Mechanism-Based Inhibition of Ribonucleoside Diphosphate Reductase from *Corynebacterium nephridii* by 2′-*C*-Methyladenosine Diphosphate†

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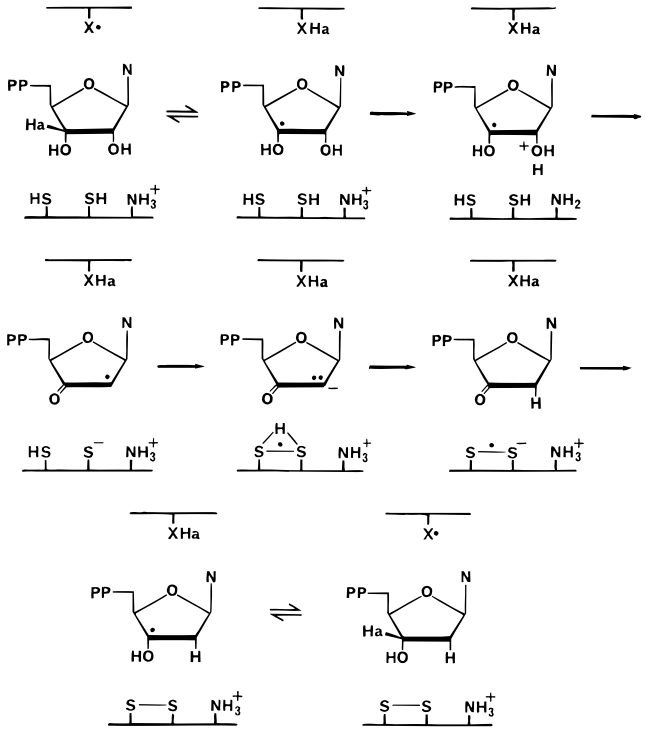
ABSTRACT: The interaction of the adenosylcobalamin-dependent ribonucleoside diphosphate reductase of *Corynebacterium nephridii* with 2′-*C*-methyladenosine diphosphate (2′-*C*-methylADP) has been investigated in more detail [Ong, S. P., McFarlan, S. C., & Hogenkamp, H. P. C. (1993) *Biochemistry 32*, 11397−11404]. This nucleotide analog partitioned between normal reduction to 2′-deoxy-2′-*C*-methyladenosine diphosphate and decomposition to adenine, 2-methylene-3(2*H*)-4-methylfuranone, and presumably pyrophosphate. Reaction of the reduced enzyme with 2′-*C*-methylADP caused the development of a chromophore at 318 nm that is characteristic of the modification of the enzyme by the furanone [Harris, G., Ator, M., & Stubbe, J. (1984) *Biochemistry 23*, 5214−5225]. Incubation of [5′-$^3H_2$]-2′-*C*-methylADP with reduced reductase resulted in the covalent incorporation of the radiolabel into the protein and into aquocobalamin. A similar incubation of the enzyme, the labeled nucleotide analog, and dithiothreitol resulted in the formation of three radioactive hydrophilic compounds. Mass spectroscopic analysis of one of these compounds showed the presence of 2-methylene-3(2*H*)-4-methylfuranone. 2′-Deoxy-2′-*C*-methylADP is a very effective promoter of the tritium exchange reaction between [5′-$^3H_2$]adenosylcobalamin and the solvent, confirming that the exchange reaction is an integral part of the overall reduction. All these observations are consistent with the proposal that 2′-*C*-methylADP serves as a substrate and a mechanism-based inhibitor of the ribonucleotide reductase of *C. nephridii*, indicating that the enzyme is able to catalyze the conversion of the nucleotide analog to a 2′-deoxy-2′-*C*-methyl-3′-ketonucleotide that can collapse to the reactive 2-methylene-3(2*H*)-4-methylfuranone. Surprisingly, 2′-*C*-methylADP did not serve as either a substrate or an inhibitor of the ribonucleoside diphosphate reductase of *Escherichia coli*.

Ribonucleotide reductases are key enzymes in all proliferating cells. They catalyze the reduction of the four common ribonucleotides to the corresponding 2′-deoxyribonucleotides, precursors for the synthesis of DNA. Heretofore, at least three different classes of ribonucleotide reductases have been identified (Reichard, 1993). These reductases differ in their protein structures and in their substrate, effector, and cofactor requirements, yet all three reductases require a protein radical for catalysis. However, each of these reductases utilizes a unique method to generate the protein radicals. The R_2 subunit of the ribonucleotide reductase from *Escherichia coli*, which is the prototype of the enzyme in higher organisms, contains a binuclear iron center and a tyrosine residue at position 122 that is the site of the radical. The iron centers of R_2 function in the generation and maintenance of this radical. The adenosylcobalamin (AdoCbl)-dependent reductases use the homolysis of the carbon−cobalt bond to generate cob(II)alamin and a 5′-deoxyadenosyl radical; the latter, in turn, generates a protein radical. Finally, the ribonucleotide reductase from anaerobically grown *E. coli* contains a Fe_4-S_4 cluster and a protein radical on a glycine residue. The generation of this radical requires adenosylmethionine.

Stubbe and co-workers (Stubbe, 1989) have proposed a mechanism for ribonucleotide reduction that involves the abstraction of a hydrogen atom from the 3′-C of the substrate by the protein radical (Scheme 1). Subsequent protonation of the 2′-hydroxyl group and loss of water generates a cation radical intermediate, which is reduced by a dithiol *via* two one-electron transfer reactions to form the 2′-deoxyribonucleotide−3′-radical. This product−radical is reduced to the 2′-deoxyribonucleotide by the protein and thus regenerates the protein radical.

Scheme 1



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The proposal is in part based on observations obtained using 2′-deoxy-2′-halonucleotides that function as mechanism-based inhibitors. Incubation of these nucleotide analogs with the reductases from *E. coli* or from *Lactobacillus leichmannii* causes the degradation of the nucleotide to pyrophosphate, halide anion, the nucleotide base, and 2-methylene-3(2*H*)-furanone. The furanone causes the covalent modification and inactivation of the enzymes. Earlier, we described the syntheses of 2′-*C*-methylADP and 2′-C-methylUDP and their interactions with the adenosylcobalamin-dependent ribonucleoside diphosphate reductase from *Corynebacterium nephridii* (Ong et al., 1993). Incubation of the nucleotide analogs with the reductase gave two products, which in the case of 2′-*C*-methylADP were identified as 2′-deoxy-2′-*C*-methylADP and adenine. Approximately 5% of the 2′-*C*-methylADP was reduced to the 2′-deoxynucleotide, while the bulk of the analog was degraded to adenine and other yet unidentified products. These results prompted us to suggest that these 2′-deoxy-2′-methylnucleotides, like the 2′-deoxy-2′-halonucleotides, function as mechanism-based inhibitors. We have now extended these earlier studies and have also prepared [5′-$^3H_2$]-2′-*C*-methylADP and followed its interaction with the ribonucleotide reductases from *C. nephridii* and *E. coli*.

MATERIALS AND METHODS

Ribonucleoside diphosphate reductase from *C. nephridii* was isolated as described by Ong et al. (1992); the specific activity = 179 nmol of dADP min^{-1} (mg of protein)$^{-1}$ (dithioreitol reductant). [5′-$^3H_2$]AdoCbl was synthesized as described before (Gleason & Hogenkamp, 1971); the specific activity = 12 869 dpm/nmol. The specific activity of the reductase in the tritium exchange reaction was 1.186×10^6 dpm min^{-1} (mg of protein)$^{-1}$ in the presence of 25 μM dGDP. The R_2 and R_1 subunits of the ribonucleoside diphosphate reductase from *E. coli* were purified separately from overproducing strains N6405/pSPS2 and C600/pMB1, respectively (Salowe & Stubbe, 1986). The *E. coli* strains as well as the purification protocols were kindly provided by Professor J. Stubbe. [2,8-^{3}H]ADP and NaB^3H_4 were purchased from NEN Research Products and Amersham Life Sciences, respectively. ADP and dGDP were from P-L Biochemicals. Dithiothreitol was obtained from Boehringer Mannheim. Silica gel 60 and trichloroacetic acid were from E. M. Science. Other materials were purchased in the highest purity available. Pyrophosphoryl chloride was synthesized as described by Imai et al. (1969). UV spectra were recorded on a Hewlett-Packard diode array spectrophotometer. High-performance liquid chromatography (HPLC) analyses were performed on a Beckman model 332 instrument equipped with an Altex Ultrasphere ODS (C18) reversed phase column (10 mm × 25 cm) and a 3 cm guard cartridge. Mass spectra were determined using a Finnigan MAT 95 high-resolution double-focusing mass spectrometer (CIMS conditions, source temperature, 150 °C; electron energy, 150 eV; NH_3 gas pressure, 1×10^{-4} Torr). The sample was introduced *via* a solids probe.

Synthesis of [5′-$^3H_2$]-2′-C-MethylADP. 2′-*C*-Methyladenosine (Ong et al., 1993) was converted to the 2′,3′-isopropylidene derivative, using the catalyst *p*-toluenesulfonic acid as described by Hampton (1961). The product was chromatographed in 10% ethanol−chloroform on a silica gel column (40 × 3 cm) and crystallized from water−ethanol. The protected nucleoside was oxidized by the dimethylsulfoxide−dicyclohexylcarbodiimide procedure of Pfitzner and Moffat (1965). The 5′-aldehyde of 2′,3′-isopropylidene-2′-*C*-methyladenosine was purified by chromatography in 10% ethanol−chloroform on silica gel. 2′,3′-Isopropylidene-2′-*C*-methyladenosine-5′-aldehyde (500 mg, 1.5 mmol) was dissolved and suspended in 10 mL of peroxide-free dioxane and 10 mL of H_2O. To this mixture, cooled in an ice bath, was added 1.25 mL of 0.1 M NaOH containing 25 mCi NaB^3H_4. The reaction mixture was stirred at room temperature for 1 h. At this time, 500 mg of $NaBH_4$ in 0.1 M NaOH was added and the mixture stirred for an additional 1 h. The mixture was then acidified with 1 N HCl to destroy excess $NaBH_4$, adjusted to pH 8 with 1 M NaOH, and evaporated to dryness. The remaining solid was triturated with 10% ethanol−chloroform and filtered and the filtrate chromatographed in 10% ethanol−chloroform on silica gel. The isopropylidene protecting group was removed by incubation of 440 μmol of [5′-$^3H_2$]-2′,3′-isopropylidene-2′-*C*-methyladenosine in 25 mL of 0.2 M HCl at room temperature for 16 h. The solvent was removed *in vacuo* and the residue dried over P_2O_5 at 60 °C. The labeled nucleoside (specific activity = 2.74×10^7 cpm/μmol) was converted to the monophosphate using pyrophosphoryl chloride in *m*-cresol as described by Imai et al. (1969), and the desired diphosphate was prepared from the monophosphate using the 1,1-carbonyldiimidazole method of Hoard and Ott (1965) as modified by Kozarich et al. (1973). The nucleotides were isolated by chromatography on QAE Sephadex (bicarbonate form; 35 × 3 cm) using a linear gradient of 0.2 to 0.6 M triethylammonium bicarbonate. [5′-$^3H_2$]-2′-*C*-MethylADP was diluted with an equal amount of 2′-*C*-methylADP and further purified by HPLC using the liquid chromatography system of Axelson et al. (1981); the specific activity = 1.72×10^7 dpm/μmol.

Reaction of [5′-$^3H_2$]-2′-MethylADP and C. nephridii Ribonucleotide Reductase. The reaction mixtures contained 100 mM dimethyl glutarate (pH 7.2), 20 mM dithiothreitol, 50 μM AdoCbl, 200 μM 2′-*C*-methylADP, 50 μM [5′-$^3H_2$]-2′-*C*-methylADP, and 5.4 μg of ribonucleotide reductase in a final volume of 100 μL. The reactions were initiated by the addition of AdoCbl and the mixtures incubated for varying time periods up to 60 min. The reactions were stopped by heating at 100 °C for 1 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 heated again at 100 °C for 1 min and centrifuged to remove denatured protein, and the supernatants were filtered through Costar SPIN-X centrifuge filter units (0.45 μm cellulose acetate). The filtrates were analyzed by HPLC on an Altex Ultrasphere ODS (C-18) reversed phase column (10 mm × 25 cm) using 20% methanol in 20 mM potassium phosphate (pH 5.6) as the eluent at a flow rate of 1.5 mL/min. The nucleosides were detected at 260 nm. One minute fractions were collected, and the radioactivity was determined by scintillation counting.

Reaction of 2′-C-MethylADP with C. nephridii Ribonucleotide Reductase and Detection of the Modified Enzyme. Ribonucleotide reductase (2.7 mg) in 1 mL of 50 mM dimethyl glutarate buffer (pH 7.6) was reduced with 100 μL of 0.5 M dithiothreitol at room temperature for 30 min. This reaction and all subsequent manipulations were carried out

under strictly anaerobic conditions in a glovebox (95% Ar, 5% H_2). Excess dithiothreitol was removed by chromatography on a Superose-12 HR 10/30 column [Pharmacia fast protein liquid chromatography (FPLC) system] using 20 mM Tris-HCl (pH 7.6) as the eluting buffer at a flow rate of 0.5 mL/min. The protein fractions were pooled (3.2 mL) and divided into four equal aliquots. The first reaction mixture contained, in a total volume of 0.88 mL, 0.80 mL of the reduced enzyme, 20 μL of 10 mM 2′-*C*-methylADP, and 60 μL of 0.05 M Tris-HCl buffer (pH 8.5). The second mixture contained the reduced enzyme, the substrate, 40 μL of 1.1 mM AdoCbl, and 20 μL of buffer. The third mixture contained the reduced enzyme, substrate, coenzyme, and 20 μL of 50 mM dithiothreitol, while the fourth reaction mixture contained the reduced enzyme and 80 μL of buffer. The four reaction mixtures were incubated at room temperature for 90 min. Each reaction mixture was then chromatographed on a Superose-12 column as described above. The protein fractions were isolated and analyzed by UV−visible spectroscopy in anaerobic cuvettes. The difference spectra from 240 to 340 nm of the proteins were determined.

In a second set of four experiments, 8.0 mg of ribonucleotide reductase was reduced with dithiothreitol at room temperature for 30 min. The reduced enzyme was then isolated as described above. Four equal aliquots of the reduced enzyme containing 1 mg/mL were used in these experiments. The first reaction mixture contained 0.60 mL of reduced enzyme, 60 μL of 2.7 mM [5′-$^3H_2$]-2′-*C*-methylADP (1.955×10^6 dpm), and 50 μL of 0.05 M Tris-HCl (pH 8.5). The second reaction mixture contained the reduced enzyme, substrate, 40 μL of 1.1 mM AdoCbl, and 10 μL of buffer. The third reaction mixture contained the reduced enzyme, substrate, coenzyme, and 10 μL of 100 mM dithiothreitol, while the fourth reaction mixture contained the reduced enzyme and 110 μL of buffer. The four reaction mixtures were incubated at room temperature for 90 min. An aliquot of 0.5 mL was then chromatographed on a Superose-12 column as described above, and the radioactivity of each 0.5 mL fraction was determined by scintillation counting. The fractions containing protein, nucleotides, and coenzyme from each chromatographic run were pooled, and the total radioactivity of each pool was determined.

Reaction of 2′-C-MethylADP with E. coli Ribonucleotide Reductase. The assay mixtures contained, in a final volume of 100 μL, 50 mM HEPES (pH 7.6), 15 mM $MgSO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 μM dGTP, 3 μg of R_1 subunit, 8 μg of R_2 subunit, 20 mM dithiothreitol, and varying concentrations of ADP and/or 2′-*C*-methylADP. The reactions were initiated by the addition of the R_1 subunit, and the mixtures were incubated for varying time periods up to 60 min at 37 °C and analyzed by HPLC as described above.

Isolation of [5′-$^3H_2$]-2′-Deoxy-2′-C-methylADP. Seven reaction mixtures each containing 20 mM dithiothreitol, 50 μM AdoCbl, 0.6 mM [5′-$^3H_2$]-2′-*C*-methylADP (4.98×10^3 dpm/nmol), 50 mM Tris-HCl buffer (pH 8.5), and 0.05 mg of *C. nephridii* ribonucleotide reductase, in a total volume of 0.2 mL, were incubated at 37 °C for 90 min. During the next 4 h, 0.05 mg of reductase, 10 nmol of AdoCbl, and 4 μmol of dithiothreitol were added at 1 h intervals. The reaction mixtures were then combined and applied to a QAE-Sephadex (Pharmacia) column (bicarbonate form, 2 mL) equilibrated in water. The column was washed with 10 mL of water, and the nucleotides were eluted with a triethylammonium bicarbonate (pH 7.8) gradient of 0.1 to 1.0 M. The fractions containing the nucleoside diphosphates were pooled and lyophilized. The ribo- and deoxyribonucleotides were then separated on a 5 mL Affi-Gel 601 boronate resin (Bio-Rad) equilibriated in 100 mM triethylammonium bicarbonate containing 15 mM magnesium acetate. The column was eluted with the same buffer until all the 2′-deoxy-2′-*C*-methylADP was removed as judged by the absorbance at 260 nm. The deoxynucleotide was lyophilized and dissolved in 1 mL of water. Analysis by HPLC of an aliquot treated with alkaline phosphatase (as described above) showed that the solution contained 92% 2′-deoxy-2′-*C*-methylADP and 8% 2′-*C*-methylADP. The nucleotide solution was desalted by HPLC on an AGMP-1 cation exchange column (14 × 0.5 cm); elution was accomplished using a water−trifluoroacetate acid (0 to 150 mM) gradient. The fractions containing the nucleotides were lyophilized, dissolved in 1 mL of water, and lyophilized again. The residue was dissolved in 0.28 mL of 50 mM Tris-HCl (pH 8.5). The yield = 33.4 mmol (1.65×10^5 dpm).

Isolation of the Dithiothreitol−Methylfuranone Adducts. The water wash of the QAE-Sephadex column, used for the isolation of the nucleotides (*vide supra*), was applied to a 2 mL AG-50W-X2 column (200−400 mesh; H^+ form) equilibrated in water. The putative dithiothreitol−methylfuranone adducts were eluted with water, while aquocobalamin and adenine were retained by the ion exchange resin. The water wash was lyophilized and the residue dissolved in 0.4 mL of water. The adducts were separated by HPLC on an Altex Ultrasphere ODS column using 15% methanol−85% water as the eluent at a flow rate of 1.5 mL/min. These radioactive fractions were collected, lyophilized, and analyzed by mass spectroscopy.

Tritium Exchange from [5′-$^3H_2$]AdoCbl. These reaction mixtures contained 50 mM dimethyl glutarate (pH 7.5), 20 mM dithiothreitol, 50 μM [5′-$^3H_2$]AdoCbl, varying concentrations of 2′-*C*-methylADP or 2′-deoxy-2′-*C*-methylADP up to 75 μM, and 2.5 μg of ribonucleotide reductase from *C. nephridii* in a final volume of 100 μL. The reactions were initiated by the addition of the labeled coenzyme and the mixtures incubated at 37 °C in the dark for 30 min. The reactions were terminated by the addition of 1 mL of 2% trichloroacetic acid containing 100 mg of activated charcoal. After the assay mixtures were mixed thoroughly, they were centrifuged for 10 min. The amount of ^3H label released to the solvent was determined by liquid scintillation counting of a 400 μL aliquot of the supernatant in 10 mL of scintillation cocktail. Because the 2′-deoxy-2′-*C*-methylADP contained 8% of the ribonucleotide, control reactions with an equivalent amount of 2′-*C*-methylADP were run in parallel and the amount of exchange was corrected for the small amount of label released in the controls.

RESULTS

Interaction of [5′-$^3H_2$]-2′-C-MethylADP with Ribonucleotide Reductase from C. nephridii. As demonstrated before (Ong et al., 1993), incubation of the ribonucleotide reductase from *C. nephridii* with 2′-*C*-methylADP yields adenine and 2′-deoxy-2′-*C*-methylADP. The effect of pH on the reaction and on the partitioning between normal reduction and

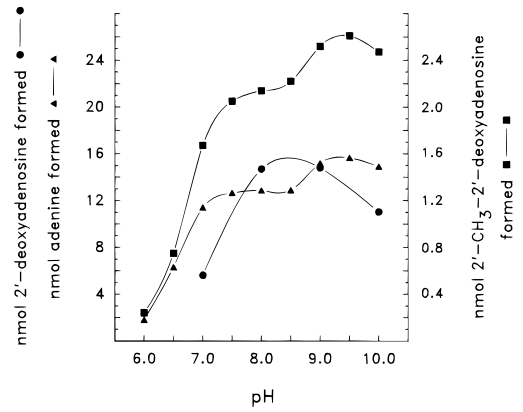

FIGURE 1: Effect of pH on the reaction of *C. nephridii* ribonucleotide reductase with ADP and 2′-methylADP. The reactions were performed and the products analyzed by HPLC as described in Materials and Methods. The buffers used were 20 mM potassium dimethyl glutarate (pH 6.0−8.0) and 100 mM Tris-HCl (pH 7.0−10.0): 2′-deoxyadenosine (●), adenine (▲), and 2′-deoxy-2′-*C*-methyladenosine (■).

decomposition is shown in Figure 1. HPLC analyses of reaction mixtures containing [5′-$^3H_2$]-2′-*C*-methylADP show (after dephosphorylation) the disappearance of the labeled substrate and the formation of adenine, [5′-$^3H_2$]-2′-*C*-methyladenosine, and three radioactive hydrophilic compounds (data not shown). No reaction occurs in the absence of either enzyme or coenzyme.

2′-C-MethylADP Does Not Interact with Ribonucleotide Reductase from E. coli. Incubation of the ribonucleotide reductase from *E. coli* with 2′-*C*-methylADP does not generate any products. HPLC analyses of reaction mixtures containing the nucleotide analog show (after dephosphorylation) only 2′-*C*-methyladenosine; no adenine or 2′-deoxy-2′-*C*-methyladenosine could be detected. The addition of the allosteric effector dGTP, varying the pH of the reaction mixtures from pH 5 to 10, or including 1 M sodium acetate did not promote the reaction. Furthermore, 2′-*C*-methylADP did not inhibit the reduction of ADP at 2′-*C*-methylADP: ADP ratios of 4:1 (Figure 2) and even at ratios up to 10:1.

2′-C-MethylADP Causes the Covalent Modification of the Ribonucleotide Reductase from C. nephridii. Several years ago, Thelander et al. (1976) reported that the inactivation of ribonucleotide reductase from *E. coli* by 2′-chloro-2′-deoxyUDP resulted in a change of the absorption spectrum of the protein with an absorbancy maximum at 320 nm. Harris et al. (1984) confirmed this finding and demonstrated an identical modification of the ribonucleotide reductase from *L. leichmannii* by 2′-chloro-2′-deoxyUTP. They also showed that this modification was caused by 2-methylene-3(2*H*)-furanone derived from the sugar moiety of the nucleotide analog. Incubation of the reduced reductase from *C. nephridii* with 2′-*C*-methylADP and AdoCbl results in a similar change in the absorption spectrum of the protein. The spectra of the ribonucleotide reductase incubated with 2′-*C*-methylADP, with and without the coenzyme, as well as their difference spectrum are presented in Figure 3. The difference spectrum clearly shows a broad maximum at 318 nm. When dithiothreitol is added to the complete reaction mixture, no change in the protein spectrum is observed (data not shown). To confirm that the reductase was indeed modified by 2′-*C*-methylADP, an identical set of experiments was carried out using [5′-$^3H_2$]-2′-*C*-methylADP as the

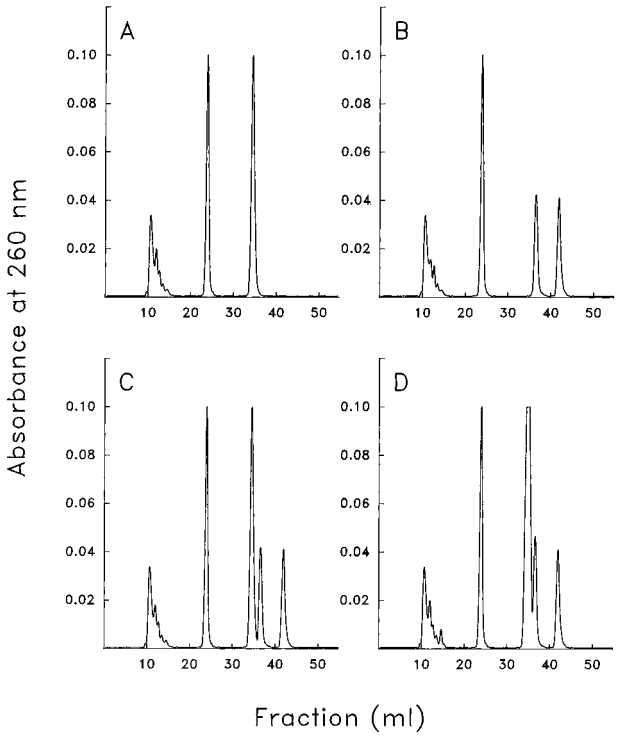

FIGURE 2: Interaction of ADP and 2′-*C*-methylADP with *E. coli* ribonucleotide reductase. HPLC profiles of the products from reaction mixtures containing 2′-*C*-methylADP (A), ADP (B), ADP + 2′-*C*-methylADP (1:1) (C), and ADP + 2′-*C*-methylADP (1:4) (D). The positive allosteric effector dGTP was added to all four reaction mixtures. The elution volumes were as follows: 2′-deoxyguanosine, 23.6 mL; 2′-*C*-methyladenosine, 34.2 mL; adenosine, 36.5 mL; and 2′-deoxyadenosine, 41.9 mL.

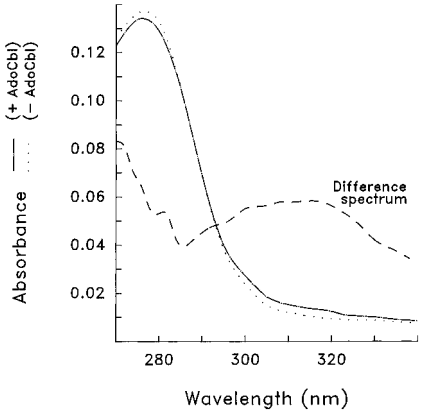

FIGURE 3: UV−visible spectra of *C. nephridii* ribonucleotide reductase after interaction with 2′-*C*-methylADP.

substrate. FPLC analysis of the reaction mixtures showed that, in a complete reaction consisting of the reduced enzyme, radioactive substrate, and coenzyme, the protein was radiolabeled (Figure 4B). Three radioactive fractions are evident. Fraction 1 (elution volume of 9 mL) contains the reductase; fraction 2 (elution volume of 15 mL) contains the nucleotide substrate and product, and fraction 3 (elution volume of 20 mL) is pink in color.

When AdoCbl is omitted from the mixture, no reaction occurs and thus the protein fraction is not labeled (Figure 4A). In contrast, when dithiothreitol is added to a complete reaction mixture, reduction proceeds further and an additional radioactive fraction with a retention volume of about 24 mL is observed. However, in this experiment, the protein fraction contains much less radiolabel (Figure 4C and Table

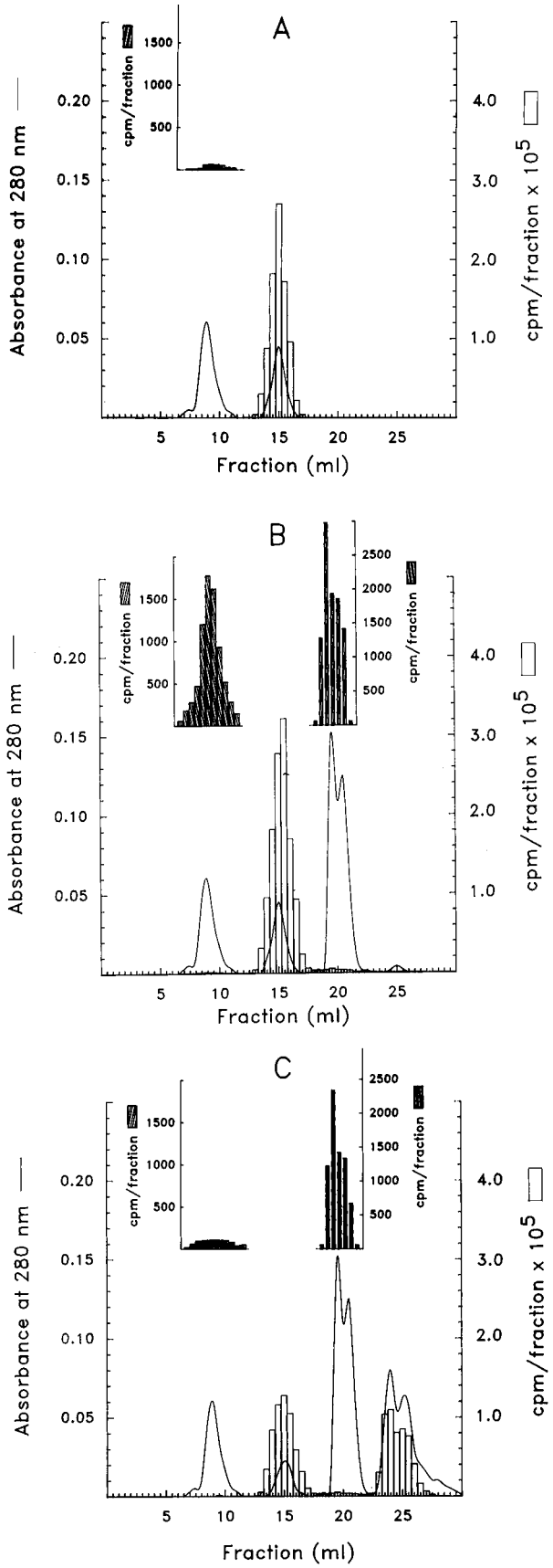

FIGURE 4: Incubation of [5′-$^3H_2$]-2′-methylADP with reduced *C. nephridii* ribonucleotide reductase. FPLC profiles of the reaction mixtures containing reduced enzyme and radiolabeled 2′-*C*-methylADP (A), reduced enzyme, radiolabeled nucleotide, and AdoCbl (B), and reduced enzyme, radiolabeled nucleotide, AdoCbl, and dithiothreitol (C).

Table 1: Modification of Ribonucleotide Reductase from *C. nephridii* by [5′-$^3H_2$]-2′-*C*-MethylADP

reaction	protein	nucleotide	cobalamin	dithiothreitol adduct	Figure
1	113[a] (0.00)[b]	1.91×10^6 (97.7)	−	−	4A
2	1.19×10^4 (0.61)	1.86×10^6 (95.1)	1.57×10^4 (0.80)	−	4B
3	1257 (0.06)	8.76×10^5 (44.8)	1.16×10^4 (0.59)	8.37×10^5 (42.8)	4C

[a] dpm ^3H in each fraction. [b] Percent of label from [5′-$^3H_2$]-2′-*C*-methylADP. 100% = 1.955×10^6 dpm.

1). In a second set of experiments using 6.135×10^4 dpm of [5′-$^3H_2$]-2′-*C*-methylADP, a very similar distribution of the radioactivity in the fractions was observed.

The protein fraction of the second reaction was lyophilized and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). All the radiolabel was found to be associated with a protein with an apparent molecular mass of 100 kDa. In order to determine the extent of the reduction, the nucleotide fraction was treated with alkaline phosphatase and the nucleosides were separated by HPLC. Measurements of the radioactivity in the nucleoside fractions showed that about 9.7% of the 2′-*C*-methylADP was reduced to 2′-deoxy-2′-methylADP. The UV−visible spectrum of the pink third FPLC fraction showed that it contained aquocobalamin. This fraction was concentrated and purified on a PEI cellulose TLC plate using *n*-butanol−acetic acid−water (5:2:3) as the solvent. Any nucleotide contaminating this fraction would stay at the origin. All of the radioactivity contained in the fraction comigrated with the cobalamin. The putative dithiothreitol−furanone adducts were isolated by HPLC and analyzed by mass spectroscopy. They were unstable under analysis conditions, and major fragmentation occurred. However, the mass spectrum of one of the fractions showed the following molecular ions: *m*/*e* (relative intensity) 202.1 (4.42), 127.1 (4.36), and 110.1 (100).

Promotion of the Exchange Reaction between [5′-$^3H_2$]-AdoCbl and the Solvent by 2′-Deoxy-2′-C-MethylADP. Earlier we reported (Ong et al., 1992) that the deoxyribonucleoside diphosphates, dGDP, dADP, and dCDP, are more effective promoters of the tritium exchange between the coenzyme and water than their corresponding ribonucleoside diphosphate substrates. Figure 5 demonstrates that 2′-deoxy-2′-*C*-methylADP is also a more effective promoter than 2′-*C*-methylADP.

DISCUSSION

Some time ago, Harris and co-workers (1987) described the interaction of a series of 2′-halogenated ribo- and arabinonucleoside triphosphates with the AdoCbl-dependent ribonucleoside triphosphate reductase from *L. leichmannii*. All these analogs acted as mechanism-based inhibitors and decomposed to the nucleotide base, triphosphate, and halide ion. In addition, the inactivated enzyme showed a change in its absorption spectrum with a maximum at 320 nm, indicative of a modification by 2-methylene-3(2*H*)-furanone derived from the sugar moiety. Because both the ribo- and arabinohalonucleotides were mechanism-based inhibitors, Harris et al. concluded that the cleavage of the 2′-carbon−halogen bond is not enzyme-catalyzed. Three of the nucle-

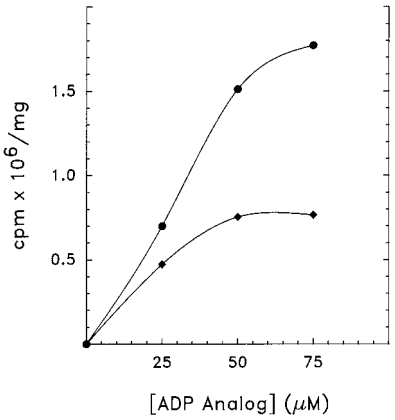

FIGURE 5: Promotion of the tritium exchange from [5′-$^3H_2$]AdoCbl to the solvent by 2′-*C*-methylADP (◆) and 2′-deoxy-2′-*C*-methylADP (●).

otide analogs tested, F-UTP, F-CTP, and Cl-UTP, were found to partition between normal reduction to the 2′-deoxyribonucleotides and formation of the 3′-keto-2′-deoxyribonucleotides which collapse to the base, triphosphate, and 2-methylene-3(2*H*)-furanone. For F-UTP, the ratio of normal reduction and abnormal turnover was about 1, while for Cl-UTP, this ratio was about 0.005. The authors suggested that the partitioning is a function of the leaving ability of the 2′-substituent. However, the partition ratio was also affected by the pH of the reaction and by the concentration of dithiothreitol. Furthermore, partitioning was only observed with the pyrimidine nucleotides; none of the halo-ATP analogs were reduced to dATP.

Earlier, we showed that the interaction of 2′-*C*-methylADP with the ribonucleoside diphosphate reductase of *C. nephridii* produced 2′-deoxy-2′-*C*-methylADP and adenine, indicating that this nucleotide analog also partitioned between normal reduction and the "abnormal" formation of 2′-deoxy-2′-*C*-methyl-3′-ketoADP which collapsed to pyrophosphate, adenine, and 2-methylene-3(2*H*)-4-methylfuranone (Ong et al., 1993). The effect of the pH of the reaction on the partitioning between normal reduction to 2′-deoxy-2′-*C*-methylADP and decomposition to adenine was investigated (Figure 1). The pH profile is complex with a broad plateau around pH 8 and a second maximum at pH 9.5. The extent of normal reduction compared to adenine formation varied from 1 in 10 at pH 6.5 to 1 in 6 from pH 8 to 9.5. These results are similar to those of Harris et al. (1987), who found that higher pH values lead to greater normal reduction of 2′-chloro-2′-deoxyUTP to dUTP by the reductase of *L. leichmannii*. The pH profile of the reaction of *C. nephridii* reductase with 2′-*C*-methylADP is distinct from that of the reduction of ADP, which shows a normal pH profile with maximum activity at pH 8.5. The complex pH profile suggests that a second functional group participates in the reaction with the nucleotide analog.

Surprisingly, the reductase system from *E. coli* does not interact with 2′-*C*-methylADP under a wide variety of conditions; 2′-*C*-methylADP is neither a substrate nor an inhibitor of this reductase (Figure 2). Is it possible that 2′-*C*-methylADP is a specific mechanism-based inhibitor of only the AdoCbl-dependent reductases?

In order to substantiate the indication that 2′-*C*-methylADP functions both as a substrate and as a mechanism-based inhibitor of the reductase from *C. nephridii*, we followed the modification of the reductase by the two approaches that were so successfully used by Stubbe and co-workers. Difference spectra of reaction mixtures containing prereduced reductase, the nucleotide analog, with and without AdoCbl, showed a new absorbance maximum at 318 nm, indicative of the covalent modification of the enzyme by a furanone (Figure 3). When dithiothreitol was included in the reaction mixtures, no modification of the enzyme was observed. In a second set of experiments, the prereduced enzyme was incubated with [5′-$^3H_2$]-2′-*C*-methylADP (Figure 4). Incubation of the labeled nucleotide analog with the enzyme and coenzyme resulted in the covalent modification of the enzyme and coenzyme. SDS−PAGE of the enzyme showed that the radiolabel comigrated with the protein. UV−visible spectroscopy of the cobalamin fraction identified it as aquocobalamin, indicating that the coenzyme decomposed either during the reaction or during the workup. Since aquocobalamin contains nucleophilic groups only in the ribazole moiety (2′-OH and 5′-OH), the corrinoid is probably modified at one or both of the hydroxyl groups. As expected, including dithiothreitol in the reaction decreased the amount of label associated with the protein and caused the appearance of an additional radioactive fraction, presumably containing the 2-methylene-3(2*H*)-4-methylfuranone−dithiothreitol adducts.

Interestingly, even in the presence of dithiothreitol, the cobalamin is still labeled, albeit somewhat less, suggesting that the coenzyme site and the substrate site are in close proximity (Table 1). Further HPLC analysis of the fraction containing the putative furanone−dithiothreitol adducts showed three radioactive hydrophilic peaks, suggesting the presence of two singly modified furanones either at the exocyclic methylene position or at C5 and one doubly substituted furanone at both positions. Although the mass spectra of the three fractions showed extensive fragmentation, suggesting that the adducts were unstable under the conditions of the analysis, the spectrum of one of the fractions showed three molecular ions at 202.1, 127.1, and 110.1. The predominant mass at 110.1 corresponds to 2-methylene-3(2*H*)-4-methylfuranone and the mass at 127.1 to its ammonia adduct. The mass at 202.1 probably corresponds to a fragment of the furanone−dithiothreitol adduct. These results demonstrate that 2-methylene-3(2*H*)-4-methylfuranone is indeed a degradation product of 2′-*C*-methylADP and that this nucleotide analog does function as a mechanism-based inhibitor of the AdoCbl-dependent ribonucleotide reductase of *C. naphridii*.

The product of the normal reduction of 2′-*C*-methylADP, 2′-deoxy-2′-*C*-methylADP, is a very effective promoter of the tritium exchange reaction, indicating that an abstractable hydrogen at the 3′-C position is essential not only for the overall reduction but also for the exchange reaction. The observation that the 2′-deoxynucleotide products promote the exchange reaction suggests that these products also interact with the enzyme in a reversible manner to generate the 3′-radical of the 2′-deoxyribonucleotides.

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