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Glucose-6-Phosphate Dehydrogenase in the Pentose Phosphate Pathway Is Localized in Vanadocytes of the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*

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ABSTRACT—Ascidians are sessile marine animals known to accumulate high levels of vanadium selectively in vanadium-containing blood cells (vanadocytes). Almost all the vanadium accumulated in the vacuoles of vanadocytes is reduced to the +3 oxidation state via the +4 oxidation state, although vanadium is dissolved in the +5 oxidation state in sea water. Some of the reducing agents that participate in the reduction have been proposed. By chemical study, vanadium in the +5 oxidation state was reported to be reduced to the +4 oxidation state in the presence of NADPH. The present study revealed the existence of glucose-6-phosphodehydrogenase (G6PDH), the first enzyme to produce NADPH in the pentose phosphate pathway, in vanadocytes of a vanadium-rich ascidian. The results suggested that G6PDH conjugates the reduction of vanadium from the +5 through to the +4 oxidation state in vanadocytes of ascidians.

INTRODUCTION

High levels of vanadium, a transition metal, were first found by Henze (1911) in the blood cells (coelomic cells) of an ascidian known alternatively as a tunicate or seasquirt. Ever since, this unusual physiological phenomenon, never before reported in other organisms, has attracted the interest of investigators including not only physiologists but analytical, bioinorganic and biological chemists. To date, various studies have been done on this phenomenon as summarized in several review articles (Goodbody, 1974; Biggs and Swinehart, 1976; Kustin *et al*., 1983; Boyd and Kustin, 1985; Michibata, 1989, 1993, 1996; Michibata and Sakurai, 1990; Wever and Kustin, 1990; Smith *et al*., 1995; Kustin and Robinson, 1995; Michibata and Kanamori, 1998). The high levels of vanadium are exclusively contained in a type of blood cell, designated vanadocytes, one of approximately ten types of blood cells in ascidians (Michibata *et al*., 1987, 1991). The highest concentration of vanadium in vanadocytes exceeds 10^7 times the concentration in sea water (Michibata *et al*., 1991). Furthermore, almost all the vanadium accumulated in the vacuoles of vanadocytes is reduced to the +3 oxidation state (V^{III}), the most reduced form in aqueous solution (Lybing, 1953; Boeri and Ehrenberg, 1954; Webb, 1956; Carlson, 1975; Tullius *et al*., 1980; Dingley *et al*., 1981; Frank *et al*., 1986; Lee *et al*., 1988; Brand *et al*., 1989; Hirata and Michibata, 1991), although vanadium is reported to be dissolved in the +5 oxidation state (V^{V}) in sea water (McLeod *et al*., 1975).

Some reducing agents must, therefore, participate in the accumulation process in vanadocytes. Several candidates for the reduction of vanadium in ascidian blood cells have been proposed. Tunichromes isolated from certain ascidian species (Bruening *et al*., 1985), glutathione, H_2S, NADPH, dithiothreitol (Ryan *et al*., 1996), and thiol such as cysteine (Frank *et al*., 1987) have all been examined for their ability to reduce V^{V} to V^{IV} and/or V^{IV} to V^{III}. However, not only has little direct evidence for involvement of these agents in the reduction in vanadocytes been obtained, but also no attention has been paid to whether these agents exist intrinsically in vanadocytes.

There is a good possibility that NADPH participates in the reduction of V^{V}. Nour-Eldeen *et al*. (1985) reported that vanadate activates the catalysis via glucose-6-phosphate dehydrogenase of the oxidation of glucose by $NADP^+$ *in vitro*. Shi and Dalal (1991, 1993) reported formation of V^{IV} in the

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The abbreviations used are: HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris (hydroxymethyl) aminomethane; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized form.

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reduction of V^V by NADPH-dependent flavoenzymes. It is known that 2 mols of NADPH are produced in the pentose phosphate pathway. One is produced by the reaction of glucose-6-phosphate dehydrogenase (G6PDH: EC1.1.1.49) and the other by that of 6-phosphogluconate dehydrogenase (6-PGDH: EC1.1.1.44).

The present experiment was therefore designed to examine whether G6PDH exists in ascidian blood cells, with the final aim being to prove the intrinsic participation of NADPH in the reduction of vanadium in the vanadocytes of ascidians. It was revealed immunocytologically that G6PDH was localized exclusively in vanadocytes and soluble extract of vanadocytes exhibited enzymatic activity of G6PDH.

MATERIALS AND METHODS

Ascidians

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

Immunocytological detection

To examine the localization of G6PDH, immunological detection was carried out in a similar manner to that described previously (Uyama *et al*., 1991, 1994). Ascidian blood, drawn by making an incision through the lower part of the tunic and puncturing the heart at 4°C, was suspended in Ca^{2+}- and Mg^{2+}-free artificial sea water containing, 460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 6 mM $NaHCO_3$ and 5 mM HEPES at pH 7.0 to avoid clotting of the blood cells and centrifuged at 300 × g for 10 min at 4°C to separate the blood cells from the serum. The blood cells were resuspended in Ca^{2+}- and Mg^{2+}- free artificial sea water and were mounted on coverslips. The coverslips were immersed in ethanol containing 5% formalin for 5 min at –15°C. After fixation, the coverslips were washed with phosphate buffered saline (PBS), which consisted of 136.9 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, at pH 7.2, for 30 min at room temperature. Next, they were immersed first in 100 μl of 10% goat normal serum for 1 hr at room temperature to reduce the non-specific background, then in 100 μl of anti-G6PDH antibody raised in rabbit against G6PDH of bakers yeast (Sigma Chemical Co., St. Louis, USA) which had been diluted at a ratio of 1 to 1000 with PBS containing 10% goat normal serum for 1 hr at room temperature. The coverslips were then washed with PBS for 30 min, before being immersed in 100 μl of fluorescein isothiocyanate-conjugated antiserum raised in goat against rabbit IgG (Organon Teknika Corporation, Philadelphia, USA) which was diluted at a ratio of 1 to 2000 with PBS for 1 hr at room temperature. Finally, they were washed with PBS for 1 hr, mounted in 80% glycerol, and observed under a microscope (Olympus Co., Ltd., Tokyo) equipped with an epifluorescence optics unit. Both bright field and fluorescence photographs of the blood cells were taken with Fuji color film (ASA 400). As a negative control, a few cover slips were immersed in preimmune rabbit serum in the same manner. Vanadocytes were identified not only by morphological appearance but by the immunoreactivity with S4D5 monoclonal antibody, specific to vanadocytes (Michibata *et al*., 1987, 1990; Uyama *et al*., 1991).

Western blot analysis

G6PDH is known to be a soluble protein composed of a dimer of identical subunit with M_r of 50-60 kDa (Takizawa *et al*., 1986; Camardella *et al*., 1988; Jeffery *et al*., 1989; Persson *et al*., 1991). Therefore, to examine whether the enzyme exists in a soluble protein fraction extracted from ascidian blood cells, Western blot analysis was applied using the antiserum against G6PDH. 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 at 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 centrifuged at 100,000 × g for 1 hr. The supernatant was collected and used as soluble protein for Western blot analysis and enzymatic assay of G6PDH. An aliquot of approx. 30 μg of the soluble protein was dissolved in a sample dissociation buffered solution consisting of 62.5 mM Tris-HCl at pH 6.8, 5%(v/v) 2-mercaptoethanol, 10%(v/v) glycerol and 2.3% (w/v) SDS. As the positive control, a purified G6PDH of the bakers yeast (Sigma) was purchased and an aliquot of 100 ng of the protein was used in the same manner. The protein content in each sample was determined by the Bradford (1976) method using a Bio-Rad Protein Assay kit (Nippon Bio-Rad Laboratories, Inc., Tokyo, Japan) and bovine serum albumin as a standard. Each sample was subjected to electrophoresis in a 10% polyacrylamide gel in the presence of 2% SDS. The proteins separated on SDS-PAGE electrophoretically were blotted onto a nitrocellulose paper for Western blot analysis as described previously (Uyama *et al*., 1997; Kanda *et al*., 1997).

Enzymatic assay

G6PDH activity in the 100,000 × g supernatant obtained from ascidian blood cells was assayed, since it was confirmed that an antigen recognized by the anti-G6PDH existed in the supernatant. Two kinds of reaction mixtures were prepared. One consisted of glucose 6-phosphate and 6-phosphogluconate ranging in concentrations from 10 μM to 200 μM (Sigma) as substrates, 0.4 mM $NADP^+$ (Oriental Yeast Co., LTD), and 5 mM $MgCl_2$ in 0.2 M Tris-HCl buffer solution at pH 8.0. The other did not contain glucose 6-phosphate. The reaction was initiated by the addition of 50 μl of enzyme solution (containing approx. 50 μg protein). Thus the final volume of the reaction mixture was 2.5 ml. Reduction of $NADP^+$ to NADPH as a result of the enzymatic reaction was recorded as the increase of absorbance at 340 nm. G6PDH activity was calculated by subtracting 6-PGDH activity from G6PDH plus 6-PGDH activities. Protein concentration was determined as described above to calculate the specific activity.

RESULTS

Immunocytological detection of G6PDH

As shown in Fig. 1, immunoreactivity of anti-G6PDH antibody was detected only in signet ring cells which had been identified as vanadocytes (Michibata *et al*., 1987, 1990). Although *A. sydneiensis samea* has at least six different types of blood cells (Michibata *et al*., 1990; Kaneko *et al*., 1995; Wuchiyama and Michibata, 1995), no immunoreactivity was observed in blood cells other than vanadocytes.

Western blot analysis of G6PDH

Western blot analysis with anti-G6PDH antibody revealed a positive band of 58 kDa in soluble proteins, as shown in Fig. 2. The corresponding molecular mass of 58 kDa is in good agreement with that previously reported in other living organisms (Takizawa *et al*., 1986; Camardella *et al*., 1988; Jeffery *et al*., 1989; Persson *et al*., 1991). This result demonstrates that G6PDH exists in the soluble protein fraction of the ascidian blood cells.

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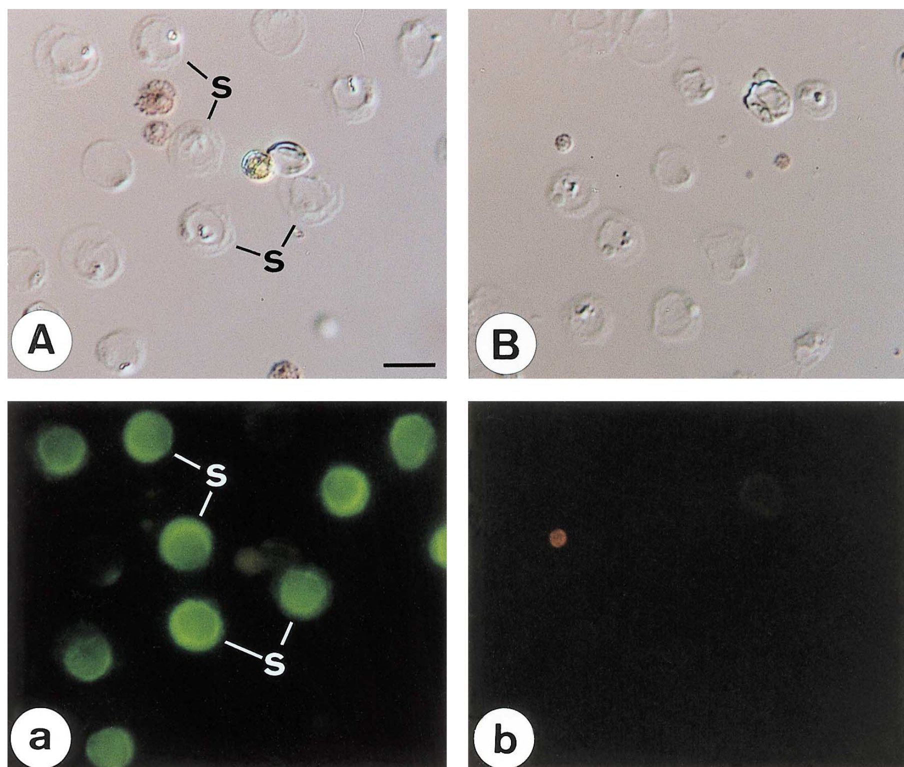

Fig. 1. Immunocytological detection of G6PDH in the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Blood cells observed in panels **A** and **a** were reacted with anti-G6PDH antibody. Blood cells in panels **B** and **b** were reacted with preimmune rabbit serum as a negative control. Upper panels (**A** and **B**) and lower panels (**a** and **b**) were visualized by a Nomarski differential-interference and by fluorescence microscopy, respectively. Vanadocytes (signet ring cells) were exclusively recognized with anti-G6PDH antibody, showing fluorescence of FITC. No immunoreactivity was observed in the other types of blood cells. s, vanadocytes (signet ring cells). Scale bar indicates 10 μm.

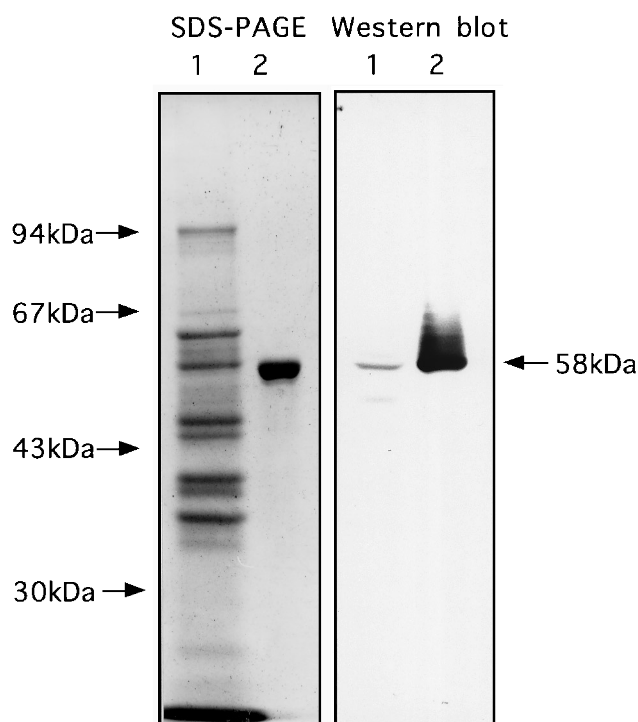

Fig. 2. Localization of a 58-kDa antigen in ascidian soluble fraction revealed by Western blot analysis using anti-G6PDH antibody. Blood cells of *Ascidia sydneiensis samea* were homogenized and proteins in the soluble fraction were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue (left). The separated proteins were blotted onto a nitrocellulose paper and reacted with anti-G6PDH antibody to examine whether G6PDH is present in the ascidian blood cells (right). A positive band of 58 kDa was detected in the soluble fraction, as well as in yeast G6PDH. This revealed that G6PDH exists in the soluble protein fraction of the ascidian blood cells. Lane 1, the soluble protein (30 μg) extracted ascidian blood cells; Lane 2, a purified G6PDH of the bakers yeast (100 ng protein).

Enzymatic assay of G6PDH

To examine whether the enzymatic activity of G6PDH is actually present in soluble extract of the blood cells, the soluble extract was assayed using glucose-6-phosphate as a substrate. A correspondingly high level of enzymatic activity of G6PDH was detected. The Lineweaver-Burk plot shows clearly that the enzymatic activity is dependent on the concentration of substrate (Fig. 3). Km for the substrate and Vmax were 99.2 μmol/l and 196 nmol/min, respectively, at pH 7.4.

DISCUSSION

The present experiments have revealed that G6PDH, the first enzyme in the pentose phosphate pathway producing 6-

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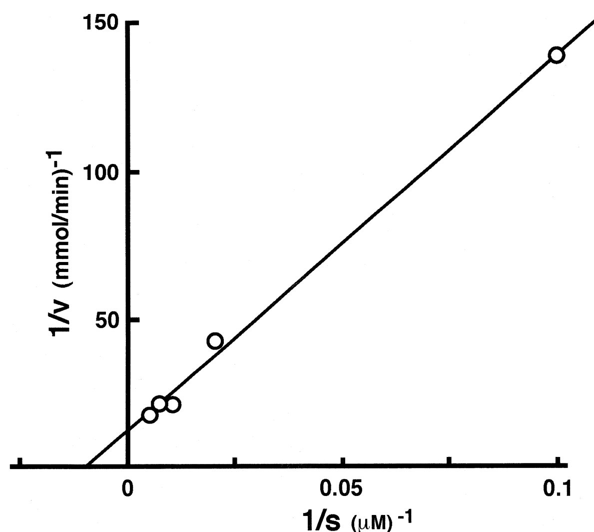

Fig. 3. Enzymatic activity of G6PDH in the ascidian blood cells. To examine whether G6PDH functions enzymatically in the soluble protein extracted from ascidian blood cells containing vanadocytes, the activity was assayed using glucose-6-phosphate (G6P). The Lineweaver-Burk plot shows clearly that the enzymatic activity is dependent on the concentration of substrate. *K*m for the substrate and *V*max are 99.2 μmol/l and 196 nmol/min, respectively.

phosphoglucono-δ-lactone and reducing $NADP^+$ to NADPH, is present in vanadocytes, vanadium-containing blood cells, of the vanadium-rich ascidian *Ascidia sydneiensis samea.* Since immunoreactivity of anti-G6PDH antibody was observed in vanadocytes but not in other types of blood cells (Fig. 1), it is clear that G6PDH is localized in vanadocytes. G6PDH was further found to be localized in the cytoplasm and not in the vacuoles of vanadocytes on close observation. By Western blot analysis anti-G6PDH antibody was revealed to have reactivity with a 58 kDa protein in the soluble fraction obtained by centrifugation at 100,000 × g (Fig. 2). Furthermore, a correspondingly high level of enzymatic activity of G6PDH was found in the soluble fraction of the blood cells (Fig. 3).

Vanadocytes, having high levels of vanadium, sulfate ions and protons in their vacuoles (Michibata *et al.*, 1991; Kanamori and Michibata, 1994; Uyama *et al.*, 1994), are not found in other living organisms. Under these conditions, vanadium is kept in the V^{III} form, the most reduced form in aqueous solution (Hirata and Michibata, 1991). Some reducing and/or chelating agents must participate in the reduction and protection against air-oxidation in vanadocytes. In fact, several candidates have been proposed, such as haemovanadin (Califano and Boeri, 1950; Webb, 1956) and tunichromes (Bruening *et al.*, 1985). However, the involvement of these compounds in reduction of vanadium is unclear (Kime-Hunt *et al.*, 1988; Michibata *et al.*, 1988, 1990; Bulls *et al.*, 1990; Tsuchida *et al.*, 1994; Ryan *et al.*, 1996).

Recently, we have revealed that 6-phosphogluconate dehydrogenase (6-PGDH), the third enzyme of the pentose phosphate pathway, was localized in vanadocytes of the ascidian using immunological methods, that the full-length cDNA encoding 6-PGDH was isolated and that soluble extract of the blood cells further exhibited a correspondingly high level of 6-PGDH enzymatic activity (Uyama *et al.*, 1998). 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 has been reported that V^V stimulates oxidation of NAD(P)H; namely, V^V is reduced to V^{IV} in the presence of NAD(P)H *in vitro.* Erdmann *et al.* (1979) first noted that V^V stimulated the oxidation of NADH by plasma membranes and attributed this effect to a membrane-containing NAD(P)H-dependent V^V reductase. Liochev and Fridovich (1990) proposed that NAD(P)H dehydrogenases or oxidases produce O_2^-, which causes V^V to stimulate NAD(P)H oxidation and endogenous superoxide plays a central role in this reaction. Shi and Dalal (1991, 1993) demonstrated that O_2^- radicals are not significantly involved in the V^{IV} generation but they pointed out that V^{IV} is generated in the microsomal reduction of V^V in the presence of NAD(P)H and the V^{IV} formation exhibits typical enzymatic kinetics. In fact, our preliminary data showed that NADPH can reduce V^V to V^{IV} *in vitro* (to be published elsewhere). These observations suggest that NADPH conjugates the reduction of V^V to V^{IV} in the vanadocytes of ascidians, although there is controversy as to the mechanism involved. While almost all vanadium ions stored in the vacuoles of vanadocytes are further reduced to V^{III}, no reducing agents that can reduce V^V or V^{IV} to V^{III} have been extracted from ascidian blood cells to date.

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