# A novel vanadium reductase, Vanabin2, forms a possible cascade involved in electron transfer

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**Summary**

The unusual ascidian ability to accumulate high levels of vanadium ions at concentrations of up to 350 mM, a $10^7$-fold increase over that found in seawater, has been attracting interdisciplinary attention for a century. Accumulated $V^V$ is finally reduced to $V^{III}$ via $V^{IV}$ in ascidian vanadocytes. Reducing agents must therefore participate in the reduction. Previously, we identified a vanadium-binding protein, Vanabin2, in which all 18 cysteines form nine disulfide bonds. Here, we report that Vanabin2 is a novel vanadium reductase because partial cleavage of its disulfide bonds results in the reduction of $V^V$ to $V^{IV}$. We propose that Vanabin2 forms a possible electron transfer cascade from the electron donor, NADPH, via glutathione reductase, glutathione, and Vanabin2 to the acceptor, and vanadium ions conjugated through thiol-disulfide exchange reactions.

## 1. Introduction

About one hundred years ago, high levels of vanadium, a transition metal, were discovered in the blood (coelomic) cells of an ascidian collected in the Bay of Naples [1]. This discovery attracted the attention of an interdisciplinary group of chemists, physiologists, and biochemists, as such extraordinarily high levels of vanadium had not been observed in other organisms [2]. The ascidians, which belong to the suborder Phlebobranchia, appear to contain high levels of vanadium [3]. Among them, *Ascidia gemmata* has the ability to accumulate extremely high levels of vanadium in the blood cells, up to 350 mM [4], a $10^7$-fold increase over its concentration in seawater [5].

Previously, we identified a family of vanadium-binding proteins, designated as Vanabins, from the vanadium-rich ascidian, *A. sydneiensis samea* [6]. The Vanabin family consists of at least five closely related small proteins, Vanabin1 through Vanabin4 and VanabinP, which are composed of about 90 amino acids including 18 cysteine residues. Recombinant Vanabin1, Vanabin2, and VanabinP, were found to bind up to 20 vanadium ions in the +4 oxidation state ($V^{IV}$) with dissociation constants of ca. $2 \times 10^{-5}$ M [2, 6]. An electron paramagnetic resonance (EPR) study not only supported the binding number but also indicated that most of the $V^{IV}$ ions were in a mononuclear state, coordinated to amine nitrogens [7]. Electrospray ionization (ESI) mass spectrometry indicated that the deconvoluted molecular mass of Vanabin2 is 10,467 Da, which is 18 mass units less than the predicted molecular mass. The complete reduction of Vanabin2 by 100 mM dithio-1,4-threitol (DTT) at 50°C for 45 min caused an increase in the molecular weight by 18 mass units, indicating that all 18 cysteine residues of Vanabin2 form nine intramolecular disulfide bonds. In fact, the three-dimensional structure of Vanabin2 determined by nuclear magnetic resonance (NMR) spectrometry indicated a single chain of four α-helices is folded in half and connected by nine disulfide bonds (Fig. 1) [8].

Under physiological conditions, vanadium ions are limited to the +3, +4, and +5 oxidation states [9]. When vanadate ions ($V^{V}$) in seawater are accumulated in the ascidians, they are first reduced to $V^{IV}$ in vanadocytes and then stored in the vacuoles where $V^{IV}$ is finally reduced to the +3 oxidation state ($V^{III}$), which appears to exist as complex cation such as $[V(H_2O)_6]^{3+}$ and $[V(H_2O)_5(HSO_4)]^{2+}$ [10,11]. Therefore, reducing agents must participate in the reduction of vanadium in vanadocytes.

In this study, we found that Vanabin2 can reduce $V^{V}$ to $V^{IV}$ in the presence of reduced glutathione (GSH). We propose a possible cascade involving Vanabin2 that may be involved in redox and electron transfer from the electron donor NADPH to the acceptor vanadium ions, conjugated through thiol-disulfide exchange reactions.

## 2. Materials and Methods

### *2.1 Reagents*

DTT, 2-mercaptoethanol (2-ME), GSH, $Na_3VO_4$, and $VOSO_4$ were purchased from Wako Pure Chemical Industries. NADPH and glutathione reductase (GR) were obtained from the Oriental Yeast Company.

### *2.2 Cloning, expression, and purification of Vanabin2*

To prepare a fusion protein of maltose binding protein (MBP) and Vanabin2, the pMAL-c plasmid containing the Vanabin2 coding region was introduced into *Escherichia coli* strain TB1. Cells harboring the fusion protein expression plasmid were incubated at 37°C for 16 h in Luria–Bertani (LB) medium containing 50 μg/ml ampicillin. For expression of the fusion protein, the cultures were diluted with nine volumes of fresh LB medium, and the diluted cultures containing 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) were incubated at 37°C for 6 h. The cells were harvested, resuspended in 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 insoluble fraction was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. 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 at 4°C for 16 h, and the released Vanabin2 was purified by anion-exchange column chromatography using DEAE-Sephagel. The eluted protein was dialyzed four times in 100 volumes of 20 mM phosphate buffer (pH 7.5) for SDS-PAGE analysis and circular dichroism (CD) measurements, or into 50 mM Tris–HCl (pH 7.4) for EPR measurement and vanadium reductase activity assay. Prior to use, the purity of the Vanabin2 was confirmed by SDS-PAGE.

*2.3 Mobility shift assay by SDS-PAGE*

DTT, 2-ME, or GSH was added at concentrations ranging from 0 to 100 mM to a purified Vanabin2 solution just before adding sample buffer containing 62.5 mM Tris–HCl, 2.3% SDS, 10% glycerol, and 0.05% bromophenol blue (BPB). As a control experiment, fully reduced Vanabin2 was prepared by incubation with 100 mM DTT at 50°C for 45 min before adding sample buffer. Vanabin2 for each treatment was separated by SDS-PAGE. After electrophoresis the gels were stained with Coomassie Brilliant Blue (CBB) for 30 min and decolorized with 7.5% acetic acid and 20% methanol.

*2.4 CD measurement*

Structural changes of Vanabin2 induced by thiol-disulfide exchange reactions were monitored by CD spectroscopy in the UV region (350–250 nm). Vanabin2 was shown to be fully reduced by incubation in the presence of 100 mM DTT for 45 min at 50°C,

and the reduced Vanabin2 was reoxidized by aerial oxygen when DTT is removed by dialysis against 100 volumes of 20 mM phosphate buffer. The CD spectra were measured at Vanabin2 concentrations of 130–300 μg/ml in 20 mM phosphate buffer (pH 7.5) in the presence or absence of reducing reagents, DTT (0–100 mM), 2-ME (0–100 mM), or GSH (0–30 mM). As GSH has weak CD intensity at 350–250 nm, the Vanabin2 spectra were estimated by subtracting the GSH spectra from those of Vanabin2/GSH mixtures at the corresponding GSH concentrations. CD spectra were measured with a Jasco J720 spectropolarimeter at 20°C for 9–16 accumulations (slit, 1.0 mm; time constant, 4 s; scan speed, 100 nm/min; and path length, 10 mm).

*2.5 Determination of reduced GSH*

Ascidian blood cells were harvested from *A. sydneiensis samea*. Giant cells with an extremely acidic vacuole and no vanadium were removed from total blood cells by density gradient centrifugation using 0.2 M sucrose. As a result, about 70% of the separated blood cell pellet consisted of vanadocytes. The blood cell pellet was resuspended in 500 μl artificial seawater (460 mM NaCl, 9 mM KCl, 32 mM $Na_2SO_4$, 5 mM HEPES, and 5 mM EDTA adjusted to pH 7.0 with $NaHCO_3$). The number and density of blood cells were measured using a Bürker–Türk hemocytometer. Samples of 250 μl of the resuspended blood cell solution were transferred to new tubes, 4 volumes of 5% (*w/v*) metaphosphate were added, and the cells were homogenized by vortexing. The solution was centrifuged at 2,800 × *g* for 10 min at 4°C. The concentration of reduced GSH in the supernatant was determined using a GSH-400 assay kit (Oxis International Inc.).

*2.6 Electron paramagnetic resonance (EPR) spectra measurement*

EPR spectrometry was used to detect $V^{IV}$ species. Experimental conditions were as follows: 1 mM $V^V$; 10 mM $V^V$ and 2 mM GSH; and 10 mM $V^V$, 2 mM GSH, and 10

μM Vanabin2. The $V^V$ solution was prepared by dissolving $Na_3VO_4$ in distilled water (DW) at a concentration of 100 mM and adjusted to neutral pH. This solution was heated at 60°C until the yellow color due to decavanadate disappeared. Aliquots of 200 μl of each solution were placed into quartz tubes and the spectra were measured on a JEOL JES-RE1X spectrometer at room temperature (scan time, 8 min; magnetic field, 3360 ± 750 G; field modulation width, 20 G; time constant, 0.03 s).

*2.7 Coupled NADPH oxidation assay*

The assay buffer contained 200 μM NADPH, 0.25 U /ml glutathione reductase (GR), and 2 mM GSH. Vanabin2 was added to a final concentration of 4 μM in each tube except for the negative control tube. $V^V$ solution was added to a final concentration ranging from 0.1 to 2.5 mM. Reductase activity was monitored at 340 nm for 5 min and 60 min, respectively, and expressed as μM of NADPH oxidized using a molar extinction coefficient of 6,200 $M^{-1}$ $cm^{-1}$ for NADPH. These experiments were performed at 20°C.

## 3. Results

*3.1 Cleavage of disulfide bonds in Vanabin2 by three reducing reagents*

We first examined the structural changes in Vanabin2 upon treatment with reducing agents using gel electrophoresis. Vanabin2 with nine fully oxidized disulfide bonds was observed on polyacrylamide gels at a position corresponding to 14 kDa. When exposed to concentrations of more than 0.6 mM DTT and 10 mM 2-mercaptoethanol (2-ME), respectively, Vanabin2 migrated to a position corresponding to 20 kDa, as shown in Figure 2A (gels 1 and 2). This suggests that the disulfide bonds of Vanabin2 were oxidized and led to the cleavage. However, the bands of Vanabin2 treated with 50 and 100 mM GSH were smeared, suggesting that some of the disulfide bonds of Vanabin2 were intact, even when exposed to high concentrations of GSH (Fig. 2A, gel 3).

CD spectroscopy clearly revealed the differences between the oxidized and reduced forms of Vanabin2. Figures 2B, 2C, and 2D display the CD spectra of Vanabin2 in the presence or absence of the reducing reagents DTT, 2-ME, and GSH. These spectra can be attributed primarily to the cysteine residues of Vanabin2 because the recombinant Vanabin2 contained no aromatic amino acid residues, except for a phenylalanine near the N-terminus. A peak in the range of 270–260 nm was observed in the fully oxidized Vanabin2, whereas this peak disappeared in the spectrum of the fully reduced Vanabin2 treated with 100 mM DTT at 50°C for 45 min; it then reappeared when reoxidized by removal of the DTT (Fig. 2B). Thus, the peak at 270–260 nm was a good indicator of the reduced state of the disulfide bonds in Vanabin2. An isoellipticity point was observed at 265 nm in the spectra of Vanabin2 treated with 2-ME at concentrations ranging from 0 to 2 mM (Fig. 2C), suggesting the existence of a stable reduced intermediate in which the nine disulfide bonds are partially reduced. The isoellipticity points disappeared with 2-ME at concentrations above 10 mM, when Vanabin2 was fully reduced as revealed by SDS-PAGE (Fig. 2A, gel 2). As shown in Figure 2D, an isoellipticity point was observed at 272 nm in the spectra of Vanabin2 treated with GSH at concentrations ranging from 0 to 4 mM, appearing slightly redshifted compared to 2-ME. At GSH concentrations above 5 mM, the isoellipticity points disappeared. These results suggest strongly that Vanabin2 will form a partially reduced intermediate structure under minimal reducing conditions, such as exposure to 0.1–2 mM 2-ME or 1–4 mM GSH. Since the concentration of reduced GSH in *A. sydneiensis samea* blood cells determined with a GSH-400 assay kit was 1.83 ± 0.94 mM, GSH is highly likely to participate in the thiol-disulfide exchange reaction of vanadium with Vanabin2 in vivo.

### *3.2 $V^{V}$ reduction by Vanabin2*

EPR spectrometry of ascidian vanadium was performed to detect $VO^{2+}$ ($V^{IV}$) species [10]. In this experiment, the typical signal consisting of eight line manifolds due to the

$V^{IV}$ species was detected, and EPR spectrometry clearly indicated that Vanabin2 could reduce $V^{V}$ to $V^{IV}$. No signal intensity due to the $V^{IV}$ species was detected in $V^{V}$ solution, as shown in Figure 3A (spectrum 1). The addition of 2 mM GSH to a 10 mM $V^{V}$ solution evoked a slight increase in EPR signal intensity after a 24 h incubation at room temperature (Fig. 3A, spectrum 2). In contrast to spectrum 2 in Figure 3A, a large signal due to $V^{IV}$ was observed 24 h after addition of 10 μM Vanabin2 to the reaction mixture containing 10 mM $V^{V}$ and 2 mM GSH at room temperature (Fig. 3A, spectrum 3). These results clearly indicated that Vanabin2 acts as a vanadium reductase.

### *3.3 $V^{V}$ reduction by Vanabin2 coupled with NADPH oxidation*

Due to the difficulty of rapidly monitoring the reduction of $V^{V}$ by Vanabin2, the activity was measured using a coupled NADPH oxidation assay with reduction of oxidized glutathione (GSSG) catalyzed by glutathione reductase (GR) [12]. As a negative control, the first assay determined whether NADPH was oxidized by 2 mM GSH and 0.25 U/ml GR. As shown in Figure 3B, little oxidation of NADPH was observed. In the second assay, 4 μM Vanabin2 was added to the first assay system, in which NADPH was slightly oxidized (Fig. 3B, dark gray bar). In the third assay, 0.1–2.5 mM $V^{V}$ was added to the first assay system, in which NADPH was oxidized a little more and the levels of oxidized NADPH increased slightly with increasing $V^{V}$ concentrations (Fig. 3B, white bars). The addition of both Vanabin2 and $V^{V}$ to the first assay system increased the amount of oxidized NADPH markedly with increasing $V^{V}$ concentrations, reaching saturation at 1.5 mM $V^{V}$ (Fig. 3B, black bars). As shown in Figure 3C, the initial rate of NADPH oxidation calculated from 5 min and monitored at 340 nm was also accelerated depending on $V^{V}$ concentration and became nearly saturated above 2 mM $V^{V}$. The initial rate of NADPH oxidation for the substrates was measured and the kinetic parameters of Vanabin2 were obtained from the Lineweaver–

Burk plots (Fig. 3D). 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.

## 4. Discussion

Thiol-disulfide exchange reactions are known to be involved in many cellular activities, such as protein folding and unfolding [13], regulation of transcription factor activity [14], activity of ribonucleotide reductase [15], maintenance of redox potentials [16], responses against oxidative stress caused by metal ions [17], and metal transfer from metalloproteins to metal-depleted enzymes (metallochaperone activity) [18] in a manner analogous to phosphorylation/dephosphorylation reactions catalyzed by protein kinases and phosphatases.

Vanabin2 is a rare protein with nine disulfide bonds per molecule (Fig. 1) [8]. The fully oxidized Vanabin2 was completely cleaved by 100 mM DTT at 50°C for 45 min [8], which migrated to a position corresponding to 20 kDa on polyacrylamide gels and resulted in the disappearance of the isoellipticity points in the CD spectra, a good indicator of the reduced state of the disulfide bonds (Figs. 2C and 2D). However, under weaker reducing conditions, such as 1–4 mM GSH, which corresponds to the intrinsic concentrations in vanadocytes, the isoellipticity points appeared, suggesting the formation of a partially reduced intermediate of Vanabin2 in vivo.

As $V^{V}$ is easily reduced to $V^{IV}$, many biologically relevant reducing agents such as ascorbate, cysteine, norepinephrine, GSH, oxalic acid, tunichromes, and NADPH have been reported to facilitate the reduction [19] .However, almost all of these processes reported previously are too slow to be biologically relevant, necessitating enzymatic mechanisms for reduction in vivo. In fact, as shown in Figure 3B, a slight reduction of $V^{V}$ to $V^{IV}$ was confirmed in the reaction mixture containing NADPH and GR, but its value was significantly less than that observed by the addition of 4 μM Vanabin2. The

apparent reduction of $V^{V}$ to $V^{IV}$ was observed immediately after the addition of Vanabin2 in the coupled assay experiments (Fig. 3B). The coupled assay showed marked increases depending on $V^{V}$ concentrations, although the amount of NADPH oxidized was limited to 10–23 μM in the absence of Vanabin2 (Fig. 3B), reflecting the reduction of $V^{V}$ to $V^{IV}$ was catalyzed by Vanabin2.

GSH, the most abundant intracellular non-protein thiol, is a potent reducing agent. In human erythrocytes, the level of GSH is typically 2–3 mM and GSSG is present at a concentration about 1–2% that of GSH [20]. In vitro studies have shown that GSH plays an important role in the biochemistry of vanadium. Costa Pessoa et al. [21] systematically studied equilibria in the $V^{IV}$-GSH system in aqueous solution. $V^{V}$ is reduced almost quantitatively to $V^{IV}$ by GSH inside cells and GSH may act as a ligand for the formed $V^{IV}$ [22].

In contrast to human erythrocytes, ascidian vanadocytes contain not only GSH but also high intrinsic levels of vanadium. In fact, 13 mM vanadium [3] and 1.83 mM GSH were found in vanadocytes of *A. sydneiensis samea* examined in the present study. An ascidian homolog encoding GR was previously found by EST analysis of vanadocytes [23]. In addition, enzymes of the pentose phosphate pathway, known to produce two molecules of NADPH per cycle, were localized in vanadocytes [24]. Therefore, the equilibrium in thiol-disulfide exchange reactions may be established among NADPH, GR, GSH, Vanabin2, and vanadium ions in ascidian vanadocytes in vivo. Among them, NADPH, GR, and GSH are components of the glutathione system, which is known to act as the electron donor systems coupled with glutaredoxin, glutathione peroxidase, and glutathione transferase [25]. Therefore, we propose a possible cascade of electron transfer involving the glutathione system, Vanabin2, and vanadium ions as shown in Figure 4, in which the order of redox reactions was estimated from the results of CD spectroscopy, EPR spectrometry, and coupled NADPH oxidation assay (Figs. 2 and 3).

In this cascade, electrons may be transferred from the donor NADPH to the acceptor vanadium ions. In turn, reduction of $V^{V}$ to $V^{IV}$ can occur via thiol-disulfide exchange reactions of Vanabin2 (Fig. 3, spectrum 3). The resultant disulfides are converted to thiols by reduced GSH (Figs. 2D and 3B, dark gray bar) and the oxidized GSH is further reduced by GR [12, 25]. The disulfides of GR are reduced to thiols by NADPH [12, 25], which may be linked to the pentose phosphate pathway (Fig. 4).

The situation in ascidians is complicated by the fact that $V^{IV}$ is further reduced to $V^{III}$. Several reducing agents possibly involved in the reduction of $V^{IV}$ to $V^{III}$ have been discussed [26]. Among them, the potentially biologically relevant reducing agents include cysteine complexes. Ascidian blood cells contain aliphatic sulfonic acids, such as cysteic acid, an oxidation product of cysteine [27], and cysteine methyl ester was reported to reduce $V^{IV}$ to $V^{III}$ with the assistance of EDTA and EDTA-like ligands of aminopolycarboxylate in water. The reduction of $V^{IV}$ to $V^{III}$ by cysteine methyl ester was found to be aided by glycylhistidine and glycylaspartic acid [26]. In addition, cleavage of the disulfide bonds of Vanabin2 resulted in the reduction of $V^{V}$ to $V^{IV}$ in the present study. Therefore, cysteine complexes are likely to participate in the reduction. To completely elucidate the electron transfer cascade from NADPH to vanadium ions, the unknown reductant involved in the subsequent reduction of $V^{IV}$ to $V^{III}$ must be clarified (Fig. 4).

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**Figure legends**

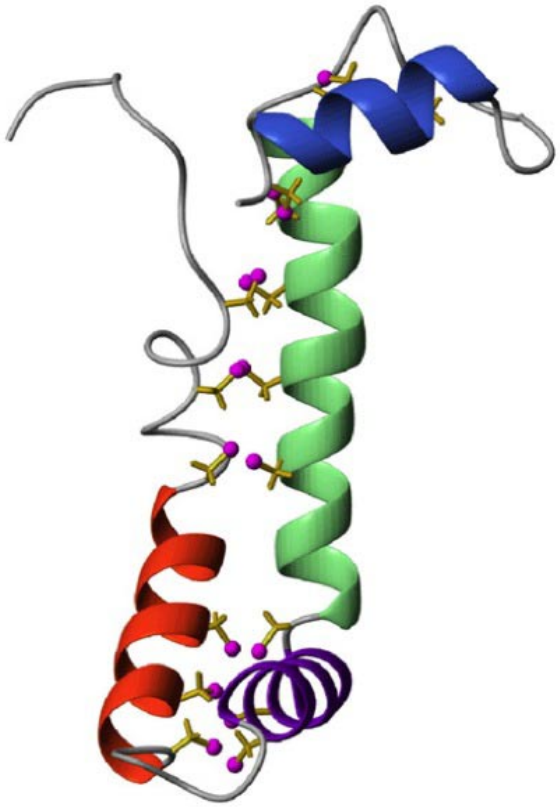

**Fig. 1.** Vanabin2, a rare protein with nine disulfide bonds per molecule composed of 91 amino acids, was revealed to be a novel vanadium reductase. The structure was modified based on the data reported in Hamada *et al.* [9].

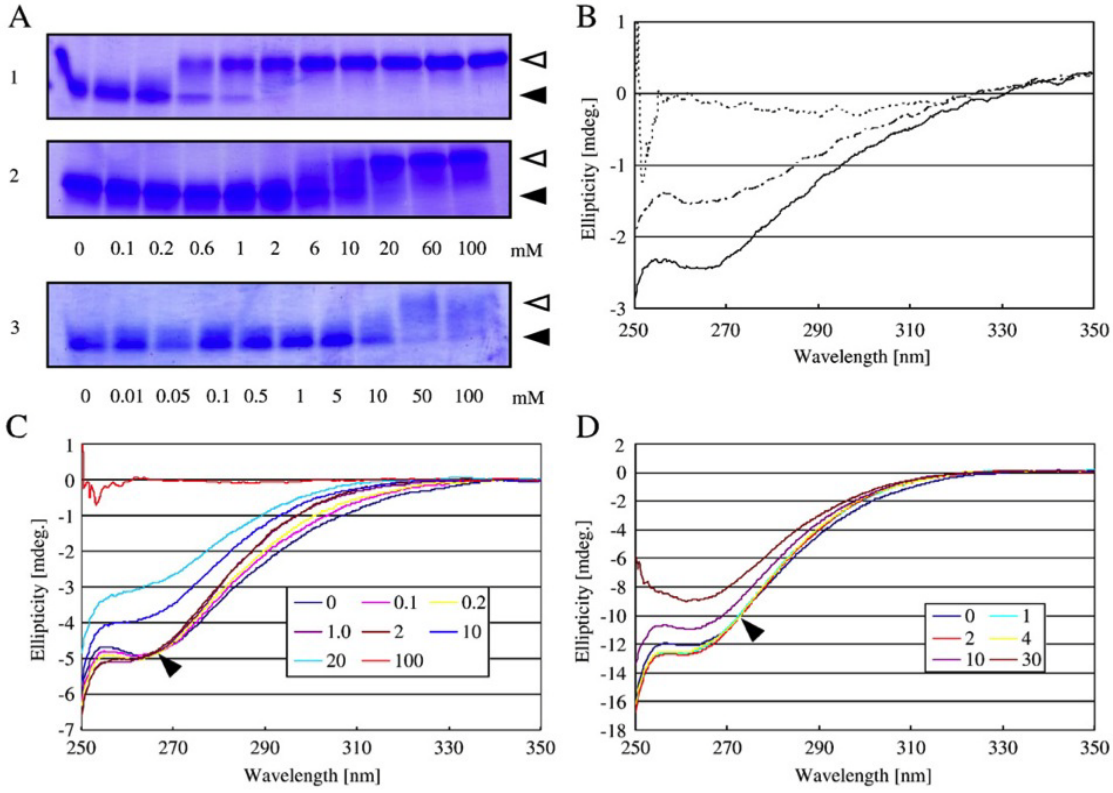


**Fig. 2.** Cleavage of nine disulfide bonds of Vanabin2. Fully oxidized and reduced Vanabin2 are shown at 14 kDa (filled arrowhead) and 20 kDa (open arrowhead), respectively, on the polyacrylamide gel (A). Vanabin2 migrated to a position corresponding to 20 kDa following treatment with more than 0.6 mM DTT (gel 1) and 10 mM 2-ME (gel 2), respectively. Vanabin2 appeared to migrate to a position corresponding to 20 kDa following treatment with 50 and 100 mM GSH, but the bands were smeared (gel 3). (B–D) Structural changes of Vanabin2 induced by thiol-disulfide exchange reactions were monitored by CD spectroscopy. (B) Purified Vanabin2 (solid line), Vanabin2 treated with 100 mM DTT at 50°C for 45 min (dotted line), and Vanabin2 reoxidized by removal of DTT (dotted-dashed line). (C) Vanabin2 treated with 2-ME at concentrations ranging from 0–100 mM. (D) Vanabin2 treated with GSH at concentrations ranging from 0–30 mM. Arrowheads in (C) and (D) indicate isoellipticity points.

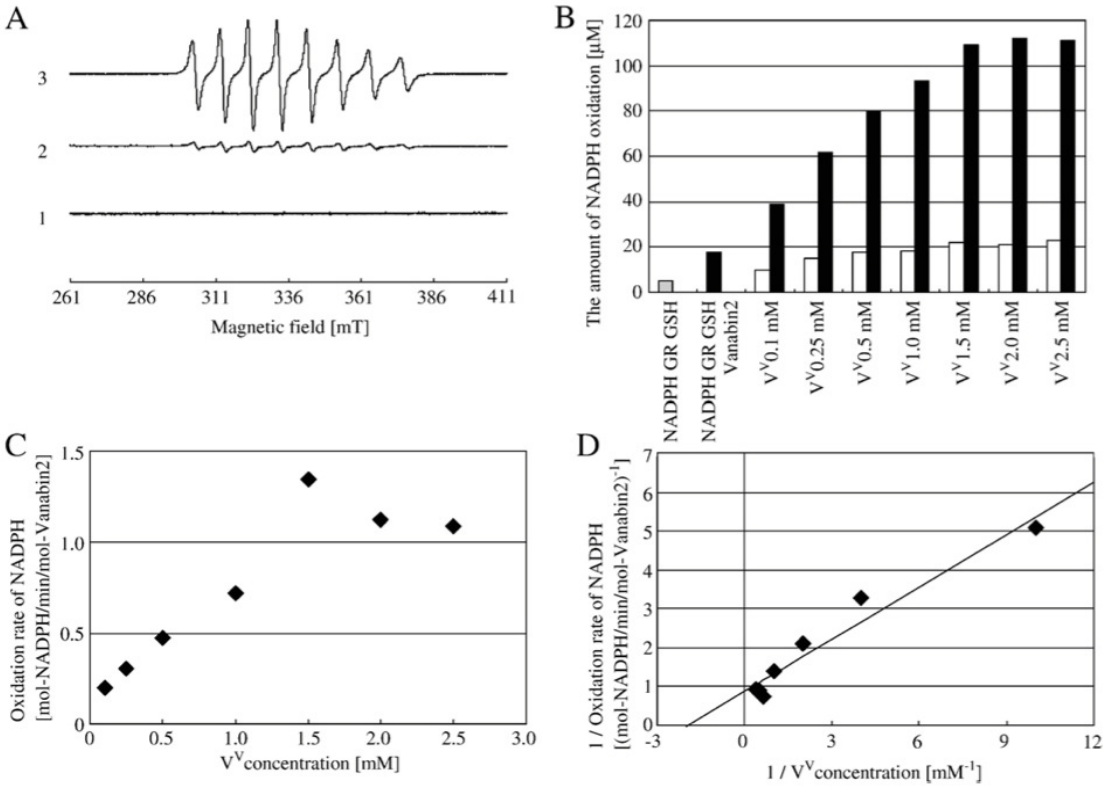


**Fig. 3.** (A) $V^V$ reduction by Vanabin2 observed by EPR spectrometry. Spectrum 1, 1 mM $V^V$; Spectrum 2, addition of 2 mM GSH to 10 mM $V^V$ 24 h after incubation at room temperature; Spectrum 3, after 24 h incubation of 10 μM Vanabin2 with a reaction mixture of 10 mM $V^V$ and 2 mM GSH at room temperature. The signal intensities are given as relative values. (B–D) $V^V$ reduction by Vanabin2 observed by coupled NADPH oxidation assay. (B) The vertical axis indicates the level of NADPH oxidation in 60 min. Light gray bar, the amount of NADPH oxidation in the absence of both $V^V$ and Vanabin2; dark gray bar, that in the presence of 4 μM Vanabin2; white bars, that in the presence of 0.1–2.5 mM $V^V$; black bars, that in the presence of both 4 μM Vanabin2 and 0.1–2.5 mM $V^V$. (C) The initial oxidation rate was calculated from the amount of NADPH oxidized in 5 min based on the data shown in B. (D) Lineweaver–Burk plots calculated from (C). Concentrations of NADPH, GR, and GSH were fixed at 200 μM, 0.25 U/ml, and 2 mM, respectively.

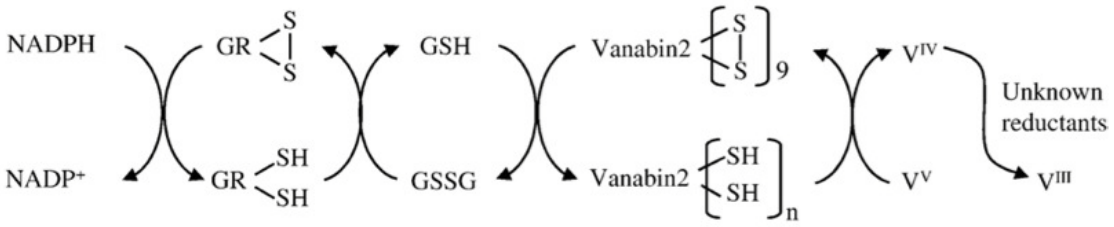


**Fig. 4.** A possible cascade for the thiol-disulfide exchange reactions conjugated with NADPH, GR, GSH, Vanabin2, and vanadium ions.

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