Redox regulation of 3'phosphoadenylylsulfate reductase from Escherichia coli by glutathione and glutaredoxins

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**Summary**

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) grows barely 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.*
**Introduction**

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 in order to get incorporated in a living cell. Prototrophic bacteria for example use inorganic sulfate ($\text{SO}_4^{2-}$, S$^{VI}$) as primary source for the biosynthesis of sulfur-containing amino acids and cofactors (1). Sulfate is first activated to adenylylsulfate (APS) and then to 3′phosphadenylylsulfate (PAPS) by ATP sulfurylase and APS kinase. Subsequently, PAPS is reduced by PAPS reductase (PR) to sulfite ($\text{SO}_3^{2-}$, S$^{IV}$) and adenosine-3′5′-bisphosphate (PAP). Sulfite is reduced to sulfide (S$^{2-}$, S$^{0}$) by sulfite reductase, and thereafter is incorporated in O-acetyl serine (OAS) by OAS (thiol)lyase, to give the primary product of sulfate assimilation, cysteine.

PAPS reductase (PR, 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, 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 reduced PR catalyzes the reduction of PAPS, leading to oxidized PR, free sulfite and PAP (equation 1). 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 (equation 2a) or glutaredoxin (equation 2b) with electrons from thioredoxin reductase (TR) and NADPH or from glutathione (GSH), glutathione reductase and NADPH.

$$\text{PR}_{SH}^{\text{SH}} + \text{PAPS} \rightarrow \text{PR}^{\text{S}} + \text{PAP} + \text{SO}_3^{2-}$$

$$\text{PR}^{\text{S}} + \text{Trx}_{SH}^{\text{SH}} \rightarrow \text{PR}_{SH}^{\text{SH}} + \text{Trx}^{\text{S}}$$

(1)  

(2a)
Thioredoxins and glutaredoxins are small (9 - 14 kDa) ubiquitous proteins that utilize their two redox-active cysteines (CxxC-motif) to catalyze reduction of disulfides (6). Whereas thioredoxins and glutaredoxins can reduce their substrates 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 of reducing intracellular disulfides, glutaredoxins may also reduce mixed disulfides forming between a protein thiol and oxidized GSH. This is a reaction not catalyzed by thioredoxins.

*E. 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 while 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, while Trx2 participates in the antioxidant response as part of the OxyR regulon. Grx1 is also a member of the OxyR regulon but not Grx2 or Grx3 (14). With levels at least 10-fold higher than those of Grx1, Grx2 and 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 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). Due to its high abundance (up to 1 % of total soluble protein) and catalytic efficiency it contributes to more than 80 % of the cellular GSH-mixed disulfides 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: 20, 21).

Combined E. coli null mutants for glutathione reductase and the three glutaredoxins (gor grxA grxB grxC) barely grow on sulfate (S\textsuperscript{VI}), but normally on sulfite (S\textsuperscript{IV}) or methionine (S\textsuperscript{II}) (22). As 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 formed upon 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). As 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\(^{-1}\); Trx1, 15,220 M\(^{-1}\) cm\(^{-1}\). SDS-PAGE was performed using the Phast-Gel system (Amersham Bioscience, Uppsala) and the Ready-Gel system (Bio-Rad, Hercules) according to the manufacturer’s instructions.

**Strains and Plasmids**

*E. coli* BI21(DE3) (Novagen, Madison) was used for the overexpression of PAPS reductase using plasmid pET16bcysH (4). DHB4gor grxA grxB grxC and the arabinose promoter-based plasmids pISCGrx2, pISCGrx2C12S, and pISCGrx2C9S/C12S for the expression of wild type Grx2 and mutants C12S and C9S/C12S were first described and characterized in (22).

**Protein expression and purification**

PAPS reductase was expressed and purified as described in (4) using a 3 liter (Meredos, Göttingen) and a 25 liter fermenter (New Brunswick Scientific, Edison). 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 in (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 of the reduced enzyme with 20 mM oxidized glutathione (GSSG, 30 min 22 °C, Tris/HCl pH
Free nucleotides, reductants, or GSSG were removed using ultrafiltration (Amicon YM3, Millipore, Bedford) and Sephadex G25-columns (Amersham Bioscience, Uppsala). The different isoforms were stored at -80 °C in 40 mM Tris/HCl pH 8, 500 mM NaCl, 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 acid-labile sulfite formation from $^{35}$[S]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 Na$_2$SO$_3$, 100 µM $^{35}$[S]PAPS, 100 µM Trx1, 1 µM thioredoxin reductase from *E. coli*, and 5 mM NADPH. Oxidized glutathione, yeast glutathione reductase (Sigma-Aldrich, St. Louis), and glutaredoxins were added as indicated. For the determination of the activity of glutaredoxin-treated oxidized, reduced, and glutathionylated enzyme the assay mixtures (5 µg PR, 50 mM Tris/HCl pH 8.0, 100 mM NaCl) were desalted on Sephadex-G25 and the activity was determined in the absence of further reductants in a single turn-over experiment (100 mM Tris/HCl pH 8, 10 mM Na$_2$SO$_3$, 100 µM $^{35}$[S]PAPS, 5 min reaction time). $^{35}$[S]PAPS (1,700 Bq nmol$^{-1}$) was prepared from $^{35}$[S]sulfite (Amersham Bioscience, Uppsala) as described by Schriek and Schwenn (25) using recombinant APS kinase from *Arabidopsis thaliana* (29). Synthesis and purity were monitored by HPLC according to (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 PR-SG 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 A$_{340}$ was used for quantification of the GSH-moieties in the...
UV-2100 photometer (Shimadzu, Kyoto) at 25°C.

**Fluorescent experiments**

5 µg PAPS reductase in 50 mM Tris/HCl pH 8.0, 100 mM NaCl, in a total volume of 20 µl was incubated with 5 µg of a reduced glutaredoxin for 30 seconds 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-Aldrich, St. Lois, solved in N,N-dimethyl formamide) for 45 min at RT in the dark before they were separated by SDS-PAGE (8 - 16 %) and analyzed on an UV-table (UVP, San-Gabriel).

**Growth of E. coli**

To determine the redox-status of PR in cell free extracts and for immuno-precipitations experiments, cells were grown overnight in LB, washed twice with cold M9-medium and inoculated in fresh M9-medium with 33 mg l⁻¹ Leu and Ile, 100 mg l⁻¹ ampicillin, and 0.1 % arabinose to an OD₆₀₀ 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, 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 Affigel 10 column (BioRad, Hercules) on which 5 mg 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, 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 hour at 100,000 g. The cell free lysate supernatant was incubated for 2 hours at 4 °C with 35 µg of affinity-purified polyclonal PAPS reductase antibodies, while shaking, and then for another hour with 350 µl of 50 % protein G-Sepharose (Amersham Bioscience, Uppsala). Cells were spun down, washed twice with 40 mM Tris-HCl pH 8.0 and 0.5 mM EDTA and resuspended in 1 % SDS and boiled for 20 min before SDS-PAGE.

**Western Blotting**

The BioRad (Hercules) system was used according to the manufacturer's protocol. After transfer the nitrocellulose membrane was blocked 20 min at room temperature with 2 % BSA in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20). It was further washed and incubated with the primary antibody over night 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 one hour with horseradish peroxidase (HRP)-goat anti rabbit antibodies (Dako, Glostrup) 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
(Boston) and visualized using the MiltiImage Light cabinet (Alpha Innotech, San Leandro).

**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 digestion was performed using sequencing grade modified Trypsin (Promega, Madison) according to the manufacturers 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, 0.1 % trifluoroacetic acid. One 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 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 (OD$_{600}$) at stationary phase (2.4). The wildtype 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 previously described 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 Mr of 30 kDa and the oxidized 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 towards 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 the incubation with GSSG, the activity of PR decreased exponentially until no PAPS reduction was detectable. This inhibition pattern is characteristic for pseudo first order kinetics and suggested a covalent modification of PAPS reductase by GSSG as the basis for the inactivation, most likely due to the formation of a mixed disulfide between the enzyme and glutathione:

$$ E + GSSG \rightarrow E - SG + GSH $$

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 addition of reductants. 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, 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 $900.413 \pm 0.017$ (n=2) corresponding to the partly cleaved C-terminal fragment $\text{REC}_{239}\text{GLHEG}$ with a calculated molecular weight of 900.399. GSSG- treatment induced a signal with the size of $m/z = 1206.455$ compatible with the formation of a disulfide between Cys 239 and glutathione (calculated $m/z$ 1206.474).
These results indicated that Cys 239 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 5-IAF 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 turn-over experiments (Fig. 5, panel I). The remaining portion of 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. 5 A, lane 9 and 11), but not the intramolecular PR disulfide (Fig. 5 A, lane 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 (panels 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 BSA (31) reacted specifically with glutathionylated PR and showed no cross reactivity with reduced or oxidized PR (Fig. 6 B, 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 ng out of 14 ng detected). 40% was the estimation for the pISCGrx2C9S/C12S-transformants (6.2 ng out of 15.9 ng detected).
**Discussion**

Regulation of biological activity by 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 Cys 199 which can be hydroxylated, nitrosylated or glutathionylated, each modification resulting in differential binding of the protein to DNA with glutathionylated OxyR having the highest transcriptional activity (32). Glutathionylation of Cys 62 of eukaryotic NF-κB subunit p50 lead to loss of DNA binding activity (33) while nitric oxide treatment of c-Jun lead to nitrosylation/glutathionylation of Cys 269 with concomitant loss of DNA binding activity (34). Deglutathionylation of Cys 374 in G actin resulted in 6-fold 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 Cα and protein tyrosine phosphatase 1B (37). Nitrosylated mammalian thioredoxin (Cys 69) has antiapoptotic properties (38), while its *in vitro* reducing activity to insulin disulfides is abolished by glutathionylation of the non active site Cys 72 (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 could 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 cysJH-operon and is transcribed in a coordinated way with other genes, all belonging to the cys regulon (reviewed in 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, derived from the cysteine precursor O-acetyl serine. No other form of kinetic regulation has been previously reported for the sulfate reduction pathway. This was demonstrated by a mutant which 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 that 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 which 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 multi level 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 apoptosis of rat neurons may be offset by glutaredoxin activity which activates NF-κB via Ref1 (42). The glutaredoxin driven signaling pathways can include both the ras phosphoinositide 3-kinase signaling cascade and the 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 (Cys 239) (fig. 7, reaction 1). Dimeric oxidized PR migrates on SDS PAGE with an apparent
molecular weight (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 Cys 239 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 µM) 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 been performed yet for *E. coli*. Glutaredoxins and their monothiol activity would be the expected molecules to key regulate deglutathionylation reactions and reverse related changes in biological activity. The identification and characterization of further proteins that undergo reversible 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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Abbreviations

APS - adenylylsulfate, Grx - glutaredoxin, GSH - (reduced) glutathione, GSSG - oxidized glutathione, HED - 2-hydroxyethydisulfide, 5-IAF - 5-(iodoacetamido)fluorescein, MALDI-TOF matrix-assisted laser desorption ionization time-of-flight, OAS - O-acetyl serine, OD - optical density, PAP - adenosine-3',5'-bisphosphate, PAPS - phosphoadenylylsulfate, ppGpp - guanosine-3',5'-tetraphosphate, PBS - phosphate buffered saline, PR - phosphoadenylylsulfate reductase, TR - thioredoxin reductase, Trx – thioredoxin.

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Figure legends

Figure 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 wildtype Grx2 (pISCGrx2), Grx2C12S (pISCGrx2C12S), and Grx2C9S/C12S (pISCGrx2C9S/C12S) (22). Cultures of 500 ml M9-medium supplemented with Leu, Ile (33 mg l-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 previously washed twice with M9-medium. The expression of Grx2 and the C12S and C9S/C12S mutants was induced by 0.1 % arabinose.

Figure 2 - Redox status of PAPS reductase in vivo

Western blots of cell extracts from E. coli DHB4gor grxA grxB grxC- pISCGrx2C9S/C12S 1: Control, 5 ng purified 10-his tagged PAPS reductase, approx. 50 % reduced, 2: 3 µg of total cell extract, 3: 30 µg of total cell extract. The cells were grown in M9-medium containing Leu, Ile (33 mg l-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).

Figure 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 in the material and methods section. 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: full circle = 0 mM, square = 1 mM, diamond = 2.5 mM, triangle = 5 mM, star = 25 mM. Out: Incubation time versus relative PAPS reductase activity, 100 % activity correspond to 7.25 µmol mg-1 min-1. Inset: Incubation time versus lg(activity). The non-linear curve fitting was done assuming pseudo
first order kinetics.

**Figure 4 - MALDI-TOF analysis of tryptic fragments from reduced (A) and glutathionylated PAPS reductase (B)**

The reduced and glutathionylated peptide masses corresponding to the cys 239-containing peptides are marked with bold/italic characters.

**Figure 5 - Reduction of PAPS reductase by glutaredoxins**

Non-reducing SDS-PAGE. All lanes contain 5 µg of reduced, oxidized or glutathionylated 10-his-PR incubated (A) with 0.5 mM GSH or (B) without GSH and different glutaredoxins for 30 s. The probes were alkylated for 30 min with 1 mM of the fluorescent 5'-IAF. (I) Activity of PR after glutaredoxin-treatment in single turn over experiments, (II) fluorescence of PR cys 239 labeled with 5'-IAF, (III), Coomassie staining of the same probes. M: Molecular marker (LMW calibration kit, Amersham Bioscience), 1: PR oxidized with PAPS, 2: Glutathionylated PR, 3: PR prereduced with Trx1, 4: Oxidized PR incubated with 5 µg Grx1, 5: Glutathionylated PR plus Grx1, 6: Oxidized PR plus 5 µg Grx1C14S, 7: Glutathionylated PR plus 5 µg Grx1C14S, 8: Oxidized PR plus 5 µg Grx3, 9: Glutathionylated PR plus 5 µg Grx3, 10: Oxidized PR plus Grx3C14S, 11: Glutathionylated PR plus Grx3C14S, 12: Glutathionylated PR after 20 min incubation with 5 mM DTT. All glutaredoxins were prereduced with 10 mM DTT and desalted using Sephadex G25-columns (Amersham Bioscience). The remaining 30 kDa protein band in the oxidized PR-samples (1) are caused by truncated PR lacking cysteines (5).

**Figure 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). Lane 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 (wildtype Grx2) (4), pISCGrx2C9s/C12S (inactive no-thiol mutant) (5), or pISCGrx2C12S (monothiol mutant) (6) (22).

*Figure 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. (R): Reduced, open form of the PAPS reductase dimer, (O): Oxidized, closed form, (G1) and (G2): Glutathionylated forms of the enzyme.
Figure 1
Figure 3
Figure 5

|     | A       | B       |
|-----|---------|---------|
| I   | PR activity | - - + + + + + - - - - + (+) - |
| II  | 66      | 66      |
|     | 45      |         |
|     | 30      |         |
|     | 20      |         |
| III |         |         |
|     | M 1 2 4 5 6 7 8 9 10 11 12 | M 1 2 3 4 5 6 7 8 9 10 11 12 |
| IV  | PR      | G R G O G O G O G O G G G      |
|     | GSH     | + + + + + + + + + + + + + + + + |
|     | DTT     | - + + - + + + + +     |
|     | Grxl    | + + + + + + + + + + + + + + + + |
|     | GxlC14S | + + + + + + + + + + + + + + + + |
|     | Grx2    | + + + + + + + + + + + + + + + + |
|     | GxlC14S | + + + + + + + + + + + + + + + + |
Figure 6
Figure 7
Redox regulation of 3' phosphoadenylsulfate reductase from Escherichia coli by glutathione and glutaredoxins

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