THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 21, Issue of May 24, pp. 18561–18567, 2002

*Printed in U.S.A.*

# Protein Levels of *Escherichia coli* Thioredoxins and Glutaredoxins and Their Relation to Null Mutants, Growth Phase, and Function*

Received for publication, February 6, 2002
Published, JBC Papers in Press, March 13, 2002, DOI 10.1074/jbc.M201225200

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**Levels of *Escherichia coli* thioredoxin 1 (Trx1), Trx2, glutaredoxin 1 (Grx1), Grx2, and Grx3 have been determined by novel sensitive sandwich enzyme-linked immunosorbent assay. In a wild type strain, levels of Trx1 increased from the exponential to the stationary phase of growth (1.5-fold to 3400 ng/mg), as did levels of Grx2 (from ~2500 to ~8000 ng/mg). Grx3 and Trx2 levels were quite stable during growth (~4500 and ~200 ng/mg, respectively). Grx1 levels decreased from ~600 ng/mg at the exponential phase to ~285 ng/mg at the stationary phase. A large elevation of Grx1 (20–30-fold), was observed in null mutants for the thioredoxin system whereas levels of the other redoxins in all combinations of examined null mutants barely exceeded a 2–3-fold increase. Measurements of thymidine incorporation in newly synthesized DNA suggested that mainly Grx1 and, to a lesser extent, Trx1 contribute to the reduction of ribonucleotides. All glutaredoxin species were elevated in catalase-deficient strains, implying an antioxidant role for the glutaredoxins. Trx1, Trx2, and Grx1 levels increased after exposure to hydrogen peroxide and decreased after exposure to mercaptoethanol. The levels of Grx2 and Grx3 behaved exactly the opposite, suggesting that the transcription factor OxyR does not regulate their expression.**

*Escherichia coli* employs two separate pathways that use NADPH to reduce cytosolic disulfides: the thioredoxin and the glutaredoxin systems. The thioredoxin system consists of thioredoxin reductase and thioredoxins 1 and 2 (Trx1 and Trx2).[1] In the glutaredoxin system electrons are transferred from NADPH to glutathione reductase (GR), then to glutathione (GSH), and finally to glutaredoxins 1, 2, and 3 (Grx1, Grx2, and Grx3). Thioredoxins reduce their substrates by employing a dithiol mechanism provided by an active site of two redox-active cysteines separated by two other amino acids (*e.g.* C*XX*C). Glutaredoxins use the dithiol mechanism and an additional monothiol (*e.g.* active site CGFS) with GSH in solution serving as the other thiol (1).

Trx1 was discovered as the reductant of ribonucleotide reductase 1a (RR1a), the essential enzyme for the reduction of ribonucleotides to deoxyribonucleotides during *E. coli* aerobic growth. Trx1 can also reduce 3′-phosphoadenylsulfate (PAPS) reductase and methionine sulfoxide reductase. PAPS reductase is the key enzyme in the reduction of sulfate to sulfite, whereas methionine sulfoxide reductase reduces methionine sulfoxide to methionine (2). The more recently discovered Trx2 can also reduce RR1a and PAPS reductase *in vitro* (3, 4), but it is unlikely that Trx2 is an *in vivo* reductant of PAPS reductase, as combined null mutants for *trxA* and *grxA* cannot grow on minimal media containing sulfate (5). Grx1 was discovered in null mutants for *trxA*, the gene encoding Trx1. Grx1 (encoded by *grxA*) can also reduce RR1a and PAPS reductase (6). Combined null mutants for *trxA* and *grxA* provided evidence for Grx2 (encoded by *grxB*) and Grx3 (encoded by *grxC*), which contributes to more than 80% of total GSH oxidoreductase activity using β-hydroxyetyl disulfide as substrate (7, 8). Grx3 is a poor *in vitro* electron donor for RR1a (about 5% of the catalytic activity of Grx1), and Grx2 lacks such activity altogether. *In vitro* experiments have shown that Grx1 can reduce the disulfide of PAPS reductase, whereas Grx2 and Grx3 were not active in this assay (4). Grx2 protects intracellular proteins from carbonylation damage occurring after exposure to hydrogen (8). All glutaredoxins are good *in vitro* electron donors for the reduction of arsenate by arsenate reductase (ArsC) (9), with Grx2 being 100-fold more efficient than Grx1 (10). In addition to their specific enzyme-linked electron donor activities, Trx1, Trx2, and to a lesser extent, Grx1 and Grx2 are involved in the general reduction of cytosolic disulfides as envisaged from experiments examining the folding of leaderless alkaline phosphatase in the *E. coli* cytosol (8, 11).

Levels of Grx1 and RR1a are up-regulated in null mutants for *trxAgshA*, presumably to maintain a balanced supply of deoxyribonucleotides (12). Grx1 is regulated at the transcriptional level, where a dramatic increase in the mRNA level was observed in a strain lacking both Trx1 and GSH (13). Apart from changes within the RR1a system, transcription of the aerobic ribonucleotide reductase 1b (RR1b) from the *nrdHIEF* operon is increased over 100-fold in strains lacking both Trx1 and Grx1 (14). The transcription factor OxyR regulates the transcription of GR, Grx1, and Trx2 under oxidative conditions (15–17). *grxA* mRNA levels are highly increased in response to oxidative stress (18). Grx1 and Trx1 are able to reduce and thus deactivate OxyR *in vitro*, but Grx1 seems to be the preferred reductant *in vivo* (19, 20). In addition to the regulation by OxyR, Trx1 and GR are growth phase-regulated by the stringent response factor ppGpp (21, 22), whereas transcription of Grx2 is up-regulated by acid stress (23).

Previous studies have reported the regulation of thioredoxins and glutaredoxins at their transcriptional level (14, 18). In this study we have determined the actual protein levels of the two

* This study was supported by grants from the Wenner-Gren Foundation, the Swedish Cancer Society (Grant 961), the Swedish Medical Research Council (Grants 13X-3529 and 03X5–13005-02B), the Karolinska Institute, and the Knut and Alice Wallenberg Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Trx, thioredoxin; ELISA, enzyme-linked immunosorbent assay; Grx, glutaredoxin; GR, glutathione reductase; PAPS, 3′-phosphoadenylsulfate; ArsC, arsenate reductase; RR, ribonucleotide reductase; PBS, phosphate-buffered saline.

TABLE I
*Strains used in this work*

| Strains | Relevant genotype | Origin (ref.) |
|---|---|---|
| Strains derived from DHB4 | See reference | (36) |
| AD494 | DHB4 *trxB*::Kan | (36) |
| WP570 | DHB4 Δ*trxA* | (36) |
| WP571 | DHB4 Δ*trxA trxB*::Kan | (36) |
| WP812 | DHB4 *grxA*::Kan | (36) |
| AV2 | DHB4 *grxB*::kan | (8) |
| WP551 | DHB4 *grxC*::Cm | (36) |
| WP840 | DHB4 *gor*::Tc | (36) |
| 523*grxB* | DHB4 *gor grxA*::Kan *grxB*::Kan. . . Tc *grxC*::Cm | (8) |
| WP612 | DHB4 *gshA*::Kan *trxA* | (36) |
| WP843 | DHB4 *gor*::Tc *trxA* | (36) |
| WP757 | DHB4 *gshA*::Kan | (36) |
| AVG9 | DHB4 *trxA grxA*::Kan *grxB*::Kan. . . Tc *grxC*::Cm | (8) |
| AVG15 | DHB4 *grxA*::Kan *grxB*::Kan. . . Tc *grxC*::Cm | (8) |
| AVG23 | DHB4 *grxA*::Kan *grxC*::Cm | (36) |
| Strains derived from Aegis | DHB4 Δ*ara-leu7967* Δ*lacX74* Δ*phoAPvull phoR* Δ*malF3 F'lac*$^+$ (*lacI*$^q$)*pro* | (37) |
| Aegis324 | DHB4 Δ*trxA* Δ*trxC* nadB::Tn10 | (37) |
| Aegis325 | DHB4 Δ*trxC* nadB::Tn10 | (37) |
| Aegis326 | DHB4 Δ*trxA* Δ*trxC trxB*::Kan nadB::Tn10 | (37) |
| Strains derived from CSH7 | See reference | (38) |
| CSH7*grxB* | CSH7 *grxB*::Kan | (8) |
| CSH7*gor* | CSH7 *gor*::Tc | (8) |
| CSH7*trxB* | CSH7 *trxB*::Kan | (8) |
| CSH7*gshA* | CSH7 *gshA*::Kan | (8) |
| UM1 | CSH7 *katE katG* | (39) |
| UM1*grxB* | UM1 *grxB*::Kan | (8) |
| UM1*gor* | UM1 *gor*::Tc | (8) |
| UM1*trxB* | UM1 *trxB*::Kan | (8) |
| UM1*gshA* | UM1 *gshA*::Kan | (8) |
| Strains derived from JM110 | See reference | (40) |
| AW10 | JM110 *ars*::Cm | (40) |

thioredoxins and the three glutaredoxins by ELISA. The aim was to obtain more information on the specific role of the five presently known cytosolic redoxins of *E. coli*.

## MATERIALS AND METHODS

*Chemicals*—Streptavidin-alkaline phosphatase-PQ was from Mabtech AB, paranitrophenyl phosphate was from Sigma, and Immunopure NHS-LC-biotin was from Pierce. All other chemicals were purchased from common commercial sources.

*Plasmids and Strains*—The plasmids carrying the genes encoding Trx1, Trx2, Grx1, Grx2, and Grx3 have been described previously (3, 7, 24–26). The bacterial strains used in this work are listed in Table I.

*Media*—For most growth purposes, cells were grown in LB liquid medium (27) or LB medium solidified with agar from Merck (15 g/liter) and supplemented (whenever needed) with ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml), or chloramphenicol (20 μg/ml). Cells were also grown in M9 minimal medium (18) containing basal medium Eagle vitamin solution (Invitrogen), leucine (50 μg/ml), isoleucine (50 μg/ml), and glucose (2 g/liter). For the experiment in which cells were treated with arsenate, low-phosphate medium was used (19).

*Treatment of Cells with Hydrogen Peroxide, Mercaptoethanol, or Arsenate*—Cells were grown to exponential phase ($A_{600}$ 0.7–0.8) in LB medium and exposed to hydrogen peroxide (5, 10 mM), arsenate (1 mM), or mercaptoethanol (10 mM) for 1 h. Cell-free extracts were prepared from these cells as described in the next paragraph.

*Preparation of Cell-free Extract*—For stationary phase experiments, overnight cultures were grown in liquid (10 ml) LB medium in a rotary shaker at 120 rpm at 37 °C in 15-ml tubes. Cells were then harvested and resuspended in 50 mM Tris-HCl, pH 8, 1 mM EDTA and disrupted by sonication. Cell-free extracts were prepared by centrifugation at 13,000 × *g* for 30 min after the addition of 1 mM phenylmethylsulfonyl fluoride. To prepare samples corresponding to the exponential phase of growth, cells were grown in LB medium to an $A_{600}$ of 0.6.

*Protein Expression and Purification*—Trx1, Trx2, Grx1, Grx2, and Grx3 from *E. coli* were prepared as described previously (3, 7, 24–26).

*Preparation and Purification of Antibodies*—Sera from rabbits immunized with serial injections of 100 μg of *E. coli* Trx1 or Grx1, -2, or -3 were collected. Sera were saturated up to 50% with ammonium sulfate and left at 4 °C overnight to precipitate the IgG fraction. The precipitate was resuspended in PBS and dialyzed extensively against PBS, pH 7.5. Affinity-purified antibodies for each specific redoxin were prepared using Affi-Gel 10 columns on which 16 mg of Trx1, 3 mg of Trx2, 5 mg of Grx1, 10 mg of Grx2, or 3 mg of Grx3 had been immobilized previously 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, pH 2.1. The eluate was immediately neutralized with 1 M Tris-HCl, pH 9, and the purified antibodies were dialyzed against PBS before being aliquoted and stored at –20 °C.

TABLE II
*ELISA sensitivity*

| ELISA test | Intra-assay CV[a] | Inter-assay CV[b] | Recovery[c] | Detection limit[d] |
|---|---|---|---|---|
| | % | % | % | *ng/ml* |
| Trx1 | 2.8 | 6.1 | 97–102 | 0.1 |
| Trx2 | 3.9 | 10.2 | 95–105 | 0.05 |
| Grx1 | 2.7 | 5.7 | 98–108 | 0.1 |
| Grx2 | 1.7 | 15.5 | 97–100 | 0.05 |
| Grx3 | 2.6 | 11.2 | 95–99 | 0.1 |

[a] Lysates were measured in duplicates 10 times in the same ELISA plate. The standard deviations of the 10 samples were divided with the mean to get the intra-assay coefficient of variation (CV).
[b] Lysates were measured in duplicates four times in different ELISA plates. The standard deviation of the four samples was divided with the mean to get the inter-assay coefficient of variation (CV).
[c] To a diluted sample with a known concentration, known amounts of the specific protein was added, and the levels were measured. The measured value was then divided with the expected value (known value + the amount of added protein) and gave the recovery in percentages.
[d] Detection limit was determined as three times the standard deviation above the blank.

*Biotinylation*—The affinity-purified antibodies (2 mg, ~1 mg/ml) were incubated on ice for 2 h with 10 μl of ImmunoPure® NHS-LC-biotin (20 mg/ml) and later were dialyzed extensively against PBS.

*Enzyme-linked Immunosorbent Assay*—Quantification of thioredoxins and glutaredoxins were carried out by sandwich ELISA. All steps were performed in a volume of 100 μl/well for Grx1, -2, and -3, and Trx1 and 50 μl/well for Trx2 ELISA. Microtiter plates (Nunc®) were coated

TABLE III
*Levels of thioredoxins 1 and 2 and glutaredoxins 1, 2, and 3 in a wild type strain*

Wild type strain (DHB4) was grown for 5 days in LB medium, and samples were taken at different time points. Redoxin levels were determined with sandwich ELISA. Values represent means of duplicates.

| Time | $A_{600}$ | Trx1 | Trx2 | Grx1 | Grx2 | Grx3 |
|---|---|---|---|---|---|---|
| *(h)* | *(ng/mg)* | *(ng/mg)* | *(ng/mg)* | *(ng/mg)* | *(ng/mg)* | *(ng/mg)* |
| 2.5 | 0.115 | 1943 | 222 | 657 | 887 | 4024 |
| 3 | 0.276 | 2693 | 235 | | 888 | 4000 |
| 4 | 0.498 | 1870 | 165 | 267 | 1841 | 4342 |
| 5 | 0.750 | 1584 | 117 | | 2493 | 4228 |
| 7 | 1.112 | 2731 | 135 | 382 | 3514 | 3589 |
| 8.5 | 1.260 | 3134 | 160 | 174 | 4594 | 3668 |
| 10.5 | 1.440 | 2341 | 193 | 282 | 6542 | 4327 |
| 24 | 2.640 | 3274 | 193 | 259 | 11370 | 3871 |
| 48 | 2.580 | 3021 | 185 | 139 | 9992 | 4954 |
| 53 | 2.400 | 2439 | 119 | 182 | 8501 | 4177 |
| 83 | 2.340 | 3024 | 174 | 129 | 8094 | 4364 |

initially with affinity-purified polyclonal antibodies (0.5 $\mu$g/ml) in carbonate buffer, pH 9.6, and incubated overnight at 4 °C. The plates were blocked with 200 $\mu$l/well PBS, 1% bovine serum albumin for 2 h and then washed four times with washing buffer (PBS, 0.05% Tween 20). Standards (0.05–50 ng/ml) or cell-free extracts in incubation buffer (PBS, 5 mg/ml bovine serum albumin, 0.05% Tween 20) of serial dilutions were allowed to react with the coated antibodies overnight at 4 °C. Plates were washed four times with washing buffer, and secondary biotinylated polyclonal antibodies (0.1 $\mu$g/ml) in incubation buffer were added (2 h at room temperature). Thereafter plates were washed four times with washing buffer and alkaline phosphatase-conjugated streptavidin, diluted 1:2000 in incubation buffer, was added (1 h at room temperature). Plates were washed four times with washing buffer before being developed with 1 mg/ml paranitrophenyl phosphate dissolved in substrate buffer (10% diethanolamine, pH 9.8, and 0.5 mM $MgCl_2$). Plates were measured at 405 nm after the addition of substrate buffer. The concentration of individual redoxins in cell-free extracts was calculated from standard curves constructed with known concentration of purified redoxins.

*Determination of ELISA Sensitivity*—The effect of cell-free extracts on the recovery of thioredoxins/glutaredoxins was examined. Known concentrations of standards were diluted in cell-free extracts, and the measured concentrations were compared with the expected values (28). Intra-assay variations were determined by measuring replicates of the same sample in the same plate and inter-assay variation by measuring the same sample repeatedly on different days (29).

*Measurements of Thymidine Incorporation*—Cultures were grown in M9 containing 0.2% glucose and basal medium Eagle vitamin solution (Invitrogen), sulfite (1 mM), and nicotinic acid (2 $\mu$g/ml). Overnight culture was spun down and resuspended in fresh M9 with a dilution of 1:100. At an $A_{600}$ of 0.2–0.3, aliquots of the culture were transferred to a microtiter plate (100 $\mu$l/well). The mini-cultures were each labeled with 8.3 $\mu$Ci/ml [*methyl*-$^3$H]thymidine (47 Ci/mmol, Amersham Biosciences) for 5 min at 37 °C. Cells were then immediately harvested with the Harvester 96®, and [*methyl*-$^3$H]thymidine incorporation was measured using a Wallac MicroBeta Plus scintillation counter.

*Protein Determination*—Total protein was determined as described by Bradford (30). The concentration of pure redoxins was determined by measuring $A_{280}$. Antibody concentration was calculated using the relation: antibody (mg/ml) = $(A_{280}–A_{310})/1.4$.

*Determination of GR Activity*—100-ml cultures were grown in LB medium in 250-ml flasks at 150 rpm, 37 °C. Samples representing the exponential phase were collected at $A_{600}$ = 0.4–0.6, whereas overnight cultures represented the stationary phase samples. 40 ml was collected each time, harvested, and stored at −80 °C. Cell-free extracts to measure GR activity were prepared on a later day as described above. GR activity was determined by monitoring the oxidation of NADPH during the reduction of oxidized glutathione at 340 nm, 25 °C in a fresh mixture of 1 mM GSSG, 0.2 mM NADPH, 2 mM EDTA, and 0.1 mg/ml bovine serum albumin in 100 mM Tris-HCl, pH 8.0. One unit of GR activity was defined as 1 $\mu$mol of NADPH oxidized/min.

## RESULTS

*Determination of ELISA Sensitivity*—Results from recovery of three different assays for the different antibodies are summarized in Table II. The detection limit was determined as three times the standard deviation above the blank (29) and was calculated to 0.05–0.1 ng/ml for all ELISAs. The parallelism tests between the sample and standard curve were performed for all samples (28). Cross-reactivity for each antibody was detected using a null mutant lacking the gene for which the specific antibody was raised (Table IV). The only antibodies that showed a small cross-reactivity were those of Trx1 (probably with Trx2).

*Levels of Thioredoxins 1 and 2 and Glutaredoxins 1, 2, and 3 in a Wild Type Strain*—Wild type (DHB4) strain was grown in 1 liter of culture for 5 days in a 3-liter Erlenmeyer flask in LB media (Table III). The levels of Grx3 and Trx2 were stable (~4.5 and 0.2 $\mu$g/mg, respectively), Grx1 levels decreased slightly (from 0.6 to 0.2 $\mu$g/mg), whereas those of Trx1 increased slightly (from 2.0 to 3.0 $\mu$g/mg). The levels of Grx2 increased during late exponential phase and were stable at late stationary phase. In late stationary phase the levels of Grx2 (8 $\mu$g/mg) were ~3-fold higher than during exponential phase (2.5 $\mu$g/mg).

*Levels of Thioredoxins 1 and 2 and Glutaredoxins 1, 2, and 3 in Different Null Mutants*—Levels of Trx1 were increased 2-fold in null mutants for the glutaredoxin system (*grxAgrxBgrxC*) at the stationary phase, with an almost 3-fold elevation in the *grxB* minus strain but also in null mutants for *gor* or *gshA* (Table IV). Trx2 was increased up to 4-fold in strains lacking all three glutaredoxins and *gor* or *trxA*. All three glutaredoxin species were up-regulated in strains deficient in the thioredoxin system (*trxAtrxBtrxC*) but at different stages of growth, with Grx1 having the highest relative increase. A 30-fold elevation of Grx1 was observed in null mutants for the thioredoxin system. Grx1 was also increased 25-fold in null mutants for *trxAgor* or *trxAgshA*. Increases in Grx1 were observed mainly at the exponential phase. Grx2 showed a 2–3-fold increase in the null mutant for *trxC*. A 3-fold increase of Grx2 was observed at the stationary phase in the *trxAtrxBtrxC* minus strain, where the levels reached 10 $\mu$g/mg. Grx3 levels were very stable during exponential phase and were not affected by any of the mutations examined at this stage of growth. At stationary phase, Grx3 was up-regulated in null mutants for *trxA*. An increase of Grx3 was also observed in strains deficient in both the glutaredoxin and thioredoxin systems ($trxA^-gor^-grxB^-$ and $trxA^-gshA^-$), suggesting a compensatory role for Grx1 and Trx1.

*Thioredoxin and Glutaredoxin Levels in Catalase (katEkatG) Minus Strains*—In *katEkatG* minus strains, the levels of Trx1 and Trx2 did not change significantly compared with those of Grx1, Grx2, and Grx3, which increased 2-fold. The effect in the levels of glutaredoxins was even more pronounced in strains lacking parts of the glutaredoxin or thioredoxin system ($gor^-$, $gshA^-$, and $trxB^-$) (Fig. 1), with Grx2 reaching >20 $\mu$g/mg in

TABLE IV
*Levels of thioredoxins 1 and 2 and glutaredoxins 1, 2, and 3 in different null mutants*
Levels were determined with sandwich ELISA. Values represent means of duplicates. NP, not performed.

| Relevant genetic characteristics | Trx1 | | Trx2 | | Grx1 | | Grx2 | | Grx3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Exponential phase | Stationary phase | Exponential phase | Stationary phase | Exponential phase | Stationary phase | Exponential phase | Stationary phase | Exponential phase | Stationary phase |
| | | | | | *ng/mg protein* | | | | | |
| *wt* | 2283 | 2443 | 154 | 209 | 598 | 289 | 3578 | 5127 | 4098 | 4673 |
| *trxA* | 5 | 0 | 149 | 184 | 827 | 379 | 6220 | 3809 | 3725 | 9349 |
| *trxB* | 2159 | 2182 | 175 | 227 | 1940 | 688 | 4404 | 1727 | 2692 | 4203 |
| *trxC* | 4886 | 4413 | NP | 1 | 793 | 1017 | 9371 | 9489 | 3329 | 5806 |
| *trxAtrxC* | NP | NP | NP | NP | 13587 | 1063 | 3341 | 5469 | 5373 | 6250 |
| *trxAtrxBtrxC* | NP | 0 | NP | NP | 17747 | 5286 | 3368 | 10376 | 3798 | 9003 |
| *grxA* | 4648 | 3034 | 194 | 203 | 0 | 0 | 6059 | 5006 | 3380 | 4028 |
| *grxB* | 2658 | 6491 | 222 | 109 | 807 | 408 | 1 | 0 | 4456 | 6564 |
| *grxC* | 2866 | 5471 | 229 | 127 | 680 | 411 | 4129 | 6470 | 1 | 0 |
| *grxAgrxC* | 4259 | 3737 | 255 | 135 | NP | NP | 3694 | 5710 | 1 | 0 |
| *grxAgrxBgrxC* | 2466 | 5909 | 185 | 170 | NP | NP | NP | NP | NP | 0 |
| *gor* | 2870 | 4229 | 168 | 512 | 1344 | 419 | 4321 | 4109 | 4938 | 6924 |
| *gorgrxAgrxBgrxC* | 1947 | 4564 | 376 | 816 | NP | NP | NP | NP | NP | NP |
| *gshA* | 4312 | 6297 | 234 | 192 | 1001 | 554 | 3838 | 2694 | 3618 | 5988 |
| *trxAgrxA* | NP | NP | 585 | 1205 | NP | NP | 3154 | 4614 | 3508 | 5589 |
| *trxAgrxAgrxBgrxC* | NP | NP | 427 | 970 | NP | NP | 0 | NP | 0 | 0 |
| *trxAgor* | NP | NP | 585 | 664 | 1340 | 3915 | 2552 | 6164 | 5264 | 6017 |
| *trxAgshA* | NP | NP | 527 | 544 | 9697 | 3898 | 2681 | 5451 | 3800 | 7433 |
| *trxAgorgrxB* | NP | NP | 404 | 103 | 1209 | 1392 | NP | NP | 4108 | 7372 |

the *katEkatGgshA* minus strain. These data correlate well with the reported up-regulation of total glutaredoxin activity in the same strains (8).

*Changes in the Thioredoxin and Glutaredoxin Levels after Treatment with hydrogen Peroxide or Mercaptoethanol*—To examine the regulation of different redoxins in reducing and oxidizing environments, cells were treated with hydrogen peroxide or mercaptoethanol. Grx1 levels increased up to 5-fold in cells treated with hydrogen peroxide, whereas Grx2 was down-regulated under the same treatment but was up-regulated under mercaptoethanol treatment (Fig. 2). Grx3 responded in the same way as Grx2 but with much lower levels of change. Trx1 and Trx2 levels changed as did those of Grx1 but with much smaller amplitude. Hydrogen peroxide and mercaptoethanol treatment of *katEkatG* minus strains gave the same pattern of changes as described above but with the redoxin levels changing much more dramatically (Fig. 3).

*Glutaredoxin Levels after Treatment with Arsenate*—Treatment of wild type cells with 1 mM Arsenate for 1 h resulted in no change in the redoxin levels compared with the untreated cells. In null mutant for arsenate reductase, the levels of thioredoxins and Grx1 remained unchanged, whereas the levels of Grx2 and Grx3 decreased (Fig. 4). The overall response of the levels of Grx2 and Grx3 after treatment with arsenate seemed to be similar to the response after treatment with hydrogen peroxide.

*Thymidine Incorporation*—Null mutants for the thioredoxins and glutaredoxins were supplied with thymidine at their exponential phase ($A_{600}$ ~0.250) of growth. Null mutants for *grxB* or *grxC* had thymidine incorporation identical to wild type. In the *grxA* null mutant, thymidine incorporation increased 5-fold (Fig. 5). The *trxA* and *trxB* null mutants showed a 2–3-fold higher incorporation rate, as did the combined null mutants *trxAtrxBtrxC* and *grxAtrxA*. The *trxC* null mutant had slightly decreased thymidine incorporation, perhaps because of the up-regulation of Trx1 in the particular strain (Table IV). Thymidine incorporation was also examined at early stationary phase ($A_{600}$ ~1.0) with cells spun down and resuspended in fresh medium to $A_{600}$ ~0.250. Under these conditions, results for the null mutants were identical to those obtained from the exponential phase (data not shown).

*Interaction between the Glutaredoxin and Thioredoxin Systems*—Oxidized Grx1 was not a good substrate for the thioredoxins (in the presence of TR and NADPH), with apparent $K_m$ values for Trx1 and Trx2 in the order of 230 and 340 μM, respectively. The apparent turnover values ($k_{cat}$) were also low (~160/min). Oxidized Trx1 was a poor substrate for glutaredoxins in a modification of the β-hydroxyetyl disulfide assay (24) (data not shown).

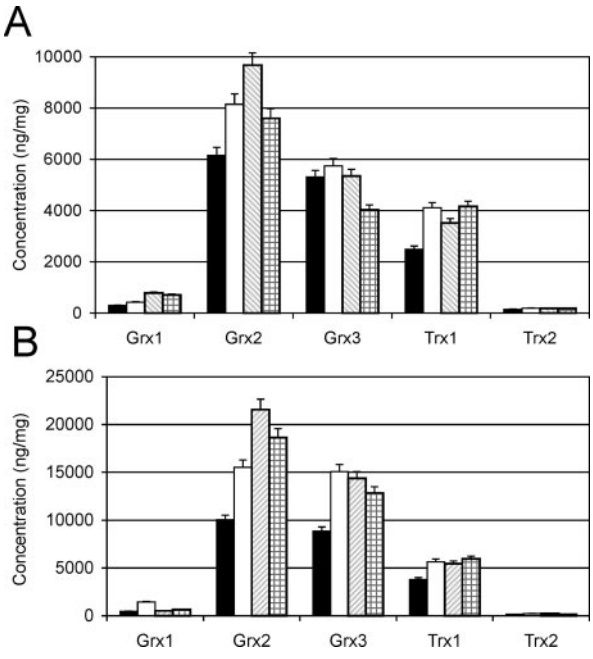


FIG. 1. **Redoxin levels in *katEG* (UM1) minus strain (*A*) and the parental wild type (CSH7) strain (*B*) and their derivatives with additional null mutants for the thioredoxin or the glutaredoxin system.** Cells were grown to stationary phase in LB medium, harvested, and lysed by sonication. Values represent the means of triplicates for the same sample. ■, wild type; □, *gor*$^-$; ▨, *gshA*$^-$; ▩, *trxB*$^-$.

*GR Activity*—The expression of GR is regulated at the exponential phase by OxyR (16) and at the stationary phase by ppGpp (31). GR levels were higher at the stationary phase compared with the exponential for all strains examined. The catalase minus strains had higher levels of GR activity in both the stationary and exponential phases. A significant elevation of GR activity was observed in the combined null mutant for

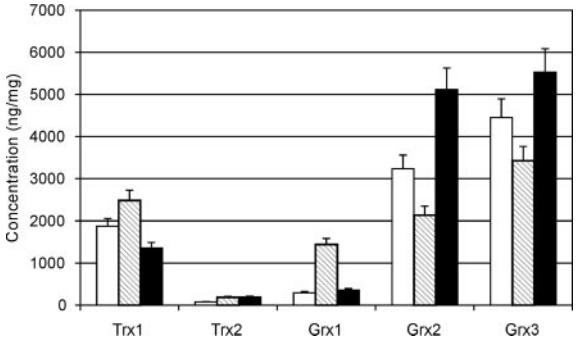


FIG. 2. **Redoxin levels after treatment with hydrogen peroxide or mercaptoethanol.** Cells (DHB4) were grown to exponential phase in LB medium ($A_{600}$ ~0.8) and treated for 1 h with 10 mM hydrogen peroxide (▨) or 10 mM mercaptoethanol (■) or left untreated (□). Data represent the ELISAs of two independent experiments.

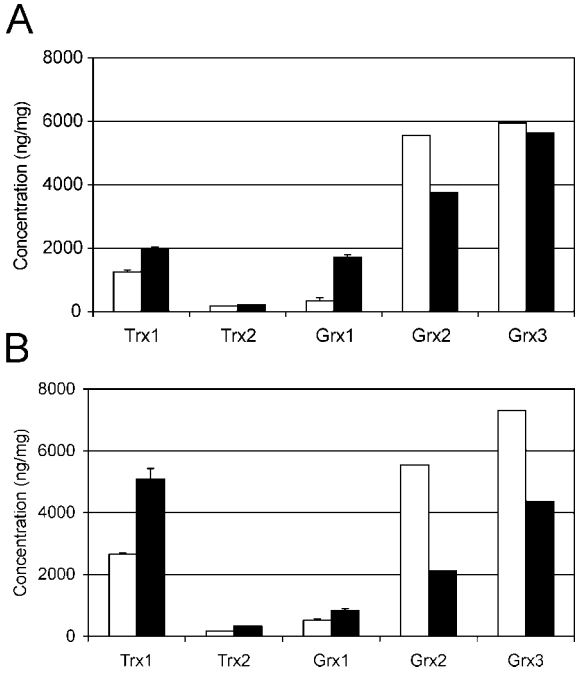


FIG. 3. **Comparison of the redoxin levels of wild type and catalase-deficient strains after treatment with hydrogen peroxide.** Wild type (CSH7) cells (*A*) and cells lacking catalase activity (UM1) (*B*) were grown to late exponential phase in LB medium ($A_{600}$ ~0.8) and treated for 1 h with 5 mM hydrogen peroxide. Data are from two independent experiments. □, untreated; ■, treated

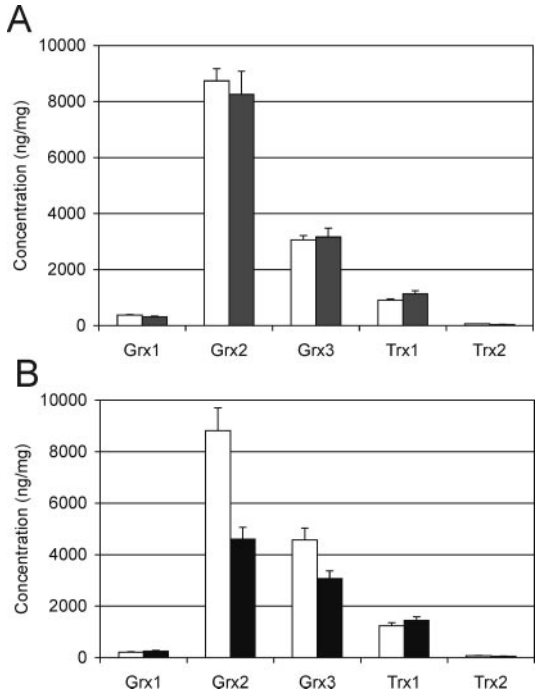


FIG. 4. **Redoxin levels after treatment of wild type and *arsC*$^-$ cells with 1 mM arsenate.** Wild type (JM110) cells (*A*) and the *arsC*$^-$ strain (AW10) (*B*) were grown to late exponential phase in low phosphate medium ($A_{600}$ ~0.8), treated with 1 mM arsenate for 1 h, and lysed by sonication. Data represent the mean of two independent experiments. □, untreated; ■, treated.

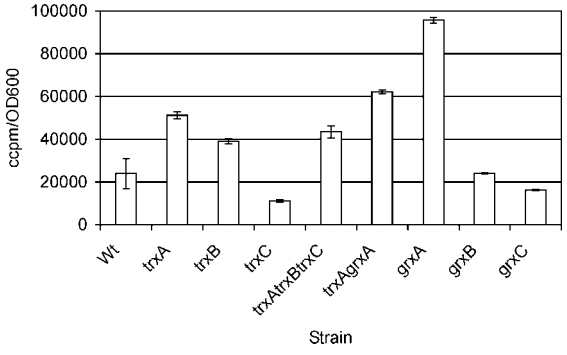


FIG. 5. **Thymidine incorporation in different null mutants.** Cells (DHB4 derivatives) were grown in M9 with the addition of sulfite (1 mM) and nicotinic acid (2 μg/ml). At $A_{600}$ of 0.2–0.3, cultures were labeled with 8.3 μCi/ml [*methyl*-$^3$H]thymidine. Values represent the means of triplicates for the same sample.

the catalases and thioredoxin reductase (*katEkatGtrxB* minus strain) (Fig 6).

## DISCUSSION

The aim of this work was to further characterize the interactions and compensations of the thioredoxin and glutaredoxin systems of *E. coli*. Measuring the relative amounts of the final effectors of the two systems, the two thioredoxins and the three glutaredoxins, could provide information on their overlapping functions. For example, the compensatory role of Grx1 as an alternative electron donor to RR1a has been suggested by measuring the levels of Grx1 in null mutants for *trxA* (12).

*General Redoxin Levels*—The striking finding is that Grx2, an atypical large glutaredoxin with structure similar to glutathione *S*-transferase (24, 32), exists in the cell in amounts as high up as 1% of total protein. Grx3 was also found at relatively high levels (0.5%), as was Trx1 especially at the stationary phase (0.3%). The best characterized Grx1 comprises at best (300–600 ng/mg) one-tenth of the amount of Grx2 and Grx3 found under normal conditions; this is worth pointing out because Grx2 and Grx3 still have a relatively unknown function(s). The levels of the redoxins under normal growth suggest that Trx1 and Grx2 are stationary phase proteins, whereas Grx1 is a protein of the exponential phase.

*Response after Treatment with Hydrogen Peroxide, Mercaptoethanol, and Arsenate*—The lack of Grx2 leads to an increase of carbonylated proteins after exposure to hydrogen peroxide (8). The same effect, but to a much lesser extent, is noticed for Grx3 but not for Grx1 (8). These findings suggest an antioxidant role for Grx2. In this work, the levels of all three glutaredoxins were elevated in catalase minus strains, suggesting an antioxidant role for all of them. However, the administration of peroxide resulted in a decrease in Grx2 (and Grx3) levels, whereas the addition of mercaptoethanol increased the amounts of both Grx2 and Grx3. What could be

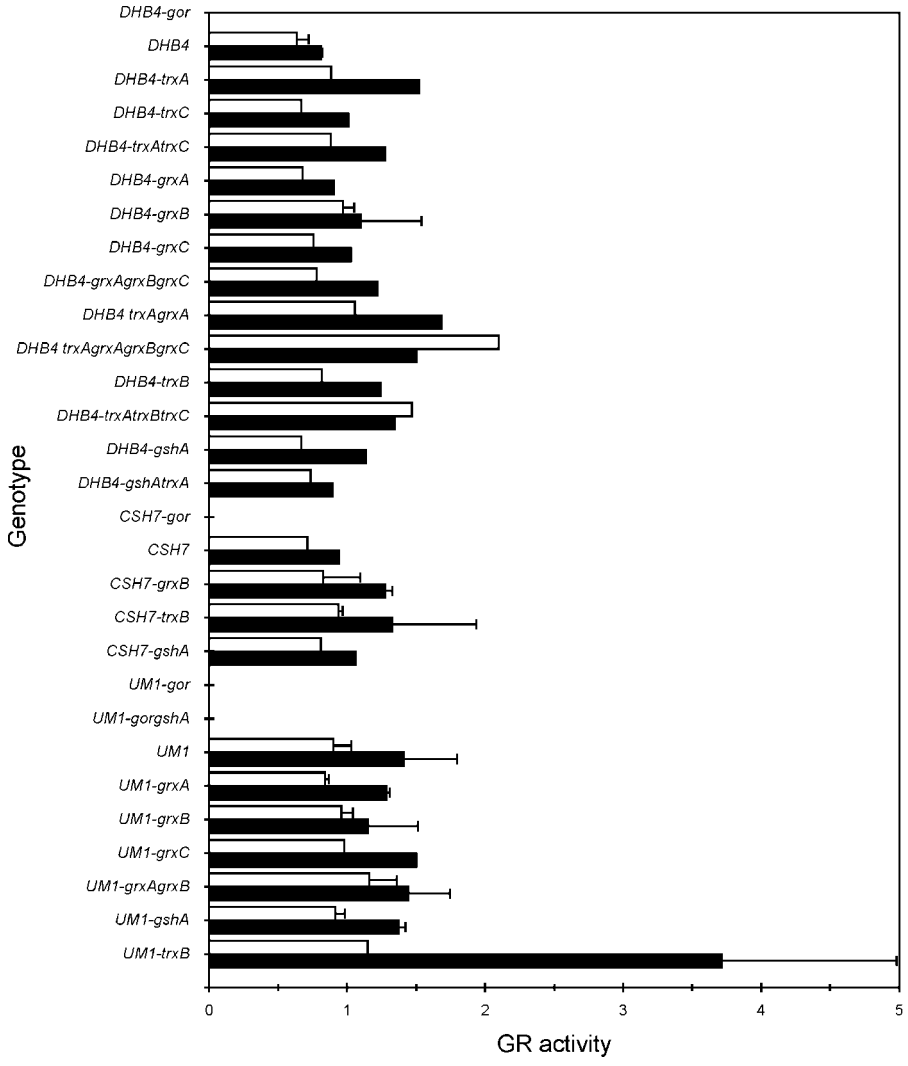


FIG. 6. **Levels of GR activity in different null mutants.** Cells were grown in LB medium and harvested at an $A_{600}$ of 0.4–0.6 (exponential phase) and after overnight growth (stationary phase). One unit of GR activity was defined as 1 $\mu$mol of NADPH oxidized/min. □, exponential phase; ■, stationary phase. *Error bars* represent ± 1 S.E. from at least two independent experiments

the explanation for this finding especially in view of the "normal" antioxidant response for Trx1, Trx2, and Grx1 after the addition of peroxide? The first explanation is that OxyR, which regulates the transcription of the other redoxins, does not control transcription of Grx2 and Grx3. There is no OxyR DNA binding consensus upstream of *grxB* or *grxC*. It could also be that Grx2 and Grx3 revert to oxidants during intense oxidative stress, making their presence deleterious for the cell. Grx1 for example can turn to a general oxidant of disulfides in null mutants for *gortrxA* (8). In addition a protein may be more rapidly degraded when exposed to oxidative stress (33), or the protein synthesis might be affected under oxidative conditions. The up-regulation of Grx2 and Grx3 after the addition of mercaptoethanol suggests that Grx2 and Grx3 can revert to oxidants/reductants according to their redox environment and that their transcription is not regulated by OxyR. The latter finding is in agreement with measurements of the global transcriptional response of *E. coli* genes after exposure to hydrogen peroxide (8, 34).

Treatment with arsenate gave similar changes in the levels of Grx2 and Grx3, as did treatment with peroxide but only in the strain lacking arsenate reductase. No changes were found in the "wild type" strain. In view of the survival of *grxAgrxB-grxC* null mutants in media with arsenate (10), our findings suggest that these dithiol glutaredoxins may not be the main *in vivo* reducers of arsenate reductase. The decrease of Grx2/Grx3 levels in the a*rsC* minus strain may reflect the turning of glutaredoxins to oxidants in the presence of arsenate.

*Levels of Redoxins in Different Genetic Backgrounds*—Apart from great increases in the levels of Grx1 in *trxAtrxC* and *trxAtrxBtrxC* null mutants, the levels of all other redoxins were relatively stable in all genetic backgrounds examined. These data suggest that only Grx1 and Trx1 have strictly overlapping and specific functions, presumably the reduction of ribonucleotides by ribonucleotide reductase.

*Thymidine Incorporation*—Measurements of thymidine incorporation in the DNA of the different mutants show that null mutants for *grxA* have the highest levels of labeled DNA, followed by *trxAgrxA*, and finally by *trxA* strains. Grx1 and Trx1 are not known to be involved directly in the synthesis of DNA, and null mutants in neither of these genes grow any faster than the wild type (8) to justify the apparent higher levels of DNA synthesis. Therefore the data for the DNA labeling must be envisaged in terms of the ability of RR1a of the different strains to provide deoxyribonucleotides for the replication of DNA. In strains with lower deoxyribonucleotide pools, there is a limited supply of available deoxythymidine for incorporation into the DNA. As a consequence, the radioactive thymidine provided for the labeling of DNA will be preferentially incorporated into newly synthesized DNA and give higher counts. Hence, these results reflect the ability of RR1a to supply deoxyribonucleotides to DNA; the more the labeling, the lower the overall activity of RR1a. Under this approach Grx1 is likely to be the major supply of electrons to RR1a followed by Trx1. A similar conclusion could be drawn from the *in vitro* reactivity of Trx1 and Grx1 with RR1a. Grx1 has a 10-fold

lower $K_m$ when compared with Trx1 for the reduction of RR1a, whereas the $V_{max}$ for the two enzymes is very similar (35). Trx2, Grx2, and Grx3 do not seem to be involved (according to our data) in the reduction of RR1a. *In vitro* data show that Grx2 does not react directly with RR1a; Grx3 has 5% of the catalytic activity of Grx1 (7), whereas Trx2 has slightly lower catalytic efficiency for the reduction of RR1a than Trx1 (3). The increased levels of Grx1 at the exponential phase of growth are in accordance with the protein having a pivotal role in ribonucleotide reduction. Cells need more DNA precursors, *i.e.* higher RR1a activity at their log phase of growth than later when they enter stationary phase. The complete lack of the thioredoxin system (*trxAtrxBtrxC* minus strain) did not seem to affect the activity of RR1a, presumably because of the 30-fold elevation of Grx1 (Table IV). However, the lack of both Grx1 and Trx1 did not inhibit the activity of RR1a as much as the lack of Grx1 alone. This suggests that there must be another system to reduce ribonucleotides that is not based on Trx1 or Grx1. Trx2 could be such a potential redoxin. Trx2 levels were elevated 2–3-fold in the *trxAgrxAgrxBgrxC* null mutant. Another system that could partially compensate for RR1a is the RR1b system from the *nrdHIEF* operon (14). This particular operon is highly transcribed in minimal media (14), where the thymidine incorporation measurements were performed.

*Glutathione Reductase Activity*—Expression of GR is regulated at the exponential phase by OxyR (16) and at stationary phase by ppGpp (31). Both factors are involved in the upregulation of antioxidant proteins. Our findings show a simultaneous elevation of GR activity and the glutaredoxins (*katEkatG* and *katEkatGtrxB* null mutants) that would fit well with the need to reduce their increased glutathione disulfide occurring as a byproduct of increased glutaredoxin activity in the particular strains.

*Flow of Electrons between the Thioredoxin and Glutaredoxin Systems*—We initiated these experiments wondering whether glutaredoxins would reduce thioredoxins and *vice versa*. This could be of importance in view of the relatively large amounts of Grx2, which is known not to reduce RR1a. Thioredoxin reductase alone or in the presence of thioredoxins was a very poor reductant of Grx1, whereas GSH and glutaredoxins gave very low rates for the reduction of oxidized Trx1 (data not shown). These data suggest that the two systems are "isolated," *i.e.* the channeling of electrons is meant to be exclusive to each system. This puts further emphasis on the discovery of the electron acceptors of Grx2 and Grx3.

*Acknowledgments*—We are grateful to Jon Beckwith, Daniel Ritz, and Eric Stewart for providing the antisera for Trx2 and the Aegis strains. We thank Antonio Miranda Vizuette for the expression vector for Trx2. Fredrik Åslund is gratefully acknowledged for useful discussions and helpful comments.

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# Protein Levels of *Escherichia coli* Thioredoxins and Glutaredoxins and Their Relation to Null Mutants, Growth Phase, and Function

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*J. Biol. Chem. 2002, 277:18561-18567.*
*doi: 10.1074/jbc.M201225200 originally published online March 13, 2002*

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