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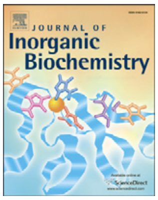

Metal-binding domains and the metal selectivity of the vanadium(IV)-binding protein VBP-129 in blood plasma

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ABSTRACT

Ascidians are well known to accumulate extremely high levels of vanadium in their blood cells. Several key proteins related to vanadium accumulation and physiological function have been isolated from vanadium-rich ascidians. Of these, vanadium(IV)-binding protein-129 (VBP-129) is a unique protein that has been identified from the blood plasma of an ascidian *Ascidia sydneiensis samea*, but its metal binding domains are not known. In this study, several deletion and point mutants of VBP-129 were generated, and their metal binding abilities were assessed by immobilized metal ion affinity chromatography (IMAC) and electron spin resonance spectroscopy (ESR). The internal partial protein, VBP-Int41, did not bind to V^{IV}, but the two constructs, VBP-N52 and VBP-Int55, added with additional 11 or 14 neighboring amino acids bound to V^{IV}. Mutations for cysteine-47 and lysine-50 in VBP-Int55 diminished V^{IV}-binding in VBP-Int55, suggesting that these amino acid residues play important roles in binding V^{IV}. ESR titration analysis revealed that VBP-129, VBP-N52 and VBP-Int55 could bind to 6, 3 and 2 V^{IV} ions, respectively. ESR spectrum analysis indicated a N_2O_2 coordination geometry, which is similar to vanabins. The cysteines may contribute to the maintenance of the three-dimensional structure that is necessary for binding V^{IV} ions. VBP-129 did not have a V^{V}-reductase activity, as expected from its tissue localization in blood plasma. This study provided the evidences that VBP-129 possesses V^{IV}-binding domains that make a similar coordination to V^{IV} as those by vanabins but VBP-129 acts solely as a V^{IV}-chaperon in blood plasma.

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 [1]. Vanadium ions are stored in the vacuoles of vanadium-containing cells called vanadocytes, where high levels of protons and sulfate ions are also found. 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 (10^7 times against its concentration in natural sea water, 35 nM). Vanadium is usually found in the +5 oxidation state (HVO_4^{2-} or $H_2VO_4^-$; V^V) in the natural aquatic environment, but most of these ions are reduced to +3 (V^{3+}; V^{III}) via the +4 state (VO^{2+}; V^{IV}) during assimilation [2,3]. Additionally, most vanadium ions are stored in the vacuoles of signet ring cells, a type of blood cell, and are often referred to as vanadocytes [4,5].

Ongoing research over 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, such as vacuolar-type H^+-ATPase [6–8], the chloride channel [9], the sulfate transporter [10], enzymes of the pentose–phosphate pathway [11–14], the glutathione transferase [15], vanabins [16–18], and a vanadium transporter [19].

Two types of vanadium-binding proteins that are expressed in blood plasma have been identified in *Ascidia sydneiensis samea*. One protein was identified as a member of the vanabin family, VanabinP, and its recombinant form binds a maximum of 13 V^{IV} ions per molecule with a K_d of 2.8×10^{-5} M [18]. The other protein was named VBP-129; it binds to V^{IV} ions as well as to Fe^{III}, Co^{II}, Cu^{II}, and Zn^{II} ions [20]. A truncated form of VBP-129 that does not bind to V^{IV} was also discovered, which has been called VBP-88 for its lack of 41 amino acid residues [20]. But, the properties such as binding number and coordination geometry in VBP-129 have not been revealed.

In this study, we sought to reveal the position and property of V^{IV}-binding domain(s) of VBP-129 as well as the contribution of crucial amino acids for the binding of V^{IV}. According to the differential sequence between full-length VBP-129 and its spontaneous truncated form VBP-88, several deletion and point mutants of VBP-129 were created using PCR amplification and in vitro site-directed mutagenesis.

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Their metal binding abilities were assessed by IMAC and ESR. ESR measurements were conducted to reveal binding number and coordination geometry of V^{IV} to VBP-129. Since reductase activity was not detected, VBP-129 seemed to act solely as a V^{IV}-carrier protein in blood plasma.

2. Material and methods

2.1. Construction of expression plasmids

Deletion mutants of VBP-129 were constructed by PCR amplification using a VBP-129 expression plasmid [20] as the template. Primer sets and templates used are listed in Table 1. PCR reactions consisted of 100 ng plasmid DNA, 200 pmol of each primer, each dNTP at 0.2 mM, 1× reaction buffer, and 2.5 units of *Taq* DNA polymerase (TaKaRa, Inc.) in a total volume of 50 μL. Amplification was performed with an initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. After purification by agarose gel electrophoresis and cloning into a pBluescript/*Eco*RV/dT vector, the nucleotide sequence was confirmed using an ABI 3130 automated DNA sequencer (Applied Biosystems Japan Ltd.) at the Natural Science Center for Basic Research and Development, Hiroshima University (N-BARD).

Each plasmid DNA was digested with restriction enzymes to excise the amplified fragments. These fragments were ligated into the multiple cloning sites of the expression vector pMAL-c2X (New England BioLabs), which contains the *lac* promoter and a coding region for MBP. Each plasmid was introduced into *Escherichia coli* BL21 for protein expression.

The plasmid vector and host *E. coli* strain for expressing another vanadium-binding protein, Vanabin1, were the same ones used in our previous work [16].

2.2. In vitro site-directed mutagenesis

Site-directed mutagenesis of the coding region of VBP-129 and VBP-Int55 was conducted by a PCR-based in vitro procedure based on the manufacturer's protocol as described previously [21], using the plasmid vectors pMal-p2X with the VBP-129 insert and pMal-c2X with the VBP-Int55 insert as templates. The combination of primer sets and template DNAs are listed in Table 1. Target residues were cysteine-47 (TGC) and lysine-50 (AAA) in the VBP-Int55 amino acid sequence, and cysteine-80 (TGC) and lysine-83 (AAA) in the VBP-129 amino acid sequence (Fig. 1). The cysteine and lysine were substituted by serine (AGC) and alanine (GCA), respectively. Each primer pair had a mutagenizing codon, which was flanked by complementary regions of 9–12 nucleotides.

The PCR reaction mix contained 50 ng of plasmid DNA, 35 pmol of each mutagenizing primer, 1× *Pfu* reaction buffer (Stratagene), 0.2 mM of each dNTP, and 2.5 units of *Pfu* turbo DNA polymerase (Stratagene) in a total volume of 50 μL. After incubation at 95 °C for 30 s, 12 cycles of 30 s at 95 °C, 60 s at 55 °C, and 14 min at 68 °C were conducted. After chilling on ice for 2 min, the restriction enzyme *Dpn*I (20 units) was added and the reaction was incubated at 37 °C for 60 min. An aliquot (1 μL) was used to transform competent *E. coli* DH5α cells. Following selection on plates of LB-Amp medium with ampicillin, nucleotide sequences were determined as mentioned above. Each plasmid was introduced into *E. coli* BL21 for protein expression.

Table 1
List of primer sets and templates used for construction of VBP-129 mutants.

Protein name	Primers[a]	Templates[b]
VBP-Int41	5′-GGAATTCCTGTCCGATTGCATCC-3′ 5′-GGAATTCCTGTCCGATTGCATCC-3′	pMAL-p2X:VBP-129
VBP-N52	5′-GGATCCCTGGGAAGCGATGCT-3′ 5′-CGTCGACTCATTTTAGGCGAAGT-3′	pMAL-p2X:VBP-129
VBP-Int55	5′-GGAATTCCTGTCCGATTGCATCC-3′ 5′-CGTCGACTCAAATTCTT TCGGTGT-3′	pMAL-p2X:VBP-129
VBP-Int55(C47S) or VBP-129(C80S)	5′-AATGCGCCAAAGCCTTAACAAAT-3′ 5′-ATTTGTTAAGGCTTTGGCGCATT-3′	pMAL-c2X: VBP-Int55 or pMAL-p2X:VBP-129
VBP-Int55(K50A) or VBP-129(K83A)	5′-ATGCCTTAACGCATACACCGAAAG-3′ 5′-CTTTCGGTGTATGCGTTAAGGCATT-3′	pMAL-c2X: VBP-Int55 or pMAL-p2X:VBP-129

[a] Primers for deletion mutants include artificial restriction sites (underlined). Primers for point mutants include mutagenizing codon in their central region (underlined).
[b] Template plasmid DNAs are derived from the previous study [20].

2.3. Recombinant protein expression

Recombinant proteins were prepared as described previously [20]. Briefly, *E. coli* cells transformed with each plasmid DNA were incubated overnight at 37 °C in 200-mL LB-Amp medium containing ampicillin (50 μg/mL). After dilution with 1800-mL fresh LB-Amp medium, 0.1 mM IPTG was added and the culture was incubated at 37 °C for 6 h. The fusion protein was purified by amylose resin column chromatography and digested with 1:500 (w/w) of factor Xa (Haematologic Technologies Inc.) at 4 °C for 18 h. The excised protein was then purified by DEAE Sephacel anion exchange chromatography (GE Healthcare Bioscience Japan). Trace metal ions were removed by dialysis against approximately 100 volumes of Tris–EDTA solution (100 mM NaCl, 25 mM Tris–HCl, 50 mM EDTA, pH 7.5). EDTA was removed by dialysis three times against approximately 100 volumes of binding buffer for each assay. The protein concentration was measured with the BioRad protein assay kit (Bio-Rad Laboratories, Inc.) using BSA as a standard.

2.4. Immobilized metal ion affinity chromatography (IMAC)

All buffers were prepared from DW and ultrapure-grade reagents, and degassed for 10 min under vacuum before use. Calcium chloride (Ca^{II}; $CaCl_2 \cdot 2\ H_2O$), cobalt sulfate (Co^{II}; $CoSO_4 \cdot 7\ H_2O$), copper chloride (Cu^{II}; $CuCl_2 \cdot 2\ H_2O$), iron chloride (Fe^{III}; $FeCl_3$), magnesium chloride (Mg^{II}; $MgCl_2 \cdot 6H_2O$), manganese chloride (Mn^{II}; $MnCl_2$), vanadyl sulfate (V^{IV}; $VOSO_4 \cdot nH_2O$, $n = 3$–4), and zinc chloride (Zn^{II}; $ZnCl_2$) were used as 99.9% pure reagents purchased from Wako Pure Chemicals, Japan. All these metal compounds were dissolved in DW at 0.1 M. Given that Fe^{III} and Zn^{II} solutions contain precipitates, only supernatants were used after settling the solutions for several minutes. Hence the concentration of Fe^{III} and Zn^{II} are slightly lower than the initial concentration of 0.1 M, but they were enough to charge the chelating resin. Chelating sepharose FF resin (GE Healthcare Bioscience Japan) was washed three times with water and mixed with each metal solution in a 2.0-mL plastic tube for 15 min (125 μL bed volume per tube). The resin was washed three times with DW and twice with binding buffer (100 mM NaCl, 20 mM Na_3PO_4, pH 7.2). The protein in the binding buffer (~300 μg/mL, 500 μL) was mixed with the resin by rotation for 30 min at 4 °C. Non-bound proteins were removed by centrifugation and the resin was washed three times with binding buffer. Bound proteins and metal ions were eluted with elution buffer (100 mM NaCl, 20 mM Na_3PO_4, 50 mM EDTA, pH 8.0, 400 μL). Non-bound and bound protein fractions were analyzed by SDS-PAGE and CBB staining.

2.5. ESR measurements

ESR measurements on Vanabin1 were done according to our previous study [22], while measurements on VBP-129 and its derivatives were done by a slightly modified protocol as described below.

In the binding assay on VBP-129, proteins were dialyzed against a buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.5; here we term it as a buffer A). Vanadyl sulfate (V^{IV}; $VOSO_4 \cdot nH_2O$, $n = 3$–4) was dissolved in DW at 1 mM immediately prior to use. After mixing the proteins with the appropriate amount of 10× concentrated stock of buffer A, the vanadium solution was added and the mixture was incubated for 5–10 min at room temperature. Longer incubation up to 3 h did