ORIGINAL RESEARCH COMMUNICATION

Protein S-Mycothiolation Functions as Redox-Switch and Thiol Protection Mechanism in *Corynebacterium glutamicum* Under Hypochlorite Stress

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Abstract

Aims: Protein S-bacillithiolation was recently discovered as important thiol protection and redox-switch mechanism in response to hypochlorite stress in *Firmicutes* bacteria. Here we used transcriptomics to analyze the NaOCl stress response in the mycothiol (MSH)-producing *Corynebacterium glutamicum*. We further applied thiol-redox proteomics and mass spectrometry (MS) to identify protein S-mycothiolation. **Results:** Transcriptomics revealed the strong upregulation of the disulfide stress σ^H regulon by NaOCl stress in *C. glutamicum*, including genes for the anti sigma factor (*rshA*), the thioredoxin and MSH pathways (*trxB1*, *trxC*, *cg1375*, *trxB*, *mshC*, *mca*, *mtr*) that maintain the redox balance. We identified 25 S-mycothiolated proteins in NaOCl-treated cells by liquid chromatography–tandem mass spectrometry (LC-MS/MS), including 16 proteins that are reversibly oxidized by NaOCl in the thiol-redox proteome. The S-mycothiolome includes the methionine synthase (MetE), the maltodextrin phosphorylase (MalP), the myo-inositol-1-phosphate synthase (Ino1), enzymes for the biosynthesis of nucleotides (GuaB1, GuaB2, PurL, NadC), and thiamine (ThiD), translation proteins (TufA, PheT, RpsF, RplM, RpsM, RpsC), and antioxidant enzymes (Tpx, Gpx, MsrA). We further show that S-mycothiolation of the thiol peroxidase (Tpx) affects its peroxiredoxin activity *in vitro* that can be restored by mycoredoxin1. LC-MS/MS analysis further identified 8 proteins with S-cysteinylations in the *mshC* mutant suggesting that cysteine can be used for S-thiolations in the absence of MSH. **Innovation and Conclusion:** We identified widespread protein S-mycothiolations in the MSH-producing *C. glutamicum* and demonstrate that S-mycothiolation reversibly affects the peroxidase activity of Tpx. Interestingly, many targets are conserved S-thiolated across bacillithiol- and MSH-producing bacteria, which could become future drug targets in related pathogenic Gram-positives. *Antioxid. Redox Signal.* 20, 589–605.

Introduction

LOW MOLECULAR WEIGHT (LMW) thiols play essential roles as redox buffers in the defense against Reactive oxygen species (ROS) to maintain the reduced state of the cytoplasm (58). Eukaryotes and Gram-negative bacteria produce glutathione (GSH) as LMW thiol redox buffer. Among the Gram-positive bacteria, *Firmicutes* utilize as thiol redox buffer bacillithiol (Cys-GlcN-Mal, BSH) (19, 41) and *Actinomyces*

produce the related redox buffer mycothiol (AcCys-GlcN-Ins, MSH) (39). MSH is involved in the detoxification of ROS, toxins and antibiotics and MSH-deficient mutants are very sensitive to thiol-reactive compounds (40). ROS cause reversible oxidations of protein thiols to intra- or intermolecular disulfides, and to mixed disulfides with LMW thiols (S-thiolations) (1).

In eukaryotes, protein S-glutathionylation has emerged as major redox-regulatory mechanism that controls the activity

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Innovation

We have studied the transcriptome and redox proteome under NaOCl stress in the mycothiol (MSH)-producing model bacterium *Corynebacterium glutamicum*. Using liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis and redox proteomics we provide for the first time evidence that protein S-mycothiolation is a widespread protein protection mechanism under NaOCl stress in Actinomycetes. The S-mycothiolome includes proteins that function in the glycolysis, biosynthesis of methionine, glycogen, nucleotides, thiamine, protein translation, and antioxidant functions, including striking conservation of some S-thiolated proteins (e.g., Tuf, MetE, GuaB) across Gram-positive bacteria. We further show that the S-mycothiolated peroxidase Tpx is inactive and reactivated by the Mrx1/MSH/Mtr electron pathway *in vitro*.

of redox sensing transcription factors and protects active site cysteine (Cys) residues from irreversible oxidation to sulfonic acids (8). Many eukaryotic proteins, like α -ketoglutarate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, ornithine δ -aminotransferase, pyruvate kinase, heat specific chaperones, and regulatory proteins (c-Jun, NF- κ B) are reversibly inactivated or activated by S-glutathionylation (8, 24). However, little is known about the regulatory role of protein S-thiolation for bacterial physiology. We have recently identified protein S-bacillithiolations as mixed BSH protein disulfides in response to NaOCl stress in *Bacillus* and *Staphylococcus* species (7). Protein S-bacillithiolation controls redox-sensing regulators and protects active site Cys residues of essential and conserved metabolic enzymes from over-oxidation. The redox-sensing MarR-family OhrR repressor is inactivated by S-bacillithiolation after NaOCl stress resulting in upregulation of the OhrA peroxiredoxin (Prx) that confers NaOCl resistance (6, 29). Mass spectrometry identified 54 S-bacillithiolated proteins in different *Bacillus* and *Staphylococcus* species that include 29 unique proteins and 8 conserved proteins. The S-bacillithiolated proteins function as metabolic enzymes in biosynthetic pathways for amino acids (methionine, branched chain and aromatic amino acids), cofactors, nucleotides; or as translation factors, chaperones, redox, and antioxidant proteins (7). The methionine synthase MetE is the most abundant S-bacillithiolated protein in *Bacillus* species under NaOCl stress. S-bacillithiolation of MetE occurs in its active site Zn center leading to enzyme inactivation and methionine auxotrophy (6). Since amino acid biosynthetic enzymes and translation factors are conserved in the S-bacillithiolome among *Firmicutes*, we propose that S-bacillithiolation could function in redox-regulation of protein synthesis.

Protein disulfides and S-thiolations are reversible Cys oxidations that are reduced by the thioredoxin (Trx)/thioredoxin reductase (TrxR) system or by the glutaredoxin (Grx)/GSH/glutathione disulfide reductase system in *Escherichia coli* (15). The Trx system is involved in reduction of inter- and intramolecular protein disulfides and the Grx proteins catalyze de-glutathionylation of S-glutathionylated proteins. In *Actinomycetes*, a Grx-like mycoredoxin1 (Mrx1) with a CGYC active site motif has been structurally and biochemical characterized. Mrx1 reduces a MSH mixed β -hydroxyethyl disulfide (HED) and exclusively uses the MSH/Mtr/NADPH

electron pathway (57). *Corynebacterium glutamicum* serves as model for pathogenic *Actinomycetes*, such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* and is also of biotechnological importance. The disulfide stress response in *Actinomycetes* is controlled by the ECF sigma factor σ^R in *Streptomyces coelicolor* or its σ^H homolog in *C. glutamicum* (26). σ^R or σ^H are sequestered at control conditions by their cognate redox-sensitive anti sigma factors, RsrA or RshA, respectively in *S. coelicolor* or *C. glutamicum*. Redox-regulation has been shown for the RsrA/ σ^R system in *S. coelicolor*. RsrA senses disulfide stress by oxidation of the Zn-binding Cys residues leading to Zn release and free σ^R (1). The RshA/ σ^H system of *C. glutamicum* controls genes for the Trx/TrxR system (*trx*B, *trx*B1, *trx*C), for MSH biosynthesis and recycling (*msh*C, *mca*) and for the mycothiol disulfide (MSSM) reductase (*mtr*) (3, 11). The regulation of the Trx/TrxR and MSH pathways by RsrA/ σ^R or RshA/ σ^H homologs is conserved among *Actinomycetes* (1, 26, 45).

However, nothing is known about proteins that are redox-controlled or protected by protein S-mycothiolation under ROS stress conditions in *Actinomycetes*. Thus, we applied here transcriptomics, redox proteomics, and mass spectrometry (MS) to analyze the NaOCl-stress response and proteome-wide protein S-mycothiolations in the MSH-producing *C. glutamicum*.

Results

NaOCl-stress elicits a RshA/ σ^H disulfide stress response in *C. glutamicum*

Previously, we identified protein S-bacillithiolation by sub-lethal NaOCl concentrations in *Firmicutes* bacteria (7). To optimize the conditions for protein S-mycothiolation, the MSH-producing *C. glutamicum* ATCC13032 wild type was grown in minimal medium and treated with sub-lethal NaOCl concentrations that reduced the growth rate about half-maximal. Treatment of exponentially growing *C. glutamicum* cells with 180 μ M NaOCl at an optical density at 500 nm (OD₅₀₀) of 8.0 resulted in a slower growth compared to untreated cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars), but cells are still able to grow and reached the stationary phase after 5–6 h. Next, we applied DNA microarray analyses to analyze if NaOCl stress causes a disulfide stress response in *C. glutamicum* as shown for *Bacillus subtilis* (6). Transcriptome analyses of *C. glutamicum* wild-type cells were performed after treatment with 180 μ M NaOCl for 10 and 30 min. The transcriptome data revealed that 224 and 411 genes are at least twofold induced and 325 and 472 genes are at least twofold repressed by NaOCl stress (p -values < 0.05) in two biological replicates after 10 and 30 min of NaOCl stress, respectively (Supplementary Tables S1 and S2). This indicates that 7.3% and 13.5% of the genome (3052 genes) were twofold induced after 10 and 30 min NaOCl stress, respectively, while 10.6% and 15.5% showed twofold reduced transcription.

The changes in the *C. glutamicum* transcriptome in response to disulfide-stress provoked by diamide were previously published (3, 11). To visualize the overlaps in the gene expression profiles between NaOCl and diamide stress, we performed a hierarchical clustering analysis. The cluster analysis revealed a strong upregulation of the disulfide stress σ^H regulon by both diamide and NaOCl (Fig. 1,

FIG. 2. NaOCl-stress causes strongly increased reversible thiol-oxidations in the redox proteome of the *C. glutamicum* wild type. The redox proteome of *C. glutamicum* wild type at control conditions (green image) was overlaid with that of cells exposed to 180 μ M NaOCl-stress (red image). For redox proteomics, reduced protein thiols in cell extracts were alkylated with IAM followed by disulfide reduction and labeling with BODIPY FL C₁-IA. NaOCl-sensitive proteins with increased redox-ratios in NaOCl-treated cells are red-labeled and the identified S-mycothiolated proteins are marked with (*). The oxidized proteins were identified by MALDI-TOF-TOF MS/MS and are listed with their ratios of reversible thiol-oxidations *versus* protein amounts ratios in Supplementary Table S4. IAM, iodoacetamide; LC, liquid chromatography. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

proteomes to identify possible S-mycothiolated proteins in the wild type. For redox proteomics, untreated and NaOCl-exposed cells were harvested in urea/thiourea buffer in the presence of 100 mM iodoacetamide (IAM) to alkylate all reduced thiols. Proteins with reversible thiol-oxidations, including S-thiolations were reduced with Tris-(2-carboxyethyl)-phosphine (TCEP) and labeled with the fluorescent dye BODIPY FL C₁-IA. The fluorescent image was overlaid with the Coomassie-stained protein amount image and the fluorescence/protein amount ratios were quantified using the Decodon software as level of reversible thiol oxidations. Proteins with basal level oxidation include the Prx Bcp (3–5-fold redox ratios) and the thiol peroxidase (Tpx) (7–10-fold redox ratios) that form intramolecular disulfides during their peroxide detoxification cycle (36). Further conserved redox-sensitive proteins with basal oxidations include the dihydrolipoyl dehydrogenase Lpd (PdhD homolog) and the glyceraldehyde-3-phosphate dehydrogenase GapA in *C. glutamicum* that are also oxidized to intramolecular disulfides (6).

Treatment of *C. glutamicum* with 180 μ M NaOCl stress resulted in strongly enhanced reversible thiol-oxidation of 30 NaOCl-sensitive proteins (Figs. 2 and 3; Supplementary Table

S4). The search for conserved Cys residues using the CDD database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that many of these proteins harbor nucleophilic Cys residues essential for catalysis, substrate or metal ion binding. These proteins are involved in the biosynthesis of carbohydrates, amino acids, cofactors, nucleotides, iron utilization, and protein translation. We were interested if the identified NaOCl-sensitive proteins are oxidized to S-mycothiolated proteins and monitored MSH-mixed protein disulfides using nonreducing MSH-specific Western blot analysis. The results showed enhanced protein S-mycothiolation specifically after NaOCl stress that was absent in the *mshC* mutant (Fig. 3B). However, the comparison of the redox proteomes between the wild type and the *mshC* mutant after NaOCl stress (Fig. 3A) revealed only little differences suggesting alternative S-thiolation in the absence of MSH. Notable was only the strong oxidation of the acetyl CoA carboxylase AccBC in the wild type by NaOCl stress that was missing in the *mshC* mutant due to lower protein amounts. In conclusion, the redox proteome and MSH Western blot results indicate that NaOCl stress causes strongly increased reversible thiol-oxidations, including protein S-mycothiolations in *C. glutamicum* wild-type cells.

FIG. 3. Close-ups of NaOCl-sensitive proteins identified in the thiol-redox proteomes of *C. glutamicum* wild type (WT) and the *mshC* mutant (A) and monitoring protein S-mycothiolation by MSH Western blot analysis (B). (A) Examples for reversibly oxidized proteins in the redox proteomes of the wild type (WT) and the *mshC* mutant are shown after NaOCl treatment. Among these NaOCl-sensitive proteins, 16 proteins are labelled with (*) that have been identified as S-mycothiolated in the wild type using LC-MS/MS analysis (Table 1 and Supplementary Table S5, Supplementary Fig. S5). Overlay images represent the redox proteome (red) compared to the Coomassie-stained protein amount image (green) at control conditions (co) and 10 and 30 min after NaOCl exposure (1–3). Column 4 represents the overlay of the redox proteomes of control cells (green) versus the redox proteome after 10 min of NaOCl stress (red). The identified proteins and their fluorescence/protein quantity ratios are listed in Supplementary Table S4 and the complete redox proteomes of the wild type and the *mshC* mutant are given in Supplementary Figures S3–S4. (B) Nonreducing MSH-specific Western blot analysis indicates widespread S-mycothiolation in *C. glutamicum* wild type. The IAM-alkylated protein extracts of the wild type, *mshC* and *malP* mutants were harvested before (co) and 15 min after 180 μ M NaOCl stress and subjected to nonreducing MSH-specific Western blot analysis. LC, liquid chromatography; MSH, mycothiol. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Shotgun-LC-MS/MS identifies 25 proteins with S-mycothiolations in NaOCl-treated cells

Proteins that form MSH mixed disulfides after NaOCl stress were identified using LTQ-Orbitrap liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. The specific S-mycothiolated Cys peptides were identified by searching the LC-MS/MS results for the 484 Da mass shift of MSH at Cys residues. We further observed that the precursor ions have lost myo-inositol during MS/MS fragmentation. These neutral loss precursor ions with a mass loss 180 Da were used as diagnostic abundant peaks for verification of S-mycothiolated peptides (Supplementary Fig. S5). In control cells, we identified two peroxidases, the Tpx S-mycothiolated at the active site Cys60 and the resolving Cys94 and the glu-

tathione peroxidase (Gpx) S-mycothiolated at the active site Cys36. Interestingly, the spectral counts (quantitative value) of these Cys-SSM peptides of Tpx and Gpx increased strongly in NaOCl-treated cells. In addition, the Prx Bcp was identified with an Cys89-SS-Cys94 intramolecular disulfide peptide and the dihydrolipoyl dehydrogenase Lpd was oxidized to the conserved Cys42-SS-Cys47 disulfide in control cells (Supplementary Fig. S6I, J).

In NaOCl-treated cells, we identified 25 S-mycothiolated proteins in *C. glutamicum* with characteristic myo-inositol-loss precursor ions in their fragment ion spectra (Table 1; Supplementary Tables S5, Supplementary Fig. S5). These 25 S-mycothiolated proteins include 16 proteins identified also in the redox proteome as NaOCl-sensitive proteins, including Tuf, GuaB1, GuaB2, SerA, and MetE as conserved

TABLE 1. IDENTIFICATIONS OF PROTEINS WITH S-MYCOTHIOLATIONS IN THE *CORYNEBACTERIUM GLUTAMICUM* WILD TYPE AND WITH S-CYSTEINYLATIONS IN THE *MSHC* MUTANT USING LC-MS/MS^a

Cg-no	Protein	Function	Conservation and function of Cys with -SSM or -SSCys	Peptide-SSM or -SSCys* sequence	m/z precursor	Peptide MW	Charge	APPM
Metabolism of carbohydrates and amino acids								
Cg1479	Malp ^b	Maltodextrin phosphorylase	Cys180 not cons.	(R)ASNQLVVVC ₁₈₀ (+484)FDDM(+16)K(T)	985.4077	1968.8008	2	0.21
Cg1290	MetE ^{b,c}	Methionine synthase	Cys713 Zn-bind. active site	(R)ASNQLVVVC ₁₈₀ (+119)FDDMK(T) (R)QLWVNPD _{C713} (+484)GLK(T) (R)QLWVNPD _{C713} (+119)GLK(T) (R)VTADDDVVC ₂₃₉ (+484)EGISNISAADIEAAQQAQGHHTIK(L)	530.2316 878.8929 464.5530 1192.2073	1587.6731 1755.7713 1390.6373 3573.6000	2 2 2 3	-0.13 0.91 -0.20 0.63
Cg1337	Hom ^b	Homoserine dehydrogenase	Cys239 not cons.	(K)VGIDLADATEASQNC ₇₉ (+484)TIK(I)	1167.0222	2332.0299	2	-0.06
Cg3323	Ino-1 ^b	Myo-inositol-1-phosphate synthase	Cys79 not cons.	(K)VGIDLADATEASQNC ₇₉ (+119)TIK(I) (R)IIESC ₃₃₂ (+484)QDLK(S)	656.6396 766.8385	1966.8969 1531.6624	2 2	-0.47 -0.71
Cg3068	Fba ^b	Fructose-bisphosphate aldolase	Cys332 not cons.	(R)GAGFDVVS _{TEPC266} (+119)TDSPLFK(L)	718.3111	2151.9115	2	-0.79
Cg1451	SerA ^b	Phosphoglycerate dehydrogenase	Cys266 not cons.	(R)RLNPELC ₃₆₇ (+484)VDPGLQFDAAVDPGVAR(K)	1012.8146	3035.4219	3	-0.01
Cg3048	Pta	Phosphate acetyltransferase	Cys367 not cons.	(R)GVLAGLNC ₃₃₈ (+484)ATTR(E)	830.3814	1658.7483	2	-0.58
Cg0147	XylB	Sugar (pentulose and hexulose) kinase	Cys338 not cons.					
Metabolism of coenzymes and nucleotides								
Cg2964	GuaB1 ^{b,c}	Inosine-5'-monophosphate dehydrogenase	Cys302 forms Thioimide, active site	(K)VGVGPGAmC ₃₀₂ (+484)TTR(M)	824.8462	1647.6778	2	-0.80
Cg0699	GuaB2 ^{b,c}	Inosine-5'-monophosphate dehydrogenase	Cys317 forms Thioimide, active site	(K)VGIGPGSIC ₃₁₇ (+484)TTR(V)	822.8771	1643.7397	2	0.78
Cg1215	NadC ^b	Nicotinate-nucleotide-pyrophosphorylase	Cys114 not cons.	(R)TSGIATLTSC ₁₁₄ (+484)YVAEVK(G)	1063.9893	2125.9640	2	-0.36
Cg2862	PurL ^b	Phosphoribosylformylglycinamide synthase 2	Cys716 not cons.	(R)TSGIATLTSC ₁₁₄ (+119)YVAEVK(G) (K)LGC ₇₁₆ (+484)TNDSAVIAVK(G)	587.9507 887.9076	1760.8302 1773.8006	3 2	-1.33 -0.39
Cg1654	TheD ^b	Thiamine biosynthesis protein	Cys451 active site, HMP substrate binding	(K)LGC ₇₁₆ (+119)TNDSAVIAVK(G) (R)VNTTNSHGTGC ₄₅₁ (+484)SLASLATK(I)	470.5635 811.6971	1408.6687 2432.0694	2 2	-0.34 0.45
Cg3409	ThiD2	Hydroxymethylpyrimidine kinase	Cys111 not cons.	(K)HVVLDPVLC ₁₁₁ (+119)K(G)	452.2452	1353.7139	2	-0.87

(continued)

TABLE 1. (CONTINUED)

C α -no	Protein	Function	Conservation and function of Cys with -SSM or -SSC _{Cys}	Peptide-SSM or -SSC _{Cys} * sequence	m/z precursor	Peptide MW	Charge	Δ ppm
Antioxidant enzymes involved in the ROS response								
Cg1236	Tpx ^{b,d}	Thiol peroxidase	Cys60 peroxidatic	(K)LVLNIFPSVDTCVC ₆₀ (+484)ATSVR(K)	1238.1064	2474.1983	2	2.51
			Cys94 resolving	(R)FC ₉₄ (+484)SAEGIENVTPVSAFR(S)	1156.0127	2310.0108	2	3.34
Cg2867	Gpx ^d	Glutathione (Myc-thiol) peroxidase	Cys36 peroxidatic	(K)C ₃₆ (+484)GLTPQYEGLEK(L)	910.9011	1819.7877	2	1.02
Cg2445	HmuO ^b	Heme oxygenase	Cys165 not cons.	(R)EYGVSEELSFYC ₁₆₅ (+484)FEDLGK(L)	1335.5519	2669.0892	2	-1.25
Cg3236	MsrA	Peptide methionine-sulfoxide reductase	Cys91 cons.	(R)EVC ₉₁ (+484)SGR(T)	567.7182	1133.4218	2	0.19
Translation factors and ribosomal proteins								
Cg0601	RpsC	30S ribosomal protein S3	Cys153 cons.	(K)VVC ₁₅₃ (+484)SGR(L)	552.7297	1103.4448	2	-2.38
Cg3308	RpsF ^b	30S ribosomal protein S6	Cys67 cons.	(K)C ₆₇ (+484)ESATVLELDR(V)	860.3685	1718.7225	2	-0.13
Cg0673	RplM	50S ribosomal protein L13	Cys50 not cons.	(K)GKPLYAPNVDC ₅₀ (+484)GDHVIVINADK(V)	706.3352	2821.3117	4	-1.28
Cg0652	RpsM	30S ribosomal protein S13	Cys86 not cons.	(R)KIEIGC ₈₆ (+484)YQGIR(H)	882.4106	1762.8066	2	-3.01
Cg0587	Tuf ^{b,c}	Elongation factor Tu	Cys277 cons.	(R)KIEIGC ₈₆ (+119)YQGIR(H)	424.2026	1269.5859	2	0.89
Cg1575	PheT ^b	Phenylalanyl-tRNA-synthetase beta sub-unit	Cys89 in tRNA binding domain	(K)LLDSAEAGDNC ₇₇ (+484)GLLLR(G)	1072.4940	2142.9735	2	3.34
				(R)HC ₈₉ (+484)HVNVDGANGTGELQSVcGAR(N)	960.0820	2877.2243	2	-3.00
Unknown function proteins								
Cg1858	WA5_1783	Uncharacterized protein (WA5_1783)	Cys322 not cons.	(R)QVFC ₃₂₂ (+484)LSSSGR(E)	784.3330	1566.6515	2	-1.70

^aCytoplasmic protein extracts of *Corynebacterium glutamicum* ATCC13032 wild type and the isogenic *misI/C* mutant were harvested at 15 min after NaOCl stress in urea-IAM-buffer, tryptic in-gel digested and the peptides were analyzed using a LTQ Orbitrap-VelosTM mass spectrometer as described in the Methods section. 25 Proteins with S-mycothiolations were identified by the additional mass of 484 Da at Cys residues and with the diagnostic neutral myo-inositol loss precursor ions that appeared as abundant ions in the MS/MS spectra of each mycithiolated peptide. Peptides with S-cysteinylation were identified with a mass difference of 119 Da at Cys residues. The table includes the protein names, Cg-accession numbers and protein functions as derived from the UniprotKB database (www.uniprot.org) and information about conservation of the mycithiolated Cys residues and Cys functions as revealed by the Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The table also includes the m/z of the precursor ions, the neutral molecular mass (MW) of the mycithiolated peptides, peptide charges and mass deviations (Δ ppm). The complete collision-induced diffraction (CID) MS/MS spectra of all S-thiolated peptides, the b and y fragment ion series, the SEQUEST Xcorr and SEQUEST Δ Cn scores, and further peptide information are given in Supplementary Figures S5 and S6, and in Supplementary Table S5.

^b16 proteins have been identified as NaOCl-sensitive proteins in the Thiol-redox proteome (Figs. 2 and 3) with increased redox/protein ratios after NaOCl stress in this study. The redox/protein amount ratios are given in Supplementary Table S5.

^cThe S-mycithiolated proteins MetE, GuaB and Tuf are also conserved targets for S-bacillithiolation in *Bacillus* or *Staphylococcus* species according to our previous study.

^dThe S-mycithiolated peroxidases Tpx and Gpx were identified in untreated and NaOCl-treated cells. All other S-mycithiolated proteins were only identified in NaOCl-exposed cells. Cys, cysteine; IAM, iodoacetamide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LTQ, linear trap quadrupole; MW, molecular weight; ROS, reactive oxygen species.

FIG. 4. Metabolic pathways for glycolysis, biosynthesis of methionine, thiamine, GMP, MSH, and glycogen metabolism that include the identified *S*-mycothiolated metabolic enzymes. The enzymatic metabolic pathways were derived from the genome sequence of *C. glutamicum* or from previous publications (37) with the identified *S*-mycothiolated or reversibly oxidized proteins labeled with colors (*S*-mycothiolated proteins are dark gray; reversibly oxidized proteins are white-gray shadowed; both reversibly oxidized and *S*-mycothiolated proteins are light gray). The selected *S*-mycothiolated metabolic enzymes include MetE, SerA, Hom (Met biosynthesis); Fba, Pta (glycolysis); MalP (glycogen utilization); Ino-1 (MSH biosynthesis); ThiD1, ThiD2 (thiamine biosynthesis); GuaB1, GuaB2 (GMP biosynthesis). Further proteins with Cys-SSM sites are involved in translation (Tuf, PheT, RpsC, RpsF, RpsM, RplM) and antioxidant functions (Tpx, Gpx, MsrA) that are not shown here. Cys, cysteine; GMP, guanosine 5'-phosphate.

targets for *S*-thiolations across Gram-positive bacteria (7). The metabolic pathways, including the functions of the main *S*-mycothiolated proteins are shown in Figure 4. The *S*-mycothiolated proteins are involved in the metabolism of carbohydrates, for example, glycolysis (Fba, Pta, XylB), glycogen and maltodextrin degradation (MalP); amino acid biosynthesis pathways for serine, Cys, methionine (MetE, SerA, Hom); nucleotide and thiamine cofactor biosynthesis (GuaB1, GuaB, PurL, NadC, ThiD1 und ThiD2); redox and antioxidant functions for peroxide detoxification (Tpx, Gpx), methionine sulfoxide reduction (MsrA), heme degradation for iron mobilization (HmuO), and protein translation, such as ribosomal proteins (RpsF, RpsC, RpsM, RplM) and translation elongation factors (Tuf). The *S*-mycothiolated proteins MetE and

MalP represent also very abundant proteins with strongly increased oxidation ratios after NaOCl stress in the redox proteome. The comparison of the *S*-mycothiolation pattern between wild type and *malP* mutant cells by nonreducing MSH-Western blots confirmed that MalP is one of the most strongly *S*-mycothiolated proteins in NaOCl-treated cells (Fig. 3B). Interestingly, also the first enzyme of the MSH biosynthesis pathways, the myo-Inositol-1-phosphate synthase (Ino-1 or Cg3323) was *S*-mycothiolated at Cys79 suggesting perhaps regulation of MSH biosynthesis under NaOCl stress.

We were interested if proteins are *S*-thiolated by alternative redox buffers, such as ergothioneine (EGT) or Cys in *C. glutamicum* in the *mshC* mutant. In previous studies, we identified few proteins that are *S*-cysteinylated in the absence of

FIG. 5. Mutants lacking MSH, σ^H and MalP are sensitive to NaOCl stress in growth assays. Growth phenotype assays of *C. glutamicum* wild type (WT) in comparison to the $\Delta mshC$ (A), $\Delta sigH$ (B) and $\Delta malP$ (C) mutant strains that were treated with 180 μM NaOCl at an OD₅₀₀ of 8 and growth was monitored. The survival assay of the $\Delta malP$ mutant (D) after 180 μM NaOCl stress indicates a stronger killing effect of NaOCl in the mutant. OD₅₀₀, optical density at 500 nm.

BSh in *B. subtilis*, but the *bshA* mutant was more sensitive to NaOCl stress indicating that S-cysteinylation cannot fully compensate for S-bacillithiolation (6, 7). In *Mycobacterium smegmatis*, the histidine-derived thiol EGT has been suggested to compensate for the loss of MSH in *mshA* mutants (56). However, we were unable to detect proteins that form mixed disulfides with EGT in the *mshC* mutant. Instead, we identified eight S-cysteinylation proteins in the *mshC* mutant, which overlapped with only six proteins detected as S-mycothiolated in the wild type (MalP, MetE, Ino-1, PurL, NadC, and RpsM) (Table 1; Supplementary Table S5, Supplementary Fig. S6). The S-cysteinylation Cys-peptides identified in the *mshC* mutant were identical with the S-mycothiolated Cys-peptides of the wild type. However, compared to S-mycothiolated peptides the number of spectra (spectral counts) was significantly lower for S-cysteinylation peptides and even for 19 S-mycothiolated proteins of the wild type no S-cysteinylation peptides were detected in the *mshC* mutant (Table 1; Supplementary Table S5B). These results indicate that Cys can be used for alternative S-thiolations in the absence of MSH, but S-cysteinylation cannot fully compensate for S-mycothiolation.

The roles of mycothiol, σ^H and MalP in protection against disulfide stress

Next, we analyzed *tpx*, *gpx*, *malP*, *mshC*, and *sigH* mutants for their sensitivity to NaOCl stress in growth phenotype assays. Previously, the *sigH* and *mshC* mutants were shown to have decreased oxidative stress resistance in *C. glutamicum* (27, 35). In our growth phenotype assays, the *mshC* and *sigH*

mutants are also very sensitive to NaOCl stress confirming previous results (Fig. 5).

Growth phenotype assays further revealed an increased sensitivity of the *malP* mutant to NaOCl-stress indicating that S-mycothiolation of MalP confers protection against NaOCl stress (Fig. 5). The sensitivity of the *malP* mutant to NaOCl stress is caused by an increased killing effect of NaOCl stress as determined in survival assays. No NaOCl-sensitive growth phenotypes were detected for *tpx* and *gpx* mutants (data not shown). In conclusion, the σ^H /RshA disulfide stress system, MSH and MalP confer protection against NaOCl stress in *C. glutamicum*.

NaOCl stress leads to reduced glycogen degradation which could involve S-mycothiolation of MalP in vivo

In *C. glutamicum*, glycogen transiently accumulates during the exponential growth. Glycogen is degraded by MalP either slowly before the onset of the stationary growth phase or rapidly after osmotic stress when glucose uptake is reduced (52). It could be possible that S-mycothiolation of MalP prevents glycogen degradation to save the energy source under oxidative stress. To test an effect of S-mycothiolation of MalP on glycogen degradation *in vivo*, the glycogen content and glucose uptake rates (GURs) were determined after NaOCl stress. The GUR decreased about threefold 10 min after addition of NaOCl (Fig. 6A). For comparison, osmotic stress caused by NaCl leads to a twofold decrease of the GUR to 15.6 ± 0.7 nmol/min/mg dw (52). The glycogen content was quickly decreased about twofold within 15 min after osmotic stress (Fig.

FIG. 6. Effects of NaOCl stress on the uptake of [^{14}C]-labelled glucose (A) and intracellular glycogen content in *C. glutamicum* wild type (B). (A) The glucose uptake rates were determined after NaOCl stress as in previous studies (34). (B) The intracellular glycogen content was measured in untreated *C. glutamicum* wild type cells (Δ) and in cells treated with NaOCl ($\blacksquare$) or NaCl ($\circ$) stress. The time point 0 indicates the time of NaOCl or NaCl addition. Glycogen contents and glucose uptake rates were determined in triplicates from 3 biological replicate experiments.

6B). However, the glycogen level of NaOCl-treated cells was only slightly reduced within 45 min of stress exposure. Since the glycogen content remains stable in NaOCl-treated cells, despite the drastically reduced glucose uptake, glycogen degradation could be inhibited by S-mycothiolation of MalP. Future studies are underway to determine the effect of S-mycothiolation on MalP activity *in vitro*.

S-mycothiolation of Tpx in vitro abolishes its peroxidase activity which can be restored by the Mrx1/MSH/Mtr electron pathway

Next, we were interested to see whether the identified S-mycothiolated proteins are substrates for Mrx1. However, redox proteome comparison between the wild type and *mrx1* mutants did not show a difference in the oxidation ratios of NaOCl-sensitive S-mycothiolated proteins (data not shown). Thus, we tested if S-mycothiolation affects the peroxidase activity of Tpx *in vitro* and if Mrx1 is able to reactivate Tpx. Tpx was expressed and purified from *E. coli* and the peroxidase activity of Tpx was analyzed in a coupled enzyme assay composed of Trx, TrxR, and NADPH. Purified Tpx has peroxidase activity when coupled to the Trx electron pathway, which is abolished when Tpx is omitted in this reaction (Fig. 7A, B). To test the effect of S-mycothiolation on the peroxidase activity, reduced Tpx was preincubated with reduced MSH before the addition of 1 mM hydrogen peroxide (H_2O_2). LC-MS/MS analysis revealed strong S-mycothiolation of all three Cys residues of Tpx, the peroxidatic Cys60, the resolving Cys94 and also the nonconserved Cys81 (Fig. 7, Supplementary Table S6). S-mycothiolation of Cys81 *in vitro* (but not *in vivo*) might be due to the much higher MSH amount applied in the *in vitro* reaction for purified Tpx.

The S-mycothiolation of Tpx most likely protects the active site from overoxidation to sulfinic and sulfonic acids during NaOCl stress, and might change its peroxidase activity. To test this, S-mycothiolated Tpx was used in this Trx/TrxR-coupled assay and no peroxidase activity was measured confirming that S-mycothiolation of the active site Cys60 inhibits the peroxidase activity (Fig. 7C) (48). To reactivate S-mycothiolated Tpx, we reconstructed the Mrx1/MSH/Mtr electron transfer pathway *in vitro* with S-mycothiolated Tpx

as substrate. Control samples without Tpx or with non-mycothiolated Tpx were included in the assay. Only the sample with 50 μM Tpx-SSM showed consumption of NADPH and the rate of NADPH consumption increased when 75 μM Tpx-SSM was applied in this assay (Fig. 7D–F). No reduction of Tpx-SSM was observed when Mrx1 was omitted, indicating that the high MSH concentration (400 μM) is not sufficient to reduce Tpx-SSM without Mrx1. MS confirmed that the S-mycothiolated Cys60, Cys81, and Cys94 peptides are completely reduced within 5–10 min by Mrx1 (Fig. 7G, H, Supplementary Tables S6A, B). During Tpx-SSM reduction, Mrx1 gets S-mycothiolated at its active site Cys12 as transient Mrx1-SSM intermediate that was also detected by MS (Supplementary Table S6D). However, most Mrx1 protein was oxidized to the Cys12-SS-Cys15 intramolecular disulfide during Tpx-SSM reduction (Supplementary Table S6E).

Discussion

Mycothiol is the major LMW thiol in Actinomycetes and functions in detoxification of oxidants, electrophiles, toxins and several antibiotics (13, 39). In *C. glutamicum*, MSH plays very broad and essential roles in the detoxification of alkylating agents, toxins (methylglyoxal), oxidants (H_2O_2 , diamide), antibiotics, glyphosate, ethanol, and toxic heavy metals (Zn, Co, Mn, Cr) (35). MSH is also an essential cofactor for the maleylpyruvate isomerase in the degradation of aromatic compounds, such as gentisate and 3-hydroxybenzoate and required also for detoxification of naphthalene and resorcinol (14, 35). Mutants in the MSH biosynthesis pathways were unable to grow in the presence of gentisate and 3-hydroxybenzoate, which indicates that MSH cannot be replaced by Cys as thiol-cofactor. In addition, MSH is required for conjugation of arsenate to a MSH-S-conjugate that is detoxified by two MSH-dependent arsenate reductases ArsC1 and ArsC2 in *C. glutamicum* (43).

In this article, we present for the first time evidence that MSH also functions in widespread protein S-mycothiolation as important thiol-protection mechanism under hypochlorite stress in *C. glutamicum*. In addition, we provide evidence that S-mycothiolation inhibits the peroxidase activity of Tpx *in vitro* which can be restored by Mrx1. Protein S-thiolation

FIG. 7. Purified Tpx is active as peroxidase, inactivated by S-mycothiolation and can be reactivated by Mrx1 *in vitro*. (A) Progress curves for the consumption of NADPH are shown. The electron transfer pathway of Tpx with Trx, TrxR and NADPH was reconstructed with 20 μM (□) or 100 μM (▲) H₂O₂ as substrate for Tpx. A control sample (○) without H₂O₂ was included. (B) An identical sample but with Tpx omitted showed no peroxidase activity. (C) The electron transfer pathway was reconstructed for S-mycothiolated Tpx showing no peroxidase activity. (D–F) The Mrx1 electron transfer pathway composed of Mrx1, MSH, Mtr and NADPH was reconstructed and reduced (▽) or S-mycothiolated (▲) Tpx was added as substrate. A control sample without Tpx (○) was included. In (D) 50 μM of reduced (▽) or S-mycothiolated (▲) Tpx was used and in (E) 75 μM of reduced (▽) or S-mycothiolated (▲) Tpx was used. (F) Mrx1 was omitted in the above pathways using 50 μM reduced (▽) or S-mycothiolated (▲) Tpx. In the absence of Mrx1, no electron transfer was observed. (G, H) Mass spectrometry revealed complete reduction of Tpx-SSM by Mrx1. The Spectral counts for the S-mycothiolated (G) and NEM-alkylated (H) Cys60-, Cys81- and Cys94-peptides of Tpx are calculated using the Scaffold software after different times of Mrx1 reduction (see Supplementary Tables S6A–C). H₂O₂, hydrogen peroxide; Mrx1, mycoredoxin1; NEM, N-ethylmaleimide; Trx, thioredoxin; TrxR, thioredoxin reductase.

was induced in our previous study in *B. subtilis* by NaOCl stress, which causes a disulfide stress response and induction of the Spx, PerR and OhrR-regulons in the transcriptome (6). Here we confirmed that NaOCl stress elicits a disulfide stress response also in *C. glutamicum* that is controlled by the σ^H /RshA regulatory system (3). In addition, similar thiol-based redox regulators respond to NaOCl stress in both bacteria, belonging to the MarR/DUF24-family that control quinone oxidoreductases or to the ArsR-family regulating metal ion or arsenate efflux systems (6). The results that NaOCl stress causes a common disulfide stress response in bacteria are in agreement with the chemistry of NaOCl. Hypochlorite is defined as reactive chlorine species that leads to chlorination of thiol groups to form sulfenyl chloride (Cys-SCl) as unstable intermediate (20, 21). Cys-SCl reacts further with proximal thiols to form disulfides or is overoxidized to Cys sulfonic acid. Thus, S-mycothiolation is

particularly important under NaOCl stress to prevent lethal overoxidation of Cys residues.

Using shotgun-LC-MS/MS analysis, we identified 25 cytoplasmic proteins that are S-mycothiolated under NaOCl stress conditions in the wild type and 16 of these are also major NaOCl-sensitive proteins in the thiol-redox proteome. In contrast, only 6 of these S-mycothiolated proteins were identified as S-cysteinylation in the absence of MSH with significantly lower spectral counts (Supplementary Table S5B). This indicates that S-cysteinylation can only partly compensate for alternative S-thiolations in the *mshC* mutant. However, due to the instability of S-thiolation modifications we cannot exclude that other redox buffers, like CoASH or EGT are also involved in S-thiolations in the absence of MSH. However, phenotype analyses of the *mshC* mutant support clearly that MSH is important for protection under NaOCl stress. The number of S-mycothiolated proteins is higher in

C. glutamicum compared to *S*-bacillithiolated proteins identified in *Bacillus* or *Staphylococcus* in our previous work (7). This may be related to the higher MSH amounts present in *Actinomyces* compared to BSH in *Firmicutes* (38). The proteins identified in the *S*-mycothiolome are functionally very similar to the identified *S*-bacillithiolated proteins in *Firmicutes* and *S*-glutathionylated proteins identified in eukaryotes (7, 33). Except for the thiol peroxidases Tpx and Gpx, all *S*-mycothiolated proteins were exclusively identified under NaOCl stress conditions, not in untreated cells, which confirms our previous results in *Firmicutes* bacteria. Thus, *S*-mycothiolation functions as redox switch and protects redox-sensitive thiols against overoxidation mainly under oxidative stress conditions.

The *S*-mycothiolated proteins were identified within the metabolic pathways for methionine biosynthesis (MetE, SerA, Hom), glycolysis (Fba, Pta, PckA), glycogen utilization (MalP), nucleotide biosynthesis (GuaB1, GuaB2, PurL, NadC), MSH biosynthesis (Ino1) and thiamine biosynthesis (ThiD1, ThiD2). Further *S*-mycothiolated proteins function in protein translation (Tuf, PheT, RpsC, RpsF, RpsM, RplM) or have antioxidant functions, such as the peroxidases Tpx and Gpx. In our previous study, we identified 8 conserved *S*-bacillithiolated proteins in *B. subtilis* and other *Firmicutes* bacteria, including the methionine synthase MetE, the inosine 5'-phosphate (IMP) dehydrogenase GuaB, the translation elongation factor Tuf, the inorganic pyrophosphatase PpaC, the phosphoglycerate dehydrogenase SerA, the chorismate mutase AroA, the ferredoxin-NADP⁺ reductase YumC and the putative bacilliredoxin YphP (6, 7). Interestingly, five of these conserved *S*-bacillithiolated proteins are also abundantly *S*-mycothiolated proteins in *C. glutamicum*, including MetE, GuaB1, GuaB2, Tuf and SerA. Thus, we hypothesize that these proteins are probably conserved targets for *S*-thiolations across bacteria. GuaB and MetE possess also conserved active site Cys residues, including the active site Zn center of MetE (Cys713 in Cg-MetE) or the thioimidate intermediate-forming active site in GuaB (Cys302 in Cg-GuaB1 and Cys317 in Cg-GuaB2). This active site of GuaB is buried in the GuaB structure from *Vibrio cholerae* (PDB code 4FEZ) (Supplementary Fig. S7). *S*-glutathionylation of GuaB has been also reported in human T-lymphocytes and endothelial cells (18, 32). It is further interesting that different enzymes of the thiamine biosynthesis pathway are *S*-thiolated in *Firmicutes* and *C. glutamicum*. For example, ThiG and ThiM were *S*-bacillithiolated in *Bacillus* and *Staphylococcus* (7), whereas the multifunctional ThiD1 protein with thiamine phosphate synthase and hydroxymethylpyrimidine (HMP) kinase domains was *S*-mycothiolated in *C. glutamicum* at Cys451 that is the conserved HMP substrate binding site.

MetE has been first shown as target for *S*-glutathionylation in *E. coli* where it is modified at the nonconserved Cys645 located at the entrance to the active site (22). The methionine synthase MetE is the major *S*-bacillithiolated protein in *Bacillus* species (6, 7) and its *S*-thiolation occurs in its active site Zn center at Cys730 and also at the nonconserved Cys719 in *B. subtilis*. This leads to MetE inactivation and Met auxotrophy in NaOCl-treated cells. Here we confirmed that MetE is also a major target for *S*-mycothiolation in *C. glutamicum* and is oxidized at the conserved active site Cys713. This Zn-binding active site Cys is buried in the MetE structures of *Thermotoga maritima* and *Streptococcus mutans* (PDB code 2NQ5) (Sup-

plementary Fig. S7), but the elastic and flexible nature of this catalytic Zn center has been demonstrated (28). However, in contrast to *E. coli* and *B. subtilis*, NaOCl stress did not cause methionine starvation since growth was not resumed by addition of Met to the growth medium after 30 and 60 min of NaOCl treatment (Supplementary Fig. S1B). Thus, the growth defect after NaOCl stress could be caused by multiple amino acid auxotrophies or by a general translation defect caused by *S*-mycothiolation of translation elongation factors and ribosomal proteins. Similar translation proteins have also been detected as reversibly oxidized in the redox proteome of *E. coli* after NaOCl stress (31), and as *S*-glutathionylated in oxidatively stressed human endothelial and yeast cells (18, 33, 54). The inhibition of protein synthesis during the time of oxidant removal is perhaps one of the conserved consequences of protein *S*-thiolation as verified in yeast cells (54).

Besides the identification of many conserved *S*-thiolated proteins across *Firmicutes* and *Actinobacteria*, there are also unique *S*-thiolated proteins in *C. glutamicum*. For example, the myo-inositol-1 phosphate synthase Ino-1, the first enzyme of the MSH biosynthesis pathway was *S*-mycothiolated at Cys79 suggesting that MSH biosynthesis itself might be feed-back redox-controlled by oxidative stress. Interestingly, the Ino-1 homolog of *M. tuberculosis* was identified as pupylated at K73, which determines its degradation by the proteasome (16), but K73 is not conserved in *C. glutamicum* Ino-1. It will be interesting to analyze the activity of *S*-mycothiolated Ino-1 and a possible cross-talk with the Pup proteasome in *C. glutamicum* in future studies. The main target for *S*-mycothiolation was the maltodextrin phosphorylase MalP in *C. glutamicum* that is involved in glycogen and maltodextrin utilization in *C. glutamicum* (53). MalP is the major reversibly oxidized protein in the thiol redox proteome of *C. glutamicum* and *S*-mycothiolated at the nonconserved Cys180, which is the only surface-exposed Cys residue in the MalP crystal structure of *Corynebacterium callunae* (PDB code 2C4M). In addition, a *malP* mutant was very sensitive to NaOCl stress suggesting that *S*-mycothiolation of MalP confers resistance to oxidative stress in *C. glutamicum*. *C. glutamicum* has the capability to accumulate transiently glycogen as energy and carbon source during the exponential growth when grown on glucose. Glycogen is degraded during the stationary phase or during osmotic stress by the glucan phosphorylase GlgP, the debranching enzyme GlgX that produces maltodextrins and the maltodextrin phosphorylase MalP (Fig. 4) (53). Thus, *S*-mycothiolation of MalP could protect and inhibit the enzyme and prevent glycogen degradation to save their energy source under oxidative stress. Indeed, the glycogen content decreased only slightly in NaOCl-treated cells despite the reduced GUR. This inhibition of glycogen degradation could be caused by a loss of MalP activity due to *S*-mycothiolation (Fig. 6). Further studies are underway to characterize the effect of *S*-mycothiolation on MalP activity. We further identified several glycolytic enzymes as targets for *S*-thiolation (e.g., Fba) or reversible thiol-oxidation (e.g., GapA, Eno) in *C. glutamicum* that were also reversibly inactivated by *S*-thiolation in yeast cells (54). It is proposed that the inhibition of the glycolytic flux by *S*-thiolation redirects glucose into the pentose phosphate pathway for NADPH regeneration to provide the reducing power for the Trx and Mrx1 systems (54). The inhibition of glycolytic

enzymes by S-thiolation could be also a consequence of the reduced GUR under NaOCl stress.

As most interesting proteins, we identified the peroxidases Tpx and Gpx as S-mycothiolated at their active sites. Tpx and Gpx have been classified as atypical 2-Cys Prxs with a conserved peroxidatic active site Cys (C_P) and a resolving Cys (C_R). Atypical Prxs react with peroxides to a transient C_P -SOH intermediate that forms an intramolecular disulfide with C_R in the same subunit that is reduced by the Trx pathway during recycling (36, 47). Since MSH-deficient mutants of *C. glutamicum* and *M. smegmatis* showed peroxide-sensitive phenotypes, the existence of MSH-dependent peroxidases was suggested earlier (13, 35). It is possible that Gpx might be the MSH-dependent peroxidase that utilizes MSH as electron donor for peroxide detoxification resulting in MSSM generation that could be recycled by Mtr. For *M. tuberculosis* Tpx (Tpx-Mtb) the catalytic cycle and structure has been resolved (PDB code 1XVQ)(17, 48). Tpx was S-mycothiolated at Cys60 and Cys94 *in vivo* that both are solvent exposed in the Tpx-Mtb structure in contrast to Cys81 that is more buried and not conserved (Supplementary Fig. S7). Tpx-Mtb shares 56.6% sequence identity to *C. glutamicum* Tpx (Tpx-Cg) and the Cys60-Cys94 intramolecular disulfide was confirmed here using MS (Supplementary Table S6C). Tpx-Cg showed also NADPH-linked peroxidase activity and reduced H_2O_2 in a Trx/TrxR-coupled assay. For *E. coli* Tpx, it was shown that the C_P -SOH was sensitive to overoxidation (2), which explains the protection of the Cys60 active site against overoxidation by S-mycothiolation in our study. We could show that S-mycothiolation of C_P and C_R inhibits the peroxidase activity which can be restored after reduction by the Mrx1/MSH/Mtr electron pathway. Thus, S-mycothiolation controls Tpx activity and protects C_P against overoxidation. S-glutathionylated Prxs have been observed as reaction intermediates in the catalytic mechanism of peroxidase activity of 2-Cys Prx from *Schistosoma mansoni*, poplar Prx, and human Prx-I (5, 46). S-glutathionylation of Prx-I induced a structural change that leads to dissociation from oligomers to dimers with a loss of chaperone activity and an increased peroxidase activity of the active dimer (5, 44, 46). For 1-Cys D-Prx, it is been shown that S-glutathionylation induced the dissociation of noncovalent homodimers to monomers that are reduced by the Grx/GSH system followed by dimerization and reactivation of the peroxidase activity (42). Also Tpx is active as homodimer and it will be exciting to unravel the structural changes of Tpx by S-mycothiolation that perhaps also regulates the quaternary structure by dimer to monomer dissociation.

Experimental Procedures

Bacterial strains and growth conditions

Bacterial strains, plasmids, and primers are listed in Supplementary Tables S7 and S8. For DNA manipulation and plasmid isolation, *E. coli* was cultivated in Luria Bertani (LB) medium at 37°C. *C. glutamicum* ATCC 13032 wild type and *sigH*, *mshC*, *malP*, *tpx* and *gpx* mutant strains were grown in CGC minimal salt medium containing 1% glucose as carbon source at vigorous agitation at 30°C (12). When appropriate, nalidixic acid (50 µg/ml for *C. glutamicum*) or kanamycin (20 µg/ml for *C. glutamicum* and 50 µg/ml for *E. coli*) were added to the media. Sodium hypochlorite (15% stock solution) and H_2O_2 were purchased from Sigma Aldrich. For

NaOCl stress experiments, *C. glutamicum* cells were grown in CGC minimal medium to an OD_{500} of 8.0 and exposed to 180 µM NaOCl that was freshly diluted in distilled water. The survival of the *malP* mutant was determined after different times of NaOCl stress by plating serial dilutions on LB plates and determining colony forming units (cfu) after 2 days. The cfu of the untreated culture was set as 100% and three replicate experiments were performed.

Construction of defined deletions in the *C. glutamicum* chromosome

Gibson assembly was applied to construct pK18*mobsacB* derivatives to perform allelic exchange of the *gpx* and *tpx* genes in the chromosome of *C. glutamicum* ATCC 13032 (50). The primers used to construct the pK18*mobsacB* derivatives are listed in Supplementary Table S8, that includes the *gpx* and *tpx* genes with internal deletions (Δgpx [480 bp] and Δtpx [447 bp]). The pK18*mobsacB* derivatives were cloned in *E. coli* JM109 (Supplementary Table S7). The pDN2 plasmid containing the $\Delta sigH$ deletion was constructed previously (3) and transformed into *C. glutamicum* ATCC 13032. The gene replacement in the chromosome of *C. glutamicum* ATCC 13032 resulted in the $\Delta sigH$, Δgpx , and Δtpx deletion mutants that were confirmed by PCR using the primers in Supplementary Table S8.

RNA isolation and DNA microarray hybridization

For RNA isolation, *C. glutamicum* ATCC 13032 cells were harvested before and 10 and 30 min after exposure to NaOCl stress from three replicate experiments and disrupted with a Precellys 24 homogenizer (Bertin Technologies). Total RNA was isolated using the RNeasy Mini Kit and treated with DNase-I as described (23). The hybridization of whole-genome oligonucleotide microarrays was performed using 15 µg of total RNA that was used for cDNA synthesis as described previously (49). The normalization and evaluation of the hybridization data was done with the software package EMMA 2 (9) using a signal intensity (a-value) cut-off of ≥ 7.0 , a signal intensity ratio (*m*-value) cut-off of ± 1 (technical variance) for hierarchical clustering and ± 1 (significance threshold) for the single analysis. Genes with at least twofold expression changes and *p*-values < 0.05 were considered as significantly changed genes.

Hierarchical clustering analysis

Clustering of gene expression profiles was performed using GENESIS (55). The transcriptome data sets included log2-fold expression changes of all genes with at least twofold expression changes (*p*-values < 0.05) at 7.5 min after 2 mM diamide stress (Busche and Kalinowski, unpublished data), 10 and 30 min after 180 µM NaOCl, respectively.

Thiol redox proteomics and MALDI-TOF-TOF MS/MS

The thiol redox proteome analysis was performed as described (6). Cells were harvested at control and NaOCl stress conditions, sonicated, alkylated in 8 M urea/1% chaps/100 mM IAM, and acetone precipitated. The pellet was resolved in urea/chaps buffer without IAM and 200 µg of the protein extract was reduced with 10 mM TCEP and labelled with BODIPY FL C₁-IA [N-(4,4-difluoro-5,7-

dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)-methyl)-iodoacetamide] (Invitrogen). The fluorescence-labeled protein extract was separated using two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), scanned for BODIPY fluorescence and stained with Coomassie as described (6). Quantitative image analysis was performed with the DECODON Delta 2D software (www.decodon.com). The oxidation ratios were calculated as fluorescence/protein amount ratios and listed in Supplementary Table S4. Tryptic digestion of proteins, peptide-spotting, and MALDI-TOF-TOF measurement was performed using a Proteome-Analyzer 5800 (Applied Biosystems) as described (6).

LTQ-Orbitrap Velos MS

IAM-alkylated cell extracts were separated by 15% nonreducing sodium dodecylsulfate (SDS)-PAGE and tryptic in-gel digested as described (6). Tryptic peptides were subjected to a reversed phase column chromatography and MS and MS/MS data were acquired with the LTQ-Orbitrap-Velos mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source as described (6). Post-translational thiol-modifications of proteins were identified by searching all MS/MS spectra in "dta" format against a *C. glutamicum* ATCC13032 target-decoy protein sequence database extracted from UniProtKB release 12.7 (UniProt Consortium, Nucleic acids research 2007, 35, D193-197) using SorcererTM-SEQUEST[®] (Sequest v. 2.7 rev. 11, Thermo Electron, including Scaffold 4.0; Proteome Software, Inc.). The Sequest search was carried out with the following parameters: parent ion mass tolerance 10 ppm, fragment ion mass tolerance 1.00 Da. Two tryptic miscleavages were allowed. Methionine oxidation (+15.994915 Da), Cys carbamidomethylation (+57.021464 Da), S-cysteinylation (+119.004099 Da for C₃H₇NO₂S), S-mycothiolations (+484.13627 Da for MSH) and S-ergothionylations (+227.07284 for EGT) were set as variable post-translational modifications in the Sequest search. Sequest identifications required ΔC_n scores of >0.10 and XCorr scores of >2.2, 3.3 and 3.75 for doubly, triply and quadruply charged peptides, respectively. Neutral loss precursor ions characteristic for the loss of inositol (−180 Da) served for verification of the S-mycothiolated peptides.

Nonreducing MSH specific Western blot analysis

Twenty-five microgram IAM-alkylated protein extracts were used for nonreducing 15% SDS-PAGE and MSH-specific Western blots as described (7). Polyclonal MSH antiserum was kindly obtained from Mamta Rawat and used at 1:500 dilution.

Analyses of the glycogen content and [¹⁴C]-glucose uptake studies

Analyses of the intracellular glycogen content were performed enzymatically as described (51). [¹⁴C]-glucose uptake studies were performed as described (34). After different times of stress exposure, the glucose uptake reaction was then started by addition 50 μ M [¹⁴C]-glucose (specific activity 250 μ Ci μ mol^{−1}; Moravsek Biochemicals). At given time intervals (15, 30, 45, 60, and 90 s), 200 μ l samples were filtered through glass fibre filters (Typ F; Millipore) and washed twice with 2.5 ml of 100 mM LiCl. Radioactivity of the samples was determined using scintillation fluid (Rotiszint; Roth) and a scintillation counter (LS 6500; Beckmann).

Cloning and purification of Tpx, Trx, TrxR, and Mrx1

Recombinant Trx, TrxR, and Mrx1 were cloned and purified as described by Ordóñez *et al.* (43). Tpx was cloned into plasmid pET28a (using primers tpx_pETfor and tpx_pETrev and the restriction sites: *Nco*I, *Xho*I). The resulting Tpx-pET28 vector, containing Tpx with a N-terminal HIS₆-tag and a PreScission protease cleavage site (GE Healthcare) was transformed into *E. coli* BL21(DE3). The transformed cells were grown overnight at 37°C in LB medium with 50 μ g/ml kanamycin. The cultures were 100-fold diluted into LB medium with 50 μ g/ml kanamycin, induced with 1 mM IPTG at an OD₆₀₀ of 0.7 and grown overnight at 30°C. Cells were harvested and resuspended in 20 mM Hepes (pH 7.5), 5 mM imidazole, 1 M NaCl, 1 mM DTT, 0.1 mg/ml 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride and 1 μ g/ml leupeptin to an OD₆₀₀ of 200. After cell crack disruption, 50 μ g/ml DNase I and 20 mM MgCl₂ were added to cell lysate, left for 30 min at 4°C before centrifugation. The supernatant was loaded on a Ni²⁺-Sephacrose (GE Healthcare) immobilized metal affinity chromatography (IMAC) column equilibrated with 20 mM Hepes (pH 7.5), 5 mM imidazole, 1 M NaCl, 1 mM DTT, and eluted with a linear gradient to 1 M imidazole in the same buffer. The eluted fractions were analyzed on SDS-PAGE (15% Tris-glycine) and the fractions with Tpx were pooled and further purified on a Superdex75PG (16/90) SEC column equilibrated in 20 mM Tris/HCl (pH 8.0), 250 mM NaCl, 1 mM DTT.

In vitro S-mycothiolation of Tpx

Tpx was incubated with a 6 M excess of reduced MSH before the addition of 1 mM H₂O₂. After an incubation of 5 min, the sample was loaded on an IMAC column and MSH and H₂O₂ were removed by a washing step with 50 mM HEPES pH 8.0, 500 mM NaCl. S-mycothiolated Tpx was eluted in the same buffer containing 300 mM imidazole.

Coupled Trx/TrxR and Mrx1/MSH/Mtr electron transfer assays

The Trx/TrxR and Mrx1/MSH/Mtr coupled enzyme assays described by Van Laer *et al.* were modified to include Tpx (57). Briefly, Tpx was reduced by 10 mM DTT for 15 min at room temperature and the excess of DTT was removed by size exclusion chromatography in 50 mM Hepes, pH 8.0, 500 mM NaCl. The Tpx/Trx/TrxR electron transfer pathway was reconstructed by incubating 500 μ M NADPH, 3 μ M TrxR, and 1500 nM Trx with 500 nM Tpx at 37°C in the assay buffer containing 50 mM Hepes pH 8.0, 500 mM NaCl. Increasing concentrations of H₂O₂ (20–100 μ M) were added as substrate to the reaction mixture. The oxidation of NADPH due to H₂O₂ reduction was monitored as a decrease in the absorption at 340 nm in function of time. The Mrx1/MSH/Mtr assay contained 500 μ M NADPH, 5 μ M Mtr, 400 μ M MSH, and 2 μ M Mrx1. Mycithiolated Tpx (50 or 75 μ M final concentration) was added and the absorption at 340 nm was monitored in function of time.

Purification and reduction of MSH

MSH was purified as described in Ordóñez *et al.* (43). Oxidized MSH was incubated with 25 mM TCEP and incubated for 20 min at room temperature. The sample was loaded on an anion exchange chromatography

(Isolute PE-AX column) and MSH was eluted in water. As a quality control, a 1D proton NMR spectra in D₂O was recorded and compared to the spectra obtained by Lee and Rosazza (30).

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

BSH = bacillithiol
Cys = cysteine
EGT = ergothioneine
GMP = guanosine 5'-phosphate
Gpx = glutathione peroxidase
Grx = glutaredoxin
GSH = glutathione
GSSG = oxidized glutathione disulfide
GST = glutathione S-transferase
GUR = glucose uptake rate
HED = β -hydroxyethyl disulfide
HMP = hydroxymethylpyrimidine
H₂O₂ = hydrogen peroxide
IAM = iodoacetamide
IMP = inosine 5'-phosphate
LB = Luria Bertani
LC = liquid chromatography
LMW = low molecular weight
LTQ = linear trap quadrupole
Met = methionine
Mrx1 = mycoredoxin1
MS = mass spectrometry
MS/MS = tandem mass spectrometry
MSH = mycothiol
MSSM = mycothione or mycothiol disulfide
Mtr = mycothiol disulfide reductase
MW = molecular weight
NEM = N-ethylmaleimide
OD₅₀₀ = optical density at 500nm
PAGE = polyacrylamide gel electrophoresis
protein-SSM = MSH protein mixed disulfide
Prx = peroxiredoxin
ROS = reactive oxygen species
SDS = sodium dodecylsulfate
TCEP = Tris-(2-carboxyethyl)-phosphine
Tpx = thiol peroxidase
Trx = thioredoxin
TrxR = thioredoxin reductase