Chapter 3
High Levels of Vanadium in Ascidians

Hitoshi Michibata and Tatsuya Ueki

Abstract Henze's discovery of high levels of vanadium in an ascidian was not only a trigger for research in vanadium science, but also aroused great interest in the question of how such extraordinarily high levels of vanadium could be accumulated and what the role of vanadium in ascidians could possibly be. Many investigators, including inorganic, catalytic, and applied chemists, as well as physiological, molecular, and pharmaceutical biologists have been involved in this interdisciplinary problem. 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: the determination of high levels of vanadium, the identification of vanadium-accumulating blood cells, the energetics of vanadium accumulation, the sulfate transport system, the redox mechanism of vanadium, and the possible physiological roles of vanadium in ascidians.

Keywords Ascidian • Vanadium • Hyper-accumulation • Redox • Metal-binding proteins

3.1 Introduction

The discovery of vanadium compounds in ascidian blood cells dates back to 1911 when the German physiologist Martin Henze discovered high levels of vanadium in an ascidian collected from the Bay of Naples [1]. It is no exaggeration to say that Henze's discovery was a catalyst for research in vanadium science, which has involved not only inorganic, catalytic, and applied chemistry, but also physiological,

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molecular, and pharmaceutical biology. From 1903 to 1921, Henze was the head of the chemistry department at the Stazione Zoologica di Napoli, which was founded in March 1872 by Anton Dohrn, a fervent defender of Darwin's theory of evolution by natural selection. Dohrn dedicated his life to collecting facts and ideas in support of Darwinism [2].

Henze's discovery concerning ascidians attracted considerable interest because of the extraordinarily high levels of vanadium, which had not been reported in any other organism, and the possible role of vanadium as an oxygen carrier, it possibly being a third prosthetic group in respiratory pigments, the others being iron and copper. Although it was later demonstrated that vanadium does not play such a role, interest continued in the ascidians because of their evolutionary position between vertebrates and invertebrates, in the subphylum Urochordata. Ascidian juveniles have a notochord during the larval stage prior to metamorphosis, during which they accumulate high levels of vanadium. Following a series of Henze's studies [1, 3–5], Callifano & Caselli isolated from ascidian blood cells a vanadium-complex that they designated "haemovanadin" [6]. Bielig et al. [7], examining the biochemistry of haemovanadin, reported that haemovanadin was a complicated complex consisting of vanadium, sulfuric acid, protein and organic ligands, with a gross composition of $[C_{16}H_{19}N_3O_{12}]VO$.

3.2 Thermal Neutron Activation Analysis

Many analytical chemists and physiologists looked for not only vanadium, but also niobium, chromium, tantalum, tungsten, and titanium, employing a variety of analytical methods such as spectrocolorimetry, emission spectrometry, and atomic absorption spectrometry. However, direct comparisons could not be made with the data obtained because of varying sensitivity and precision of the methods used and because the data were reported variously, in terms of dry weight, wet weight, ash weight, and protein amount.

The most sensitive method for determining vanadium in those days was thermal neutron activation analysis, which is the method we used at the start of our studies. Seven species belonging to the suborder Phlebobranchia and eight species belonging to the suborder Stolidobranchia were analyzed for vanadium content. Samples were irradiated with thermal neutrons in the TRIGA MARK II nuclear reactor at Rikkyo University. Species belonging to the suborder Phlebobranchia were shown to have a higher vanadium content than those in the suborder Stolidobranchia. Of the ascidian tissues examined, blood cells contained the highest amounts of vanadium [8]. The highest concentration of vanadium (350 mM) was found in the blood cells of *Ascidia gemmata*, belonging to the suborder Phlebobranchia [9]. Levels of iron and manganese, determined simultaneously, did not vary much between the members of the two suborders (Table 3.1).

Table 3.1 Concentrations of vanadium in the tissues of several ascidians (mM)

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

The vanadium content in each tissue was quantitatively determined mainly by neutron-activation analysis [8, 9]
N.D. not determined

3.3 Identification of Vanadocytes

Ascidian blood cells (coelomic cells) are morphologically classified into 9–11 different types, grouped into six categories: hemoblasts, lymphocytes, leukocytes, vacuolated cells, pigment cells, and nephrocytes [10]. The vacuolated cells are classified into at least four types: morula cells, signet ring cells, compartment cells, and small compartment cells. Among them, morula cells had been thought to be the vanadium-accumulating cells, the so-called "vanadocytes" [11–15].

At the end of the 1970s, with the increasing availability of scanning transmission electron microscopes equipped with an energy-dispersing X-ray detector, it became possible to determine which cell type was the true vanadocyte. An Italian group first reported that it was not the morula cells, but rather the granular amoebocytes, signet ring cells, and compartment cells that emitted the X-rays characteristic of vanadium. Thus, identification of the true vanadocytes became a high priority matter to researchers concerned with the mechanism by which vanadium was accumulated in ascidians. Using density gradient centrifugation to isolate specific types of blood cells and using thermal neutron activation analysis to quantify vanadium in isolated subpopulations of blood cells, we showed that vanadium was accumulated in the signet ring cells of *Ascidia ahodori* [16]. In *Phallusia mammillata*, analysis with the chelating reagent 2,2-bipyridine, which is known to complex with vanadium in the +3 oxidation state, revealed that many types of blood cells, including signet ring cells, vacuolated amoebocytes, bivacuolated cells, and type-II compartment cells, were stained brown, indicating the presence of vanadium [17]. We also found evidence of vanadium in the signet ring cells of *Phallusia nigra* by transmission X-ray microscopy at the Synchrotron Radiation Center of Ritsumeikan University, Kyoto, Japan [18].

However, using the above methods it was not possible to obtain direct evidence of vanadium localization in vanadocytes. What could provide direct evidence for the location of vanadium was the scanning X-ray microscope installed at the

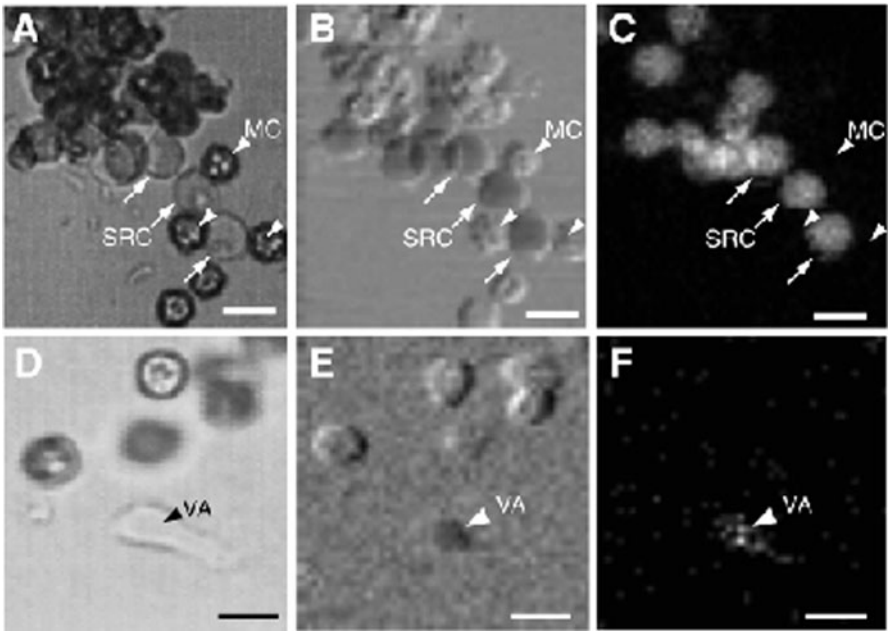

Fig. 3.1 *Phallusia mammillata* blood cells observed by differential interference contrast optical microscopy (**a**, **d**) and X-ray microscopy in the transmission mode (**b**, **e**) and fluorescence mode for vanadium (**c**, **f**). Photographs **a**–**c** are from the same field of view; photographs **d**–**f** are from another field. Vanadium is accumulated in signet ring cells (SRC, shown by *arrows*) and a vacuolated amoebocyte (VA, shown by *arrowheads* in **d**–**f**), but not in morula cells (MC, shown by *arrowheads* in **a**–**c**). Each scale bar = 10 mm (Reproduced from reference [21]. Copyright 2002 Zoological Society of Japan)

ESRF in the ID 21 beam line. This microscope is dedicated to X-ray imaging and spectromicroscopy in the 0.2–7 keV range, in both absorption and fluorescence modes [19, 20]. Because X-ray microscopy in this energy range is useful for observing hydrated specimens up to 10 μm thick, we used this technology to successfully visualize vanadium in living ascidian blood cells. The vanadium image obtained by integrating the fluorescence signal in only the vanadium window clearly showed that the signet ring cells and vacuolated amoebocytes contained vanadium, but the morula cells and compartment cells did not [21]. This study provided conclusive evidence that the true vanadocytes were the signet ring cells (Fig. 3.1).

3.4 Oxidation State of Vanadium

In seawater, the average concentration of vanadium is approximately 35 nM and the oxidation state of vanadium ions dissolved in seawater is the +5 oxidation state [22, 23]. When vanadium ions are taken in by ascidians, most of them are

reduced to the +3 oxidation state (V^{III}) via the +4 oxidation state (V^{IV}). Henze [1] first suggested that the blood cells of *P. mammillata* contained vanadium in the +5 oxidation state (V^{V}). Later, Lybing [24], Bielig et al. [25], Boeri and Ehrenberg [26] and Webb [27] reported the +3 oxidation state of vanadium in ascidians. Thereafter, studies using noninvasive physical methods, such as EPR, EXAFS, XAS, NMR, and SQUID, revealed that the intracellular oxidation state of vanadium is predominantly in the +3 oxidation state, with a small amount in the +4 oxidation state [28–31]. However, as mentioned above, ascidians have at least 9–11 different types of blood cells. The above physical methods were applied to whole blood cells, but not to isolated vanadocytes. Thus, we reexamined, using noninvasive EPR, the oxidation state of vanadium in fractionated vanadocytes of *Ascidia gemmata* under a reducing atmosphere. The results confirmed that 97.6% of the vanadium ions were in the +3 oxidation state with small amounts (2.4%) of V^{IV} ions [32].

3.5 Vanadium Reducing Agents

When vanadium ions are reduced to V^{III}, some reducing agent(s) must participate in the process. Several candidates for the reduction of vanadium in ascidian blood cells were proposed: tunichromes, a class of hydroxy-DOPA containing tripeptides [33], GSH, H_2S, NADPH, DTT [34], and thiols, such as cysteine [35].

As described later in detail, it is highly probable that NADPH and glutathione act as intrinsic reducing agents in ascidian blood cells. The enzymes of the pentose phosphate pathway, which is known to produce two molecules of NADPH per cycle, were identified in ascidian blood cells and shown to localize exclusively to the vanadocytes [36–39]. The enzymes identified were 6-PGDH (EC1.1.1.44), G6PDH (EC1.1.1.49), TKL (EC2.2.1.1), and GP (EC2.4.1.1). Glutathione was also reported to be localized in the vanadocytes, with a concentration estimated to be in the mM range [40]. Based on their redox potentials, these agents can reduce V^{V} to V^{IV} but cannot reduce V^{IV} to V^{III}. Thus, other agents are required for the reduction of V^{IV} to V^{III}.

Several reducing agents that may be involved in the reduction of V^{IV} to V^{III} have been discussed [41]. Among the biologically relevant molecules that may be reducing agents are cysteine complexes. Ascidian blood cells contain aliphatic sulfonic acids, such as cysteic acid, an oxidation product of cysteine [35], and cysteine methyl ester has been reported to reduce V^{IV} to V^{III} with the assistance of EDTA and EDTA-like ligands of aminopolycarboxylate in water [41, 42]. The reduction of V^{IV} to V^{III} by cysteine methyl ester was found to be aided by glycylhistidine and glycylaspartic acid [41]. Additionally, cleavage of the disulfide bonds of Vanabin2 resulted in the reduction of V^{V} to V^{IV} [40]. Thus, cysteine complexes are likely to participate in the reduction. To completely elucidate the electron transfer cascade from NADPH to vanadium ions, the unknown reductant involved in the reduction of V^{IV} to V^{III} must be clarified (Fig. 3.2).

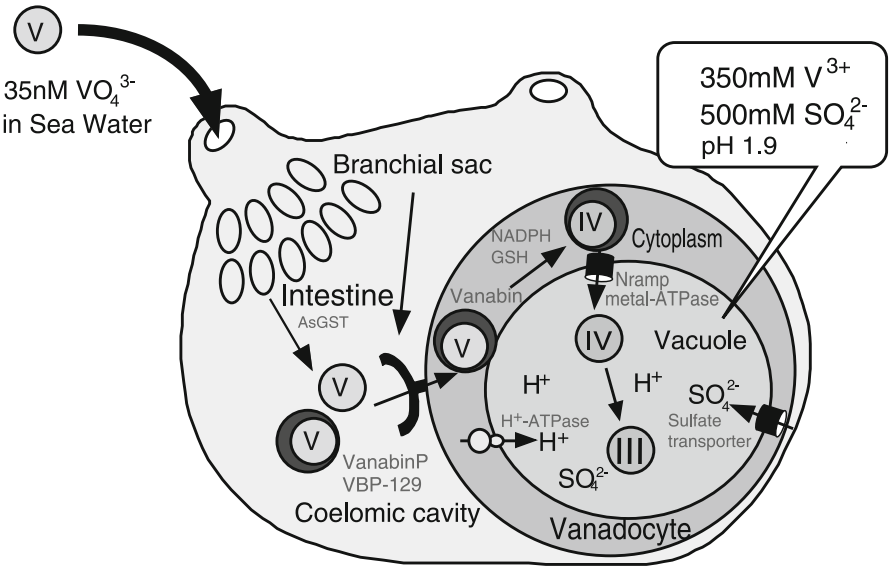

Fig. 3.2 Schematic representation of vanadium accumulation and reduction in ascidians. The concentration of vanadium in the +5 oxidation state is only 35 nM in seawater; in contrast, the highest concentration of vanadium in ascidian blood cells is 350 mM [9], and the concentration of sulfate is 500 mM [62]. The vacuole interior is maintained at an extremely low pH (1.9) by H^+-ATPases [9, 48]. In this environment, almost all of the vanadium accumulated is reduced to V^{III} via V^{IV} [32]. The first step in vanadium uptake may occur at a branchial sac or digestive organ (intestine), where glutathione-S-transferase was identified as a major vanadium carrier protein [91]. We discovered vanadium-binding proteins, designated Vanabins, in the blood plasma and cytoplasm of vanadocytes [68–69, 73, 75]. 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} [36–39]. A metal-ATPase that might be involved in vanadium transport has been found in the vacuolar membrane

3.6 Energetics of Accumulation

In addition to reporting a high level of vanadium, Henze [1] also reported extreme acidity in ascidian blood cells. The mysterious fact that the cells containing high levels of vanadium show extremely low pH has attracted the interest of investigators. The chemical species of vanadium are dependent on pH and the redox potential [43]. Thus, the pH values in ascidian blood cells are of interest to researchers in the vanadium sciences. In the 1980s, it became controversial whether ascidian blood cells have a low pH; some investigators insisted that the intracellular pH was neutral on the basis of measurements made by an improved transmembrane equilibrium using ^{14}C-labeled methylamine and on the basis of the ^{31}P chemical shift observed using NMR [44]. Nevertheless, 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.

Table 3.2 Correlation between the vanadium concentration and pH in ascidian blood cells

Species	Vanadium concentration (mM)	pH
A. gemmata	350	1.86
A. ahodori	60	2.67
A. sydneiensis samea	13	4.20

The vanadium content in each tissue was quantitatively determined mainly by neutron-activation analysis [8, 9]. pH values were measured by both a microelectrode and ESR under anaerobic conditions and converted into $[H^+]$ [9]

In our studies, we focused on this phenomenon from the perspective of the energetics of vanadium accumulation. In *Ascidia gemmata*, in which the signet ring cells contain the highest concentration of vanadium known (350 mM), the vacuolar pH (1.86) is also the lowest [9]. In *A. ahodori*, in which the signet ring cells contain 60 mM vanadium, the vacuolar pH is 2.67, and in *A. sydneiensis samea*, in which the signet ring cells contain 13 mM vanadium, the vacuolar pH is 4.20 [9]. Thus, a comparative analysis of the intracellular levels of vanadium versus pH values in the signet ring cells (vanadocytes) of three different species suggests a correlation between a high level of vanadium and a low pH (i.e., a higher concentration of protons) (Table 3.2).

V-ATPases are proton pumps that play an important 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 [45–47]. V-ATPases create electrochemical gradients between the plasma and vacuolar membranes. These proton gradients are used to drive secondary transport processes. In ascidian blood cells, the steep proton gradient is thought to be the energy source for the accumulation of vanadium ions, which, at maximum accumulation, exhibit a 10^7-fold concentration gradient across the membrane.

Immunocytological analysis revealed that V-ATPases are localized in the vacuolar membranes of vanadocytes. Additionally, a specific inhibitor of V-ATPases, bafilomycin A_1, inhibits the proton pump in vanadocyte vacuoles, resulting in neutralization of the vacuolar contents [48]. Thus, V-ATPases were ascertained to function in vanadocytes. V-ATPases are composed of many subunits that are clustered into a membrane-associated domain (V_0 domain) and a peripherally associated domain (V_1 domain). Subunit C of the V-ATPases was reported to play an important role in the regulation of V-ATPases; when isolated cDNA encoding subunit C was expressed in the corresponding pH-sensitive budding yeast mutant *vma5*, which is normally viable only in a low pH medium, the transformed yeast mutant could grow in a neutral pH medium [49], suggesting it may have acquired the ability to acidify its intracellular contents. As a follow-up to this study, a functional assay to examine whether protons concentrated by V-ATPases are linked to the accumulation of vanadium should be conducted.

In the vacuoles of vanadocytes, almost all vanadium ions are reduced to V^{III} [32]. In such an environment, the possibility cannot be excluded that hydrolysis of the coordinating water molecules contributes to the extremely low pH.

3.7 Sulfate Ions

The co-existence of high levels of both vanadium and sulfate ions has attracted investigators since Henze first discovered this unusual phenomenon [1]. Considerable amounts of sulfate and sulfur compounds have been reported to be associated with vanadium in ascidian blood cells [5, 30, 35, 50–61]. We reported that the blood cells of the most vanadium-rich ascidian, *A. gemmata*, contain 350 mM vanadium, and that 97% of this vanadium is reduced to V^{III} [32]. Raman spectroscopy revealed that the ratio of the levels of sulfate and vanadium in blood cells from *A. gemmata* is approximately 1.5, which is the expected value if sulfate ions are present as the counterions of vanadium ions in the +3 oxidation state [62]. In *A. sydneiensis samea*, the ratio was 2.2 (86 mM sulfate vs. 38 mM V^{III}) in the blood cell fraction without giant cells [63]. This value is within an appropriate range for the existence of sulfate as counter ions, although the ratio is beyond the expected ratio of 1.5. In another ascidian, *A. ceratodes*, X-ray absorption spectrometry of whole blood cells in comparison with inorganic models revealed the V^{III}: sulfate ratios to be 1.0:1.1 and 1.9:1.0 in two specimens; however, the combined concentration of sulfate and sulfonate (SO_3^-) ions was sufficient to balance the ionic charge [30]. Together, these results support the hypothesis that sulfate ions are actively accumulated in ascidian blood cells and their concentration is sufficient to balance the charges in the vacuoles of vanadocytes.

Kinetic studies of sulfate transport and the metabolic pathways of sulfate incorporation have been examined in various living organisms [64], 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 is then used in the synthesis of organic sulfur metabolites (mostly cysteine, methionine, and *S*-adenosylmethionine) in a process that requires considerable amounts of NADPH. The reduction of sulfate to sulfide proceeds via adenylation, which lowers the electropotential of sulfate so that it can be reduced to sulfite and sulfide by means of NADPH oxidation [65, 66]. The correlation 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 [67].

To identify cDNA encoding a sulfate transporter from blood cells of *A. sydneiensis samea*, a PCR using degenerate primers corresponding to the conserved region among known sulfate transporters was performed. The deduced amino acid sequence of one of the representative cDNAs encoding a putative sulfate transporter had striking similarities to Slc13-type sulfate transporters from

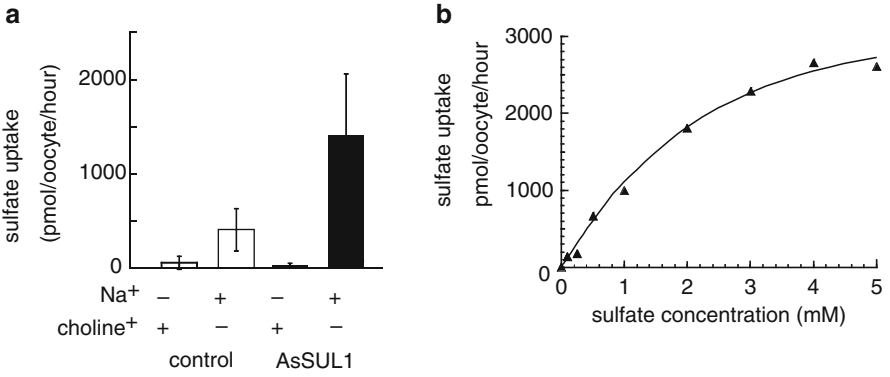

Fig. 3.3 *As*SUL1 expression in Xenopus oocytes. (**a**) Na^+ dependency of sulfate uptake by AsSUL1. The initial sulfate concentration was 1 mM. (**b**) Kinetic properties of *As*SUL1. Sulfate uptake was measured with increasing concentrations of sulfate in the presence of (^{35}S) sulfate in uptake buffer containing 100 mM NaCl. The K_m and V_{max} of *As*SUL1 were 1.75 mM and 2,500 pmol/oocyte/h, respectively (Reproduced from reference [63]. Copyright 2009 Elsevier B.V.)

various organisms [63]. The putative protein product was calculated to possess 12 transmembrane domains and a C-terminal signature sequence, which are also common characteristics of the Slc13 family. Thus, the corresponding gene was designated *A. sydneiensis samea* sulfate transporter 1 (*As*SUL1). The isolated *As*SUL1 cDNA was used for a transport assay using the *Xenopus* oocyte expression system. The *Xenopus* oocyte expressing *As*SUL1 was observed to take up about threefold higher amounts of SO_4^{2-} in the presence of Na^+ than that of the control [63]. Thus, it was concluded that *As*SUL1 encoded a protein of the Slc13 family of Na^+-dependent sulfate transporters. The significance of the co-existence of high levels of vanadium and sulfate ions in the vacuoles of ascidian vanadocytes should be clarified as a next step (Fig. 3.3).

3.8 Vanabins: Vanadium-Binding Proteins

Although it stands to reason that some proteins must participate in the accumulation process of vanadium in ascidians, no protein had been reported until we first identified a vanadium-associated protein from the vanadium-rich ascidian, *A. sydneiensis samea* [68]. When a homogenate of blood cells was applied to a DEAE-Sephacel anion-exchange column, one major peak containing both vanadium and proteins was obtained. Resolved by SDS-PAGE, the peak included at least two major proteins with apparent molecular masses of 12.5 and 15 kDa, and a minor 16-kDa protein. Using immunoscreening or PCR, cDNAs encoding 12.5 and 15 kDa proteins were identified [69]. Recombinant proteins, designated as Vanabin1 and Vanabin2, of these two independent but related cDNAs, were shown to bind, respectively, 10

and 20 V^{IV} ions, with dissociation constants (K_d) of 2.1×10^{-5} and 2.3×10^{-5} M [69]. The binding of V^{IV} (VO^{2+}) to these Vanabins was inhibited by the addition of Cu^{2+} ions, but not by Mg^{2+} or MoO_4^{2-} ions. The conserved motif of the Vanabins can be described as the consensus sequence {C}−{X2–5}−{C}. The minor 16-kDa protein band was revealed to correspond to sequence variants of Vanabin2 [70]. Vanabins are rich in charged residues, such as arginine (3/87 and 5/91 in Vanabin1 and Vanabin2, respectively), aspartate (6/87 and 6/91), glutamate (2/87 and 7/91), and lysine (12/87 and 14/91), whereas metallothioneins are rich in serine and lysine. By a mutagenesis study, two regions where positively charged amino acids are gathered have recently been reveled to be responsible for V^{IV} binding [71]. 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 so far [72]. Vanabin2 was shown to selectively bind V^{IV}, Fe^{3+}, and Cu^{2+} ions under acidic conditions. In contrast, Co^{2+}, Ni^{2+}, and Zn^{2+} 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 [72]. Additionally, EST analysis of vanadocytes revealed that two other novel Vanabins, designated as Vanabin3 and Vanabin4, have primary structures that are similar to those of Vanabin1 and Vanabin2, bind qualitatively to V^{IV} ions, and are expressed in vanadocytes [73, 74]. These Vanabins, Vanabin1–4, were shown to be localized in the cytoplasm of vanadocytes (Fig. 3.4).

Although vanadium ions are supposedly taken up from seawater through the branchial sac or alimentary canal, transferred to the coelom, and concentrated in vanadocytes, it is not known if carrier proteins are involved in the transport of vanadium from the coelomic fluid (blood plasma) into the vanadocytes. Using IMAC, we identified several vanadium-associated proteins in the coelomic fluid (blood plasma) and cloned the cDNA for the major protein. Sequence analysis indicates that this protein is a novel Vanabin, which we have designated "VanabinP" (Vanabin in plasma) [75]. RT-PCR analysis and *in situ* hybridization indicated that the VanabinP gene was transcribed in some cell types localized to peripheral connective tissues of the alimentary canal, muscle, blood cells, and a portion of the branchial sac. Recombinant VanabinP bound a maximum of 13 V^{IV} ions per molecule with a K_d of 2.8×10^{-5} M. These results suggest that VanabinP is produced in several types of cell, including blood cells, and is immediately secreted into the blood plasma, where it functions as a V^{IV} carrier.

A better understanding of the functions of Vanabins and the mechanism of vanadium accumulation in ascidians requires high-quality 3D structures of the proteins in the presence and absence of vanadium ions. We reported the solution structure of Vanabin2 by multidimensional NMR experiments [75]. Vanabin2 is composed of 91 amino acids, including 18 cysteines. The electrospray ionization (ESI) mass spectrum of Vanabin2 showed a deconvoluted molecular mass of 10,467 Da, which is 18 mass units lower than the expected molecular mass of the protein in which all of the cysteine residues are reduced. Indeed, the complete reduction of Vanabin2 by DTT caused the molecular weight (at 10,485 Da) to increase by 18 mass units, indicating that all 18 cysteine residues of Vanabin2

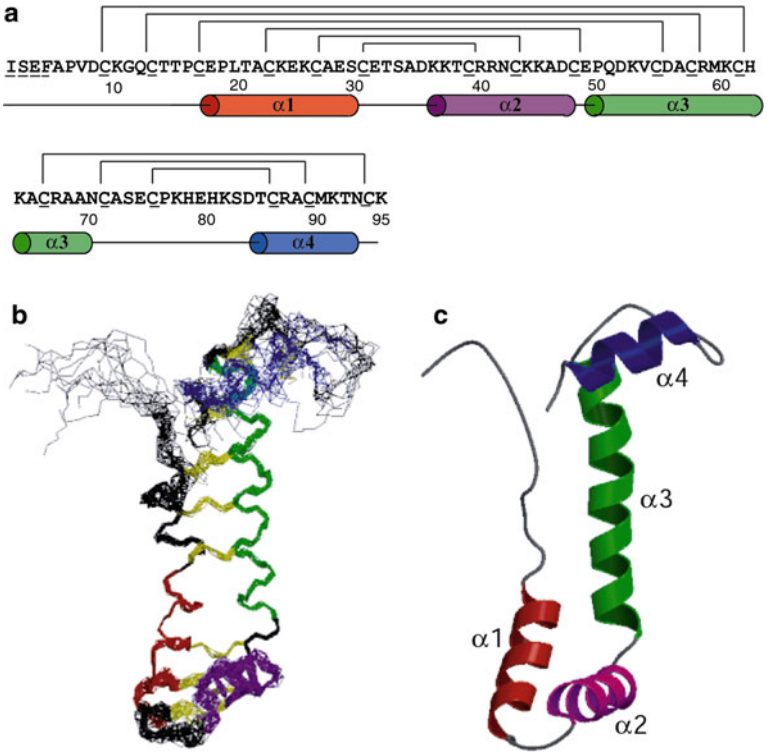

Fig. 3.4 Structure of Vanabin2 from **a**. sydneiensis samea. (**a**) Amino acid sequence of Vanabin2. The amino-terminal tag is *italicized*. Disulfide bond pairings, as determined by the CYANA calculation, are indicated at the *top* of the sequence. The secondary structure elements of Vanabin2 are indicated at the *bottom* of the sequence and are colored correspondingly in all panels. (**b**) The final ten 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 reference [76]. Copyright 2005 American Chemical Society)

are involved in intramolecular disulfide bonds [76]. Thus, the NMR study was carried out under nonreducing conditions. The structural analysis revealed a novel bow-shaped conformation, with four α-helices connected by nine disulfide bonds. No structural homolog has been reported to date. The ^{15}N-HSQC perturbation experiments of Vanabin2 indicate that V^{IV} ions, which are exclusively localized on the same face of the molecule, are coordinated by amine nitrogens derived from amino acid residues, such as lysines, arginines, and histidines, as suggested by the EPR results [77].

Structural modeling of Vanabins 1, 3, and 4, based on the NMR data of Vanabin2, resulted in fairly homologous structures, whereas the modeled structure of VanabinP differed from that of Vanabin2, especially in the long loop domain between the third and fourth helices. Generally, metal-binding proteins can interact with

other metal-binding proteins and proteins, such as membrane metal transporters, membrane anchor proteins, or metal-reducing/oxidizing proteins [78]. Thus, using the Far Western blotting method, several proteins that interact with Vanabins were obtained. Among these, Vanabin interacting protein 1 (VIP1) was shown to be localized in the cytoplasm of vanadocytes and to clearly interact with Vanabins 1–4, but not with VanabinP, based on two hybrid analyses [79]. To determine whether ascidian species other than *A. sydneiensis samea* have Vanabin-like genes, we searched for the genes in a database and found five groups of cDNAs that encoded Vanabin-like proteins in another ascidian, *Ciona intestinalis* [80]. The genes encoding *C. intestinalis* Vanabins, *Ci*Vanabin1 to *Ci*Vanabin5, were clustered in an 8.4-kb genomic region. All the *C. intestinalis* Vanabins were cysteine-rich, and the repetitive pattern of cysteines closely resembled that of *A. sydneiensis samea* Vanabins. Using immobilized metal ion affinity chromatography (IMAC), we found that a recombinant protein of at least one of the *C. intestinalis* Vanabins (*Ci*Vanabin5) bound to V^{IV} ions [80].

3.9 Vanabin as Vanadium Reductase

Vanabin2 is a rare protein with nine disulfide bonds per molecule. The fully oxidized disulfide bonds were observed on polyacrylamide gels at a position corresponding to 14 kDa. When exposed to concentrations of more than 0.6 mM DTT and 10 mM 2-ME, Vanabin2 migrated to a position corresponding to 20 kDa with a concomitant disappearance of the isoellipticity points in the CD spectra, suggesting that the disulfide bonds of Vanabin2 were reduced and cleaved [40]. The treatment of Vanabin2 with an intrinsic reducing reagent, 1–4 mM GSH, which corresponds to the intrinsic concentrations in vanadocytes, resulted in mild migration, indicating that Vanabin2 was partially reduced.

On the other hand, thiol–disulfide exchange reactions are known to be involved in many cellular activities, such as protein folding and unfolding [81], regulation of transcription factor activity [82], activity of ribonucleotide reductase [83], maintenance of redox potentials [84], responses against oxidative stress caused by metal ions [85], and metal transfer from metalloproteins to metal-depleted enzymes (metallochaperone activity) [86] in a manner analogous to phosphorylation/dephosphorylation reactions catalyzed by protein kinases and phosphatases.

We examined whether thiol–disulfide exchange reactions in Vanabin2 were involved in reduction of V^{V} to V^{IV} [40]. EPR spectrometry was performed to detect VO^{2+} (V^{IV}) species, which exhibit a typical signal consisting of eight-line manifolds. Within 24 h after addition of 10 μM Vanabin2 to a reaction mixture containing 10 mM V^{V} and 2 mM GSH at room temperature, a large signal due to V^{IV} was observed. In contrast, when 2 mM GSH was added to a 10 mM V^{V} solution without Vanabin2, only a slight increase in EPR signal intensity was observed after a 24 h incubation. Based on these results, we concluded that Vanabin2 acts as a vanadium reductase.

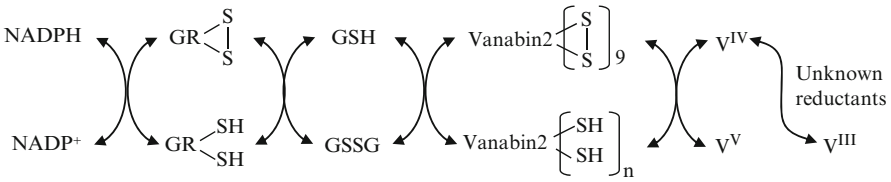

Fig. 3.5 A possible cascade for the thiol-disulfide exchange reactions conjugated with NADPH, GR (glutathione reductase), GSH, Vanabin2, and vanadium ions (Reproduced from reference [40]. Copyright 2009 Elsevier B.V.)

Due to the inherent difficulty of rapidly monitoring by EPR the reduction of V^V by Vanabin2, activity of the reductase was instead measured using a coupled NADPH oxidation assay with reduction of GSSG catalyzed by glutathione reductase (GR) [40]. As a negative control, the first assay determined that little NADPH was oxidized by 2 mM GSH and 0.25 U/mL GR in the absence of Vanabin2 and V^V ions. When 4 μM Vanabin2 was added to the assay system, NADPH was slightly oxidized. When 0.1–2.5 mM V^V was added to 2 mM GSH and 0.25 U/mL GR, the levels of oxidized NADPH increased slightly with increasing V^V concentration. Finally, addition of both Vanabin2 and V^V to 2 mM GSH and 0.25 U/mL GR increased the amount of oxidized NADPH markedly with increasing V^V concentration, reaching saturation at 1.5 mM V^V. The V_{app} and K_{app} values for Vanabin2-catalyzed V^V reduction were 1.15 mol-NADPH/min/mol-Vanabin2 and 0.51 mM, respectively. Thus, Vanabin2 is a novel vanadium reductase, because partial cleavage of its disulfide bonds by GSH results in the reduction of V^V to V^{IV}.

Vanabin2 forms a possible electron transfer cascade from the electron donor, NADPH, via GR, GSH, and Vanabin2 to the acceptor vanadium ions conjugated through thiol–disulfide exchange reactions [40] (Fig. 3.5). In this cascade, electrons are ultimately transferred from the donor NADPH to the acceptor vanadium ions. In this model, reduction of V^V to V^{IV} occurs via thiol–disulfide exchange reactions of Vanabin2. The resulting disulfides are converted to thiols by reduced GSH and the oxidized GSSG is then re-reduced by GR [87, 88]. The resulting disulfides of GR are reduced to thiols by NADPH [87, 88], which may be linked to the pentose-phosphate pathway. We are currently in the process of confirming the intrinsic existence of the components, such as glutaredoxin. The situation in ascidians is complicated, however, by the fact that V^{IV} is further reduced to V^{III}.

3.10 Other Proteins Involved in Vanadium Accumulation

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 V-ATPase [48, 49, 89], chloride channel [90], enzymes of the

pentose-phosphate pathway [36–39], glutathione transferase [91, 92], VBP-129 [93], and *As*Nramp [94]. In this section we briefly describe the last three proteins recently isolated.

Seawater containing plankton as food is taken by ascidians through their branchial siphon and branchial sac into the digestive tract, which consists of a narrow tube commencing with the opening of the esophagus at the base of the branchial sac. The molecular mechanisms underlying the uptake of vanadium from seawater in the ascidian digestive system had not been investigated. Thus, using a V^{IV}-chelating column, we investigated whether there were vanadium-binding proteins in the ascidian digestive system. Consequently, vanadium-binding proteins with striking homology to GSTs, designated *As*GST-I and *As*GST-II, were isolated [91]. The recombinant *As*GST-I formed a dimer, exhibited GST activity, and was able to bind both V^{IV} (VO^{2+}) and V^{V} ($VO_4{}^{3-}$). *As*GST-I bound approximately 16 vanadium ions per dimer as either V^{IV} or V^{V}, with K_d values of 1.8×10^{-4} and 1.2×10^{-4} M, respectively. *As*GST-I also bound Fe^{3+} and Cu^{2+} with high affinity, in the order $Cu^{2+} > VO^{2+} > Fe^{3+}$, and it bound Co^{2+}, Ni^{2+}, and Zn^{2+} with low affinity [92]. The expression levels of *As*GSTs are exceptionally high in the digestive system relative to the other major organs and tissues, as determined by immunoblotting. Because *As*GSTs may be among the first molecules involved in the influx of vanadium ions through the digestive system, it is important to understand them in analyzing the first step of the ten million-fold vanadium-accumulating process in ascidians.

Vanadium ions taken in through the branchial sac and digestive tract must be discharged into the body cavity where the blood plasma is filled. At least two vanadium-binding proteins, VanabinP [75] and VBP-129 [93], have been isolated from ascidian plasma. VBP-129, designated as a vanadium-binding protein consisting of 129 amino acid residues, was identified in blood plasma of the vanadium-rich ascidian *A. sydneiensis samea*. Although VBP-129 mRNA was transcribed in all tissues examined, the VBP-129 protein was exclusively localized in the blood plasma and muscle cells of this ascidian. It bound not only VO^{2+} but also Fe^{3+}, Co^{2+}, Cu^{2+}, and Zn^{2+}; on the other hand, a truncated form of VBP-129, designated VBP-88, bound only Co^{2+}, Cu^{2+} and Zn^{2+}. In a pull-down assay, an interaction between VanabinP and VBP-129 occurred both in the presence and absence of VO^{2+}. These results suggest that VBP-129 and VanabinP function cooperatively as metallochaperones in ascidian blood plasma [93].

As previously mentioned, Vanabins1–4 were shown to be localized in the cytoplasm of vanadocytes. Thus, some membrane metal transporter(s) seem to be necessary to transport vanadium ions into the cytoplasm through the membrane. Because the Nramp/DCT family of membrane metal transporters is known to transport a broad range of divalent cations (Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+}) across the membrane using a proton gradient as the motive force, localization of a homolog of the Nramp/DCT family in the membrane of ascidian vanadocytes was examined, using immunological methods. Consequently, a cDNA encoding a protein closely related to the Nramp family of proteins was cloned from the cDNA library of *A. sydneiensis samea* blood cells. We found that the product

of this gene, *As*Nramp, was localized on the vacuolar membrane and operated as an antiporter of VO^{2+} ions and H^+ ions. In contrast, a rat homolog of Nramp/DCT, rDCT1, could not transport VO^{2+} under any of the conditions examined [94]. The results of this study indicated that *As*Nramp was a $VO^{2+} \cdot H^+$ 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. These results will be reported in detail as an original paper.

Several other pathways may exist that function together. Another possible mechanism for VO^{2+} transport into the vacuole is a P_{1B}-type ATPase, *As*HMA1, which we have already cloned from blood cells of *A. sydneiensis samea* and the metal selectivity and transport activity of which we have examined (Ueki et al. unpublished data).

3.11 Possible Role of Vanadium in Ascidians

Our final objective is to clarify the physiological function of vanadium in ascidian blood cells. As described in Sect. 3.9, thiol–disulfide exchange reactions in Vanabin2 are involved in the reduction of V^V to V^{IV}. Vanabin2 may act as an electron carrier in an electron transfer cascade from the electron donor, NADPH, to the acceptor vanadium ions, via GR, GSH, and Vanabin2, which both conjugates and reduces the vanadium ions through thiol–disulfide exchange reactions. Thus, ascidians may accumulate the metal ions as a source of oxidizing energy that can drive redox reactions in blood cells or in other tissues.

One approach to identifying the physiological function of vanadium is the comprehensive study of the effect of vanadium on gene expression. A draft genome of the ascidian species *Ciona intestinalis* has been read and inferred to contain approximately 16,000 protein-coding genes [95]. This species accumulates vanadium to a concentration of 0.6 mM in its blood cells [8] and is a model organism for the study of vanadium accumulation. cDNAs for transcripts of 13,464 genes have been characterized and compiled as the "*Ciona intestinalis* Gene Collection Release I" [96, 97], and a custom oligo-based DNA microarray is available [98]. We have performed a study to identify genes regulated by excess vanadium ions, using DNA microarrays (Kume et al. unpublished data). Among 39,523 gene-specific probes on the microarray slide, 550 spots were up-regulated and 820 spots were down-regulated by treatment with 1 mM V^{IV} or V^V ions for 24 h, suggesting that the overall change in gene expression was similar between V^{IV}- and V^V-treated individuals. Vanabins, subunits of V-ATPase, and several metal transporters were up-regulated. Among the major metabolic pathways, expression of enzymes in the glutathione-related pathway, such as glutaredoxin, thioredoxin reductase, peroxiredoxin, and sulfiredoxin was affected by treatment with V^V or V^{IV}. The results will be reported in detail as an original paper.

Another comprehensive approach to identify the function of vanadium in ascidians could be a mutational analysis to find a mutant strain that cannot accumulate vanadium. In *C. intestinalis*, such an approach is available by using transposon mutagenesis [99–101].

3.12 Outlook

It has been a century since the first finding that ascidian species contain high levels of vanadium and sulfate ions by Henze [1, 3–5]. Since then, various hypotheses about the functional role of vanadium have been proposed, but most of them are not supported by sufficient evidence. From the middle of the 1990s, many genes and proteins have been shown to be involved in the process of accumulating and reducing vanadium in ascidians. Vanabins are one family of such genes and proteins found only in vanadium-rich ascidians and are thought to be the key molecule in this process. In our model, Vanabins participate in an electron transfer cascade, in which electrons are transferred from the donor (NADPH) to the acceptor (vanadium ions), via GR, GSH, and Vanabin2, which acts as a vanadium-reducing enzyme via thiol-disulfide exchange reactions, which, in turn, may accelerate the accumulation of vanadium (Fig. 3.5). Our recent comprehensive studies, such as DNA microarray experiments, have suggested that various genes and proteins participate in redox systems 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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