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6-Phosphogluconate Dehydrogenase Is a 45-kDa Antigen Recognized by S4D5, a Monoclonal Antibody Specific to Vanadocytes in the Vanadium-Rich Ascidian *Ascidia sydneiensis samea*[1]

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We previously prepared a monoclonal antibody, S4D5, specific to vanadocytes, vanadium-containing blood cells, in the vanadium-rich ascidian *Ascidia sydneiensis samea*. Here, we demonstrate that a 45-kDa antigen recognized by S4D5 is 6-phosphogluconate dehydrogenase (6-PGDH), an enzyme of the pentose phosphate pathway, based on cDNA isolation of RNA samples from blood cells of the ascidian. Western blot analysis confirmed an abundance of 6-PGDH protein in the vanadocytes and localization of 6-PGDH in the soluble extract of the blood cells. Soluble protein exhibited a correspondingly high level of 6-PGDH enzymatic activity. Ascidians are known to selectively accumulate high levels of vanadium in vanadocytes, and the highest recorded concentration of accumulated vanadium is 350 mM, which is 10^7 times the concentration in sea water. Almost all vanadium ions are reduced to the +3 oxidation state *via* the +4 oxidation state in vanadocytes, indicating that reducing agents must participate in the accumulation. On the other hand, vanadium ions in the +5 oxidation state are reduced to the +4 oxidation state by the presence of NADPH *in vitro*. Together, these observations suggest that NADPH produced in the pentose phosphate pathway may conjugate the reduction of vanadium from the +5 oxidation state through the +4 oxidation state in vanadocytes of ascidians.

Key words: ascidian, NADPH, 6-PGDH, tunicate, vanadium.

The discovery by Henze (*1*) of high levels of vanadium in the blood cells (coelomic cells) of an ascidian has attracted the attention of not only analytical chemists, but also physiologists, biochemists, and chemists of natural products, partly because such levels have never before reported in living organisms but also because of the possible participation of vanadium in oxygen transport as a third metal after iron and copper, constituting respiratory pigment. Since the discovery, many scientists have endeavored to elucidate the mechanism of accumulation and physiological function of vanadium at high levels in ascidians (for reviews, see Refs. *2* and *3*).

Consequently, a lot is known about the vanadium in ascidians, which are also known as tunicates or sea squirts. For example: (i) the concentration of vanadium attains 350 mM in the blood cells of *Ascidia gemmata* belonging to the suborder Phlebobranchia, which is 10^7 times higher than the concentration in sea water (*4*), (ii) among approximately ten types of blood cells, signet ring cells are true vanadocytes, vanadium-containing blood cells (*5, 6*), (iii) almost all vanadium is reduced to the +3 oxidation state (V^{III}) *via* the +4 oxidation state (V^{IV}) and stored in vacuoles of vanadocytes (*7, 8*), although vanadium is in the +5 oxidation state (V^{V}) in seawater (*9*), (iv) the contents of the vacuoles have low pH values ranging from 1.9 to 4.2 (*4*), and (v) the acidity is maintained by a vacuolar-type H^+-ATPase localized in the vacuolar membranes of vanadocytes (*10*).

Previous results indicate that vanadocytes must have a key role in the unusual highly selective accumulation and reduction of vanadium. We, therefore, prepared a monoclonal antibody against vanadocytes in ascidian blood cells, due to the difficulties encountered in distinguishing morphologically the different types of blood cells (*11*). The vanadocyte-specific monoclonal antibody, designated S4D5, was revealed to react with a single polypeptide of about 45 kDa extracted from not only *Ascidia sydneiensis samea*, which provided the antigen, but also other vanadium-rich ascidians, *A. ahodori*, *A. gemmata*, and *Ciona intestinalis*. The antigen of the 45-kDa peptide was shown by immunocytological studies to be localized in the cytoplasmic region and around the intravacuolar vesicle of the vanadocytes. This polypeptide is predominant among many

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Abbreviation: CsTFA, cesium trifluoroacetate.

peptides extracted from a subpopulation of vanadocytes in *A. sydneiensis samea* (*11*). However, its function is unknown. The present experiments demonstrate that the antigen of 45 kDa localized in vanadocytes is 6-phosphogluconate dehydrogenase (6-PGDH: EC 1.1.1.44), an enzyme of the pentose phosphate pathway, based on cDNA isolation of RNA samples from blood cells of the ascidian. Soluble protein of the blood cells exhibited a correspondingly high level of 6-PGDH enzymatic activity.

Since almost all vanadium ions are reduced to V^{III} *via* V^{IV} in vanadocytes (*8*), reducing agents must participate in the accumulation. On the other hand, V^{V} is reported to stimulate oxidation of NAD(P)H: *i.e.*, V^{V} is reduced to V^{IV} by the addition of NAD(P)H *in vitro* (*12-14*). Together, these observations suggest that NADPH produced in the pentose phosphate pathway may conjugate one step of reduction of vanadium from V^{V} through V^{IV} in vanadocytes of ascidians.

MATERIALS AND METHODS

Ascidians—Specimens of the vanadium-rich ascidian *A. sydneiensis samea* were collected in the vicinity of the Asamushi Marine Biological Station of Tohoku University, Asamushi, Aomori Prefecture, and the Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, Otsuchi, Iwate Prefecture. The ascidians were maintained in an aquarium that contained circulating natural sea water at 18°C.

Immunocytological Detection—A monoclonal antibody, S4D5, was previously reported to react exclusively with signet ring cells, vanadium-containing blood cells (vanadocytes), among about ten types of blood cell (*11*). In the present experiment, to reconfirm the specific immunoreactivity of S4D5, immunological detection was carried out in a similar manner to that described previously (*11*).

Construction and Immunoscreening of cDNA Library—Ascidian blood was centrifuged at $300 \times g$ for 10 min to separate blood cells from serum. The blood cells were suspended in Ca^{2+}- and Mg^{2+}-free artificial sea water containing 0.2 M sucrose, 368 mM NaCl, 7.2 mM KCl, 26.4 mM Na_2SO_4, 4.8 mM $NaHCO_3$, and 4 mM HEPES at pH 7.0 to avoid clotting and were centrifuged at $100 \times g$ for 10 min at 4°C. The precipitated blood cells were composed of two layers of cells. The upper layer, predominantly consisting of a subpopulation of giant cells, was discarded. An aliquot of 600 mg wet weight of the lower one, mainly consisting of signet ring cells (vanadocytes), was used for preparation of total RNA in a manner similar to the method described by Glisin *et al.* (*15*). Total RNA obtained was used for purification of poly(A)$^+$ RNA by Oligotex-dT30 beads (Japan Roche, Tokyo). The cDNA library was

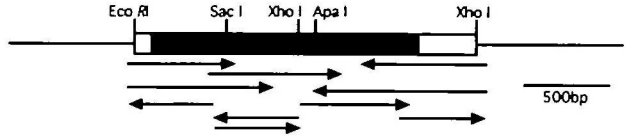

Fig. 2. **Strategy for sequencing the cDNA encoding a 45-kDa antigen recognized by S4D5 monoclonal antibody.** A diagram illustrating the structure of clone sd45 encoding S4D5 cDNA. S4D5 cDNA (2 kbp) was inserted in pBluescript SK(−) between the *Eco*RI and *Xho*I site. The solid line represents the vector region. The open boxes represent non-coding regions. The closed box represents the coding region. Subclones were constructed for subcloning of clone sd45. Arrows indicate the extent and direction of the sequenced strands. bp, base pairs.

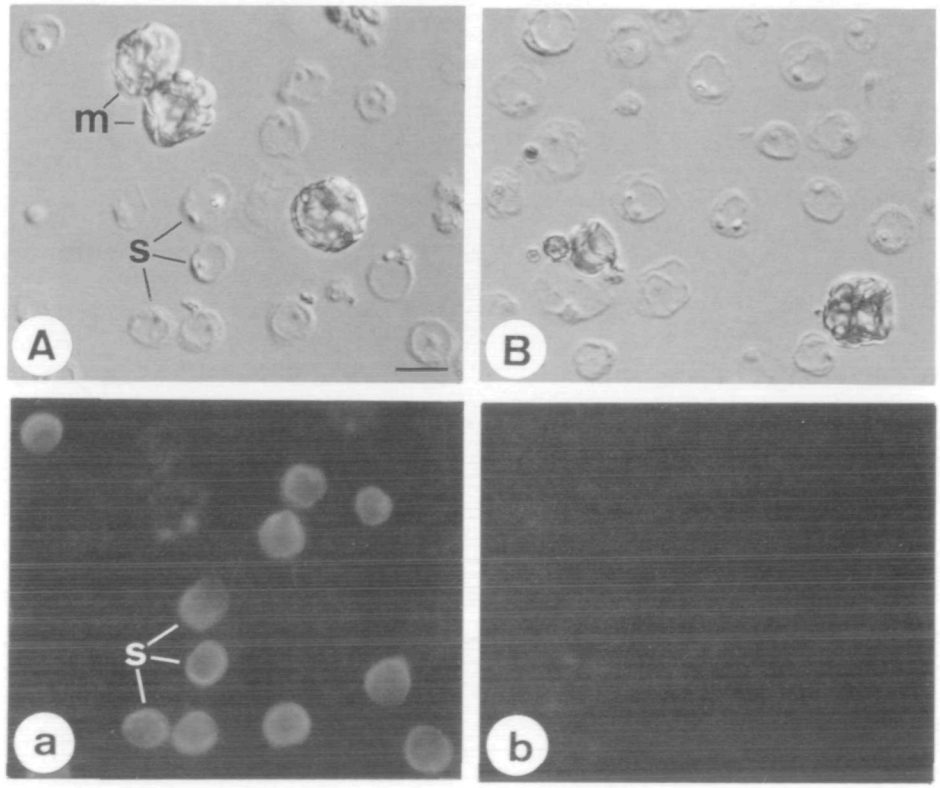

Fig. 1. **Immunocytological detection of S4D5 in blood cells of the vanadium-rich ascidian *Ascidia sydneiensis samea*.** Blood cells observed in panels A and a were reacted with S4D5. Blood cells in panels B and b were reacted with nonimmune mouse serum as a negative control. Upper panels (A and B) and lower panels (a and b) were visualized by Nomarski differential-interference and fluorescence microscopes, respectively. Vanadocytes (signet ring cells) were exclusively recognized by S4D5, showing fluorescence of FITC. No immunoreactivity was observed in the other types of blood cells. Autonomous fluorescence was faintly emitted from morula cells (*6, 28*). s, vanadocytes (signet ring cells); m, morula cells. Scale bar indicates 10 μm.

prepared using the Uni-ZAP XR vector (Stratagene, La Jolla, USA).

Western Blot Analysis—To examine the localization of the 45-kDa antigen in subcellular fractions, an aliquot of 200 mg wet weight of blood cells of *A. sydneiensis samea* was homogenized in 6 ml of 0.2 M Tris-HCl buffer, pH 8.0, containing protease inhibitors [leupeptin, pepstatin A, chymostatin, phenylmethylsulfonyl fluoride (PMSF), each at a concentration of 10 μg/ml] using a Potter-Elvehjem homogenizer. The homogenate was divided into two aliquots. One was subjected to SDS-PAGE (lane 1 in Fig. 4) and the other centrifuged at 1,000×g for 10 min. The

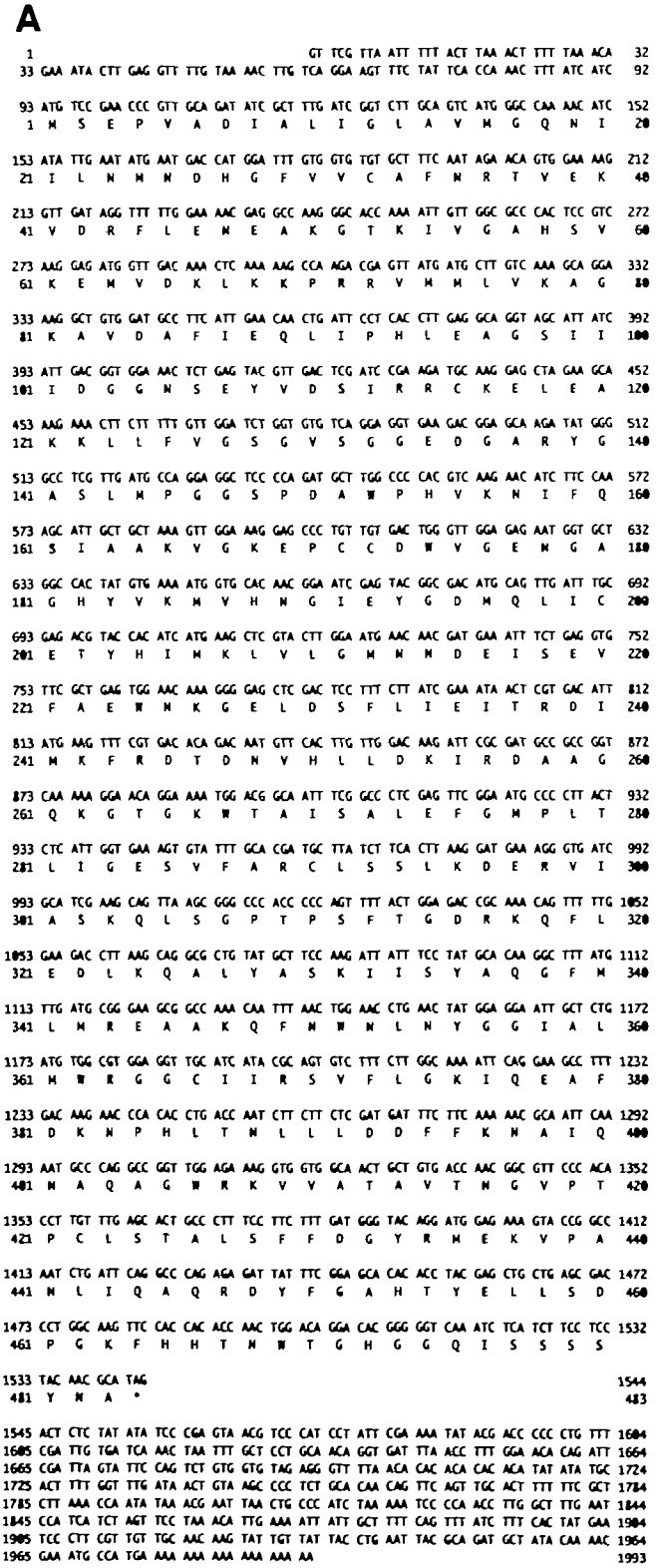

Fig. 3. **Sequence alignments of the 5′-end of sd45 gene encoding a 45-kDa antigen (A) and of deduced amino acids (B).** sd45 gene encompassed 92 bp of the 5′ untranslated region, a 1,452-bp open reading frame, and 449 bp of the 3′ untranslated sequence. The stop codons are indicated by asterisks (A). A search of the SwissProt sequence data base for similarities with sd45 gene detected a match with 6-phosphogluconate dehydrogenase (6-PGDH). The open reading frame encoded a protein of 483 amino acids with 74.4% identity and 87.6% similarity to that of sheep. Alignments between amino acids deduced from the nucleotide sequence of sd45 gene and those of 6-PGDH derived from sheep liver, the fly *Drosophila*, and the yeast *Saccharomyces cerevisiae* were compared. Amino acids that are identical among four sequences are marked by an asterisk, while those that are similar are marked by a period (B).

precipitate obtained was used as the fraction of nuclei and plasma membranes (lane 2) and the supernatant was centrifuged again at 10,000 × *g* for 10 min. The 2nd precipitate was the mitochondria-rich fraction (lane 3). The 2nd supernatant was further centrifuged at 100,000 × *g* for 1 h to obtain a microsome fraction in the precipitate (lane 4) and a soluble protein fraction in the supernatant (lane 5). Samples containing approx. 30 μg of protein were dissolved in a sample-dissociation buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 2.3% (w/v) SDS. The dissolved sample was applied to 12.5% uniform SDS-PAGE, then subjected to Western blot analysis to examine the localization of antigens using the monoclonal antibody S4D5 as a probe. Content of protein in each sample was determined by the Bradford method (*16*). Western blot analysis was also carried out to examine whether monoclonal antibody S4D5 specifically reacted with 6-PGDH, the 45-kDa antigen. The 3rd supernatant of ascidian blood cells and 6-phosphogluconate dehydrogenase purified from sheep liver, purchased from Sigma Chemical, St. Louis, USA, were dissolved, respectively, in sample-dissociation buffer. Samples containing 20 μg of protein were subjected to electrophoresis in 10% polyacrylamide gel in the presence of 2% SDS.

Enzymatic Assay—To assay 6-PGDH activity, the 3rd supernatant obtained from ascidian blood cells was used, since preliminary experiments confirmed that the enzymatic activity existed only in the 3rd supernatant. The reaction mixture consisted of 6-phosphogluconate (Sigma Chemical) ranging in concentration from 10 to 200 μM as a substrate, 0.4 mM $NADP^+$ (Oriental Yeast, Tokyo), and 5 mM $MgCl_2$ in 0.1 M Tris-HCl buffer, pH 8.9. A total volume of 2,375 μl of the reaction mixture in a quartz cuvette was preincubated for 10 min at 25°C. The reaction was initiated by the addition of 125 μl of enzyme solution containing 70 μg of protein. Reduction of $NADP^+$ to NADPH by the enzymatic reaction was successively recorded as the increase of absorbance wavelength at 340 nm.

RESULTS

Immunocytological Detection of S4D5—As shown in Fig. 1, immunoreactivity of the monoclonal antibody S4D5 was exclusively detected in signet ring cells, which had been identified as vanadocytes, vanadium-containing blood cells. Although *A. sydneiensis samea* has about ten types of blood cell (*6, 17*), no immunoreactivity was observed in blood cells other than vanadocytes.

cDNA Cloning and Sequence Analysis—The monoclonal antibody was used as a probe to screen a gene encoding a 45-kDa antigen from a cDNA library prepared from the blood cells of the vanadium-rich ascidian, *A. sydneiensis samea*. Three positive clones were isolated. The longest insert was found to cover the other two clones by nucleotide sequencing. A full-length cDNA, designated sd45, for the newly cloned molecule was subcloned into the expression vector according to the sequencing strategy of the cDNA shown in Fig. 2. Consequently, sd45 encompassed 92 bp of the 5′ untranslated region, a 1,452-bp open reading frame, and 449 bp of the 3′ untranslated sequence, as shown in Fig. 3. A search of the SwissProt sequence data base for similarities with sd45 detected a match with 6-PGDH both in levels of nucleotide and amino acid sequences. The open reading frame encoded a protein of 483 amino acids with 74.4% identity and 87.6% similarity to 6-PGDH of sheep.

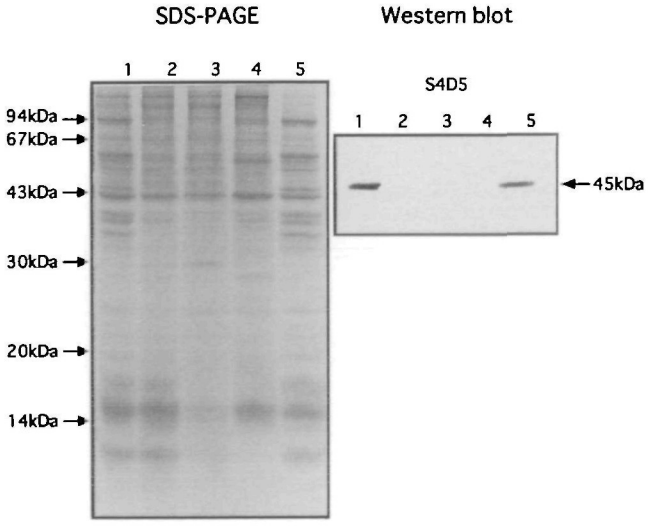

Fig. 4. **Localization of a 45-kDa antigen in cell-free fractions by SDS-PAGE and Western blot analysis.** Blood cells of *A. sydneiensis samea* were homogenized and proteins in the subcellular fractions were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (left). The separated proteins were blotted onto nitrocellulose paper and reacted with S4D5 monoclonal antibody (right). Lane 1, homogenate of blood cells; lane 2, fraction of nuclei and plasma membranes; lane 3, mitochondrial fraction; lane 4, fraction of microsomes; lane 5, soluble proteins. Western blot analysis revealed positive bands recognized by the monoclonal antibody S4D5 in lanes of homogenate and soluble proteins but not in the other lanes, demonstrating that the 45-kDa antigen is a soluble protein localized in the supernatant obtained by centrifugation at 100,000 × *g*.

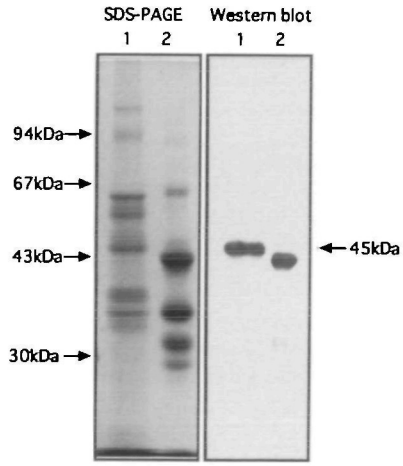

Fig. 5. **Western blot analysis of 6-PGDH.** To examine whether S4D5 monoclonal antibody specifically reacted with the 6-PGDH revealed to be a 45-kDa antigen, Western blot analysis was carried out using S4D5. Soluble proteins extracted from the blood cells of *A. sydneiensis samea* and 6-PGDH of sheep liver purchased from Sigma were separated by SDS-PAGE (left) and the separated proteins were subjected to Western blot analysis (right). S4D5 monoclonal antibody reacted with each 45 kDa protein of the ascidian and 6-PGDH. Lane 1, soluble proteins of ascidian blood cells; lane 2, 6-PGDH of sheep liver.

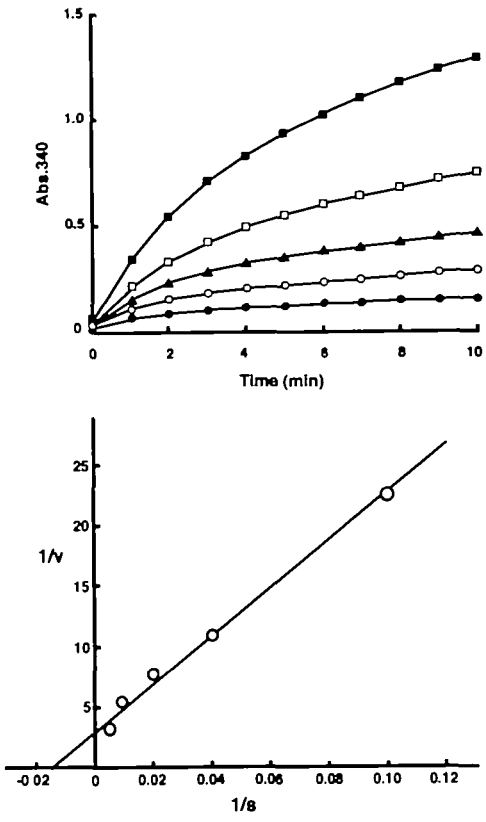

Fig. 6. **Enzymatic activity of 6-PGDH in the ascidian blood cells.** To examine whether 6-PGDH functions enzymatically in the soluble protein extracted from ascidian blood cells, the activity was assayed using 6-phosphogluconate (6-PG) as a substrate. The Lineweaver-Burke plot shows clearly that the enzymatic activity is dependent on the concentration of substrate. K_m for the substrate and V_{max} are 62.5 μmol/liter and 129 nmol/min, respectively. ●, 10 μM 6-PG; ○, 25 μM 6-PG; ▲, 50 μM 6-PG; □, 100 μM 6-PG; ■, 200 μM 6-PG.

The 45-kDa antigen recognized by the monoclonal antibody S4D5 is, therefore, shown to be 6-PGDH, an enzyme of the pentose phosphate pathway, based on cDNA isolation of RNA samples from blood cells of the ascidian.

Western Blot Analysis—We have demonstrated that the monoclonal antibody S4D5 specifically recognizes vanadocytes among about ten types of blood cells in the vanadium-rich ascidian *A. sydneiensis samea* (*11*). In the present study, we examined whether the 45-kDa antigen is localized in the soluble extract of blood cells, because 6-PGDH is known to be a soluble protein. As shown in Fig. 4, a positive band of 45 kDa appeared in the lanes of homogenate and soluble proteins, but not in the other lanes.

We also confirmed that S4D5 reacted with 6-PGDH derived from sheep liver. Western blot analysis confirmed that the monoclonal antibody reacted with soluble proteins of ascidian blood cells and 6-PGDH of sheep liver, as shown in Fig. 5.

Enzymatic Assay—To examine whether the soluble protein of the blood cells contains enzymatic activity of 6-PGDH, the soluble protein was assayed using 6-phosphogluconate as a substrate. As shown in Fig. 6, a correspondingly high level of enzymatic activity of 6-PGDH was found. The Lineweaver-Burke plot shows clearly that the enzymatic activity is dependent on the concentration of substrate. K_m for the substrate and V_{max} were calculated to be 62.5 μmol/liter and 129 nmol/min at pH 8.9, corresponding with those reported in rat liver (*18*).

DISCUSSION

The present study revealed the existence of 6-PGDH in vanadocytes, vanadium-containing blood cells, of the vanadium-rich ascidian *A. sydneiensis samea*. The gene encoding a 45-kDa antigen was screened using S4D5 monoclonal antibody as a probe from a cDNA library prepared from the blood cells of the ascidian, and the DNA sequence resolved was compared against the SwissProt sequence data base. Consequently, the gene encoding the 45-kDa antigen that was exclusively expressed in the vanadocytes was revealed to be approximately 66.6, 65.4, and 61.4% homologous to that of 6-PGDH derived from the sheep, the fly, and the yeast, respectively (*19-21*). In amino acid sequence, the antigen shows approximately 74.4% identity and 87.6% similarity with the 6-PGDH in sheep liver (Fig. 3). By Western blot analysis, S4D5 monoclonal antibody prepared from the vanadocytes of the ascidian was shown to react with 6-PGDH isolated from sheep (Fig. 5). Although the molecular mass of 6-PGDH of sheep liver recognized by S4D5 monoclonal antibody was estimated by Western blot analysis to be 43 kDa (Fig. 5), as opposed to the 45 kDa of the ascidian protein, it has been pointed out that purified 6-PGDH has a tendency to show apparently lower molecular mass on the SDS-polyacrylamide gel (*22*). It was, furthermore, confirmed that the soluble protein of the ascidian blood cells exhibited a correspondingly high level of enzymatic activity of 6-PGDH (Fig. 6). Expression of the 45-kDa antigen was also confirmed to be localized in the vanadocytes but not in the other types of blood cells by immunocytological analysis (Fig. 1). From the results described above, the 45-kDa antigen localized in the cytoplasm of the vanadocytes and recognized by S4D5 monoclonal antibody is considered to be 6-PGDH, the third enzyme in the pentose phosphate pathway, which is known to catalyze the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate, with release of CO_2 and the reduction of $NADP^+$.

The pentose phosphate pathway is the major supplier of reducing agents in the form of NADPH and is tightly coupled to cellular processes which require NADPH and other reductase systems. It, therefore, may play a critical role in maintaining a reducing environment. On the other hand, a consensus has been reached that almost all the vanadium accumulated in the vacuoles of vanadocytes is reduced to V^{III} *via* V^{IV}, although the mechanism underlying the reduction of V^V in ascidian blood cells is a matter of controversy. Whatever the case a reducing agent must participate in the accumulation of vanadium. Haemovanadin, a nitrogenous compound containing vanadium and sulfate (*23*), and tunichromes, isolated from the blood cells of certain ascidian species and identified as a class of hydroxy-dopa-containing tripeptides, have been studied as possible reducing agents in ascidians (*24*), but the possibility that these compounds are involved in the reduction of vanadium was ruled out (*3*).

We propose here the possibility that the NADPH produced in the pentose phosphate pathway in vanadocytes

is responsible for the reduction of V^{V} to V^{IV} in the cytoplasm of the vanadocytes, although this proposal requires further study. It is known that V^{V} stimulates oxidation of NAD(P)H: *i.e.*, V^{V} is reduced to V^{IV} in the presence of NADPH *in vitro* (*13, 14*). For example, Nour-Eldeen *et al.* (*25*) reported that vanadate is associated with the activation of glucose-6-phosphate dehydrogenase in the oxidation of glucose by $NADP^{+}$. Shi and Dalal (*14, 26*) reported the formation of V^{IV} in the reduction of V^{V} by NADPH-dependent flavoenzymes. These observations strongly suggest that NADPH produced in the pentose phosphate pathway might conjugate the reduction of V^{V} to V^{IV} in vanadocytes of ascidians. In addition to the 6-PGDH, glucose-6-phosphate dehydrogenase (G6PDH: EC 1.1.1.49), the first enzyme to produce NADPH in the pentose phosphate pathway, has been found in vanadocytes of the ascidian (*27*), supporting the above possibility. The coexistence of enzymes in the pentose phosphate pathway and high levels of vanadium in vanadocytes appear to be implicated the reduction of vanadium in ascidians.

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