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

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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 antioxidiant 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). 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. CXXC). 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 sulf oxide reductase. PAPS reductase is the key enzyme in the reduction of sulfate to sulfite, whereas methionine sulf oxide reductase reduces methionine sulf oxide 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, 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-hydroxyethyl 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 trxA, 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 ndrHI'E' 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 Grx1 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...
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Materials and Methods

Chemicals—Streptavidin-alkaline phosphatase-PQ was from Mabtech AB, paranitrophenyl phosphate was from Sigma, and Immuno-nurep 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, -2, or -3 have been described previously (3, 7, 26). The bacterial strains used in this work are listed in Table I.

Strains used in this work

| Strains used in this work | Relevant genotype | Origin (ref.) |
|--------------------------|------------------|---------------|
| Strains derived from DHB4 | See reference    | (36)          |
| AD494                    | DHB4 trxB::Kan   | (36)          |
| WP570                    | DHB4 ΔtrxA       | (36)          |
| WP571                    | DHB4 ΔtrxA trxB::Kan | (36) |
| WP812                    | DHB4 grxA::Kan   | (36)          |
| AV2                      | DHB4 grxB::kan   | (8)           |
| WP551                    | DHB4 grx::Cm     | (36)          |
| WP840                    | DHB4 gor:Te      | (36)          |
| 523grxB                  | DHB4 gor grxA::Kan grxB::Kan... Te grxC::Cm | (8) |
| WP612                    | DHB4 gshA::Kan trxA | (36) |
| WP843                    | DHB4 gor:Te trxA | (36)          |
| WP757                    | DHB4 gshA::Kan   | (36)          |
| AVG9                     | DHB4 trxA grxA::Kan grxB::Kan... Te grxC::Cm | (8) |
| AVG15                    | DHB4 grxA::Kan grxB::Kan... Te grxC::Cm | (8) |
| AVG23                    | DHB4 grxA::Kan grxB::Kan... Te grxC::Cm | (8) |
| Strains derived from Aegis324 | DHB4 ΔtrxA ΔtrxC nadB::Tn10 | (37) |
| Strains derived from CSH7 | See reference    | (38)          |
| CSH7gtrB                 | CSH7 gtrB::Kan   | (8)           |
| CSH7gor                  | CSH7 gor::Te     | (8)           |
| CSH7trxB                 | CSH7 trxB::Kan   | (8)           |
| CSH7gshA                 | CSH7 gshA::Kan   | (8)           |
| UM1                      | CSH7 katE katG   | (39)          |
| UMG1gxB                  | UMG1 gxB::Kan    | (8)           |
| UMG1gor                  | UMG1 gor::Te     | (8)           |
| UMItrxB                  | UMI trxB::Kan    | (8)           |
| UMIgshA                  | UMI gshA::Kan    | (8)           |
| Strains derived from JM110 | See reference    | (40)          |
| AW10                     | JM110 ars::Cm    | (40)          |

Strains derived from Aegis324 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.

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Table II

| ELISA test | Intra-assay CV | Inter-assay CV | Recovery | Detection limit |
|------------|----------------|----------------|----------|----------------|
| 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            |

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). 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). 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. Detection limit was determined as three times the standard deviation above the blank.

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. Micropattern plates (Nunc®) were coated 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 7.5, and the purified antibodies were dialyzed against PBS before being aliquoted and stored at −20 °C.

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. Micropattern plates (Nunc®) were coated 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 7.5, and the purified antibodies were dialyzed against PBS before being aliquoted and stored at −20 °C.

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initially with affinity-purified polyclonal antibodies (0.5 μg/ml) in carbonate buffer, pH 9.6, and incubated overnight at 4 °C. The plates were blocked with 200 μl/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 alkaline phosphatase-conjugated streptavidin, diluted 1:2000 in incubation buffer, was added (1 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 MgCl₂). 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 allowed to react with the coated antibodies overnight at 4 °C. Levels of TRX and GR levels were determined using ELISA. Values represent means of duplicates.

**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 μg/mg, respectively), Grx1 levels decreased slightly (from 0.6 to 0.2 μg/mg), whereas those of Trx1 increased slightly (from 2.0 to 3.0 μg/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 μg/mg) were ~3-fold higher than during exponential phase (2.5 μg/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 (grxAgrxB-grxC) 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 (trxAtxA-trxB) 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 trxAshA. 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 μg/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 (trxAgor-trxB and trxAgor-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 μg/mg in

| Time (h) | A₁₀₀ (ng/mg) Trx1 (ng/mg) | A₁₀₀ (ng/mg) Trx2 (ng/mg) | A₁₀₀ (ng/mg) Grx1 (ng/mg) | A₁₀₀ (ng/mg) Grx2 (ng/mg) | A₁₀₀ (ng/mg) Grx3 (ng/mg) |
|---------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 2.5     | 0.115                    | 1943                     | 222                      | 657                      | 887                      | 4024                    |
| 3       | 0.276                    | 2693                     | 235                      | 267                      | 1841                     | 4342                    |
| 4       | 0.498                    | 1870                     | 165                      | 382                      | 2493                     | 4225                    |
| 5       | 0.750                    | 1584                     | 117                      | 174                      | 4594                     | 3668                    |
| 7       | 1.112                    | 2731                     | 135                      | 282                      | 6542                     | 4327                    |
| 8.5     | 1.260                    | 3134                     | 160                      | 259                      | 11370                    | 3871                    |
| 10.5    | 1.440                    | 2341                     | 193                      | 139                      | 9992                     | 4954                    |
| 24      | 2.640                    | 3274                     | 119                      | 182                      | 8501                     | 4177                    |
| 48      | 2.580                    | 3021                     | 185                      | 129                      | 8094                     | 4364                    |
| 53      | 2.400                    | 2439                     | 119                      | 129                      | 8094                     | 4364                    |
| 8.5     | 1.260                    | 3134                     | 160                      | 259                      | 11370                    | 3871                    |
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| 53      | 2.400                    | 2439                     | 119                      | 129                      | 8094                     | 4364                    |

**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).

**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.
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 the 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 $\mu$M, 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 $\beta$-hydroxyethyl disulfide assay (24) (data not shown).

**Table IV**

| Relevant genetic characteristics | Exponential phase | Stationary phase | Exponential phase | Stationary phase | Exponential phase | Stationary phase | Exponential phase | Stationary phase |
|---------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| 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               | NP               | 793              | 1017            | 9371             | 9489             | 3329             | 5806             |
| trxAtrxC                        | NP               | NP               | NP               | NP               | 13387            | 1063            | 3341             | 5469             | 5373             | 6250             |
| trxAtrBtrxC                     | NP               | 0                | NP               | NP               | 17747            | 5286            | 3368             | 10376            | 3798             | 9003             |
| grxA                            | 4648             | 3034             | 194              | 203              | 0                | 0               | 0                | 0                | 6059             | 5006             | 3380             | 4028             |
| grxB                            | 2658             | 6491             | 222              | 109              | 807              | 408             | 1                | 0                | 0                | 4456             | 6564             |
| grxC                            | 2866             | 5471             | 229              | 127              | 680              | 411             | 4129             | 6470             | 1                | 0                |
| grxAgrxC                        | 4259             | 3737             | 255              | 135              | NP               | NP              | 3684             | 5710             | 1                | 0                |
| grxAgrxBgrxC                    | 2466             | 5909             | 185              | 170              | NP               | NP              | NP               | NP               | NP               | 0                |
| gor                              | 4270             | 4229             | 168              | 512              | 1344             | 419             | 4321             | 4109             | 4938             | 6924             |
| gorgrxAgrxBgrxC                 | 1947             | 4564             | 376              | 816              | NP               | NP              | NP               | NP               | NP               | NP               |
| gorgrxAgrxB                     | 4312             | 6297             | 234              | 192              | 1001             | 554             | 3388             | 2694             | 3618             | 5988             |
| trxAgrxA                        | NP               | NP               | 585              | 1205             | NP               | NP              | 3154             | 4614             | 3508             | 5589             |
| trxAgrxAgrxBgrxC                | NP               | NP               | 427              | 970              | NP               | NP              | NP               | NP               | 0                | 0                |
| trxAgor                         | NP               | NP               | 585              | 664              | 1340             | 3915            | 2552             | 6164             | 5264             | 6017             |
| trxAgorhA                       | NP               | NP               | 527              | 544              | 9697             | 3898            | 2681             | 5451             | 3800             | 7433             |
| trxAgorgrxB                     | NP               | NP               | 404              | 103              | 1209             | 1392            | NP               | NP               | 4108             | 7372             |

**Fig. 1.** Redoxin levels in katE (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.

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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
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 gortxA (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).

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 supplier 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 $V_{\text{max}}$ 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 ($trxAtrxBtrxC$ 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 $trxAgrxAgrxB$ null mutant. Another system that could partially compensate for RR1a is the RR1b system. This puts further emphasis on the discovery of 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 up-regulation of antioxidant proteins. Our findings show a simultaneous elevation of GR activity and the glutaredoxins (katEkatAT and katEkatGtrxB null mutants) that would fit well with the need to reduce their increased glutathione disulfide ring as a byproduct of increased glutathione 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.

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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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