Synthesis of 3′-*C*-Methyladenosine and 3′-*C*-Methyluridine Diphosphates and Their Interaction with the Ribonucleoside Diphosphate Reductase from *Corynebacterium nephridii*†

Seng Poon Ong, Lowell S. Nelson, and Harry P. C. Hogenkamp*

Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

Received July 9, 1992; Revised Manuscript Received August 28, 1992

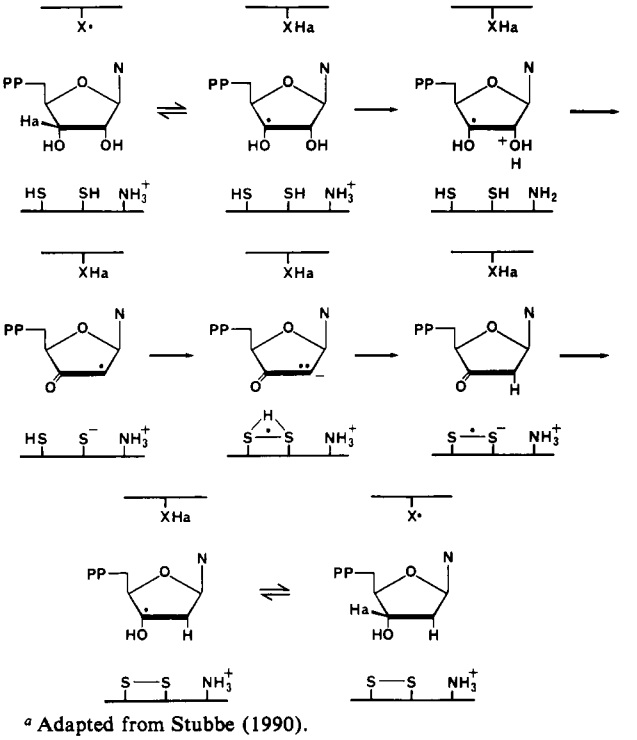
ABSTRACT: Two nucleoside diphosphate analogs, 3′-*C*-methyl-ADP and 3′-*C*-methyl-UDP, have been tested as substrate and/or allosteric effectors using the adenosylcobalamin-dependent ribonucleoside diphosphate reductase of *Corynebacterium nephridii*. Neither analog was a substrate for the reductase. However, they did function as allosteric effectors and as inhibitors of the reduction of ADP and UDP, respectively. The nucleotide analogs did not stimulate the hydrogen exchange reaction between [5′-$^3H_2$]adenosylcobalamin and the solvent, indicating that the cleavage of the 3′-carbon–hydrogen bond is a prerequisite for the exchange reaction. A reinvestigation of the requirements for the exchange reaction revealed that the deoxyribonucleoside diphosphate products are very effective promoters of this reaction. Indeed, the deoxyribonucleoside diphosphates were found to be more effective in promoting the exchange reaction than the ribonucleoside diphosphate substrates. In contrast, the deoxyribonucleoside triphosphate effectors, dATP, dUTP, and dTTP, were only marginally effective as promoters of this reaction.

Ribonucleotide reductase catalyzes the irreversible reduction of ribonucleotides to the corresponding 2′-deoxyribonucleotides concomitant with the reduction of a pair of sulfhydryl groups to a disulfide within the active site of the enzyme. The first evidence pointing to the mechanism of the reduction was reported by Thelander et al. (1976). They observed that incubation of 2′-chloro-2′-deoxynucleoside 5′-diphosphates with *Escherichia coli* reductase resulted in inactivation of the enzyme, release of chloride ion, cleavage of the glycosidic bond, and release of the purine or pyrimidine base. These observations prompted Stubbe and Kozarich (1980a,b) to examine the interaction of this reductase with several 2′-substituted 2′-deoxyribonucleoside 5′-diphosphates. They observed that, in addition to the halide ion and the base, pyrophosphate was released. On the basis of these results, they postulated the formation of 2-methylene-3-furanone from the deoxyribose moiety and suggested that this reactive furanone causes the inactivation of the enzyme. A pivotal observation that led to the proposed mechanism (Scheme I) was made by Stubbe and Ackles (1980). They found that during catalysis the 3′-carbon–hydrogen bond of the nucleotide substrate was cleaved. They also showed that a small but significant amount of tritium from [3′-^{3}H]uridine diphosphate was released to the solvent. Their proposed mechanism is based on analogies with chemical model systems (Walling & Johnson, 1975), and the enzymatic studies with both the *E. coli* ribonucleoside diphosphate reductase (Stubbe et al., 1983) and the *Lactobacillus leichmannii* ribonucleoside triphosphate reductase (Ashley et al., 1986). In spite of the significant differences in the physical structures, substrate, effector, and cofactor requirements, the proposed mechanism probably applies to all the ribonucleotide reductases (Ashley & Stubbe, 1989).

The first key step in this mechanism is the abstraction of the 3′-hydrogen of the ribonucleotide by a protein-based radical. In order to provide additional evidence for the cleavage of the 3′-C–H bond, we synthesized two 3′-*C*-methylribonucleoside diphosphates and tested their ability to act as substrates, inhibitors, or effectors of the ribonucleoside diphosphate reductase isolated from *Corynebacterium nephridii*. This adenosylcobalamin- (AdoCbl-) dependent enzyme also catalyzes the exchange of the 5′-hydrogens of the coenzyme with the solvent. This dimeric enzyme is more complex in structure and allosteric control than the monomeric *L. leichmannii* enzyme but more primitive than the tetrameric reductase from *E. coli*.

As expected, neither 3′-*C*-methyl-ADP (3′-MeADP) nor 3′-*C*-methyl-UDP (3′-MeUDP) acts as a substrate, but

Scheme I [a]



[a] Adapted from Stubbe (1990).

† This work was supported by NIH Grant GM-33776.

* Address correspondence to this author.

0006-2960/92/0431-11210$03.00/0

surprisingly, neither nucleotide analog is able to promote the tritium exchange from [5′-$^3H_2$]adenosylcobalamin to the solvent. Overall, both nucleotide analogs were found to inhibit the reduction and the exchange reactions, but at low concentrations they were found to act as allosteric effectors in both processes.

MATERIALS AND METHODS

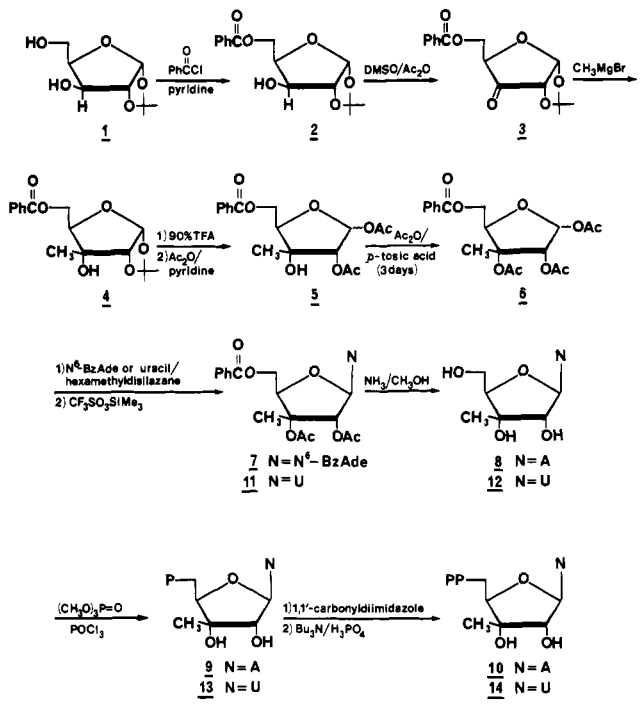
1,2-*O*-Isopropylidene-α-D-xylofuranose, uracil, N^6-benzoyladenine, diatomaceous earth, 3,3-dimethylglutaric acid, AdoCbl, bovine intestinal mucosa alkaline phosphatase (2800 units/mg of protein), [^{14}C]UDP, tris[hydroxymethyl]aminomethane (Tris), and dGTP were purchased from Sigma Chemical Co. Dry pyridine, dry dimethyl sulfoxide, dry toluene, dry 1,2-dichloroethane, dry dimethylformamide, benzoyl chloride, acetic anhydride, methylmagnesium bromide, trifluoroacetic acid, *p*-toluenesulfonic acid monohydrate, hexamethyldisilazane, (trimethylsilyl)trifluoromethanesulfonate, trimethyl phosphate, phosphorous oxychloride, tributylamine, and 1,1′-carbonyldiimidazole were from Aldrich Chemical Co. Dithiothreitol (DTT) was obtained from Boehringer Mannheim. Methanol (HPLC grade), water (HPLC grade), and activated charcoal were from Mallinckrodt, Inc. UDP and ADP were from P-L Biochemicals. [^{14}C]ADP was a product of Amersham Corp. dTTP and DEAE-Sepharose CL-6B were purchased from Pharmacia LKB Biotechnology, Inc. Dowex 1-X2 and Dowex 50-X8 were products of Bio-Rad. Spin-X centrifuge filter units were purchased from Costar. [5′-$^3H_2$]AdoCbl was prepared as described before (Gleason & Hogenkamp, 1972).

Ribonucleotide reductase from *C. nephridii* was purified as described before (Tsai & Hogenkamp, 1980), modified as follows. Frozen cell paste (120 g) was suspended in 130 mL of 100 mM 3,3-dimethylglutarate (pH 7.2), and the cells were disrupted by one pass through a chilled French pressure cell at 20 000 psi. Cell debris was removed by centrifugation at 140000*g* for 60 min, and to the supernatant solution was added ammonium sulfate to a final concentration of 40%. The suspension was centrifuged at 8000*g* for 60 min. The pellet (703 mg) was suspended in 70 mL of 20 mM Tris-HCl/1 mM EDTA (pH 7.6) and applied to a DEAE-Sepharose CL-6B column (2.5 × 88 cm). The enzyme was eluted with a 2-L gradient of 0–0.4 M NaCl in the Tris-HCl/EDTA buffer. The fractions containing ribonucleotide reductase activity were pooled and concentrated by using an Amicon PM30 membrane, and the concentrate was dialyzed against 100 mM 3,3-dimethylglutarate buffer (pH 7.2). The enzyme preparation (79 mg) was then applied to a dGTP-Sepharose affinity column (1 × 20 cm) and the reductase was eluted with a gradient of 0–0.5 M NaCl in dimethylglutarate buffer (pH 7.2). The reductase (7 mg) was desalted by dialysis against dimethylglutarate buffer (pH 7.2) and concentrated to about 300 μg/mL. Stored at 4 °C, this preparation retains full activity for many months.

HPLC analyses were performed using a Beckman Model 332 instrument equipped with either a Brownlee Aquapore RP-300 C-8 (20 × 0.46 cm) or C-18 (22 × 1 cm) reversed-phase column and a 3-cm guard cartridge. The absorbance was monitored at 260 nm. UV–visible spectra were recorded on a Hewlett-Packard diode array spectrophotometer. NMR spectra were obtained on a GE Omega 500-MHz spectrometer.

Synthesis of 3′-MeADP (**10**) *and 3′-MeUDP* (**14**). These nucleotide analogs were synthesized in nine steps starting from 1,2-*O*-isopropylidene-α-D-xylofuranose (**1**) by a combination of different published procedures (Scheme II). Benzoylation

Scheme II



of **1** (10 g, 53 mmol) with benzoylchloride in pyridine (Tong et al., 1967) gave 5-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-xylofuranose (**2**): yield 10.2 g, 35 mmol, 66%, mp 80–84 °C. Oxidation of **2** with dimethyl sulfoxide in acetic anhydride (Tong et al., 1967) gave 5-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-*erythro*-3-pentulofuranose (**3**). This keto sugar (4.6 g, 15.8 mmol) was dissolved in 80 mL of dry ether and treated with 10 mL of 3.0 M methylmagnesium bromide to yield 2.4 g (7.8 mmol, 49%, mp 107–110 °C) of 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*C*-methyl-α-D-ribofuranose (**4**) (Nutt et al., 1968). The key intermediate, 1,2,3-tri-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-α-D-ribofuranose (**6**), was prepared from **4** (1.85 g, 6 mmol) following the procedures of Mikhailov et al. (1983). The syrupy triacetate **6** (1.55 g, 3.9 mmol) was condensed with N^6-benzoyladenine in the presence of hexamethyldisilazane and (trimethylsilyl)trifluoromethanesulfonate as described by Beigelman et al. (1988) to yield 9-(2,3-di-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-β-D-ribofuranosyl)-N^6-benzoyladenine (**7**) in 54% yield (1.2 g, 2.1 mmol). Condensation of **6** (1.55 g, 3.9 mmol) with uracil using the same procedure gave 950 mg (2.1 mmol, 53%) of 1-(2,3-di-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-β-D-ribofuransoyl)uracil (**11**). The protected nucleosides (**7** and **11**) were deacylated with methanolic ammonia and the nucleosides were purified using Dowex 1-X2 (hydroxide form) as described by Dekker (1965). 3′-*C*-Methyladenosine (**8**) was eluted with 60% aqueous methanol, while the elution of 3′-*C*-methyluridine (**12**) required 0.1 M ammonium bicarbonate.

The 3′-*C*-methylnucleosides **8** and **12** (0.5 mmol) were converted to the corresponding 5′-monophosphates **9** and **13** by the procedure of Yoshikawa and Takenishi (1967). They were purified by chromatography on Dowex 1-X2 (chloride form) and isolated as their lithium salts. The 3′-*C*-methylnucleoside 5′-diphosphates **10** and **14** were prepared from **9** and **13** (0.25 mmol) using the 1,1′-carbonyldiimidazole method of Hoard and Ott (1965). The UV spectra of these nucleotides were identical to those of ADP and UDP, respectively. 3′-Methyl-ADP: ^{1}H NMR (500 MHz) (D_2O) 8.57 (s, 1 H, C_8H), 8.20 (s, 1 H, C_2H), 6.07 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, $C_{1'}H$), 4.27 (br s, 1 H, $C_{4'}H$), 4.09 (m, 2 H, $C_{5'}H_{a,b}$), and 1.45 (s,

3 H, $C_{3'}$-Me); ^{31}P NMR (202 MHz) (D_2O, pH 7.0) −5.15 (d, $J_{\beta,\alpha}$ = 21.6 Hz, P_β) and −9.99 (d, $J_{\alpha,\beta}$ = 21.7 Hz, P_α). 3′-Methyl-UDP: ^{1}H NMR (500 MHz) (D_2O) 8.04 (d, 1 H, $J_{6,5}$ = 8.1 Hz, C_6H), 6.03 (d, 1 H, $J_{1',2'}$ = 7.9 Hz, $C_{1'}H$), 5.96 (d, 1 H, $J_{5,6}$ = 8.1 Hz, C_5H), 4.17–4.14 (m, 2 H, $C_{2',4'}H$), 4.12–4.05 (m, 2 H, $C_{5'}H$, $C_{5'}H_{a,b}$), and 1.38 (s, 3 H, $C_{3'}$–Me); ^{31}P NMR (202 MHz) (D_2O, pH 6.0) −9.76 (d, $J_{\beta,\alpha}$ = 22.1 Hz, P_β) and −10.98 (d, $J_{\alpha,\beta}$ = 20.6 Hz, P_α).

Reaction of 3′-MeADP and 3′-MeUDP with Ribonucleotide Reductase. The reaction mixtures contained 100 mM dimethylglutarate (pH 7.2), 11 mM DTT, 83 μM AdoCbl, 100 μM 3′-MeADP or 3′-MeUDP, and 15 μg of ribonucleotide reductase in a final volume of 180 μL. Positive control reactions were identical except that the 3′-*C*-methylnucleotides were replaced by 100 μM ADP or UDP. The reactions were initiated by the addition of AdoCbl and the mixtures were incubated at 37 °C for 1 h in the dark. The reactions were stopped by boiling for 2 min. Each cooled assay mixture was treated with 15 units of alkaline phosphatase in 50 μL of 0.5 M Tris-HCl (pH 8.5) containing 20 mM $MgCl_2$ and incubated for 1 h at 37 °C. The mixtures were then boiled for 2 min and centrifuged to remove denatured protein, and the supernatant was filtered through a costar SPIN-X centrifuge filter unit (0.45-μm cellulose acetate). The filtrate was analyzed by HPLC on a C-8 reversed-phase column either with 10% methanol in 20 mM potassium phosphate (pH 5.6) as eluent for the adenosine/3′-*C*-methyladenosine samples or with 20 mM potassium phosphate (pH 5.6) as eluent for the uridine/3′-*C*-methyluridine samples. The production of 2′-deoxynucleosides was measured at 260 nm.

Tritium Exchange from [5′-$^3H_2$]AdoCbl. The reaction mixtures containing 3′-MeADP and 3′-MeUDP consisted of 100 mM dimethylglutarate (pH 7.2), 16 mM DTT, 50 μM [5′-$^3H_2$]AdoCbl, varying concentrations of the nucleotide analogs, and 15 μg of ribonucleotide reductase in a final volume of 200 μL. The reactions were initiated by the addition of the labeled coenzyme and incubated at 37 °C for 1 h in the dark. The reaction was terminated with 1 mL of 2% trichloroacetic acid containing 100 mg of activated charcoal. The assays were mixed thoroughly and centrifuged for 5 min. The amount of ^{3}H released was determined by liquid scintillation counting of a 400-μL aliquot in 10 mL of scintillation cocktail.

Other reaction mixtures contained 100 mM dimethylglutarate (pH 7.2), 16 mM DTT, 50 μM [5′-$^3H_2$]AdoCbl (specific activity 7840 cpm/nmol), varying concentrations of ribo- or deoxyribonucleotides, and 2 μg of ribonucleotide reductase in a total volume of 250 μL. The assay mixtures, without AdoCbl, were preincubated for 20 min at 37 °C. AdoCbl was then added and the final mixture was incubated for 10 min at 37 °C in the dark. The assays were then terminated as described above and the amount of ^{3}H released was determined by liquid scintillation counting of a 500-μL aliquot of the supernatant in 10 mL of scintillation cocktail.

Effect of 3′-MeADP and 3′-MeUDP on the Reduction of ADP and UDP. The assay mixtures contained, in a final volume of 100 μL, 100 mM dimethylglutarate (pH 7.2), 20 mM DTT, 100 μM AdoCbl, fixed concentrations of [^{14}C]-ADP at 10, 25, and 75 μM with varying concentrations of 3′-*C*-methyl-ADP up to 500 μM, and 70 ng of ribonucleotide reductase. These experiments were then repeated with 1 mM dGTP in the reaction mixtures. The reactions were started by the addition of AdoCbl and incubated in the dark at 37 °C for 10 min. They were then dephosphorylated as described before and [^{14}C]adenosine and [^{14}C]deoxyadenosine were separated by HPLC on a 200- × 4.6-mm C-8 reversed-phase column equipped with a 3-cm guard cartridge. The eluent was 6% methanol in 20 mM potassium phosphate (pH 5.6), at a flow rate of 1 mL/min. One-minute fractions were collected and the radioactivity was determined by scintillation counting.

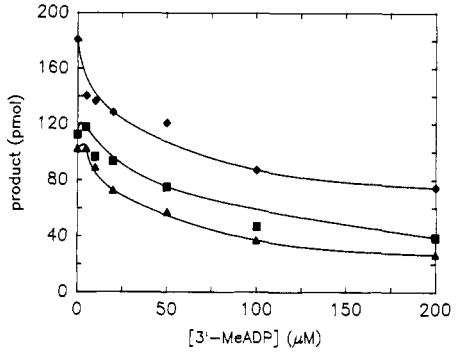


FIGURE 1: Effect of 3′-MeADP on ADP reduction. Experimental conditions are described under Materials and Methods. ▲, 10 μM ADP; ■, 25 μM ADP; ◆, 75 μM ADP.

The effect of 3′-MeUDP and UDP reduction was determined in reaction mixtures containing 100 mM dimethylglutarate (pH 7.2), 20 mM DTT, 100 μM AdoCbl, fixed concentrations of [^{14}C]UDP at 20 and 100 μM with varying concentrations of 3′-MeUDP up to 500 μM, and 150 ng of ribonucleotide reductase in a total volume of 100 μL. These experiments were repeated with 1 mM dTTP as a positive allosteric effector of UDP reduction. The reaction mixtures were processed as described before. [^{14}C]Uridine and [^{14}C]deoxyuridine were separated by HPLC on a 22- × 1-cm C-18 reversed-phase column equipped with a 3-cm guard cartridge, using 8% methanol in 20 mM potassium phosphate (pH 5.6) as the eluting buffer at a flow rate of 2 mL/min. One-minute fractions were collected and the radioactivity was determined by scintillation counting.

Effect of 3′-MeADP and 3′-MeUDP on the Tritium Exchange from [5′-$^3H_2$]AdoCbl Promoted by ADP and UDP. The incubation mixtures (200 μL) contained 100 mM dimethylglutarate (pH 7.2), 10 mM DTT, 50 μM [5′-$^3H_2$]-AdoCbl, fixed concentrations of ADP at 25, 75, and 200 μM or of UDP at 20 and 100 μM with varying concentrations of either 3′-MeADP or 3′-MeUDP up to 300 μM, and 7.5 μg of ribonucleotide reductase. Reactions were initiated and processed as described above.

RESULTS

3′-C-Methylnucleotides Are Not Substrates and Do Not Promote the Exchange Reaction. Neither 3′-MeADP nor 3′-MeUDP functions as a substrate for the ribonucleotide reductase of *C. nephridii*. HPLC analysis of reaction mixtures containing either analog showed, after dephosphorylation, no evidence of 2′-deoxy-3′-methyladenosine or 2′-deoxy-3′-methyluridine after a 1-h incubation period even when the enzyme concentration was increased 3–10-fold over an enzyme concentration that catalyzed nearly complete (≥95%) reduction of ADP or UDP in 15 min. Additionally, neither nucleotide analog was able to promote tritium exchange between [5′-$^3H_2$]AdoCbl and the solvent. However, 3′-MeADP does interact with the reductase.

At low concentrations (5–10 μM), 3′-MeADP consistently produced a small stimulation of ADP reduction, while at higher concentrations this analog inhibited the reduction of ADP (Figure 1). In an attempt to confirm the role of 3′-MeADP as an effector, similar reduction experiments were repeated

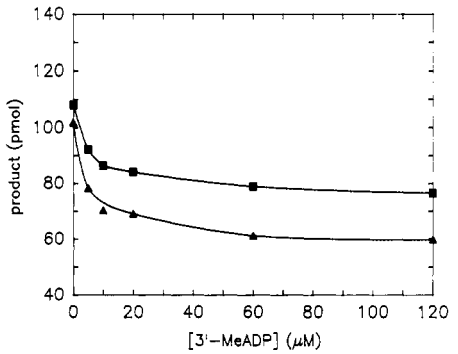


FIGURE 2: Effect of 3′-MeADP on ADP reduction in the presence of 1 mM dGTP. See Materials and Methods for details. ▲, 10 μM ADP; ■, 25 μM ADP.

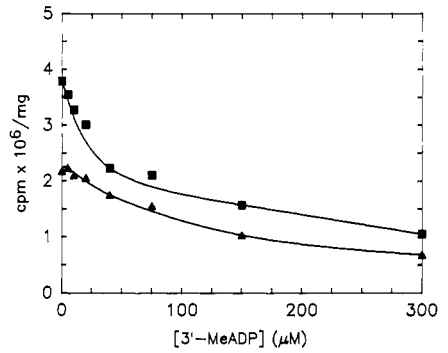


FIGURE 3: Effect of 3′-MeADP on the tritium exchange from [5′-$^3H_2$]AdoCbl to solvent stimulated by ADP. See Materials and Methods for details. ▲, 25 μM ADP; ■, 75 μM ADP.

in the presence of 1 mM dGTP, a known positive allosteric effector for ADP reduction. A large excess of dGTP was used so that it would occupy the effector site(s) on the enzyme, limiting 3′-MeADP to interact at the reduction site. In the presence of 1 mM dGTP, the stimulatory effect of 3′-MeADP on ADP reduction was not observed (Figure 2). Dixon plots could not be constructed to determine a K_i value for the effect of the analog on ADP reduction with or without dGTP. This was not unexpected because of the complicated kinetics arising from the concomitant binding of the analog to other site(s) of the enzyme.

Similar to its effect on ADP reduction, stimulation at low concentrations and inhibition at higher concentrations of 3′-MeADP was observed in the ADP-promoted tritium exchange reaction (Figure 3).

The effect of 3′-MeUDP on UDP reduction and on the UDP-promoted exchange reaction was very similar to that of 3′-MeADP on those two reactions. 3′-MeUDP also acted as a positive allosteric effector of UDP reduction at low concentrations (5–15 μM) and inhibited at higher concentrations (Figure 4). In the presence of dTTP, a positive allosteric effector of UDP reduction, the stimulation by 3′-MeUDP was abolished. The nucleotide analog also had a small but reproducible stimulatory effect on the UDP-promoted tritium exchange reaction (data not shown).

Effects of Deoxyribonucleoside Diphosphates on the Exchange Reaction. The inability of the two nucleotide analogs to stimulate the exchange reaction prompted us to reassess the effects of substrates, products, and allosteric effectors on this reaction. Early studies on the effect of nucleotides on the tritium exchange reaction between [5′-$^3H_2$]AdoCbl and water were carried out using the ribonucleotide triphosphate reductase from *L. leichmannii* (Hogenkamp et al., 1968). In these early studies the ribonucleoside triphosphate substrates

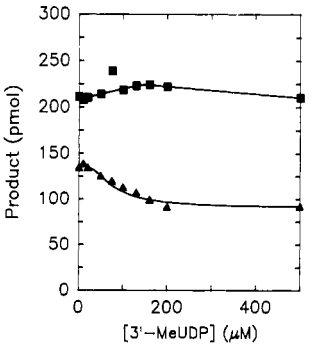


FIGURE 4: Effect of 3′-MeUDP on UDP reduction. See Materials and Methods for details. ▲, 20 μM UDP; ■, 100 μM UDP.

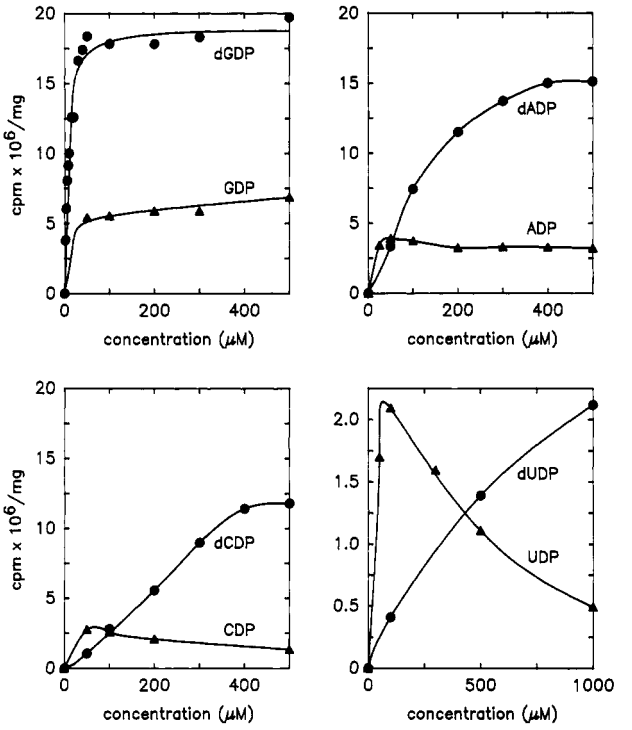


FIGURE 5: Effect of ribonucleoside diphosphates and 2′-deoxyribonucleoside diphosphates on the tritium exchange reaction. Assay mixtures with GDP, dGDP, ADP, dADP, CDP, and dCDP were incubated for 10 min, while those containing UDP and dUDP were incubated for 20 min. See Materials and Methods for further details.

and deoxyribonucleoside triphosphate effectors were found to promote the exchange reaction. In this enzyme system, it was impossible to distinguish between the deoxyribonucleoside triphosphates as products or as allosteric effectors. The ribonucleotide reductase from *C. nephridii* is a ribonucleoside diphosphate reductase, and thus, the effect of the nucleoside diphosphate products and the triphosphate effectors can be distinguished.

The results presented in Figure 5 show that the deoxyribonucleoside diphosphate products, dGDP, dADP, and dCDP, are much more effective in promoting the exchange reaction than their corresponding ribonucleoside diphosphate substrates. The behavior of the uridine nucleotides is anomalous; at low concentrations (<100 μM) UDP is a better promoter than dUDP, and at higher concentrations UDP is less effective than dUDP. The deoxyribonucleoside diphosphate products were all better stimulators of the exchange reaction than their corresponding deoxyribonucleoside triphosphates (Table I). Of the deoxyribonucleoside triphosphates, only dGTP and dCTP were effective promoters of the exchange reaction,

Table I: dNTP/dNDP Stimulation of the Tritium Exchange Reaction between [5′-$^3H_2$]AdoCbl and Solvent[a]

nucleotide	concn (μM)	activity (cpm × 10^5/mg of protein)	nucleotide	concn (μM)	activity (cpm × 10^5/mg of protein)
dGTP	10	14.9	dADP	50	29.2
dGDP	10	54.7	dUTP	500	2.9
dCTP	100	10.9	dUDP	500	4.4
dCDP	100	16.7	dTTP	500	0
dATP	50	0	dTDP	500	4.5

[a] Tritium exchanged to the solvent was kept to less than 20% of the total tritium in each assay.

consistent with the earlier study by Tsai and Hogenkamp (1980).

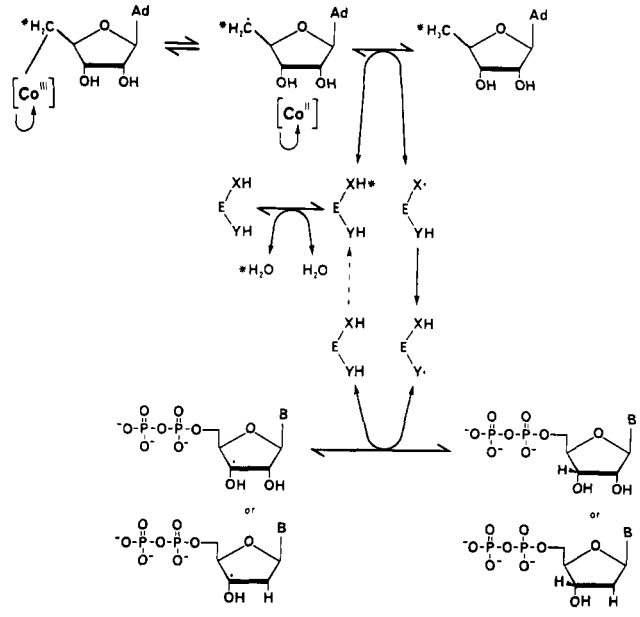
DISCUSSION

The initial step in the mechanism of ribonucleotide reduction formulated by Stubbe (1990) is the homolytic cleavage of the 3′-carbon–hydrogen bond mediated by a protein radical (Scheme I). Such carbon–hydrogen bond cleavage is not likely in the 3′-methylnucleotides, 3′-MeADP and 3′-MeUDP, and thus it is not surprising that these two nucleotide analogs do not function as substrates. Indeed, the results support the proposed mechanism and also show that the stereochemistry in the active site is strict, so that cleavage of the 2′-carbon–hydrogen bond instead of the 3′-carbon–hydrogen bond, if the latter is blocked, does not occur. Both nucleotide analogs do interact with the enzyme, functioning as inhibitors of ADP and UDP reduction, respectively. At low and nonsaturating substrate concentrations they stimulate the reduction of the substrates slightly but reproducibly, suggesting that they are also able to bind to the allosteric binding site of the enzyme. In the presence of the usual allosteric effectors (dGTP for ADP reduction and dTTP for UDP reduction), this site is blocked and the stimulation by the nucleotide analogs is no longer observed.

The two 3′-methylnucleotides are not able to promote the exchange between [5′-$^3H_2$]AdoCbl and the solvent. In contrast, as shown in Figure 5, the deoxyribonucleoside diphosphates, dGDP, dADP, and dCDP, are very effective promoters of this exchange reaction. Indeed, they are more effective than their respective substrates or the deoxyribonucleoside triphosphates that function as allosteric effectors. These observations suggest that the homolytic cleavage of the 3′-carbon–hydrogen bond of either the substrate or the product is a prerequisite for the hydrogen exchange between the coenzyme and the solvent. Such a conclusion could have been reached earlier when it was observed that the triphosphates of 9-(β-D-arabinofuranosyl)adenine and 1-(β-D-arabinofuranosyl)cytosine were promoters of the exchange reaction catalyzed by the ribonucleotide reductase from *L. leichmannii* (Follmann & Hogenkamp, 1971). In the arabino nucleotides the 3′-hydrogen is positioned correctly for potential cleavage, while in a xylo nucleotide [e.g., 9-(β-D-xylofuranosyl)adenine] a hydroxyl group is in this position. This xylo compound did not stimulate hydrogen exchange.

Stubbe et al. (1981) have demonstrated that the 3′-carbon–hydrogen bond of the substrate is cleaved in the reduction reaction and that the hydrogen abstracted from the 3′-position by the protein radical is returned to the 3′-position of the 2′-deoxyribonucleotide product. Their findings have shown that the 3′-hydrogen of the substrate does not exchange with the 5′-hydrogen of the coenzyme. In a scheme describing the exchange reaction by Ashley et al. (1986), the homolytic

Scheme III



cleavage of the carbon–cobalt bond of the coenzyme generates a 5′-deoxyadenosyl radical and cob(II)alamin. The adenosyl radical then abstracts a hydrogen from an amino acid residue in the active site of the enzyme to produce 5′-deoxyadenosine and a protein-based radical. The reversal of this process leads to the exchange of the 5′-hydrogens of the coenzyme with an acidic hydrogen of the protein.

Our observations suggest that the mechanism of the exchange reaction between the coenzyme and solvent is more complex and should be modified to include the homolytic cleavage of the 3′-carbon–hydrogen bond of either the ribonucleotide substrate or the 2′-deoxyribonucleotide product (Scheme III). In the absence of a ribonucleotide substrate or 2′-deoxynucleotide product, the equilibria of the homolytic cleavage of the carbon–cobalt bond of the coenzyme and the generation of a protein radical by the 5′-deoxyadenosyl radical lie predominantly to the left (upper reaction). In the presence of a substrate, product, or analog with an abstractable hydrogen at C-3′, those equilbria are shifted to the right, generating more protein-based radical X (upper line of Scheme III) and thereby stimulating the exchange of hydrogen between coenzyme and solvent (second line of Scheme III).

Stubbe and Ackles (1980) observed that a very small amount of the 3′-hydrogen of the substrate exchanged with the solvent, indicating either that the overall reduction rate is much faster than the hydrogen exchange between coenzyme and solvent and/or that different protein radicals are involved in the reduction and in the exchange reaction. We suggest that a second protein radical at residue Y is formed by abstraction of hydrogen by the radical at X (middle reaction of Scheme III). It is this radical at Y that catalyzes the reversible 3′-carbon–hydrogen cleavage of substrates or products (lower reaction of Scheme III). In these two reactions, protein radical Y abstracts the 3′-hydrogen of either a ribonucleotide or a 2′-deoxyribonucleotide.

In our scheme, the proton on amino acid residue X is readily exchangeable with the solvent. In contrast, the hydrogen on residue Y, "the working radical", is not accessible to the solvent and, as shown by Stubbe and Ackles (1980), does not exchange to an appreciable extent.

It should be stressed that this scheme does not include stimulation of the exchange reaction by deoxyribonucleoside triphosphates interacting at the allosteric site(s), and indeed,

our results indicate that the allosteric effectors (dATP and dTTP) do not promote this exchange. In earlier studies (Abeles & Beck, 1967; Hogenkamp et al., 1968) with the nucleoside triphosphate from *L. leichmannii*, it was impossible to distinguish between stimulation of the exchange reaction by deoxyribonucleotides acting as products or at allosteric sites. In the present study with the nucleoside diphosphate reductase from *C. nephridii* such a distinction between product effects and allosteric effectors can be made.

Our proposal involving the presence of a second protein-based radical has precedent in the recent literature. The three-dimensional structure of the B2 subunit of the *E. coli* ribonucleotide reductase has been determined (Nordlund et al., 1990). The crystal structure shows that tyrosine-122, which harbors the stable free radical is buried in the protein 10 Å from the surface, indicating that this radical cannot participate directly in the hydrogen abstraction from the substrate. The authors suggest that an electron transfer has to occur to generate a second radical at the interface between the B2 and B1 subunits. A similar electron transfer has been proposed by Wagner et al. (1992) to generate a "working radical" in pyruvate–formate lyase. They visualize a reversible hydrogen transfer, induced by substrate binding, between glycine-734, which carries the stable radical, and cysteine-418 to generate a thiyl radical. Such a hydrogen transfer in AdoCbl-dependent reductases may well be possible and fits with our Scheme III involving two different radicals in the reduction and exchange reactions.

A survey of the earlier literature of the AdoCbl-dependent ribonucleotide reductases from *L. leichmannii* (Hogenkamp et al., 1968), *C. nephridii* (Tsai & Hogenkamp, 1980), and *Euglena gracilis* (Gleason & Hogenkamp, 1970) demonstrates that the order of substrate activity parallels that of substrate-stimulated exchange. In accord with those findings, our present results indicate that the exchange reaction between the coenzyme and the solvent is an integral part of the overall reduction.

ACKNOWLEDGMENT

We thank Gerald T. Bratt for providing the NMR analyses of the analogs and their intermediates.

REFERENCES

Abeles, R. H., & Beck, W. S. (1967) *J. Biol. Chem. 242*, 3589.

Ashley, G. W., & Stubbe, J. (1989) in *Inhibitors of Ribonucleoside Diphosphate Reductase Activity* (Cory, J. G., & Cory, A. H., Eds.) pp 55–87, Pergamon Press, New York.

Ashley, G. W., Harris, G., & Stubbe, J. (1986) *J. Biol. Chem. 261*, 3958–3964.

Beigelman, L. N., Gurskaya, G. V., Tsapkina, E. N., & Mikhailov, S. N. (1988) *Carbohydr. Res. 181*, 77–88.

Dekker, C. A. (1965) *J. Am. Chem. Soc. 87*, 4027–4029.

Follmann, H., & Hogenkamp, H. P. C. (1971) *Biochemistry 10*, 186–192.

Gleason, F. K., & Hogenkamp, H. P. C. (1970) *J. Biol. Chem. 245*, 4894–4899.

Gleason, F. K., & Hogenkamp, H. P. C. (1972) *Methods Enzymol. 18C*, 65–71.

Hoard, D. E., & Ott, D. G. (1965) *J. Am. Chem. Soc. 87*, 1785–1788.

Hogenkamp, H. P. C., Ghambeer, R. K., Brownson, C., Blakley, R. L., & Vitols, E. (1968) *J. Biol. Chem. 243*, 799–808.

Mikhailov, S. N., Beigelman, L. N., Gurskaya, G. V., Padyukova, N. S., Yakovlev, G. I., & Karpeisky, M. Y. (1983) *Carbohydr. Res. 124*, 75–96.

Nordlund, P., Sjöberg, B.-M., & Eklund, H. (1990) *Nature 345*, 593–598.

Nutt, R. F., Dickinson, M. J., Holly, F. W., & Walton, E. (1968) *J. Org. Chem. 33*, 1789–1795.

Stubbe, J. (1990) *J. Biol. Chem. 265*, 5329–5332.

Stubbe, J. & Ackles, D. (1980) *J. Biol. Chem. 265*, 8027–8030.

Stubbe, J. & Kozarich, J. W. (1980a) *J. Am. Chem. Soc. 102*, 2505–2507.

Stubbe, J. & Kozarich, J. W. (1980b) *J. Biol. Chem. 255*, 5511–5513.

Stubbe, J., Ackles, D., Segal, R., & Blakley, R. L. (1981) *J. Biol. Chem. 256*, 4843–4846.

Stubbe, J., Ator, M., & Krenitsky, T. (1983) *J. Biol. Chem. 258*, 1625–1631.

Thelander, L., Larrson, A., Hobbs, J., & Eckstein, F. (1976) *J. Biol. Chem. 251*, 1398–1405.

Tong, G. L., Lee, W. W., & Goodman, L. (1967) *J. Org. Chem. 32*, 1984–1986.

Tsai, P. K., & Hogenkamp, H. P. C. (1980) *J. Biol. Chem. 255*, 1273–1278.

Wagner, A. F. V., Frey, M., Neugebauer, F. A., Schäfer, W., & Knappe, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 996–1000.

Walling, C., & Johnson, R. A. (1975) *J. Am. Chem. Soc. 97*, 2405–2407.

Yoshikawa, M., & Takenishi, T. (1967) *Tetrahedron Lett. 50*, 5065–5068.