Reaction Mechanism of Ribonucleoside Diphosphate Reductase from Escherichia coli

OXIDATION-REDUCTION-ACTIVE DISULFIDES IN THE B1 SUBUNIT*

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SUMMARY

Ribonucleoside diphosphate reductase consists of two nonidentical subunits, proteins B1 and B2. The enzyme catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides. The electrons required in this reduction are transported from NADPH via a flavoprotein, thioredoxin reductase, to a low molecular weight protein, thioredoxin. The reduced form of thioredoxin acts as hydrogen donor in ribonucleotide reduction. Both thioredoxin and thioredoxin reductase contain oxidation-reduction active disulfides participating as electron carriers during catalysis.

In this paper, data are presented which show that in the absence of hydrogen donor, ribonucleotide reductase reduces a limited amount of ribonucleotides at the expense of sulfhydryls of protein B1. A maximal value of 3 moles of deoxyribonucleotide was obtained per mole of B1 and at the same time 6 moles of sulfhydryls were oxidized. The reaction requires the presence of protein B2. The same initial rate of cytidine diphosphate reduction was obtained in the presence and in the absence of reduced thioredoxin. Electron transfer occurred readily between fully reduced B1 and the oxidation-reduction active disulfide of thioredoxin. Steady state kinetics of ribonucleotide reductase indicated that the enzyme acted by a ping-pong mechanism, i.e. alternated between two stable forms during catalysis. Based on these results it is proposed that in the reduction of ribonucleotides, electrons flow from thioredoxin to oxidation-reduction active disulfides of protein B1. The dithiols formed interact by unknown mechanisms with a free radical species in protein B2 (Atkin, C. L., Thelander, L., Reichard, P., and Lang, G. (1973) J. Biol. Chem. 248, 7464-7472) to reduce the ribonucleotide.

Deoxyribonucleotides are synthesized by a direct reduction of the corresponding ribonucleotides. The ultimate hydrogen donor is NADPH. In Escherichia coli four proteins participate in the electron transport from NADPH to the ribonucleotide (1). The reaction sequence can be depicted in scheme 1.

Reactions 1, 2 and 3 are mediated by a flavoprotein, thioredoxin reductase, whose active center contains an oxidation-reduction active disulfide and a flavin adenine dinucleotide (2-4). Thioredoxin is a low molecular weight protein which also transports electrons via an oxidation-reduction active disulfide (5). Reactions 4 and 5 are mediated by ribonucleoside diphosphate reductase (EC 1.17.4.1). $X_{ox}/X_{red}$ represents one or several hypothetical oxidation-reduction couples in the enzyme which transport electrons from reduced thioredoxin to the ribonucleotide.

Ribonucleotide reductase consists of two nonidentical subunits, proteins B1 and B2. In the presence of magnesium ions 1 molecule of B1 (mol wt 160,000) together with 1 molecule of protein B2 (mol wt 78,000) forms the active enzyme (6). Each subunit alone has no known catalytic activity. Protein B1 contains binding sites for ribonucleoside diphosphate substrates and nucleoside triphosphate effectors. Protein B2 contains non-heme bound iron and in addition an organic free radical of unknown structure which is directly correlated to enzymatic activity (8).

The mechanism by which B1 interacts with B2 to effect ribonucleotide reduction is unknown. This paper presents data which indicate that the first step in the reduction is the transfer of electrons from reduced thioredoxin to oxidation-reduction active disulfides of protein B1.

EXPERIMENTAL PROCEDURE

Materials

Determination of Sulfhydryl Groups—Sulfhydryl determinations were performed as previously (6) using 5,5'-dithiobis(2-nitro-

1 U. von Döbeln, unpublished results.
benzoic acid) in guanidine hydrochloride. The decrease in volume during evacuation and equilibration with argon was calculated by weighing the Thunberg cuvette before and after the evacuation. The loss amounted to about 3% and all figures are corrected for this.

**Assay of Deoxyribonucleotide Formation—Formation of dCDP** from [3H]CDP was determined as described in Ref. 11, after addition of 1.0 ml of 1 M perchloric acid to aliquots of reaction mixtures (65 to 100 µl). The radioactivity present in the dCMP pool from the Dowex columns was determined in a Packard scintillation counter using Bray’s solution (12). The identity of the product as a deoxy compound was confirmed by chromatographing the dCMP pool on Whatman No. 3MM paper in 1-butanol-NH₄OH (13) after digestion with alkaline phosphatase. The formation of dADP from [3H]ADP (specific activity, 20,000 cpm per nmole) was determined after precipitating the protein in aliquots of the reaction mixtures with ethanol (final concentration about 75%) and chromatographing the supernatant on paper using a borate medium (14). The nucleotide-containing spots were cut out and radioactivity was determined in a Packard scintillation counter after addition of 100 µl of 4 M H₂O and 10 ml of Bray’s solution (12).

**Kinetic Measurements** Initial reaction rates of CDP reduction were determined at 25° by using either the NADPH-oxidation method in a Zeiss recording spectrophotometer equipped with a water-jacketed cuvette holder and adjusted to give a full scale sensitivity of 0 to 0.5 absorbance units or the [3H]CDP method (9).

In both cases the reaction was started by the addition of prewarmed solutions of CDP. In the [3H]CDP assay the prewarmed samples were incubated in a water bath for 5 min with CDP and then the reaction was stopped by the addition of 1 M perchloric acid. A typical reaction mixture contained 6 nmoles of dTTP, 1.6 µmoles of MgCl₂, 0.1 µmole of thioribonuclease, 65 nmoles of NADPH, 5 µmoles of Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 7.6, about 0.01 nmole of B₁, a 5-fold molar excess of B₂, and thioribonuclease and CDP as indicated, in a final volume of 130 µl.

**RESULTS**

**Thiol Groups in Ribonucleotide Reductase**—As mentioned in the introduction, electron transfer via oxidation-reduction active disulfides plays a central role in the reduction of thioribonuclease by NADPH. Before attempts were made to investigate whether ribonucleotide reductase also contained oxidation-reduction active disulfides, repeated analyses of the cysteine and cysteine content of proteins B₁ and B₂ were performed. The results are summarized in Table I. Protein B₁ contained about 21 half-cystines and most if not all of these existed as cysteines in the fully active molecule. Protein B₂ contained 10 half-cystines and in contrast to B₁, two of these might form a disulfide in the native B₂.

**Reduction of CDP by Sulphydryl Groups of Protein B₁**—Incubation mixtures were prepared containing B₁ and B₂ in varying molar ratios, magnesium ions, and dTTP as positive allosteric effector (7). After taking aliquots for —SH determinations each mixture was incubated with [3H]CDP at 25° for 30 min under argon. Aliquots were again taken for sulphydryl analyses, and the remainder of the mixture was analyzed for [3H]CDP. The results of a series of such experiments are shown in Table II.

**Scheme 1**

**Table I**

<table>
<thead>
<tr>
<th>Method</th>
<th>Total</th>
<th>Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteic acid measured after per-</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>formic acid oxidation (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conversion to S-carboxymethyl-</td>
<td>16.9-18.5</td>
<td>(3)</td>
</tr>
<tr>
<td>cysteine after reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrophotometric titration</td>
<td>9.3 (1)</td>
<td>(6)</td>
</tr>
<tr>
<td>Spectrophotometric titration</td>
<td>7.7-8.3</td>
<td>(3)</td>
</tr>
<tr>
<td>after reduction with dithiothreitol</td>
<td>9.9 (1)</td>
<td></td>
</tr>
<tr>
<td>B₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteic acid measured after per-</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>formic acid oxidation (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conversion to S-carboxymethyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cysteine after reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrophotometric titration</td>
<td>9.3 (1)</td>
<td>(6)</td>
</tr>
<tr>
<td>Spectrophotometric titration</td>
<td>7.7-8.3</td>
<td>(3)</td>
</tr>
<tr>
<td>after reduction with dithiothreitol</td>
<td>9.9 (1)</td>
<td></td>
</tr>
</tbody>
</table>

a Carboxymethylation was performed as described in Ref. 6 after reduction with mercaptoethanol or dithiothreitol in 6 M guanidine hydrochloride.

b See “Experimental Procedure” for details.

When both B₁ and B₂ were present in the incubation mixtures, there was an oxidation of sulphydryl groups on the addition of CDP and at the same time about stoichiometric amounts of dCDP were formed. The results, obtained from varying the molar ratio B₁:B₂, showed that approximately the same amount of sulphydryls, 4.5 to 6.6 moles, were oxidized per mole of B₁, indicating that the oxidized sulphydryls were located exclusively on B₁. In all experiments 2.4 to 3.0 moles of dCDP were formed per mole of B₁. Therefore about 2 moles of sulphydryl groups were oxidized per mole of dCDP formed.

No oxidation of sulphydryl groups and no formation of deoxyribonucleotides were observed in mixtures containing either B₁ or B₂ alone. Even after preincubation in dithiothreitol to increase its sulphydryl content, B₂ alone could not reduce the added ribonucleotide.

**Reduction of Adenosine Diphosphate** Mixtures of B₁ and B₂ in the absence of thioribonuclease could also reduce ADP. A 1.5:1 mixture of B₁ and B₂ in the presence of dGTP as effector (7) reduced 1.5 moles of ADP per mole of B₁. Simultaneously, 2.7 moles of sulhydryl groups were oxidized per mole of B₁. This showed that purine as well as pyrimidine ribonucleotides were reduced by ribonucleotide reductase in the absence of thioribonuclease.

**Rate of Ribonucleotide Reduction**—Measurements of the rate of ribonucleotide reduction in the presence and absence of re-
Reduced thioredoxin were made in order to investigate whether the reduction in the absence of hydrogen donor was fast enough to be part of the catalytic process. Two sets of reaction mixtures were prepared, each containing approximately equimolar amounts of proteins B1 and B2 and also magnesium ions and dTTP. No additions were made to the first set while thioredoxin and diithiothreitol were added to the second. One series of mixtures was incubated at 0°C and another at 25°C. After addition of [3H]CDP, aliquots of the mixtures were analyzed for dCDP at different time points.

The results presented in Fig. 1A show that the rate of CDP reduction measured at 0°C was about the same in the presence (Curve 3) and in the absence (Curve 4) of reduced thioredoxin. Reduction of thioredoxin by NADPH and thioredoxin reductase (not shown) gave the same result as when diithiothreitol was used. These experiments indicate that the reaction rate in the absence of hydrogen donor was sufficiently high to form part of the catalytic process. The reactions at 25°C in the presence (Curve 1) and absence (Curve 2) of reductant were both too rapid to allow determination of initial rates since aliquots could not be taken in less than 10 s.

**Dependence on Effector**—When the negative effector dATP (7) was used instead of dTTP the rate of CDP reduction at 25°C was very slow compared to the rate in the presence of dTTP (Fig. 1B). At 0°C no reduction was observed after 10 min in the presence of dATP (not shown). This showed that like CDP reduction in the presence of hydrogen donor, the reaction in the absence of hydrogen donor was subject to regulation by the allosteric effectors of ribonucleotide reductase (7).

**Electron Transfer between Protein B1 and Thioredoxin**—To test the ability of electron transfer between thioredoxin and B1, fully reduced B1 was mixed with oxidized thioredoxin and the formation of reduced thioredoxin detected in the following way. In an anaerobic system thioredoxin can specifically reduce the FAD of thioredoxin reductase resulting in a decrease in the flavin absorbance at 456 nm (2). Consequently, reduction of thioredoxin could be monitored in an anaerobic system containing thioredoxin reductase by registering the absorbance at 456 nm.

Addition of protein B1 to a mixture of oxidized thioredoxin and thioredoxin reductase resulted in an immediate decrease in \( A_{456} \) (Table III). Addition of B1 plus B2 gave the same result as B1 alone. All spectral changes occurred in less than 14 min. Further addition of thioredoxin had no effect. Similarly, no effect was seen on addition of B2 instead of B1, or on addition of B1 to a system lacking thioredoxin. Admission of air immediately restored the original spectrum (2). This showed that electrons could be transported from B1 to thioredoxin and supports the hypothesis that the reverse reaction occurs during ribonucleotide reduction.

**Steady State Kinetics of Ribonucleotide Reductase**—Measurements of the kinetics of ribonucleotide reduction were performed in a manner similar to that described in Table II.

### Table II

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Molar ratio B1:B2</th>
<th>Sulphydryl oxidized per B1</th>
<th>dCDP formed per B1</th>
<th>Sulphydryl oxidized per dCDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.5:1</td>
<td>4.7</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>4.5</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>5.8</td>
<td>2.9</td>
<td>2.0</td>
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<tr>
<td></td>
<td>4:1</td>
<td>6.6</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>II</td>
<td>0.5:1</td>
<td>6.1</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>5.1</td>
<td>3.0</td>
<td>1.7</td>
</tr>
<tr>
<td>III</td>
<td>4:1</td>
<td>5.1</td>
<td>3.0</td>
<td>1.7</td>
</tr>
<tr>
<td>IV</td>
<td>2:1</td>
<td>5.5</td>
<td>2.4</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Table III

Electron transfer between protein B1 and thioredoxin

In Experiment I a reaction mixture containing 5.2 nmoles of thioredoxin reductase (10.3 nmoles of protein-bound FAD), 1.6 nmoles of thioredoxin, and 72 μmole of Tris Cl, pH 7.6, in a total volume of 1.44 ml was pipetted into a Thunberg cuvette. The side arm contained 8.3 nmoles of protein B1 (158 nmoles of sulphhydryl groups) in 175 μl of 0.05 M Tris-Cl, pH 7.6. The cuvette was equilibrated with oxygen-free argon and the spectrum between 310 and 600 nm registered at 25°C in a Cary 14 spectrophotometer using the 0 to 0.1 absorbance unit full scale slide wire. After mixing the contents of the cuvette, the absorbance at 456 nm was recorded during 3 min and then the complete visible spectrum was recorded at intervals during 1 hour. Finally, air was admitted to the cuvette and the spectrum recorded once more.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Reductant</th>
<th>Δ 456 nm (corrected for dilution)</th>
<th>Amount of protein-bound FAD reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8.3 of B1</td>
<td>0.019</td>
<td>0.010</td>
</tr>
<tr>
<td>II</td>
<td>3.1 of B1</td>
<td>0.007</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>3.1 of B1 but thioredoxin omitted from the cuvette</td>
<td>0.007</td>
<td>3.2*</td>
</tr>
<tr>
<td></td>
<td>13.7 of B2</td>
<td>0.007</td>
<td>3.2*</td>
</tr>
<tr>
<td></td>
<td>3.3 of B1 plus 2.8 of B2</td>
<td>0.007</td>
<td>3.2*</td>
</tr>
</tbody>
</table>

* The initial A456 nm in this case was 0.075. Total reduction of the protein-bound FAD of thioredoxin reductase decreased the A456 nm to 18% of the initial value (2). Accordingly, total reduction in this case would give a Δ 456 nm of 0.062. The observed Δ 456 nm was 0.010 which is 01% of 0.062. This means that 31% of the protein-bound FAD were reduced which equals 3.2 nmoles (see above).

to determine the enzymatic mechanism of ribonucleotide reductase. In the following experiments the concentration of one of the substrates, thioredoxin, was kept constant at different levels while the concentration of the second substrate, CDP, was varied. Initial reaction rates were measured in the presence of an excess of thioredoxin reductase and NADPH. Plots of the reciprocal velocities of dCDP formation against the reciprocal of CDP concentration gave a series of parallel lines (Fig. 2). Similar results were obtained using the NADPH-oxidation method and the [3H]CDP method. These experiments suggest that the reaction mechanism of ribonucleotide reductase is of the ping-pong type (15).

**DISCUSSION**

Protein B1 contained 21 half-cystines per molecule and more than 90% of these occurred in the form of cysteine residues in the fully active protein. It was shown that oxidation of protein B1 in air resulted in a reversible dissociation and inactivation of the molecule due to a gradual formation of cystine residues from cysteines (6). Therefore, B1 was always prepared and stored in the presence of dithiothreitol which might explain the high content of sulfhydryls. In contrast, protein B2 contained on the average 1 cystine and 8 cysteine residues per molecule and in this case the specific activity was not influenced by reduction of the disulfide.

Proteins B1 and B2 without exogenous reductant could reduce both purine and pyrimidine ribonucleoside diphosphates to the corresponding deoxyribonucleotides at the expense of sulfhydryl groups in B1. The reaction required both B1 and B2 and also an active conformation of the enzyme—the rate of CDP reduction in the presence of the negative effector dATP was much lower than that in the presence of the positive effector dTTP. The low figure of about 1.5 moles of dADP formed per mole of B1 as compared to about twice this amount in CDP reduction might be explained by the fact that dADP functions as a negative effector (16).

The observation that the rate of ribonucleotide reduction at 0°C was about the same whether reduced thioredoxin was present or not supported the hypothesis that the reaction really was part of the catalytic mechanism. The reason for the pronounced temperature dependence of the ribonucleotide reduction shown in Fig. 1A is not known.

Protein B1 contains binding sites for all ribonucleoside diphosphate substrates and nucleoside triphosphate effectors (7). Binding of thioredoxin has not yet been demonstrated. However, the fact that B1 alone can reduce thioredoxin indicates that thioredoxin also interacts with protein B1.

The kinetics of ribonucleotide reduction agrees with a ping-pong mechanism (15). This means that the enzyme alternates between two stable forms. The data presented here suggest strongly that these are represented by the oxidized and reduced
form of B1. A mechanism compatible with all data is the following (Fig. 3). In the reduction of ribonucleotides, thioredoxin binds to B1, reduces it, and then leaves the enzyme. The ribonucleotide is now bound to the reduced B1-B2 complex and is reduced while B1 is reoxidized. Finally, the deoxyribonucleotide is released from the enzyme. The ping-pong mechanism might explain the difficulties in observing any binding between thioredoxin and protein B1 and/or protein B2.

The reduction of about 3 moles of ribonucleotide per mole of B1 must not be taken as final evidence that there are three active sites on ribonucleotide reductase. These data were all obtained after 30-min incubations at 25°. Fig. 1B shows that the reduction of CDP at 25° in the absence of thioredoxin seems to consist of two reactions, a rapid one leading to the reduction of about 2 moles of CDP per mole of B1 and a slower one leading to formation of 1 additional mole of dCDP. Only the first reaction might be of catalytical significance. This would agree with results from binding experiments which indicate that there are two binding sites for ribonucleotide substrates per molecule of protein B1. However, the final solution of this problem has to await further studies.

Vitols et al. showed that the vitamin B12 coenzyme-dependent ribonucleoside triphosphate reductase from Lactobacillus leichmannii contained a thioredoxin-reducible disulfide which was reoxidized by the ribonucleotide substrate (17). This was explained by assuming that the reduction of the ribonucleotide by the reduced enzyme was mediated by catalytic amounts of thioredoxin present in the preparation. However, Tamao and Blakley have recently suggested that the reducing agent in the Lactobacillus system is a deoxyadenosyl radical-thiol system with the probable involvement of a second thiol group (18).

The B1 and B2 preparations in this study were not contaminated with thioredoxin. Instead, earlier data (8) and those presented in this paper indicate that the reducing agents in the E. coli ribonucleotide reductase system are the oxidation-reduction active diithiols of protein B1 in combination with the free radical species of protein B2. Data to be presented elsewhere from studies using ribonucleotide analogues have shown that both these structures are in close proximity in the active site of ribonucleotide reductase and that this site is formed both from B1 and B2. The iron in protein B2 does not seem to participate as electron carrier in the enzyme reaction (8).

REFERENCES

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1 A. Holmgren, unpublished results.

2 L. Theander and F. Eckstein, to be published.
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Lars Thelander


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