

**Glutathione transferases with vanadium-binding activity isolated
from the vanadium-rich ascidian *Ascidia sydneiensis samea***

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Summary

Some ascidians accumulate vanadium in vanadocytes, which are vanadium-containing blood cells, at high levels and with high selectivity. However, the mechanism and physiological significance of vanadium accumulation remain unknown. In this study, we isolated novel proteins with a striking homology to glutathione transferases (GSTs), designated AsGST-I and AsGST-II, from the digestive system of the vanadium-accumulating ascidian *Ascidia sydneiensis samea*, in which the digestive system is thought to be involved in vanadium uptake. Analysis of recombinant AsGST-I confirmed that AsGST-I has GST activity and forms a dimer, as do other GSTs. In addition, AsGST-I was revealed to have vanadium-binding activity, which has never been reported for GSTs isolated from other organisms. AsGST-I bound about 16 vanadium atoms as either V(IV) or V(V) per dimer, and the apparent dissociation constants for V(IV) and V(V) were 1.8×10^{-4} M and 1.2×10^{-4} M, respectively. Western blot analysis revealed that AsGSTs were expressed in the digestive system at exceptionally high levels, although they were localized in almost all organs and tissues examined. Considering these results, we postulate that AsGSTs play important roles in vanadium accumulation in the ascidian digestive system.

1. Introduction

Several species of ascidians, so-called sea squirts, accumulate high levels of vanadium [1, 2]. In the most remarkable case, the cellular vanadium concentration reaches 350 mM, or roughly 10^7 times the concentration in seawater [3]. Through the

accumulation process, almost all vanadium ions, thought to be present in the +5 oxidation state [V(V)] in seawater, are reduced to the +3 oxidation state [V(III)] via the +4 oxidation state [V(IV)] and are stored in the vacuole of vanadocytes, the vanadium-containing cells [4-6]. Several proteins that are likely involved in the vanadium-accumulation process have already been isolated from the blood cells of the vanadium-rich ascidian *A. sydneiensis samea* [7-15]. Of these, two similar proteins, Vanabin1 and Vanabin2, have attracted attention because of their binding ability and selectivity for vanadium [13-15]. In fact, Vanabin1 and Vanabin2 can bind 10 and 20 V(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively, and the binding with V(IV) is barely inhibited in the presence of magnesium(II) or molybdate(V) ions [15]. Three-dimensional structural analysis using nuclear magnetic resonance (NMR) [16] revealed that Vanabin2 has a novel bow-shaped conformation consisting of four helices connected by nine disulfide bonds, which has no reported structural homologs. V(IV) ions, which are exclusively localized on the same face of the Vanabin2 molecule, are mostly coordinated by amine nitrogens derived from amino acid residues, such as lysines and arginines, as suggested by electron paramagnetic resonance (EPR) results [17]. In addition, two Vanabin homologs, designated Vanabin3 and Vanabin4, were identified from the cytoplasm of vanadocytes in an expressed sequence tag (EST) analysis [18], and one Vanabin homolog, designated VanabinP, was isolated from the blood plasma (coelomic fluid) using V(IV)-chelating column chromatography [19]. Since all five of these Vanabins are rich in charged residues and have conserved motifs described as the consensus

sequence {C}-{X₂₋₅}-{C}, they have been placed in the Vanabin family, and should provide a clue to resolving the problem of the selective accumulation of vanadium in ascidians [15, 18, 19].

The vanadium-accumulating pathway from seawater into the ascidian coelom has never been investigated. Therefore, in this study, we used a V(IV)-chelating column to isolate vanadium-binding proteins from the digestive system, which is thought to be involved in vanadium uptake. Consequently, we isolated and identified two similar, but slightly different, vanadium-binding proteins with striking homology to GSTs, designated AsGST-I and AsGST-II. Like other GSTs, recombinant AsGST-I formed a dimer, and it not only showed GST activity, but also bound to both V(IV) and V(V). Moreover, using immunoblotting, the expression level of these two AsGSTs in the digestive system was found to be exceptionally high compared to the major organs and tissues. Therefore, AsGSTs may play important roles in vanadium accumulation in the ascidian digestive system.

2. Materials and methods

2.1. Specimens

Specimens of the vanadium-rich ascidian *A. sydneiensis samea* were collected at the International Coastal Research Center, Ocean Research Institute, The University of Tokyo, Otsuchi, Iwate, Japan. They were maintained in an aquarium that contained circulating natural seawater until use.

2.2. *Screening vanadium-binding proteins from the ascidian digestive system using V(IV)-chelating column chromatography*

To avoid the analytical difficulty that arises from individual differences, especially in the analysis of amino acid sequences, we used one individual per preparation. After the tunic was removed from the ascidian, the body wall was dissected. The digestive system was separated and washed thoroughly with artificial sea water (ASW; 460 mM NaCl, 9 mM KCl, 32 mM Na₂SO₄, 5 mM HEPES, 6 mM NaHCO₃, pH 7.0) to remove the contents of the digestive system. The specimen was then homogenized in five volumes (ml per gram wet weight) of a homogenizing buffer (200 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0) using a Teflon homogenizer. The homogenate was centrifuged at 21,600×g for 10 min, and the resulting supernatant was filtered through a cellulose acetate filter (0.45-μm pores) to remove lipidic floats, enclosed in a dialysis tube (6-8,000 Da cut-off), and dialyzed against 100 volumes of a chelating buffer (50 mM Tris-HCl, 500 mM NaCl, 25 mM EDTA, pH 7.2) to remove any metal ions that might interfere with the efficiency of V(IV)-chelating column chromatography. Next, the sample was dialyzed three times in 100 volumes of a binding buffer (20 mM Na₂HPO₄, pH 7.2) to remove any EDTA, which might also interfere with chromatography. The protein solution after dialysis was centrifuged at 21,600×g for 10 min and filtered through a cellulose acetate filter (0.45-μm pores) to remove the proteins insolubilized during dialysis.

Chelating Sepharose Fast Flow (Amersham Biosciences) was packed into a polypropylene column (bed size, 1 cm ϕ × 10 cm). V(IV) ions were chelated to the

Sepharose by adding twice the bed volume of 200 mM $\text{VOSO}_4 \cdot x\text{H}_2\text{O}$ (Wako). The Sepharose column was washed with ten bed volumes of distilled water (DW) to remove the unbound excess V(VI) ions, and then equilibrated to the binding buffer. The prepared protein solution was loaded onto the V(IV)-chelating column. After the application, the Sepharose was washed thoroughly using binding buffer, and the absorbed proteins were eluted using an elution buffer (20 mM Na_2HPO_4 , 500 mM NaCl, 50 mM EDTA, pH 7.2). Portions of the protein samples from each chromatography step were analyzed using SDS-PAGE on a 12.5% gel.

2.3. Determining the N-terminal partial amino acid sequence of vanadium-binding proteins

The chromatography eluent was concentrated by ultrafiltration using a Centriplus YM-3 (Millipore), and the proteins were further separated using reverse-phase high performance liquid chromatography (HPLC). HPLC was performed using a 5C18-AR-300 column (2.0×150 mm, Nacalai Tesque) with water and acetonitrile (both containing 0.1% trifluoroacetic acid). The proteins were eluted at a flow rate of 0.2 ml/min using a linear gradient of 10-70% acetonitrile over 60 min. Two major protein peaks detected 53 and 55 min after injection were collected, dried under vacuum in a rotatory concentrator, and dissolved in a sample buffer for SDS-PAGE. The samples were subjected to SDS-PAGE on a 12.5% gel and electro-blotted onto polyvinylidene fluoride (PVDF) membrane. The N-terminal

partial amino acid sequences of the protein bands corresponding to the two major protein peaks were determined using the Edman degradation method [20].

2.4. Isolation of cDNA clones for the two major vanadium-binding proteins

A degenerate primer was designed based on the sequence common to the N-terminal partial amino acid sequences obtained from the two major protein peaks as follows: 28K/F, 5'-GTN AAR TTY TAY TTY AAY GAY-3' for Val-Lys-Phe-Tyr-Phe-Asn-Asp. Using a cDNA library of vanadocytes from the ascidian *A. sydneiensis samea* constructed with UniZap XR vector [18] as the template, PCR was performed with the degenerate primer and primer T7 using Eazy A™ High-Fidelity PCR Cloning Enzyme (Stratagene). The PCR mixture was denatured at 95°C for 2 min and then cycled 30 times at 95°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min, with a final 7-min extension at 72°C. The amplified fragments were TA-cloned in pBluescript SK⁻ vector (Stratagene) according to the manufacturer's instructions. The constructed plasmids were used for sequencing with Thermosequenase using an ALF Express II DNA sequencer (Amersham Biosciences).

2.5. Expression and purification of recombinant AsGST-I

A specific primer was designed based on the coding region for the C-terminus of the AsGSTs containing the termination codon and a restriction enzyme site for *SalI* as follows; AsGST/R sal, 5'-GGT CGA CTT ATT CGG TTC TC-3'. Using primers AsGST/R sal and T3, PCR was performed as described above changing the DNA

annealing temperature from 45 to 50°C. The PCR products were sequenced as described above to determine the sequence of the degenerate primer region and the location of the initiation methionine. Based on the sequences, another specific primer was designed to amplify the coding regions of the putative mature AsGSTs, including the initiation methionine, using primer AsGST/R sal; this primer was AsGST/F, 5'-ATG ACA GTC AAA TTT TAT TTC AAC G-3'. Using primers AsGST/F and AsGST/R sal, PCR was performed as described above with an annealing temperature of 50°C. The PCR products were TA-cloned in pETblue1 expression vector (Novagen). The constructed vector containing the AsGST-I or AsGST-II gene was introduced into *Escherichia coli* Tuner™ (DE3)pLacI strain (Novagen).

The *E. coli* cells bearing the AsGST-I-expressing vector were cultured in LB medium containing 0.5% glycerol, 50 µg/ml ampicillin, and 34 µg/ml chloramphenicol at 37°C to an OD₆₀₀ of approximately 0.6-1.0. After storing at 4°C for 4-8 h, the culture was diluted approximately 30 times in fresh LB medium containing 0.5% glycerol, 50 µg/ml ampicillin, and 34 µg/ml chloramphenicol. The culture was incubated with shaking at 25°C until the OD₆₀₀ was approximately 0.5-1.0. After adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, the cells were incubated with shaking at 25°C for 8 h. The cells were collected by centrifugation at 4,000×g for 10 min, resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 7.2), and sonicated on ice until clear. The lysate was centrifuged at 10,000×g for 10 min. The resulting supernatant was enclosed in dialysis tubing (6-8,000 Da cut-off) and dialyzed three times against 100 volumes of

the binding buffer. The lysate after the dialysis was subjected to V(IV)-chelating column chromatography to purify the AsGST-I recombinant protein as described above. The recombinant protein absorbed to the V(IV)-chelating column was eluted using binding buffer containing 100 mM NaCl. Portions of the protein samples from each purification step were analyzed using SDS-PAGE on a 12.5% gel.

Although the same experiment was performed using *E. coli* cells bearing the AsGST-II-expressing vector, little AsGST-II recombinant protein was obtained because of its low expression level. Therefore, in this study, we used recombinant AsGST-I as AsGST in all of the following experiments.

2.6 Characterization of AsGST-I as a GST enzyme

The GST activity of AsGST-I was determined by using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, according to Habig et al. [21]. The supernatant after the ultrasonication of *E. coli* cells expressing AsGST-I prepared as described above was diluted 100-fold using a reaction buffer (100 mM K₂HPO₄, 1 mM reduced glutathione, 1 mM CDNB, pH 6.5) and the A₃₄₀ was monitored. The supernatant prepared from *E. coli* cells bearing the empty pETBlue1 vector after ultrasonication was used as a control.

Gel filtration was used to investigate whether AsGST-I forms a dimer as do other GSTs. A gel filtration column (bed size, 7 mm ϕ \times 50 cm) filled with Biogel P-60 gel (Medium, Bio-Rad) was equilibrated with the buffer for the Hummel-Dreyer method (HD buffer; 10 mM Tris-HCl, 100 mM NaCl, pH 7.2; see below). For

molecular weight calibration, one vial of protein mixture of a low-molecular-weight calibration kit for SDS electrophoresis (Amersham Biosciences) was diluted in 500 μ l of HD buffer, and 100 μ l of the protein mixture was loaded onto the prepared column and separated at a flow rate of 0.9 ml/h. The eluted fractions were collected every 10 min. Each fraction was analyzed by SDS-PAGE to examine the correlation between molecular weight and the fraction number of eluted peaks. The same experiment was performed using AsGST-I recombinant protein (\approx 10 μ g) to confirm the dimerization of AsGST-I based on the determined correlation. The protein concentration of each fraction was determined with Bio-Rad reagent (Bio-Rad) to detect the peak fraction.

2.7. Vanadium-binding assay of AsGST-I

The vanadium-binding activity of AsGST-I was assayed using the Hummel-Dreyer method [22] as described previously [15], with some modifications. All prepared buffers were degassed for 10 min under vacuum before use. $\text{VO}_2\text{SO}_4 \cdot x\text{H}_2\text{O}$ (Wako) was dissolved in water and mixed with an equal molar ratio of iminodiacetic acid (IDA). After the pH was adjusted to 7.0 by adding NaOH, the resulting solution was used as the V(IV) solution. Na_3VO_4 (Wako) was dissolved in water at 10 mM, and the pH was adjusted to 7.0 by adding HCl and incubating it at 60°C to decompose the yellow decavanadate until it became colorless; this was then used as the V(V) solution.

The AsGST-I recombinant protein purified as described above was concentrated using Centriplus YM-3 and dialyzed once against 100 volumes of the

chelating buffer, and three times against 100 volumes of HD buffer. After dialysis, the protein concentration was adjusted to $\approx 1\text{mg/ml}$. HD buffer containing twice the concentration of vanadium ions was added to the protein solution such that the vanadium concentration in the protein solution equaled the operating concentration (10-100 μM).

A gel filtration column (bed size, 7mm $\phi \times 50$ cm) filled with Biogel P-6 DG gel (Bio-Rad) was equilibrated with HD buffer containing vanadium ions at the same concentration as the prepared protein solution. The protein solution (100 μg in 200 μl) was loaded onto the prepared column and separated at a flow rate of 0.3-0.35 ml/min. The eluted fractions were collected each min. The vanadium concentration in each fraction was determined using atomic absorption spectrophotometry (AAS 220Z, Varian) with the application software Spectra AA (Varian), and the protein concentration was determined as described above. To determine the vanadium concentration using AAS, each sample diluted with HNO_3 to a final concentration of 0.1 M was placed in a graphite tube, which was heated according to the following program: 85°C for 5 sec, from 85 to 95°C in 40 sec, from 95 to 120°C in 10 sec, from 120 to 1000°C in 5 sec, kept at 1000°C for 1 sec with an influx of argon (3 L/min), kept at 1000°C for 2 sec, from 1000 to 2700°C in 1.3 sec, kept at 2700°C for 2 sec with no influx of argon, and again with an influx of argon at 2700°C for 2 sec. The molecular ratio of the bound vanadium per AsGST-I molecule (as the dimer) was calculated for the fraction with the protein peak. The results were analyzed using a Scatchard plot [23].

2.8. Preparation of antiserum to AsGST-I

Purified and concentrated AsGST-I recombinant protein ($\approx 100 \mu\text{g}$) in 500 μl of phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.3) was mixed with an equal volume of complete Freund's adjuvant and injected into a male Wistar rat (10.0 weeks old, 350 g). Two weeks after the first injection, a second injection was given using incomplete Freund's adjuvant instead of complete adjuvant. Three additional injections using incomplete Freund's adjuvant were given at 2-week intervals. The rat was euthanized 1 week after the last injection, and the blood was collected, incubated at 37°C for 1 h, and then kept at 4°C for 2 days. The blood was then centrifuged at 1,500 $\times g$ for 5 min and the resulting supernatant was filtered through a cellulose acetate filter (0.45- μm pores). The prepared serum was used as antiserum to AsGST-I.

2.9. Localization analysis of AsGSTs using antiserum to AsGST-I

Using antiserum to AsGST-I, an immunoblot analysis was performed to examine the localization of the AsGSTs. An individual ascidian was dissected into four components: the digestive system, body wall, endostyle, and branchial sac. Blood cells were collected from several individual ascidians, as described previously [13]. Each specimen was homogenized as described above. Each homogenate mixed with sample buffer for SDS-PAGE was fractionated using SDS-PAGE on a 12.5% gel. The purified AsGST-I recombinant protein was also loaded on the gel as

a positive control. The separated proteins were transferred electrophoretically to a nitrocellulose membrane, which was then incubated with TEN buffer (20 mM Tris-HCl, 1 mM EDTA, 140 mM NaCl, pH 7.4). After treating the membrane with TEN-BSA buffer (TEN buffer containing 1% BSA) for 1 h, the membrane was placed in TEN-BSA buffer containing 1:5000 antiserum to AsGST-I for 1 h. The membrane was washed with TEN-Tween buffer (TEN buffer containing 0.05% Tween20), and then treated with TEN-Tween buffer containing 1:5000 anti-rat-IgG peroxidase-labeled secondary antibodies (Kirkegaard & Perry Laboratories) for 30 min. After washing the membrane with TEN-Tween buffer sufficiently to remove nonspecifically bound antibodies, the immune complexes were detected on Hyperfilm ECL (Amersham Bioscience) with enhanced chemiluminescence reagents (Amersham Bioscience).

3. Results

3.1. Isolation and identification of novel vanadium-binding proteins from the ascidian digestive system

To search for factors involved in vanadium accumulation of ascidians, we attempted to isolate novel vanadium-binding proteins from the digestive system, which is thought to be involved in vanadium uptake. Soluble proteins prepared from the digestive system of an individual vanadium-rich ascidian, *A. sydneiensis samea*, were separated using a V(IV)-chelating column (Fig.1). V(IV) is one of the assumed chemical forms of vanadium ingested through the digestive system. Another

chemical form, V(V), could not be used for a chelating column because of its low affinity for the column. As a result, several proteins were identified in the vanadium-absorbed fraction (Fig. 1A, lane 5). The most strongly expressed protein in the digestive system comprised almost the entire vanadium-absorbed fraction (Fig. 1A, arrowhead). For further separation, the vanadium-absorbed fraction was applied to reverse-phase HPLC (Fig. 1B). After each of the two major peaks detected 53 and 55 min after injection was confirmed to be a single band (Fig. 1B), the N-terminal partial amino acid sequence of each peak was determined. The two sequences were almost identical, except for the ninth residue (Fig. 1C). The leucine in the protein from peak 1 was replaced by methionine in peak 2 (Fig. 1C, open letters).

Using a degenerate primer designed based on the common sequence, cDNAs encoding those proteins were screened from a cDNA library of *A. sydneiensis samea* vanadocytes [18]. As a result, we obtained two similar, though slightly different, clones predicted to encode mature proteins of 221 amino acids (Fig. 2). The predicted amino acid sequences were compared with known protein sequences registered in public protein databases using the program BLASTP and were revealed to have $\approx 50\%$ similarity with theta-class GSTs of insects and mammals. Therefore, they were designated AsGSTs (ascidian GSTs). The AsGST with the methionine at residue 9 was designated AsGST-I because the cDNAs encoding this protein constituted the majority of the screened cDNAs, and the AsGST with leucine at residue 9 was designated AsGST-II (Fig. 2). AsGST-I and AsGST-II correspond to the peak 2 and peak 1 proteins obtained on HPLC-separation (Fig. 1B, C).

3.2. Purification of recombinant AsGST-I using a V(IV)-chelating column

For a functional assay of AsGSTs, an expression system for recombinant AsGSTs was constructed in *E. coli*. Since the level of AsGST-II expression in *E. coli* was very low, we used the AsGST-I expression system. Recombinant AsGST-I was specifically absorbed to a V(IV)-chelating column, and the absorbed fraction could be eluted with 100 mM NaCl (Fig. 3, lane 5), indicating that AsGST-I clearly binds to V(IV) ions, but the affinity was weak. When recombinant AsGST-I was subject to HPLC, a single peak was eluted at 55 min (data not shown). The elution time corresponded to that of native AsGST-I (Fig.1B). On SDS-PAGE, recombinant AsGST-I migrated with native AsGST-I (Fig. 3, lanes 5 and 7). These results indicate that the recombinant AsGST-I corresponds to native AsGST-I.

3.3. Characterization of AsGST-I as a GST enzyme

First, we examined whether AsGST-I has enzymatic activity using CDNB as a substrate. A significantly higher level of activity was detected in lysate prepared from *E. coli* cells expressing AsGST-I than in lysate prepared from control cells bearing the empty vector (Fig. 4A), indicating that AsGST-I acts as a GST enzyme.

As GSTs are known to form dimers [24], we examined whether AsGST-I formed a dimer using the purified AsGST-I recombinant protein. AsGST-I was subjected to gel-filtration chromatography, and the elution pattern was compared to that of low-molecular-weight marker proteins. As shown in Fig. 4B, the elution peak

of AsGST-I was detected fraction No.29 which was immediately after the elution peak of a 45-kDa marker protein. If AsGST-I was a monomer, the peak should be detected after fraction No. 34 because the molecular weight of the AsGST-I subunit is 25.6 kDa. Therefore, AsGST-I may form a dimer although the estimated size of the dimer was 40.7kDa that is smaller than the calculated size of 51.2 kDa (Fig. 4B inset).

3.4. Vanadium-binding assay of AsGST-I

To assess the vanadium-binding ability of AsGST-I, the Hummel-Dreyer method was used [22]. Measurements were made using solutions of free V(IV) or V(V) ions ranging from 10 to 100 μ M. The recombinant AsGST-I bound with both oxidation states of vanadium (Fig. 5A). Scatchard plot analysis of the data revealed that AsGST-I bound to about 16 vanadium atoms of either V(IV) or V(V) per dimer, and the apparent dissociation constants against V(IV) and V(V) were 1.8×10^{-4} M and 1.2×10^{-4} M, respectively (Fig. 5B). These values suggest that AsGST-I binds to both oxidation states of vanadium ion with weak affinity. These results demonstrate that AsGST-I not only has characteristics of a GST enzyme, but also the novel property of vanadium-binding activity, which likely leads to the vanadium accumulation in ascidians.

3.5. Localization analysis of AsGSTs

To examine in which organs or tissues AsGSTs are localized, an immunological analysis was conducted using antiserum to AsGST-I (Fig. 6).

Homogenates of the digestive system, body wall, endostyle, branchial sac, and blood cells prepared from adult individuals of *A. sydneyensis samea* were used in Western blot analysis. Purified AsGST-I recombinant protein was used as a positive control. As result, AsGSTs were detected from all of the organs and tissues examined, and very dense bands due to AsGSTs were detected from the digestive system (Fig. 6B, lane1, arrowhead), which suggests that AsGSTs play an especially important role in the digestive system.

4. Discussion

The study of the mechanism of vanadium accumulation by ascidians has attracted interdisciplinary attention over the century since its discovery [1]. However, the mechanism and physiological significance of vanadium accumulation in ascidians remain poorly understood. In this decade, we have introduced biochemical and molecular biological methods to the investigation of these questions and have succeeded in identifying various key factors [2, 7-15, 18, 19]. For example, Vanabins that are expressed strongly in vanadocytes and blood plasma of vanadium-accumulating ascidians play important roles in vanadium accumulation [13-15, 18, 19].

In contrast, few studies have examined the pathway of vanadium accumulation from seawater into the ascidian coelom. In this study, novel vanadium-binding proteins with striking homology to GSTs, named AsGST-I and AsGST-II, were isolated from the digestive system for the first time using V(IV)-chelating column

chromatography (Figs. 1 and 2). GSTs are a superfamily of enzymes that utilize glutathione (GSH) in reactions contributing to the detoxification of a wide range of toxic compounds. GSTs are found in all eukaryotes and have various functions [24]. Notwithstanding their versatility, GSTs have never been considered to be involved in metal homeostasis. Using AsGST-I recombinant protein, we have demonstrated that AsGST-I forms a dimer, as do other GSTs (Fig. 4B), and that it not only has GST activity (Fig. 4A), but it also has vanadium-binding activity (Fig. 5). These activities could not be examined in AsGST-II because it was impossible to obtain sufficient recombinant protein due to the low expression level of AsGST-II in *E. coli*. However, since there is only a 5% difference in the amino acid sequences of AsGST-I and AsGST-II (Fig. 2), we believe that these proteins have similar characteristics and functions. AsGSTs were the most strongly expressed proteins in the digestive system (Fig. 1A), and the expression there was much higher than in the other organs and tissues (Fig. 6). Therefore, it is very likely that AsGSTs play an important role in the digestive system of ascidians.

We have already localized Vanabin1 and Vanabin2 to the cytoplasm of vanadocytes and have demonstrated that they bind to 10 and 20 V(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively [15]. Further, VanabinP, which is localized in the blood plasma, binds 13 V(IV) ions with a dissociation constant of 2.8×10^{-5} M [19]. These values are comparable to those of the nickel chaperone protein UreE (1.0×10^{-5} M for Ni^{2+}), which assists in the insertion of Ni^{2+} in the active site of urease [25], the copper-binding site of Menkes

protein (4.6×10^{-5} M for Cu^{2+}) [26], and the periplasmic molybdate-binding protein ModA (3×10^{-6} M for molybdate and 7×10^{-6} M for tungstate) [27]. In comparison, AsGST-I bound to about 16 atoms of either V(IV) or V(V) per dimer, and the apparent dissociation constants for V(IV) and V(V) were 1.8×10^{-4} M and 1.2×10^{-4} M, respectively (Fig. 5B). AsGST-I resembles Vanabins in its binding with vanadium ions, although the binding affinity of AsGST-I was about ten times lower than that of Vanabins. In addition, the expression of AsGSTs in the digestive system was considerably higher than in the other organs and tissues (Fig. 6) and was comparable to that of Vanabins in the blood cells (Fig. 6A, lane5, asterisk) [13-15]. Therefore, AsGSTs may play an alternative role as a vanadium carrier protein in the digestive system, although further study of its function is required.

In seawater, vanadium is in the +5 oxidation state, while in ascidians, almost all the vanadium is reduced to the +3 oxidation state via the +4 oxidation state and is stored in the vacuoles of the vanadocytes [4-6]. During this process, NADPH produced by the pentose phosphate pathway localized in the vanadocyte cytoplasm is likely involved in the reduction of V(V) to V(IV) [7-10, 28]. However, the compound reducing V(IV) to V(III) has not been identified in ascidians. Nevertheless, there is no doubt that the accumulation of vanadium by ascidians is inevitably intertwined with the reduction of vanadium. One function of GSTs is to promote the conjugation of GSH to the electrophilic center of toxic compounds [24], and GSH, the cofactor of GSTs, reduces V(V) to V(IV) and may act as a ligand for the generated V(IV) [29]. Recently, hGSTO1-1 (human GST Omega class) was

demonstrated to have activity as a reductase catalyzing the reduction of the metalloid compound monomethylarsonate (MMA^{V}) to monomethylarsenous acid (MMA^{III}) [30]. Further, Gtt2 (a yeast GST) [31] and hGSTP1-1 (human GST Pi class) [32] were suggested to participate in the conjugation of GSH to cadmium and arsenic, which results in the production of GSH-complexes with cadmium [$\text{Cd}(\text{GS})_2$] and arsenic [$\text{As}(\text{GS})_3$], respectively. Therefore, we hypothesize that AsGSTs may directly or indirectly be involved in the reduction of V(V) to V(IV) and the conjugation of V(IV) with GSH in the digestive system and the possibility should be examined in future.

Based on the results of our experiments, it is likely that the main function of AsGSTs in the vanadium-accumulating process is vanadium-carrier in the digestive system. Vanadium ions taken up by ascidians via the digestive system might be captured by AsGSTs, vanadium-binding proteins that are locally abundant. Since AsGSTs might be one of the first molecules involved in the influx of vanadium ions through the digestive system, these seem to be a quite important clue to elucidate the first step of the 10 million-fold vanadium-accumulating process in ascidians.

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Figure legends

Fig. 1. Isolation and N-terminal amino acid sequence analysis of vanadium-binding proteins from the digestive system of an ascidian. (A) Screening vanadium-binding proteins from the digestive system of an ascidian using V(IV)-chelating column chromatography. Portions of the protein samples from each of the chromatography steps were analyzed using SDS-PAGE. Lane 1, low-molecular-weight markers; lane 2, homogenate of the digestive system; lane 3, soluble protein from the digestive system; lane 4, flow-through fraction; and lane 5, eluent with EDTA. The arrowhead indicates the most strongly expressed protein in the digestive system. (B) Further separation of the vanadium-absorbed proteins. The eluent with EDTA (approximately 11.5 μ g of protein) was loaded onto a C18 reverse-phase HPLC column and eluted using a linear gradient of 10-70% acetonitrile over 60 min. Two major protein peaks detected 53 (peak 1) and 55 (peak 2) min after injection were collected and analyzed using SDS-PAGE (lanes 1 and 2 in the inset, respectively). The most strongly expressed protein in the digestive system actually consists of two proteins. (C) The N-terminal partial amino acid sequences of the HPLC peaks. The two N-terminal sequences were identical, except for the ninth (open letters), twelfth and fourteenth residues (boxed letters). Boxed residues were confirmed to be identical by cDNA sequencing (see Fig. 2).

Fig. 2. Alignment of the amino acid sequences deduced from the cDNA sequences of the AsGSTs. The AsGST with methionine as the ninth residue was designated AsGST-I; the other, which had leucine as the ninth residue, was designated AsGST-II. Identical residues are boxed; “m” indicates the initiation methionine. Open letters indicate the amino acid residues identical to those determined in the N-terminal amino acid sequence analysis. An approximately 5% overall difference between their amino acid sequences is observed.

Fig. 3. Purification of AsGST-I recombinant protein using a V(IV)-chelating column. Portions of protein samples from each purification step were analyzed using SDS-PAGE. Native AsGSTs obtained from HPLC (Fig. 1B) were also analyzed to identify the expressed recombinant protein by comparing their migration on the gel. Recombinant AsGST-I could be purified using a V(IV)-chelating column, which confirmed the vanadium-binding activity of AsGST-I. Lane 1, low-molecular-weight markers; lane 2, *E. coli* cell lysate after ultrasonication; lane 3, soluble protein of the lysate; lane 4, flow-through fraction; lane 5, eluent with 100 mM NaCl; lane 6, native AsGST-II obtained from the HPLC analysis; lane 7, native AsGST-I obtained from the HPLC analysis.

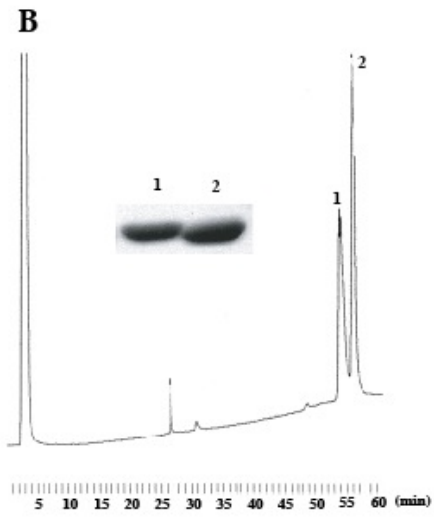
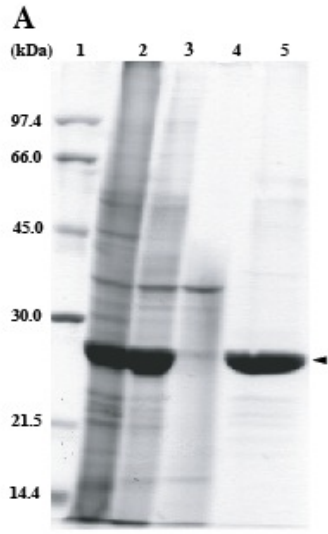
Fig. 4. Characterization of AsGST-I as a GST enzyme. (A) The GST activity of

AsGST-I with CDNB was determined by monitoring the changes in A_{340} . The initial increase in A_{340} for 1 minute after the reaction represents GST activity (vertical axis). The lysate prepared from *E. coli* cells expressing recombinant AsGST-I (AsGST-I) showed much greater GST activity than the lysate prepared from non-expressing cells (Control). Bars represent the means of triplicate determinations (\pm S.D.) in a single experiment. (B) Dimerization of AsGST-I was examined using gel filtration chromatography. The elution pattern of AsGST-I recombinant protein (line graph) was compared to that of low-molecular-weight marker proteins. The vertical axis indicates the protein concentration of AsGST-I. Inverted triangles indicate the elution peaks of the marker proteins. The elution peak of AsGST-I was detected immediately after that of a 45-kDa marker protein, suggesting that AsGST-I forms a dimer. Inset, The mass of AsGST-I was determined from its elution position (arrow) on the chromatography. The elution positions of the marker proteins are indicated for albumin (a, 66kDa), ovalbumin (b, 45kDa), carbonic anhydrase (c, 30kDa), trypsin inhibitor (d, 20.1kDa), and α -lactalbumin (e, 14.4kDa).

Fig. 5 Assay for the vanadium-binding activity of AsGST-I using the Hummel-Dreyer method. (A) The relationship between the concentration of free vanadium ions and the ratio of bound vanadium per AsGST-I dimer. V(IV) ions (black diamonds) or V(V) ions (gray triangles) were used for the assay. The logarithmic fit of each dataset is indicated using black or gray solid lines,

respectively. (B) Scatchard plot of the results shown in (A). Black diamonds indicate the results with V(IV) and gray triangles indicate the results with V(V). The linear fit for each dataset is indicated using black or gray dashed lines, respectively. K_d and n are the apparent dissociation constant of AsGST-I against vanadium (M) and the maximum number of vanadium atoms bound per AsGST-I dimer (mol/mol), respectively. These results suggest that AsGST-I can bind with multiple vanadium ions in both the +4 and +5 oxidation states.

Fig. 6. Localization of AsGSTs using antiserum to AsGST-I. Homogenates prepared from major organs or tissues of the ascidian *A. sydneiensis samea* were analyzed using (A) SDS-PAGE and (B) Western blotting using antiserum to AsGST-I. Purified AsGST-I recombinant protein was used as a positive control. Lane 1, digestive system; lane 2, body wall; lane 3, endostyle; lane 4, branchial sac; lane 5, blood cells; lane 6, purified AsGST-I recombinant protein. AsGSTs were detected in all specimens (arrowhead). The expression in the digestive system was exceptionally high and rivaled the level of Vanabins in blood cells (asterisk).



C

Peak 1: TVKIFYND₁SPXSR

Peak 2: TVKIFYND₁SPXSPorRSVM

Fig. 1

Figure 2

A.

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1 ATGACAGTCAAATTTTATTTCACAGCATGAGTCCGCCGAGCCGAAGCGTTATGATGGTAATCAGAGAACTAGGG 75
1 M T V K F Y F N D M S P P S R S V M M V I R E L G 25
76 GTCCTTGCGAAGTGAAGGATGTGGACATGATGACCAGACAACAAAAACGCCGGAATTCCTGAGGATCAACCCA 150
26 V P C E V K D V D M M T R Q Q K T P E F L R I N P 50
151 CGTGGAAGAGTTCGGCGCTGGTCGATGGCGATTGCAAATTCGGAAAGCCGATCGCTTGTACCTTTGC 225
51 R G R V P A L V D G D L Q I S E S Q A I A C Y L C 75
226 AACAGTTCGCGAAGGCCAAAAACGTGCTTTACCCAACGGGCCCGACCGCGGAATGGTTGATATGTGG 300
76 N K F A K G A K T S L Y P T G P G P R E M V D M W 100
301 CTGTACAGAAGCGTATGGATTAACGACCTGGTCGAAGCTTATCTTAACACGCATCGCATTCTTTCGACAACGAA 375
101 L Y R S V W I N D L V E A Y L N T H R I L F D N E 125
376 ACGATGAAGTACGACGCTTGCACGAACTCAAGGAGTCTCCATATTTTCGACAAATGGCTGAGAAATCTTTG 450
126 T M N Y D A L H E L K E G L H I F D K W L R N S L 150
451 TACCTGACCGGCAACACGTGACAATTCAGATTACTTTAATGCTGTTGCAATTCACCTCGACCAATCTGGA 525
151 Y L T G N N V T I A D Y F N A V A I L H L D Q S G 175
526 AGAAGGTTTATAACGAGTTTCCGAGGCTGAAGGAGTGGATCTCGAGGATGAGAAGCATTTCCTTGTGACGAG 600
176 R K V Y N E F P R L K E W I S R M R S I S L F D E 200
601 ATCAATCAACCGGGATCGGACAACTCACGAAAATCATTCAAGACACCTTGCGGAAGAGAACCGAATAA 669
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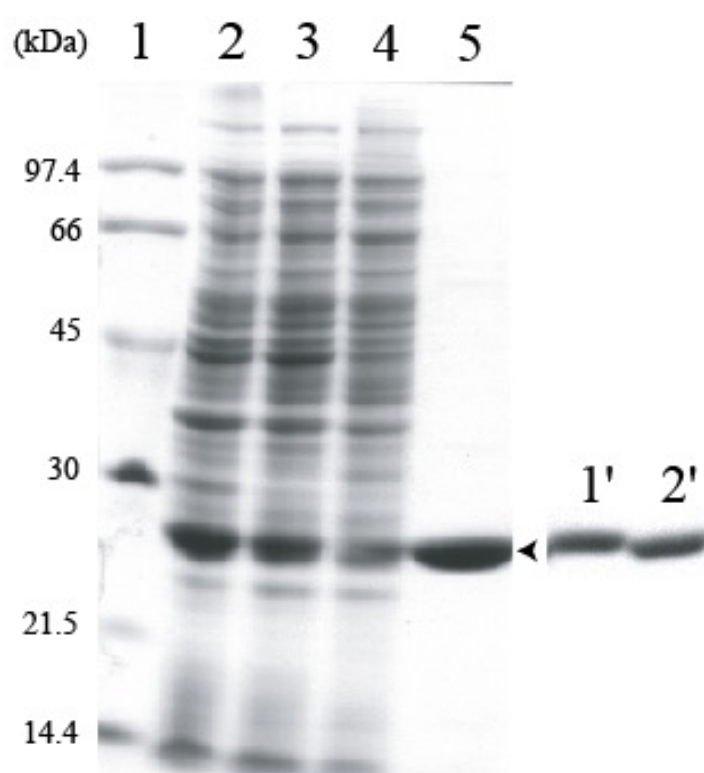
B.

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AsGST-I 1 M T V K F Y F N D M S P P S R S V M M V I R E L G V P C E V K D V D M M T R Q Q K T P E 44
AsGST-II 1 M T V K F Y F N D L S P P S R S V M M V I R E L G V A C E V K D V D M M T R Q Q K T P E 44
45 F L R I N P R G R V P A L V D G D L Q I S E S Q A I A C Y L C N K F A K G A K T S L Y P 88
45 F L R I N P R G R V P A L V D G D W Q I S E S Q A I A C Y L C N K F A K G A K T S L Y P 88
89 T G P G P R E M V D M W L Y R S V W I N D L V E A Y L N T H R I L F D N E T M N Y D A L 132
89 T G P G P R A M V D M W L Y R S V W I N N L V E A Y L N T H R I L F H N E T M N D D A L 132
133 H E L K E G L H I F D K W L R N S L Y L T G N N V T I A D Y F N A V A I L H L D Q S G R 176
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177 K V Y N E F P R L K E W I S R M R S I S L F D E I N Q P G S D K L T K I I Q D T L A K R 220
177 K V Y H E F P R L K E W I S R M R S I S L F D E I N Q P G S D K L T K I I Q D T L A K R 220
221 T E 222
221 T E 222

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Figure 3



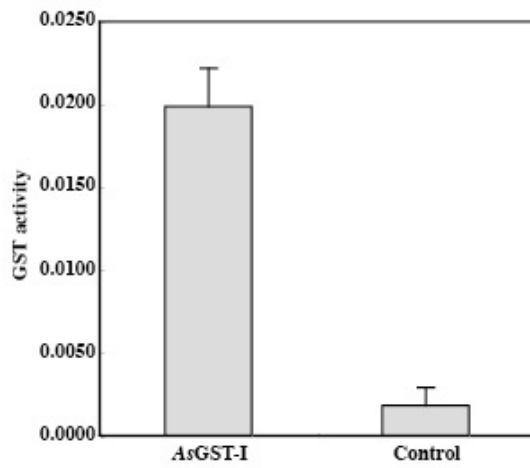
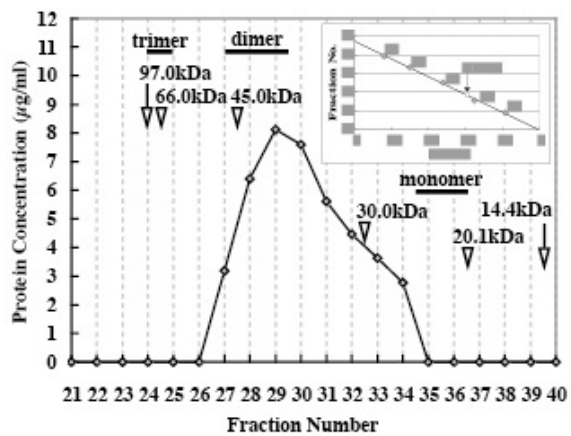
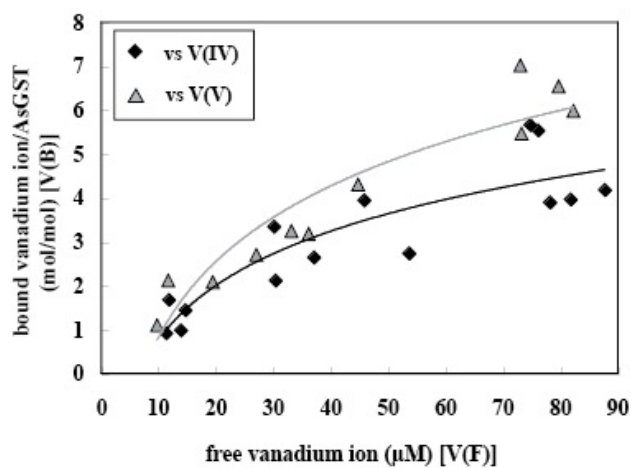
A**B****Fig. 4**

Figure 5

A.



B.

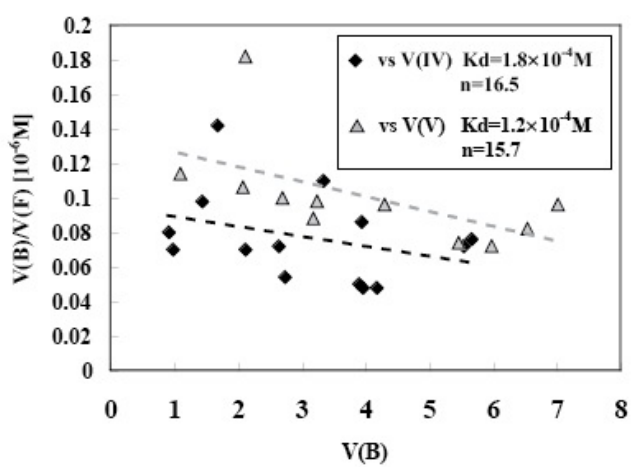


Figure 6

