Mechanism of Inactivation of *Escherichia coli* Ribonucleotide Reductase by 2′-Chloro-2′-deoxyuridine 5′-Diphosphate: Evidence for Generation of a 2′-Deoxy-3′-ketonucleotide via a Net 1,2 Hydrogen Shift†

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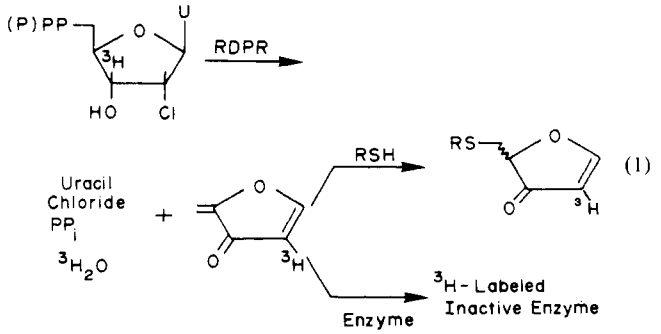
ABSTRACT: Sodium borohydride or ethanethiol protects the *Escherichia coli* ribonucleoside-diphosphate reductase (RDPR) from inactivation by 2′-chloro-2′-deoxyuridine 5′-diphosphate (ClUDP). Incubation of [3′-^{3}H]ClUDP with RDPR in the presence of $NaBH_4$ allowed trapping of [^{3}H]-2′-deoxy-3′-ketouridine 5′-diphosphate. Degradation of the reduced ketone by a combination of enzymatic and chemical methods indicated that the hydrogen originally present in the 3′-position of ClUDP is transferred to the β-face of the 2′-position of 2′-deoxy-3′-keto-UDP. RDPR therefore catalyzes a net 1,2 hydrogen shift. Incubation of RDPR with ClUDP in the presence of ethanethiol allowed trapping of 2-methylene-3(2*H*)-furanone, the species responsible for inactivation of RDPR. Trapped 2-[(ethylthio)methyl]-3(2*H*)-furanone was identical by ^{1}H NMR spectroscopy with material synthesized chemically. Both subunits of the enzyme are covalently radiolabeled in the reaction of RDPR with [5′-^{3}H]ClUDP. Studies with [3′-^{3}H]ClUDP and prereduced RDPR in the absence of a reductant and with oxidized RDPR indicated that the redox-active thiols of the B1 subunit are not involved in inactivation of the enzyme by ClUDP.

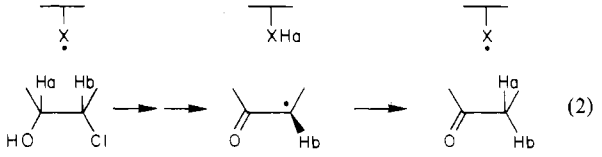
The observation that 2′-chloro-2′-deoxyuridine 5′-diphosphate (ClUDP)[1] inactivates the *Escherichia coli* ribonucleoside-diphosphate reductase (RDPR) with concomitant production of uracil and chloride was originally reported by Thelander et al. (1976). On the basis of these unusual results, we began a detailed study of the mechanism of the reaction of this analogue with the *E. coli* ribonucleotide reductase. In previous papers in this series (Stubbe & Kozarich, 1980; Harris et al., 1984), we have shown that [3′-^{3}H]ClUDP undergoes a remarkable series of transformations upon incubation with RDPR, which results in production of uracil, inorganic pyrophosphate, [^{3}H]-2-methylene-3(2*H*)furanone, ^3H_2O, and ^{3}H bound to the inactivated protein (eq 1).



(1)

These previous studies have led us to propose the following detailed working hypothesis for this inactivation (Scheme I) (Harris et al., 1984). This scheme predicts that a radical on the protein abstracts the hydrogen atom from the 3′-position of ClUDP to produce the 3′-nucleotide radical **1**. This intermediate could then rapidly lose Cl^-, a process not requiring catalysis, to generate the radical cation intermediate **2**. Since the active site thiols of the enzyme are not in the proper protonation state to carry out the normal reduction reaction, the enzyme instead generates a 2′-deoxy-3′-ketonucleotide radical, **3**. This intermediate then reabstracts the hydrogen atom from the protein radical to generate a 2′-deoxy-3′-ketonucleotide, **4**. Ketone **4** decomposes to produce PP_i, uracil, and methylene-3(2*H*)-furanone (**5**), which inactivates the enzyme by alkylation.

Evidence is presented in this paper that 2′-deoxy-3′-keto-UDP is, in fact, an intermediate in the ClUDP inactivation of ribonucleotide reductase. Furthermore, evidence obtained with [3′-^{3}H]ClUDP is presented that indicates that the hydrogen abstracted from the 3′-position of the analogue is either exchanged with solvent or returned to the β face of the 2′-position of the nucleotide to produce the 2′-deoxy-3′-ketonucleotide (eq 2). These intriguing results indicate that in



(2)

the case of ClUDP, ribonucleotide reductase is capable of catalyzing a net 1,2 hydrogen rearrangement reaction. Finally, the role of the active site thiols of RDPR in its reaction with ClUDP has been examined.

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[1] Abbreviations: ClUDP, 2′-chloro-2′-deoxyuridine 5′-diphosphate; RDPR, ribonucleoside-diphosphate reductase; PP_i, inorganic pyrophosphate; 2′-deoxy-3′-epiuridine, 1-(2′-deoxy-β-D-*threo*-pentofuranosyl)uracil; $TPDSCl_2$, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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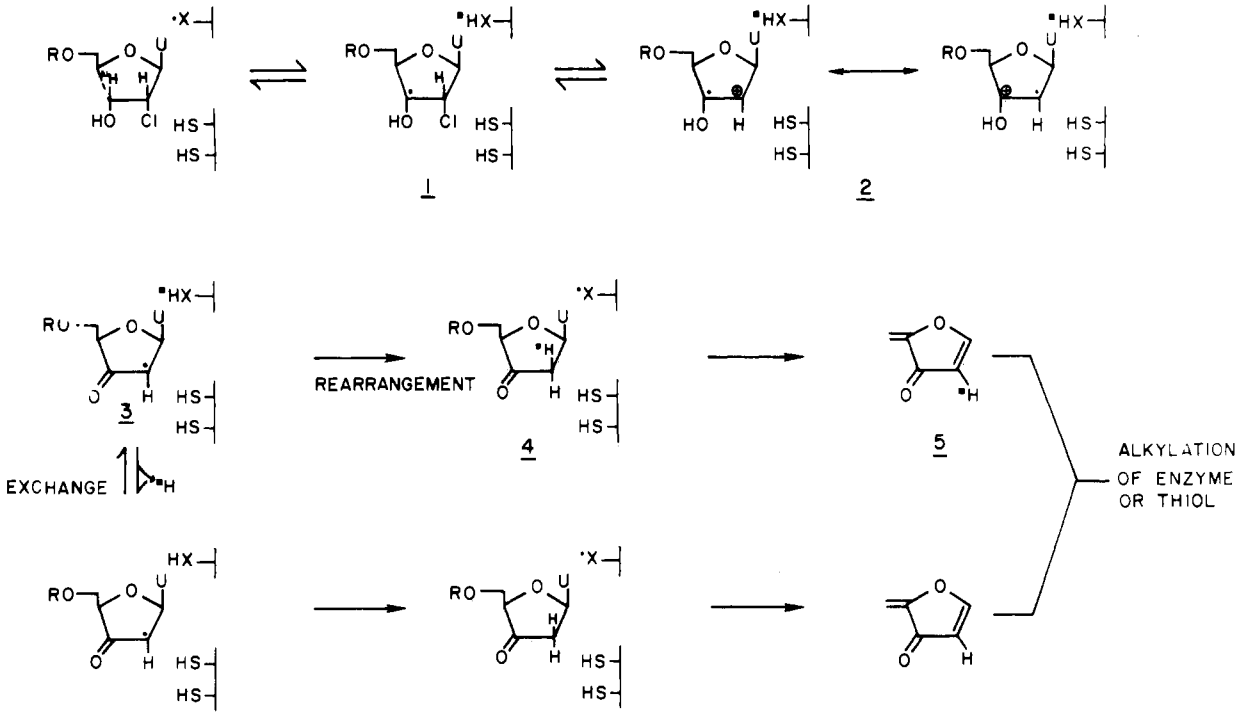
Scheme I: Proposed Mechanism of Inactivation of Ribonucleotide Reductase by 2′-Chloro-2′-deoxyuridine 5′-Diphosphate[a]



[a] The thiol groups represent the active site thiols on the B1 subunit, and X represents the tyrosyl radical of the B2 subunit.

Materials and Methods

Ribonucleoside-diphosphate reductase was isolated from *E. coli* strain KK546 by the procedure of Eriksson et al. (1977) using a dATP–Sepharose affinity column synthesized by extensive modifications of the procedure of Berglund & Eckstein (1977) as described by Knorre et al. (1976). B1 and B2 had specific activities of 0.34 and 3.0 μmol min^{-1} mg^{-1}, respectively. Both subunits were greater than 95% pure on the basis of SDS gel electrophoresis (Laemmli, 1970). RDPR concentrations were determined by the absorbance at 280 nm, using previously published extinction coefficients (Thelander, 1973). Thioredoxin and thioredoxin reductase were isolated by extensive modifications of published procedures (Pigiet & Cooley, 1977; Moore et al., 1964). Deoxyuridine 2′-hydroxylase was isolated from *Rhodotorula glutinis* by the procedure of Stubbe[2] and catalyzed the conversion of 0.35 μmol of 2′-deoxyuridine to uridine per minute per milligram of protein at 25 °C.

[3′-^{3}H]ClUDP was prepared by the procedure of Stubbe et al. (1983b). Location of the ^{3}H at the 3′-position was established by synthesis of the corresponding ^{2}H-labeled material by an identical procedure and by use of ^{1}H NMR spectroscopy. [5′-^{3}H]ClUDP was synthesized as described by Harris et al. (1984). [^{14}C]CDP was obtained from New England Nuclear. NADPH, ATP, CDP, $NaBH_4$, and bovine intestine alkaline phosphatase were purchased from Sigma. DTT was obtained from U.S. Biochemical Corp., and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane was purchased from Aldrich. All other materials were purchased in the highest quality available.

UV spectra were recorded on a Cary 210 spectrophotometer, and NMR spectra were obtained on a Bruker 270-MHz or Nicolet 200-MHz spectrometer. Fourier-transform infrared spectra were recorded on a Nicolet MX-1 FT-IR spectrometer. Scintillation counting was done on a Packard 300C scintillation counter with Scint-A (Packard) as scintillation fluid. Reverse-phase HPLC was performed on an Altex 110A system equipped with a microprocessor, using Alltech C_{18} reverse-phase columns.

RDPR Assay Mixture. A typical assay mixture contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1 mM NADPH, 1.25 mM [^{14}C]CDP (sp act. 5 × 10^5 cpm/μmol), 20 μg of thioredoxin, 5 μg of thioredoxin reductase, and RDPR in a volume of 0.1 mL. Each assay was incubated for 20 min at room temperature and was then placed in a boiling water bath for 1 min to terminate the reaction. Each sample was treated with 50 μL of 0.5 M Tris-HCl (pH 8.5) and 1 unit of bovine intestine alkaline phosphatase for 1 h at 37 °C. The resulting deoxycytidine was determined by the procedure of Steeper & Stewart (1970).

$NaBH_4$ Protection against Inactivation of RDPR by ClUDP. The reaction mixture contained 100 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 0.17 mg (2.2 nmol) of B1, 0.03 mg (0.4 nmol) of B2, 2 μg of thioredoxin, 1 μg of thioredoxin reductase, 100 mM $NaBH_4$, and 0.5 mM ClUDP in a volume of 75 μL. The reaction was initiated by addition of $NaBH_4$ (dissolved in 0.1 N NaOH) followed immediately by ClUDP. Aliquots of 10 μL were withdrawn at 0, 0.5, 1, 2, and 5 min after addition of inactivator and diluted into 90 μL of [^{14}C]CDP assay mixture. The assays were allowed to proceed for 10 min at room temperature and were terminated by incubation for 1 min in a boiling water bath. The amount of dCDP produced was determined as described under assay conditions.

Two control reactions were run simultaneously with the above experiment. One control mixture contained $NaBH_4$ but no ClUDP, while the second contained ClUDP and no $NaBH_4$.

Reaction of ClUDP with RDPR in the Presence of $NaBH_4$: Isolation of 1-(2′-Deoxy-β-D-threo-pentofuranosyl)uracil 5′-Diphosphate and dUDP and Characterization by NMR

[2] [2′-α-^{3}H]Deoxyuridine and [2′-β-^{3}H]deoxyuridine have been prepared. Incubation of the former with deoxyuridine 2′-hydroxylase results in the complete release of ^{3}H to the solvent at 100% conversion to product. There is no measurable isotope effect on this reaction. Incubation of the latter with the hydroxylase results in no release of ^3H_2O, and the specific activity of the product is identical with that of the starting material at late extents of reaction (Stubbe, 1985).

Spectroscopy. The reaction mixture contained 250 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 2.3 mg (30 nmol) of B1, 0.96 mg (12.3 nmol) of B2, 20 μg of thioredoxin, 5 μg of thioredoxin reductase, 100 mM $NaBH_4$, and 2.5 mM ClUDP in a final volume of 0.4 mL. Ten similar reactions were run, and at the end of 10 min the pH of the solutions was adjusted to 7.6 with 1 N HCl. The samples were incubated for 1 min in a boiling water bath. The denatured protein was removed by centrifugation, and the supernatants were pooled. The pellets were washed with 2.0 mL of 0.5 M Tris-HCl (pH 8.5), and the wash was combined with the supernatant. Bovine intestine alkaline phosphatase (15 units) was added to the sample, which was then incubated at 37 °C for 1 h. The volume of the sample was reduced in vacuo to 1.5 mL, and the mixture was incubated for 1 min in a boiling H_2O bath, cooled, and centrifuged to remove protein. The supernatant was chromatographed on a 7 mm × 25 cm reverse-phase HPLC column equilibrated in 5% methanol (flow rate 2.0 mL/min; compounds and retention times: 2′-deoxy-3′-epiuridine and 2′-deoxyuridine, 7.8 min). The isolated epimers of deoxyuridine were concentrated in vacuo to 0.5 mL and chromatographed on a 1 × 46 cm column of Sephadex G-10 equilibrated in H_2O. The fractions containing the 3′-epimers of deoxyuridine were pooled, exchanged into D_2O, and examined by NMR spectroscopy. NMR (D_2O) for 2′-deoxyuridine showed the following: δ 7.40 (1 H, d, $J_{5,6}$ = 8 Hz), 5.86 (1 H, t, $J_{1',2'} = J_{4',2''}$ = 6.6 Hz), 5.45 (1 H, br s), 4.2–3.3 (4 H, m), and 1.94 (2 H, m). NMR (D_2O) for 2′-deoxy-3′-epiuridine showed the following: δ 7.58 (1 H, d, $J_{5,6}$ = 8 Hz), 5.71 (1 H, dd, $J_{1',2'}$ = 1.5 Hz, $J_{1',2''}$ = 8.1 Hz), 5.45 (1 H, br s), 4.07 (1 H, m), 3.70 (1 H, m), 3.51 (2 H, m), 2.30 (1 H, m), and 1.71 (1 H, d, $J_{2',2''}$ = 15.4 Hz).

Synthesis of 2′-Deoxy-3′-epiuridine and 2′-Deoxyuridine. 2′-Deoxy-5′-trityluridine was prepared by the procedure of Michelson & Todd (1953) and was oxidized to 2′-deoxy-3′-keto-5′-trityluridine by the procedure of Bergstrom et al.,[3] IR ($CDCl_3$) 1769 cm^{-1}.

The 3′-ketone (0.1 g, 2 mmol) was dissolved in 3 mL of ethanol, and $NaBH_4$ (0.757 g, 20 mmol) was added. After 1 h at room temperature, the reaction mixture was partitioned between 20 mL of $CHCl_3$ and 20 mL of H_2O. The organic layer was then reextracted with 20 mL of H_2O and dried over sodium sulfate. The $CHCl_3$ layer was filtered and the solvent removed in vacuo. The residue was redissolved in 3 mL of 80% acetic acid and heated for 1 h at 100 °C. The acetic acid was removed in vacuo, and the residue was redissolved in 5 mL of $CHCl_3$ and extracted with 3 × 5 mL of H_2O. The H_2O layers were pooled, evaporated to dryness, and characterized by NMR spectroscopy. NMR (D_2O) for 2′-deoxyuridine showed the following: δ 7.40 (1 H, d, $J_{5,6}$ = 8 Hz), 5.90 (1 H, t, $J_{1',2'} = J_{1',2''}$ = 7 Hz), 5.44 (1 H, d, $J_{5,6}$ = 8 Hz), 4.1 (1 H, m), 3.9–3.2 (3 H, m), and 1.96 (2 H, m). NMR (D_2O) for 2′-deoxy-3′-epiuridine showed the following: δ 7.59 (1 H, d, $J_{5,6}$ = 8 Hz), 5.72 (1 H, dd, $J_{1',2'}$ = 1.5 Hz, $J_{1',2''}$ = 8.1 Hz), 5.47 (1 H, d, $J_{5,6}$ = 8 Hz), 4.1 (1 H, m), 3.9–3.2 (3 H, m), 2.32 (1 H, m), and 1.71 (1 H, d, $J_{2',2''}$ = 15 Hz).

Reaction of [3′-^{3}H]ClUDP with RDPR in the Presence of $NaBH_4$: Location of ^3H in 2′-Deoxy-3′-keto-UDP. The reaction mixture contained 250 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 6.4 mg (82 nmol) of B1, 3.2 mg (41 nmol) of B2, 24 μg of thioredoxin, 5 μg of thioredoxin reductase, 100 mM $NaBH_4$, and 1.4 mM [3′-^{3}H]ClUDP (sp act. 6.16 × 10^6 cpm/μmol) in a volume of 1.0 mL, which was divided into three plastic centrifuge tubes. The reaction was initiated with $NaBH_4$, followed immediately by ClUDP. The sample was incubated at room temperature for 10 min. The pH of the solutions was readjusted to 7.6 by addition of 14 μL of 1 N HCl to each tube, and the samples were incubated for 1 min in a boiling water bath. The samples were pooled, and the phosphates were removed from nucleotides by adding 0.45 mL of 0.5 M Tris-HCl (pH 8.5) containing 5 units of bovine intestine alkaline phosphatase to the sample and incubating it at 37 °C for 1 h. The mixture was then incubated for 1 min in a boiling water bath, cooled, and centrifuged to remove protein. The solvent was removed by bulb to bulb distillation and examined for ^3H_2O by scintillation counting of the distillate. The residue was redissolved in 0.2 mL of H_2O and injected onto a reverse-phase HPLC column equilibrated in 2% methanol (flow rate 1.5 mL/min; compounds and retention times: 2′-deoxyuridine and 2′-deoxy-3′-epiuridine, 8.5 min; 2′-chloro-2′-deoxyuridine, 15 min). Fractions of 1.0 min were collected, and the amount of radioactivity in each was determined. The 2′-deoxyuridine- and 2′-deoxy-3′-epiuridine-containing fractions were pooled, and the specific activity of the isolated material was determined.

Deoxyuridine was converted to uridine in a reaction mixture that contained 25 mM sodium phosphate (pH 7.5), 1 mM α-ketoglutarate, 1 mM ascorbic acid, 1 mM $Fe(NH_4)_2(SO_4)_2$, 3 mg (1.2 × 10^5 units) of bovine liver catalase, 1.5 mM 3′-epimers of [^{3}H]deoxyuridine, and 0.15 unit of deoxyuridine hydroxylase in a volume of 0.625 mL. The reaction was run at room temperature for 1 h, and the mixture was then shell-frozen in a pear-shaped flask. The solvent was removed by bulb to bulb distillation, and the distillate was examined for ^3H_2O by scintillation counting. The residue was redissolved in 0.2 mL of H_2O and chromatographed on reverse-phase HPLC in H_2O (flow rate 1.5 mL/min; compounds and retention times: uridine, 8.6 min; 2′-deoxy-3′-epiuridine, 11 min). Fractions of 1.0 min were collected and analyzed for radioactivity. The uridine-containing fractions were pooled, and the specific activity of the material was determined by UV spectroscopy and scintillation counting. The 2′-deoxy-3′-epiuridine-containing fractions were also pooled, and its specific activity was measured.

The [^{3}H]uridine (93 nmol, 195 000 cpm) was dried by repeated evaporation in vacuo from dry *N,N*-dimethylformamide (distilled in vacuo from CaH_2) and diluted with 25 mg (102 μmol) of unlabeled uridine. The nucleoside was converted to 3′,5′-*O,O*-TPDS-uridine by the procedure of Robins et al. (1983) and used without further purification. The product was dissolved in methanol and its specific activity measured by UV spectroscopy and scintillation counting.

The 3′,5′-*O,O*-TPDS-uridine was oxidized with a complex of CrO_3, pyridine, and acetic anhydride in dichloromethane as described by Hansske et al. (1984). The product, 2′-keto-3′,5′-*O,OTPDS*-uridine, which was approximately 90% pure by NMR spectroscopy, was dissolved in methanol, and its specific activity was determined.

Reduction of 2′-keto-3′,5′-*O,OTPDS*-uridine with $NaBH_4$ was accomplished by the method of Hansske et al. (1984). The major product, 3′,5′-*O,O*-TPDS-arauridine, was purified by normal-phase HPLC on a Waters μPorasil column equilibrated in CH_2Cl_2–MeOH (49:1) (flow rate 1.7 mL/min; compounds and retention times: 3′,5′-*O,O*-TPDS-uridine, 7 min; 3′,5′-*O,O*-TPDS-arauridine, 11 min). The purified 3′,5′-*O,O*-TPDS-arauridine was concentrated to drynes in vacuo and

[3] D. Bergstrom, Department of Chemistry, University of North Dakota, personal communication.

dissolved in methanol and its specific activity determined by UV spectroscopy and scintillation counting.

Protection by Ethanethiol against Inactivation of RDPR by ClUDP. The reaction mixture contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 0.17 mg (2.2 nmol) of B1, 0.03 mg (0.4 nmol) of B2, 10 μg of thioredoxin, 2 μg of thioredoxin reductase, 30 mM ethanethiol, and 0.5 or 2.0 mM ClUDP in a volume of 75 μL. Aliquots were taken at various times and diluted into 90 μL of [^{14}C]CDP assay mixture as described under assay conditions. The amount of dCDP produced was determined as described above.

Trapping of 2-Methylene-3(2H)-furanone by Ethanethiol in the Reaction of RDPR with ClUDP. The reaction mixture contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 20 μg of thioredoxin, 5 μg of thioredoxin reductase, 1.56 mg (20 nmol) of B1, 0.78 mg (10 nmol) of B2, 230 mM ethanethiol, and 5 mM ClUDP in a volume of 0.5 mL. The reaction was run for 30 min at room temperature, followed by extraction with 6 × 3 mL of $CHCl_3$. The solvent was removed under reduced pressure, and the residue was redissolved in freshly distilled $CDCl_3$ and examined by NMR spectroscopy. The solvent was again removed, the sample was redissolved in $CHCl_3$, and a FT-IR spectrum was obtained, IR ($CHCl_3$) 1704 (major) and 1762 (minor) cm^{-1}. NMR ($CDCl_3$) showed the following: δ 8.20 (1 H, d, $J_{1,2}$ = 2 Hz), 5.67 (1 H, d, $J_{1,2}$ = 2 Hz), 4.48 (1 H, dd, $J_{4,5'}$ = 3.7 Hz, $J_{4,5''}$ = 7.3 Hz), 3.04 and 2.76 (2 H, m, $J_{5,5}$ = 15 Hz, $J_{4,5'}$ = 3.7 Hz, $J_{4,5''}$ = 7.3 Hz), 2.56 (2 H, q, J, = 7.3 Hz), and 1.20 (3 H, t, J = 7.3 Hz).

A control incubation was run in parallel, which was identical except for the omission of enzymes. In experiments to determine the efficiency of ClUDP turnover and $CHCl_3$ extraction, [5′-^{3}H]ClUDP (sp act. 2.8 × 10^5 cpm/μmol) replaced unlabeled inactivator.

Stoichiometry of Labeling of Each Subunit of RDPR by [5′-^{3}H]ClUDP. The reaction mixture contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 60 μg of thioredoxin, 12 μg of thioredoxin reductase, 2.8 mg (36 nmol) of B1, 1.5 mg (19 nmol) of B2, and 0.6 mM [5′-^{3}H]ClUDP (sp act. 2.0 × 10^7 cpm/μmol) in a volume of 0.4 mL. The reaction was run at room temperature for 10 min, and the mixture was chromatographed on a column of Sephadex G-50 equilibrated in 50 mM Tris-HCl (pH 7.6) at 4 °C. Fractions of 1 mL were collected and analyzed for radioactivity and protein. The protein-containing fractions were pooled and dialyzed overnight against the same buffer.

The subunits of RDPR were then separated by loading the protein onto a column of dATP–Sepharose equilibrated in 50 mM Tris-HCl (pH 7.6). The B2 subunit was eluted by washing the column with the same buffer, while the B1 subunit was eluted with 50 mM Tris-HCl (pH 7.6) and 10 mM ATP. The ATP was removed from B1 by chromatography on a 1 × 25 cm column of Sephadex G-50 in the same buffer. The amount of ^{3}H bound to each subunit was determined by scintillation counting and UV spectroscopy, using the previously published extinction coefficients for B1 and B2 (Thelander, 1973).

Oxidation of RDPR. Dithiothreitol was removed from RDPR by centrifugation through a 1-mL column of Sephadex G-25 (Penefsky, 1977) equilibrated in 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, and 1 mM EDTA. The protein was collected, and its concentration was determined by its absorbance at 280 nm. The enzyme was oxidized with a mixture that contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 1.0 mM CDP, 66 μM B1, and 22 μM B2 in a final volume of 90 μL. After incubation for 5 min at room temperature, the enzyme was again centrifuged through Sephadex G-25 to remove the CDP.

The efficiency of the oxidation of RDPR was determined by addition of 2.75 nmol of B1 and B2 to a mixture that contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, and 0.3 mM [^{14}C]CDP (sp act. 1.4 × 10^7 cpm/μmol) in a final volume of 0.1 mL. The sample was maintained at room temperature for 20 min followed by incubation for 1 min in a boiling H_2O bath. The sample was treated with 50 μL of 0.5 M Tris-HCl (pH 8.5) and 1 unit of bovine intestine alkaline phosphatase for 1 h at 37 °C. The amount of deoxycytidine produced was determined by the method of Steeper & Stewart (1970).

Time-Dependent Inactivation of Oxidized RDPR by ClUDP. The reaction mixture contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 2 mM ClUDP, 55 μM (5.5 nmol) oxidized B1, and 10 μM (1.0 nmol) B2 in a volume of 0.1 mL. At various times, 10-μL aliquots were withdrawn and diluted into 90 μL of assay mixture, and the amount of dCDP produced was determined as described under assay conditions.

Reaction of Oxidized RDPR with [3′-^{3}H]ClUDP: ^{3}H Bound to RDPR and Release of ^3H_2O. The reaction mixture was identical with that described for time-dependent inactivation of oxidized RDPR by ClUDP except [3′-^{3}H]ClUDP (sp act. 6.1 × 10^5 cpm/μmol) replaced unlabeled ClUDP and the reaction was run in a volume of 0.275 mL. At various times, 25-μL aliquots were withdrawn, diluted into 0.475 mL of cold H_2O in a pear-shaped flask, and shell-frozen. The solvent was removed by bulb to bulb distillation, and the amount of ^{3}H in the distillate was determined by scintillation counting.

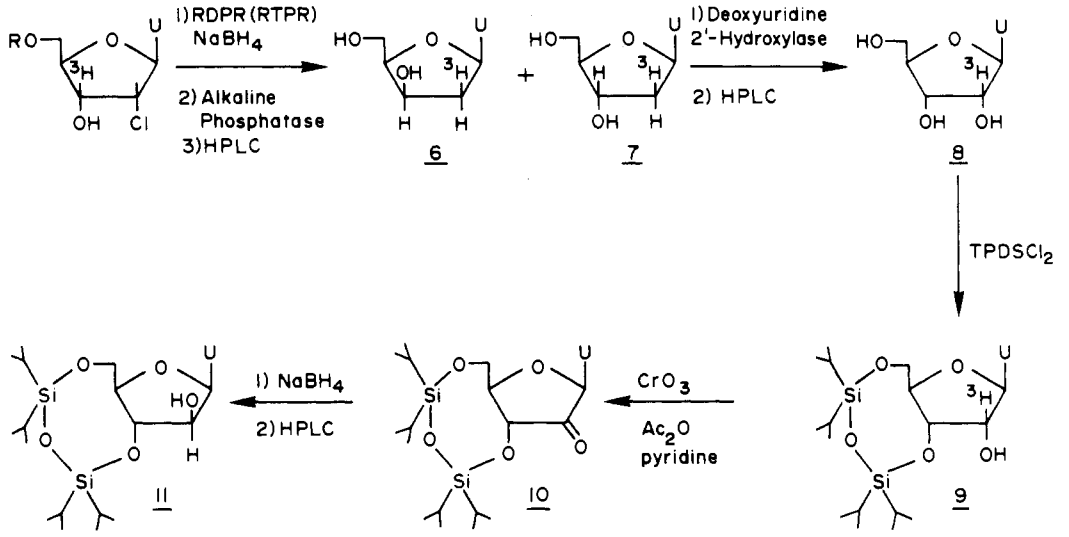
The remainder of the reaction mixture (0.150 mL) was chromatographed on a Sephadex G-50 column equilibrated in 50 mM Tris-HCl (pH 7.6). Fractions of 0.5 mL were collected, and protein (A_{280}) and radioactivity in each fraction were measured. The protein-containing fractions were pooled, the UV spectrum of the protein was recorded, and the amount of ^{3}H bound was determined by scintillation counting.

RESULTS

Protection by $NaBH_4$ against Inactivation of RDPR by ClUDP. As indicated in Figure 1, 0.5 mM ClUDP rapidly inactivates RDPR with a $t_{1/2}$ of less than 20 s. Our working hypothesis for the mechanism of this inactivation (Scheme I) predicts that RDPR catalyzes the conversion of ClUDP to 2′-deoxy-3′-keto-UDP and that this species is released into solution. Nonenzymatic decomposition of the ketonucleotide generates 2-methylene-3(2*H*)-furanone, which is responsible for enzyme inactivation. Addition of $NaBH_4$ to this reaction mixture might reduce the 2′-deoxy-3′-keto-UDP, prevent its conversion to the reactive alkylating agent, and retard enzyme inactivation. Inactivation of RDPR with 0.5 mM ClUDP in the presence of 0.1 M $NaBH_4$ does result in protection against enzyme inactivation, increasing the $t_{1/2}$ to greater than 5 min (Figure 1). Control experiments indicate that the reduction of CDP by RDPR is unaffected by the presence of 0.1 M $NaBH_4$.

Identification of the Products of the Reaction of ClUDP with RDPR in the Presence of $NaBH_4$. The observation of protection by $NaBH_4$ against inactivation of RDPR by ClUDP is consistent with trapping of 2′-deoxy-3′-keto-UDP. This hypothesis was confirmed by identification of the products of

Scheme II: Analysis of the Position of ^3H in $NaBH_4$-Trapped 2′-Deoxy-3′-keto-UDP Obtained from the Reaction of [3′-^{3}H]ClUDP with RDPR



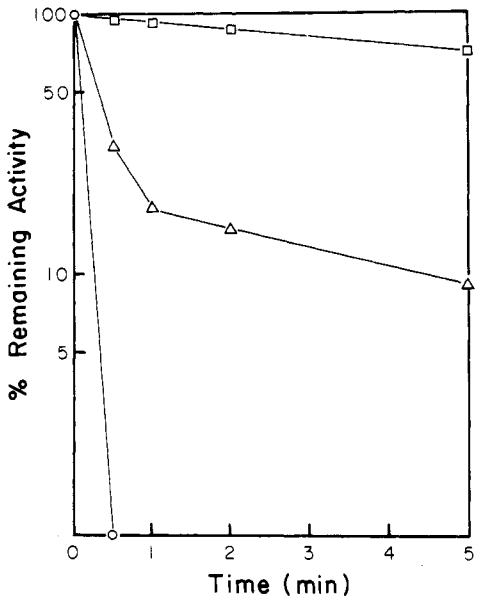


FIGURE 1: Time-dependent inactivation of RDPR by ClUDP. RDPR was incubated with 0.5 mM ClUDP in the presence of 0.1 M $NaBH_4$ (□), in the presence of 30 mM ethanethiol (△), or with no protecting agent (○), and the rate of enzyme inactivation was measured.

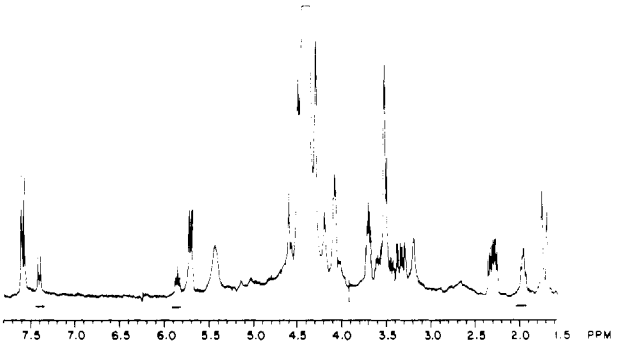


FIGURE 2: ^{1}H NMR spectrum of the mixture of 2′-deoxyuridine and 2′-deoxy-3′-epiuridine isolated from the reaction of RDPR with ClUDP in the presence of $NaBH_4$. The underlined resonances at 7.40, 5.86, and 1.94 ppm are due to the 6, 1′-, and 2′-hydrogens of 2′-deoxyuridine.

this reduction reaction. RDPR was incubated with [5′-^{3}H]-ClUDP and 0.1 M $NaBH_4$, the solution was treated with alkaline phosphatase to convert nucleotides to nucleosides, and the mixture was chromatographed on reverse-phase HPLC. Approximately 60% of the radioactivity initially present co-migrated with 2′-deoxyuridine and 2′-deoxy-3′-epiuridine. The remaining 40% of the radioactivity was reisolated as 2′-chloro-2′-deoxyuridine.

A similar experiment was run on large scale in order to further characterize the products by NMR spectroscopy. The NMR spectrum of the material isolated from the reaction mixture by HPLC is shown in Figure 2. Two species are present: 2′-deoxyuridine and 2′-deoxy-3′-epiuridine in a ratio of approximately 1:5. The spectrum is identical with the spectrum of these materials synthesized chemically by reduction of 2′-deoxy-3′-keto-5′-trityluridine and removal of the 5′-trityl group (data not shown). These results confirm the intermediacy of 2′-deoxy-3′-keto-UDP in the ClUDP inactivation of RDPR.

Determination of the Location of ^3H in 2′-Deoxyuridine from Reaction of RDPR with [3′-^{3}H]ClUDP. Our previous studies demonstrated that inactivation of RDPR with [3′-^{3}H]- and [β-^{32}P]ClUDP resulted in production of 6 equiv of PP_i, 5 equiv of ^3H_2O, and 1 equiv of ^3H covalently bound to the protein per equivalent of B1 subunit of RDPR (Harris et al., 1984). These results were interpreted to indicate that the hydrogen abstracted from the 3′-position of [3′-^{3}H]ClUDP is exchanged with the solvent 5 times for every time it is returned to the 2′-position of intermediate **3** (Scheme I) to produce 2′-deoxy-3′-keto-UDP. The location of the tritium and its stereochemistry have now been determined by enzymatic analysis and chemical degradation of the [^{3}H]-2′-deoxyuridine isolated from the incubation of [3′-^{3}H]ClUDP and RDPR with $NaBH_4$ (Scheme II).

Table I: Reaction of RDPR with [3′-^{3}H]ClUDP: Determination of the Location of ^3H in 2′-Deoxy-3′-keto-UDP

compound	specific activity (cpm/μmol)[a]
[3′-^{3}H]ClUDP	6.16×10^6
2′-deoxyuridine epimers	2.13×10^6
uridine	2.10×10^6
2′-deoxy-3′-epiuridine	1.93×10^6
3′,5′-*O*,*O*-TPDS-uridine[b]	1530
3′,5′-*O*,*O*-TPDS-arauridine	11

[a] RDPR was incubated with [3′-^{3}H]ClUDP in the presence of $NaBH_4$, the mixture was treated with alkaline phosphatase, and the mixture was chromatographed on reverse-phase HPLC. The isolated deoxyuridine was analyzed as described in Scheme II, and the specific activity of the nucleoside was determined at each step. [b] Diluted 1100-fold with unlabeled uridine.

[3′-^{3}H]ClUDP was incubated with RDPR and $NaBH_4$ until the ClUDP was consumed. The phosphates were removed from nucleotides with alkaline phosphatase, and the resulting mixture was chromatographed on reverse-phase HPLC. A 5:1 mixture of 2′-deoxy-3′-epiuridine and 2′-deoxyuridine (**6** and **7**, Scheme II) was isolated. The specific activity of this material was 35% that of the ClUDP (Table I), reflecting the previously reported partitioning between return of the abstracted 3′-hydrogen to the nucleotide and exchange of that hydrogen with the solvent (Harris et al., 1984). Previous labeling experiments with [3′-^{3}H]- and [5′-^{3}H]ClUDP predicted that the specific activity of the epimers of deoxyuridine should be 22% that of the ClUDP (Harris et al., 1984). The discrepancy between these values may be due to a change in the partitioning of the 3′-hydrogen due to the presence of $NaBH_4$ in the reaction mixture.

The model in Scheme I and eq 2 predicts that the ^{3}H in deoxyuridine should be located on the β face at the 2′-position and that the hydrogen originally present in the 2′-position of ClUDP should have undergone an inversion of configuration. The stereochemistry of this 1,2 hydrogen shift therefore must be established. This was accomplished by using the Fe^{2+}-requiring enzyme deoxyuridine 2′-hydroxylase from *Rhodotorula glutinis*, which converts deoxyuridine, α-ketoglutarate, and O_2 to uridine, CO_2, and succinate. The stereochemistry of this transformation has been established unequivocally to be retention of configuration.[2] Incubation of the mixture of ^{3}H-labeled epimers of 2′-deoxyuridine with deoxyuridine 2′-hydroxylase, analysis for ^3H_2O and isolation of the products of the reaction by HPLC resulted in recovery of a 1:5 mixture of uridine (**8**, Scheme II) and 2′-deoxy-3′-epiuridine. The latter is not a substrate for deoxyuridine 2′-hydroxylase. The specific activity of the uridine was essentially identical with that of the starting material (Table I), and the amount of ^{3}H found in the solvent corresponded to only 0.2% of the amount of deoxyuridine converted to uridine. These results show that if the ^{3}H originally present in the 3′-position of ClUDP is returned to the 2′-position of 2′-deoxy-3′-keto-UDP, it is on the β face of the sugar moiety.[4]

Chemical analysis of the uridine obtained from the deoxyuridine 2′-hydroxylase reaction was performed to demonstrate that the ^{3}H was attached to the 2′ carbon of the sugar moiety. To facilitate handling of the material during the required chemical transformations, 102 μmol of unlabeled uridine was added to the ^{3}H-labeled nucleoside. The 3′- and 5′-positions of the uridine were blocked with the bifunctional reagent 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (Robins et al., 1983), generating 3′,5′-*O*,*O*-TPDS-uridine (**9**). The 2′-position of the blocked nucleoside was then oxidized by the procedure of Hansske et al. (1984), to produce 2′-keto-3′,5′-*O*,*O*-TPDS-uridine (**10**, Scheme II). The specific activity of this material was 4% that of the protected uridine prior to oxidation. Because of the possibility that the remaining radioactivity resulted from a small amount of unoxidized TPDS uridine, purification of the 2′-ketonucleoside on normal-phase HPLC was attempted. The ketone rapidly decomposed under these conditions, necessitating stabilization of the molecule by reduction to 3′,5′-*O*,*O*-TPDS-arauridine (**11**) with $NaBH_4$. Separation of this material from unoxidized TPDS uridine was accomplished by normal-phase HPLC and resulted in isolation of 3′,5′-*O*,*O*-TPDS-arauridine with a specific activity of less than 0.7% that of the starting blocked uridine.[5] These results demonstrate that the hydrogen originally present in the 3′-position of ClUDP is abstracted by RDPR, is exchanged with the solvent 5 of 6 times, and is returned 1 of 6 times to the β face of the 2′-position of the nucleotide, resulting in formation of 2′-deoxy-3′-keto-UDP (Scheme I).

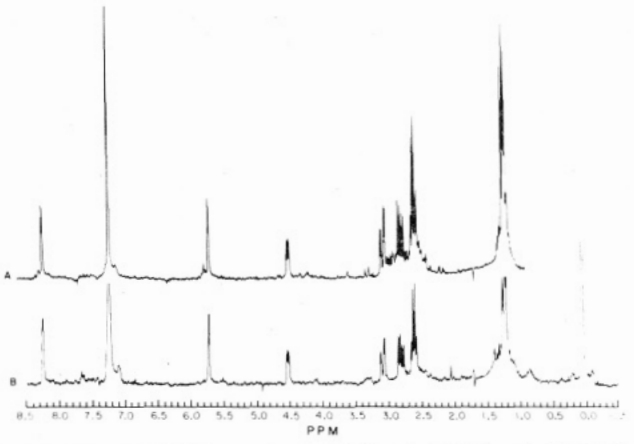


FIGURE 3: ^{1}H NMR spectrum of 2-[(ethylthio)methyl]-3(2*H*)-furanone: (A) isolated from the reaction of RDPR with ClUDP in the presence of ethanethiol by $CHCl_3$ extraction; (B) synthesized chemically as described by Harris et al. (1984).

Ethanethiol Trapping of 2-Methylene-3(2H)-furanone from the Reaction of RDPR with ClUDP. Previous studies have suggested that the product of the RDPR-catalyzed degradation of ClUDP is 2′-deoxy-3′-keto-UDP, which nonenzymatically decomposes to generate 2-methylene-3(2*H*)-furanone by elimination of uracil and inorganic pyrophosphate (Harris et al., 1984). This reactive species is proposed to inactivate the enzyme by covalent modification. Trapping of the furanone in enzymatic reactions with DTT has allowed characterization of thiol–sugar adducts by spectroscopic methods. The NMR spectrum of the sugar–DTT adducts was complicated by an excess of DTT in the $CHCl_3$-extracted material as well as by the instability of the products isolated, preventing further purification. These experiments have, therefore, been repeated by utilizing ethanethiol as a trapping agent in place of DTT. Ethanethiol was chosen for several reasons: (1) Recent studies have established that redox active thiols are unnecessary for ClUDP inactivation of RDPR. This eliminates the need for the presence of a reductant such as DTT. (2) Ethanethiol derivatives of 2-methylene-3(2*H*)-furanone have been synthesized chemically and characterized by NMR and IR spectroscopy (Harris et al., 1984). (3) Excess ethanethiol can easily be removed in vacuo following its extraction into $CHCl_3$.

[4] This result predicts that [2′-β-^{3}H]ClUDP should be converted to [2′-α-^{3}H]-2′-deoxyuridine 5′-diphosphate and [2′-α-^{3}H]-2′-deoxy-3′-epiuridine 5′-diphosphate in the presence of RDPR and $NaBH_4$. This possibility is presently being investigated by the deoxyuridine 2′-hydroxylase methodology described in the text.

[5] Even after rechromatography on normal-phase HPLC, the specific activity of this material was still 0.7% that of 3′,5′-*O*,*O*-TPDS-uridine prior to oxidation. A possible explanation for this result is that RDPR may catalyze partitioning of ClUDP between formation of 2′-deoxy-3′-keto-UDP and production of a small amount of the normal reduction product, 2′-dUDP. If ClUDP were reduced to 2′-dUDP, the hydrogen originally present in the 3′-position of the substrate analogue would be expected to be returned to the same location of the product, in analogy with normal substrate reduction (Stubbe et al., 1983a). In the analysis for the location of ^{3}H, 2′-dUDP produced in this manner would undergo the same chemical transformations as material resulting from $NaBH_4$ trapping, but oxidation of the 2′-position would result in no loss of label. Experiments designed to measure the amount of dUDP produced by RDPR-catalyzed reduction of ClUDP have shown that one turnover in about 10^4 may result in formation of dUDP.[6] This amount of turnover would be sufficient to account for the amount of ^{3}H observed in 3′,5′-*O*,*O*-TPDS-arauridine.

[6] M. Ator and J. Stubbe, unpublished observations.

Addition of 30 mM ethanethiol to a reaction mixture containing RDPR and 0.5 mM ClUDP results in protection against enzyme inactivation (Figure 1). Furthermore, if the concentration of ethanethiol is increased to 230 mM, no inactivation is observed and all of the ClUDP is consumed (data not shown). Control experiments indicate that ethanethiol has no effect on the rate of reduction of CDP by RDPR.

Incubation of [5′-^{3}H]ClUDP with RDPR in the presence of 230 mM ethanethiol results in essentially quantitative isolation of the radioactivity by extraction into $CHCl_3$. A similar reaction was performed on a larger scale with unlabeled ClUDP, and the $CHCl_3$-extractable material was examined by NMR and IR spectroscopy. The spectrum of the product of the enzymatic reaction (Figure 3A) is virtually identical with that of 2-[(ethylthio)methyl]-3(2*H*)-furanone, which was synthesized chemically (Figure 3B) (Harris et al., 1984). Several small peaks in the region of 2.4–3.2 ppm in the spectrum of the enzymatically synthesized material cannot be assigned to either mono- or disubstituted furanones. The origin of these signals has not been determined. The IR spectrum of the products of the enzymatic reaction contains a major absorption at 1704 cm^{-1} consistent with the presence of 2-[(ethylthio)methyl]-3(2*H*)-furanone.

Stoichiometry of Radiolabeling of Each Subunit of RDPR by [5′-^{3}H]ClUDP. Incubation of RDPR with [5′-^{3}H]ClUDP in the absence of thiols has been demonstrated previously to result in radiolabeling of the protein with 4.6 equiv of ^{3}H per equivalent of B1 inactivated (Harris et al., 1984). The location of the label on RDPR was not determined, but it was assumed that the B1 subunit was modified, since Thelander et al. (1976) demonstrated that it was inactivated during the reaction, while the B2 subunit was not affected. The high stoichiometry of labeling indicates that 2-methylene-3(2*H*)-furanone nonspecifically alkylates the enzyme and suggests that both subunits might be labeled. This hypothesis was tested by incubating the enzyme with [5′-^{3}H]ClUDP, separating the subunits of the inactive enzyme, and determining the amount of ^{3}H bound to each.

A stoichiometry of 4.2 equiv of ^{3}H bound per equivalent of B1 inactivated was observed initially. This value is slightly lower than that reported previously (Harris et al., 1984), which may reflect the different ratio of the two subunits utilized in the experiments. Dialysis for 12 h against 50 mM Tris-HCl (pH 7.6) at 4 °C decreased the stoichiometry to 3.6 equiv of ^{3}H bound per equivalent of B1. Separation of the subunits on a dATP–Sepharose affinity column revealed that both subunits were covalently modified during the reaction. The B1 subunit contained 2.5 equiv of ^{3}H per equivalent of protein, and 1.7 equiv of ^{3}H was bound per equivalent of B2 subunit. These results are consistent with the generation of 2-methylene-3(2*H*)-furanone in solution and the nonspecific modification of the enzyme by the alkylating agent.

Role of Active Site Thiols of RDPR in Its Reaction with ClUDP. Reduction of NDP substrates by RDPR results in oxidation of two thiols of the enzyme, which must be reduced prior to subsequent turnover (Thelander, 1974). The active site thiols have been postulated to be required for protonation of the leaving hydroxyl group as well as reduction of the 2′-position to produce dNDP (Stubbe et al., 1983a). In the hypothesis for the mechanism of inactivation of RDPR by ClUDP presented in Scheme I, the thiols play no active chemical role. The role of the active site thiols has been examined by incubation of ClUDP with prereduced RDPR in the absence of a reductant and with RDPR, which contains oxidized active site thiols.

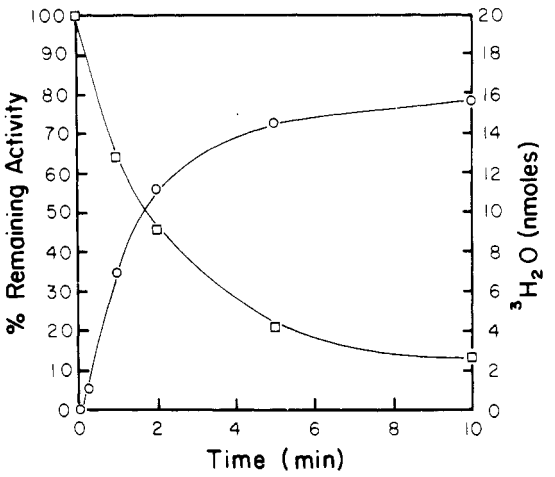


FIGURE 4: Measurement of the rate of enzyme inactivation (□) and of release of ^3H_2O (○) in the reaction of [3′-^{3}H]ClUDP with RDPR that contains oxidized active site thiols.

In the first experiment, RDPR was prereduced with DTT, the thiol was removed, and the enzyme was incubated with [3′-^{3}H]ClUDP in the absence of a reducing system. The enzyme was rapidly inactivated with a $t_{1/2}$ of less than 10 s. Under these conditions, 1.9 equiv of ^3H_2O was released per equivalent of B1, and the protein was radiolabeled with 0.7 equiv of ^{3}H/equiv of B1. Multiple turnovers of substrate were therefore observed, indicating that oxidation of the active site thiols does not occur in the reaction of RDPR with ClUDP or that RDPR, which contains oxidized active site thiols, is still capable of reaction with ClUDP.

The reaction of oxidized RDPR with ClUDP was then examined. RDPR was oxidized by incubation with excess CDP in the absence of a reductant, followed by removal of the substrate by Sephadex chromatography. Assay of the enzyme with [^{14}C]CDP indicated that it was greater than 98.5% oxidized. The assay for the amount of reduced enzyme was sufficiently sensitive such that 5% remaining activity would have resulted in 500 cpm above background in the assay. The oxidized enzyme was then incubated with [3′-^{3}H]ClUDP, and concomitant time-dependent inactivation and release of ^3H_2O were observed (Figure 4). The half-lives of these processes were approximately 1.7 min, which was at least 10 times slower than the rates observed with reduced enzyme in either the presence or absence of a reductant. A total of 2.6 equiv of ^3H_2O was produced, compared to 4.5 equiv obtained in the presence of a reducing system (Harris et al., 1984). Isolation of the inactive enzyme by chromatography on Sephadex G-50 followed by analysis for radioactivity revealed 1 equiv of ^{3}H bound per equivalent of B1. An increase in the absorbance of the protein at 320 nm was also noted, consistent with alkylation of the protein by [^{3}H]-2-methylene-3(2*H*)-furanone. Thus, two products of the reaction between ClUDP and RDPR are identical regardless of whether the thiols of RDPR are oxidized or reduced, although the other products of the reaction with oxidized enzyme have not been isolated. The results with prereduced enzyme in the absence of reductant and with oxidized enzyme indicate that the redox active thiols of RDPR are not required for conversion of ClUDP to species **5** (Scheme I) responsible for enzyme inactivation.

Discussion

In 1976, Thelander et al. reported the interesting observation that incubation of ClUDP with *E. coli* RDPR resulted in the production of uracil and chloride concomitant with enzyme inactivation. Extensive studies from our laboratory with

specifically radiolabeled ClUDPs have proven particularly informative in elucidation of the sequence of events responsible for this enzyme inactivation (Stubbe & Kozarich, 1980; Stubbe et al., 1983b; Harris et al., 1984). Incubation of RDPR with [5′-^{3}H]ClUDP resulted in covalent labeling of the protein with 4.6 equiv of ^{3}H/equiv of B1. Similar studies with [3′-^{3}H]-ClUDP resulted in partitioning of the ^{3}H between radiolabeling of the protein (1.0 equiv of ^{3}H/equiv of B1) and release to solvent (4.7 equiv of ^{3}H/equiv of B1) (Harris et al., 1984). These observations led to the proposal shown in Scheme I. The hydrogen atom abstracted from the 3′-position of ClUDP by the tyrosyl radical of B2 is exchanged with the solvent in five of every six turnovers. In one out of every six turnovers, however, the hydrogen atom originally in the 3′-position of ClUDP must be returned to the 2′-position of intermediate **3**, resulting in formation of 2′-deoxy-3′-keto-UDP (**4**). This hypothesis is also consistent with the observation that the tyrosyl radical of RDPR is not affected during the enzyme inactivation by ClUDP (Thelander et al., 1976).[6] The observation of protection by $NaBH_4$ against inactivation of RDPR by ClUDP and the identification of 2′-deoxyuridine and 2′-deoxy-3′-epiuridine as the products of the trapping reaction confirm that 2′-deoxy-3′-keto-UDP is an intermediate in the RDPR-catalyzed destruction of ClUDP.

The ability to trap many equivalents of 2′-deoxy-3′-keto-UDP with $NaBH_4$ in the reaction between RDPR and [3′-^{3}H]ClUDP has allowed determination of the fate of the 3′-hydrogen of the substrate analogue. Chemical and enzymatic analysis (Scheme II, Table I) of the [^{3}H]deoxyuridine obtained from the trapping reaction unambiguously establishes that the ^{3}H is located in the β-hydrogen of the 2′-position of 2′-deoxy-3′-keto-UDP. Therefore, RDPR catalyzes a net 1,2 hydrogen shift during its reaction with ClUDP.

This hydrogen shift allows definition of the location of the proposed protein radical X, which performs the hydrogen atom abstraction. This residue must reside on the top face of the substrate with access to both the 2′- and 3′-positions. Furthermore, knowledge of the stereochemistry of the ^{3}H at the 2′-position also assigns the stereochemistry of elimination of uracil from 2′-deoxy-3′-keto-UDP. Tritium from [3′-^{3}H]-ClUDP is known to be present in 2-methylene-3(2*H*)-furanone (Harris et al., 1984). Therefore, the unlabeled α-hydrogen of the 2′-position of the ketonucleoside must be lost, indicating that release of uracil occurs through a trans elimination.[4]

It is important to compare the mechanism of reduction of substrates by RDPR with the mechanism of inactivation of RDPR by ClUDP. In the reduction of nucleotides to 2′-deoxynucleotides, the hydrogen abstracted from the 3′-position of the substrate is returned to that position in the product (Stubbe et al., 1983a). In the case of ClUDP, however, the 3′-hydrogen is returned to the 2′-position of the product, leading ultimately to destruction of the substrate analogue and inactivation of the enzyme. These differences have been rationalized in terms of the protonation state of the thiol groups at the active site (Harris et al., 1984). The hypothesis in Scheme I predicts that the active site thiols have no role in the reaction of RDPR with ClUDP, while they are postulated to protonate the leaving group and reduce the radical cation intermediate in reduction of the substrate (Stubbe et al., 1983a; Harris et al., 1984).

The role of the active site thiols of RDPR in its reaction with ClUDP was examined by incubation of [3′-^{3}H]ClUDP with (1) prereduced RDPR in the absence of a reductant or (2) RDPR that contained oxidized active site thiols. In both cases, time-dependent inactivation, release of ^{3}H to the solvent, and covalent radiolabeling of the enzyme were observed, and multiple equivalents of tritiated products were obtained. These results are consistent with the lack of involvement of the active site thiols of RDPR in its reaction with ClUDP, as proposed in Scheme I, and may provide an explanation for the unusual chemistry observed with this analogue.

Summary

Trapping experiments have demonstrated the intermediacy of 2′-deoxy-3′-keto-UDP in the reaction of RDPR with ClUDP. Enzymatic and chemical analyses of the products of this reaction indicate that both enzymes catalyze a net 1,2 hydrogen shift, with the hydrogen initially present at the 3′-position of the substrate analogue transferred to the β face of the 2′-position of the product 2′-deoxy-3′-ketonucleotide. Studies on the reaction of ClUDP with RDPR containing oxidized active site thiols are consistent with the lack of a role for the enzyme thiols in the ClUDP reaction. These results suggest that differences in thiol protonation state at the active site of RDPR lead to the remarkably different reactions observed with NDPs and ClUDP.

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