Interactions of glutaredoxins, ribonucleotide reductase, and components of the DNA replication system of *Escherichia coli*

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A strain of *Escherichia coli* missing three members of the thioredoxin superfamily, thioredoxins 1 and 2 and glutaredoxin 1, is unable to grow, a phenotype presumed to be due to the inability of cells to reduce the essential enzyme ribonucleotide reductase. Two classes of mutations can restore growth to such a strain. First, we have isolated a collection of mutations in the gene for the protein glutaredoxin 3 that suppress the growth defect. Remarkably, all eight independent mutations alter the same amino acid, methionine-43, changing it to valine, isoleucine, or leucine. From the position of the amino acid changes and their effects, we propose that these alterations change the protein so that its properties are closer to those of glutaredoxin 1. The second means of suppressing the growth defects of the multiply mutant strain was by mutations in the DNA replication genes, *dnaA* and *dnaN*. These mutations substantially increase the expression of ribonucleotide reductase, most likely by altering the interaction of the regulatory protein DnaA with the ribonucleotide reductase promoter. Our results suggest that this increase in the concentration of ribonucleotide reductase in the cell allows more effective interaction with glutaredoxin 3, thus restoring an effective pool of deoxyribonucleotides. Our studies present direct evidence that ribonucleotide reductase is the only *essential* enzyme that requires the three reductive proteins missing in our strains. Our results also suggest an unexpected regulatory interaction between the DnaA and DnaN proteins.

The thioredoxin superfamily consists of proteins that carry out oxidation and reduction reactions using the redox chemistry of cysteine residues. Members of the family are defined by a common "thioredoxin fold" and an active site that most often contains a Cys-Xaa-Xaa-Cys sequence involved in redox reactions. In *Escherichia coli*, the family includes the thioredoxins themselves, the glutaredoxins, a number of proteins involved in disulfide bond formation and isomerization, and proteins involved in other reductive processes such as peroxide inactivation and cytochrome biogenesis (ref. 1; for review see refs. 2 and 3).

The prototypical member of this family, thioredoxin 1 of *E. coli*, has a molecular weight of only 12,000. Comparably sized proteins of the family include thioredoxin 2, glutaredoxins 1 and 3, and the thioredoxin-like domains of proteins such as glutaredoxin 2, DsbD, CcmG, DsbC, and DsbG. Given the shared features of these proteins, it is not surprising that some of them can efficiently act on the same substrates (4). For example, the enzymes glutaredoxin 1, thioredoxin 1, and thioredoxin 2 are each capable of reducing the essential enzyme ribonucleotide reductase to regenerate its activity *in vivo* and *in vitro* (5, 6).

Thus, any one of these proteins can suffice for growth of *E. coli* in the absence of the two others. Nevertheless, there are often instances when the proteins differ significantly in their substrate specificity. Glutaredoxin 3 is a case in point, exhibiting only a low capacity to reduce oxidized ribonucleotide reductase *in vitro*. Despite being expressed at approximately 10 times the protein levels of glutaredoxin 1, it is not able to support the growth of the bacteria on its own (5, 6). Similarly, thioredoxin 1 can maintain methionine-sulfoxide reductase in the reduced state, whereas thioredoxin 2, when expressed at levels similar to those of thioredoxin 1, does so only very inefficiently (7). What determines the variations in specificity between these very similar molecules? Is it the redox potential of the proteins, the specificity of protein–protein interactions, or some other factors?

While the glutaredoxins and thioredoxins exhibit similar three-dimensional structures and active sites, the sources of electrons required for maintaining their reductive activities differ. The pathway for the reduction of oxidized glutaredoxins initially utilizes NADPH, which maintains the enzyme glutathione oxidoreductase in the reduced state. This enzyme, in turn, transfers electrons to oxidized glutathione, generating reduced glutathione, which then transfers electrons to oxidized glutaredoxins. In the case of the thioredoxins, the initial source of electrons is also NADPH, but in this case the electrons are transferred to the enzyme thioredoxin reductase, which then directly reduces oxidized thioredoxins (for review see ref. 2).

To begin to answer questions about the variations in specificity of the thioredoxin superfamily members, we have initiated a study of the specificity differences between glutaredoxins 1 and 3, two proteins that share 33% (8) amino acid identity (Fig. 1). Our results show that changes in a single critical amino acid of glutaredoxin 3 can confer the ability of this protein to effectively substitute for glutaredoxin 1 *in vivo* in the reduction of ribonucleotide reductase. In addition, we have sought other mechanisms by which the specificity differences can be overcome. In the process, we have discovered an unexpected feature of the regulation of ribonucleotide reductase expression.

Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown routinely in NZ medium at 37°C (9). Growth on minimal medium, M63, was achieved as described (7). When necessary, cysteine was added at a final concentration of 50 μg/ml. Antibiotic selection was maintained for all markers either on plasmids or on the chromosome, at the following concentrations: ampicillin, 200 μg/ml (plasmid) or 25 μg/ml (chromosome); chloramphenicol, 10 μg/ml; kanamycin, 40 μg/ml; tetracycline, 15 μg/ml.

Medium was supplemented with 0.2% L-arabinose or 0.2% D-glucose to induce or repress, respectively, expression of *trxC* and *trxB* alleles under the control of the P_{BAD} promoter (10). Induction of *nrdAB* from the *trc* promoter was accomplished by addition of 0–1 mM isopropyl β-D-thiogalactopyranoside.

Abbreviation: PAPS, 1,1,3′-phosphoadenylylsulfate.

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Fig. 1. Sequence alignments of glutaredoxin 1 (Grx1) and glutaredoxin 3 (Grx3). Black boxes indicate identical amino acids. The modified Met-43 in the Grx3 mutants is shaded in gray.

Plasmid Construction. The *grxC* coding sequence was amplified by PCR using pFA16 (pBAD33-*grxC*) plasmid as template. We used primer P1, which introduces a *Nde*I restriction site overlapping with the ATG start codon of the *grxC* gene, and primer P2, which anneals to the region 131 bp, downstream of pBAD33 polylinker *Hin*dIII site and introduces an *Afl*III restriction site at the 3′ end of the amplified DNA fragment. The PCR product was digested with *Nde*I and *Afl*III and cloned into the same sites of pJAH01 plasmid (11). The resulting plasmid was designated pRO1. It contains *grxC* gene under control of *lacUV5* promoter and the tetracycline-resistance gene as a selectable marker.

The *nrdAB* coding sequence was amplified by PCR using DHB4 chromosomal DNA as template. We used primers that introduce an *Eco*RI and a *Hin*dIII restriction site, respectively. The product was digested with *Eco*RI and *Hin*dIII and ligated into the same sites of the pDSW204 (12) to create plasmid pSMG6.

The DNA fragment containing the tetracycline-resistance gene and its promoter region was amplified by PCR using pACYC184 (13) as template. We used primers that introduce a *Sca*I and an *Nco*I restriction site, respectively. The product was digested with *Sca*I and *Nco*I and ligated into the same sites of pEJS62 (pBAD33-*trxC*) to create plasmid pSMG7.

Genetic and Molecular Biology Procedures. Standard techniques were used for cloning and analysis of DNA, PCR, electroporation, transformation, and P1 transduction (14, 15).

Strains were constructed by P1 transduction. The mutant alleles of the genes encoding components of the thioredoxin and glutaredoxin systems (see Fig. 3) used to construct the strains for this study are *trxB*::Km (16), *grxB*::Kan (8), *grxC*::Cm (6), and *nrdH*::spc (F. Åslund, this laboratory).

Oligonucleotide site-directed mutagenesis was carried out by using a QuikChange Kit (Stratagene) as recommended by the supplier. The DNA sequences were determined by the Micro Core Facility at the Department of Microbiology and Molecular Genetics, Harvard Medical School.

The PCR mutagenesis of the *grxC* gene was carried out as described (17) with P1 and P2 primers and pRO1 plasmid as a template DNA. Nucleotide ratios of 5:1 (cytosine + thymine):(adenine + guanine) or (adenine + thymine):(cytosine + guanine) at final concentrations of 1 mM and 0.2 mM each, respectively, were used. The resulting PCR products (457 bp) were digested with *Nde*I and *Afl*III and inserted into pRO1 to replace the original *grxC* gene.

Suppressor Mapping and Linkage Analysis. λNK1324, carrying the Tn*10* transposon, was used to obtain random insertions into the suppressor strain. A P1 lysate was prepared from the library of the random transposon insertions and was used to transduce to chloramphenicol-resistance the RO36 strain. The transductants were selected on NZ plates supplemented with glucose (to shut down expression of the *trxC* gene from the complementing plasmid) and chloramphenicol (transposon marker). This selection should yield transductants that received the suppressor mutation along with a closely linked transposon insertion. All recombinants were restreaked on the same plates to confirm the mutant phenotype. The transposon insertion sites in all recombinants were established by arbitrary PCR as described (18). The summary of the insertion sites and the linkage analysis is presented in Fig. 2.

Construction of Strains Containing a *nrdA′–`lacZ Fusion.* To obtain a translational fusion of the N-terminal part of ribonucleotide reductase 1 α subunit, R1 (encoded by *nrdA* gene), with β-galactosidase, a DNA fragment was amplified by PCR using chromosomal DNA from DHB4 as template and primers that hybridize to a position 686 nucleotides upstream of the translation start site of the *nrdA* gene and contain a *Hin*dIII site and a primer that anneals within the *nrdA*-coding region and contains

Table 1. Strains and plasmids used in this work

Strains/plasmids	Relevant genotype	Ref.
Plasmids		
pRO1	pJAH01-*grxC*	This work
pRO2	pJAH01-$grxC_{M43V}$	This work
pSMG6	pBAD33-Tc^r-*trxC*	This work
pSMG7	pDSW204-*nrdAB*	This work
pDR1024	pBAD18-*trxB*	31
Strains		
DHB4*	Δ(*ara–leu*)7697 *araD139* Δ*lacX74 galE galK rpsL phoR* Δ(*phoA*)*Pvu*II Δ*malF3 thi*	32
FÅ173	DHB4 Δ*trxA* Δ*trxC nrdH*::spc/pBAD18-*trxC*	This work
FÅ174	DHB4 Δ*trxA* Δ*trxC grxA*::kan *nrdH*::spc/pBAD39-*trxC*	This work
RO36	FÅ173 *grxA*::kan	This work
RO34	RO36 $dnaA^{sup1}$	This work
RO50	RO36 $dnaN^{sup}$	This work
RO51	RO36 $dnaA^{sup2}$	This work
RO48	DHB4 Δ(λ*attL-lom*)::*bla nrdA′–′lacZ*	This work
SMG234	DHB4 Δ*trxA* Δ*trxC grxA*::kan *nrdH*::spc/pSMG7 pSMG6	This work
SGM235	SGM234 *grxC*::Cm	This work
SMG237	RO48 $dnaA^{sup1}$ . . . MiniTn*10*Cm^r	This work
SMG238	RO48 $dnaN^{sup}$ . . . MiniTn*10*Cm^r	This work
KK450	*nrdA*(Ts) *nrdB1 thyA thr leu thi deo tonA lacY supE44 gyrA*	33

*FA variant of DHB4 that had been cured of the F′ plasmid was used throughout this study.

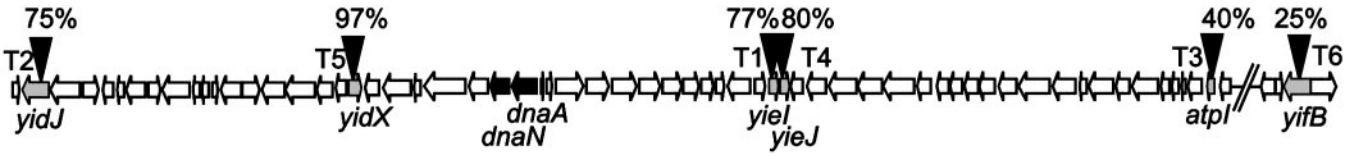

Fig. 2. The *dnaAN* locus is responsible for the restoration of disulfide bond reduction in a *trxA, trxC, grxA, nrdH* strain. The RO36 suppressor locus was identified by analysis of randomly inserted λNK1324 transposons. The ORFs that were disrupted by the transposon insertions (▼) are shown in gray, and the *dnaAN* locus is marked in black. The percentages represent the P1 linkage between the transposon and the RO36 suppressor mutation.

a *Bam*HI site. The amplified DNA fragment was cut with *Hin*dIII and *Bam*HI and ligated into pNG102 plasmid (19) in the same sites to give plasmid pRO3. The construct expresses a hybrid protein with the first 13 amino acids of R1 subunit fused to β-galactosidase. The fusion was then integrated into the chromosome of DHB4 by using specialized transduction with lambda phage InCh (20) to give RO48. β-Galactosidase activity assays in liquid media were performed as described (14).

Analytical Procedure. Proteins were analyzed by SDS/PAGE. For Western blotting, proteins were transferred to a nitrocellulose membrane after electrophoresis; the ECL-Western blotting system (Amersham Pharmacia) was used for detection.

Results

Isolation of Glutaredoxin 3 Mutants That Restore Growth to a Strain Missing Glutaredoxin 1 and the Thioredoxins. To study the differences between glutaredoxins 1 and 3 that define their differing specificities, we first sought mutants of glutaredoxin 3 that would allow it to substitute for a physiological role of glutaredoxin 1. In a triple mutant *E. coli* strain missing thioredoxins 1 and 2 (*trxA, trxC*) and glutaredoxin 1 (*grxA*), growth is abolished on both rich and minimal medium. Because the only known essential substrate of these proteins is ribonucleotide reductase, it is postulated that the growth defect is due to the inactivation of this enzyme, resulting in the bacterium's inability to synthesize deoxyribonucleotides. Ribonucleotide reductase reduces ribonucleotides by using its active site cysteines and must be regenerated by reduction using one of the three missing thioredoxin family members. The introduction of a plasmid expressing glutaredoxin 1 or one of the thioredoxins restores growth to these cells. In addition, the expression of a third thioredoxin (NrdH), which under normal growth conditions is not expressed, also restores growth to the triple mutant (7).

Therefore, to look for alterations of glutaredoxin 3 that would allow it to substitute for the missing thioredoxin family members, we began with a strain carrying deletions of the *trxA*, *trxC*, *grxA*, and *nrdH* genes. Because a plasmid containing the *nrdH* gene expressed at high levels restores growth to the triply mutant strain, we worried that selection for restoration of ribonucleotide reductase activity would yield mainly mutants that caused overexpression of *nrdH*. To obtain the quadruple mutant strain, we first constructed a Δ*trxA* Δ*trxC nrdH*::spc triple mutant carrying a plasmid (pBAD18-*trxC*) conditionally expressing the *trxC* gene (FÅ173). The *trxC* gene is under the control of the pBAD promoter and, thus, is induced by the presence of arabinose in the growth medium. This strain was transduced to kanamycin-resistance by using a P1 lysate from strain FÅ174, which contains a kanamycin-resistance marker inserted into the *grxA* gene. The resulting strain was designated RO36. The Δ*trxA* Δ*trxC grxA*::kan *nrdH*::spc/pBAD18-*trxC* genotype was confirmed by PCR. As expected, the RO36 strain grows on rich medium containing arabinose, where *trxC* is expressed, but not on rich medium containing glucose.

We used error-prone PCR to mutagenize the gene for glutaredoxin 3, *grxC*. We introduced the mutagenized gene into plasmid pRO1, where it is under control of the *lacUV5* promoter. The pools of mutagenized plasmid DNA were transformed into strain RO36, and cells were spread onto rich medium containing glucose and tetracycline to detect mutant cells to which growth had been restored. To ensure that the growth was not due to mutations increasing the expression of *trxC* carried by the other plasmid, the potential glutaredoxin 3 mutants were streaked on rich medium without ampicillin to test whether the pBAD18-*trxC* could be lost. To further narrow down the mutations to those that were carried by pRO1, plasmid was prepared from each of the mutants and retransformed into the parent strain. Plasmids that passed these tests were then subjected to sequencing of the plasmid-encoded *grxC* gene.

Each of the plasmids sequenced contained a single nucleotide change conferring an amino acid change on the protein. We have found three different mutations in the *grxC* gene that allow glutaredoxin 3 to suffice for at least some growth of the quadruple mutant strain. Remarkably, all three changes alter amino acid 43 of the protein, from methionine to valine, leucine or isoleucine (Fig. 1). These three mutant glutaredoxins allowed near-normal growth rates of the bacteria, although the leucine change was somewhat less effective than the valine and isoleucine changes. The Met43Val mutant was found in three independent PCR mutageneses and the Met43Leu mutant was found only once. In an attempt to obtain glutaredoxin 3 suppressors other than the Met43Val mutant, we changed the nucleotide ratio in the PCR mutagenesis reaction (see *Materials and Methods*). Two PCRs carried out with the altered ratios each yielded a Met43Val mutation and a Met43Ile mutation. Combining data from all of the PCRs carried out, we find that these glutaredoxin 3 mutations occur at about a frequency of 1/1,500 mutagenized plasmids.

To verify that a mutational change at Met-43 was indeed responsible for the restoration of growth to the quadruple-mutant strain, we chose the Met43Val change and introduced the same base change into a plasmid-encoded wild-type copy of the *grxC* gene (pRO1) by oligonucleotide-directed mutagenesis. The plasmid (pRO2) containing this altered *grxC* also restored growth. As ribonucleotide reductase is one of the substrates of these reductive proteins that are essential for growth, we conclude that these changes allow sufficient reduction of that enzyme to permit growth.

Extragenic Suppressors of the *trxA, trxC, grxA, nrdH* Quadruple Mutant. At the same time that we were studying the specificity of glutaredoxin 3, we also sought other means by which the growth deficiency in the *trxA, trxC, grxA, nrdH* quadruple mutant could be overcome. These studies have provided further insights into the interactions of glutaredoxin 3 with ribonucleotide reductase and revealed an unanticipated regulatory mechanism for ribonucleotide reductase.

We selected for mutants of strain RO36 that would grow on rich solid media containing glucose. Three independently isolated mutants, designated RO34, RO51, and RO52, were obtained. These mutant bacteria readily segregated the plasmid expressing the *trxC* gene, indicating that the suppressor strains no

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longer required thioredoxin 2 for growth and that the suppressor mutations must map to the bacterial chromosome.

To locate the suppressor mutations on the *E. coli* genetic map, we used a collection of transposon insertions into the chromosome of the RO34 suppressor strain (see *Materials and Methods*). We identified a series of insertions, each of which was linked by P1 transduction to the suppressor mutation at around the 83-min region of the *E. coli* genetic map (Fig. 2). One of these insertions (T5) was greater than 90% linked to the suppressor. We used this insertion to ask whether suppressor mutations in strains RO51 and RO52 map to the same region. A P1 lysate prepared from a strain that contains the T5 insertion but does not exhibit suppressor phenotype was used to transduce the T5 insertion into the RO51 and RO52 suppressor strains in the presence of the complementing plasmid pBAD18-*trxC*. In both cases more then 90% of the transductants lost the suppressor phenotype: their growth was dependent on the expression of *trxC* from the complementing plasmid. This finding indicates that the all three suppressors map to the same region of the chromosome.

As there were no clear-cut candidate genes for the suppressor mutations in this region, we decided to sequence 20-kb regions upstream and downstream of the T5 insertion in each suppressor as well as in the parental RO36 strain. The sequence analysis showed single mutations in each of the suppressor strains. Two of them contained a mutation in the *dnaA* gene; RO34 contains a change of Ala-345 (GCC) to Ser (TCC), and RO52 has a change of Thr-174 (CCA) to Pro (CCG). The third suppressor mutation, RO51, is located in the *dnaN* gene, causing the replacement of Gly-157 (GGT) with Cys (TGT). DnaA is a protein involved in initiation of chromosome replication and DnaN is a part of the replication machinery, acting as a sliding clamp anchoring DNA polymerase during DNA synthesis (for a review, see ref. 21).

Perplexed by these results, as no simple connection could be seen between the function of the two proteins (DnaA and DnaN) and the restoration of ribonucleotide reductase activity, we pursued several lines of experimentation to understand the mechanism of this suppression. First, we carried out genetic studies to see whether the growth of the suppressor strains was dependent on remaining members of either thioredoxin or glutaredoxin pathways. We did this by generating strains that, in addition to the quintuple mutations (the starting quadruple mutation plus the suppressor mutation) in our starting suppressor strain, carried null mutations of *trxB* (encoding thioredoxin reductase) or *grxC* or *grxB* (encoding glutaredoxin 2) (see *Materials and Methods*). These additional alterations were made in the suppressor strains in the presence of complementing plasmids conditionally expressing either *trxB* or *trxC*. Whereas the *trxB* and *grxB* mutations had no effect on the growth of the suppressor strains, the *grxC* null allele prevented the suppressor mutation from restoring growth to the cells.

These findings are most easily explained by proposing that the mutations in *dnaA* and *dnaN* genes have altered the cell in such a way as to allow glutaredoxin 3 to reduce ribonucleotide reductase to such an extent that normal growth is possible (Fig. 3). However, it still remained a possibility that the suppressor had altered cellular physiology so that another enzyme replaced the ribonucleotide reductase that is normally used during logarithmic-phase aerobic growth of *E. coli*. The normal aerobic ribonucleotide reductase is composed of two subunits encoded by the *nrdAB* operon. To determine whether the *dnaA* suppressor mutation in RO36 eliminated the requirement for the *nrdAB*-encoded enzyme, we introduced the suppressor by P1 transduction into an otherwise wild-type strain carrying an *nrdAts* (temperature-sensitive lethal) mutation (KK450). The strain remained temperature-sensitive in the presence of the suppressor mutation. This result shows that the suppressor mutation does not eliminate the dependence on the *nrdAB*-encoded ribonucleotide reductase for growth.

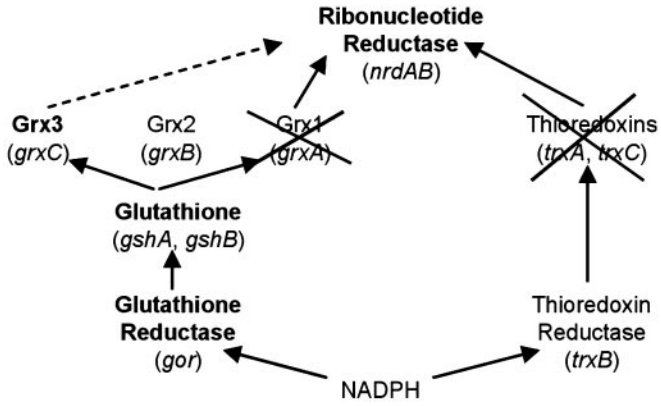

Fig. 3. Model of electron flow in *trxA, trxC, grxA, nrdH* suppressor strains. Arrows represent the path of reduction of disulfide bonds. The mutant form of either DnaA or DnaN allows glutaredoxin 3 to reduce ribonucleotide reductase. The effect depends on the presence of glutathione reductase, glutathione, and glutaredoxin 3 (Grx3). The channeling of electrons between Grx3 and ribonucleotide reductase has not been shown biochemically (dashed arrow).

These findings led us to hypothesize that the suppressor mutations activate a regulatory mechanism that increases ribonucleotide reductase expression, thus allowing sufficient reduction of the enzyme to take place. One reason for proposing this explanation is that, in addition to having a direct role in DNA replication, DnaA is also a regulatory protein, binding to DnaA boxes and activating or repressing the expression of genes for proteins such as DnaA itself, RpoH, UvrC, GlpD, FliC, and PolA (for review see ref. 22). The upstream region of *nrdAB* itself contains two DnaA boxes (23). We considered the possibility that the suppressor mutations in *dnaA* and *dnaN* were causing an increase in the levels of ribonucleotide reductase by promoting DnaA-based activation or releasing DnaA-based repression of the *nrdAB* genes. Such an increase in the amounts of ribonucleotide reductase might provide sufficient amounts of the enzyme to allow reduction by the normally weakly active glutaredoxin 3 and, thus, permit growth of cells.

To determine whether *nrdAB* expression was affected in the suppressor mutant backgrounds, we used both an *nrdA′–'lacZ* fusion generated in this laboratory and two antibody preparations, one raised against NrdA and the other against NrdB. We first examined the original suppressor strain by Western blotting and by introducing an *nrdA′–'lacZ* fusion to assess regulatory effects. These results clearly showed significant increases (2- to 3-fold) in *nrdAB* expression in these backgrounds when the strains were compared with the parent strain expressing TrxC from the plasmid (results not shown). However, interpretation of these results is somewhat complicated by the fact that mutant strains defective in these redox pathways already show derepression of ribonucleotide reductase (ref. 24 and our unpublished results). While there were clear-cut differences between the control strain and the suppressor strains, the different redox states of the strains is a confounding factor. Therefore, we examined the effect of two of the suppressor mutations, *dnaA*(Ala345Ser) and *dnaN*(Gly157Cys), in an otherwise wild-type background, strains SMG237 and SMG238, respectively. Here, using *nrdA′–'lacZ* fusions and Western blots, we observed substantially increased levels of NrdA and NrdB (Fig. 4). By dilution experiments with the Western blots, we estimate 8-fold and 4-fold increases of ribonucleotide reductase subunits in the SMG237 and SMG238 strains, respectively.

While these results show a correlation between the increased

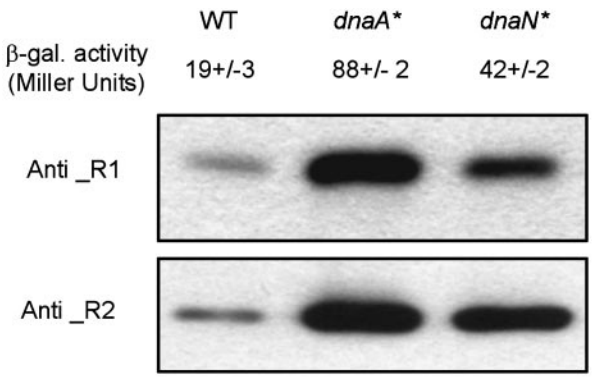

Fig. 4. Mutations in the *dnaAN* locus increase the expression of the *nrdAB* operon. β-Galactosidase activity of the *nrdA′–`lacZ* fusion in the DHB4 (WT) and SMG237 (*dnaA**) and SMG238 (*dnaN**) mutant strains is expressed in Miller units (14). Equal amounts of total cellular proteins from the same strains were separated by SDS/10% polyacrylamide gel electrophoresis. Subunits R1 (*nrdA*) and R2 (*nrdB*) of the ribonucleotide reductase were detected by Western blotting.

expression of ribonucleotide reductase in the suppressor strains and the restoration of growth, they do not show a cause–effect relationship. We wished to show that overexpression of this enzyme was sufficient to suppress the growth defect. To this end, we constructed strain SMG234, Δ*trxA* ΔtrxC *grxA*::kan *nrdH*::spc, carrying *trxC* under pBAD control and plasmid pSMG7 expressing NrdAB under the control of *trc* promoter, induced by isopropyl β-D-thiogalactopyranoside (IPTG). This derivative of the quadruple mutant showed growth in the presence of glucose as well as arabinose when IPTG was added, indicating that the plasmid-expressed *nrdAB* genes had suppressed the growth defect. We showed by Western blots that the ribonucleotide reductase subunits were substantially increased (data not shown). Finally, we constructed a derivative of SMG234 that carried a *grxC* mutation, in addition to all of the mutations and plasmids of the original strain. This strain was able to grow only on media containing arabinose, not glucose, whether or not IPTG was added. These results show that it is possible to overcome the growth defect in the multiple mutant by overexpressing ribonucleotide reductase and that this suppression requires the presence of glutaredoxin 3.

A *dnaA* Suppressor Mutation Provides a Means of Further Testing the Altered Specificity of the Glutaredoxin 3 Mutants. We wished to determine whether the glutaredoxin 3 mutants altered in their ability to reduce ribonucleotide reductase were also able to reduce a different substrate of glutaredoxin, 1,1,3′-phosphoadenylylsulfate (PAPS) reductase (25), an enzyme required for cysteine biosynthesis. However, results of such *in vivo* tests for restoration of cysteine prototrophy by these mutants in strain RO36 were difficult to interpret because the strain requires both ribonucleotide reductase and PAPS reductase for cells to grow in the absence of cysteine. The properties of strain RO34 eliminated this problem, as the suppressor has restored functional ribonucleotide reductase without resort to a mechanism that affects the glutaredoxins. We introduced plasmids containing each of the three glutaredoxin 3 mutants into RO34 and found that they restored cysteine prototrophy to differing extents, with the Met43Val change giving the best growth and the Met43Ile giving the worst. Wild-type glutaredoxin 3, of course, gave only extremely weak growth.

Discussion

These studies report on suppressor mutations that restore functional redox activities important for the growth of *E. coli* to strains that are defective in components of the thioredoxin and glutathione/glutaredoxin pathways. They were initiated (*i*) to deepen our understanding of the variations in specificities of members of the thioredoxin superfamily and (*ii*) to reveal novel aspects of redox pathways.

Glutaredoxin 3 Mutants. For the first goal of these studies, we chose to study the difference in specificities between glutaredoxins 1 and 3. Whereas the former can very efficiently reduce ribonucleotide reductase, the latter does so only inefficiently. From biochemical studies on these reactions, the differences can be readily explained by the differences in kinetic constants. Glutaredoxins 1 and 3 show K_m values for ribonucleotide reductase of 0.13 μM and 0.35 μM, respectively. The V_{max} of glutaredoxin 1 is 20-fold higher than that of glutaredoxin 3 in this reaction, thus providing perhaps the major explanation for the ineffectiveness of glutaredoxin 3 *in vivo*. The redox potentials of glutaredoxins 1 and 3 are similar, −233 and −198 mV, respectively (5). These *in vitro* studies suggest that, even though glutaredoxin 3 is capable of reducing ribonucleotide reductase, the rate of generation of deoxyribonucleotides *in vivo* is not sufficient for growth.

We sought and obtained mutants of glutaredoxin 3 that compensate for the absence of three proteins (glutaredoxin 1 and thioredoxins 1 and 2) and provide enough reductive capacity to cells to allow growth of *E. coli*. Because evidence discussed below supports the conclusion that ribonucleotide reductase is the only essential enzyme of the bacteria that requires this reductive activity, we conclude that the glutaredoxin 3 mutants are altered so as to allow more efficient reduction of this enzyme. While the most likely explanation for this finding is that the altered glutaredoxin 3 molecules are interacting directly with ribonucleotide reductase, we cannot eliminate the possibility that glutaredoxin 3 mutant proteins are acting through some intermediary to pass electrons on to the enzyme. These mutant glutaredoxins also allow reduction of the protein PAPS reductase.

None of the results presented here illuminate how the altered amino acids have enhanced the activity of this protein. Several possibilities should be considered. Even though the K_m values of glutaredoxin 1 and 3 and their redox potentials are quite similar, slightly increased affinity for ribonucleotide reductase or a change in redox potential might suffice. However, an increase in the V_{max} of the protein seems most attractive, because wild-type glutaredoxin 3 has a 20-fold higher V_{max} for ribonucleotide reductase than glutaredoxin 3. Perhaps the greatest opportunity for enhancement of glutaredoxin 3 is in this kinetic constant.

The three-dimensional structures of glutaredoxins 1 and 3 are quite similar. Significant differences can be observed in the length and positions of some loops (Fig. 5, which is published as supporting information on the PNAS web site). One of the regions that differs has been implicated in the binding of glutaredoxin 1 to ribonucleotide reductase (26). This region includes helix 2 in the thioredoxin fold and is part of the loop that connects the thioredoxin fold N-terminal motif to the C-terminal motif. This loop corresponds to Asp-37 through Ala-52 in glutaredoxin 1 and Asp-34 through Arg-46 in glutaredoxin 3 and includes the methionine that is altered in the mutants we obtained. Helix 2 in glutaredoxin 1 is kinked and the loop is longer by three amino acids than the loop of glutaredoxin 3. These two dissimilarities might allow a higher degree of freedom to this region. Structural data show that upon binding of ribonucleotide reductase to glutaredoxin 1 the loop undergoes significant structural changes (26) and the helix 2 portion upstream of the kink loses its secondary structure. The methionine-to-valine change at amino acid 43 introduces at this position a valine that one could deduce is at the same position in glutaredoxin 1. Until completion of further biochemical

studies we will not speculate further on the significance of this change.

Whatever the mechanistic explanation, our results show that, *in vivo*, glutaredoxin 3 is capable of providing effective reduction of ribonucleotide reductase with only slight changes. These findings are consistent with the *in vitro* studies on its weak activity toward the enzyme. We point out that glutaredoxin 3 and its mutants in these experiments are expressed from a plasmid at higher levels than the chromosomally expressed gene. It may be that the mutants when incorporated into the chromosome would not suffice for growth.

The *dnaA* and *dnaN* Mutations. In the second part of this study, we sought extragenic suppressors that would restore growth to the mutant strain multiply defective for members of the thioredoxin superfamily. Hoping to find mutations in alternative redox pathways, we were surprised to find mutations in the *dnaA* and *dnaN* genes, whose protein products are essential for DNA replication. Our results suggest that the restoration of growth to strain RO36 resulting from these mutations is due to increased levels of ribonucleotide reductase. We show that the mutations do cause this increase and that overexpression of ribonucleotide reductase from a plasmid is also sufficient to overcome the cell's redox deficiency for this enzyme. This suppression, whether caused by the suppressor mutations or by overexpression of ribonucleotide reductase from a plasmid, is dependent on the reductant glutaredoxin 3. This leads us to postulate that the increased concentration of ribonucleotide reductase in the cells, in the presence of the already highly expressed glutaredoxin 3 from its chromosomal locus, provides enough reduced ribonucleotide reductase to allow growth. These results again cohere with the properties of glutaredoxin 3 *in vitro*.

Our results also provide direct support for the long-held supposition that the reason for the essentiality of the glutaredoxins and thioredoxins is their role in reducing ribonucleotide reductase. That is, because the plasmid-expressed ribonucleotide reductase is sufficient to restore growth to the multiply mutant strain, it appears that it is the only essential enzyme in aerobically growing *E. coli* that requires these reductive proteins to maintain its activity.

This study also reveals a regulatory effect on ribonucleotide reductase expression. The *dnaA* and *dnaN* mutations cause substantial increases in the levels of NrdA and NrdB. One possible explanation for this result is based on the existence of DnaA boxes in the region upstream of the *nrdAB* operon. According to this explanation, the *dnaA* mutations alter the structure of DnaA so that it is shifted more to a form that allows increased expression of the operon. How the *dnaN* mutation has its effects is more mysterious. However, recent findings suggest that DnaN, by interaction with an intermediary protein, Hda, can alter the conformation of DnaA so that the equilibrium shifts from the ATP-binding form to the ADP-binding form (27). This change may affect its regulatory properties. Alternatively, these mutations may slow down DNA synthesis, which, in turn, calls into play some other regulatory mechanism. Little is known about the regulation of the *nrdAB* operon. It is derepressed in strains that are missing various combinations of the redox components of the thioredoxin and glutathione pathways (5, 28). However, *nrdAB* derepression is not mediated by OxyR, a regulatory protein that responds to oxidative stress, even though it is often activated by defects in these redox pathways (29, 30). Redox-dependent regulation of NrdAB and the regulatory effect reported here may reflect a common mechanism. Factors that interfere with DNA replication, less directly by reducing the production of deoxyribonucleotides, or more directly by affecting the activity of proteins involved in DNA replication, may induce a common response.

Our findings suggest an unexpected complexity in the regulatory mechanisms leading to DNA replication. Variations in the interactions between cellular redox components, ribonucleotide reductase, and protein components of the DNA replication machinery may be important in the response to oxidative stress as well as under normal growth conditions. Studies such as these, revealing intricately evolved networks involved in physiologically important pathways, raise questions about current attempts to model even such a well characterized organism as *E. coli*. They suggest, instead, a need to devote even greater efforts to detecting and characterizing these networks.

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