Exclusive Expression of Transketolase in the Vanadocytes of the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea**

Tatsuya Ueki [a,#,], Taro Uyama [a,#], Kazuhiro Yamamoto [a], Kan Kanamori [b], and Hitoshi Michibata [a]**

[a] *Marine Biological Laboratory, Graduate School of Science, Hiroshima University, Mukaishima-cho 2445, Hiroshima 722-0073, Japan*

[b] *Department of Chemistry, Faculty of Science, Toyama University, Toyama 930-8555, Japan*

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# These two authors contributed equally to this work.

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** Corresponding author. Phone: +81-848-44-1143, Fax: +81-848-44-5914, e-mail: ueki@sci.hiroshima-u.ac.jp.

Abstract

Ascidians, especially those belonging to the Ascidiidae, are known to accumulate extremely high levels of vanadium in vanadocytes, one type of blood (coelomic) cell. Vanadium, which exists in the +5 oxidation state in seawater, is accumulated in the vanadocytes and reduced to the +3 oxidation state. We have been trying to characterize all of the polypeptides specific to vanadocytes and to specify the proteins that participate in the accumulation and reduction of vanadium. To date, we have localized three enzymes in vanadocytes: 6-phosphogluconate dehydrogenase (6-PGDH: EC 1.1.1.44), glucose-6-phosphate dehydrogenase (G6PDH: EC 1.1.1.49), and glycogen phosphorylase (GP: EC 2.4.1.1), all of which are involved in the pentose phosphate pathway. In the current study, we cloned a cDNA for transketolase, an essential and rate-limiting enzyme in the non-oxidative part of the pentose phosphate pathway, from vanadocytes. The cDNA encoded a protein of 624 amino acids, which showed 61.8% identity to the human adult-type transketolase gene product. By immunocytochemistry and immunoblot analyses, the transketolase was revealed to be a protein that was expressed only in vanadocytes and not in any of the more than ten other types of blood cell. This finding, taken together with the localized expression of the other three enzymes, strongly supports the hypothesis that the pentose phosphate pathway functions exclusively in vanadocytes.

1.Introduction

Ascidians, known as tunicates or sea squirts (Chordata, Urochordata, Ascidiacea), accumulate extremely high levels of vanadium in vanadocytes, one of their approximately ten types of blood cell [1-5]. The vanadium concentration in vanadocytes can reach 350 mM, corresponding to 10^7 times the level in seawater, where vanadium is dissolved at a concentration of 35 nM [6, 7]. This is thought to be the highest rate of concentration of a metal in any living organisms. Vanadium, a multivalent transition metal, is generally present in the biosphere in the +5, +4, and +3 oxidation states [8, 9]. Vanadium dissolved in seawater usually occurs as vanadate anions in the +5 oxidation state (V(V)) [10], whereas most vanadium in vanadocytes is reduced to the +3 oxidation state (V(III)) with a small amount in the +4 oxidation state (V(IV)) [11-17]. Some reducing agents must, therefore, be involved in the reduction and accumulation of vanadium in ascidian blood cells. *In vitro* experiments have shown that some biologically relevant reducing agents can reduce V(V) to V(IV), including ascorbate [18], cysteine [19, 20], norepinephrine [21], and glutathione [22]. However, no information has been obtained on the reducing agents that participate in the reduction in ascidian vanadocytes.

About ten years ago, we prepared several monoclonal antibodies to distinguish vanadocytes from the other types of blood cell. During the course of these experiments, a monoclonal antibody, S4D5, recognized a 45 kDa antigen specific to the cytoplasm of vanadocytes [23]. This was revealed to be 6-phosphogluconate dehydrogenase (6-PGDH: EC1.1.1.44) based on cDNA isolation

of RNA samples from the blood cells of the ascidian by immunoscreening [24]. 6-PGDH from the pentose phosphate pathway is localized massively in vanadocytes. Therefore, we examined whether other enzymes related to the pentose phosphate pathway are also localized in vanadocytes. As a result, two other enzymes related to the pentose phosphate pathway were also revealed to be exclusively and strongly expressed in vanadocytes by immunocytochemistry and by measuring enzymatic activity [25, 26]. One is glucose-6-phosphate dehydrogenase (G6PDH: EC1.1.1.49), the first enzyme of the pentose phosphate pathway. The other is glycogen phosphorylase (GP: EC 2.4.1.1), which catalyzes the phosphorylation of glycogen to produce glucose 1-phosphate, which is further interconverted into glucose 6-phosphate, the first substrate in both the pentose phosphate and Embden-Meyerhof pathways.

The pentose phosphate pathway consists of an oxidative part and a nonoxidative part. The oxidative part converts glucose 6-phosphate to ribulose 5-phosphate and CO_2, and at the same time generates NADPH for use in reductive biosynthesis. The nonoxidative part isomerizes ribulose 5-phosphate to xylulose 5-phosphate and ribose 5-phosphate, which are converted into fructose 6-phosphate and glyceraldehyde 3-phosphate by a sugar rearrangement system. Therefore, the present study examines whether transketolase (TKT: EC2.2.1.1), the rate-limiting enzyme of the nonoxidative part of this pathway, is also exclusively localized in vanadocytes. It is worthwhile that the pentose phosphate pathway is fully functional in vanadocytes, because the coexistence of enzymes in the pentose phosphate pathway and high levels of vanadium in vanadocytes implicate the pathway in the reduction and accumulation of

vanadium by ascidians.

2. Materials and Methods

2.1 PCR amplification

A cDNA library of blood cells of *Ascidia sydneiensis samea* has been constructed in Uni-Zap XR vector (Stratagene, La Jolla, USA) [24]. Lambda phage DNA was extracted from the amplified cDNA library. Degenerate primers were designed from the conserved amino acid sequences of transketolases of several animals. The forward primer was 5'- AT(A/C/T) GG(A/C/G/T) GA(A/G) GA(A/C/T) GG(A/C/G/T) CC -3' and the reverse one was 5'- CC (A/G)TC (A/C/G/T)(C/G)(A/T) (A/C/G/T)GG (A/G)TA (A/G)AA (A/C/G/T)AC -3'. PCR conditions were as follows: 0.2 μg lambda phage DNA, 200 pmole each primer, 0.2 mM dNTP, 25 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. The reaction volume was 50 μl. 30 cycles of PCR were run; each cycle consisted of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec. This was followed by a final extension at 72°C for 5 min. Amplified DNA fragments of the expected length were directly cloned into pBluescript vector at the *Eco*RV site with T-overhangs.

2.2 Screening the cDNA library

The same cDNA library was screened with digoxigenin-labeled random-primed DNA probes derived from the cloned PCR fragments. Hybridization was

done at 65°C for at least 16 hrs in 0.25 M disodium hydrogen phosphate buffer (Na-Pi buffer, pH 7.2), 7% SDS, 1 mM EDTA, and 10 ng/ml probes. The membranes were washed three times in 20 mM Na-Pi buffer (pH 7.2), 1% SDS at 65°C. They were treated with anti-digoxigenin-AP conjugate (F. Hoffmann-La Roche Ltd, Switzerland). The signals were detected by chemiluminescence with CSPD substrates (F. Hoffmann-La Roche Ltd) and Hyper-ECL film (Amersham Pharmacia Biotech, Sweden). Positive plaques were screened repeatedly until they were cloned. The cDNAs were excised as plasmids *in vivo* according to the manufacturer's protocol. Nucleotide sequences were determined using a ThermoSequenase dye-prime sequencing kit and an ALFexpress automated sequencer (Amersham Pharmacia Biotech).

2.3 Preparation of anti-transketolase antiserum

The partial fragment of ascidian transketolase cDNA that corresponded to the amino acid residues from the 27th to the 264th residue (Fig. 1, double arrows) was amplified from cDNA clone #106 with the following primers: Forward, 5'- GGA ATT CAT TCT GTC TAC T -3'; Reverse, 5'- GGT CGA CTC AAG ATA CAG GTT TCC -3'. The amplified cDNA fragment was cloned in the *Eco*RV site of pBluescript SK(-) vector, and the nucleotide sequence was confirmed to be the same as the corresponding part of cDNA clone #106. The cDNA fragment was excised by *Eco*RI and *Sal*I, and ligated into the corresponding site of pMAL-c2 plasmid vector (New England Biolabs, Inc., MA, U.S.A.). By nucleotide sequence determination, the open reading frame of the

ascidian transketolase was confirmed to be continuous with that of maltose binding protein (MBP). The resulting plasmid was designated pTKT-MBP.

Six ml of an overnight culture of E. coli TB1 cells transformed with pTKT-MBP were diluted in 250 ml LB medium supplemented with 100 μg/ml ampicillin and 0.3 mM IPTG, and then cultured at 37°C for 6 hrs. The cells were pelleted, suspended in lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25% Tween20, 10 mM 2-mercaptoethanol, 10 mM EDTA, and 10 mM EGTA) containing 6M urea, and lysed with a sonicator (Vibra Cell; Sonic & Materials, Inc., CT, U.S.A.). The solubilized protein fraction was recovered by centrifugation at 10,000 ×g for 10 min at 4°C, and dialyzed against 100 volumes of lysis buffer at 4°C overnight. Then, the TKT-MBP fusion proteins obtained were purified with amylose resin according to the supplier's protocol (New England Biolab, Inc.).

One hundred micrograms of purified TKT-MBP fusion proteins were injected intraperitoneally into an 8-week-old female BALB/c mouse. The injection was repeated two and four weeks after the first injection. Three days after the last injection, blood was collected, allowed to stand at 37°C for 1 hr, and incubated at 4°C for 12 hrs. 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. The supernatant obtained was stored at -20°C.

2.4 Immunoblotting and immunohistochemistry

Immunoblot analysis and immunocytochemistry with anti-transketolase

antiserum were carried out as described previously (19, 20). Briefly, the TKT-MBP fusion proteins were electrophoresed and blotted onto a nitrocellulose membrane. The antibodies were purified with the immobilized fusion proteins. The purified antibodies were used for both the immunoblot analysis and the immunocytochemistry at 4,000 and 1,000 times dilution calculated from the original antiserum, respectively. For the immunoblot analysis, HRP-conjugated anti-mouse IgG antibodies (PI-2000; Vector, Inc., CA, U.S.A.) were used and the signals were detected with ECL reagents (Amersham Pharmacia Biotech). Fluorescein-labelled anti-mouse IgG antibodies were absorbed by fixed ascidian blood cells before use in immunocytochemistry.

3. Results

3.1 Cloning cDNAs for transketolase from ascidian blood cells

Using a pair of degenerate primers corresponding to conserved amino acid sequences of known transketolase proteins, we amplified cDNA fragments related to a transketolase gene from an *A. sydneiensis samea* blood cell cDNA library. Several amplified fragments were cloned, sequenced, and revealed to have almost identical nucleotide sequences (data not shown). Using one of the PCR fragments as a probe, we isolated eight independent cDNA clones of a transketolase homologue out of 9×10^4 phages of the cDNA library of *A. sydneiensis samea* blood cells. They contained almost identical nucleotide sequences, although their lengths varied from 1.1 kbp to 2.5 kbp. We chose the

longest cDNA clone, #106, subcloned it by restriction digestion, and sequenced both strands of the subclones completely (Fig. 1). The nucleotide length of cDNA clone #106 was 2,545 bp including the cloning sites (*Eco*RI at the 5' terminal and *Xho*I at the 3' terminal). This is consistent with the apparent length of the transcript detected by Northern blot analyses (data not shown), suggesting that the cDNA clone corresponded to full-length mRNAs. The cDNA contained a single, long open reading frame of 1,875 nucleotides including the termination codon, which encoded a protein of 624 amino acids (Fig. 1).

The deduced amino acid sequence of ascidian cDNA clone #106 showed striking similarities to transketolases from various organisms (Fig. 2). There was 61.8% amino acid identity between the ascidian and human adult-type transketolase sequences, and 61.7% identity between the ascidian and mouse transketolase sequences. A thiamine diphosphate-binding motif and a transketolase motif, which were pointed out by Schenk *et al.* (23), were found in the predicted amino acid sequence of cDNA clone #106 (Fig. 2). Within the transketolase motif, an NADH-binding-like motif was also completely conserved (Fig. 2). The calculated molecular weight of the amino acid sequence predicted from cDNA clone #106 was 67.8 kDa, which is close to those of mammals (ranging from 67.7 to 67.8 kDa) and a nematode (66.0 kDa). Therefore, we concluded that cDNA clone #106 corresponded to a transketolase homologue in *A. sydneiensis samea*.

3.2 Expression of transketolase proteins in vanadocytes

More than ten types of blood cell have been distinguished in *A. sydneiensis samea* on the basis of their morphology. These include hemoblasts, lymphocytes, leukocytes, pigment cells, morula cells, signet ring cells, compartment cells, small compartment cells, and giant cells [27-30]. Of these, signet ring cells have been revealed to be the vanadium-accumulating cells, also known as vanadocytes, and these generally account for more than 70% of the total blood cells [29, 31, 32]. To examine the localization of transketolase, we prepared a fusion protein of the N-terminal part of the ascidian transketolase and a maltose binding protein, in order to obtain anti-transketolase antiserum. The antiserum detected a single band of 65 kDa on Western blots of blood cell extracts of *A. sydneiensis samea*, showing good agreement with the expected molecular weight predicted from its nucleotide sequence (Figs. 3A, B). By immunocytochemistry, the antiserum detected the transketolase protein only in the cytoplasm of signet ring cells (Figs. 4A, C). No detectable protein was expressed in the other blood cells, demonstrating that the transketolase is exclusively expressed in the vanadocytes among the blood cells.

4. Discussion

The first clue to the exclusive localization of enzymes in the pentose phosphate pathway in vanadocytes was the identification of monoclonal antibody S4D5, which was prepared to distinguish vanadocytes from the approximately ten types of ascidian blood cell [23]. A 45-kDa antigen, recognized by S4D5 monoclonal antibody, was found to localize in the cytoplasm of vanadocytes and

produce a clear band on SDS-PAGE of the homogenate of ascidian blood cells. The antigen was unexpectedly revealed to be 6-PGDH [24], which is generally thought to be expressed ubiquitously. The specific expression of glucose-6-phosphate dehydrogenase (G6PDH), another enzyme in the pentose phosphate pathway, in the cytoplasm of vanadocytes, has also been detected by both immunocytochemistry and enzymatic activity [25]. Furthermore, a 100 kDa antigen recognized by a newly prepared monoclonal antibody, S8E4, was exclusively localized in the cytoplasm of vanadocytes and identified as glycogen phosphorylase (GP), by sequencing the encoded cDNA [26]. The fact that glycogen phosphorylase and three other enzymes in the pentose phosphate pathway, including TKT found in this study, are abundant in the cytoplasm of vanadocytes, while undetectable in the other blood cells, suggests that the pentose phosphate pathway is closely linked with the reduction and accumulation of vanadium ions in vanadocytes.

Most of the vanadium accumulated in ascidians is reduced to V(III), with a small amount of vanadium also present as V(IV) [12-17]. A consensus has been reached on the process of vanadium accumulation in ascidians. First, vanadium dissolved in seawater as V(V) is accumulated in ascidians. Then, the vanadium is reduced to V(IV) by some reducing agent contained in the ascidians. Finally, almost all the vanadium is accumulated in the vacuoles of vanadocytes in the cationic form of V(III) under high sulfate concentrations at an extremely low pH [32-35]. In the vacuoles, the vanadium seems to be in a free form and not bound to macromolecules.

Thus, the pentose phosphate pathway is the major supplier of reducing

agents in the form of NADPH, and is tightly coupled to cellular processes that require NADPH and other reductase systems. In fact, we have confirmed that NADPH directly reduces V(V) to V(IV) *in vitro*, in the presence of EDTA at neutral pH [36]. It is important to consider whether the reduction of V(V) observed *in vitro* occurs *in vivo*. It is reasonable to assume that V(V) ion binds to a peptide or protein in the cytoplasm of ascidian vanadocytes. We have isolated a vanadium-associated protein that is composed of at least 12.5 and 15 kDa peptides from vanadocytes [37] and cloned the cDNA encoding these peptides (unpublished). If such a peptide or protein plays the role that EDTA does *in vitro*, V(V) could be reduced to V(IV) directly by NADPH. This assumption is likely true, since peptides and proteins bind more strongly to d^1 V(IV) ion than to d^0 V(V) ion, according to known chemistry, and as a result V(V) species tend to be reduced more easily.

TKT is known to be a rate-limiting enzyme of the pentose phosphate pathway. Although essentially most types of cell in all organisms examined have this enzyme, its expression is regulated in accordance with its requirement. For example, transketolase activity in human white blood cells is extremely high, and a loss of its activity occurs in several diseases, such as a chronic lymphatic leucemia [38]. The activity is 100-200 times higher than that of red blood cells, suggesting the specific localization and function of pentose phosphate pathway in white blood cells. Another example is that the mammalian cornea contains particularly high levels of TKT, consistent with the proposal that NADPH produced by the pentose phosphate pathway activity has a role in the removal of light-generated radicals [39-42]. Therefore, in ascidian vanadocytes, the specific

expression of TKT suggests an requirement for NADPH to reduce V(V) to V(IV).

As shown schematically in Fig. 5, we have identified several proteins specific to vanadocytes. Two polypeptides, of 58 and 45 kDa, have been identified as G6PDH and 6-PGDH, respectively [24, 25]. A 100 kDa protein has been identified as GP [26]. In addition, this study found that TKT in the pentose phosphate pathway was exclusively expressed in vanadocytes. The localization of these enzymes strongly supports the hypothesis that the pentose phosphate pathway functions in vanadocytes and generates NADPH, which may be used as a reducing agent during the course of vanadium accumulation. On the other hand, smaller molecules of 12.5, 15, and 16 kDa were identified as vanadium-associated proteins (VAPs) [37]. Their characterization is on going. The vanadium ions are accumulated in the vacuoles of vanadocytes, where protons and sulfate ions at extremely high concentration coexist with V(III) ions. We have already identified and cloned several subunits of a vacuolar-type proton ATPase localized in the vanadocytes [43, 44], and a search for other subunits and other membrane proteins is in progress.

The exclusive presence of these peptides in vanadocytes has attracted our attention and must be the key to elucidating the mechanism of the unusual ascidian ability to accumulate vanadium at extremely high levels, which has never been reported in other living organisms, and reduce it to V(III).

The function of the accumulated vanadium ions in the ascidian still remains to be explained. Several researchers proposed that the vanadocytes use vanadium ions for the synthesis of tunic [48-53], while others proposed that the vanadium-containing vanadocytes may trap oxygen [54]. Another hypothesis is

proposed that vanadium ions may be used as antimicrobial agents [55-56]. However, these proposals are not supported by sufficient experimental evidences.

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Figure Legends

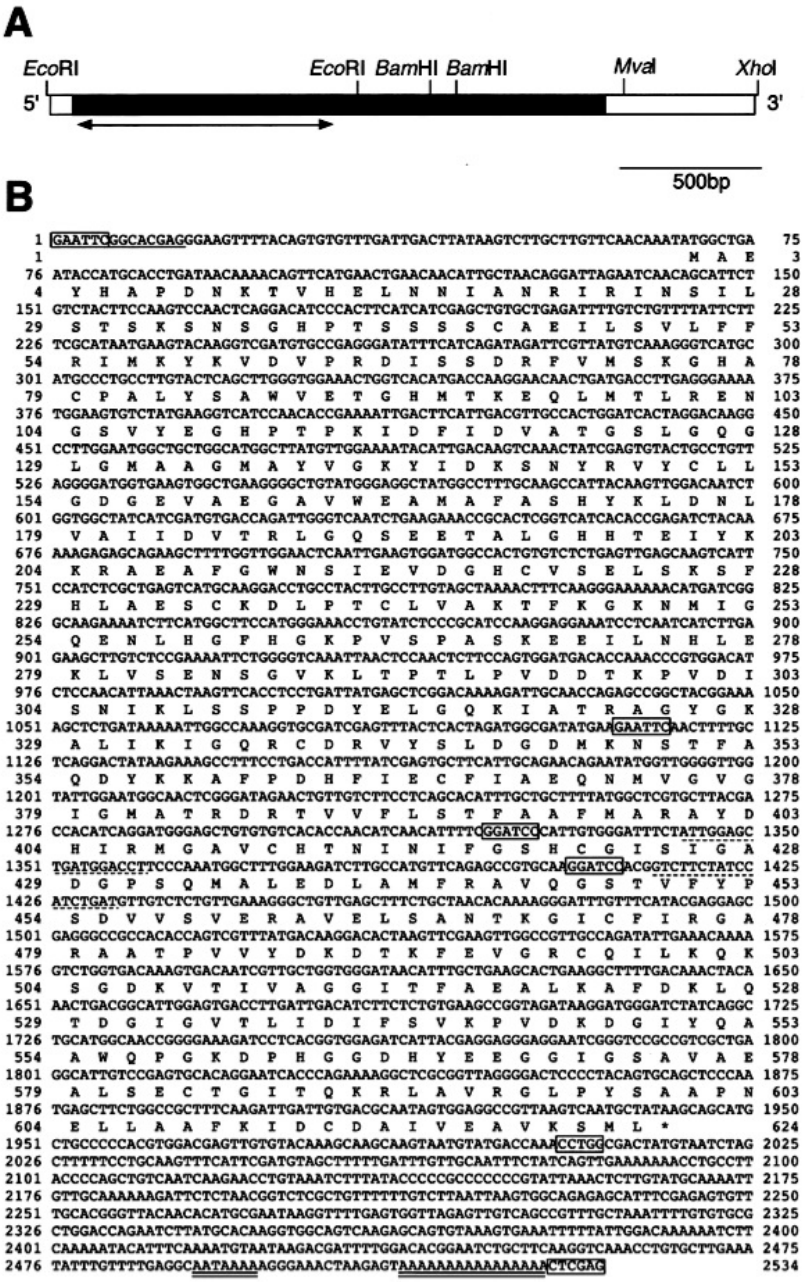

Fig. 1. (A) Restriction map of an ascidian transketolase cDNA clone. Open bars show non-translated regions and the solid bar shows the coding region. The cDNA fragment corresponding to the 27th through 264th amino acid residues of the predicted protein was used for constructing pTKT-MBP plasmid (double arrows). Scale bar, 500 bp. (B) Nucleotide and amino acid sequences of the

cDNA clone. Boxes show the restriction sites used for subcloning. Regions corresponding to the PCR primers are shown by dotted lines. The polyadenylation signal and poly (A) tail are doubly underlined. The *Eco*RI linker is underlined. These sequences are stored in the DDBJ, GenBank, and EMBL nucleotide databases under accession number AB016786.

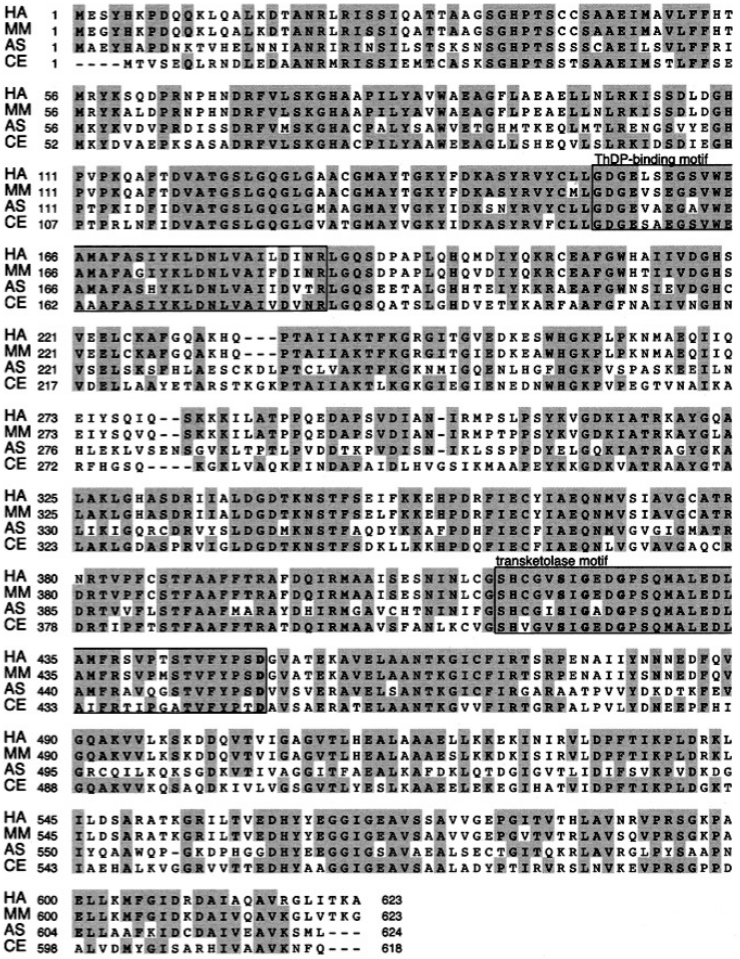

Fig. 2. Alignment of the amino acid sequences of transketolases from various animals. Identical residues are shaded. A thiamine diphosphate (ThDP)-binding motif and a transketolase motif are boxed. Within the latter motif, the NADH-binding-like motif is shown in bold face. Abbreviations: HA, human (*Homo sapiens*), adult-type isoform [45]; MM, mouse (*Mus musculus*) [46]; As,

ascidian (*Ascidia sydneiensis samea*) (this study); CE, nematode (*Caenorhabditis elegans*) [47].

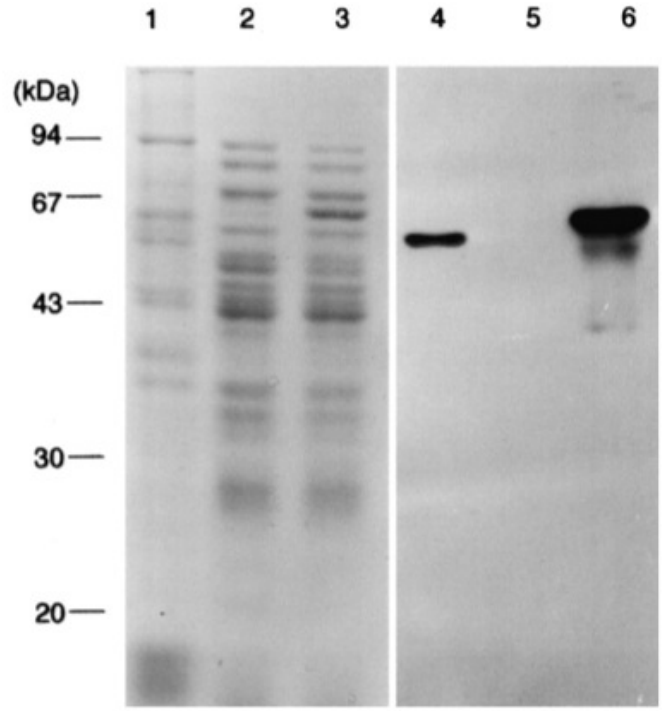

Fig. 3. Expression of transketolase proteins identified by anti-ascidian transketolase antiserum. Lanes 1 and 4, 20 μg of whole cell extracts from blood cells of *Ascidia sydneiensis samea*; lanes 2 and 5, 10 μg of whole cell extracts of *E. coli* TB1 transformed with pTKT-MBP; lanes 3 and 6, 10 μg of whole cell extracts of *E. coli* TB1 transformed with pTKT-MBP after the induction of the expression of fusion proteins. Samples were loaded and separated using 10% SDS-PAGE gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (lanes 1-3), or blotted on a nitrocellulose membrane and reacted with anti-transketolase antiserum (lanes 4-6). Note that 65-kDa transketolase proteins in the blood cells (lane 4) and 67-kDa TKT -MBP fusion proteins (lane 6) are detected by the anti-transketolase antiserum.

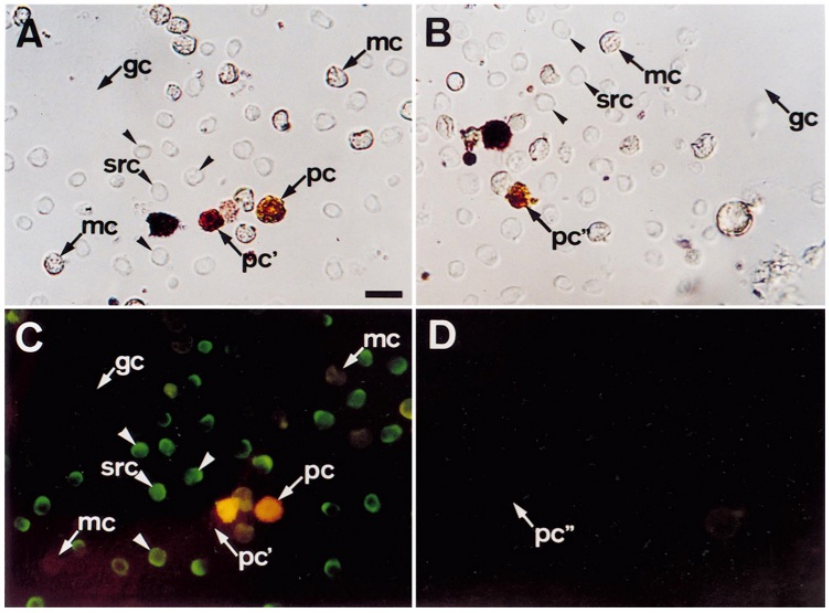

Fig. 4. Immunocytochemistry of blood cells of *A. sydneiensis samea*. The cells were fixed and reacted with (A, C) or without (B, D) the anti-transketolase antiserum. The cells were then reacted with FITC-conjugated secondary antibody and observed with Nomarski microscopy (A, B) or fluorescent microscopy (C, D). Note that staining was detected only in the signet ring cells (SRC, arrowheads), also known as vanadocytes, but not in the other blood cells such as giant cells (GC), morula cells (MC), or pigment cells (PC). Some pigment cells show autonomous fluorescence. Scale bar, 20 μm.

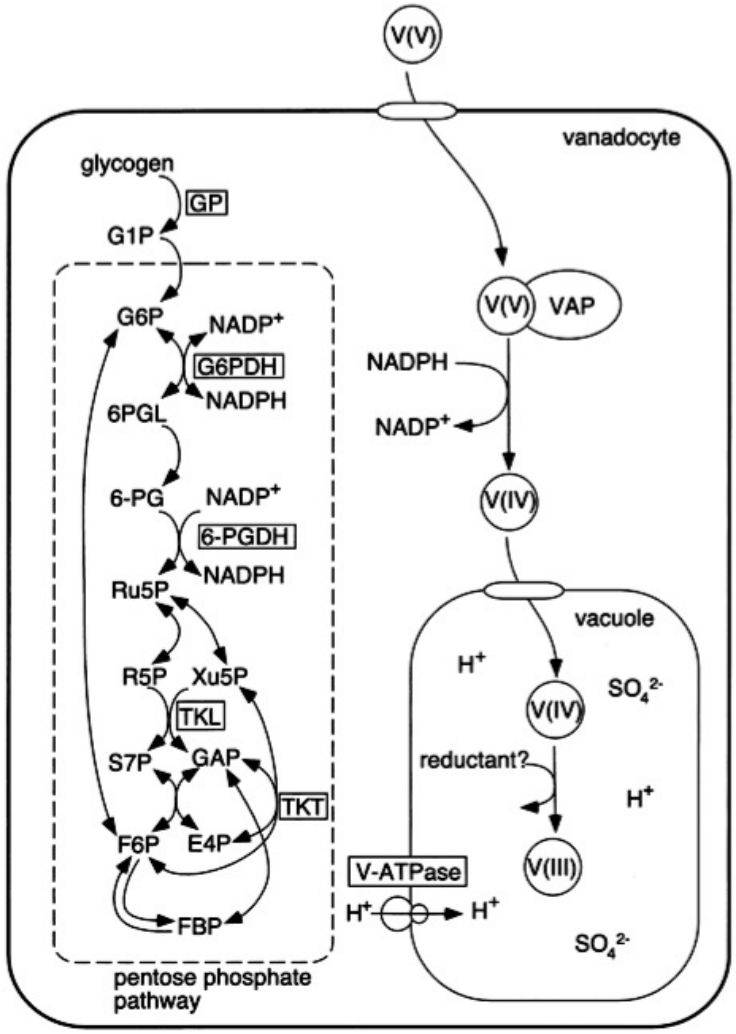

Fig. 5. A model for the pathway for the reduction and accumulation of vanadium in ascidian vanadocytes. The vanadium taken into the vanadocytes is bound by vanadium-associated proteins (VAPs). The pentose phosphate pathway exists in the vanadocytes and functions to generate NADPH, which may reduce V(V) to V(IV). The V(IV) ions are transported into the vacuole and reduced to V(III) by unknown reductant(s). The vacuole has an extremely high levels of H^+ and SO_4^{2-}. Vacuolar proton ATPase (V-ATPase) accumulates protons in the vacuole. Abbreviations: 6-PG, 6-phosphogluconate; 6-PGDH, 6-phosphogluconate dehydrogenase (EC1.1.1.44); 6PGL, 6-phosphogluconolactone; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-

bisphosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase (EC1.1.1.49); GAP, glyceraldehyde 3-phosphate; GP, glycogen phosphorylase (EC2.4.1.1); R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; TKT, transketolase (EC2.2.1.1); Xu5P, xylulose 5-phosphate.