Properties of Ribonucleoside Diphosphate Reductase in Nucleotide-Permeable Cells

HUBER R. WARNER

Medical Nobel Institute, Department of Biochemistry, Karolinska Institutet, Stockholm, Sweden, and Department of Biochemistry University of Minnesota, St. Paul, Minnesota 55108

Received for publication 12 January 1973

Ribonucleoside diphosphate (RDP) reductase activity can be readily assayed in ether-treated Escherichia coli cells. The rate of cytidine 5'-diphosphate (CDP) reduction observed in ether-treated cells by using saturating substrate concentrations is about 25% of the rate of de novo deoxyribonucleotide synthesis required to account for in vivo deoxyribonucleic acid synthesis. Optimal activity is observed in the presence of magnesium ions and a positive effector. Adenosine 5'-triphosphate (ATP), deoxy ATP (dATP), and deoxythymidine triphosphate serve as positive effectors, and dATP also serves as a negative effector. These effects on the activity in ether-treated cells resemble those observed in vitro with highly purified enzyme. When the RDP reductase activity in these cells is assayed by using high specific activity 3H-CDP as substrate, even at nonsaturating substrate concentrations, the sensitivity of the assay is sufficient to make it useful for the assay of the low levels of reductase activity in cells not derepressed by thymine starvation or in cells containing mutationally altered RDP reductase. This assay is much easier to perform than the usual in vitro assay, since thioredoxin, thioredoxin reductase, and enzyme subunits B1 or B2 need not be first purified and added to the reaction mixtures.

The ribonucleoside diphosphate (RDP) reductase system of Escherichia coli has been extensively studied by Reichard and co-workers (3, 4, 7, 8, 11, 12) and shown to consist of at least four proteins: proteins B1 and B2, thioredoxin, and thioredoxin reductase. The activity of RDP reductase is difficult to detect in crude fractions because of competing reactions, and optimal activity requires the addition of highly purified B1 or B2 protein as “helper enzymes” (3). Furthermore, the molecular activity of the purified B1 and B2 proteins appears to be far too low to support the observed rate of in vivo deoxyribonucleic acid (DNA) synthesis (3).

DNA synthesis in toluene- and ether-treated cells is dependent upon an exogenous supply of deoxyribonucleoside triphosphates (10, 15), suggesting that these nucleotides readily enter these cells without excessive alteration. We used ether-treated cells to assay the reduction of cytidine 5'-diphosphate (CDP) and to determine whether this activity approaches physiological levels of activity in such cells. The latter might be expected if the ether treatment preserves some intracellular structural features needed for optimal activity of the RDP reductase system.

MATERIALS AND METHODS

E. coli B/5 was obtained from D. P. Snustad (Dept. of Genetics and Cell Biology, Univ. of Minnesota, St. Paul, Minn.). A thymine-requiring mutant was isolated from this strain (14) and was used for some experiments. 3H-CDP was obtained from Amersham and was checked for purity by chromatography on polyethylenimine-cellulose plates run in 0.8 M LiCl.

Growth of E. coli and preparation of ether-treated cells and crude extracts. The E. coli B/5 cells were grown at 30 C in Davis medium (5) with vigorous aeration. For the experiment described in Table 2, the medium was supplemented with 0.5% Casamino Acids. These cells are considered to have repressed levels of RDP reductase. The E. coli B/5 T cells were also grown in medium supplemented with 0.5% Casamino Acids and 10 mg of thymidine per ml. The cells were harvested after growth had ceased due to thymine starvation (absorbancy at 650 nm [A650] = 0.5). The RDP reductase in these cells was considered to be derepressed due to thymine starvation (1). Harvested cells were treated with ether as described by Vosberg and Hoffman-Berling (15), and the treated cells were suspended in basic medium (15) corresponding to 1/200 to 1/100 of their original culture volume. The concentrations of the ether-treated, derepressed E. coli B/5T cells used in Fig. 1, 4, and 5 and the repressed E. coli B/5 cells (A650 = 0.8) used in Fig. 2 are 3.9 and 19.6 mg of protein per ml, respectively.
Extracts were prepared from harvested cells by grinding the cells with twice their weight of alumina and extracting the paste with five volumes of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 7.0) containing 10⁻³ M mercaptoethanol. This suspension was centrifuged for 30 min at 20,000 × g and the supernatant fluid was designated crude extract.

**Assay of RDP reductase activity in ether-treated cells.** In the standard assay, reaction mixtures containing 1 µmol of MgCl₂, 5 µmol of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 8.4), 5 µmol of dithiothreitol, 1.25 nmol of [³H]-CDP (40-80 counts per min per pmol), 120 nmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH), 50 nmol of adenosine 5'-triphosphate (ATP), and ether-treated cells in a final volume of 0.125 ml were incubated for 10 min at 30 C. The reaction was stopped with HClO₄, and the amount of product formed was determined as previously described (11), except that 7 ml of the 70-ml column eluate was routinely evaporated to dryness, the residue was taken up in 0.2 ml of 1 M NH₄OH, 10 ml of Bray solution (2) was added, and the amount of radioactivity was determined by scintillation counting.

For the determination of the Kₘ for CDP, unlabelled CDP was added to standard reaction mixtures to vary the CDP concentration. For the comparison of reductase activity with the in vivo rate of DNA synthesis, the CDP concentration was 4.2 × 10⁻⁴ M.

**Assay of RDP reductase activity in crude extracts.** The radioactive assay described by Brown et al. (3) was used. The reaction mixtures contained 200 nmol of ATP, 1.8 µmol of MgCl₂, 100 nmol of ethylenediaminetetraacetic acid, 10 µmol of HEPES buffer (pH 7.6), 1.35 µmol of dithiothreitol, 70 nmol of [³H]-CDP (1–2 counts per min per pmol), 80 nmol of NADPH, excess thioredoxin and thioredoxin reductase, and crude extract in a final volume of 0.125 ml. Purified B1 or B2 was added as indicated. The mixtures were incubated at 30 C, and the reaction rate was constant for at least 10 min under these conditions. The amount of product formed was determined as described above.

**Assays for DNA and protein.** Samples (5 ml) were removed at various times and added to 0.5 ml of cold 50% trichloroacetic acid. The precipitates were collected by centrifugation, washed twice with 2 ml of cold 5% trichloroacetic acid, and resuspended in 1 ml of 1 M NH₄OH. Samples were assayed for DNA and protein by the methods of Short et al. (13) and Lowry et al. (9), respectively, by using salmon sperm DNA and bovine serum albumin as standards.

**RESULTS AND DISCUSSION**

In reactions using nonsaturating CDP concentration (10⁻⁴ M), ribonucleoside diphosphate reductase activity can be readily detected in ether-treated cells (Fig. 1). The reaction is approximately linear with respect to time (Fig. 1A) and amount of enzyme (Fig. 1B), and requires magnesium ions for maximum activity (Fig. 1C). The optimal magnesium ion concentration in ether-treated cells is about 10⁻² M, which is comparable to that reported for the reduction of CDP and guanosine 5'-diphosphate with purified enzyme (7, 8). Inhibition of product formation occurs at higher magnesium ion concentrations, as reported earlier for the reduction of guanosine 5'-diphosphate (8). The pH optimum in ether-treated cells is 8.2 to 8.4 with either Tris or HEPES buffer, and equal activity is detected with the two buffers (data not shown). In contrast, the reaction with purified enzyme has been routinely assayed at pH 7.6 (3).

To determine the rate of reduction with saturating substrate concentrations, the effect of CDP concentration on initial rate was determined (Fig. 2). From these data the apparent Kₘ for CDP was calculated to be about 8 × 10⁻⁴ M. This value corresponds well with the value of 4 × 10⁻⁴ M reported earlier by using purified enzyme (7).

For comparison of RDP reductase activity in ether-treated cells with that required to support in vivo DNA synthesis, a culture of *E. coli* B/5 was sampled at various times and assayed for DNA, protein content, and reductase activity. This culture grew exponentially throughout the experiment, as shown by the plot of A₅₅₀ against time (Fig. 3); the DNA and protein content also increased exponentially. The rates of DNA synthesis were estimated from the slope of the DNA curve at the time of harvesting cells for ether treatment and assay for reductase activity (designated by arrows in Fig. 3), and these rates are shown in Table 1. The RDP reductase activity, determined in the presence of a saturating concentration of CDP (4.2 × 10⁻⁴ M), is about 25% of the corresponding rate of DNA synthesis.
synthesis at all three cell densities. Thus, near-physiological rates of CDP reduction can be obtained by using these conditions in ether-treated cells, providing further evidence that this enzyme system is responsible for de novo deoxyribonucleotide synthesis in *E. coli*.

Assays were also carried out with crude extracts prepared from *E. coli* B/5 (Table 2). Although DNA synthesis was not directly measured, the theoretical required rate of deoxyribonucleotide synthesis in the repressed cells at the time of harvest (optical density at 650 nm was 2.0) can be estimated to be at least 800 to 1,000 pmol per min per ml of culture. When reductase assays were carried out with crude extracts in the presence of thioredoxin and thioredoxin reductase but were unsupplemented with either B1 or B2 "helper enzymes," the activity corresponded to only 1% of this theoretical value. This activity is enhanced five- to eightfold by the addition of excess B1 or B2 protein. The activity in unsupplemented extracts of derepressed cells is tenfold higher than in extracts of repressed cells and is enhanced only two- to fourfold by the addition of excess B1 or B2 protein. These results suggest that the low activity observed in crude extracts is at least partly due to dissociation of the B1 and B2 proteins upon dilution, and that this dissociation either does not occur or is reduced in ether-treated cells. In addition, ether-treated cells may actually preserve some intracellular structural feature essential for optimal ribonucleotide reduction.

The reducing power for the reaction can be supplied by either NADPH or by dithiothreitol, although optimal activity is observed when both

![Figure 2](http://jb.asm.org/)

**FIG. 2.** Lineweaver-Burk plot. Standard reaction mixtures containing a constant amount of \(^{3}H\)-CDP (1.4 x 10^4 counts/min), varying amounts of unlabeled CDP, and 10 ml of repressed ether-treated *E. coli* B/5 cells were incubated for 10 min at 30 C.

![Figure 3](http://jb.asm.org/)

**FIG. 3.** Growth of *E. coli* B/5. A 1,500-ml culture was aerated vigorously at 30 C and 100- to 200-ml samples were removed at times indicated by the arrows and treated with ether. Samples (5 ml) were removed at more frequent intervals and assayed for DNA (○) and protein (●) content. The increase in cell mass was followed by determining the absorbance at 650 nm (●) in a Beckman DB spectrophotometer.

Table 1. Comparison of rates of deoxyribonucleotide synthesis in vivo and in ether-treated, repressed *E. coli* B/5 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate of DNA synthesis</th>
<th>RDP reductase activity</th>
<th>RDP reductase/DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.30</td>
<td>1.2 x 10^4</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>0.63</td>
<td>2.5 x 10^4</td>
<td>230</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>4.6 x 10^4</td>
<td>430</td>
</tr>
</tbody>
</table>

*Expressed as picomoles of deoxyribonucleotide per minute per milliliter of culture. The average molecular weight of a deoxyribonucleotide in DNA was assumed to be 310.

Table 2. Ribonucleoside diphosphate reductase activity in crude extracts

<table>
<thead>
<tr>
<th>Subunit added</th>
<th>RDP reductase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repressed cells*</td>
</tr>
<tr>
<td></td>
<td>Derepressed cells*</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
</tr>
<tr>
<td>B1</td>
<td>40</td>
</tr>
<tr>
<td>B2</td>
<td>61</td>
</tr>
</tbody>
</table>

*Expressed as picomoles of deoxy CDP synthesized per minute per milliliter of culture.

*The optical density at 650 nm at time of harvest was 2.0 and 0.5 for the repressed *E. coli* B/5 and derepressed *E. coli* B/5 T*-* cells, respectively.
are present (Fig. 4). These results in ether-treated cells are comparable to those obtained with purified enzyme (3), except that 10-fold-higher concentrations of dithiothreitol are required for optimal activity in these cells. Perhaps leakage of the thioredoxin from these cells is responsible for this difference, although the addition of thioredoxin did not stimulate the reaction.

The sensitivity of the assay using low concentrations (10⁻⁴ M) of high specific activity CDP (40-80 counts per min per pmol) suggests that ether-treated cells might be useful for detecting and partially characterizing various RDP reductase mutants of E. coli and other bacteria. If the activity in ether-treated cells is sensitive to the same effectors that regulate the activity in vitro with purified enzyme, then mutants with defects in allosteric regulation might be detected with this assay. ATP, deoxythymidine triphosphate (dTTP), and deoxy ATP (dATP) stimulate activity optimally at 10⁻⁴ M, 5 × 10⁻⁴ M, and 10⁻⁴ M, respectively (Fig. 5); optimal stimulation of the reduction with purified proteins has been reported at concentrations of ATP > 10⁻⁴ M, dTTP > 10⁻⁴ M, and dATP = 10⁻⁴ M (3, 4). The differences between optimal values in ether-treated cells and those obtained with purified proteins are probably due largely to nucleotide interconversions within the cells which make the actual intracellular concentrations of all added nucleotides uncertain. Very high concentrations of ATP inhibit, but this is probably due to conversion of CDP to cytidine triphosphate, since similar concentrations of ATP do not inhibit when the CDP concentration is 4 × 10⁻⁴ M (data not shown). dATP becomes a potent inhibitor at dATP concentrations above 5 × 10⁻⁴ M, and nearly complete inhibition is obtained at 5 × 10⁻³ M. With purified enzyme, 10⁻⁴ M dATP almost completely inhibits CDP reduction (3). Thus, the reductase activity in ether-treated cells responds to positive and negative effectors in a pattern quite similar to that observed with purified enzyme.

The use of this assay for measuring the heat sensitivity of RDP reductase in the dnaF mutant of E. coli has already been reported (6). The activity measured both in the ether-treated cells and in assays using the purified defective B1 protein was heat sensitive, suggesting that the two assays measure the activity of the same ribonucleotide reductase system.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research career development award GM-45729 from the National Institute of General Medical Sciences and research grant AI-07986 from the National Institute of Allergy and Infectious Diseases, and by a grant (to Peter Reichard) from the Swedish Medical Research Council.

LITERATURE CITED

5. Davis, B. D., and E. S. Mingioli. 1950. Mutants of


