Full Paper

Functional comparison of methionine sulfoxide reductase A and B in *Corynebacterium glutamicum*

(Received December 13, 2016; Accepted January 18, 2017; J-STAGE Advance publication date: September 12, 2017)

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Methionine sulphoxide reductases (Msr) are able to reduce methionine sulfoxide to methionine and protect bacteria against reactive oxygen species (ROS). Many organisms express both methionine sulfoxide reductase A (MsrA), specific for methionine-S-sulfoxide and methionine sulfoxide reductase B (MsrB), active against methionine-R-sulfoxide. *Corynebacterium glutamicum* expresses MsrA, the function of which has been well defined; however, the function of MsrB has not been studied. Whether MsrB and MsrA play an equally important role in the antioxidant process is also poorly understood. In this study, we identified MsrB encoded by *ncgl1823* in *C. glutamicum*, investigated its function and made a comparison with MsrA. The *msrB* gene showed a slight effect on utilizing methionine sulfoxide (MetO) as the sole Met source; however, the survival rates showed no sensitivity to oxidants. MsrB showed catalytic activity using thioredoxin/thioredoxin reductase (Trx/TrxR) reducing system as electron donors, but independent from the mycoredoxin 1/mycothione reductase/mycothiol (Mrx1/Mtr/MSH) system. Therefore, MsrB plays a limited role in resisting oxidative stress and it could reduce MetO to Met by the Trx/TrxR reducing system, which is useful for expanding the understanding of the functions of Msr in this important industrial microbe.

Key Words: *Corynebacterium glutamicum*; MsrB; oxidative stress; Trx/TrxR reducing system

Introduction

*Corynebacterium glutamicum* is not only well known as a very important industrial microorganism (mainly used in the production of amino acids and vitamins), but also as a model organism in microbial research, and for the disclosing of catabolism mechanisms of aromatic compounds in high G+C content Gram-positive bacteria (Shen et al., 2012). It is inevitable that bacteria will be subject to a variety of environmental stresses, resulting in the production and accumulation of various reactive oxygen and nitrogen species (ROS, RNS), causing the oxidation of biological macromolecules, breaking the intracellular reduct balance, resulting in cell dysfunction and leading to cell damage and even apoptosis (Davies, 2005; Si et al., 2014). To survive under hostile conditions, bacteria adopt a variety of resistant mechanisms, including several antioxidant enzymes, such as catalases, superoxide dismutases, peroxiredoxins, alkyl hydroperoxide reductases, organic hydroperoxide resistance protein and thiol peroxidase, which have been characterized to resist oxidative stresses (An et al., 2010; Bayssse et al., 2000; Hassett et al., 1995; Liu et al., 2016; Ochsner et al., 2001).

Amino acid residues in proteins represent one of the major targets of ROS and cellular oxidants (Vogt, 1995). Methionine (Met) ranks as one of the most sensitive amino acids to oxidation and it can be oxidized into methionine sulfoxide (MetO) which contains two classes, methionine-S-sulfoxide (Met-S-O) and methionine-R-sulfoxide (Met-R-O), leading to changes in protein conformation and causing a loss of biological activities (Couturier et al., 2012; Stadtman et al., 2002, 2003). Methionine sulfoxide reductases (Msr) present in most living organisms are antioxidant repair enzymes that catalyze the reduction of MetO...
to Met in free and protein-bound forms, which contain two unrelated classes, methionine sulphoxide reductase A (MsrA) and methionine sulphoxide reductase B (MsrB). MsrA is very specific for the reduction of the S-isomer of MetO, and the other, MsrB, is specific for the reduction of the R-isomer of MetO (Grimaud et al., 2001; Kryukov et al., 2002). These enzymes catalyze the reduction at the expense of NADPH using thioredoxin (Trx) or glutaredoxin systems and repair some oxidatively damaged proteins, and also protect cells against oxidative stress (Levine et al., 1996; Tarrago et al., 2009a, 2012; Zhang et al., 2009b).

In a previous study, we have shown that MsrA plays an important role in hostile conditions, and not only can it reduce MetO to Met via the thioredoxin/thioredoxin reductase (Trx/TrxR) reducing system, but also the mycoredoxin 1/mycothione reductase/mycothiol (Mrx1/Mtr/MSH) system (Si et al., 2015b). The purpose of the present study was to investigate the function of MsrB and compare it with MsrA. Our results show that MsrB is limited to reduce MetO in oxidative stress and it could reduce MetO to Met by the Trx/TrxR reducing system.

### Materials and Methods

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. *Escherichia coli* and *C. glutamicum* strains were cultured in Luria-Bertani (LB) broth or on LB plates. The culture temperatures for *E. coli* and *C. glutamicum* strains were 37°C and 30°C, respectively, and a shaking speed of 220 rpm for liquid culture. Antibiotics were used as follows: kanamycin, 50 µg ml⁻¹ for *E. coli* and 25 µg ml⁻¹ for *C. glutamicum*; chloramphenicol, 20 µg ml⁻¹ for *E. coli* and 10 µg ml⁻¹ for *C. glutamicum*; nalidixic acid, 20 µg ml⁻¹ for *C. glutamicum*.

**Plasmids construction.** Plasmids and primers used in this study are listed in Table 1. The genes coding for MsrB (ncgl1823) were amplified by PCR using *C. glutamicum* genomic DNA as a template with primers MsrBF/MsrBR. The amplified DNA fragments were digested and then subcloned into similar digested pET28a and pXMJ19 plasmids, obtaining the plasmids pET28a-msrB and pXMJ19-msrB, respectively. To construct the deletion...
Functional comparison of MsrA and MsrB

Amino acid sequences alignment of Ncgl1823 with MsrB proteins from selected microorganisms.

Alignment was performed with MEGA 6. The marker showed the strong conserved Cys in positions 66 and 122. The highly conserved catalytic motif RXCXN of MsrB has also been marked out. Accession numbers: C. glutamicum ATCC 13032 (NP_601104); M. tuberculosis (KF91903); Saccharomyces cerevisiae (EDV09680.1); E. coli (PA0A748.1); Neisseria gonorrhoeae (1L1D_B); Salmonella enterica subsp. (P65449.1), Klebiella pneumoniae subsp. (A677R0.1).

Recombinant protein expression and purification. To express and purify His-tagged recombinant proteins, pET28a derivatives were transformed into E. coli BL21 (DE3). For protein production, bacteria were grown at 37°C in an LB medium to an OD 600 of 0.4, then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and cultivated for an additional 12 h at 26°C. Harvested cells were disrupted by sonification and purified with the His•Bind Ni-NTA resin (Novagen, Madison, WI, USA) according to the manufacturer’s instructions. Protein concentrations were determined by the Bradford assay.

Spotting assay. A deletion of msrB and mrsa was made in the ΔmetHΔmetE mutant which was reported to be auxotrophic for Met, respectively (Lee and Hwang, 2003; Rückert et al., 2003). The ability to utilize MetO as the sole Met source was tested on mineral salts medium with 10 mg ml−1 MetO or Met (Ezraty et al., 2005; Jacob et al., 2011). The difference between the indicated Msr mutants was determined by viability on a mineral salts medium. After serial dilutions, each mutant was spotted onto agar plates and incubated at 30°C for 3 days, and photographed. This experiment was repeated three times.

Stress assay. The stress assay was conducted based on the methods of Wang et al. (2015) described with minor modifications. Overnight-grown cultures of C. glutamicum (LB broth, 30°C) were diluted 100-fold with LB medium and exposed to 80 mM hydrogen peroxide, 8 mM cumene hydroperoxide (CHP) and 0.3 mM of CdCl2, at 30°C, 100 rpm for 30 min. After treatment, the cultures were serially diluted, spread on LB plates and incubated at 30°C for 36 h. Each bacterial survival percentage was calculated as [(CFU ml−1 with stress)/(CFU ml−1 without stress)] × 100.

Enzyme assay. Enzyme assays of MsrA and MsrB were conducted according to the method of Si et al. (2015b).

Formation and separation of heterodimers. Assays were performed based on the methods of Rouhier et al. (2002) described with minor modifications. MsrB (15 μM) and Trx or Mrx1 (wild-type and its variants; 10 μM) were mixed in TE buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final volume of 20 μl. This reaction mixture was incubated at room temperature for 15 min before the addition of 40 μM MetO. The mixture was incubated at room temperature for another 60 min and then subjected to 15% SDS-PAGE in the presence of a non-reducing sample buffer.

Statistical analysis. The results shown represent the average of three independent experiments; error bars indicate the standard deviation (SD) from three independent experiments. Statistical analysis was carried out using the Student’s t-test. Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego, California, USA).
Identification of C. glutamicum MsrB

ncgl1823 is a 411-bp gene annotated as encoding a conserved hypothetical protein. We performed a multiple alignment against MsrB proteins from numerous other microorganisms (Fig. 1). ncgl1823 is 73% identical in amino acid sequence to MsrB from M. tuberculosis (Lee et al., 2009) and E. coli (Grimaud et al., 2001) as well as other MsrB sequences from numerous other microorganisms (Fig. 1). ncgl1823 is 73% identical in amino acid sequence to MsrB from M. tuberculosis and 44% identical to MsrB from E. coli, and includes two conserved cysteine residues (Cys 66 and Cys 122) as well as adjacent clusters of residues that form the presumptive active site (Lowther et al., 2002; Moskovitz et al., 2002; Ranavinson et al., 2009).

The deletion of msrB slightly affects the ability of C. glutamicum to utilize MetO as the sole Met source

In the spotting assay, the ΔmetHΔmetEΔmsrB mutant did not show a growth defect under Met-depleted conditions with MetO as the sole Met source compared with that in the presence of Met. Then we deleted both msrA and msrB in the ΔmetHΔmetE mutant to test the ability to utilize MetO as the sole Met source (Fig. 2). This double knockout mutant showed the most growth defect under Met-depleted conditions with MetO as the sole Met source compared with the complementation and the complementation of the mutant with msrA gene grew the best. The growth of the complementation mutant with msrB gene was slightly better than ΔmetHΔmetEΔmsrAΔmsrB but worse than the complementation of the mutant with msrA gene. These results indicate that MsrB could reduce MetO to Met, but, however, could only slightly affect the ability of C. glutamicum to utilize MetO as the sole Met source, which was weaker than MsrA.

MsrB plays a limited role under oxidative stresses compared with MsrA

We have earlier reported that the msrA deletion mutant showed a significant sensitivity in a hostile environment (Si et al., 2015b). To test whether MsrB has the same effect as MsrA does, the sensitivity of wild-type, ΔmsrA mutant, the complementary strain ΔmsrA (pXMJ19-ΔmsrA), ΔmsrB and the complementary strain ΔmsrB (pXMJ19-ΔmsrB) to H2O2 (80 mM), cumene hydroperoxide (CHP, 8 mM) and CdCl2 (0.3 mM) were tested (Fig. 3). The survival rates of the ΔmsrA mutant decreased by about 26.7–30.1% compared with the wild-type, and the sensitive phenotype of ΔmsrA mutant to oxidative stresses was completely reversed by complementation with the msrA gene. However, the survival rates of the ΔmsrB mutant showed no significant decrease.

MsrB can use Trx as electron donors

To characterize the kinetics of MsrB and MsrA, Mr activity was measured after NADPH oxidation at 340 nm using MetO as substrates (Couturier et al., 2012). The catalytic constants of MsrB and MsrA with Trx reducing system as the recycling reductant were determined under steady-state conditions at a saturating concentration of reductants (40 μM) and different concentrations of MetO (0–150 mM). As shown in Table 2, The Km value, Kcat value, and the catalytic efficiency of MsrA were calculated to be 3.3 ± 0.2 mM, 2.4 ± 0.3 s−1 and 727.27 M−1 s−1, and the Km value, Kcat value, and the catalytic efficiency of MsrB were 11.5 ± 1.4 mM, 0.19 ± 0.05 s−1 and 16.52 M−1 s−1.

Table 2. Kinetics parameters of MsrA and MsrB toward MetO by using the Trx/TrxR reducing pathway.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Km (mM)</th>
<th>kcat (s−1)</th>
<th>kcat/Km (M−1 s−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MsrA</td>
<td>3.3 ± 0.2</td>
<td>2.4 ± 0.3</td>
<td>727.27</td>
</tr>
<tr>
<td>MsrB</td>
<td>11.5 ± 1.4</td>
<td>0.19 ± 0.05</td>
<td>16.52</td>
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</tbody>
</table>

For determination of the Michaelis constant, activities were measured in the reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 250 μM NADPH, 2 μM MsrA, 2 μM MsrB, and the Trx/TrxR reducing system (4 μM Trx and 40 μM of Trx). The concentrations for MetO ranged from 0–150 mM. The data are presented as the means of the values obtained from three independent assays.
confirmed that MsrB could use Trx as electron donors. This result also indicated that the regeneration mechanism of MsrB is similar to MsrA: a transient intermolecular disulfide bond is formed between MsrB and Trx, then Trx attacks the intermolecular disulfide bond to realize the regeneration of MsrB. These results provide direct evidence that the Trx/TrxR system reduces the disulfide bond of MsrB formed during the regeneration process, indicating that the regeneration mechanism of MsrB is dependent of the Mrx1/Mtr/MSH reducing system, which was different from MsrA. MsrA still has a certain ability to reduce MetO. However, MsrB still has a certain ability to reduce MetO.

Table 3. Kinetics parameters of MsrA and MsrB for reduction of MetO by the Trx/TrxR and Mrx1/Mtr/MSH pathways.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Trx/TrxR</th>
<th>Mrx1/Mtr/MSH</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$K_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>MsrA</td>
<td>14.5 ± 2.6</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>MsrB</td>
<td>27.9 ± 5.8</td>
<td>0.14 ± 0.07</td>
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For determination of the Michaelis constant, activities were measured in the reaction mixtures containing 50 nM Tris-HCl (pH 8.0), 2 nM EDTA, 250 µM NADPH, 2 µM MsrA, 2 µM MsrB, 100 mM MetO. Trx system (4 µM TrxR and 0–120 µM of Trx) or Mrx1 system (4 µM Mtr, 500 µM MSH and 0–120 µM of Mrx1). The data are presented as the means of the values obtained from three independent assays. ND, not detectable under the conditions used.

Next, MsrA and MsrB activity was determined at a fixed concentration of MetO (100 nM) with different concentrations of Trx or Mrx1 reductants (0–120 µM). As shown in Table 3, the $K_m$ value, $K_{cat}$ value, and the catalytic efficiency of MsrA for Trx were 14.5 ± 2.6 µM, 2.2 ± 0.2 s$^{-1}$ and 15.2 × 10$^4$ M$^{-1}$ s$^{-1}$. The $K_m$ value, $K_{cat}$ value, and the catalytic efficiency of MsrA for Mrx1 were 26.8 ± 0.1 µM, 0.8 ± 0.1 s$^{-1}$ and 3.0 × 10$^7$ M$^{-1}$ s$^{-1}$. The $K_m$ values, $K_{cat}$ values, and catalytic efficiency of MsrB for Trx were 27.9 ± 5.8 µM, 0.14 ± 0.07 s$^{-1}$ and 5.01 × 10$^7$ M$^{-1}$ s$^{-1}$, and MsrB did not show activity when Mrx1 was used as the reductant. These data indicate that MsrB could reduce MetO to Met by the Trx/TrxR reducing system, but independent of the Mrx1/Mtr/MSH reducing system, which was different from MsrA. In addition, although there was activity shown in the presence of MsrB using Trx as the electron donor, the catalytic efficiency of MsrA were 30–45 fold higher than that of MsrB when using Trx as electron donors.

MsrB forms heterodimers with Trx during regeneration

When MsrA reduces oxidized Met to reduced Met, oxidized MsrA can form intramolecular disulfide bridges or sulfenic acid (Boschi-Muller et al., 2000). During MsrA regeneration, the intramolecular disulfide bond is subject to attack by Trx and switches to a transient intermolecular disulfide bond to link MsrA with Trx (Kim et al., 2009a; b; Si et al., 2015b; Tarrago et al., 2009a). To clarify the mechanism for regenerating MsrB activity, Trx variants (TrxC32S and TrxC35S), MsrB variants (MsrB1C12S and MsrB1C15S) and their wild-type were incubated with MsrB, respectively, in the presence of MetO. A control sample with just one type of protein was included in the assay system. As shown in Fig. 4, no heterodimer was detected between any one of the Mrx1 (wild-type and its variants) and MsrB. An additional polypeptide occurred in the MetO-containing mixture of TrxC35S and MsrB which did not exist in MetO-containing TrxC35S or MsrB solution, indicating that Trx can reduce disulfide bonds formed in MsrB. These results provide direct evidence that the Trx/TrxR system reduces the disulfide bond of MsrB formed during the regeneration process, indicating that the regeneration mechanism of MsrB is similar to MsrA: a transient intermolecular disulfide bond is formed between MsrB and Trx, then Trx attacks the intermolecular disulfide bond to realize the regeneration of MsrB. This result also confirmed that MsrB could use Trx as electron donors.

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<table>
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<th>Mrx1/Mtr/MSH</th>
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Discusson

This work establishes that C. glutamicum, like many other organisms, expresses at least two Msr proteins, each stereo specific for the reduction of methionine sulfoxide. Most organisms contain MsrA and MsrB existing independently of each other as separate enzymes (Lee et al., 2009; Tarrago et al., 2012). In yeast, MsrA efficiently reduces oxidized proteins and free MetO, whereas MsrB is specialized for the reduction of oxidized proteins (Tarrago et al., 2012). In M. tuberculosis, only the absence of both MsrA and MsrB will result in sensitivity to reactive nitrogen intermediates (RN1) (Lee et al., 2009). Besides, MsrA and MsrB can also exist as domains in a single fused protein (MsrAB) in some bacteria, such as Streptococcus pneumoniae and Shewanella oneidensis (Chen et al., 2007; Kim et al., 2009b). However, C. glutamicum is different. Although MsrA and MsrB exist independently, deletion of the msrB gene does not make C. glutamicum more sensitive to oxidative stresses, and only MsrA displays significant sensitivity. However, MsrB still has a certain ability to reduce MetO.

Our observations may indicate that MsrB in C. glutamicum is not very critical for the reduction of methionine sulfoxide. One possibility could be the differences of substrate specificity. Previous studies have demonstrated that MsrA is very specific to the reduction of Met-R-O (Couturier et al., 2012). In C. glutamicum, like many other organisms, expresses at least two Msr proteins, each stereo specific for the reduction of methionine sulfoxide. Most organisms contain MsrA and MsrB existing independently of each other as separate enzymes (Lee et al., 2009; Tarrago et al., 2012). In yeast, MsrA efficiently reduces oxidized proteins and free MetO, whereas MsrB is specialized for the reduction of oxidized proteins (Tarrago et al., 2012). In M. tuberculosis, only the absence of both MsrA and MsrB will result in sensitivity to reactive nitrogen intermediates (RN1) (Lee et al., 2009). Besides, MsrA and MsrB can also exist as domains in a single fused protein (MsrAB) in some bacteria, such as Streptococcus pneumoniae and Shewanella oneidensis (Chen et al., 2007; Kim et al., 2009b). However, C. glutamicum is different. Although MsrA and MsrB exist independently, deletion of the msrB gene does not make C. glutamicum more sensitive to oxidative stresses, and only MsrA displays significant sensitivity. However, MsrB still has a certain ability to reduce MetO.

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2012; Stadtman et al., 2002, 2003). Therefore, it is possible that, like Mycobacterium smegmatis, oxidation of Met with R-epimers in C. glutamicum is more infrequent than the oxidation of Met with S-epimers, thus requiring less or no MsrB to reduce MetO (Dhandayuthapani et al., 2009).

The physiological function of the msrB gene in C. glutamicum has been investigated here for the first time. We have further described the kinetic analyses of MsrB and have made a comparison with MsrA. Our studies suggest a limited protective role for C. glutamicum MsrB under oxidative stress conditions; however, MsrB still has a certain ability to reduce MetO via the Trx/TrxR system. This observation may provide additional insights into the study of Msr of C. glutamicum.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 31270078 and 31500087), the Key Science and Technology R&D Program of Shaanxi Province, China (2014K02-12-01), the Natural Science Foundation of Shandong Province, China (ZR2015CM012), the Laboratory Open Fund-project of Qufu Normal University (SK201503) and the Key Scientific and Technological Project of Henan Province, China (152300410229).

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