Inactivation of the Ribonucleoside Triphosphate Reductase from *Lactobacillus leichmannii* by 2′-Chloro-2′-deoxyuridine 5′-Triphosphate: A 3′–2′ Hydrogen Transfer during the Formation of 3′-Keto-2′-deoxyuridine 5′-Triphosphate†

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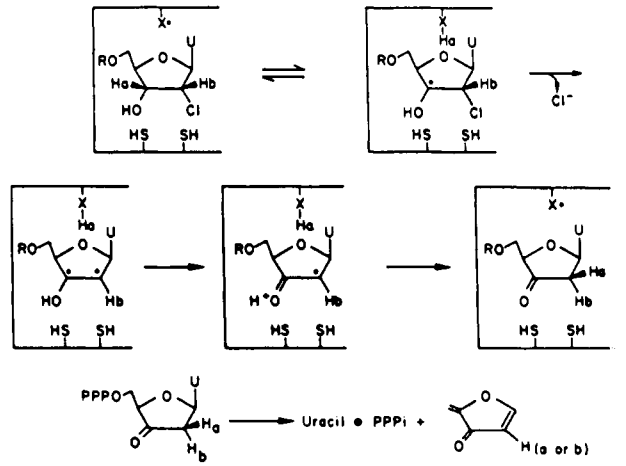
ABSTRACT: The ribonucleoside triphosphate reductase of *Lactobacillus leichmannii* converts the substrate analogue 2′-chloro-2′-deoxyuridine 5′-triphosphate (ClUTP) into a mixture of 2′-deoxyuridine triphosphate (dUTP) and the unstable product 3′-keto-2′-deoxyuridine triphosphate (3′-keto-dUTP). This ketone can be trapped by reduction with $NaBH_4$, producing a 4:1 mixture of *xylo*-dUTP and dUTP. When [3′-^{3}H]ClUTP is treated with enzyme in the presence of $NaBH_4$, the isomeric deoxyuridines isolated after alkaline phosphatase treatment retained 15% of the ^{3}H in ClUTP. Degradation of these isomeric nucleosides has established the location of the ^{3}H in 3′-keto-dUTP as predominantly 2′(*S*). The *xylo*-dU had 98.6% of its label at the 2′(*S*) position and 1.5% at 2′(*R*). The isolated dU had 89.6% of its label at 2′(*S*) and 1.4% at 2′(*R*), with the remaining 9% label inferred to be at the 3′-carbon, this resulting from the direct enzymic production of dUTP. These results are consistent with enzymic production of a 1:1000 mixture of dUTP and 3′-keto-dUTP, where the 3′-hydrogen of ClUTP is retained at 3′ during production of dUTP and is transferred to 2′(*S*) during production of 3′-keto-dUTP. The implications of these results and the unique role of the cofactor adenosylcobalamin (Ashley et al., 1986) are discussed in terms of reductase being a model for the B_{12}-dependent rearrangement reactions.

Ribonucleotide reductases play a central role in DNA biosynthesis, catalyzing the conversion of nucleotides to deoxynucleotides concomitant with oxidation of protein thiols to a disulfide. Recent efforts from our laboratory have focused on elucidation of the chemical mechanism of the ribonucleoside triphosphate reductase (RTPR)[1] from *Lactobacillus leichmannii* and the ribonucleoside diphosphate reductase (RDPR) from *Escherichia coli*. RTPR is a single polypeptide (M_r 76K) and utilizes coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl) as a cofactor. RDPR, in contrast, is composed to two subunits B_1 (α, α'; M_r 143K) and B_2 (β, β; M_r 87K) and utilizes a binuclear Fe(III) center associated with a stable protein–tyrosine radical as a cofactor. Despite the dissimilarities in cofactor requirements and protein structures, these enzymes have been recently shown to possess amazing similarities in their catalytic capabilities and in the primary sequence of the peptide containing their active site thiols (Lin et al., 1987).

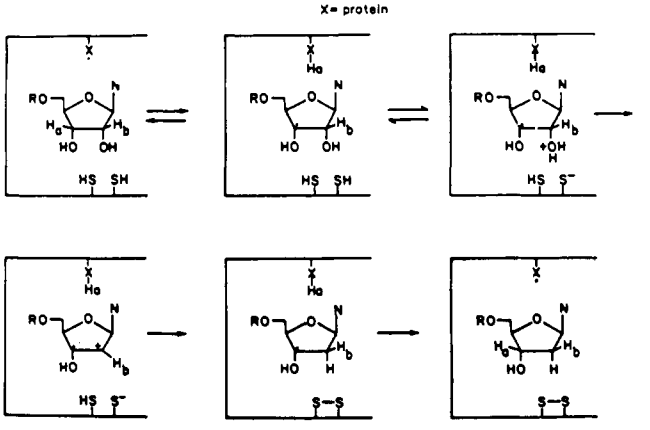
The most dramatic example of the similarities in their chemistry has been derived from mechanistic studies using isotopically labeled 2′-chloro-2′-deoxynucleotides. Both proteins catalyze the conversion of 2′-chloro-2′-deoxynucleotides into an unstable 3′-keto-2′-deoxynucleotide which decomposes to produce uracil, a phosphate moiety (PP_i or PPP_i), and 2-methylene-3(2*H*)-furanone. The furanone alkylates a protein residue(s), resulting in enzyme inactivation (Harris et al., 1984; Ator & Stubbe, 1985; Ashley et al., 1988).

The mechanism shown in Scheme I has been proposed to account for the observed products and is a variation of our proposed mechanism for the normal reduction process (Scheme II). In both cases (Schemes I and II) catalysis is initiated

Scheme I: Proposed Mechanism of Inactivation of RTPR by ClUTP



Scheme II: Proposed Mechanism for RTPR-Catalyzed Reduction of NTPs to dNTPs



by homolytic cleavage of the 3′ C–H bond by a radical protein residue, X•. Loss of OH^- or Cl^- to form a cation radical would

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require acid catalysis in the former case, perhaps by one of the redox-active thiols, and not in the latter case. The altered protonation state of these thiols, in the case of the 2′-chloronucleotides in comparison with the normal substrates, permits alternative reaction chemistry to predominate.

Support for the relatedness of the normal reduction reaction and the formation of 3′-keto-2′-deoxynucleotides is provided by experiments examining the product distribution from the interaction of RTPR with a variety of 2′-halo-NTPs. Although only dUTP is observed with UTP (2′-OH), a 2:1 mixture of dUTP and 3′-keto-2′-deoxynucleotide is observed with FUTP (2′-F). This ratio changes to about 1:200 with ClUTP (2′-Cl). Thus, there appears to be a correlation between the pK_a of the 2′-leaving group and the ratio of normal to abnormal products. A similar effect has been observed with RDPR, although no dUDP has been observed during destruction of ClUDP (Ator and Stubbe, unpublished results).

The mechanism in Scheme I proposes that the hydrogen atom originally removed from the 3′-carbon of 2′-chloronucleotide may, in contrast with the normal reduction process, be returned to the 2′-carbon, resulting in formation of 3′-keto-2′-deoxynucleotide. [3′-^{3}H]-2′-Chlorodeoxynucleotides were therefore prepared to test this thesis. During conversion of [3′-^{3}H]ClUTP and [3′-^{3}H]ClUDP to products, the majority of the ^{3}H label is exchanged with solvent. With RDPR, however, a significant amount (35%) of the label remains in the 3′-keto-dUDP, resulting in ultimate attachment of radiolabel to the inactivated RDPR. By use of $NaBH_4$ to trap 3′-keto-dUDP and by means of a combined enzymatic and chemical degradation procedure, previous experiments have demonstrated that this label is present only at the 2′(*S*) position of 3′-keto-dUDP (Ator & Stubbe, 1985). This is the anticipated result based on the hypothesis presented in Scheme I. Similar experiments were undertaken with RTPR, where only 15% of the label originally present in [3′-^{3}H]ClUTP remains in 3′-keto-dUTP; however, the observed partitioning of ClUTP between dUTP and 3′-keto-dUTP led to difficulties in assigning the position of the ^{3}H label. We now report the results of an extended degradation sequence that demonstrates the transfer of hydrogen from the 3′-carbon of ClUTP to the 2′(*S*) position of 3′-keto-dUTP.

Materials and Methods

General. RTPR was isolated from *L. leichmannii* ATCC 7830, assayed, and prereduced as described by Ashley et al. (1986) and had a specific activity of 1.3 μmol/(min·mg). Deoxyuridine hydroxylyase was isolated from *Rhodotorula glutinis* (Stubbe, 1985). AdoCbl, $NaBH_4$, diethyl azodicarboxylate, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDS-Cl_2), and *E. coli* alkaline phosphatase were from Sigma. [3′-^{3}H]ClUTP was prepared according to the procedure of Stubbe et al. (1983). SepPak C_{18} cartridges were from Waters Associates. NMR spectra were obtained at 270 MHz. For samples in D_2O, residual HOD was used as standard (δ 4.65), while Me_4Si was used as standard for samples in $CDCl_3$. Flash chromatography was performed according to Still et al. (1978). HPLC was performed on an Altex gradient system with either an Alltech ODS reversed-phase column (10 μm) or an Alltech SiO_2 column. Samples containing protein were deproteinized before injection onto the HPLC by heating for 1 min in a boiling water bath followed by removal of precipitated protein by centrifugation.

Trapping of 3′-Keto-dUTP from [3′-^{3}H]ClUTP and RTPR. A mixture of prereduced RTPR (7.6 mg, 100 nmol) and [3′-^{3}H]ClUTP (1000 nmol, 6.0×10^6 cpm/μmol) in 1.0 mL of 0.3 M Tris-HCl, pH 7.4, was warmed to 37 °C and treated with AdoCbl (1000 nmol) and 50 μL of a 2 M solution of $NaBH_4$ in 10 mM NaOH. The mixture was immediately centrifuged for 1 min to settle the protein froth. Additional 50-μL aliquots of the $NaBH_4$ solution were added at 2 and 5 min after addition of AdoCbl. After a total reaction time of 30 min, a 50-μL aliquot of the reaction mixture was removed and chromatographed on a 1.5 × 20 cm column of Sephadex G-50 using 50 mM potassium phosphate, pH 7.3. The protein fractions were pooled and analyzed: there was 10% remaining RTPR activity by the ATP assay, and the ^{3}H specific radioactivity was 9.8×10^4 cpm/μmol. The remainder of the reaction mixture (1000 μL) was frozen and bulb-to-bulb distilled. A 100-μL aliquot of the distillate was analyzed by scintillation counting, revealing 408 200 cpm; thus, 720 nmol of ^3H_2O was formed during the reaction. The distillation residue was dissolved in H_2O and passed through a SepPak C_{18} cartridge to remove nonpolar materials. The eluate was evaporated to dryness and redissolved in 0.9 mL of 0.2 M Tris-HCl, pH 8.7, 5 mM $MgCl_2$, and 5 mM $ZnCl_2$, containing 4 units of *E. coli* alkaline phosphatase. After 5 h at 37 °C, the mixture was deproteinized, neutralized by addition of 2 drops of concentrated HCl, and analyzed by HPLC (ODS reversed phase, H_2O). Products recovered (retention time, amount) were uracil (3.5 min, 195 nmol) and a 4:1 mixture of *xylo*-dU and dU (12 min, 342 nmol, 9.2×10^5 cpm/μmol); further elution with 20% MeOH/H_2O gave 2′-chloro-2′-deoxyuridine (18 nmol).

Analysis of Label Location in Deoxyuridine. The mixture of *xylo*-dU and dU from the above trapping experiment (342 nmol, 315 000 cpm) was treated by 0.002 unit of deoxyuridine hydroxylase in 0.5 mL of 40 mM Tris-HCl, pH 7.4, 2 mM 2-oxoglutarate, 1 mM ascorbate, 1 mM $Fe(NH_4)_2SO_4$, and 0.3 mg/mL catalase. After 20 min at ambient temperature, the mixture was frozen and bulb-to-bulb distilled. A 450-μL aliquot of the distillate was analyzed by scintillation counting, showing 704 cpm; thus, 0.8 nmol of ^3H_2O was released. The distillation residue was dissolved in 0.5 mL of H_2O, deproteinized, and analyzed by HPLC (ODS reversed phase, H_2O). Products recovered (retention time, amount) were uracil (4 min, trace), uridine (8 min, 41 nmol, 9.0×10^5 cpm/μmol), and *xylo*-dU (12 min, 228 nmol, 9.2×10^5 cpm/μmol).

The uridine sample (36 nmol) was mixed with unlabeled uridine (24 mg, 100 μmol) and dried by repeated evaporation from pyridine (freshly distilled from CaH_2). After the dried uridine was dissolved in 0.5 mL of pyridine, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (48 μL, 150 μmol) was added and the mixture was stirred for 12 h. After dilution with Et_2O, the organic phase was washed successively with H_2O, cold 1 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by flash chromatography on SiO_2 by using 1:1 ethyl acetate/hexanes, yielding 88 μmol of 3′,5′-*O*-TIPDS-uridine (182 cpm/μmol). The 3′,5′-*O*-TIPDS-uridine was oxidized according to the procedure of Hansske et al. (1984), using 215 μmol of the CrO_3/

[1] Abbreviations: RTPR, ribonucleoside triphosphate reductase from *Lactobacillus leichmannii*; RDPR, ribonucleoside diphosphate reductase; AdoCbl, 5′-deoxyadenosylcobalamin; ClUTP, 2′-chloro-2′-deoxyuridine 5′-triphosphate; ClUDP, 2′-chloro-2′-deoxyuridine 5′-diphosphate; 3′-keto-dUTP, 1-(2-deoxy-β-D-*glycero*-pentofuran-3-ulosyl)uracil 5′-triphosphate; 3′-keto-dUDP, 1-(2-deoxy-β-D-*glycero*-pentofuran-3-ulosyl)-uracil 5′-diphosphate; dUTP, 2′-deoxyuridine 5′-triphosphate; *xylo*-dUTP, 1-(2-deoxy-β-D-*threo*-pentofuranosyl)uracil 5′-triphosphate; TIPDS, 1,1,3,3-tetraisopropyl-1,3-disilanediyl; FUTP, 2′-deoxy-2′-fluorouridine 5′-triphosphate; DEADCAT, diethyl azodicarboxylate.

pyridine/acetic anhydride reagent in 0.5 mL of CH_2Cl_2. The crude ketone product was dissolved in 1.0 mL of tetrahydrofuran and cooled on ice. A solution of $NaBH_4$ (35 mg, 1 mmol) in 1.0 mL of 1:1 2-propanol/H_2O was added, and the mixture was stirred for 1 h at 0 °C. After addition of 1 mL of saturated aqueous NaCl, the mixture was extracted with Et_2O. The organic phase was dried with Na_2SO_4, filtered, and evaporated. Final purification was achieved by HPLC (SiO_2, 2% MeOH/CH_2Cl_2). The 3′,5′-*O*-TIPDS-*arabino*-uridine (retention time 15 min) was readily separated from 3′,5′-*O*-TIPDS-uridine (retention time 10 min). Scintillation counting of 2.8 μmol of the 3′,5′-*O*-TIPDS-*arabino*-uridine revealed a specific radioactivity of 19 cpm/μmol. The identities of the final materials were confirmed by NMR.

Analysis of Label Location in xylo-Deoxyuridine. The labeled *xylo*-dU from the deoxyuridine hydroxylase reaction (228 nmol) was mixed with unlabeled *xylo*-dU (86 μmol), giving a specific radioactivity of 1700 cpm/μmol. This was dried in vacuo over P_2O_5. The nucleoside was dissolved in 0.5 mL of hexamethylphosphoric triamide and treated with benzoic acid (32 mg), triphenylphosphine (68 mg), and diethyl azodicarboxylate (42 μL). After 5 h at 60 °C, a second identical portion of these reagents was added and the mixture was heated for an additional 2 h. After dilution with ethyl acetate, the mixture was washed successively with H_2O, 1 N HCl, and saturated aqueous $NaHCO_3$. After drying over $MgSO_4$, the organic phase was filtered and evaporated. The residue was dissolved in 5 mL of MeOH containing 100 mg of sodium methoxide and kept for 30 min. Dowex-50W (H^+) was added to neutralize the base, and the mixture was filtered and evaporated. The residue was partitioned between Et_2O and H_2O, and the aqueous phase was evaporated. The crude dU was purified by HPLC (ODS reversed phase, 2% MeOH/H_2O), yielding 21.2 μmol of dU (1900 cpm/μmol). NMR analysis indicated a 70:30 mixture of dU and *xylo*-dU.

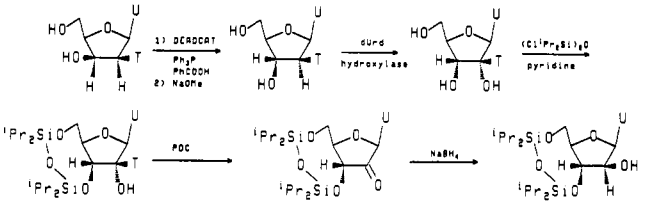
This material was treated with 0.04 unit of deoxyuridine hydroxylase in 2.0 mL of the reaction mixture described above, but containing 25 mM 2-oxoglutarate and 10 mM ascorbate. After 6 h, the mixture was analyzed as described above. Distillation indicated formation of 0.12 μmol of ^3H_2O, and the recovered uridine (9.0 μmol, 2000 cpm/μmol) had not lost any significant specific radioactivity.

The labeled uridine was mixed with carrier and degraded by the protocol described above. The 3′,5′-*O*-TIPDS-uridine (28.7 μmol, 244 cpm/μmol) was oxidized and then treated with $NaBH_4$. HPLC purification afforded 3′,5′-*O*-TIPDS-*arabino*-uridine having a specific radioactivity of <0.6 cpm/μmol. Again, NMR was used to verify the identities of the products.

Synthesis of xylo-2′-Deoxyuridine. 1-(5-Trityl-2-deoxy-β-D-*threo*-pentofuranosyl)uracil was prepared according to the procedure used by Hansske et al. (1984) for the synthesis of 1-(5-trityl-2-deoxy-β-D-*threo*-pentofuranosyl)thymine. Purification by flash chromatography on SiO_2 (5% MeOH/$CHCl_3$) separated 5′-trityl-2′-deoxyuridine from the desired product.

The tritylated nucleoside (100 μmol) was deblocked by refluxing in 1 mL of 80% aqueous acetic acid for 30 min. The solvent was evaporated and the residue partitioned between H_2O and Et_2O. The aqueous phase was washed three times with Et_2O and evaporated, yielding 87 μmol of *xylo*-2′-deoxyuridine: NMR (D_2O) δ 7.88 (d, 1 H, J = 8 Hz), 5.96 (d, 1 H, J = 8 Hz), 5.70 (d, 1 H, J = 8 Hz), 4.35 (m, 1 H), 3.95 (m, 1 H), 3.78 (m, 2 H), 2.59 (ddd, 1 H, J = 7, 8, 15 Hz), 2.00 (d, 1 H, J = 15 Hz).

Scheme III: Analysis of the Position of ^3H in $NaBH_4$-Trapped 2′-Deoxy-3′-keto-dUTP Obtained from the Reaction of [3′-^{3}H]ClUTP with RTPR



RESULTS

Trapping of 3′-Keto-dUTP from [3′-^{3}H]ClUTP. When [3′-^{3}H]ClUTP was treated with prereduced RTPR and AdoCbl in the presence of $NaBH_4$ and the resulting nucleotides were dephosphorylated with alkaline phosphatase, 3.4 equiv/RTPR of a 4:1 mixture of *xylo*-dU and *ribo*-dU, having 15% the specific radioactivity of the [3′-^{3}H]ClUTP, was isolated by chromatography on HPLC. As expected, the majority of the total label (72%) was volatilized as ^3H_2O. A significant amount of uracil (2 equiv/RTPR) was also observed. The isolated RTPR was 90% inactive and was found to have 2% the specific activity of [3′-^{3}H]ClUTP, indicating that some alkylation by 2-methylene-3(2*H*)-furanone had occurred even in the presence of $NaBH_4$.

Analysis of Label Position in dU Produced from [3′-^{3}H]-ClUTP. The initial steps in the analysis of the location of the ^3H in the dU produced during the reaction of RTPR with [3′-^{3}H]ClUTP in the presence of $NaBH_4$ (Scheme III) followed the sequence used for similar experiments on RDPR (Ator & Stubbe, 1985). The *xylo*-dU/*ribo*-dU mixture was treated with deoxyuridine hydroxylase. This enzyme oxidizes the 2′-position of *ribo*-dU to give uridine with retention of configuration at 2′ and without a noticeable kinetic isotope effect (Stubbe, 1985). The *xylo*-dU is unaffected by the enzyme. Thus, any ^3H present at the 2′(*R*) position of dU will be released as ^3H_2O upon conversion of *ribo*-dU to uridine, while the *xylo*-dU will be recovered unchanged. Oxidation of 342 nmol of the dU mixture gave 41 nmol of uridine, 0.8 nmol of ^3H_2O, 228 nmol of *xylo*-dU, and 12 nmol of uracil formed by contaminating uridine phosphorylase present in the deoxyuridine hydroxylase. As the uracil arises by destruction of uridine, the total amount of uridine formed was 53 nmol. Thus, only 1.5% of the radiolabel present in the *ribo*-dU was present at the 2′(*R*) position.

The uridine isolated from the deoxyuridine hydroxylase reaction was further degraded by chemical methods to establish the location of the remaining 98.6% of ^3H. After addition of unlabeled uridine as carrier, the 3′- and 5′-hydroxyls were blocked as the 1,1,3,3-tetraisopropyl-1,3-disiloxanediyl derivative. The 3′,5′-*O*-TIPDS-uridine produced was oxidized at 2′ by using the CrO_3/pyridine/acetic anhydride reagent (Hansske et al., 1984). As some stability problems were experienced with the 2′-ketone, the crude product was treated with $NaBH_4$. The 3′,5′-*O*-TIPDS-*arabino*-uridine produced was separated from 3′,5′-*O*-TIPDS-uridine by HPLC and found to have a specific radioactivity 10% that of the starting 3′,5′-*O*-TIPDS-uridine.

Analysis of Label Position in xylo-dU Produced from [3′-^{3}H]ClUTP. The location of the ^3H in the *xylo*-dU remaining from the deoxyuridine hydroxylase reaction was determined by an extension of the degradation sequence described above. Inversion of the 3′-hydroxyl of *xylo*-dU was effected by treatment with diethyl azodicarboxylate/Ph_3P/PhCOOH (Mitsunobu, 1981). Displacement of the initially formed triphenylphosphonium salts of the 3′- and 5′-hydroxyl

groups by benzoate yields 3′,5′-dibenzoyl-2′-deoxyuridine. Removal of the benzoate esters with sodium methoxide provided *ribo*-dU. Due to the steric hindrance to nucleophilic substitution at the 3′-position, some competing benzoylation of *xylo*-dU was observed which resulted in a 70:30 mixture of *ribo*-dU and uninverted *xylo*-dU.

Treatment of the inversion product with deoxyuridine hydroxylase resulted in formation of 9.0 μmol of uridine, with only a trace of uracil (0.12 μmol) being formed in this case. Again, about 1.4% of the label in the *ribo*-dU was volatilized, indicating that it was present at the 2′(*R*) position in *xylo*-dU.

The uridine produced was degraded as described above. In this case, the 3′,5′-*O*-TIPDS-*arabino*-uridine end product was found to contain less than 0.3% of the ^3H originally present in the 3′,5′-*O*-TIPDS-uridine. Thus, 98.6% of the ^3H in *xylo*-dU was present at the 2′(*S*) position.

Discussion

Inactivation of the *E. coli* RDPR by ClUDP was first noted by Thelander et al. (1976). The realization that the initial product of RDPR action on ClUDP was likely to be 3′-keto-dUDP led to formulation of a hypothesis for the mechanism of this reaction by Stubbe and Kozarich (1980) (Scheme I). Work in our laboratory has verified several predictions made by this hypothesis: (1) cleavage of the 3′ C–H bond, (2) production of 3′-keto-dUDP, (3) transfer of ^3H from the 3′-position of ClUDP to the 2′(*S*) position of 3′-keto-dUDP, (4) production of 2-methylene-3(2*H*)-furanone, and (5) inactivation of RDPR by covalent modification by 2-methylene-3-(2*H*)-furanone. Stubbe et al. (1983) subsequently noted that the coenzyme B_{12} dependent RTPR of *L. leichmannii* was inactivated by the triphosphate analogue ClUTP. In analogy with results from the RDPR described above, RTPR converts ClUTP into 3′-keto-dUTP, from which PPP_i and uracil are eliminated to give 2-methylene-3(2*H*)-furanone (Harris et al., 1984; Ashley et al., 1988).

While retention of radioactivity in 3′-keto-dUTP from [3′-^3H]ClUTP was observed, initial experiments parallel to those performed with RDPR (Ator & Stubbe, 1985) failed to unequivocally demonstrate the location of the radiolabel. When the *ribo*-dU produced from $NaBH_4$ trapping of the 3′-keto-dUTP was degraded according to Scheme III, a significant portion of the ^3H was retained in the final 3′,5′-*O*-TIPDS-*arabino*-uridine. The most likely location of the remaining tritium in this molecule is the 3′-position. This hypothesis is based on our previous observations that (1) RTPR mediates a partitioning of ClUTP between dUTP and 3′-keto-dUTP production (Harris et al., 1987) and (2) in the normal RTPR-catalyzed reduction of [3′-^2H]UTP the hydrogen abstracted from the 3′-position in the starting material is returned to the 3′-position in the product (Stubbe et al., 1981). The partitioning of [3′-^3H]ClUTP should therefore yield [2′(*S*)-^3H]-3′-keto-dUTP and [3′-^3H]dUTP. Subsequent $NaBH_4$ reduction of the 3′-keto-dUTP should give a mixture of *xylo*-dUTP and *ribo*-dUTP; thus, the total *ribo*-dUTP produced should be a mixture of [2′(*S*)-^3H]dUTP and [3′-^3H]dUTP, with the proportions depending upon the partition ratio and the relative amounts of label exchange with solvent in the two pathways. The amount of label retained at the end of the degradation of *ribo*-dU varied with the exact experimental conditions used in the trapping reaction. This is not unexpected, as we have previously found that the partition ratio varies substantially (Harris et al., 1987).

This proposal was tested by determining the ^3H positions in both the *ribo*-dU and *xylo*-dU obtained from the trapping reaction after treatment with alkaline phosphatase. Analysis of the *xylo*-dU should provide a clear view of the stereochemistry of the labeling in 3′-keto-dUTP, as this isomer only arises from the $NaBH_4$ reduction. Treatment of RTPR with [3′-^3H]ClUTP in the presence of $NaBH_4$ followed by dephosphorylation of the nucleotides with alkaline phosphatase gave a 4:1 mixture of *xylo*-dU and *ribo*-dU which had a specific radioactivity 15% that of the starting [3′-^3H]ClUTP. Both the isomers had approximately 1.4% of their label in the 2′(*R*) position as determined by the degradation method using deoxyuridine hydroxylase. In the *ribo*-dU, only 89.6% of the ^3H was at the 2′(*S*) position; the location of the remaining 9% was undetermined by the degradation. However, the *xylo*-dU had 98.6% of its ^3H at the expected 2′(*S*) position. As the origins of the hydrogen atoms of *ribo*-dU and *xylo*-dU differ only at the 3′-position, and the *xylo*-dU is derived solely from $NaBH_4$ while the *ribo*-dU is derived from $NaBH_4$ and from enzymic production of dUTP, the 9% label remaining in the *ribo*-dU isomer must be located at the 3′-position in accord with our hypothesis.

Two factors could be responsible for what appears to be a high amount of labeling at the 3′-position of *ribo*-dU. First, reduction of 3′-keto-dUTP with $NaBH_4$ gives a preponderance of *xylo*-dUTP. The 20% of 3′-keto-dUTP converted to *ribo*-dUTP by this route is thus disproportionately diluted with enzymically produced dUTP. Second, the normal reduction of ribonucleotides to deoxyribonucleotides proceeds with very little exchange of the 3′-hydrogen with solvent (Ashley et al., 1986). On the other hand, destruction of [3′-^3H]ClUTP results in exchange of 85% of the 3′-label with solvent. If the enzymic production of dUTP from ClUTP is mechanistically similar to the normal reduction process, the dUTP produced enzymically from [3′-^3H]ClUTP will have a much higher specific activity than that produced by $NaBH_4$ reduction of the 3′-keto-dUTP. From the current experiment, assuming that there are no differential kinetic isotope effects on the two processes, a partition ratio of 1000:1 in favor of 3′-keto-dUTP can be estimated. Although this is higher than previous determinations (Harris et al., 1987), it must be noted that earlier measurements were necessarily performed in the presence of dithiothreitol (DTT). It was found that the partition ratio between 3′-keto-dUTP and dUTP formation is sensitive to the DTT concentration, with 30 mM DTT giving 110:1 and 3 mM DTT giving 220:1. As no DTT was used in the present experiments, a higher partition ratio may be anticipated.

These studies have unequivocally demonstrated that RTPR catalyzes a 3′ to 2′ hydrogen shift in the conversion of ClUTP to 3′-keto-dUTP. While our previous studies have indicated that this reaction is not catalytic in cofactor, subsequent studies with a large number of 2′-halonucleotides (Ashley et al., 1987), which on the basis of product identification appear to behave in an analogous fashion to the ClUTP reaction, demonstrate a catalytic requirement of cofactor. Therefore, while an analogous 3′ to 2′ hydrogen shift experiment needs to be carried out on one of these halonucleotides, present evidence supports the interpretation that in general the reaction in which 3′-ClNTP is converted to 3′-keto-dNTP is catalytic in enzyme and cofactor.

These results are quite intriguing given the unique role postulated for AdoCbl in the RTPR-catalyzed reduction reaction and the "superficial" similarity of this ClUTP reaction to that of the AdoCbl-dependent enzymes catalyzing rearrangement reactions: diol dehydrase (R = CH_3, X = OH, eq 1) and ethanolamine ammonia lyase (R = H, X = NH_2, eq 1). In addition, the recent observation of Hartmanis and Stadtman (1987) that there is a non-B_{12}-dependent diol de-

$$RCHXC^{*}HHOH \rightarrow [RCH^{*}HCHOHX] \rightarrow RCH^{*}HCHO \quad (1)$$

hydrase which appears to contain a hydroxyurea-sensitive protein radical suggests that the enzymes catalyzing AdoCbl-dependent rearrangements and RTPR may share more common mechanistic features than previously thought.

Our hypothesis at present is that the function of AdoCbl is as a radical chain initiator. Homolytic cleavage of the C–Co bond produces cobalamin(II) and a 5′-deoxyadenosyl radical. The latter abstracts a hydrogen atom from an amino acid residue at the enzyme's active site to produce a protein radical X•. We postulate that it is X• which mediates the chemistry outlined in Scheme II. A major difference between the diol dehydrase catalyzed rearrangement and the RTPR-catalyzed reduction is that the former involves intramolecular and the latter intermolecular redox chemistry. However, in the case of RTPR-catalyzed reaction on ClUTP, the reaction is formally identical with the diol dehydrase reaction in that the conversion to 3′-keto-dUTP is an internal redox reaction.

Elegant studies on diol dehydrase by Essenberg et al. (1971) have clearly demonstrated that the 5′-deoxyadenosyl radical is chemically and kinetically competent to function as a hydrogen atom mediator between substrate and product. This mechanism, however, does not satisfactorily explain the extremely large selection effect against transfer of ^{3}H from [5′-^{3}H]AdoCbl to product ($k_H/k_T = 125$). In 1982 Cleland proposed a mechanism for diol dehydrase in which the major function of the 5′-deoxyadenosyl radical was to generate a new radical species which in the majority of turnovers of propanediol to propanal served as the hydrogen atom mediator (Cleland, 1982). On the basis of the findings reported in this paper and elsewhere (Ashely et al., 1987), we postulate that the protein radical is a common feature of all AdoCbl-dependent enzymes. Recent studies that map the active site of RTPR suggest that a thiyl radical may serve this function (Lin et al., 1987). Alternatively, a tyrosyl radical, in analogy with *E. coli* RDPR, must also be considered. Present efforts are directed toward identifying the putative X•.

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