Oxygen-sensitive ribonucleoside triphosphate reductase is present in anaerobic *Escherichia coli*

(deoxyribonucleotides/hydroxyurea/evolution)

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ABSTRACT Ribonucleoside diphosphate reductase from *Escherichia coli* and mammalian cells provides the deoxyribo-

nucleoside triphosphates for DNA synthesis. The active enzyme contains a tyrosyl free radical whose formation requires oxygen. Earlier genetic evidence suggested that the enzyme is not required for anaerobic growth of *E. coli*, implicating the activity of a different enzyme or enzyme system for deoxyribo-

nucleotide synthesis in the absence of oxygen. We now conclude from isotope incorporation experiments that *E. coli* during anaerobiosis obtains its deoxyribonucleotides by reduct-

ion of ribonucleotides. Extracts from anaerobically grown bacteria contain a different enzyme activity capable of reducing CTP to dCTP. To obtain an active enzyme, strict anaerobiosis must be maintained during extract preparation and during assay of the enzyme. The reaction is stimulated by NADPH, Mg\(^{2+}\), and ATP. Inhibition by deoxyribonucleoside triphos-

phates suggests that the anaerobic enzyme has allosteric properties. Antibodies raised against the aerobic enzyme do not inhibit the new activity, and hydroxyurea, a potent scavenger of the tyrosyl radical of the aerobic enzyme, only weakly inhibits the anaerobic enzyme. The anaerobic enzyme has interesting evolutionary aspects since it might reflect on an activity that in the absence of oxygen made possible the transition from an “RNA world” into a “DNA world.”

*Escherichia coli* obtains the deoxyribonucleotides required for DNA synthesis from ribonucleotides through the activity of the enzyme ribonucleoside diphosphate reductase (1–3). A closely related enzyme has the same function in mammalian cells. Both enzymes consist of two nonidentical subunits, proteins B1 and B2 (4) of *E. coli* and the corresponding mammalian proteins M1 and M2 (5). B2 and M2 contain a tyrosyl radical (6–8) that has arisen by oxidation of a specific tyrosine residue of the polypeptide chain. An oxygen-requiring enzyme system catalyzes the formation of this radical in *E. coli* (9, 10). However, *E. coli* grows well also under anaerobic conditions. This poses the question how deoxyribonucleotides are synthesized in the absence of oxygen. One could imagine that during anaerobiosis an electron acceptor other than oxygen might function in radical generation. Or, deoxyribonucleotides might be formed by other enzymes. The second alternative was favored strongly from genetic experiments (11–13) demonstrating that mutations in the *nrDA* or *nrDB* gene, coding for protein B1 or B2, did not affect the anaerobic growth of the bacteria. These experiments did, however, not distinguish between a different ribonucleotide reductase or a completely different mode of synthesis—e.g., via the deoxyribose 5-phosphate aldolase pathway (15, 16).

Here we first establish from isotope experiments that *E. coli* during anaerobiosis obtains its deoxyribonucleotides by reduction of ribonucleotides. We then describe the existence of a different reductase in extracts from anaerobically grown bacteria. The enzyme is not inhibited by antibodies raised against protein B1 or B2 and is only moderately sensitive to hydroxyurea, a drug that strongly inhibits the aerobic reductase, but is rapidly inactivated by air. The substrate of the reaction appears to be CTP and not CDP, as for the aerobic reaction.

MATERIALS AND METHODS

Bacterial Strains. The *E. coli* K-12 strain AB1157 and its oxygen-sensitive derivative Oxy\(^{−}\)-11 (11) were obtained from H. I. Adler (Biology Division, Oak Ridge National Labora-

tory, Oak Ridge, TN). *E. coli* Sö1452, a strain with an insertion mutation in the gene for cytidine deaminase, was a gift from B. Mygind (Institute for Biological Chemistry, University of Copenhagen, Denmark).

Preparation of Bacterial Extracts. Five-liter cultures of AB1157 or Oxy\(^{−}\)-11 were grown anaerobically in Luria broth containing 1 g of glucose per liter under continuous bubbling with 4% CO\(_2\)/96% N\(_2\) in a Microferm New Brunswick fermentor with constant pH control. When the bacteria had reached midlogarithmic phase (OD = 1.0 at 640 nm) they were harvested by centrifugation in twelve 0.5-liter bottles in cold Sorvall centrifuges, resuspended under a flow of argon in a small volume of an anaerobic 50 mM Tris buffer (pH 7.5), and transferred under argon to four preweighed capped Beckman no. 355603 thick-walled polycarbonate tubes. After centrifugation and removal of the supernatant solution, the weighed bacteria (1–2 g per tube) were stored under liquid nitrogen. The bacteria were extracted in the cold room under a stream of argon for 45 min with an equal volume of oxygen-free 50 mM Tris (pH 7.5) containing 50 mM KCl, 10 mM dithiothreitol, and 0.6 mg of egg white lysozyme per ml + 0.4 µg of phage T4 lysozyme per ml, frozen twice in liquid nitrogen, and thawed each time in an ice bath. The argon-filled tubes were capped and centrifuged for 45 min at 4°C and at 45,000 rpm in the Ty 65 rotor of a Beckman ultracentrifuge. After centrifugation the bacterial extract was aspirated with a syringe, previously flushed with oxygen-free buffer, and transferred through a rubber septum into an argon-filled tube attached to an anaer-

obic manifold. All further transfers were made with syringes to argon-filled tubes attached to the manifold. Portions of the extract were stored under liquid nitrogen.

Assays for Ribonucleotide Reduction. Anaerobic incubations with [\(^{3}H\)]CDP were made at room temperature under a stream of moist argon in small glass tubes attached to the anaerobic manifold. Oxygen-free reagents were first added to the tubes, which were then maintained under a stream of argon for at least 1 hr. The reaction was started by addition

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of the bacterial extract and stopped by addition of 0.5 ml of 1 M perchloric acid. Ribonucleotide reduction was quanti-
tized as described earlier (16) from the amount of radioactive dCMP obtained after acid hydrolysis of all deoxycytidine phosphates formed. In one experiment when we wished to determine separately isotope incorporation into dCMP, dCDP, and dCTP, perchloric acid was removed by extraction with octylamine/Freon 113 (1,1,2-trichloro-1,2,2-trifluoro-
ethane) (17), and the three nucleotides were separated by chromatography on DEAE-Sephadex (18) before the acid hydrolysis step. One unit of activity is defined as 1 nmol of dCMP formed per min; specific activity is units/mg of protein (19).

To determine the relative amounts of labeled CMP, CDP, and CTP present during incubation, portions of the solutions were separated by thin-layer chromatography (20). The areas corresponding to each nucleotide were cut out, eluted overnight with 1 ml of 0.5 M HCl, and assayed for radioactivity after addition of Instagel in a liquid scintillation counter.

RESULTS

In Vivo Evidence for Ribonucleotide Reduction in E. coli During Anaerobic Growth. Strong genetic evidence indicates that the enzyme coded for by the nrdA and nrdB genes is not required for deoxribonucleotide synthesis during anaerobic growth of E. coli (11–13). We conducted the following experiment to investigate whether E. coli during anaerobiosis also synthesizes deoxynucleotides by reduction of ribo-
nucleotides. Two cultures of E. coli Sö1452, a strain lacking the enzyme cytidine deaminase, were grown in parallel with [3H]cytidine either in air or during anaerobic conditions. In air, cytidine is phosphorylated efficiently to CDP, further reduced to dCDP, and finally incorporated into RNA and DNA (21). Table 1 shows for both cultures the incorporation of isotope into RNA and DNA and also gives the specific activities of the CTP and dCTP pools after 15 and 30 min of incubation. Furthermore, the anaerobic bacteria incorporated cytidine not only into the CTP pool and RNA but also into dCTP and DNA. In effect, the ratios between the incorporations into DNA and RNA as well as between the specific activities of the dCTP and CTP pools at both time points were identical during aerobic and anaerobic growth.

These data provide compelling evidence for the metabolism from cytidine to DNA being the same in the two cultures and demonstrate that the anaerobic bacteria also used the reduc-
tion of ribonucleotides to provide the deoxyribonucleotides required for DNA synthesis.

An Oxygen-Sensitive Ribonucleotide Reductase in Extracts from Anaerobic E. coli. In our attempts to find reduction of ribonucleotides in a soluble system from anaerobic bacteria we used crude extracts from cells of an oxygen-sensitive bacterial strain (Oxy+11) (11). This strain cannot grow in air since it has a mutation in the nrdB gene (G. Lindahl, B.-M. Sjöberg, and P.R., unpublished) and lacks a functional aerobic reductase. Initially we could not demonstrate the reduction of cytidine ribonucleotides by such extracts under conditions similar to those used successfully with extracts from aerobic E. coli. In these early experiments extracts of the anaerobically grown cells and incubations were made in air. Consistent positive results were finally obtained only by strictly adhering to the anaerobic techniques described in Materials and Methods. We could then follow a wide concentra-
tion range demonstrate a linear dependence of the formation of deoxycytidine phosphates (analyzed as dCMP after hydrolysis of dCDP and dCTP) on the amount of enzyme (Fig. 1). This is in contrast to S-shaped curves found in similar experiments with the aerobic enzyme (4) that depended on the dissociation of two loosely bound nonidentical subunits. The present result suggests either that the anaerobic enzyme consists of only one subunit or that subunits are tightly associated. A time curve of the reaction is also shown in Fig. 1. The rate of the reaction decreased after 10 min even though incubation was carried out at room temperature and only a small percentage of the substrate was transformed into product.

Fig. 2 shows the dependence of dCMP synthesis on various additions to the incubation mixture. Optimal synthesis occurred at 4 mM ATP and 8 mM Mg2+. NADPH gave a small but consistent stimulation. Addition of phosphoenolpyruvate at a final concentration of 20 mM increased dCMP formation ≈2-fold (data not shown). Through the action of pyruvate kinase, present in the bacterial extract, phosphoenolpyruvate continuously regenerates nucleoside triphosphates and phosphoylates most of the initially added labeled CDP to CTP. Within a few minutes an equilibrium was established between CTP, CDP, and CTP and then largely maintained during the 20 min of the experiment (data not shown). Addition of phosphoenolpyruvate strongly affected the equilibrium. When it was added, the CTP/CDP ratio was close to 10; in

Table 1. Comparison of the metabolism of [3H]cytidine in aerobic and anaerobic E. coli

<table>
<thead>
<tr>
<th>Condition</th>
<th>DNA</th>
<th>RNA</th>
<th>DNA/RNA</th>
<th>cpm/pmol</th>
<th>dCTP</th>
<th>dCTP/CTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>7.5</td>
<td>110</td>
<td>0.07</td>
<td>175</td>
<td>320</td>
<td>1.8</td>
</tr>
<tr>
<td>30 min</td>
<td>19.5</td>
<td>250</td>
<td>0.08</td>
<td>227</td>
<td>360</td>
<td>1.6</td>
</tr>
<tr>
<td>Anaerobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>8.3</td>
<td>92</td>
<td>0.09</td>
<td>138</td>
<td>260</td>
<td>1.9</td>
</tr>
<tr>
<td>30 min</td>
<td>16</td>
<td>170</td>
<td>0.09</td>
<td>131</td>
<td>210</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Two 15-ml cultures of E. coli Sö1452 in Luria broth with 0.1% glucose were grown in parallel and vigorously gassed at 37°C with either 96% N2/4% CO2 or 95% air/5% CO2. When the cultures had reached an OD of 0.22 (640 nm), [3H]cytidine (5000 cpm/pmol) was added to a final concentration of 6 μM. Five-milliliter portions were removed from each culture after 15 min (OD = 0.36) and 30 min (OD = 0.53) and centrifuged, and the pellet was used to measure incorporation of radioactivity into RNA and DNA as well as for the determination of the specific radioactivities of the CTP and dCTP pools by earlier described methods (22).

![Fig. 1](attachment:image.png)

**FIG. 1.** Dependence of dCMP formation on amount of extract and time. Increasing amounts of an anaerobic extract were incubated anaerobically at room temperature in a final volume of 0.09 ml at 4 mM ATP, 8 mM MgCl2, 1 mM NADPH, 1 mM [3H]CDP (52 cpm/ pmol), 20 mM phosphoenolpyruvate, and 5 mM dithiothreitol for between 10 and 30 min. Portions of the incubation mixtures were removed at the indicated time intervals and analyzed for formation of [3H]CMP. The enzyme concentration curve (●) was obtained after 20 min; the time curve (○) was made with 1.6 mg of enzyme protein.
its absence the ratio was 1.5–2.5. The finding that phosphoenolpyruvate increased the reaction rate may be linked to this effect and thus indicates that CTP is the preferred substrate.

The dependence of the anaerobic reaction on substrate concentration in the presence of phosphoenolpyruvate is shown in Fig. 3. \(^{3}H\)CDP added as substrate was largely phosphorylated to \(^{3}H\)CTP by the pyruvate kinase and nucleoside kinase activities present in the extract. An equilibrium was rapidly established between the di- and triphosphates and maintained during the course of the experiment. The position of this equilibrium and the concentrations of CDP and CTP were determined by thin-layer chromatography at different intervals during incubation. In Fig. 3 the rate of the reaction, expressed as specific activity of the enzyme, is plotted against the concentration of either CTP or CDP. Plotted against the CTP concentration the reaction obeys Michaelis–Menten kinetics with a high \(K_m\) for the substrate in the millimolar range. With CDP as the putative substrate the curve would be highly sigmoidal. The aerobic reductase does not show a sigmoidal substrate concentration curve (1). On balance, these data again suggest that CTP, and not CDP, is the substrate for the anaerobic enzyme. More decisive evidence on this point comes from the next experiment.

In this case our aim was to determine whether dCDP or dCTP is the immediate product of the reaction. An excess of nonlabeled dCDP and dCTP was added in the experiment to trap the labeled product before isotope equilibrated between dCMP, dCDP, and dCTP. The immediate product of the reaction then contains the highest specific radioactivity, with the largest difference found at early time points. The results summarized in Table 2 demonstrate that dCTP has the highest specific activity after 5 and 10 min.

**Anaerobic Reductase Is Probably an Allosteric Enzyme.** Addition of phosphoenolpyruvate sufficed to transform >90% of CDP to CTP, also in the absence of added ATP. Nevertheless, addition of ATP stimulated CTP reduction (Table 3), without affecting the CTP/CDP ratio. With some extracts the stimulation was >10-fold. Deoxynucleoside triphosphates could not substitute for ATP but, instead, inhibited the reaction (Table 3). A closer analysis of these effects is postponed until a pure preparation of the enzyme is available. Nevertheless, the present results are related to similar observations with the aerobic enzyme that are explained by protein B1 being an allosteric protein (1). This suggests that the anaerobic enzyme also has allosteric properties.

**Anaerobic and Aerobic Reductases Differ in Their Inhibition by Hydroxyurea and Antibodies.** Hydroxyurea can act as a radical scavenger and in this capacity inhibits the aerobic ribonucleotide reductase by destroying its tyrosyl radical (23). Fig. 44 compares the effect of hydroxyurea on ribonucleotide reduction catalyzed by an extract from the aerobically grown AB1157 and the anaerobic Oxy-11. The aerobic extract was inhibited strongly, whereas there was only a minor but reproducible effect on the anaerobic enzyme. The difference in the sensitivity of the two systems to hydroxyurea was almost of order of magnitude. A similar comparison concerning the inhibition of the two systems by polyclonal antibodies raised against the two subunits of the aerobic

### Table 2. Product of the anaerobic reductase is dCTP

<table>
<thead>
<tr>
<th>Specific activity, cpm/nmol</th>
<th>5 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCMP</td>
<td>460</td>
<td>1800</td>
</tr>
<tr>
<td>dCDP</td>
<td>750</td>
<td>1750</td>
</tr>
<tr>
<td>dCTP</td>
<td>1060</td>
<td>2000</td>
</tr>
</tbody>
</table>

An anaerobic extract (3.3 mg of protein) was incubated as described in the legend to Fig. 1, but without phosphoenolpyruvate, together with 0.25 mM (each) dCDP and dCTP in a final volume of 0.23 ml. Samples were removed after 5 and 10 min, precipitated with 0.5 ml of 1 M perchloric acid, and centrifuged. After extraction with Freon to remove the acid, CMP + dCMP, CDP + dCDP, and CTP + dCTP were separated by chromatography on DEAE with a volatile triethylamine/CO\(_2\) buffer. The buffer was removed in a vacuum from each nucleotide fraction, and after acid hydrolysis each fraction was chromatographed on Dowex-50 to separate CMP from dCMP to permit determinations of the specific activities of the three deoxyribonucleoside phosphates.

### Table 3. Effects of nucleoside triphosphates on the reaction rate

<table>
<thead>
<tr>
<th>Added dNTP</th>
<th>Conc., mM</th>
<th>Specific activity (\times 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>dATP</td>
<td>0.03</td>
<td>52</td>
</tr>
<tr>
<td>0.1</td>
<td>44</td>
<td>499</td>
</tr>
<tr>
<td>0.3</td>
<td>32</td>
<td>183</td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>183</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.1</td>
<td>33</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>65</td>
</tr>
<tr>
<td>dCTP</td>
<td>1</td>
<td>62</td>
</tr>
<tr>
<td>dGTP</td>
<td>1</td>
<td>22</td>
</tr>
</tbody>
</table>

Incubations were carried out with 0.44 mg of protein for 10 min as described in the legend to Fig. 1 and with ATP and deoxyribonucleoside triphosphates added as indicated.
enzyme (proteins B1 and B2) showed no effect on the anaerobic enzyme at antibody concentrations that strongly inhibited the aerobic enzyme (Fig. 4B).

"Wild-Type" E. coli also Contains an Anaerobic Reductase.

The enzyme experiments described so far were obtained with an extract from OxyS-11 that lacks aerobic ribonucleotide reductase activity. It was important to investigate whether the anaerobic activity could be demonstrated also in the parent strain that has normal nrdA and nrdB genes and thus during aerobic growth synthesizes deoxyribonucleotides via "normal" CDP reduction. To this purpose we prepared an anaerobic extract from anaerobically grown parent AB1157 and tested its ability to reduce CTP during anaerobic and aerobic incubation. Enzyme activity was found only during anaerobic conditions and then was not inhibited by an antibody against protein B2 nor did it show the sensitivity for hydroxyurea expected from this aerobic enzyme (Table 4). From these results we conclude that the same enzyme is present in anaerobic extracts from anaerobically grown AB1157 and OxyS-11.

**DISCUSSION**

Three well-characterized classes of ribonucleotide reductases are known (2): (i) the iron-tyrosyl radical enzyme of E. coli and mammalian cells; (ii) the cobalamin-dependent enzyme of Lactobacillus leichmannii, present in many microorganisms; and (iii) the manganese-dependent enzyme of Brevibacterium ammoniagenes, also present in some other microorganisms. An additional ribonucleotide reductase activity recently found in extracts from the strict anaerobic Methanobacterium thermoautotrophicum may represent a fourth class (24). The properties of the enzyme activity described here differ from those of the earlier characterized E. coli enzyme. The enzyme activity described here is in all probability due to a different protein, not coded for by either the nrdA or nrdB gene. The enzyme appears to reduce triphosphates and not diphosphates. This is suggested from the findings that the triphosphate dCTP is labeled before dCDP when isotopic cytidine ribonucleotides are reduced and from the stimulation of the reaction by addition of phosphoenolpyruvate. The pyruvate kinase reaction was reported to be largely responsible for the regeneration of nucleoside triphosphates from diphosphates during anaerobic growth of E. coli (25), and in our experiments addition of phosphoenolpyruvate greatly decreased the concentration of CDP with a corresponding increase in CTP. In similar earlier experiments with the aerobic enzyme, addition of a regenerating system inhibited ribonucleotide reduction (16).

Addition of ATP gave a strong stimulation of the anaerobic reaction, also in the presence of phosphoenolpyruvate—i.e., when ATP did not increase the phosphorylation of CDP. We suggest that this represents an allosteric effect, similar to the effect of ATP on the aerobic E. coli enzyme (1). Also the inhibition by deoxyribonucleoside triphosphates is reminiscent of the allosteric behavior of the aerobic enzyme. Taken together these results suggest that the anaerobic reductase is also an allosteric protein with regulatory properties not too different from the aerobic enzyme.

A major difference between the two E. coli reductases lies in the rapid inactivation of the anaerobic enzyme by air. In this respect and in view of its moderate sensitivity to hydroxyurea the newly characterized enzyme appears related to the M. thermoautotrophicum enzyme. This enzyme also has similar allosteric properties. Such a relation is not unreasonable since both enzymes are required for anaerobic DNA replication, but obviously more work is required to substantiate this point. It is not likely that the anaerobic enzyme is a cobalamin-dependent reductase even though we cannot yet rule this out completely. Mutants of Salmonella typhimurium (and this presumably also applies to E. coli) that lack the ability to synthesize cobalamin are apparently not inhibited in their anaerobic growth (14).

Recently Barlow (13) claimed that extracts of the E. coli strain KK 535 grown under anaerobic conditions had the ability to reduce GTP to deoxyguanosine phosphates. KK 535 contains mutations in the nrdA and nrdB genes and the observed activity was shown not to be caused by the normal reductase. In contrast to our results, this activity was not sensitive to oxygen; extraction of bacteria and incubation were done in air. The activity was also completely resistant to hydroxyurea. Differences in experimental conditions concern the E. coli strain and the substrate. It remains to be shown whether these or other differences explain the conflicting results or whether separate enzymes or enzyme systems are involved.

The anaerobic reductase might be of special interest for our understanding of evolution. Conceivably ribonucleotide reduction was a prerequisite for the evolution of the "DNA world" from the "RNA world." The transition would have occurred at a time when oxygen was scarce and would thus require an anaerobic ribonucleotide reductase.

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