Role of cysteine residues in the V(V)-reductase activity of Vanabin2

Sohei Yamamoto [1], Koichi Matsuo [2], Hitoshi Michibata [1], Tatsuya Ueki [1,3,*]

*[1] Molecular Physiology Laboratory, Department of Biological Science, and [2] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan. [3] Marine Biological Laboratory, Graduate School of Science, Hiroshima University, 2445 Mukaishima, Onomichi, Hiroshima 722-0073, Japan. *Corresponding author: ueki@hiroshima-u.ac.jp TEL: +81-848-44-1434, FAX: +81-848-44-5914.*

ABSTRACT

Ascidians (tunicates or sea squirts) accumulate extremely high levels of vanadium as the reduced form V(III) in extremely acidic vacuoles in their blood cells. Several key proteins related to vanadium accumulation have been isolated from vanadium-rich ascidians and their physiological functions characterized. Of these, vanabins are small, cysteine-rich proteins that have been identified only in vanadium-rich ascidians. Our previous study revealed that Vanabin2 can act as a V(V)-reductase. The current study examines the role of cysteine and several other amino acid residues of Vanabin2 in V(V)-reduction. When all eighteen cysteine residues of Vanabin2 were substituted with serine residues, the V(V)-reductase activity was lost. Substitutions of three, structurally clustered cysteines in three different regions resulted in a moderate decrease in reductase activity, suggesting that more than a single cysteine pair is responsible for the V(V)-reductase activity of Vanabin2. Mutations in the V(IV)-binding domains caused either an increase or decrease in activity but no mutation caused the complete

loss of activity. These results suggest that some pairs, but more than a single pair, of cysteine residues are necessary for the V(V)-reductase activity of Vanabin2.

Keywords

Transition metal; Disulfide bonds; Vanadium; Reductase; Ascidians.

1. Introduction

The unusual ability of ascidians 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, which is thought to be the highest metal accumulation 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 in ascidians [1, 2]. 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) [1]. 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. Among these, a class of vanadium-binding proteins named vanabins may be responsible for the selective transport of vanadium.

Vanabins commonly possess 18 conserved cysteine residues and constitute a unique protein family present only in vanadium-rich ascidians [3-6]. The most-studied vanabin is Vanabin2, which has been isolated from *Ascidia sydneiensis samea* [7, 8, 6]. The three-dimensional structure of Vanabin2 was determined by NMR spectroscopy, which revealed eighteen cysteine residues that form nine disulfide (SS) bonds between specific amino acid residues [7]. Although Vanabin2 was originally isolated as a V(IV)-binding

protein, it adopts an SS/SH intermediate structure and can act as a V(V)-reductase [9]. In recent experiments, Vanabin2 was shown to act only as a V(V)-reductase [10]. This high selectivity may account for the metal ion selectivity of vanadium accumulation in ascidians.

The actual reaction mechanism of Vanabin2 as a V(V)-reductase is still unclear. We hypothesize the mechanism shown in Scheme 1 [9, 10]. In this redox cascade mechanism, electrons may be transferred from donor NADPH to acceptor vanadium ions. In turn, reduction of V(V) to V(IV) can occur via thiol–disulfide exchange reactions with Vanabin2.

According to Scheme 1, one or some of the nine cysteine pairs must be responsible for V(V)-reduction but it is unclear which cysteine pair(s) is(are) an active site(s). Therefore, this study examined the contribution of each pair of cysteine residues by site-directed mutagenesis. In addition, according to another model hypothesizing that V(IV)-binding sites accelerate the reduction of V(V) [11], we examined the effect of mutagenesis on V(IV)-binding sites.

2. Experimental Section

2.1 Reagents

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. NADPH and glutathione reductase (GR) were obtained from the Oriental Yeast Company.

2.2 In vitro site-directed mutagenesis

Most of the Vanabin2 mutants were synthesized as described previously [11]. The numbering of amino acid residues was done in accordance with Hamada *et al.* [7], where isoleucine, serine, glutamate, and phenylalanine derived from the junction region between the pMal-c vector and the Vanabin2 cDNA were deemed positions 1–4 (I1–S2–E3–F4). Lysine (AAA or AAG), arginine (CGN), and histidine (CAT or CAC) residues were substituted with

alanines (GCN). Cysteine residues (TGT or TGC) were substituted by serines (TCN, AGT or AGC). Several additional mutants were generated in the same way. Nucleotide sequences were confirmed using an ABI 3130 automated DNA sequencer (Applied Biosystems Japan Ltd.) at the Natural Science Center for Basic Research and Development at Hiroshima University (N-BARD).

2.3 Preparation of recombinant proteins

Recombinant Vanabin2 protein was prepared in accordance with procedures published previously [11]. Briefly, the pMAL-c plasmid containing the Vanabin2 coding region [6] was introduced into *Escherichia coli* strains TB1, BL21 or KRX. The transformed cells were incubated in LB medium containing 50 μg/mL ampicillin and 0.5 mM IPTG. The cells were harvested, re-suspended in a lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 10 mM EDTA, 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). For further purification, when necessary, 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.

2.4. NADPH-coupled oxidation assays

Metal reductase activity was measured using an NADPH-coupled oxidation assay as described previously [9]. The assay buffer contained 50 mM Tris-HCl (pH 7.4), 200 μM NADPH, 0.25 U/mL GR, and 2 mM GSH. With the exception of the negative control tube, Vanabin2 or the control protein was added to a final concentration of 2 μM in each tube and

the solutions were pre-heated at 20°C for at least 15 min. V(V) stock solution was prepared by dissolving sodium orthovanadate in ultra-pure water at 10 mM and the pH was adjusted to 7.4. The solution was heated at 65°C until colorless. V(V) was added to the reaction tube to a final concentration of 2.5 mM. NADPH has a peak absorbance at 340 nm, and its oxidation coupled with GSH/GSSG can be monitored at this wavelength [12]. NADPH oxidation was monitored by measuring the solution absorbance at 340 nm for 30 min at 20°C using a U-2900 spectrophotometer equipped with a thermoelectric cell holder (Hitachi Co. Ltd.).

2.5. Circular dichroism spectrometry

The secondary structure of Vanabin2 was examined by circular dichroism (CD) spectroscopy as in a previous study with minor modifications [11]. Prior to CD measurements, the purity of the protein was confirmed by SDS-PAGE and the protein concentration was adjusted to 100 μg/mL in 50 mM Tris-HCl buffer (pH 7.4). CD spectra were measured using a Jasco J720W spectropolarimeter in a 1.0-mm or 10.0-mm pathlength cell at RT with a 1.0-mm slit, a 4-s time constant, a 100-nm/min scan speed, and 6–10 accumulations.

3. Results

3.1 Coupled assays for V(V)-reductase activity in Vanabin2 mutants

According to Scheme 1, disulfide bonds are necessary for the V(V)-reductase activity of Vanabin2. Vanabin2 possesses nine cysteine pairs that make disulfide bonds, and they are numbered as per our previous study (SS1 to 9) (Fig. 1). The 3D structure revealed that these cysteine pairs exist as three spatially dispersed clusters [7]. Therefore, we first created three disulfide mutants of Vanabin2. Serine was chosen as the substituent because it is structurally similar to cysteine but contains a hydroxyl (-OH) group in place of the sulfhydryl, or thiol (-SH) group. SSm1-3 incorporates cysteine-to-serine mutations at SS1, SS2, and SS3. SSm4-6 and SSm7-9 contain substitutions in SS4 to 6 and SS7 to 9, respectively.

The reductase activity of the three disulfide mutants was measured and compared to the reductase activity of wild-type Vanabin2. As shown in Fig. 2, wild-type Vanabin2 produced 18 μmol $NADP^+$ per μmol enzyme in 30 min. Typical absorbance spectra are shown in Supplementary Fig. 1. This result is reproducible relative to the results of a previous report [9]. All three disulfide mutants maintained their reductase activity. This indicates that more than a single disulfide bond is involved in the V(V)-reducing activity of Vanabin2. Furthermore, SSm1-3 and SSm4-6 mutations exhibited elevated reductase activity over a similar catalytic time course, but with different catalytic velocities. The possible reason for elevated activity is discussed below.

Three additional mutants, K10AR60A, K24AK38AR41AR42A, and H64A, which contain mutations of V(IV)-binding sites, were also examined [11]. The results are summarized in Fig. 3, which shows the amount of NADPH oxidation after 30 min under identical reaction conditions. Similar results for SSm1-3, SSm4-6, and SSm7-9 mutants from the same experiment are shown in Fig. 2. Bovine serum albumin (BSA) was used as the negative control. Two of the disulfide mutants, SSm1-3 and SSm4-6, yielded an elevated reductase activity, while the activity of SSm7-9 did not differ significantly from that of wild-type Vanabin2. Two of the proteins with mutant V(IV)-binding sites resulted in a slightly elevated reductase activity, although the H64A mutant did not alter reductase activity. These results suggest that more than a single cysteine pair is critical for the reductase activity of Vanabin2. They also indicate that any single V(IV)-binding site may be solely responsible for V(V)-reductase activity. The possible reason for elevated activity is also discussed below.

We then examined a mutant lacking all nine pairs of disulfide bonds (SSm1-9). Since this mutant protein in easily degraded after removing the carrier MBP, MBP fusion proteins were used in these assays. As a comparison, wild-type Vanabin2 was also fused to MBP. MBP itself was used as a control.

The V(V)-reductase activity of the Vanabin2-MBP fusion protein did not differ significantly from that of Vanabin2 itself (Figs. 3 and 4). MBP, as a negative control, gave a low but significant reductase activity in this assay system, reaching an activity about twice that of the control reaction without MBP. The SSm1-9 mutant fused with MBP yielded a decreased (V)-reductase activity level not significantly different from that of the MBP control. Thus, Vanabin2 lacking cysteine residues could not reduce V(V), which supports the cascade mechanism shown in Scheme 1.

3.2 Secondary structure of SSm1-9 mutant Vanabin2

Since the artificial mutations described above might affect the overall protein structure, the effects of mutations on the secondary structure of Vanabin2 were examined by CD spectroscopy. In these assays, wild-type Vanabin2 and SSm1-9 mutants were prepared separate from the carrier MBP protein. The purity of this wild-type Vanabin2 was almost 100%. The SSm1-9 mutant was a mixture of 70% full-length segments with the remainder of shorter fragments.

Fig. 5A shows the CD spectra of wild-type Vanabin2 and the SSm1-9 mutant in 50 mM Tris-HCl buffer (pH 7.4). In this wavelength range, the CD spectra indicated the presence of α-helices and β-sheets. The spectra were normalized to the ellipticity at 222 nm. These spectra suggest that the secondary structure of SSm1-9 was not significantly altered from that of wild-type Vanabin2.

The peak at 270–260 nm in the CD spectra of Vanabin2 is a good indicator of the reduced state of the disulfide bonds in Vanabin2 [9]. CD spectra were also acquired for the SSm1-9 mutant, which lacks all of the native SS bonds. Fig. 5B shows the CD spectra of wild-type Vanabin2 and the SSm1-9 mutant in the absence of reducing agents. The data show that wild-type Vanabin2 formed SS-bonds while SSm1-9 completely lacked SS-bonds. Thus, the

SSm1-9 mutant retained the secondary structure of the wild-type protein but lost its V(V)-reductase activity due to the loss of SS-bonds.

4. Discussion

Vanabin2, a vanadium-binding protein isolated from the cytoplasm of vanadocytes, acts as a V(V)-reductase [9]. As of yet, Vanabin2 has been reported as only a V(V)-reductase [10]. In the reaction cascade shown in Scheme 1, the disulfide bond is important for the reduction of V(V). Vanabin2 contains nine disulfide bonds [7] that are thought to be important for its reductase activity. In this study, we examined the contribution of cysteine and several other amino acid residues to the V(V)-reductase activity of Vanabin2. The first series of mutants, SSm1-3, SSm4-6, and SSm7-9, lacked three of the nine cysteine pairs and retained reductase activity. In contrast, the SSm1-9 mutant, which lacked all nine cysteine pairs, lost all reductase activity. These results suggest that cysteine pairs are indispensable in the V(V)-reductase activity of Vanabin2 but that more than a single pair of cysteine residues is responsible for the activity.

In some of mutants for cysteines and vanadium-binding sites, reductase activity was significantly elevated (Figs. 2 and 3). This was controversy to our expectation that reductase activity must be decreased by amino acid substitution. Generally, disulfide bridges play an important role in the construction of the tertiary structure of proteins. Most proteins containing disulfide bridges unfold when they are completely reduced even in the absence of a denaturant [13, 14]. In case of Vanabin2, the nine disulfide bridges maintain its rigid structure as supposed from the NMR study [7], but the SSm1-9 mutant that lacks all disulfide bonds retains some secondary structures as suggested from the CD spectrum of Fig 5A. Although we need further experimental evidences about the structure-function relationships of Vanabin2, these findings indicate the reductase activity would be complicatedly affected by

the secondary structure and structural flexibility around the reductase reaction sites.

4.1 Role of cysteine residues in the reductase activity of Vanabin2

Thiol–disulfide exchange reactions are involved in many cellular activities, such as protein folding and unfolding [15], maintenance of redox potentials [16], activation of oxygen-sensitive transcription factors [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. In our model system, thiol/disulfide exchange reactions link the reducing agent NADPH to the V(V)-reductase activity of Vanabin2. In this model, the free thiols of Vanabin2 reduce V(V) by direct electron transfer.

The direct involvement of disulfide bonds in metal redox reactions is not often reported. Electron transfer has been observed between protein thiols and metal ions such as Cu^{2+} and Fe^{3+}. The reduction of metallothionine by a heme protein, cytochrome *c*, is thought to occur through a direct electron transfer from iron to cysteine [19], while other cysteine oxidation pathways in the presence of metal ions involve radical species. Cysteine residues are particularly susceptible to reactive oxygen species, such as superoxide and hydrogen peroxide, generated in the presence of redox-active metal ions. As observed by Crans [20] and discussed in our recent paper [10], V(V) cannot be reduced to V(IV) ($E^0 = -0.341$ V) by thiol compounds whose reduction potentials ($E^0 \sim -0.26$ V) are higher than that of the V(V)/V(IV) redox couple. However, specific interactions between thiols and V(V)/V(IV) can occur under physiological conditions [10, 20]. It is therefore necessary to show whether the reduction of V(V) to V(IV) in our model system occurs via direct or indirect reaction between thiols and vanadium ions.

Vanabin2 reduces vanadate anions (VO_4^{3-}; V(V)) to vanadyl cations (VO^{2+}; V(IV)).

Arsenate reductases are interesting in that their substrates are also anions (AsO_4^{3-}; As(V)) and it is meaningful to compare the relative properties of these two systems. *E. coli* ArsC and *S. cerevisiae* Acr2p have been identified earlier and investigated extensively [21]. The only substrate of *E. coli* ArsC was arsenate (K_m=0.8 mM) with a V_{max} that typically ranges from 0.8 to 1.5 pmol/min/mg protein[22]. A recent study on *Synechocystis* ArsC revealed a V_{max} of 3.1 μmol/min/mg protein[23]. The latter value corresponds to 45 mol/min/mol of ArsC, which is about 40-fold greater than that of wild-type Vanabin2 (1.15 mol/min/mol protein) [9]. ArsC has a single catalytic cysteine residue [24], while *Synechocystis* ArsC contains three essential cysteine residues [23]. The position of the catalytic cysteine residue is to be determined in a future study.

4.3 Possible in vivo reduction cascade

In the ascidian *A. sydneiensis samea*, the Vanabin family consists of at least five closely related proteins, Vanabins 1–4 and VanabinP. Vanabins 1–4 are expressed in the cytoplasm of vanadocytes, while VanabinP is in blood plasma [25, 3]. All five of these Vanabins possess 18 cysteine residues; the intervals between these cysteines are very well conserved. Vanabin1 and Vanabin4 can also reduce V(V) to V(IV) (unpublished data). Therefore, it is possible that four cytoplasmic vanabins act together to reduce V(V) to V(IV). Since the concentration of GSH is 1.83 mM in the vanadocytes of *A. sydneiensis samea*, and the enzymes involved in the pentose phosphate pathway are expressed exclusively in the cytoplasm of these vanadocytes, the existence of a redox cascade from NADPH to V(V) ions in the cytoplasm of vanadocytes is likely.

4.4 Detoxification or energy source?

In our model, V(V) ions are readily reduced to V(IV) in the cytoplasm, V(IV) ions are stabilized by Vanabins, and a proton electrochemical gradient generated by vacuolar H^+-ATPase (V-ATPase) gives the driving force for V(IV) transport from the cytoplasm into

the vacuole using the V(IV)/H^+ antiporter, *As*Nramp [26]. Export of stored vanadium out of the vacuole has not been reported to date.

It is usually accepted that vanadium toxicity increases with an increasing oxidation state, with V(V) being the most toxic. Vanadate also inhibits ATPases because vanadate and phosphate are structurally similar [27]. V(IV) should be toxic to cellular components because it can cause Fenton reactions, which produce reactive oxygen species [28]. Storage of V(III) in an acidic vacuole is, therefore, an appropriate strategy for storing high concentrations of vanadium.

Considering the cost of maintaining such high levels of vanadium, which often reach up to 10^7 times higher than that in sea water, vanadium accumulation must benefit ascidians. One should therefore consider whether the reverse reactions, accompanying the oxidations of V(III) to V(IV) and V(IV) to V(V), occur in the proposed cascade, perhaps resulting in the release of energy as in a vanadium redox flow battery. Thus, another possibility is that ascidians may accumulate metal ions as an energy source.

5. Conclusions

Vanabin2 contains nine disulfide bonds. When all of these disulfide bonds were removed by site-directed mutagenesis, the resulting SSm1-9 mutant lost its V(V)-reductase activity. Three partial mutants, SSm1-3, SSm4-6, and SSm7-9, which lack three of the nine cysteine pairs, retained their reductase activity. These results suggest that cysteine pairs are indispensable in the V(V)-reductase activity of Vanabin2, but that more than a single pair of cysteines is required. In contrast, all V(IV)-binding site mutants examined retained their reductase activity. Thus, V(IV)-binding sites are not required for the reductase activity of Vanabin2.

Abbreviations

ATPase, adenosine triphosphatase; CD, circular dichroism; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GR, glutathione reductase; IPTG, β-D-1-thiogalactopyranoside;2-ME , 2-mercaptoethanol; MBP, maltose-binding protein; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized form of nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; Nramp, natural resistance associated macrophage protein; RT, room temperature (20~25°C);SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris-HCl, tris hydroxymethyl aminomethane hydrogen chloride salt.

Acknowledgements

DNA sequencing was done at the Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (nos. 22224011, 25120508 and 25440170).

References

[1] H. Michibata, Y. Iwata, J. Hirata, J. Exp. Zool. 257 (1991) 306–313.

[2] J. Hirata, H. Michibata, J. Exp. Zool. 257 (1991) 160–165.

[3] M. Yoshihara, T. Ueki, T. Watanabe, N. Yamaguchi, K. Kamino, H. Michibata, Biochim. Biophys. Acta 1730 (2005) 206–214.

[4] N. Yamaguchi, K. Kamino, T. Ueki, H. Michibata, Mar. Biotechnol. 6 (2004) 165-174.

[5] S. Trivedi, T. Ueki, N. Yamaguchi, H. Michibata, Biochim. Biophys. Acta 1630 (2003) 64–70.

[6] T. Ueki, T. Adachi, S. Kawano, M. Aoshima, N. Yamaguchi, K. Kanamori, H. Michibata, Biochim. Biophys. Acta 1626 (2003) 43–50.

[7] T. Hamada, M. Asanuma, T. Ueki, F. Hayashi, N. Kobayashi, S. Yokoyama, H. Michibata, H. Hirota, J. Am. Chem. Soc. 127 (2005) 4216–4222.

[8] K. Fukui, T. Ueki, H. Ohya, H. Michibata, J. Am. Chem. Soc. 125 (2003) 6352–6353.

[9] N. Kawakami, T. Ueki, Y. Amata, K. Kanamori, K. Matsuo, K. Gekko, H. Michibata, Biochim. Biophys. Acta 1794 (2009) 674–679.

[10] H. Kitayama, S. Yamamoto, H. Michibata, T. Ueki, Dalton. Trans. 42 (2013) 11921–11925.

[11] T. Ueki, N. Kawakami, M. Toshishige, K. Matsuo, K. Gekko, H. Michibata, Biochim. Biophys. Acta 1790 (2009) 1327–1333.

[12] E. Racker, J. Biol. Chem. 217 (1955) 855–865.

[13] T.E. Creighton, J. Mol. Biol. 144 (1980) 521–550.

[14] C.B. Anfinsen, E. Haber, M. Sela, F.H. White Jr, Proc. Natl. Acad. Sci. U. S. A. 47 (1961) 1309–1314.

[15] A.J. Doig, D.H. Williams, J. Mol. Biol. 217 (1991) 389–398.

[16] F. Aslund, K.D. Berndt, A. Holmgren, J. Biol. Chem. 272 (1997) 30780–30786.

[17] M. Zheng, Science 279 (1998) 1718–1722.

[18] Y.-F. Lin, J. Yang, B.P. Rosen, J. Bioenerg. Biomembr. 39 (2007) 453–458.

[19] C. Simpkins, P. Eudaric, C. Torrence, Z. Yang, Life Sciences 53 (1993) 1975–1980.

[20] D.C. Crans, B. Zhang, E. Gaidamauskas, A.D. Keramidas, G.R. Willsky, C.R. Roberts, Inorg. Chem. 49 (2010) 4245–4256.

[21] B.P.R. Rita Mukhopadhyay, Environ. Health Perspect. 110 (2002) 745.

[22] T.B. Gladysheva, K.L. Oden, B.P. Rosen, Biochemistry 33 (1994) 7288–7293.

[23] R. Li, J.D. Haile, P.J. Kennelly, J. Bacteriol. 185 (2003) 6780–6789.

[24] J. Liu, T.B. Gladysheva, L. Lee, B.P. Rosen, Biochemistry 34 (1995) 13472–13476.

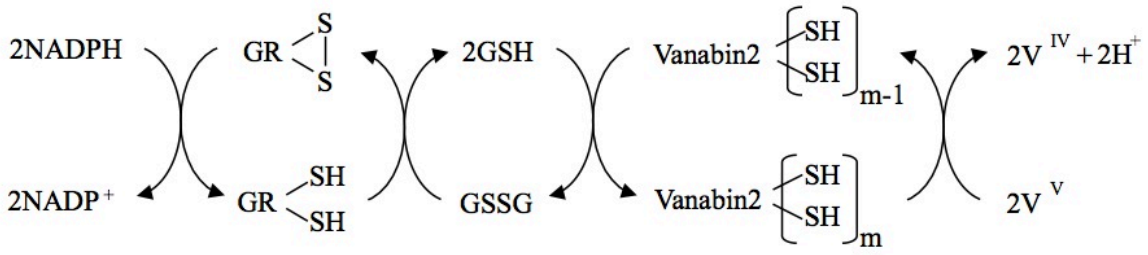
[25] N. Yamaguchi, Y. Amakawa, H. Yamada, T. Ueki, H. Michibata, Zool. Sci. 23 (2006) 909–915.

[26] T. Ueki, N. Furuno, H. Michibata, Biochim. Biophys. Acta 1810 (2011) 457–464.

[27] L.C. Cantley, L. Josephson, R. Warner, M. Yanagisawa, C. Lechene, G. Guidotti, J. Biol. Chem. 252 (1977) 7421-7423.

[28] R.J. Keller, R.P. Sharma, T.A. Grover, L.H. Piette, Arch. Biochem. Biophys. 265 (1988) 524–533.

Figure legends



Scheme 1. A proposed redox cascade mechanism for the V(V) reductase activity of Vanabin2.

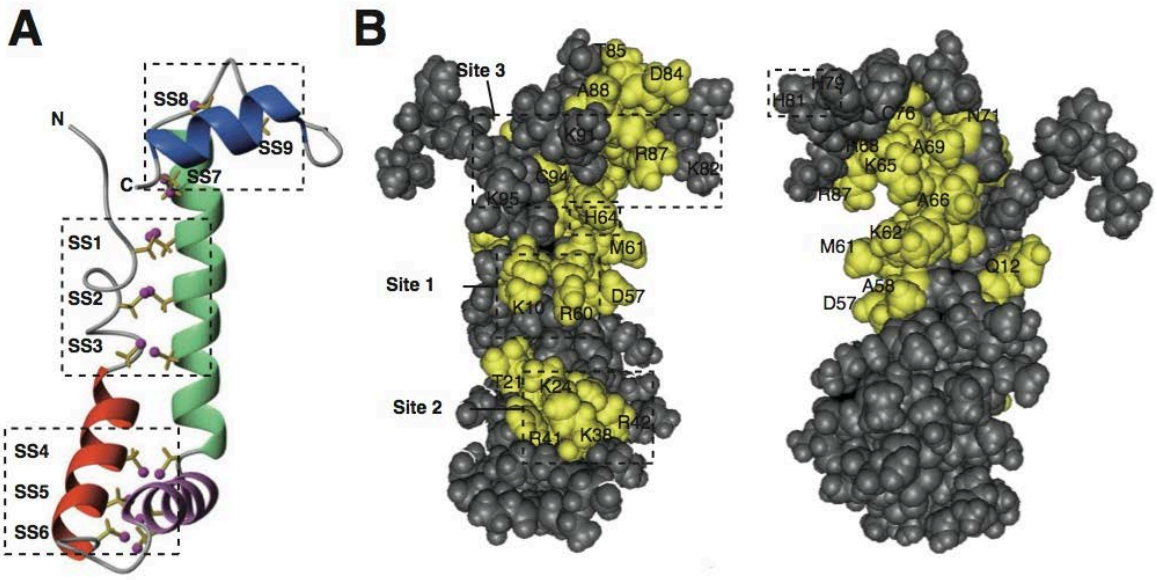


Fig. 1. The three-dimensional structure of Vanabin2 is shown with mutation sites as indicated. (A) The ribbon model of Vanabin2 shows nine cysteine pairs (SS1–9). (B) The spherical model of Vanabin2 shows possible VO^{2+}-binding sites in light gray and are denoted by amino acid numbers on the two faces, looking in opposite directions [7]. PDB data (ID: 1vfi) were used to compute these structures, which included the four additional amino acids (I–S–E–F) originating from the junction region of the pMal-c vector. PyMol software was used to generate the spherical images. Images were modified from our previous work [11].

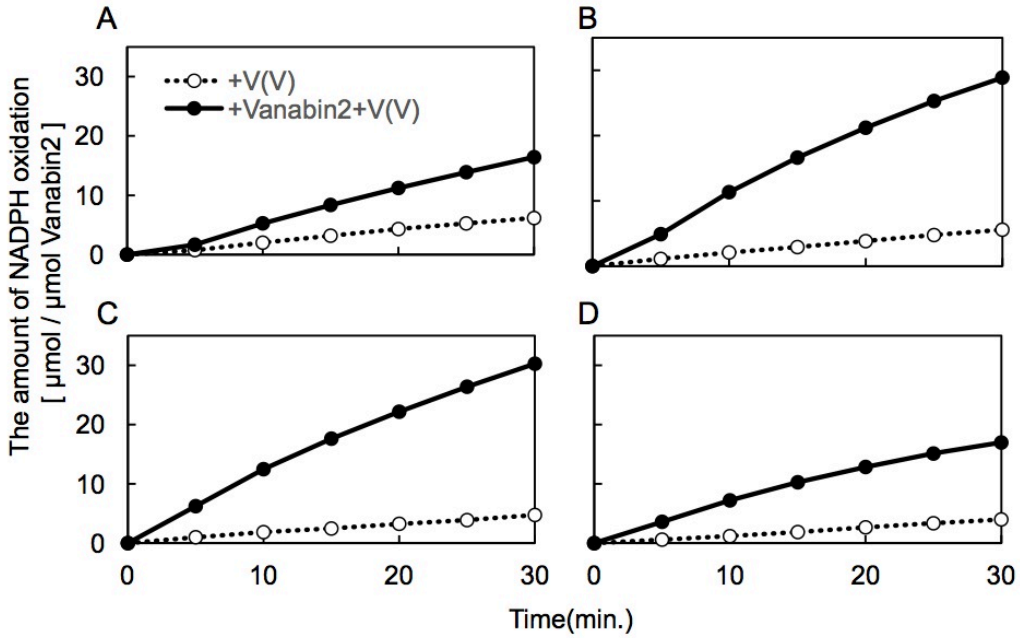


Fig. 2. The reductase activities of wild-type and disulfide mutants of Vanabin2 are shown as a function of time. Representative results are given for (A) wild-type Vanabin2 and (B) SSm1-3, (C) SSm4-6 and (D) SSm7-9 mutants of Vanabin2. The solid line with solid circles corresponds to the activity of 2 µM wild-type or mutant Vanabin2. The dotted line with open circles corresponds to the activity without Vanabin2. The horizontal axis indicates the time after the initiation of catalysis. The vertical axis indicates the amount of NADPH oxidation.

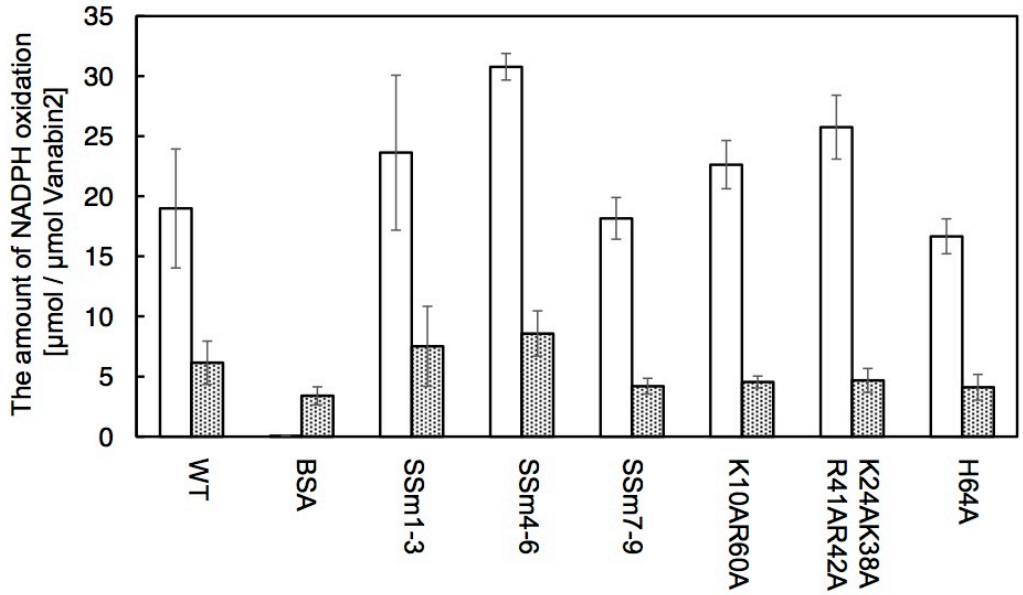


Fig. 3. The reductase activities of wild-type, disulfide mutants, and V(IV)-binding-site mutants of Vanabin2 are shown. WT and BSA correspond to wild-type Vanabin2 and bovine serum albumin, respectively. SSm1-3, SSm4-6, and SSm7-9 correspond to disulfide mutants of Vanabin2. K10AR60A, K24AK38AR41AR42A, and H64A contain mutations in V(IV)-binding sites. The vertical axis indicates the amount of NADPH oxidation after 30 min under the same reactions conditions as those used in Fig. 2. Open bars indicate the values for experiments including each protein at a concentration of 2 μM. Gray bars indicate negative controls excluding each protein. BSA was included as a negative control.

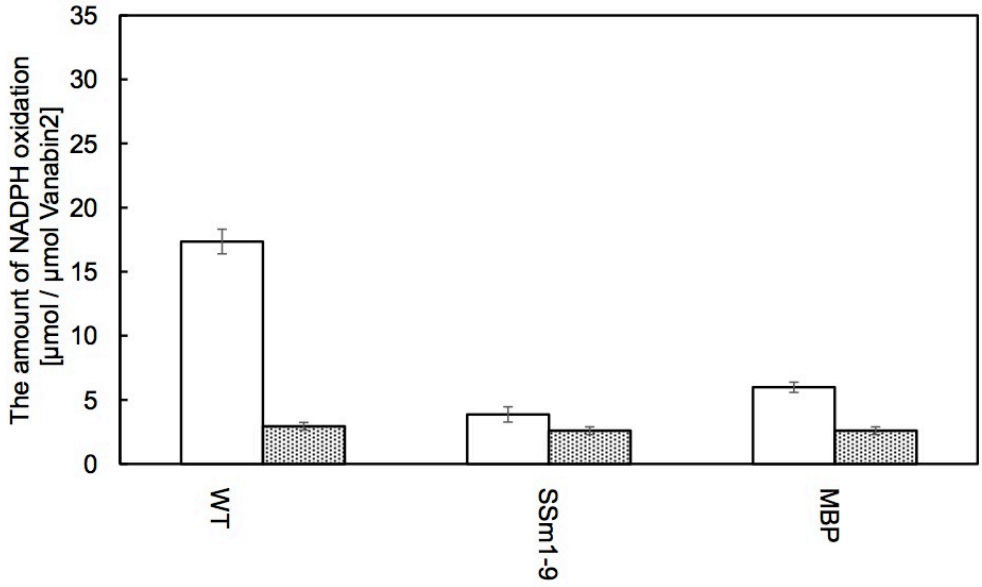
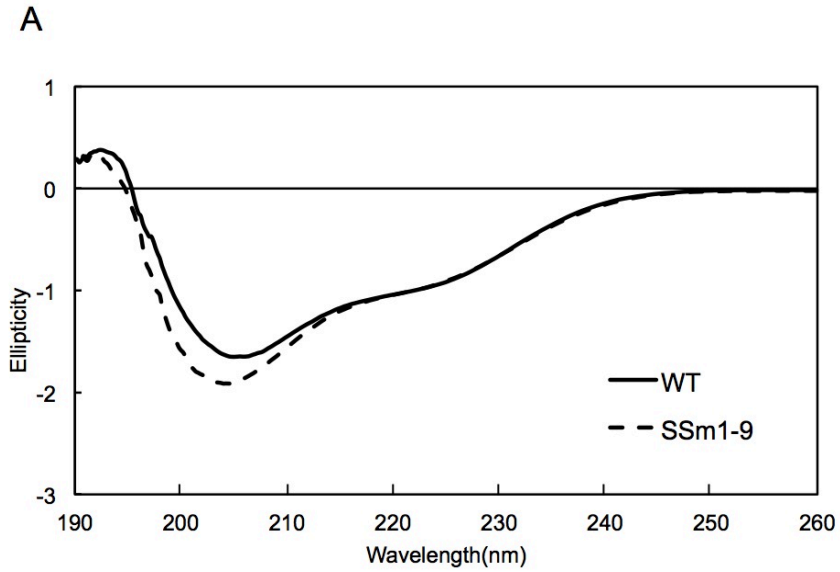


Fig. 4. The reductase activity of wild-type and mutants of Vanabin2, including the activity of maltose-binding protein (MBP), is shown. WT corresponds to wild-type Vanabin2. SSm1-9 is the mutant Vanabin2 with serine residues replacing all of the native cysteine residues. MBP was used as a negative control. The vertical axis indicates the amount of NADPH oxidation after 30 min using the same conditions used to generate the data in Fig. 2. Open bars indicate values obtained with each protein at a concentration of 2 μM. Gray bars indicate data obtained with the negative controls.



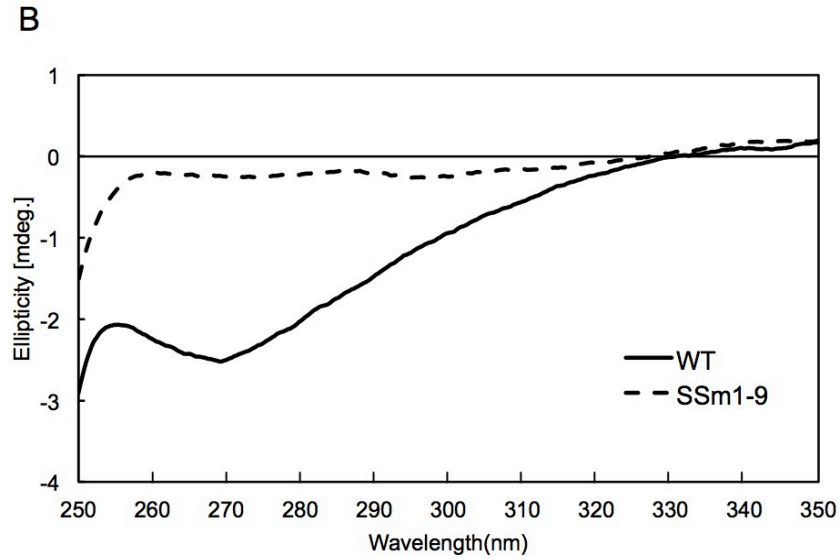


Fig. 5. CD spectra of wild-type (WT) Vanabin2 and the SSm1-9 mutant. CD spectra were measured in 50 mM Tris-HCl buffer (pH 7.4) at 20°C for WT Vanabin2 (solid line) and the SSm1-9 mutant (dotted line). (A) The secondary structure of wild-type and mutant Vanabin2. The spectra were normalized to the ellipticity at 222 nm. No significant changes to the secondary structure were induced by the mutation. (B) Disulfide bond formation as observed by CD spectroscopy. A peak between 270 and 260 nm was observed with fully oxidized Vanabin2. In contrast, this peak was not observed in the CD spectrum of the SSm1-9 mutant.