Biochimica et Biophysica Acta 1840 (2014) 3238–3245

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Participation of thioredoxin in the V(V)-reduction reaction by Vanabin2

Tatsuya Ueki [a,b,*], Masayuki Uwagaki [a], Sohei Yamamoto [a], Hitoshi Michibata [a,c]

[a] Molecular Physiology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan
[b] Marine Biological Laboratory, Graduate School of Science, Hiroshima University, 2445 Mukaishima, Onomichi, Hiroshima 722-0073, Japan
[c] Hiroshima University, Japan

ARTICLE INFO

Article history:
Received 2 May 2014
Received in revised form 25 July 2014
Accepted 30 July 2014
Available online 7 August 2014

Keywords:
Ascidian
Vanadium
Reductase
Metal
Redox

ABSTRACT

Background: It is well-understood that ascidians accumulate high levels of vanadium, a reduced form of V(III), in an extremely acidic vacuole in their blood cells. Vanabins are small cysteine-rich proteins that have been identified only from vanadium-rich ascidians. A previous study revealed that Vanabin2 can act as a V(V)-reductase in the glutathione cascade.
Methods: *As*Trx1, a thioredoxin gene, was cloned from the vanadium-rich ascidian, *Ascidia sydneiensis samea*, by PCR. *As*Trx1 and Vanabin2 were prepared as recombinant proteins, and V(V)-reduction by Vanabin2 was assessed by ESR and ion-exchange column chromatography. Site-directed mutagenesis was performed to examine the direct involvement of cysteine residues. Tissue expression of *As*Trx1 was also examined by RT-PCR.
Results: When reduced *As*Trx1 and Vanabin2 were combined, Vanabin2 adopted an SS/SH intermediate structure while V(V) was reduced to V(IV). The loss of cysteine residues in either Vanabin2 or *As*Trx1 caused a significant loss of reductase activity. V_{app} and K_{app} values for Vanabin2-catalyzed V(V)-reduction in the thioredoxin cascade were 0.066 mol-V(IV)/min/mol-Vanabin2 and 0.19 mM, respectively. The K_{app} value was 2.7-fold lower than that observed in the glutathione cascade. The *As*Trx1 gene was expressed at a very high level in blood cells, in which Vanabins 1–4 were co-expressed.
Conclusions: *As*Trx1 may contribute to a significant part of the redox cascade for V(V)-reduction by Vanabin2 in the cytoplasm of vanadocytes, but prevails only at low V(V) concentrations.
General significance: This study is the first to report the reduction of V(V) in the thioredoxin cascade.

1. Introduction

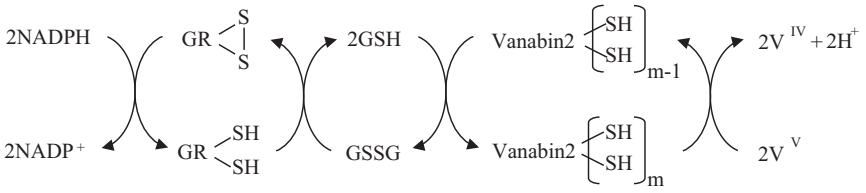
The unusual ability of ascidians to accumulate high levels of vanadium ions has been attracting attention in biological and chemical disciplines for over a century since the original finding by Henze (1911) [1]. The maximum concentration of vanadium can reach 350 mM in vanadocytes of *Ascidia gemmata*, belonging to the class Ascidiidae, which is believed to be the highest metal accumulation of any living organism [2]. 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 [3]. Most vanadium ions are stored in the vacuoles of one type of blood cell, specifically signet ring cells, called vanadocytes (vanadium-accumulating cells) [2]. 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, the proteins that could be responsible for the selective transport of vanadium are the vanadium-binding proteins, vanabins.

Vanabins generally contain 18 conserved cysteine residues and constitute a unique protein family present only in vanadium-rich ascidians [4–8]. The most studied vanabin is Vanabin2, which was isolated from *Ascidia sydneiensis samea* [7,9,10]. The three-dimensional structure of Vanabin2 was determined by nuclear magnetic resonance (NMR), which revealed 18 cysteine residues that comprise nine disulfide (SS) bonds between specific pairs of amino acid residues [11]. Although Vanabin2 was originally isolated as a V(IV)-binding protein, it was later determined to adopt an SS/SH intermediate structure and one that can act as a V(V)-reductase [12,13]. Based on previous studies, we hypothesized that the mechanism of action of Vanabin2 is similar to the cascade shown in Scheme 1. In this cascade, electrons are transferred from the donor NADPH to glutathione (GSH) via glutathione reductase (GR), and then to the acceptor V(V) ions via thiol–disulfide exchange reactions of Vanabin2.

On the other hand, another universally important redox factor in biological systems is thioredoxin (Trx). Trx was first identified as a low-molecular-weight redox protein in the deoxycytidine diphosphate synthesis pathway in *Escherichia coli*, together with thioredoxin reductase (TrxR) [14]. The Trx, TrxR and NADPH systems are, thereafter, found in all types of organisms from Archaea to humans. Reduced Trx serves as an electron donor for enzymes such as ribonucleotide reductases, peroxiredoxins, and methionine sulfoxide reductases, and is

* Corresponding author at: Marine Biological Laboratory, Graduate School of Science, Hiroshima University, 2445 Mukaishima, Onomichi, Hiroshima 722-0073, Japan. Tel.: +81 848 44 1434; fax: +81 848 44 5914.
E-mail address: ueki@hiroshima-u.ac.jp (T. Ueki).

http://dx.doi.org/10.1016/j.bbagen.2014.07.023
0304-4165/



Scheme 1. Redox cascade including glutathione (GSH).

common to many organisms and organism-specific functions in higher organisms [15]. Thus, we hypothesize that Trx may participate in the redox cascade for V(V)-reduction by Vanabin2 (Scheme 2).

In this study, we first isolated a homolog of Trx from the vanadium-rich ascidian *A. sydneiensis samea* to examine the participation of Trx in V(V)-reduction. We examined the redox cascade shown in Scheme 2 using recombinant Vanabin2 and Trx in *in vitro* reductase assays. Tissue localization of Trx was also examined by reverse-transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Reagents

Dithiothreitol (DTT), 2-mercaptoethanol (2-ME), and reduced GSH were purchased from Wako Pure Chemical Industries. Sodium orthovanadate (Na_3VO_4; >99.9%) was purchased from Sigma-Aldrich Co. Human thioredoxin (*Hs*Trx1) was purchased from Oriental Yeast Co., Ltd., as a recombinant protein.

2.2. Animals

Ascidian *A. sydneiensis samea* adults were collected at Yamada Bay, Iwate, Japan, and Kojima Port, Okayama, Japan. Blood was extracted and diluted with Ca^{2+}- and Mg^{2+}-free artificial seawater (460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 6 mM $NaHCO_3$, 5 mM HEPES, and 5 mM EDTA, pH 7.0). Blood cells were collected by centrifugation at 300 ×*g* for 10 min at 4 °C. Giant cells were removed by sucrose density gradient centrifugation, as this type of cell contains highly acidic materials that adversely affect protein and RNA extraction. Additional tissues were manually excised from each body part using scissors and tweezers.

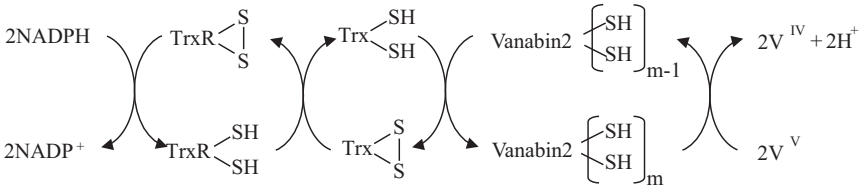
2.3. Molecular cloning of the AsTrx1 gene

Four degenerate primers from the conserved regions of known Trx family genes were designed: Trx-f, 5′-AAY GTN GGN TGY ATH CCN AA-3′; and Trx-r, 5′-TGN ACN CKR TRN CCC CAR TT-3′. Phage DNA amplified from the blood cell cDNA library from *A. sydneiensis samea* [16] was used as the PCR template. PCR was performed as follows: 100 ng DNA, Trx-f and Trx-r primers (200 pmol each), deoxynucleotide triphosphate (dNTP, 2 nmol each), 1 × reaction buffer, and 2.5 U *Taq* DNA polymerase (TaKaRa, Inc.) in a final reaction volume of 50 μL. After denaturation at 94 °C for 2 min, 30 cycles of PCR were performed (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s), followed by a final extension at 72 °C for 5 min. After cloning the DNA fragment into a plasmid vector, the PCR product was sequenced by the dideoxy termination method using an ALFexpress DNA sequencer and Thermo Sequenase kit (GE Healthcare). Rapid amplification of cDNA ends (RACE) was performed using the following primers: TrxR2-3RACE-1, 5′-GAA GAA TCT ATT TCA AAA TG-3′ for the 3′-end; and TrxR2-5RACE-1, 5′-TTG AAC TGC AGT TGA TAA TG-3′ for the 5′ end of the target DNA sequence using the same phage library DNA as the template. PCR fragments were then used for DNA sequencing. The combined full-length cDNA sequence of *As*Trx1 was submitted to DDBJ/EMBL/Genbank (accession number AB911105). Predicted amino acid sequences were compared with known Trx family proteins by the neighbor-joining method using the ClustalW software.

2.4. Preparation of recombinant proteins

Recombinant wild-type (WT) and mutant Vanabin2 proteins were prepared in accordance with procedures published previously [7,17]. Preparation of the *As*Trx1 protein was essentially identical to the published procedures. In some experiments, recombinant proteins were used as fusion proteins, which were fused to *E. coli* maltose-binding protein (MBP).

The cDNA region corresponding to the *As*Trx1 full-length coding region was amplified by PCR using a specific primer set with artificial restriction sites, as follows: *As*Trx1-F-*Eco*RI, 5′-GAA TTC ATG CCT TTG ATT TTA A-3′; and *As*Trx1-R-*Sal*I, 5′-GTC GAC TTA CTT GTG GGT-3′. Amplified fragments were then digested with *Eco*RI and *Sal*I, and ligated into the corresponding site of the pMAL-c2X expression vector (New England BioLabs Inc.). This vector contains a *lac* promoter and a coding region for MBP, to which the *As*Trx1 coding region was ligated to produce a fusion protein. The plasmid was introduced into *E. coli* BL21 cells. An overnight culture of non-induced *E. coli* cells bearing the expressed plasmid was diluted 1:10 in Luria broth (LB) medium containing 50 μg/mL ampicillin. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added to the medium, and cells were then cultured at 37 °C for 7 h. The fusion protein was purified by amylose resin column chromatography according to the manufacturer's protocol (New England BioLabs). The junction between MBP and *As*Trx1 was cut using Factor Xa (1/200 wt/wt) at 4 °C for 7 days. After dialysis against 50 mM Tris–HCl, pH 7.4, *As*Trx1 was purified by DEAE-Sephacel anion exchange column chromatography (GE Healthcare) and high-performance liquid chromatography (HPLC) using a 5C18-AR300 widepore column (Nakalai Tesque, Inc.). The resulting recombinant protein had four additional amino acids (I–S–E–F), which were derived from the junction region at the N-terminus. Protein concentration was measured using the protein assay CBB reagent (Nakalai) with bovine serum albumin (BSA, Pierce Inc.) as a standard.



Scheme 2. Possible redox cascade including thioredoxin (Trx).