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

Tatsuya Ueki[1,2,*], Masayuki Uwagaki[1], Sohei Yamamoto[1], and Hitoshi Michibata[1, 3]

[1] Molecular Physiology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan.

[2] Marine Biological Laboratory, Graduate School of Science, Hiroshima University, 2445 Mukaishima, Onomichi, Hiroshima 722-0073, Japan.

[3] Professor Emeritus, Hiroshima University, Japan.

**Corresponding author: ueki@hiroshima-u.ac.jp*

TEL: +81-848-44-1434, FAX: +81-848-44-5914.

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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 Vanbin2 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 system is, 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 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 real-time 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 sea water (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 *Escherichia 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, pH7.4, *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.

Cysteine residues at positions 33 and 36 in the putative redox active site were subjected to *in vitro* mutagenesis. A set of mutagenesis primers (5'-TTT GCA GAC TGG AGT GGA CCG AGC AAA GTT ATT-3' and 5'-AAT AAC TTT GCT CGG TCC ACT CCA GTC TGC AAA-3') were designed to mutate the two cysteines into serines. Each reaction mixture contained plasmid DNA (WT *As*Trx1 in pMAL-c2X, 10 ng), 12.5 pmol each primer, and 1 × KAPA HiFi Reaction Mix (KAPA Biosystems) in a total volume of 50 μL. After denaturation at 95°C for 2 min, 16 cycles PCR were performed (20 s at 98°C, 30 s at 57°C, and 6 min at 72°C). The restriction enzyme *Dpn*I (20 U) was added to the reaction, and the mixture was incubated at 37°C for 60 min. An aliquot (5 μL) was used to transform competent *E. coli* DH5α cells. Following selection on LB plates with ampicillin, nucleotide sequences were determined 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). A representative plasmid (clone #uk43) was introduced into *E. coli* BL21 cells for protein expression, as well as the WT.

2.5. Mobility shift assay by SDS-PAGE

The mobility shift assay was performed as described previously by [12]. Briefly, each reaction contained 1.5 μM Vanabin2, 0–3.0 μM *As*Trx1, 0.1 mM DTT, and 50 mM Tris-HCl, pH 7.4 in a final volume of 100 μL. Immediately after mixing each reaction, 20-μL aliquots were mixed with loading dye lacking a reducing agent (62.5 mM Tris–HCl, 2.3% SDS, 10% glycerol, and 0.05% bromophenol blue), and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Brilliant Blue (CBB) staining.

2.6. Reductase assay using ESR spectrometry

Reaction mixtures for the reductase assays were as follows: 1.25 µM Vanabin2, 0–2.5 µM *As*Trx1, 0.1 mM DTT, 8.3 mM V(V), 3.3 mM EDTA, and 50 mM Tris-HCl, pH 7.4. The concentrations of V(V) and ethylenediaminetetraacetic acid (EDTA) were proportionally reduced where indicated. The final reaction volume was 120 µL. Vanabin2 and *As*Trx1 were not added to negative controls, as described in the Figure legends. After incubation at 20°C for 2 h, 50-µL aliquots were removed from each reaction, placed in a quartz tube (5 × 100 mm, ST-X-5, Agri Co.), and frozen immediately in liquid nitrogen. ESR signals were recorded using the ELEXSYS-II E500 CW-ESR (Bruker BioSpin) with the ER 4112V temperature control system at the N-BARD. Samples were inserted into the quartz dewar, which was held in a steam of temperature-controlled nitrogen gas. ESR measurements were read at 79 K with a frequency of 9.4 GHz and a modulation (100 kHz) of 1 mT. ESR intensities were calculated by integrating the peak at $3/2_{\perp}$ peak in each ESR spectrum. Vanadyl sulfate (0–50 µM) mixed with 50 mM EDTA was used as the standard solution to calibrate the concentration of V(IV) products after enzymatic reactions.

2.7. Reductase assay using ion-exchange chromatography

Reaction mixtures were the same as those described in Section 2.6 in a final volume of 120 µL except for protein concentrations.

After incubation at 20°C for 2 h, 20 µL aliquots were removed from each reaction and injected into an ion exchange column (SI-50G, Shodex Inc.), equilibrated with 12 mM sodium hydrogen carbonate, 4 mM sodium carbonate, and 20 mM EDTA at a flow rate of 0.7 mL/min. Vanadium ions were detected by ultraviolet (UV) absorption at 282 nm and recorded by a D-7500 integrator (Hitachi). Under these conditions, V(IV) eluted 30 s after injection, while V(V) eluted 1 min following injection. The amount of reduced V(IV) was calculated as the relative area ratio calculated from each peak profile.

2.8. Semi-quantitative RT-PCR

Total RNA extraction from ascidian tissues was performed as described previously [5]. Reverse transcription was performed using ReverTra Ace reverse transcriptase (TOYOBO) and the dT15 primer. PCR primers for each gene were as follows: *As*Trx1, 5’-TTG CAG ACT GGT GTG GAC-3’ and 5’-CCA CGT TGC ATA TAC AAA TGT-3’; VanabinP, 5’-GAA TTC

CGA AAG AAA AAG AAG-3’ and 5’-CTC GAG TCA ACC TTC AAA CAA-3’; β-actin, 5’-GAC CTA TGC TGC TCT TGA-3’ and 5’-CAG GAT GGA TCC TCC AAT-3’; and EF-1α, 5’-TTT GCG CCA ACC AGT CTT AC-3’ and 5’-AGC TCC TGA AAC TTG CAA GC-3’. Each PCR mixture contained reverse-transcribed DNA from 2 ng of total RNA, forward and reverse primers (10 pmol each), dNTPs (2 nmol each), 1 × PCR buffer, and 0.5 U HybriPol DNA Polymerase (Bioline, Inc) in a total reaction volume of 10 μL. After incubation at 94°C for 2 min, 27 cycles of PCR were performed (30 s at 94°C, 30 s at 50°C, and 30 s at 72°C). Amplified fragments were examined by 1.8% agarose gel electrophoresis and ethidium bromide staining. Relative band strength was calculated by densitometry using a FAS III gel documentation system (TOYOBO) and the ImageJ 1.46r software (http://imagej.nih.gov/ij/). Under these conditions, amplification of each gene was significant and did not reach saturation, as judged from the densitometric data by varying cycle number from 23 to 30.

3. Results

3.1. Cloning of the AsTrx1 gene

cDNA fragments related to a Trx gene from the *A. sydneiensis samea* blood cell cDNA library were initially amplified by PCR using a pair of degenerate primers corresponding to conserved amino acid sequences of known Trx1 family proteins. We then performed 5’- and 3’-RACE to obtain the full-length cDNA sequence from the same cDNA library. The full-length cDNA sequence contained a single, long open reading frame (ORF) 324 nucleotides in length, including the stop codon, which encoded a protein 107 amino acids in length. The amino acid sequence deduced from the cDNA clone showed striking similarities to Trx1 proteins from various organisms (Fig. 1). The predicted amino acid sequence contained a putative redox active motif (C-G-P-C), which is commonly found in all Trx1 proteins. Therefore, we concluded that the cDNA clone corresponded to a Trx1 homolog in *A. sydneiensis samea*, which we refer to as *As*Trx1.

3.2. Mobility shift assay

Vanabin2 changes its conformation in the presence of reducing agents, and these changes can be detected as a shift in mobility on SDS-PAGE gels [12]. Here, we examined the structural changes of Vanabin2 upon addition of the reduced form of *As*Trx using gel

electrophoresis. In the present study, 0.1 mM DTT was used as the reducing agent since we have been unable to clone TR, and our previous study revealed that Vanabin2 mobility did not change in the presence of 0.1 mM DTT [12].

Using an *E. coli* expression system, we prepared recombinant Vanabin2 and *As*Trx1 proteins (Fig. 2A). Recombinant Vanabin2, containing nine fully oxidized disulfide bonds, was observed by SDS-PAGE at a position corresponding to 14 kDa [12]. In the presence of 0.1 mM DTT, Vanabin2 migrated to a position corresponding to a much larger size in response to increasing concentrations of *As*Trx1 (Fig. 2B). The apparent size of Vanabin2 peaked in the presence of *As*Trx1 (1:1 ratio), but the apparent size did not achieve that of the fully reduced form (20 kDa) [12], suggesting that the disulfide bonds of Vanabin2 were partially reduced to facilitate cleavage in the presence of a low concentration of DTT and a stoichiometric ratio of 1:1 with *As*Trx1.

3.3 Coupled assays for V(V)-reductase activity in Vanabin2–AsTrx1 cascade

TrxR is necessary to examine V(V)-reduction by the cascade described in Scheme 2, but we have been unsuccessful cloning TrxR from *A. sydneiensis samea*. Therefore, we utilized DTT as the initial reducing agent. As described in the previous section, the disulfide bonds of Vanabin2 were not cleaved by 0.1 mM DTT, but were partially reduced to cleave in the presence of 0.1 mM DTT and at a 1:1 stoichiometric ratio with *As*Trx1. Thus, in the presence of DTT and *As*Trx1, the redox cascade in Scheme 3 was examined. To measure the rate of V(V) reduction, V(IV) was directly measured by ESR and ion-exchange chromatography.

Results are summarized in Figure 3. Briefly, when 0.1 mM DTT was added to V(V) without *As*Trx1 and Vanabin2, no reduction occurred. In the presence of either *As*Trx1 or Vanabin2, a low level of V(V)-reduction was observed. When both *As*Trx1 and Vanabin2 were mixed in the presence of 0.1 mM DTT, *As*Trx1-dependent reduction of V(V) was observed.

The reduction of V(V) by Vanabin2 was catalyzed by SH/SS exchange between Vanabin2 and V(V) [17]. Here, we determined that the cascade including *As*Trx1 is also dependent on cysteine residues in Vanabin2. As shown in Figure 4, the V2mSS1-9 mutant lacking all nine cysteine pairs did not catalyze the reduction of V(V). Thus, the last step of the Trx cascade (Schemes 2 and 3) is similar to that in the GSH cascade (Scheme 1).

The C-X-X-C motif, which is commonly found in Trxs, has been revealed to be the redox active site that reduces disulfide bonds by a ping-pong mechanism [18]. In *As*Trx1, the

C-G-P-C sequence from positions 33 to 36 corresponds to this motif (Fig. 1). To assess the contribution of this motif to the reduction of Vanabin2, the possible redox active cysteines were deleted from *As*Trx1 by substitution to serines. This C33SC36S mutant contributed approximately half of the V(V)-reduction by Vanabin2 when the initial concentration of V(V) was 8.3 mM (Fig. 5A).

Kinetic analysis was performed by varying the V(V) concentration from 0.25 to 8.3 mM (Fig. 5B and C). V_{app} and K_{app} values for Vanabin2-catalyzed V(V) reduction in this system were 0.066 mol-V(IV)/min/mol-Vanabin2 and 0.19 mM, respectively. As compared to the observed V_{app} and K_{app} values in the GSH system (1.15 mol-NADPH/min/mol-Vanabin2 and 0.51 mM, respectively) [12], V_{app} was ~20-fold lower than that of the GSH system, but the K_{app} was 2.7-fold lower than that of the GSH system. These results suggest that the Trx system may act in blood cells, but prevails only at low V(V) concentrations.

3.4 Coupled assays for V(V)-reductase activity in Vanabin2–human Trx1 cascade

In order to assess whether the redox coupling reaction is specific to ascidian thioredoxin or not, we performed an coupled assay using human Trx1 (*Hs*Trx1). The purity of commercially obtained *Hs*Trx1 was examined by SDS-PAGE (Fig. 6A), and the protein was used without any further purification. As a result, *Hs*Trx1-dependent reduction of V(V) was observed (Fig. 6B). The final product V(IV) was about 50% more than that using *As*Trx1 as a component. This result suggests that the reductase-coupling function of thioredoxin is conserved between ascidians and humans.

3.5 Tissue localization of AsTrx1

To examine the tissue localization of *As*Trx1, we performed semi-quantitative RT-PCR analysis. Two housekeeping genes, β-actin and elongation factor-1α (EF-1α), were tested to determine whether they could be used as controls. The results revealed that expression of these housekeeping genes was lower in blood cells than in the other three tissues examined (Fig. 6). Since the expression profiles were similar, these genes were used as controls.

*As*Trx1 expression was similar to that of the housekeeping genes in blood cells, but was undetected in the brachial sac, and very low in the intestine and muscle. When expression of *As*Trx1 was normalized to that of β-actin, the expression level in blood cells was 20- or

10-fold higher than in the brachial sac and muscle, respectively. When normalized to EF-1α, its expression in blood cells was 30- or 15-fold higher than that in the brachial sac and muscle, respectively. Thus, the expression of *As*Trx1 in blood cells is relatively high.

4. Discussion

This study is the first to report the reduction of vanadium in the Trx cascade. In this study, we first isolated a homolog of Trx from blood cells of the vanadium-rich ascidian *A. sydneiensis samea*. The cDNA clone, *As*Trx1, encoded a protein 107 amino acids in length, which is closely related to Trx1 in animals (Fig. 1). Generally, most animals possess two Trxs and several Trx-related proteins [19,20]. Trx1 is expressed in the cytoplasm, while Trx2 is expressed in mitochondria [21,22]. Thus far, we have been unable to isolate Trx2 from this species of ascidian, although we attempted to obtain it using PCR with several pairs of degenerate primers. This problem could be due to increased sequence diversity in Trx2 compared with Trx1, and is an important limitation in our trials.

Both Trx1 and Trx2 have been identified in genome and EST analyses on another ascidian, *Ciona intestinalis*, and are registered in public databases under the GenBank accession numbers AK112286 and AK114174.1, respectively. A previous report indicated that Trx1 is expressed in *C. intestinalis* blood cells, and its expression is constant during development [23]. In our previous microarray study, we determined that the expression of Trx1 and Trx2 was not significantly affected by treatment with excess vanadium [24]. To our knowledge, detailed studies on Trx in *C. intestinalis* have yet to be published.

This study is also the first to report metal reduction by the Trx cascade in eukaryotes. Reduction of metal ions by the Trx system has been reported for U(VI) and Cr(VI) in bacteria. In the *mre* operon of the *Desulfovibrio desulfuricans* bacterium, a novel oxidoreductase MreG was demonstrated to reduce U(VI) and Cr(VI) in the presence of Trx, TrxR and NADPH [25]. It is proposed that MreG functions to protect cells against oxidation by metal ions. A similar reduction cascade was reported for As(V), although it is not a metal. A family of ArsC reductase proteins, including a typical protein from the *E. coli* plasmid R773 ArsC, can reduce As(V) to As(III) using Grx and GSH as reductants [26]. Another family represented by the *Staphylococcus aureus* plasmid, pI258 ArsC, also uses Trx as a reductant [27]. Similar reduction cascades could exist in other eukaryotes. Since vanabins are only found in vanadium-rich ascidians, another type of reductase could participate in such cascades.

In this study, it was revealed that reduction of V(V) was observed when human Trx1 was

added as a component (Fig. 6B). This must not occur in nature, but the fact suggests the highly conserved structure and function of *As*Trx and human Trx1. Structural modeling indicated that their structures were predicted to be highly conserved (RMSD = 0.088) (Fig. 8). This high level of structural homology could explain the combination of Vanabin2 from the ascidian and human Trx1 works *in vitro*.

In our previous work [12], we hypothesized that Vanabin2 is reduced by GSH (Scheme 1). In this study, we hypothesized that Vanabin2 is reduced by Trx (Scheme 2). It was proved that the cascade including *As*Trx1 is also dependent on cysteine residues in Vanabin2 (Fig.4). Thus, the last step of the Trx cascade is similar to that in the GSH cascade. When the possible redox active cysteines were deleted from *As*Trx1, the reductase activity fell down to about half (Fig. 5A), suggesting the possibility of participation of other cysteines in *As*Trx. In both cascades, the reducing potential is provided by NADPH, which is produced in the pentose phosphate pathway that is exclusively localized in vanadocytes [16,28,29]. Thus, these two cascades could exist and function together in the cytoplasm of vanadocytes. Potential differences between these two cascades may be: (1) the availability of GSH and Trx, and (2) kinetic parameters.

Cysteines with low p*K*a values are ionized and can be highly reactive in buffers at physiological pH [30,31]. As summarized in the review by Cheng et al., p*K*a values for Cys32 in *E. coli* Trx range from 6.3 to 10.0, while that of Cys35 is less well-defined, somewhere between 7.0 and 14.0 [30]. The p*K*a value for the thiol in GSH is 8.7. Judging from these p*K*a values, it is difficult to conclude which cascade is prevailing in the cytoplasm of vanadocytes. The most probable hypothesis is that these two cascades coexist and work together, but the relative contribution depends on other factors, such as redox state, concentrations of GSH and Trx, and the concentration of V(V) in the cytoplasm.

A link between Trx and GSH systems has been reported in yeast and bacteria [32]. Yeast lacking the glutathione reductase gene (*GLR1*) requires either one of the two Trx genes for growth under aerobic conditions [33]. Moreover, loss of both Trx genes results in elevated levels of GSSG, indicating a link between the Trx and GSH systems [33]. In *E. coli*, both the Trx and glutaredoxin systems contribute to reduction of disulfide bonds in alkaline phosphatase, and these systems can partially replace each other *in vivo* [34]. Thus, at least in these microorganisms, Trx and GSH systems have overlapping functions and can compensate for each other. The contribution of Trx and GSH on V(V)-reductase function of vanabins in ascidian blood cells should be examined *in vivo* using gene-knockout experiments.

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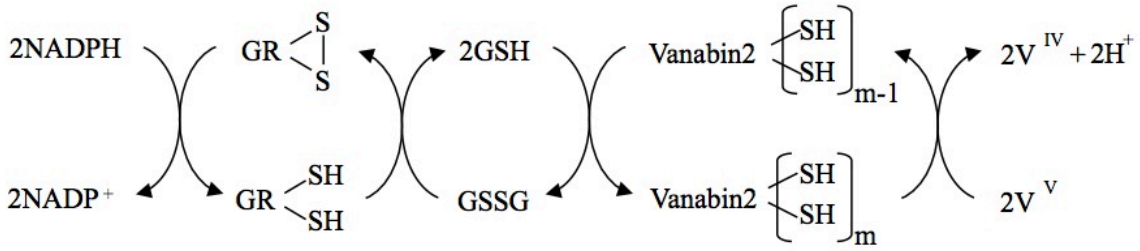
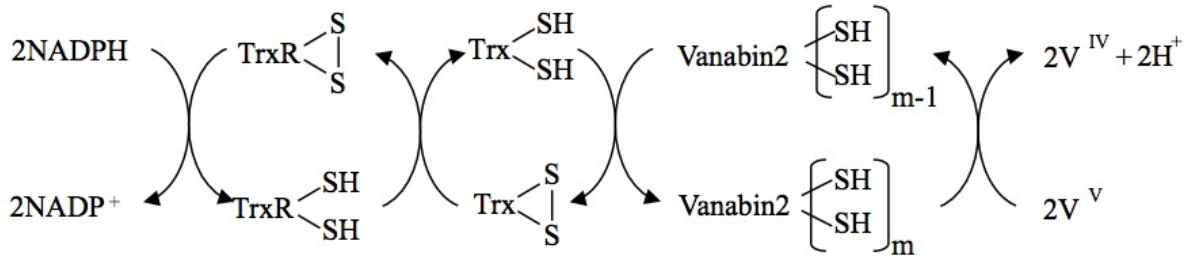
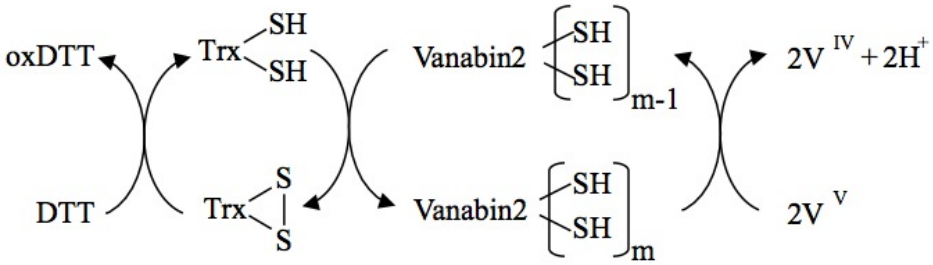
Figure and Scheme legends



Scheme 1. Redox cascade including glutathione (GSH).



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



Scheme 3. Experimental cascade used to examine the cascade in Scheme 2. Reduced DTT supplies the reduction potential in this cascade.

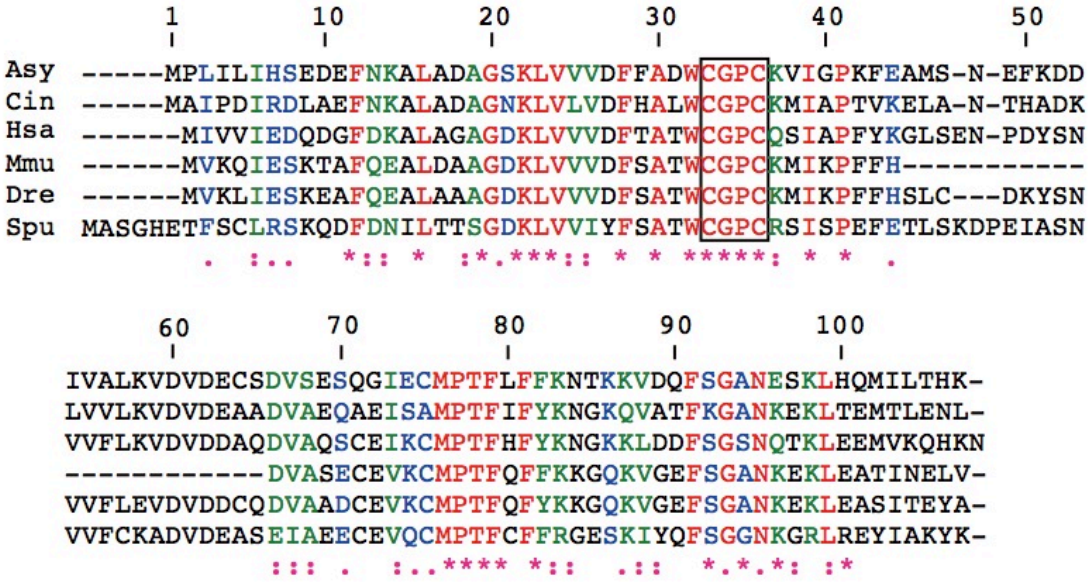


Fig. 1. Amino acid sequences of Trxs from *Ascidia sydneiensis samea* and closely related Trxs from several representative animals. Asy, *As*Trx1 from *Ascidia sydneiensis samea* (this study); Cin, *Ciona intestinalis* Trx1 (KEGG ID: 100183957); Hsa, *Homo sapiens* Trx1 (7295); Mmu, *Mus musculus* Trx1 (22166); Dre, *Danio rerio* Trx1 (zebrafish) (336637); Spu, *Strongylocentrotus purpuratus* Trx1 (purple sea urchin) (752709). Numbers indicate amino acid positions in Asy. Identical or conserved residues are indicated in red (6/6), green (5/6) and blue (4/6). The redox active site (C-G-P-C) is boxed.

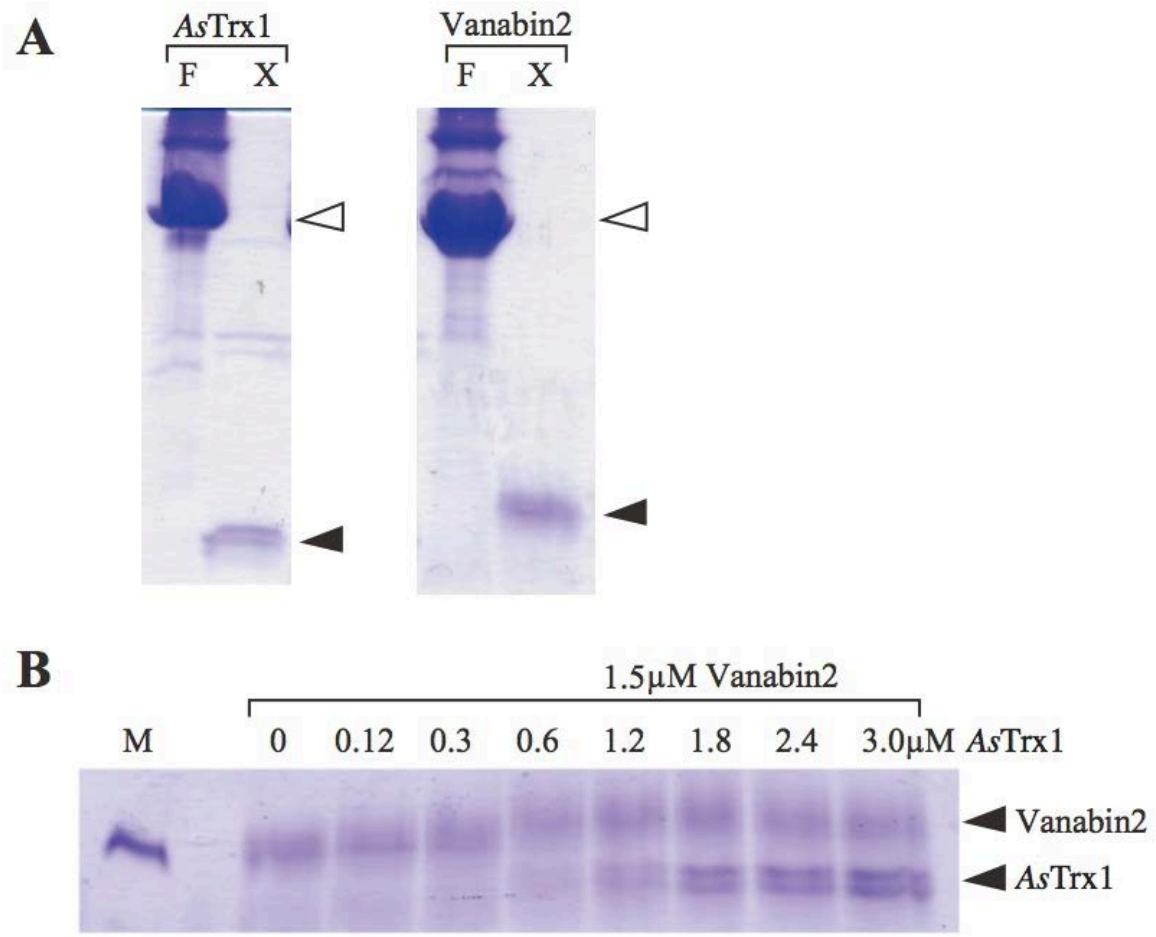


Fig. 2. *As*Trx1 and Vanabin2: protein expression, purification and mobility shift assay. (A) *As*Trx1 and Vanabin2 were purified using amylose resin as a fusion protein (open arrowheads, lane F), and further purified by anion-exchange column chromatography after digestion with Factor Xa (solid arrowheads, lane X). (B) Mobility shift assay on Vanabin2 by the addition of increasing amounts of *As*Trx1. M, 14-kDa marker protein. 0–3.0 μM *As*Trx1 was added to a constant amount of Vanabin2 (1.5 μM) in the presence of 0.1 mM DTT. Note that the mobility of Vanabin2 shifted from 14 kDa (apparent size) to a larger size, depending on the concentration of *As*Trx1.

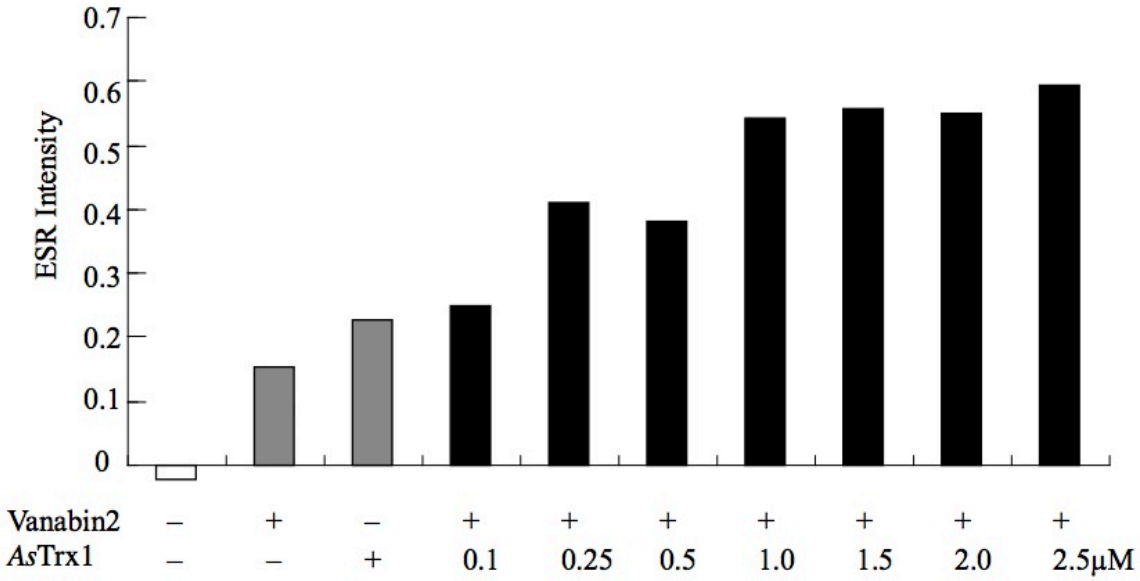


Fig. 3. Reduction of V(V) by Vanabin2 in the presence of *As*Trx1 and DTT. Results from a representative experiment. Common reaction components included 0.1 mM DTT, 8.3 mM V(V), 3.3 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Vanabin2 and *As*Trx1 were not added to negative controls. Incubation temperature and time were 20°C and 2 h, respectively. Plus symbols indicate the addition of 1.25 μM Vanabin2 or *As*Trx1, and increasing concentrations of *As*Trx1 were added as indicated. The vertical axis indicates relative differential ESR peak height.

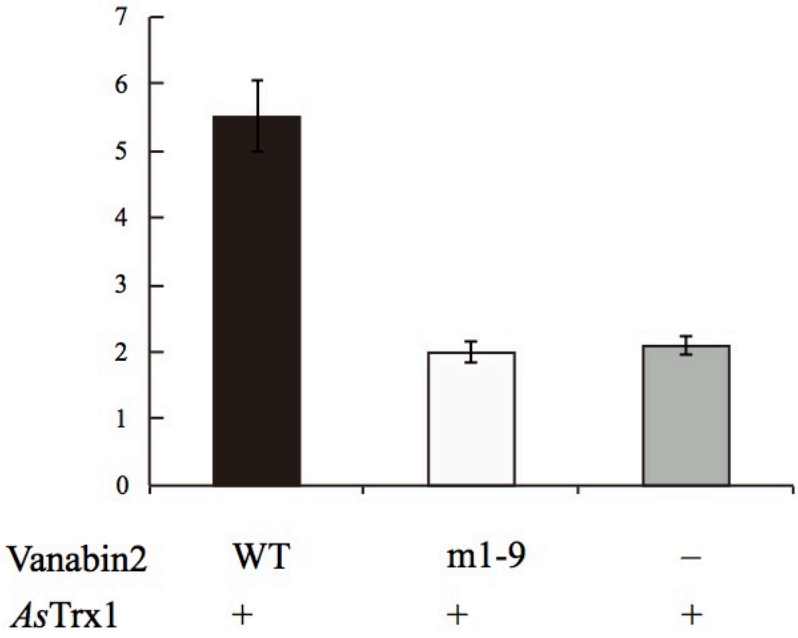


Fig. 4. Reduction of V(V) by a combination of wild-type (WT) or mutant Vanabin2 with WT *As*Trx1. Common reaction components included 2.5 μM WT *As*Trx1, 0.1 mM DTT, 8.3 mM V(V), 3.3 mM EDTA, and 50 mM Tris-HCl, pH 7.4 in a final volume of 120 μL. WT Vanabin2-MBP or a mutant Vanabin2-MBP lacking all 18 cysteine residues (m1-9) were added at a concentration of 1.25 μM, as indicated. Incubation temperature and time were 20°C and 2 h, respectively. The vertical axis indicates relative area ratio for V(IV) calculated from each peak profile by ion-exchange chromatography. Results are presented as the means ± standard deviation (S.D.), n = 4.

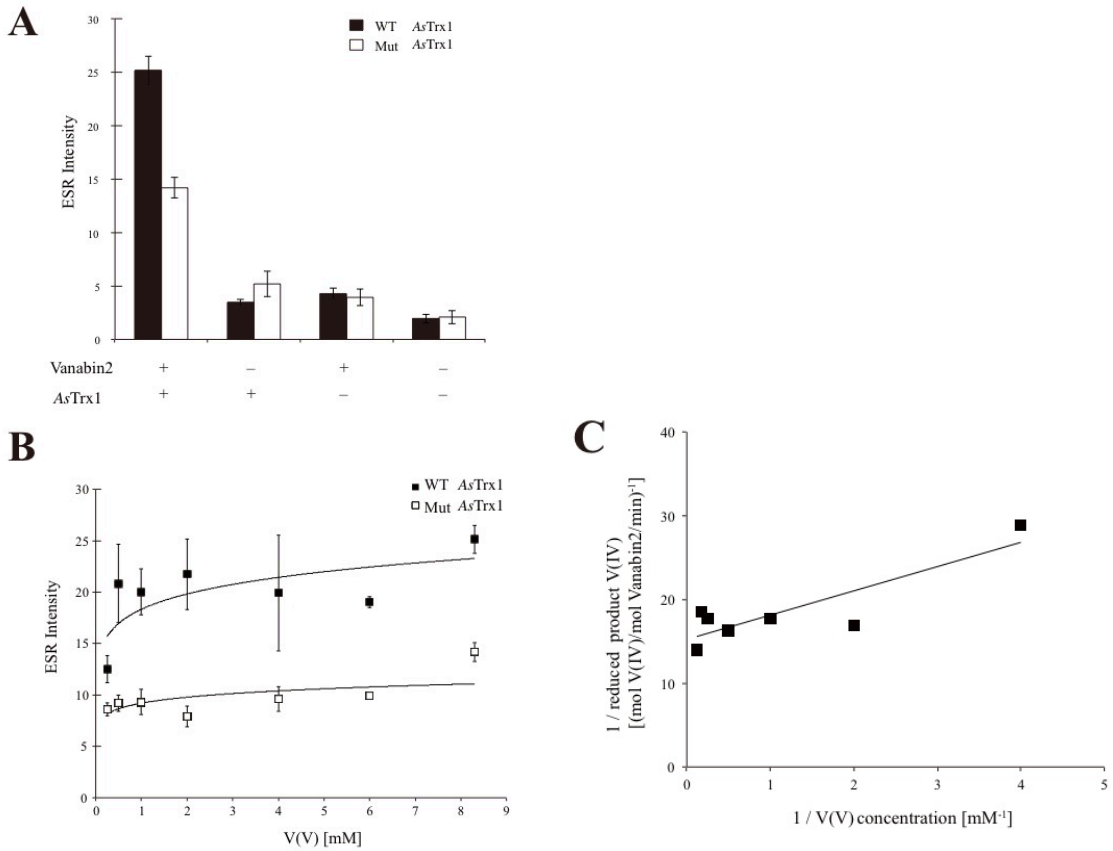


Fig. 5. Reduction of V(V) by a combination of WT Vanabin2 and WT or C33SC36S mutant *As*Trx1. Common reaction components included 0.1 mM DTT and 50 mM Tris-HCl, pH 7.4. Incubation temperature and time were 20°C and 2 h, respectively in a final reaction volume of 120 µL. (A) Comparison of the reduction by WT Vanabin2 combined with WT or mutant *As*Trx1. Vanabin2 was added at a concentration of 1.25 µM, as indicated by a plus sign. WT or mutant *As*Trx1 was added at a concentration of 2.5 µM, as indicated by a plus sign. V(V) (8.3 mM) and EDTA (3.3 mM) were also added. The vertical axis indicates ESR intensity, and results are presented as the means ± S.D., n = 3. (B) Kinetic analysis of V(V)-reduction by Vanabin2 (1.25 µM) in the presence of WT or mutant *As*Trx1 (2.5 µM). The horizontal axis indicates the initial concentration of V(V). The vertical axis indicates ESR intensity, and results are presented as the means ± S.D., n = 2-4. (C) Lineweaver–Burk plots calculated from (B). In these experiments, ESR intensity was calculated as an integrated value for $3/2_{\perp}$ peak, and the actual V(IV) concentration was determined using the equation [V(IV)] (µM) = 0.44 × ESR intensity - 0.31.

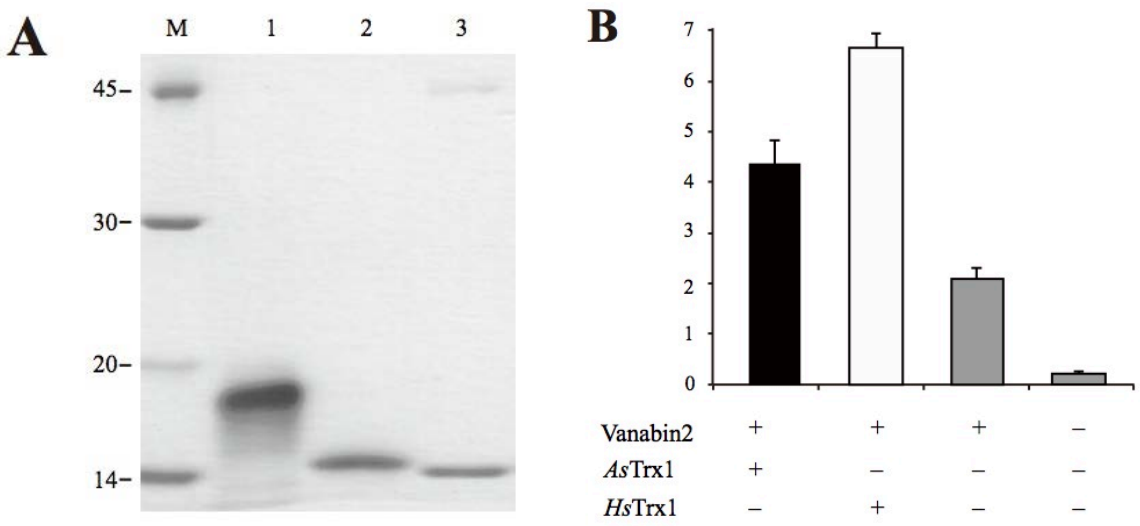


Fig. 6. Reduction of V(V) by a combination of wild-type Vanabin2 with *As*Trx1 or human Trx1 (*Hs*Trx1). (A) Synthesized or commercially obtained proteins. lane M, molecular weight marker. lane1, Vanabin2. lane2, *Hs*Trx1. lane 3, *As*Trx1. Note that *As*Trx was finally purified by HPLC. A small amount of carrier MBP remains (~45 kDa) but it was neglected in this assay. (B) Results of V(V)-reduction assays. Common reaction components included 0.4μM Vanabin2, 0.1 mM DTT, 8.3 mM V(V), 3.3 mM EDTA, and 50 mM Tris-HCl, pH 7.4 in a final volume of 120 μL. *As*Trx1 or *Hs*Trx1 was added at a concentration of 0.8 μM, as indicated. Incubation temperature and time were 20°C and 2 h, respectively. The vertical axis indicates relative area ratio for V(IV) calculated from each peak profile by ion-exchange chromatography. Results are presented as the means ± standard deviation (S.D.), n = 3 - 4.

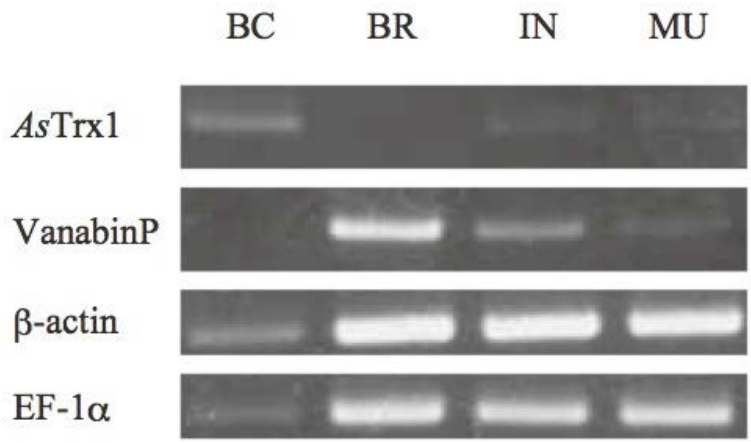


Fig. 7. Expression of *As*Trx1 in tissues/organs of *Ascidia sydneiensis samea* examined by conventional semi-quantitative RT-PCR. Results from a representative experiment. PCR products amplified from the same amount of cDNA under the same cycle conditions were loaded. VanabinP, β-actin and EF-1α were used as controls. Under these conditions, the band strength was not saturated and was comparable among genes and tissues. BC, blood cells; BR, branchial sac; IN, intestine; MU, muscle.

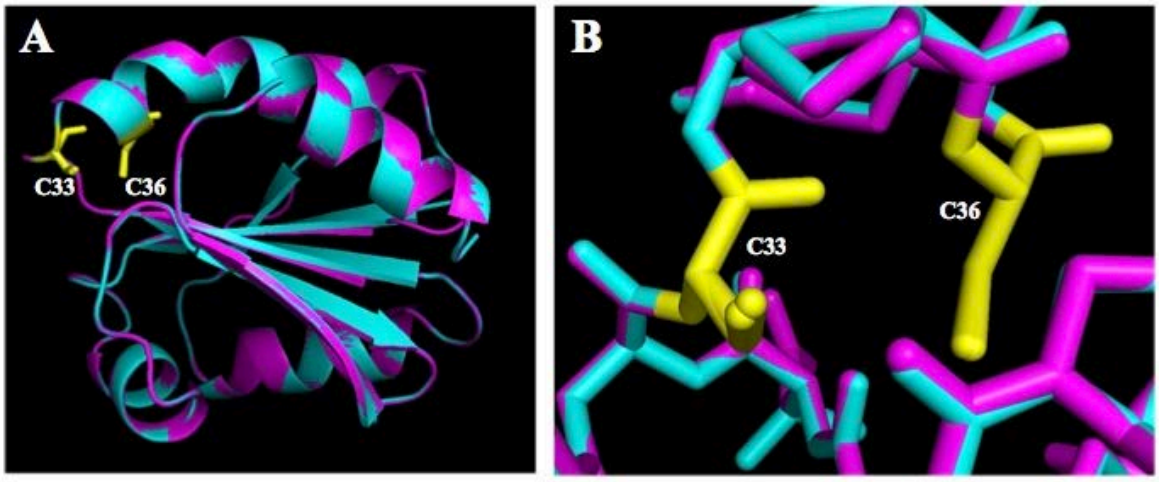


Fig. 8. Homology modeling of *As*Trx1. (A) Superimposed structures of *As*Trx1 (magenta) and *Hs*Trx1 (cyan). (B) Close-up image of the redox-active cysteine motifs (C33 and C36 in *As*Trx1, and C31 and C34 in *Hs*Trx1). Structures were calculated by SwissModel workspace (http://swissmodel.expasy.org/workspace/) based on the structure of *Hs*Trx1 (PDB ID: 1ertA), and aligned and pixilated by MacPyMol software. RMSD was 0.088 (571 to 571 atoms).