Glutathione-dependent Synthesis of Deoxyribonucleotides

CHARACTERIZATION OF THE ENZYMATIC MECHANISM OF ESCHERICHIA COLI GLUTAREDOXIN*

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In the presence of the glutaredoxin system, the enzymatic formation of deoxyribonucleotides was shown to occur with reduced glutathione (GSH) and ribonucleoside 5'-diphosphates as substrates. This paper describes studies of the reaction:

2 GSH + CDP → GSSG + dCDP + H2O

This reaction in vitro requires the combined function of three pure Escherichia coli proteins, namely the B1 and B2 subunits of ribonucleotide reductase and glutaredoxin. The stoichiometry was demonstrated by determinations of the products $[^3H]dCDP$ and GSSG. The standard assay couples oxidation of NADPH to the reduction of GSSG by glutathione reductase. The apparent $K_m$ value of glutaredoxin with ribonucleotide reductase at 4 mM GSH was 1.3 × 10^{-3} M. The molecular activity of glutaredoxin in CDP reduction was about 10-fold higher than that of thioredoxin and similar to the corresponding values for the B1 and B2 subunits of ribonucleotide reductase. The apparent $V_{max}$ values in vitro of ribonucleotide reductase with the thioredoxin or glutaredoxin systems were similar. The apparent $K_m$ value for GSH was 0.40 mM in the absence of excess NADPH, glutathione reductase, and CDP. The dithiol or reduced form of glutaredoxin, isolated after chemical reduction with dithiothreitol, reduced a stoichiometric amount of CDP to dCDP in the presence of ribonucleotide reductase. In the absence of NADPH and glutathione reductase, the velocity of the reduction of CDP to dCDP by GSH was highly influenced by the ratio of GSH to GSSG and even small amounts of GSSG inhibited strongly. Inhibition by GSSG is a possible physiological control mechanism for deoxyribonucleotide synthesis.

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The recent identification of the protein glutaredoxin in Escherichia coli (1) has established a novel coenzyme function for glutathione (GSH) in the biosynthesis of the precursors for DNA synthesis. Glutaredoxin couples the reducing capacity of the monothiol of GSH to the enzymatic formation of deoxyribonucleotides by the enzyme ribonucleotide reductase (1). In the preceding paper (2), we described a 40,000-fold purification of E. coli glutaredoxin to an apparent homogeneous state. Glutaredoxin is a small (Mr = 12,000) acidic protein which, in addition to its specific function in the ribonucleotide reductase reaction, is also a powerful GSH-disulfide transhydrogenase enzyme (2).

The molecular weight of E. coli glutaredoxin is similar to that of another small protein, thioredoxin-(SH)$_2$ which until recently (1, 3) is known as the natural hydrogen donor substrate for ribonucleotide reductase. The characterization of an E. coli mutant lacking detectable thioredoxin (3) but with no decreased capacity to reduce ribonucleotides (1) showed that thioredoxin is a nonobligatory intermediate in ribonucleotide reduction. In fact, its true physiological role is presently unclear and the only firmly established physiological function of thioredoxin is to be an essential subunit of a virus-induced DNA polymerase (4) formed after T7 phase infection. Thioredoxin-S$_2$ is a well characterized protein which contains its oxidation-reduction active disulfide (5) in a protrusion of the three-dimensional structure (6). The disulfide is reduced to a dithiol with NADPH by a specific flavoprotein, thioredoxin reductase (7). Thioredoxin-(SH)$_2$ is the direct substrate for ribonucleotide reductase in vitro and thioredoxin-S$_2$ is formed in the reaction.

Ribonucleotide reductase from E. coli consists of two nonidentical subunits, protein B1 (Mr = 160,000) and protein B2 (Mr = 78,000) (7). Each subunit is completely inactive by itself. In the presence of Mg$^{2+}$, the subunits combine to yield the active ribonucleotide reductase (Mr = 240,000). The enzyme can reduce all four ribonucleotide diphosphate substrates, but both the substrate specificity and overall activity are controlled by allosteric effectors which are nucleoside triphosphates (7). The B1 subunit contains two ribonucleotide substrate binding sites and all four binding sites for effectors (8). B1 also contains oxidation-reduction active sulfhydryl groups which, in the presence of B2, reduce a stoichiometric amount of substrate in the absence of an external hydrogen donor (9). Protein B2 contains two nonheme iron and varying amounts of an organic-free radical which are required for enzyme activity (7). The radical has recently been localized to the benzene ring of a tyrosine residue (10). The catalytic site of ribonucleotide reductase is formed from both subunits and appears to include the organic-free radical of B2 and the dithiol of R1 (11).

To determine what the natural hydrogen donor for ribonucleotide reductase is, it is essential to understand the role of the glutaredoxin system (GSH, glutaredoxin, NADPH, and glutathione reductase) in deoxyribonucleotide synthesis. In the present study, the enzymatic mechanism of the glutaredoxin-dependent ribonucleotide reductase reaction was examined.
EXPERIMENTAL PROCEDURES

If not otherwise indicated, materials and methods were as specified in the accompanying paper (2).

Glutaredoxin

Most experiments were done with E. coli B glutaredoxin, Fraction X (2), which is homogeneous. In some experiments, glutaredoxin from E. coli tsnC7004 or E. coli B3 was used. These preparations were 40 and 70% pure as judged by specific activity in the GSH-transhydrogenase assay (2) and by analytical polyacrylamide gel electrophoresis (2).

Materials

N-ethylmaleimide, isodecic acid, and GSSG were from Sigma. Ribonucleotide reductase was a preparation from E. coli K144 (12) containing 90 mg/ml (25,000 units of B1 and 32,300 units of B2/ml) in 50 mM Tris-Cl, pH 7.6, 15 mM MgCl2, 10 mM dithiothreitol, and 20% glycerol. The specific activities of B1 and B2 were 500 and 3000 units/mg where 1 unit corresponds to the formation of 1 nmol of dCDP/min (12). The enzyme was diluted 5-fold with 50 mM Tris-Cl, pH 7.6, and stored at -70 °C as a large number of 15-μl aliquots. For each experiment, a 10-μl aliquot was thawed and diluted with 50 μl of 50 mM Tris-Cl, pH 7.6, and 3 or 5 μl was used in a final volume of 120 μl. In some experiments, the enzyme was freed from traces of dithiothreitol by passage through a small column (2 ml) of Sephadex G-25 fine equilibrated with 50 mM Tris-Cl, pH 7.5. The enzyme concentration was determined from the absorbance at 280 nm. Glutathione reductase from E. coli was prepared by affinity chromatography on 2′,5′-ADP-Sepharose 4BZ from Fraction IV (2) and was around 300-fold more active than the enzyme present in crude E. coli. Routinely, highly purified (240 units/mg) yeast glutathione reductase was used.

Determination of GSSG

GSSG was determined spectrophotometrically at 340 nm using 0.5 ml of 0.4 mM NADPH in 0.20 M potassium phosphate, pH 7.0, 5 mM EDTA. The change in absorbance at 340 nm after addition of excess yeast glutathione reductase (5 μg) was used to calculate the GSSG concentration (15).

RESULTS

Activity of Glutaredoxin with Ribonucleotide Reductase—The standard assay for glutaredoxin measures its ability to catalyze the formation of [3H]dCDP from [3H]CDP and GSH (4 mM) by ribonucleotide reductase. The dependence of the reaction rate on glutaredoxin concentration in this assay shows saturation kinetics. This allows determination of the apparent KM value for the homogeneous E. coli B glutaredoxin in the ribonucleotide reductase reaction. A Lineweaver-Burk plot gave a value of 0.15 μM. In other experiments, glutaredoxin from E. coli tsnC7004 (2) and E. coli B3 cells (2) gave values of 0.13 and 0.10 μM, respectively.

In these experiments, glutaredoxin was present in concentrations similar to that of the active ribonucleotide reductase enzyme.

Comparison of the Activity of Thioredoxin and Glutaredoxin Systems—To further study the activity of glutaredoxin with ribonucleotide reductase in vitro, it was compared with the activity of the well known thioredoxin system (thioredoxin, NADPH, and thioredoxin reductase). Glutaredoxin was 8-fold more active on a molar basis in this reaction (Fig. 1). Apparent KM values for thioredoxin of 1.25 × 10⁻⁶ M and for glutaredoxin of 0.15 × 10⁻⁶ M were obtained. The Vmax values for ribonucleotide reductase were essentially identical (within ±10%) with the two systems. The molecular activities of glutaredoxin and thioredoxin were calculated and compared with those of subunits B1 and B2 of ribonucleotide reductase (Table 1). It is apparent from this calculation that glutaredoxin has a turnover number which is intermediate between proteins B1 and B2.

Effects of Dithiothreitol—Ribonucleotide reductase from E. coli is stabilized by dithiothreitol since the B1 subunit is inactivated by oxidation of SH groups (16). At high concentration (1 to 50 mM), dithiothreitol can act as direct hydrogen donor for the enzyme. The small amounts of dith-
Assay of Glutaredoxin by NADPH Oxidation—In the experiments described above, the glutaredoxin activity was measured by the reduction of $[^3H]CDP$ to $[^3H]dCDP$. The reaction mixture also contained NADPH and glutathione reductase which will reduce GSSG formed in the reaction. Because the equilibrium of the glutathione reductase reaction at pH 7.5 in the presence of excess NADPH is shifted towards reduction of all GSSG, the assays of glutaredoxin may also be performed spectrophotometrically by following the oxidation of NADPH as shown in Fig. 2. The specific activity of glutaredoxin expressed as nanomoles of dCDP/min/mg of protein was the same when calculated from assays with $[^3H]dCDP$ formation and oxidation of NADPH spectrophotometrically, demonstrating that the overall stoichiometry of the reaction is 1 mol of UCDP produced/1 mol of NADPH consumed as previously observed (1). The specific activity of glutaredoxin expressed as nanomoles of dCDP/min/mg of protein was the same when calculated from assays with $[^3H]dCDP$ formation and oxidation of NADPH spectrophotometrically, demonstrating that the overall stoichiometry of the reaction is 1 mol of UCDP produced/1 mol of NADPH consumed as previously assumed. The spectrophotometric assay is quick and suitable for kinetic measurements. With less pure enzyme preparations, it is essential to record the background NADPH-oxidation carefully and to start the reaction with addition of CDP.

As shown in Fig. 3, thioredoxin from E. coli gave no activity in the glutaredoxin assay system which is an indirect demonstration of the inability of GSH to reduce thioredoxin-S2 (17). Assays for glutaredoxin may thus be performed in the presence of thioredoxin provided that all thioredoxin reductase has been inactivated. This may be accomplished by heating samples to 85°C to 100°C, a treatment that inactivates both thioredoxin reductase and glutathione reductase (1).

Stoichiometry of the GSH-dependent CDP Reduction—The GSH-dependent reduction of CDP may be described as occurring in two steps with NADPH as the ultimate hydrogen donor (Reactions 1 to 3 below).

$$\text{CDP} + 2 \text{GSH} \rightarrow \text{dCDP} + \text{GSSG} + \text{H}_2\text{O} \quad (1)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+ \quad (2)$$

$$\text{Sum: NADPH} + \text{H}^+ + \text{CDP} \rightarrow \text{NADP}^+ + \text{dCDP} + \text{H}_2\text{O} \quad (3)$$

Reaction 1 was studied by removing NADPH and glutathione reductase from the standard incubation mixture. The stoichiometry was established to be 1.21 mol of GSSG produced/mol of ribonucleotide reductase in the presence of glutaredoxin (1). The specific activity of glutaredoxin expressed as nanomoles of dCDP/min/mg of protein was the same when calculated from assays with glutaredoxin and thioredoxin in the presence of excess of the B1 or B2 subunit, respectively, in the presence of excess glutaredoxin or thioredoxin.

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Turnover number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaredoxin</td>
<td>110–150</td>
</tr>
<tr>
<td>B1*</td>
<td>40–80</td>
</tr>
<tr>
<td>B2*</td>
<td>110–240</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>13–15</td>
</tr>
</tbody>
</table>

* Molecules of substrate reduced/protein molecule/min.

a Values from literature (Ref. 12) and also calculated from experiments with excess of the B1 or B2 subunit, respectively, in the presence of excess glutaredoxin or thioredoxin.
both yeast glutathione reductase and E. coli glutathione reductase to exclude a species specificity interaction. No activity was observed with E. coli thioredoxin reductase (data not shown) confirming the absence of reactivity of glutaredoxin with thioredoxin reductase (1, 2).

**Reaction of Chemically Reduced Glutaredoxin with Ribonucleotide Reductase—**The presence of a single disulfide bridge in glutaredoxin which is reducible by GSH, NADPH, and glutathione reductase was demonstrated in the previous paper (2). It may be assumed that this disulfide is at the active site of glutaredoxin and is participating in the function of glutaredoxin. In agreement with this, glutaredoxin, which was reduced and carboxymethylated (2) or treated with N-ethylmaleimide, was completely inactive in ribonucleotide reduction. The fully reduced forms of glutaredoxin and thioredoxin were isolated after chemical reduction by excess dithiothreitol followed by desalting with argon-equilibrated buffer. This reduced glutaredoxin contained 1.47 mol of sulfhydryl groups/molecular of protein as measured with 5,5'-dithiobis(2-nitrobenzoic acid) (14). As shown in Fig. 4A, the addition of reduced glutaredoxin to ribonucleotide reductase resulted in a stoichiometric reduction of CDP to dCDP. In a parallel experiment, the same result was obtained with chemically reduced E. coli thioredoxin (Fig. 4B). Thus, the fully reduced glutaredoxin is a direct hydrogen donor for ribonucleotide reductase.

**Thiol Specificity of the Glutaredoxin System—**To study the thiol specificity in Reaction 1, GSH was exchanged for 2-mercaptoethanol or dithiothreitol. The activity was determined as a function of time and compared to that of GSH (Fig. 5). No activity was obtained with mercaptoethanol. As observed previously, the activity with dithiothreitol as substrate is high. From the results of Fig. 5, it is apparent that the rate of the reaction with GSH is strongly influenced by the presence of NADPH and glutathione reductase. Two explanations for this are possible. Either glutaredoxin is directly reduced by glutathione reductase and GSH or GSSG, present in the GSH solutions, is an inhibitor of the overall reaction. The last explanation appears most likely.

**TABLE III**

**Stoichiometry of the reduction of CDP by GSH**

The standard incubation mixture was modified by removing bovine serum albumin, NADPH, and glutathione reductase. The [3H]CDP concentration was increased to 1.2 mM. The total volume was 240 μl. After 15 min at room temperature, 100 μl was taken to 1.0 ml of 1 M perchloric acid and the amount of [3H]CDP was determined as in the standard assay. To the residual 140 μl was added 290 μl of 0.50 M potassium phosphate, 5 mM EDTA, pH 7.0, and the amount of GSSG was determined as described under "Experimental Procedures." Blank values of GSSG were subtracted. Of the GSH, 3% was GSSG at the start of the incubation.

<table>
<thead>
<tr>
<th>Addition</th>
<th>GSSG (nmol/15 min)</th>
<th>dCDP (nmol/15 min)</th>
<th>Glutaredoxin (μmol/molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaredoxin, 300 pmol</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteins B1 and B2, 100 pmol</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutaredoxin, 300 pmol + proteins B1 and B2, 100 pmol</td>
<td>24.9</td>
<td>20.6</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Molecules of substrate converted/min/mole of protein.

*1 Protein B1 contains dithiols which reduce 2 to 3 eq of CDP (9).

![Fig. 3. Assay of activity of glutaredoxin by NADPH oxidation.](http://www.jbc.org/)

![Fig. 4. A, stoichiometry of formation of dCDP by fully reduced glutaredoxin. B, stoichiometry of formation of dCDP by fully reduced thioredoxin.](http://www.jbc.org/)

![Fig. 5.](http://www.jbc.org/)
changed this simple picture and questioned the physiological role of thioredoxin. The results obtained here show that in the presence of glutaredoxin, the substrates for ribonucleotide reductase are GSH and each of the four ribonucleoside-5'-diphosphates. Glutaredoxin, together with the subunits D1 and B2 of ribonucleotide reductase, thus has GSH-ribonucleotide-oxidoreductase activity. Each of the three proteins are catalytically inactive by themselves in this overall reaction. The molecular activity of glutaredoxin in vitro is similar to that of the subunits D1 and B2 of ribonucleotide reductase. Furthermore, as shown in the preceding paper (2), glutaredoxin is also a powerful GSH-disulfide oxidoreductase enzyme. These facts, together with the substrate nature of GSH and ribonucleotides, suggest that glutaredoxin may bind as an additional enzyme subunit of the ribonucleotide reductase multienzyme complex.

In vitro, both thioredoxin and glutaredoxin can act as hydrogen donors for ribonucleotide reductase. What is the situation in vivo? Several possibilities exist concerning the role of thioredoxin and glutaredoxin in ribonucleotide reduction: (i) thioredoxin is not involved in this process in vivo; (ii) thioredoxin and glutaredoxin are both natural hydrogen donors for ribonucleotide reductase but are utilized for different purposes in the cells; (iii) thioredoxin is utilized for some other aspect of ribonucleotide reduction, i.e. regulation or substrate phosphorylation (18). The final answers to which of these suggestions is correct probably requires careful analysis of mutants in the gene for glutaredoxin as well as thioredoxin. The gene for thioredoxin, called trxA, has been mapped in E. coli K12 (19). It is located at 84 min on the E. coli linkage map, between ural E and melE, and close to the origin of replication. As yet, no mutants in the glutaredoxin gene are known.

Independent evidence for the nonessential nature of the thioredoxin system in ribonucleotide reduction is the recent isolation by Fuchs (20) of an E. coli mutant lacking detectable in vitro activity of thioredoxin reductase. This mutant has no in vivo defect in the reduction of ribonucleotides. Evidence was also obtained that in such cells permeabilized by ether treatment, ribonucleotide diphosphate reduction can utilize glutathione as an alternate reducing system (20). The system described by Fuchs (20) must be identical with the glutaredoxin system studied here. In a previous paper, Fuchs and Warner (21) described the isolation of an E. coli mutant defective in glutathione synthetase. The specific activity of the B1 subunit of ribonucleotide reductase was greatly reduced in the mutant and the conclusion was drawn that one role for GSH in the cell is to maintain at least this protein in an active state (21, 22). The mutant in glutathione synthesis was the then unexpected result of an original attempt to find a mutant deficient in thioredoxin. These results provide strong circumstantial evidence for the importance of the glutaredoxin pathway in the synthesis of deoxyribonucleotides.

Another recent result questions the role of thioredoxin in ribonucleotide reduction. Piget and Conley (23) suggested a role for E. coli thioredoxin in phosphate transfer reactions. Thioredoxin, rapidly isolated from log phase cells by antibody affinity chromatography (24), has phosphate linked to both cysteine-32 (60%) and cysteine-35 (40%) via a novel thiol-phosphate bond (23). Moreover, this phosphate could be transferred from phosphothioredoxin to water or nucleotides in reactions catalyzed by L0 oxidized glutathione, NADPH-thioredoxin reductase, or crude E. coli cell extracts (25). Later measurements suggest that up to 96% of thioredoxin occurs in vivo as the phosphorylated species (26). This form of thioredoxin should not be a reductant until after dephosphorylation. As yet, the full interpretation of the biological significance of...
these results is not clear. We have observed\(^4\) that thioredoxin in fresh extracts of gently lysed cells (1) is not active as hydrogen donor for ribonucleotide reductase in vitro in the presence of thioct or NADPH until after heat treatment, suggesting the presence of an unstable modification such as phosphorylation.

The comparison of the relative activity of glutaredoxin and thioredoxin in the ribonucleotide reductase reaction favors glutaredoxin as being the natural hydrogen donor. A schematic comparison of the two systems is shown in Fig. 6. Glutaredoxin is almost 10-fold more active than thioredoxin on a molar basis. Thioredoxin-(SH)\(_2\) is not bound in any complex with ribonucleotide reductase.\(^3\) Its function may be not to reduce directly the ribonucleotide but rather to reduce a disulfide on the Bl subunit of ribonucleotide reductase and then to leave the enzyme before the substrate is bound (9). This "ping-pong" mechanism of action (9) should limit the turnover rate of the thioredoxin-catalyzed ribonucleotide reduction. The oxidized thioredoxin in turn must bind to thioredoxin reductase to become reduced (27) before its renewed interaction with ribonucleotide reductase.

Thioredoxin is a powerful general reductant of protein disulfides (28, 29). It seems likely that thioredoxin-(SH)\(_2\) in vitro may be a regulator of ribonucleotide reductase which has -SH groups which are critical for activity and is stabilized with diethylthreitol. The results with the partially inactivated ribonucleotide reductase (Table II) strongly suggest that thioredoxin can act both as hydrogen donor in vitro and as activator of the enzyme, similar to diethylthreitol. It should also be pointed out that the addition of diethylthreitol which is used in different methods to assay ribonucleotide reductase (7, 30) will measure the hydrogen donor activity of both glutaredoxin and thioredoxin.

E. coli cells have a high concentration of GSH ranging from 4 mM in log phase to 6 mM in stationary phase (31). Since GSH is one of the substrates for ribonucleotide reductase, the concentration of this tripeptide in the cell wall be of importance for the rate of deoxyribonucleotide and DNA synthesis. Moreover, as shown here, the uncoupled reaction, i.e. in the absence of NADPH and glutathione reductase, is strongly inhibited by even low concentrations of the product GSSG. The mechanism behind this is most likely a strong product inhibition by GSSG rather than an unfavorable equilibrium in the reaction. The oxidation-reduction potential of 2 GSH/ GSSG is \(-0.26\, \text{V} \) at \(25^\circ\text{C}\) and pH 7.0 (32) which is close to the value of \(-0.26\) V for the couple \(E, \text{coli} \) thioredoxin-(SH)\(_2\)/ thioredoxin-S\(_2\) (5). No value has as yet been assigned the oxidation-reduction active dithiol/disulfide of glutaredoxin.

Glutathione, which is widely distributed in biological materials, has been implicated in various theories of control of growth (33-35). Ribonucleotide reductase catalyzes the first unique step in DNA synthesis and the enzyme provides the cell with a balanced supply of the four deoxyribonucleotides. A close correlation of the regulation of DNA synthesis and ribonucleotide reduction can be expected and has been observed in many systems. The glutaredoxin pathway may constitute the coupling of ribonucleotide reduction to GSH-dependent control mechanisms. In this context, it is of special interest that all the spermidine and a large part of the GSH form the covalent adduct, glutathionylspermidine, at the end of logarithmic growth in E. coli (36, 37). Glutathionylspermidine is in equilibrium with free GSH and spermidine through enzymatic processes (37). Spermidine has been implicated in DNA synthesis and nucleic acid metabolism and glutathionylspermidine may play an integral role in the regulation of growth and nucleic acid metabolism (37).

Glutaredoxin is not unique for E. coli. Recently, the protein called phase T4 thioredoxin, which is induced after T4 virus infection of E. coli was shown to have glutaredoxin activity (17). We have also identified a glutaredoxin system from calf thymus\(^4\) with the aid of a calf thymus ribonucleotide reductase.

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Functional Properties of Glutaredoxin


A Holmgren


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