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Mechanism of Vanadium Accumulation and Possible Function of Vanadium in Underwater Adhesion in Ascidians

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Abstract. Ascidians are marine animals that belong to the same phylogenetic group (Phylum Chordata) as human beings do. One of the three suborders in ascidians can accumulate a high level of vanadium ions in blood cells. *Ascidia gemmata* has been reported to accumulate the highest levels of vanadium at 350 mM, which is 10^7-fold higher than the vanadium concentration in seawater. In the last two decades, many genes and proteins related to vanadium accumulation and reduction have been revealed by molecular biological and biochemical methods. Modern omics approach enhanced the comprehensive identification of factors related to this phenomenon. In this review article, first, we would like to summarize the history of studies on vanadium accumulation in ascidians briefly. Then, we would like to overview recent advances by omics studies. How ascidians selectively accumulate vanadium is discussed from biochemical properties of proteins responsible for each step, and why ascidians accumulate vanadium is discussed in relation to the underwater adhesion.

INTRODUCTION

Ascidians (sea squirts or tunicates) are well known to accumulate extremely high levels of vanadium in their blood cells. The highest amount was found in blood (coelomic) cells of an ascidian *Ascidia gemmata* (Fig. 1A). The vanadium concentration in this species reaches 350 mM [1-2], which is 10^7 times the concentration found in seawater (35 nM) [3-4]. This is thought to be the highest degree of accumulation of metal in any living organism. How and why ascidians accumulate vanadium in a highly selective manner and at such an extremely high levels? To address these questions, our research group has been trying to identify genes and proteins responsible for the accumulation and reduction of vanadium in blood cells, as well as the process of vanadium transport from seawater to blood cells through the branchial sac, intestine and blood plasma.

In this review article, first, we would like to summarize the history of the studies on vanadium accumulation in ascidians briefly. Next, we would like to overview recent advances by omics studies on this phenomenon. How ascidians selectivity accumulate vanadium is discussed from biochemical properties of proteins responsible for each step, and why ascidians accumulate vanadium is discussed in relation to underwater adhesion function.

HISTORY OF STUDIES ON VANADIUM ACCUMULATION IN ASCIDIANS

Ascidians are marine sessile invertebrate animal belonging to the Phylum Chordata. They have a swimming larval stage with a body plan conserved with other chordates, but after metamorphosis, they adhere to some substrate, drastically reconstruct the body plan and become a round-shape adult form. Reflecting their phylogenetic position, they are pretty good models for studying genome evolution, early development, immune system and nervous system [5-6]. A cosmopolitan species, *Ciona intestinalis* or *Ciona robusta* (Fig. 1C), is the best model, whose whole genome was determined as the 7th animal species so far determined [7]. On the contrary, they possess unique features that are

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not found in other chordates; cellulose synthesis [8-12], asexual reproduction [13,14], and metal accumulation [15-18].

About a hundred years ago, the German physiological chemist Dr. Martin Henze discovered high levels of vanadium in the blood cells of an ascidian *Phallusia mammillata* (Fig. 1E) 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 the tissues of many ascidians has been determined by neutron activation analysis, electron paramagnetic resonance (EPR), or atomic absorption spectrometry (AAS). Ascidians belonging to the suborder Phlebobranchia contain higher levels of vanadium than those of the suborder Stolidobranchia [2]. Of the tissues examined, blood cells contain the highest amounts of vanadium (Table 1).

Ascidian blood cells can be classified into 9–11 different types, which are classified based on their morphology [21]. For many years, morula cells were thought to be the vanadium-accumulating cells or vanadocytes. But, by the direct measurement of vanadium in each type of blood cell, it was probed that morula cells are not cells that accumulate vanadium, but signet ring cells and some of the vacuolated cells are true vanadocytes [22-23]. The more detailed history is given in previous reviews [24-26].

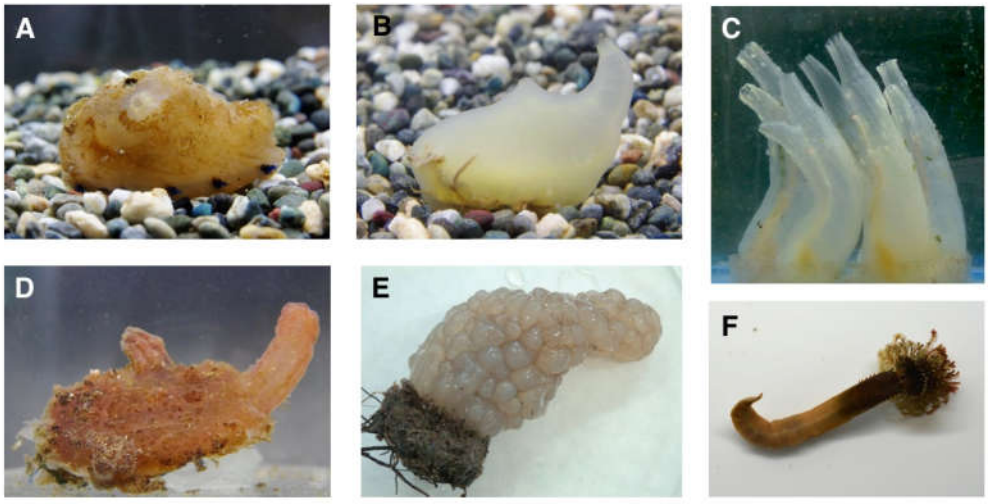

FIGURE 1. Vanadium-rich ascidians and a Polychaeta worm. (A) *Ascidia gemmata*, (B) *Ascidian ahodori*, (C) *Ciona intestinalis* or *Ciona robusta*, (D) *Ascidia sydneiensis samea*, (E) *Phallusia mammillata*, and (F) *Pseudopotamilla occelata*. Reproduced from [27] with the permission from YODOSHA Co., Ltd..

TABLE 1. Vanadium concentrations in tissues/organs in representative ascidian species. Data are expressed as millimolar concentration. Species belonging to the suborder Phlebobranchia accumulate more vanadium than those belonging to Stolidobranchia [1-2].

Suborder	Species	Tunic	Mantle	Branchial sac	Blood plasma	Blood cells
Phlebobranchia	*Ascidia gemmata*	-	-	-	-	347.2
	Ascidia ahodori	2.4	11.2	12.9	1.0	59.9
	Ascidia sydneiensis samea	0.06	0.7	1.4	0.05	12.8
	Ciona intestinalis	0.003	0.7	0.7	0.008	0.6
Stolidobranchia	*Styela plicata*	0.005	0.001	0.001	0.003	0.007
	Halocynthia roretzi	0.01	0.001	0.004	0.001	0.007
	Halocynthia aurantium	0.002	0.002	0.002	-	0.004

MECHANISM OF VANADIUM ACCUMULATION

Accumulation and Reduction of Vanadium in Vanadocytes

Vanadium ions outside the cell generally exist as vanadate anions (V^{V}). In vanadocytes, most of the vanadium is reduced to V^{III} via V^{IV}. V^{III}-aqua ions are only stable at low pH, and the acidic vacuole is the suitable compartment for storage of V^{III} ions. V^{IV}-aqua ions are also stable at low pH, but the cytoplasm is not acidic. Therefore, V^{IV}-binding proteins must exist in the cytoplasm to absorb and stabilize V^{IV} ions at a physiological pH. Reducing agents must participate in the accumulation of vanadium in vanadocytes. Vanadium-binding proteins named Vanabins are key proteins.

Vanabins were first isolated from *Ascidia sydneiensis samea* (Fig. 1D) as a novel vanadium-binding proteins by anion exchange column chromatography [28]. In this species, the Vanabin family consists of six closely related proteins. Among them, Vanabins 1–4 and VanabinP have been reported [29-31], while the other has been identified by a recent transcriptome analysis on blood cells (Tri *et al.,* unpublished data). All of these Vanabins possess 18 cysteine residues, and the intervals between cysteines are very well conserved. A homology search of public DNA and protein databases revealed homologous proteins with striking similarities only in the two ascidian species, *Ciona intestinalis* and *C. savignyi* [32]. In addition, EST analysis yielded two Vanabin homologs (*Ag*Vanabin1 and *Ag*Vanabin2) in *A. gemmata* [33]. Vanabins therefore seem 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.

Vanabin2 catalyzes the reduction of V^{V} to V^{IV} in the presence of glutathione reductase and glutathione [34] or the presence of thioredoxin [35] as intermediates of redox cascades starting from NADPH. An NADPH-coupled redox assay yielded no evidence for reduction of the two 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 [36]. Thus, so far, Vanabin2 acts only as a V^{V}-reductase. This high selectivity may be the key factor for the metal ion selectivity of vanadium accumulation in ascidians.

In our model, V^{V} ions are readily reduced to V^{IV} in the cytoplasm by Vanabins, and V^{IV} ions are stabilized by Vanabins, which act as both V^{V}-reductases and V^{IV}-chaperones. Ascidians possess several vanadium-binding proteins, vanadium-transporters and vanadium reductases in their genome and combine them to selectively accumulate and reduce vanadium in blood cells. Some are new invention during the course of ascidian evolution, while the others are common to other groups of organisms. The combination of this protein as a system should make it possible to accumulate vanadium selectively. More detailed review of accumulation and reduction mechanism can be found in our recent review article [37].

Uptake of Vanadium from Seawater: Direct or Indirect?

Vanadium absorption by ascidians was first experimentally demonstrated by Goldberg *et al*. [38]. They administrated individuals of two ascidian species *Ascidia ceratodes* and *Ciona intestinalis* with radioactive V^{48} as a form of vanadate anions (V^{V}). Radioautographic analysis indicated that vanadium is seen primarily in the ovary, gut wall, eggs, and branchial basket. They also observed that a high concentration of phosphate ions competed with vanadium uptake. Thereafter, Dingley *et al.* pointed out that the phosphate anion transporter is a candidate for the specific transporter of vanadate anions [39]. However, for a rebuttal on Goldberg's experiments where phosphate ions cannot completely inhibit the uptake of vanadate ions, Kalk pointed out the possibility of uptake of V^{IV} ions complexed with sulfate in the mucosa [40]. Thus, both the direct absorption of V^{V} and indirect absorption of V^{IV} must be considered. Our preliminary studies on *C. intestinalis* suggested that at least one of the eight Na^{+}-dependent phosphate transporters can transport V^{V} (Ueki et al., unpublished data). This could be a candidate for the uptake of V^{V} from the outer environment, but no definite evidence so far. More detailed review of V^{V} transport can be found in our recent review article [37].

What is the possible mechanism for indirect uptake of V^{IV}, that is, how V^{V} is reduced to V^{IV} before uptake by ascidians? One such idea is a bacterial reduction of V^{V} ions. The hint comes from the studies on intestinal bacteria in ascidians. Antipov et al. reported that molybdenum- and molybdenum cofactor-free nitrate reductases isolated from vanadate-reducing bacteria *Pseudomonas isachenkovii* are likely to mediate V^{V} reduction [41]. Carpentier et al.

reported that *Shewanella oneidensis* was also capable of growth in the presence of V^{V} as the sole electron acceptor and reduced V^{V} to V^{IV} ions [42-43].

The vanadium concentration in the intestinal content of *A. sydneiensis samea* reaches 0.67 mM [44]. This suggests that the intestinal cells of ascidians uptake vanadium from this vanadium-rich microenvironment which is kept by symbiotic bacteria, and we successfully isolated nine vanadium-resistant bacteria that symbioses in the intestinal content in *A. sydneiensis samea* [44]. The bacteria can accumulate vanadium and may cooperate with ascidians to uptake vanadium from the outer environment.

A more comprehensive study on microbial diversity in the intestinal content, as well as those associating with branchial sac and intestinal cells, was recently done by our laboratory. We performed a comparative 16S rRNA amplicon sequence analyses on samples from three tissues isolated from two vanadium-rich (*Ascidia ahodori* and *Ascidia sydneiensis samea*) and one vanadium-poor species (*Styela plicata*) [45]. The results suggested that specific selective forces maintain the bacterial population in the three ascidian tissues, and symbiotic bacteria enriched in vanadium-rich ascidians may contribute to vanadium accumulation by ascidians. This study furthers the understanding of the relationship between bacterial communities and metal accumulation in vanadium uptake from the outer environment by ascidians.

UNDERWATER ADHESION

Ascidians attach to underwater substrate such as rocks, shells, plastic buoys, ship bottoms, and seawalls during the larval period. Ascidian tadpole larvae have three adhesive papillae that are typically situated at the anterior end of the head. The sticky adhesive papillae are used for the primary “rapid adhesion” process. After settlement and metamorphosis, vascular-like ampullae form in the vicinity of the attached juvenile ascidian epidermis. The ampullae expand over the substratum, and “slow adhesion” subsequently occurs. After this secondary slow adhesion, the ampullae disappear and are reabsorbed into the tunic. Adhesion is mostly limited to the ventral side or lower part of the ascidian tunic.

The tunic covering ascidian body is a living tissue that contains unique animal cellulose fibers, other polysaccharides, and proteins. Recent genomic analysis has shown that the tunicate cellulose synthase gene was introduced by a horizontal gene transfer from bacteria during tunicate evolution [11]. The tunic is known to increase ascidian strength and protect against predators, injury, pathogens, and UV radiation. However, the adhesive mechanism of the ascidian tunic has not yet been well clarified.

We have recently published a work on the ultrastructures related to adhesion and putative adhesive materials in the ascidian tunic [46]. To investigate ascidian adhesive materials, we focused on the “slow adhesion” of solitary ascidians, because there is an interesting positive correlation between the adhesive area and vanadium concentrations. We used transmission electron microscopy (TEM), low-temperature scanning electron microscopy (Cryo-SEM), energy-dispersive X-ray spectrometry (EDS), and imaging mass spectrometry to investigate the ultrastructures and metal distribution of adhesive and non-adhesive regions of solitary ascidians. Epigenetically developed large projections were found in the peripheral adhered tunic of some solitary ascidians. As this tissue has more adhesive activity than other adult tunic regions, we named it “adhesive projection” to distinguish it from the adhesive papillae and ampullae. We succeeded in artificially inducing adhesive projections and analyzing the structure, function, and metal distribution of these adhesive projections [46]. We have also performed a comparative proteome analysis on adhesion and non-adhesion sites and adhesive projections. By using transcriptome data on blood cells, we found a possible metal-binding adhesive protein that is solely expressed in the adhesion site, which is expected to be one of the adhesive materials (Ueki et al., unpublished data). Information on these adhesives may have potential applications related to creating underwater adhesives, biodegradable agents, and antifouling paints by biomimetic methods.

CONCLUSION AND PERSPECTIVE

The accumulation, reduction and transport of vanadium ions and their biological functions are expected to be tightly linked. In this review, we tried to illustrate ascidians as such a sophisticated system. In the first step of vanadium uptake from the outer environment, it is probable that bacteria on the branchial sac or in the intestinal content assist the absorption of vanadium from outer environment. The metal selectivity of this system is based on a combination of vanadium-binding proteins, vanadium transporters and vanadium reductase. How ascidians selectively accumulate vanadium is discussed from biochemical properties of proteins responsible for each step, and why ascidians

accumulate vanadium is discussed in relation to the underwater adhesion. Transcriptome and proteome analyses depicted a possible function of vanadium in underwater adhesion.

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