

## Review

# Advances in research on the accumulation, redox behavior, and function of vanadium in ascidians

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## Abstract

The discovery of high levels of vanadium-containing compounds in ascidian blood cells goes back to 1911. Ascidians, which are also known as tunicates or sea squirts, belong to a subphylum of the Chordata, between the vertebrates and invertebrates. This discovery attracted the attention of an interdisciplinary group of chemists, physiologists, and biochemists, in part because of interest in the possible role of vanadium in oxygen transport as a prosthetic group in respiratory pigments, which was later shown not to be such a role, and in part because of the fact that high levels of vanadium were unknown in other organisms. The intracellular concentration of vanadium in some ascidian species can be as high as 350 mM, which is  $10^7$  times that in seawater. Vanadium ions, which are thought to be present in the +5 oxidation state in seawater, are reduced to the +3 oxidation state via the +4 oxidation state and are stored in the vacuoles of vanadium-containing cells called vanadocytes, where high levels of protons and sulfate ions are also found. Recently, many proteins and genes that might be involved in the accumulation and reduction of vanadium have been isolated. In this review, we not only trace the history of vanadium research but also describe recent advances in our understanding of the field from several viewpoints: (i) vanadium-accumulating blood cells, (ii) the energetics of vanadium accumulation, (iii) the redox mechanism of vanadium, (iv) the possible role of sulfate, and (v) the physiological roles of vanadium.

**Keywords:** ascidian; hyperaccumulation; metal-binding proteins; redox; vanadium.

## Introduction: chemistry of vanadium

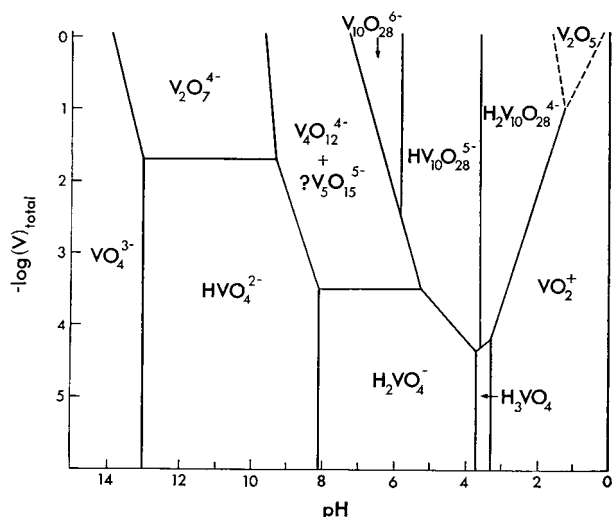
Vanadium, atomic number 23, is the 22nd most abundant element on Earth. Its average crustal abundance is estimated at 100  $\mu\text{g/g}$ , which is approximately twice that of copper, 10 times that of lead, and 100 times that of molybdenum (1). However, vanadium is considered a rare metal in many

countries because vanadium ores tend to be lean and localized. The major vanadium ores are vanadinite ( $\text{Pb}_5[\text{VO}_4]_3\text{Cl}$ ) and carnotite (also an important uranium ore,  $\text{K}_2[\text{UO}_2]_2[\text{V}_2\text{O}_8]\cdot 3\text{H}_2\text{O}$ ). In seawater, the average concentration of vanadium is approximately 35 nM (2, 3). Vanadium, which is known to be present in crude oil, is a source of environmental pollution, catalytic poisoning during the petroleum cracking process, and the corrosion of refinery equipment. Western Venezuelan crude oil contains approximately 257  $\mu\text{g/g}$  of vanadium (4). Vanadium can be recovered as  $\text{V}_2\text{O}_5$  from smoke dust after the combustion of oil. Approximately 50% of the vanadium in oil is in the form of a vanadyl porphyrin complex, which resembles chlorophyll and hemoglobin. Vanadium is also contained in coal and tar sand (5).

Vanadium forms diverse chemical compounds with a wide range of oxidation states (from -3 to +5). Vanadium ions under ordinary aqueous conditions are, however, limited to +2, +3, +4, and +5. Of these oxidation states, +3, +4, and +5 are biologically relevant under physiological conditions. Vanadium compounds in the +2 oxidation state are unusable by living organisms because they are strongly reduced and easily oxidized, even by water. In particular, a reduced form of vanadium has been reported in nitrogenases from *Azotobacter* (6). Vanadium ions in the +3 oxidation state ( $\text{V}^{\text{III}}$ ) are usually unstable in the presence of air or moisture, and  $\text{V}^{\text{III}}$  ions are hydrolyzed to  $(\text{V}(\text{H}_2\text{O})_6)^{3+}$  at and above pH 2.2. In neutral and alkaline solutions, no chemical species with vanadium in the +3 oxidation state have been reported. Vanadium compounds with oxidation states from +3 to +5 have been found to play various important roles in living organisms (7). Figure 1, which is adapted from Pope (8), shows the predominant vanadium species in water as functions of pH and redox.

## Biological significance of vanadium

From the early 1970s to the 1980s, the biological significance of vanadium was the focus of several reports. In 1972, Bayer and Kneifel (9) isolated a pale-blue compound containing vanadium, and Bayer et al. (10) characterized a complex of N-hydroxyimino- $\alpha, \alpha'$ -dipropionic acid containing  $\text{V}^{\text{IV}}$ , which they designated amavadin, from the toadstool *Amanita muscaria*. High levels of vanadium (3.3 mg/kg) were found to be contained in this species by Ter Meulen (11); however, the physiological function of amavadin in mushrooms remains to be resolved.



**Figure 1** Speciation diagram showing the oxidation state and major species of vanadium as a function of pH and reduction potential.

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In 1977, Josephson and Cantley (12) reported that a potent inhibitor of the  $Na^+K^+$ -ATPase was contained in ATP purified from both equine and rabbit muscle. Subsequently, Cantley et al. (13) determined that a low level of vanadium ions in the +5 oxidation state (vanadate) in muscle acts as a potent inhibitor of the  $Na^+K^+$ -ATPase. This finding attracted the attention of investigators from various fields, including biochemistry, chemistry, and biology, aiming to explain the physiological functions of vanadium. The inhibition of the  $Na^+K^+$ -ATPase by quabain was found to activate glucose transport and glucose oxidation in rat adipocytes (14); later, Shechter and Karlish (15) found new inhibitors in the form of vanadium salts (vanadate and vanadyl) that stimulated glucose oxidation in rat adipocytes. In 1985, Heyliger et al. (16) reported that vanadate appeared to have insulin-like effects. Meyerovitch et al. (17) showed that the oral administration of vanadate normalized blood glucose levels in streptozotocin-treated rats, which are a model of type I diabetes. Vanadate was also revealed to lower glucose levels in animal models of type II diabetes (18). Inorganic and organic vanadium-containing compounds have been of interest as insulin mimics and have been studied in clinical trials. Among them, the vanadium compound, BEOV [bis(ethylmaltolato)oxovanadium(IV)], has passed clinical test phase II (19). Vanadium compounds have been further found to be effective as anti-cancer agents in several variants of leukemia (20).

In 1984, Vilter first revealed the presence of vanadium bromoperoxidase in the marine macroalga *Ascophyllum nodosum* (21). Peroxidases comprise a large family of enzymes that use  $H_2O_2$  or organic hydroperoxides as an electron acceptor in the catalysis of various oxidative reactions. Although heme and non-heme peroxidases were first reported long ago, a peroxidase containing vanadium in its active site was first found by Vilter (21). Bromoperoxidase is

known to be involved in the biosynthesis of bromometabolites such as the halogenated compounds  $CH_2Br_2$ ,  $CHBr_2Cl$ , and  $CHBr_3$ . This finding triggered the discovery of various vanadate-dependent haloperoxidases, including iodo-, bromo-, and chloro-peroxidases not only in marine algae but also in terrestrial fungi and lichens, which harbor enzymes that catalyze the halogenation of organic substrates by  $H_2O_2$  and halides (22–24). In 1986, Robson et al. (25) reported that the alternative nitrogenase of *Azotobacter chroococcum* was a vanadium enzyme. Subsequent genetic and physiological studies showed that V-nitrogenases are widely distributed but are only synthesized when molybdenum is a limiting nutrient (26).

### Finding of high levels of vanadium in ascidians

By contrast, the discovery of vanadium compounds in ascidian blood (coelomic) cells dates back to 1911 when the German physiologist M. Henze (27) discovered high levels of vanadium in the blood cells of an ascidian, *Phallusia mammillata*, collected from the Bay of Naples. Ascidians, which are also known as tunicates or sea squirts, are marine animals that live under water, attached to stones, rocks, or other solid surfaces. Ascidians have been assigned to a subphylum of the Chordata, between vertebrates and invertebrates, mainly based on the fact that ascidian juveniles have a notochord during the larval stage prior to metamorphosis. The discovery of high levels of vanadium in ascidians attracted the attention of an interdisciplinary group of chemists, physiologists, and biochemists, in part because of interest in the possible role of vanadium in oxygen transport as a third possible prosthetic group in respiratory pigments (in addition to iron and copper), although such a role was subsequently disproved, and in part because of strong interest in the fact that high levels of vanadium have not been reported in other organisms. Since then, this unusual phenomenon in ascidians has been studied by investigators with inorganic chemical, biochemical, physiological, and molecular biological backgrounds. Therefore, we will not only trace the history of research on vanadium but also describe recent advances in the field: (i) vanadium-accumulating blood cells, (ii) the energetics of vanadium accumulation, (iii) the redox mechanism of vanadium, (iv) the possible role of sulfate, and (v) the physiological roles of vanadium.

### Vanadium-accumulating blood cells

After Henze's finding of vanadium in ascidian blood cells (27), many analytical chemists joined in attempts to characterize the metals in other living organisms. The presence of vanadium was reported in some mollusks and holothurians, but was later shown to be a specific idiosyncrasy and latent impurity in the graphite electrodes used in these studies (28). However, the finding that a polychaeta, *Pseudopotamilla ocellata*, contained levels of vanadium corresponding to those in ascidians is credible (29). It is still unclear

how and why the polychaeta accumulate such high levels of vanadium.

By contrast, many ascidian species have been analyzed for the presence not only of vanadium but also of other transition metals. As a result, manganese was shown to be the second most common metal in ascidians (30–33). Thereafter, niobium, chromium, tantalum, tungsten, and titanium were also reported to be present in ascidians (34–37), but the presence of these metals has not been reproducible in most cases because transition metals can be difficult to measure quantitatively. Thus, we attempted to determine the vanadium contents of several tissues from several ascidian species collected from the Mediterranean and waters around Japan using thermal neutron-activation analysis, which is an extremely sensitive method for quantifying metals. Ascidians belonging to two suborders, Phlebobranchia and Stolidobranchia, in the order Enterogona were analyzed. Vanadium was detected in almost all of the species examined, but those species belonging to the suborder Phlebobranchia contained higher levels of vanadium than did those belonging to Stolidobranchia. Furthermore, the blood (coelomic) cells of the tissues examined were confirmed to contain the highest amount of vanadium (33). The highest concentration of vanadium detected (350 mM), found in the blood cells of *Ascidia gemmata* (38), which belong to the suborder Phlebobranchia, corresponds to  $10^7$  times the vanadium concentration in seawater (2, 3). The levels of iron and manganese did not vary significantly among the species of the two suborders (Table 1) (33, 38).

Ascidian blood cells, or, more precisely, coelomic cells, can be classified into nine to 11 different types that could be further grouped into six categories morphologically: hemoblasts, lymphocytes, leukocytes, vacuolated cells, pigment cells, and nephrocytes (39). The vacuolated cells are in turn divided into at least four types: morula cells, signet ring cells, compartment cells, and small compartment cells. Among these, morula cells are referred to vanadocytes (28) because the dense granules observed in morula cells by electron microscopy were assumed to be deposits of vanadium (40). However, the de Vincentiis group found that morula cells contain little or no vanadium mainly using X-ray micro-

analysis (41–45); today, signet ring cells rather than morula cells are considered to be vanadocytes based on the discovery of vanadium in isolated subpopulations of blood cells (46) and the detection of X-ray fluorescence owing to vanadium in blood cells by scanning X-ray microscopy (Figure 2) (47).

### Energetics of accumulation

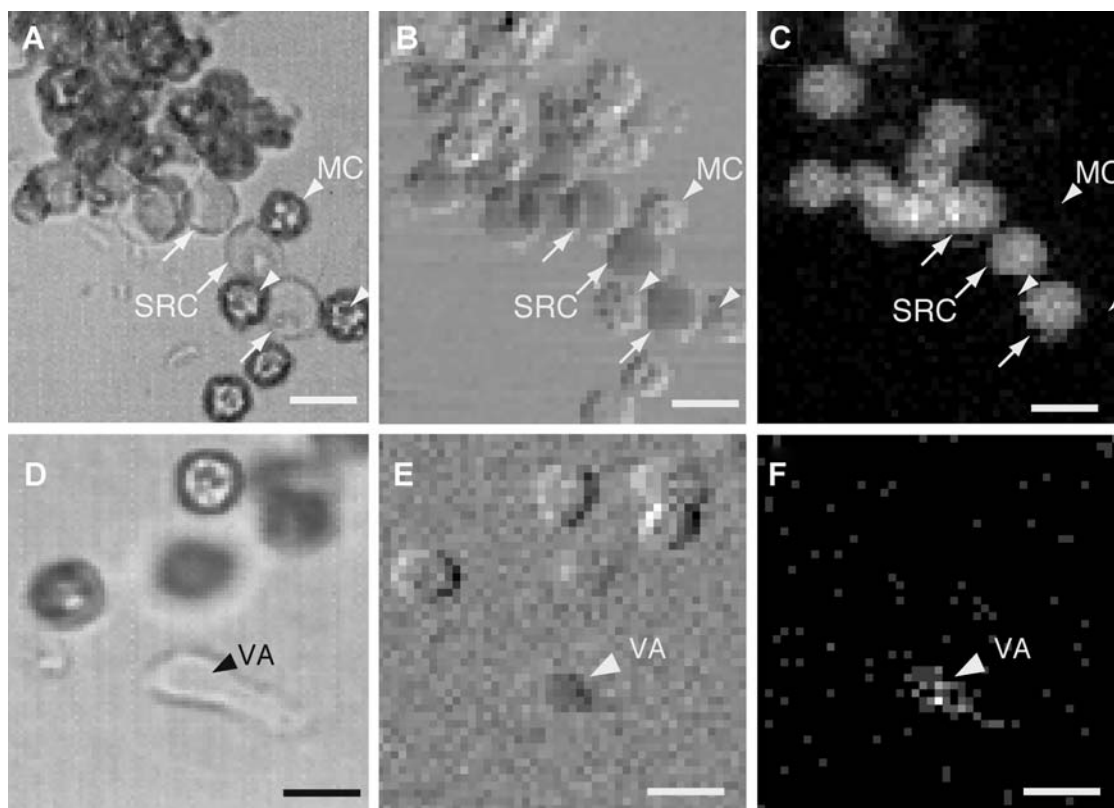
Henze first reported that a homogenate of ascidian blood cells was extremely acidic (27, 48–50). Thereafter, whether ascidian blood cells carry a low pH became a source of controversy (51); regardless, this unusual phenomenon has attracted the interest of investigators because of the possible role of a highly acidic environment in changing or maintaining the redox potential.

In our own studies, we have focused on this phenomenon from the perspective of the energetics of vanadium accumulation. *Ascidia gemmata*, which contains the highest concentration of vanadium (350 mM), has the lowest vacuolar pH (1.86). The vacuoles of *A. ahodori*, which contain 60 mM vanadium, have a pH of 2.67, whereas those of *A. sydneiensis samea* (containing 13 mM vanadium) have a pH of 4.20 (38). Thus, comparative analyses of the pH values and levels of vanadium in the signet ring cells of three different species suggest a close correlation between a high level of vanadium and a low pH (i.e., a higher concentration of protons). Vacuolar-type  $H^+$ -ATPases play a role in pH homeostasis in various intracellular organelles, including clathrin-coated vesicles, endosomes, lysosomes, Golgi-derived vesicles, multivesicular bodies, and chromaffin granules, which belong to the central vacuolar system (52–54). By immunocytological analysis, vacuolar-type  $H^+$ -ATPases were found to be localized in the vacuolar membranes of vanadocytes, and inhibition of the proton pump using the specific vacuolar-type  $H^+$ -ATPases inhibitor bafilomycin  $A_1$  resulted in neutralization of the contents of vacuoles (55). Therefore, vacuolar-type  $H^+$ -ATPases were ascertained to function in vanadocytes. Vacuolar-type  $H^+$ -ATPases are composed of several subunits containing either a  $V_o$  domain (membrane-

**Table 1** Concentrations of vanadium in the tissues of several ascidians (mM).

Species	Tunic	Mantle	Branchial sac	Serum	Blood cells
Phlebobranchia					
<i>Ascidia gemmata</i>	N.D.	N.D.	N.D.	N.D.	347.2
<i>A. ahodori</i>	2.4	11.2	12.9	1.0	59.9
<i>A. sydneiensis</i>	0.06	0.7	1.4	0.05	12.8
<i>Phallusia mammillata</i>	0.03	0.9	2.9	N.D.	19.3
<i>Ciona intestinalis</i>	0.003	0.7	0.7	0.008	0.6
Stolidobranchia					
<i>Styela plicata</i>	0.005	0.001	0.001	0.003	0.003
<i>Halocynthia roretzi</i>	0.01	0.001	0.004	0.001	0.007
<i>H. aurantium</i>	0.002	0.002	0.002	N.D.	0.004

N.D., not determined. The vanadium content in each tissue was quantitatively determined mainly by neutron-activation analysis (33, 38). Reproduced from Ref. (51). Copyright 1998 John Wiley & Sons, Inc.



**Figure 2** *Phallusia mammillata* blood cells observed by differential interference contrast optical microscopy (A, D) and X-ray microscopy in the transmission mode (B, E) and fluorescence mode for vanadium (C, F).

Photographs (A–C) are from the same field of view; photographs (D–F) are from another field. Vanadium is accumulated in signet ring cells (SRCs, shown by arrows) and a vacuolated amoebocyte (VA, shown by arrowheads in D–F), but not in morula cells (MCs, shown by arrowheads in A–C). Each scale bar=10  $\mu\text{m}$ . Reproduced from Ref. (47). Copyright 2002 Zoological Society of Japan.

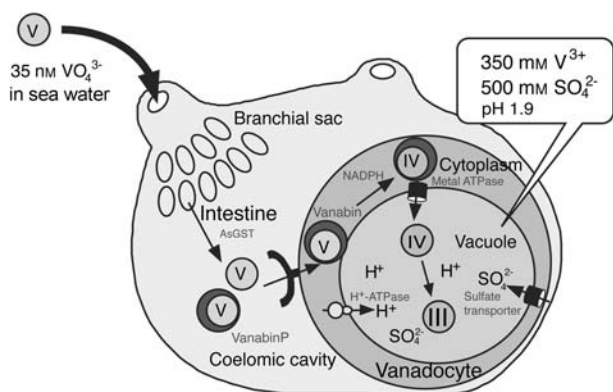
associated subunits) or  $V_1$  domain (peripherally associated subunits) (56). Notably, the expression of subunit *C* from ascidian cDNA in the pH-sensitive budding yeast mutant *uma5* resulted in a successful rescue (57). As a follow-up to this study, a functional assay to examine whether the protons concentrated by vacuolar-type  $\text{H}^+$ -ATPases are linked to the accumulation of vanadium should be conducted.

### Redox mechanism of vanadium

A debate exists concerning which oxidation state of vanadium is predominant in ascidian blood cells. Non-invasive physical methods, including electron paramagnetic resonance (EPR), X-ray absorption spectrometry (XAS), nuclear magnetic resonance (NMR), and superconducting quantum interference device (SQUID), have shown that the vanadium ions in ascidian blood cells are predominantly in the +3 oxidation state, with a small amount in the +4 oxidation state (58–63). We confirmed that vanadocytes contain vanadium predominantly in the +3 oxidation state with a small amount in the +4 oxidation state at a ratio of 97.6:2.4 (64). By contrast, vanadium was reported to be present in seawater in the +5 oxidation state ( $\text{H}_2\text{VO}_4^-$ ) (65). Therefore, reducing agents must play an important role in the accumulation of vanadium, because during the accumulation process  $\text{V}^{\text{V}}$  is reduced

to  $\text{V}^{\text{III}}$  via  $\text{V}^{\text{IV}} \cdot \text{V}^{\text{V}}$  can be reduced to  $\text{V}^{\text{IV}}$  without difficulty owing to its redox potential ( $\text{VO}_2^+/\text{VO}^{2+}$ : 1.00 V in a strongly acidic solution) (66). In fact, a number of biologically relevant reducing agents, including ascorbate (67–69), cysteine (70), norepinephrine (71), glutathione (69, 72), oxalic acid (73), and tunichromes, as well as blood pigments isolated from a tunicate (74, 75) have been found to reduce  $\text{V}^{\text{V}}$  to  $\text{V}^{\text{IV}}$ .

The vanadocytes of vanadium-rich ascidians express enzymes belonging to the pentose-phosphate pathway (76–79), which is an alternative metabolic pathway of glycolysis. During the process, NADPH and pentose sugars are generated. NADPH provides the reducing equivalents for the biosynthetic and oxidation-reduction reactions involved in protection against the toxicity of reactive oxygen species through the regeneration of reduced glutathione (GSH). Shi et al. (80) showed that  $\text{V}^{\text{V}}$  is reduced by GSH reductase and flavoenzymes in the presence of NADPH under aerobic conditions (81). Furthermore, we previously showed that NADPH could reduce  $\text{V}^{\text{V}}$  to  $\text{V}^{\text{IV}}$  in the absence of enzyme catalysis when EDTA, which has a large stability constant for  $\text{VO}^{2+}$  [ $\log K=18.63$ :  $K=(\text{complex})/(\text{VO}^{2+})(\text{EDTA})$ ] (82), was present in the reaction mixture. In addition, NADPH was revealed to be able to only partially reduce  $\text{V}^{\text{V}}$  in the absence of EDTA (83). NADPH could also play a role as a reducing agent for vanadium in ascidians (Figure 3).



**Figure 3** Schematic representation of vanadium accumulation and reduction in ascidians.

The concentration of vanadium in the +5 oxidation state is only 35 nM in seawater; in contrast, the highest concentration of vanadium in ascidian blood cells is 350 nM (38), and the concentration of sulfate is 500 mM (95). The vacuole interior is maintained at an extremely low pH (1.9) by  $H^+$ -ATPases (55). In this environment, almost all of the vanadium accumulated is reduced to  $V^{III}$  via  $V^{IV}$  (64). The first step in vanadium uptake can occur at a branchial sac or digestive organ (intestine), where glutathione-S-transferase was identified as a major vanadium carrier protein (103). We discovered vanadium-binding proteins, designated Vanabins, in the blood plasma and cytoplasm of vanadocytes (105–108, 110). The pentose-phosphate pathway, which produces NADPH, was shown to be localized in the cytoplasm by *in vitro* experiments, and NADPH has been found to reduce  $V^V$  to  $V^{IV}$  (76–79). A metal-ATPase that might be involved in vanadium transport has been found in the vacuolar membrane (submitted).

By contrast, biologically relevant reducing agents involved in the reduction of  $V^{IV}$  to  $V^{III}$  have not yet been reported because the reduction of  $V^{IV}$  to  $V^{III}$  requires a rather low redox potential ( $V^{IV}/V^{III}$ : 0.337 V) (66). Tunichromes, which are a class of hydroxy-dopa-containing tripeptides, have been isolated from some ascidian species (74, 75, 84, 85). Because tunichromes contain pyrogallol and catechol moieties that can have a reducing functionality, it was proposed that tunichromes participate in the reduction of vanadium in ascidians (86). In fact, pyrogallol (a model tunichrome) reduces  $V^{IV}$  to  $V^{III}$  in tetrahydrofuran (87). However, whether tunichromes directly participate in the reduction of vanadium in ascidians is unclear (88–90). Ascidian blood cells contain aliphatic sulfonic acids, such as cysteic acid, which is an oxidation product of cysteine (91), and cysteine methyl ester, which was reported to reduce  $V^{IV}$  to  $V^{III}$  with the assistance of EDTA and EDTA-like ligands of aminopolycarboxylate in water. The reduction of  $V^{IV}$  to  $V^{III}$  by cysteine methyl ester was found to be aided by glycylhistidine and glycylaspartic acid (92).

### The possible role of sulfate

After the first finding of sulfate in ascidian blood cells (50), a considerable amount of sulfate has been reported in association with vanadium in ascidian blood cells (41–45), which

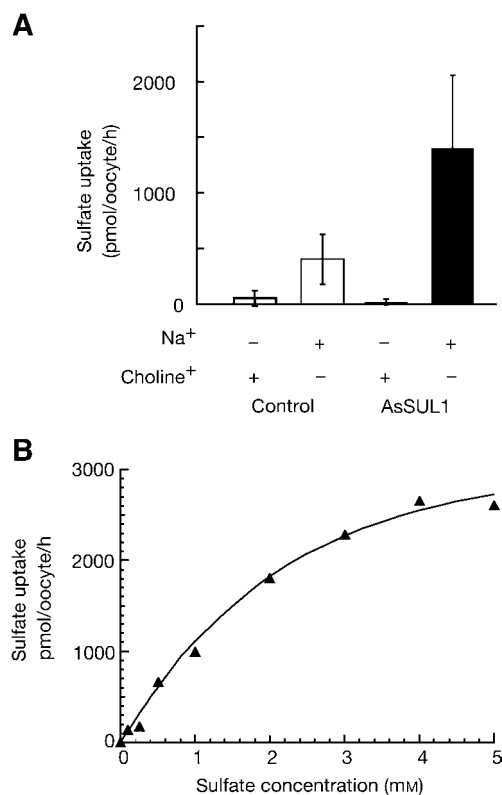
suggests that sulfur compounds might be involved in the biological function of vanadium. Frank et al. (91, 93) reported that large quantities of intracellular sulfate as well as aliphatic sulfonic acids such as cysteic acid were present in ascidian blood cells. As cysteic acid is an oxidation product of cysteine, cysteine might be the reducing agent responsible for the reduction of  $V^{IV}$  to  $V^{III}$  in ascidians. Although cysteine itself cannot reduce  $V^{IV}$  to  $V^{III}$ , we have shown that cysteine methyl ester can reduce  $V^{IV}$  to  $V^{III}$  with the assistance of EDTA and EDTA-like ligands (94). Therefore, if cysteine does work as a reducing agent in ascidians, biogenic ligands promoting the reduction of  $V^{IV}$  are expected to participate in the reaction.

In fact, high levels of sulfate ions and protons have been shown to be localized in the vacuoles of vanadocytes. The pH in the vacuole correlates with that of vanadium ions, with the lowest pH value (1.9) in *A. gemmata* (38). The highest concentration of sulfate ions found in the blood cells of *A. gemmata* is 500 mM, which suggests that  $V^{III}$  and sulfate ions coexist in the vacuole (95). The sulfate ions incorporated into ascidian blood cells exist as complex with vanadium ions such as  $(V(HSO_4)(H_2O)_5)^{2+}$  or  $(V(SO_4)(H_2O)_5)^+$  at least in *A. ceratodes*, although the speciation of vanadium in the vanadocytes seems to be much more complex (93, 96). To maintain the concentration of sulfate ions in the blood cells of ascidians, an active transporter must exist. In our recent study, a sulfate transporter gene, *AsSUL1*, expressed in the blood cells of *A. sydneiensis samea* was isolated, and its sulfate ion transport activity was examined using a functional expression system in *Xenopus* oocytes (Figure 4) (97).

Kinetic studies of sulfate transport and the metabolic pathways of sulfate incorporation have been examined in various living organisms (98), and several genes involved in these processes, including *SUL1*, *SUL2*, *MET3*, *MET4*, and *MET6*, have been isolated. Assimilated sulfate is first reduced to sulfite using reducing equivalents produced by the oxidation of NADPH and used in the synthesis of organic sulfur metabolites (mostly cysteine, methionine, and *S*-adenosyl-methionine) in a process that requires considerable amounts of NADPH. The reduction of sulfate to sulfite proceeds via adenylation, which lowers the electropotential of sulfate so that it can be reduced to sulfite and sulfide by means of NADPH oxidation (99, 100). The relationship between high levels of sulfate ions assimilated in the vacuole and the expression of enzymes involved in the pentose-phosphate pathway in the cytoplasm of vanadocytes is noteworthy.

### Vanadium-binding proteins

Several proteins that are probably involved in vanadium accumulation and the redox process have already been isolated from a vanadium-rich ascidian, *A. sydneiensis samea*, including a vacuolar-type  $H^+$ -ATPase (55, 57, 101), chloride channel (102), enzymes of the pentose-phosphate pathway (76–79), glutathione transferase (103, 104), and vanadium-binding proteins such as Vanabins (105–110), and VBP-129



**Figure 4** AsSUL1 expression in *Xenopus* oocytes. (A) Na<sup>+</sup> dependency of sulfate uptake by AsSUL1. The initial sulfate concentration was 1 mM. (B) Kinetic properties of AsSUL1. Sulfate uptake was measured with increasing concentrations of sulfate in the presence of (35S) sulfate in uptake buffer containing 100 mM NaCl. The  $K_m$  and  $V_{max}$  values of AsSUL1 were 1.75 mM and 2500 pmol/oocyte/h, respectively. Reproduced from Ref. (97). Copyright 2009 Elsevier B.V.

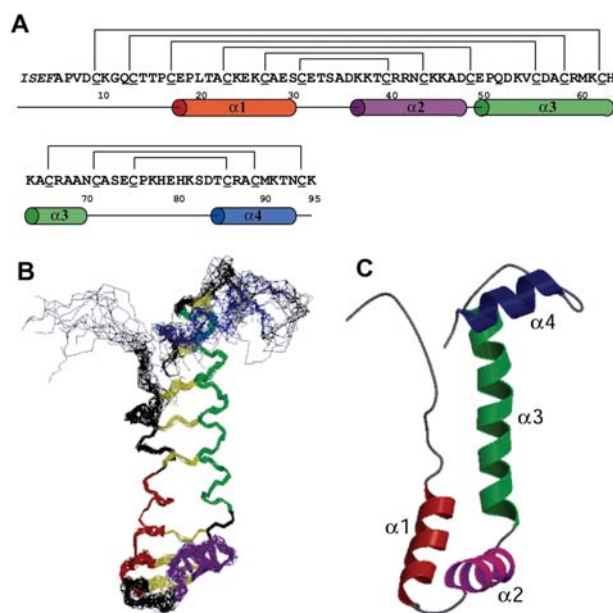
(111). Given the isolation of five homologs of Vanabins from another vanadium-rich ascidian species, *Ciona intestinalis* (112), but not from other organisms, Vanabins can only exist in vanadium-rich ascidians.

Structural and biochemical analyses have been largely conducted using Vanabin2, which possesses a unique bow-shaped structure with nine disulfide bonds (113). The Vanabin family consists of at least five closely related small proteins (Vanabin1 through Vanabin4 and VanabinP), which are composed of approximately 90 amino acids, including 18 cysteine residues. Recombinant Vanabin1, Vanabin2, and VanabinP were found to bind up to 20 vanadium ions in the +4 oxidation state ( $V^{IV}$ ) with dissociation constants of approximately  $2 \times 10^{-5}$  M (106, 111). An EPR study not only supported the binding number but also indicated that most of the  $V^{IV}$  ions were in the mononuclear state, coordinated to amine nitrogens (114). Electrospray ionization mass spectrometry indicated that the deconvoluted molecular mass of Vanabin2 is 10 467 Da, which is 18 mass units less than the predicted value. The complete reduction of Vanabin2 by 100 mM dithio-1,4-threitol at 50°C for 45 min caused an increase in the molecular weight by 18 mass units, indicating that the 18 cysteine residues in Vanabin2 form nine intra-

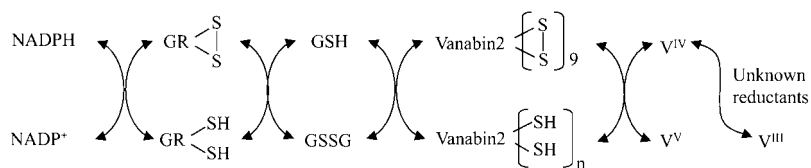
molecular disulfide bonds. In fact, the three-dimensional structure of Vanabin2 as determined by NMR spectrometry indicated a single chain of four  $\alpha$ -helices folded in half and connected by nine disulfide bonds (Figure 5, Vanabin2 3D) (113). Recently, Vanabin2 was shown to bind selectively to V(IV), Fe(III), and Cu(II) ions (115). Replacing the lysines and arginines with alanines at three sites, where lysine and arginine residues are clustered in Vanabin2, diminished the  $VO^{2+}$ -binding ability of Vanabin2 depending on the number of mutated residues at each site. Triple disulfide mutants near each site also affected the  $VO^{2+}$ -binding ability. These results indicate that the site (K10/R60) has high affinity for  $VO^{2+}$  (116).

### Thiol-disulfide exchange reactions

Recently, we showed that Vanabin2 was able to reduce  $V^V$  to  $V^{IV}$  in the presence of GSH. Therefore, we proposed a cascade including Vanabin2 that could be involved in redox and electron transfer from the electron donor (NADPH) to the acceptor (vanadium ions), conjugated through thiol-disulfide exchange reactions (117) (Figure 6). Thiol-disulfide exchange reactions are known to be involved in many cellular activities, such as protein folding and unfolding (118), the regulation of transcription factor activity (119),



**Figure 5** Structure of Vanabin2 from *A. sydneiensis samea*. (A) Amino acid sequence of Vanabin2. The amino terminal tag is italicized. Disulfide bond pairings, as determined by the CYANA calculation, are indicated at the top of the sequence. The secondary structure elements of Vanabin2 are indicated at the bottom of the sequence and are colored correspondingly in all panels. (B) The final 10 structures superposed over the backbone heavy atoms of residues 18–70. The side chains of the half-cysteine residues are shown as yellow lines. (C) Ribbon representation of a single structure in the same orientation as in panel (B). Reproduced from Ref. (113). Copyright 2005 American Chemical Society.



**Figure 6** A possible cascade for the thiol-disulfide exchange reactions conjugated with NADPH, GR (glutathione reductase), GSH, Vanabin2, and vanadium ions. Reproduced from Ref. (115). Copyright 2009 Elsevier B.V.

the activity of ribonucleotide reductase (120), the maintenance of redox potentials (121), responses against oxidative stress caused by metal ions (122), and metal transfer from metalloproteins to metal-depleted enzymes (metallochaperone activity) (123) in a manner analogous to the phosphorylation/dephosphorylation reactions catalyzed by protein kinases and phosphatases.

Ascidian vanadocytes contain not only GSH but also high intrinsic levels of vanadium. In fact, 13 mM vanadium (33) and 1.83 mM GSH were found in the vanadocytes of *A. sydneiensis samea* (117). An ascidian homolog encoding glutathione reductase (GR) was previously found by expressed sequence tags (EST) analysis in vanadocytes (108). In addition, enzymes of the pentose-phosphate pathway, which produces two molecules of NADPH per cycle, were localized in vanadocytes (76–79). Therefore, the equilibrium in thiol-disulfide exchange reactions could be established among NADPH, GR, GSH, Vanabin2, and vanadium ions in ascidian vanadocytes *in vivo*. Among them, NADPH, GR, and GSH are components of the glutathione system, which is known to act as an electron donor system coupled with glutaredoxin, glutathione peroxidase, and glutathione transferase (124). Therefore, we propose a possible cascade of electron transfer involving the glutathione system, Vanabin2, and vanadium ions as shown in Figure 6, in which the order of redox reactions was estimated from the results of circular dichroism (CD) spectroscopy, EPR spectrometry, and coupled NADPH oxidation assays.

In this cascade, electrons can be transferred from the donor (NADPH) to the acceptor (vanadium ions). In turn, the reduction of  $V^V$  to  $V^{IV}$  can occur via thiol-disulfide exchange reactions using Vanabin2. The resultant disulfides are converted to thiols by reduced GSH, and the oxidized GSH is further reduced by GR (124, 125). The disulfides of GR are reduced to thiols by NADPH, which could be linked to the pentose-phosphate pathway. Moreover, it is worth considering whether the reverse reactions accompanying the oxidation of  $V^{III}$  to  $V^{IV}$  and  $V^{IV}$  to  $V^V$  occur in the proposed cascade, perhaps resulting in a release of energy, just like the vanadium redox flow battery (126). Thus, ascidians can accumulate metal ions as an energy source.

## Outlook

It has been a century since the first finding that ascidian species contain high levels of vanadium and sulfate ions by Henze (27, 48–50). Since then, various hypotheses about the

functional role of these levels have been proposed, but most of them are not supported by sufficient evidence. However, the findings of reducing agents in ascidian blood cells by Califano and Caselli (127) and Bielig et al. (128) deserve greater attention. In our model, Vanabins participate in the cascade as vanadium-reducing enzymes via a thiol-disulfide exchange reaction and can accelerate the accumulation of vanadium (Figures 3 and 5); they can also play a physiological role in vanadium accumulation as  $V^{III}$  ions in the vacuole. Our recent DNA microarray experiments have suggested that various genes and proteins participate in redox systems, including those involving vanadium ions, via thiol-disulfide exchange reactions (submitted). We hope that the role of vanadium in ascidians will be clarified in the near future.

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