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

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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

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

28

29 **Keywords**

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

31

32 **1. Introduction**

33 The unusual ability of ascidians to accumulate high levels of vanadium ions has been
34 attracting attention in biological and chemical disciplines for a century. The maximum
35 concentration of vanadium can reach 350 mM in vanadocytes of *Ascidia gemmata*, belonging
36 to the class Ascidiidae, which is thought to be the highest metal accumulation of any living
37 organism [1]. Vanadium usually exists as V(V) in HVO_4^{2-} or H_2VO_4^- in natural aquatic
38 environments. These ions are reduced to V(III) via a V(IV) state (VO^{2+}) during assimilation in
39 ascidians [1, 2]. Vanadium ions are stored in the vacuoles of signet ring cells, which are a type
40 of blood cell often referred to as vanadocytes (vanadium-accumulating cells) [1]. Ongoing
41 research during the last two decades has identified many proteins involved in the process of
42 accumulating and reducing vanadium in vanadocytes, blood plasma, and the digestive tract of
43 ascidians. Among these, a class of vanadium-binding proteins named vanabins may be
44 responsible for the selective transport of vanadium.

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

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

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

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

63

64 **2. Experimental Section**

65 *2.1 Reagents*

66 Dithiothreitol (DTT), 2-mercaptoethanol (2-ME), and reduced glutathione (GSH) were
67 purchased from Wako Pure Chemical Industries. Sodium orthovanadate (Na_3VO_4 ; >99.9%)
68 was purchased from Sigma-Aldrich Co. NADPH and glutathione reductase (GR) were
69 obtained from the Oriental Yeast Company.

70 *2.2 In vitro site-directed mutagenesis*

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

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

81 *2.3 Preparation of recombinant proteins*

82 Recombinant Vanabin2 protein was prepared in accordance with procedures published
83 previously [11]. Briefly, the pMAL-c plasmid containing the Vanabin2 coding region [6] was
84 introduced into *Escherichia coli* strains TB1, BL21 or KRX. The transformed cells were
85 incubated in LB medium containing 50 µg/mL ampicillin and 0.5 mM IPTG. The cells were
86 harvested, re-suspended in a lysis buffer (10 mM Na₂HPO₄, 30 mM NaCl, 10 mM EDTA,
87 0.25% Tween 20, 10 mM 2-ME, pH 7.0) containing 4 M urea and then sonicated using a
88 UH-150 ultrasonic homogenizer (SMT Company). The fusion protein was purified from the
89 soluble cellular fraction by affinity chromatography using amylose resin in accordance with
90 the manufacturer's protocol (New England BioLabs). For further purification, when necessary,
91 the protein was cleaved at the MBP junction by incubation with Factor Xa and the released
92 Vanabin2 was purified through an anion-exchange column filled with DEAE-Sephacel resin
93 (GE Healthcare). The eluted protein was dialyzed four times in 100 volumes of 50 mM
94 Tris-HCl (pH 7.4) for use in vanadium reductase activity assays. Prior to use, the purity of the
95 Vanabin2 was confirmed by SDS-PAGE.

96 *2.4. NADPH-coupled oxidation assays*

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

101 the solutions were pre-heated at 20°C for at least 15 min. V(V) stock solution was prepared
102 by dissolving sodium orthovanadate in ultra-pure water at 10 mM and the pH was adjusted to
103 7.4. The solution was heated at 65°C until colorless. V(V) was added to the reaction tube to a
104 final concentration of 2.5 mM. NADPH has a peak absorbance at 340 nm, and its oxidation
105 coupled with GSH/GSSG can be monitored at this wavelength [12]. NADPH oxidation was
106 monitored by measuring the solution absorbance at 340 nm for 30 min at 20°C using a
107 U-2900 spectrophotometer equipped with a thermoelectric cell holder (Hitachi Co. Ltd.).

108 *2.5. Circular dichroism spectrometry*

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

115

116 **3. Results**

117 *3.1 Coupled assays for V(V)-reductase activity in Vanabin2 mutants*

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

126 The reductase activity of the three disulfide mutants was measured and compared to the
127 reductase activity of wild-type Vanabin2. As shown in Fig. 2, wild-type Vanabin2 produced
128 18 $\mu\text{mol NADP}^+$ per $\mu\text{mol enzyme}$ in 30 min. Typical absorbance spectra are shown in
129 Supplementary Fig. 1. This result is reproducible relative to the results of a previous report [9].
130 All three disulfide mutants maintained their reductase activity. This indicates that more than a
131 single disulfide bond is involved in the V(V)-reducing activity of Vanabin2. Furthermore,
132 SSm1-3 and SSm4-6 mutations exhibited elevated reductase activity over a similar catalytic
133 time course, but with different catalytic velocities. The possible reason for elevated activity is
134 discussed below.

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

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

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

158 *3.2 Secondary structure of SSm1-9 mutant Vanabin2*

159 Since the artificial mutations described above might affect the overall protein structure,
160 the effects of mutations on the secondary structure of Vanabin2 were examined by CD
161 spectroscopy. In these assays, wild-type Vanabin2 and SSm1-9 mutants were prepared
162 separate from the carrier MBP protein. The purity of this wild-type Vanabin2 was almost
163 100%. The SSm1-9 mutant was a mixture of 70% full-length segments with the remainder of
164 shorter fragments.

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

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

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

177

178 **4. Discussion**

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

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

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

201

202 *4.1 Role of cysteine residues in the reductase activity of Vanabin2*

203 Thiol–disulfide exchange reactions are involved in many cellular activities, such as
204 protein folding and unfolding [15], maintenance of redox potentials [16], activation of
205 oxygen-sensitive transcription factors [17], and metal transfer from metalloproteins to
206 metal-depleted enzymes (metallochaperone activity) [18] in a manner analogous to
207 phosphorylation/dephosphorylation reactions catalyzed by protein kinases and phosphatases.
208 In our model system, thiol/disulfide exchange reactions link the reducing agent NADPH to
209 the V(V)-reductase activity of Vanabin2. In this model, the free thiols of Vanabin2 reduce
210 V(V) by direct electron transfer.

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

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

225 Arsenate reductases are interesting in that their substrates are also anions (AsO_4^{3-} ; As(V)) and
226 it is meaningful to compare the relative properties of these two systems. *E. coli* ArsC and *S.*
227 *cerevisiae* Acr2p have been identified earlier and investigated extensively [21]. The only
228 substrate of *E. coli* ArsC was arsenate ($K_m=0.8$ mM) with a V_{\max} that typically ranges from 0.8
229 to 1.5 pmol/min/mg protein[22]. A recent study on *Synechocystis* ArsC revealed a V_{\max} of 3.1
230 $\mu\text{mol}/\text{min}/\text{mg}$ protein[23]. The latter value corresponds to 45 mol/min/mol of ArsC, which is
231 about 40-fold greater than that of wild-type Vanabin2 (1.15 mol/min/mol protein) [9]. ArsC
232 has a single catalytic cysteine residue [24], while *Synechocystis* ArsC contains three essential
233 cysteine residues [23]. The position of the catalytic cysteine residue is to be determined in a
234 future study.

235 4.3 Possible *in vivo* reduction cascade

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

246 4.4 Detoxification or energy source?

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

250 the vacuole using the V(IV)/H⁺ antiporter, AsNramp [26]. Export of stored vanadium out of
251 the vacuole has not been reported to date.

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

258 Considering the cost of maintaining such high levels of vanadium, which often reach up to
259 10⁷ times higher than that in sea water, vanadium accumulation must benefit ascidians. One
260 should therefore consider whether the reverse reactions, accompanying the oxidations of
261 V(III) to V(IV) and V(IV) to V(V), occur in the proposed cascade, perhaps resulting in the
262 release of energy as in a vanadium redox flow battery. Thus, another possibility is that
263 ascidians may accumulate metal ions as an energy source.

264

265 **5. Conclusions**

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

274

275 **Abbreviations**

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

284

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290

291 **References**

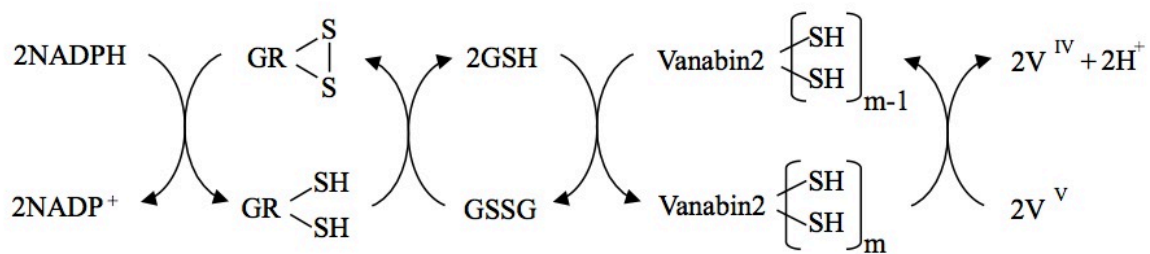
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334 **Figure legends**

335

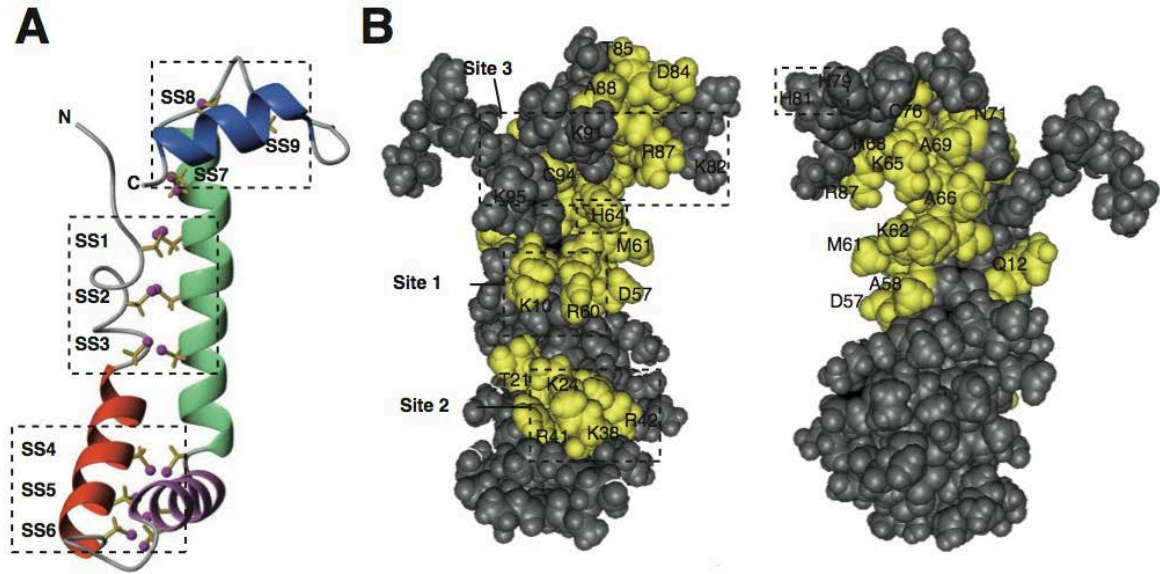


336

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

338

339



340

341 Fig. 1. The three-dimensional structure of Vanabin2 is shown with mutation sites as indicated.

342 (A) The ribbon model of Vanabin2 shows nine cysteine pairs (SS1–9). (B) The spherical

343 model of Vanabin2 shows possible VO^{2+} -binding sites in light gray and are denoted by amino

344 acid numbers on the two faces, looking in opposite directions [7]. PDB data (ID: 1vfi) were

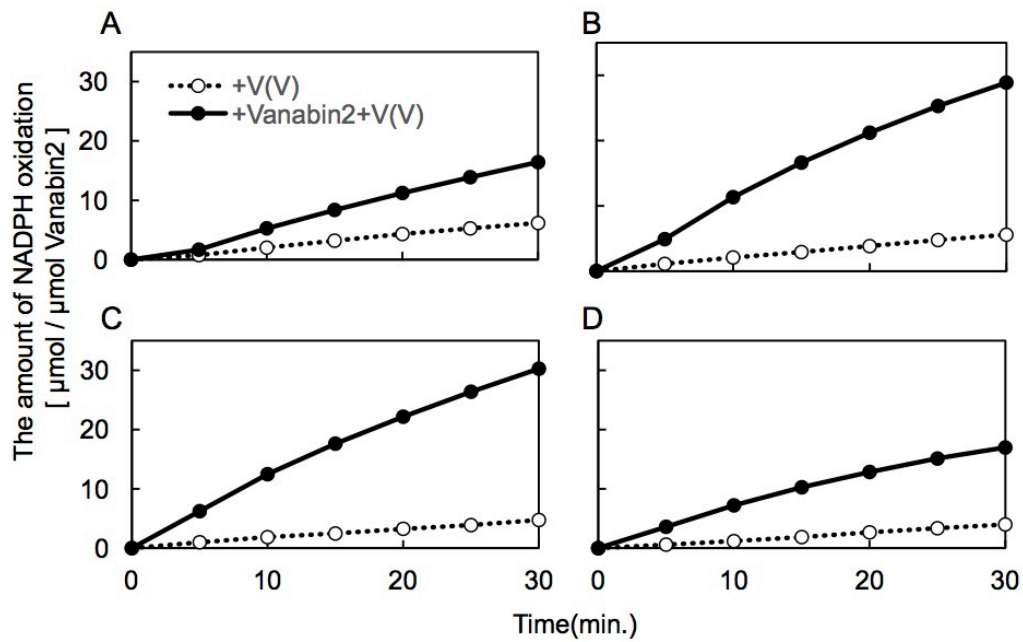
345 used to compute these structures, which included the four additional amino acids (I–S–E–F)

346 originating from the junction region of the pMal-c vector. PyMol software was used to

347 generate the spherical images. Images were modified from our previous work [11].

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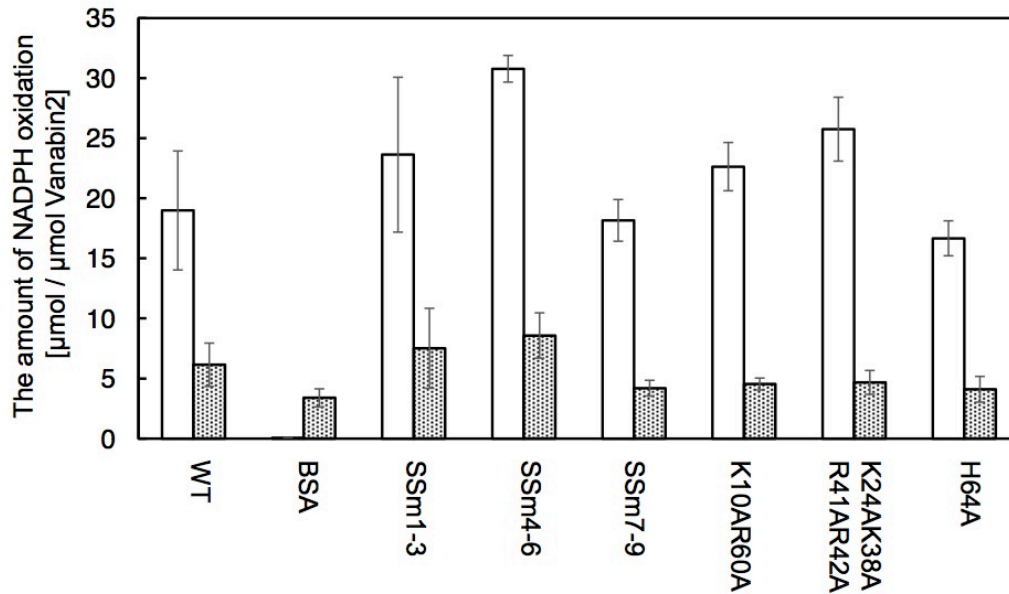


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351 Fig. 2. The reductase activities of wild-type and disulfide mutants of Vanabin2 are shown as a
 352 function of time. Representative results are given for (A) wild-type Vanabin2 and (B) SSm1-3,
 353 (C) SSm4-6 and (D) SSm7-9 mutants of Vanabin2.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

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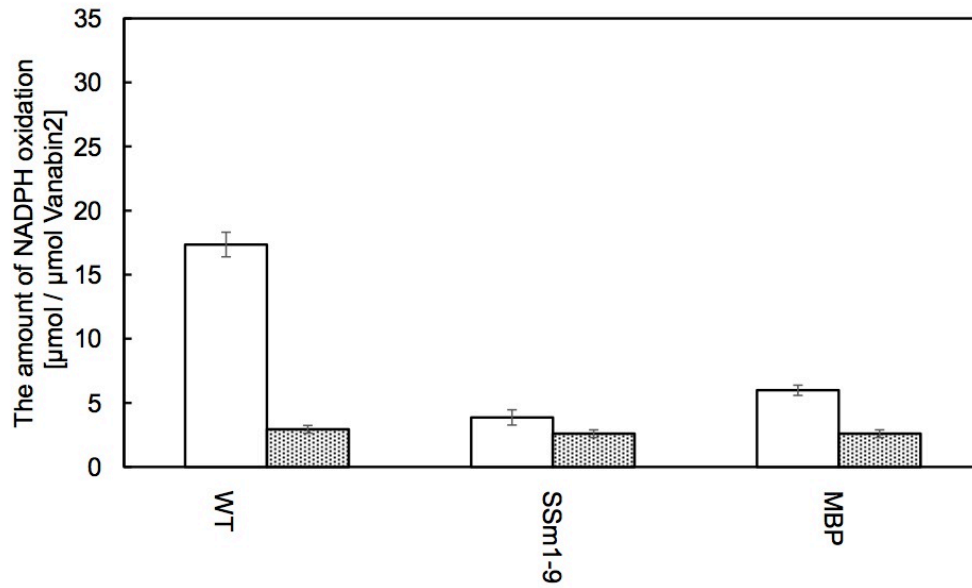


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360 Fig. 3. The reductase activities of wild-type, disulfide mutants, and V(IV)-binding-site
 361 mutants of Vanabin2 are shown. WT and BSA correspond to wild-type Vanabin2 and bovine
 362 serum albumin, respectively. SSm1-3, SSm4-6, and SSm7-9 correspond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

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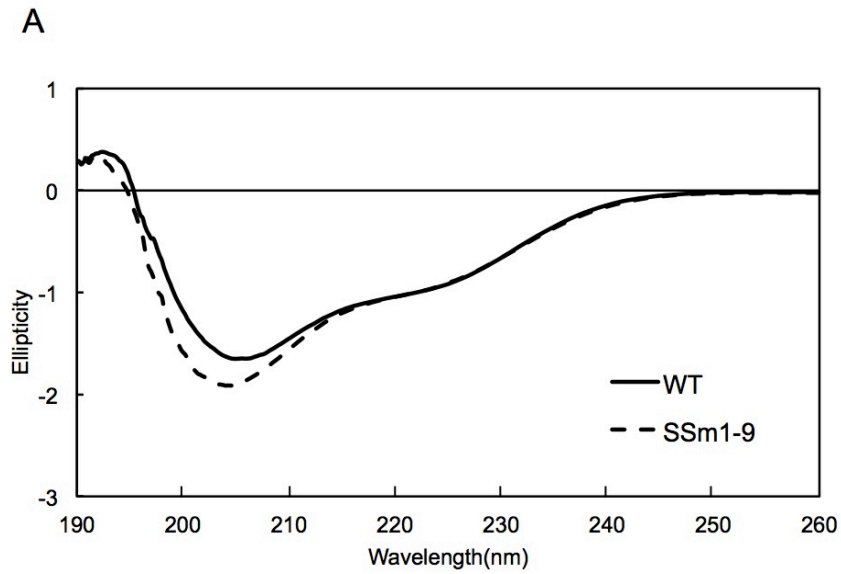


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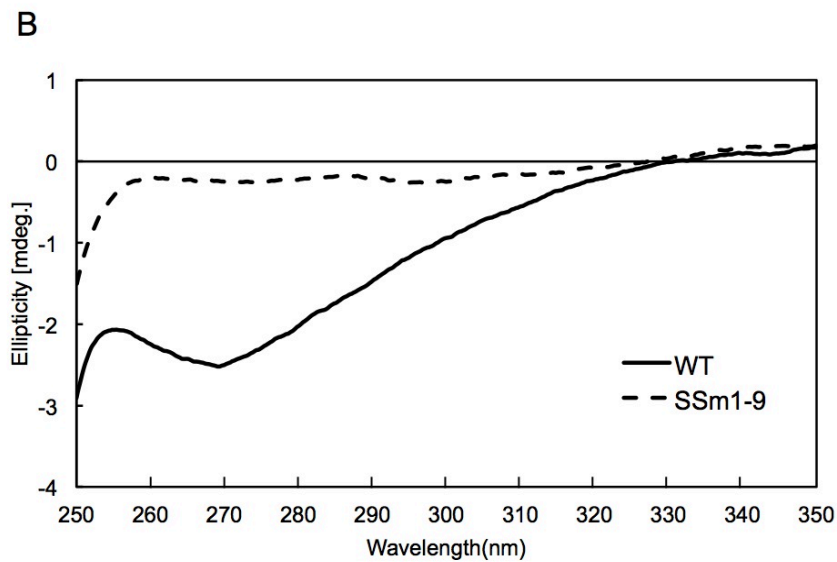
371 Fig. 4. The reductase activity of wild-type and mutants of Vanabin2, including the activity of
 372 maltose-binding protein (MBP), is shown. WT corresponds to wild-type Vanabin2. SSm1-9 is
 373 the mutant Vanabin2 with serine residues replacing all of the native cysteine residues. MBP
 374 was used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

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

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