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# Hydrogen donor system for *Escherichia coli* ribonucleoside-diphosphate reductase dependent upon glutathione

(DNA synthesis/thioredoxin)

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ABSTRACT *E. coli* B *tsnC** 7004, an *E. coli* B/1 mutant with normal phenotype unable to replicate phage T7 DNA [Chamberlin, M. (1974) *J. Virol.* 14, 509–516], contained no detectable level of thioredoxin when assayed with ribonucleotide reductase (2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1). Gently lysed *E. coli tsnC* 7004 cell extracts reduced CDP when supplemented with NADPH as efficiently as the parent strain *E. coli* B/1 despite the lack of thioredoxin, indicating the presence of another hydrogen transport system. This could be divided into two parts by heat treatment at 85°; one heat-stable fraction, which was active in the presence of dithiothreitol or glutathione, and one heat-labile fraction. Addition of yeast glutathione reductase [NAD(P)H:oxidized-glutathione oxidoreductase, EC 1.6.4.2] to the heated extracts restored full activity. The results demonstrate a novel hydrogen transport system in *E. coli* consisting of NADPH, glutathione, glutathione reductase, and a heat-stable enzyme called "glutaredoxin". Reduced glutathione at physiological concentrations functions as hydrogen donor for ribonucleotide reduction only in the presence of glutaredoxin. Glutaredoxin was not reduced by *E. coli* thioredoxin reductase (NADPH:oxidized-thioredoxin oxidoreductase, EC 1.6.4.5) and showed no crossreaction with antibodies against thioredoxin. These results demonstrate the existence of two different electron transfer systems from NADPH to deoxyribonucleotides and provide a function for glutathione in DNA synthesis.

Deoxyribonucleotides for DNA synthesis in *Escherichia coli* are formed from the corresponding ribonucleotides in a reaction catalyzed by the essential enzyme ribonucleoside-diphosphate reductase (2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1) (1). This enzyme requires certain dithiols as hydrogen donors, whereas monothiols like reduced glutathione (GSH) or cysteine are inactive (2). Thioredoxin is a small electron transport protein, which originally was isolated as the natural hydrogen donor for ribonucleotide reductase (3). The oxidation-reduction active group of thioredoxin consists of a single cystine residue which is reduced to sulfhydryl form by NADPH in the presence of *E. coli* thioredoxin reductase (NADPH:oxidized-thioredoxin oxidoreductase, EC 1.6.4.5) (4). *E. coli* thioredoxin has been extensively characterized in previous studies (5). Thus, both the complete amino acid sequence (6) and a three-dimensional structure to 2.8-Å resolution for thioredoxin-$S_2$ by x-ray crystallography have been determined (7).

Mutants of *E. coli* with apparent normal phenotype that were unable to support the intracellular growth of bacteriophage T7 (*tsn*) were isolated by Chamberlin (8). One class of *tsn* mutants, *tsnC*, was defective in a component provided by the host that was essential for viral duplex DNA replication *in vivo* and *in vitro* (8). Extracts of phage T7-infected *tsnC* mutants were deficient in an essential T7 DNA nucleotidyltransferase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) activity (9, 10). However, a small, heat-stable protein purified from uninfected *E. coli tsnC*$^+$ cells by complementation *in vitro* was able to restore full phage T7 DNA nucleotidyltransferase activity and the capacity of infected *tsn* cell extracts to replicate duplex T7 DNA (9, 10). Furthermore, the highly purified T7 DNA nucleotidyltransferase was found to be an enzyme composed of phage- and host-specified subunits (9, 10). The enzyme was a 1:1 complex of the gene 5 protein of phage T7 (84,000 daltons) and the *E. coli tsnC* protein (12,000 daltons) (10). Recent evidence shows that the *tsnC* protein is identical with *E. coli* thioredoxin (11, 12).

In a study of three *tsnC* mutants, we found that *E. coli tsnC* 7004 contained no detectable levels of thioredoxin by immunochemical methods (A. Holmgren, I. Ohlsson, and M. Grankvist, manuscript in preparation). In this paper I describe studies of the hydrogen transport system for ribonucleotide reduction in *E. coli tsnC* 7004. In the absence of any measurable thioredoxin activity, a hydrogen transport system, dependent upon physiological concentrations of reduced glutathione, was discovered.

## MATERIALS AND METHODS

NADPH, CDP, and ATP were from Sigma. Dithiothreitol was from Calbiochem. [$^3$H]CDP was from Schwarz/Mann. Thioredoxin from *E. coli* was a homogeneous preparation from *E. coli* (13). Thioredoxin reductase from *E. coli* was a preparation of better than 80% purity (14). Proteins B1 and B2 of ribonucleotide reductase were 60–100% pure preparations from *E. coli* B3 (15) obtained from the laboratory of Dr. Peter Reichard. The gamma-globulin fraction of several pooled rabbit antisera against *E. coli* thioredoxin were obtained as described (16). GSH and crystalline yeast glutathione reductase [NAD(P)H:oxidized-glutathione oxidoreductase, EC 1.6.4.2] were from Sigma. Lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) was from Nutritional Biochemicals Corporation.

**Bacterial Strains.** *E. coli* B/1 Su$^-$ and the derivative *tsnC* mutant 7004 (8), were kindly provided by Dr. M. Chamberlin and Dr. C. C. Richardson.

**Growth of Cells.** *E. coli* B/1 and *tsnC* 7004 were grown in L-broth (10 g/liter of Bacto-tryptone, 5 g/liter of yeast extract, and 10 g/liter of NaCl) supplemented with 0.1% glucose (8). Parallel cultures in aerated flasks at 37° were grown to an absorbance of 0.8–1.0 at 650 nm (Zeiss PMQIII spectrophotometer), chilled to 10°, harvested by centrifugation, and stored at −20°.

**Preparation of Crude Extract by Gentle Lysis.** The bacterial cells were mixed with 3.75 times their weight of 10% sucrose, 50 mM Tris·HCl, pH 7.6, and 0.1 M NaCl; lysozyme was added to a final concentration of 0.2 mg/ml. The suspension was incubated in an ice bath for 40 min, warmed for 2 min in a 37° bath, and then centrifuged for 100 min at 20,000 rpm at

Abbreviation: *tsnC* mutant, phage *T* seven negative mutant of *Escherichia coli* B belonging to class C (8); GSH, reduced glutathione.

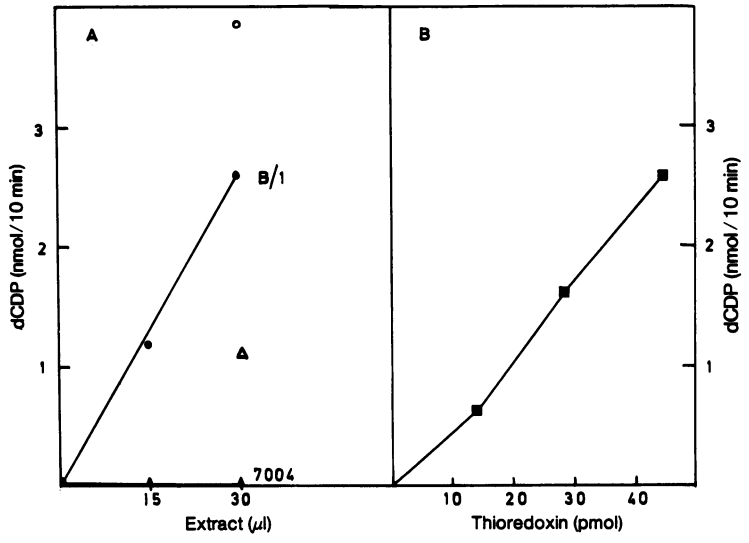


FIG. 1. (Panel A) Thioredoxin activity in heated crude extracts from *E. coli* B/1 (●) and *E. coli tsnC* 7004 (▲). Effect of added thioredoxin, 15 pmol, to *E. coli tsnC* 7004 extract (△) and to *E. coli* B/1 extract (○). (Panel B) Standard curve for homogeneous thioredoxin (■). Incubations were carried out as described in *Materials and Methods* using the spectrophotometric assay with ribonucleotide reductase, NADPH, and thioredoxin reductase.

+4° (Sorvall RC2B, SS34 rotor). The supernatant (E1) was collected and used immediately for assay of ribonucleotide reductase activity.

**Protein Purification.** All steps were carried out at +4°. Centrifugations were performed at 15,000 rpm for 20 min.

To fraction E1 was slowly added streptomycin sulfate (2 ml of 5% solution per 10 ml of E1) and the mixture was stirred for 15 min. After centrifugation, the supernatant fraction, E2, was withdrawn.

To fraction E2 was slowly added 1 M acetic acid to pH 5.0. The supernatant after centrifugation was neutralized with 1 M $NH_3$ to give fraction E3. The precipitate was also dissolved in a small volume after neutralization to give fraction E4.

To fraction E3 was added EDTA to a final concentration of 0.005 M, and the mixture was heated to 85° in a boiling-water bath under vigorous stirring and then rapidly cooled in an ice bath. The precipitated protein was filtered off, and the filtrate, fraction E5, was saved. Aliquots of fractions E1–E5 were frozen at −20° and assayed after a few days.

**Enzyme Assays.** The activity of ribonucleotide reductase was determined either by measurement of the formation of [$^3$H]dCDP from [$^3$H]CDP (15) or by spectrophotometric determination of NADPH oxidation in the presence of CDP (17).

The incubation mixture for thioredoxin determination contained in a final volume of 0.13 ml: 200 nmol of ATP, 1.5 μmol of $MgCl_2$, 80 nmol of NADPH, 5 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.6, 100 nmol of EDTA, and 5 μg of thioredoxin reductase. The two subunits of ribonucleotide reductase (15), B2 (15 μg) and B1 (15 μg), were added separately. The B1 protein, which is stored as an ammonium sulfate precipitate in 0.01 M dithiothreitol in liquid nitrogen (15), was collected by centrifugation and dissolved in 0.05 M Tris·HCl, pH 8.0, without dithiothreitol to 5 mg/ml immediately before use. Thioredoxin or extract was added to one cuvette, and the absorbance at 340 nm was recorded continuously in an automatic recording spectrophotometer (Zeiss PMQ III) with another cuvette with identical composition without thioredoxin as blank. The reaction was started by addition of 3 μl of 30 mM (90 nmol) CDP to both cuvettes and followed at 25°.

For assay of [$^3$H]CDP reduction with dithiothreitol as chemical reductant of an electron transport protein, the same incubation mixture as above was used with 5–15 μg of B1 and B2 subunits. NADPH and thioredoxin reductase were exchanged for dithiothreitol, 1.67 mM final concentration. The reaction was started with 90 nmol of [$^3$H]CDP (10,000–50,000 cpm/nmol), and the mixtures were incubated for 10 min at 25°. The reaction was stopped by addition of 1 ml of 1 M $HClO_4$ and the amount of [$^3$H]dCDP was determined by chromatography on Dowex-50 columns, as described by Reichard (2). Radioactivity was determined in 10 ml of Instagel (Packard) with a Packard liquid scintillation counter.

The assay of ribonucleotide reductase activity dependent upon NADPH in gently lysed high-speed supernatants of bacteria was a slight modification of the method described by Eriksson (18). The incubation mixture contained in a final volume of 0.120 ml: 1 μmol of $MgCl_2$, 5 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 8.1, 250 nmol of ATP, 160 nmol of NADPH, and 90 nmol of [$^3$H]CDP (specific activity 50,000 cpm/nmol). Incubations were performed at 37° for 20 min and stopped by addition of 1 ml of 1 M $HClO_4$; the amount of [$^3$H]dCDP formed was determined as described above.

**Protein Determination.** Protein was determined by the method of Lowry *et al.* (19) or by reading the absorbance at 280 and 310 nm in a Zeiss PMQ III spectrophotometer.

## RESULTS

### Thioredoxin activity in *E. coli* B/1 and *tsnC* 7004

Extracts prepared from exponentially growing *E. coli* B/1 and *tsnC* 7004 were heated to 85°, and the supernatants assayed for thioredoxin activity in the reduction of CDP by ribonucleotide reductase, NADPH, and thioredoxin reductase. The result is shown in Fig. 1A. The extract from *E. coli* B/1 contained the expected concentration of thioredoxin (18 μg/ml) as determined from a standard curve with pure thioredoxin (Fig. 1B), whereas no detectable thioredoxin activity could be found in the extract from the mutant *tsnC* 7004. Addition of 15 pmol of thioredoxin to the assay cuvettes of B/1 and 7004 gave the expected stimulation in both extracts and demonstrated that the lack of activity in *E. coli tsnC* 7004 was not due

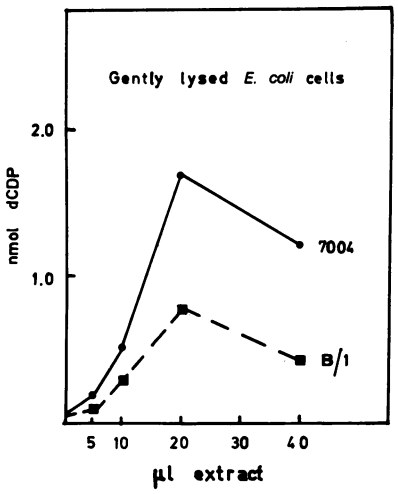


FIG. 2. Ribonucleotide reductase activity of gently lysed *E. coli* B/1 (■- - -■) and *E. coli tsnC* 7004 cells (●—●). Incubations were carried out as described in *Materials and Methods* with [$^3$H]CDP and NADPH.

to the presence of an inhibitor. The result demonstrated that *E. coli tsnC* 7004 must have a mutation affecting thioredoxin. Furthermore, any hydrogen transport protein specific for ribonucleotide reductase present in the heated *E. coli tsnC* 7004 extract was not reduced by NADPH and thioredoxin reductase.

### Ribonucleotide reductase activity in gently lysed cells

The ultimate hydrogen donor for the reduction of ribonucleotides to deoxyribonucleotides in *E. coli* is NADPH (1). Since no thioredoxin could be demonstrated in *E. coli tsnC* 7004, it was essential to determine if NADPH-dependent ribonucleotide reduction was present in this mutant. The activity of ribonucleotide reductase in *E. coli* in crude extracts prepared from mechanically disrupted cells is very low. Recently, Eriksson showed (18) that concentrated high-speed supernatants from *E. coli* treated with lysozyme contained a highly active form of the enzyme which, however, was unstable and could not be purified. In the assay system used by Eriksson (18), dithiothreitol was used as hydrogen donor at optimal concentration (6 mM) and dithiothreitol was also added to the supernatants at a final concentration of 5 mM. I have modified this system by removing dithiothreitol from the supernatant and the incubation mixture. As the ultimate hydrogen donor, only NADPH was added at 1.2 mM concentration in order to measure a physiological electron transport system dependent upon this coenzyme. Assay of ribonucleotide reductase activity in high-speed supernatants from parallel exponential cultures of *E. coli* B/1 and *E. coli tsnC* 7004 are shown in Fig. 2. The unexpected finding was that the extract of mutant *E. coli tsnC* 7004 had a higher NADPH-dependent ribonucleotide reductase activity than the parent *E. coli* B/1, despite the lack of thioredoxin in the former. The calculated specific activity for ribonucleotide reductase (nmol of dCDP per mg of protein) in the *E. coli* B/1 lysate was similar to the values reported by Eriksson (18) for wild-type *E. coli*.

### Evidence for an unknown hydrogen transport protein specific for ribonucleotide reductase in *E. coli tsnC* 7004

Thioredoxin can be kept in the reduced state chemically by using 1–2 mM dithiothreitol in the assay of ribonucleotide reductase (15), which allows measurements of thioredoxin independent of thioredoxin reductase and NADPH. Dithiothreitol at this concentration will, however, act as direct chemical hydrogen donor for ribonucleotide reductase to give some activity (15). Table 1 shows the hydrogen donor activity for ribonucleotide reductase of fractions from purification steps applied to a crude extract of *E. coli tsnC* 7004. The crude extract contained a hydrogen donor activity that stimulated the reduction of CDP to dCDP by ribonucleotide reductase in the presence of dithiothreitol. The activity could be recovered through streptomycin sulfate precipitation and acid precipitation and was stable to heating at 85°. It was not affected by addition of anti-thioredoxin gamma globulin in amounts that completely inhibited a similar amount of added thioredoxin (Table 1). Furthermore, it was obviously not reacting with thioredoxin reductase and NADPH, as shown in Fig. 1.

Table 1. Evidence for an unknown hydrogen transport protein specific for ribonucleotide reductase in fractions of *E. coli tsnC* 7004

| Addition | Ribonucleotide reductase activity (nmol of dCDP/ 10 min) |
|---|---|
| E1 (crude extract), 50 µl | 2.41 |
| E2 (streptomycin supernatant), 50 µl | 3.23 |
| E3 (pH 5 supernatant), 50 µl | 2.79 |
| E5 (85° supernatant), 50 µl | 2.57 |
| E1, 50 µl, + anti-thioredoxin gamma globulin, 20 µl | 2.38 |
| E1, 50 µl, omit ribonucleotide reductase | 0.01 |
| Thioredoxin, 14 pmol | 1.88 |
| Thioredoxin, 28 pmol | 2.95 |
| Thioredoxin, 28 pmol, + anti-thioredoxin gamma globulin, 20 µl | 1.15 |
| None | 1.11 |

For details of the incubation conditions and for the preparation of fractions E1–E5, see *Materials and Methods*.

### NADPH-dependent hydrogen transport system for ribonucleotide reductase in *E. coli tsnC* 7004

In order to find the unknown physiological NADPH-dependent hydrogen transport system, dithiothreitol was exchanged for NADPH in the ribonucleotide reductase assay (Table 2).

Table 2. Hydrogen transport system for ribonucleotide reductase in *E. coli tsnC* 7004 with NADPH or dithiothreitol

| Addition | Ribonucleotide reductase activity (nmol of dCDP/ 10 min) |
|---|---|
| E2, 25 µl, + 1.67 mM dithiothreitol | 1.92 |
| E2, 50 µl, + 1.67 mM dithiothreitol | 3.04 |
| Only 1.67 mM dithiothreitol | 0.98 |
| E2, 25 µl, + 1.2 mM NADPH | 0.83 |
| E2, 50 µl, + 1.2 mM NADPH | 1.65 |
| Only 1.2 mM NADPH | 0.32 |

Assays were carried out with [$^3$H]CDP and ribonucleotide reductase. For preparation of fraction E2 and experimental details, see *Materials and Methods*.

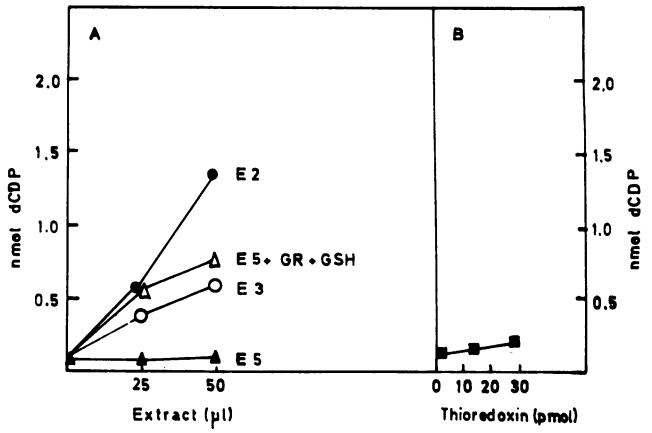


FIG. 3. (Panel A) Hydrogen donor activity for ribonucleotide reductase of fractions of *E. coli tsnC* 7004 in the presence of NADPH. Fraction E2 (●), fraction E3 (○), fraction E5 (85° supernatant) (▲). Fraction E5 in the assay was supplemented with 5 µg of yeast glutathione reductase (GR) and 0.5 µmol of GSH (4 mM) (△). (Panel B) Hydrogen donor activity for ribonucleotide reductase of thioredoxin in the presence of NADPH, 5 µg of yeast glutathione reductase, and 0.5 µmol of GSH (4 mM) (■).

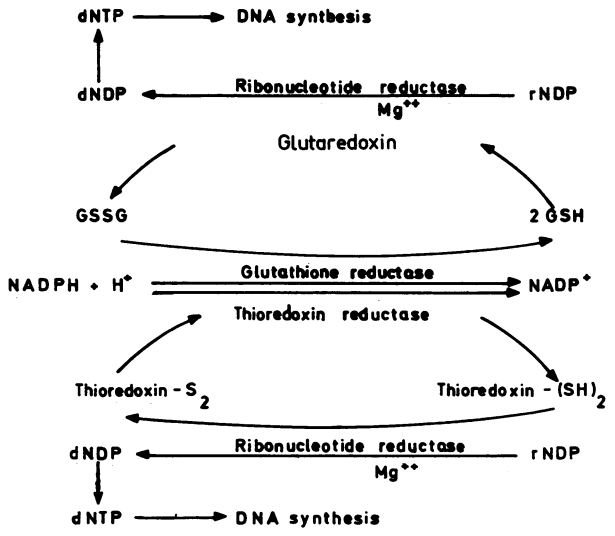


FIG. 4. Metabolic pathways from NADPH to the biosynthesis of deoxyribonucleotides and DNA in *E. coli*; rNDP (ribonucleoside diphosphates), dNDP and dNTP (deoxyribonucleoside diphosphates and triphosphates). GSSG is glutathione.

NADPH could support ribonucleotide reduction almost as efficiently as dithiothreitol when fraction E2 was used. A clue to the nature of the physiological system came when fractions E1–E5 were assayed with NADPH.

As shown in Fig. 3, the NADPH activity was completely lost when the heated fraction E5 was used, demonstrating that a heat-labile enzyme constituted the electron transport from NADPH. Thioredoxin reductase is inactivated by heating to 85° (3, 4), but addition of this enzyme to fraction E5 did not restore the activity, a result already evident from the experiment shown in Fig. 1. Fraction E5 still contained a heat-stable, unknown hydrogen donor for ribonucleotide reductase, as shown by the results in Table 1.

A second electron transport system in *E. coli* is NADPH, glutathione, and glutathione reductase. Heat treatment to 85° will inactivate glutathione reductase. To test if this system was involved in the unknown hydrogen transport system, I added crystalline yeast glutathione reductase and 4 mM GSH to the heated fraction E5. As shown in Fig. 3A, this restored full activity. Glutathione reductase, 4 mM GSH, and NADPH did not act as hydrogen donor for ribonucleotide reductase, in agreement with previous results (2) showing that the monothiol GSH is not hydrogen donor for ribonucleotide reductase. Furthermore, thioredoxin did not function as hydrogen donor for ribonucleotide reductase in the presence of NADPH, glutathione reductase, and 4 mM GSH, as shown in Fig. 3B and Table 3.

Table 3. Hydrogen donor activity for ribonucleotide reductase of purified enzyme from *E. coli tsnC* 7004

| Addition | GSH (mM) | Ribonucleotide reductase activity (nmol dCDP/ 10 min) |
|---|---|---|
| Purified enzyme, 1.2 µg | 0 | 0.6 |
| Purified enzyme, 1.2 µg | 0.8 | 9.4 |
| Purified enzyme, 1.2 µg | 4.0 | 10.1 |
| Thioredoxin, 3 µg | 4 | 0.7 |
| Thioredoxin, 3 µg, + thioredoxin reductase, 1 µg | 4 | 8.9 |

Incubations were carried out under standard conditions in a final volume of 120 µl with [$^3$H]CDP, NADPH, glutathione reductase (1 µg), and ribonucleotide reductase. GSH was added in the incubation mixture to the final concentration indicated.

These results clearly demonstrated that the unknown, heat-stable, hydrogen transport protein for ribonucleotide reduction in *E. coli tsnC* 7004 was different from thioredoxin and suggested that it was an enzyme that enables GSH to supply its reducing power to ribonucleotide reductase.

We have now purified this enzyme to an essentially homogeneous state from *E. coli tsnC* 7004 cells. The assay of the enzyme depended on its ability to stimulate the reduction of CDP catalyzed by ribonucleotide reductase in the presence of NADPH, 4 mM GSH, and yeast glutathione reductase. The purification procedure will be described in detail elsewhere (A. Holmgren, manuscript in preparation). As shown in Table 3, the highly purified enzyme in the presence of NADPH, 4 mM GSH, and glutathione reductase gave the same activity *in vitro* with ribonucleotide reductase as when saturating concentrations of NADPH, thioredoxin, and thioredoxin reductase were used. The enzyme was not active in the absence of GSH.

## DISCUSSION

The results in this paper show that the *E. coli* mutant, *tsnC* 7004, has a mutation in the gene for thioredoxin. Since the tsnC protein is identical with thioredoxin (11, 12), this demonstrates that the *tsnC* gene codes for thioredoxin. No thioredoxin activity with ribonucleotide reductase could be demonstrated in *E. coli tsnC* 7004 by the thioredoxin reductase-coupled assay using heated extracts. The most straightforward interpretation of this result is that thioredoxin is missing in the *E. coli* 7004 cells or present in greatly reduced amounts. Alternatively, the cells contain a thioredoxin gene product that is heat labile or mutationally changed so that *in vitro* it does not react with thioredoxin reductase or ribonucleotide reductase.

The thioredoxin-deficient mutant, *E. coli tsnC* 7004, can grow normally, and the only phenotypical difference between it and its intact parent, *E. coli* B/1, is the failure of the mutant strain to support the growth of phages $\phi$2, T3, and T7 (8). We have estimated that normal *E. coli* contain 10,000–20,000

molecules of thioredoxin per cell (5), which is in large excess over the number of ribonucleotide reductase molecules present. Why the normal cell has so many thioredoxin molecules remains to be elucidated. The reduction of ribonucleotides and of thioredoxin shows practically no connection with other metabolic pathways in the cell. The ribonucleotide reduction is not reversible. Only the participation of the thioredoxin system in enzymatic reduction of methionine sulfoxide and sulfate in yeast (20, 21) has been detected.

Ribonucleotide reductase is an essential enzyme, since temperature-sensitive mutants, which contain mutations in the cistrons coding for this enzyme, show temperature-sensitive DNA synthesis (22). The thioredoxin system (thioredoxin, thioredoxin reductase, and NADPH) is so far the only natural hydrogen donor system for ribonucleotide reductase and has been isolated from microorganisms (3, 4, 23), yeast (20, 21), and mammalian liver (24, 25). The apparent absence of thioredoxin in *E. coli tsnC* 7004, coupled with the highly active form of NADPH-dependent ribonucleotide reductase in these cells (Fig. 2), is a demonstration of an alternative metabolic pathway from NADPH to a deoxyribonucleotide. This led to the postulation of the existence of a second hydrogen transport system normally masked in *E. coli* extracts by the high activity of the thioredoxin system. The results in this paper demonstrate that glutathione can supply its reducing power to ribonucleotide reductase, provided that it is coupled with a novel enzyme. The name "glutaredoxin" appears appropriate for this enzyme. Glutaredoxin has recently been identified in wild-type *E. coli* cells (A. Holmgren, in preparation).

The tripeptide glutathione is known to be present in all kinds of cells (26, 27) and comprises up to 90% of the nonprotein thiols in mammalian cells (up to 10 mM). In *E. coli*, 25% of the sulfur is found in glutathione and up to 95% of the nonprotein thiols may be in glutathione (28). Although this compound has been implicated in numerous metabolic processes (29), few solid biochemical facts are known. Some authors have even questioned a physiological function for glutathione. The results presented in this paper provide a function for glutathione and glutathione reductase in DNA synthesis and demonstrate the existence of two metabolic pathways from NADPH to the formation of deoxyribonucleotides, as shown in Fig. 4. Further studies are required to understand the relative contributions and functions of the two systems in the biosynthesis of deoxyribonucleotides and DNA in normal cells.

For gifts of *E. coli tsnC* mutants I thank Drs. C. C. Richardson and M. Chamberlin. I am grateful to Dr. C. C. Richardson for sending preprints of his results showing that thioredoxin is a subunit of phage T7 DNA polymerase. The excellent technical assistance of Mrs. Maja-Lena Grankvist is gratefully acknowledged. This investigation was supported by grants from the Swedish Medical Research Council (Project nos. 13X-3529 and 13P-4292), Magnus Bergvalls Stiftelse, and the Medical Faculty of Karolinska Institutet.

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