Gemcitabine 5′-Triphosphate Is a Stoichiometric Mechanism-Based Inhibitor of *Lactobacillus leichmannii* Ribonucleoside Triphosphate Reductase: Evidence for Thiyl Radical-Mediated Nucleotide Radical Formation†

Domingos J. Silva‡ and JoAnne Stubbe*,‡,§

Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

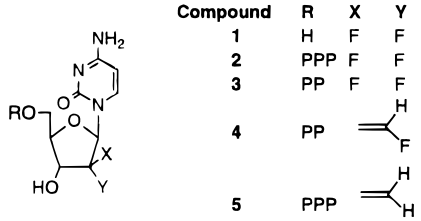
Vicente Samano and Morris J. Robins

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602

Received December 1, 1997; Revised Manuscript Received February 13, 1998

ABSTRACT: Ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* utilizes adenosylcobalamin and catalyzes the conversion of nucleoside triphosphates to deoxynucleoside triphosphates. One equivalent of 2′,2′-difluoro-2′-deoxycytidine 5′-triphosphate, F_2dCTP, rapidly inactivates RTPR. Analysis of the reaction products reveals that inactivation is accompanied by release of two fluoride ions and 0.84 equiv of 5′-deoxyadenosine and attachment of 1 equiv of corrin covalently to an active-site cysteine residue of RTPR. No cytosine release was detected. Proteolysis of corrin-labeled RTPR with endoproteinase Glu-C and peptide mapping at pH 5.8 revealed that C419 was predominantly modified. The kinetics of the inactivation have been examined by stopped-flow (SF) UV−vis spectroscopy and rapid freeze quench (RFQ) electron paramagnetic resonance (EPR) spectroscopy. Monitoring ΔA_{525} nm shows that cob(II)alamin is formed with an apparent k_{obs} of 50 s^{-1}, only 2.5-fold slower than a similar experiment carried out with cytidine 5′-triphosphate (CTP). The same reaction mixture was thus quenched at times from 22 ms to 30 s and examined by EPR spectroscopy. At early time points the EPR spectrum resembled a thiyl radical exchange coupled to cob(II)alamin. From 22 to 255 ms the total spin concentration remained unchanged at 1.4 spins/RTPR, twice that predicted by the amount of cob(II)alamin determined by SF. However, with time the signal attributed to the thiyl radical-cob(II)alamin disappears and new signal(s) with broad feature(s) at $g = 2.33$ and a sharp feature at $g = 2.00$ appeared, suggesting formation of cob(II)alamin and a nucleotide-based radical with only dipolar interactions. These studies have been interpreted to support the proposal that an RTPR-based thiyl radical can give rise to a nucleotide-based radical.

Gemcitabine, 2′-deoxy-2′,2′-difluorocytidine (F_2dC)[1] (**1**, Figure 1), has recently been shown to possess potent chemotherapeutic efficacy against a variety of solid tumors (*1*, *2*). The mode of cytotoxicity is thought to be related to the ability of the triphosphate **2** (F_2dCTP) to inactivate DNA polymerase by chain termination and the ability of the diphosphate **3** (F_2dCDP) to inactivate ribonucleotide reductase (RNR) (*3*, *4*), responsible for the conversion of nucleotides to deoxynucleotides. Previously, we have shown that F_2dCDP is a stoichiometric mechanism based inactivator of *E. coli* ribonucleotide reductase with a unique mode of inactivation (*4*). In the present paper, F_2dCTP is shown to be a stoichiometric mechanism-based inactivator of the adenosylcobalamin- (AdoCbl-) dependent *Lactobacillus le-*



Compound	R	X	Y
1	H	F	F
2	PPP	F	F
3	PP	F	F
4	PP		
5	PPP		

FIGURE 1: Structures of F_2dC (**1**), F_2dCTP (**2**), F_2dCDP (**3**), FMdCDP (**4**), and MdCTP (**5**).

† This research was supported by grants from the National Institutes of Health to J.S. (GM 29595) and by a postdoctoral fellowship from the Cancer Research Fund of the Damon Runyon−Walter Winchell Foundation to D.J.S. (DRG-1333).

* Corresponding author: Tel (617) 253-1814; Fax (617) 258-7247; e-mail stubbe@mit.edu.

‡ Department of Chemistry, MIT.

§ Department of Biology, MIT.

[1] Abbreviations: F_2dC, 2′-deoxy-2′,2′-difluorocytidine; F_2dCTP, 2′-deoxy-2′,2′-difluorocytidine-5′-triphosphate; F_2dCDP, 2′-deoxy-2′,2′-difluorocytidine 5′-diphosphate; AdoCbl, adenosylcobalamin; AqCbl, aquocobalamin; RTPR, ribonucleoside triphosphate reductase; SF, stopped flow; RFQ, rapid freeze quench; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); GlutCbl, glutathionylcobalamin; EDTA, ethylenediaminetetraacetic acid; TR, thioredoxin; TRR, thioredoxin reductase; dATP, 2′-deoxyadenosine 5′-triphosphate; NTP, nucleoside triphosphate; CTP, cytidine 5′-triphosphate; 5′-dA, 5′-deoxyadenosine; MS-EI, mass spectrometry by electrospray ionization; RNR, ribonucleotide reductase; N_3NDP, 2′-deoxy-2′-azidonucleoside 5′-diphosphate; FMdCDP, 2′-deoxy-2′-fluoromethylenecytidine 5′-diphosphate; FMdCTP, 2′-deoxy-2′-fluoromethylenecytidine 5′-triphosphate; MdCTP, 2′-deoxy-2′-methylenecytidine 5′-triphosphate; RDPR, ribonucleoside diphosphate reductase.

S0006-2960(97)02934-6 CCC: $15.00
Published on Web 04/02/1998

ichmannii ribonucleoside triphosphate reductase (RTPR). Stopped-flow (SF) UV−vis spectroscopy and rapid freeze quench (RFQ) EPR spectroscopy have revealed that, as in the case with the normal nucleoside triphosphate substrates, a thiyl radical and cob(II)alamin is generated with a k_{obs} of 50 s^{-1} and that disappearance of this signal occurs concomitant with formation of a new intermediate: cob(II)alamin and what is proposed to be a nucleotide-based radical. Analysis of the fate of the F_2dCTP and AdoCbl are presented, providing new insight into the catalytic capabilities of this RTPR.

MATERIALS AND METHODS

Materials. F_2dCTP was prepared as previously described. Glutathionylcobalamin (GlutCbl) was obtained as a kind gift from Professor Richard Finke (Colorado State University). Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim. "Hepes buffer" corresponds to 25 mM Hepes, 4 mM EDTA, and 1 mM $MgCl_2$, pH 7.5. RTPR was overexpressed and isolated as previously described (*5*) and had a specific activity of 1.0−1.5 μmol min^{-1} mg^{-1}. Prereduced RTPR was prepared as previously described (*6*). Thioredoxin (TR) (*7*) and thioredoxin reductase (TRR) (*8*) were purified using standard procedures. Isopentane was purchased from Aldrich. Suprasil quartz EPR sample tubes (o.d. 4 mm, i.d. 2.4 mm, 7 in. long) were obtained from Wilmad Glass Co. Endoproteinase Glu-C, nucleotides, nucleosides, and NADPH were obtained from Sigma.

Methods. UV−vis spectroscopy was performed on a Cary 3 spectrophotometer equipped with a temperature-controlled water bath. Centricon-30 microconcentrators were obtained from Millipore. HPLC analyses were carried out on a Model SD-200 Dynamax Rainin system. Fluoride analyses were carried out with an Orion 96-09 fluoride combination electrode (*9*). SF UV−vis spectroscopy was performed on an Applied Photophysics DX.17MV sequential stopped-flow spectrofluorometer coupled to a variable-temperature water bath. RFQ-EPR samples were prepared using an Update Instrument System 1000 chemical/freeze quench apparatus, in conjunction with an Update Instrument isopentane bath. The 8-L isopentane bath was kept at −140 °C by adding liquid N_2 to the outer container. EPR spectra at 9.47 GHz were recorded on a Bruker ESP-300 spectrometer at 20 K. Spin quantitation was achieved with a 1.0 mM $CuSO_4$, 2 M $NaClO_4$, 0.01 M HCl, and 20% (v/v) glycerol standard (*10*).

Peptide sequencing was performed at the MIT Biopolymers Lab. Mass spectrometry and total amino acid analyses were performed at the Harvard Microchemistry Facility. GlutCbl and labeled peptide were analyzed by direct infusion using a Harvard syringe pump on a Finnigan MAT TSQ 7000 triple-quadrupole mass spectrometer scanning from *m*/*z* 300 to 1800 in 1.7 s. Labeled peptide treated with cyanide ion was run as above and analyzed on a Finnigan MAT LCQ ion trap mass spectrometer scanning from *m*/*z* 300 to 1800 in 3.0 s. Mass spectra were acquired at pH 5.8 giving the following calculated masses: cobalamin lacking an upper axial ligand, $C_{62}H_{88}N_{13}O_{14}PCo$, M_r 1329.35, net charge +1; glutathione, $C_{10}H_{16}N_3O_6S$, M_r 306.33, net charge +1; the peptide of residues 411−423 of RTPR, M_r 1403.56, net charge −2.

The following extinction coefficients were used: RTPR, ϵ_{280} = 101 000 M^{-1} cm^{-1}; AdoCbl, ϵ_{525} = 8000 M^{-1} cm^{-1} and ϵ_{475} = 5800 M^{-1} cm^{-1}; cob(II)alamin, ϵ_{525} = 3200 M^{-1} cm^{-1} and ϵ_{475} = 9200 M^{-1} cm^{-1}; F_2dCTP, ϵ_{272} = 9100 M^{-1} cm^{-1}. All of the assays were performed with deoxygenated solutions. In the case of small volumes (<1.0 mL), the deoxygenation was achieved by placing the solutions in a septum-sealed 1.5 mL Eppendorf tube and purging with argon for 20 min. For larger volumes (between 1.0 and 3.0 mL), the solutions were transferred to round-bottom flasks containing stirring bars and purged with argon for 20 min with moderate stirring. Operations involving AdoCbl were performed in the dark or under dim light to avoid cofactor photolysis.

Time-Dependent Inactivation of RTPR by F_2dCTP. All time-dependent inactivation studies were performed at 37 °C. Solutions contained, in a final volume of 200 μL, 15 μM prereduced RTPR, 15 μM F_2dCTP, 120 μM dATP, 30 μM AdoCbl, and Hepes buffer. The incubation solution, containing all components except for AdoCbl and F_2dCTP, was kept at 37 °C for 5 min. AdoCbl was added and a 15 μL aliquot was removed (sample corresponding to t = 0) and assayed for activity. The reaction was started by adding inhibitor. Aliquots, 15 μL, were removed at defined time points between 0.25 and 15 min and assayed for activity. In a typical activity protocol, a 15 μL aliquot was added to 140 μL of the assay solution, previously equilibrated to 37 °C. The solution contained AdoCbl, 20 μM; [U-^{14}C]CTP (1.43×10^6 cpm/μmol), 1 mM; dATP, 120 μM; TR, 20 μM; TRR, 0.12 μM; NADPH, 0.2 mM; and Hepes buffer. The solutions were incubated at 37 °C for 5 min; a 100 μL aliquot was removed and added to 50 μL of 2% (v/v) perchloric acid. This solution was treated with 50 μL of 0.4 M KOH and 30 μL of 0.5 M Tris (pH 8.5). Dephosphorylation of nucleotides was performed by adding alkaline phosphatase (5 units) to the solutions and incubating them at 37 °C for 90 min. The solutions were loaded onto Dowex AG1×2 columns (borate form, 0.6 × 10 cm) (*11*). The columns were washed with 12 mL of water. A portion of the eluent (2 mL) was analyzed by scintillation counting.

Analysis of Fluoride Release. All measurements were performed at 37 °C in a final volume of 500 μL containing prereduced RTPR (1.0 unit/mg), 300 μM; AdoCbl, 450 μM; dATP, 1 mM; Hepes buffer; and F_2dCTP, 300 μM. An aliquot of stock F^- solution was added to the assay solution to provide a constant initial electrode reading. At t = 0, AdoCbl was added to the reaction mixture, and electrode readings were recorded as a function of time. After 20 min, 400 μL of the reaction mixture was transferred to a Centricon 30 unit and concentrated to ≈50 μL. The retentate was diluted with 1 mL of Hepes buffer and the dilution−concentration process was repeated three times to ensure maximum separation of small molecules and protein fraction. The filtrates were combined and analyzed for small molecules. The retentate was used to identify labeled RTPR.

Analysis of Small Molecules. The pH of the combined filtrate (4.0 mL) was adjusted to 8.5 with 1 N NaOH. The solution was treated with 20 units of alkaline phosphatase and incubated at 37 °C for 2 h. One-fourth of the reaction mixture (1.0 mL) was analyzed by HPLC on an Econosil C_{18} column (4 mm × 25 cm). The products were eluted at a flow rate of 1 mL/min, isocratically with water for 8 min, followed by a linear gradient over 13 min to 20% methanol, followed by a second linear gradient from 20% to 100%

methanol over 45 min. Under these conditions, the retention times of the following standards were determined: cytosine, 4.5 min; 5′-deoxyadenosine (5′-dA), 24 min; 2′-deoxyadenosine, 28 min; AdoCbl, 48.5 min; aquocobalamin, 50 min. The void volume was ≈3.5 min. Products were collected, and the volume and UV−vis absorbance of the pooled fractions were measured in order to allow quantitation.

Analysis of the Stability of Labeled RTPR to Denaturant and pH. A series of solutions (total volume of 1.0 mL) containing labeled RTPR (30 μM), KP_i (100 mM), and 6 M guanidine hydrochloride were prepared at different pH values (5.0, 6.0, 7.0, 8.0, and 9.0). The solutions were kept for 16 h at 4 °C in the dark and concentrated using a Centricon 30. The filtrates (0.80 ± 0.05 mL) were collected and their UV−vis spectra were recorded.

Digestion of Cobalamin-Labeled RTPR with Endoproteinase Glu-C. Stock solutions of endoproteinase Glu-C (1 mg/mL) were prepared by dissolving the lyophilized enzyme in water and used immediately. The reaction mixture contained labeled RTPR (30 μM), urea (2 M), and endoproteinase Glu-C (0.04 mg/mL) in 140 mM KP_i (pH 5.8). Under these conditions the ratio of Glu-C to labeled protein was 1:62.5 (w/w). The solution was incubated for 3 h at 37 °C in the dark, and additional Glu-C (1 mg/mL) was added to bring the ratio of Glu-C to labeled protein to a final value of 1:40 (w/w). The solution was then kept for 12 h at 37 °C.

HPLC Analysis of the Digested Labeled Protein. After digestion, the solution was filtered through a 0.2 μm filter to remove any precipitate. HPLC analysis was performed on a C_{18} Vydac column (0.46 × 23 cm). For stability reasons the HPLC analysis, flow rate of 1.0 mL/min, was carried out in NH_4OAc (pH 5.8) using solvent A (1 mM NH_4OAc, pH 5.8) and solvent B (80% acetonitrile and 20% solvent A). The peptides were eluted with a linear gradient from 2% to 30% solvent B over 50 min, followed by a second linear gradient to 98% solvent B over 70 min.

Kinetic Studies with Cyanide Ions. A quartz cuvette containing 38.4 μM labeled peptide in 100 mM NH_4OAc, pH 5.8, was placed in the light path of a UV−vis spectrophotometer thermostated at 30 °C. At $t = 0$, 400 μM CN^- solution was added. The reaction was monitored from 300 to 650 nm every minute for 30 min. This assay was repeated using a 40 μM solution of GlutCbl in place of the labeled peptide. (For both labeled peptide and GlutCbl, concentrations were estimated using $\epsilon_{525} = 8000\ M^{-1}\ cm^{-1}$).

Stopped-Flow UV−Vis Analysis of Interaction of CTP and F_2dCTP with RTPR. The experiment was performed under anaerobic conditions: the loading syringes were sealed with rubber septa and soaked overnight in 50 mM dithionite. Immediately before use, the reducing solution was removed. Each syringe and its corresponding instrument line were flushed with 20 mL of argon-saturated Hepes buffer. Reaction mixtures were deaerated and transferred to the loading syringes using gastight syringes. All exposed parts of the instrument were wrapped in aluminum foil to avoid photolysis of AdoCbl. Argon was bubbled through the water bath for 3 h before data acquisition and throughout the experiment. Syringe A (4.00 mL) contained AdoCbl, 900 μM; dATP, 1 mM; and Hepes buffer. Syringe B (1.25 mL) contained RTPR (0.75 unit/mg), 600 μM; CTP, 600 μM; dATP, 1 mM; Hepes buffer; TR, 20 μM; TRR, 1 μM; and NADPH, 2 mM. For experiments with F_2dCTP the conditions were identical to those described above except that CTP was replaced with 600 μM F_2dCTP.

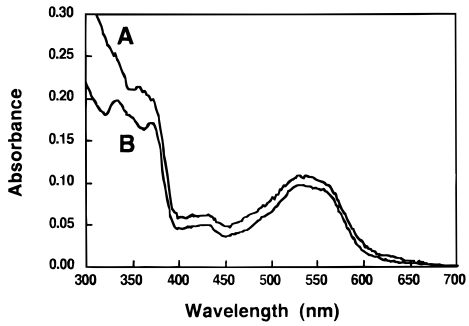


FIGURE 2: (A) UV−Vis spectrum of RTPR inactivated by F_2dCTP after passage through a Sephadex G25 column. (B) UV−Vis spectrum of GlutCbl.

Preparation of Samples for EPR Analysis. Samples were prepared using RFQ methods and conditions identical to those described for the SF and F^- release studies to allow direct comparison of the results (*12*).

Preparation of EPR Hand-Quenched Sample. The assay solution contained in a final volume of 275 μL RTPR (0.75 unit/mg), 300 μM; F_2dCTP, 300 μM; AdoCbl, 450 μM; dATP, 1 mM; TR, 10 μM; TRR, 0.5 μM; NADPH, 1 mM; and Hepes buffer. The solution, containing all components except inhibitor, was degassed and incubated at 37 °C for 15 min. The reaction was initiated by adding F_2dCTP and run at 37 °C for 20 s. The solution was then quickly transferred via a gastight syringe to an EPR tube (previously purged with argon) and frozen in liquid N_2. The sample was wrapped in aluminum foil and stored in liquid N_2.

EPR Experiments. EPR analysis was performed at 20 K. Samples were loaded in dim light to avoid photolysis. Spectra were recorded at different irradiation powers to study field-dependent line shape and saturation behavior. Acquisition conditions: microwave frequency, 9.4 GHz; modulation amplitude, 5.12 G; modulation frequency, 100 kHz; time constant, 163.84 ms.

RESULTS

Time-Dependent Inactivation of RTPR. F_2dCTP was examined as a potential mechanism-based inhibitor of RTPR. Incubation of prereduced RTPR with 1 equiv of F_2dCTP led to rapid time-dependent inactivation of the enzyme with >90% of the activity lost within 15 s.

Covalent Modification of the Protein. Previous studies with 2′-substituted 2′-deoxynucleotides that function as mechanism-based inhibitors of RNR have revealed that inactivation is accompanied by release of nucleic acid base, the leaving group at the 2′-position, and 2-methylene-3(2*H*)-furanone (*13*). The latter has been shown to label the protein, resulting in a new protein-associated chromophore with a λ_{max} of 320 nm (*14*, *15*). In addition, oftentimes the inactivation is accompanied by AdoCbl decomposition to 5′-dA and cob(II)alamin (*16*). To determine whether the inactivated RTPR is labeled by a similar mechanism, inactivated enzyme was separated from the small molecules using a Sephadex G25 column. Surprisingly, the protein-containing fraction was red, showing absorbance maxima at 355, 370, 430, and 535 nm, consistent with the presence of a cobalamin species (Figure 2A). This spectrum is very

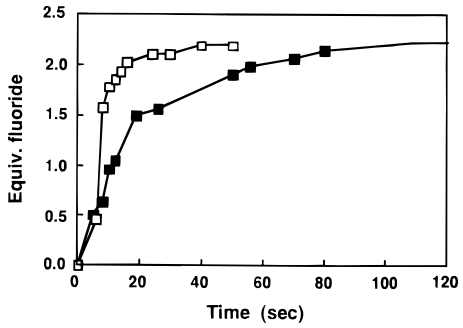


FIGURE 3: Fluoride release from F_2dCTP accompanying inactivation of RTPR (300 μM): (■) enzymatic reaction in the presence of 300 μM F_2dCTP; (□) control incubation, to determine the electrode dead time, in the presence of 660 μM NaF.

similarto that of GlutCbl, a model compound containing a Co−S bond (Figure 2B), suggesting that a corrin may be attached to a cysteine in the labeled protein. The ratio A_{280}/A_{525} is 0.080 in the labeled RTPR. Assuming that the A_{280} is due primarily to RTPR (ϵ_{280} = 101 000 M^{-1} cm^{-1}) and ϵ_{525} is similar to that of GlutCbl (ϵ_{525} = 8000 M^{-1} cm^{-1}), RTPR appears to be quantitatively labeled with this chromophore. This adduct, as determined subsequently, possesses a cobalt−thiol bond to RTPR.

Quantitation of Cytosine and 5′-Deoxyadenosine Produced during Inactivation. Unfortunately, F_2dCTP is not yet available in radiolabeled form. Despite this limitation, the small molecules from the inactivation mixture were isolated by Centricon 30 centrifugation, which maintains the protein in its native state, and analyzed by HPLC. 5′-dA, ≈0.84 equiv/equiv of RTPR, was observed. No cytosine was detected despite the fact that as much as 20 nmol in principle could have been produced. This contrasts with the results with *E. coli* RDPR, where under similar conditions 1 equiv of cytosine is released (*4*). A caveat in this latter case is that, as in the case of many mechanism-based inhibitors, more than one pathway is responsible for inactivation and a minor pathway may in fact have no cytosine associated with its occurrence. It is thus possible that a commonality of mechanism between the class I and class II RNRs exists, despite the differences in the observed cytosine release.

Fluoride Release during Inactivation. Release of F^- during RTPR inactivation was monitored using a fluoride-sensitive electrode. The conditions of the F^- assay are similar to those used in the SF UV−vis and RFQ-EPR spectroscopic studies described subsequently, to allow a direct comparison. Stepwise addition of reagents to the reaction mixture indicated that neither F_2dCTP nor RTPR interferes with electrode readings. Approximately 2.2 equiv of fluoride ion/RTPR are released after 1 min of reaction (Figure 3). The fluoride electrode has a long equilibration time (20−40 s), which is a function of initial and final F^- concentrations. To obtain an estimate of the electrode dead time under the reaction conditions, a control experiment was performed where NaF solution was added to generate similar variations in F^- concentration as those observed in the enzymatic reaction (Figure 3). In the first 10 s of reaction the two curves are indistinguishable. At later time points (between 20 and 60 s) the changes in reading in the actual reaction are slower than in the case of direct addition of F^-. Examination of the data suggests that release of the first F^- equivalent occurs within the instrument deadtime and that the second equivalent of fluoride is released at a much slower rate. (This analysis assumes that once F^- is eliminated from the substrate it equilibrates with bulk solution and is detected by the electrode; this assumption may or may not be valid.).

Studies of the Cobalamin−RTPR Adduct: *Stability of the Label to Denaturation*. As discussed above, inactivation of prereduced RTPR with F_2dCTP leads to its labeling with a cobalamin moiety. Since the X-ray structure of RTPR is not available, there is great interest in obtaining information about the cofactor binding site. Therefore, considerable effort was spent trying to establish that the protein is covalently labeled and the exact position of labeling.

To determine the optimal pH for protease digestion, solutions of labeled protein in 6 M guanidine hydrochloride were prepared at pHs from 5.0 to 9.0. They were incubated for 12 h at 4 °C and concentrated using a Centricon 30. The UV−vis spectra of the filtrates (which contained hydrolyzed label in the form of hydroxocobalamin/aquocobalamin) showed that label loss increases with increasing pH (data not shown). While no attempt was made to study the kinetics of label hydrolysis, the results suggested that minimization of label loss could be achieved by digestion at low pH (5−6).

The labeled protein was then incubated for 12 h in 2 M urea and 100 mM KP_i, pH 5.8 at 37 °C, conditions required for protease digestion, and concentrated using a Centricon 30. Only 75% of the protein is recovered in a soluble form under these conditions, with 80% of the label associated with RTPR. These conditions were thus chosen to digest the partially denatured, labeled RTPR and to isolate a labeled peptide that could be sequenced.

Digestion Studies of the Labeled Peptide. Based on these stability studies, endoproteinase Glu-C from *Staphylococcus aureus* V8 was selected for RTPR digestion. RTPR (30 μM) was treated with endoproteinase Glu-C (1:40 w/w) in 2 M urea in phosphate buffer (pH 5.8) for 16 h at 37 °C in the dark. The peptides were then chromatographed on a Vydac C_{18} column. Standard methods using 1% trifluoroacetic acid led to release of the labeled chromophore as aquocobalamin during the chromatography. The HPLC separation was thus run using 1 mM NH_4OAc, pH 5.8 (solvent A), and 80% CH_3CN and 20% solvent A (solvent B). Under these conditions the HPLC chromatogram shows a major peak (t_r = 44.7 min) with an UV−vis spectrum similar to that of the adduct (Figure 4A). The fractions containing this material were reanalyzed by HPLC using a shallower elution profile and once again a single symmetric peak (t_r = 45.1 min) was observed (Figure 4B). From the digestion of 72 nmol of labeled RTPR (quantitated assuming ϵ_{524} = 8000 M^{-1} cm^{-1}), through the two chromatographic steps, 6.0 nmol of a single peptide was recovered.

The peptide(s) containing the chromophore was submitted for sequencing. In this experiment, cycles 1−8 indicated the sequence ISLANGEP, identical to residues 411−418 of RTPR, and cycle 9 gave no amino acid, consistent with the presence of an unmodified or labeled cysteine. Cycle 10 indicated an N, consistent with the predicted N420. Taken together, these results suggest that the labeled peptide is composed of residues 411−423 of RTPR. The most likely site of modification by the corrin would be C419 and, as described above, the UV−vis spectrum of the modified peptide is consistent with a Co−S adduct.

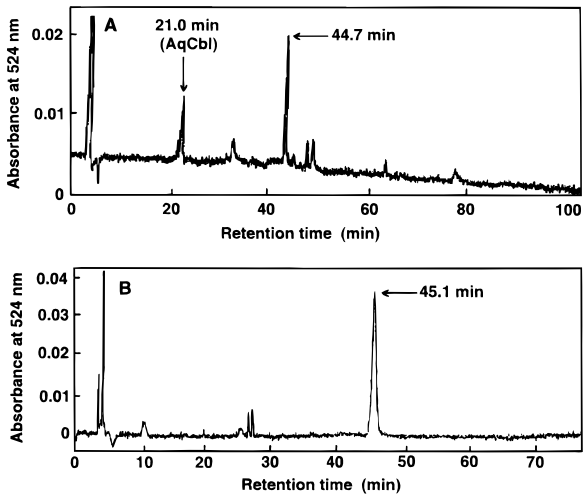


FIGURE 4: (A) HPLC analysis of the Glu-C digest of RTPR inactivated by F_2dCTP. The chromatography conditions are described in the Methods section and the effluent was monitored at 524 nm. The peak at 21 min is AqCb1. (B) HPLC purification of the peak observed at 44.7 min in trace A.

The labeled peptide was submitted for electrospray ionization (EI) mass spectrometric analysis (*17*), to minimize fragmentation. GlutCbl was also analyzed to determine whether this method is suitable for compounds containing Co−S bonds. Both GlutCbl and labeled peptide underwent significant fragmentation upon ionization and desorption. However, analysis of the spectra still proved informative. The spectrum of GlutCbl gave rise to a low-intensity $(M + 2H)^+$ peak at 1637.3 (predicted *m*/*z* of 1637.7) and a doubly charged peak $(M + 3H)^{2+}$ at $m/z = 819.2$ (expected *m*/*z* of 819.4). The spectrum of the labeled peptide gave a peak at $m/z = 2735$, consistent with the $(M + 3H)^+$ for the conjugate of cobalamin and peptide 411−423 (expected *m*/*z* value of 2735.9). Peaks for $(M + 4H)^{2+}$ ($m/z = 1368$; expected 1368.5) and $(M + 5H)^{3+}$ ($m/z = 912$; expected 912.6) were also observed. These results are consistent with the presence of an adduct between cobalamin and peptide 411−423. The spectrum showed no sign of uncomplexed peptide 411−423, which has an expected $(M + 3H)^+$ of 1406.6. This peak should have been observed if the unmodified peptide 411−423 was present in the sample.

To determine if the site of attachment of cobalamin to peptide 411−423 is through cysteine 419, the UV−vis spectrum and the kinetics of the interaction of labeled peptide and GlutCbl with CN^- (10 equiv) were examined at pH 5.8 and 30 °C. Both compounds were converted to cyanocobalamin with λ_{max} at 356 and 550 nm. Furthermore, the kinetics of its formation were identical, adding further support that cofactor is attached to the peptide through C419 and that GlutCbl is a good model for the labeled peptide. The products of these reactions were analyzed by HPLC. In both cases a new peak with retention time identical to that of vitamin B_{12} was detected. The reaction mixture resulting from CN^- treatment of the labeled peptide was examined by MS-EI and the analysis indicated a peak at $m/z = 1406.3$, consistent with the expected $(M + 3H)^+$ peak for peptide 411−423 ($m/z = 1406.6$). A peak consistent with $(M + 4H)^{2+}$ peak was also observed at $m/z = 703$ (expected value 703.8). The presence of these peaks is consistent with the model where peptide 411−423 is liberated once it is treated with CN^-. As noted above, these peaks are absent from the EI spectrum of intact labeled peptide.

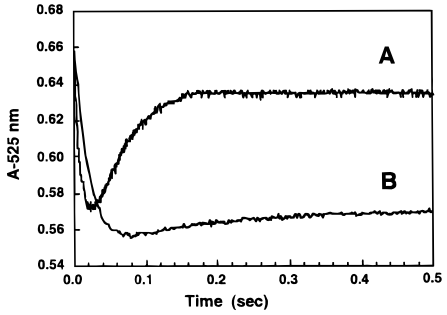


FIGURE 5: (A) SF experiment monitoring cob(II)alamin formation on interaction of CTP with RTPR and AdoCbl. The reaction was monitored at 525 nm. (B) SF experiments monitoring cob(II)alamin formation on interaction of F_2dCTP with RTPR and AdoCbl. The reaction was monitored at 525 nm from 0 to 0.5 s.

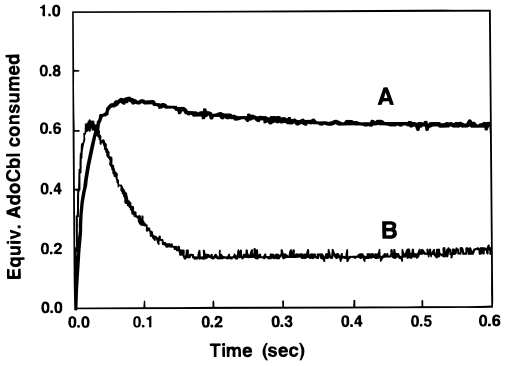


FIGURE 6: Equivalents of AdoCbl consumed during interaction of RTPR with (A) F_2dCTP and (B) CTP at 37 °C. This normalization assumes that only a single reaction is occurring in the first 200 ms.

Spectroscopic Studies: *Stopped-Flow UV−Vis Spectroscopy*. The detection of 5′-dA in the inactivation mixture of RTPR with F_2dCTP, suggested that SF experiments could be insightful in the investigation of the kinetics of AdoCbl consumption during the reaction. To allow comparison of the chemistry with the normal substrate to that with the inhibitor, the SF experiments were performed with CTP as well as F_2dCTP, using dATP as an allosteric effector. Typical experiments contained AdoCbl and dATP in one syringe and prereduced RTPR and CTP (or F_2dCTP) in the second syringe. The formation of cob(II)alamin was monitored at 525 nm (Figure 5). Assuming that $\Delta\epsilon_{525} = 4800$ M^{-1} cm^{-1} for each reaction, an assumption that is valid only in the early stages of the reaction (through 200 ms), the changes in absorbances at 525 nm can be converted to equivalents of consumed AdoCbl, as shown in Figure 6.

In the case of CTP, cob(II)alamin is formed with a k_{obs} of 130 s^{-1}, reaches a maximum, and then decreases to a steady-state level with a rate constant of 20 s^{-1} (Figure 5A). Similar studies monitoring cob(II)alamin with F_2dCTP are more complex (Figure 5B). As demonstrated above, a Co−S bond is formed at some time during the inactivation process, apparent from our isolated peptide. However, this process most probably occurs on a slow time scale after the initial formation of cob(II)alamin. A single-exponential fit to the 2−50 ms time frame of this trace, gives a rate constant of 50 s^{-1}. The kinetics are, however, clearly complex, and since multiple reactions are occurring concurrently, additional fits

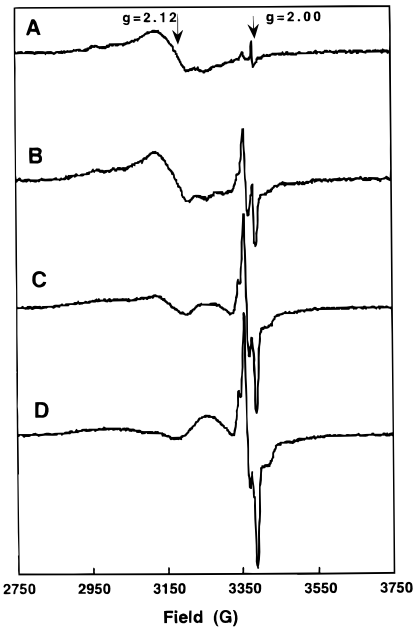


FIGURE 7: Overlay of RFQ-EPR spectra from the reaction of F_2-dCTP with RTPR. Experimental conditions are given in the Methods section and the quenching times were as follows: (A) 22 ms; (B) 78 ms; (C) 141 ms; (D) 255 ms.

Table 1: Spin Quantitation Derived from EPR Experiments

sample	spin quantitation (μM)	predicted spin[a] (μM)	% spin
22 ms	130	150	87
78 ms	199	215	93
141 ms	187	203	92
255 ms	181	193	94
20 s	108	300	36

[a] Predicted spin is derived from the SF trace, assuming that consumption of 1 equiv of AdoCbl leads to formation of 2 equiv of new radicals.

have thus far not been attempted. The kinetic complexity of the reaction is apparent when the same reactions are monitored at 475 nm (data not shown). A graph of the ratio of $\Delta A_{525}/\Delta A_{475}$ as a function of time (after 100 ms) (data not shown), differs from the expected value of 1.41 for AdoCbl conversion to cob(II)alamin, suggesting that one or more species different from "free" cob(II)alamin are formed upon AdoCbl consumption.

RFQ-EPR Experiments. F_2dCTP is the first mechanism-based inhibitor identified where the cofactor is converted to cob(II)alamin and additional species at such a rapid rate. RFQ-EPR experiments were therefore carried out with quenching at 22, 78, 141, and 255 ms, in an attempt to follow the evolution of radical species in the system. The spectra are represented in Figure 7. A sample at 20 s was also generated. The spin concentration of each sample was determined by the double integration method using a $CuSO_4$ standard and assuming a packing factor of 0.7 for the RFQ samples. The packing factor is derived from a control experiment where EPR samples of $CuSO_4$ were prepared by the RFQ method and their experimental spin concentrations were compared to the known values. The results are summarized in Table 1.

The EPR spectrum of the 22 ms RFQ-EPR sample is very similar to that previously observed in both the exchange reaction and the normal reduction reaction. The g_{eff} of 2.12 and the cobalt hyperfine of 48 G is indicative of the previously characterized thiyl radical exchange coupled to cob(II)alamin (*18*, *20*). Even more exciting is the observation that the putative thiyl radical-cob(II)alamin disappears, and at 140 and 255 ms, two new signals with features associated with cob(II)alamin (broad absorption at $g = 2.3$) and an organic radical ($g = 2.0$) are detected. The total number of spins does not appear to change between the first time point and the 255 ms time point, suggesting that the first species gives rise to the second species (Figure 7). The second species could be a nucleotide-based radical and cob(II)alamin, where the two compounds experience only dipolar interactions. At the 20 s time point the number of spins has decreased (Table 1), consistent with the "slow" rate of conversion of a cob(II)alamin-type species into the observed covalently bound adduct with C419.

DISCUSSION

F_2dCTP is unique with respect to other 2′-substituted nucleotides previously studied with RTPR in that the carbon−cobalt bond of AdoCbl is homolyzed with an apparent rate constant of 50 s^{-1}, very close to that observed with CTP, 130 s^{-1}, under similar conditions. Furthermore, given that the experiments have not yet been carried out with saturating F_2dCTP, the observed apparent rate constant is a lower limit for homolysis. The requirement that cob(II)alamin formation must be accompanied by formation of a second paramagnetic species suggested that EPR spectroscopic analysis of this system could be mechanistically informative and could facilitate detection of the elusive nucleotide radical intermediate. RFQ EPR studies have revealed this to be the case. By 78 ms, 0.7 equiv of cob(II)alamin/RTPR are present (1.4 equiv of paramagnetic species) by SF UV−vis spectroscopic analysis, and the predominant species observed by EPR spectroscopy appears to be thiyl radical exchange coupled to cob(II)alamin. The signal has a g value of 2.12, with cobalt hyperfine of ≈48 G. A similar signal has previously been reported by Orme-Johnson et al. (*19*) and Licht et al. (*20*) in studies on RTPR mediated washout of ^{3}H from [5′-^{3}H]AdoCbl, the exchange reaction, and in the nucleotide reduction process. Studies of both these reactions using RTPR containing (β-^{2}H) cysteines and simulation of the observed signal provided the first direct assignment of this species to a thiyl radical exchange coupled with cob(II)alamin (*20*, *18*). In the case of F_2dCTP, the amount of cob(II)alamin is approximately equal to that observed with CTP with the maximum amount of signal appearing at 78 ms rather than at 10 ms. In addition, the signal associated with the CTP reaction decreases to a steady-state level of 0.1 equivalents within 150 ms, while that with F_2dCTP changes slowly into a nonparamagnetic species with an unusual chromophore derived from cobalt−sulfur bond formation. In the F_2dCTP reaction, from the initial time point of 22 ms until 255 ms, the spin quantitation does not change, but the signal associated with the thiyl radical and cob(II)alamin disappears and is replaced with new signals with features at $g = 2.3$ and 2.00, similar to those of free cob(II)alamin and an organic radical. From 250 ms to 20 s the signal does not change in appearance, but the total amount of paramagnetic species is reduced by ≈70%. The simplest interpretation

of these results is that the thiyl radical gives rise to a nucleotide-based radical and cob(II)alamin, both of which become quenched.

We hoped that assignment of a structure to the new radical associated with the feature at $g = 2.00$ would be facilitated by determination of the structure of the nucleotide radical generated by the interaction of F_2dCDP with the class I *E. coli* RDPR. *E. coli* RDPR is also stoichiometrically inactivated by F_2dCDP (*4*). Inactivation with prereduced RDPR in the absence of reductant results from loss of the essential tyrosyl radical and, among other events, formation of a new nucleotide radical. If the radical(s) generated by class I and class II reductases are identical, then structural identification of the radical associated with the class I enzyme should facilitate simulation of the species detected with the class II RTPR, which is of necessity more complex due to the presence of cob(II)alamin. Unfortunately at present, the detailed mechanism(s) of inactivation of neither class of RNR is sufficiently well understood to allow a direct comparison.

Determination of the fate of the nucleotide and the structure of the new radical(s) in the case of both RNRs requires synthesis of isotopically labeled F_2dCTP (F_2dCDP) and use of high-field, 140 GHz, EPR spectroscopic analysis. The rapidity with which the *L. leichmannii* RNR carries out the inactivation holds promise that we may be looking at a radical closer in chemistry to the proposed 3′-nucleotide radical thought to initiate the inactivation process. This contrasts with the results from the inactivation of the *E. coli* RNR by F_2dCDP, which occurs on the second scale. Recently, using 2′-deoxy-2′-fluoromethylenecytidine 5′-diphosphate (FMdCDP; Figure 1, **4**) and the *E. coli* RDPR, we have detected a 3′-nucleotide radical for the first time. These observations, and the amazing similarities between the class I and class II reductases, make it likely that the initial step in catalysis for nucleotides or nucleotide analogues are very similar. Thus we believe our EPR data are providing the first direct evidence that a thiyl radical can generate in a kinetically competent fashion a nucleotide radical. Only isotopic labeling and simulation of the observed signals will provide unambiguous confirmation of this proposal. We believe that our data with F_2dCTP provide compelling support for our mechanistic model that the function of the thiyl radical is to generate a nucleotide radical. This is an important step forward mechanistically in that similar studies with normal substrates NTPs and a variety of allosteric effectors at differing pHs have failed to reveal, thus far, any direct observation of nucleotide radicals. This result is perhaps not surprising given the rapidity of the proposed steps in nucleotide reduction (*21*) and hence the unlikelihood of the accumulation of any intermediate, a requirement for its detection.

One additional observation, the formation of an adduct with a Co−S bond, requires comment. Similar adducts, defined spectroscopically, have recently been observed with 2′-deoxy-2′-fluoromethylenecytidine 5′-triphosphate (FMdCTP) and 2′-deoxy-2′-methylenecytidine 5′-triphosphate (MdCTP; Figure 1, **5**). What is unique to F_2dCTP is our ability to recover in 20% yield a peptide containing the adduct attached to C419. The lability of the Co−S linkage and the observation of a single peptide containing an active-site residue is intriguing and suggests that chemistry to generate this linkage occurs within the active site. These results contrast with those observed during the inactivation of RTPR by FMdCTP and MdCTP, where the label is much more randomly distributed (*22*) and where the most abundant labeled peptide contains C736, known by analogy with *E. coli* RDPR to reside on the surface of RTPR. In these cases formation of the Co−S bond appears to occur from solution. While a clear interpretation of these results awaits a structure of RTPR, these studies help define the architecture of the active site of RTPR, specifically that cob(II)alamin has access to at least one cysteine (419) that provides the reducing equivalents and is located on the α face of the nucleotide. This is an unexpected and intriguing result in that simulation of the RFQ-EPR spectra of the initially formed radical pair also suggest that cob(II)alamin is ≈6.5 Å from C408 on the β face of the nucleotide.

Our previous studies using site-directed mutants and mechanism-based inhibitors with both class I and II RNRs have allowed us to propose that, despite the lack of primary sequence homology, the active site of RTPR may well be in a very similar secondary and tertiary architecture to that observed with RDPR. Thus we can draw upon the recent structural data on the R1 subunit of RDPR to provide us with a rationalization of the above results. From the X-ray structure of oxidized *E. coli* RDPR R1, the three active-site cysteines, with C225 and C462 present as a disulfide, are within 6 Å of each other (*23*). Recent studies with the reduced form of RDPR R1 suggest a flexiblity within this active site, especially for C462, which is found 6 Å from C225 (*24*). Since C419 in RTPR is expected to be the equivalent of C462 of RDPR, this suggests that this class II RNR may also possess conformational flexibility within the active site. While the thiol of C419 must be within bonding distance of cob(II)alamin, the slow rate of formation of the Co−S bond could be associated with a slow conformational change consistent with this proposed flexibility. The combination of the present results and our previous results limits the location of the cofactor relative to the active-site cysteines and provides further support for direct involvement of the cofactor within the active site in the B12-dependent system.

REFERENCES

1. Plunkett, W., Huang, P., Xu, Y.-Z., Heinemann, V., Grunewald, R., and Gandhi, V. (1995) *Semin. Oncol. 22*, 3−10.
2. Hertel, L. W., Kroin, J. S., Grossman, C. S., Grindey, G. B., Dorr, A. F., Storniolo, A. M. V., Plunkett, W., Gandhi, V., and Huang, P. (1996) *ACS Symp. Ser. 639*, 265−278.
3. Baker, C. H., Banzon, J., Bollinger, J. M., Jr., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. (1991) *J. Med. Chem. 34*, 1879−1884.
4. van der Donk, W. A., Yu, G., Perez, L., Sanchez, R. J., and Stubbe, J. (1998) *Biochemistry*, in press.
5. Booker, S., and Stubbe, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8352−8356.
6. Booker, S., Licht, S., Broderick, J., and Stubbe, J. (1994) *Biochemistry 33*, 12679−12685.
7. Lunn, C. A., Kathju, S., Wallace, B. J., Kushner, S., and Pigiet, V. (1984) *J. Biol. Chem. 259*, 10469−10474.
8. Russel, M., and Model, P. (1985) *J. Bacteriol. 163*, 238−242.
9. Stubbe, J., and Kozarich, J. W. (1980) *J. Biol. Chem. 255*, 5511−5513.
10. Malmström, B., Reinhammar, B., and Vänngård, T. (1970) *Biochim. Biophys. Acta 205*, 48.
11. Steeper, J. R., and Steuart, C. D. (1970) *Anal. Biochem. 34*, 123−130.

12. Bollinger, J. M., Jr., Tong, W. H., Ravi, N., Huynh, B. H., Edmondson, D. E., and Stubbe, J. (1995) *Methods Enzymol. 258*, 278−303.
13. Harris, G., Ator, M., and Stubbe, J. (1984) *Biochemistry 23*, 5214−5225.
14. Ashley, G. W., Harris, G., and Stubbe, J. (1986) *J. Biol. Chem. 261*, 3958−3964.
15. Ashley, G. W., Harris, G., and Stubbe, J. (1988) *Biochemistry 27*, 4305−4310.
16. Harris, G., Ashley, G. W., Robins, M. J., Tolman, R. L., and Stubbe, J. (1987) *Biochemistry 26*, 1895−1902.
17. Nelson, R. M., and Long, G. M. (1989) *Anal. Biochem. 180*, 147−150.
18. Gerfen, G. J., Licht, S. L., Willems, J.-P., Hoffman, B. M., and Stubbe, J. (1996) *J. Am. Chem. Soc. 118*, 8192−8197.
19. Orme-Johnson, W. H., Beinert, H., and Blakley, R. L. (1974) *J. Biol. Chem. 249*, 2338−2343.
20. Licht, S. L., Gerfen, G. J., and Stubbe, J. (1996) *Science 271*, 477−481.
21. Licht, S., and Stubbe, J. (1998) in *Comprehensive Natural Products Chemistry* (Barton, D., and Nakanishi, K., Eds.) Pergamon press, in press.
22. Lawrence, C., and Stubbe, J. (1998), manuscript in preparation.
23. Uhlin, U., and Eklund, H. (1994) *Nature 370*, 533−539.
24. Ericksson, M., Uhlin, U., Ramaswamy, S., Ekberg, M., Regnstrom, K., Sjöberg, B. M., and Eklund, H. (1997) *Structure 5*, 1078−1092.

BI972934E