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# CHARACTERIZATION OF THE METHIONINE SULFOXIDE REDUCTASES OF *SCHISTOSOMA MANSONI*

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ABSTRACT: Schistosomiasis, also known as Bilharzia, is an infectious disease caused by several species of *Schistosoma*. Twenty million individuals suffer severe symptoms and 200,000 people die annually from the disease. The host responds to the presence of *S. mansoni* by producing reactive oxygen species that cause oxidative stress. We hypothesized that schistosomes produce antioxidants in response to oxidative stress. A known antioxidant protein is methionine sulfoxide reductase (Msr). Methionine residues can be oxidized to methionine sulfoxide in the presence of oxidizing agents, and the process is readily reversed by the action of the Msr system. Two *S. mansoni* MsrB genes (MsrB1 and MsrB2) were cloned and the recombinant proteins were expressed in bacteria and purified. The *S. mansoni* MsrB proteins contained the common conserved catalytic- and zinc-coordinating cysteines. Analysis of the proteins showed that both proteins promote the reduction of both free methionine sulfoxide (Met[O]) and dabsyl-Met(O) to free methionine (Met) and dabsyl-Met, respectively, while exhibiting differences in their specific activities toward these substrates. Using real-time polymerase-chain reaction (RT-PCR), both proteins were found to be expressed in all stages of the parasite's life cycle, with the highest level of expression of both proteins in the egg stage. This is the first description of MsrB proteins from a parasite.

Schistosomiasis is an important tropical parasitic disease, with over 200 million human infections in more than 70 countries (Steinmann et al., 2006; Hotez et al., 2008). Twenty million individuals suffer severe symptoms from this disease (van der Werf et al., 2003). Schistosomiasis is caused by several species of *Schistosoma*, which have a complex life cycle that involves freshwater snails and humans as hosts. In the human host, female worms deposit eggs that are encapsulated in a host-derived granuloma composed of immune cells and fibroblasts. The parasite's egg is subjected to intense immune-generated oxidative stress in the granuloma (Damian, 1987). The granulomas also cause liver and intestinal damage, the main pathology of the disease. Eggs in the intestinal wall are eventually excreted in feces back into the water where they hatch, releasing a miracidia that will infect a snail host.

Host immune cells respond to the presence of *S. mansoni* worms and eggs by producing reactive oxygen species (ROS) such as superoxide radical ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which cause oxidative stress. ROS may provide the host with a defensive mechanism against parasites. These ROS are also produced during normal cellular metabolism such as aerobic respiration; ROS generation is widespread in all aerobic biological systems, even under basal metabolic conditions. ROS can damage proteins, nucleic acids, and membranes sufficiently to kill cells and even whole organisms. However, parasites are not always killed by host responses and have evolved a number of immune evasion mechanisms, such as the production of antioxidant enzymes, that may play a crucial role in protecting the parasite against ROS host responses (Callahan et al., 1988; Mkoji et al., 1988; LoVerde, 1998).

Defense against ROS is mediated by several enzymatic processes in most eukaryotic cells. In vertebrates, there are 2 largely independent systems available to detoxify ROS: 1 is based on glutathione and the other on thioredoxin. These systems have a dedicated NADPH-dependent flavoenzyme to maintain glutathione or thioredoxin in their reduced state, i.e., glutathione reductase and thioredoxin reductase (TrxR), respectively (Townsend et al., 2003; Lillig and Holmgren, 2007). $H_2O_2$ is neutralized by 3 distinct enzymatic activities in humans, i.e., catalase, glutathione peroxidases, and peroxiredoxins.

*Schistosoma mansoni* has a simplified and biochemically distinct redox system compared to its human host. The glutathione and thioredoxin pathways in *S. mansoni* are dependent on a single multifunctional enzyme, thioredoxin-glutathione reductase (TGR), which replaces both glutathione reductase and TrxR in the parasite (Alger and Williams, 2002; Kuntz et al., 2007). TGR has been validated as a new and promising target for anti-schistosome drug development (Kuntz et al., 2007; Sayed et al., 2008), and specific lead compounds targeting TGR have been identified (Simeonov et al., 2008). Catalase is absent from *S. mansoni* (Mkoji et al., 1988) and only lipid hydroperoxide glutathione peroxidases are present; the latter enzymes occur in low abundance and exhibit low activity with $H_2O_2$ (LoVerde, 1998). Peroxiredoxins are the main activity-reducing $H_2O_2$ in *S. mansoni* (Sayed and Williams, 2004; Sayed et al., 2006). Because of the unusual organization of the schistosome defense against ROS, the parasite redox pathway appears to be an effective target for the development of new anti-schistosome chemotherapies (Cioli et al., 2008).

Other methods by which organisms are protected from ROS are through a variety of repair mechanisms. The presence of $H_2O_2$ causes oxidative stress, in part by oxidizing Met residues Met(O). The oxidation of methionine by ROS generates a diastereomeric mixture of methionine-*S*-sulfoxide (Met-*S*-SO) and methionine-*R*-sulfoxide (Met-*R*-SO). Oxidation of Met can lead to the loss or altered properties of a protein. For instance, α-1-proteinase inhibitor, a methionine-containing protein that regulates elastase activity, loses its functions when oxidized. This protein is important for preventing the breakdown of tissues in the lungs (Abrams et al., 1981). Inactivation of α-1-proteinase inhibitor, for example, leads to excessive destruction of lung tissue, which leads to emphysema (Janoff et al., 1983). Studies by Weissbach and colleagues showed that biological inhibitory activity of canine α-1-proteinase inhibitor was restored by an enzyme from *Escherichia coli* (Brot et al., 1981). This enzyme was later identified as MsrA and was shown to reduce Met-*S*-SO (Moskovitz et al., 1996, 2000). Another Msr enzyme, MsrB, was shown to reduce Met-*R*-SO (Moskovitz et al., 2002; Rodrigo et al., 2002). Both Msr enzymes use reduced thioredoxin for their activities.

Msr proteins have been shown to protect bacterial, yeast, animal, and plant cells from the cytotoxic effects of ROS, thereby

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preventing excessive accumulation of oxidized proteins and premature death (Moskovitz et al., 1995, 1997; Rodrigo et al., 2002; Romero et al., 2004). In mammalian cells, overexpression of MsrA in cultured human T cells enhanced their resistance to hydrogen peroxide–mediated oxidative stress (Moskovitz, Flescher et al., 1998). Overexpression of MsrA in fruit flies increased their survival, especially under elevated oxidative stress conditions (Ruan et al., 2002). In mammals, *MsrA* knock-out mice exhibited a shortened life span under normoxia and hyperoxia (100% oxygen), while accumulating carbonylated proteins (Moskovitz et al., 2001). These findings provide supportive evidence for the antioxidant capability of the Msr system, which is mediated by its enzymatic reduction of Met(O) residues in proteins.

To date, no Msr enzymes have been characterized from a parasitic organism. In this study, we hypothesized that *S. mansoni*, which lives in an aerobic environment and under oxidative stress from host-generated ROS, is expected to have Msr proteins to maintain its cellular redox balance. To test this hypothesis, genomic and expressed sequence databases were queried to identify Msr genes; 2 MsrB genes were identified. Full-length clones of the *S. mansoni* Msr genes were obtained and the recombinant proteins were expressed in bacteria and purified for characterization. In addition, the expression of *MsrB* mRNAs during the parasite's life cycle was studied, using real-time PCR.

## MATERIALS AND METHODS

### Parasite preparation

Percutaneous infection of outbred Swiss-Webster mice with *S. mansoni* cercariae (NMRI strain) obtained from infected *Biomphalaria glabrata* snails, perfusion of adult worms (6–7 wk) and juvenile worms (23 days), and preparation of schistosomula from mechanically transformed cercariae, were performed as described by Lewis (1998). This study was approved by the Institutional Animal Care and Use Committee of Illinois State University (08-2002; DHHS animal welfare assurance number A3762-01).

### Cloning and analysis of first *S. mansoni* MsrB genes

A query of the *S. mansoni* expressed sequence tag (EST) database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=s_mansoni) for genes similar to the human MsrB3 gene from GenBank (XP_170724) resulted in the identification of 1 *S. mansoni* MsrB gene. Querying the EST database at GenBank with XP_170724 identified 2 *S. japonicum* sequences with similarity to Msr. The *S. japonicum* sequences were then used to query the *S. mansoni* EST database, resulting in the identification of a second *S. mansoni* MsrB gene. The entire open reading frame of *S. mansoni* MsrB1 was amplified by PCR, using an adult worm cDNA library as the template. The oligonucleotide primers were modified to contain *Bam*HI and *Hind*III restriction sites to facilitate subcloning afterward. The 5′ end of the forward primer contains the *Bam*HI site (5′-TAGGATCCATGAATG TATTG-CAGTTG-3′) and the 3′ end, or the reverse primer, contains the *Hind*III site (5′-GCAAGCTTAAGCAGTTGGCAAATTTA-3′). The PCR product was cloned into PCR II using the TOPO TA cloning kit (Invitrogen, San Diego, California) and transformed into Top 10 cells (Invitrogen). The open reading frame of the second *S. mansoni* MsrB gene was obtained by PCR, using oligonucleotide primers modified to contain the 4 base pair sequence CACC necessary for directional cloning into the Topo-pET100 vector (Invitrogen), using Vent DNA polymerase (New England Biolabs, Beverly, Massachusetts): Forward primer (5′-CACCTCTACGAAAAATCAGAC-GAA-3′) and reverse primer (5′-GCAAGCTTTTATTCTGCTTTT-TAAAT-3′). The nucleotide sequences were determined by fluorescent dideoxynucleotide sequencing on an AB1 automated DNA sequencer.

### Expression of MsrB1 protein

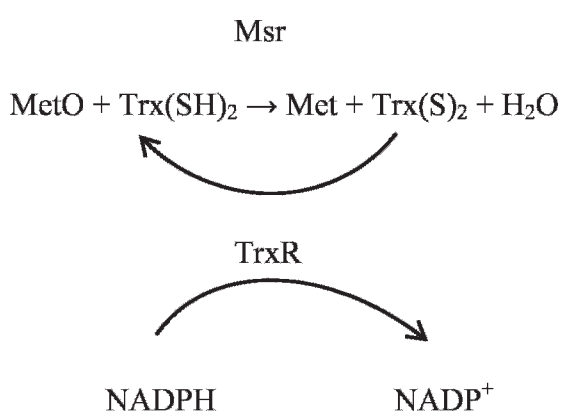
Recombinant MsrB1 protein was expressed in bacterial cells after subcloning into the pRSETA vector to express the recombinant protein with a 6-histidine tag. Cells (BLR(DE3)pLysS) were grown in LB with 50 μg/ml ampicillin at 37 C to $A_{600} = 0.5$. Then, isopropyl-β-D-thiogalactoside (IPTG, 0.3 mM) was added and the cells were cultured for 3 hr at 37 C. In an attempt to increase the solubility of the recombinant protein, MsrB1 was also expressed at different temperatures (20, 25, and 30 C), with different concentrations of IPTG (25 μM, 50 μM, 100 μM) for 3–24 hr and in the presence of 2% ethanol, 2.5% 1-thioglycerol, or 5 μM zinc. MsrB1 was purified by His-Trap nickel-affinity chromatography (Amersham Biosciences, Piscataway, New Jersey), as previously described (Kwatia et al., 2000), with the inclusion of 8 M of urea in all buffers to solubilize the protein. The purified protein was analyzed by SDS-PAGE and protein concentration was determined with the BioRad protein assay (Biorad, Hercules, California). Refolding of MsrB1 was done using the refolding CA kit (Takara Bio Inc., Otsu, Shiga, Japan). Eight refolding reactions with different detergents were set up to determine which conditions allowed for the successful refolding of the protein. The conditions tested were 1% (v/v) Tween 40, 1% (v/v) Tween 60, 1% (v/v) cetyltrimethylammonium bromide (CTAB), or 1% (v/v) myristylsulfobetaine (SB3-14), all with and without the addition of 200 mM DL-cysteine. One microliter of 4 M DTT was added to 99 μl of MsrB1 in 8 M urea and allowed to stand at room temperature for 1 hr. To 20 μl of the unfolded protein, 1.4 ml of a surfactant solution was added and allowed to stand at room temperature for 1 hr. The surfactant solutions were prepared by adding 70 μl of the various detergents to 1.33 ml of phosphate-buffered saline. Four of the surfactant solutions had 14 μl of 200 mM DL-cysteine added. After an hour, 100 μl of 3% cycloamylose solution was added to 400 μl of the reaction mixture and incubated overnight at room temperature. The solutions were centrifuged at 12,000 *g* for 10 min and the supernatants were collected and tested for Msr activity.

### Expression of MsrB2 protein

The recombinant protein was expressed in *E. coli* strain BL21 Star (DE3), using the Champion pET Directional TOPO Expression kit (Invitrogen) as an N-terminally tagged His6 protein. MsrB2 was also expressed in Origami cells, Rosetta cells, and BLR (DE3)pLysS cells. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% (v/v) glycerol, 0.5% (wt/v) Triton X-100, 10 mM imidazole), sonicated, and purified as described (Kwatia et al., 2000). The purified protein was analyzed by SDS-PAGE and protein concentration was determined with the BioRad protein assay. Purity of the MsrB protein was further analyzed by Western blotting, using antibodies against the hexa-histidine tag (Sigma, St. Louis, Missouri).

### Determination of Msr activity

*Coupled Met(O) assay:* Msr activity was measured in a coupled reaction shown below by consumption of NADPH, using a Shimadzu UV-1601 spectrometer (Shimadzu Scientific Instruments, Kyoto, Japan). The reaction mixture contained 500 mM Tris-HCl (pH 7.4), 100 μM L-Met(O), 3 μM *E. coli* Trx, 100 μM NADPH, and 60 nM *E.coli* TrxR (Moskovitz et al., 1996; Sayed and Williams, 2004). NADPH consumption was monitored at 340 nm. Data were reported in terms of specific activity (units/mg protein). One unit MsrB activity was defined as the oxidation of 1 μmol of NADPH ($\varepsilon = 6{,}220\ M^{-1}\ cm^{-1}$) per min at 25 C.



*Dabsylated Met(O) assay:* The activities of *S. mansoni* MsrB proteins were further analyzed using dabsylated Met(O) as the substrate and dithiothreitol (DTT) as the reducing agent. The reduction of dabsyl-Met(O) to dabsyl-Met was determined by high-pressure liquid chromatography (HPLC), using a octadecylsilane (ODC) $C_{18}$ column. Preparation of dabsylated derivative L-Met(O) was as previously described (Moskovitz, Berlett, et al., 1998). The reaction mixture (100 µl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 mM KCl, 10 mM DTT, 200 µM dabsylated L-Met(O), and 5 µg of the Msr proteins. The reaction mixture was incubated at 37 C for 1 hr and the reaction was stopped by adding 200 µl of acetonitrile. After centrifugation, 10 µl of the supernatant was applied and the column was pre-equilibrated with 0.14 M sodium acetate, 0.5 ml/L triethylamine (pH 6.1), and 30% (v/v) acetonitrile. The column was developed using a linear gradient (from 30% to 70%) of acetonitrile in the equilibration buffer and the dabsyl derivatives were monitored by absorbance at 436 nm (Moskovitz, Berlett et al., 1998).

### Determination of Msr gene expression

Quantitative RT-PCR method ($2^{-[delta][delta]Ct}$ method; Livak and Schmittgen, 2001) was used to determine the expression of Msr mRNAs at different stages of the parasite's life cycle. Parasite preparation, RNA isolation, and cDNA synthesis were as described (Sayed et al., 2006). Purity of the RNA samples was checked by running the samples on an RNA agarose gel; the concentration was determined at an absorbance of 260 nm and purity was evaluated by $A_{260}/A_{280}$. The primers used in the real-time reverse transcription PCR (RT-PCR) experiment were designed on Primer Express software (Table I). The primers were selected if they had a melting temperature ($T_m$) between 58 and 60 C and to produce amplicons or PCR products of 50–150 bp. RT-PCR was carried out on Applied Biosystems 5700 (Life Technologies, Carlsbad, California), using SYBR green dye as the detection dye. cDNA samples from different stages were diluted 1:5 (for target genes) and 1:500 (for control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) with sterile, nuclease-free water. The reaction mixture contained the following: 125 nM of each of the gene-specific primers; 0.2 mM each of dATP, dCTP, and dGTP; and 0.4 mM of dUTP, 0.08 µl of 1:400 dilution of SYBR green (Molecular Probes, Eugene, Oregon) in DMSO, 0.225 µM ROX (5-carboxy-X-rhodamine) reference dye (Invitrogen), and 1.5 mM $MgCl_2$. The cycle conditions used were 95 C for 10 min, 40 cycles at 95 C for 15 sec, and 1 min at 60 C. Samples were analyzed in triplicate and product purity was checked through dissociation curves at the end of the cycles. For validation of the primer pairs, female adult worm cDNA was diluted 1:5 (for MsrB1) and 1:500 (for GAPDH) with sterile nuclease-free water. Male adult worm cDNA was diluted 1:5 (for MsrB2) and 1:500 (for GAPDH). RT-PCR was carried out as described above, using SYBR green dye as the detection dye. To make a validation curve, the delta $C_t$ was plotted against the log of RNA concentration.

### Statistical analysis

RT-PCR data were statistically analyzed using a *t*-test to determine whether any differences seen in the results were real. Data from each parasite life-cycle stage were compared with all of the other stages.

## RESULTS

### Cloning *Schistosoma mansoni* Msr genes

Querying the *S. mansoni* EST database identified 2 potential Msr genes. These sequences were submitted to GenBank with accession numbers AY669149 and AY669150. Mapping AY669149 and AY669150 genes to genome contigs at the *S. mansoni* genome project (www.sanger.ac.uk/ Projects/S_mansoni) allowed us to identify their gene structure. AY669149 has 3 exons and 2 introns, while AY669150 has 4 exons and 3 introns (Fig. 1).

AY669149 has a 525 base pair open reading frame encoding 175 amino acids with a predicted molecular weight of 23.3 kDa, while AY669150 has a 411 base pair open reading frame encoding 137 amino acids with a predicted molecular weight of 15.4 kDa.

TABLE I. Sequences of primers used for RT-PCR analysis.

| Gene | Primer direction | Sequence |
|---|---|---|
| GAPDH | Forward | 5′-AATTATGGCGAGATGGCCGT-3′ |
| | Reverse | 5′-TTTGGCAGCACCAGTGGAA-3′ |
| MsrB1 | Forward | 5′-GTCGTTGGCCTGCATTTAACA-3′ |
| | Reverse | 5′-ACGGACTTCACCGATGACAAA-3′ |
| MsrB2 | Forward | 5′-TGGATCAGGTTGGCCATCAT-3′ |
| | Reverse | 5′-TGAGAATCATCACGTCCGTTTG-3′ |

The protein encoded in AY669149, *S. mansoni* (Sm)MsrB1, is 37% identical to human MsrB1, 48% identical to human MsrB2, and 46% identical to human MsrB3, while the protein encoded in AY669150, SmMsrB2, is 38% identical to human MsrB1, 55% identical to human MsrB2, and 52% identical to human MsrB3. SmMsrB1 and SmMsrB2 are 47% identical and 62% similar to each other. Figure 1 shows the alignment of SmMsrB1 and SmMsrB2 sequences with human MsrB protein sequences. This arrangement shows that the catalytic Cys that directly attacks the sulfur in Met(O) is conserved. The zinc coordinating residues, which are involved in the structure of the protein, are also conserved in both SmMsrB proteins. The recycling amino acids involved in the reaction mechanism of the protein are also conserved. In SmMsrB1, the recycling amino acid is Cys, which is suggested to form a disulfide bond with the catalytic Cys. In SmMsrB2, there is no resolving Cys, but there is a threonine, while in the human MsrB proteins, serine is present in this position. The electronegativity of the threonine and serine residue probably contributes to lowering the pKa of the catalytic Cys residue. However, it has been shown that Cys residues located in other positions in Msr proteins can act as the resolving Cys (Neiers et al., 2004), and therefore, the possibility that Cys12 in SmMsrB2 is the resolving Cys residue cannot be excluded.

### Expression and purification of SmMsrB1 and SmMsrB2

SmMsrB1 was expressed in *E. coli* as a His-tagged protein. The recombinant protein was found in inclusion bodies (results not shown). Solubilization of the inclusion bodies in urea allowed for affinity purification of SmMsrB1 (results not shown). However, the purified proteins precipitated upon removal of urea (results not shown). Further attempts to express soluble SmMsrB1 included expression at lower temperatures, lower IPTG concentrations, in the presence of 3% ethanol, 2.5% monothioglycerol, or 5 µM zinc, none of which resulted in soluble protein (results not shown).

To obtain soluble SmMsrB1 protein, the protein purified from inclusion bodies was re-folded in the presence of different detergents and cysteine. The denatured protein (dissolved in urea) was diluted to reduce the urea concentration and a detergent was used to maintain the protein in solution. A cyclodextrin was added that initiates folding by stripping the detergent from the protein-detergent complex (Daugherty et al., 1998). Eight refolding reactions with different detergents were conducted to determine which conditions allowed for the successful refolding of the protein (Table II). Refolding conditions resulting in the generation of the most active SmMsrB1 was 1% Tween 40 + 200 mM DL-cysteine.

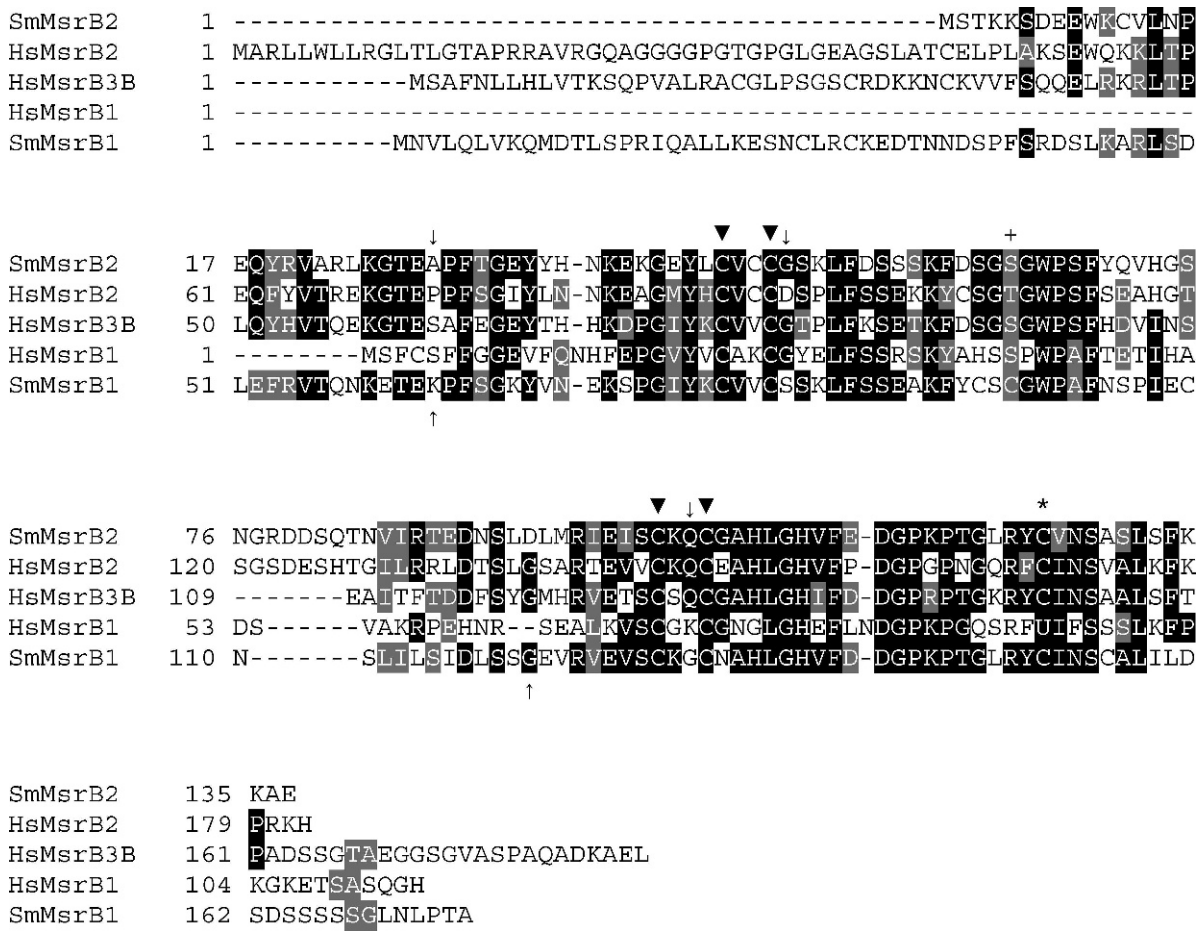



FIGURE 1. Alignment of *Schistosoma mansoni* MsrB amino acid sequences with human MsrB proteins: HsMsrB1(NP_057416), HsMsrB2 (NP_036360), and HsMsrB3B (NP_001026849), (36). The conserved catalytic cysteine (C) and selenocysteine (shown as U) residues are indicated with an asterisk (*), the serine (S) and threonine (T) residues that replaced the cysteine (except for MsrB1) are indicated with a cross (+), and the 4 zinc coordinating Cys residues are indicated with arrowheads (▼). The black blocks represent identical amino acids. Amino acids with similar chemical properties are shown in gray. Arrows (↓) indicate intron positions in the *S. mansoni* MsrB genes.

SmMsrB2b was expressed in *E. coli* strains BL21 Star (DE3), Origami, Rosetta, and BLR (DE3)pLysS as a His-tagged protein. Highest expression was seen in Rosetta cells from which SmMsrB2 was expressed and purified by Ni-affinity chromatography (results not shown).

TABLE II. Specific activities (μmol/min/mg protein) of *S. mansoni* MsrB1 with various refolding conditions to determine the optimal condition for refolding MsrB1. Specific activities were determined using the L-Met(O) assay. Cetyltrimethylammonium bromide (CTAB) or myristylsulfobetaine (SB3-14) were used in the refolding assays.

| Refolding conditions | MsrB1 activity |
|---|---|
| 1% (v/v) Tween 40 | 0.036 |
| 1% Tween 60 | 0.32 |
| 1% (v/v) CTAB | 0.32 |
| 1% (v/v) SB3-14 | 0.18 |
| 1% Tween 40 + 200 mM DL-cysteine | 1.94 |
| 1% Tween 60 + 200 mM DL-cysteine | 1.4 |
| 1% CTAB + 200 mM DL-cysteine | 0.36 |
| 1% SB3-14 + 200 mM DL-cysteine | 1.8 |

### Activity of *S. mansoni* MsrB1 and MsrB2 proteins

The activities of the recombinant *S. mansoni* MsrB proteins were measured using 2 methods. The first was a coupled reaction with *E. coli* thioredoxin and thioredoxin reductase, followed by the consumption of NADPH. In this assay, refolded MsrB1 (using 1% Tween 40 + 200 mM DL cysteine) had a specific activity of 1.94 unit/mg protein and MsrB2 had a specific activity of 0.064 unit/mg. The activities of both proteins were also assayed using dabsylated Met(O) as the substrate and DTT as the reducing agent. In this assay, MsrB1 had a specific activity of 0.226 unit/mg and MsrB2 had a specific activity of 2.48 unit/mg. In the dabsyl-Met(O) assay (with DTT used as the reducing agent), SmMsrB1 had a specific activity that was only one seventh of the specific activity monitored using L-Met(O) assay (with Trx as the reducing agent). In contrast, in the dabsyl-Met(O) assay, SmMsrB2 had a specific activity of ~38-fold higher than with the L-Met(O) assay. Thus, by comparison, the

SmMsrB2 was 9.3 times more active than SmMsrB1 in the dabsyl-Met(O) assay.

### Stage expression of *S. mansoni* MsrB1 and MsrB2

The expression of *S. mansoni* MsrB1 and MsrB2 was determined in free-living cercariae; 4-hr, 24-hr, and 5-day schistosomula; 10-day lung stage; 23-day liver stage; and adult female, adult male, and egg stages by quantitative RT-PCR. A validation experiment was performed to determine that gene expression was unaffected by experimental treatment or RNA/cDNA concentrations. Serial dilutions (31.25–1,000 ng) of cDNA from adult female worms (for MsrB2a) and adult male (for MsrB2b) worms were used in the RT-PCR experiment. The delta $C_t$ values ($C_t$ value is the cycle threshold value at which the target amplification is first detected in the PCR cycle) for the development stages for each gene were calculated by subtracting the average $C_t$ values from the average $C_t$ values of GAPDH (Livak and Schmittgen, 2001), as shown below:

$$\Delta C_t = \text{Avg } C_{t,\text{target gene}} - \text{Avg } C_{t,}\, \Delta C_t$$
$$= \text{Avg } C_{t,\text{target gene}} - \text{Avg } C_{t,\text{control gene}}$$

A graph of the delta $C_t$ versus the log ng RNA was plotted. A validation curve with an absolute value of its slope less than 0.1 indicates that gene expression is not affected by experimental treatment. The validation curve for the MsrB2a gene had a slope of 0.0901 and MsrB2b had a slope of −0.0971. The calibrator, or reference, stage was 4-hr schistosomula, because it had the lowest MsrB mRNA expression levels and the other stages were upregulated when compared to this stage. All other stages were compared to the calibrator stage by using the $2^{-[\Delta\Delta Ct]}$ method (Livak and Schmittgen, 2001) as shown below:

$$\Delta\Delta C_t = \Delta C_{t,\text{target gene}} - \Delta C_{t,\text{reference gene}}$$

Here, $\Delta C_{t,\text{target gene}}$ is the $C_t$ value for any sample normalized to the endogenous housekeeping gene and $\Delta C_{t,\text{ reference}}$ is the $C_t$ value for the calibrator also normalized to the endogenous housekeeping gene. The results were expressed as the fold change in the expression of MsrB2a and MsrB2b at the other stages relative to the reference stage (Figs. 2A, 3). Both *S. mansoni* MsrB genes were expressed at all stages of development, with the highest levels seen in eggs. Statistical analysis showed that the egg stage was significantly different ($P < 0.05$) from all other stages.

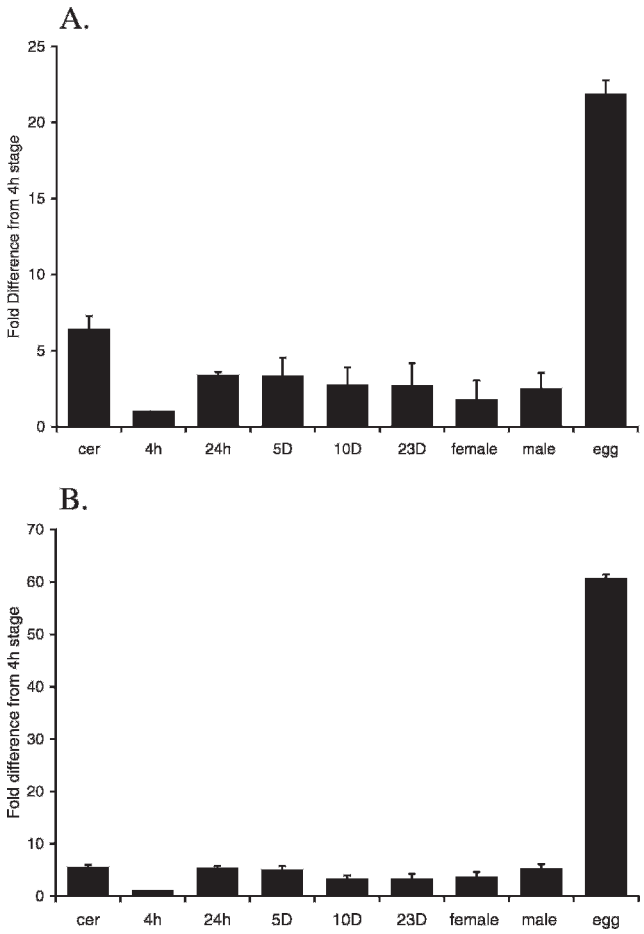



FIGURE 2. Stage RT-PCR. Expression of MsrB1 (**A**) and MsrB2 (**B**) was monitored through parasite life stage using RT-PCR. All of the data were normalized using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Four-hour schistosomula were used as the calibrator stage. Cer, cercariae; 4h, 4-hr schistosomula; 24h, 24-hr schistosomula; 5D, 5-day schistosomula, lung stage; 10D, 10-day schistosomula, lung stage; 23D, 23-day, liver stage; female, female adult worm; male, male adult worm; egg, egg stage. Bars show the standard deviation (n = 3).

## DISCUSSION

Schistosomes can survive in the human host's blood stream for up to 30 yr (Vermund et al., 1983). In order to survive, the parasites and their eggs have developed defense mechanisms to cope with the host's immune response. One such mechanism is the production of antioxidant enzymes. These enzymes, e.g., superoxide dismutase, glutathione peroxidases, and peroxiredoxin, can remove the reactive oxygen species produced by the host's immune system, thereby protecting the parasite. In other organisms, the Msr system has been found to function as an antioxidant that reduces oxidized methionine residues in proteins (Moskovitz, 2005). We hypothesized that *S. mansoni* expresses Msr proteins in response to oxidative stress and that these enzymes may be needed by the organism to survive. Msrs have not yet been characterized in schistosomes, or other parasites for that matter; thus, our goal was to determine the presence and activity of this antioxidant enzyme in *S. mansoni*. In this study, 2 MsrB-family genes (MsrB1 and MsrB2) from the *S. mansoni* that were similar to human MsrB genes were identified, sequenced, and recombinantly expressed.

Different forms of MsrB have been identified in other organisms. Mammalian MsrB proteins exist in 2 forms, i.e., selenocysteine (Sec)-containing MsrB (Sec-MsrB) and Cys-containing MsrB (Singh et al., 2001; Bar-Noy and Moskovitz, 2002; Moskovitz et al., 2002; Kim and Gladyshev, 2004). Humans have 3 MsrB proteins: 1 Sec-MsrB protein (HsMsrB1) and 2 Cys-containing proteins (HsMsrB2 and HsMsrB3) (Rodrigo et al., 2002). We have identified 2 Cys-containing Msr proteins in *S. mansoni*. There is no evidence for a Sec-containing Msr in *S. mansoni*.

Previous studies have shown that the catalytic Cys or Sec located in the C-terminal portion of the Msr protein directly

attacks Met(O), with the formation of a sulfenic acid intermediate on the catalytic cysteine residue and the release of 1 mol of Met per mol of enzyme (Boschi-Muller et al., 2000; Bar-Noy and Moskovitz, 2002; Boschi-Muller et al., 2005). There is then a nucleophilic attack of the recycling Cys on the sulfur atom of the sulfenic acid intermediate. This leads to the formation of an intramolecular disulfide bond between the 2 cysteines, with the release of $H_2O$ (Boschi-Muller et al., 2005). The last step of this process involves the reduction of the Msr disulfide bond by thioredoxin (Trx). The active site of Msr is returned to its fully reduced state and Trx is oxidized. In some Msr proteins, the recycling Cys is replaced with a serine and the formation of the disulfide-bonded intermediate is not possible. However, studies have shown that the recycling Cys is not essential and the sulfenic acid intermediate may be directly reduced by Trx (Kumar et al., 2002).

In SmMsrB1, the recycling amino acid is proposed to be Cys, while in SmMsrB2, there is no recycling Cys, but instead a threonine. Therefore, the MsrB proteins of *S. mansoni* may differ slightly in their reaction mechanism. We propose that the reaction process of MsrB1 requires the formation of a disulfide bond between the catalytic Cys and the recycling Cys. This bond is then reduced by Trx, leading to regeneration of the enzyme. For MsrB2, there is no disulfide intermediate due to the absence of the recycling cysteine, suggesting that Trx must directly reduce the sulfenic acid intermediate. Other potential physiological reductants of Msr proteins are glutaredoxins (Vieira Dos Santos et al., 2007) and TGR, which has a glutaredoxin-like domain (Alger and Williams, 2002). However, Cys residues located in other positions in Msr proteins can act as the resolving Cys (Neiers et al., 2004), and the possibility that Cys12 in SmMsrB2 is the resolving Cys residue cannot be excluded without further experimentation.

MsrB proteins have also been separated into 2 groups that differ by the presence or absence of zinc (Kumar et al., 2002). The first group, known as Form 1 MsrB, contains 4 cysteine residues that are organized in 2 CXXC motifs. Form 2 MsrB proteins, which lack the 2 CXXC motifs, are more closely related to other Form 2 Msr proteins and are present in pathogenic bacteria. Form 1 MsrB proteins are present in all MsrB-containing archaeans and eukaryotes (Kumar et al., 2002). Mutation of any one, or a combination, of these residues results in the complete loss of metal-binding and catalytic activity (Kumar et al., 2002). All 4 potential Zn-binding Cys residues are conserved in both *S. mansoni* Msr proteins.

We also mapped *S. mansoni* MsrB1 and MsrB2 genes to determine their gene structure. SmMsrB2 has 1 more intron and exon when compared with SmMsrB1. Alignment of the amino acid sequence revealed that the first introns of both proteins are located in the same positions. Usually, genes that are related by evolution have some of their intron positions conserved (Fedorov et al., 2002). It is probable that both MsrB genes share a common ancestral gene, but since the position of only 1 intron is conserved, this suggests an ancient gene duplication event.

We have characterized the enzymatic abilities of recombinant *S. mansoni* MsrB proteins and shown that both proteins promote the thiol-dependent reduction of Met(O) to Met. We tested the activities of both proteins using 2 assays, i.e., the Met(O) assay and the dabsylated Met(O) assay. The Met(O) assay indirectly measures the activity of the MsrB proteins by assessing the consumption of NADPH during the reduction reaction of $Trx(S)_2$ to $Trx(SH)_2$. The oxidation of $Trx(SH)_2$ back to $Trx(S)_2$ is coupled with the reaction that converts Met(O) to Met. Clear disparities in specific activities between the tested MsrB1 and MsrB2 proteins were seen. The differences may be due to the reducing agent, or the substrate, or both. Both Trx and DTT can reduce the sulfenic acid intermediate or the disulfide-bonded intermediate formed during the reaction and regenerate the MsrB protein. It has been proposed that Trx is a better electron donor to the disulfide than is DTT (Kumar et al., 2002). The recycling Cys in SmMsrB1 forms a disulfide bond with the catalytic Cys, so we suspect that this is the reason we saw higher activity of SmMsrB1 with the Met(O) assay and lower activity of SmMsrB2.

An alternative explanation is that, in the Met(O) assay, we used free Met(O) as the substrate. SmMsrB2 may have a low affinity for free Met(O) and a higher affinity for peptide-bound Met(O). Dabsyl-Met(O) has a larger structure than Met(O) and may better represent peptide-bound Met(O) (Vieira Dos Santos et al., 2005). The lower enzyme activity of SmMsrB1 in the dabsyl-Met(O) assay may be attributed to the enzyme's low affinity for peptide-bound Met(O).

The specific activities of SmMsrB1 and SmMsrB2 are comparable to those of mammalian MsrB proteins. In the dabsyl-Met(O) assay, mammalian MsrB1-Sec, MsrB2, and MsrB3b had specific activities of 1.56 unit/mg, 0.353 unit/mg, and 0.423 unit/mg, respectively (Kim and Gladyshev, 2004). The higher enzyme activity seen in MsrB1 may be due to the presence of Sec (Bar-Noy and Moskovitz, 2002; Kim and Gladyshev, 2004). Our results from the dabsylated Met(O) assay showed that SmMsrB2 was 1.6 times more active than MsrB1 and 7 and 6 times more active than mammalian enzymes MsrB2 and MsrB3b, respectively.

The 2 Msr proteins were expressed in all parasite stages tested. The highest level of mRNA expression for both genes was seen in the eggs. Two other redox proteins, Trx1 and peroxiredoxin1, have been found at high levels in schistosome eggs (Williams et al., 2001; Alger et al., 2002). However, unlike Trx1 and peroxiredoxin1, neither of the Msr proteins was found in egg secretions (Cass et al., 2007). The parasite's egg is subjected to intense immune-generated oxidative stress in the host-derived granuloma (Damian, 1987). This could explain why elevated levels of MsrB mRNA transcripts were seen in eggs and why elevated levels of Trx and peroxiredoxin have also been found in the eggs.

Reports of antioxidant levels in *S. mansoni* show that the highest levels of superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione *S*-transferase, and peroxiredoxin were seen in the adult stages and the presence of these antioxidants at high levels made the worms resistant to oxidative killing (Mkoji et al., 1988; Mei and LoVerde, 1997; Sayed et al., 2006). Our results show that expression of both MsrB genes was lowest in 4 hr schistosomula; shortly after transformation from the cercaria to the schistosomule, both MsrB1 and MsrB2 expression dropped rapidly. Expression of both genes increased significantly at the 24-hr stage and remained unchanged through to the adult. It is not clear why MsrB expression remains constant during development—perhaps the MsrB proteins are present in adequate amounts to ensure parasite survival.

Currently, treatment of schistosomiasis involves the use of a single drug, praziquantel. Praziquantel does not prevent reinfection and recent findings suggest that resistance to the drug may be developing (Doenhoff et al., 2008). Therefore, there is a

need to identify new ways of dealing with the parasite via therapeutic methods. Redox biology of the parasite has proven to be distinctive and provides promising targets for the development of new anti-schistosome drugs (Cioli et al., 2008; Sayed et al., 2008). The identification and characterization of other redox enzymes, such as Msr, may help in the development of new chemotherapies to combat this important disease.

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