The Levels of Ribonucleotide Reductase, Thioredoxin, Glutaredoxin 1, and GSH Are Balanced in Escherichia coli K12*

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The dithiol forms of thioredoxin and glutaredoxin are hydrogen donors for ribonucleotide reductase. We have determined the intracellular levels of ribonucleotide reductase (RR), thioredoxin (Trx), glutaredoxin 1 (Grx1), and glutathione (GSH) and the glutathione redox status in new Escherichia coli K12 strains lacking thioredoxin (trxA), glutaredoxin 1 (grxA), and/or GSH (gshA) or overproducing Trx or Grx1 from multicopy plasmids. We propose a regulatory network in which RR activity is controlled by the levels of Trx, Grx1, and GSH; therefore, the absence of thioredoxin is lethal, as is the absence of glutaredoxin, even under exposure to lipoic acid. Thus, GSH may be the only substitute for lipoate in the reduction of ribonucleotides to deoxyribonucleotides.

The reduction of ribonucleotides to deoxyribonucleotides is catalyzed by the enzyme ribonucleotide reductase (RRase) (Reichard, 1988). Two dithiol-dependent hydrogen donor systems for this enzyme have been discovered separately (Holmgren, 1989). One is the NADPH-dependent thioredoxin system (Holmgren, 1985a), and the other is a glutathione-dependent system where the protein glutaredoxin couples the oxidation of GSH to the reduction of ribonucleotides (Holmgren, 1985b). Thioredoxin and glutaredoxin are also important for the reduction of sulfate to sulfite via 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, which is the first step in the cysteine biosynthesis and is essential for sulfur metabolism (Tsang, 1981; Kredich, 1987).

A double mutant lacking thioredoxin and glutaredoxin was constructed in Escherichia coli K38 in order to establish the physiological relevance of these two proteins (Russel and Holmgren, 1988). The best growing mutant selected (named A410 strain) was not viable on minimal medium unless a source of reduced sulfur was added. It was concluded that thioredoxin and glutaredoxin were essential for the reduction of sulfate (Russel et al., 1990) but not for deoxyribonucleotide biosynthesis, implying the existence in E. coli of a third unknown hydrogen donor system for ribonucleotide reductase.

To investigate whether a mutator phenotype was associated with the simultaneous deficiency in thioredoxin and glutaredoxin, new double defective bacteria carrying trxA and grx null mutations were isolated in the genetic background of E. coli K12 (Miranda-Vizuete et al., 1994). In contrast to previous data (Russel and Holmgren, 1988; Russel et al., 1990), the new defective bacteria made colonies of normal size on rich plates and grew as rapidly as wild-type cells. Thus, it (i) grows on minimal medium plates; (ii) shows normal mutagenic sensitivity toward a wide variety of DNA-damaging agents; (iii) displays 14% of GSH-dependent and 30% of NADPH-dependent ribonucleotide reduction capacity with CDP as substrate in the presence or the absence of exogenous RRase, respectively; and (iv) has very high levels (20-fold increase) of ribonucleotide reductase activity. These properties indicate (i) that thioredoxin and glutaredoxin might not be essential for sulfate reduction, in contrast to previous data; (ii) that a balanced dNTP synthesis is maintained in the absence of thioredoxin and glutaredoxin, even under exposure to mutagens; and (iii) the existence of a third GSH-dependent hydrogen donor system for ribonucleotide reductase in E. coli.

Initial attempts to purify the presumed hydrogen donor for RRase from the original mutant strain A410 were not success-
ful. The newly constructed E. coli K12 UC647 was then used on the assumption that any hydrogen donor might be induced to higher levels in this strain. From UC647 two independent GSH-disulfide oxidoreductases (using hydroxyethyl disulfide as a substrate) were purified to homogeneity and named glutaredoxin 2 (Grx2) and glutaredoxin 3 (Grx3), respectively (Åslund et al., 1994). Grx3 showed a low but significant activity as hydrogen donor for E. coli ribonucleotide reductase, whereas Grx2 was inactive. Therefore, glutaredoxin 3 was considered to be the substitute for thioredoxin (Trx) and the first discovered glutaredoxin (Grxl) in the double mutant strain UC647 (Åslund et al., 1994).

This study investigates the association among the intracellular levels of RRase, their two main hydrogen donors, thioredoxin and glutaredoxin 1, and glutathione and its redox status. To this end new multicopy plasmids have been constructed in the genetic background of UC647. The nutrient medium was LB medium (Davis et al., 1977; Wallace and Kushner, 1984; Hög et al., 1986). Strains overproducing Trx (UC872 and UC919) or Grx1 (UC873) were isolated by transformation of UC5710 or UC827 with pBHk8 and pBR322ECG, respectively. Control strains UC871 and UC928 carrying the pBR322 vector were also obtained. Plasmids were isolated using the Magic2 miniprep DNA purification system. Competent bacteria and transformation were as described by Hanahan (1983). E. coli K38 and derivatives (A307, A407, and A410) were used for comparison with UC E. coli K12 strains.

### MATERIALS AND METHODS

#### Chemicals and Proteins—[3H]CDP was from Amersham Corp. GSGS, GSH, lipic acid (4-6,8-thiodic acid), and yeast glutathione reductase were from Sigma. Perchloric acid, orthophosphoric acid, and salts for the mobile phase used in HPLC determinations were from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade. Thioredoxin, glutaredoxin, and thioredoxin reductase from E. coli were prepared as described previously (Dyson et al., 1989; Björnberg and Holmgren, 1991; Luthman and Holmgren, 1982). E. coli ribonucleotide reductase was a gift from Prof. Britt-Marie Sjöberg, Stockholm University.

#### Bacterial Strains and Plasmids—The bacterial strains and plasmids used in this work are listed in Table I. All UC strains are E. coli K12, UC5710 being the parental wild type (wt). Strains UC844 (trxA::), UC848 (grx2::), UC946 (grx3::trxA::, unable to grow in minimal medium), and UC827 (spontaneous derivative of UC946 growing in minimal medium) are equivalent to UC518, UC525, UC646, and UC647, respectively (Miranda-Vizuete et al., 1994) but without the mutagenesis-enhancing plasmid pKM101. The grx2:: bacteria were constructed by P1 (ghsa::Tn10kan) transduction and selection for kanamycin resistance and subsequently screened for diamide sensitivity. Strains carrying the ghsA::Tn10kan allele showed undetectable levels of glutathione, as determined by HPLC. The triple ghsA::trxA::lipA:: defective strain (UC954) was constructed by cotransducing to UC859 the lipA2 mutation and the linked Tn10 insertion (zbe279::Tn10). Tetraacycline-resistant transductants were first selected on rich LB medium and then scored for growth on minimal plates supplemented with either lipoic acid or acetate plus succinate. The vector plasmid (pBR322) and those carrying the gene encoding the Trx (pBHk8) or the Grx1 (pBR322ECG) proteins have been previously described (Bölvívar et al., 1977; Wallach and Kushnir, 1984; Hög et al., 1986). Strains producing Trx (UC872 and UC919) or Grx1 (UC873 and UC929) were isolated by transformation of UC5710 or UC827 with pBHk8 and pBR322ECG, respectively. Control strains UC871 and UC928 carrying the pBR322 vector were also obtained. Plasmids were isolated using the Magic2 miniprep DNA purification system. Competent bacteria and transformation were as described by Hanahan (1983). E. coli K38 and derivatives (A307, A407, and A410) were used for comparison with UC E. coli K12 strains.

#### Purification of Antibodies and Enzyme Immunoassay—The formation of [3H]CDP from [3H]CDP in a reaction mixture containing 30 mM Hepes, pH 7.6, 10 mM MgCl2, 1.3 mM ATP, 0.5 mg/ml bovine serum albumin, 0.5 mM [3H]CDP (31,000 cpm/nmol), 2 mM NADPH, 1.2 μM E. coli Trx, and 0.2 μM E. coli thioredoxin reductase in 0.12 M final volume. Three different concentrations of bacterial extract were used in the assay, and average activity was calculated by linear regression. All experiments were conducted at least three times. Glutathione reductase and thioredoxin reductase activities were measured as described previously (Mata et al., 1984; Holmgren, 1977). No differences in the activities of these two enzymes were observed among the bacterial strains described in this paper.

#### Purification of Antibodies and Enzyme Immunoassay—Purification of anti-Trx and anti-Grx1 antibodies by immunoaffinity chromatography and quantification of Trx and Grx1 protein levels by indirect competitive enzyme immunoassay were carried out as described previously (Miranda-Vizuete et al., 1994). Antibody titers were 0.183 and 0.531 nmol/ml for anti-Trx and anti-Grx1, respectively.

#### Glutathione Determination—Bacteria from LB overnight cultures were harvested, washed twice in VB salts, reincubated in VB medium with casamino acids, and grown to stationary phase in a rotary incubator. Cells were centrifuged at 15,000 g for 15 minutes, and the pellets aliquoted (200 mg/ml) and frozen at −80 °C. Bacteria were thawed, resuspended
Enzyme-linked immunosorbent assay determination of Trx and Grx1 levels in deficient or overproducing strains of E. coli

Trx and Grx1 levels were determined in cell-free extracts of bacteria grown in LB nutrient medium, as described under "Materials and Methods." All bacterial strains are derived from UC5710, which is considered the parental wild type. UC827 (able to grow in minimal medium) was the gshA trxA- mutant strain. The overproduced proteins (Trx or Grx1) are indicated in parentheses. Data show the mean values ± S.D. of at least three independent determinations. The relative values compared with those of wild type are shown in italics.

| Relevant genetic characteristics | Trx level | Grx1 level |
|---------------------------------|-----------|------------|
|                                  | ng/mg protein | ng/mg protein |
| wt                               | 1911 ± 142 | 1.00 |
| trxA-                           | <0.54 | 0.0002 |
| grxA-                           | 2325 ± 140 | 1.22 |
| gshA                            | 3624 ± 280 | 1.90 |
| gshA trxA-                      | <0.54 | 0.0002 |
| gshA grxA-                      | <0.54 | 0.0002
| wt/pBR322                       | 4216 ± 500 | 1.00 |
| grxA-                           | 2885 ± 150 | 1.51 |
| wt/pBH K8 (Trx)                 | 43887 ± 6171 | 10.4 |
| grxA-                           | 2105 ± 517 | 0.50 |

(5 ml/g) in 1 M HClO4 containing 2 mM EDTA, and disrupted at room temperature in an ultrasonic bath for 15 min (Alonso-Moraga et al., 1987). The extracts were centrifuged at 31,000 × g for 20 min at 4°C, and the pellets were extracted again with HClO4. The protein-free supernatants were filtered through 0.2-

Protein was determined as described by Bradford (1976) (activity assays) or by bicinchoninic acid protein assay (Pierce) (immunological quantifications).

RESULTS

The Levels of Trx, Grx1, and GSH Are Compensated—The levels of Trx and Grx1 were determined by specific competitive enzyme-linked immunossay methods in E. coli strains defective in thioredoxin (trxA-), glutaredoxin 1 (grxA-), and/or GSH biosynthesis (gshA-) (Table I, upper part). A small but consistent increase in Trx was observed in the absence of Grx1 and vice versa. These results suggested the existence of a regulatory network in which the levels of Trx and Grx1 were balanced. This was further addressed by measuring the concentrations of these two proteins in bacteria carrying multiplicity plasmids for overproduction of Trx or Grx1 (Table II, lower part). Overproduction of one component decreased the level of the other.

Glutaredoxin activity is coupled to cellular glutathione. Thus, the putative effect of GSH deficiency on Grx1 level was investigated in bacteria carrying a null gshA::Tn10 allele blocking the first step in the GSH biosynthetic pathway (Greenberg and Demple, 1986). An important increase of 5.53-fold in Grx1 was observed in gshA- single mutant, the increase raising to 54.9-fold when the GSH deficiency was combined with the trxA- mutation (Table II). Comparativcly, the effect of GSH deficiency on Trx levels was much less pronounced, as would be expected. To see whether lipoic acid is involved in an alternative pathway in reducing glutaredoxins, we constructed a triple gshA trxA lipA- mutant strain by P1 transduction of the lipA2 (lipoate synthase) mutation to UC859 (gshA::Tn10kan, ΔtrxA). The resulting bacterial strain (UC954) grew on minimal plates supplemented with lipoic acid, but no growth could be observed when replacing the lipoic acid requirement by acetate and succinate.

Ribonucleotide Reductase Activity Is Balanced by the Levels of Its Hydrogen Donors—Table III shows the ribonucleotide reductase activity of bacteria containing variable amounts of Trx and Grx1, its two main electron donors. The absence of Trx or Grx1 increased RRase activity of wt cells by a factor of 3.32- and 1.60-fold, respectively, whereas bacteria simultaneously lacking Trx and Grx1 exhibited very high levels of RRase activity (× 23.08), as previously reported (Miranda-Vizuete et al., 1994). The link between these three proteins was further investigated by measuring RRase activity in both wt and grxA trxA- bacteria overproducing Trx or Grx1. Oppositely to the increased RRase activity observed in Trx- or Grx1-defective bacteria, overproduction of these two electron donors decreased RRase activity of wt bacteria to 41 and 33%, respectively. Much more extensive effects were observed in grxA trxA- bacteria, in which Trx or Grx1 overproduction diminished RRase activity to 8 or 12% of the control (grxA trxA- /pBR322), thus showing approximately wt levels. These drastic decreases are explained by the high RRase activity of grxA trxA- /pBR322 bacteria as compared with the corresponding control strain wt/pBR322 (3.83 versus 0.27 nmol dCDP/20 min/mg protein). Deficiency in GSH, electron donor of all glutaredoxins, increased RRase activity to a higher extent than the absence of Grx1, 4.68-versus 1.60-fold. Nevertheless, GSH deficiency did not show

\[ \text{TABLE II} \]

| Hydrogen Donor Systems of E. coli Ribonucleotide Reductase |
|------------------------------------------------------------|
| RRase was measured by the reduction of \(^{3}H\)CDP to \(^{3}H\)dCDP, as described under "Materials and Methods." Measurements were carried out in cell-free extracts of bacteria grown in LB nutrient medium. All bacterial strains are derived from UC5710, which is considered the parental wild type. UC827 (able to grow in minimal medium) was the grxA trxA- mutant strain. The overproduced proteins (Trx or Grx1) are indicated in parentheses. Data show the mean values ± S.D. of at least three independent determinations. The relative values compared with those of wt, wt/pBR322, or grxA trxA- /pBR322 are shown in italics. |
|------------------------------------------------------------|
| Strain | RRase activity |
|--------|----------------|
| wt     | 0.25 ± 0.10 |
| wt/pBR322 | 0.40 ± 0.11 |
| grxA trxA- | 0.17 ± 0.43 |
| gshA trxA- | 0.97 ± 0.48 |
| gshA grxA- | 1.22 ± 0.17 |

| TABLE III |

| Ribonucleotide reductase activity in mutant or overproducing strains of E. coli |
|-------------------------------------------------------------------------------|
| RRase was measured by the reduction of \(^{3}H\)CDP to \(^{3}H\)dCDP, as described under "Materials and Methods." Measurements were carried out in cell-free extracts of bacteria grown in LB nutrient medium. All bacterial strains are derived from UC5710, which is considered the parental wild type. UC827 (able to grow in minimal medium) was the grxA trxA- mutant strain. The overproduced proteins (Trx or Grx1) are indicated in parentheses. Data show the mean values ± S.D. of at least three independent determinations. The relative values compared with those of wt, wt/pBR322, or grxA trxA- /pBR322 are shown in italics. |
|-------------------------------------------------------------------------------|
synergistic effect with Trx deficiency on RRase activity (4.68 ± 3.32 vs 3.88-fold increase). It is worth noticing that gshA\textsuperscript{-} trxA\textsuperscript{-} bacteria exhibited extremely high levels of Grx1 (see Table II).

Role of Trx and Grx1 in Glutathione Redox Status—The glutathione content and redox status of trxA\textsuperscript{-} and/or grxA\textsuperscript{-} mutant bacteria are presented in Table IV. Glutathione redox status was expressed as the [protein-glutathione mixed disulfide]/[GSH] ratio, because UC bacterial strains showed detectable levels of GSSG (<0.1 nmol/g dry weight). The grxA\textsuperscript{-} trxA\textsuperscript{-} bacteria unable to grow in minimal medium plates showed very low concentrations of total intracellular GSH (13% of wt level). This decreased GSH concentration was accompanied by a shift toward a more oxidized redox status, which increased 2.47-fold as compared with wild type. Equivalent or even higher effects were observed in the E. coli K38 genetic background included for comparison with UC K12 strains. The UC grxA\textsuperscript{-} trxA\textsuperscript{-} derivative strain able to grow in minimal medium plates had recovered nearly wild type levels of both total intracellular GSH concentrations and glutathione redox status.

**DISCUSSION**

Here we show that trxA\textsuperscript{-} or grxA\textsuperscript{-} mutants have increased levels of Grx1 or Trx, respectively, in agreement with previous results in bacteria carrying the pKM101 plasmid (Miranda-Vizuete et al., 1994). The inverse relation between the intracellular levels of Trx and Grx1 was further supported by using bacteria overproducing one of these two proteins. Trx concentration seemed to have a higher effect on the Grx1 level than Grx1 on Trx, as expected from the existence of two other glutaredoxins, Grx2 and Grx3 (Åslund et al., 1994). The present paper demonstrates also that bacteria deficient in GSH biosynthesis has an elevated amount (×5.53) of Grx1; this effect was higher than that of Trx deficiency (×2.22), in agreement with the fact that GSH is the physiological hydrogen donor of all known glutaredoxins (Holmgren, 1989; Åslund et al., 1994).

In spite of the pivotal physiological role of GSH, bacteria simultaneously defective in both GSH and thioredoxin were viable, as previously reported (Fuchs et al., 1983). This suggests that glutaredoxins might be reduced independent of GSH. The putative involvement of lipoic acid in glutaredoxin reduction in vivo was investigated because dihydrolipoamide is a good reductant of E. coli Trx in vitro (Holmgren, 1979b). The inability of the gshA\textsuperscript{-} trxA\textsuperscript{-} lipoA\textsuperscript{-} triple mutant strain (UC954) to grow on minimal plates supplemented with acetate plus succinate suggests that lipoic acid could be the only substitute of GSH for glutaredoxin reduction in the gshA\textsuperscript{-} trxA\textsuperscript{-} bacteria (UC859). The low efficiency of lipoate as hydrogen donor of glutaredoxins would explain the extremely high increase in Grx1 content (×54.9) observed in UC859.

We have previously reported that deficiencies in Trx, Grx1, and particularly in both hydrogen donors result in enhanced ribonucleotide reductase activity (Miranda-Vizuete et al., 1994). Here we confirm the inverse relation existing between Trx or Grx1 and RRase activity in overproducing bacterial strains. The RRase activity diminished in cells with high intracellular levels of Trx or Grx1, this effect being clearly noticeable in grxA\textsuperscript{-} trxA\textsuperscript{-} bacteria where RRase is induced ×20-fold. This work demonstrates further that deficiency in GSH biosynthesis (gshA\textsuperscript{-} bacteria) also increases the activity of ribonucleotide reductase, actually to a larger extent than Trx deficiency. No synergism between RRase activity and the simultaneous deficiency in Trx and GSH was detected in contrast to that reported for Trx and Grx1. This might be explained by the fact that gshA trxA\textsuperscript{-} defective bacteria contained much higher (55-fold) levels of Grx1 than wild type cells. This large increase in Grx1 and conceivably also in other glutaredoxins might exclude the need for inducing RRase.

E. coli trxA\textsuperscript{-} or grxA\textsuperscript{-} single mutants exhibited nearly wild type glutathione levels and redox status, including no increases in GSH-protein mixed disulfides. Thus, E. coli must have an additional system to reduce low molecular weight disulfides, as previously suggested from studies with glutathione reductase and thioredoxin or thioredoxin reductase defective bacterial strains (Tuggle and Fuchs, 1985). This proposal is supported further by the nearly normal glutathione redox state of grxA\textsuperscript{-} trxA\textsuperscript{-} double mutant selected to grow in minimal medium plates.

The thioredoxin and glutaredoxin 1 defective E. coli K12 strain (UC946) unable to grow in minimal medium plates has very low glutathione content with a markedly oxidized redox status. The glutathione concentration of UC946 could even be overestimated because it was measured in cells grown in minimal medium supplemented with 0.2% casamino acids, thus containing cystine at 3.2 mg/liter. The impairment of sulfate assimilation pathway and the oxidized redox status of glutathione is considered the most reliable explanation for the inability of UC946 to grow in minimal medium, based on the previous proposal that accumulation of PAPS is toxic to the cells (Russell et al., 1990) and that relatively small oxidation of glutathione essentially inactivates the reducing capacity of glutaredoxin (Holmgren, 1979b). The ability to grow in minimal medium plates of E. coli grxA\textsuperscript{-} trxA\textsuperscript{-} defective bacteria (UC827) was accompanied by the recovery of nearly wild type levels of both glutathione content and redox status. The functioning of the sulfate reduction pathway in UC827 would require the induction of PAPS reductase and/or of new electron
donors for this enzyme, distinct from Trx and Grx1. Glutaredoxin 2 is probably not the electron donor for PAPS reductase, leaving as alternative the induction of Grx3 or expression of a new redoxin from E. coli (Jordan et al., 1996). This would additionally explain the recovery of nearly wild type glutathione redox status in strain UC827, because no differences in glutathione reductase activity were found among the different bacterial strains (data not shown).

Now that the E. coli gene (grxC) for Grx3 has been cloned and the Grx2 and Grx3 proteins have been purified to homogeneity (Åslund et al., 1994), it should be possible to quantify in different bacterial constructions the levels of Grx2 and Grx3 by specific competitive enzyme-linked immunoassays and the expression of grxC gene by Northern analysis. These studies will give clues about the roles of glutaredoxins in the physiology of E. coli, thus facilitating the characterization of their function in a physiological system.

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