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The Role of the Thioredoxin and Glutaredoxin Pathways in Reducing Protein Disulfide Bonds in the *Escherichia coli* Cytoplasm*

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In *Escherichia coli*, two pathways use NADPH to reduce disulfide bonds that form in some cytoplasmic enzymes during catalysis: the thioredoxin system, which consists of thioredoxin reductase and thioredoxin, and the glutaredoxin system, composed of glutathione reductase, glutathione, and three glutaredoxins. These systems may also reduce disulfide bonds which form spontaneously in cytoplasmic proteins when *E. coli* is grown aerobically. We have investigated the role of both systems in determining the thiol-disulfide balance in the cytoplasm by determining the ability of protein disulfide bonds to form in mutants missing components of these systems. We find that both the thioredoxin and glutaredoxin systems contribute to reducing disulfide bonds in cytoplasmic proteins. In addition, these systems can partially substitute for each other *in vivo* since double mutants missing parts of both systems generally allow substantially more disulfide bond formation than mutants missing components of just one system. Some of these double mutants were found to require the addition of a disulfide reductant to the medium to grow well aerobically. Thus, *E. coli* requires either a functional thioredoxin or glutaredoxin system to reduce disulfide bonds which appear after each catalytic cycle in the essential enzyme ribonucleotide reductase and perhaps to reduce non-native disulfide bonds in cytoplasmic proteins. Our results suggest the existence of a novel thioredoxin in *E. coli*.

Disulfide bridges play an important structural role in many proteins (1, 2). While these bonds often occur in extracytoplasmic proteins, they are rarely found in cytoplasmic proteins (3, 4). It has been suggested that the cytoplasm is too reducing for many disulfide bonds to form (5, 6). In fact, when many exported proteins that ordinarily form disulfide bonds are expressed in the cytoplasm, they do not form these bonds (7, 8) (for an exception, see Ref. 9).

A number of factors are thought to determine the thiol-disulfide balance in the cytoplasm of *Escherichia coli*. The principle thiol-disulfide redox buffer in the cytoplasm is constituted by the cysteine containing tripeptide glutathione. *E. coli* contains high levels of glutathione (the intracellular concentration is approximately 5 mM) that is kept almost entirely reduced (10). The ratio of reduced to oxidized glutathione in the *E. coli* cytoplasm is roughly 50:1 to 200:1 (11). *In vitro*, similar levels of oxidized and reduced glutathione are not conducive to disulfide bond formation in many proteins (11–14).

In addition to glutathione, the *E. coli* cytoplasm contains at least four thiol-disulfide oxidoreductases that may help reduce protein disulfide bonds in the cytoplasm via their redox active disulfides: thioredoxin, glutaredoxin 1, glutaredoxin 2, and glutaredoxin 3 (15–17). Thioredoxin is the best characterized of these and belongs to a superfamily of proteins that contain an active site CX_1X_2C motif and share a similar fold, in those cases where structure is known (18). The redox potential of thioredoxin is low (−270 mV) (19) and, *in vitro*, thioredoxin efficiently reduces disulfide bonds in a wide variety of proteins (15, 20). Although they have not been as extensively tested as thioredoxin, the glutaredoxins are generally less efficient reductants of disulfide bonds than thioredoxin (16, 21). This may be partially explained by the higher redox potentials of the glutaredoxins (glutaredoxin 1 = −233 mV; glutaredoxin 3 = −198 mV).[1]

Upon reducing disulfide bonds, the thiol-disulfide oxidoreductases become oxidized. In order for these proteins to be functional, they in turn must be kept reduced. The flavoenzyme thioredoxin reductase uses NADPH to reduce thioredoxin but is unable to reduce any of the glutaredoxins (16, 22).[2] Instead, the glutaredoxins are reduced by glutathione, which in turn is reduced by glutathione reductase. Like thioredoxin reductase, glutathione reductase is a flavoenzyme that uses NADPH to reduce its substrate. Since glutathione does not efficiently reduce thioredoxin (22) and the glutaredoxins are not substrates of thioredoxin reductase, it has been presumed that *E. coli* has two separate pathways for using NADPH to reduce disulfide bonds in the cytoplasm: the thioredoxin system (which consists of thioredoxin reductase and thioredoxin) and the glutaredoxin system (glutathione reductase, glutathione, and the three glutaredoxins) (Fig. 1) (16, 23).

Similar systems are thought to reduce protein disulfide bonds in the cytoplasm of eukaryotic cells as well. Most eukaryotic cells contain high levels of reduced glutathione as well as thioredoxin and glutaredoxin (6, 15, 24, 25).

In *E. coli*, the thioredoxin and glutaredoxin systems are known to participate in the reduction of disulfide bonds in essential cytoplasmic enzymes which require this step to complete their catalytic cycles. These include ribonucleotide reduc-

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[1] F. Åslund, K. D. Berndt, and A. Holmgren, submitted for publication.

[2] F. Åslund and A. Holmgren, unpublished results.

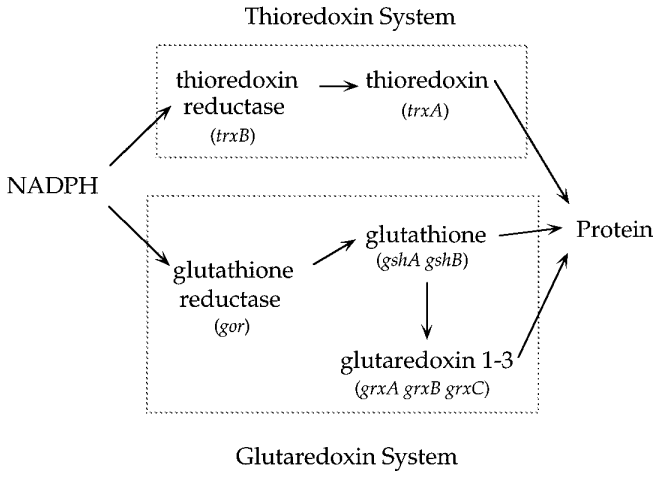

FIG. 1. **Known components of the thioredoxin system (*top*) and glutaredoxin system (*bottom*).** The genes encoding the components of these systems are shown in *parentheses*.

tase, PAPS[3] reductase, and methionine sulfoxide reductase (16). However, the ability of the thioredoxin and glutaredoxin systems to function *in vivo* as general reductants of cytoplasmic protein disulfide bonds has not been fully determined. We have previously demonstrated that the thioredoxin system has a role in this process (26). In this study, we have investigated the role of the glutaredoxin system and both systems together in determining the thiol-disulfide equilibrium in the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials—Media and chemical reagents were prepared or purchased as described previously (27). Diazenedicarboxylic acid bis(*N,N′*-dimethylamide) (diamide) and DTT were purchased from Sigma.

Strains and Plasmids—The strains and plasmids used in this study are listed in Table I. Strains were constructed by P1 transduction (28). The mutant alleles of the genes encoding components of the thioredoxin and glutaredoxin systems (Fig. 1) used to construct the strains for this study are: *trxB*::Km (29), Δ*trxA* (30), *gor522* (lab collection), *gshA20*::Tn*10*Km (31), *grxA*::Km (32), and *grxC*::Cm (this study). Three of the mutants (WP861, WP863, and WP864) contain *trxB36* (26) in place of *trxB*::Km.

A strain in which the coding region of *grxC*, which encodes glutaredoxin 3, was replaced with a gene encoding chloramphenicol resistance (*grxC*::Cm) was constructed by linear transformation (33). For this purpose, we constructed a plasmid pGrxC::Cm, which contains *grxC*::Cm and the chromosomal DNA flanking *grxC*. We made pGrxC::Cm from pGrxC, which contains a 1723-base pair fragment of chromosomal DNA that includes *grxC* cloned into the *Eco*RI site of pGEM-3Z.[4] pGrxC was cut with *Bal*I and *Bam*HI, which removes all but 20 base pairs of the coding sequence of *grxC*. A *Bam*HI linker was added to the *Bal*I site and the ends of the resulting fragment were ligated together. Into the *Bam*HI site of the resulting plasmid, we cloned DNA encoding chloramphenicol resistance obtained from pHP45Ω-Cm (34) after digestion with *Bam*HI. The resulting plasmid is called pGrxC::Cm. The fragment of chromosomal DNA containing *grxC*::Cm obtained from pGrxC::Cm after digestion with *Eco*RI was then used to transform JCB495 (*recD*) to chloramphenicol resistance. The replacement of *grxC* by *grxC*::Cm in the resulting strain was confirmed by P1 transduction, polymerase chain reaction, and Western blot analysis using anti-glutaredoxin 3 antibody (data not shown).

Construction and Growth of WP759 and WP778—Two of the strains constructed for this study, WP759 (*trxB gshA*) and WP778 (*trxB gor*), grew very poorly unless they were grown in medium that contains a disulfide reductant like DTT. To construct WP759, WP758 (*gshA*) was transduced with P1 grown on A305 (*trxB*::K_m. . . . Tn*10*) and plated on NZ-amine-A plates containing 20 μg/ml tetracycline. A 1-cm filter disk containing 25 μl of 1 M DTT was placed on the plate. After incubated for 24 h at 37 °C, two types of colonies were observed: large colonies evenly distributed on the plate and small colonies that were only present roughly 1 to 2 cm from the filter disk. The large colonies did not require DTT for growth and were found by P1 transduction not to contain *trxB*::Km. The small colonies required DTT for growth and contained *trxB*::Km (by P1 transduction). The same procedure was used to construct WP778 except that AD494 (*trxB*::Km) was transducted to tetracycline resistance with P1 grown on WP522 (*gor522*. . . . mini-Tn*10*Tc).

WP759 and WP778 were grown on NZ-amine-A plates with 8 mM DTT. 4 and 2 mM DTT was used to grow these strains in liquid NZ-amine-A and liquid M63 minimal medium, respectively. In minimal medium, these strains require supplementation with cysteine.

Growth rates of WP759 and WP778 (Fig. 2) were determined as follows. Overnight cultures of both strains and DHB4 were grown at 37 °C in NZ-amine-A medium supplemented with 4 mM DTT. The cultures were diluted 1:100 in the same medium and their growth followed for 3 h by determining their optical density at 600 nm. The cells were then pelleted and resuspended in NZ-amide-A medium without DTT and their growth was followed for another 3 h. Growth rates were calculated from the average of three determinations and differed from one another by less than 1%.

WP759 and WP778 accumulate suppressing mutations that allow them to grow about as rapidly as DHB4 (wild-type) in media that does not contain a disulfide reductant. Since these mutations arise at a high frequency (approximately 10^{-6} or 10^{-7}), it was necessary to monitor the growth rate of these strains when they were grown without DTT (*e.g.* to determine AP activity or urokinase activity) to make sure that they grew slowly. Cultures that did not grow as slowly as those shown in Fig. 2*B* were discarded.

Alkaline Phosphatase Assays—The strains shown in Table II were grown at 37 °C in M63 minimal medium containing 0.2% glucose, 50 μg/ml each of all amino acids except methionine, 200 μg/ml ampicillin, and 5 mM isopropyl-1-thio-β-D-galactopyranoside to a final optical density at 600 nm of approximately 0.4. They were then incubated on ice for 20 min in the presence of 100 mM iodoacetamide. The remainder of the assay was performed as described in Ref. 27, except that 100 mM iodoacetamide was used instead of 1 mM iodoacetamide in the wash buffer. The assays were performed in duplicate and varied by less than 5%.

The strains shown in Table III were grown at 37 °C to an optical density at 600 nm of approximately 0.6 in NZ-amine-A containing 200 μg/ml ampicillin and 4 mM DTT. They were pelleted, resuspended in NZ-amine-A containing 200 μg/ml ampicillin and 5 mM isopropyl-1-thio-β-D-galactopyranoside, and grown at 37 °C for 3 h. The AP assays were then performed as above.

Zymography of Urokinase—Cells were grown in NZ-amine-A plus 200 μg/ml ampicillin and 4 mM DTT at 37 °C to an optical density at 600 nm of approximately 0.6. They were pelleted and resuspended in NZ-amine-A plus 200 μg/ml ampicillin and 5 mM isopropyl-1-thio-β-D-galactopyranoside and grown for 3 h at 37 °C. Zymography using casein plasminogen agar underlays was performed as described previously (26) except that after incubation of the samples on ice for the times indicated in Fig. 3, iodoacetamide was added to 100 mM and the samples were incubated for an additional 20 min on ice.

Treatment of Cells with Diamide—To determine the affect of diazenedicarboxylic acid bis(*N,N′*-dimethylamide) (diamide) on growth rate, cells were grown to an optical density of 0.2 at 600 nm in NZ-amine-A medium at 37 °C and diamide was then added to a final concentration of 250 mM. The growth of the cells was then followed by determining their optical density at 600 nm.

RESULTS

To investigate the role of the thioredoxin and glutaredoxin systems in maintaining the thiol-disulfide balance in the cytoplasm, we constructed a set of mutants missing various components of these systems. We then assessed the ability of disulfide bonds to form in the cytoplasm of these strains by determining the extent to which *E. coli* alkaline phosphatase (AP) is able to form disulfide bonds in the cytoplasm of the mutants. AP is a periplasmic homodimeric enzyme that contains two intrachain disulfide bonds in each monomer (35). These bonds are required for AP to be enzymatically active. AP is synthesized with an N-terminal signal sequence which targets it for export to the periplasm. When AP is expressed with

[3] The abbreviations used are: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; DTT, dithiothreitol; Km, kanamycin resistance; Cm, chloramphenicol resistance; Tc, tetracyclin resistance; AP, alkaline phosphatase.

[4] F. Åslund, K. D. Berndt, G. Spyrou, and A. Holmgren, manuscript in preparation.

TABLE I
Strains and plasmids used in this work

Plasmids	Description	Source or reference
pΔA-uPA	ΔA-uPA under control of tac promoter	D. Belin
pAID135	APΔ2–22 under control of tac promoter	27
pGrxC	pGEM-3Z containing a 1723-bp fragment of the E. coli chromosome which includes *grxC*	F. Åslund
pGrxC::Cm	pGrxC but with *grxC*::Cm	This work
Strains	**Relevant genotype**	**Source or reference**
DHB4	F′ *lac-pro lacI^{Q}*/Δ*(ara-leu)7697 araD139* Δ*lacX74 galE galK rpsL phoR* Δ*(phoA)*PvuII Δ*malF3 thi*	Lab collection
WP551	DHB4 + pAID135	This work
WP591	WP551 Δ*trxA*	This work
WP552	WP551 *trxB*::Km	This work
WP838	WP551 *grxA*::Km	This work
WP823	WP551 *grxC*::Cm	This work
WP776	WP551 *gshA20*::Tn10Km	This work
WP841	WP551 *gor522*. . . . mini-Tn10Tc	This work
WP898	WP551 *gshA20*::Tn10Km Δ*trxA*	This work
WP786	WP551 *gshA20*::Tn10Km *trxB*::Km. . . . Tn10	This work
WP843	WP551 *gor522*. . . . mini-Tn10Tc Δ*trxA*	This work
WP782	WP551 *gor522*. . . . mini-Tn10Tc *trxB*::Km	This work
WP861	WP551 *trxB36 grxA*::Km	This work
WP824	WP551 *trxB*::Km *grxC*::Cm	This work
WP592	WP551 *trxB*::Km Δ*trxA*	This work
WP863	WP551 *trxB36 grxA*::Km *grxC*::Cm	This work
WP826	WP551 *trxB*::Km *grxC*::Cm Δ*trxA*	This work
WP864	WP551 *trxB36 grxA*::Km *grxC*::Cm Δ*trxA*	This work
WP839	WP551 Δ*trxA grxA*::Km	This work
WP825	WP551 Δ*trxA grxC*::Cm	This work
WP860	WP551 Δ*trxA grxA*::Km *grxC*::Cm	This work
WP859	WP551 *grxA*::Km *grxC*::Cm	This work
WP759	DHB4 *gshA20*::Tn10Km *trxB*::Km. . . . Tn10	This work
WP778	DHB4 *gor522*. . . . mini-Tn10Tc *trxB*::Km	This work
WP613	DHB4 *trxB36* Δ*$_1$fbp*. . . . Tn10Km	Lab collection
AD494	DHB4 *trxB*::Km	This work
WP840	DHB4 *gor522*. . . . mini-Tn10Tc	This work
WP822	DHB4 Δ*trxA grxA*::Km *grxC*::Cm	This work
WP758	DHB4 *gshA20*::Km	This work
WP843	DHB4 Δ*trxA gor522*. . . . mini-Tn10Tc	This work
WP612	DHB4 Δ*trxA gshA20*::Km	This work
JCB495	*recD*	J. Bardwell
WP522	DHB4 *gor522*. . . . mini-Tn10Tc	Lab collection
JTG10	*gshA20*::Tn10Km	31
A304	*trxB*::Km	29
A307	Δ*trxA*	30
A407	*grxA*::Km	32
A305	*trxB*::Km. . . . Tn10	M. Russel

a defective or missing signal sequence, it is not exported to the periplasm, but remains in the cytoplasm (36). In this compartment, AP does not form disulfide bonds and cannot fold into an enzymatically active conformation (8). However, in the cytoplasm of *E. coli* mutants missing thioredoxin reductase a fraction of a signal sequenceless version of AP (APΔ2-22) does forms disulfide bonds and folds into an active conformation (26). Thus, APΔ2-22 can be used to assess the potential for disulfide bond formation in the cytoplasm of the mutants missing components of the thioredoxin and glutaredoxin systems.

Construction of Mutants Lacking Components of the Thioredoxin and Glutaredoxin Systems—Some mutants lacking components of the thioredoxin and glutaredoxin systems have been previously constructed. However, these mutants are in different genetic backgrounds. To facilitate comparisons between the mutants, a set of isogenic strains was constructed by P1 transduction (Table I). The mutant alleles of each gene used in the construction of these strains do not make any detectable functional product of that gene (Refs. 29, 31, and 37; data not shown).

In addition to the previously described mutants lacking glutaredoxin 1 (encoded by *grxA*), we wished to obtain mutants lacking either glutaredoxin 2 or glutaredoxin 3. A mutant missing glutaredoxin 2 has only recently been constructed,[5] and was not used in these studies.

We constructed a strain missing glutaredoxin 3 using linear transformation (33). The plasmid pGrxC contains a fragment of chromosomal DNA that includes *grxC* (which encodes glutaredoxin 3). The coding region of *grxC* in this plasmid was removed and replaced with DNA from pHP45Ω-Cm (34) which confers chloramphenicol resistance. The fragment of chromosomal DNA was then removed from the resulting plasmid and used to transform a *recD* strain (JCB495) to chloramphenicol resistance. The replacement of *grxC* with *grxC*::Cm was confirmed by polymerase chain reaction and P1 transduction (data not shown). Western blot analysis revealed that *grxC*::Cm cells contained no detectable glutaredoxin 3 (data not shown). *grxC* is in an operon with *secB*, which encodes an export-specific cytoplasmic chaperone. Western blots revealed that strains containing *grxC*::Cm did not make detectable amounts of SecB (data not shown).

Growth Requirements of the Mutants—Seven mutants constructed for this study could not grow in M9 or M63 minimal medium unless it was supplemented with cysteine or glutathi-

[5] A. Vlamis-Gardikas and A. Holmgren, manuscript in preparation.

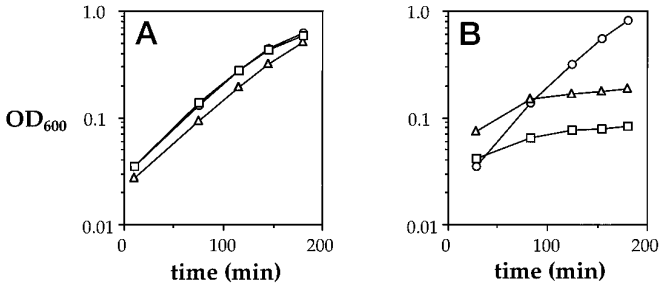

FIG. 2. **WP759 (*trxB gshA*) and WP778 (*trxB gor*) grow poorly without DTT.** The growth rate of DHB4 (wild-type, ○), WP759 (*trxB gor*, △), and WP778 (*trxB gshA*, □) was followed by determining the optical density (at 600 nm) of cells grown in NZ-amine-A medium. *A*, cultures were grown for 3 h with 4 mM DTT. *B*, these cultures were pelleted, resuspended in media without DTT, and grown for 3 h.

one: WP839 (*trxA grxA*), WP860 (*trxA grxA grxC*), WP864 (*trxB trxA grxA grxC*), WP843 (*trxA gor*), WP898 (*trxA gshA*), WP824 (*trxB grxA*), and WP863 (*trxB grxA grxC*) (data not shown). DHB4, the wild-type parent of these strains, does not require these supplements. However, these additives were not required if the M9 medium was prepared with sulfite instead of sulfate (data not shown). None of these strains required cysteine or glutathione to grow in NZ-amine-A medium. Similar findings have been reported for *trxB grxA* and *trxA grxA* mutants and were attributed to the inability of these strains to reduce PAPS reductase, an enzyme that is required for growth when sulfate is the only source of sulfur in the medium (32, 38, 39).

E. coli Requires Either a Functional Thioredoxin or Glutaredoxin System to Grow Well Aerobically but Not Anaerobically—Two strains constructed for this study, WP759 (*trxB gshA*) and WP778 (*trxB gor*), grew extremely poorly. They formed microscopic colonies on rich medium (NZ-amine-A) after incubation for 1 day and small colonies after 4 days at 37 °C (wild-type cells form large colonies in 1 day). Since we suspected that these strains grew poorly because they were not able to efficiently reduce cytoplasmic protein disulfide bonds, we determined whether their growth rate could be improved by adding a disulfide reductant to the medium. Both strains grew at about the same rate as wild-type cells when 4 mM DTT was added to the medium (Fig. 2). The doubling time of WP759 and WP778 in NZ-amine-A medium without DTT is about 300 min, while this rate increases to 30 min when 4 mM DTT is added to the medium. The growth defect of these mutants could also be complemented by the addition of β-mercaptoethanol, but not oxidized DTT, to the medium (data not shown). Interestingly, WP759 and WP778 were able to grow about as rapidly as wild-type cells, even in medium that did not contain DTT or another disulfide reductant, when the cells were grown anaerobically (data not shown).

Mutations that allow WP759 and WP778 to grow well aerobically without a disulfide reductant in the medium arose at a relatively high frequency (approximately 10^{-6} or 10^{-7}). These mutations have not been characterized further.

Disulfide Bond Formation in the Cytoplasm of the Mutant Strains—APΔ2-22 becomes active in the cytoplasm of the mutants missing components of either the thioredoxin or the glutaredoxin systems (Table II). We confirmed that the AP activity in some of these mutants in fact reflects an increase in the extent of disulfide bond formation in APΔ2-22 by using the finding that oxidized and reduced APΔ2-22 can be separated with nonreducing SDS-PAGE (8) to determine the redox status of APΔ2-22 in the mutants (data not shown). As previously reported, mutants missing thioredoxin reductase allow a substantial amount of APΔ2-22 (approximately 25%) to form disulfide bonds in the cytoplasm. Cells missing thioredoxin or one of the components of the glutaredoxin system allow less APΔ2-22 to form disulfide bonds. The amount of AP activity in many of these mutants is only slightly higher than that found in wild-type cells. However, even these small differences probably indicate a substantial increase in the ability of APΔ2-22 to form disulfide bonds in the cytoplasm of these mutants. We suggest this since, as far as we can tell, all of the AP activity in wild-type cells (WP551) reflects the 1–2% of APΔ2-22 that is exported to the periplasm; no disulfide bonds form in APΔ2-22 in the cytoplasm of wild-type cells. Thus, the increase in activity in the mutants is effectively relative to zero rather than to the activity seen in WP551.

TABLE II
AP activity of various strains expressing APΔ2–22

Strains were grown in M63 minimal medium containing 0.2% glucose, 200 μg/ml ampicillin, 5 mM isopropyl-thio-β-D-galactopyranoside, and 50 μg/ml each of all amino acids except methionine.

Strain	Relevant genotype	AP activity
WP551	Wild-type	90
WP552	*trxB*	870
WP591	*trxA*	130
WP841	*gor*	260
WP776	*gshA*	130
WP838	*grxA*	80
WP823	*grxC*	290
WP592	*trxB trxA*	310
WP861	*trxB grxA*	420
WP824	*trxB grxC*	860
WP843	*trxA gor*	860
WP898	*trxA gshA*	760
WP839	*trxA grxA*	63
WP825	*trxA grxC*	32
WP863	*trxB trxA grxA*	230
WP826	*trxB trxA grxC*	140
WP860	*trxA grxA grxC*	200
WP864	*trxB trxA grxA grxC*	290

We point out that all of these strains were grown in cysteine-containing medium (see Table II), since several of them required that amino acid for growth (see above) and we wished to grow all strains under the same conditions. The addition of cysteine, we discovered, resulted in some differences in results from those reported before (26). In particular, our previous study reported that combining the *trxA* mutation with the *trxB* mutation did not reduce the potency of the *trxB* mutation in allowing high levels of alkaline phosphatase activity. In the presence of cysteine, we now observe some effect of *trxA* on the *trxB* phenotype (Table II). However, during these studies, we also showed that removal of cysteine from the growth media restored the properties of the *trxB trxA* double mutant to those observed previously (data not shown).

Double mutants that lack either glutathione or glutathione reductase in combination with one of the proteins of the thioredoxin system allow a substantial amount of disulfide bond formation in the cytoplasm. Thus WP843 (*trxA gor*) and WP898 (*trxA gshA*) allow about as much APΔ2-22 to form disulfide bonds as cells missing thioredoxin reductase (WP552) (Table II). Even more active APΔ2-22 was found in the cytoplasm of WP759 (*trxB gshA*) and WP778 (*trxB gor*) when they were grown without DTT (Table III). We found that the rate of disulfide bond formation in APΔ2-22 in these strains is faster than in cells missing only thioredoxin reductase (data not shown).

Since the cytoplasm of WP759 and WP778 appears to be a relatively oxidizing environment and these strains require DTT in the medium for high growth rates, we wondered whether other proteins also formed disulfide bonds efficiently in the cytoplasm of these strains. The serine protease domain of murein urokinase (ΔA-uPA) requires six disulfide bonds to fold

TABLE III
AP activity of strains expressing APΔ2–22 after growth for 3 h without DTT
Strains were grown as described under "Experimental Procedures"

Strain	Relevant genotype	AP activity
WP551	Wild-type	38
WP552	*trxB*	240
WP782	*trxB gor*	1200
WP786	*trxB gshA*	1700

into an active conformation (40). Like APΔ2-22, when ΔA-uPA is expressed in the cytoplasm of wild-type cells, it cannot form its native disulfide bonds and, therefore, is not enzymatically active. It is also not active when it is expressed in a mutant missing thioredoxin reductase, even though APΔ2-22 is active in the cytoplasm of this mutant. However, we showed previously that slight amounts of urokinase activity are observed when cells are allowed to sit on ice for several hours (26). The ability of ΔA-uPA to form disulfide bonds and hence become active in the cytoplasm of WP759 and WP778 was assessed by zymography with casein plasminogen agar underlays (Fig. 3). Substantially more active ΔA-uPA was found in WP759 and WP778 than in the cells lacking only thioredoxin reductase or wild-type cells. Thus, both APΔ2-22 and ΔA-uPA form disulfide bonds more efficiently in the cytoplasm of WP759 and WP778 than in cells missing only thioredoxin reductase. Some of the active ΔA-uPA in these strains runs more slowly on the gel than native ΔA-uPA (Fig. 3). This material may be active ΔA-uPA that has formed disulfide bonds with other cytoplasmic proteins.

The Mutants Missing Components of the Thioredoxin or Glutaredoxin Systems Are Defective in Reducing Cytoplasmic Disulfide Bonds—We used a second approach to assess the ability of the mutants to reduce cytoplasmic disulfide bonds. Mutants with defects in disulfide reduction should be more sensitive to a thiol-specific oxidant than wild-type cells. The membrane-permeant thiol-specific oxidizing agent diamide (diazenedicarboxylic acid bis(*N,N′*-dimethylamide)) temporarily inhibits the growth of *E. coli* when it is added to mid-log phase cultures (41, 42). It has been assumed that this inhibition is due to the formation of disulfide bonds in the cytoplasm. Growth resumes after a period of time which is proportional to the amount of diamide added and is thought to be determined by the rate of disulfide bond reduction in the cytoplasm (43).

We determined the affect of diamide on the growth rate of some of the mutants (Fig. 4). In six of the seven strains tested, there is a good correlation between the length of diamide-induced growth inhibition and the ability of APΔ2-22 to form disulfide bonds in the cytoplasm. Thus the mutants that allow APΔ2-22 to form disulfide bonds in the cytoplasm also have a lower capacity to reduce cytoplasmic disulfide bonds generally. One exception to this pattern was the mutant AD494 (*trxB*), which did not display a very pronounced growth lag after diamide addition, but allowed a substantial amount of APΔ2-22 to form disulfide bonds in the cytoplasm.

SecB Has no Effect on the Ability of APΔ2-22 to Fold in the Cytoplasm—As described above, the strains we constructed missing glutaredoxin 3 also lack SecB, since *grxC*::Cm is polar on *secB*. Since genes encoding proteins with unrelated functions are sometimes found in the same operon, we think it unlikely that there is any functional relationship between glutaredoxin 3 and SecB. However, SecB is likely to interact with APΔ2-22 in the cytoplasm and has been found to slow the folding of the proteins it interacts with (27, 44). We wanted to rule out the possibility that the effects on AP activity we found in cells missing glutaredoxin 3 were due to the fact that they were also missing SecB. A mutant allele of *secB* (*secB*::Tn*5*) was

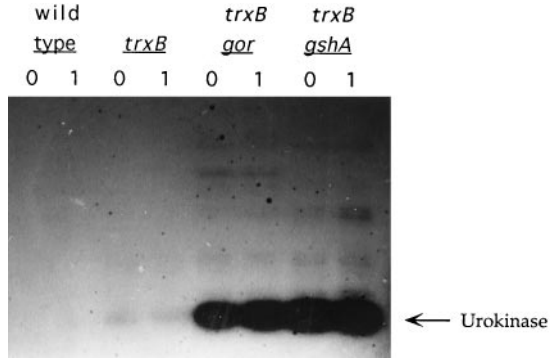

FIG. 3. **Urokinase becomes active in the cytoplasm of WP759 and WP778.** Zymography with casein plasminogen underlays was performed as described under "Experimental Procedures." Protein extracts from the same numbers of cells were run in each lane. The *numbers above* the lanes indicate the amount of time the cells were held on ice before the addition of iodoacetamide.

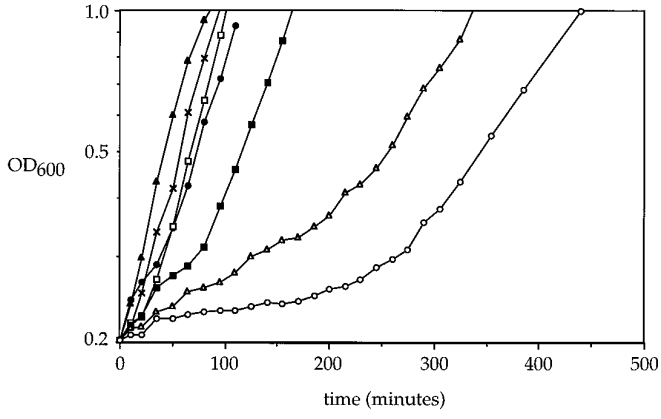

FIG. 4. **Affect of diamide on the growth rate of mutants missing components of the thioredoxin and glutaredoxin systems.** DHB4 (wild-type, ▲), AD494 (*trxB*, ×) WP840 (*gor*, ●), WP822 (*trxA grxA grxC*, □), WP758 (*gshA*, ■), WP843 (*trxA gor*, △), and WP612 (*trxA gshA*, ○) were grown to an optical density at 600 nm of 0.2 in NZ-amide-A. Diamide was then added to a final concentration of 250 μM.

introduced into WP551 (wild-type) and WP552 (*trxB*) by P1 transduction. Eliminating SecB in WP551 and WP552 had no affect of the amount of AP activity in these strains (data not shown). In addition, the expression level, stability, and rate of oxidation of APΔ2-22 was unaffected by the absence of SecB (data not shown).

DISCUSSION

In this paper, we have investigated the role of the thioredoxin and glutaredoxin systems in determining the thiol-disulfide balance in the cytoplasm. This balance is too reducing for many protein disulfide bonds to form in the cytoplasm of wild-type cells. We have shown that eliminating certain combinations of the components of the thioredoxin and glutaredoxin systems by mutation results in high levels of disulfide bond formation. We demonstrated this alteration of the redox environment of the cytoplasm by assaying for disulfide bond formation in a cytoplasmic version of the normally periplasmic alkaline phosphatase, and by sensitivity of cells to the thiol-specific oxidant, diamide.

A Second Function for Thioredoxin Reductase—To better interpret our results, it is first necessary to summarize our evidence suggesting a second thioredoxin molecule in the *E. coli* cytoplasm. We previously showed that mutants lacking thioredoxin reductase (*trxB*) exhibited high levels of cytoplasmic AP activity due to efficient formation of disulfide bonds in the protein (26). This disulfide bond formation was not simply

due to the absence of the reduced form of the known thioredoxin, since mutants lacking thioredoxin alone had little effect on AP activity. Furthermore, the properties of the *trxB* mutant could not be explained by its allowing the accumulation of oxidized thioredoxin, since the double mutant *trxB trxA* exhibited the same activity as the *trxB* mutant alone. These findings raised the possibility that the phenotype of *trxB* was due to a second function of the enzyme.

Results presented here strongly support that conclusion. Since *trxA gor* and *trxA gshA* mutants grow normally, while *trxB gor* and *trxB gshA* mutants are barely viable aerobically, it appears that thioredoxin reductase must be doing something in addition to its role in reducing the known thioredoxin. We suspect that our results are indicative of the existence of at least one additional thioredoxin in the *E. coli* cytoplasm for the following reasons. First, we expect that this second function of thioredoxin reductase must be such that it also contributes to the disulfide reducing environment of the cytoplasm. This expectation is based on the effects of the *trxB* mutants on disulfide bond formation in AP and on the finding that *trxB gor* and *trxB gshA* mutants require a disulfide reductant in the media to grow at a normal rate (Fig. 2). Second, thioredoxin reductase exhibits high substrate specificity for thioredoxins, and does not, for instance, act on glutaredoxins (16, 22).[6] Consistent with these results, we have recently found two open reading frames in the *E. coli* chromosome that appear to correspond to new cytoplasmic thioredoxins. We are currently seeking mutants of these open reading frames to determine whether their properties fulfill predictions from the reasoning presented here.[7,8] It should be noted that biochemical methods have failed to detect additional thioredoxins in *E. coli*.[6]

Which Are the Important Contributors to Maintaining Cysteines Residues in the Reduced State?—Those of our strains that are normally viable and that permit high levels of disulfide bond formation in AP are the *trxB* strain and the two double mutants, *trxA gor* and *trxA gshA*. We propose that, in the former strain, eliminating the reduction of the putative two thioredoxins is sufficient to dramatically increase disulfide bond formation in AP. With the latter two strains, while eliminating the known thioredoxin alone has little effect, combining the *trxA* mutation with mutations that totally inactivate the glutathione-glutaredoxin pathway does have a dramatic effect also. While this finding shows that it is not necessary to eliminate the function of the putative second thioredoxin to achieve high levels of disulfide bond formation, this molecule is probably still contributing to the thiol-disulfide redox environment, since the *trxB gor* and *trxB gshA* double mutants exhibit even higher disulfide bond formation than the three strains described above (Table III, Fig. 3). Thus, in the absence of any contribution from the two reductive pathways, disulfide bond formation is at its highest. Our results show that both the thioredoxin and glutaredoxin pathways contribute to keeping protein disulfide bonds reduced in the cytoplasm and that individual components of each pathway make some contribution to this redox activity.

Disulfide Bond Isomerization in the Cytoplasm?—We have assayed disulfide bond formation in the cytoplasm both by measuring the amount of active AP and, in some cases, by determining the amount of active urokinase. The latter protein requires the formation of six disulfide bonds for its activation. Elsewhere, we have shown that urokinase, when expressed in the *E. coli* periplasm is strongly dependent on DsbC, a known protein disulfide isomerase (46). This dependence is presumably due to the partial formation of incorrect disulfide bonds in urokinase. This finding raises the question of how formation of the proper disulfide bonds in urokinase can occur in the cytoplasm. It is not inconceivable that the cytoplasm also contains an isomerase. However, more likely is the explanation that the reducing environment of the cytoplasm in these mutants is sufficient to reduce disulfide bonds, particularly if the protein is not assembled into its most stable configuration. Thus, bonds may be reduced and reformed until the appropriate set of disulfide bonds has been assembled. Clearly, the cytoplasm of even the strongest mutant pairs we have examined must still contain disulfide reductants to maintain any cell viability (see discussion in next section).

Why Does E. coli Lacking Both the Thioredoxin and Glutaredoxin Pathways Grow so Poorly Aerobically?—One way to explain the poor aerobic growth of the *trxB gor* and *trxB gshA* strains is that an essential enzyme(s) is strongly dependent on the presence of at least one of the two reductive pathways, the glutaredoxin and thioredoxin systems. One candidate for such an enzyme is aerobic ribonucleotide reductase, which requires the reduction of a disulfide bond to complete its catalytic cycle and which, *in vitro*, can use thioredoxin or certain of the glutaredoxins for this purpose (16, 17). Since the anaerobic ribonucleotide reductase does not use the thioredoxin or glutaredoxin systems (47), the ability of these double mutants to grow well anaerobically would be predicted.

Alternatively, the *trxB gor* and *trxB gshA* mutants may grow poorly aerobically without a reductant because non-native disulfide bonds accumulate in a number of cytoplasmic proteins. Such "unwanted" disulfide bonds could affect the function and stability of cytoplasmic proteins, thus affecting cell physiology. However, our attempts to detect disulfide bonds in cytoplasmic proteins acquired in these strain backgrounds have yielded no indication that such bonds exist (data not shown). It may be that, in fact, many thiols of cytoplasmic proteins are buried and not available to form disulfide bonds (48).

Previous reports suggested that *trxB gor* and *trxB gshA* strains do not exhibit an aerobic growth defect, although the *trxB gor* strain described was not viable at 42 °C (38, 49). Suppressor mutations may have been unwittingly selected during the manipulations of these strains. We have shown that such suppressor mutations are quite common.

E. coli Likely Contains Other Protein Disulfide Reducing Pathways—In addition to our speculation that *E. coli* contains a second thioredoxin, the results with the strains constructed here indicate that there is likely to be a pathway or pathways in addition to the thioredoxin and glutaredoxin systems that can substitute for them. While the double mutants completely defective for both pathways grow very poorly aerobically, they do exhibit some growth. Furthermore, these poorly growing double mutants readily throw off suppressor mutations that allow rapid growth. These findings could be explained if there is, at least, one more lower activity system that maintains the thiol-disulfide balance in the cytoplasm and permits the slow growth of the double mutants. The suppressor mutations may increase the activity or the expression of such a system.

Further evidence that undiscovered disulfide reducing proteins are likely to exist in *E. coli* is provided by the fact that we have constructed a mutant lacking all three of the know reductants of the essential enzyme ribonucleotide reductase (thioredoxin, glutaredoxin 1, and glutaredoxin 3 (16, 17)). Thus, *E. coli* must contain at least one other protein that is capable of reducing ribonucleotide reductase *in vivo*.

Eliminating Some Components of the Thioredoxin and Glu-

[6] W. A. Prinz, F. Åslund, A. Holmgren, and J. Beckwith, unpublished results.
[7] E. Stewart and J. Beckwith, unpublished results.
[8] A. Jordan, F. Åslund, E. Pontis, P. Reichard, and A. Holmgren, submitted for publication.

taredoxin Systems Alters the Ability of Proteins to Form Disulfide Bonds in the Cytoplasm in Unexpected Ways—While it is clear that both the thioredoxin and glutaredoxin systems contribute to determining the thiol-disulfide balance in the cytoplasm, not all the differences in AP activity shown in Table II are explicable by a simple model. For instance, while cells missing glutaredoxin 3 allow some APΔ2-22 to become oxidized in the cytoplasm, cells missing both glutaredoxin 3 and thioredoxin do not allow any oxidation of APΔ2-22. However, it is known that mutations in certain genes of these pathways can alter the expression of the remaining components and thus change the thiol-disulfide balance in unexpected ways (45). Such compensatory responses to alteration of the redox balance might be expected to yield some confounding results in the assay for APΔ2-22 activation.

Another unexpected finding is that cells missing thioredoxin reductase were only slightly more sensitive to diamide than wild-type cells, but allowed a substantial amount of APΔ2-22 to form disulfides in the cytoplasm. Thus, there is probably some specificity in the disulfide bond reducing capability of the thioredoxin and glutaredoxin systems. The protein or proteins that are oxidized by diamide may be more susceptible to reduction by the glutaredoxin system than APΔ2-22. Diamide preferentially oxidizes GSH to GSSG (10) and may raise the level of GSH-mixed disulfides. The finding that glutaredoxins show high activity with such substrates (25) may explain why mutants lacking thioredoxin reductase, but containing an active glutaredoxin system, are only slightly more sensitive to diamide than wild-type cells.

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The Role of the Thioredoxin and Glutaredoxin Pathways in Reducing Protein Disulfide Bonds in the *Escherichia coli* Cytoplasm

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