

New Thioredoxins and Glutaredoxins as Electron Donors of 3'-Phosphoadenylylsulfate Reductase*

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Reduction of inorganic sulfate to sulfite in prototrophic bacteria occurs with 3'-phosphoadenylylsulfate (PAPS) as substrate for PAPS reductase and is the first step leading to reduced sulfur for cellular biosynthetic reactions. The relative efficiency as reductants of homogeneous highly active PAPS reductase of the newly identified second thioredoxin (Trx2) and glutaredoxins (Grx1, Grx2, Grx3, and a mutant Grx1C14S) was compared with the well known thioredoxin (Trx1) from *Escherichia coli*. Trx1, Trx2, and Grx1 supported virtually identical rates of sulfite formation with a V_{\max} ranging from 6.6 units mg^{-1} (Trx1) to 5.1 units mg^{-1} (Grx1), whereas Grx1C14S was only marginally active, and Grx2 and Grx3 had no activity. The structural difference between active reductants had no effect upon K_m PAPS (22.5 μM). Grx1 effectively replaced Trx1 with essentially identical K_m -values: K_m Trx1 (13.7 μM), K_m Grx1 (14.9 μM), whereas the K_m Trx2 was considerably higher (34.2 μM). The results agree with previous *in vivo* data suggesting that Trx1 or Grx1 is essential for sulfate reduction but not for ribonucleotide reduction in *E. coli*.

Prototrophic bacteria or fungi mainly use inorganic sulfate as the only supply of sulfur for the biosynthesis of amino acids and essential cofactors. Assimilation of sulfate occurs in five enzymatic steps. First, it is activated to adenylylsulfate (APS)¹ and 3'-phosphoadenylylsulfate (PAPS) by ATP sulfurylase and APS kinase. PAPS is then reduced to sulfite by PAPS reductase and sulfite is reduced to sulfide by sulfite reductase. Finally, cysteine is formed when sulfide is incorporated into *O*-acetyl-L-serine (OAS) by OAS-(thiol)lyase. The enzyme 3'-phosphoadenylylsulfate (PAPS) reductase (EC 1.8.99.4) catalyzes the first reductive step in this sequence. It uses thioredoxin (Trx) or glutaredoxin (Grx) as *in vitro* hydrogen donor for the reduction of 3'-phosphoadenylylsulfate to free sulfite.



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¹ The abbreviations used are: APS, adenylylsulfate; Grx, glutaredoxin; OAS, *O*-acetyl-L-serine; PAP, adenosine 3',5'-bis-phosphate; PAPS, 3'-phosphoadenylylsulfate; Trx, thioredoxin; PAGE, polyacrylamide gel electrophoresis.

The requirement for a low weight dithiol in sulfite formation from PAPS was originally described by Wilson *et al.* (1). Gonzalez-Porqué *et al.* (2) identified thioredoxin as reductant while investigating methionine sulfoxide and sulfate reduction in yeast. Glutaredoxin as the alternate cofactor was found by Tsang and Schiff (3) in a thioredoxin-negative mutant. In *Escherichia coli*, thioredoxin or glutaredoxin is essential for sulfate reduction but not for the reduction of ribonucleotides as deletion or inactivation of both the genes *trxA* and *grxA* caused cysteine auxotrophy but allowed growth on minimal medium (4). When supplied with cysteine the Trx-Grx double mutants were still viable implying that they contained a reductant that could substitute for thioredoxin or glutaredoxin in ribonucleotide reduction. The search for alternate reductant(s) led to the isolation of two new glutaredoxins, termed Grx2 and Grx3 (5) and later a hitherto unrecognized larger heat labile thioredoxin, Trx2 (6). The yet to be explored function of the new redoxins as alternate reductants of ribonucleotide reductases (7), their role in the maintenance of compartmental homeostasis (8), and role in the protection against oxidative stress (9) re-addresses also the question to their possible function as electron donors of the PAPS reductase. In addition to the original observations by Gonzalez-Porqué *et al.* (2) and Tsang (10), that thioredoxin and glutaredoxin can serve as reductant in partially purified preparations, cumulative evidence from earlier investigations suggested that PAPS reductase from *E. coli* could in fact be used to detect thioredoxins from different origins (11) and of different biochemical properties (12).

More recently a homogeneous PAPS reductase from *E. coli* was investigated in greater detail defining its reaction mechanism (13) and its three-dimensional structure (14). Structural and functional data established a ping-pong mechanism for the enzyme homodimer in which thioredoxin reduced the enzyme in a first reaction forming a stable reduced enzyme isoform that is oxidized by PAPS as a second reactant to give sulfite and adenosine 3',5'-bis-phosphate (PAP). A highly conserved cysteine residue in an ECGLH-motif near the C terminus was recognized as the reactive nucleophile of the enzyme. No evidence for an intermediary thioredoxin S:SO₃ or a catalytically competent enzyme substrate complex was obtained making thioredoxin operate as a protein disulfide reductase. PAPS reductase is specific for PAPS and thioredoxin as the electron donor cannot be replaced by monothiols like glutathione or artificial dithiols like dithiothreitol. Until today, all previous data concerning thioredoxin as the electron donor of the PAPS reductase were obtained with a protein that is now designated as thioredoxin 1 (Trx1). In this study, we could extend these investigations using glutaredoxin1 (Grx1) and the newly discovered glutaredoxin 2 (Grx2), glutaredoxin 3 (Grx3), and thioredoxin 2 (Trx2). The major aim was to compare their function

as substrate of PAPS reductase and to evaluate their specificity as hydrogen donor.

EXPERIMENTAL PROCEDURES

Bacteria Strains, Plasmids, and Oligonucleotides—*E. coli* TG1: *supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB⁺ lacI^q lacZΔM15)*; BL21(DE3): *F', ompT, hsdS_B (r_B⁻rM⁻), gal, dcm* (DE3) (Novagen, Heidelberg). Plasmid pET16b_{cysH} is a derivative of pET16b (Novagen) used for cloning of a 733-base pair *cysH* polymerase chain reaction fragment from pUB5 (13) amplified by polymerase chain reaction using the oligonucleotides PRNdeI (5'-GTGAGGAACATATGTCCAAAC-3') and PRBglIIrev (5'-CCGGCAAGA-TCTACCTTCG-3').

Growth of Bacteria, Expression, and Purification of PAPS Reductase—Transformed *E. coli* BL21(DE3) cells were grown in complete LB medium at pH 7.5 containing 100 μg · ml⁻¹ ampicillin (15). The medium was supplemented with 2% glycerol when the bacteria were grown in a fermenter. The fermenter (Meredos, Göttingen) was inoculated with a mid log phase culture of *E. coli* BL21(DE3) at a concentration of 2% (v/v) harboring plasmid pET16b_{cysH}. Cells were grown under vigorous aeration at 32 °C. Expression of *cysH* was induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 0.5 mM when the cells had reached a density of A₅₉₅ 0.7. The cells were collected within 2–3 h after induction by centrifugation at 10,000 × *g* for 20 min and washed in 20 mM Tris/HCl, 100 mM NaCl, pH 8.0. The yield was 2.5 g liter⁻¹. Bacteria (0.06 g ml⁻¹ in 20 mM Tris/HCl, 100 mM NaCl, pH 8.0) were disrupted by two passages through a French press (Ribi Cell Fractionator) applying 14.2 MPa at 5 °C. The homogenate was clarified by centrifugation at 15,000 × *g* for 20 min and applied to TALON affinity resin (CLONTECH, Heidelberg). His₁₀-tagged protein was eluted with 200 mM imidazole. Its purity was examined by SDS-PAGE according to Laemmli (16) and laser mass spectroscopy (17). The average yield per liter of broth was 14 mg PAPS reductase using LB medium. Recombinant Trx1 was purchased from MBI Fermentas (St.Leon-Roth). Trx2 was expressed and purified using the method of Miranda-Vizuete *et al.* (6). Grx1, Grx1C14S, Grx2, and Grx3 were purified as described earlier (18–21).

PAPS Reductase—PAPS reductase activity was measured as formation of [³⁵S]-SO₃²⁻ from [³⁵S]PAPS (12). Each individual determination of the reaction velocity was averaged from two identical samples. The kinetic constants were calculated from three sets of data with the lowest error derived from six different concentrations of PAPS *versus* five different concentrations of reductant. The assay mixture contained 100–750 ng ml⁻¹ of purified PAPS reductase, 100 mM Tris/HCl, pH 8.0, 10 mM Na₂SO₃, 0.5–60 μM [³⁵S]PAPS (specific radioactivity: 1700 Bq × nmol⁻¹), 0.5–50 μM thioredoxin kept reduced by 10–25 mM dithiothreitol, or 0.5–50 μM glutaredoxin kept reduced by 10–25 mM reduced glutathione. [³⁵S]PAPS was prepared enzymatically from [³⁵S]SO₄²⁻ (Amersham-Buchler, Braunschweig) using recombinant His₁₀-APS kinase from *Arabidopsis thaliana* (22). The reaction system contained ATP-sulfurylase and inorganic pyrophosphatase from Sigma (München), pyruvate kinase and phosphoenolpyruvate from Boehringer Mannheim as described by Schriek and Schwenn (23). PAPS synthesis was monitored by high performance liquid chromatography as described by Schwenn and Jender (24).

General Methods—Isolation, restriction, and cloning of DNA, agarose gel electrophoresis, and polymerase chain reaction were performed according to Sambrook *et al.* (15). *E. coli* BL21(DE3) cells were transformed according to Hanahan (25). DNA sequencing was done with an automated DNA sequencer using Auto Read sequencing kit (Pharmacia, Freiburg) with fluorescein-labeled primers. Annealing temperatures for oligonucleotides were calculated depending on their G:C content. The concentration of proteins was determined colorimetrically using Coomassie Brilliant Blue (26) or from a Warburg and Christian nomogram (27). SDS-PAGE was run on 12.5% gels replacing β-mercaptoethanol by dithiothreitol. Polyclonal anti-PAPS reductase antibodies from rabbit (28) were used to detect the protein by Western immunoblotting (29). Immunoprecipitates were visualized on nitrocellulose BA-S 85 (Schleicher & Schuell) by peroxidase-conjugated goat anti-rabbit antibodies. 4-Chloro-1-naphthol in methanol was used for staining.

RESULTS AND DISCUSSION

PAPS reductase as a His₁₀-tag fusion protein was purified by chelate affinity chromatography yielding a homogeneous and enzymatically active protein in a single step. The method used previously by Berendt *et al.* (13) involved several chromatographic steps including hydrophobic interaction chromatogra-

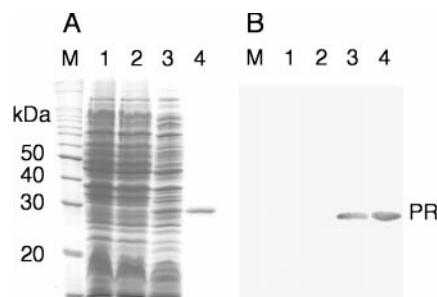


FIG. 1. Recombinant expression of PAPS reductase. A, SDS-PAGE (12.5% Coomassie Blue) of cell extracts from *E. coli* BL21(DE3). B, Western blotting of the SDS gel using anti-PAPS reductase antibodies. Lane 1, host organism without plasmid; lane 2, pET16b; lane 3, pET16b with *cysH* inserted; lane 4, 3 μg of authentic PAPS reductase (PR).

phy on phenyl-Sepharose and dye matrix affinity chromatography on blue- and red-Sepharose. The His-tagged fusion protein is pure as judged by SDS-gel electrophoresis and Western immunoblotting using polyclonal antibodies against the recombinant protein (Fig. 1, A and B). By laser mass spectroscopy (17), we could confirm that the His₁₀-tagged PAPS reductase was expressed as unmodified protein with a mono-isotopic mass of 30634.4 as expected (data not shown). The mass spectroscopy also indicated the presence of a by-product with a mass peak that differed by 149.3 daltons. It is assumed that the difference in isotopic mass of this by-product very likely was caused by a His₁₀-tag PAPS reductase lacking the N-terminal methionine.

Optimized reaction conditions for the PAPS reductase were found at a pH value of 9.2–9.3. The rate of reaction increased by a factor of 1.93 per Δ10 °C interval from 18 to 35 °C provided that the concentration of enzyme was maintained below 750 ng ml⁻¹ of purified protein. Under these conditions, less than 20% of the substrate is converted if the reaction time is kept ≤180 s (data not shown). Rates obtained under these conditions were taken as initial velocity. The molecular activity of the His₁₀-tagged protein was 3.5 s⁻¹ per catalytic center compared with 0.05 s⁻¹ reported earlier for the recombinant untagged enzyme (13). We know now that the lower specific activity of the recombinant untagged enzyme was due to a high proportion of inactive protein, which copurified with the intact enzyme protein. Laser mass spectroscopy and refinement of the three-dimensional structure showed that this truncated protein lacked fourteen amino acids of the C terminus including the functionally important cysteine, whereas thirteen residues at the truncated terminus were disordered and could not be located (14). We have reason to believe that this truncated protein was already produced by the host TG1 transformed with the pBTac derivative pUB5. The new His₁₀-tagged protein described here was produced in BL21(DE3) using a pET16b vector (Novagen) giving rise to a complete and enzymatically active PAPS reductase without removal of the His₁₀-tag fusion.

The Michaelis constants were determined with the concentration of PAPS near saturating (2.5–30 μM) and that of Trx1 slightly limiting to near saturating (1–20 μM). Double reciprocal plots produced a set of parallel lines indicating a ping-pong type reaction mechanism. The set of lines produced a common intersect on the *y* axis above the origin when the data were plotted in a Hanes-Woolf plot. The slopes were replotted to give *V*_{max} and *K*_m (Fig. 2, A and B). Kinetic constants for Grx1 were determined accordingly (Fig. 3, A and B). *In vitro*, Trx1 is effectively replaced by Grx1, which supported the rate of sulfite formation at a comparable rate (6.8–6.6 units mg⁻¹ *versus* 5.4–4.8 units mg⁻¹ using Grx1). A lack of effect upon the Michaelis constant for PAPS may have been expected, because only the reduced enzyme isomer can react with the substrate but it is also noteworthy that the *K*_m values for Trx1 or Grx1

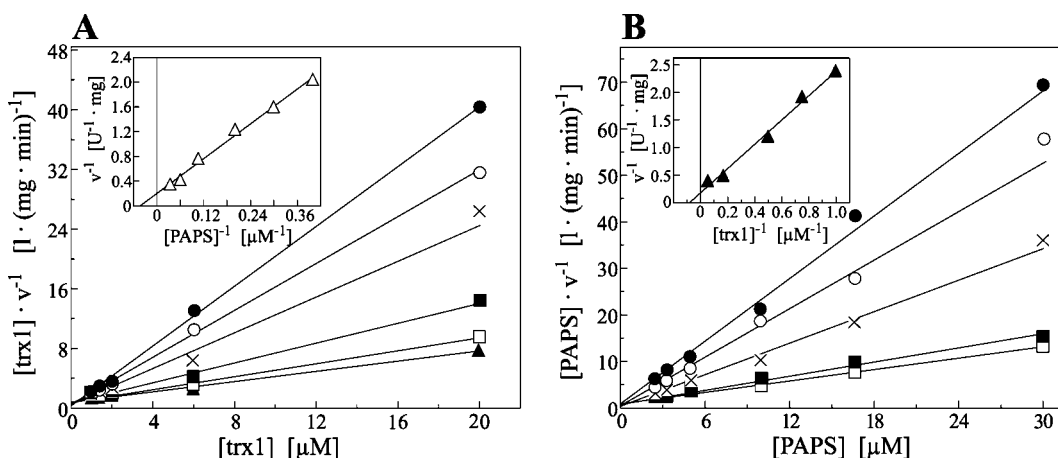


FIG. 2. **Initial velocity kinetics of the PAPS reductase.** A, Hanes-Woolf plot of $[\text{Trx1}] \cdot v^{-1}$ versus $[\text{Trx1}]$ varied at different PAPS concentrations. $[\text{PAPS}]$ (μM): $\bullet = 2.5$, $\circ = 3.3$, $\times = 5$, $\blacksquare = 10$, $\square = 16.7$, $\blacktriangle = 30$. The lines show a common intersect on the y axis above the origin. *Inset*, secondary plot of the slopes of the primary plot versus $[\text{PAPS}]^{-1}$ to obtain V_{\max} and K_m trx1 . B, Hanes-Woolf plot of $[\text{PAPS}] \cdot v^{-1}$ versus $[\text{PAPS}]$ varied at different Trx1 concentrations. $[\text{Trx1}]$ (μM): $\bullet = 1$, $\times = 1.3$, $\circ = 2$, $\blacksquare = 6$, $\square = 20$. *Inset*, secondary plot of the slopes of the primary plot versus $[\text{Trx1}]^{-1}$ to obtain V_{\max} and K_m PAPS.

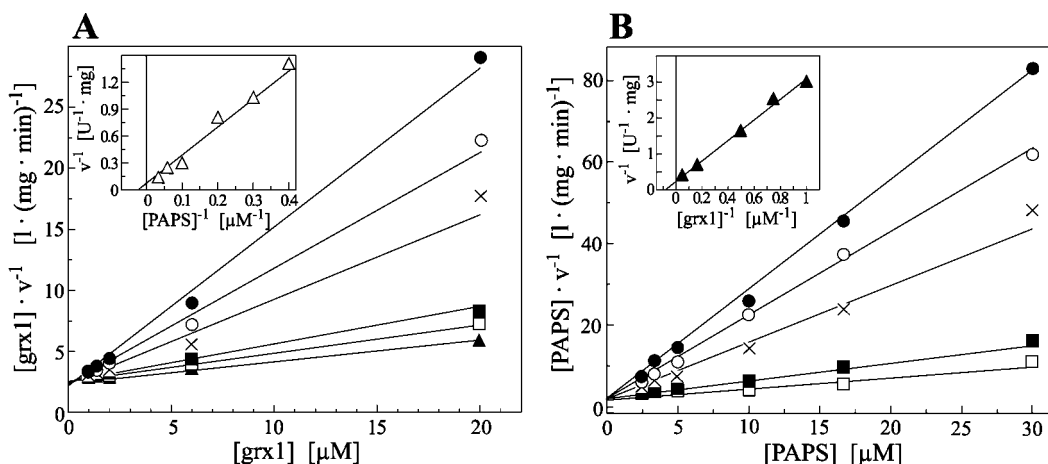


FIG. 3. **Initial velocity kinetics of the PAPS reductase.** A, Hanes-Woolf plot of $[\text{Grx1}] \cdot v^{-1}$ versus $[\text{Grx1}]$ varied at different PAPS concentrations. $[\text{PAPS}]$ (μM): $\bullet = 2.5$, $\circ = 3.3$, $\times = 5$, $\blacksquare = 10$, $\square = 16.7$, $\blacktriangle = 30$. The lines show a common intersect with the y axis above the origin as in Fig. 2. *Inset*, secondary plot of the slopes of the primary plot versus $[\text{PAPS}]^{-1}$ to obtain V_{\max} and K_m grx1 . B, Hanes-Woolf plot of $[\text{PAPS}] \cdot v^{-1}$ versus $[\text{PAPS}]$ varied at different Grx1 concentrations. $[\text{Grx1}]$ (μM): $\bullet = 1$, $\circ = 1.3$, $\times = 2$, $\blacksquare = 6$, $\square = 20$. *Inset*, secondary plots of the slopes of the primary plots versus $[\text{Grx1}]^{-1}$ to obtain V_{\max} and K_m PAPS.

were virtually identical. When thioredoxin and glutaredoxin are compared as reductants of the ribonucleotide reductase, Grx1 with its 10-fold lower K_m , appeared to be more predominant *in vivo*. It also appears more specific because it did not reduce structural disulfides though both reductants were reported to support a similar V_{\max} (30). Yet, the estimated intracellular concentration of Trx (10 μM) is higher compared with a 10-fold lower one for Grx1 (30, 31) suggesting a preference *in vivo* for thioredoxin as hydrogen donor for sulfate reduction. The kinetic constants for thioredoxins and the glutaredoxins in the reaction catalyzed by PAPS reductase are compared in Table I.

When Trx2 was used as reductant, V_{\max} was not significantly affected, but K_m increased 2.4-fold to 34.2 μM . The higher K_m for Trx2 may be seen as an indication of a slightly disturbed contact in the charge transfer complex between enzyme and reductant. Hence, the enzyme rate supported by Trx2 may be sufficient for heterotrophic growth, but the low level of expression of Trx2 reported by Miranda-Vizuetes *et al.* (6) may be the reason why Trx1/Grx1 double mutants score as cysteine auxotrophs. This explanation is supported with the recent finding that overexpression of Trx2 enables a *trxA grxA* double mutant to grow on minimal medium without the supplement of cysteine (33). Miranda-Vizuetes *et al.* (6) also described a 5-fold

TABLE I
Kinetic parameters of PAPS, Trx1, Trx2, Grx1, and Grx1C14S for PAPS reductase

The measurements were repeated three times with three enzyme preparations for each K_m value using five concentrations of reductant versus six concentrations of PAPS. The K_m values were extracted from slope replots of the corresponding Hanes-Woolf plots assuming a ping-pong bireactant enzyme mechanism. The deviation is given as standard deviation.

	K_m	V_{\max}
	μM	units mg^{-1}
PAPS	22.5 ± 0.6	6.8 ± 2.6
Trx1	13.7 ± 1.6	6.6 ± 1.2
Trx2	34.2 ± 5.4	6.3 ± 1.2
Grx1	14.9 ± 1.8	5.1 ± 0.9
Grx1C14S	7.2 ± 1.2	0.3 ± 0.1

lower activity of Trx2 in comparison with Trx1 in the insulin disulfide reductase assay. As pre-reduction of Trx2 with dithiothreitol or deletion of an N-terminal extension containing two additional CXXC motifs increased its activity considerably the authors proposed that the two CXXC motifs present in Trx2 control the disulfide reductase activity by their redox state. In the PAPS reductase assay the redox state of Trx2 had no effect

upon the enzymatic activity, because pre-incubation with dithiothreitol did not stimulate the rate of sulfite formation. Grx2 and Grx3 were both inactive as hydrogen donors for PAPS reductase, whereas the Grx1 mutant C14S retained 6% of the wild type activity (330 milliunits mg^{-1}) with a slightly increased affinity as reflected in a lower K_m of 7.2 μM . A reduction of the homodimeric PAPS reductase by Grx1C14S seems noteworthy, because it would lead to a possibly transient glutaredoxin enzyme-mixed disulfide that becomes accessible to reduced glutathione. Indeed, the monothiol mutant retained 38% of the wild-type protein in the glutathione disulfide oxidoreductase assay but was completely inactive as hydrogen donor for ribonucleotide reductase (19). The lack of activity observed with Grx2 is not surprising because of its unusual structure (20). Grx3, however, shares a 33% overall identity with Grx1 and contains several patches of identical residues that have been identified as indispensable for redox functions (34, 35). Moreover, the secondary and tertiary structure of both glutaredoxins were found to be very similar (35). However, the vicinal active site cysteine residues in Grx3 are surrounded by charged amino acids that differ significantly from the residues in Grx1 (34). This may have an influence on the redox potential of Grx3, which is more positive than Grx1 and on the area of contact in hydrophobic protein-protein interactions. A positive potential surrounding the negative charge of the thiolate of the active site most certainly is necessary to guide the protruding end of the Grx molecule toward its substrate buried in the catalytic site of the enzyme.

Our results give an explanation for the essential role of Trx1 or Grx1 for the growth of *E. coli* on minimal medium containing sulfate as the only source of sulfur (4). Because the apparent K_m values of Trx1 and Grx1 are similar (approximately 14 μM) and much higher than the corresponding values for ribonucleotide reductase (30, 31), the sulfate reduction pathway would seem less favored than that of ribonucleotide reduction, which is essential for DNA synthesis. Furthermore, the even higher apparent K_m value for Trx2 and its relatively low expression level (6) makes this new thioredoxin a poor electron donor to PAPS reductase in particular when the full demand is on to supply DNA replication with the essential deoxyribonucleotides.

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