu/annotation/ciona/index.html). In addition, expressed sequence tag (EST) analysis yielded several Vanabin homologs in *A. gemmata*, which accumulates the highest levels of vanadium ions (Samino *et al*., unpublished data). Vanabins therefore seem to be ubiquitous among the vanadium-accumulating ascidians and may hold the key to resolving the mechanism underlying the highly selective accumulation of vanadium ions.

3.3. Transport of V^{IV} into the vacuole

As described in Section 2.3, the pH of the vanadocyte vacuole is kept extremely low by V-ATPase. We hypothesized that a proton gradient generated by V-ATPase provides the energy to transport vanadium across the vacuolar membrane. To test this hypothesis, we cloned a cDNA encoding a member of the Nramp/DCT1 family from vanadocytes of *A. sydneiensis samea* because the Nramp/DCT1 family is known to transport a broad range of divalent cations (Fe^{II}, Cu^{II}, Zn^{II}, Mn^{II}, Cd^{II}, Co^{II}, Ni^{II}, and Pb^{II} ions) across the membrane using a proton gradient as the motive force [93, 94]. We found that the product of this gene, *As*Nramp, is localized on the vacuolar membrane and operates as an antiporter of V^{IV} ions and H^+ ions. In contrast, a rat homolog of Nramp/DCT, rDCT1, could not transport V^{IV} under any of the conditions examined (Ueki *et al*., unpublished

data). The results of this study indicated that *As*Nramp is a V^{IV}/H^{+} antiporter expressed on the vacuolar membrane of vanadocytes. These findings support the proposed model holding that a proton electrochemical gradient generated by V-ATPase is the driving force for V^{IV} transport from the cytoplasm into the vacuole.

The direction of transport and metal selectivity is well studied for mammalian Nramps. Nramp2 is reported to be a proton/divalent cation symporter that uses a proton electrochemical gradient as an energy source. Transport studies using *Xenopus* oocytes or mammalian cell lines indicated that Nramp2 transports a broad range of divalent cations, including Fe^{II}, Zn^{II}, Cd^{II}, Mn^{II}, Cu^{II}, and Co^{II}, but not Ca^{II} or Mg^{II} ions, in a pH-dependent manner [95]. In contrast, the direction of proton/metal transport by Nramp1 is controversial. One study used *Xenopus* oocytes to examine metal transport by Nramp1 and revealed that Nramp1 acts as an antiporter for protons and the divalent cations Fe^{II}, Zn^{II}, and Mn^{II} ions [96]. The antiport activity and vacuolar localization of *As*Nramp are similar to that of mammalian Nramp1.

Another candidate protein for vanadium transport is a P-type metal ATPase, which is able to import or export heavy metal ions into cellular organelles. P-type ATPases are classified into 12 types, depending on the primary amino acid sequence and substrate specificity [97, 98]. The heavy metal-transporting P_{1B}-type ATPases (metal ATPases) typically possess one to six metal-binding domains (MBDs) in the N-terminal region of the protein. These MBDs allow the transport of monovalent and divalent transition metal ions such as Cu^{I}, Cu^{II}, Co^{II}, Zn^{II}, Cd^{II}, and Pb^{II} ions, either into cellular organelles or out of the cell [99]. We recently cloned the cDNA for a P_{1B}-type ATPase, *As*HMA1, from blood cells of *A. sydneiensis samea* and are now examining its metal selectivity and transport activity (Ueki *et al*., unpublished data).

3.4. Reduction of V^{IV} to V^{III} in the vacuole

In ascidians, V^{IV} is further reduced to V^{III}. Several candidates have been suggested as reductants of V^{IV} [100]. Among them, the potential biologically relevant reducing agents include cysteine complexes. Frank *et al.* reported that large quantities of intracellular sulfate, as well as aliphatic sulfonic acids such as cysteic acid, are present in ascidian blood cells [49, 50]. Since cysteic acid is an oxidation product of cysteine, cysteine may be the reducing agent responsible for the reduction of V^{IV} to V^{III} in ascidians. If cysteine does function as a reducing agent in ascidians, some biogenic ligands promoting the reduction of V^{IV} are expected to participate in the reaction. Although cysteine by itself cannot reduce V^{IV} to V^{III}, cysteine methyl ester was reported to reduce V^{IV} to V^{III} with the assistance of EDTA and EDTA-like ligands of aminopolycarboxylate in water [101]. Glycylhistidine and glycylaspartic acid are two biological ligands that were shown to assist the reduction of V^{IV} to V^{III} by cysteine methyl ester [79].

XAS studies have suggested the reduction of V^{V} to V^{III} in a single coordination environment [102, 103]. A new complexed form of $[V^{IV}O(EDTA)]^{-}$ was observed, with an increase in the amount of $[V^{III}(H_2O)_6]^{3+}$ in blood cells of *A. ceratodes* exposed to DTT plus $V^{IV}O^{2+}$ ions. The proposal was made that two reduction steps from V^{V} to V^{III} can occur sequentially in an EDTA-like 7-coordinate ligand array under mild cytoplasm-like conditions of an aqueous pH 6.5 solution. This model is suggestive of the reaction mechanism and the structure of the active center of vanadium reductase. Glutamic acid and aspartic acid residues of Vanabins could provide such a coordination environment, although our model (Section 3.2) based on the identification of *As*Nramp is apparently inconsistent with the model. Frank et al. [103] suggested the presence of V^{III} transporter on the vacuolar membrane, and search for such transporter is necessary to substantiate the model.

3.5. Regulation of intracellular vanadium via the glutathione pathway

The draft genome of an ascidian species, *C. intestinalis*, has been read and inferred to contain approximately 16,000 protein-coding genes. The cDNAs for transcripts of 13,464 genes have been characterized and compiled as the "*Ciona intestinalis* Gene Collection Release I" [92, 104, 105]. This species is known to accumulate up to 0.6 mM vanadium in its blood cells [19] and is therefore a good model organism to study vanadium accumulation.

We recently performed microarray experiments to identify genes that are regulated by excess vanadium ions in *C. intestinalis*. The overall change in gene expression was similar between V^{IV}- and V^{V}-treated individuals, but the responses were not identical. Among the major metabolic pathways, expression of enzymes in the glutathione-related pathway was significantly affected by V^{IV}-treatment. This response was further examined using reverse-transcription polymerase chain reaction (RT-PCR) from the perspective of redox activity and accumulation of metal ions in each tissue (Kume *et al*., unpublished data). Excess vanadium ions could induce the glutathione-related pathway in *C. intestinalis* to stabilize the vanadium concentration in tissues or cells. Another possible regulator of vanadium ion concentration is the GS-X pump, which plays an important role in the excretion or compartmentalization of excess metal ions, such as Cd^{II} or Pt^{II} [106, 107]. The vanadyl–glutathione conjugate could be a possible target of the export pathway [108, 109]. Adamis *et al*. [110] reported that Cd^{II} activates γ-GT and glutathione transferase2 (GST2) in yeast and that this activation is necessary for the regulation of cytoplasmic Cd^{II} levels, the recycling of glutamate, and regeneration of GSH. Similar mechanisms could act in *C. intestinalis* to regulate the concentration of vanadium in each tissue.

4. Function of accumulated V^{III}

Although the unusual phenomenon whereby some ascidians accumulate vanadium to levels

more than 10 million times higher than those in seawater has attracted researchers in various fields, the physiological role(s) of vanadium remains to be explained. Endean [27, 111, 112] and Smith [113, 114] proposed that the cellulose of the tunic might be produced by vanadocytes. Carlisle [115] suggested that vanadium-containing vanadocytes might reversibly trap oxygen under conditions of low oxygen tension. However, no direct evidence exists in support of these hypotheses [18]. Also, vanadium was proposed to protect ascidians from fouling or predation, or act as an antimicrobial agent [116-118]. Antifouling and anti-predation are the most relevant physiological function of acid contained in the tunic, but the contribution of vanadium for these functions is not conclusive [119, 120].

Since we have suggested that Vanabin2 catalyzes a redox cascade connecting NADPH to V^{V}/V^{IV}, one should consider whether the reverse reactions, accompanying the oxidations of V^{III} to V^{IV} and V^{IV} to V^{V}, occur in the proposed cascade, perhaps resulting in the release of energy, just like the vanadium redox flow battery [121]. Thus, the ascidians may accumulate the metal ions as an energy source.

A more comprehensive survey for the function of vanadium could be achieved by mutational analysis to find an ascidian strain that cannot accumulate vanadium. In *C. intestinalis*, such an approach is available by using transposon mutagenesis [122-124].

5. Conclusion and perspectives

The accumulation, reduction, and transport of vanadium ions are expected to be tightly linked. The ascidians that accumulate extremely high levels of vanadium in a reduced form, V^{III} make good model systems. The physiological function of accumulated vanadium ions in a reduced form, V^{III}, is presumably exerted through redox-coupled reactions. An analysis of vanadium reductase and vanadium transporters should provide the key to understand the function of vanadium in ascidians. The study of vanadium assimilation in ascidians should also provide a fundamental insight into the physiological roles of vanadium, not only in ascidians but also in mammals, in which vanadium is an essential element [125].

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Table 1. Correlations among vanadium concentration, pH, and sulfate in ascidian blood cells.

Species	tissue	vanadium	pH	sulfate	ref.
Ascidia gemmata	whole blood cells	350 mM	1.86	500 mM	[20]
Ascidia ahodori	whole blood cells	60 mM	2.67	N. D.	[20]
Ascidia sydneiensis samea	whole blood cells	13 mM	4.20	N. D.	[20]
	blood cells without giant cells	38 mM	N. D.	86 mM	[55]
Ascidia ceratodes	whole blood cells	99 mM	1.8	0.25 M	[54]

Figure Legends

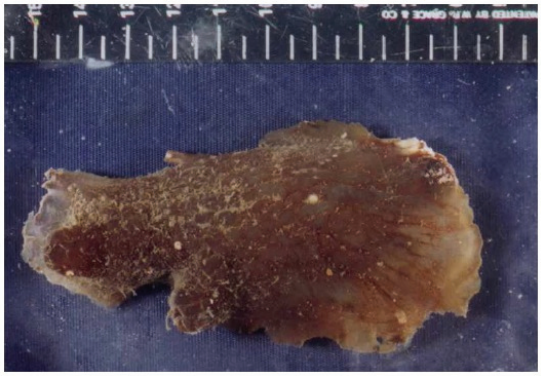

Fig. 1. *Ascidia gemmata*, the most vanadium-rich ascidian species found so far. A specimen collected at Mukaishima Marine Biological Laboratory, Hiroshima, Japan.

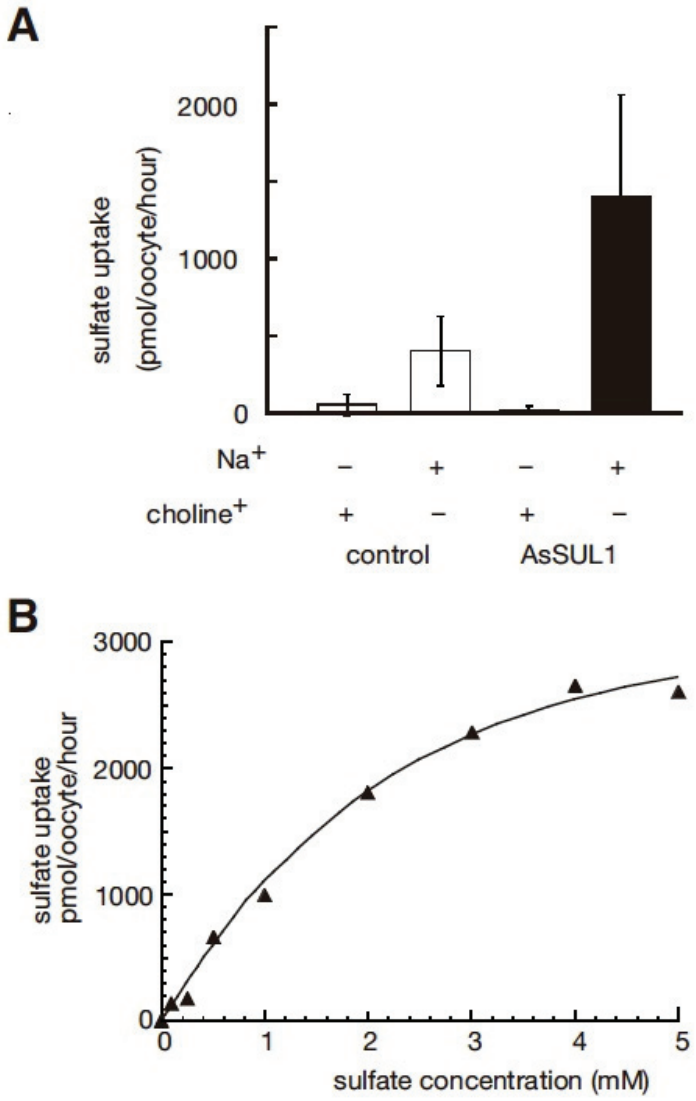

Fig. 2. Specificity of *As*SUL1 expressed in *Xenopus* oocytes. (A) Na^+ dependency of sulfate uptake by *As*SUL1. Oocytes were injected with (solid bars) or without (white bars) *As*SUL1 mRNA and incubated for 3–5 days before the uptake experiment. Sulfate uptake was examined in the presence (+) or absence (–) of 100 mM sodium chloride or choline chloride at 21°C. The initial sulfate concentration was 1 mM. (B) Kinetic properties of *As*SUL1. Sulfate uptake was measured with increasing concentrations of sulfate in the uptake buffer containing 100 mM NaCl. Each data point represents the mean value for three experiments. The K_m and V_{max} of *As*SUL1 were 1.75 mM and 2500 pmol/oocyte/h, respectively. Reproduced from [55] with permission from the copyright holders.

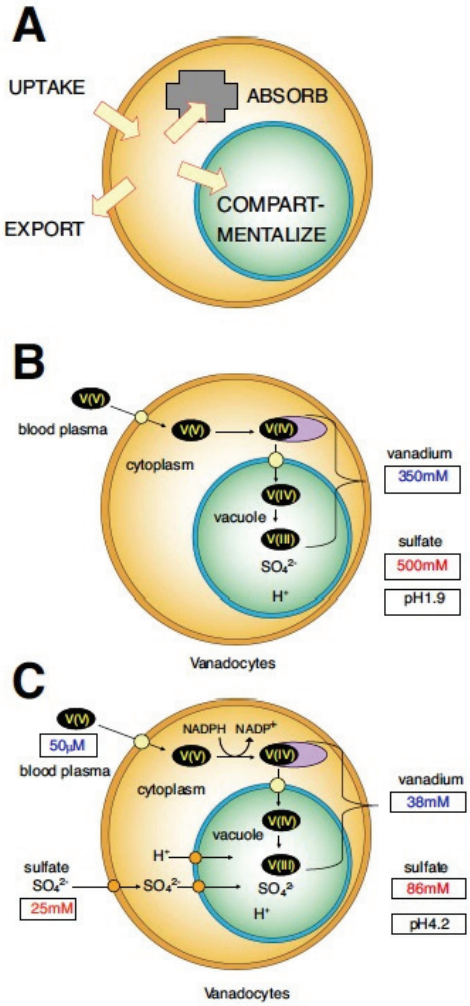

Fig. 3. (A) General scheme of homeostatic regulation of concentrations of metal ions and other substances in a cell. (B, C) Putative pathway of vanadium accumulation and the concentration of vanadium, sulfate, and protons in *Ascidia gemmata* (B) and *A. sydneiensis samea* (C).

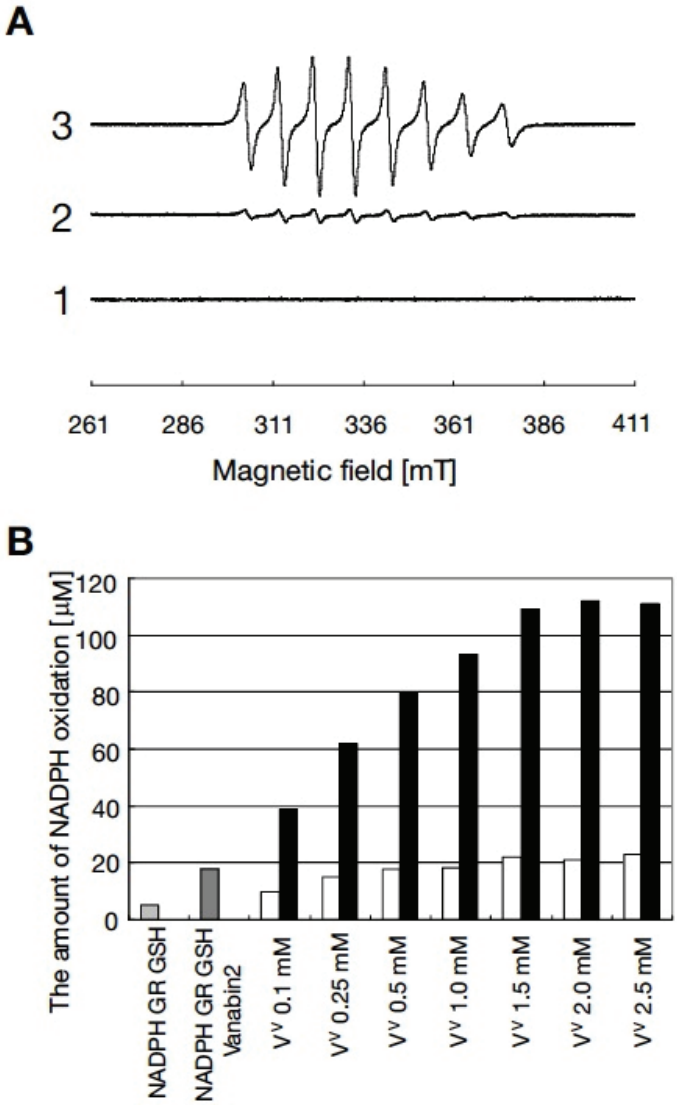

Fig. 4. (A) V^V reduction by Vanabin2 observed by EPR spectrometry. Spectrum 1, 1 mM V^V; Spectrum 2, addition of 2 mM GSH to 10 mM V^V 24 h after incubation at room temperature; Spectrum 3, after 24 h incubation of 10 μM Vanabin2, with a reaction mixture of 10 mM V^V and 2 mM GSH at room temperature. The signal intensities are given as relative values. (B) V^V reduction by Vanabin2 observed by coupled NADPH oxidation assay. The vertical axis indicates the level of NADPH oxidation after 60 min. Light gray bar, the amount of NADPH oxidation in the absence of both V^V and Vanabin2; dark gray bar, that in the presence of 4 μM Vanabin2; white bars, that in the presence of 0.1–2.5 mM V^V; black bars, that in the presence of both 4 μM Vanabin2 and 0.1–2.5 mM V^V. Reproduced from [81] with permission from the copyright holders.

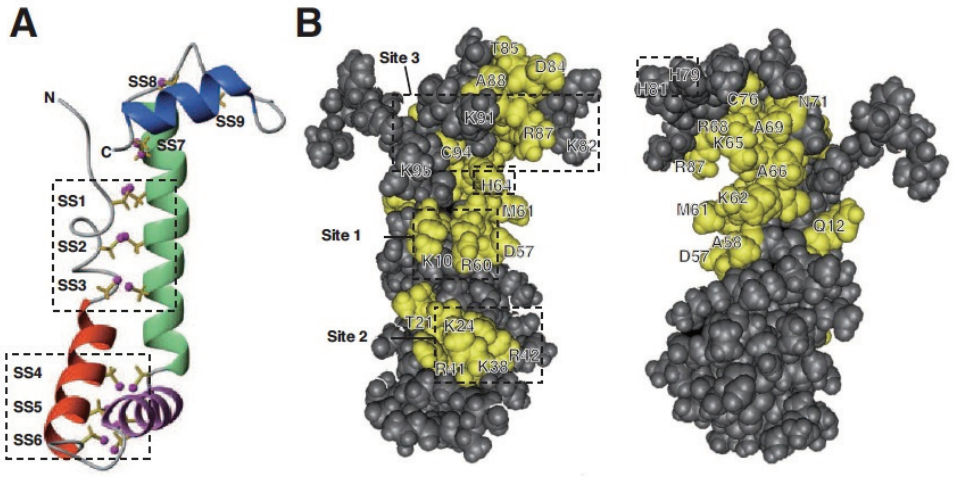

Fig. 5. Three-dimensional structure of Vanabin2 (PDB ID: 1vfi) and the mutated sites in ref. [89]. (A) Ribbon model of Vanabin2. Nine disulfide bonds are indicated (SS1–9). The cysteine residues in two sites were mutated (shown by dotted rectangles). (B) Spherical model of Vanabin2. Possible V^{IV}-binding sites are shown in yellow and are denoted by amino acid numbers on the two faces, looking in opposite directions [86]. The three boxed residues were mutated in ref. [89]. Reproduced from the original color figure for [89] with permission from the copyright holders.