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Isolation of an *Escherichia coli* Mutant Deficient in Thioredoxin Reductase

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A mutant of *Escherichia coli* defective in thioredoxin reductase has been isolated and partially characterized. This mutant has no detectable thioredoxin reductase activity in vitro and yet it exhibits no in vivo defect in reduction of ribonucleotides. Evidence is presented that indicates that, in cells permeabilized via ether treatment, ribonucleoside diphosphate reduction can utilize glutathione as an alternate reducing system.

Ribonucleoside diphosphate (RDP) reduction in *Escherichia coli* constitutes the first metabolic reaction unique to deoxyribonucleic acid (DNA) synthesis. This reaction is catalyzed by the enzyme RDP reductase which consists of two nonidentical subunits, proteins B1 and B2, which form an active complex in the presence of magnesium ions (5). The physiological hydrogen donor for this reaction is believed to be reduced nicotinamide adenine dinucleotide phosphate (NADPH). For NADPH to serve as a hydrogen donor, two additional proteins, thioredoxin and thioredoxin reductase, are required (1, 15). Thioredoxin reductase, a flavoprotein that contains an active center oxidation-reduction disulfide, catalyzes the transfer of reducing potential from NADPH to thioredoxin. RDP reductase catalyzes the reduction of ribonucleoside diphosphates using reduced thioredoxin as the hydrogen donor. This reaction was recently shown to involve an oxidation-reduction active disulfide of subunit B1 and to occur by a ping-pong mechanism (22). The overall reaction sequence for the transfer of electrons from NADPH to ribonucleotides is shown in Fig. 1.

Brown et al. found that direct reduction of subunit B1 by dithiothreitol can bypass the requirement for this "thioredoxin system"; however, the rate of ribonucleotide reduction was greatly decreased in the absence of thioredoxin (4). The rate of reduction of ribonucleotides using direct reduction of thioredoxin by dithiothreitol was equal to the rate in the presence of thioredoxin, thioredoxin reductase, and NADPH. In conditions more closely approximating physiological conditions, cells made permeable to nucleotides were found to have maximal ribonucleotide reductase activity in the presence of both dithiothreitol and NADPH (24).

Thioredoxin systems similar to the *E. coli* system have been purified from yeast (18), regenerating rat liver cells (11, 12), ascites hepatoma cells (8), and *Lactobacillus* (17). A different type of thioredoxin system was recently isolated from *Euglena* (16). In addition to its participation in the reduction of ribonucleotides, the "thioredoxin system" from yeast has been shown to participate in the in vitro reduction of sulfate and in the in vitro reduction of methionine sulfoxide (19). The thioredoxin system isolated from *E. coli* was able to substitute for the yeast thioredoxin system in these reactions (19). Yeast appear to have separate methionine sulfoxide reductase enzymes for the reduction of the two isomers of L-methionine sulfoxide since partial purification of the enzyme causes a loss of the ability to reduce L-methionine-*d*-sulfoxide (2). Although methionine sulfoxide reductase has not been purified from *E. coli*, intact cells can reduce all four isomers of methionine sulfoxide (7). Recently, Tsang and Schiff (23) reported that a protein apparently identical to thioredoxin participates in sulfate reduction in *E. coli*. Host thioredoxin has also been found to constitute one of the subunits of the *E. coli* bacteriophage T7 DNA polymerase (14).

In this paper the isolation of an *E. coli* mutant deficient in thioredoxin reductase is reported. The mutant does not appear to be defective in ribonucleotide reduction in vivo in spite of having little or no detectable thioredoxin reductase activity in vitro, and utilization of methionine sulfoxide is only slightly diminished. The mutant supports the growth of T7 phage and can use sulfate as its sole sulfur source.

MATERIALS AND METHODS

Materials. [^{3}H]uridine 5'-diphosphate was purchased from Schwarz/Mann and was purified before

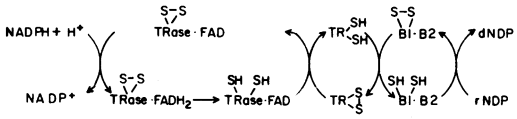

FIG. 1. *Transfer of electrons from NADPH to ribonucleotides. TRase, thioredoxin reductase. TR, thioredoxin. B1 · B2, subunits of RDP reductase.*

use. The [^{3}H]uridine 5'-diphosphate was concentrated and streaked on a thin-layer plate (20 by 20 cm) of polyethyleneimine cellulose and chromatographed as follows: 1 M ammonium acetate containing 2.5% boric acid was allowed to migrate to 1 cm above the origin, the plate was immediately placed in a solvent of 2 M ammonium acetate containing 5% boric acid, and the solvent was allowed to migrate to 15 cm above the origin. Solvent salts were removed by washing the polyethyleneimine cellulose plate in 100 ml of anhydrous methanol containing 125 mg of tris(hydroxymethyl)aminomethane and then in 100 ml of anhydrous methanol. The [^{3}H]uridine 5'-diphosphate was eluted with 1 M triethylammonium bicarbonate (pH 8.1). The triethylammonium bicarbonate was removed by lyophilizing twice.

L-[^{14}C]methionine was purchased from Amersham/Searle Corp. and was converted to L-[^{14}C]methionine-*d,l*-sulfoxide by the method of Greene (7) except that the procedure was scaled down 1,000-fold and the precipitates were collected by centrifugation rather than by filtering. Greater than 99.9% of the ^{14}C product co-chromatographed with authentic L-methionine-*d,l*-sulfoxide.

A phase-combining system was obtained from Amersham/Searle.

Thioredoxin and thioredoxin reductase were prepared from *E. coli* K-12 as described previously (6).

Methionine sulfoxide reductase was prepared from yeast as described by Block et al. (2) except that the product of their purification scheme was further purified by gel filtration on Sephadex G-50. The methionine sulfoxide reductase was free of any detectable yeast thioredoxin or thioredoxin reductase activity.

Bacterial strains. The strains used in this study are derivatives of *E. coli* KK1004 (6) which is *metB upp udk thyA* (Ts). KK1006 used as the parental strain in this study was obtained from strain KK1004 without mutagenesis by selecting a derivative that would form distinct colonies on the background growth that occurs in minimal agar supplemented with 2 μg of thymine per ml. Strain KK1006 is not sensitive to deoxyadenosine and thus is presumably a *drm* derivative of strain KK1004.

Growth of bacteria. The media used and growth of cells were as described previously (6). When methionine sulfoxide was used in place of methionine, it was at a concentration of 30 μg/ml. Mutagenesis and penicillin treatment were carried out as described by Karlström (10).

Thioredoxin reductase assays. Thioredoxin reductase activity was determined in a coupled assay. In a total volume of 25 μl the following components were added: 2.4 μg of yeast L-methionine-*l*-sulfoxide reductase, 250 pmol of *E. coli* thioredoxin, 2.5 μl of 0.5 M Tris-chloride (pH 7.7), 10 nmol of NADPH, 10.6 nmol (55 nCi) of L-[^{14}C]methionine-*d,l*-sulfoxide, and crude *E. coli* extract. Incubation at 37°C for 15 min was followed by heating for 2 min at 100°C to stop the reaction. A total of 10 μl of the reaction mix was spotted on cellulose-thin layer plates and chromatographed in a solvent containing isopropanol-formic acid-water (80:5.4:20). The areas corresponding to ninhydrin-reacting L-methionine in parallel channels of the plate were cut out and placed in a scintillation vial. One milliliter of water was added to elute the L-[^{14}C]methionine from the cellulose, and 10 ml of a 2:1 mixture of phase-combining system-xylene scintillation solution was added and counted in a Beckman LS235 liquid scintillation spectrometer.

Thioredoxin assay. Thioredoxin assays were conducted in a manner similar to thioredoxin reductase assays. Thioredoxin and NADPH were omitted from the above assay and 32 nmol of dithiothreitol was added.

RDP reductase assay of ether-treated cells. Cells were grown, harvested, ether treated and assayed as described previously (6) with the following modifications: [^{3}H]uridine 5'-diphosphate was used as substrate at a concentration of 2 mM (1.4×10^4 cpm/nmol) and adenosine triphosphate was replaced by thymidine triphosphate (0.4 mM). To prevent breakdown of the product and to serve as a carrier for chromatography, 3 mM deoxyuridine 5'-monophosphate was added to the reaction mix. Essentially all of the deoxyuridine produced in these assays was recovered as deoxyuridine 5'-monophosphate presumably due to the action of nucleoside diphosphate kinase and deoxyuridine 5'-triphosphate pyrophosphatase. A total of 10 μl of reaction mix was spotted onto polyethyleneimine-impregnated cellulose thin-layer plates at 5-min intervals. The deoxyuridine 5'-monophosphate was separated from uridine ribonucleotides by chromatography in a solvent containing 30 ml of ethylene glycol, 70 ml of water, 6 g of sodium borate and 3 g of boric acid. The deoxyuridine 5'-monophosphate spot was cut out, eluted, and counted as described previously (6).

DNA synthesis. An exponential culture was grown in minimal media containing methionine (30 μg/ml), deoxyadenosine (100 μg/ml), and [^{14}C]thymidine (2 μg/ml [0.5 μCi/ml]). At indicated times, 50 μl of culture was transferred to 1-inch (2.54-cm) diameter disks of Whatman no. 1 filter paper, and the disks were immersed in 5% trichloroacetic acid containing 1% $Na_4P_2O_7 \cdot 10H_2O$. The disks were washed twice in the trichloroacetic acid, then twice in 95% ethanol, and then twice in ether, and air dried (3). The disks were counted in a scintillation solution containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2]-(5-phenyloxazolyl)benzene in toluene.

RESULTS

Rationale for selection. Exogenous deoxynucleosides cannot be utilized by *E. coli* for DNA synthesis, with the exception of thymidine and deoxyuridine, due to the absence of deoxynucleoside kinases. Thus, mutants unable to syn-

thesize deoxynucleotides can only be obtained as conditional mutants. Since *E. coli* appeared to require thioredoxin and thioredoxin reductase for ribonucleotide reduction as well as for the reduction of methionine sulfoxide, conditional mutants deficient in thioredoxin or thioredoxin reductase were sought. In a methionine auxotrophic strain, a mutant with a partially defective thioredoxin or thioredoxin reductase, that had sufficient activity for ribonucleotide reduction but not sufficient activity to simultaneously support both reduction of ribonucleotides and the methionine sulfoxide required for growth, could exist. Such a mutant might be expected to grow when supplied with methionine rather than with methionine sulfoxide, thus sparing the thioredoxin system for use in ribonucleotide reduction. If such a mutant had a defective thioredoxin reductase with increased thermolability, no growth would be expected at higher temperatures even on enriched media.

The methionine auxotroph, KK1006, was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, phenotypically expressed and then subjected to counterselection by penicillin in minimal media containing methionine sulfoxide as the sole source of methionine. Three hundred survivors were tested for their ability to grow on minimal media containing either methionine or methionine sulfoxide. Three mutants that grew better on methionine than on methionine sulfoxide at 30°C were isolated. Two of these mutants failed to grow at 42°C on enriched plates. One of these, KK1048, was studied in detail.

Thioredoxin activity in KK1048 and its parent, KK1006, was assayed by coupling oxidation of dithiothreitol via thioredoxin to reduction of methionine sulfoxide. Excess yeast L-methionine-*l*-sulfoxide reductase was added to make the rate of reduction of L-methionine sulfoxide dependent on the amount of thioredoxin present. No significant difference in thioredoxin activity was observed.

Thioredoxin reductase activity was quantitated in a similar manner, except that NADPH replaced dithiothreitol, and excess *E. coli* thioredoxin was added to make the rate of reduction of methionine sulfoxide proportional to the amount of thioredoxin reductase activity present. Even at concentrations of crude extract sufficient to saturate the reaction mix of the parent with thioredoxin reductase, the mutant, KK1048, has negligible thioredoxin reductase activity (Fig. 2). This mutant also had negligible thioredoxin reductase when assayed at different temperatures (Table 1). Since the mutant thioredoxin reductase activity may be unstable and may not survive lysing of the cells, whole cells made permeable to NADPH by treatment with 1% toluene were assayed for thioredoxin reductase activity. Again, essentially no activity was detected in KK1048, in contrast to an activity of 6.5 nmol of methionine formed per minute per milligram of protein in KK1006.

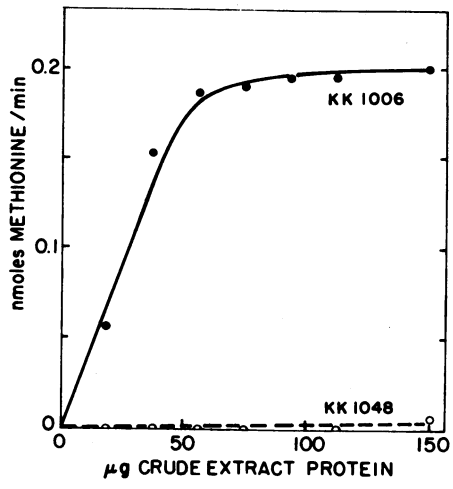

FIG. 2. *Thioredoxin reductase activity in crude extracts of strains KK1006 and KK1048. Incubation mixture contained yeast methionine sulfoxide reductase, E. coli thioredoxin, [^{14}C]methionine sulfoxide, and NADPH as reducing agent. Symbols:* (●) *strain KK1006;* (○) *strain KK1048.*

TABLE 1. *Thioredoxin reductase activity at various temperatures*

Strain	Temp (°C)	Activity[a]
KK1006	25	2.48
KK1048	25	<0.01
KK1006	30	4.47
KK1048	30	<0.01
KK1006	37	5.25
KK1048	37	<0.01
KK1006	40	6.50
KK1048	40	<0.01

[a] Nanomoles of methionine formed per minute per milligram of protein.

To verify that the KK1048 extract did not contain an altered protein or factor that would inhibit the thioredoxin reductase reaction, an extract of KK1048 was mixed with an extract of its parent KK1006 as well as with purified thioredoxin reductase. No inhibition was observed in either case (Table 2).

Warner (24) found that cells made permeable to nucleotides via ether treatment could utilize NADPH as the reductant for RDP reductase.

TABLE 2. *Thioredoxin reductase activity in KK1048 in the presence of KK1006 extract or purified thioredoxin reductase*

Source of thioredoxin reductase	Activity[a]
16.7 μg of KK1006	36.0
12.0 μg of KK1048	<0.1
16.7 μg of KK1006 + 12.0 μg of KK1048	37.5
12.0 μg of KK1048 + purified thioredoxin reductase	124.0
Purified thioredoxin reductase	106.2

[a] Picomoles of methionine formed per minute.

Ether-treated cells of KK1048 and its parent, KK1006, were assayed for ribonucleotide reduction by using either dithiothreitol or NADPH as the reductant. Table 3 indicates that KK1048 has activity similar to its parent with dithiothreitol as reductant but it has greatly reduced activity with NADPH as reductant. Dithiothreitol can be used as a reductant without the requirement for thioredoxin reductase, whereas NADPH can only be used via thioredoxin reductase. These results again indicate that the mutant is deficient in thioredoxin reductase activity.

KK1048 was selected as a mutant unable to grow at 42°C after penicillin enrichment at 30°C for mutants deficient in their ability to use methionine sulfoxide as a methionine source, and yet the thioredoxin reductase activity of KK1048 appears to be greatly deficient at all temperatures. If the inability of KK1048 to grow at 42°C is due to the thioredoxin reductase mutation, and thioredoxin reductase is essential for DNA synthesis as anticipated, one would predict that (i) DNA synthesis would decrease after a shift to 42°C and, (ii) revertants able to grow at 42°C would regain thioredoxin reductase activity. Figure 3 indicates that DNA synthesis does not decrease at 42°C. Five revertants that grow at 42°C are still lacking in assayable thioredoxin reductase, indicating that the temperature-sensitive phenotype must be due to a secondary mutation. These results indicate that thioredoxin reductase does not appear to be an essential enzyme in *E. coli*.

In a recent paper, Tsang and Schiff (23) found that thioredoxin appears to be required for in vitro reduction of sulfate in *E. coli*. To determine whether thioredoxin reductase participates in this reaction in vivo, KK1048 and its parent KK1006 were converted to methionine prototrophs by P1 transduction, and these derivatives were grown with sulfate as the sole sulfur source. No difference in growth was observed (not shown) indicating that thioredoxin reductase is not required in vivo for growth on sulfate.

TABLE 3. *RDP reductase activity in ether-treated cells*

Strain	Reducing agent	Activity[a]
KK1006	Dithiothreitol	0.60
KK1048	Dithiothreitol	0.75
KK1006	NADPH	0.16
KK1048	NADPH	0.01

[a] Nanomoles of deoxyuridine 5'-diphosphate formed per minute per milligram of protein.

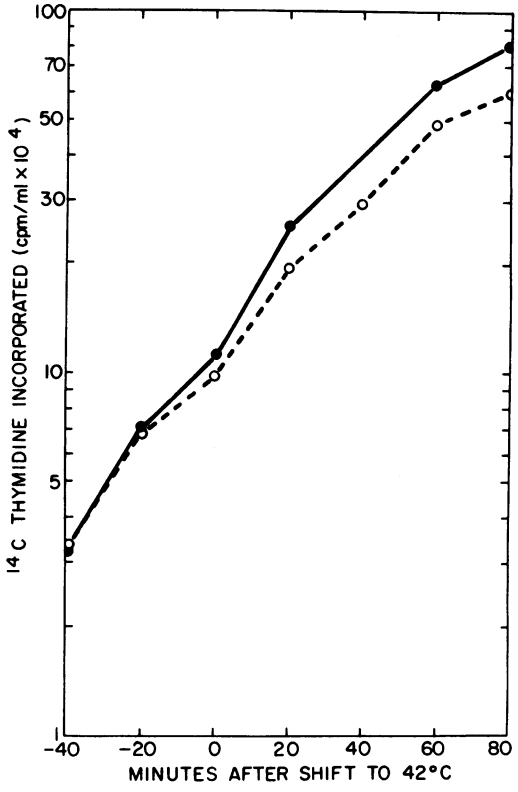

FIG. 3. *Incorporation of [^{14}C]thymidine into DNA after shift to 42°C. Symbols:* (●) *strain KK1006;* (○) *strain KK1048.*

Bacteriophage T7 has been found to require thioredoxin as an essential subunit of its DNA polymerase (14). The possible involvement of thioredoxin reductase was not investigated. The ability of T7 phage to form plaques on KK1006 and KK1048 was investigated by observing the size and turbidity of plaques. No differences were observed.

Thus, the apparent loss of thioredoxin reductase activity seems to have little effect on the mutant harboring this defect. If the lack of in

vitro thioredoxin reductase activity reflects a corresponding lack of in vivo activity, how is KK1048 able to function without thioredoxin reductase? A possible alternate pathway of coupling oxidation of NADPH to generation of thiols is the glutathione system which can form monothiols rather than dithiols formed by the thioredoxin system. In the preparation of ether-treated cells used for RDP reductase assays, the intracellular glutathione would be lost due to diffusion out of the permeable cells. KK1048 and its parent were assayed after ether-treatment for ribonucleoside diphosphate reductase activity using various reducing agents. Glutathione at a 1 mM concentration (a concentration approximating the intracellular concentration) stimulates the activity of KK1048 (Table 4). In the presence of both NADPH and 1 mM glutathione, KK1048 and KK1006 have equivalent RDP reductase activities. The increased activity of KK1048 in the presence of both NADPH and glutathione suggest that glutathione reductase is using NADPH to keep the glutathione reduced. The failure of a 1 mM concentration of mercaptoethanol to significantly stimulate RDP reductase in KK1048 indicates specificity of the monothiol used. The physiological monothiol, glutathione, satisfies this requirement.

Table 5 indicates that in toluene-treated cells, 1 mM reduced glutathione as the reductant also increases the rate of reduction of methionine sulfoxide but the rate attained is still much less than the parental rate. However, this increased rate may be sufficient to explain how KK1048 can grow, although at a decreased rate, with methionine sulfoxide as a methionine source.

DISCUSSION

In early studies on RDP reductase, it was found that several dithiols could be used as reducing agents for the reaction (20), but none of these appeared to be the physiological reducing agent. The discovery of two proteins, thioredoxin and thioredoxin reductase, that could couple NADPH oxidation to reduction of ribonucleotides, appeared to solve the problem. However, extensive experiments designed to exclude other systems able to couple NADPH oxidation to ribonucleotide reduction were not conducted. The evidence presented in this paper strongly suggests that thioredoxin reductase is not essential for ribonucleotide reduction. Furthermore, the observation that addition of physiological concentrations of reduced glutathione to ether-treated cells restores ribonucleotide reduction suggests that glutathione may well serve as an alternate system able to couple oxidation of NADPH to reduction of ribonucleotides. Recently, Holmgren (9) found that a protein, glutaredoxin, in the presence of reduced glutathione, can replace the thioredoxin system in vitro using purified RDP reductase.

Previously, we (6) as well as others (1) found that glutathione is not essential for *E. coli*, indicating that a glutathione system cannot be the sole source of in vivo coupling of NADPH oxidation to reduction of ribonucleotides. We did find, however, that a mutant of *E. coli* unable to synthesize glutathione had a B1 subunit of RDP reductase that was only partially active (6). We attributed this to possible oxidation of thiols needed to keep the proper tertiary structure of the enzyme. In view of the evidence presented in this paper, a better interpretation may be that glutathione is important in keeping the two thiols in the active site in a reduced state.

Conclusive evidence that the thioredoxin system is not essential in vivo can be obtained by deleting the gene coding for one of these proteins. Experiments to accomplish this are in progress. Experiments to construct a strain defective in both the glutathione synthesis and

TABLE 4. *RDP reductase activity in ether-treated cells using various reducing agents*

Reducing agent	Activity[a]	
	Strain KK1006	Strain KK1048
NADPH	0.17	0.01
NADPH + 1 mM mercaptoethanol	0.21	0.03
NADPH + 1 mM glutathione	0.26	0.28
1 mM glutathione	0.04	0.14

[a] Nanomoles of deoxyuridine 5'-diphosphate formed per minute per milligram of protein.

TABLE 5. *Methionine sulfoxide reductase activity in toluene-treated cells with various reducing agents*

Reducing agent	Activity[a]	
	Strain KK1006	Strain KK1048
NADPH	6.50	0.01
NADPH + 1 mM mercaptoethanol	8.27	0.03
NADPH + 1 mM glutathione (reduced)	7.94	0.19
NADPH + 1 mM glutathione (oxidized)	5.20	0.14

[a] Nanomoles of methionine formed per minute per milligram of protein.

thioredoxin reductase are also in progress to determine whether these are the only two systems that can couple NADPH oxidation to ribonucleotide reduction in vivo and to evaluate the importance of each system.

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