Characterization of Escherichia coli Null Mutants for Glutaredoxin 2*

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Three Escherichia coli glutaredoxins catalyze GSH-disulfide oxidoreductions, but the atypical 24-kDa glutaredoxin 2 (Grx2, grxB gene), in contrast to the 9-kDa glutaredoxin 1 (Grx1, grxA gene) and glutaredoxin 3 (Grx3, grxC gene), is not a hydrogen donor for ribonucleotide reductase. To improve the understanding of glutaredoxin function, a null mutant for grxB (grxB−) was constructed and combined with other mutations. Null mutants for grxB or all three glutaredoxin genes were viable in rich and minimal media with little changes in their growth properties. Expression of leaderless alkaline phosphatase showed that Grx1 and Grx2 (but not Grx3) contributed in the reduction of cytosolic protein disulfides. Moreover, Grx1 could catalyze disulfide formation in the oxidizing cytosol of combined null mutants for glutathione reductase and thioredoxin 1. grxB− cells were more sensitive to hydrogen peroxide and other oxidants and showed increased carbonylation of intracellular proteins, particularly in the stationary phase. Significant up-regulation of catalase activity was observed in null mutants for thioredoxin 1 and the three glutaredoxins, whereas up-regulation of glutaredoxin activity was observed in catalase-deficient strains with additional defects in the thioredoxin pathway. The expression of catalases is thus interconnected with the thioredoxin/glutaredoxin pathways in the antioxidant response.

Glutaredoxins are ubiquitous proteins that catalyze reductions of disulfides (protein-S-S) or mixed disulfides formed between proteins and glutathione (protein-S-SG) in a coupled system with GSH, NADPH, and glutathione reductase (1). Glutaredoxins can thus be envisaged as reductants of disulfides via GSH. To perform their redox reactions, glutaredoxins have evolved two catalytic mechanisms. The dithiol mechanism uses the two adjacent Cys residues of the active site (usually CPTC) to reduce protein-S-S or protein-S-SG substrates (2). The alternative monothiol mechanism (usual active site CGFS) can only reduce protein-S-SG substrates (2). A good in vitro substrate for glutaredoxin activity is the mixed disulfide composed of GSH and mercaptoethanol. This molecule forms spontaneously when β-hydroxyethyl disulfide (HED)1 or di-mercaptoethanol and GSH are mixed and is a good substrate for both monothiol and dithiol glutaredoxins (HED assay) (1).

The numerous isoforms of glutaredoxins in different organisms seem to reflect involvement in distinct functions. Yeast, for example, contains five different glutaredoxins (3, 4). The first two employ a dithiol mechanism (CPYC active site) (3), whereas the other three have a monothiol active site (CGFS) (4). Although all yeast glutaredoxins play a role in their response against oxidative stress (3, 4), yGrx1 mainly participates in the protection against the superoxide anion, and yGrx2 against hydrogen peroxide (3). Null mutants for yGrx5 grow slowly and are highly sensitive to oxidative damage (carbonylation) or hyperosmotic treatment (4). Null mutants for yGrx2 and -5 or yGrx3, -4, and -5 are not viable, suggesting that a specific minimal monothiol glutaredoxin is essential for yeast survival (4).

Escherichia coli has three glutaredoxins (5) and two thioredoxins (thioredoxin 1 (Trx1) and thioredoxin 2 (Trx2)) (6). The first glutaredoxin (Grx1; 9 kDa, encoded by grxA) was discovered as a glutathione-dependent hydrogen donor for E. coli class 1a ribonucleotide reductase (RR1a) in mutants lacking Trx1 (7). The other two glutaredoxins, Grx2 (encoded by grxB) and Grx3 (9 kDa, encoded by grxC) were purified from an E. coli null mutant for Grx1 and Trx1 (5). Grx1 and Grx3 have 33% sequence identity and similar structures (thioredoxin/glutaredoxin fold) (8, 9). The larger Grx2 (24.3 kDa) has little homology with the other glutaredoxins (10) and a structure resembling that of GSH S-transferases (11). Grx3 can reduce RR1a with 5% catalytic efficiency of Grx1, whereas Grx2 cannot reduce RR1a at all (5). Grx2 and Grx3 do not seem to participate in the reduction of sulfate (SO42−) to sulfite (SO32−), which is catalyzed in E. coli by the enzyme 3′-phosphoadenylyl sulfate (PAPS) reductase (1). Trx1, Trx2, and Grx1 can reduce the intramolecular disulfide of PAPS reductase in vitro, whereas Grx2 and Grx3 cannot (13).

Using HED as a substrate, Grx3 and especially Grx2 have higher catalytic activities in vitro compared with Grx1 (10). In the case of the mixed disulfide of arsenate reductase with glutathione as a substrate (14), Grx2 is a 100-fold more active than any of the other two glutaredoxins. Arsenate reductase catalyzes the reduction of arsenate to arsenite (14). Grx2 is

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1 The abbreviations used are: HED, β-hydroxyethyl disulfide; AP, alkaline phosphatase Δ2–22; gor, gene encoding glutathione reductase; Grx, glutaredoxin; grxB, gene encoding glutaredoxin 2; Kan, kanamycin; PAPS, 3′-phosphoadenylyl sulfate; Te, tetracyclin resistance; Tn, transposon; Trx, thioredoxin; TrxR, thioredoxin reductase; RR1a, class 1a ribonucleotide reductase; HPI and HPII, catalase I and catalase II.
Additional Functions for E. coli Glutaredoxins

thus very good in reducing mixed disulfides between a protein thiol and GSSG and is an abundant protein (5), and yet its biological function is still largely unknown. To elucidate more functions of the E. coli glutaredoxins, we constructed E. coli null mutants for the Grx2 gene (grxB) as well as different combinations of other genes of the glutaredoxin/thioredoxin pathways and characterized their properties.

EXPERIMENTAL PROCEDURES

Materials—β-Hydroxyethyl disulfide was from Tokyo Kasei Kogyo Co. NADPH and glutathione reductase (yeast) was from Sigma. Oligonucleotides were synthesized by Amersham Biosciences Inc. Restriction enzymes, T4 DNA ligase, and Taq polymerase were from Promega. M9 media were supplemented with 50 μg/ml Leu-Ile and 1× basal medium Eagle’s vitamin solution (Invitrogen).

Bacterial Strains and Plasmids—XL-1 blue and DH5α were used for plasmid propagation and cloning purposes. Other strains and plasmids of this study and their origin are listed in Table I.

Inhibition of Growth Assays—An aliquot (100 μl) of cells grown overnight in M9 minimal medium was applied to 4 ml of minimal top agar, and 5 μl of stress compound was pipetted on the top agar, and 5 μl of stress compound was pipetted on the discs. The oxidant used were cumene hydroperoxide (1.5%), diamide (250 mM), and hydrogen peroxide (0.2%), and mandelone (580 mM). Inhibition of growth was measured after 16–24 h at 37 °C as the diameter of the disc of clear cells around the oxidant. Every plate contained four different oxidants, and average values for inhibition zones were calculated from three different plates.

Protein Determinations—In the measurement of total glutaredoxin activity in cell-free lysates from different mutants, total protein was measured in supernatants prepared as described (10) after sonication and centrifugation using the Bradford protein assay (15). In the estimation of carbonylation content, total protein was measured using intact cells (no sonication) by the method of Peterson (16).

Construction of a Null Mutant for Glutaredoxin 2—The E. coli gene for Grx2 (grxB) was deleted using a constructed recombinant p235 plasmid from the Kohara library in which the Grx2 coding sequence was replaced by a kanamycin resistance cassette (17). At a first stage, a 1.2-kb fragment flanking upstream grxB was amplified using primers gBdelF3 (5′-CCT GAT CAT CGA ATT CAT GCT GC-3′) and gBdelR1 (5′-GAT CCT AAA TGG ATA GCT CTA G-3′), and a 1.2-kb downstream flanking region was amplified using primers gBdelF2 (5′-AGG TGA GCC CTC ATT ACA TTT C-3′) and gBdelR3 (5′-GGT CAT CAA TGG AAT CGC TTT AAT CC-3′), which were used to produce a 2.4-kb fragment. After digestion with EcoRI and BamHI, the sticky ends were filled with T4 DNA polymerase, and the 1.2-kb fragment was cloned into the BamHI site in the middle and one EcoRI site at each end. The fragment was digested with EcoRI and cloned in vector pGEM-3Z in which the BamHI site had been previously eliminated after cutting by BamHI and then filling and religaturing. The new vector (pGEM-3Z-1.2-trgsB-B1.2) was digested with BamHI, the sticky ends were filled with T4 DNA polymerase, and the 1.4-kb kanamycin resistance cassette from vector PUC-4K (Amersham Biosciences Inc.) was ligated after it had been previously excised with PstI and filled in with T4 DNA polymerase. The new plasmid (pGEM-3Z-1.2-Kan-1.2) was used to transform strain MC1061, and the grxB::kan deletion was transferred to λ phase 233 according to a described method (17), which resulted in the formation of MC1061 grxB::kan. The absence of grxB in MC1061 was confirmed by Southern blot of total chromosomal DNA using amplified grxB as a probe. The grxB::kan genotype was transferred to DHB4 and derivatives by P1 transduction (18). As some of DHB4 derivatives also contained Kan resistance cassettes, Tn10 (19) was placed close to the grxB::kan mutation to create grxB::kan::Tn10. P1 lysogens from this genotype were used to transduce DHB4 derivatives selecting for Tc resistance. All Tc resistance transductants from this procedure were screened by PCR amplification of the gBdelB1.2 fragment. In this way, the Kan-containing derivative of the primary PCR product to be used as a template for primers G2EcoF3 and gBdelRC1 giving a 2.4-kb fragment was used as a template for primers G2EcoF4 and gBdelRC2 giving a 2.4-kb fragment was used as a template for primers G2EcoF5 and gBdelRC3. The resulting 2.4-kb fragment was purified on an agarose gel and was used as templates for primers gBdelF3 and gBdelR1. The resulting 2.4-kb fragment was purified on an agarose gel and was used as templates for primers gBdelF3 and gBdelR1. The resulting 2.4-kb fragment was purified on an agarose gel and was used as templates for primers gBdelF3 and gBdelR1. The resulting 2.4-kb fragment was purified on an agarose gel and was used as templates for primers gBdelF3 and gBdelR1. The resulting 2.4-kb fragment was purified on an agarose gel and was used as templates for primers gBdelF3 and gBdelR1.
determine HPII activity. The activity level of each one of the catalase isozymes was calculated based on their pH optima. At pH 6.3 both E. coli cytosol (22, 25, 26). Strains CSH7 and UM1 were also used to previously to assess conditions affecting the folding of AP in the series of mutants (Table I). Strain DHB4 was generally used as the wild type and null mutants for grxB, trxAgorB, trxB, trxBgrxA, trxBgrxC, and trxBgrxG grew also at similar rates. However strains trxAgor2, gor trxA gorB grew very slowly and reached lower final levels of growth (Fig. 1) in M9. A possible explanation is that lack of TrxA combined with partial neutralization of the glutathione/glutaredoxin pathway reduced the ability of cells to utilize sulfate. For example, trxAgorA cells cannot grow at all in minimal media as they cannot reduce SO4 \(^{2-}\) to SO3 \(^{-}\) (12).

Null mutants for gorxABC could barely grow on solid minimal medium unless supplemented with sulfite, methionine, or cysteine. The term “barely” means that very small colonies were observed after 2 days at 37 °C and that these colonies did not increase in size after extended incubation at 37 °C. However, null mutants for gorxABC, gorxAC, or gorxBC, which have a single functional glutaredoxin, grew well on minimal plates lacking reduced forms of sulfate (data not shown). Therefore at least one glutaredoxin was essential for the reduction of sulfate to sulfite in gor mutants. gor gorxAgorBC strains transformed with plasmids encoding wild type Grx2 and Grx2 C12S (monothiol) could grow in minimal liquid M9 better than the Grx2 C10S/C12S (inactive double mutant) transformants (Fig. 2). These results demonstrate that Grx2 and its monothiol mutant could regulate the activity of PAPS reductase.

Levels of Glutaredoxin Activity—Levels of total glutaredoxin activity were determined for different strains using the HED assay (1). Less than 1% glutaredoxin activity remained in strain gorxABC (Fig. 3), indicating the presence of another
glutaredoxin(s). Grx2 contributed to 80% of total glutaredoxin activity. A similar contribution to glutaredoxin activity from Grx2 was calculated previously from mutants and extrapolated to the wild type strain using the kinetic data for the pure protein (5). Total glutaredoxin activity was not elevated in null mutants for trxB or at 42 °C or between strains growing in rich or minimal media.

Sensitivity of Strains to Oxidative Stress—To examine the role of Grx2 and other redoxins for survival in oxidative stress, the inhibitory effect on the growth of different strains exposed to different oxidants (27) was examined in M9 medium. The first set of experiments (Table II) was performed using the strain employed for the expression of intracellular AP (Table I). Almost all trxA strains showed increased sensitivity to the organic peroxide cumene hydroperoxide. The exception was strain trxA, which was significantly less sensitive than trxA. This was a somewhat unexpected result, as grxC alone was more sensitive to cumene hydroperoxide than the wild type. Null mutants for trxB and its derivatives were more resistant than the wild type. trxB− strains were more sensitive to the thiol oxidant diamide, whereas all gor derivatives and null mutants for γ-glutamylcysteine synthetase (gshA strain, lacking GSH) were very sensitive, especially the gor strain. Furthermore, trxA, gor, trxA gor, and trxB gor− strains were more sensitive to hydrogen peroxide, whereas the trxA and trxA gor− mutants were less sensitive than the wild type. Sensitivity to the superoxide anion generator menadione was increased in combined null mutants for katE, katE gshA, and katE trxB− strains. Table IV summarizes the results of the experiments with the four different oxidants for all strains.

Disulfide Bond Formation in the Cytoplasm—To assess the contribution of glutaredoxins in reducing cytosolic disulfides, levels of cytosolic AP were measured in null mutants for the individual glutaredoxins. High levels of intracellular AP activity are considered an indication of an oxidizing cytosol (22, 25, 26). The wild-type and null mutants for gshB and gshABC had similar levels of intracellular AP activities (Fig. 4) suggesting that the lack of glutaredoxins in E. coli with intact thioredoxin/thioredoxin reductase (TrxR) and GSH reducing pathways did not significantly affect the intracellular redox environment. The slightly higher levels of AP activity for the grxC strain have been reported previously (22). gor null mutants combined with grxA and grxB (but not grxC) knockouts gave significantly higher AP activity levels than the gor knockout alone (Fig. 4). Therefore, a lack of Grx1 or -2 resulted in increasing formation of intracellular disulfides in gor− derivatives.

The levels of intracellular AP activity were higher in null mutants for gortrxA (Fig. 5) (22), gortxAgrxB and gortxAgrxC null mutants had marginally lower levels of intracellular AP activity, whereas gortxAgrxA null mutants had significantly lower levels (Fig. 5). The addition of reduced sulfur in the form of Met did not seem to influence the levels of intracellular AP activity in any of the gor− derivatives (Fig. 5). All mutants had similar to identical amounts of AP expression as determined by Western blots (data not shown). Differences in AP levels therefore represent alterations in the oxidative folding of AP.

Measurements of Carbonylation of Intracellular Proteins—Western blots with total cell lysates showed that the lack of Grx2 rendered cells more sensitive to carbonylation damage induced by hydrogen peroxide than did lack of Grx1 or Grx3 (Fig. 6A). Lack of GSH (gshA null mutant) gave the highest carbonylation effect (Fig. 6B). No significant increase in carbonylation was observed in extracts from cells lacking Grx1, Grx3, TrxR, or glutathione reductase (Fig. 6, A and B). The protective role of Grx2 was markedly enhanced at the stationary phase of growth (Fig. 6C).

Total Glutaredoxin (HED) Activity in Catalase-deficient Strains—Strains CSH7 (wild type parental), UM1 (catalase-deficient), and null mutant derivatives of these strains for gor, gshA, or trxB were grown until stationary phase in LB. Cells were then harvested, lysed by sonication, and centrifuged, and the HED reducing activity of their culture supernatants was determined. Total glutaredoxin activity was similar in null mutants for single genes. However, in combined null mutants for catalases and TrxR or GSH or glutathione reductase, glutaredoxin activity was elevated significantly (Fig. 7).

Catalase Activity Measurements—As shown previously (Fig. 3) the trxA grxA B C− mutant had less than 1% of total glutaredoxin activity compared with the wild type parental strain. It is assumed that this strain was able to survive by using GSH and TrxR/Trx2 for the maintenance of a reducing cytosol (25). To examine whether other pathways were affected, we measured the activity levels of the two catalases. In Fig. 8 it can be seen that HPI levels were dramatically elevated in the mutant strain.

**DISCUSSION**

Glutathione and glutaredoxins constitute a major supply of reducing power in almost all living cells. The role of glutaredoxins in the reduction of ribonucleotides has been established for one class of glutaredoxins that share the classical thioredoxin/glutaredoxin fold (29). The presence of five different
yeast glutaredoxins, or even isoforms of “classical” glutaredoxins with largely different catalytic properties (e.g., human glutaredoxin 2 (30)), suggests that reduction of ribonucleotides is only part of the discovered glutaredoxin functions. In E. coli, Grx1 and Grx3 belong to the classical superfamily in terms of fold, but Grx2 has different catalytic properties and a three-dimensional structure that brings another dimension to the field; proteins with no significant amino acid homology may have identical folds and perform similar functions. For example, E. coli Grx2 folds like a human protein (p28) (33), and the human CLIC1 (chloride intracellular channel 1) (34), whereas at the same time these proteins have very limited homology. The functions of these proteins have as a common denominator the response to stress. From this point of view E. coli Grx2 is the paradigm of a structure/function concept, “the Grx2 structural superfamily,” found in many different organisms. We therefore consider that the investigation of the function of Grx2 is of general importance. In view of its high level and amount of catalytic activity, E. coli Grx2 would appear to be the enzyme that performs the most oxidoreductions related to GSSG-protein mixed disulfides.

**Glutaredoxins and the Formation of Cytosolic Disulfides**—The contribution of glutaredoxins in reducing cytosolic disulfides has not been investigated thoroughly. Instead, the thioredoxin system has been well established as the major factor of the reducing effect of the glutaredoxin system. Null mutants (trxB strains) have disulfide-promoting cytosols as manifested by the levels of intracellularly expressed AP (22, 25, 26). This has been explained in terms of thioredoxins constantly being in an oxidized state because of lack of TrxR, with thioredoxins thus appearing to be the enzyme that performs the most oxidoreductions related to GSSG-protein mixed disulfides.

**TABLE II**

| Strain            | Cumene hydroperoxide | Diamide | H$_2$O$_2$ | Menadione |
|-------------------|----------------------|---------|------------|-----------|
| DHB4 (wild type)  |                      |         |            |           |
| grxA$^-$          | 15                   | 17      | 17         | 12        |
| grxB$^-$          | 14                   | 17      | 17         | 13        |
| grxC$^-$          | 19                   | 15      | 15         | 15        |
| grxA$^-$ B$^-$    | 15                   | 16      | 17         | 13        |
| grxA$^-$ C$^-$    | 14                   | 16      | 16         | 13        |
| grxB$^-$ C$^-$    | 14                   | 15      | 16         | 11        |
| gor$^-$           | 14                   | 17      | 17         | 10        |
| gor grxA$^-$      | 15                   | 32      | 17         | 15        |
| gor grxB$^-$      | 15                   | 27      | 17         | 10        |
| gor grxC$^-$      | 15                   | 21      | 17         | 11        |
| trxA$^-$          | 20                   | 15      | 15         | 14        |
| trxA gor grxB$^-$ | 25                   | 38      | 18         | 12        |
| trxA gor grxC$^-$ | 21                   | 35      | 14         | 18        |
| trxA gshA$^-$     | 16                   | 18      | 17         | 10        |
| trxB$^-$          | 14                   | 30      | 18         | 17        |
| trxB grxB$^-$     | 13                   | 17      | 17         | 12        |
| trxB grxC$^-$     | 12                   | 17      | 17         | 11        |
| trxB grxB C$^-$   | 13                   | 18      | 18         | 14        |
|                   |                      |         |            |           |

**FIG. 2.** A, growth of DHB4gorgrxA ABC on M9 minimal plate. Strain DHB4gor grxA B$^-$ C$^-$ transformed with pISCGrx2 (top right), pISCGrx2C12S (top left), or pISCGrx2C9S/C12S (bottom) was grown on M9 medium, supplied with Leu-Ile-Met, 50 μg/ml. Single colonies were then streaked on the same medium lacking Met but supplied with 0.2% arabinose and left at 37 °C for 48 h. B, growth of DHB4gor grxA B$^-$ C$^-$ on M9 minimal medium. Strain DHB4gorgrxA ABC transformed with pISCGrx2 (thick solid line), pISCGrx2C12S (thick dashed line), or pISCGrx2C9S/C12S (thin dashed line) was grown on LB medium with ampicillin, 100 μg/ml, to stationary phase and then washed two times with water and resuspended to equal $A_{600}$ in M9 medium supplied with Leu, Ile, and ampicillin, 100 μg/ml (no Met), and 0.1% arabinose. The cultures were then moved to a rotary shaker at 37 °C.

**FIG. 3.** Levels of glutaredoxin activity in DHB4 derivatives. Values correspond to the total glutaredoxin activity of sonicated crude extracts from overnight cultures of different genetic backgrounds. One unit of glutaredoxin activity corresponds to 1 μmol of NADPH oxidized/min using the HED assay for the measurement of GSH-disulfide oxidoreductase activity (1). Mean values shown represent the mean of three measurements for the same lysate, and error bars represent the corresponding S.D.
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**TABLE III**

Sensitivity of CSH7 strains to different oxidants

The diameters of the inhibition zones represent the means of at least three different measurements.

| Strain                  | Cumene hydroperoxide | Diamide | H$_2$O$_2$ | Menadione |
|-------------------------|----------------------|---------|------------|-----------|
| CSH7 wild type          | 14                   | 17      | 5          | 7         |
| grxB$^-$                | 15                   | 19      | 8          | 7         |
| gor$^-$                 | 14                   | 18      | 10         | 8         |
| gshA$^-$                | 14                   | 13      | 24         | 8         |
| trxB$^-$                | 13                   | 14      | 17         | 9         |
| katE$^-$ G$^-$          | 15                   | 19      | 17         | 10        |
| katE$^-$ G grxA$^-$     | 15                   | 20      | 16         | 7         |
| katE$^-$ G grxB$^-$     | 14                   | 19      | 14         | 7         |
| katE$^-$ G grxC$^-$     | 14                   | 25      | 15         | 9         |
| katE$^-$ G gor$^-$      | 15                   | 30      | 16         | 7         |
| katE$^-$ G gshA$^-$     | 14                   | 23      | 15         | 10        |
| katE$^-$ G trxB$^-$     | 13                   | 19      | 14         | 12        |

**TABLE IV**

Summary for sensitivity to different oxidants

A plus sign indicates that the specific gene contributes to antioxidant defense, whereas a minus sign means that the absence of the gene confers increased resistance. Lack of either sign means no difference from the wild type. Signs surrounded by parentheses correspond to conclusions drawn from combined gene deletions.

| Genotype | Cumene hydroperoxide | Diamide | H$_2$O$_2$ | Menadione |
|----------|----------------------|---------|------------|-----------|
| wild type| +                    | +       | +          | +         |
| grxA     | +                    | +       | +          | +         |
| grxB     | +                    | +       | +          | +         |
| grxC     | +(-)                 | +       | +(-)       | +(-)      |
| gor      | +                    | +       | +          | +         |
| gshA     | +                    | +       | +          | +         |
| trxA     | -                    | +       | -          | -         |
| trxB     | -                    | +       | +          | +         |
| katEG    | -                    | -       | -          | -         |

**Fig. 4.** Measurement of intracellular AP activity in different mutants. Cells transformed with plasmid pAD135 encoding leaderless AP (21) were grown in M63 medium, Leu-Ile-Met, 50 μg/ml, 100 μg/ml ampicillin at 37 °C to an A$_{600}$ of 0.3–0.5. Cells were then induced for 1 h with 1 mM isopropyl-1-thio-D-galactopyranoside, and intracellular AP activity was measured as described (21). The results represent the means of at least three different cell lysates. Error bars represent the standard deviation.

The reductive function of individual glutaredoxins. Levels of intracellular AP activity increased in gor strains lacking grxA and grxB (but not grxC). In the more oxidizing environment of the gor-trxA$^-$ strain, Grx2 and Grx3 had a marginal effect in contributing to a reducing cytosol. However, lack of Grx1 in null mutants for gor-trxA resulted in a significant decrease of intracellular disulfides suggesting that Grx1 contributed to the formation rather than the reduction of cytosolic disulfides in

**Fig. 5.** Carbonylation in null mutants for catalases and individual glutaredoxins. Different null mutants growing exponentially in LB were exposed to H$_2$O$_2$ for 1 h at room temperature. Cells were harvested and their lysates analyzed after derivatization with 2,4-dinitrophenylhydrazine and Western blotting with an antibody to the 2,4-dinitrophenyl moiety. Results are shown for the different null mutants in exponential (A and B, chemiluminescence detection) or stationary phase (C, detection by alkaline phosphatase conjugates).

**Fig. 6.** Measurements of intracellular AP activity in gor-trxA$^-$ derivatives. Conditions were the same as described in the legend for Fig. 4. □, gor-trxA$^-$; ▼, gor-trxA grxA$^-$; ◆, gor-trxA grxB$^-$; ◆, gor-trxA grxC$^-$.
some form of reduced sulfur (SO$_2^-$, Met, or Cys). Reduction of SO$_2^-$ to SO$_4^{2-}$ is catalyzed in E. coli by PAPS reductase (13). The enzyme has a single Cys at its C terminus, which becomes oxidized after reduction of PAPS. Trx1, Trx2, and Grx1 can reduce the disulfide of the PAPS reductase, whereas Grx2 and Grx3 cannot (13). Therefore, E. coli should not need any glutaredoxin to reduce sulfate to sulfite; thioredoxins should be able to compensate fully. Our results suggest that in conditions with perturbed reduction of GSSG (gor$^-$ strain), glutaredoxin activity is essential to maintain viability. Because gor$^-$gor$^+$B$^-$/C$^-$ cells could be rescued by monothiol Grx2, it seems that a mechanism for the activation of PAPS reductase involves a thiol that when forming a mixed disulfide with GSSG renders the enzyme inactive. In other words, PAPS reductase is subject to a mixed disulfide mechanism for the regulation of its activity. This mechanism would render the enzyme inactive in higher GSSG concentrations. Apparently, the remaining glutaredoxin activity in grx$^+$B$^-$/C$^-$ strains cannot restore the activity of PAPS reductase. Our data suggest the involvement of all three glutaredoxins in the reduction of PAPS reductase, presumably via a monothiol mechanism.

**Glutaredoxins and Oxidative Stress**—Significant carbonylation of cytosolic proteins occurred in knockouts for grxB, or gshA but not for the grxA, grxC, gor, or trxB genes. Grx2 and GSH therefore constitute the major thiol system for the protection of proteins against H$_2$O$_2$-induced carbonylation. Grx2 is also a potent antioxidant against dopamine-induced oxidative stress in rat neurons, preventing their apoptosis by activating the binding activity of NF-$k$B via Rel-1 (38). Grx2 could also affect both the Ras/phosphoinotiside 3-kinase/Akt/NF-$k$B and the JNK1/JNK2/AP1 cascades (37). The antioxidant role of Grx2 was further confirmed in E. coli with assays measuring inhibition of growth after exposure to oxidants (Table VI). The protection of Grx2 against hydrogen peroxide was observed only in catalase negative strains and was not in proportion to its increased protective effect against protein carbonylation. This is not unexpected, as increased protein carbonylation but with no direct link to cell viability has been described for cells entering stationary phase (38). Transcription of grxB was not increased after treatment with H$_2$O$_2$ (39) but was increased after exposure to acid stress (40), suggesting that the antioxidant effects of Grx2 observed in this work may not constitute its primary function.

Grx1 did not protect proteins from carbonylation, but its absence decreased the survival of cells against H$_2$O$_2$. In comparison, the absence of Trx1 improved survival. Exposure of cells to H$_2$O$_2$ activates the transcription of antioxidant genes such as katG (catalase HPI), grxA, trxC (Trx2), gor, and ahpCF (alkyl hydroperoxide reductase) via OxyR (39, 41), which is also controlled by the cellular redox environment (43). The improved viability of the trxB$^-$ after exposure to H$_2$O$_2$ (or cumene hydroperoxide for trxB$^-$/B$^-$) could be explained in terms of up-regulation of other antioxidant genes via OxyR (39).

Grx3 participated in the detoxification of organic peroxides and in the defense against the superoxide anion radical (menadione). Transcriptional analysis of the response to superoxide anions has not identified grxC as an involved gene (44). These findings demonstrate the complexity of the biological systems and the need for thorough investigations to determine the function and transcriptional regulation of individual genes.

Apart from the catalase genes, all of the other genes examined provided protection against diamide. Diamide, therefore, is an oxidant of thiols. This finding is in agreement with the thiol-promoting role of the glutaredoxin and thioredoxin systems.

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\[ F. \text{ Aslund, personal communication.} \]

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**FIG. 7.** Total glutaredoxin (HED) activity in CSH7 derivatives. See Fig. 1 legend for details.

**FIG. 8.** Levels of the activity of catalases in the wild type and a trxA$^-$grxA$^-$/B$^-$/C$^-$/mutant. The levels of catalases HPI and HPII were measured at different time points after inoculation. Circles correspond to HPI activity and triangles to the activity of HPII. Open circles and squares correspond to the wild type and closed circles and triangles to the trxB$^-$grxA$^-$/B$^-$/C$^-$/null mutant.
Evidence for Additional Glutaredoxin(s)—Significant glutaredoxin activity was measured in crude extracts from grx−ABC null mutants, indicating the presence of an additional glutaredoxin. Similar low levels of glutaredoxin activity were determined in trxAgrxA−B−C− mutants (data not shown). A protein with low HED oxidoreductase activity, which could represent the remaining glutaredoxin activity in the grx−ABC knockout, is currently being characterized in our laboratory.3

Glutaredoxins and Catalases—Strain trxA−grxA−B−C− was used to identify potential responses to stress other than by the up-regulation of Trx2. trxB− strains, for example, have increased transcription of the catalase gene katG (45). E. coli has two catalases: HPI, encoded by katG, and HPII, encoded by katE (28, 46). Catalases dismutate H2O2 to water and molecular oxygen to avoid the formation of the extremely toxic hydroxyl radicals from H2O2. HPI is readily induced by hydrogen peroxide, whereas HPII is up-regulated in stationary phase (35, 36). This work has shown increased activity of HPI in the trxA−grxA−B−C− strain. Such a response may represent a compensating mechanism to reinforce the antioxidative capacity of the cells. HPI is induced by H2O2, and higher cellular activities of this enzyme suggest that the steady state concentrations of hydrogen peroxide in the cells are elevated. In catalase-deficient strains with intact thioredoxin/glutaredoxin pathways, there was a slight increase in total glutaredoxin activity. However in catalase-deficient strains (UM1) lacking TrxR or GSH, a significant up-regulation of total glutaredoxin activity was observed. These results and previous findings (31) show the existence of a concerted response of catalases and the thioredoxin and glutaredoxin systems to oxidative stress. Further studies are needed to identify the electron acceptors for Grx2 and Grx3 and to analyze their participation in the antioxidant mechanisms of the cell.

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