On the Mechanism of Ribonucleoside Diphosphate Reductase from *Escherichia coli*

EVIDENCE FOR 3'-C—H BOND CLEAVAGE*

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The 3'-carbon—hydrogen bond of [3'-H]uridine 5'-diphosphate is cleaved during its conversion to 2'-deoxyuridine 5'-diphosphate catalyzed by *Escherichia coli* ribonucleoside diphosphate reductase. A selection against 3H of approximately 3.3 is observed on this reduction reaction. During the course of this reaction, a small but significant amount of 3H is observed on the solvent.

Ribonucleoside-diphosphate reductase (EC 1.17.4.1) catalyzes the reduction of ribonucleoside 5'-diphosphates to their corresponding 2'-deoxyribonucleoside 5'-diphosphates. The *Escherichia coli* reductase has been characterized extensively by Reichard and co-workers (for review, see Ref. 1) and has been shown to consist of two nonidentical subunits, B1 (Mr = 78,000) and B2 (Mr = 39,000) (2). The B1 subunit binds substrates and allosteric effectors and contains sulfhydryl groups which are oxidized upon substrate reduction (1). The B2 subunit contains two nonheme irons (Fe(III)) and an unusual organic free radical localized on a tyrosine residue (3-5).

Recent results from this laboratory (6, 7) and the laboratory of Thelander et al. (8) indicated that incubation of ribonucleoside diphosphate reductase with 2'-chloro-2'-deoxyuridine 5'-diphosphate (2'-ClUDP) or 2'-deoxy-2'-fluorocytidine 5'-diphosphate resulted in release of Cl- or F-, cleavage of the base from the sugar, release of inorganic pyrophosphate, loss of sulfhydryl groups, and inactivation of the B2 subunit. We proposed that these observations could be accounted for by generation of a 3'-ketone intermediate I:

![Scheme 1](image)

Generation of the 3'-ketone I would labilize 2'-H and 4'-H of the sugar moiety and permit elimination of base and pyrophosphate, respectively. In addition, the resulting reactive unsaturated ketone could explain the concomitant loss of sulfhydryl groups and enzyme activity of B2 (6-8).

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The mechanism shown in Scheme 1 demonstrates how such a 3'-ketone could be generated during inactivation of ribonucleoside diphosphate reductase with 2'-ClUDP. 1-3

This hypothetical reaction (Scheme 1), based on analogy with chemical model systems (9), involves enzyme-catalyzed cleavage of the 2'-carbon—chlorine bond via a radical cation mechanism (7). In Scheme 1, the tyrosyl radical of B2 abstracts the 3'-H atom of 2'-ClUDP to generate the 3'-nucleotide radical II. This initial radical abstraction facilitates cleavage of the 2'-C-Cl bond to generate radical cation III. Radical cation III can then collapse to form 3'-ketone I.

**MATERIALS AND METHODS**

Ribonucleoside diphosphate reductase was isolated using a dATP-Sepharose affinity column (10). Thioredoxin and thioredoxin reductase were isolated by modifications of procedures previously described (11-13). Protein concentrations were determined by the procedure of Bradford (14).

[3'-H]NaBH4 and uniformly labeled [14C]uridine were purchased from New England Nuclear. NADPH, ATP, and dithiothreitol were purchased from Sigma Chemical Co. All other materials were purchased in the highest purity available.

Isolation of all compounds was accomplished using an Altex HPLC system with a Whatman ODS-1 column. All solutions were analyzed for radioactivity using Biofluor mixture and a Beckman model LS 7500 scintillation counter.

**Synthesis of 3'-3H/UDP**

2',5'-Di-O-trityl-3'-ketouridine was prepared by the proce-

1. J. W. Kozarich and J. A. Stubbe, unpublished observations. A 3'-ketone intermediate could also be generated upon inactivation of ribonucleoside diphosphate reductase by 2'-ClUDP by abstraction of 1'-H atom of 2'-ClUDP. Results reported in this communication eliminate this as a viable alternative.

2. The abbreviations used are: 2'-ClUDP, 2'-chloro-2'-deoxyuridine 5'-diphosphate; HPLC, high performance liquid chromatography.
dure of Cook and Moffatt (15). The 3'-ketone (0.680 g, 1.07 mmol) was placed in a 50-ml round bottom flask in 30 ml of absolute ethanol. [3H]NaBH₄ (13 mg, 0.3 mmol) 100 mCi) was added to this solution and the reaction was allowed to proceed for 20 min at room temperature. At the end of the period, 600 mg (0.016 mol) of unlabeled NaBH₄ was added to the reaction vessel and the reaction proceeded for an additional 2 h at room temperature. The EtOH was removed in vacuo and the product was extracted with 3 × 50 ml of CHCl₃ from 50 ml of H₂O. The CHCl₃ layer was dried over MgSO₄, filtered, and concentrated in vacuo. In our hands, this procedure resulted in production of 65:35 mixture of 1-(2,5-di-O-trityl-β-D-xylofuranosyl)uracil and 1-(2,5-di-O-trityl-β-D-ribofuranosyl)-uracil. The desired compound was isolated by a modification of the procedure described by Cook and Moffatt using CCl₄/acetone (5:1) and Merck Silica Gel G (Rf 0.08 and 0.12, respectively). The compound was then repurified using TLC grade silica Gel 60H column chromatography. The peak fractions were pooled and rechromatographed by column on the TLC grade silica gel.

The desired compound was then deblocked using 80% acetic acid described by Cook and Moffatt (15). The pmr spectrum showed uridine with no contaminating xylo isomer (specific activity, 8 × 10⁵ cpm/µmol).

The compound was then isotonically diluted 1:8 with unlabeled uridine and converted to the corresponding 5'-monophosphate by the procedure of Yoshikawa and Takenishi (18) and to the diphosphate by activation of the monophosphate with carbonyldiimidazole (19) (specific activity, 9.7 × 10⁶ cpm/µmol). A second batch of [3'-3H]UDP prepared analogously showed a specific activity of 9.1 × 10⁶ cpm/µmol. At each stage, the nucleotide was purified by DEAE-Sephadex G-25 chromatography by elution with the appropriate triethylammonium bicarbonate linear gradients (18, 19). [14C]UDP was analogously synthesized from uniformly labeled uridine and had a specific activity of 5 × 10⁶ cpm/µmol.

**Selection Effect Studies**

[3'-3H]UDP—A typical incubation mixture of 1 ml total volume contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.6), 50 mM; Mg(OAc)₂, 15 mM; EDTA, 1.5 mM; NADPH, 4 mM; ATP, 1.5 mM; thioredoxin, 51 µg; thioredoxin reductase, 5.5 µg; [3'-3H]UDP, 1 mM (specific activity, 9.1 × 10⁶ cpm/µmol); and ribonucleoside diphosphate reductase (0.65 mg = 8 mmol passed through a Sephadex G-50 column directly before use to remove dithiothreitol). At fixed time intervals, 100-µl aliquots were removed from the incubation mixture and boiled for 1 min to stop the reaction.

To each fraction, 50 µl of 0.5 M Tris (pH 8.5) and 5 µl of alkaline phosphatase E. coli (1.75 units) were added and incubated for 1 h at 37°C. The samples were then boiled for 1 min and the protein was removed by centrifugation. The supernatant was removed with a disposable pipette and placed in a 5-ml round bottom flask. The protein precipitate was washed with 0.845 ml of H₂O and the H₂O wash was placed in the same flask. The H₂O was then removed by bulb to bulb distillation.

The dried residue was redissolved in 100 µl of H₂O and injected directly onto the Whatman ODS-1 reverse phase column (flow, 1.7 ml/min, H₂O eluant) and 0.4-min fractions collected. Compounds and retention times: uridine, 3.25 min; deoxyuridine, 5.5 min. The uridine and deoxyuridine peaks were purified to constant specific activity by HPLC. All specific activities are reported in counts per min/µmol, as all samples were counted with 48% efficiency. Extent of reaction was determined by weighing the appropriate HPLC traces.

The specific activities of uridine and deoxyuridine were determined by counting a known amount of sample (λmax 260, ε = 1 × 10⁴ M⁻¹ cm⁻¹).

**Double Label Experiments:** [3'-3H]UDP/[14C]UDP—1) The protocol was identical with that described above except that 0.25 µmol of [3'-3H]UDP (specific activity, 9.7 × 10⁶ cpm/µmol) and 0.75 µmol of [14C]UDP (specific activity, 5.0 × 10⁶ cpm/µmol) were used.

After the initial isolation by HPLC of the products from each individual time point, the appropriate uridine and deoxyuridine peaks were pooled and recounted. H⁺/¹⁴C windows were set such that 20,000 cpm in the ³H channel overlapped 12% into the ¹⁴C channel and 5,000 cpm in the ¹⁴C channel overlapped 11% into the ³H channel. A typical specific activity of uridine or deoxyuridine was determined with 20,000 counts in the ³H channel.

2) Protocol was identical with that described in 1) except that solutions were not treated with alkaline phosphatase. Instead, UDP and dUDP were separated by ion pair reverse phase chromatography directly on the Whatman reverse phase ODS-1 column using an isocratic elution with 0.055 M KP (pH 5.5) and 5 mM tetrabutylammonium hydroxide, flow rate, 1.5 ml/min. Compounds and retention times: UDP, 4.5 min; dUDP, 7.0 min. A late migrating impurity was observed (retention time, 10 to 12 min) accounting for 1.5% of [3'-³H]-UDP and 5.6% of [¹⁴C]UDP.

**RESULTS**

Three independent methods were used to determine the ³H selection effect on the conversion of UDP to dUDP catalyzed by ribonucleoside diphosphate reductase. These methods are:

1) Single label experiment using [3'-³H]UDP and analysis of uridine and deoxyuridine (Fig. 1A), 2) a double label experiment using [3'-³H]UDP/[¹⁴C]UDP and analysis of uridine and deoxyuridine (Table 1), and 3) a double label experiment using [3'-³H]UDP/[¹⁴C]UDP and analysis of UDP and dUDP (Table 1). Methods 1 and 2 provide more accurate determination of the specific activity of uridine during 5 to 50% conversion to product. Method 3 provides more accurate determination of the specific activity of uridine at >70% reaction. Errors involved with each method are discussed subsequently.

1) Single Label [3'-³H]UDP—[3'-³H]UDP was incubated with ribonucleoside diphosphate reductase for fixed time intervals. Aliquots of the reaction mixture were treated with

### TABLE I

**Selection effect determined with double label: 3'-³H]-UDP/[¹⁴C]UDP**

| Extent of Reaction | ³H/¹⁴C (uridine) | ³H/¹⁴C (d-uridine) |
|--------------------|-----------------|------------------|
| %                  |                 |                  |
| 1a                 | 7               | 7.08             |
| 2                 | 7.53            | 2.15             |
| 3                | 8.49            | 2.54             |
| 4                 | 10.99           | 3.06             |
| 5                | 11.48           | 3.76             |
| 6                 | 11.28           | 5.16             |

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a Determined using ¹⁴C data.

b Experimental, "selection effect" a for details.

c Experimental "selection effect" c for details.
alkaline phosphatase and the resulting uridine and deoxyuridine were separated via HPLC. The results shown in Fig. 1A indicate that the specific activity of deoxyuridine is clearly less than the specific activity of starting uridine. For example, at 5% reaction, the specific activity of deoxyuridine is 2.5 × 10^6 cpm/μmol, while that of uridine is 9.6 × 10^6 cpm/μmol. This observed difference in specific activity of starting material and product at early time points indicates a selection against [3H]UDP during enzyme-catalyzed reduction. Fig. 1A indicates that this selection against [3H] is approximately 3.8.

Also indicated in Fig. 1A is that the specific activities of both uridine and deoxyuridine increase with the extent of the reaction. The increase in specific activity of uridine with time is a reflection of the selection against [3H]UDP during reduction. The increase in specific activity of the deoxyuridine is a reflection of the increased specific activity of uridine. Furthermore, at 100% reaction, the specific activity of deoxyuridine should be identical with that of the starting material if no [3H] is lost during this reduction. As indicated in Fig. 1A, the specific activity of deoxyuridine at 90% reaction is 7.5 × 10^6 cpm/μmol and approaches that of the starting material, which is 9.1 × 10^6 cpm/μmol.

The greatest inaccuracy of this single label method for determining the selection against [3H]UDP involves measurement of the specific activity of uridine at greater than 70% conversion to products. Decreased uridine specific activity is due to ultraviolet absorbing materials and radiolabeled materials which migrate with similar retention times to uridine upon HPLC analysis. These impurities result from several sources. 1) [3H]UDP is contaminated with an unknown phosphorylated uridine (1.5 to 3.2% depending on the [3H]-UDP preparation) which migrates with a retention time of 10 to 12 min on ion pairing reverse phase column chromatography. This impurity, when treated with alkaline phosphatase, resulted in a compound which migrated in the uridine region on analysis by reverse phase chromatography, H2O eluant. 2) Uridine has a short retention time (3.5 min) by HPLC analysis and thus migrates near the void volume of the column (2 to 2.5 ml). Small amounts of UV absorbing material (<5%) eluted in the void volume, therefore, may contaminate the isolated uridine. 3) Analysis of the specific activity of uridine at >90% reaction is complicated by the fact that H2O is released during the enzyme-catalyzed reduction (Fig. 1B).

It should be emphasized that the above problems, while increasing the uncertainty of the specific activity of uridine at late times, are not a problem at early times.

Results from this single label experiment indicated that the reduction reaction catalyzed by ribonucleoside diphosphate reductase is accompanied by release of a small but significant amount of H2O (Fig. 1B). At 5% reaction, when we observe a selection against [3H]UDP of ~3.8, only a very small amount of total counts (0.003%) has been released as H2O. Thus, under initial reaction conditions, the selection effect should not be significantly altered by H2O release. At late reaction times where, at 80% reaction, 1.8% of total counts have been volatilized, analysis of specific activity of uridine becomes more complicated.

Because the percentage of counts volatilized was small compared with the total counts in each experiment, control experiments with heat-inactivated ribonucleoside diphosphate reductase were run under identical conditions. In a typical experiment containing 4.6 × 10^6 cpm, at 85% reaction, 8.8 × 10^4 cpm volatilized in the experiment was accompanied by 2.1 × 10^7 cpm (0.045% total counts) volatilized in the control. These background counts remained constant with time. Each time point recorded in Fig. 1B has been corrected for this background.

This H2O formation also reflects the selection against [3H]UDP. If there were no selection effect on cleavage of the 3'-C—H bond, a linear relationship would be expected between extent of reaction and percentage of H2O released. The fact that the H2O release deviates from linearity with the extent of reaction reflects the observed selection against [3H]UDP (Fig. 1A).

2) Double Label Experiment I—To avoid the problems with UV absorbing impurities, [3H]UDP and [14C]UDP were incubated with ribonucleoside diphosphate reductase and the reaction mixture was analyzed for uridine and deoxyuridine. The results of this experiment are indicated in Table 1a. At 7% reaction, a selection against [3H]UDP of 3.3 is observed. This reduction reaction is also accompanied by H2O release. No 14C material is volatilized. The release of H2O as a function of extent of reaction is similar to that described in the single label experiment (data not shown).

Analysis of this double label experiment by HPLC revealed that [3H]uridine and [3H]deoxyuridine eluted from the reverse phase column slightly ahead of the corresponding 14C-labeled nucleosides. Appropriate fractions were therefore pooled and
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recounted. Finally, at late times of reaction, the specific activity of uridine again did not increase as rapidly as predicted. Analysis of [14C]UDP by ion pair reverse phase chromatography revealed a late migrating 14C-phosphorylated uridine species, retention time (10 to 12 min) similar to that described for [3'-3H]UDP. This contamination represented 5.6% of [14C]UDP. Incubation of this "late" peak with alkaline phosphatase followed by analytical HPLC (H2O eluant) indicated that most of these counts migrated in the "uridine" region (3.5-min retention time). This contamination, again, only provides difficulty in determining the specific activity of uridine at late times of reaction.

3) Double Label Experiment II—To avoid 3H and 14C impurity problems, [3'-3H]UDP and [14C]UDP were incubated with ribonucleoside diphosphate reductase and analysis of the reaction mixture was performed directly using ion pair reverse phase chromatography to analyze for UDP and dUDP. Results of this experiment are reported in Table 1b. At 14% reaction, a selection effect of 2.9 is observed. This reaction was also accompanied by release of 3H2O as in the previous two experiments.

**DISCUSSION**

By three independent methods, we have shown that ribonucleoside diphosphate reductase from E. coli cleaves the 3'-C—H bond of UDP during catalysis of this unusual reduction reaction. Furthermore, during this reaction, 3H2O is released to the media to the extent of 2.5% at 90% product formation.

These results provide the first evidence that cleavage of the 3'-C—H bond is important in this reduction reaction and render unlikely the possibility that the cleavage of the 2'-C—OH bond occurs via either an S1 or S2 reaction at the 2'-carbon. While the details of this mechanism are currently under investigation, one speculative mechanism we will propose for this enzyme-catalyzed reduction can account for all the observations made to date and is based on a chemical system (9). The proposed mechanism is as follows. The tyrosyl radical on the B1 subunit may abstract a 3'-H atom of the ribose ring of UDP to generate a radical similar to that described with 2'-ClUDP (11) in Scheme 1. Reduction of this radical cation at the 2' position by the thiol on B2 would regenerate the 3'-nucleotide radical. The hydrogen atom originally on the 3'-carbon is reabstracted from the tyrosyl moiety by the nucleotide radical to form the reduced ribonucleotide and regenerate the original tyrosine radical.

This speculative mechanism indicates that a hydrogen atom is abstracted from substrate. However, we have also observed that this abstraction is accompanied by a small but significant release of 3H to solvent, probably as a proton. No definitive statements about the mechanism by which this exchange occurs can be made at present.

Finally, it is interesting to note that ribonucleoside triphosphate reductase from Lactobacillus leichmannii is a B12-dependent enzyme. Abeles and Dolphin (16) proposed a radical cation mechanism for the B12-dependent rearrangements of diol dehydrase and ethanolamine ammonia lase.

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