Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1760 (2006) 495 – 503
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Glutathione transferases with vanadium-binding activity isolated from the vanadium-rich ascidian *Ascidia sydneiensis samea*

Masafumi Yoshinaga [a], Tatsuya Ueki [a], Nobuo Yamaguchi [b], Kei Kamino [c], Hitoshi Michibata [a,*]

[a] *Molecular Physiology Laboratory, Graduate School of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8526, Japan*
[b] *Marine Biological Laboratory, Graduate School of Science, Hiroshima University, Mukaishima 2445, Hiroshima 722-0073, Japan*
[c] *Marine Biotechnology Institute Co, Ltd., 3-75-1 Heita, Kamaishi City, Iwate 026-0001, Japan*

Received 4 November 2005; received in revised form 16 January 2006; accepted 18 January 2006
Available online 9 February 2006

Abstract

Some ascidians accumulate vanadium in vanadocytes, which are vanadium-containing blood cells, at high levels and with high selectivity. However, the mechanism and physiological significance of vanadium accumulation remain unknown. In this study, we isolated novel proteins with a striking homology to glutathione transferases (GSTs), designated *As*GST-I and *As*GST-II, from the digestive system of the vanadium-accumulating ascidian *Ascidia sydneiensis samea*, in which the digestive system is thought to be involved in vanadium uptake. Analysis of recombinant *As*GST-I confirmed that *As*GST-I has GST activity and forms a dimer, as do other GSTs. In addition, *As*GST-I was revealed to have vanadium-binding activity, which has never been reported for GSTs isolated from other organisms. *As*GST-I bound about 16 vanadium atoms as either V(IV) or V(V) per dimer, and the apparent dissociation constants for V(IV) and V(V) were 1.8×10^{-4} M and 1.2×10^{-4} M, respectively. Western blot analysis revealed that *As*GSTs were expressed in the digestive system at exceptionally high levels, although they were localized in almost all organs and tissues examined. Considering these results, we postulate that *As*GSTs play important roles in vanadium accumulation in the ascidian digestive system.

Keywords: Ascidian; Vanadium; Metal accumulation; Digestive system; Glutathione transferase

1. Introduction

Several species of ascidians, so-called sea squirts, accumulate high levels of vanadium [1,2]. In the most remarkable case, the cellular vanadium concentration reaches 350 mM, or roughly 10^7 times the concentration in seawater [3]. Through the accumulation process, almost all vanadium ions, thought to be present in the +5 oxidation state [V(V)] in seawater, are reduced to the +3 oxidation state [V(III)] via the +4 oxidation state [V(IV)] and are stored in the vacuole of vanadocytes, the vanadium-containing cells [4–6]. Several proteins that are likely involved in the vanadium-accumulation process have already been isolated from the blood cells of the vanadium-rich ascidian *A. sydneiensis samea* [7–15]. Of these, two similar proteins, Vanabin1 and Vanabin2, have attracted attention because of their binding ability and selectivity for vanadium [13–15]. In fact, Vanabin1 and Vanabin2 can bind 10 and 20 V (IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively, and the binding with V(IV) is barely inhibited in the presence of magnesium(II) or molybdate(VI) ions [15]. Three-dimensional structural analysis using nuclear magnetic resonance (NMR) [16] revealed that Vanabin2 has a novel bow-shaped conformation consisting of four helices connected by nine disulfide bonds, which has no reported structural homologs. V(IV) ions, which are exclusively localized on the same face of the Vanabin2 molecule, are mostly coordinated by amine nitrogens derived from amino acid residues, such as lysines and arginines, as suggested by electron paramagnetic resonance (EPR) results [17]. In addition, two Vanabin homologs, designated Vanabin3 and Vanabin4, were identified from the cytoplasm of vanadocytes in an expressed sequence tag (EST) analysis [18], and one Vanabin homolog, designated VanabinP, was isolated from the blood plasma

* Corresponding author. Tel./fax: +81 82 424 7438.
E-mail address: hmichi@hiroshima-u.ac.jp (H. Michibata).

0304-4165/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2006.01.008

(coelomic fluid) using V(IV)-chelating column chromatography [19]. Since all five of these Vanabins are rich in charged residues and have conserved motifs described as the consensus sequence {C}−{X_{2-5}}−{C}, they have been placed in the Vanabin family, and should provide a clue to resolving the problem of the selective accumulation of vanadium in ascidians [15,18,19].

The vanadium-accumulating pathway from seawater into the ascidian coelom has never been investigated. Therefore, in this study, we used a V(IV)-chelating column to isolate vanadium-binding proteins from the digestive system, which is thought to be involved in vanadium uptake. Consequently, we isolated and identified two similar, but slightly different, vanadium-binding proteins with striking homology to GSTs, designated *As*GST-I and *As*GST-II. Like other GSTs, recombinant *As*GST-I formed a dimer, and it not only showed GST activity, but also bound to both V(IV) and V(V). Moreover, using immunoblotting, the expression level of these two *As*GSTs in the digestive system was found to be exceptionally high compared to the major organs and tissues. Therefore, *As*GSTs may play important roles in vanadium accumulation in the ascidian digestive system.

2. Materials and methods

2.1. Specimens

Specimens of the vanadium-rich ascidian *A. sydneiensis samea* were collected at the International Coastal Research Center, Ocean Research Institute, The University of Tokyo, Otsuchi, Iwate, Japan. They were maintained in an aquarium that contained circulating natural seawater until use.

2.2. Screening vanadium-binding proteins from the ascidian digestive system using V(IV)-chelating column chromatography

To avoid the analytical difficulty that arises from individual differences, especially in the analysis of amino acid sequences, we used one individual per preparation. After the tunic was removed from the ascidian, the body wall was dissected. The digestive system was separated and washed thoroughly with artificial sea water (ASW; 460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 5 mM HEPES, 6 mM $NaHCO_3$, pH 7.0) to remove the contents of the digestive system. The specimen was then homogenized in five volumes (ml per gram wet weight) of a homogenizing buffer (200 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0) using a Teflon homogenizer. The homogenate was centrifuged at 21,600×*g* for 10 min, and the resulting supernatant was filtered through a cellulose acetate filter (0.45-μm pores) to remove lipidic floats, enclosed in a dialysis tube (6–8000 Da cut-off), and dialyzed against 100 volumes of a chelating buffer (50 mM Tris–HCl, 500 mM NaCl, 25 mM EDTA, pH 7.2) to remove any metal ions that might interfere with the efficiency of V(IV)-chelating column chromatography. Next, the sample was dialyzed three times in 100 volumes of a binding buffer (20 mM Na_2HPO_4, pH 7.2) to remove any EDTA, which might also interfere with chromatography. The protein solution after dialysis was centrifuged at 21,600×*g* for 10 min and filtered through a cellulose acetate filter (0.45-μm pores) to remove the proteins insolubilized during dialysis.

Chelating Sepharose Fast Flow (Amersham Biosciences) was packed into a polypropylene column (bed size, 1 cm ϕ × 10 cm). V (IV) ions were chelated to the Sepharose by adding twice the bed volume of 200 mM $VOSO_4{\cdot}xH_2O$ (Wako). The Sepharose column was washed with ten bed volumes of distilled water (DW) to remove the unbound excess V(IV) ions, and then equilibrated to the binding buffer. The prepared protein solution was loaded onto the V(IV)-chelating column. After the application, the Sepharose was washed thoroughly using binding buffer, and the absorbed proteins were eluted using an elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, 50 mM EDTA, pH 7.2). Portions of the protein samples from each chromatography step were analyzed using SDS-PAGE on a 12.5% gel.

2.3. Determining the N-terminal partial amino acid sequence of vanadium-binding proteins

The chromatography eluent was concentrated by ultrafiltration using a Centriplus YM-3 (Millipore), and the proteins were further separated using reverse-phase high performance liquid chromatography (HPLC). HPLC was performed using a 5C18-AR-300 column (2.0 × 150 mm, Nacalai Tesque) with water and acetonitrile (both containing 0.1% trifluoroacetic acid). The proteins were eluted at a flow rate of 0.2 ml/min using a linear gradient of 10–70% acetonitrile over 60 min. Two major protein peaks detected 53 and 55 min after injection were collected, dried under vacuum in a rotatory concentrator, and dissolved in a sample buffer for SDS-PAGE. The samples were subjected to SDS-PAGE on a 12.5% gel and electro-blotted onto polyvinylidene fluoride (PVDF) membrane. The N-terminal partial amino acid sequences of the protein bands corresponding to the two major protein peaks were determined using the Edman degradation method [20].

2.4. Isolation of cDNA clones for the two major vanadium-binding proteins

A degenerate primer was designed based on the sequence common to the N-terminal partial amino acid sequences obtained from the two major protein peaks as follows: 28K/F, 5′-GTN AAR TTY TAY TTY AAY GAY-3′ for Val–Lys–Phe–Tyr–Phe–Asn–Asp. Using a cDNA library of vanadocytes from the ascidian *A. sydneiensis samea* constructed with UniZap XR vector [18] as the template, PCR was performed with the degenerate primer and primer T7 using Eazy A™ High-Fidelity PCR Cloning Enzyme (Stratagene). The PCR mixture was denatured at 95 °C for 2 min and then cycled 30 times at 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min, with a final 7-min extension at 72 °C. The amplified fragments were TA-cloned in pBluescript SK⁻vector (Stratagene) according to the manufacturer's instructions. The constructed plasmids were used for sequencing with Thermosequenase using an ALF Express II DNA sequencer (Amersham Biosciences).

2.5. Expression and purification of recombinant AsGST-I

A specific primer was designed based on the coding region for the C-terminus of the *As*GSTs containing the termination codon and a restriction enzyme site for *Sal*I as follows; *As*GST/R sal, 5′-GGT CGA CTT ATT CGG TTC TC-3′. Using primers *As*GST/R sal and T3, PCR was performed as described above changing the DNA annealing temperature from 45 to 50 °C. The PCR products were sequenced as described above to determine the sequence of the degenerate primer region and the location of the initiation methionine. Based on the sequences, another specific primer was designed to amplify the coding regions of the putative mature *As*GSTs, including the initiation methionine, using primer *As*GST/R sal; this primer was *As*GST/F, 5′-ATG ACA GTC AAA TTT TAT TTC AAC G-3′. Using primers *As*GST/F and *As*GST/R sal, PCR was performed as described above with an annealing temperature of 50 °C. The PCR products were TA-cloned in pETblue1 expression vector (Novagen). The constructed vector containing the *As*GST-I or *As*GST-II gene was introduced into *Escherichia coli* Tuner™(DE3)pLacI strain (Novagen).

The *E. coli* cells bearing the *As*GST-I-expressing vector were cultured in LB medium containing 0.5% glycerol, 50 μg/ml ampicillin, and 34 μg/ml chloramphenicol at 37 °C to an OD_{600} of approximately 0.6–1.0. After storing at 4 °C for 4–8 h, the culture was diluted approximately 30 times in fresh LB medium containing 0.5% glycerol, 50 μg/ml ampicillin, and 34 μg/ml chloramphenicol. The culture was incubated with shaking at 25 °C until the OD_{600} was approximately 0.5–1.0. After adding isopropyl-beta-D-thiogalacto-pyranoside (IPTG) to a final concentration of 0.5 mM, the cells were incubated with shaking at 25 °C for 8 h. The cells were collected by centrifugation at 4000×*g* for 10 min, resuspended in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, pH 7.2), and sonicated on ice until clear. The lysate was centrifuged at 10,000×*g* for 10 min. The resulting supernatant was enclosed in