A NEW RIBONUCLEOTIDE REDUCTASE SYSTEM AFTER INFECTION WITH PHAGE T4*

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Abstract.—The ribonucleotide reductase system of Escherichia coli B participates in the biosynthesis of DNA by reducing ribonucleoside diphosphates to the corresponding deoxyribonucleotides. The enzyme is regulated in a complicated way by allosteric modifiers. We now find that infection of E. coli with the bacteriophage T4 results in the appearance of a new ribonucleotide reductase system which shows a somewhat different pattern of regulation.

Two new proteins, provisionally called fractions A and B, were purified from the extracts of infected bacteria. The reduction of ribonucleotides by these two fractions required the addition of either dithiothreitol or TPNH and E. coli thioredoxin reductase. Mutants of T4 which lacked fraction A activity were obtained. Fraction B may be a virus-induced thioredoxin.

Escherichia coli contains an enzyme system that catalyzes the reduction of the four ribonucleoside diphosphates to the corresponding deoxyribonucleotides. The enzyme system consists of four proteins, which participate in the following reactions:

1. Ribonucleoside 5'-diphosphate + thioredoxin-(SH)₂ → deoxyribonucleoside 5'-diphosphate + thioredoxin-S₂.

2. Thioredoxin-S₂ + TPNH + H⁺ → thioredoxin-(SH)₂ + TPN⁺.

Escherichia coli contains an enzyme system that catalyzes the reduction of the four ribonucleoside diphosphates to the corresponding deoxyribonucleotides. The enzyme system consists of four proteins, which participate in the following reactions:

\[ \text{Ribonucleoside 5'-diphosphate} + \text{thioredoxin-(SH)₂} \xrightarrow{\text{proteins B₁ and B₂}} \text{Mg}^{2⁺} \xrightarrow{\text{Mg}^{2⁺}} \text{deoxyribonucleoside 5'-diphosphate} + \text{thioredoxin-S₂}. \quad (1) \]

\[ \text{Thioredoxin-S₂} + \text{TPNH} + \text{H⁺} \xrightarrow{\text{thioredoxin reductase}} \text{thioredoxin-(SH)₂} + \text{TPN⁺}. \quad (2) \]

Thioredoxin is a protein that is low in molecular weight and that contains two half-cystine residues which exist either in the reduced (\(=\text{thioredoxin-(SH)₂}\)) or oxidized (\(=\text{thioredoxin-S₂}\)) state. Thioredoxin-(SH)₂ reduces the ribosyl moiety of the ribonucleotide as depicted in reaction (1). The regeneration of thioredoxin-(SH)₂ by TPNH in reaction (2) is catalyzed by the flavoprotein thioredoxin reductase. Reaction (1) is catalyzed by the enzyme ribonucleotide reductase, which contains two nonidentical subunits (proteins B₁ and B₂) bound together in the presence of Mg ions.

The substrate specificity of ribonucleotide reductase is determined by nucleoside triphosphates acting as allosteric effectors. ATP stimulates the reductions of pyrimidine ribonucleotides; dGTP stimulates that of purine ribonucleotides; and deoxythymidine triphosphate (dTTP) stimulates and dATP inhibits the reduction of all four ribonucleotides.

The reactions catalyzed by ribonucleotide reductase supply the cell with deoxyribonucleotide precursors required for the synthesis of DNA. Infection...
of *E. coli* with T-even phages results in the formation of several early enzymes related to DNA synthesis. Some of these phage enzymes, e.g., thymidylate synthetase and dihydrofolate reductase, are formed despite the presence of isoenzymes existing previously in uninfected cells. The induction of ribonucleotide reductase activity by T-even phage has been described by Cohen and Barner (T6) and Biswas, Hardy, and Beck (T2). Both groups demonstrated increased enzyme activities in crude extracts from phage-infected bacteria.

In the present report we describe a new ribonucleotide reductase system that is present in *E. coli* B after infection with T4. Our results indicate that the T4-induced enzymes differ from those of uninfected *E. coli* with respect to composition of protein components, Mg²⁺ requirement, and behavior toward allosteric effectors, and we have identified two T4 mutants that lack the ability to induce ribonucleotide reductase. In a separate communication, Yeh, Dubovi, and Tessman describe independent experiments concerning genetic and functional aspects of T4-induced ribonucleotide reductase.

**Materials and Methods.**—Nucleotides were obtained from sources mentioned previously. *H*-CMP, *H*-CDP, and *H*-CTP were purified on DEAE-Sephadex according to the method of Wehrli, Verheyden, and Moffat. Thioredoxin and thioredoxin reductase were 80% pure preparations from *E. coli*. Proteins B1 and B2 were purified from *E. coli B3.*

**Enzyme assays:** The standard incubation mixture for the ribonucleotide reductase assay contained in a volume of 0.060 ml: MgCl₂, 0.35-0.70 μmole; hepes (N-2-hydroxyethylpiperazine-N'₂-2-ethane sulfonic acid) buffer, pH 7.6, 1.8 μmoles; ATP, 0.09 μmole; EDTA, 0.06 μmole; *H*-CDP (specific activity 2.0 × 10⁶ cpm/μmole) 0.05 μmole; TPNH, 0.03 μmole; thioredoxin, 1.3 × 10⁻⁴ μmoles; thioredoxin reductase, 2 × 10⁻⁴ μmoles; dithiothreitol, 0.7 μmole, and enzyme. After incubation for 10 min at 22-24°C, the reaction was stopped by the addition of 1 ml of M HClO₄, and the amount of dCDP formed was determined. One unit of enzyme is defined as that amount of enzyme which catalyzes the formation of 1 μmole of dCDP under the above conditions. Specific activity is defined as units per milligram of protein. Fraction A was assayed in the presence of an excess of fraction B and vice versa. In some experiments, the reaction rate was measured spectrophotometrically by determinations of the disappearance of TPNH with an incubation volume of 0.13 ml. Dithiothreitol was omitted in this assay, since it interferes with the oxidation of TPNH. However, this omission resulted in a destabilization of the enzyme system, and only relative reaction rates could be obtained with this second assay. The dCTPase assay was a radioactive (¹⁴C-dCTP) modification of that of Wiberg et al.

**Strains:** *E. coli* B, *E. coli* CR63, and T4 am 122 were supplied by Dr. O. Sköld; T4D o and T4D am A453 were from Dr. I. Tessman.

**Mutagenesis:** T4D o was treated extensively with hydroxylamine as described by Hall and Tessman. Out of 16 clones tested, two failed to induce ribonucleotide reductase activity. These clones (T4D nrd101 and 102) were backcrossed once to T4D am A453.

**Phage infection:** Cells of *E. coli* B were grown in glycerol-casamino acids medium to a density of 5 × 10⁶/ml. T4 was added twice at a multiplicity of four (4-min interval). At various times, aliquots of the culture were removed and chilled rapidly with crushed ice. The cells were harvested by centrifugation and stored at −20°C.

**Enzyme purification:** All steps were carried out at 0-4°C. All buffer solutions contained 1 mM dithiothreitol.

Frozen bacteria were disintegrated in a modified Hughes press and extracted with five times their weight of 0.05 M Tris-HCl buffer, pH 7.0, containing 0.001 M mercaptoethanol. The extract after centrifugation (25 mg of protein/ml) was precipitated with 5% strepto-
mycin sulfate (1 ml per 5 ml of extract) and centrifuged. The supernatant solution was then fractionated with solid ammonium sulfate. The fractions precipitated between 0–35% saturation (fraction A) and 45–80% saturation (fraction B), respectively, were used. Fraction A, after being desalted on Sephadex G-25, was chromatographed on DEAE-cellulose with a phosphate gradient (pH 7.0) from 0.2 to 0.5 M. The activity appeared around 0.35 M. Fraction B was also chromatographed on DEAE-cellulose. After removal of inactive protein by 0.01 M phosphate buffer, pH 7.0, fraction B was eluted with 0.05 M phosphate buffer, pH 7.0.

Both DEAE fractions were precipitated with ammonium sulfate, dissolved in 0.05 M Tris-HCl, pH 7.0, and stored frozen. The final yield of the 30-fold purified fraction A was 15%; the yield of the 60-fold purified fraction B was 30%.

Results.—Formation of ribonucleotide reductase in E. coli B infected with T4 am 122: Extracts of E. coli B infected with T4 am 122 were prepared from samples taken at intervals after infection and assayed for CDP-reducing activity (Fig. 1). At time zero, essentially no enzyme activity was detectable under the assay conditions used. Activity began to appear five minutes after infection and increased almost linearly with time up to about 20 minutes after infection. In a control experiment, also shown in Figure 1, the extracts were assayed for dCTPase activity. The formation of dCTPase was induced with normal kinetics,7 which indicated that infection of the nonpermissive bacterial host was successful.

Evidence for the presence of two new proteins: Attempts to purify the T4-induced CDP reductase activity by procedures developed for the purification of proteins B1 and B2 from uninfected E. coli were unsuccessful, and a completely different purification scheme had to be worked out (see Materials and Methods). The enzyme activity separated into two protein fractions (fractions A and B) on precipitation with two different ammonium sulfate concentrations, and each of the two fractions could be purified further by chromatography on DEAE cellulose. The simultaneous presence of both fractions was required for enzyme activity.

The dependence of the reduction of CDP on the amounts of fractions A and B is shown in Figure 2. The reaction rate was directly proportional to the concentration of the limiting fraction. Fraction B was completely inactive in the absence of fraction A, but most preparations of fraction A showed about 5 per cent activity in the absence of fraction B.

Table I demonstrates that the A and B activities could not be detected in an extract from uninfected bacteria; the extract did not inhibit the phage-induced reductase.

Table I also shows experiments with extracts from bacteria infected with two mutants of the phage (T4D nrd101 and nrd102; for their isolation, see Materials and Methods). Both these extracts reduced CDP when fortified with fraction A, but showed no activity after addition of fraction B.

Further evidence for the nonidentity of the purified fractions A and B with either of the components of the ribonucleotide reductase system from uninfected E. coli was obtained from mixing experiments (Table 2). It is clear that in the presence of TPNH and thioredoxin reductase from uninfected bacteria, no cross reaction was observed between either fractions A or B and any of the three com-
components (B1, B2, or thioredoxin) from uninfected bacteria. Together with the results of Table 1, these experiments strongly indicate that the formation of both fraction A and fraction B activities is induced by T4 am 122 in E. coli B, and that fractions A and B are different from proteins B1, B2, and thioredoxin.

**Requirements for CDP reduction:** Some of the requirements for the reduction of CDP are summarized in Table 3. The reaction showed absolute requirements for either dithiothreitol or two components of the thioredoxin system: thioredoxin reductase and TPNH. On the other hand, no requirement for thioredoxin could be demonstrated. Neither fraction A nor fraction B contained significant amounts of E. coli thioredoxin as a contaminant, and it therefore appears that E. coli thioredoxin does not participate in the phage-induced

**Table 1.** Ribonucleotide reductase activities in crude extracts from uninfected and phage-infected* E. coli B.

<table>
<thead>
<tr>
<th>Phage used for infection</th>
<th>Purified fraction added</th>
<th>Enzyme activity (mumoles dCDP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>A</td>
<td>0.1†</td>
</tr>
<tr>
<td>Uninfected</td>
<td>B</td>
<td>0.1</td>
</tr>
<tr>
<td>Uninfected</td>
<td>A, B†</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>A, B†</td>
<td>9.2</td>
</tr>
<tr>
<td>T4D nrd101</td>
<td>A</td>
<td>4.8†</td>
</tr>
<tr>
<td>T4D nrd101</td>
<td>B</td>
<td>0.1</td>
</tr>
<tr>
<td>T4D nrd102</td>
<td>A</td>
<td>5.1†</td>
</tr>
<tr>
<td>T4D nrd102</td>
<td>B</td>
<td>0.1</td>
</tr>
<tr>
<td>T4D o</td>
<td>A</td>
<td>6.4†</td>
</tr>
<tr>
<td>T4D o</td>
<td>B</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Incubations were performed under standard conditions with 10 µl of extract (containing 25 mg of protein per ml) and fraction A (24 µg) or fraction B (7 µg).

* 20-min infection.
† In these experiments, only 6 µg of fraction A and 3 µg of fraction B were used.
‡ These values are corrected for residual fraction A activity (1.2 mumoles).
were slightly except as which demonstrates
TABLE 3.

\[
\begin{array}{cccc|c}
\text{Normal } E. coli & \text{Thioredoxin} & \text{Phage-Induced} & \text{Enzyme activity} \\
\hline
\text{B1} & \text{B2} & \text{Fraction A} & \text{Fraction B} & (\text{mmoles dCDP}) \\
+ & + & + & - & 11.9 \\
- & - & + & - & 0.3 \\
+ & + & + & + & <0.1 \\
+ & + & + & - & 0.1 \\
+ & + & - & - & 0.3 \\
+ & + & - & + & 0.3 \\
- & - & + & + & 14.9 \\
\end{array}
\]

Incubations were carried out under standard conditions, except that dithiothreitol was omitted.
Amounts of enzyme protein used: 8 \( \mu \text{g} \) of protein B1, 6 \( \mu \text{g} \) of protein B2, 1.5 \( \times 10^{-4} \) \( \mu \text{mole} \) of thioredoxin, 18 \( \mu \text{g} \) of fraction A, and 7 \( \mu \text{g} \) of fraction B.

**TABLE 3.** Requirements for the reduction of CDP.

<table>
<thead>
<tr>
<th>Conditions of incubation</th>
<th>dCDP (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>12.8</td>
</tr>
<tr>
<td>Thioredoxin system (thioredoxin, thioredoxin reductase, and TPNH) omitted</td>
<td>12.6</td>
</tr>
<tr>
<td>Dithiothreitol omitted</td>
<td>6.5</td>
</tr>
<tr>
<td>&quot; and thioredoxin omitted</td>
<td>6.4</td>
</tr>
<tr>
<td>&quot; and thioredoxin reductase omitted</td>
<td>0.3</td>
</tr>
<tr>
<td>&quot; and TPNH omitted</td>
<td>0.4</td>
</tr>
<tr>
<td>MgCl(_2) omitted</td>
<td>5.9</td>
</tr>
<tr>
<td>ATP omitted</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Incubations were done under standard conditions (12 \( \mu \text{g} \) of fraction A and 3 \( \mu \text{g} \) of fraction B) except as indicated.

ribonucleotide reductase system. We shall return to this point in the discussion. The stimulatory effect of dithiothreitol in the presence of the thioredoxin system may be explained by the ability of the dithiol to stabilize fraction A.

Both ATP and \( \text{Mg}^{2+} \) clearly stimulated the reaction; the requirement for \( \text{Mg}^{2+} \) was not absolute, not even in the presence of 3 \( \times 10^{-4} \) \( \text{M} \) EDTA. Maximal conversion was obtained at 5 \( \times 10^{-3} \) \( \text{M} \) \( \text{MgCl}_2 \), while higher concentrations were slightly inhibitory.

CDP was the preferred substrate for the reaction. This is shown in Figure 3, which demonstrates an experiment where CMP, CDP, and CTP were compared as substrates. Only CDP was reduced, and saturation of the enzyme was achieved at concentrations above 5 \( \times 10^{-4} \) \( \text{M} \).

The stimulation of CDP reduction by ATP was not specific for this nucleotide; both dTTP and dATP gave similar effects (Fig. 4). Maximal activity with the latter nucleotides required concentrations around \( 10^{-5} \) \( \text{M} \), while the maximal stimulation by ATP required concentrations above \( 10^{-3} \) \( \text{M} \). Thus, with dTTP and dATP, only catalytic amounts of nucleotides were required; and it seems reasonable to propose that the nucleoside triphosphates act as allosteric effectors, as with the "normal" reductase. The finding that dATP stimulated the reaction in the whole concentration range is particularly interesting, since higher concentrations of dATP (above \( 10^{-5} \) \( \text{M} \)) inhibit the reduction of CDP by the bacterial enzyme.\(^5\)

**Reduction of UDP and GDP:** The phage-induced enzyme also catalyzed the reduction of other ribonucleoside diphosphates. In Table 4 the reductions of
CDP, UDP, and GDP in the presence of dTTP are compared. About equal reaction rates were obtained with all three substrates. In these experiments, the reduction of substrates was coupled to the oxidation of TPNH. ADP causes special problems and was not included in this experiment. When dATP was added to the reaction mixtures, the reduction of GDP was completely inhibited, while the reductions of CDP and UDP were unaffected (Table 4). This experiment again demonstrates the dissimilarity between the normal and the phage-induced enzymes with respect to inhibition by dATP.

**Discussion.**—The kinetics of ribonucleotide reductase formation after infection of *E. coli* B with T4 am 122 are characteristic for those of early enzyme synthesis induced by mutants unable to form DNA. The formation of both fraction A and fraction B activities appears to be induced by T4, and neither of the two fractions was present in extracts from uninfected cells. The evidence is strongest for fraction A, since two mutants defective in the ability to induce fraction A activity could be isolated. Much more extensive genetic evidence for the existence of two cistrons of T4 involved in the reduction of ribonucleotides was obtained by Yeh, Dubovi, and Tessman.17

The reduction of CDP with fractions A and B required the addition of either dithiothreitol or TPNH and *E. coli* thioredoxin reductase. However, no evidence for the participation of *E. coli* thioredoxin was obtained. These results

**Table 4.** Substrate specificity of ribonucleotide reductase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Δ TPNH (mumoles/10 min)</th>
<th>Δ TPNH after addition of dATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>~</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>CDP</td>
<td>9.9</td>
<td>10.9</td>
</tr>
<tr>
<td>UDP</td>
<td>9.1</td>
<td>7.8</td>
</tr>
<tr>
<td>GDP</td>
<td>8.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Incubations were carried out in microcuvettes under standard conditions for TPNH oxidation (incubation volume 0.13 ml), except that ATP was replaced by dTTP (4.5 × 10⁻⁵ M). The cuvettes contained 30 μg of fraction A and 7.5 μg of fraction B. Ten minutes after addition of substrates, 46 mumoles of dATP were added to each cuvette.
would be explained if in the virus-induced ribonucleotide reductase system either fraction A or fraction B were the functional counterpart of \textit{E. coli} thioredoxin.

We have recently found that fraction B is a small, heat-stable protein and that very high concentrations of dithiothreitol can substitute in part for the fraction B requirement. These findings lead us to believe that fraction B is indeed a T4 thioredoxin. In that case, fraction A would be the actual T4 ribonucleotide reductase. This explanation would require that the reduction of the viral thioredoxin be catalyzed by \textit{E. coli} thioredoxin reductase and that fraction A use the viral thioredoxin—but not \textit{E. coli} thioredoxin—for the reduction of CDP. Further purification of fraction B is required, however, to settle these questions more definitely.

A comparison between the viral and bacterial ribonucleotide reductases brings out several similarities and dissimilarities. The similarities concern the fact that both enzymes use ribonucleoside diphosphates as substrates, are stimulated by magnesium ions, and are modified by nucleoside triphosphates.

On the other hand, the phage-induced enzyme shows only a moderate stimulation by Mg$^{2+}$, while the normal reductase shows an absolute requirement for a divalent cation which appears to function in the formation of the active reductase from its subunits (proteins B1 and B2).4 One further dissimilarity concerns the effect of modifiers. Only a few experiments were carried out on this point with the viral enzyme, but it is already apparent that DATP in this system is not a \textit{general} negative effector, as has been found with the bacterial enzyme. However, a detailed study of the effect of modifiers must await the availability of pure viral enzymes.

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6 \textit{Ibid.}, p. 2540.