

Redox Regulation of 3'-Phosphoadenylylsulfate Reductase from *Escherichia coli* by Glutathione and Glutaredoxins*

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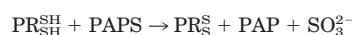
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Inorganic sulfate (SO_4^{2-} , S^{+VI}) is reduced *in vivo* to sulfite (SO_3^{2-} , S^{+IV}) via phosphoadenylylsulfate (PAPS) reductase. *Escherichia coli* lacking glutathione reductase and glutaredoxins (*gor⁻grxA⁻grxB⁻grxC⁻*) barely grows on sulfate. We found that incubation of PAPS reductase with oxidized glutathione leads to enzyme inactivation with simultaneous formation of a mixed disulfide between glutathione and the active site Cys-239. A newly developed method based on thiol-specific fluorescent alkylation and gel electrophoresis showed that glutathionylated PAPS reductase is reduced by glutaredoxins via a monothiol mechanism. This glutathionylated species was also observed in poorly growing *gor⁻grxA⁻grxB⁻grxC⁻* cells expressing inactive glutaredoxin 2 (Grx2) C9S/C12S. However, it was absent in better growing cells expressing monothiol Grx2 C12S or wild type Grx2. Reversible glutathionylation may thus regulate the activity of PAPS reductase *in vivo*.

Sulfur is an ingredient of all living organisms. The first, most common form of sulfur in nature is inorganic sulfate, which needs to be further reduced for incorporation in a living cell. Prototrophic bacteria, for example, use inorganic sulfate (SO_4^{2-} , S^{+VI}) as primary source for the biosynthesis of sulfur-containing amino acids and cofactors (1). Sulfate is first activated to adenylylsulfate (APS)¹ and then to 3'-phosphoadenylylsulfate (PAPS) by ATP sulfurylase and APS kinase. Subsequently, PAPS is reduced by PAPS reductase (PR) to sulfite (SO_3^{2-} , S^{+IV}) and adenosine-3'-5'-bisphosphate (PAP). Sulfite is reduced to sulfide (S^{2-} , S^{-II}) by sulfite reductase, and, thereafter, is incorporated in *O*-acetyl serine (OAS) by OAS-(thiol)ylase to give the primary product of sulfate assimilation, cysteine.

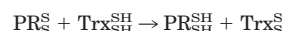
PAPS reductase (EC 1.8.99.4) is composed of two identical subunits of 28 kDa. It is devoid of chromophores and contains a single cysteine per subunit in a highly conserved ECGLH motif, which is identified as the redox-active center of the enzyme (2). Reduction of sulfate to sulfite by PR requires two

electrons during which the cysteines of PR are oxidized to a disulfide. The oxidized enzyme is inactive and needs to be reduced for the reduction of PAPS to continue. Kinetic data (2–4) and the crystal structure of PR (5) implicate a ping-pong mechanism for its reduction. In the first step, shown here in Reaction 1,



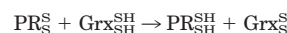
REACTION 1

reduced PR catalyzes the reduction of PAPS, leading to oxidized PR, free sulfite, and PAP. Upon oxidation the enzyme dimer undergoes conformational changes leading to a significant decrease in apparent molecular weight and the inability to bind PAPS (2, 3). In the second step, oxidized PR is reduced by thioredoxin as shown in Reaction 2,



REACTION 2

or glutaredoxin, which is depicted in Reaction 3,



REACTION 3

with electrons from thioredoxin reductase (TR) and NADPH or glutathione (GSH), glutathione reductase, and NADPH.

Thioredoxins and glutaredoxins are small (9–14 kDa) ubiquitous proteins that utilize their two redox-active cysteines (CXXC motif) to catalyze the reduction of disulfides (6). Whereas thioredoxins and glutaredoxins can reduce their substrates by using both active site cysteines (dithiol mechanism) (7), glutaredoxins can also utilize the thiols from GSH in solution together with the glutaredoxin N-terminal cysteine (monothiol mechanism) (8). In addition to their ability to reduce intracellular disulfides, glutaredoxins may also reduce the mixed disulfides that form between a protein thiol and GSH. This is a reaction that is not catalyzed by thioredoxins.

Escherichia coli contains two thioredoxins (Trx1 and Trx2) and three glutaredoxins (Grx1, Grx2, and Grx3) (9). Trx1, Trx2, and Grx1 can reduce the disulfide that forms on ribonucleotide reductase 1a (RNR1a) upon the reduction of ribonucleotides with comparative efficiencies, whereas Grx3 is only a weak reductant *in vitro* (10–13). Trx1, Trx2, and Grx1 participate in the *in vitro* reduction of PAPS by PR (4). Other functions for Trx1 include the reduction of methionine sulfoxide via methionine sulfoxide reductase, whereas Trx2 participates in the antioxidant response as part of the OxyR regulon. Grx1 is also a member of the OxyR regulon, but Grx2 and Grx3 are not (14). With levels at least 10-fold higher than those of Grx1, Grx2 and

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¹ The abbreviations used are: APS, adenylylsulfate; DTT, dithiothreitol; Grx, glutaredoxin; HED, 2-hydroxyethyl disulfide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PAP, adenosine-3',5'-bisphosphate; PAPS, phosphoadenylylsulfate; PR, phosphoadenylylsulfate reductase; TR, thioredoxin reductase; Trx, thioredoxin.

Grx3 are highly abundant proteins in *E. coli* (15) and contribute up to 98% of the GSH-dependent oxidoreductase activity, using the disulfide between β -mercaptoethanol and GSH as a substrate (HED assay) (11). Grx2 is an atypical glutaredoxin with a molecular mass of 23.4 kDa and structural similarities to mammalian GSH *S*-transferases (16). Because of its high abundance (up to 1% of total soluble protein) and catalytic efficiency, it contributes to >80% of the cellular GSH-mixed disulfide reducing activities (in the HED assay) (17, 18). The enzyme is also highly active in the reduction of the mixed disulfide between glutathione and arsenate reductase (19). Grx2 is involved in the antioxidant response, as mutants lacking Grx2 have increased levels of carbonylation of their intracellular proteins after exposure to hydrogen peroxide. Glutaredoxins and thioredoxins are not only direct antioxidants, but may also participate in the signal transduction of redox-induced cellular responses (overviews in Refs. 20 and 21).

Combined *E. coli* null mutants for glutathione reductase and the three glutaredoxins (*gor⁻grxA⁻grxB⁻grxC⁻*) barely grow on sulfate (S^{+VI}) but grow normally on sulfite (S^{+IV}) or methionine (S^{-II}) (22). Because these mutants contain sufficient amounts of thioredoxin to reduce PR (15), this disturbed growth must represent some sort of inhibition of PR activity not based on the reduction of the enzyme's disulfide that is formed upon the reduction of PAPS. Growth of *gor⁻grxA⁻grxB⁻grxC⁻* could be restored with monothiol or wild type Grx2 *in trans* but not with the inactive C9S/C12S species (22). Because Grx2 cannot reduce the disulfide of oxidized PR (4), this finding raises the possibility that the activity of PAPS reductase *in vivo* may be regulated by oxidized glutathione and glutaredoxins.

EXPERIMENTAL PROCEDURES

General Methods—Materials, chemicals, and enzymes were purchased from different companies in the highest available purity. *E. coli* cells were transformed according to Hanahan (23). The concentration of proteins in crude extracts was determined as described by Bradford (24). Pure proteins were quantified using the following molar absorbance coefficients at 280 nm: PAPS reductase, 52,630 $M^{-1} cm^{-1}$ (monomer); Grx1, 10,810 $M^{-1} cm^{-1}$; Grx2, 21,620 $M^{-1} cm^{-1}$; Grx3, 3,840 $M^{-1} cm^{-1}$; and Trx1, 15,220 $M^{-1} cm^{-1}$. SDS-PAGE was performed using the Phast-Gel system (Amersham Biosciences) and the Ready-Gel system (Bio-Rad) according to each manufacturer's instructions.

Strains and Plasmids—*E. coli* Bl21(DE3) (Novagen, Madison, WI) was used for the overexpression of PAPS reductase using plasmid pET16bcysH (4). DHB4*gor⁻grxA⁻grxB⁻grxC⁻* and the arabinose promoter-based plasmids pISCGrx2, pISCGrx2C12S, and pISCGrx2C9S/C12S for the expression of wild type Grx2 and the mutants C12S and C9S/C12S, respectively, were first described and characterized in Ref. 22.

Protein Expression and Purification—PAPS reductase was expressed and purified as described (4) using a 3-liter (Meredos, Göttingen, Germany) and a 25-liter fermenter (New Brunswick Scientific, Edison, NJ). Oxidized PAPS reductase was prepared by incubation with an excess of PAPS (15 min, 22 °C, 50 mM Tris/HCl, pH 8.0) essentially as described (25). The reduced protein was prepared by treatment with 10 mM dithiothreitol (DTT) and 1 μM Trx1 from *E. coli* (15 min, 22 °C, 50 mM Tris/HCl, pH 8.0). The glutathionylated form of PR was produced by incubating 200 μM reduced enzyme with 20 mM oxidized glutathione (GSSG, 30 min 22 °C, Tris/HCl, pH 8.0). Free nucleotides, reductants, or GSSG were removed using ultrafiltration (Amicon YM3, Millipore) and Sephadex G25-columns (Amersham Biosciences). The different isoforms were stored at -80 °C in 40 mM Tris/HCl, pH 8, 500 mM NaCl, and 10% glycerol. Grx1, Grx1C14S, Grx2, Grx2C12S, Grx3, Grx3C15S, and thioredoxin reductase from *E. coli* were expressed and purified as described previously (8, 17, 26, 27).

Enzymatic Assays—PAPS reductase activity was measured as an acid-labile sulfite formation from [³⁵S]PAPS at 30 °C (28). The assay mixture (100 μl) contained 50–500 ng ml^{-1} PAPS reductase, 100 mM Tris/HCl, pH 8, 10 mM Na₂SO₃, 100 μM [³⁵S]PAPS, 100 μM Trx1, and 1 μM thioredoxin reductase from *E. coli*, and 5 mM NADPH. Oxidized glutathione, yeast glutathione reductase (Sigma), and glutaredoxins were added as indicated. For determination of the activity of glutaredoxin-treated oxidized, reduced, and glutathionylated enzyme, the as-

say mixtures (5 μg of PR, 50 mM Tris/HCl, pH 8.0, and 100 mM NaCl) were desalted on Sephadex-G25, and the activity was determined in the absence of further reductants in a single turnover experiment (100 mM Tris/HCl pH 8, 10 mM Na₂SO₃, and 100 μM [³⁵S]PAPS; 5-min reaction time). [³⁵S]PAPS (1,700 Bq $nmol^{-1}$) was prepared from [³⁵S]sulfite (Amersham Biosciences) as described by Schriek and Schwenn (25) using recombinant APS kinase from *Arabidopsis thaliana* (29). Synthesis and purity were monitored by high pressure liquid chromatography (30).

GSH-mixed disulfides were assayed in a reaction mixture (500 μl) containing 100 mM Tris/HCl, pH 8.0, 100 mM reduced glutathione, 100 μM NADPH, yeast glutathione reductase, 1 μM glutaredoxin, and glutathionylated PR as indicated. Glutaredoxins catalyze the reduction of mixed disulfides using GSH as electron donor. The resulting GSSG is reduced by glutathione reductase with electrons from NADPH. The reaction was initiated by the addition of glutaredoxin. The decrease in A₃₄₀ was used for quantification of the GSH-moieties in the UV-2100 photometer (Shimadzu, Kyoto) at 25 °C.

Fluorescent Experiments—5 μg of PAPS reductase in 50 mM Tris/HCl, pH 8.0, and 100 mM NaCl in a total volume of 20 μl was incubated with 5 mg of a reduced glutaredoxin for 30 s in the presence or absence of 0.5 mM reduced glutathione. The samples were alkylated and labeled with 0.5 mM 5-(iodoacetamido)-fluorescein (5-IAF, Sigma; solved in *N,N*-dimethylformamide) for 45 min at room temperature in the dark before they were separated by SDS-PAGE (8–16%) and analyzed on a UV table (Ultraviolet Products, San Gabriel, CA).

Growth of *E. coli*—To determine the redox status of PR in cell-free extracts and for immunoprecipitation experiments, cells were grown overnight in LB, washed twice with cold M9 medium, and inoculated in fresh M9 medium with 33 mg liter⁻¹ Leu and Ile, 100 mg liter⁻¹ ampicillin, and 0.1% arabinose to an A₆₀₀ of 0.14. Cells were grown in 300-ml cultures at 170 rpm and 37 °C until they reached stationary phase. The cells were then collected, incubated with 100 mM iodoacetamide for 20 min on ice to stop further reactions of thiol groups, harvested by centrifugation, resuspended in TE buffer (50 mM Tris/HCl, pH 8.0, and 1 mM EDTA), and frozen at -20 °C.

Purification of Antibodies—Rabbit sera were adjusted with ammonium sulfate to 50% of saturation and left stirring at 4 °C overnight. The precipitated IgG fraction was resuspended in phosphate-buffered saline (PBS) and dialyzed extensively against PBS, pH 7.5. Affinity-purified antibodies for PAPS reductase were prepared using an Affi-Gel 10 column (Bio-Rad) on which 5 mg of PAPS reductase had been previously immobilized using the procedure recommended by the manufacturer. Prior to the application of the IgG fraction, columns were equilibrated with 20 mM Tris-HCl, pH 7.5, followed by 20 mM Tris-HCl, pH 7.5, with 500 mM NaCl and, finally, 20 mM Tris-HCl, pH 7.5. After sample loading, columns were subsequently washed with the same buffers, and bound antibodies were eluted with a pulse of 0.1 M acetic acid-formic acid, pH 2.1. The eluate was immediately neutralized with 1 M Tris-HCl, pH 9, aliquoted and stored at -20 °C.

Immunoprecipitation—Frozen cells were washed once with 40 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA and resuspended in 3 ml of the same buffer. Cells were incubated for 45 min with 1 mg ml^{-1} lysozyme on ice. Cells were sonicated, treated with 1 mM phenylmethanesulfonyl fluoride (PMSF), and centrifuged for 1 h at 100,000 $\times g$. The cell-free lysate supernatant was incubated for 2 h at 4 °C with 35 mg of affinity-purified polyclonal PAPS reductase antibodies while shaking, and then for another hour with 350 ml of 50% protein G-Sepharose (Amersham Biosciences). Cells were spun down, washed twice with 40 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA, resuspended in 1% SDS, and boiled for 20 min before SDS-PAGE.

Western Blotting—The BioRad system was used according to the manufacturer's protocol. After transfer, the nitrocellulose membrane was blocked for 20 min at room temperature with 2% bovine serum albumin in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). It was further washed and incubated with the primary antibody overnight at 4 °C (1:500 for anti-PR-antibodies and 1:2000 for anti-GSH-antibodies). The membrane was washed with 150 mM NaCl for 20 min and then with TBST for additional 20 min, followed by incubation for 1 h with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Dako) using a dilution of 1:4000 for PR antibodies and 1:2000 for GSH antibodies in TBST. The blots were developed by chemiluminescence using the Western Lightning kit from PerkinElmer Life Sciences and visualized using the MultiImage Light cabinet (Alpha Innotech, San Leandro, CA).

Mass Spectrometry—Mass spectrometry was performed on a PE Biosystems Voyager 6061 (Applied Biosystems) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) system. Tryptic diges-

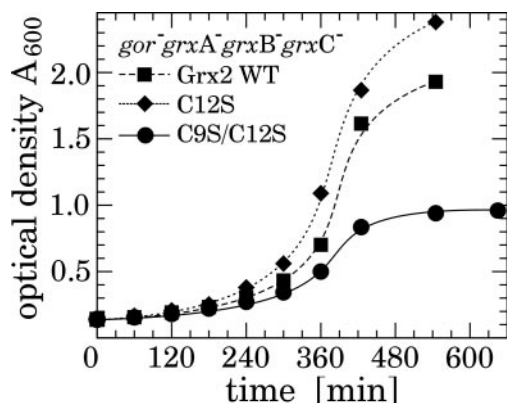


FIG. 1. Growth of transformed *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻ in M9 minimal medium. *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻ was transformed with plasmids expressing wild type (WT) Grx2 (pISCGrx2), Grx2C12S (pISCGrx2C12S), and Grx2C9S/C12S (pISCGrx2C9S/C12S) (22). Cultures of 500 ml of M9 medium supplemented with Leu, Ile (33 mg liter⁻¹), and 100 μg ml⁻¹ ampicillin were inoculated to an optical density (A₆₀₀) of 0.14 using overnight cultures grown in LB medium that were washed twice previously with M9 medium. The expression of Grx2 and the C12S and C9S/C12S mutants was induced by 0.1% arabinose.

tion was performed using sequencing grade-modified trypsin (Promega) according to the manufacturer's protocol. The peptides were diluted in 75% acetonitrile containing 1% trifluoroacetic acid and mixed with an equal amount of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. 1 μl of this solution was allowed to crystallize on the applicator plate before ionization.

RESULTS

Growth Properties of *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻—Combined *E. coli* null mutants for glutathione reductase and Grx1, Grx2, and Grx3 (DHB4gor⁻grxA⁻grxB⁻grxC⁻) barely grow in the presence of sulfate, but they grow well in the presence of sulfite, cysteine, or methionine (22) (Fig. 1). Consistent with previous findings (22), transformants with the monothiol Grx2 grew faster and reached the highest optical density (A₆₀₀) at stationary phase (2.4). The wild type Grx2-containing cells reached an OD of 1.9, and the no-thiol Grx2-containing cells only an OD of 1.0 at stationary phase, as described previously for the non-transformed strain (22).

Redox Status of PAPS Reductase *in Vivo*—To investigate whether the limited growth of the null mutant was caused by an arrest of PR in the oxidized state, we determined the redox state of the enzyme *in vivo*. PR is a homodimeric enzyme whose active site is formed by an intermolecular dithiol-disulfide couple between the only cysteines at position 239 (2). As there are no other covalent links between the two subunits, the reduced and oxidized forms of the enzyme can be separated by non-reducing SDS-PAGE, where the reduced enzyme corresponds to an apparent M_r of 30 kDa, and the oxidized enzyme to 60 kDa. No oxidized PR could be detected in the null mutant transformed with the no-thiol Grx2 (Fig. 2, lanes 2 and 3) or in any other strain (data not shown). Therefore, the inhibition of cell growth in the particular strain was not caused by an arrest of PR in its oxidized conformation.

Reversible Inhibition of PAPS Reductase by Oxidized Glutathione—As the GSH/GSSG ratio in the gor⁻ strain would be expected to shift more toward oxidized glutathione, we investigated whether GSSG affects PR activity. The enzyme was incubated with different amounts of GSSG before reduction of PAPS was performed with electrons delivered from Trx1, Trx reductase (TrxR), and NADPH (Fig. 3). Following incubation with GSSG, the activity of PR decreased exponentially until no PAPS reduction was detectable. This inhibition pattern is char-

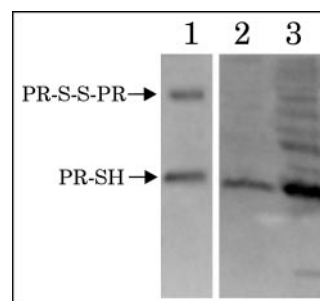


FIG. 2. Redox status of PAPS reductase *in vivo*. Western blots of cell extracts from *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻ pISCGrx2C9S/C12S. Lane 1, control, 5 ng of purified His₁₀-tagged PAPS reductase, ~50% reduced; lane 2: 3 μg of total cell extract; lane 3, 30 μg of total cell extract. The cells were grown in M9 medium containing Leu, Ile (33 mg liter⁻¹), 100 μg ml⁻¹ ampicillin, and 0.1% arabinose for 10 h. Further reactions of thiol groups were blocked by incubating the culture with 100 mM iodoacetamide (Sigma) for 30 min on ice. The cells were lysed by sonication, and the extract was cleared by centrifugation (30 min, 28,000 × g).

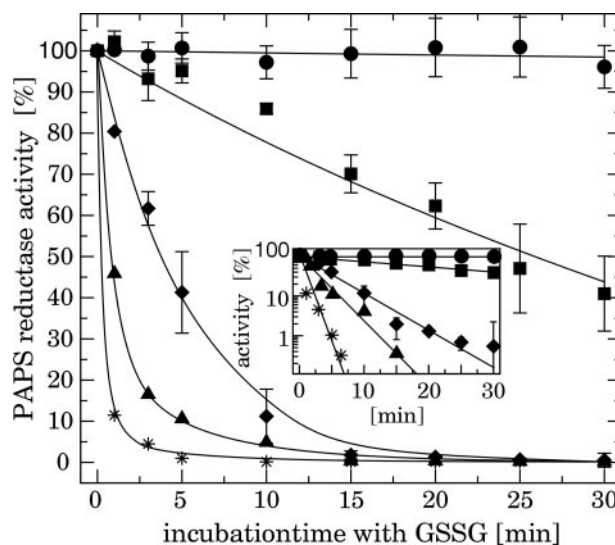
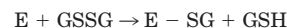


FIG. 3. Inhibition of PAPS reductase by oxidized glutathione (GSSG). PAPS reductase was incubated with different concentrations of GSSG before it was assayed for reduction of PAPS as described under "Experimental Procedures." The assay contained 100 mM Tris/HCl, pH 8, 10 mM Na₂SO₃, 100 μM Trx1, 1 μM thioredoxin reductase, 10 mM NADPH, 100 ng ml⁻¹ PAPS reductase, and GSSG. Circle, 0 mM; square, 1 mM; diamond, 2.5 mM; triangle, 5 mM; and asterisk, 25 mM. Main panel, incubation time versus relative PAPS reductase activity; 100% activity corresponds to 7.25 μmol mg⁻¹ min⁻¹. Inset, half-logarithmic representation. The non-linear curve fitting was done assuming pseudo first-order kinetics.

acteristic for pseudo first order kinetics and suggested a covalent modification of PAPS reductase by GSSG as the basis for the inactivation, which, as shown in Reaction 4,



REACTION 4

is most likely due to the formation of a mixed disulfide between the enzyme and glutathione. The non-linear curve fitting of these results was made assuming pseudo-first order kinetics. Calculated from the first-order rates obtained for the different GSSG-concentrations, the second-order rate constant was $80.4 \pm 5.6 \text{ M}^{-1} \text{ min}^{-1}$, indicating a rapid reaction between PAPS reductase and GSSG.

As the inhibition of PR was likely due to formation of a mixed disulfide between the active site thiol and glutathione, we tried to restore enzymatic activity by the addition of reductants. The

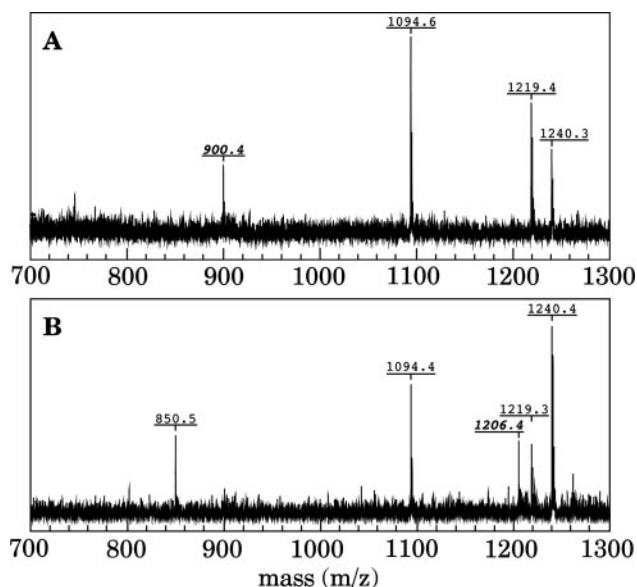


FIG. 4. MALDI-TOF analysis of tryptic fragments from reduced (A) and glutathionylated PAPS reductase (B). The reduced and glutathionylated peptide masses corresponding to the Cys²³⁹-containing peptides are marked with *bolditalic* characters.

addition of DTT or glutathione reductase could not restore the enzymatic activity of PR. When an additional glutaredoxin was added to the reaction mixture, the activity of the enzyme was restored to the former extent in less than 3 min (data not shown). All *E. coli* glutaredoxins (Grx1, Grx2, and Grx3) as well as their monothiol (CXXS)-mutants (Grx1C14S, Grx2C12S, and Grx3C15S) were capable of reactivating PR in that time period.

Glutathionylation of Cysteine 239—To confirm the glutathionylation of the active site cysteine 239, reduced and GSSG-treated PAPS reductase were analyzed by MALDI-TOF (Fig. 4). Tryptic digestion of the reduced protein generated a peptide mass of 900.413 ± 0.017 ($n = 2$) corresponding to the partly cleaved C-terminal fragment Arg-Glu-Cys²³⁹-Gly-Leu-His-Glu-Gly with a calculated molecular weight of 900.399. GSSG-treatment induced a signal with the size of $m/z = 1206.455$, which is compatible with the formation of a disulfide between Cys²³⁹ and glutathione (calculated m/z 1206.474). These results indicated that Cys²³⁹ can form a mixed disulfide with glutathione.

Oxidation and Reduction of Glutathionylated PR—Highly purified oxidized and glutathionylated PR (1.93 ± 0.08 GSH per PR) were analyzed using thiol-specific alkylation with fluorescent mM 5-(iodoacetamido)-fluorescein and non-reducing SDS-PAGE (Fig. 5). Oxidized and glutathionylated PR exhibited virtually no fluorescence or activity (Fig. 5, lanes 1 and 2), whereas the Cys²³⁹ thiol of reduced PR was accessible for alkylation (Fig. 5, lane 3) and active in single turnover experiments (Fig. 5, panel I). The remaining portion of the 30-kDa protein in the oxidized protein (lane 1) corresponds to redox-inactive C-terminal truncated protein without any cysteine (5). Reduced Grx1 reduced both the PR disulfide and the mixed disulfide between PR and GSH in the presence and absence of GSH (Fig. 5, lanes 4 and 5). Reduced monothiol Grx1C14S reduced the two PR species in the presence of GSH, but formed a stable mixed disulfide with PR in the absence of GSH (Fig. 5, lanes 6 and 7). Grx3, as well as its monothiol mutant Grx3C15S, reduced the glutathionylated form of PR in the presence of GSH (Fig. 5A, lane 9 and 11) but not the intramolecular PR disulfide (Fig. 5A, lanes 8 and 10). As seen in the reactivation assays, DTT or GSH alone could not reduce glutathionylated PR, as they did not increase the amount of free thiols of the glutathionylated form (Fig. 5, lanes 2 and 12). Remarkably, in all preparations the existence of free thiols in PR corresponded to protein in the active conformation (sections I and II).

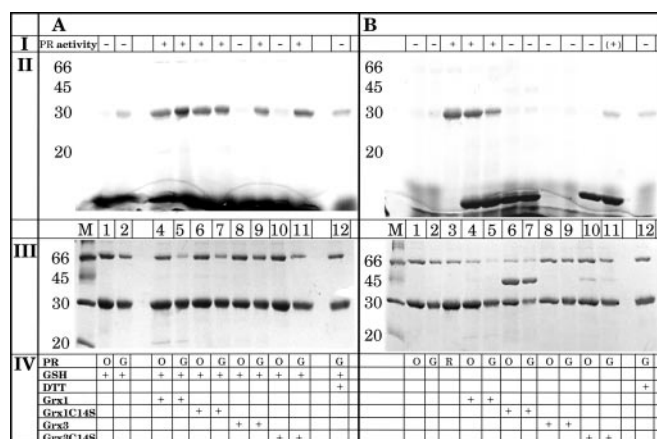


FIG. 5. Reduction of PAPS reductase by glutaredoxins. Non-reducing SDS-PAGE was performed. All lanes contain 5 μ g of reduced, oxidized, or glutathionylated His₁₀-PR incubated with 0.5 mM GSH (A) or without GSH and different glutaredoxins (B) for 30 s. The probes were alkylated for 30 min with 1 mM fluorescent 5'-IAF. Section I, activity of PR after glutaredoxin treatment in single turnover experiments. Section II, fluorescence of PR Cys²³⁹-labeled with 5'-IAF. Section III, Coomassie Blue staining of the same probes. Lane M, molecular weight marker (LMW calibration kit, Amersham Biosciences); lane 1, PR oxidized with PAPS; lane 2, glutathionylated PR; lane 3, PR pre-reduced with Trx1; lane 4, oxidized PR incubated with 5 μ g of Grx1; lane 5, glutathionylated PR plus Grx1; lane 6, oxidized PR plus 5 μ g of Grx1C14S; lane 7, glutathionylated PR plus 5 μ g of Grx1C14S; lane 8, oxidized PR plus 5 μ g of Grx3; lane 9, glutathionylated PR plus 5 μ g of Grx3; lane 10, oxidized PR plus Grx3C14S; lane 11, glutathionylated PR plus Grx3C14S; lane 12, glutathionylated PR after 20 min of incubation with 5 mM DTT. All glutaredoxins were pre-reduced with 10 mM DTT and desalted using Sephadex G25 columns (Amersham Biosciences). The remaining 30-kDa protein band in the oxidized PR samples (lane 1) are caused by truncated PR lacking cysteines (lane 5).

thionylated PR, as they did not increase the amount of free thiols of the glutathionylated form (Fig. 5, lanes 2 and 12). Remarkably, in all preparations the existence of free thiols in PR corresponded to protein in the active conformation (sections I and II).

Glutathionylation of PR in Vivo—Was glutathionylated PR the reason for the limited growth of the *gor*⁻*grxA*⁻*grxB*⁻*grxC*⁻ strain in M9 media free of reduced sulfur? Antibodies raised against GSH-moieties on bovine serum albumin (31) reacted specifically with glutathionylated PR and showed no cross reactivity with reduced or oxidized PR (Fig. 6B, lanes 1–3). No glutathionylated PR was detected in extracts from *gor*⁻*grxA*⁻*grxB*⁻*grxC*⁻ transformed with Grx2C12S (Fig. 6, lane 6). The mixed disulfide species was detected in extracts from transformants encoding wild type Grx2 or the no thiol Grx2C9S/C12S mutant (Fig. 6, lanes 4 and 5). From the density of the bands on the blot, 20% of the PR in the pISCGrx2 transformants was glutathionylated (2.8 of 14 ng detected). 40% was the estimation for the pISCGrx2C9S/C12S-transformants (6.2 of 15.9 ng detected).

DISCUSSION

Regulation of biological activity by the reversible modification of protein thiols is a growing concept in cell signaling. A fine tuning in the DNA binding properties of the transcription factor OxyR centers on Cys¹⁹⁹, which can be hydroxylated, nitrosylated, or glutathionylated, with each modification resulting in differential binding of the protein to DNA; glutathionylated OxyR has the highest transcriptional activity (32). Glutathionylation of Cys⁶² of the eukaryotic NF- κ B subunit p50 lead to loss of DNA binding activity (33), whereas nitric oxide treatment of c-Jun lead to nitrosylation/glutathionylation of Cys²⁶⁹ with concomitant loss of DNA binding activity (34). Deglutathionylation of Cys³⁷⁴ in G-actin resulted in a 6-fold

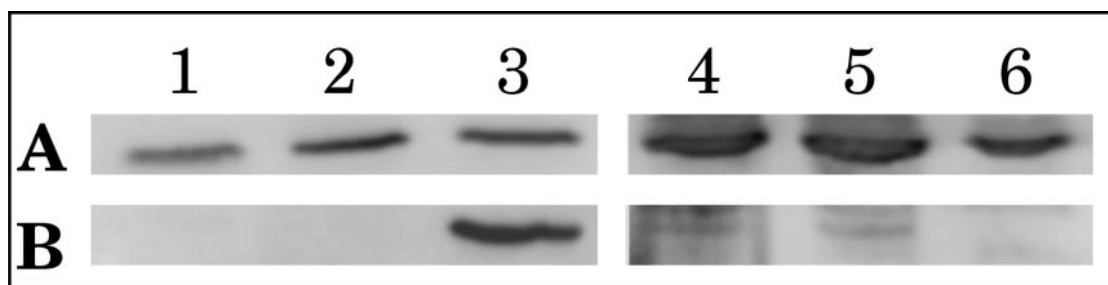


FIG. 6. Glutathionylation of PR in *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻ *in vivo*. Western-blots of recombinant PAPS reductase (30.6 kDa, lanes 1–3) and anti-PR immunoprecipitates from *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻ (lanes 4–6). The membranes were developed using anti-PR antibodies in A, anti-GSH antibodies in B (31), and peroxidase-coupled anti-rabbit antibodies as secondary antibodies in A and B. Lanes 1–3 contain oxidized PR, reduced PR, and glutathionylated PR, respectively. 10 ng of the enzyme were applied to A and 200 ng to B. Lanes 4–6, identification of native PR (27.8 kDa) (A) and glutathionylated PR (B) in immunoprecipitates from extracts of *E. coli* DHB4gor⁻grxA⁻grxB⁻grxC⁻ transformed with pISCGrx2 (wild type Grx2) (lane 4), pISCGrx2C9s/C12S (inactive no-thiol mutant) (lane 5), or pISCGrx2C12S (monothiol mutant) (lane 6) (22).

increase in the rate of its polymerization (35). The activity of tyrosine hydroxylase, the rate-limiting enzyme for the biosynthesis of dopamine, was inhibited by reversible glutathionylation (36). The same has been reported for protein kinase C α and protein tyrosine phosphatase 1B (37). Nitrosylated mammalian thioredoxin (Cys⁶⁹) has antiapoptotic properties (38), whereas its *in vitro* reducing activity to insulin disulfides is abolished by glutathionylation of the non-active site Cys⁷² (39). Consistent with the concept of glutathionylation in modifying biological activity, we propose that PR is inhibited by the formation of a mixed disulfide between cysteine 239 and GSH. *E. coli* glutaredoxins can reverse this inhibition by reducing the mixed disulfide. As precedent for this hypothesis, human glutaredoxin can reduce the mixed disulfide for both G-actin and protein tyrosine phosphatase 1B, thus reversing the effects of glutathionylation and restoring biological activity (35, 37).

The gene encoding PR (*cysH*) is located in the *cysJIH* operon and transcribed in a coordinated way with other genes, all belonging to the *cys* regulon (reviewed in Ref. 40). Genes within this regulon are only transcribed when sulfur is limiting and no forms of reduced sulfur are available for the cell. This regulation is controlled by the negatively auto-regulated transcription activator CysB and the inducer *N*-acetyl serine, which is derived from the cysteine precursor *O*-acetyl serine. No other form of kinetic regulation has been reported previously for the sulfite reduction pathway. This was demonstrated by a mutant that cannot repress this pathway and accumulates large amounts of sulfide (41). Regulation of PR activity by glutathionylation introduces another level of complexity in the overall regulation of sulfite biosynthesis. What could be the biological relevance of this control? Reduction of PAPS is linked to loss of electrons, which means loss of reducing equivalents. Under conditions favoring protein glutathionylation (*e.g.* oxidative stress), the unhindered continuation of electron flow via the PR pathway would further deteriorate cell homeostasis by using electrons that would have otherwise been used to reverse undesired oxidations. Therefore, stopping the activity of PR under conditions favoring its glutathionylation could be considered an adaptation of the cell to severe oxidative stress. Neurons constitute another example in which a multilevel regulation in the production of an oxidant and the responses against it could be regulated in a coordinated manner at many levels. The activity of tyrosine hydroxylase, the rate-limiting enzyme for the biosynthesis of dopamine, a potent oxidant, is inhibited by reversible nitrosylation of a structural cysteine (36). At the same time, dopamine-induced oxidative stress leading to the apoptosis of rat neurons may be offset by glutaredoxin activity, which activates NF- κ B via Ref1 (42). The glutaredoxin-driven signaling pathways can include both the Ras phosphoinositide

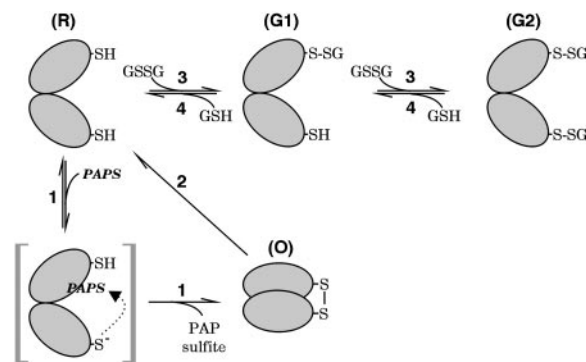


FIG. 7. Model of the thiol-based mechanism and the conformational changes of PAPS reductase. 1, oxidation; 2, reduction; 3, glutathionylation; and 4, deglutathionylation of PAPS reductase. Details are discussed in the text (under “Discussion”). R, reduced, open form of the PAPS reductase dimer. O, oxidized, closed form. G1 and G2, glutathionylated forms of the enzyme.

3-kinase signaling cascade and the c-Jun N-terminal kinase pathway (43).

In view of the findings of this work, a more complete picture emerges for the catalytic mechanism of PR (Fig. 7). In a “normal” reducing cell environment, reduced PR is a homodimer. PAPS can bind to this reduced form (Fig. 7, R), to yield sulfite, PAP, and oxidized PR with an intra-molecular disulfide bridge between the active site cysteines (Cys²³⁹) (Fig. 7, reaction 1). Dimeric oxidized PR migrates on SDS-PAGE with an apparent molecular mass (60 kDa) higher than that of the reduced form (30 kDa). The disulfide of the oxidized enzyme can be reduced by Trx1, Trx2, or Grx1 but not the other glutaredoxins (Fig. 7, reaction 2) (4, 44). If the intracellular environment is somewhat oxidizing (*e.g.* the *gor*⁻ strain), a mixed disulfide may form between Cys²³⁹ and glutathione, rendering the enzyme inactive. All glutaredoxins can catalyze the reduction of this mixed disulfide (Fig. 7, reaction 4).

Formation of protein-glutathione-mixed disulfides is of physiological relevance for *E. coli*. Up to 2% of the total glutathione content (10–20 μ M) is in the form of protein-mixed disulfides, and this value can be increased, as for example in *trxA*⁻*grxA*⁻ mutants (5–7%) (45). In mammalian cells, extensive glutathionylation of protein substrates has been identified to include chaperons, cytoskeletal proteins, cell cycle regulators, and enzymes participating in the intermediary metabolism (46). Such a study has not, to date, been performed for *E. coli*. Glutaredoxins and their monothiol activity would be the molecules expected to key regulate deglutathionylation reactions and reverse related changes in biological activity. The identification and characterization of further proteins that undergo revers-

ble S-glutathionylation and are specifically related to the glutaredoxin species will be necessary for a deeper understanding of cellular redox regulation and signaling.

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REFERENCES

- Schwenn, J. D. (1994) *Z. Naturforsch.* **49c**, 531–539
- Berendt, U., Haverkamp, T., Prior, A., and Schwenn, J. D. (1995) *Eur. J. Biochem.* **233**, 347–356
- Krone, F. A., Westphal, G., and Schwenn, J. D. (1991) *Mol. Gen. Genet.* **225**, 314–319
- Lillig, C. H., Prior, A., Schwenn, J. D., Åslund, F., Ritz, D., Vlamis-Gardikas, A., and Holmgren, A. (1999) *J. Biol. Chem.* **274**, 7695–7698
- Savage, H., Montoya, G., Svensson, C., Schwenn, J. D., and Sinning, I. (1997) *Structure* **5**, 895–906
- Holmgren, A. (1989) *J. Biol. Chem.* **264**, 13963–13966
- Holmgren, A. (1995) *Structure*, **3**, 239–243
- Bushweller, J. H., Åslund, F., Wüthrich, K., and Holmgren, A. (1992) *Biochemistry* **31**, 9288–9293
- Vlamis-Gardikas, A., and Holmgren, A. (2002) *Methods Enzymol.* **347**, 286–296
- Laurent, T. C., Moore, E. C., and Reichard, P. (1964) *J. Biol. Chem.* **239**, 3436–3444
- Holmgren, A. (1979) *J. Biol. Chem.* **254**, 3664–3671
- Miranda-Vizuete, A., Damdimopoulos, A. E., Gustafsson, J., and Spyrou, G. (1997) *J. Biol. Chem.* **272**, 30841–30847
- Åslund, F., Ehn, B., Miranda-Vizuete, A., Pueyo, C., and Holmgren, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9813–9817
- Potamitou, A., Neubauer, P., Holmgren, A., and Vlamis-Gardikas, A. (2002) *J. Biol. Chem.* **277**, 17775–17780
- Potamitou, A., Holmgren, A., and Vlamis-Gardikas, A. (2002) *J. Biol. Chem.* **277**, 18561–18567
- Xia, B., Vlamis-Gardikas, A., Holmgren, A., Wright, P. E., and Dyson, H. J. (2001) *J. Mol. Biol.* **310**, 907–918
- Vlamis-Gardikas, A., Åslund, F., Spyrou, G., Bergmann, T., and Holmgren, A. (1997) *J. Biol. Chem.* **272**, 11236–11243
- Lundström-Ljung, J., Vlamis-Gardikas, A., Åslund, F., and Holmgren, A. (1999) *FEBS Lett.* **443**, 85–88
- Shi, J., Vlamis-Gardikas, A., Åslund, F., Holmgren, A., and Rosen, B. P. (1999) *J. Biol. Chem.* **274**, 36039–36042
- Cartmel-Harel, O., and Stortz, G. (2000) *Annu. Rev. Microbiol.* **54**, 439–461
- Holmgren, A. (2001) *Antioxid. Redox Signal.* **2**, 811–820
- Vlamis-Gardikas, A., Potamitou, A., Zarivach, R., Hochman, A., and Holmgren, A. (2002) *J. Biol. Chem.* **277**, 10861–10868
- Hanahan, D. (1985) in *DNA Cloning* (Glover, D. M., ed) Vol. 1, pp. 109–135, IRL Press, Oxford, UK
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 254–284
- Schriek, U., and Schwenn, J. D. (1986) *Arch. Microbiol.* **145**, 32–38
- Åslund, F., Nordstrand, K., Berndt, K. D., Nikkola, M., Bergman, T., Ponstingl, H., Jörnvall, H., Otting, G., and Holmgren, A. (1996) *J. Biol. Chem.* **271**, 6736–6745
- Luthman, M., and Holmgren, A. (1982) *Biochemistry*, **21**, 6628–6633
- Schwenn, J. D., and Schriek, U. (1987) *Z. Naturforsch.* **42c**, 93–102
- Lillig, C. H., Schiffmann, S., Berndt, C., Berken, A., Tischka, R., and Schwenn, J. D. (2001) *Arch. Biochem. Biophys.* **392**, 303–310
- Schwenn, J. D., and Jender, H. G. (1980) *J. Chromatogr.* **139**, 285–290
- Hjelle, O. P., Chaudhry, F. A., and Ottersen, O. P. (1994) *Eur. J. Neurosci.* **6**, 793–804
- Kim, S. O., Merchant, K., Nudelman, R., Beyer, W. F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Stamler, J. S. (2002) *Cell* **109**, 383–396
- Pineda-Molina, E., Klatt, P., Vazquez, J., Martina, A., de Lacoba, M. G., Perez-Sala, D., and Lamas, S. (2001) *Biochemistry* **40**, 14134–14142
- Klatt, P., Pineda-Molina, E., de Lacoba, M. G., Padilla, A. C., Martinez-Galisteo, E., Barzena, J. A., and Lamas, S. (1999) *FASEB J.* **13**, 1481–1490
- Wang, J., Boja, E. S., Tan, W., Tekle, E., Fales, H. M., English, S., Mielal, J. J., and Chock, P. B. (2001) *J. Biol. Chem.* **276**, 47763–47766
- Borges, C. R., Geddes, T. J., Watson, J. T., and Kuhn, D. M. (2002) *J. Biol. Chem.* **277**, 48295–48302
- Barett, W. C., DeGnoro, J. P., König, S., Fales, H. M., Keng, Y. F., Zhang, Z. Y., Yim, M. B., and Chock, P. B. (1999) *Biochemistry* **38**, 6699–6705
- Haendeler, J., Hoffmann, J., Tischler, V., Berk, B. C., Zeiher, A. M., and Dimmeler, S. (2002) *Nat. Cell Biol.* **4**, 743–749
- Casagrande, S., Bonetto, V., Fratelli, M., Gianazza, E., Eberini, I., Massignan, T., Salmona, M., Chang, G., Holmgren, A., and Ghezzi, P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 9745–9749
- Kredich, N. M. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) pp. 514–527, ASM Press, Washington, D. C.
- Borum, P. R., and Monty, K. J. (1976) *J. Bacteriol.* **125**, 53–55
- Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) *J. Biol. Chem.* **276**, 1335–41334
- Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) *J. Biol. Chem.* **276**, 21618–21626
- Russel, M., Model, P., and Holmgren, A. (1990) *J. Bacteriol.* **172**, 1923–1929
- Miranda-Vizuete, A., Rodriguez-Ariza, A., Toribio, F., Holmgren, A., Lopez-Barea, J., and Pueyo, C. (1996) *J. Biol. Chem.* **271**, 19099–19103
- Lind, C., Gerdes, R., Hammell, Y., Schuppe-Koistinen, I., von Löwenhielm, H., Holmgren, A., and Cotgreave, I. (2002) *Arch. Biochem. Biophys.* **406**, 229–240