Pergamon

Tetrahedron, Vol. 53, No. 35, pp. 12005-12016, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00713-8

MECHANISM BASED INACTIVATION OF THE ADENOSYLCOBALAMIN-DEPENDENT RIBONUCLEOTIDE REDUCTASE FROM *L. LEICHMANNII* BY 2'-ARA-2'-AZIDO-2'-DEOXY ADENOSINE-5'-TRIPHOSPHATE: OBSERVATION OF PARAMAGNETIC INTERMEDIATES

Gary W. Ashley, Christopher C. Lawrence and JoAnne Stubbe*

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

Morris J. Robins*

Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602-5700

Abstract:
Ribonucleotide reductases catalyze the reduction of nucleotides to deoxynucleotides in all organisms. 2'-Ara-2'-azido-2'-deoxyadenosin-5'-triphosphate ($araN_3ATP$) is a potent mechanism based inhibitor of the AdoCbl-dependent enzyme from *Lactobacillus leichmannii* (RTPR). Incubation of RTPR with [3'-^{3}H]- or [β-^{32}P]- or [8-^{3}H]-$araN_3ATP$ reveals that inactivation is accompanied by covalent labeling, formation of cob(II)alamin and 5'-deoxyadenosine in a ratio of 0.4:0.4:0.4 equivalent/equivalent of RTPR. During this inactivation at least three paramagnetic species are detected by EPR spectroscopy. RTPR inactivation by $araN_3ATP$, in contrast to all other mechanism based inhibitors, does NOT appear to involve 3'-carbon-hydrogen bond cleavage. © 1997 Elsevier Science Ltd.

INTRODUCTION

Ribonucleotide reductases play an essential role in DNA biosynthesis catalyzing the conversion of nucleotides to deoxynucleotides.[1,2] The cofactors required for this reduction are unique chemically and distinctive phylogenetically. They share in common, however, a way to initiate a radical dependent mechanism for nucleotide reduction, through a distinct protein radical that they generate. The reductase from *E. coli*, mammals, and Herpes Simplex virus utilize nucleoside diphosphates as substrates (RDPRs). They contain a dinuclear-iron center and a tyrosyl radical cofactor[3] located on one subunit whose function is proposed to generate a thiyl radical on the second subunit.[4,5] The reductase from *L. leichmannii* (RTPR) utilizes nucleoside triphosphates as substrates and adenosylcobalamin (AdoCbl) as a cofactor. RTPR catalyzes the conversion of this cofactor to cob(II)alamin and a thiyl radical in a kinetically competent fashion.[6,7] In both systems, the thiyl radical is then proposed to mediate hydrogen atom abstraction from the 3'-position of the nucleotide to initiate the nucleotide reduction process.[1,4]

Over the past decade three approaches have been taken to investigate the mechanism of these unusual reduction processes. The first involves using the normal substrate, wild-type enzyme, and standard physical organic chemistry methods. This approach has established the requirement for 3'-carbon-hydrogen bond cleavage of the nucleotide and that the hydrogen abstracted from the 3'-position in the nucleotide is returned to the same position of the deoxynucleotide.[8,9]

The second involves use of protein analogs, site directed mutants. This approach, in conjunction with biochemical studies, has suggested that five cysteines are required for catalysis: two that provide the reducing

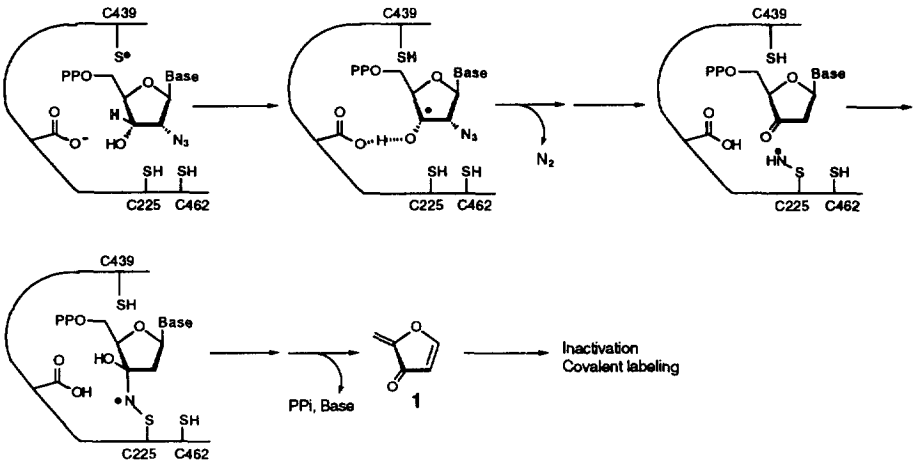
equivalents required for substrate reduction, two on the surface of the protein that shuttle reducing equivalents in and out of the active site from thioredoxin, the protein reductant used *in vivo*, and a fifth thiol which is converted to a thiyl radical by the metallocofactor and initiates nucleotide reduction via 3'-hydrogen atom abstraction.[10-15]

The third approach uses substrate analogs, most of which are mechanism based inhibitors. Studies with 2'-chloro-2'-deoxynucleotides have suggested that 3'-ketodeoxynucleotides may be intermediates during the reduction of normal nucleotide substrates.[16,17] Studies on the inactivation of RDPR with 2'-azido-2'-deoxynucleoside diphosphates,[17-21] gemcitabine diphosphate[22] and 2'-fluoromethylene-2'-deoxycytidine diphosphate[23] have provided evidence that radical intermediates are generated during nucleotide reduction. With the RTPRs, however, such evidence has been lacking. In the case of nucleotide analogs examined thus far, all become inhibitors only after enzyme mediated 3'-hydrogen atom abstraction. The present study reports an unprecedented mechanism of inhibition of the *L. leichmannii* RTPR by 2'-ara-2'-azido-2'-deoxyadenosine-5'-triphosphate (araN$_3$ATP). Two equivalents of inhibitor result in ~90 % loss of the catalytic activity and a major mode of inactivation does not appear to be accompanied by 3'-carbon-hydrogen bond cleavage. In addition, several new radical species have been detected during the inactivation, thus providing evidence for RTPR's ability to mediate radical-dependent chemistry.

RESULTS

Time-dependent Inactivation of RTPR by 2'-Azido-2'-deoxynucleotides. 2'-Substituted nucleoside-5'-diphosphates have provided much information about the catalytic capabilities of the *E. coli* RDPR. Many of these nucleotides cause inactivation, requiring enzyme-mediated hydrogen atom abstraction from their 3'-position. The subsequent chemistry then deviates from the normal reduction process in a fashion dependent on the 2'-substitution. Previous studies with 2'-azido-2'-deoxyuridine- and 2'-azido-2'-deoxycytidine-5'-diphosphates (N$_3$UDP, N$_3$CDP) revealed that they are potent mechanism based inhibitors of *E. coli* RDPR (Scheme 1).[17-21] Based on the similarities between RDPR and the RTPR from *L. leichmannii*, we decided to examine the interaction of the corresponding triphosphates with RTPR in the hope of obtaining evidence for nucleotide radical intermediates. Both compounds exhibit time-dependent inactivation at millimolar concentrations with multiphasic kinetics (Figure 1). The slow rates of inactivation and the kinetic complexity, however, made us unenthusiastic about pursuing detailed mechanistic analyses of these reactions.

Time-dependent Inactivation of RTPR by araN$_3$ATP. Whereas *E. coli* RDPR does not accomodate the presence of substituents at the 2' position on the β face of the nucleotide, RTPR appears to be much more tolerant.[24] We therefore decided to investigate the interaction of araN$_3$ATP with RTPR. Incubation of RTPR with a five-fold excess of araN$_3$ATP and AdoCbl resulted in rapid time-dependent inactivation (Figure 1). The rate of inactivation was enhanced in the presence of dGTP, a typical allosteric effector for ATP reduction, and was insensitive to inclusion of a thioredoxin, thioredoxin reductase, NADPH reducing system in the inactivation mixture. In contrast to results with the well characterized 2'-halo-2'-deoxynucleotides,[16] the presence of dithiothreitol (DTT, 30 mM), that can be used in place of thioredoxin as a reductant, had no effect on the rate of inactivation. The biphasic nature of the inactivation has thus far precluded a detailed kinetic analysis, but suggests multiple modes of inactivation.



Scheme 1. Proposed mechanism of inactivation of the *E. coli* RDPR by 2'-azido-2'-deoxynucleotides.

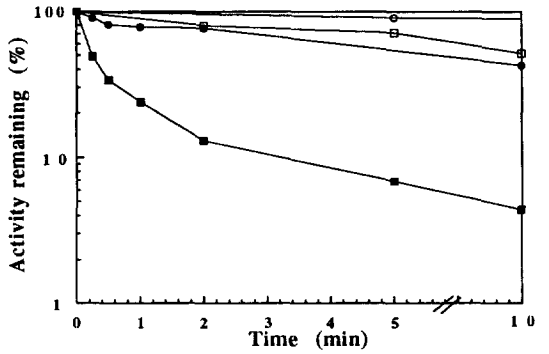


Figure 1. Time dependent inactivation of RTPR (25 μM) by 2'-azido-NTPs. ○, Control; ●, 260 μM N_3ATP; □, 260 μM N_3UTP; ■, 125 μM araN_3ATP; the reductant in each case is 3 mM DTT, other conditions as in the Experimental section.

Synthesis of [3'-^{3}H]-araN_3ATP and [β-^{32}P]-araN_3ATP. In order to investigate the mechanism of inactivation, isotopically labeled araN_3ATPs were prepared. Previous studies with ClUDP and N_3UDP and the *E. coli* reductase and with ClUTP and araClUTP with RTPR, suggested that 3'-carbon-hydrogen bond cleavage was required for catalysis (Scheme 1) and that PPi (PPPi) was released during enzyme inactivation.[16,20,24] Thus the targets for synthesis were [3'-^{3}H]-araN_3ATP and [β-^{32}P]-araN_3ATP.

Although araN_3A has been prepared previously,[25] the requirement for specifically radiolabeled material led us to a new synthetic approach. [3'-^{3}H]-Adenosine is readily prepared from [3'-^{3}H]-uridine as previously described.[9] Following the procedure of Wagner *et al.*, 2'-tosyladenosine was prepared from adenosine.[26] Treatment of the

tosylate with sodium azide gave a 1:1 mixture of adenine and araN$_3$A, whereas use of lithium azide gave only adenine. Purification of the nucleoside was achieved using a Sephadex LH-20 column, giving product of sufficient purity such that it could be crystallized. The material isolated was identical by ^{1}H-NMR, TLC on silica and reverse phase HPLC to that previously prepared.[25]

Phosphorylation to the monophosphate and subsequently to the di- and triphosphates, was carried out by standard procedures.[27,28] The diphosphate was incubated with ^{32}Pi and polynucleotide phosphorylase to make [β-^{32}P]-araN$_3$ADP, and subsequently with UTP and NDP kinase to give [β-^{32}P]-araN$_3$ATP. Purification was achieved using DEAE-Sephadex A25 followed by ion-pairing C_{18}-reverse phase HPLC and a second chromatography on DEAE-Sephadex A25, giving [β-^{32}P]-araN$_3$ATP in 30 % yield (specific radioactivity *ca.* 1 x 10^7 cpm/μmol).

Species Bound to RTPR after Inactivation. In order to determine if 3'-carbon-hydrogen bond cleavage, detected as ^3H_2O, was required for inactivation, and if release of inorganic triphosphate accompanied inactivation, RTPR was treated with [3'-^{3}H/β-^{32}P]-araN$_3$ATP (^{3}H/^{32}P = 1.02) and the products of inactivation were examined. One portion of the inactivation mixture was placed on a Sephadex G-50 column and the isolated protein assayed for activity and analyzed by scintillation counting. RTPR was >90 % inactivated and contained ~0.4 equivalents of each radiolabel (^{3}H/^{32}P = 1.04). This is a surprising result indicating that the bound species has retained its 3'-hydrogen. When [3'-^{3}H/β-^{32}P]-araN$_3$ATP-inactivated RTPR was denatured using 6 M guanidine hydrochloride, ~60 % of the bound ^{3}H and ^{32}P were released from the protein; treatment of a separate ^{32}P labeled sample with SDS buffer and β-mercaptoethanol at 100 °C for four minutes resulted in loss of ~75 % of the label. Thus far we have been unable to identify the labeled material(s) released. These data suggest, in contrast to expectations, that a major mode of inactivation does not involve 3'-carbon-hydrogen bond cleavage.

A second portion of the inactivation mixture was analyzed for ^3H_2O using bulb-to-bulb distillation. A small quantity of ^3H_2O (0.03 - 0.07 equivalents per mole of RTPR) was observed in eight different experiments. The observed radioactivity was *ca.* five fold above the control experiment done in the absence of RTPR. The same sets of samples were analyzed for P^{32}PPi release. These experiments always have radioactivity in the control done in the absence of RTPR and these results suggest that if PPPi is formed, it is produced in quantities less than 0.15 equivalents with respect to RTPR.[16,24] The difficulties associated with the analytical chemistry and the reproducibility of the small amounts of ^3H_2O, suggest the possibility that a minor pathway for inactivation could be occurring that involves chemistry similar to that observed in Scheme 1.

Is Adenine Released? Previous studies with mechanism based inhibitors such as ClNTPs have shown that the nucleic acid base is readily released during the inactivation process.[16,17] To investigate this possibility, [8-^{3}H]-araN$_3$ATP was prepared. Once again, inactivation with RTPR resulted in 0.4 equivalents of label bound per equivalent of RTPR. HPLC analysis of the fractions containing small molecules, obtained by Centricon-30 filtration, resulted in recovery of only araN$_3$ATP and adenine in quantities approximately equivalent to a control experiment. Thus less than 0.25 equivalents of adenine appears to be produced. The adenine, triphosphate, and the 3'-hydrogen of araN$_3$ATP appear to be retained by the protein suggesting that the nucleotide remains intact. The

detection of small and variable amounts of adenine suggest, with the ^3H_2O, that a minor pathway could be occurring as well.

Analysis for Azide Release and Chromophore Formation on RTPR at 320 nm. If inactivation of RTPR by araN$_3$ATP proceeds via a mechanism similar to ClUTP or N3UDP for the *E. coli* reductase, it should be accompanied by formation of a new chromophore at 320 nm and azide release. Inactivated protein, subsequent to Sephadex G-50 chromatography, was therefore examined for absorbance at 320 nm. With RTPR inactivated by ClUTP the $A_{280}/A_{320} = 3.0$. In the case of araN$_3$ATP the $A_{280}/A_{320} = 42$ (data not shown). Thus 2-methylene-3-furanone (**1**, Scheme 1) does not appear to be produced during the inactivation consistent with the low levels of ^3H_2O produced and our inability to detect any substantial amounts of adenine and triphosphate above background. Azide release was monitored using several variations of formate dehydrogenase assays previously described by Salowe *et al.*[20] and, once again, none was detected above background levels.

Destruction of Adenosylcobalamin during the Inactivation: Formation of Cob(II)alamin and 5'-Deoxyadenosine. Previous studies of the inactivation of RTPR by ClUTP and araClNTPs, indicated that under certain conditions, the inactivation (performed anaerobically) was accompanied by destruction of AdoCbl and formation of cob(II)alamin and 5'-deoxyadenosine (5'-dA).[24] When a 1:1 complex of RTPR and AdoCbl was treated with araN$_3$ATP under anaerobic conditions, the UV-VIS spectrum of the cofactor rapidly changed to that of cob(II)alamin (data not shown). The time course for cofactor destruction was monitored at 525 nm and showed biphasic kinetics. The initial rapid phase was analyzed by subtraction of the linear slow component followed by plotting the resulting decay in semi-log form. These data indicated a rate constant for decay of 2.6 min^{-1} which was approximately the same as the rate of inactivation under the same conditions. The slow linear phase of decay occurred with a rate constant of 0.27 min^{-1}. This value is roughly equal to the slow rate of cofactor destruction observed with RTPR in the presence of DTT and dGTP.[29] At the conclusion of the experiment the reaction mixture was analyzed for 5'-dA. After addition of [5'-^{3}H]-5'-dA as a tracer, the mixture was separated on HPLC and the 5'-dA quantitated by A_{260} using [^{3}H] as a measure of % recovery. The results suggest that the amount of cob(II)alamin is equivalent to the amount of 5'-dA and is ~0.4 equivalents per equivalent of RTPR.

Detection of Paramagnetic Species during Inactivation of RTPR: EPR Spectroscopy. Formation of cob(II)alamin must be accompanied by production of a second paramagnetic species. Evidence for this new radical(s) was sought using EPR spectroscopy. A solution of araN$_3$ATP, RTPR, AdoCbl, dGTP as an allosteric effector and TR, TRR and NADPH as reductant were incubated for 30 s and quenched in a liquid nitrogen bath. The EPR spectrum of this mixture is shown in Figure 2A. The spectrum is unusual in that it contains features similar to the previously detected spectrum of a thiyl radical exchange coupled to cob(II)alamin[6] (Figure 2B) or a carbon centered radical exchange coupled to cob(II)alamin.[30] In addition, a broad feature at g = 2.33 and a sharper feature at g = 2.00 appear to be associated with two additional species, cob(II)alamin associated with an organic radical by dipolar interactions. This signal is very similar to one recently detected by inactivation of RTPR by 2'-spirocyclopropyl-2'-deoxyCTP (CPdCTP) (Figure 2C).[31] Spin quantitation of the species generated with araN$_3$ATP, using a $CuSO_4$ standard, reveals 88 μM in spin relative to 100 μM RTPR. Given that 0.4 equivalents

(40 μM) of cob(II)alamin are detected by UV-VIS spectroscopy, these results suggest that for every cob(II)alamin formed, there is a second paramagnetic species generated.

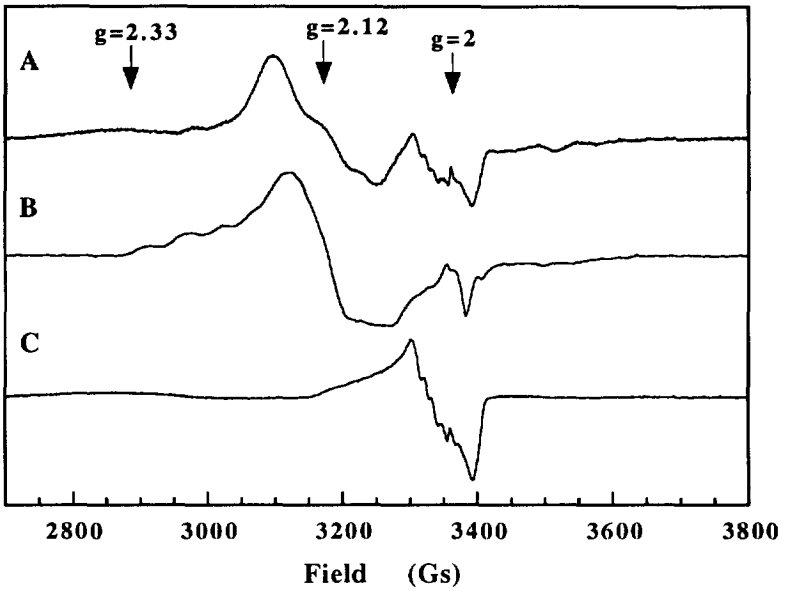


Figure 2. EPR spectra of paramagnetic species generated on interaction of RTPR with a variety of nucleotide analogs. **A** RTPR/araN$_3$ATP inactivation mixture quenched after 30 s incubation; **B** RTPR/ATP reaction mixture quenched at 28 ms by rapid-freeze quenching;[6,7] **C** RTPR/CPdCTP inactivation mixture quenched after 10 min incubation.

DISCUSSION

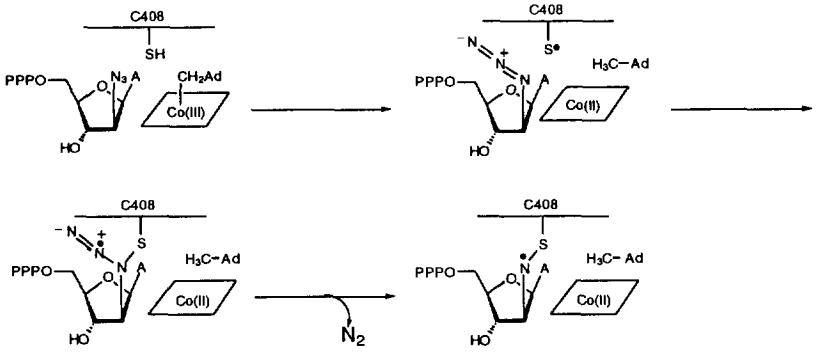
Given the similar behavior of the *E. coli* RDPR and the *L. Leichmannii* RTPR towards other mechanism based inhibitors such as 2'-deoxy-2'-halonucleotides,[16,24] we decided to examine the interaction of RTPR with 2'-azido-2'-deoxynucleotides. Results from studies with the *E. coli* reductase and N$_3$UDP, N$_3$CDP, N$_3$ADP have been reported previously.[17-21] These studies have provided insight into the catalytic capabilities of the RDPRs, specifically their abilities to mediate radical-dependent transformations on the nucleotides.

We examined the interaction of RTPR with N$_3$ATP and N$_3$UTP as well as araN$_3$ATP to determine if they function as inactivators of the enzyme. AraN$_3$ATP, with the azido moiety on the β-face of the nucleotide, inactivates RTPR in time-dependent fashion. N$_3$ATP and N$_3$UTP, with the azido moiety on the α-face, also inactivate RTPR in a time-dependent fashion but at much higher concentrations and at slower rates (Figure 1). We decided, therefore, to

examine the interaction of araN$_3$ATP in some detail given that the rapid inactivation, if accompanied by cob(II)alamin formation, would allow us to look for new paramagnetic species.

Isotopically labeled araN$_3$ATP (3'-^{3}H, β-^{32}P, 8-^{3}H) made it possible to look for product production accompanying inactivation. The results were unexpected and, while still not understood in detail, are thought provoking in nature. First, the requirement for two equivalents of inhibitor to obtain 90 % inactivation and the biphasic nature of this process require multiple pathways for inactivation. Second, in contrast to all the other nucleotide analog mechanism based inhibitors examined to date, araN$_3$ATP's main mode of inactivation does not appear to be initiated by 3'-carbon hydrogen bond cleavage. While very small amounts of ^3H_2O (0.03 - 0.07 equiv) are released when [3'-^{3}H]-araN$_3$ATP is incubated with RTPR, less than 0.25 equivalents of adenine and less than 0.15 equivalents of triphosphate are detected. These results make it unlikely that the major mode of inactivation is associated with a mechanism similar to the paradigm described for all other mechanism based inhibitors (Scheme 1).[23] Third, [^{3}H] is retained on the protein in amounts equivalent to phosphate and adenine with a total of 0.4 equivalents per equivalent of RTPR. This stoichiometry is approximately equivalent to the amount of cob(II)alamin and 5'-dA produced which suggested that at least 0.4 equivalents of a new protein radical could be generated. The observation that 0.4 equivalents of label results in 100 % inactivation of RTPR, requires again multiple modes of inactivation. If there are two pathways, equally represented, then the production of 0.4 equivalents of cob(II)alamin may or may not be associated with the 0.4 equivalents of the label attached to the protein.

An additional clue to the chemistry leading to inactivation of RTPR is provided by detection of several paramagnetic species during this process (Figure 2A). The identity of these species is at present unknown and analysis is complicated by the presence of two paramagnetic species adjacent to one another in the same active site cavity, cob(II)alamin and an organic radical that can potentially interact with each other by exchange coupling and dipolar interactions. In fact, the spectrum suggests, based on the recently characterized spectra of thiyl radical/cob(II)alamin in RTPR[6,7] (Figure 2B) and carbon centered radical/cob(II)alamin in glutamate mutase,[30] that the signal at g = 2.12 is arising from an exchange coupled system. There appears to be at least two additional features in the spectrum: one at g = 2.33 and one at g = 2.00, that could arise from cob(II)alamin interacting with an organic radical via dipolar interactions where the two species are farther removed from one another than in the



Scheme 2. One possible mechanism of RTPR inactivation by araN$_3$ATP leading to formation of paramagnetic species.

exchange coupled system. Identification of the organic contributor(s) to these signals requires isotope labeling experiments of both the protein, specifically with [β-^{2}H] cysteines, and the substrate analog.

One possible model for one of the mechanisms of inactivation is shown in Scheme 2 and assumes that all the products produced are associated with a single pathway (as discussed above, this may or may not be a valid assumption). In this model, binding of araN$_3$ATP triggers cleavage of the carbon cobalt bond of AdoCbl to generate a thiyl radical on C408 and cob(II)alamin. In contrast to all of the other 2'-substituted mechanism based inhibitors thus far examined, this thiyl radical does not appear to abstract a hydrogen atom from C-3' of the nucleotide. Perhaps due to an altered sugar pucker and the steric constraints imposed by the azido moiety on the β-face of the nucleoside, we propose that the thiyl radical adds to the alkyl azide to form a covalent complex. This reaction could liberate nitrogen gas and generate a nitrogen centered radical. This chemistry is similar to that previously proposed to account for the inactivation of *E. coli* RDPR by N$_3$UDP[21] (Scheme 1) and has been postulated to occur in a model system in which a putative thiyl radical interacts with alkyl azides.[32,33] The intriguing possibility exists, therefore, that the azido moiety is acting as a radical trap for the protein thiyl radical that initiates catalysis. Studies using [β-^{2}H]-cysteine RTPR and [^{15}N]-araN$_3$ATP should provide support for or against this model.

EXPERIMENTAL

Materials.

RTPR was isolated from *L. leichmannii* and over-producing strain pSquire/HB101 as reported previously.[34,35] The specific activity of RTPR was 0.8 - 1.5 U/mg (ε_{280} = 101000 M^{-1}cm^{-1}). Pre-reduced RTPR was prepared as previously described.[10] TR and TRR were isolated from over-producing strains SK3981 and K91/pMR14 with specific activities of 36 U/mg and 1000 U/mg respectively.[36,37] 'Carrier-free' $H_3{}^{32}PO_4$ was obtained from New England Nuclear. [3'-^{3}H]-Uridine was prepared as described previously.[38] $POCl_3$, triethylphosphate and carbonyldimidazole were purchased from Aldrich, polynucleotide phosphorylase and dGTP were purchased from P-L Biochemicals. NDP-kinase and all other reagents were purchased from Sigma. EPR Suprasil quartz tubes were purchased from Wilmad.

Methods.

UV-VIS spectra were recorded on either a Hewlett-Packard 8452A diode array spectrophotometer or a Cary 3 spectrophotometer. HPLC analysis was performed on an Altex HPLC system with an Econosil C-18 Alltech reverse phase column (4.6 x 250 mm). Solutions were analyzed for radioactivity using a Packard 310 scintillation counter. NMR spectra were recorded on a Bruker 270 MHz spectrometer. EPR spectra were recorded at 100 K on a Bruker ESP-300 spectrometer at a microwave frequency of 9.41 GHz. Spin quantitation was achieved by comparison to a standard solution of 1.03 mM $CuSO_4$, 2 M $NaClO_4$, 0.01 M HCl, 20 % (v/v) glycerol.[39] All operations involving AdoCbl were performed in the dark or in dim red light. The following extinction coefficients were used for quantitation of cob(II)alamin: AdoCbl, ε_{524} = 8000 M^{-1}cm^{-1}, ε_{478} = 5800 M^{-1}cm^{-1}; cob(II)alamin, ε_{524} = 3200 M^{-1}cm^{-1}, ε_{478} = 9200 M^{-1}cm^{-1}. Standard activity assays were carried out in 500 µl of a mixture containing 10 mM ATP, 30 mM DTT, 12 µM AdoCbl, 1 M sodium acetate and 50 mM potassium phosphate pH 7.8. The dATP

formed was quantitated by the diphenylamine procedure.[40] Formate dehydrogenase (FDHase) assays were performed as described previously.[20]

Phosphorylation of Nucleosides. All nucleosides were phosphorylated to the monophosphates with $POCl_3$ in triethylphosphate by the procedure of Yoshikawa *et al.*[27] and then to the di- or triphosphates with carbonyldiimidazole and tributylammonium phosphate or pyrophosphate according to the procedure of Hoard and Ott.[28]

Synthesis of [β-^{32}P]-araN$_3$ATP. A mixture of araN$_3$ADP (10 μmol), $H_3{}^{32}PO_4$ (1 mCi) and polynucleotide phosphorylase (20 units) in 2.5 ml of 0.1 M Tris-HCl pH 8.7 containing 5 mM $MgCl_2$ and 0.3 mM EDTA, was kept for 12 hr at room temperature. To this was added 250 μl of 1.0 M Tris-HOAc pH 7.2 containing NDP kinase (100 units) and UTP (30 μmol). After 1 hr the mixture was diluted into 75 ml of cold H_2O and chromatographed on a 1.5 x 10 cm column of DEAE-Sephadex A25 using a gradient of 0 to 0.8 M Et_3N-H_2CO_3. The triphosphates were pooled and chromatographed by HPLC (50 mM potassium phosphate pH 6, 5 mM Bu_4NBr, 32.5 % (v/v) MeOH). Fractions containing [β-^{32}P]-araN$_3$ATP were pooled yielding 3.8 μmol of product (38 %). The ion-pairing buffer components were removed by a second chromatography on DEAE-Sephadex A25 as before yielding product having a specific radioactivity of 1 x 10^7 cpm/μmol. ^{1}H-NMR analysis of this product was difficult due to the small amounts of material, but the features associated with H(1') and the base protons indicated that a single nucleotide was present.

Separation of ^{3}H and ^{32}P Counts on the ^{3}H/^{32}P Channel. Samples of [3'-^{3}H]-araN$_3$ATP (7000 - 30500 cpm) and [β-^{32}P]-araN$_3$ATP (10000 cpm) were counted using the preset ^{3}H/^{32}P channel of the scintillation counter (^{3}H window 0-16, ^{32}P window 16-1700). Overlap of ^{3}H into the upper ("A") channel was negligible (0.07 %) while overlap of ^{32}P into the lower ("B") channel was 2.26 %. Thus observed "A" and "B" channel counts were corrected for overlap by the following formulae:

^{32}P cpm = ("B" cpm) /0.9774 ^{3}H cpm = ("A" cpm) - (0.0226)*(^{32}P cpm)

Inactivation of RTPR by [3'-^{3}H/β-^{32}P]-araN$_3$ATP. In a typical experiment, RTPR (20 nmol, 1.38 U/mg) was treated with [3'-^{3}H/β-^{32}P]-araN$_3$ATP (100 nmol, ^{3}H/^{32}P = 1.02, ^{3}H specific radioactivity = 3.1 x 10^6 cpm/μmol) and AdoCbl (30 nmol) in 250 μl of 50 mM potassium phosphate pH 7.8 containing 1.0 M NaOAc and 3 mM DTT. After 5 min at 37 °C the protein was 94 % inactive.[40] The mixture was centrifuged for 10 min (5000 x *g*, 4 °C) in a Centricon-30 ultra-filtration device. The protein concentrate was chromatographed on a 1.4 x 20 cm column of Sephadex G-50 (10 mM potassium phosphate pH 7.3). The isolated protein had a ^{3}H specific radioactivity of 1.11 x 10^6 cpm/μmol and a ^{3}H/^{32}P = 1.09 indicating *ca.* 0.4 equiv of both ^{3}H and ^{32}P bound per equivalent of inactivated RTPR (average of eight experiments). Assay of the reisolated RTPR, indicated no recovery of enzymatic activity. A 150 μl aliquot of the filtrate from the concentration step was added to 350 μl of H_2O, frozen and bulb-to-bulb distilled. Scintillation counting of a 400 μl aliquot of the distillate showed 2610 cpm: a control omitting RTPR was run simultaneously and showed 587 CPM at this stage. The difference (2023 CPM) indicates formation of 1.4 nmol of ^3H_2O or 0.07 equivalents/equivalent of inactivated RTPR. The residue from the distillation was dissolved in 400

µl of 50 mM potassium phosphate pH 6 containing 5 mM Bu_4NBr and analyzed by HPLC (50 mM potassium phosphate pH 6, 5 mM Bu_4NBr, 30 % (v/v) MeOH). The experiment and control traces were identical showing two peaks of radioactivity, $araN_3ATP$ eluting at 8 min, and an unidentified peak at 3.5 min ($^3H/^{32}P = 1.10$).

Stability of Bound araN3ATP-Product to Denaturation. RTPR which had been inactivated by [3'-^{3}H/β-^{32}P]-$araN_3ATP$ ($^3H/^{32}P = 1.40$, 10 nmol) was treated with 6 M guanidine-HCl in 0.2 M potassium phosphate pH 8.0 in a total volume of 500 µl. After 10 min this mixture was centrifuged in a Centricon-30 unit. A 50 µl aliquot of the filtrate was analyzed by scintillation counting indicating 477 cpm of ^3H and 315 cpm of ^{32}P. A 400 µl portion of the filtrate was injected onto HPLC (50 mM potassium phosphate pH 6, 5 mM Bu_4NBr) but interpretation of the trace was thwarted by the effects of 6 M guanidine on peak migration. Analysis of a 50 µl portion of the protein concentrate after dilution with 450 µl of the guanidine buffer gave 340 cpm of ^3H and 229 cpm of ^{32}P. Thus 60 % of both radiolabels were recovered in small molecules after denaturation.

Preparation of [8-^{3}H]-araN3ATP. A modification of the procedure of Shelton & Clark was used to incorporate ^3H from ^3H_2O into $araN_3AMP$.[41] To a 200 µl solution of $araN_3AMP$ (100 nmol) in 75 mM potassium phosphate pH 8.0, was added 50 µl of ^3H_2O (500 mCi/0.5 g) and the reaction vessel was sealed. The reaction mixture was heated in a boiling H_2O bath for 5.5 hr. The reaction mixture was cooled and the ^3H_2O removed, subsequent to freezing, via bulb-to-bulb distillation. The residue was redissolved in 1 ml of H_2O and the distillation repeated. This procedure was repeated two additional times. The residue was then purified on a 1.4 x 10 cm column of DEAE-Sephadex A25 by standard procedures. The specific radioactivity of the isolated material was 3.6×10^5 cpm/µmol. The monophosphate was converted to the triphosphate by standard procedures.[28]

Inactivation of RTPR by [8-^{3}H]-araN3ATP. The reaction was carried out as described above using 20 nmol of RTPR, 30 nmol of AdoCbl and 60 nmol of [8-^{3}H]-$araN_3ATP$ (specific radioactivity 2.1×10^5 cpm/µmol). A control reaction in the absence of RTPR was carried out in parallel. After incubation for 10 min at 37 °C, the reaction was split into two parts. One part was analyzed by Sephadex G-50 chromatography as described above, the second part was concentrated using a Centricon-30 and the filtrate analyzed by HPLC for adenine and $araN_3ATP$, after addition of 200 nmols of adenine as carrier. Via elution with 30 % (v/v) methanol, adenine eluted at 18 min while $araN_3ATP$ eluted in the void volume of the column. The nucleotide containing fractions were pooled, the phosphates were removed using *E. coli* alkaline phosphatase and the resulting products subjected to HPLC using elution with 20 % (v/v) methanol. Only $araN_3A$ was recovered (specific radioactivity 2.1×10^5 cpm/µmol).

Destruction of AdoCbl During Inactivation by araN3ATP. A mixture of 15 nmol of RTPR and 15 nmol of AdoCbl in 1 M NaOAc, 50 mM KPi pH 7.3 and 3 mM DTT in a total volume of 750 µl was treated with 60 nmols of inactivator and the UV-VIS spectrum was scanned repeatedly. The extent of cofactor destruction was estimated by the change in absorbance at 525 nm ($\Delta\varepsilon = 4800\ M^{-1}cm^{-1}$). The inactivation mixtures were analyzed by HPLC at the conclusion of the experiment in order to look for formation of 5'-dA. To allow quantitation of recovery, 5200 cpm of [5'-^{3}H]-5'-dA (specific radioactivity = 8.7×10^6 cpm/µmol)was added to the mixture as a tracer. The solution

was heated in a boiling water bath for 1 min and centrifuged to remove protein. The supernatant was evaporated, redissolved in H_2O and injected onto the HPLC. From this, 5.4 nmols of 5'-dA were recovered (as determined by A_{260}) giving 3600 CPM (69 % recovery). This indicates that 8 nmols of 5'-dA were present in the inactivation mixture.

Assay for Azide Release with Formate Dehydrogenase. RTPR (0.5 ml) was dialyzed against two changes of 1000 ml of 10 mM KPi, pH 7.2, to remove NaN_3. Addition of 10 µl of RTPR stock solution (0.84 nmol) to the FDHase assay resulted in an initial velocity of 11.1 nmol min^{-1}. The control assay with no additions gave 10.1 nmol min^{-1}. An inactivation mixture of 1 M NaOAc, 50 mM KPi, pH 7.3, 3 mM DTT, 75 nmol of $araN_3ATP$, 34 nmol of AdoCbl, and 15 nmol of RTPR was incubated at 37 °C for 20 min. Aliquots of 10 µl were removed at t = 0 and 20 min and added to the standard RTPR assay. These assays indicated complete inactivation of RTPR. A 50 µl aliquot of the inactivation mixture was then added to the FDHase and the initial velocity measured as 5.8 nmol min^{-1} (average of two determinations). A control consisting of all inactivation components except RTPR was performed as above, giving an initial velocity of 5.9 nmol min^{-1}. From the Dixon plot, release of 1 equiv of N_3^- in this experiment (0.84 nmol in FDHase assay) would have resulted in 80 % inactivation of the FDHase reaction (initial velocity = 1.2 nmol min^{-1}).

To look for time-dependent production of azide, the inactivation was run in the presence of the FDHase assay system. The reaction contained, in a volume of 1 ml, 50 mM Hepes pH 7.5, 10 mM NAD, 5 µl of FDHase, 1 M NaOAc, 11.2 µM AdoCbl, 0.84 µM of dialyzed RTPR and 30 mM DTT. The cuvette was equilibrated at 37 °C, and $NaHCO_3$ was added to a concentration of 1.8 mM. After measuring the initial velocity, 5 µl of 2.2 mM $araN_3ATP$ (11 nmol) was added and the reaction progress curve was recorded at 340 nm. After 5 min, the rate of formation of NADH was still linear, indicating no N_3^- formation.

Preparation of EPR Sample of RTPR Inactivated by $araN_3ATP$. The reaction mixture contained in 300 µl: 100 µM pre-reduced RTPR, 200 µM AdoCbl, 300 µM $araN_3ATP$, 0.5 mM dGTP, 20 µM TR, 0.12 µM TRR, 0.4 mM NADPH, 4 mM EDTA, 1 mM $MgCl_2$, 50 mM Hepes pH 7.5. All components except $araN_3ATP$ were combined in a 10 ml pear shaped flask and purged with argon for 20 min at 4 °C while stirring. The argon inlet and outlets were then removed and replaced with a balloon of argon and the flask was equilibrated in a 37 °C water bath for 10 min. Argon-purged $araN_3ATP$ solution was then added via gas tight syringe, the contents mixed, and the flask replaced in the water bath. After *ca.* 30 s, 250 µl of solution were transferred to an argon-purged EPR tube and frozen immediately in liquid nitrogen. The EPR conditions were as follows: microwave frequency 9.41 GHz; temperature 100 K; microwave power 10 mW; modulation amplitude 3.9 Gs; modulation frequency 100 kHz; time constant 0.164 s Ten scans were performed.

Inactivation of RTPR by 2'-Azido-2'-deoxynucleotides. A typical reaction mixture contained in a final volume of 140 µl: 26 µM RTPR, 0.26 mM AdoCbl, 50 mM potassium phosphate pH 7.8, 3 mM DTT or 20 µM TR/0.12 µM TRR/0.2 mM NADPH or no reductant, 1 M sodium acetate and 260 µM N_3UTP, N_3ATP, or 26-260 µM $araN_3ATP$ at 37 °C. Aliquots were removed at various time periods and added to 500 µl of the standard assay mixture.[40]

Inactivation of RTPR by CPdCTP. A 500 μl reaction mixture containing 50 μM pre-reduced RTPR, 200 μM AdoCbl, 1 mM CPdCTP, 1 mM dATP, 20 μM TR, 0.12 μM TRR, 0.2 mM NADPH, 4 mM EDTA, 1 mM $MgCl_2$, 50 mM Hepes pH 7.5 was incubated at 37 °C for 10 min under an argon atmosphere. Under these conditions, the assay revealed that the enzyme was >90 % inactivated.[31] The solution was exposed to air for *ca.* 1 min to oxidize non-active site bound cob(II)alamin, and 250 μl of this mixture was transferred to an EPR tube and frozen in liquid nitrogen. The EPR conditions were as above.

Acknowledgments. This work was funded by NIH grant GM 29595. We thank Georgi Harris for studies on the interaction of RTPR with N_3ATP and N_3UTP.

REFERENCES

1. Stubbe, J. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1990,** 63, 349-417.
2. Reichard, P. *Science* **1993,** 260, 1773-1777.
3. Sjöberg, B.-M.; Reichard, P.; Gräslund, A.; Ehrenberg, A. *J. Biol. Chem.* **1977,** 252, 536-541.
4. Stubbe, J. *J. Biol. Chem.* **1990,** 265, 5329-5332.
5. Uhlin, U.; Eklund, H. *Nature* **1994,** 370, 533-539.
6. Licht, S.; Gerfen, G. J.; Stubbe, J. *Science* **1996,** 271, 477-481.
7. Gerfen, G. J.; Licht, S.; Willems, J.-P.; Hoffman, B. M.; Stubbe, J. *J. Am. Chem. Soc.* **1996,** 118, 8192-8197.
8. Stubbe, J.; Ackles, D.; Segal, R.; Blakley, R. L. *J. Biol. Chem.* **1981,** 256, 4843-4846.
9. Stubbe, J.; Ator, M.; Krenitsky, T. *J. Biol. Chem.* **1983,** 258, 1625-1630.
10. Booker, S.; Licht, S.; Broderick, J.; Stubbe, J. *Biochemistry* **1994,** 33, 12676-12685.
11. Mao, S. S.; Holler, T. P.; Yu, G. X.; Bollinger, J. M.; Booker, S.; Johnston, M. I.; Stubbe, J. *Biochemistry* **1992,** 31, 9733-9743.
12. Mao, S. S.; Yu, G. X.; Chalfoun, D.; Stubbe, J. *Biochemistry* **1992,** 31, 9752-9759.
13. Aberg, A.; Hahne, S.; Karlsson, M.; Larsson, A.; Ormö, M.; Ahgren, A.; Sjöberg, B. M. *J. Biol. Chem.* **1989,** 264, 2249-2252.
14. Mao, S. S.; Johnston, M. I.; Bollinger, J. M.; Stubbe, J. *Proc. Natl. Acad. Sci. USA* **1989,** 86, 1485-1489.
15. Mao, S. S.; Holler, T. P.; Bollinger, J. M.; Yu, G. X.; Johnston, M. I.; Stubbe, J. *Biochemistry* **1992,** 31, 9744-9751.
16. Harris, G.; Ator, M.; Stubbe, J. *Biochemistry* **1984,** 23, 5214-5225.
17. Thelander, L.; Larsson, B.; Hobbs, J.; Eckstein, F. *J. Biol. Chem.* **1976,** 251, 1398-1405.
18. Sjöberg, B.-M.; Gräslund, A.; Eckstein, F. *J. Biol. Chem.* **1983,** 258, 8060-8067.
19. Ator, M.; Salowe, S. P.; Stubbe, J.; Emptage, M. H.; Robins, M. J. *J. Am. Chem. Soc.* **1984,** 106, 1886-1887.
20. Salowe, S. P.; Ator, M.; Stubbe, J. *Biochemistry* **1987,** 26, 3408-3416.
21. van der Donk, W. A.; Stubbe, J.; Gerfen, G. J.; Bellew, B. F.; Griffin, R. G. *J. Am. Chem. Soc.* **1995,** 117, 8908-8916.
22. Baker, C. H.; Banzon, J.; Bollinger, J. M.; Stubbe, J.; Samano, V.; Robins, M. J.; Lippert, B.; Jarvi, E.; Resvick, R. *J. Med. Chem.* **1991,** 34, 1879-1884.
23. van der Donk, W. A.; Yu, G.; Silva, D. J.; Stubbe, J.; McCarthy, J. R.; Jarvi, E. T.; Matthews, D. P.; Resvick, R. J.; Wagner, E. *Biochemistry* **1996,** 35, 8381-8391.
24. Harris, G.; Ashley, G. W.; Robins, M. J.; Tolman, R. L.; Stubbe, J. *Biochemistry* **1987,** 26, 1895-1902.
25. Robins, M. J.; Hawrelak, S. D.; Hernández, A. E.; Wnuk, S. F. *Nucleosides Nucleotides* **1992,** 11, 821-834.
26. Wagner, D.; Verheyden, J. P. H.; Moffatt, J. G. *J. Org. Chem.* **1974,** 39, 24-30.
27. Yoshikawa, M.; Kato, T.; Takenishi, T. *Tetrahedron Lett.* **1967,** 50, 5065-5068.
28. Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965,** 87, 1785-1788.
29. Hamilton, J. A.; Yamada, R.; Blakley, R. L.; Hogenkamp, H. P. C.; Looney, F. D.; Winfield, M. E. *Biochemistry* **1971,** 10, 347-355.
30. Zelder, O.; Beatrix, B.; Leutbecher, U.; Buckel, W. *Eur. J. Biochem.* **1994,** 226, 577-585.
31. Lawrence, C. C.; Booker, S.; Samano, V.; Robins, M. J.; Stubbe, J. Unpublished results.
32. Shingaki, T. *Sci. Rep. Coll. Gen. Educ. Osaka Univ.* **1963,** 11, 67-79.
33. Shingaki, T. *Sci. Rep. Coll. Gen. Educ. Osaka Univ.* **1963,** 11, 81-92.
34. Ashley, G. W.; Harris, G.; Stubbe, J. *J. Biol. Chem.* **1986,** 261, 3958-3964.
35. Booker, S.; Stubbe, J. *Proc. Natl. Acad. Sci. USA* **1993,** 90, 8352-8356.
36. Lunn, C. A.; Kathju, S.; Wallace, B. J.; Kushner, S. R.; Pigiet, V. *J. Biol. Chem.* **1984,** 259, 10469-10474.
37. Russel, M.; Model, P. *J. Bacteriol.* **1985,** 163, 238-242.
38. Stubbe, J.; Ackles, D. *J. Biol. Chem.* **1980,** 255, 8027-8030.
39. Malmström, B. G.; Reinhammar, B.; Vänngård, T. *Biochim. Biophys. Acta* **1970,** 205, 48-57.
40. Blakley, R. L. *Methods Enzymol.* **1978,** 51, 246-259.
41. Shelton, K. R.; Clark, J. M. *Biochemistry* **1967,** 6, 2735-2739.

(*Received* 19 *December* 1996; *accepted* 20 *January* 1997)