Mechanism of Inactivation of *Escherichia coli* and *Lactobacillus leichmannii* Ribonucleotide Reductases by 2′-Chloro-2′-deoxynucleotides: Evidence for Generation of 2-Methylene-3(2*H*)-furanone†

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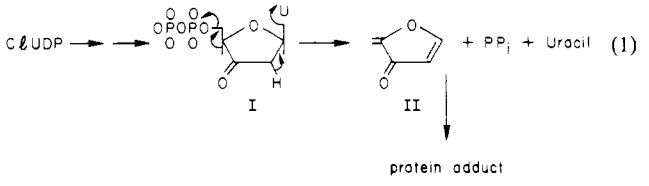
ABSTRACT: Incubation of 2′-chloro-2′-deoxy[3′-^{3}H]uridine 5′-diphosphate ([3′-^{3}H]ClUDP) with *Escherichia coli* ribonucleotide reductase (RDPR) and use of thioredoxin–thioredoxin reductase as reductants result in release of 4.7 equiv of ^3H_2O/equiv of B_1 protomer, concomitant with enzyme inactivation. Inactivation is accompanied by the production of 6 equiv of inorganic pyrophosphate [Stubbe, J. A., & Kozarich, J. W. (1980) *J. Am. Chem. Soc. 102*, 2505–2507] and by the release of uracil as previously shown [Thelander, L., Larsson, A., Hobbs, J., & Eckstein, F. (1976) *J. Biol. Chem. 251*, 1398–1405]. Reisolation of RDPR by Sephadex chromatography and analysis by scintillation counting indicate that 0.96 equiv of ^{3}H is bound per protomer of the B_1 subunit of the inactivated enzyme. Incubation of [5′-^{3}H]ClUDP with RDPR followed by similar analysis indicates that 4.6 mol of ^{3}H is bound per protomer of the B_1 subunit of the inactivated enzyme. No ^3H_2O is released, and 6 equiv of inorganic pyrophosphate is produced during the inactivation. RDPR is protected against inactivation when dithiothreitol (DTT) is used as a reductant in place of thioredoxin–thioredoxin reductase. Incubation of [5′-^{3}H]ClUDP with RDPR and DTT results in the isolation of $CHCl_3$-extractable material that exhibits infrared absorptions at 1710 and 1762 cm^{-1}. The infrared spectrum and the NMR spectrum of the $CHCl_3$-extracted material are very similar to model compounds prepared by the interaction of 2-methylene-3(2*H*)-furanone with ethanethiol. Incubation of ribonucleoside-triphosphate reductase (RTPR) from *Lactobacillus leichmannii* with [3′-^{3}H]ClUTP and 3 mM DTT also results in time-dependent ^3H_2O release concomitant with enzyme inactivation. Reisolation of the inactive protein by Sephadex chromatography followed by radiochemical analysis indicates that 0.4 equiv of ^{3}H is bound covalently per mol of inactivated enzyme. Similar studies with [5′-^{3}H]ClUTP indicate that 2.9 equiv of ^{3}H is bound covalently per mol of inactivated enzyme. No ^3H_2O is released. High concentrations of DTT protect the enzyme against inactivation. Extraction of the enzymatic reaction mixture with $CHCl_3$ and analysis of the isolated products result in an infrared spectrum and an NMR spectrum remarkably similar to those observed with the *E. coli* RDPR. Data presented are consistent with the proposal that both the *E. coli* and *L. leichmannii* enzymes are able to catalyze the breakdown of the appropriate 2′-chloro-2′-deoxynucleotide to a 3′-keto-2′-deoxynucleotide that can collapse to form the reactive sugar intermediate 2-methylene-3(2*H*)-furanone. The latter can then react either with nucleophilic amino acid residues in these enzymes resulting in their inactivation or with DTT resulting in the formation of adducts that are soluble in $CHCl_3$.

Ribonucleotide reductases catalyze the conversion of ribonucleotides to deoxynucleotides (Thelander & Reichard, 1979; Stubbe, 1983; Follmann, 1974; Lammers & Follmann, 1982; Reichard & Ehrenberg, 1983). The *Escherichia coli* enzyme, studied extensively by Reichard, Thelander, Sjöberg, and collaborators, has been shown to consist of two types of subunits designated B_1 and B_2. The B_1 subunit (α–α' M_r 160000) binds the NDP substrates and contains the redox active thiols and the binding sites for the allosteric effectors. The B_2 subunit (β–β M_r 78000) contains the unusual cofactor that is composed of two Fe^{3+} and one tyrosine radical, an integral part of the B_2 polypeptide chain. The *Lactobacillus leichmannii* enzyme, studied extensively by Blakley, Hogenkamp, Follmann, and collaborators, consists of a single polypeptide chain (M_r 76000), which uses the cofactor coenzyme B_{12} (AdoCbl).[1] This enzyme uses NTPs as substrates. Our working hypothesis in studying the mechanism of these reduction reactions is that, even though the structure of the cofactors is unique, the mechanisms of reduction are probably quite similar.

Evidence will be presented that these enzymes upon interaction with 2′-chloro-2′-deoxynucleotides (ClUDP, ClUTP) catalyze formation of the same reactive species that results in enzyme inactivation. The impetus for these studies arose from the exciting observations of Thelander et al. with the *E. coli* reductase that the interaction of ClUDP or ClCDP with enzyme resulted in release of Cl^- and uracil (cytosine) concomitant with inactivation of B_1 (Thelander et al., 1976). We extended these studies by showing that incubation with [β-^{32}P]ClUDP resulted in the production of PP_i (Stubbe & Kozarich, 1980), and we proposed that the mechanism of inactivation might proceed as described in eq 1.



In eq 1, ClUDP is converted to 3′-keto-2′-deoxyuridine diphosphate (I) by a mechanism that may involve formation of a cation radical intermediate. I, whose 2′- and 4′-hydrogens

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[1] Abbreviations: AdoCbl, adenosylcobalamin; ClUDP, 2′-chloro-2′-deoxyuridine 5′-diphosphate; ClUTP, 2′-chloro-2′-deoxyuridine 5′-triphosphate; ClCDP, 2′-chloro-2′-deoxycytidine 5′-diphosphate; DTT, dithiothreitol; RDPR, ribonucleoside-diphosphate reductase; RTPR, ribonucleoside-triphosphate reductase; SDS, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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are now reasonably acidic, should rapidly decompose to form the reactive Michael acceptor 2-methylene-3(2H)-furanone (II). Compound II would then alkylate the B_1 subunit, resulting in enzyme inactivation.

Recently, we have examined the interaction of [3′-^{3}H]-ClUTP and [β-^{32}P]ClUTP with *L. leichmannii* ribonucleoside-triphosphate reductase (RTPR) and made similar observations to those made with the *E. coli* enzyme (Stubbe et al., 1983). RTPR catalyzed the formation of tripolyphosphate, uracil, and ^3H_2O, concomitant with inactivation of enzyme. Furthermore, AdoCbl was also destroyed during this reaction, producing 5′-deoxyadenosine and cobalamin II. We proposed a similar sequence of events to those described in eq 1 to account for these results.

Our working hypothesis indicated in eq 1 makes a number of predictions that are experimentally testable: (1) the 3′-carbon hydrogen bond of ClUDP should be cleaved during inactivation; (2) the enzyme(s), upon incubation with [5′-^{3}H]ClUDP ([5′-^{3}H]ClUTP), should be radiolabeled; (3) (a) ketone(s) should be generated during reductase-catalyzed destruction of ClUDP (ClUTP). This paper reports the results of experiments that examined these predictions. Our results support the above hypothesis (eq 1) and further substantiate the remarkable similarities between these two enzyme that utilize totally different cofactors.

Materials and Methods

Ribonucleoside-diphosphate reductase from *E. coli* strain KK546 was isolated on a dATP–Sepharose affinity column synthesized by extensive modifications of the procedure of Berglund & Eckstein (1974), as described by Knorre et al. (1976). B_1 had a specific activity of 0.340 μmol min^{-1} mg^{-1}. B_2, after further purification by hydroxylapatite, had a specific activity of 3.0 μmol min^{-1} mg^{-1}. B_1 and B_2 are greater than 95% pure on the basis of SDS gel electrophoresis (Laemmli, 1970). However, a specific activity for B_1 of 0.59 μmol min^{-1} mg^{-1} has been reported by Eriksson et al. (1977). Whether our B_1 contains inactive B_1 is presently unknown. Thioredoxin–thioredoxin reductase were isolated by modification of procedures previously described (Pigiet & Cooley, 1977; Moore et al., 1964). Ribonucleoside-triphosphate reductase from *L. leichmannii* was isolated by the procedure of Chen et al. (1974) and had a specific activity with ATP (DTT reductant) of 1.5 μmol min^{-1} mg^{-1}. The enzyme was judged to be homogeneous by gel electrophoresis (Laemmli, 1970). Protein concentrations were determined by the procedure of Lowry et al. (1951).

NaB^3H_4 and [^{14}C]CDP were purchased from New England Nuclear. [3′-^{3}H]ClUDP and [3′-^{3}H]ClUTP were prepared by the procedure of Stubbe et al. (1983). NADPH, ATP, DTT, and isopropylideneuridine were purchased from Sigma. All other materials were purchased in the highest purity available.

Fourier-transform infrared spectra were recorded on a Nicolet MX-1 FT IR spectrometer. UV spectra were recorded on a Cary 210 spectrophotometer, and all scintillation counting was done on a Packard 300C scintillation counter with Aquasol as scintillation fluid. NMR spectra were recorded on a Brucker 270-MHz or Nicolet 200-MHz spectrometer.

Synthesis of 2′-Chloro-2′-deoxy[5′-^{3}H]uridine 5′-Monophosphate. The 5′-aldehyde of isopropylideneuridine was synthesized from isopropylideneuridine by using the Me_2SO (distilled from CaH)–Ac_2O oxidation procedure of Pfitzner & Moffatt (1965). All attempts to isolate a crystalline aldehydic product failed, and hence, the aldehyde was rereduced without extensive purification. The aldehyde (600 mg, 2 mmol) was dissolved in 20 mL of CH_3OH, and the reaction mixture was neutralized with saturated $NaHCO_3$ and cooled in an ice bath. NaB^3H_4 (100 mCi) was added to this mixture, and the reaction was removed from the ice bath and allowed to proceed for 20 min at room temperature. At this time, the reaction mixture was cooled again, 142 mg (4 mmol) of $NaBH_4$ was added, and then the mixture stirred for an additional 0.5 h at room temperature. The mixture was acidified with Dowex 50 H^+ to pH 4–5 and the Dowex removed by filtration. The filtrate was evaporated to dryness and triturated several times with CH_3OH. The product was chromatographed in 6% ethanol–$CHCl_3$ on 120 g of silica gel. The crystalline product was recovered in 68% yield. The NMR spectrum was identical with that of the starting isopropylideneuridine.

The isopropylidene group was removed by placing 0.45 mmol of isopropylideneuridine in 5 mL of 10% HOAc and heating the reaction mixture for 1 h at 100 °C. The solution was cooled and the solvent removed in vacuo to give 0.41 mmol of [5′-^{3}H]uridine (90% yield), sp act. 1.2×10^7 cpm/μmol.

The [5′-^{3}H]uridine was converted to anhydrouridine by the procedure of Hampton & Nichol (1966) and was then converted to chloro[5′-^{3}H]uridine by heating it in dioxane saturated with HCl for 18 h (Codington et al., 1964). The NMR spectrum and melting point of the isolated 2′-chloro-2′-deoxyuridine were identical with those previously reported in the literature. The nucleoside was converted to the monophosphate with $POCl_3$ in triethyl phosphate (Yoshikawa et al., 1967) and to the di- and triphosphates with carbonyldiimidazole and P_i or PP_i, respectively (Kozarich et al., 1973).

The 2′-chloro-2′-deoxynucleotides were further purified by ion-paired reverse-phase HPLC on a C_{18} column with 50 mM potassium phosphate (pH 4.8), 5 mM tetrabutylammonium bromide, and 30% CH_3OH as eluent. The retention times were as follows: ClUDP 5.0 min, impurity 16 min; ClUTP 7.5 min, impurity 19 min. The impurity has been identified as the 3′-methyl carbonate derivative of ClUDP (ClUTP).

^3H_2O Release from [3′-^{3}H]ClUDP by RDPR. Each 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, 10 μg of thioredoxin reductase, 2.8 mg (18 nmol) of B_1, 0.5 mg (6 nmol) of B_2, and 0.48 mM [3′-^{3}H]ClUDP (sp act. 6.7×10^5 cpm/μmol) in a final volume of 0.75 mL. One reaction also contained 3 mM DTT. A control reaction was identical except RDPR was inactivated by 20 mM hydroxyurea prior to addition of ClUDP.

The reactions were initiated by addition of ClUDP and were incubated at 25 °C. At various times, 0.1-mL portions were withdrawn and added to 0.4 mL of ice-cold H_2O in a 5-mL round-bottom flask. The sample was immediately frozen in dry ice–acetone and the volatile material removed by bulb-to-bulb distillation. A total of 450 μL of the lyophilized H_2O was added to 8 mL of scintillation fluid, and the amount of radioactivity present was determined by scintillation counting.

Rate of Inactivation of RDPR by ClUDP. The reaction mixture was identical with that described above for ^3H_2O release from ClUDP. At various time intervals, 10-μL portions of the reaction mixture were diluted into an enzyme assay mixture. The assay mixture contained in a final volume of 100 μL 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), thioredoxin, and thioredoxin reductase. Each assay was incubated for 20 min at 25 °C and was then placed in a boiling H_2O 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 calf intestine alkaline phosphatase and was incubated at 37 °C for 1 h. The resulting deoxycytidine was determined on Dowex 1 borate columns by the procedure of Steeper & Stewart (1970).

Formation of A_{320} on RDPR following Inactivation with ClUDP. RDPR was incubated with ClUDP for 10 min as described above, in a volume of 0.5 mL. This time was sufficient for total enzyme inactivation. A control incubation was identical except for the omission of ClUDP. Both samples were chromatographed on 1 × 40 cm columns of Sephadex G-50 in Tris-HCl (pH 7.6), 50 mM, and $MgSO_4$, 15 mM, at 4 °C. The A_{280} of the inactivated and control RDPR samples was balanced by appropriate addition of the same buffer, and the samples were placed in the sample and reference beams, respectively, at 25 °C. The difference spectrum of the protein from 400 to 230 nm was recorded. When the difference spectrum was recorded 10 min after the initial scan, a new absorbance appeared at 320 nm; therefore, the spectrum was recorded periodically until no further increase at 320 nm was observed.

Quantitation of ^3H_2O, ^3H on Protein, and [^{32}P]PP_i Release from [3′-^3H]ClUDP or [5′-^3H]ClUDP and [β-^{32}P]ClUDP. A typical reaction mixture contained 50 mM HEPES (pH 7.6), 16 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 0.5 mM NADPH, 8 mM NaF, 50 μg of thioredoxin, 6 μg of thioredoxin reductase, 0.86 mg (11 nmol) of B_1, 0.16 mg (2 nmol) of B_2, and 0.5 mM [3′-^{3}H]- and [β-^{32}P]ClUDP ([^{3}H]ClUDP sp act. 6.2 × 10^5 cpm/μmol; [^{32}P]ClUDP sp act. 8.4 × 10^6 cpm/μmol) in a volume of 250 μL. A second reaction was identical except [3′-^{3}H]ClUDP was replaced with [5′-^{3}H]-ClUDP (sp act. 5.7 × 10^5 cpm/μmol). Prior to addition of ClUDP, a 5-μL aliquot was withdrawn and diluted into 95 μL of a [^{14}C]CDP assay mixture. Enzymatic activity was measured on Dowex 1 borate columns as described previously. The inactivation was initiated by addition of ClUDP and was allowed to proceed for 10 min at room temperature. A second 5-μL aliquot was taken to determine remaining activity. Inorganic pyrophosphate (100 μL of 0.1 M) was added, and the reaction was divided into three portions: (1) A 100-μL portion of the reaction was added to 10 ml of 75 mM NH_4OAc (pH 5.0) and 0.25 g of acid-washed charcoal in a plastic centrifuge tube. After 10 min at room temperature, the charcoal was removed by centrifugation. The supernatant was filtered through glass wool, adjusted to pH 7.5 with NaOH, and loaded onto a 0.5 × 11 cm DEAE-Sephadex A-25 column. Inorganic pyrophosphate was eluted with a 60 × 60 mL linear gradient of 0–0.6 M triethylammonium bicarbonate. Radioactivity and phosphate were quantitated, and the specific activity of the pyrophosphate was determined. (2) A 50-μL portion of the reaction was added to 450 μL of cold H_2O in a round-bottom flask. The solution was frozen and bulb-to-bulb distilled. Quantitation of ^3H_2O was determined by scintillation counting of the distillate. (3) The remainder of the reaction mixture was chromatographed at 4 °C on a 0.7 × 18 cm column of Sephadex G-50 equilibrated in 50 mM Tris-HCl (pH 7.6). Fractions of 0.6 mL were collected, and protein and radioactivity were determined in each fraction. The protein-containing fractions were pooled, and the amount of radioactivity bound was determined by scintillation counting and quantitation of the protein by UV spectroscopy.

A second set of reactions was run in D_2O. All reagents were dissolved in D_2O, and the protein was exchanged into D_2O by centrifugation through a 1-mL column of Sephadex G-25 equilibrated with 50 mM HEPES (pD 8.0), 15 mM $MgSO_4$, and 1 mM EDTA in D_2O.

3′-^3H/β-^{32}P Isotope Effect on Reaction of ClUDP with RDPR. 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, 3 μg of thioredoxin reductase, 0.34 mg (4.4 nmol) of B_1, 0.06 mg (0.8 nmol) of B_2, and 0.36 mM [3′-^{3}H]- and [β-^{32}P]ClUDP ([^{3}H]ClUDP sp act. 5.9 × 10^5 cpm/μmol; [^{32}P]ClUDP sp act. 1.3 × 10^5 cpm/μmol) in a volume of 100 μL. A control reaction contained no enzymes. The reaction was run for 1 min at room temperature and was terminated by incubation in a boiling H_2O bath. Remaining ClUDP was reisolated by ion-pairing reverse-phase HPLC on an Alltech C_{18} column in 23% methanol, 77% 50 mM potassium phosphate (pH 4.8), and 5 mM tetrabutylammonium bromide (flow rate 1.5 mL/min; ClUDP retention time 10.2 min). Fractions of 1.0 min were collected, and the radioactivity in each fraction was determined. Fractions containing ClUDP were pooled, and the $^3H/^{32}P$ ratio was determined by scintillation counting. Extent of reaction was determined from the number of ^{32}P counts in each sample.

Infrared Spectroscopy of the Adduct Isolated upon Incubation of RDPR with ClUDP and DTT. The inactivation mixture contained 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM EDTA, 1.6 mM ATP, 3 mM DTT, 1.5 mM [5′-^{3}H]-ClUDP, and 4 mg of RDPR (90% B_1, 10% B_2) in a volume of 3.4 mL. The reaction was allowed to proceed for 30 min at room temperature and was then transferred to a 15-mL Corex tube. Cold chloroform (3 mL) was added, and the sample was vortexed and then centrifuged for 5 min at 27000*g*. Protein that had precipitated at the interface was removed with a copper wire. The protein was washed with 0.75 mL of H_2O twice. The wash was added to the aqueous layer of the extraction, and the sample was extracted 7 more times with 3 mL of chloroform. The chloroform layers were pooled, concentrated to dryness, and dissolved in 0.3 mL of chloroform. The IR spectrum was taken by using NaCl plates. A control reaction containing no RDPR was also examined by Fourier-transform IR spectroscopy. An NMR spectrum in $CDCl_3$ was also recorded.

Time-Dependent Inactivation of RTPR by ClUTP. The incubation mixture contained in a total volume of 140 μL 3 mM (or 10 mM) DTT, 1 mM EDTA, 1 M NaAc, 0.05 M potassium phosphate (pH 7.8), 1.3 mM AdoCbl, and 0.14 mg of RTPR. Incubation was at 37 °C, and DTT was added at 15 min to bring the final concentration to 4.5 mM. Aliquots, 20 μL, were removed at various times and dilutd into a 500-μL assay mixture containing 30 mM DTT, 1 mM EDTA, 10 mM ATP, 1 M NaAc, 0.05 M potassium phosphate (pH 7.8), and 12 μM AdoCbl. The assay mixtures were incubated for 10 min at 37 °C, and formation of dATP was measured by the method of Blakley (1973) with diphenylamine.

Release of ^3H_2O from [3′-^3H]ClUTP. The reaction mixture contained in a total volume of 0.75 mL 0.5 M potassium phosphate (pH 7.8), 3 mM DTT, 1 M NaAc, 1 mM EDTA, 10 nmol (0.78 mg) of RTPR, 1.3 mM AdoCbl, and 2 mM ClUTP (sp act. 3.8 × 10^5 cpm/μmol). The reaction was incubated at 37 °C, and at 10 min, a second aliquot of DTT was added, bringing the final concentration to 4.5 mM.

Aliquots, 100 μL, were removed at various times, diluted into 400 μL of H_2O, shell frozen, and bulb to bulb distilled. The amount of volatilized ^3H was determined by scintillation counting.

Formation of A_{320} on RTPR following Inactivation with ClUTP. RTPR was incubated with ClUTP for 45 min as described above, in a volume of 0.5 mL. At the end of this incubation, the enzyme was inactive. A control contained

everything except for AdoCbl. Both samples were chromatographed through a Sephadex G-50 column equilibrated in 10 mM potassium phosphate (pH 7.3) and 1 mM EDTA at 4 °C. The protein from the control and the experiment were diluted so that the absorbance at 280 nm was equal. The difference spectrum of the protein from 400 to 230 nm was recorded. A new absorption at 320 nm was observed; therefore, spectra were recorded every 10 min until no further spectral changes occurred.

Isolation of Adduct from the Incubation of RTPR with ClUTP. The reaction mixture contained in a final volume of 10 ml 3 mM DTT, 1 mM EDTA, 1 M NaAc, 2 mM ClUTP, 1.3 mM AdoCbl, 0.05 M potassium phosphate (pH 7.8), and 12 μM RTPR. The reaction was started by the addition of AdoCbl and incubated at 37 °C. At 15 and 45 min additional DTT was added to bring the final concentration to 5.25 mM. After 1.25 h, the reaction was stopped by extraction with 8 × 10 mL $CHCl_3$. The protein precipitated at the interface of the $CHCl_3$ and H_2O layers and was carefully removed. The combined $CHCl_3$ layers were evaporated to dryness and the residue was redissolved in 5% CH_3OH–$CHCl_3$ for characterization by NMR and IR spectroscopy.

Determination of ^3H_2O, ^3H on Protein, and ^3H in $CHCl_3$-Extracted Adducts from [3′-^{3}H]- or [5′-^{3}H]ClUTP. A typical reaction mixture contained 0.05 M potassium phosphate (pH 7.8), 3 mM DTT, 1 mM EDTA, 1 M NaAc, 3 mM [5′-^{3}H]ClUTP (sp. act. 4.5 × 10^6 cpm/μmol), 9.2 nmol of RTPR, and 1.6 mM AdoCbl in a volume of 700 μL. A second reaction was identical except [5′-^{3}H]ClUTP was replaced by [3′-^{3}H]ClUTP (sp act. 3.6 × 10^5 cpm/μmol). The reaction was begun by the addition of DTT. The samples were incubated at 37 °C for 60 min. DTT was added at 15 and 45 min, bringing the final concentration to 5.25 mM.

Portions of 20 μL were removed before the reactions were begun and after the 60-min incubation. They were diluted into 480 μL of cold H_2O, frozen, and bulb-to-bulb distilled. Quantitation of ^3H_2O was determined by scintillation counting of the distillate.

After the 60-min incubation, 100 μL of each reaction mixture was diluted to 1 mL with cold H_2O and extracted with 8 × 1-mL portions of $CHCl_3$. The pooled $CHCl_3$ extracts were evaporated to dryness and redissolved in 1 mL of $CHCl_3$. The amount of ^3H extracted into $CHCl_3$ was determined by scintillation counting.

After the 60-min incubation, 500 μL of each reaction mixture was loaded onto a Sephadex G-50 column (0.7 × 27 cm), which was equilibrated in 10 mM potassium phosphate (pH 7.3). Fractions of 0.5 mL were collected, and 0.4 mL of each fraction was added to 4.0 mL of scintillation fluid to determine the amount of ^3H in each fraction. The reisolated protein was assayed for residual activity by the method of Blakley (1973).

A second set of reactions was run in D_2O. The reaction mixtures contained 0.05 M potassium phosphate, 3 mM DTT, 1 mM EDTA, 1 M NaAc, 8.6 nmol of RTPR, 1.5 mM AdoCbl, and 2 mM [5′-^{3}H]- or [3′-^{3}H]ClUTP ([5′-^{3}H]ClUTP sp act. 2.6 × 10^6 cpm/μmol; [3′-^{3}H]ClUTP sp act. 3.6 × 10^5 cpm/μmol) in a final volume of 650 μL. The potassium phosphate, DTT, EDTA, NaAc, and AdoCbl were lyophilized in a foil-covered vessel and redissolved in D_2O. The [5′-^{3}H]ClUTP and [3′-^{3}H]ClUTP were lyophilized into separate Ependorff tubes and were redissolved by the addition of the reaction components dissolved in D_2O. The reactions were begun by the addition of RTPR. The samples were incubated at 37 °C for 60 min. DTT was added at 15 and 45 min, bringing the final concentration to 5.25 mM. The workup of the experiment was carried out as described for the reaction in H_2O.

Preparation of 3′-Keto-5′-tosylthymidine. Tosylthymidine (0.5 g, 12.5 mmol) was oxidized by the pyridinium dichromate method of Bergstrom et al.[2] The isolated brown solid (0.65 g) was dissolved in 7 mL of CH_2Cl_2 to which 56 mL of diethyl ether was added. The resulting brown precipitate was removed by filtration, and the filtration was concentrated in vacuo to give 0.2 g of a beige solid, mp 115 °C dec. This beige solid could not be further purified due to its instability toward a variety of chromatographic procedures. It was contaminated with small amounts of chromium salts: IR ($CHCl_3$) 1773 cm^{-1}; NMR ([$^2H_6$]acetone) δ 7.46, 7.56, 7.79, and 7.83 (4 H, dd), 7.50 (1 H, s), 6.3 (1 H, dd, $J_{1',2'}$ = 7.8 Hz, $J_{1',2''}$ = 4 Hz), 4.43 (1 H, 4′-H, m), 4.36 (2 H, 5′- and 5″-H, m), 3.16 (1 H, 2′-H, m, $J_{2',2''}$ = 19 Hz), 2.88 (1 H, 2″-H, m), 2.47 (3 H, s), 1.89 (3 H, s).

Incubation of RTPR with 2-Methylene-3(2H)-furanone. 3′-Keto-5′-tosylthymidine (3 mg, 10 μmol) was dissolved in 150 μL of acetone, and 1.5 μL of triethylamine (distilled from acetic anhydride, followed by BaO) was added. After 15 min at room temperature, the solid that precipitated was removed by centrifugation. This solution was diluted with 450 μL of acetone, and 4.5 μL (.075 μmol) of II was then added to 3.9 nmol (0.3 mg) of RTPR and 0.027 μmol of AdoCbl in 0.2 M potassium phosphate (pH 7.3). Acetone (4.5 μL) was added to a similar sample of RTPR and AdoCbl as a control. The enzyme was immediately passed through a Sephadex column (0.7 × 18 cm) equilibrated in 10 mM potassium phosphate (pH 7.3). The appropriate fractions were pooled, examined spectrophotometrically, and assayed for activity.

Preparation of 2-Methylene-3(2H)-furanone (II). Generation of II was catalyzed by the addition of 5 μL of triethylamine to 15 mg (0.038 mmol) of 3′-keto-5′-tosylthymidine in 0.5 mL of [$^2H_6$]acetone: NMR ([$^2H_6$]acetone) δ 8.22 (1 H, d, J = 2.42 Hz), 5.67 (1 H, d, J = 2.4 Hz), 5.25 (1 H, d, J = 2.0 Hz), 5.06 (1 H, d, J = 2.0 Hz).

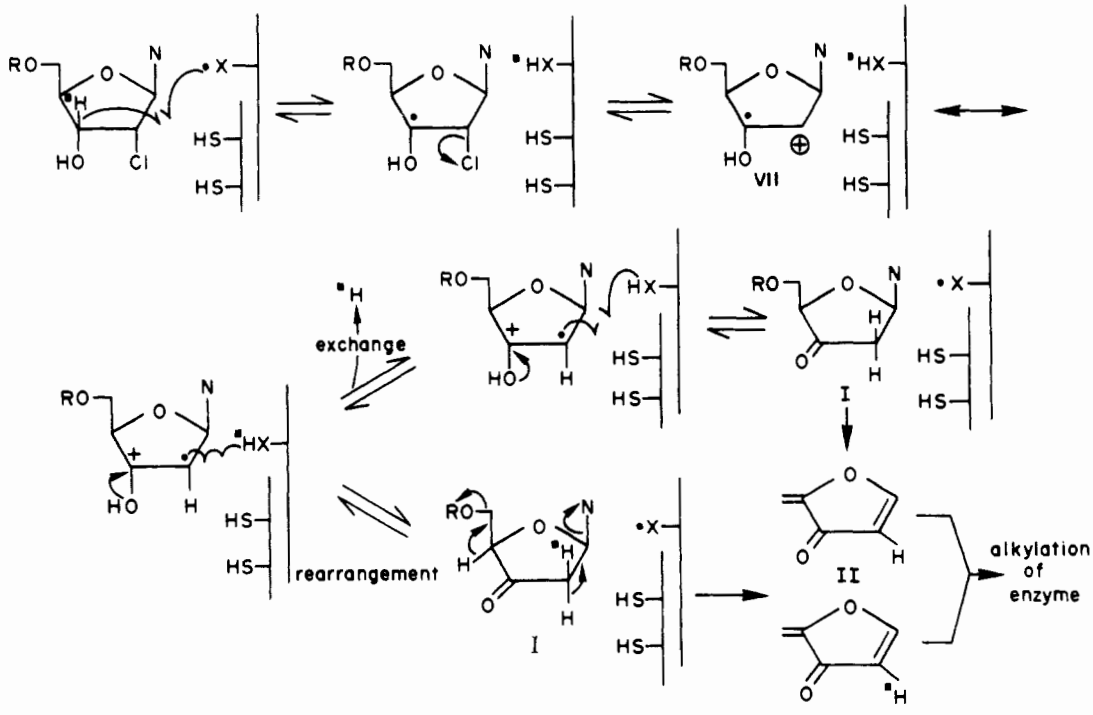
Preparation of 2-[(Ethylthio)methyl]-3(2H)-furanone (V) and 5-(Ethylthio)-2-[(ethylthio)methyl]-4,5-dihydro-3-(2H)-furanone (VI). 3-Keto-5′-tosylthymidine (100 mg, 2.5 mmol) was dissolved in 2.0 mL of chloroform, and 10 μL of purified triethylamine was added. After 10 min at room temperature, 40 μL (3 mmol) of ethanethiol and 20 μL of triethylamine were added. The $CHCl_3$ solution was then extracted with 3 × 2.0 mL of H_2O. The products in the $CHCl_3$ layer were purified by silica gel column chromatography in $CHCl_3$. Two compounds were isolated. Compound V showed the following: IR ($CDCl_3$) 1706 cm^{-1}; NMR ($CDCl_3$) δ 8.26 (1 H, d, $J_{1,2}$ = 3 Hz), 5.70 (1 H, d, $J_{1,2}$ = 3 Hz), 4.50 (1 H, dd, $J_{4,5'}$ = 4 Hz, $J_{4,5''}$ = 7 Hz), 3.00 and 2.70 (2 H, m, $J_{5,5'}$ = 14.8 Hz, $J_{4,5'}$ = 4 Hz, $J_{4,5''}$ = 7 Hz), 2.50 (2 H, t, J = 7.4 Hz), 1.2 (3 H, t, J = 7.4 Hz). Compound VI showed the following: IR ($CDCl_3$) 1763 cm^{-1}; NMR ($CDCl_3$) δ 5.83 (1 H, d, 8.1 Hz), 4.37 (1 H, t, 4 Hz), 2.37–3.04 (m), 1.2–1.45 (m).

Results

[3′-^{3}H]ClUDP and RDPR: Time-Dependent Inactivation and ^3H_2O Release. Incubation of a 20-fold excess of [3′-^{3}H]ClUDP with RDPR, thioredoxin, and thioredoxin reductase resulted in rapid time-dependent inactivation of the en-

[2] Don Bergstrom, University of North Dakota, personal communication.

Scheme I: Proposed Mechanism of Inactivation of Reductase by ClNDP



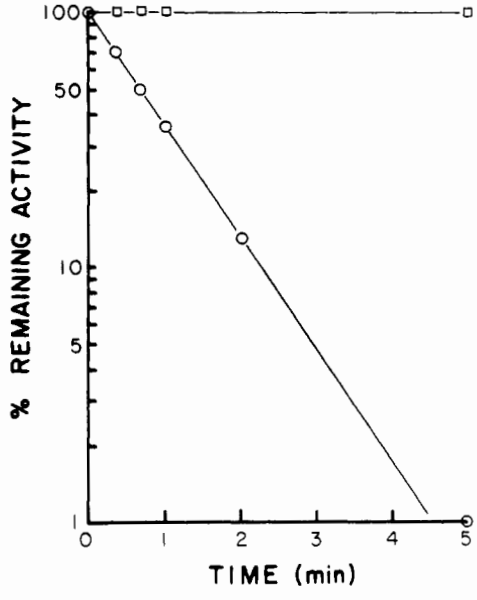


FIGURE 1: Inactivation of RDPR by ClUDP. The experimental details are described under Materials and Methods: (○) thioredoxin–thioredoxin reductase–NADPH used as reductant; (□) 10 mM DTT used as reductant.

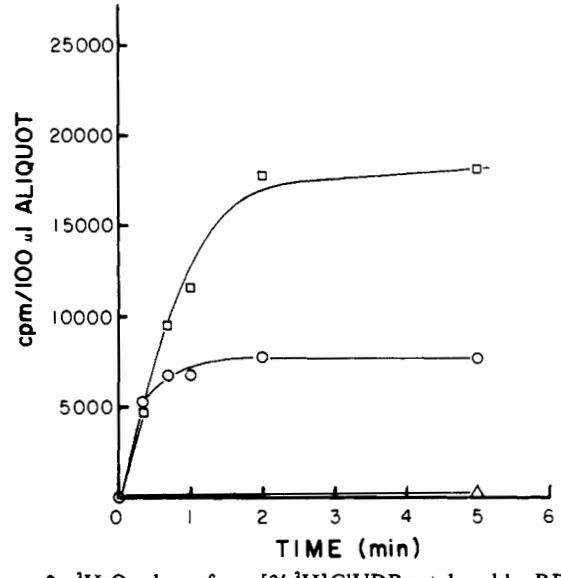


FIGURE 2: ^3H_2O release from [3′-^{3}H]ClUDP catalyzed by RDPR. Conditions are as in Figure 1: (○) thioredoxin–thioredoxin reductase–NADPH used as reductant; (□) 10 mM DTT used as reductant; (△) hydroxyurea-inactivated control.

zyme with $t_{1/2} \sim 45$ s (Figure 1). Furthermore, if DTT replaced thioredoxin–thioredoxin reductase as reductant, the enzyme remained greater than 90% active, and all of the ClUDP was consumed. DTT thus protected the enzyme against inactivation, presumably by trapping the reactive intermediate (II) generated at the active site or in solution. Inactivation and adduct formation were accompanied by ^3H_2O release (Figure 2). When thioredoxin–thioredoxin reductase were used as reductant, assuming no isotope effect on 3′ C–H bond cleavage, 5 mol of ^{3}H was released per mol of B_1 inactivated.[3] When DTT was the reductant, ClUDP breakdown was concomitant with ^3H_2O release, and 76% of the ^{3}H from [3′-^{3}H]ClUDP had been converted to ^3H_2O. The remainder of the ^{3}H was presumably distributed between the adduct that alkylates DTT and the protein. These results are consistent with our working hypothesis (eq 1) that inactivation of enzyme is accompanied by an enzyme-catalyzed oxidation of the 3′ C–H bond of ClUDP.

Incubation of [3′-^{3}H]-, [5′-^{3}H]-, or [β-^{32}P]ClUDP with RDPR: Measurement of Release of ^3H_2O, ^{3}H Bound to RDPR, and Production of PP_i. A detailed working hypothesis, Scheme I, indicates that the number of turnovers per inactivation of RDPR can be determined by the amount of PP_i produced, that ^{3}H from [5′-^{3}H]ClUDP will be bound to RDPR or trapped by a component of the reaction mixture, and that ^{3}H from [3′-^{3}H]ClUDP will be released as ^3H_2O, bound to the protein or trapped by a component of the reaction mixture.

[3] By use of [3′-^{3}H]ClUDP and [β-^{32}P]ClUDP in a double-label experiment, an isotope effect of 1.17 was calculated.

Table I: Incubation of [3′-^{3}H]ClUDP, [5′-^{3}H]ClUDP, and [β-^{32}P]ClUDP with RDPR: Fate of ^{3}H and ^{32}P[a]

medium	ClUDP	equiv $^3H_2O/B_1$	equiv ^{3}H bound/B_1	equiv $[^{32}P]PP_i/B_1$
H_2O	3′-^{3}H	4.5	1.0[b]	6.2
	5′-^{3}H	0.23[c]	4.6	5.2
^2H_2O	3′-^{3}H	4.7	3.1[d]	9.4
	5′-^{3}H	0.4[c]	5.9	8.7

[a] In three parallel experiments, ClUDP (3′- or 5′-^{3}H or β-^{32}P) (125 nmol) was incubated for 10 min with 11 nmol of B_1, 2 nmol of B_2, thioredoxin, thioredoxin reductase, and NADPH. The reaction mixture was then analyzed for release of ^3H_2O, ^{3}H bound to the protein, and production of PP_i. The experimental details are described under Materials and Methods. [b] The average of four determinations: 1.0, 1.05, 0.96, and 1.09. [c] Hydroxyurea-inactivated RDPR incubated with [5′-^{3}H]ClUDP showed no ^3H_2O release. At present, we cannot explain this observation. [d] The average of three determinations: 3.2, 3.0, and 3.1.

In three parallel experiments, labeled ClUDP (3′- or 5′-^{3}H or β-^{32}P) (125 nmol) was incubated for 10 min with 11 nmol of B_1 and 2 nmol of B_2, with the thioredoxin–thioredoxin reductase system as reductant. At the end of this incubation, the enzyme was inactive. The [3′-^{3}H]ClUDP reaction mixture was analyzed for release of ^3H_2O and ^{3}H bound to RDPR. Similar analyses were undertaken with the [5′-^{3}H]ClUDP reaction mixture. The [β-^{32}P]ClUDP reaction mixture was analyzed for production of PP_i. The results of these experiments run in H_2O and in D_2O are indicated in Table I. The amount of inorganic pyrophosphate produced is taken as a measure of the number of RDPR-catalyzed turnovers per inactivation: six in H_2O and nine in D_2O. Moreover, results with [3′-^{3}H]ClUDP indicate that 1.0 equiv of ^{3}H is covalently bound to RDPR and 4.5 equiv of ^{3}H is found as volatilized material (^3H_2O). The total number of ^{3}H equivalents (5.5) is within experimental error the same as ^{32}P equivalents (6.0). The parallel experiment with [5′-^{3}H]ClUDP indicates that 4.6 equiv is bound covalently to RDPR and almost no ^{3}H is volatilized. The results from [3′-^{3}H]ClUDPs suggest that a partitioning is occurring between the exchange of ^{3}H to the medium and retritiation of an intermediate generated during RDPR-catalyzed breakdown of ClUDP (Scheme I).

In order to examine this partitioning hypothesis, the enzyme inactivation was run in D_2O in an attempt to perturb the enzyme conformation and consequently the partitioning. The results from the D_2O experiment (Table I) support this partitioning hypothesis. The experiments with [3′-^{3}H]ClUDP in D_2O indicate that 3 times more ^{3}H is bound to the protein than in H_2O and that proportionately less ^{3}H, on the basis of total ClUDP turnover, is found in the solvent. A similar experiment with [5′-^{3}H]ClUDP indicates that more ^{3}H is bound to the protein in D_2O than in H_2O, consistent with the increased number of turnovers per inactivation. The implications of these finding in terms of a postulated mechanism are indicated in Scheme I and will be discussed subsequently.

UV Change Subsequent to RDPR Inactivation. Thelander et al. (1976) reported that inactivation of RDPR with ClUDP resulted in a slow change in the protein absorption spectrum at 320 nm. To investigate this observation in more detail, [β-^{32}P]ClUDP was incubated with RDPR, thioredoxin, and thioredoxin reductase for 10 min followed by Sephadex G-50 chromatography at 0 °C. A control experiment was run under identical conditions, minus ClUDP. Upon isolation of the protein from the columns, a difference spectrum was recorded at 25 °C. This spectrum showed an absorption with a λ_{max} at 254 nm (ϵ = 5200 M^{-1} cm^{-1}) and a second absorption at 320 nm. This difference spectrum, monitored as a function

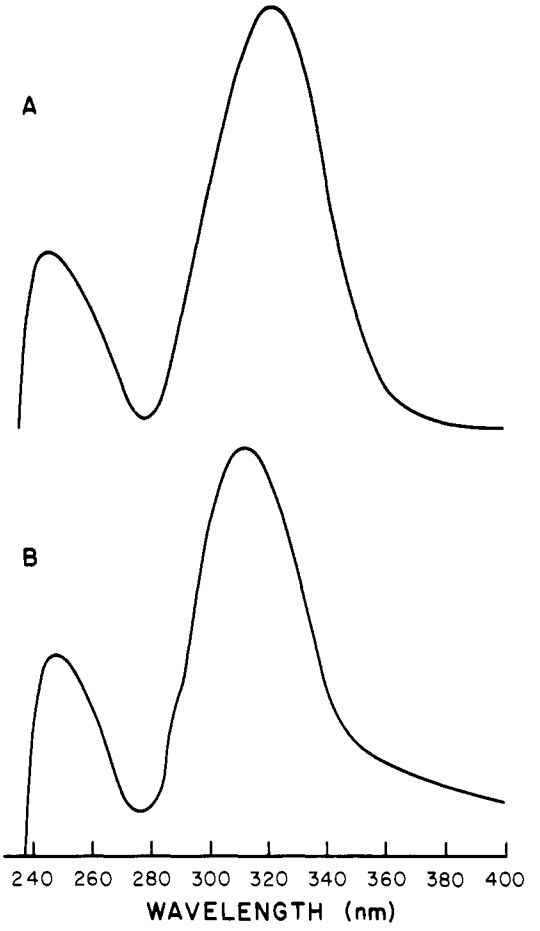
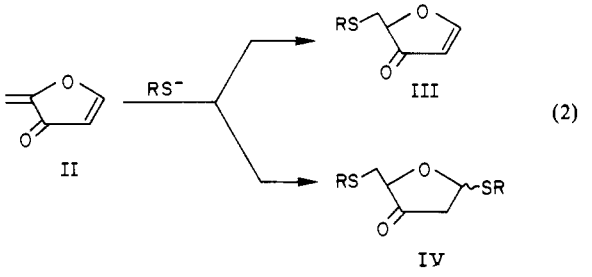


FIGURE 3: (A) Difference spectrum of ClUDP-inactivated RDPR vs. native enzyme. The spectrum was recorded after Sephadex isolation when no further change at 320 nm occurred. (B) Difference spectrum of ClUTP-inactivated RTPR vs. native enzyme. The spectrum was recorded after Sephadex isolation when no further change at 314 nm occurred.

of time, indicated a decrease in A_{254} concomitant with an increase in A_{320} (data not shown). The reaction was monitored until no further change in A_{320} occurred. At this time, the difference spectrum was again recorded and is shown in Figure 3A: A_{320} (ϵ = 10 900 M^{-1} cm^{-1}).[4] The $t_{1/2}$ for this change is approximately 30 min at 25 °C, and at this time, the A_{280}/A_{320} = 2.2. As indicated above, the [β-^{32}P]ClUDP is used to inactivate RDPR, no radioactivity coelutes with the protein. Furthermore, if uracil were present on the protein (5 nmol) at the first time point, an additional A_{260} of 0.05 ought to have been observed. The rearrangement, monitored by the change in optical spectra, must be due to the sugar moiety alone. The stoichiometry of labeling during this change remains unchanged.

Fourier-Transform Infrared Spectra of Isolated Adduct Trapped by DTT. Our working hypothesis also predicts that RDPR converts ClUDP to a number of ketone species (I and II, eq 1). To investigate this prediction, [5′-^{3}H]ClUDP was incubated with RDPR and with 3 mM DTT as reductant. We reasoned that if a reactive species such as II were generated and trapped by DTT at the active site of the enzyme to produce III or IV, these products might in fact be extractable with $CHCl_3$ (eq 2). Since DTT protects the enzyme against inactivation, RDPR could catalyze multiple turnovers and

[4] In order to emphasize the remarkable similarities between RDPR and RTPR, Figures 3, 4, and 7 contain spectra derived from both enzyme systems, even though the RTPR results will be presented subsequently.



(2)

produce enough material to be observable by FT-IR and NMR spectroscopy.

Incubation of [5′-^{3}H]ClUDP and RDPR for 40 min followed by $CHCl_3$ extraction produced the FT-IR indicated in Figure 4A. At least two ketone species with absorptions at 1762 and 1710 cm^{-1} were observed. Control experiments in the absence of enzyme or with enzyme inactivated by hydroxyurea indicated no absorption in this region. The material extracted with $CHCl_3$ represented 50% of the products produced upon ClUDP breakdown.

[3′-^{3}H]ClUTP with RTPR: Time-Dependent Inactivation and ^3H_2O Release. Incubation of [3′-^{3}H]ClUTP with RTPR and 3 mM DTT as reductant resulted in time-dependent inactivation with a $t_{1/2}$ = 9 min (Figure 5). If the concentration of DTT was increased to 10 mM, the enzyme was protected against inactivation. Inactivation of the enzyme and formation of DTT-trapped adducts were accompanied by time-dependent ^3H_2O release (Stubbe et al., 1983). If one uses 3 mM DTT as reductant and assumes no isotope effect on C–H bond cleavage, approximately 150 turnovers occurred per mol of enzyme inactivated.[5] These results are analogous to those described with RDPR and are consistent with enzyme-catalyzed oxidation of the 3′ C–H bond being required for inactivation.

Change in UV Spectrum upon Interaction with ClUTP. Inactivation of *E. coli* reductase is accompanied by a slow increase in absorbance of the protein spectrum at 320 nm. RTPR was examined to see if a similar spectral change accompanied inactivation. The reaction of ClUTP with RTPR was allowed to proceed for 45 min. After Sephadex chromatography at 4 °C, a difference spectrum was recorded (Figure 3B). An increase in absorbance at 314 nm was observed to be concomitant with a decrease in absorbance at 249 nm (data not shown). The ratio of A_{280}/A_{320} is 3.0 at the end of the reaction. The difference spectrum is remarkably similar to that observed for the *E. coli* reductase (compare spectra A and B of Figure 3).

Fourier-Transform Infrared Spectrum. $CHCl_3$ extraction of the reaction mixture in which [5′-^{3}H]ClUTP, RTPR, and 3 mM DTT have been incubated for 45 min followed by FT-IR of the extractable material gave the results indicated in Figure 4B. Approximately 25–30% of the RTPR-catalyzed breakdown products are extractable into $CHCl_3$. Two absorptions are observed: 1762 and 1707 cm^{-1}. Control experiments run under identical conditions in the absence of either enzyme, cofactor, or reductant showed no absorption in this region and no radioactive material extracted into the $CHCl_3$ layer. This infrared spectrum is remarkably similar to that recorded for the *E. coli* reductase under analogous conditions (compare parts A and B of Figure 4).

Model Reactions. Our working hypothesis predicts that ClUDP (ClUTP) is converted by reductase to II, which alkylates the enzyme or reacts with DTT to produce III and/or IV (eq 2). We have recently succeeded in synthesizing 2-[(ethylthio)methyl]-3(2*H*)-furanone (V) and 5-(ethylthio)-2-[(ethylthio)methyl]-4,5-dihydro-3(2*H*)-furanone (VI). The Fourier-transform IR spectrum of V shows absorption at 1706 cm^{-1}. Furthermore, V can be converted to VI by addition of excess thiol and triethylamine in $CHCl_3$ containing 10% CH_3OH, resulting in a new absorption at 1764 cm^{-1}.

On the basis of these results, we hypothesized that the species extracted by $CHCl_3$ in both reductase reactions might be similar to V and VI with the $-SCH_2CH_3$ moiety replaced by DTT. If this prediction is correct, then addition of tri-

[5] Harris and Stubbe have shown that an isotope effect of <1.2 is observed on this reaction (unpublished results).

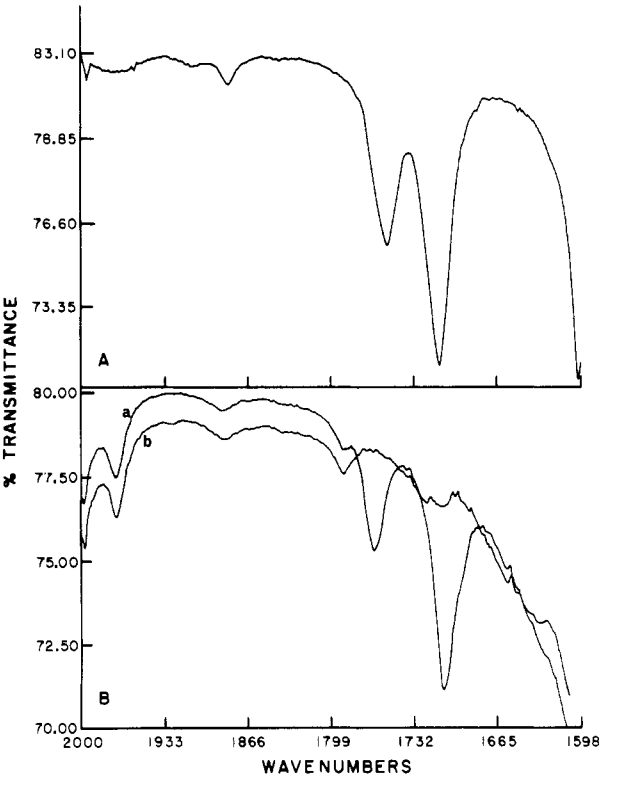


FIGURE 4: (A) Infrared spectrum of $CHCl_3$-extractable adducts generated on interaction of ClUDP with RDPR. DTT was used as reductant. (B) Infrared spectrum of $CHCl_3$-extractable adducts generated on interaction of ClUTP with RTPR: (a) DTT (3 mM) used as reductant; (b) control in the absence of AdoCbl. Similar results were obtained with a control run either without enzyme or without reductant.

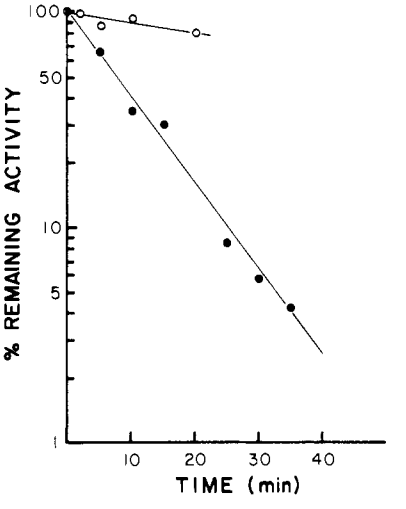


FIGURE 5: Interaction of [3′-^{3}H]ClUTP with RTPR. The experimental details are described under Materials and Methods: (●) in the presence of 3 mM DTT as reductant; (○) in the presence of 10 mM DTT as reductant.

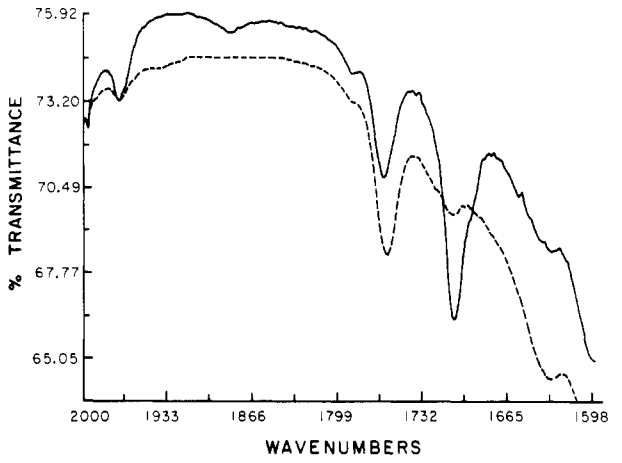


FIGURE 6: Infrared spectrum of $CHCl_3$-extractable adducts generated on interaction of ClUTP with RTPR: (—) spectrum of initial extraction mixture; (--) spectrum after addition of ethanethiol and triethylamine to initial extraction mixture.

ethylamine and thiol to the $CHCl_3$-extracted material should convert the absorption at 1707 cm^{-1} to a new absorption at 1762 cm^{-1}. Results of this experiment with RTPR reductase indicate this to be the case (Figure 6).

The NMR spectrum of the $CHCl_3$-extracted RTPR reaction can also be compared with those of model compounds V and VI (Figure 7D,B,A). For clarity, only the region between 4 and 9 ppm is indicated. Compound V shows two vinyl protons at 8.26 and 5.70 ppm and the 4′-H at 4.50 ppm (Figure 7B). Compound VI (stereochemistry unknown) shows a 1′-hydrogen multiplet at 5.83 ppm and a second multiplet of the 4′-hydrogen at 4.37 ppm (Figure 7A). The adducts isolated from the enzymatic reaction mixture show vinyl protons at 8.26 and 5.69 ppm, remarkably similar to the vinyl protons of V (Figure 7D). Furthermore, as in the case of V, these protons are coupled to one another (data not shown). The enzymatic adduct mixture also has an absorption at 4.50 ppm in the same region as the 4′-H of V. Finally, the RTPR reductase reaction mixture also shows resonances at 5.78 and 4.44 ppm similar to those observed for the 1′- and 4′-hydrogens of VI. While the splitting patterns in the 1′ and 4′ regions are much more complex than those of the model compound (Figure 7A), in the case of the enzyme reaction product a mixture of stereoisomers exists, compared with a single stereoisomer in the case of VI. Furthermore, DTT has several asymmetric centers that could further complicate the observed splitting. An NMR spectrum of the $CHCl_3$-extracted *E. coli* RDPR reaction can also be compared with model compounds V and VI and is indicated in Figure 7C. The spectrum is remarkably similar to that observed with RTPR (Figure 7D). These results in conjunction with the IR data are suggestive of the intermediacy of II, in the reductase-catalyzed reactions. All attempts to isolate and further purify the adducts extracted from the enzymatic reactions have thus far failed. This failure probably results from the free thiol of the adduct undergoing further reaction.

Preparation of 2-Methylene-3(2H)-furanone and Inactivation of RTPR. Incubation of 3′-keto-5′-tosylthymidine in acetone and a catalytic amount of triethylamine resulted in the production of II whose structure was established by NMR spectroscopy. Incubation of RTPR with a 20-fold excess of II, followed by rapid Sephadex chromatography, resulted in inactivated reductase with the spectrum indicated in Figure 8. When this was allowed to stand in the cold, a new absorbance appeared at 314 nm. The difference spectrum (Figure 8, inset) of the resulting product is remarkably similar

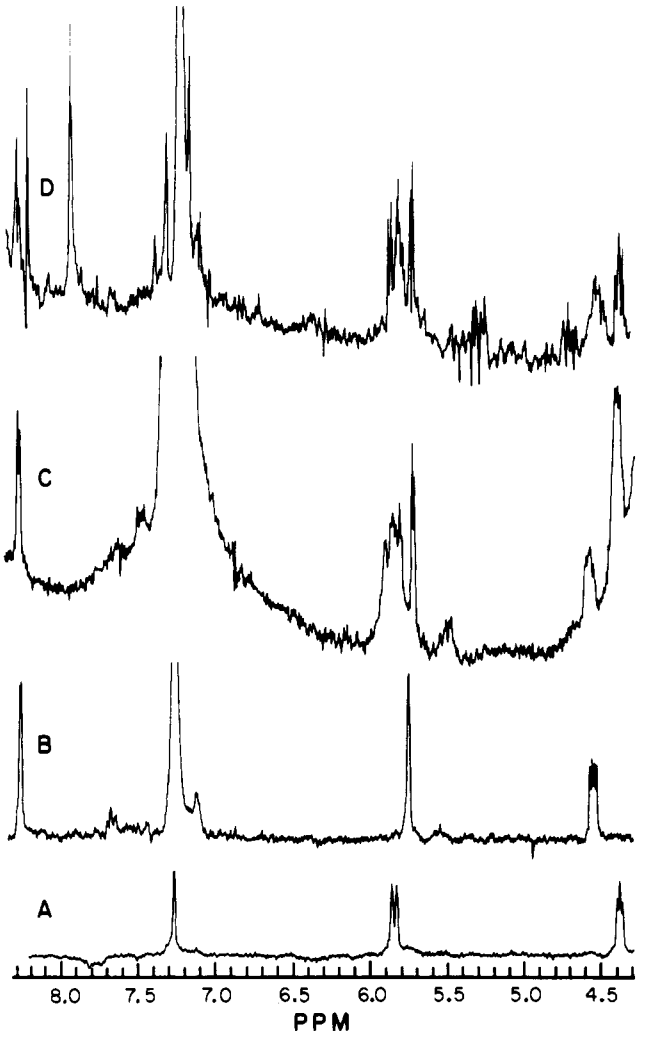


FIGURE 7: NMR spectrum of $CHCl_3$-extractable adducts generated on interaction of 2-chloro-2′-deoxynucleotides with reductases: (A) model compound 2-[(ethylthio)methyl]-3(2*H*)-furanone (V); (B) model compound 5-(ethylthio)-2-[(ethylthio)methyl]-4,5-dihydro-3-(2*H*)-furanone (VI); (C) $CHCl_3$ extracts of enzymatic reaction mixture containing RDPR and ClUDP; (D) $CHCl_3$ extracts of enzymatic reaction mixture containing ClUTP and RTPR.

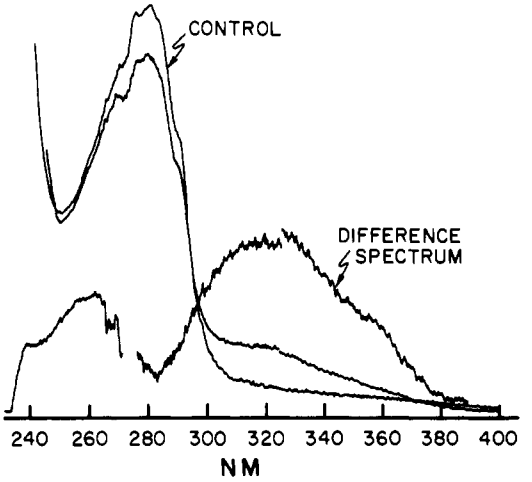


FIGURE 8: Spectrum of RTPR upon interaction with 2-methylene-3(2*H*)-furanone.

to that produced upon ClUTP enzyme inactivation (Figure 3B).

Incubation of [3′-^{3}H]- or [5′-^{3}H]ClUTP with RTPR: Measurement of Release of ^3H_2O, ^{3}H Bound to RTPR, and ^{3}H Found in $CHCl_3$-Extractable Products. Our working

Table II: Incubation of [3′-^{3}H]ClUTP or [5′-^{3}H]ClUTP with RTPR: Fate of the ^{3}H Label[a]

medium	ClUTP	^{3}H released (μmol)	equiv of ^{3}H/equiv of protein	^{3}H adduct extracted with $CHCl_3$ (μmol)
H_2O	3′-^{3}H	1.3	0.41	0.09
	5′-^{3}H		2.91	0.75
^2H_2O	3′-^{3}H	0.89	0.44	0.12
	5′-^{3}H		3.20	0.67

[a] In two parallel experiments, ClUTP (3′-^{3}H or 5′-^{3}H), 1.95 μmol, was incubated for 60 min with 8.6 nmol of RTPR and 3 mM DTT. The reaction mixtures were then analyzed for ^3H_2O release, ^{3}H bound to the protein, and ^{3}H that was extractable into $CHCl_3$. The experimental details are described under Materials and Methods.

hypothesis detailed in Scheme I indicates that ^{3}H from [5′-^{3}H]ClUTP could have two fates: labeling of RTPR and labeling of products produced by trapping of 2-methylene-3-(2*H*)-furanone (II). The fate of ^{3}H from [3′-^{3}H]ClUTP is more complicated since ^{3}H could also be released to the solvent. In two parallel experiments, labeled ClUTP (3′-^{3}H or 5′-^{3}H) was incubated for 60 min with RTPR and 3 mM DTT as reductant. Both reaction mixtures were then analyzed for ^3H_2O release, ^{3}H bound to the protein, and ^{3}H that was extractable into $CHCl_3$ and indicative of DTT-trapped 2-methylene-3(2*H*)-furanone (II). The results of these experiments run in H_2O and in D_2O are indicated in Table II. Results with [3′-^{3}H]ClUTP indicate that 1.3 μmol of ^{3}H is volatilized, that 0.09 μmol of ^{3}H is found in $CHCl_3$-extractable adducts, and that 0.4 equiv of ^{3}H is bound covalently per equiv of RTPR. Results with [5′-^{3}H]ClUTP indicate that no ^{3}H is volatilized, that 0.75 μmol of ^{3}H is found in $CHCl_3$-extractable adducts, and that 2.9 equiv of ^{3}H is bound per equiv of RTPR. Similar results are observed with D_2O as the solvent. The ratio of ^{3}H on the protein derived from [3′-^{3}H]ClUTP compared to [5′-^{3}H]ClUTP is 0.14 and of ^{3}H in $CHCl_3$-extractable adducts from [3′-^{3}H]ClUTP compared to [5′-^{3}H]ClUTP is the same, ~0.12. These results suggest protein and DTT are alkylated by ^{3}H-labeled 2-methylene-3(2*H*)-furanone (II) and that release of ^3H_2O to solvent from [3′-^{3}H]ClUTP occurred prior to generation of 3′-keto-2-deoxyuridine triphosphate (I), Scheme I.

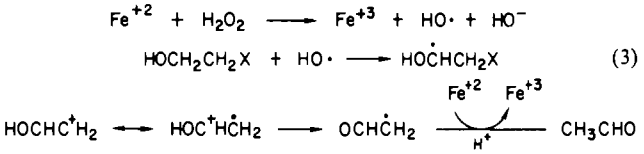
Discussion

Ribonucleotide reductases are uniquely responsible for the reduction of nucleotides to deoxynucleotides and hence play an essential role in DNA synthesis. It seems unusual, therefore, that this reduction requires an elaborate set of cofactors that have not been evolutionarily preserved. The AdoCbl and tyrosine phenoxy Fe^{3+} reductases have been most extensively studied and are the focal point of this paper.

Our working hypothesis for the investigation of these reductases is that the mechanism by which their substrates are reduced might be very similar even though the cofactor structures are different. Furthermore, both classes of enzymes are irreversibly inactivated by 2′-chloro-2′-deoxynucleotides, which act as mechanism-based inhibitors. Similarities of the products produced and distribution of radiolabels in these products produced by reductase-catalyzed breakdown of [3′-^{3}H]- or [5′-^{3}H]ClUDP (ClUTP) substantiate the amazing similarities in catalytic capabilities of the two classes of reductases.

Mechanism of Inactivation of Reductase by ClUDP. One possible mechanism for the conversion of ClNDP to 3′-keto-deoxynucleoside 5′-diphosphate (I) and subsequently to a 2-methylene-3(2*H*)-furanone (II) is proposed in Scheme I. A radical on the protein (X·) abstracts a hydrogen atom from the 3′-position of ClUDP. This abstraction is followed by rapid loss of chloride ion, which produces the radical cation intermediate VII. This intermediate (VII) now reabstracts a H· from the protein residue XH to generate the 3′-keto-2′-deoxy-NDP (I). This mechanistic proposal predicts that the hydrogen atom abstracted from the 3′-position by the protein radical X· is capable of exchanging this hydrogen with solvent and, in contrast with the proposal for the normal substrates (NDPs), returning this H· to the β-face of the 2′-position of intermediate VII to give 3′-keto-2′-deoxy-NDP (I). Compound I can now dissociate from the active site of the enzyme and undergo buffer-catalyzed abstraction of the 2′-hydrogen on the α-face of I to eliminate uracil and abstraction of the 4′-hydrogen to eliminate PP_i. The 2-methylene-3(2*H*)-furanone generated can now alkylate the enzyme, resulting in its inactivation, or alkylate a nucleophile in the reaction mixture. Scheme I is consistent with all of the known experimental observations as subsequently discussed in detail.

The proposed intermediacy of radical cation VII (Scheme I) is based on the work of Walling & Johnson (1975) and Gilbert et al. (1972). These workers have investigated the interaction of HO·, generated from Fenton's reagent, with chloroethanol and ethylene glycol, which are analogues of ClNDPs and NDPs, respectively. These workers concluded that a radical cation mechanism similar to that proposed in Scheme I is involved in cleavage of the C–X (X = Cl, OH) bond and generation of one of the products, acetaldehyde, produced from both substrates (eq 3). In contrast with the



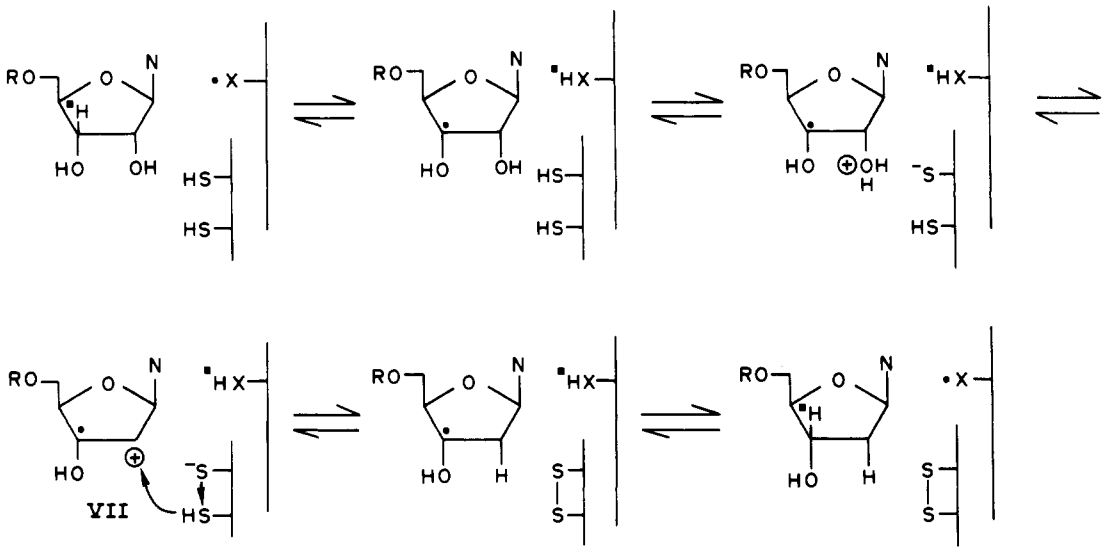
results found with ethylene glycol, however, the chloroethanol reaction does not need to be acid catalyzed.

Scheme I predicts that the products produced on interaction of ClNDP with reductase are PP_i, uracil, and 2-methylene-3(2*H*)-furanone and that the entire process is initiated by cleavage of the 3′ C–H bond. Previous studies substantiated the production of PP_i (Stubbe & Kozarich, 1980) and uracil (Thelander et al., 1976). Studies reported in this paper are consistent with 3′ C–H bond cleavage (Figure 2) and production of the highly reactive 2-methylene-3(2*H*)-furanone (Figures 4 and 7).

Comparison of IR and NMR data of model compounds V and VI with those of $CHCl_3$-extracted adducts III and IV isolated from the reductase reaction mixture (Figures 4 and 7) strongly suggests that 2-methylene-3(2*H*)-furanone is their precursor. Furthermore, synthesis of II and incubation with RTPR result in rapid inactivation and a modification of protein at 320 nm (Figure 8). The spectrum is remarkably similar to that produced when reductase is incubated with 3′-chloro-2′-deoxynucleotides (Figure 3).

Any mechanistic proposal for inactivation of reductase by ClUDP must also account for the observation of Thelander et al. reported in 1976 that during this inactivation no loss of the protein tyrosine radical was observed. Therefore, in Scheme I, the H atom abstracted from the 3′-position in ClUDP is of necessity returned to the 2′-position to produce I, regenerating the protein radical X·. Scheme I also indicates that the hydrogen atom abstracted from the 3′-position of ClUDP may be capable of exchange from XH with solvent at numerous stages along the reaction pathway. The results reported in Table I are consistent with this proposal. Tritium from [3′-^{3}H]ClUDP is found in solvent as well as covalently bound to the protein, while ^{3}H from [5′-^{3}H]ClUDP, which

Scheme II



cannot exchange with the solvent, is found in substantially higher quantities on the protein. The observed stoichiometry of 6 equiv of PP_i produced during enzyme inactivation with 5 equiv of ^3H found in solvent and 1 equiv of ^3H bound to the protein strongly suggests that both events (release of ^3H_2O and radiolabeling of protein) are the result of a single reaction pathway such as that proposed for generation of ketone I. This stoichiometry also suggests there is a small or no isotope effect on 3′ C–H bond cleavage of ClUDP. These results are consistent with our double-label [3′-^{3}H,β-^{32}P]ClUDP experiment ($k_H/k_T \leq 1.17$) designed to measure this isotope effect directly.

Subtle changes in protein structure may produce a change in the partitioning of ^3H from [3′-^{3}H]ClUDP between solvent and protein. To further substantiate the proposal in Scheme I, experiments with [3′-^{3}H]- and [5′-^{3}H]ClUDP were also carried out in ^2H_2O (Table I). These results indicate that more ^3H from [3′-^{3}H]ClUDP is bound to the protein in ^2H_2O vs. H_2O and that proportionally less ^3H is found in the solvent (based on the number of turnovers per inactivation). The fact that in ^2H_2O 9 equiv of PP_i is produced and 8 equiv of ^3H is accounted for as ^3H_2O or ^3H bound to protein is again strongly supportive of a single reaction pathway being responsible for the different fates of the ^3H label.

The stoichiometry of labeling of RDPR with [5′-^{3}H]ClUDP of 4.6 ^3H per B_1 protomer is suggestive of nonspecific labeling. We propose, therefore, that ketone I (Scheme I) may dissociate from the active site of RDPR and that nonenzymatic processes are involved in generation of II and subsequent enzyme alkylation. Ample chemical precident exists for nonenzymatic elimination of both uracil and PP_i for compounds such as I (Binkley et al., 1977, 1978; Pfizner & Moffat, 1965).[6] In fact, there is no mechanistic reason why RDPR should catalyze conversion of I to 2-methylene-3(2*H*)-furanone (II), although at present we cannot totally rule out this possibility.

Comparison of the Mechanism of Reduction of Substrate (NDP) by RDPR with the Mechanism of Inactivation of RDPR by ClUDP. The mechanism we have previously proposed for NDP reduction is shown in Scheme II. The subtle differences between the mechanism of substrate reduction by RDPR and the mechanism of inactivation of RDPR by ClNDP are important to address.

In the case of the normal substrate, on the basis of model studies discussed in the previous section, the 2′-hydroxyl of NDP needs to be protonated to produce radical cation VII. Our hypothesis (Scheme II) is that one of the thiols involved in the redox chemistry may also be involved in the protonation of the 2′-OH to make it a better leaving group. Therefore, the radical cation (VII) is now at the active site with thiol and thiolate protein residues, which can carry out reduction to produce a disulfide and the 3′-deoxynucleotide radical. The role of the thiols in Scheme II contrasts with that proposed for the role of thiols in Scheme I, because on the basis of the model studies, ClNDP does not require protonation of the leaving group Cl^- to produce radical cation VII. Thus, the protonation state of the thiols of RDPR is different in the case of normal substrate reduction and inactivation by ClUDP. In the latter case, where two protonated thiols exist at the active site, the rate of disulfide formation may be sufficiently slow to allow alternative chemistry to occur.

A second very important difference exists between the proposed mechanism of reduction of NDP and inactivation by ClUDP (Scheme II, Scheme I): the fate of the 3′-hydrogen. In the former case, we have shown that the hydrogen abstracted from the 3′-position in the starting material is returned to the 3′-position in the product (Stubbe et al., 1983). Moreover, it is interesting to note that incubation of [3′-^{3}H]UDP or [3′-^{3}H]ADP with *E. coli* reductase results in a very small amount of ^3H_2O release during turnover. In the latter case, inactivation by ClUDP, we are proposing that the H atom abstracted from the 3′-position of ClNDP may be exchanged with solvent (similar to the observations with NDP) and is then returned to the 2′-position to produce the 3′-keto-2′-deoxynucleotide (I). The H atom is returned to the same face, but to a different position, of the nucleotide from which is was abstracted. We are proposing that a subtle change in conformation, due to the protonation state of the thiols, is capable of allowing a rearrangement reaction to occur. This rearrangement reaction has important implications for the AdoCbl-requiring reductase.

Inactivation of RTPR by ClNTPs: Mechanistic Implications. Our recent work with the *L. leichmannii* reductase (Stubbe et al., 1981, 1983) is consistent with the hypothesis that 5′-deoxyadenosyl radical generated by homolytic cleavage of the carbon–cobalt bond of AdoCbl may be involved in generation of a protein radical X·, which could then function as a hydrogen abstractor from NTP. Therefore, the mechanisms proposed in Scheme II for substrate reduction and in Scheme I for inactivation by ClUTP are also consistent with

[6] G. Harris and J. Stubbe, unpublished observations.

experimental observations with the AdoCbl RTPR. The proposed mechanism for RTPR-catalyzed inactivation by ClNTPs takes on special significance with this enzyme. RTPR is the only AdoCbl-dependent enzyme that does not catalyze a rearrangement reaction. Scheme I would indicate that very subtle changes in active site structure would allow a rearrangement to occur.

Evidence consistent with a rearrangement reaction catalyzed by the *L. leichmannii* reductase is presented in Table II. The AdoCbl reductase undergoes ~150 turnovers per inactivation as measured by ^3H_2O release and by assuming a small (<1.2) isotope effect on this reaction.[6] Because all of the reactions with RTPR were run in the presence of 3 mM DTT, ^3H from [3′-^{3}H]ClUTP has three fates: it may be released to the solvent, bound to the protein, or trapped in sugar adducts (eq 2). The mechanism in Scheme I suggests that when the rearrangement occurs, ^3H bound to RTPR and ^3H trapped in a sugar adduct are derived from a common intermediate, 2-methylene-3(2*H*)-furanone (II). Furthermore, ^3H on the protein can also exchange with solvent prior to generation of I. In the case of [5′-^{3}H]ClUTP, since no exchange with solvent occurs, the total ^3H present in II should be greater than that in II derived from [3′-^{3}H]ClUTP. However, the distribution of II between alkylation of protein and DTT ought to be the same as with [3′-^{3}H]ClUTP. If Scheme I is valid, therefore, measurement of the ratio of ^3H bound to RTPR from the [3′-^{3}H]- and [5′-^{3}H]ClUTPs should be analogous to that observed in the sugar adducts isolated by the $CHCl_3$ extraction procedure. As indicated in Table II, (^3H protein bound from [5′-^{3}H]ClUTP)/(^3H protein bound from [3′-^{3}H]ClUTP) = 7, and (^3H sugar adduct from [5′-^{3}H]ClUTP)/(^3H sugar adduct from [3′-^{3}H]ClUTP) = 8. These results are consistent with ^3H exchange prior to generation of an intermediate I and with the fact that ^3H from the 3′-position of [3′-^{3}H]ClUTP is in some cases returned to the β-face of the 2′-position to generate I. Compound I can nonenzymatically collapse to generate II by trans elimination of base, thus leaving the ^3H still present in the 2′-position of II, which now partitions between alkylation of RTPR and alkylation of DTT.

This rearrangement proposal is based on the supposition, which is difficult to prove, that ^3H on RTPR and ^3H in the solvent are the result of the same reaction pathway. We feel that this hypothesis is reasonable for two reasons. (1) RTPR is inactivated by [3′-^{3}H]ClUTP when only 0.4 equiv of ^3H is bound, and presumably, 2.5 equiv of unlabeled species is bound per M_r 76 000, while 2.9 equiv of ^3H is bound with [5′-^{3}H]-ClUTP. (2) Products produced by RTPR-catalyzed decomposition of ClUTP are remarkably similar to those produced by the *E. coli* reductase: PPP_i, uracil, ^3H_2O from [3′-^{3}H]-ClUTP (Stubbe et al., 1983), and 2-methylene-3(2*H*)-furanone. In the case of RDPR, quantitation of PP_i stoichiometrically to ^3H_2O and ^3H-labeled protein allows a strong case to be made for connection of ^3H_2O and ^3H on the protein to a single reaction pathway. Scheme I is consistent with all of the information available for inactivation of RTPR by ClUTP.

Present studies in our laboratory are focused on experiments to support or disprove this rearrangement hypothesis (Scheme I). Specifically, if 3′-keto-2′-deoxynucleotide (I) is generated enzymatically and dissociates into solution, it might be trapped by the presence of $NaBH_4$ in the reaction mixture. If trapping is successful, then experiments carried out with [3′-^{2}H]-ClNTPs, followed by isolation of the $NaBH_4$-trapped product, ought to allow detection by mass spectrometry of ^2H in the trapped product. Confirmation of the proposed enzyme-catalyzed chemistry in Scheme I, specifically the rearrangement, may be of importance in understanding AdoCbl-catalyzed rearrangement reactions.

Summary. Results presented in this paper demonstrate the remarkable similarities of the *E. coli* and *L. leichmannii* enzymes upon interaction with suicide inhibitor 2′-chloro-2′-deoxynucleotides. Labeling studies with [5′-^{3}H]ClUDP (ClUTP) and [3′-^{3}H]ClUDP (ClUTP) indicate that both enzymes catalyze cleavage of the 3′ C–H bond and that ^3H from both the 5′- and the 3′-positions of the sugar can be found bound to the protein. The differences in the amount of ^3H bound to the protein, which depends on the original location of the label in the sugar, have been interpreted to indicate both enzymes' ability to catalyze a rearrangement reaction. This interpretation is particularly intriguing for the AdoCbl-dependent reductase. Evidence substantiated by model studies is also presented that both reductases catalyze the production of 2-methylene-3(2*H*)-furanone. This furanone is thought to be responsible for enzyme inactivation.

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Inhibition of Angiotensin Converting Enzyme: Mechanism and Substrate Dependence†

Robert Shapiro and James F. Riordan*

ABSTRACT: The interaction of angiotensin converting enzyme with six metal-coordinating [(D-3-mercapto-2-methylpropanoyl)-L-Pro (captopril), *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro (MK-422), *N*-(phenylphosphoryl)-L-Phe-L-Phe, N^{α}-(3-mercaptopropanoyl)-L-Arg, N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-Ala-L-Lys, and *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly] and three dipeptide inhibitors (Gly-L-Trp, L-Phe-L-Arg, and L-Ala-L-Pro) was examined at pH 7.5 in the presence of 300 mM NaCl. Inhibition modes, apparent K_i [K_i(app)] values, and shapes of $1/v$ vs. [I] plots were found to vary with the substrate employed. All inhibitors except Phe-Arg were competitive with the substrate furanacryloyl (Fa)-Phe-Gly-Gly, while five of seven tested with Fa-Phe-Phe-Arg as substrate produced mixed patterns. K_i(app) values for *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly, *N*-(phenylphosphoryl)-L-Phe-L-Phe, Gly-Trp, and MK-422 were 8.3-, 5.5-, 4.7-, and 2.6-fold lower, respectively, when Fa-Phe-Gly-Gly was substrate, compared with values measured with Fa-Phe-Phe-Arg. In contrast, K_i(app) values for Phe-Arg and (3-mercaptopropanoyl)-Arg were lower (2.8- and 2.2-fold, respectively) when Fa-Phe-Phe-Arg was the substrate. Plots of $1/v$ vs. [I] for most of the inhibitors were nonlinear, to an extent which was also substrate dependent. By curve fitting, these plots were shown to be consistent with a rate equation of the form $v/[E_0] = (1 + d[I])/(a + b[I] + c[I]^2)$, suggesting that inhibitor can bind to more than one enzyme form and that there are alternative pathways to product. Two inhibition mechanisms are described which incorporate these features and may account for the observed substrate dependence. These mechanisms attribute the unusual kinetics to (i) inhibitor binding to an enzyme–product complex or (ii) interaction of the enzyme with activating anions. A third mechanism, consistent with the kinetic observations, involves multiple inhibitor binding and appears unlikely on the basis of equilibrium dialysis measurements.

During the past several years, considerable effort has been devoted to the development of tight-binding, specific inhibitors of angiotensin converting enzyme (peptidyl dipeptidase, EC 3.4.15.1) (ACE)[1] for use as antihypertensive drugs. The effectiveness of these compounds in the management of hypertension, which has been demonstrated in numerous clinical studies (Gavras et al., 1978; Cushman & Ondetti, 1980), is presumably a consequence of the known physiological actions of ACE: it catalyzes both the generation of the potent, vasoconstricting octapeptide angiotensin II from the decapeptide angiotensin I and the inactivation of the vasodilating nonapeptide bradykinin (Soffer, 1976; Erdös, 1976; Peach, 1977).

The first orally active ACE inhibitor employed for control of blood pressure, (D-3-mercapto-2-methylpropanoyl)-L-proline (captopril), was designed on the basis of the active-site structure of the analogous zinc exopeptidase, carboxypeptidase A (Ondetti et al., 1977; Cushman et al., 1977). Its strength of binding to ACE (K_i = 1.7 nM) is thought to derive largely from the interaction of its sulfhydryl group with the zinc atom at the active site of the enzyme. Subsequently, Patchett et al. (1980) synthesized a series of *N*-(carboxyalkyl)dipeptide inhibitors containing a carboxylate group which presumably coordinates to the zinc atom. At least two of these, the *N*-[1(*S*)-carboxy-3-phenylpropyl] derivatives of Ala-Pro (MK-422) and Lys-Pro, have nanomolar IC_{50} values and are orally effective antihypertensive agents (Patchett et al., 1980; Gavras et al., 1981). Inhibitors incorporating a $-PO^-$ anion as the metal ligand have also been synthesized (Holmquist & Vallee, 1979; Galardy, 1980; Thorsett et al., 1982; Galardy et al., 1983), and some of these exhibit nanomolar K_i values.

Hundreds of potential ACE inhibitors have now been synthesized, resulting in the discovery of at least six classes of compounds effective at nanomolar concentrations. Despite this intense effort, relatively few reports have examined the mode of action of these inhibitors. Most of the nonclinical ACE inhibition literature concerns structure–activity relationships; inhibition mechanisms have received only cursory attention. Such work has been limited, in part, by the almost universal use of a discontinuous assay system, with reaction

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[1] Abbreviations: ACE, angiotensin converting enzyme; captopril, (D-3-mercapto-2-methylpropanoyl)-L-proline; MK-422, *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro; IC_{50}, the concentration of inhibitor producing 50% inhibition at the particular substrate and enzyme concentrations employed; Tris, tris(hydroxymethyl)aminomethane; Bz, *N*-benzoyl; SDS, sodium dodecyl sulfate; CA-Phe-Gly, *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly; PPPP, *N*-(phenylphosphoryl)-L-Phe-L-Phe; MP-Arg, N^{α}-(3-mercaptopropanoyl)-L-Arg; CP-Ala-Lys, N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Lys; Fa, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; *t*-BOC, *tert*-butyloxycarbonyl.

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