Vanadium accumulation in ascidians: A system overview

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Abstract

Several families of ascidians are known to accumulate extremely high levels of vanadium in their blood cells. The concentration of vanadium has been determined in each species; the highest concentration, found in *Ascidia gemmata*, reaches 350 mM, corresponding to 10^7 times that of sea water. How and why ascidians accumulate vanadium in a highly selective manner and at such extremely high levels have yet to be determined. To address these questions, our research group sought to identify the genes and proteins responsible for the accumulation and reduction of vanadium in vanadocytes, a type of blood cell, as well as the process of vanadium transport from sea water to blood cells through the branchial sac, intestine, and blood plasma. Here, we review the accumulation steps as a system, especially those related to the concentration and chemical species of vanadium at each step. A comprehensive analysis on each organ has already revealed several categories of protein families, such as vanadium-binding proteins and vanadium transporters. Herein, we also discuss the mechanisms by which ascidians selectively accumulate vanadium ions from a biochemical viewpoint.

Keywords: Vanadium; Ascidian; Metal-Binding Proteins; Membrane Transporters; Reductase.

Abbreviations: AAS, atomic absorption spectrometry; CW, constant-wave; DCT, divalent cation transporter; DTT, dithiothreitol; EPR, electron paramagnetic resonance; EST, expressed sequence tag; FISH, fluorescence *in situ* hybridization; GR, glutathione reductase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; IMAC, immobilized metal ion affinity chromatography; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; Nramp, natural resistance-associated macrophage protein; OTU, operational taxonomic unit; V-ATPase, vacuolar H^+-ATPase.

Contents

1. Introduction

Several families of ascidians (sea squirts or tunicates) are known to accumulate extremely high levels of vanadium in their blood cells. The concentration of vanadium has been determined in each species; the highest concentration, in *Ascidia gemmata*, was 350 mM, corresponding to 10^7 times that of sea water. How and why ascidians accumulate vanadium in a highly selective manner and at such extremely high levels have yet to be determined. To address these questions, our research group sought to identify the genes and proteins responsible for the accumulation and reduction of vanadium in vanadocytes, a type of blood

cell, as well as the process of vanadium transport from sea water to blood cells through the branchial sac, intestine, and blood plasma.

In this review article, we first briefly summarize previous studies on vanadium accumulation in ascidians. Next, we overview the accumulation steps as a system, especially those related to the concentration and chemical species of vanadium at each step. How an ascidian system takes up vanadium selectively is also discussed from biochemical properties of proteins responsible for each step. A more comprehensive review of the molecular mechanism for each step of accumulation was published in a previous issue of this journal [1].

2. Prior studies on vanadium accumulation in ascidians

Ascidians are marine sessile invertebrate animals belonging to Urochordata, Phylum Chordata. At the larval stage, the body plan is quite well-conserved with other chordates, but after metamorphosis, it is drastically reconstructed and takes on a unique shape (Fig. 1). Reflecting their phylogenetic position, they are good models for studying genomic evolution, early development, as well as the immune and nervous systems [2,3]. A cosmopolitan species, *Ciona intestinalis*, is an extremely good model; it was the seventh animal species whose whole genome was sequenced [4], and millions of expressed sequence tag (EST) data have been deposited in public databases [5-7]. In contrast, they possess unique features that are not found in other chordates, including cellulose synthesis [8-12], asexual reproduction [13,14], and metal accumulation [15-18].

Approximately 100 years ago, the German physiological chemist Dr. Martin Henze discovered high levels of vanadium in the blood (coelomic) cells of the ascidian *Phallusia mammillata* collected from the Bay of Naples, Italy [19]. 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 [20].

The concentration of vanadium within tissues of many ascidians has been determined by neutron activation analysis, EPR, or AAS. Ascidians belonging to the suborder

Phlebobranchia contain higher levels of vanadium than those of the suborder Stolidobranchia [21] (Fig. 2). Of the tissues examined, blood cells contained the highest amount of vanadium. The greatest concentration was found in blood cells of the ascidian *Ascidia gemmata*, at up to 350 mM [21,22], which is 10^7 times that in seawater (35 nM) [23,24]; this is believed to be the highest degree of accumulation of a metal in any living organism.

Ascidian blood cells can be classified into 9–11 types, mainly on the basis of their morphology [25]. Morula cells were long believed to be vanadium-containing cells, or vanadocytes. However, by direct measurement of vanadium in each blood cell type, it was determined that morula cells did not accumulate vanadium; rather, signet ring cells and some vacuolated cells are true vanadocytes [26,27]. A more detailed history is provided in previous reviews [1,15,28,29].

Dr. Henze, in addition to discovering very high levels of vanadium in blood cells, also reported that blood cell homogenate is extremely acidic and contains high levels of sulfur [19,30-32]. Reported pH values varied considerably in early studies. It is probable that one of the reasons for the variation in pH values is that the measurement was made without fractionation of the population of blood cells. Thus, an elegant study was designed to isolate acidic blood cells among several types of blood cell to determine whether acidic blood cells were identical to vanadocytes [22]. Microelectrode measurement of blood cell lysates and non-invasive EPR measurements on intact cells under anaerobic conditions revealed a correlation between the concentration of V^{III} ions and pH within the vacuoles of three *Ascidia* species [22,26]. Sulfate concentration was also measured by Raman spectroscopy in two *Ascidia* species [33,34]. Analytical and EPR spectral measurements have been carried out in vanadocytes from *Ascidia ceratodes* [35] to reveal vanadium, proton and sulfate concentrations. Reported values are summarized in Table 1.

3. How do intestinal cells absorb vanadium?

Vanadium is a multivalent transition metal. Under physiological aqueous conditions, vanadium ions are limited to the +3, +4 and +5 oxidation states [36]. In nature, vanadium is usually in the +5 state (HVO_4^{2-} or $H_2VO_4^-$; V^V) [37], but the majority is reduced to the +3 state (V^{3+} ; V^{III}) via vanadyl ions in the +4 state (VO^{2+} ; V^{IV}) during assimilation and

hyperaccumulation by ascidians [38-40]. Thus, the first questions to consider are how and where ascidians absorb vanadium as V^V anions.

The chemical features of V^V ions at low concentration resemble those of phosphate anions (HPO_4^{2-} or $H_2PO_4^-$; P^V) [16,41]. Figure 3 shows a set of speciation diagrams at high ionic strength (0.45 M) simulating the situation in sea water calculated by Visual MINTEQ ver. 3 based on MINTEQA2 [42]. The speciation is a function of both pH and concentration, and to a lesser extent, ionic strength. At low concentration (1 μM), both V^V and P^V behave as monomeric anions (Fig. 3B, D). The protonated state of V^V and P^V anions are somehow different at each pH. Phosphate anions exist as monomers at high concentration (1 mM) (Fig. 3C), although V^V anions form dimeric, tetrameric and decameric species in a pH-dependent manner at the same concentration (Fig. 3A).

Vanadium absorption by ascidians was first experimentally demonstrated by Goldberg et al. [43]. Two ascidian species, *Ascidia ceratodes* and *Ciona intestinalis*, were administered to individuals with radioactive ^{48}V as a form of vanadate, and the remaining amount of vanadium was measured in culture medium. Radioautographic analysis indicated that vanadium is primarily found in the ovary, gut wall, eggs, and branchial basket. They also observed that a high concentration of phosphate ions competed with vanadium for uptake. Thereafter, Dingley et al. pointed out that the phosphate anion transporter is a candidate for the specific transport of vanadate anions, since influx of vanadate into cells is a rapid process that can become saturated (K_m = 1.4 mM); phosphate also competes with vanadate for transport, and is taken up by the cell [44]. However, in contrast to Goldberg's experiments, in which phosphate ions did not completely inhibit the uptake of vanadate ions, Kalk pointed out the possibility of uptake of V^{IV} ions complexed with sulfate in mucosa [45]. Thus, both direct absorption of V^V and indirect absorption of V^{IV} must be considered.

3.1. Phosphate transporters

Phosphate anions are involved in a large number of biochemical processes and contribute to the structure of nucleic acids, proteins, phospholipids, and the energy molecule adenosine triphosphate (ATP), as well as signaling or structural modifications, such as phosphorylation. Bacteria, yeast, plants, and animals have developed their own strategies to control phosphate

homeostasis using different membrane transporters [46-49].

For example, in unicellular organisms, when *Saccharomyces cerevisiae* cells were exposed to high concentrations of V^V (1 or 5 mM), V^V became cell-associated and was converted to V^{IV}, which then accumulated in the culture medium with time [50], suggesting the existence of a V^V uptake system and V^{IV} export system on the plasma membrane. Two systems have been shown to be involved in the active transport of phosphate into *S. cerevisiae* cells from the medium [51], one of which is a high affinity derepressible system (K_m = 8.2 μM), and the other, a low-affinity system (K_m = 770 μM). PHO84 and PHO89 have been identified as the high- and low-affinity phosphate transporters, respectively [52,53]. Although phosphate transporters are believed to be the key players in arsenate (As^V) transport in yeast [54], to our knowledge, there is no evidence to act as a V^V-transporter. Two phosphate transporters were identified in the most common laboratory bacterium, *Escherichia coli* [55,56], but there has been no biochemical analysis of vanadium transport in this species.

One of the most extensively investigated unicellular species is the filamentous fungus, *Neurospora crassa*. This species has two phosphate transport systems: V^V is taken up by a high-affinity phosphate transport system II (K_m = 0.62 μM) [57], whereas activation of the vanadate uptake system (K_m = 8.2 μM; V_{max} = 0.15 mmol/min per liter cell water) occurs under conditions identical to those required for derepression of a high-affinity phosphate transport system II. The *van* mutants are unable to take up vanadate from the medium and are also deficient in the uptake of phosphate via a derepressible, high affinity phosphate transport system II [58].

In vertebrates, the mechanism by which cells reduce cytoplasmic V^V to V^{IV} was first investigated using the human erythrocyte as a model system [59]. The uptake of V^V by erythrocytes occurs in two phases: an initial fast phase representing equilibration across the membrane via the anion-exchange system, and a subsequent slower phase. Formation of V^{IV} parallels the slow phase of uptake, suggesting that reduction is the rate-limiting step in the process. In vertebrates, including humans, three types of Na^+-dependent phosphate transporter, NaPi-I, NaPi-II, and NaPi-III, have been identified [60,61]. These transporters are classified into two large groups, the slc34 and slc20 families, depending on their amino acid sequence similarity [60,61]. However, no study of the direct contribution of phosphate transporters to

vanadium transport in vertebrates has been conducted.

With respect to ascidians, there has been no published functional analysis of phosphate transporters. According to our preliminary analysis, the *C. intestinalis* genome contains eight Na^+-dependent phosphate transporters, five of which belong to the slc34 family, while the rest belong to the slc20 family (Fig. 4). Our preliminary studies also suggest that at least one of these eight transporters is able to transport V^V (Ueki et al., unpublished data). Therefore, this could be a candidate for V^V uptake from the extracellular environment.

3.2. Contribution of intestinal bacteria

What is the mechanism for indirect uptake of vanadium; that is, how is V^V reduced to V^{IV} before uptake by ascidians? One possibility is bacterial reduction of V^V ions. The evidence comes from studies of intestinal bacteria in ascidians. Antipov et al. demonstrated that molybdenum- and molybdenum cofactor-free nitrate reductases isolated from vanadate-reducing bacteria *Pseudomonas isachenkovii* are likely to mediate vanadate reduction [62]. In the following study, a vanadium-binding protein was isolated from the culture medium of vanadium-reducing bacterium that utilized vanadate as the terminal electron acceptor upon anaerobic respiration [63]. The protein was associated with vanadium at a molar ratio of 1:20, although no further biochemical analysis has been published. Such bacteria might be responsible for vanadate reduction before uptake of V^{IV} by ascidians.

The concentration of vanadium in sea water is 35 nM (Table 2), which suggests that high affinity vanadium ion transporter is necessary to uptake vanadium ions directly across the cytoplasmic membrane. As mentioned previously, influx of vanadate into cells is a rapid process that can become saturated (K_m = 1.4 mM) [44]. Our preliminary measurement of vanadium in a vanadium-rich ascidian, *Ascidia sydneiensis samea*, indicated that the intestinal vanadium concentration could reach 0.4 mM, and 2 mM in intestinal cells (Table 2). These facts suggest that the intestine is the first location of vanadium accumulation, and intestinal cells then take up vanadium (as V^V or V^{IV} ions) from this vanadium-rich microenvironment. We are currently engaged in isolation of vanadium-resistant or -reducing bacterial symbionts in the microenvironment of the intestine that cooperate with ascidians to take up vanadium from the extracellular sea-water environment.

In addition to ascidians, two research groups demonstrated that the Mn-reducing *Shewanella oneidensis* MR-1 strain can also reduce V^{V} [64,65]. Carpentier et al. reported that the strain can reduce V^{V} (initial concentration of 2 or 10 mM) to V^{IV} under anaerobic conditions by a biochemical process, and anaerobic reduction results in a granular precipitate containing predominantly V^{IV} [64]. The latter also reported that the strain showed V^{V}-reduction ability, as assessed by EPR (initial concentration of 2 mM), and several cell components required for the reduction of V^{V} were determined, such as an electron transport chain that includes cytochromes on both the cytoplasmic membrane and the outer membrane [65]. This strain can grow on vanadate as the sole electron acceptor [66]. Another bacterial species, *Geobacter metallireducens*, has also been reported to reduce V^{V} [67]. The reduction of U^{VI} to U^{IV} occurs as a biochemical cell process and can support cell growth of this species [68,69], which is consistent with the geochemical and chemical similarities between these two elements [70-73].

A more general and comprehensive study of the intestinal bacterial flora in a vanadium-rich ascidian *C. intestinalis* has been reported [74]. The composition of bacterial communities associated with the intestine of this species from three geographically divergent populations and under starved and unstarved conditions was examined by 16S ribosomal RNA sequencing using a next-generation DNA sequencer. The intestinal bacterial communities from the three geographically isolated populations were strikingly similar in terms of the abundance of operational taxonomic units (OTUs), consistent with selection of a core community by the gut ecosystem. In addition, a study on the bacteria flora in tunic of this species has been performed [75]; a combination of 454 pyrosequencing of 16S ribosomal RNA genes, catalyzed reporter deposition fluorescence *in situ* hybridization (FISH), and bacterial culture were used to characterize the bacteria living inside and on the tunic. Such a comprehensive study combined with biochemical analysis may reveal the relationship between ascidians and bacteria, especially in relation to vanadium uptake from the outer environment.

4. How do ascidians selectively accumulate vanadium in blood cells?

4.1. Selectivity of vanadium transport in the blood plasma and uptake by blood cells

How are vanadium ions absorbed in the intestine or branchial sac transported into the blood plasma (coelomic fluid) and then accumulated in blood cells? To our knowledge, there is no cellular or molecular evidence of the mechanism of transport out of the intestine or branchial sac. As the vanadium concentration in intestinal cells is extremely high, passive transport by a channel protein is sufficient to export vanadium into the blood plasma, where the vanadium concentration is relatively low (2 mM vs. 50 μM) (Table 2). Therefore, we propose two possible mechanisms: export of V^{V} from these tissues into the blood plasma, or export of V^{IV} after reduction in these tissues or at the previous step. In the latter case, vanadium transport proteins must exist in the blood plasma since it is a physiologically aerobic environment.

How are vanadium ions transported within the blood plasma? Our research group previously identified two key proteins, VanabinP and VBP-129, which are involved in vanadium ion transport [76,77]. Both are vanadium-binding proteins present in blood plasma of *A. sydneiensis samea*. Recombinant VanabinP binds a maximum of thirteen V^{IV} ions per molecule, with a $K_d = 2.8 \times 10^{-5}$ M [77]. VanabinP can also bind V^{V}, but it was not possible to determine the binding constant or the number of binding sites of VanabinP for V^{V} ions; this may be due to the complex behavior of V^{V} speciation in near-neutral aqueous solutions as a result of protonation and oligomerization (Fig. 3), and buffer interaction [78]. In contrast, VBP-129 can bind to six V^{IV} ions per molecule, as well as Fe^{III}, Co^{II}, Cu^{II}, and Zn^{II} ions [76,79]. VanabinP and VBP-129 interact with each other [76], but the mechanisms by which VanabinP and VBP-129 capture vanadium in the blood plasma and subsequently transfer vanadium ions have yet to be resolved.

How do blood cells uptake vanadium from blood plasma? In our model, it is probable that V^{V} in blood plasma is transported by an anion transporter on the plasma membrane. Na^{+}-dependent phosphate transporters are strong candidates because the concentration of Na^{+} in blood plasma is also extremely high and so the mechanism described in Section 3.1 could also be in play.

4.2. Selectivity of vanadium assimilation within blood cells

In vanadocytes, the majority of vanadium is reduced to V^{III}. V^{III}-aqua ions are stable only at low pH, and the acidic vacuole is the most suitable compartment for V^{III} ion storage. V^{V} ions

outside of 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 are needed to participate in vanadium accumulation in vanadocytes as the reduction of V^{V} to V^{IV} and/or V^{IV} to V^{III} is presumed to accelerate this accumulation.

It was recently revealed that the vanadium-binding protein Vanabin2 catalyzes the reduction of V^{V} to V^{IV} in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase (GR), and glutathione (GSH) [80]. *In vitro* studies indicate that nine disulfide bonds are partially cleaved at 1–4 mM GSH, forming intermediate structures [80], and more than a single pair of cysteine residues participate in the reductase activity of Vanabin2 [81]. These results suggest that electrons may be transferred from the donor NADPH to the acceptor V^{V} ions via thiol-disulfide exchange reactions in Vanabin2 (Fig. 5). The resultant disulfides in Vanabin2 are converted to thiols by reduced GSH, and oxidized GSH is re-reduced by GR. The disulfides of GR are then reduced to thiols by NADPH [82], which is linked to the pentose phosphate pathway. Since the concentration of GSH is 1.83 mM in vanadocytes of *A. sydneiensis samea* [80], the existence of a redox cascade from NADPH to V^{V} ions in ascidians is likely. Our recent study assessed whether Vanabin2 can reduce transition metal ions other than vanadium *in vitro*. An NADPH-coupled oxidation assay and re-oxidization assay yielded no evidence for the reduction of hexavalent transition metal anions, $Mo^{VI}O_4^{2-}$ and $W^{VI}O_4^{2-}$, or with four divalent cations, Mn^{II}, Ni^{II}, Co^{II}, and Cu^{II}, by Vanabin2 [83]. Thus far, Vanabin2 acts only as a V^{V}-reductase. This high selectivity may be the key factor in the metal ion selectivity of vanadium accumulation in ascidians.

Vanabin2 was first isolated from *A. sydneiensis samea* as a novel vanadium-binding protein by anion-exchange column chromatography [84]. In this species, the Vanabin family consists of at least five closely related proteins, Vanabins1–4 and VanabinP [77,85,86]. All five Vanabins possess 18 cysteine residues, and the intervals between cysteines are well-conserved (Fig. 6). A homology search of public DNA and protein databases, using both Vanabin1 and Vanabin2 amino acid sequences, revealed no proteins with striking similarities, other than those from two ascidian species, *Ciona intestinalis* and *Ciona savignyi*. We identified five Vanabins (*Ci*Vanabin1 to *Ci*Vanabin5) from *C. intestinalis* [87]. We also found five Vanabin homologs from the related species *C. savignyi*, although its genome assembly is incomplete

(http://www.broad.mit.edu/annotation/ciona/index.html). In addition, EST analysis yielded two Vanabin homologs (*Ag*Vanabin1 and *Ag*Vanabin2) in *A. gemmata*, which accumulates the highest levels of vanadium ions [88]. Therefore, Vanabins appear to be ubiquitous among the vanadium-accumulating ascidians and may hold the key to resolving the mechanism underlying the highly selective and extremely high-level accumulation of vanadium ions.

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 equal to 2.1×10^{-5} and 2.3×10^{-5} M, respectively, as determined by a gel filtration column chromatography [86]. X-band CW EPR studies revealed that the V^{IV}–Vanabin complex invariably exhibits a usual mononuclear-type V^{IV} signal up to a 15:1 molar ratio of V^{IV}:Vanabin1 and a 24:1 molar ratio of V^{IV}:Vanabin2, and no allosteric effects were observed in the process of binding multiple vanadium ions [79,89]. EPR spectrum analysis indicated a N_2O_2 coordination geometry common to V^{IV}:Vanabins and V^{IV}:VBP-129 coordination [79].

The metal selectivity in Vanabins has been investigated by immobilized metal ion affinity chromatography (IMAC). Vanabin2 was shown to selectively bind V^{IV}, Fe^{III} and Cu^{II} ions in the pH range 4.5–7.5 [90]. 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 unaffected by acidification. It can also bind Co^{II} and Zn^{II} at pH 7.5. Thus, under physiological conditions, Vanabin2 can bind not only V^{IV} but also several divalent cations. Site-directed mutagenesis of Vanabin2 has been performed to reveal the functional contribution of each binding site. An *in vitro* site-directed mutagenesis study identified a high affinity V^{IV}-binding site and a low-affinity binding site, and mutation of both sites caused an apparent loss of V^{IV} binding, as assessed by IMAC [91]. Therefore, we proposed that V^{V} is reduced to V^{IV} by thiol-disulfide exchange reactions using cytoplasmic Vanabins as reductases, with the resultant V^{IV} ions bound to these two V^{IV}-binding sites of Vanabins and stabilized in the cytoplasm. Vanabin2 is highly abundant in the cytoplasm of vanadocytes in *A. sydneiensis samea*. Sequence variants of Vanabin2 coexist in blood cells, and sequence variations have been under strict evolutionary constraints, with the high-affinity binding sites for V^{IV} ions conserved among all Vanabin2 variants [92].

The pH of the vanadocyte vacuole is kept extremely low by V-ATPase [93-95]. Therefore, we hypothesized that a proton gradient generated by V-ATPase provides the energy to transport vanadium across the vacuolar membrane. We previously identified a member of the Nramp/DCT1 family, *As*Nramp, from vanadocytes of *A. sydneiensis samea* [96]. 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}) across the membrane using a proton gradient as the motive force [97-99]. We found that *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 [96]. V^{IV}-transport activity is suppressed by the addition of excess Cu^{II}, Fe^{II}, Mn^{II}, and Zn^{II} ions, but not Ni^{II} ions, suggesting that *As*Nramp can also transport these divalent cations. These findings support the proposed model that a proton electrochemical gradient generated by V-ATPase is the driving force for V^{IV} transport from the cytoplasm into the vacuole, and that V^{IV}-transport activity is a novel additional feature of the ascidian homolog of Nramp/DCT1.

In summary, the genomes of ascidians encode several vanadium-binding proteins, vanadium transporters, and vanadium reductases. Some were introduced during the course of ascidian evolution, while others are common to similar groups of organisms. With the exception of the V^{V}-reductase activity of Vanabin2, none of these proteins are specific for vanadium. The combination of membrane transporter proteins, metal-binding proteins, and a metal reductase facilitate selective accumulation of vanadium (Table 3).

Finally, it is of particular interest how V^{IV} ions are reduced to V^{III}. V^{III} ions are usually unstable in the presence of air or moisture, and V^{III} ions are hydrolyzed to $V(OH)^{2+}$ at above pH 2.2. In neutral and alkaline solutions, chemical species with vanadium in the +3 oxidation state have not been reported. Thus the reduction could occur after V^{IV} ions are transported into the vacuole, but there are no direct evidences so far. As described in Section 2, vanadium ions in the blood cells of ascidians are stable under the strongly acidic conditions in these cells. It is of interest to coordination chemists or biochemists to determine whether any molecular ligands participate in the stabilization of vanadium ions in the +3 oxidation state in ascidian blood cells.

5. Conclusion and perspectives

The accumulation, reduction, and transport of vanadium ions are expected to be tightly linked. In this review, the authors described the ascidian as a sophisticated system. Possible models of vanadium assimilation pathways are provided in Figure 7. In the first step of vanadium uptake from the outer environment, it is probable that a phosphate transporter directly takes up vanadate from the outer environment. In addition, intestinal bacteria may assist the absorption of vanadium from the environment. The metal selectivity of this system is due to a combination of vanadium-binding proteins, vanadium transporters and vanadium reductases. The whole system mediates selective accumulation and reduction of vanadium ions. The pentose phosphate and glutathione pathways are the key sources of the reducing force, and the glutathione pathway is important for regulation of gene transcription in response to excess vanadium [100].

The physiological function of accumulated vanadium ions in a reduced form (V^{III}) is presumably exerted through redox-coupled reactions. The necessity of vanadium is still unclear, but through use of a modern biological technique, such gene-knockout methods [101-105], it is possible to create ascidians that cannot accumulate vanadium. Further studies on vanadium assimilation in ascidians should provide fundamental insights into the physiological roles of vanadium not only in ascidians but also in mammals, in which vanadium is an essential element.

Acknowledgements

We would like to thank Mr. T. Morita and staff at the International Coastal Research Center, Ocean Research Institute, University of Tokyo at Otsuchi, Iwate, Japan, and Kojima Port, Okayama, Japan, for their help collecting adult ascidians. We also thank Ms. K. Hirayama and other staff at the Graduate School of Science, Kyoto University, Maizuru Fisheries Research Station, Kyoto University, and Misaki Marine Biological Station, Graduate School of Science, the University of Tokyo for providing *C. intestinalis* through the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. DNA sequencing, mass spectrometry, and EPR analyses were performed at the Natural Science Center for Basic Research and Development (N-BARD), Hiroshima

University. This work was supported by Grants-in-Aid from the Japan Society for Promotion of Science (JSPS) (Nos. 22224011, 25120508 and 25440170) and an Environmental Research Grant from the Nippon Life Insurance Foundation (2012).

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Figures and Tables

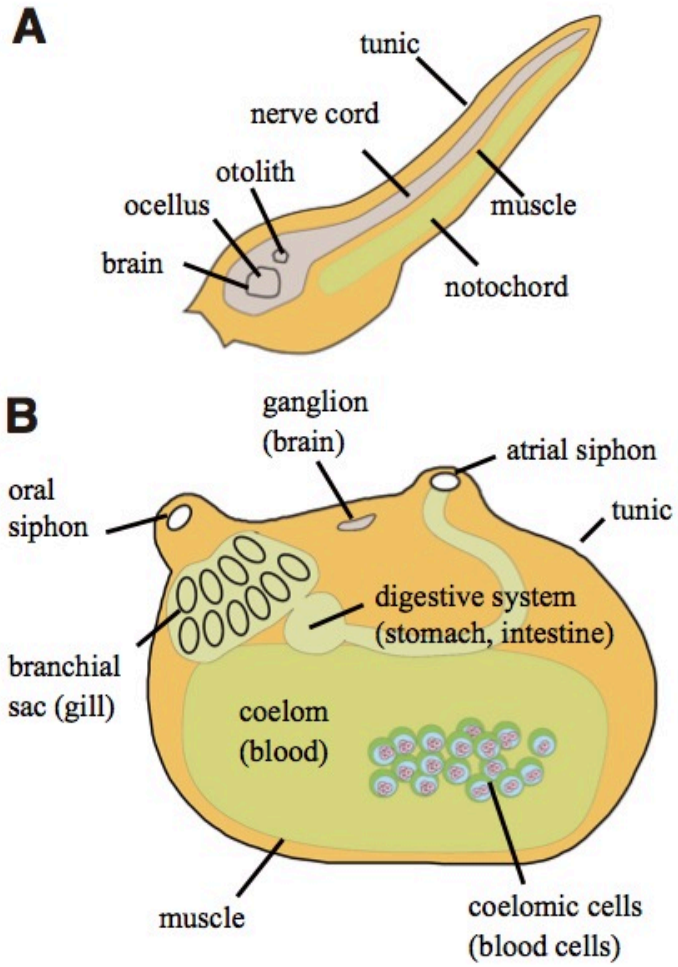

Fig. 1. Schematic illustration of the larval and adult forms of ascidians. (A) A typical larval form. The swimming larva possess head/trunk and tail regions, and can swim by the tail movement. Brain and sensory organs are located in head/trunk region. The larva attaches to a substrate, and metamorphose to the adult form by losing tails and rearranging the internal organs. (B) A typical adult form. It cannot locomote by itself. A single cerebral ganglion lies between two siphons.

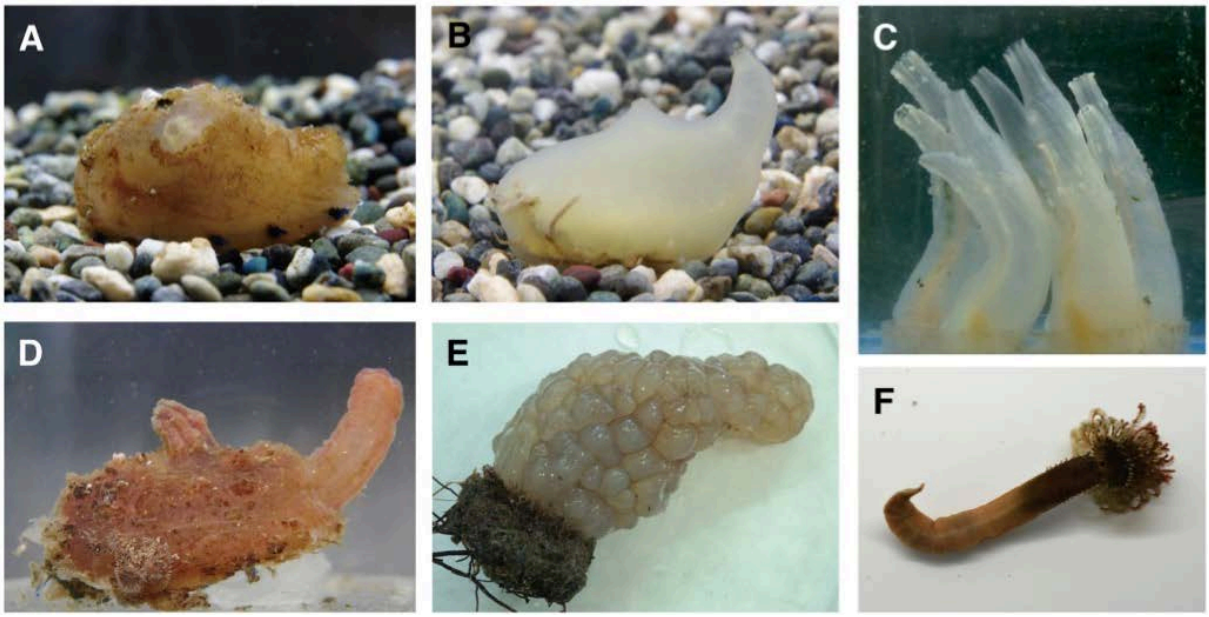

Fig. 2. Vanadium-rich ascidians and a Polychaeta worm. (A) *Ascidia gemmata*, (B) *A. ahodori*, (C) *Ciona intestinalis*, (D) *A. sydneiensis samea*, (E) *Phallusia mammillata*, (F) *Pseudopotamilla occelata*. Reproduced from [106] with permission from Yodosha Co., Ltd.

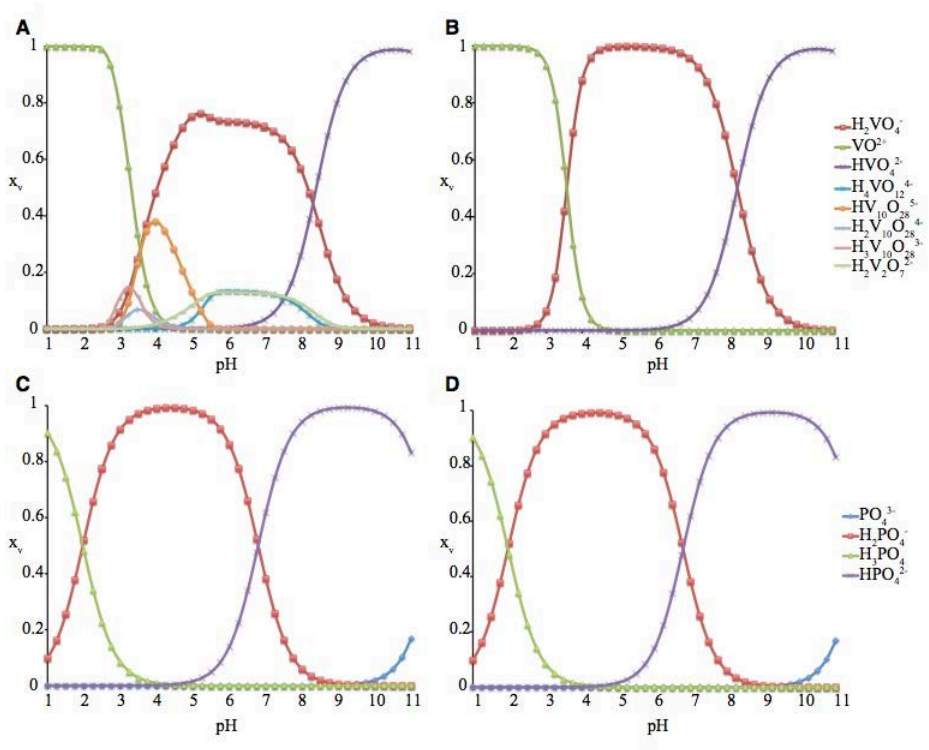

Fig. 3. Speciation diagram for aqueous vanadate (A, B) and phosphate (C, D) solutions calculated by Visual MINTEQ ver. 3 based on MINTEQA2 [42]. Data are normalized to total concentration and expressed as molar fraction x_V vs. pH. Ionic strength: 0.45 M. Vanadium concentration and species: 1 mM V^V (A) or 1 µM V^V (B). Phosphorous concentration and species: 1 mM P^V (A) or 1 µM P^V (B). Temperature: 25°C. Species that comprised less than 3% are not shown. As compared with experimentally determined speciation diagrams [16], the ratios of polymeric vanadate species are relatively low.

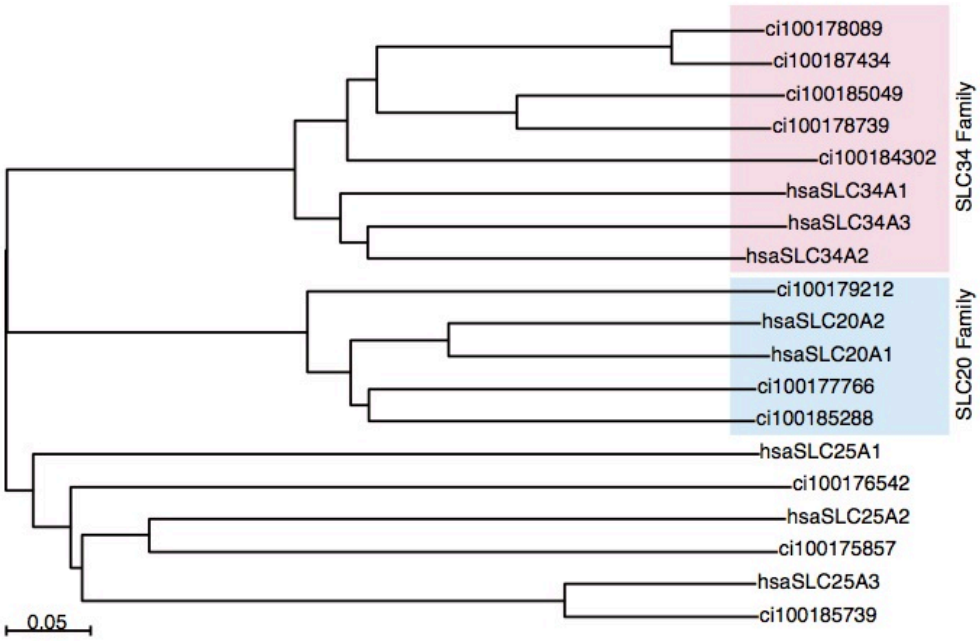

Fig. 4. Molecular phylogenetic tree of Na^+-dependent phosphate transporters from *Ciona intestinalis* and *Homo sapiens*. IDs are given as entry numbers and gene definitions registered in the KEGG database (http://www.kegg.jp/). Full-length amino acid sequences were aligned using the ClustalW software [107], and the neighbor-joining method was used to draw the tree [108].

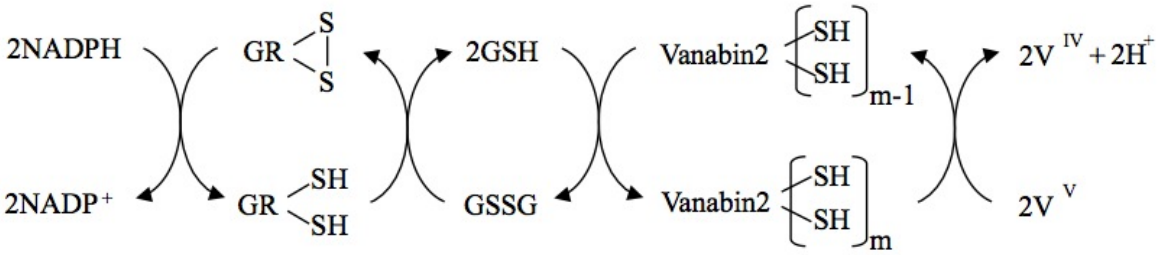

Fig. 5. A proposed redox cascade mechanism for the V^V reductase activity of Vanabin2, reproduced from [81] with permission from copyright holders.

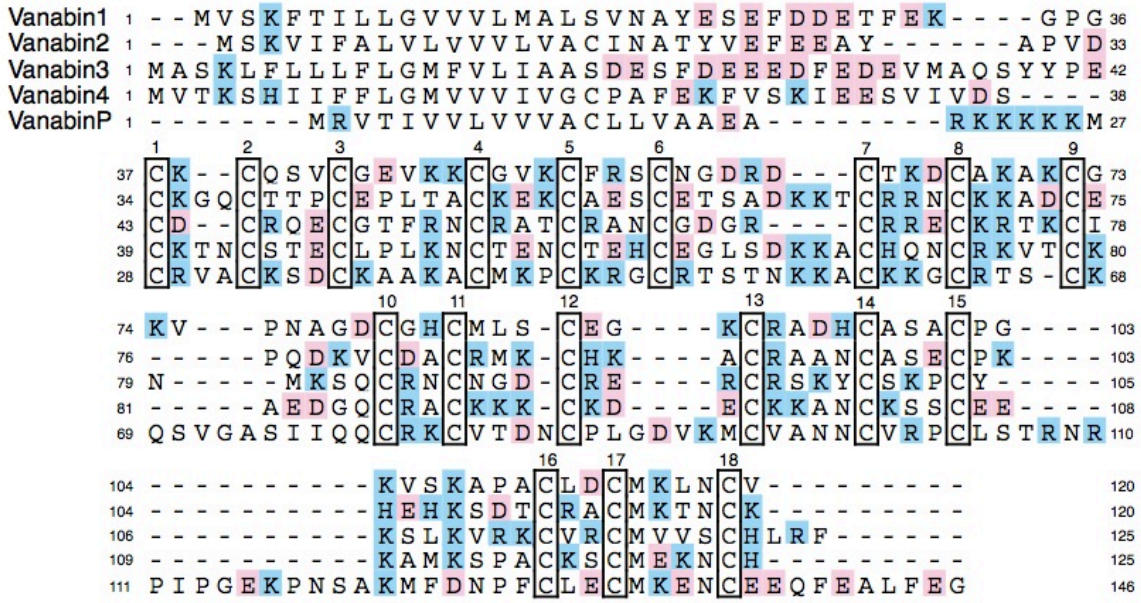

Fig. 6. Amino acid sequences of Vanabins isolated from *Ascidia sydneiensis samea*. Eighteen conserved cysteine residues are boxed. Positively and negatively charged residues are shown in blue and red, respectively. Reproduced from [106] with permission from Yodosha Co., Ltd. For a reference, see [77,85,86].

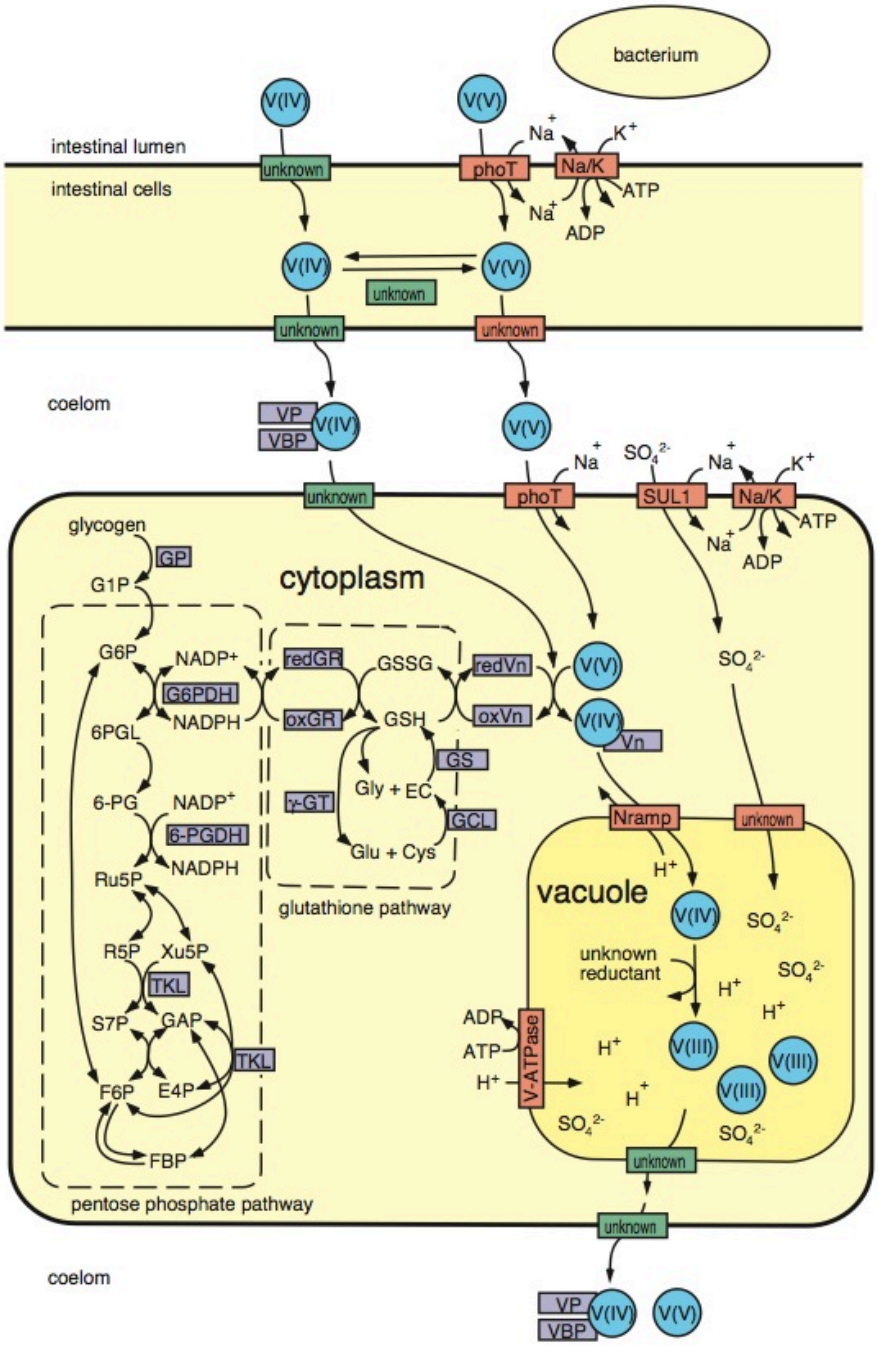

Fig. 7. Proposed model for vanadium uptake, reduction, and excretion by vanadocytes. Purple, cytoplasmic proteins. Green and orange, membrane proteins. Blue, vanadium ions. Vn, Vanabin1–4. VP, VanabinP. VBP, VBP-129.

Table 1. Concentration of vanadium, proton and sulfate in ascidians.

ascidian species	vanadium	pH	sulfate	references
Ascidia gemmata [a]	347.2 mM	1.86	500 mM	[22,34]
Ascidia ceratodes [a]	99mM	1.8	250 mM	[35]
Ascidia ahodori [a]	59.9 mM	2.67	–	[22]
Ascidia sydneiensis samea [a]	12.8 mM	4.20	210mM	[22,33]
Ascidia sydneiensis samea [b]	38mM	~5	86mM	[33]

[a] Values obtained from whole blood cells

[b] Values obtained from blood cells with the exception of giant cells

Table 2. Concentrations and chemical species in sea water and the tissues of vanadium-rich ascidians.

location/tissue	vanadium concentration	major vanadium species	reference
sea water	35 nM	V(V)	[23,24]
branchial sac	1.4 mM	n.d.	[21]
intestinal content	0.4 mM	n.d.	Ueki et al., unpublished data
intestine	1.8 mM	n.d.	Ueki et al., unpublished data
blood plasma	50 μM	n.d.	[21]
blood cells	38 mM	–	[33]
cytoplasm	low	V(V) ---> V(IV)	-
vacuole	high	V(III)	[27, 38]

n.d., Not determined

Table 3. Metal selectivity of vanadium-related proteins in vanadium-rich ascidians.

Localization	**Protein (function)**	**Metal ion selectivity** [b]	reference
intestinal cells	phoT [a] (transporter)	**P^{V}**, **V^{V}**	Ueki et al., unpublished data
blood plasma	VBP-129 (binding protein)	$\underline{Ca^{II}}$, **Co^{II}**, **Cu^{II}**, **Fe^{III}**, $\underline{Mg^{II}}$, $\underline{Mn^{II}}$, **V^{IV}**, **Zn^{II}**	[79]
cytoplasm of vanadocytes	Vanabin2 (reductase)	$\underline{Co^{II}}$, $\underline{Cu^{II}}$, $\underline{Mn^{II}}$, $\underline{Mo^{VI}}$, $\underline{Ni^{II}}$, **V^{V}**, $\underline{W^{VI}}$	[83]
cytoplasm of vanadocytes	Vanabin2 (binding protein)	$\underline{Ca^{II}}$, **Co^{II}**, **Cu^{II}**, **Fe^{III}**, $\underline{Mg^{II}}$, $\underline{Mn^{II}}$, **V^{IV}**, **Zn^{II}**	[91]
vacuolar membrane	Nramp (transporter)	**Cu^{II}**, **Fe^{II}**, **Mn^{II}**, $\underline{Ni^{II}}$, **V^{IV}**, **Zn^{II}**	[96]

[a] Tentative name

[b] Bold characters indicate the metal ions transported, bound or reduced by each proteins, while underlined characters indicate the metal ions <u>not</u> transported, bound or reduced by each proteins