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Relation	

Metal Ion Selectivity of the Vanadium(V)-Reductase Vanabin2

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In a previous study, Vanabin2, a member of a family of V(IV)-binding proteins, or Vanabins, was shown to act as a V(V)-reductase. The current study assesses the ability of Vanabin2 to reduce various transition metal ions *in vitro*. An NADPH-coupled oxidation assay yielded no evidence of reduction activity with the hexavalent transition metal anions, $Mo^{VI}O_4^{2-}$ and $W^{VI}O_4^{2-}$, or with three divalent cations, Mn(II), Ni(II), and Co(II). Although Cu(II) is readily reduced by glutathione and is gradually oxidized in air, this process was not affected by the presence of Vanabin2. In the experiments conducted thus far, Vanabin2 acts only as a V(V)-reductase. This high selectivity may account for the metal ion selectivity of vanadium accumulation in ascidians.

1. Introduction

The unusual ability of ascidians (sea squirts) to accumulate high levels of vanadium ions has been attracting attention in biological and chemical disciplines for a century. The maximum concentration of vanadium can reach 350 mM in vanadocytes of *Ascidia gemmata*, belonging to the class Ascidiidae, and is thought to be the highest metal accumulation factor of any living organism [1]. Vanadium usually exists as V^V in HVO_4^{2-} or $H_2VO_4^-$ in natural aquatic environments. These ions are reduced to V^{III} via a V^{IV} state (VO^{2+}) during assimilation [2,1]. Usually, vanadium ions are stored in the vacuoles of signet ring cells, which are a type of blood cell often referred to as vanadocytes (vanadium-accumulating cells) [3]. One of the biggest unknowns is how and why ascidians accumulate only vanadium at these extremely high levels. Of particular interest is how ascidians select vanadium out of the many transition metal ions.

Ongoing research during the last two decades has identified many proteins involved in the process of accumulating and reducing vanadium in vanadocytes, blood plasma, and the digestive tract of ascidians. These proteins include vacuolar-type H^+-ATPase [4], a chloride channel [5], a sulfate transporter [6], enzymes of the pentose-phosphate pathway [7-10], a vanadium transporter Nramp [11], and V^{IV}-binding proteins Vanabins [12-15].

Among these, the proteins that could be responsible for the selective transport of vanadium are the Vanabins and Nramp. Vanabins commonly possess 18 conserved cysteine residues and constitute a unique protein family present only in vanadium-rich ascidians. The most studied Vanabin is Vanabin2, which has been isolated from *Ascidia sydneiensis samea* [15]. The three-dimensional structure of Vanbin2 was determined by NMR, which revealed 18 cysteines that form nine disulfide (SS) bonds between specific amino acid residues [16]. Nramp is expressed on the vacuolar membrane of vanadocytes and acts as a VO^{2+}/H^+-antiporter [11]. Vanadium transport by Nramp is inhibited by excess amounts of Fe^{II}, Mn^{II}, Cu^{II}, or Zn^{II}. Another possible mechanism of metal ion selectivity would be a transporter expressed on the cytoplasmic membrane of vanadocytes. Several ideas related to such a transporter have been post[17] but as of yet there have been no confirmative results.

Although Vanabin2 was originally isolated as a V^{IV}-binding protein, it was found to adopt an SS/SH intermediate structure and can act as a V^V-reductase [18]. Vanabin2 can also bind Fe^{III}, Cu^{II}, Co^{II}, Mn^{II}, and Zn^{II} [19,20] although it has not been determined whether Vanabin2 can reduce any metal ions other than vanadium. Therefore, the current report assesses the hypothesis that Vanabins determine the specificity of vanadium accumulation and reduction in ascidians. Specifically, the ability of Vanabin2 to reduce alternate transition metal ions, including Mn^{II}, Co^{II}, Ni^{II}, Cu^{II}, $Mo^{VI}O_4^{2-}$, and $W^{VI}O_4^{2-}$ was evaluated.

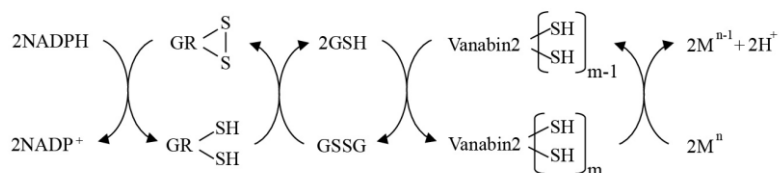## 2. Materials and methods

2.1. Reagents

Fig. 1. Reduction of metal ions by Vanabin2 coupled with the oxidation of NADPH.

Dithiothreitol (DTT), 2-mercaptoethanol (2-ME), and reduced glutathione (GSH) were purchased from Wako Pure Chemical Industries. Sodium orthovanadate (Na_3VO_4; >99.9%) was purchased from Sigma-Aldrich Co. Other metal reagents were all ultrapure reagents ($MnCl_2 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and $Na_2WO_4 \cdot 2H_2O$; >95%) purchased from Wako. NADPH and glutathione reductase (GR) were obtained from the Oriental Yeast Company. Bathocuproine disulfonic acid disodium salt (BCS) was obtained from Nacalai Tesque, Inc.

2.2 Preparation of recombinant protein

Recombinant Vanabin2 protein was prepared in accordance with procedures published previously [18]. Briefly, the pMAL-c plasmid containing the Vanabin2 coding region [15] was introduced into *Escherichia coli* strain TB1. The transformed cells were incubated in Luria–Bertani (LB) medium containing 50 μg/mL ampicillin and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested, resuspended in a lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 10 mM EDTA, 10 mM EGTA, 0.25% Tween 20, 10 mM 2-ME, pH 7.0) containing 4 M urea and then sonicated using a UH-150 ultrasonic homogenizer (SMT Company). The fusion protein was purified from the soluble cellular fraction by affinity chromatography using amylose resin in accordance with the manufacturer's protocol (New England Biolabs). The protein was cleaved at the MBP junction by incubation with Factor Xa and the released Vanabin2 was purified through an anion-exchange column filled with DEAE-Sephacel resin (GE Healthcare). The eluted protein was dialyzed four times in 100 volumes of 50 mM Tris–HCl (pH 7.4) for use in vanadium reductase activity assays. Prior to use, the purity of the Vanabin2 was confirmed by SDS-PAGE.

2.3. NADPH-coupled oxidation assays

Metal reductase activity was measured using an NADPH-coupled oxidation assay as described previously [18]. The assay buffer contained 50 mM Tris-HCl (pH 7.4), 200 μM NADPH, 0.25 U/mL glutathione reductase (GR), and 2 mM GSH. In assays using cobalt, the buffer was replaced with citrate/sodium citrate buffer (2:3 mixture of 100 mM stock; pH 4.7 adjusted by NaOH) as described in the Results and Discussion section. Vanabin2 was added to a final concentration of 2 or 4 μM in each tube, except for the negative control tube, and the solutions were pre-heated at 18°C for at least 15 min. Metal ion solutions were prepared by dissolving ultra pure reagent in ultra pure water at 10 mM. For Na_3VO_4, the solution was heated at 65°C until it became colorless. Metal solutions were added to a final concentration of 1 mM. NADPH oxidation was monitored at 340 nm for 60 min at 18°C using a U-2900 spectrophotometer equipped with a thermoelectric cell holder (Hitachi Co. Ltd.).

2.4. Measurement of Cu^I using BCS

Concentrations of Cu^I was determined using bathocuproine (BCS) [21]. Vanabin2 was mixed with NADPH, GR, and GSH in Tris buffer in each tube, except for the negative control tube, and pre-heated at 18°C for at least 15 min. $Cu^{II}Cl_2$ solution was added to a final concentration of 2 mM and incubated at 18°C. The assay buffer contained 50 mM Tris-HCl (pH 7.4), 200 μM NADPH, 0.25 U/mL glutathione reductase (GR), 2 mM GSH, 2
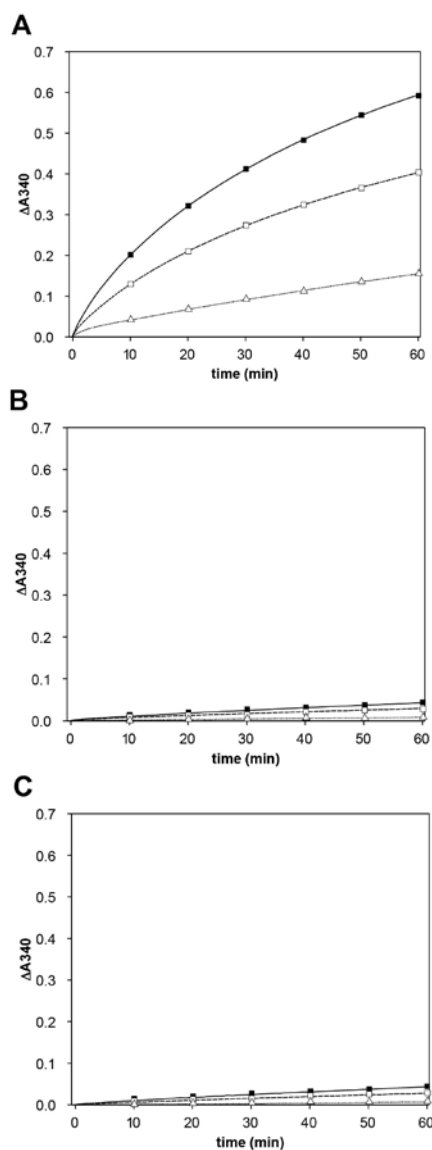

Fig 2. NADPH-coupled assays for the (A) reduction of V^V, (B) reduction of Mo^{VI}, and (C) reduction of W^{VI}. Solid line with solid rectangles correspond to the reaction with 4 μM Vanabin2. Dashed line with open rectangles corresponds to the reaction with 2 μM Vanabin2. Dotted line with open triangles shows data acquired without Vanabin2. The horizontal axis indicates the time after initiation of the reaction. The vertical axis indicates the decrease in absorbance at 340 nm due to NADPH oxidation.

μM Vanabin2 and 2 mM $Cu^{II}Cl_2$ in a 1-mL volume. At specific time intervals, aliquots (125 μL) were removed and mixed with 40–μL BCS (5 mg/ml), 100 μl EDTA (1.2 mg/mL), 400–μL McIlvaine's buffer (a mixture of 0.2 M Na_2HPO_4 and 0.1 M citric acid, pH 7.2) and 960-μL water. The solution absorbance due to the formation of Cu^I-BCS was measured at 480 nm within 15 min using the U-2900 spectrophotometer.
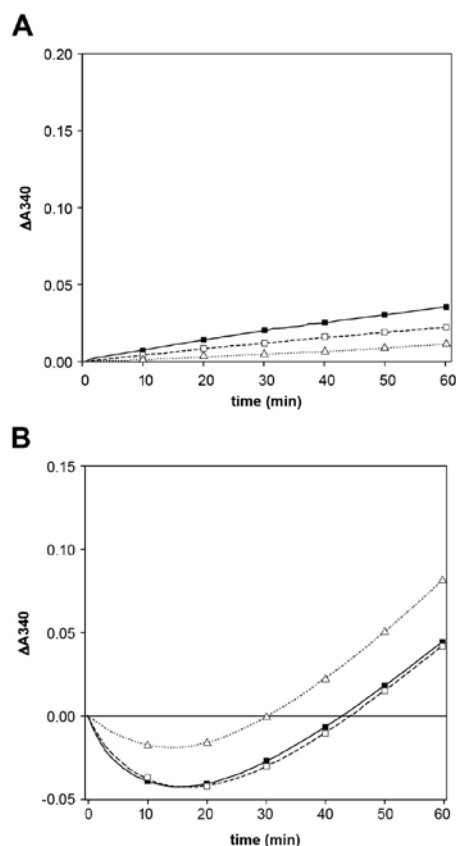
Fig 3. NADPH-coupled assays for the (A) reduction of Mn^{2+} and (B) reduction of Ni^{II}. Solid line with solid rectangles corresponds to the reaction with 4 µM Vanabin2. Dashed line with open rectangles corresponds to the reaction with 2 µM Vanabin2. Dotted line with open triangles shows data acquired without Vanabin2. The horizontal axis indicates the time after initiation of the reaction. The vertical axis indicates the decrease in absorbance at 340 nm due to NADPH oxidation.

3. Results and discussion

3.1. Reduction of penta- or hexavalent transition metal anions by Vanabin2 coupled with NADPH oxidation

As reported previously, Vanabin2 was shown to reduce pentavalent vanadium ($V^{V}O_4^{3-}$) to a tetravalent form ($V^{IV}O^{2+}$) when coupled with NADPH, glutathione reductase (GR), and glutathione (GSH/GSSG) [18]. We assessed whether Vanabin2 can reduce other transition metal ions according to the reaction cascade shown in Fig. 1.

First, the reproducibility of the previously reported vanadium ($V^{V}O_4^{3-}$)-reductase activity was verified using an identical method [18]. The indicated components were mixed and incubated for 60 min, and changes in solution absorbance at 340 nm due to NADPH oxidation were recorded for 60 min (Fig. 2A). When 2 or 4 µM Vanabin2 was added, NADPH oxidation was significantly enhanced in a dose-dependent manner. Thus, the V^{V}-reductase activity of Vanabin2 was confirmed (Fig. 2A).

When molybdate ($Mo^{VI}O_4^{2-}$) ions were included as the final electron acceptor, there was no significant NADPH oxidation
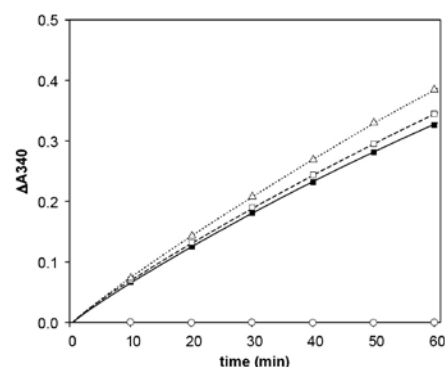

Fig.4. NADPH-coupled assays of the reduction of Co^{II}. The dashed line with open rectangles shows data acquired with 200 µM NADPH, 0.25 U/mL glutathione reductase (GR), 2 mM GSH, 1 mM Co^{II} ion, and 2 µM Vanabin2. The solid line with solid rectangles shows data acquired without Vanabin2. The dashed-dotted line corresponds with open triangles to 200 µM NADPH only. The dotted line with open circles shows data acquired with 2 mM GSH and 1 mM Co^{II} only. Note that the dotted line with open circles is overlaid on the horizontal axis and is difficult to discern. The horizontal axis indicates the time after initiation of the reaction. The vertical axis indicates the decrease in absorbance at 340 nm due to NADPH oxidation.

with or without Vanabin2 (Fig. 2B). The same results were obtained when tungstate ($W^{VI}O_4^{2-}$) ions were used (Fig. 2C). These results suggest that Vanabin2 does not act as a Mo- or W-reductase.

3.2. Reduction of divalent transition metal cations by Vanabin2 coupled with NADPH oxidation

The ability of Vanabin2 to reduce divalent transition metal ions was also evaluated. When manganese (Mn^{II}) ions were included as the final electron acceptor, there was no significant NADPH oxidation with or without Vanabin2 (Fig. 3A).

With nickel (Ni^{II}) ions and Vanabin2, the solution absorbance at 340 nm gradually increased and then began to decrease after approximately 15 min (Fig. 3B). The same trend was observed in the absence of Vanabin2, but the absolute values of absorbance were always slightly higher than those obtained with Vanabin2.

Since cobalt (Co^{II}) ions form a conjugate complex with Tris and GSH with significant absorbance at 340 nm, a citrate/sodium citrate buffer at pH 4.7 was used to examine the reduction of Co^{II}. At pH 4.7, Vanabin2 can still form the required SS/SH intermediate [20]. Auto-oxidation of NADPH was observed in this buffer, resulting in a gradual increase in the absorbance at 340 nm (Fig. 4). When GSH and Co^{II} were mixed and incubated in the citrate/sodium citrate buffer, no significant change in absorption was observed. When Co^{II} ions were included as the final electron acceptor, the absorbance at 340 nm increased gradually at a rate not significantly different from that observed with NADPH auto-oxidation.

Thus, no evidence of reduction of these three divalent metal cations by Vanabin2 was observed.

3.3. Reduction of copper (Cu^{II}) ions by Vanabin2

Copper (Cu^{II}) ion is readily reduced by glutathione (GSH) to Cu^{I} [21] and forms a Cu^{I}-GS^{-} complex [22]. This reaction is extremely rapid, taking less than 200 ms[23]. Therefore, in the coupled assay system used here, it was impossible to observe the reduction of Cu^{II} to Cu^{I} by Vanabin2. However, a decrease in solution absorbance at 340 nm was observed when Cu^{II} and GSH were combined and incubated over a period of 1-6 h (data not shown). This indicates the reverse reaction due to the re-oxidation of Cu^{I} to Cu^{II} by dissolved oxygen in the assay buffer. This re-oxidation was exploited in an assay system to examine the Cu-reductase activity of Vanabin2.

In the Cu-reductase assay, bathocuproine (BCS) was used to measure the concentration of Cu^{I} directly. BCS is known to form a complex with Cu^{I}, but not with Cu^{II}. The complex with Cu^{I} exhibits an absorbance peak at 480 nm [21]. GSH and Cu^{II} (2 mM each) were mixed together with NADPH and GR and the Cu^{I} concentration was measured every 15 min. The initial solution absorbance at 480 nm was about 0.6 (Fig. 5, 0 h). Every hour up to 4 h, aliquots were removed from the reaction mixture, mixed with BCS assay reagents, and the absorbance at 480 nm was recorded (Fig. 5, 1–4 h). The absorbance decreased around 2–3 h but no differences associated with the presence or absence of Vanabin2 could be discerned at any time point. Thus, no evidence of the Cu-reductase activity of Vanabin2 was obtained.

3.4. Reduction potentials

When standard apparent reduction potentials of each transition metal ions were considered, V^{V} could not be reduced to V^{IV} (E^{0} = -0.341 V) by thiol compounds whose reduction potentials (E^{0} ~ -0.26 V) were higher than that of V^{V}/V^{IV} [24]. The values for Mn^{II}/Mn^{0} (E^{0} = -1.18 V), Ni^{II}/Ni^{0} (E^{0} = -0.257 V) and Co^{II}/Co^{0} (E^{0} = -0.277 V) are similar or lower than those of thiols. Reduction of Mo^{VI}/Mo^{IV} or W^{VI}/W^{IV} could occur by thiols, but the behavior of molybdate and tungstate are complicated depending on pH. When Vanabin2 was included as the cascade shown in Fig. 1 at slightly basic physiological pH of 7.4, only reduction of V^{V} to V^{IV} was significantly enhanced in a dose-dependent manner, suggesting that a specific interaction of Vanabin2 and V^{V}/V^{IV} occurred, although its nature is unclear. In case of copper, reduction potential of Cu^{II}/Cu^{I} is higher that that of thiols, and thus copper (Cu^{II}) ion is readily reduced by GSH. As far as examined in this study, we could not observe any specific interaction of Vanabin2 and Cu^{II}/Cu^{I} as assessed by the prevention of reoxidation of Cu^{I}.

3.5. Enzyme kinetics

The V_{app} and K_{app} values for Vanabin2 catalyzed V^{V} reduction are 1.15 mol-NADPH/min/mol-Vanabin2 and 0.51 mM, respectively [18]. V_{app} for other metal ions examined in this work were about 12 to 18 times lower than that for V^{V}. One electron reduction of V^{V} has been reported for several flavoenzymes [25, 26]. Since kinetic parameters were not reported, it is difficult to compare the enzyme activity among Vanabin2 and these flavoenzymes. Further analysis may need to clarify the physiological significance of the kinetics of reduction for V^{V}.

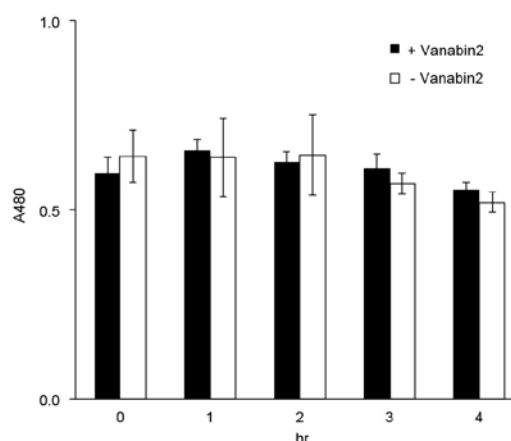## 4. Conclusions

The vanadium-binding protein, Vanabin2, isolated from the

Fig. 5. BCS assay of Cu^{II} reduction by Vanabin2 in a NADPH-coupled cascade. Solid and open bars show data acquired with and without Vanabin2, respectively. The vertical axis shows absorbance at 480 nm as means ± one standard deviation (n = 3).

vanadium-rich ascidian *Ascidia sydneiensis samea*, was evaluated for reductase activity using two hexavalent transition metal anions, Mo^{VI}O$_4^{2-}$ and W^{VI}O$_4^{2-}$, and four divalent cations, Mn^{II}, Ni^{II}, Co^{II}, and Cu^{II}. In NADPH-coupled assays, no evidence of the reduction of these six metal ions by Vanabin2 was obtained. Thus far Vanabin2 has shown only V^{V}-reductase activity. This specificity may account for the specificity of vanadium accumulation in ascidians.

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