Identification of a Vanadium-Associated Protein from the Vanadium-Rich Ascidian, Ascidia sydneiensis samea

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ABSTRACT—Ascidians are known to accumulate vanadium in their blood cells (vanadocytes) at extremely high levels which correspond to about 10^6 to 10^7 times the levels of vanadium ions in seawater. The route for the accumulation of vanadium ions from the outside environment into the blood system in ascidians has not yet been discovered. In the present experiments, using a combined technique of anion exchange column and atomic absorption spectrometry, we first extracted a vanadium-associated protein (VAP) from the blood cells of the ascidian *Ascidia sydneiensis samea*. VAP was estimated to associate with vanadium at an approximate ratio of 1 mol : 16 mols. SDS-PAGE and a polyclonal antibody against VAP (anti-VAP) revealed that VAP is composed of at least two types of peptides estimated to be 12.5 kDa and 15 kDa with a minor peptide of 16 kDa and that VAP is localized in the cytoplasm of the vanadocytes.

INTRODUCTION

Ascidians (tunicates), in particular those belonging to the family Ascidiidae, are known to selectively accumulate vanadium ions at extremely high levels in their blood cells, the so-called vanadocytes, from seawater. The highest levels of vanadium ions in the blood cells of members of the family Ascidiidae are in excess of 350 mM, corresponding to about 10^6 to 10^7 times the levels of vanadium ions in seawater (Michibata *et al.*, 1986, 1991a; Michibata, 1993, 1996).

The route for the accumulation of vanadium ions from the outside environment into the blood system in ascidians has not yet been discovered. The mechanism for the uptake of vanadium ions by ascidians was studied using radioactive vanadium ions (^{48}V), for which previous studies were commonly designed to clarify the direct uptake of vanadium ions from the surrounding seawater and were, therefore, limited to examining how much of the vanadium ions were incorporated into some tissues (Goldberg *et al.*, 1951; Bielig *et al.*, 1963; Dingley *et al.*, 1981; Michibata *et al.*, 1991b).

In general, under physiological conditions, heavy metal ions, including vanadium ions, incorporated into the tissues of living organisms are known to bind with macromolecules, such as proteins (cf. Chasteen, 1995). We have, therefore, searched vanadium-associated proteins in the blood cells of ascidians. Using a combined technique of anion exchange column and atomic absorption spectrometry, we first extracted a vanadium-associated protein (VAP) from the blood cells of the ascidian, *Ascidia sydneiensis samea* and raised a polyclonal antibody against VAP (anti-VAP).

MATERIALS AND METHODS

Preparation of blood cells

Specimens of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, were collected at the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Otsuchi, Iwate, Japan and at the Ushimado Marine Biological Station of Okayama University, Ushimado, Okayama, Japan. They were maintained in an aquarium that contained circulating natural seawater at each laboratory at 18°C until use.

After the tunic was removed, blood was drawn by puncturing the heart and pooled in 20 volumes of ice-cold buffer solution (buffer A) that contained 400 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Tris-HCl (tris-hydroxymethyl aminomethane) at pH 8.5. The pooled blood was centrifuged at 400 × g for 10 min at 4°C. The precipitated blood cells were composed of two layers of cells: The upper layer, dominantly consisting of a subpopulation of giant cells, was discarded. The lower one, consisting of the other types of blood cells including vanadocytes, was resuspended in buffer A and again centrifuged at 400 × g for 10 min at 4°C. The pelleted cells were suspended in the other buffer solution (buffer B) that contained 1 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl-fluoride) and 10% (v/v) glycerol in buffer A. The suspended sample was rapidly frozen in liquid nitrogen and then kept in a deep freezer at -85°C until use.

Isolation of vanadium-associated protein (VAP)

After the frozen blood cells were thawed on ice, they were centrifuged at 400 × g for 10 min at 4°C to remove the cell debris. The supernatant was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant obtained was dialyzed against a 50 mM Tris-HCl buffer containing 1 mM PMSF, 20 µg/ml of pepstatin A, 10 µg/ml of leupeptin

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and 10 μg/ml of chymostatin at pH 7.4 for 24 hr at 10°C in order to remove the inorganic vanadium ions which did not associate with the proteins. The dialyzed sample was centrifuged at 100,000 × g for 60 min at 4°C, and the supernatant was applied to a DEAE-Sephacel anion-exchange column (ø1.0 × 10 cm length) which had been equilibrated with a 50 mM Tris-HCl buffer at pH 7.4. After the column was washed with the same buffer solution at 0.5 ml/min and the non-adsorbed substances were eluted, proteins were eluted in 3.4 ml-fractions from the column with increasing NaCl solution, step-wise from 0 to 400 mM in the buffer solution. The concentrations of the protein and vanadium in each fraction were determined by the method of Bradford (1976) using BSA (bovine serum albumin) as the standard and by an atomic absorption spectrometry (Michibata et al., 1990), respectively.

SDS-PAGE

The SDS-PAGE sample was dissolved in a sample dissociation buffered solution containing 62.5 mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 2.3% (w/v) SDS at pH 6.8. It was subjected to electrophoresis on a 14% uniformed SDS-PAGE for 12 hr with a 10 mA constant current. Coomassie Brilliant Blue R-250 was used to stain the proteins.

Preparing antibodies

The anti-VAP antibody was prepared by the immunization of an albino rabbit. A 3.5 ml-aliquot of peak 1 fractions that contained vanadium-associated proteins (ca. 100 μg protein/3.5 ml) after elution from a DEAE-Sephacel column was dialyzed against a 5 mM NaCl solution for 6 hr at 4°C. The sample obtained was then lyophilized; it was dissolved in 0.7 ml of physiological saline and mixed with 0.7 ml of Freund's complete adjuvant. The mixture was hypodermically injected into a rabbit, and the injection was repeated two weeks after the first injection to boost the titer of the antibody. Three days after the last injection, blood was collected from the ear. The blood was used for the preparation of the anti-VAP serum, which was allowed to stand at 37°C for 1 hr, and was then incubated at 4°C overnight. The serum was removed from the clot, and any remaining insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4°C. After the preparation, the serum was stored at -30°C until use.

The anti-VAP antibody was purified as follows: Peak 1 fractions containing about 500 μg protein were subjected to SDS-PAGE. The proteins separated on SDS-PAGE were electrophoretically blotted onto nitrocellulose paper and sliced into several bands which corresponded to the VAP. The paper sliced was then washed with Tris-buffered saline (TBS), which consisted of 140 mM NaCl and 10 mM Tris-HCl buffer at pH 7.2, for 20 min and soaked in TBS containing 1% (w/v) BSA for 1 hr. The paper was then exposed to the anti-VAP serum, which was diluted 20-fold with TBS containing 1% BSA, for 2 hr at room temperature. After washing three times with TBS containing 0.01% (v/v) Tween 20 for 10 min, the paper was incubated in a 0.1 M glycine-HCl buffer (pH 3.0) and vigorously agitated in order to release the antibodies from the antigen. The solution was immediately neutralized with 1 M Tris-HCl (pH 9.0) containing 100 μg BSA. The solution was then dialyzed against 10 mM NH$_4$HCO$_3$ for 3 hr at 4°C and concentrated with Centricon 10 (Amicon, Inc., Mass. USA) to use as purified antibodies (purified anti-VAP).

Immunoblot analysis

Homogenates of blood cells containing 40 μg protein and peak 1 fractions containing 5 μg protein were dissolved in a sample dissociation buffered solution and subjected to SDS-PAGE. For immunoblot analysis, each sample subjected to SDS-PAGE was transferred to nitrocellulose paper. The paper was then soaked in TEN buffer, which consisted of 150 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl buffer at pH 7.4 containing 1% BSA, for 3 hr and exposed to purified anti-VAP antibody, which was diluted at a ratio of 1 to 500 in TEN buffer containing 1% BSA, for 1 hr. The nitrocellulose paper was

twice washed with TEN buffer containing 0.01% Tween 20 for 30 min and incubated with the anti-rabbit IgG (H + L)-HRP conjugate (Organon Teknika Corporation, Philadelphia, USA), which was diluted at a ratio of 1 to 2,500 with TEN buffer containing 0.01% Tween 20, for 30 min. After washing six times with the TEN buffer containing 0.01% Tween 20 for 15 min, the nitrocellulose paper was incubated with ECL Western blotting detection reagents (Amersham International, Plc., Buckinghamshire, England). Finally, the nitrocellulose blot was exposed to Hyperfilm-ECL (Amersham International, Plc.).

Hypotonic, LiCl and digitonin treatments

To examine whether or not vanadium ions are associated with the membranes of blood cells and whether or not the VAP is derived from the cytoplasm of blood cells, the blood cells were treated with hypotonic, LiCl and digitonin solutions, respectively. The hypotonic treatment was carried out as follows: An aliquot of 200 mg of the pelleted blood cells was suspended in 40 μl of hypotonic solution (1 mM Tris-HCl at pH 7.4 containing 10 μg/ml PMSF, 10 μg/ml leupeptin and 10 μg/ml chymostatin) for 30 min at 4°C and then centrifuged at 100,000 × g for 60 min at 4°C. The total amount of the supernatant obtained was mixed with 10 μl of a sample dissociation buffer solution containing 25 mM Tris, 125 mM glycine, 5% (v/v) β-mercaptoethanol, 2.4% (w/v) SDS and 20% (v/v) glycerol and subjected to SDS-PAGE. An aliquot of the blood cells which had undergone the hypotonic treatment was further treated with LiCl as follows: The total amount of pelleted sample after treatment with hypotonic solution were further treated with LiCl. The sample was mixed with 1 ml of LiCl solution (1 mM Tris-HCl and 1 M LiCl at pH 7.4) and centrifuged at 100,000 × g for 60 min at 4°C. The supernatant obtained was mixed with a sample dissociation buffer solution and subjected to SDS-PAGE as same as the hypotonic treated sample. For the treatment with digitonin, an aliquot of 200 mg of the pelleted blood cells was resuspended in 1 ml of 1 mM Tris-HCl at pH 7.4 containing 0.5% digitonin, and the subsequent treatments were carried out in the same manner as described above. As the control, isotonic treament was done as follows; an aliquot of 200 mg of the pelleted blood cells was suspended in 40 μl of isotonic solution contained 460 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 50 mM Tris-HCl at pH 8.5 and the subsequent treatments were done in the same manner as described above.

RESULTS

Isolation of vanadium-associated proteins (VAP)

Approximately 50% of the vanadium contained in the initial precipitation of the blood cells was lost after the treatment of freezing and thawing, and approximately 90% of the initial vanadium was further lost from the sample after the subsequent dialysis, as shown in Table 1. When the dialyzed sample was loaded onto a DEAE-Sephacel anion-exchange column, one major peak designated as peak 1, containing both proteins and vanadium, was obtained in fractions 3-9, and the other major peak designated as peak 2, containing only vanadium, was found in fractions 30-55 followed by gradients of 200 mM to 400 mM NaCl, as shown in Fig. 1. Peak 1 fractions contained 25 μg /ml of proteins and 0.95 μg /ml of vanadium. On the other hand, to confirm that the vanadium in peak 2 fractions was an inorganic chemical species, an inorganic solution of 6 μM vanadate (Na$_3$VO$_4$) was applied to the column under the same conditions. Consequently, the elution pattern of peak 2 was essentially indistinguishable from that of the inorganic solution (data not shown).
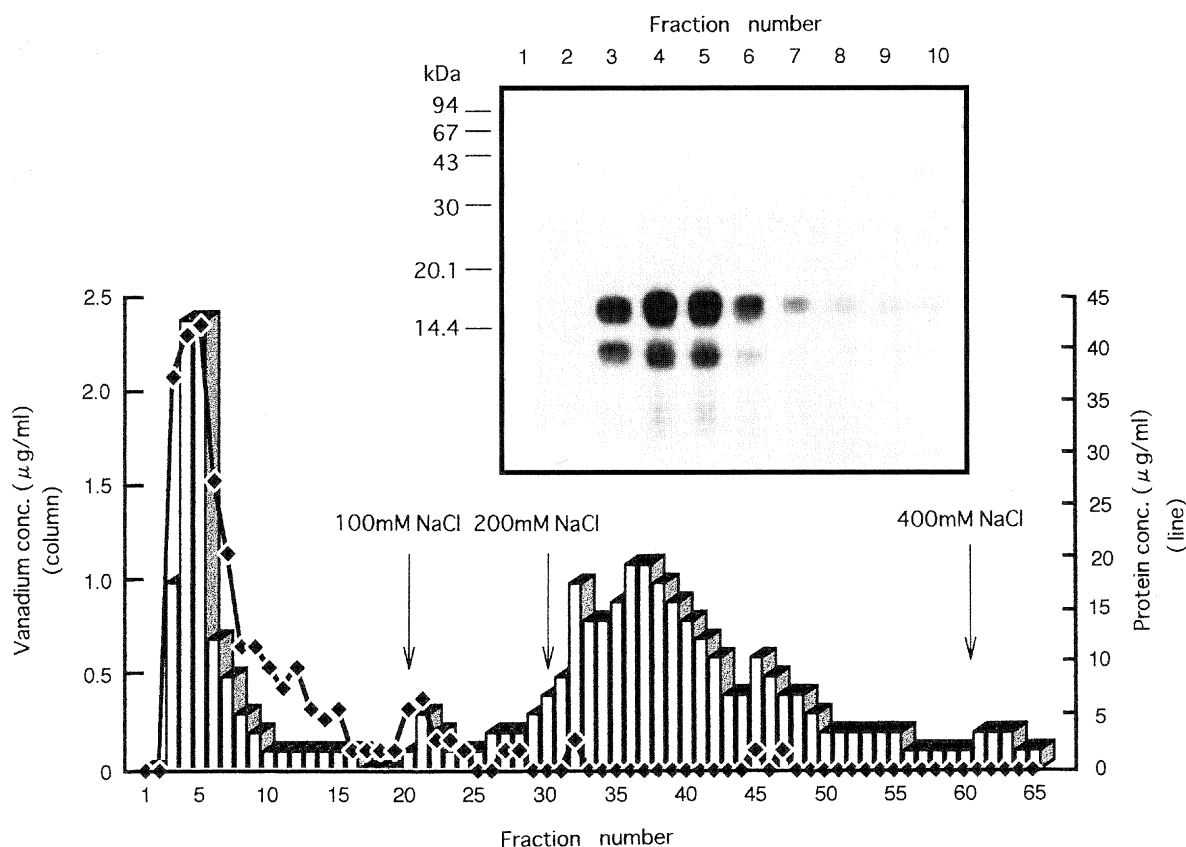
Fig. 1. Isolation of a vanadium-associated protein (VAP) from the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. When the supernatant of the blood cells was applied to a DEAE-Sephacel anion-exchange column, one major peak designated as peak 1, containing both proteins and vanadium, was obtained in fractions 3-9, and the other major peak designated as peak 2, containing only vanadium, was found in fractions 30-55 followed by gradients of 200 mM to 400 mM NaCl. Peak 1 fractions contained 25 μg /ml of proteins and 0.95 μg /ml of vanadium. Peak 2 fractions contained an inorganic vanadium species. The insertion is SDS-PAGE indicating that peak 1 contained at least two types of peptides whose molecular weights were estimated to be 12.5 kDa and 15 kDa, respectively.

SDS-PAGE and immunoblot analysis

SDS-PAGE revealed that peak 1 contained at least two types of peptides whose molecular weights were estimated to be 12.5 kDa and 15 kDa, respectively, as shown in Figs. 1 (insertion) and 2A. The comparison of the SDS-PAGE patterns of peak 1 fractions with those of the homogenate of the blood cells, supernatant after freezing and thawing, and the dialyzed sample shows that the treatment of freezing and thawing removed the higher molecules of proteins and resulted in the desirable purification of the vanadium-associated protein. Purification of the vanadium-associated protein was carried out in the same manner but without Ca^{2+} to examine the effects of Ca^{2+}-dependent proteases on extraction of peptides from ascidian blood cells, consequently, several bands more than 45 kDa appeared on SDS-PAGE but little effects were observed around 15 kDa (data not shown).

When the blood cells were treated with a hypotonic solution and digitonin, around 15 kDa bands were stained much more deeply than those in the control (Fig. 3), which indicates that the 15 kDa proteins contained in the cytoplasm of blood cells were released by the treatments, namely, VAP existed intrinsically in the cytoplasm of the blood cells. The

treatment with LiCl also released some amounts of proteins, suggesting that the VAP might bind with cell membranes by ionic interaction.

Immunoblot analysis confirmed that at least two main peptides of 12.5 kDa and 15 kDa with a minor peptide of 16 kDa contained in peak 1 fractions were recognized by a polyclonal antibody, anti-VAP (Fig. 2).

DISCUSSION

This is the first report that a vanadium-associated protein (VAP) was isolated from the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. SDS-PAGE and immunoblot analysis revealed that VAP was at least composed of 12.5 kDa and 15 kDa peptides with a minor peptide of 16 kDa (Fig. 2). With respect to the pathway for the accumulation of vanadium ions by ascidians from seawater, it seems likely that some proteins might participate in the process of the accumulation. However, few investigations on the extraction of vanadium-associated protein from ascidians have previously been reported. Ascidians, differing from the other organisms, have vanadium-containing blood cells, the so-called
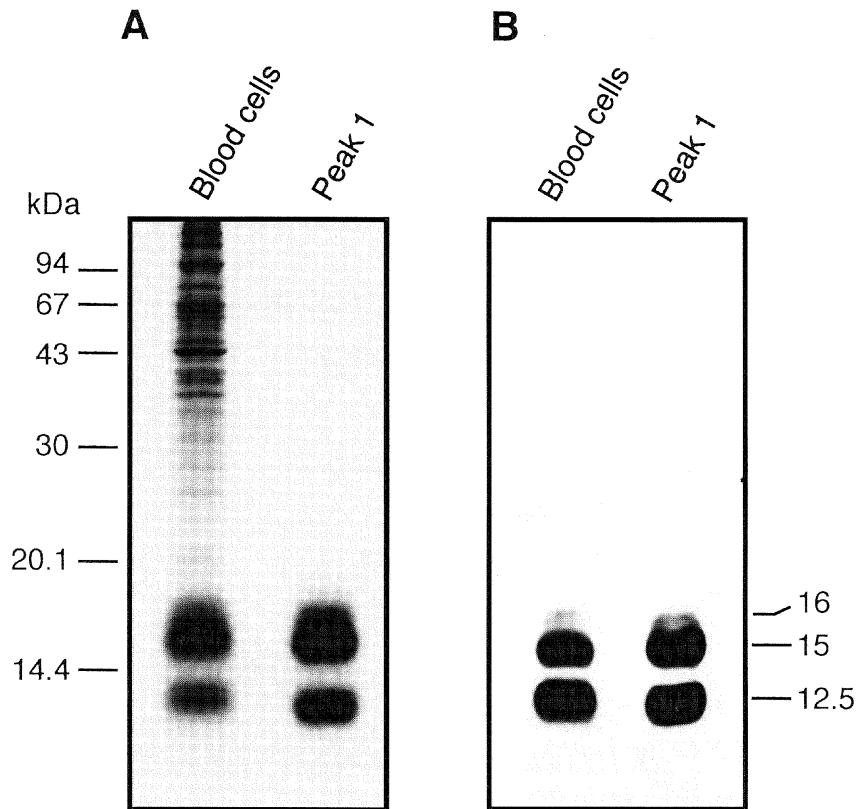
Fig. 2.   SDS-PAGE and immunoblot analysis. SDS-PAGE (**A**) revealed that peak 1 contained at least two types of peptides whose molecular weights were estimated to be 12.5 kDa and 15 kDa, respectively. The comparison of the SDS-PAGE patterns of peak 1 fractions with those of the homogenate of the blood cells, supernatant after freezing and thawing, and the dialyzed sample shows that the treatment of freezing and thawing removed the higher molecules of proteins and resulted in the desirable purification of the vanadium-associated protein. Immunoblot analysis (**B**) shows that a polyclonal antibody, anti-VAP, recognized at least two main peptides of 12.5 kDa and 15 kDa with a minor peptide of 16 kDa in the homogenate of blood cells and in peak 1 fractions.

vanadocytes, having single and fluid-filled large vacuoles whose pH values are extremely low from 1.8 to 4.2 (Michibata *et al.*, 1991a). Thus, the majority of the vanadium incorporated by the ascidians might be finally accumulated in their vacuoles in an ionic form or associated with the low-molecular substances rather than with the proteins under such extremely low pH conditions (Michibata *et al.*, 1991a; Michibata, 1996), so that the extraction of vanadium-associated protein was difficult. In fact, in the present experiment, approximately 90% of the vanadium that had been accumulated in the vacuoles was lost during the process of extraction (Table 1).

A multivalent transition metal, such as vanadium, is limited to the +3, +4, and +5 oxidation states under physiological conditions (Chasteen, 1983; Kustin *et al.*, 1983; Michibata, 1996). Vanadium dissolved in the +5 oxidation state (VO_4^{3-}) in seawater (McLeod *et al.*, 1975) is known to be finally accumulated in the vacuoles of the vanadocytes of ascidians in the cationic form of the +3 oxidation state (V^{3+}) via the vanadyl species of the +4 oxidation state (VO^{2+}), under strong sulfate and extremely low pH conditions (Michibata *et al.*, 1991a; Kanamori and Michibata, 1994). Under these conditions, it is reasonable that vanadium is in a free and not bound form with the macromolecules, corresponding to the

fact that almost all vanadium in the blood cells was lost during the process of extraction.

However, the remaining part of the vanadium was revealed to be associated with the protein in the present experiments (Fig. 1). Furthermore, hypotonic, LiCl and digitonin treatments disclosed that VAP was not in the vacuoles of the vanadocytes but in the cytoplasm and might bind with cell membranes by ionic interaction (Fig. 3). The VAP found in the cytoplasm and associated with the cell membranes of the vanadocytes might participate in the transient binding of the vanadium ions in the cytoplasm of the vanadocytes and transfer them to the vacuoles. Of course, further evidence would be needed to determine the role of the VAP. Assuming that the average molecular weight of VAP is 15 kDa, on the basis of the contents of proteins and vanadium in peak 1 fractions eluted from a DEAE column (Table 1), VAP was estimated to associate with vanadium at an approximate ratio of 1 mol : 16 mols. However, further biochemical analysis of VAP is required to clarify the association constant and stoichiometry. Sequence analysis for the amino acids of VAP are also needed to know the location and number of binding sites with vanadium in VAP.

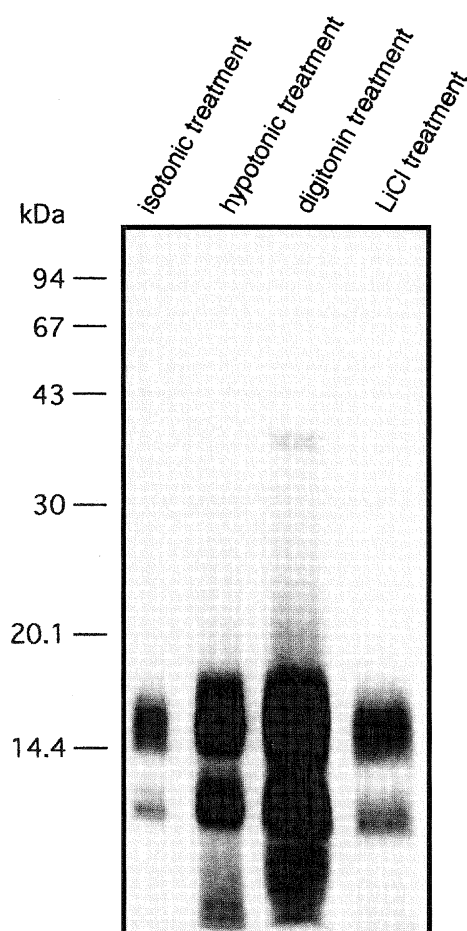A few years ago, besides the ascidians, the polychaete

Fig. 3. Treatment with hypotonic solution, digitonin, and LiCl. The blood cells treated with hypotonic solution, digitonin, or LiCl were submitted for SDS-PAGE. Around 15 kDa bands were stained much more deeply than those in the control by the treatment with hypotonic solution or with digitonin, indicating that VAP existed intrinsically in the cytoplasm of the blood cells. The treatment with LiCl also released some amounts of proteins, suggesting that the VAP might bind with cell membranes by ionic interaction.

Pseudopotamilla occelata was demonstrated to contain high levels of vanadium (Ishii *et al.*, 1993). We recently disclosed that *P. occelata* had the same antigens as those in the ascidian, which were recognized by two types of antibodies: a polyclonal antibody anti-VAP and a monoclonal antibody S4D5 against the vanadocytes in the vanadium-rich ascidian *A. sydneiensis samea* (Uyama *et al.*, 1997). The discovery of common antigens between two phylogenetically different species becomes a matter of primary concern. It is, therefore, quite possible that VAP has the key to solving the mechanism of the selective accumulation of vanadium by the ascidians and the polychaete.

ACKNOWLEDGMENTS

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Table 1. Contents of vanadium and proteins during isolation of the vanadium-associated proteins

	volume (ml)	vanadium (μg)	protein (mg)	vanadium/protein (μg/mg)
supernatant after the frozen-thawed treatment	10	2127	6.6	322.3
supernatant after the dialysis	10	273	3.6	75.8
peak 1 fraction eluted from DEAE Sephacel	28	26.6	0.7	36.9
fraction No. 5 in peak 1	3.5	8.3	0.2	55.0

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