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The *Methanosarcina acetivorans* thioredoxin system activates DNA binding of the redox-sensitive transcriptional regulator MsvR

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Abstract

The production of biogas (methane) by anaerobic digestion is an important facet to renewable energy, but is subject to instability due to the sensitivity of strictly anaerobic methanogenic archaea (methanogens) to environmental perturbations, such as oxygen. An understanding of the oxidant-sensing mechanisms used by methanogens may lead to the development of more oxidant tolerant (i.e. stable) methanogen strains. MsvR is a redox-sensitive transcriptional regulator that is found exclusively in methanogens. We show here that oxidation of MsvR from *Methanosarcina acetivorans* (MaMsvR) with hydrogen peroxide oxidizes cysteine thiols, which inactivates MaMsvR binding to its own promoter (P_{msvR}). Incubation of oxidized MaMsvR with the *M. acetivorans* thioredoxin system (NADPH, MaTrxR, and MaTrx7) results in reduction of the cysteines back to thiols and activation of P_{msvR} binding. These data confirm that cysteines are critical for the thiol-disulfide regulation of P_{msvR} binding by MaMsvR and support a role for the *M. acetivorans* thioredoxin system in the *in vivo* activation of MaMsvR. The results support the feasibility of using MaMsvR and P_{msvR} , along with the *Methanosarcina* genetic system, to design methanogen strains with oxidant-regulated gene expression systems, which may aid in stabilizing anaerobic digestion.

Keywords

anaerobe; methane; methanogen; oxidative stress; transcription; disulfide

INTRODUCTION

Methane-producing archaea (methanogens) are strict anaerobes that are the rate-limiting step in biogas production [25]. Members of the genus *Methanosarcina* are particularly important in biogas production, due to their ability to produce methane with multiple substrates,

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including CO₂, methylated compounds (ex. methanol), and acetate, where all other methanogens are restricted to hydrogenotrophic (H₂ + CO₂) methanogenesis [4]. Environmental perturbations can significantly impact the ability of methanogens to produce methane. For example, many methanogenesis enzymes are oxygen sensitive resulting in a loss of energy conservation and methane production upon exposure to oxygen [9]. The partial reduction of oxygen by flavoenzymes and metalloenzymes results in the production of reactive oxygen species (ROS), including superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), which target many macromolecules within cells [13]. For example, O₂⁻ and H₂O₂ oxidize Fe-S cluster containing enzymes and thiols of cysteine residues in proteins, causing Fe-S cluster degradation and formation of deleterious disulfides, which leads to loss of metabolism [14]. For anaerobes, such as methanogens, to overcome oxygen exposure requires mechanisms to decrease the production of ROS and/or actively remove ROS, as well as repair oxidatively-damaged proteins. Indeed, many methanogens can survive oxygen exposure; however, methane production is severely inhibited [10,18,1,2]. In particular, members of the *Methanosarcinales* appear to be some of the most aerotolerant methanogens [1], which is likely due to a large number of putative antioxidant and repair proteins encoded in the genomes of sequenced *Methanosarcinales*. [5,11,19,7] However, information on the mechanisms used by methanogens, including the *Methanosarcinales*, to sense and respond to oxygen (oxidative stress) is limited. An understanding of the oxidant-sensing mechanism used by *Methanosarcina* sp., along with their robust genetic system [12], may lead to the development of strains with increased oxidant tolerance.

Many cells use the formation of disulfides in proteins to monitor the cellular redox state and the presence of deleterious ROS. For example, OxyR is a transcriptional regulator found in *Escherichia coli* and other bacteria that is used to monitor the presence of H₂O₂ [26]. Redox-sensing thiols of cysteine residues in OxyR are oxidized in the presence of H₂O₂, resulting in OxyR activation which increases the expression of H₂O₂ scavengers, Fe-S cluster repair enzymes, and thiol redox buffer systems. Once H₂O₂ levels have been reduced, OxyR is inactivated by the reduction of the disulfides by glutaredoxin 1, with reductant supplied by glutathione/glutathione reductase and NADPH [6]. H₂O₂-sensing transcriptional regulators have also been identified in eukaryotes [3]. Recent evidence has revealed the presence of a redox-sensing transcriptional regulator (MsvR) in methanogens. In *Methanothermobacter thermautotrophicus*, MsvR regulates expression of F₄₂₀H₂ oxidase (FpaA) by redox-sensitive binding to the promoter region of *fpaA* [17]. MsvR also regulates its own expression in *M. thermautotrophicus*. MsvR functions as a negative regulator to repress expression of *fpaA* and *msvR* under reducing conditions. Oxidation of MsvR results in the induction of *fpaA* and *msvR*. More recently, MsvR from *Methanosarcina acetivorans* (MaMsvR), a member of the *Methanosarcinales*, was shown to bind to its own promoter (P_{msvR}) only under reducing conditions [15]. Formation of disulfide(s) within the C-terminal V4R effector domain of MaMsvR were shown to abrogate binding of MaMsvR to the P_{msvR} promoter region. For MaMsvR to function as a redox-sensing transcriptional regulator, P_{msvR} binding by MaMsvR would need to be restored by disulfide reduction to reset the system once oxygen/ROS is removed. Reduction of MaMsvR disulfides and activation of P_{msvR} binding can be accomplished *in vitro* by the addition of the disulfide-reducing agent dithiothreitol (DTT); however, the physiological reducing system is unknown. In the

majority of organisms, disulfide reduction is mediated by thioredoxin and/or glutaredoxin systems [22].

Methanogens lack glutathione and therefore likely do not possess functional glutaredoxin systems [8,21]. However, recent evidence indicates the majority of methanogens contain thioredoxins (Trx) [20,24], which are small (~12 kDa) proteins that possess a CXXC active site motif necessary for disulfide oxidoreductase activity [22]. In the canonical system, Trx receives reducing equivalents from thioredoxin reductase (TrxR) with NADPH as the electron donor. Thus, the thioredoxin system could serve as the MsvR disulfide reducing system. *M. acetivorans* contains seven putative Trx homologs (MaTrx1-7) and a single TrxR homolog (MaTrxR). Recent evidence revealed that *M. acetivorans* contains at least three functional Trxs (MaTrx2, MaTrx6, and MaTrx7) and a complete NADPH-dependent thioredoxin system comprised of MaTrxR and MaTrx7 [20]. We show herein that the *M. acetivorans* NADPH-dependent thioredoxin system can reduce disulfides in oxidized MaMsvR and restore P_{msvR} -binding activity, indicating that the thioredoxin system is the physiological MaMsvR disulfide reducing system. Thus, it may be feasible to use P_{msvR} along with the *Methanosarcina* genetic system to design *Methanosarcina* strains with oxidant-responsive genes, which may increase the stability of biomethanation.

MATERIALS AND METHODS

Protein purification and manipulation

Recombinant MaTrxR and MaTrx7 were expressed in *E. coli* and purified to homogeneity as previously described [20]. Strep-tagged MaMsvR was expressed in *E. coli* and purified to homogeneity as previously described [15]. H_2O_2 -oxidized MaMsvR (MaMsvR_{ox}) was prepared by incubation of MaMsvR with 100-fold molar excess of H_2O_2 in buffer A (20 mM Tris pH 8, 15 mM $MgCl_2$, 120 mM KCl, 12.5 μ g/mL heparin, 10% glycerol) for 30 mins. Residual H_2O_2 was removed by buffer exchange into buffer A using a NAP5 column (GE Healthcare). DTT-reduced samples of MaMsvR were prepared by incubating 100 μ M MaMsvR_{ox} in buffer A containing 10 mM DTT for 20 min at room temperature. Residual DTT was removed using a NAP5 column. The ability of the thioredoxin system to reduce MaMsvR_{ox} was assayed by incubation of 10 μ M MaMsvR_{ox} with 1 mM NADPH, 0.5 μ M MaTrxR, and 2.5 μ M MaTrx7 for 1 hour at 37 °C in buffer A. Protein concentrations were determined by both the Bradford assay and using fluorescence with a Qubit protein assay following the manufacturer's instructions (Invitrogen).

Electrophoretic mobility shift assay (EMSA)

Complimentary 50-bp oligonucleotides containing P_{msvR} were synthesized (Integrated DNA technologies) and annealed to generate the P_{msvR} DNA probe used in all EMSAs [15]. DNA-binding reactions were prepared by incubating 100 nM P_{msvR} with 8 μ M MaMsvR in buffer A for 20 min at 37°C. Binding reactions were loaded onto a pre-run 6% polyacrylamide gel in 0.5X TBE buffer and electrophoresed for 75 min at 75 V at 10 °C. Gels were stained using SYBR gold (Life Technologies) and visualized using a Gel-Doc XR+ system (Bio-Rad Technologies).

Quantitation of thiols in MaMsvR

Aliquots of MaMsvR-containing samples used in EMSAs were analyzed for total thiol content using DTNB [23]. MaMsvR was denatured and thiols quantified by the addition of 10 μ L of MaMsvR-containing sample to 90 μ L of 6M guanidine-HCl in 100 mM KPO₄, pH 7.8 containing 175 μ M DTNB. Samples were incubated anaerobically for 15 min at room temperature and the absorbance at 412 nm was recorded. The number of thiols per MaMsvR monomer was calculated based on the concentration of TNB using $\epsilon_{412} = 13,700 \text{ M}^{-1} \text{ cm}^{-1}$ [23]. All samples were analyzed in triplicate. The background amount of thiols contributed by the denatured thioredoxin system was determined in samples containing NADPH, MaTrxR, and MaTrx7, but without MaMsvR.

RESULTS AND DISCUSSION

Reduction of MaMsvR disulfides and activation of MaMsvR DNA-binding by thioredoxin

MaMsvR contains ten cysteine residues, with two located in the DNA-binding domain, four in the V4R domain, and the remaining four located in the linker domain [15]. The cysteines within the V4R domain (C206, C225, C232, and C240) are postulated to function in redox-sensing, whereby thiol-disulfide exchange causes conformation changes which alter the ability of MaMsvR to bind an inverted repeat sequence motif (TTCGN₇₋₉CGAA) upstream of *P_{msvR}*. Three of the residues (C206, C232, and C240) are conserved in all MsvR homologs [17]. Specifically, C206 was shown to be critical for redox-sensitive binding of MaMsvR to *P_{msvR}*, because a MaMsvR C206A variant was able to bind to *P_{msvR}* under non-reducing conditions, whereas the wild-type MaMsvR is unable [15]. Previous results also revealed C225 was not involved in redox-sensing, while C232 and C240 impact MsvR binding to *P_{msvR}*, but the precise role of these cysteines is unclear. Thus, C206 is likely, and C232/C240 are possibly, involved in thiol-disulfide formation which serves to control DNA-binding by MaMsvR.

EMSA and thiol quantitation experiments were used to examine the role of thiol-disulfide exchange in controlling DNA binding by MaMsvR. First, MaMsvR was incubated with 100-fold molar excess of H₂O₂ to generate H₂O₂-oxidized MaMsvR (MaMsvR_{ox}). Quantitation of the thiol content of MaMsvR_{ox} under denaturing conditions revealed that four of the cysteines were not oxidized by H₂O₂ (Table 1), indicating some cysteines are inaccessible to H₂O₂, and likely do not participate in redox-sensing. Importantly, MaMsvR_{ox} was incapable of binding to the *P_{msvR}* region as revealed by the lack of shift when examined by EMSA (Fig. 1, **lane 2**). This result indicates that oxidation of the thiols of six cysteine residues is sufficient to inactivate MaMsvR DNA binding. The subsequent treatment of MaMsvR_{ox} with DTT resulted in detection of approximately nine thiols (Table 1), consistent with the total number of cysteines present in MaMsvR. Moreover, incubation of MaMsvR_{ox} with DTT restored binding to *P_{msvR}* (Fig. 1, **lane 3**). This result is consistent with H₂O₂ causing the oxidation of six thiols to disulfides, which causes reversible inactivation MaMsvR binding to *P_{msvR}*. The remaining four thiols are likely buried within the folded protein and are inaccessible to H₂O₂ or DTT, and therefore do not participate in thiol-disulfide exchange.

Similar experiments were performed to determine if the *M. acetivorans* thioredoxin system could also activate DNA-binding of MaMsvR_{ox}. Incubation of MaMsvR_{ox} with NADPH, MaTrxR, and MaTrx7 (complete thioredoxin system) activated binding of MaMsvR_{ox} to P_{msvR} (Fig. 1, **lane 6**). The complete thioredoxin system alone did not cause a shift of P_{msvR} in the EMSA (Fig. 1, **lane 4**) and NADPH/MaTrxR in the absence of MaTrx7 also failed to activate binding of MaMsvR_{ox} to P_{msvR} (Fig. 1, **lane 5**). Moreover, incubation of MaMsvR_{ox} with the complete thioredoxin system resulted in the detection of ten thiols (Table 1), consistent with all the H₂O₂-generated disulfides in MaMsvR being surface exposed and accessible to reduction by MaTrx7. Taken together these results demonstrate that the *M. acetivorans* NADPH-dependent thioredoxin system can activate P_{msvR} binding in oxidized MaMsvR and that MaTrx7 is required for the reduction of disulfides in oxidized MaMsvR. The reduction of MaMsvR by MaTrx7 is the first evidence of thioredoxin playing a role in the regulation of the activity of a transcription regulator in a methanogen. The activation of MaMsvR DNA binding by MaTrx7 also integrates P_{msvR} regulation by MsvR into the physiology of *M. acetivorans*, which supports the future use of P_{msvR} in engineering oxidant-responsive gene expression strains. For example, we have previously demonstrated that increased expression of catalase protects *M. acetivorans* from H₂O₂ [16].

Proposed model of MaMsvR regulation in *M. acetivorans*

Based on results from previous studies [15,17] and herein, we propose the following model (Fig. 2) for the regulation of the P_{msvR} binding activity of MaMsvR by thiol-disulfide exchange involving the thioredoxin system. Exposure of *M. acetivorans* to oxidants (ex. H₂O₂) results in oxidation of critical cysteines in MaMsvR to disulfides. Based on previous studies, C206 plays a crucial role, likely forming an intermolecular disulfide between MaMsvR monomers [15]. However, under the conditions tested here, at least six cysteines are involved in H₂O₂-induced disulfide formation, which may generate three intra-molecular, six inter-molecular, or some combination of intra/inter-molecular disulfides. Nonetheless, the formation of disulfides likely causes a conformational change in MaMsvR, such that MaMsvR is no longer able to bind P_{msvR}, which allows for RNAP to bind and transcription to proceed. Removal of oxidant and/or an influx of electron donor would allow for the reduction of MaMsvR disulfides by MaTrx7, with reducing equivalents supplied by MaTrxR and NADPH. The *in vitro* results presented here demonstrate that MaTrx7 can specifically reduce disulfides in MaMsvR, but we cannot rule out that the additional MaTrxs or other proteins also participate in the *in vivo* reduction of disulfides in MaMsvR and may do so under different conditions. However, the target specificity and the redox partner(s) of the other MaTrxs is currently unknown [20]. The data presented here link the regulation of MaMsvR to the redox status of *M. acetivorans* and the availability of reducing equivalents (e.g. NADPH). The results also reveal that methanogens have oxidant sensing systems which are integrated into metabolism in a manner similar to systems identified in bacteria and eukaryotes. Ultimately, due to the thioredoxin-dependent reversible P_{msvR} binding by MaMsvR it may be feasible to engineer strains with oxidant-inducible genes (e.g. catalase) using P_{msvR} in an effort to generate oxidant tolerant strains without an increased energy demand that would come from constitutive gene expression.

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Fig. 2. Proposed model of MaMsvR activation by the NADPH-dependent MaTrxR-MaTrx7 thioredoxin system in *M. acetivorans*

H₂O₂ causes the oxidation of thiols (SH) to disulfides which inactivates MaMsvR DNA binding, allowing transcription by RNAP. MaTrx7 receives reducing equivalents from NADPH/MaTrxR to reduce the disulfides to thiols and restore MaMsvR DNA binding.

Table 1

Quantitation of MaMsvR thiols.

Sample ^a	Thiols
MaMsvR _{ox}	4.0 ± 0.6
MaMsvR _{ox} + DTT	9.0 ± 1.5
MaMsvR _{ox} + NADPH/MaTrxR	4.8 ± 0.1
MaMsvR _{ox} + NADPH/MaTrxR/MaTrx7	9.9 ± 1.0

^a samples were processed and thiols quantified using DTNB as described in the Materials and Methods