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Thioredoxin or Glutaredoxin in *Escherichia coli* Is Essential for Sulfate Reduction but Not for Deoxyribonucleotide Synthesis

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We have shown previously that *Escherichia coli* cells constructed to lack both thioredoxin and glutaredoxin are not viable unless they also acquire an additional mutation, which we called X. Here we show that X is a *cysA* mutation. Our data suggest that the inviability of a *trxA grx* double mutant is due to the accumulation of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), an intermediate in the sulfate assimilation pathway. The presence of excess cystine at a concentration sufficient to repress the sulfate assimilation pathway obviates the need for an X mutation and prevents the lethality of a novel *cys*$^+$ *trxA grx* double mutant designated strain A522. Mutations in genes required for PAPS synthesis (*cysA* or *cysC*) protect cells from the otherwise lethal effect of elimination of both thioredoxin and glutaredoxin even in the absence of excess cystine. Both thioredoxin and glutaredoxin have been shown to be hydrogen donors for PAPS reductase (*cysH*) in vitro (M. L.-S. Tsang, J. Bacteriol. 146:1059–1066, 1981), and one or the other of these compounds is presumably essential in vivo for growth on minimal medium containing sulfate as the sulfur source. The cells which lack both thioredoxin and glutaredoxin require cystine or glutathione for growth on minimal medium but maintain an active ribonucleotide reduction system. Thus, *E. coli* must contain a third hydrogen donor active with ribonucleotide reductase.

Thioredoxin and glutaredoxin are small, heat-stable, redox-active proteins that have been isolated as hydrogen donors for ribonucleotide diphosphate reductase, an essential *Escherichia coli* enzyme that reduces ribonucleotides to deoxyribonucleotides (17, 18, 28). Glutaredoxin was discovered in a mutant of *E. coli* that lacked thioredoxin (*trxA*) (14); it has glutathione (GSH)-disulfide transhydrogenase activity (15, 16) which enables GSH to serve as a hydrogen donor for ribonucleotide reductase. Glutaredoxin, with 85 amino acid residues, has essentially no similarity in primary structure to thioredoxin, with 108 amino acid residues (13, 19); however, both of these proteins have a 14-member disulfide ring in the active center with the sequence -CXYC-, and it has been suggested that they have similar three-dimensional structures (2).

Glutaredoxin is markedly less abundant than thioredoxin (15), but it shows a lower K_m for ribonucleotide reductase (16), and it has been presumed that either glutaredoxin is the actual in vivo cofactor or glutaredoxin can substitute for thioredoxin in vivo as well as in vitro (17). Thioredoxin and glutaredoxin can participate in the following reactions as well (10): in the reduction of sulfate to sulfite by 3′-phosphoadenosine 5′-phosphosulfate (PAPS) reductase (29), an enzyme of the sulfate assimilation pathway leading to cysteine synthesis, and in the reduction of methionine sulfoxide to methionine by methionine sulfoxide reductases (5). Only in the latter case has the in vivo hydrogen donor been established; an *E. coli* methionine-requiring mutant cannot utilize exogenous methionine sulfoxide if it lacks thioredoxin, showing that thioredoxin is the main reductant (26).

Recently, we constructed a glutaredoxin null mutant (*grx*::*kan*) which grew as well as its wild-type parent under all conditions tested (25). Our initial efforts to isolate a strain lacking both thioredoxin and glutaredoxin failed, suggesting that one or the other of these proteins is required for viability. However, we finally succeeded in constructing such a strain; genetic data indicated that this Δ*trxA grx*::*kan* mutant, designated strain A410, was not viable until an additional mutation, which we referred to as X, had arisen. In this paper we show that X is a *cysA* mutation. We present data that suggest that the inviability of the Δ*trxA grx*::*kan* mutant is due to the accumulation of an intermediate in the sulfate assimilation pathway, PAPS. The presence of cystine at a concentration sufficient to inhibit the pathway, as well as mutations in genes required for PAPS synthesis (*cysA* or *cysC*), protects cells from the otherwise lethal effect of elimination of both thioredoxin and glutaredoxin.

Strains that lack thioredoxin and glutaredoxin maintain an active ribonucleotide reduction system. Thus, *E. coli* must contain a third hydrogen donor active with ribonucleotide reductase.

MATERIALS AND METHODS

Bacterial strains, media, and genetic techniques. The bacterial strains used in this study are listed in Table 1. P1 transductions were done as described previously (23, 25), and cotransduction of linked markers was determined by replica plating unless otherwise indicated. Transformation of cells, growth media, and plating of cells were as previously described (25, 26). Either cystine or cysteine satisfied the growth requirements of the mutants used in this study; cystine was used routinely. Tetracycline-sensitive cells were selected by the method of Maloy and Nunn (22).

As noted previously, strain A410 cells grow on minimal medium to which methionine (50 μg/ml) is added. This was seen also for an established *cysA* mutant and is a result that was obtained independently in both of our laboratories. This result is not in accord with presently known metabolic pathways in *E. coli* but has been observed previously (20). Contamination of the methionine with cystine or cysteine might account for this result. Alternatively, conversion of

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TABLE 1. *E. coli* strains

Strain	Relevant genotype	Source, reference, or construction
K38	HfrC *tonA22 garB10 ompF relA1 pit-10 spoT1* T2^r *phoA6*	Standard laboratory strain
IT1022	*ilvY864*::Tn*10*	27
A223	*ilvY864*::Tn*10 trxA*$^+$	K38 transduced to Tet^r by P1(IT1022)
A307	Δ*trxA*	K38 derivative (26)
A407	*grx*::*kan zbi*::Tn*10*	K38 derivative (25)
A408	*zbi*::Tn*10*	K38 derivative (25)
A410	Δ*trxA grx*::*kan* X1[a] *zbi*::Tn*10*	K38 derivative (25) X1 is *cysA*(X1)
A410-2[b]	Δ*trxA grx*::*kan* X2 *zbi*::Tn*10*	K38 derivative; X2 is *cysA*(X2)
A434	Δ*trxA grx*::*kan* X1	Tet^s derivative of A410
A435	Δ*trxA* X1 *zbi*::Tn*10*	A434 transduced to *grx*$^+$ by P1(A408)
A436	*grx*::*kan* X1 *ilvY864*::Tn*10*	A434 transduced to *trxA*$^+$ by P1(A223)
A437	Δ*trxA* X1	Tet^s derivative of A435
A438	*grx*::*kan* X1	Tet^s derivative of A436
A439	X1 *ilvY864*::Tn*10*	A437 transduced to *trxA*$^+$ by P1(A223)
A440	X1	Tet^s derivative of A439
A452	Δ*trxA cysC95*::Tn*10*	A307 transduced by P1(K1137)
A454	Δ*trxA cysC*	Cys$^-$ Tet^s derivative of A452
A522	*cys*$^+$ Δ*trxA grx*::*kan*	A307 transduced by P1(A407); see text
K1136	*nupC510*::Tn*10*	CGSC 6569 (SΦ1024)
K1137	*cysC95*::Tn*10*	CGSC 6656 (N3002)
K1138	Δ(*ptsI-cysA*)	CGSC 5914 (DG37)
JTG10	*gshA*::*kan*	(11)
A396	*gshA*::*kan*	K38 transduced by P1(JTG10)
A397	Δ*trxA gshA*::*kan*	A307 transduced by P1(JTG10)

[a] The X mutation present in A410 is referred to as X1 [i.e., *cysA*(X1)] to distinguish X-like mutations of independent origin.
[b] An equivalent series of *trxA*$^+$ or *grx*$^+$ derivatives were also constructed by P1 transduction.

methionine to cysteine as in eucaryotic cells could conceivably occur.

Preparation of extracts and in vitro assays. Cultures of strains A407, A408, and A410 were grown at 37°C with shaking in 500 ml of FB medium (25). The cells were harvested in log phase at an A_{650} of about 0.7 and were frozen at −20°C overnight. Fresh cleared lysates of gently lysed cells were prepared by treatment with lysozyme and osmotic shock of spheroplasts, followed by high-speed centrifugation. The activity of ribonucleotide reductase in the extract was determined with [^{3}H]CDP and NADPH, essentially as previously reported (14).

Log-phase cells (A_{650}, 0.7) from 200-ml cultures were also permeabilized with ether as described by Warner (30) and were assayed for endogenous ribonucleotide reductase activity with [^{3}H]CDP and dithiothreitol (final concentration, 40 mM). The cell suspensions (10^{10} cells per ml) were stored frozen at −70°C.

RESULTS

Properties of the mutant strains. The properties of *E. coli* A410, which lacks thioredoxin and glutaredoxin, are summarized in Table 2. Because A410 has several puzzling phenotypes (mucoidy and a cell separation defect) in addition to the cysteine requirement, a second derivative was isolated by transducing thioredoxin deletion strain A307 with a *zbi*::Tn*10 grx*::*kan* P1 lysate and selecting on rich (Ty) plates, as described previously (25). The properties of this strain, A410-2, are also presented in Table 2, along with the properties of parental control strains A307 (Δ*trxA*), A407 (*grx*::*kan zbi*::Tn*10*), and A408 (*zbi*::Tn*10*). The three control strains grew on defined medium in the absence of amino acids, while A410 and A410-2 did not. Addition of cystine, cysteine, or glutathione enabled these strains that lack thioredoxin and glutaredoxin to grow. Strain A410 formed filaments of septated, but unseparated cells, and the colonies that it produced were mucoid, especially on minimal medium. In contrast, A410-2 formed neither filaments nor mucoid colonies. The basis for these differences is not understood, but these properties are not obligatorily coupled to the survival of Δ*trxA grx*::*kan* cells. Additional Δ*trxA grx*::*kan* transductants, which, like A410, were derived from strain K38, also required cystine for growth; this requirement was not a fortuitous result of mutations present in the K38 background (Table 1), since Δ*trxA grx*::*kan* derivatives of strain W3110 also had a cystine requirement (data not shown).

TABLE 2. Some growth properties of *trxA grx*::*kan* mutant strains

Strain	Genotype			Phenotype		
	trxA	*grx*	X	Mucoidy[a]	Cell separation defect	Cys required[b]
K38	+	+	+	No	No	No
A307	−	+	+	No	No	No
A407	+	−	+	No	No	No
A408	+	+	+	No	No	No
A410	−	−	−	Yes	Yes	Yes
A410-2	−	−	−	No	No	Yes

[a] On Ty plates.
[b] On minimal medium containing 1 mM sulfate.

To determine whether the inability of A410 and A410-2 to grow on defined medium without a reduced sulfur source is a physiological consequence of having neither thioredoxin nor glutaredoxin or whether it reflects a genetic change, thioredoxin or glutaredoxin or both were restored. Introduction of the wild-type *trxA* gene on a high-copy-number plasmid did not eliminate the requirement, nor did it alter the cell separation or mucoidy phenotypes of A410 derivatives (Table 3). The wild-type genes were also restored by P1 transduction (Fig. 1). Introduction of the wild-type *grx* gene (and elimination of the mutant allele) into A410 did not alter these properties, but replacement of the Δ*trxA* allele by transduction to *trxA*$^+$ eliminated the cell separation defect of A410 (Table 3). This suggests that the cell separation defect is genetically, but not functionally, linked to the Δ*trxA* allele. The derivatives of A410 and A410-2 which carried *trxA*$^+$ or *grx*$^+$ or both still required cystine for growth. Thus, the inability of parental strains A410 and A410-2 to grow without cystine reflects a genetic change.

A *trxA gshA* double mutant, strain A397, which lacked thioredoxin and glutathione synthase, also required cystine and showed a growth defect even in rich medium (Table 3) (7); in contrast to the results obtained with mutants that lacked thioredoxin and glutaredoxin, restoration of thiore-

TABLE 3. Properties of mutants after restoration of *trxA*$^+$ or *grx*$^+$

Strain	Genotype				Phenotype		
	trxA	*grx*	*gsh*	X[a]	Mucoidy[b]	Cell separation defect	Cys required[c]
A410	−	−		−	Yes	Yes	Yes
A410-2	−	−		−	No	No	Yes
A434	−	−		−	Yes	Yes	Yes
A437	−	+		−	Yes	Yes	Yes
A438	+	−		−	Yes	No	Yes
A440	+	+		−	Yes	No	Yes
A440-2	+	+		−	No	No	Yes
A434(pPMR18)[d]	+	−		−	Yes	Yes	Yes
A437(pPMR18)	+	+		−	Yes	Yes	Yes
A396	+		−	+	No	No	No
A397	−		−	+	No	No	Yes
A397(pPMR18)	+		−	+	No	No	No

[a] −, Mutant allele of the X gene.
[b] On Ty plates.
[c] On minimal medium containing 1 mM sulfate.
[d] pPMR18 contains the *trxA*$^+$ gene (26).

doxin reversed the cystine requirement and cured the growth defect of A397 (Table 3) (7).

Characterization of the cystine requirement in Δ*trxA grx*::*kan* mutants. Strain A410 cells grew on sulfite as a sulfur source, which is consistent with a block in sulfate conversion to PAPS via the known metabolic reactions in the cysteine biosynthetic pathway (Fig. 2). Gillespie et al. (9) reported that *cysH* mutants of *Salmonella typhimurium*, which were blocked in the reduction of PAPS, accumulated secondary mutations in *cysA*, *cysC*, and *cysD*, genes involved in steps in cysteine biosynthesis that precede the reaction controlled by *cysH* (Fig. 2). These authors suggested that *cysH* mutant cultures might accumulate a biosynthetic intermediate or by-product that is deleterious and that mutations which prevent this accumulation might be selected. The *cysH* gene encodes PAPS reductase (20), the enzyme whose in vitro activity requires thioredoxin or glutaredoxin (29). We reasoned that in the absence of thioredoxin and glutaredoxin, the toxic intermediate postulated by Gillespie et al. (9) might accumulate in *cysH*$^+$ cells, creating selection for mutants blocked earlier in the pathway, and so the mutation responsible for the cystine requirement of A410 was mapped. The *nupC* gene of *E. coli*, at ca. 52 min, is closely linked to *cysA* (sulfate permease) and to *cysM* and *cysK* (cysteine synthase isozymes) (20, 24). Strains A437 and A437-2, the *grx*$^+$ derivatives of A410 and A410-2, were transduced to Tet^r by using a lysate prepared on a *cysA*$^+$ *nupC*::Tn*10* strain. In each case, about 60% of the Tet^r transductants had become Cys$^+$, the same frequency that was obtained when a known *cysA* mutant strain (K1138) was used as the recipient. A plasmid (pRSM18) containing the *cysA* gene, but not one (pRSM28) containing the *cysK* gene, from *Salmonella typhimurium* (24) eliminated the cystine requirement of A437 and A437-2, as well as that of K1138 (data not shown). Thus, these strains are *cysA* mutants.

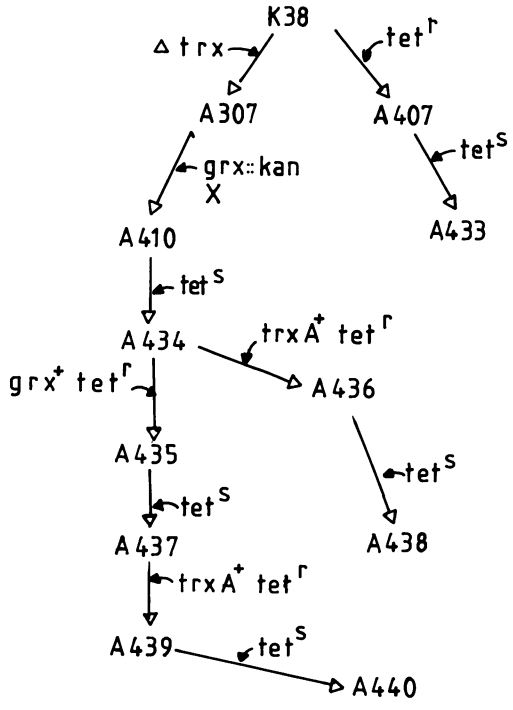

FIG. 1. Genealogy of *E. coli* strains constructed. See also Table 1.

Inhibition of PAPS synthesis enables Δ*trxA grx*::*kan* mutants to survive. The sulfate assimilation pathway is subject to feedback inhibition by cyst(e)ine (20). If the lethality due to the absence of thioredoxin and glutaredoxin is due to PAPS accumulation, cyst(e)ine at high concentration should inhibit the pathway and eliminate the lethality. Table 4 shows that this is the case. The *zbi*::Tn*10* and *grx*::*kan* mutations are very closely linked; as shown previously, almost all of the Tet^r transductants of a *trxA*$^+$ strain become *grx*::*kan*. When transductants were selected on defined medium containing 0.1% Casamino Acids (conditions permissive for *cysA* and *cysC* mutants), the number of Tet^r transductants of Δ*trxA* strain A307 was low with the *zbi*::Tn*10* *grx*::*kan* donor (compared with the control transduction with a *zbi*::Tn*10* *grx*$^+$ donor), and none of the transductants became *grx*::*kan*. This linkage distortion confirmed our previous observation that Δ*trxA grx*::*kan* mutant strains are not viable under these conditions (25). However, when Tet^r transductants of Δ*trxA* strain A307 were selected on the same defined medium that contained additional cystine (15 μg/ml), normal linkage was restored. One of these new Δ*trxA grx*::*kan* transductants was saved as strain A522. Although this strain also tended to accumulate better-growing derivatives, it was genetically *cys*$^+$ even though it required high concentrations of cystine for growth; unlike what happened with A410, when thioredoxin or glutaredoxin was restored to A522 (by introducing the genes on a plasmid), A522 grew in the absence of cystine (data not shown). Presumably, while the cystine concentration in 0.1% Casamino Acids is sufficient to support growth of a *cys* mutant, it is not sufficient to inhibit the sulfate assimilation pathway. Similar results were obtained with a Δ*trxA* derivative of strain W3110 (data not shown).

To further test the idea that accumulation of a toxic intermediate is lethal to cells that lack thioredoxin and glutaredoxin, a Δ*trxA cysC* double mutant strain was constructed, and this strain was transduced to Tet^r (without excess cystine) by using the *zbi*::Tn*10* and *zbi*::Tn*10* *grx*::*kan* donors. The *cysC* mutation protected *trxA grx* mutant cells (Table 5). Unlike the linkage distortion that occurs with a Δ*trxA* mutant, the Δ*trxA cysC* double mutant strain was efficiently transduced to Tet^r regardless of which

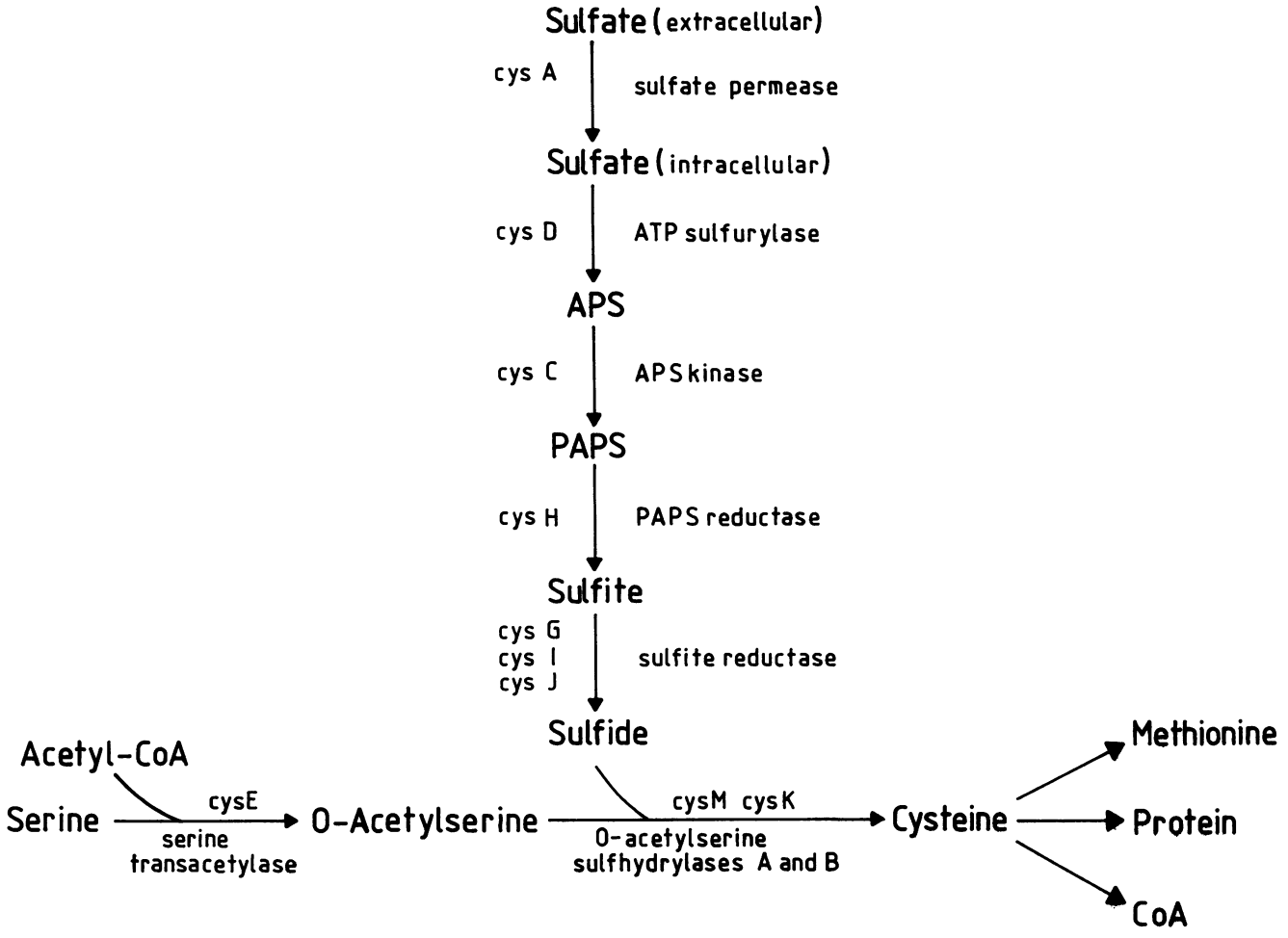

FIG. 2. Outline of cysteine biosynthesis in *E. coli*. The pathway is modified from the pathway described in reference 20. APS, Adenosine 5'-phosphosulfate; CoA, coenzyme A.

donor was used, and most of the transductants had become *grx*::*kan*. The protective effect of a *cysC* mutation, which blocks the conversion of adenosine 5'-phosphosulfate to PAPS (Fig. 2), suggests that PAPS is the toxic intermediate.

Cells lacking thioredoxin and glutaredoxin have an active system for the reduction of ribonucleotides. Previously, it was shown that a heated (85°C) extract of *E. coli* A410 cells did not function as a hydrogen donor for ribonucleotide reductase in vitro with NADPH, 4 mM GSH, and glutathione reductase or with NADPH and thioredoxin reductase (25). This result was consistent with the total absence of both thioredoxin and glutaredoxin, which was also demonstrated by using a radioimmunoassay. Furthermore, it was consistent with the absence of any major heat-stable hydrogen donor activity that could be reduced either by GSH and glutathione reductase or by thioredoxin reductase. To test whether *E. coli* A410 cells had active ribonucleotide reductase, either ether-treated cells or high-speed supernatants were used. The ether-permeabilized cells all had active ribonucleotide reductase when they were assayed with a high dithiothreitol concentration (Table 6). Thus, they contained an active ribonucleotide reductase system, whether or not thioredoxin or glutaredoxin or neither was present.

High-speed supernatants of lysozyme-lysed, exponentially growing *E. coli* cells contain a labile, high-activity form

TABLE 4. High cystine concentration prevents lethality of *trxA grx* mutations[a]

Recipient	Donor	Results in medium containing:			
		CAA[b]		CAA + 0.062 mM cystine	
		No. of Tet^r transductants	% Kan^r	No. of Tet^r transductants	% Kan^r
K38 (*trxA*$^+$)	*zbi*::Tn*10*	44		40	
A307 (Δ*trxA*)	*zbi*::Tn*10*	34		21	
K38	*zbi*::Tn*10* *grx*::*kan*	35	100	31	97
A307	*zbi*::Tn*10* *grx*::*kan*	2	0	41	95

[a] The recipient strains were transduced by approximately equal titers of P1 phage lysates prepared on strain A408 (*zbi*::Tn*10*) or on strain A407 (*zbi*::Tn*10* *grx*::*kan*). Tetracycline-resistant transductants were selected on minimal plates containing 0.2% glucose, 5 μg of thiamine per ml, and 0.1% Casamino Acids with or without additional cystine (0.062 mM).

[b] CAA, Casamino Acids.

[c] % Kan^r, Cotransduction of *grx*::*kan* was determined by scoring the growth of each transductant on plates that contained kanamycin and tetracycline.

TABLE 5. *cysC* mutation prevents lethality of *trxA grx* double mutations[a]

Recipient	Donor	No. of Tet^r transductants	No. of Kan^r transductants/ no. of Tet^r transductants (%)
K38 (*trxA*$^+$)	*zbi*::Tn*10*	161	
A307 (Δ*trxA*)	*zbi*::Tn*10*	152	
A454 (Δ*trxA cysC*)	*zbi*::Tn*10*	35	
K38	*zbi*::Tn*10 grx*::*kan*	294	35/38 (92)
A307	*zbi*::Tn*10 grx*::*kan*	18[b]	0/14 (<7)
A454	*zbi*::Tn*10 grx*::*kan*	32	20/21 (95)

[a] The recipient strains were transduced by approximately equal titers of P1 phage lysates prepared on strain A408 (*zbi*::Tn*10*) or on strain A407 (*zbi*::Tn*10 grx*::*kan*). Tetracycline-resistant transductants were selected on minimal plates containing 0.2% glucose, 5 μg of thiamine per ml, and 0.1% Casamino Acids; cotransduction of *grx*::*kan* was determined by picking transductants onto plates that contained kanamycin and tetracycline.

[b] After several days of incubation at 37°C, additional, very small colonies appeared on the selective plates; they were Tet^r Kan^r *cys*.

of ribonucleotide reductase when they are assayed with NADPH and dithiothreitol as the ultimate hydrogen donors (3) or with only NADPH (14). The wild-type and mutant extracts gave similar activities with NADPH as the ultimate hydrogen donor (Table 7); if anything, the mutant (A410) lacking both thioredoxin and glutaredoxin was more active in ribonucleotide reduction, strongly suggesting that an unknown third hydrogen donor was present.

DISCUSSION

The construction of a double mutant without thioredoxin and glutaredoxin would a priori be expected to result in a strain without functional ribonucleotide reductase, an essential enzyme (6–8, 28). Quite unexpectedly, we found previously (25) that we could obtain a double mutant (A410) but that it had to acquire a compensating mutation, X, before it could grow. Since strain A410 cells can grow on glucose minimal medium to which only glutathione, cystine, or methionine has been added, they must be using endogenous nucleotides and thus be dependent on ribonucleotide reductase. In this paper we show that X is *cysA* and that the cystine requirement of A410 is due to this genetic defect. Selection for *cysA* mutations probably occurs because of the toxic effects of PAPS accumulation. Thus, since each single mutant does not require cystine, thioredoxin and glutaredoxin can effectively substitute for each other in sulfate reduction.

By adding excess cystine during the P1 transduction, a true *trxA grx* double mutant (strain A522) was obtained. The A522 cells grow only when minimal medium is supplemented with excess cystine and are thus phenotypically cystine auxotrophs. However, they are strictly *cys*$^+$ genetically since they grow on minimal medium when thioredoxin or glutaredoxin is provided by a plasmid. The A522 cells should be useful for analysis of the functional redox properties of thioredoxin or glutaredoxin mutants. In addition, strain A522 may be useful for cloning glutaredoxin or thioredoxin genes from other species or in studies of PAPS metabolism.

Kren et al. (21) have isolated a strain with reduced glutaredoxin activity in a thioredoxin reductase (*trxB*) mutant background. This strain requires cystine for growth and shows a growth defect at high temperatures. Both defects are relieved by introduction of a plasmid which expresses normal glutaredoxin. On the basis of these results, Kren et al. (21) postulated that *grx* is a required gene. This explanation cannot be correct, since we have shown that a strain which contains an internal deletion and a kanamycin resistance cassette within the *grx* gene and lacks glutaredoxin, as determined by enzymatic activity and by radioimmunoassay, grows normally (25). An alternative explanation for the results of Kren et al. (21) is that glutaredoxin deficiency in the strain lacking thioredoxin reductase leads to the cystine requirement and that the toxic effects of the accumulation of PAPS account for the growth defect. The same explanation can account for the properties of cells lacking thioredoxin and glutathione synthase. The requirement for cystine in these two cases could reflect both a direct nutritional need

TABLE 6. Activity of ribonucleotide reductase in ether-permeabilized *E. coli* cells[a]

Strain or prepn	Amt (μl) of ether-treated cells	Amt of dCDP (pmol/20 min)
A407	5	43
	10	61
	20	84
A408	5	76
	10	120
	20	199
A410	5	142
	10	231
	20	400
No cells		9

[a] The cell suspensions (10^{10} cells per ml) were permeabilized with ether (30) and stored at −70°C before they were assayed in a final volume of 125 μl containing 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 8.4), 8 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM [^{3}H]CDP (40,000 cpm/nmol), and 40 mM dithiothreitol. The preparations were incubated at 37°C for 20 min, and dCDP levels were determined (14).

TABLE 7. Activity of ribonucleotide reductase in high-speed supernatants of *E. coli* cells[a]

Strain or prepn	Amt (μl) of extract added	Amt of dCDP (pmol/20 min)
A407	10	138
	20	336
A408	10	244
	20	421
A410	10	421
	20	698
No extract		127

[a] Cells were thawed in 3.75 times their weight of 50 mM Tris hydrochloride (pH 7.5)–10% sucrose and again frozen in an ethanol-dry ice bath. After the addition of 0.1 M NaCl and 0.5 mg of hen egg white lysozyme (Sigma Chemical Co.) per ml, mixtures were incubated for 1 h at 0°C and for 6 min at 37°C. Then 10 μg of DNase I (Sigma) per ml and 10 mM $MgSO_4$ were added, and incubation was continued for 30 min at 4°C. High-speed supernatants were prepared by centrifugation in a model TL-100 benchtop ultracentrifuge (Beckman Instruments, Inc.), using a model TL 100-2 rotor (50,000 rpm for 30 min). The supernatants were withdrawn and frozen at −70°C; they contained, for strains A407, A408, and A410, 12.2, 15.3, and 20.3 mg of protein per ml, respectively. Ribonucleotide reductase activity was determined by 20-min incubations at 37°C in a final volume of 120 μl containing 83 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.6), 16 mM $MgCl_2$, 5 mM ATP, 1.25 mM [^{3}H]CDP (40,000 cpm/nmol), and 1 mM NADPH. The amount of dCDP formed was determined after acid hydrolysis as described previously (15).

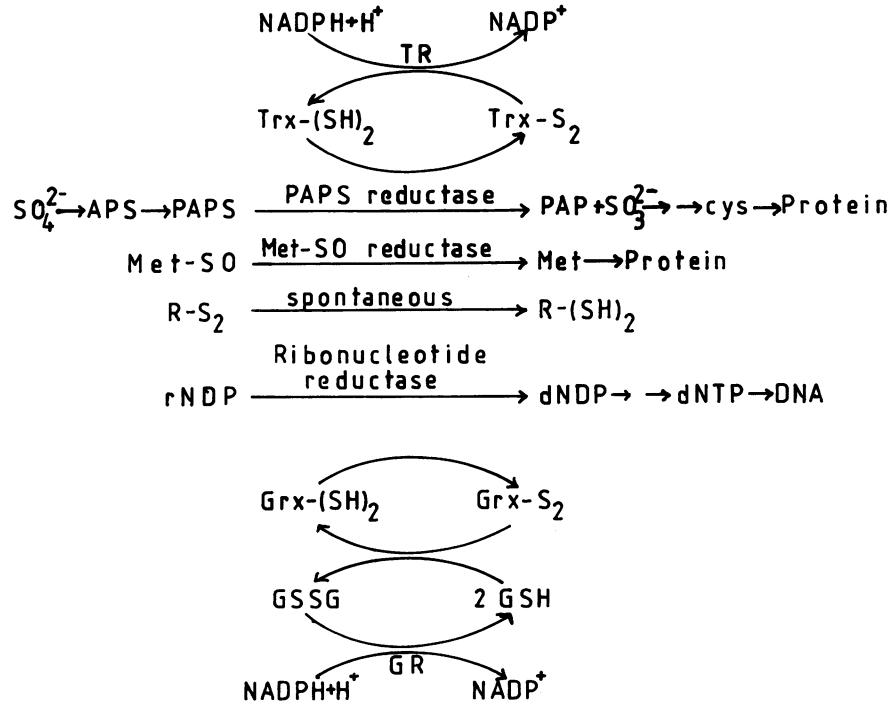

FIG. 3. Reactions in which the thioredoxin or glutaredoxin systems are implicated in *E. coli*, including sulfate reduction, methionine sulfoxide reduction (either free or in peptide linkage), protein or low-molecular-weight ($R-S_2$) disulfide reduction, and reduction of ribonucleotides to deoxyribonucleotides. TR, thioredoxin; APS, adenosine 5′-phosphosulfate; Met-SO, methionine sulfoxide; rNDP, ribonucleoside diphosphate; dNDP, deoxyribonucleoside diphosphate; dNTP, deoxyribonucleoside triphosphate; GSSG, glutathione (oxidized); GR, glutathione reductase.

and a role for cystine as a negative regulator of the PAPS biosynthesis pathway (20).

With respect to ribonucleotide reductase, there must be a third hydrogen donor system even in nonmutant cells. However, glutaredoxin and thioredoxin are likely to be important for ribonucleotide reduction in normal cells. Support for this hypothesis comes from studies of DNA metabolism in bacteriophage T4. T4 encodes a ribonucleotide reductase and a thioredoxin (28). T4 thioredoxin is homologous to *E. coli* glutaredoxin in structure (2, 19) and has glutaredoxin activity by catalyzing GSH-dependent reduction of T4 ribonucleotide reductase; it is also a substrate for *E. coli* thioredoxin reductase and therefore maximizes the transfer of reducing equivalents to T4 DNA precursor synthesis (17).

Ribonucleotide reduction in *E. coli* is more complex than has hitherto been thought. The ribonucleotide reductase encoded by the *nrdA* and *nrdB* genes was defined as essential by the isolation of *nrd* mutants that are not viable at high temperatures (6–8). Hantke (12) has recently isolated *E. coli* mutants with large insertions in *nrdB*; these mutants grow anaerobically and, if depleted of iron, aerobically. Barlow (1) has found that a temperature-sensitive *nrdA nrdB* double mutant grows anaerobically at the nonpermissive temperature even in the presence of hydroxyurea, an inhibitor of the tyrosyl radical-containing *nrdB*-encoded B2 subunit. Furthermore, an oxygen-sensitive new ribonucleoside triphosphate reductase activity has been discovered in anaerobically grown *E. coli* by Fontecave et al. (4).

The reactions in which thioredoxin and glutaredoxin are known to participate are shown in Fig. 3. For most purposes the two proteins can substitute for each other. The reduction of methionine sulfoxide is specifically dependent on thioredoxin in vivo (26), but if increased levels of glutaredoxin are provided from a high-copy-number plasmid, slow growth of a methionine auxotroph on methionine sulfoxide can be obtained (unpublished data). Both ribonucleotide reduction and sulfate reduction are essential reactions in *E. coli* grown on minimal medium containing sulfate; without the first reaction no DNA synthesis is possible, and without the second no synthesis of proteins or coenzymes with reduced sulfur (coenzyme A, biotin, lipoic acid, etc.) would occur. Since either a *trxA* mutant or a *grx* mutant is viable, thioredoxin and glutaredoxin can replace each other in sulfate reduction reactions; however, one of the two is essential. Ribonucleotide reduction, either aerobically or anaerobically (25), can occur in the absence of these proteins. Thus, a third hydrogen donor for ribonucleotide reduction exists. In this paper we present evidence that there is an NADPH-linked system in crude extracts that remains to be characterized. It is possible that this third hydrogen donor is coupled with either the activity found in anaerobically grown cells (1, 4) or the classical *nrd*-encoded ribonucleotide reductase.

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