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Epimerization at Carbon-5′ of (5′*R*)-[5′-^{2}H]Adenosylcobalamin by Ribonucleoside Triphosphate Reductase: Cysteine 408-Independent Cleavage of the Co−C5′ Bond[†]

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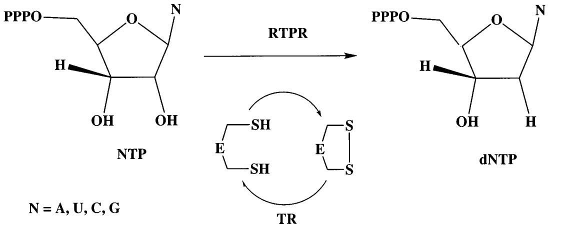
ABSTRACT: The adenosylcobalamin-dependent ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* catalyzes the reduction of ribonucleoside triphosphates to deoxyribonucleoside triphosphates. RTPR also catalyzes the exchange of the C5′-hydrogens of adenosylcobalalamin with solvent hydrogen. A thiyl radical located on Cys 408 is generated by reaction of adenosylcobalamin at the active site and is proposed to be the intermediate for both the nucleotide reduction and the 5′-hydrogen exchange reactions. In the present research, a stereochemical approach is used to study the mechanism of the Co−C5′ bond cleavage of adenosylcobalamin in the reaction of RTPR. When stereoselectively deuterated coenzyme, (5′*R*)-[5′-$^2H_1$] adenosylcobalamin (5′*R*/*S* = 3:1), was incubated with RTPR or the Cys 408 viariants, C408A-RTPR and C408S-RTPR in the presence of dGTP, the deuterium at the 5′-carbon was stereochemically scrambled, leading to epimerization of the (5′*S*)-[5′-$^2H_1$]- and (5′*R*)-[5′-$^2H_1$]-isotopomers. Observation of epimerization with mutated RTPR proves that transient cleavage of the Co−C5′ bond occurs in the absence of the thiol group on Cys 408. The rate constants for epimerization by RTPR, C408A-RTPR, and C408S-RTPRs in the presence of dGTP are 5.1, 0.28, and 0.42 s^{-1}, respectively. Only the wild-type RTPR catalyzes the 5′-hydrogen exchange reaction. Both epimerization and 5′-hydrogen exchange reactions are stimulated by the allosteric effector dGTP, and epimerization is not detected in the absence of the effector. Mechanistic implications with respect to wt-RTPR-mediated carbon cobalt bond homolysis and the intermediacy of the 5′-deoxyadenosyl radical will be presented.

In vivo, deoxyribonucleotides are produced by reduction of ribonucleotides in reactions catalyzed by ribonucleotide reductases. The *Lactobacillus leichmannii* ribonucleoside triphosphate reductase (RTPR[1]), a class II ribonucleotide reductase, requires adenosylcobalamin and catalyzes the reaction shown in eq 1 in which reduction is accompanied by active site disulfide formation (*1*).



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Thioredoxin (TR) is essential for multiple turnovers, rereducing the active site thiols indirectly by disulfide interchange through two additional C-terminal thiols (*2*, *3*). RTPR catalyzed reduction also requires deoxyribonucleoside triphosphates and/or ATP as allosteric effectors to control the rate of reduction and the substrate specificity (*4*).

RTPR is unique among adenosylcobalamin requiring proteins in that in addition to the reduction reaction, it catalyzes the exchange of 5′-methylene hydrogens of adenosylcobalamin with solvent (*5*, *6*). The rate constant for the exchange reaction is similar to that of nucleotide reduction (*7*). The exchange reaction also requires the presence of an allosteric effector, and both reactions require adenosylcobalamin dependent formation of a transient thiyl radical. Rapid freeze quench EPR experiments have identified the transient thiyl radical and shown it to be kinetically competent (*8*, *9*). Site-directed mutagenesis experiments, and more recently crystallographic studies, have identified C408 as the locus of this protein radical (*3*, *11*). The role of adenosylcobalamin is thus unique relative to other B_{12}

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[1] Abbreviations: RTPR, ribonucleoside triphosphate reductase; C408A-RTPR, C408A variant of ribonucleoside triphosphate reductase; C408S-RTPR, C408S variant of ribonucleoside triphosphate reductase; adenosylcobalamin, 5′-deoxyadenosylcobalamin; dGTP, 2′-deoxyguanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; NADPH, β-nicotinamide adenine dinucleotide; DTT, dithiothreitol; TR, thioredoxin; TRR, thioredoxin reductase; D_2O, deuterium oxide; ^1H NMR, proton nuclear magnetic resonance spectroscopy; TFA, trifluoroacetic acid.

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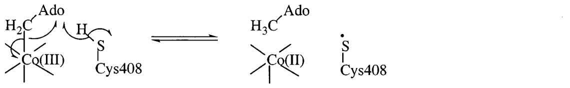
requiring proteins; its role is to generate a transient protein radical, not a carbon centered radical on a substrate.

In recent presteady state kinetic studies, which were based on the original experiments of Blakley and Tamao (*12*), Stubbe and co-workers proposed that both the exchange reaction and the reduction reaction proceed through a common intermediate: a thiyl radical, cob(II)alamin, and 5′-deoxyadenosine (*7*, *8*). Analysis of the kinetics and isotope effects on the exchange reaction led to the proposal that formation of this intermediate from adenosylcobalamin and C408-RTPR occurred in a concerted fashion, eq 2 (*7*). However, an alternative stepwise mechanism (eq 3) in which a high energy 5′-deoxyadenosyl radical intermediate and cob(II)alamin is generated followed by rapid formation of thiyl radical and 5′-deoxyadenosine could not be excluded.



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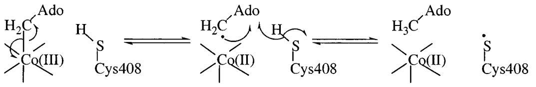

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The present work was undertaken in a search for the 5′-deoxyadenosyl radical intermediate and to better understand the mechanism of Co−C5′ bond cleavage in the reaction catalyzed by RTPR.

EXPERIMENTAL PROCEDURES

Materials. Adenosylcobalamin, dGTP, ATP, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), dithiothreitol (DTT), TR, and thioredoxin reductase (TRR) were purchased from Sigma. Deuterium oxide (D_2O, 99.9 atom % D) was from Aldrich. HPLC grade methanol was from Fisher Scientific. Microcon-30 centrifugal filter devices were obtained from Millipore. The filter devices employ Amicon low-binding, hydrophilic regenerated cellulose membranes of low absorption characteristics. Sep-Pak C_{18} cartridges were from Waters. *Eschericia coli* cells (strain HB101) transformed with the expression plasmid carrying the RTPR gene from *L. leichmannii* and *E. coli* cells (strain BL21) transformed with the expression plasmid carrying the genes for expression of C408S-RTPR and C408A-RTPR were as described (*3*, *10*).

Synthesis of Stereoselectively Deuterated [5′-^{2}H] Adenosylcobalamin. Synthesis of (5′*R*)-[5-$^2H_1$]adenosylcobalamin [(5′*R*)/(5′*S*) = 3:1] was carried out in the dark as described elsewhere (*13*). Stereochemical analysis by ^{1}H NMR showed it to consist of a 3:1 mixture of 5′*R*/5′*S* epimers. This material is hereafter referred to as (5′*R*)-[5′-$^2H_1$]adenosylcobalamin. The product was purified in the dark by reverse-phase chromatography on a Superformance glass cartridge (2.5 × 30 cm, E. Merck, Germany, packed with octadecyl functionalized silica gel from Aldrich, equilibrated in 0.02% trifluoroacetic acid (TFA)/water). The reaction mixture was acidified with concentrated TFA to pH 1, diluted to 150 mL, and transferred into a 150 mL Super-Loop (Pharmacia, Sweden). The Super-Loop was connected to a FPLC system (Pharmacia, Sweden), and the sample was loaded at a flow rate of 5 mL/min. The loop was removed from the system, and the column was washed with 200 mL of 0.02% TFA (A). A linear gradient starting at 100% A to 75% B (80% methanol, 0.02% TFA) was applied at 5 mL/min and over 220 min. Fractions (20 mL) were collected, and aliquots were examined by UV/Vis spectroscopy. (5′*R*)-[5-$^2H_1$]Adenosylcobalamin emerged at 28% B. Pure fractions containing (5′*R*)-[5-$^2H_1$]adenosylcobalamin were pooled, evaporated under reduced pressure, and redissolved in 5 mL of water. The desired product was absorbed on a SepPak C18 cartridge (3 mL, Waters), and the cartridge was washed with water until the flow-through was neutral. (5′*R*)-[5-$^2H_1$]Adenosylcobalamin was then eluted with methanol. The (5′*R*)-[5-$^2H_1$]adenosylcobalamin in methanol was diluted to 1.5 mg/mL, and 1 mL aliquots were evaporated in vacuo in amber 1.5 mL centrifuge tubes and stored at −20 °C.

General Methods. Solution concentrations were measured spectrophotometrically using the following molar extinction coefficients: ϵ_{280} of 101 000 M^{-1} cm^{-1} for RTPR (*1*), ϵ_{525} = 8000 M^{-1} cm^{-1} for adenosylcobalamin (*14*), and ϵ_{260} = 15 400 M^{-1} cm^{-1} for dGTP and ATP (*15*). All experiments involving adenosylcobalamin were carried out in dark or under dim light. In D_2O solutions, all measured pHs were corrected to pD by addition of 0.4 to the reading. Enzyme activities were assayed by the coupled assay method using TRR/TR/NADPH as the reducing agents (*1*). The assay mixture (1.0 mL) contained 50 mM HEPES (pH 7.4), 1.0 mM dGTP, 2.0 mM ATP, 200 μM NADPH, 12 μM TR, 0.16 μM TRR, and 0.5 μM RTPR or 10 μM C408A-RTPR. The reaction was initiated by the addition of adenosylcobalamin (5.0 μM), and the decrease in A340 nm was monitored. Background was corrected by running a control reaction in the absence of the added enzyme. Rates were calculated using $\Delta\epsilon_{340} = 6220$ M^{-1} cm^{-1}. A specific activity of 0.41 ± 0.05 μmol min^{-1} mg^{-1} ($k_{cat} = 0.55 \pm 0.07$ s^{-1}) was measured at 37 °C. No activity for C408A-RTPR was detected.

Measurement of Adenosylcobalamin Binding to C408A-RTPR. Measurement of adenosylcobalamin binding was carried out by ultrafiltration using Microcon-30 centrifugal filter devices (Millipore) and a 30 K cutoff filter as described (*16*). In one set of experiments, the solution contained 50 mM HEPES (pH 7.4), 105 μM adenosylcobalamin, 2.0 mM dGTP, 20 mM DTT, and from 40 to 520 μM of C408A-RTPR in a total volume of 500 μL. The mixtures were transferred to the filter devices after being incubated at 37 °C for 10 min and centrifuged for 3−5 min so that less than 15% of the solution had passed into the filtrate. Controls excluding protein revealed that the amount of adenosylcobalamin bound to the filters was small (<1%) under the experimental conditions, and it was corrected by subtraction. The concentration of adenosylcobalamin in the filtrate was accepted as the concentration of free adenosylcobalamin. The concentration of the adenosylcobalamin−C408A-RTPR was calculated by subtracting the concentration of free adenosylcobalamin from total coenzyme. The ratio of $[\text{C408A-RTPR}]_B/[\text{ adenosylcobalamin}]_T$ (α) was then plotted against the concentrations of C408A-RTPR. The dissociation constant, K_d, was calculated from least-squares fitting of the data to eq 4

$$\alpha = \frac{C\,[\text{enzyme}]}{(K_d + [\text{enzyme}])} \quad (4)$$

where C is a constant defining the maximal degree of binding.

In a second set of experiments, fixed concentrations of C408A-RTPR (100 μM) were incubated with various concentrations of adenosylcobalamin (from 40 to 700 μM) in 50 mM HEPES (pH 7.4), 2.0 mM dGTP, and 20 mM DTT in a total volume of 500 μL under the same conditions described above. The workup and analysis were as described above (eq 4).

Assay for Epimerization at C5′ of (5′R)-[5′-$^2H_1$]Adenosylcobalamin Catalyzed by C408A-RTPR and WT- RTPR. Epimerization of (5′*R*)-[5′-$^2H_1$]adenosylcobalamin catalyzed by mutant or wt-RTPR was analyzed by monitoring the scrambling of the deuterium at the 5′-carbon using NMR spectroscopy. In reactions catalyzed by C408A-RTPR, the assay mixture contained 0.6 mM (5′*R*)-[5′-$^2H_1$]adenosylcobalamin, and 0.0−10.0 μM C408A-RTPR. In reactions catalyzed by RTPR, conditions were the same except that buffer and reagents were exchanged with D_2O, and the concentrations of enzyme and (5′*R*)-[5′-$^2H_1$]adenosylcobalamin were 2.5 μM and 0.5 mM, respectively. The reactions were preincubated at 37 °C for 5 min before the addition of (5′*R*)-[5′-$^2H_1$]adenosylcobalamin and enzyme. After the preincubation, (5′*R*)-[5′-$^2H_1$]adenosylcobalamin was added, and a 0.85 mL aliquot of the reaction mixture was removed (t = 0) and loaded onto a Sep-Pak C_{18} cartridge that had been previously washed with 10 mL of methanol followed by 15 mL of ddH_2O. The reactions were initiated by the addition of enzyme. At 1−8 min time intervals, 0.85 mL aliquots were removed and loaded onto the cartridge followed by a quick wash with 2.0 mL of ddH_2O to quench the reactions. The cartridges were then further washed with 10 mL of ddH_2O, and the coenzyme was eluted with methanol. The solvent was removed in vacuo, and adenosylcobalamin was redissolved in 0.6 mL of D_2O for analysis by ^{1}H NMR spectroscopy. In some experiments DTT was replaced with NADPH/TR/TRR at 0.5 mM, 65 μM, and 0.2 μM, respectively.

Assay for Exchange of 5′-Hydrogens of Adenosylcobalamin with D_2O. The hydrogen exchange reactions were carried out using buffers and reagents exchanged in D_2O. The assay mixtures were contained in a volume of 7.0 mL:50 mM HEPES at pD 7.4, 320 μM dGTP, 20 mM DTT, 0.6 mM adenosylcobalamin or 0.5 mM (5′*R*)-[5′-$^2H_1$]adenosylcobalamin, and 2.5 μM RTPR or 10.0 μM C408A-RTPR. The reactions were carried out and worked up as described above.

Spectrometry. UV/visible spectrophotometry was performed on a Hewlett-Packard model 8452A diode array spectrometer with a thermostatically controlled cuvette holder. ^{1}H NMR spectra were acquired on a Bruker DMX 500 MHz spectrometer at 300 K. The probe was tuned for each sample, and the 90° pulse was calibrated. Spectral conditions were 16 K time domain points and 256 scans, with a 3.0 s delay between acquisitions. Spectral data were processed with a Silicon Graphics workstation using the XWINNMR 2.6 software package. Peaks of interest were integrated manually using multiple polynomial baseline corrections. Data from the integration of the C-5′ proton peaks derived from (5′*R*)-[5′-$^2H_1$]adenosylcobalamin, (5′*S*)-[5′-$^2H_1$]adenosylcobalamin, or adenosylcobalamin were plotted against reaction times and fitted to single exponentials using the nonlinear least-squares fitting program in the KaleidaGraph software.

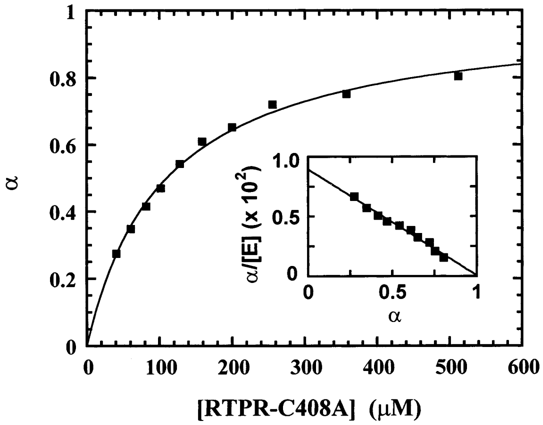


FIGURE 1: Determination of the dissociation constant for the complex of adenosylcobalamin and C408A-RTPR. Adenosylcobalamin solution (~105 μM) was titrated with various concentrations of C408A-RTPR (from 40 to 520 μM) in 50 mM HEPES buffer (pH 7.4) containing 2.0 mM dGTP and 20 mM DTT in a total volume of 0.5 mL at 37 °C. The binding mixtures were then briefly centrifuged in Microcon-30 so that less than 15% of the binding solution had passed the filter. The concentrations of free adenosylcobalamin in the filtrates were measured using $\epsilon_{525} = 8000$ M^{-1} cm^{-1}. The bound C408A-RTPR were calculated by subtracting the concentrations of free adenosylcobalamin from that of the total adenosylcobalamin. A dissociation constant of 132 ± 10 μM was determined by plotting α (where $\alpha = [\text{C408A-RTPR}]_B/[\text{adenosylcobalamin}]_T$) vs C408A-RTPR concentrations and fitting the data to eq 4. The inset is the Scatchard plot of the data.

RESULTS

Measurement of Adenosylcobalamin Binding to C408A-RTPR. As a prelude to the investigation of whether RTPR can catalyze the Co−C5′ homolytic cleavage of adenosylcobalamin in the absence of Cys 408, the binding of adenosylcobalamin to C408A-RTPR was first evaluated using the ultrafiltration method (*16*). Adenosylcobalamin (105 μM) was mixed with various concentrations of C408A-RTPR and dGTP and DTT under the conditions described in the General Methods section. Figure 1 shows the plot of the degree of binding (α) versus the concentration of C408A-RTPR from one set of experiments. A value of 132 ± 10 μM for K_d was calculated by nonlinear least-squares fitting of the data to eq 4. A Scatchard plot (inset) reveals one adenosylcobalamin binding site per enzyme molecule. Similar results were obtained when the concentration of adenosylcobalamin was varied and that of C408A-RTPR was constant (data not shown). This value of K_d is within error the same as the value of 200 ± 67 μM obtained in the previous studies with the wild-type enzyme (*17*), and it is larger than that reported by Brown and Li (*21*). Therefore, the binding affinity between the adenosylcobalamin and the enzyme active site appears to be only slightly if at all affected by deleting the thiol group from Cys 408.

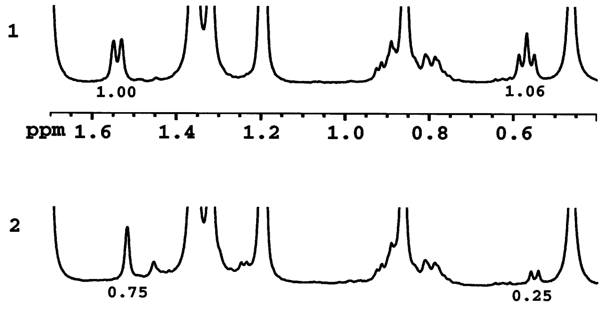


FIGURE 2: High-field portion of the ^{1}H NMR spectra of adenosylcobalamin and (5′*R*)-[5′-$^2H_1$]adenosylcobalamin in D_2O. In the spectrum of adenosylcobalamin (spectrum 1), the pro-*R* and the pro-*S* diastereotopic protons of the 5′-methylene group give rise to a triplet at 0.57 ppm and a doublet at 1.54 ppm, respectively. In the spectrum of [5′-$^2H_1$]adenosylcobalamin (spectrum 2), the coupling patterns were changed because of the deuteration at one of the C-5′ positions. The doublet at 0.55 ppm and the singlet at 1.52 ppm (doublet not resolved in figure, $J \leq 2$ Hz) integrate to 0.25 and 0.75, respectively, indicating (5′*R*)-[5′-$^2H_1$]adenosylcobalamin/(5′*S*)-[5′-$^2H_1$]adenosylcobalamin = 3:1. In either case, signals of the pro-*S* and pro-*R* 5′-protons of adenosylcobalamin are well-resolved from other nearby signals. The spectra were taken with a 500-MHz NMR spectrometer at a probe temperature of 300 K.

Catalysis of Epimerization by C408A-RTPR and C408S-RTPR. The thiyl radical responsible for nucleotide reduction and the 5′-hydrogen exchange has been located on residue Cys 408 (*3*, *11*). Previous studies have shown that C408S-RTPR does not catalyze substrate reduction or the exchange of tritium from the 5′-carbon of adenosylcobalamin with water ($k_{obs} < 1.25 \times 10^{-5}$ s^{-1}). Furthermore, formation of cob(II)alamin from adenosylcobalamin could not be detected in reactions of C408S-RTPR under presteady state conditions (*7*), in contrast with wt-RTPR, in which cob(II)alamin is formed quickly ($k_{obs} = 40$ s^{-1}) to the extent of 0.2 equiv of cob(II)alamin per equivalent of RTPR. These observations do not, however, bear directly on the question of whether Co−C5′ bond cleavage by RTPR requires the thiol group of Cys 408. To determine whether Cys 408 is required for this homolysis, and to obtain information bearing on the concerted versus the stepwise mechanism for this process (eqs 2 and 3), the ability of C408A-RTPR to catalyze the Co−C5′ bond cleavage of adenosylcobalamin was examined by a stereochemical method. (5′*R*)-[5′-$^2H_1$]Adenosylcobalamin (5′*R*/*S* = 3:1) was synthesized and incubated with C408A-RTPR. Transient cleavage of the Co−C5′ bond would convert the 5′-carbon of the coenzyme into a torsiosymmetric 5′-deoxyadenosyl radical. Rotation of the methylene group about the C4′−C5′ bond followed by reformation of the carbon cobalt bond would lead to (5′*S*)-[5′-$^2H_1$]adenosylcobalamin. Epimerization resulting from transient cleavage of the Co−C5′ bond can be observed by ^{1}H NMR spectroscopy (*13*) as the pro-*S* and pro-*R* hydrogens on the 5′-carbon of adenosylcobalamin display distinct chemical shifts and coupling interactions (*18*, *19*).

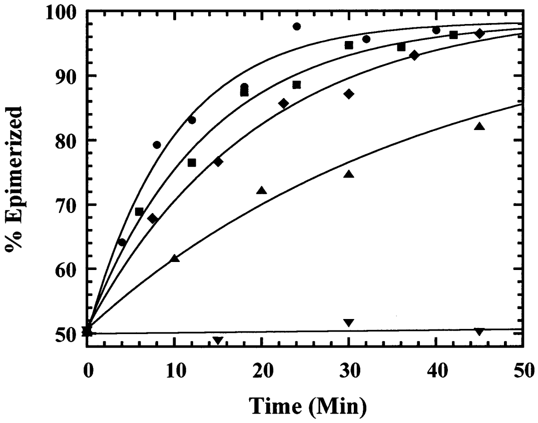


FIGURE 3: C5′-epimerization catalyzed by C408A-RTPR. Epimerization at C5′ was measured by monitoring the scrambling of the chirally deuterated adenosylcobalamin from (5′*R*)-[5′-$^2H_1$]/(5′*S*)-[5′-$^2H_1$] = 3:1 to (5′*R*)-[5′-$^2H_1$]/(5′*S*)-[5′-$^2H_1$] = 1:1 using ^{1}H NMR spectroscopy. The reaction conditions were 50 mM HEPES (pH 7.4), 320 μM dGTP, 20 mM DTT, 0.6 mM [5′-$^2H_1$]adenosylcobalamin, and various amounts of C408A-RTPR at 37 °C. The concentrations of the enzyme used are 0 μM (inverse triangles), 2.5 μM (triangles), 5.0 μM (diamonds), 7.5 μM (squares), and 10.0 μM (circles), respectively. The epimerization data were fitted to single exponentials. The observed rate constants (0.03 ± 0.01, 0.05 ± 0.01, 0.07 ± 0.01, and 0.11 ± 0.01 min^{-1}, respectively, for the different enzyme concentrations) are proportional to the enzyme concentration.

Scheme 1

$$\mathrm{E + A} \xrightleftharpoons{K} \mathrm{EA} \xrightleftharpoons[k]{k} \mathrm{EA^*} \xrightleftharpoons{K} \mathrm{E + A^*}$$

A = (5′*R*)-[5′-^{2}H]adenosylcobalamin
A* = (5′*S*)-[5′-^{2}H]adenosylcobalamin

The ^{1}H NMR spectra of adenosylcobalamin and (5′*R*)-[5′-$^2H_1$]adenosylcobalamin in the upfield region are shown in Figure 2. The signals at 0.57 and 1.54 ppm are the pro-*R* and pro-*S* C-5′ protons, respectively (*18*), and are well-resolved from other signals. Owing to the change of coupling between the C-5′ protons in (5′*R*)-[5′-$^2H_1$]adenosylcobalamin, the signal of the C-5′*R* proton appears as a doublet at 0.55 ppm, in place of the triplet in the parent coenzyme, and the C-5′*S* proton appears as a broadened singlet at 1.52 ppm. The doublet at 0.55 ppm of the deuterated coenzyme arises from coupling between the C-5′*R* proton and the C-4′ proton (*18*). The signal for the C-5′*S* proton of the deuterated adenosylcobalamin appears as an unresolved singlet because of a much smaller coupling constant with the C-4′ proton (<2 Hz as calculated from a Lorentzian curve fitting routine within the XWINNMR software).

After incubation of the stereoselectively deuterated adenosylcobalamin with various concentrations of C408A-RTPR in the presence of dGTP and DTT, the coenzyme was re-isolated and examined by ^{1}H NMR spectroscopy. The data, such as those presented in Figure 3, indicate epimerization. Therefore, transient cleavage of the Co−C5′ bond of the coenzyme must occur in these reactions of C408A-RTPR, and Cys 408 is not required for this cleavage. Epimerization requires the presence of C408A-RTPR, and the extent of epimerization depends on the reaction time and the concentration of enzyme. The epimerization reaction also requires the presence of the allosteric effector dGTP. No epimerization was detected in the absence of dGTP under the experimental conditions and within the time frame of our experiments.

The simplest kinetic mechanism for epimerization would be that of Scheme 1. On the basis of this mechanism, the observed pseudo-first-order rate constants for epimerization, which display a linear relationship with enzyme concentration, can be calculated from nonlinear least-squares fittings of the data to eq 5

$$R = 1 - \frac{1}{2}\exp(-k_{\mathrm{obs}}t) \tag{5}$$

where R is the percentage of epimerization. The rate constant ($k_{\mathrm{cat}}{}^{\mathrm{ep}}$) for epimerization catalyzed by C408A-RTPR can be calculated using eq 6, where $k_{\mathrm{cat}}{}^{\mathrm{ep}} = 2k$ in Scheme 1.

$$k_{\mathrm{cat}}{}^{\mathrm{ep}} = \frac{k_{\mathrm{obs}}([\mathrm{A}]_{\mathrm{T}} + K_{\mathrm{d}})}{[\mathrm{E}]_{\mathrm{T}}} \tag{6}$$

The k_{obs} is the observed first-order rate constant obtained from fitting data to eq 5, $[\mathrm{A}]_{\mathrm{T}}$ is the concentration of the stereoselectively labeled adenosylcobalamin, K_{d} is the dissociation constant for the enzyme−coenzyme complex, and $[\mathrm{E}]_{\mathrm{T}}$ is the total enzyme concentration. The value of $k_{\mathrm{cat}}{}^{\mathrm{ep}} = 0.28 \pm 0.04\ \mathrm{s}^{-1}$ was obtained for C408A-RTPR.

Similar experiments with C408S-RTPR also led to epimerization of (5′*R*)-[5′-^{2}H$_{1}$]adenosylcobalamin with dependence on the presence of dGTP. A control experiment showed that no cob(II)alamin formation could be detected spectrophotometrically, and no organic radical could be detected by EPR spectrometry in the reaction mixtures. Thus, the mutated RTPR is not catalyzing decomposition of the cofactor on the time scale of the experiment. The catalytic rate constant ($k_{\mathrm{cat}}{}^{\mathrm{ep}}$) for epimerization by C408S-RTPR is $0.42 \pm 0.1\ \mathrm{s}^{-1}$, similar to that calculated for C408A-RTPR.

Although a reducing agent is not required for epimerization, DTT greatly stimulates epimerization. Epimerization is also supported by TR/TRR/NADPH as the reducing system (data not shown). At 10.0 μM enzyme in the absence of any added reductant, little epimerization could be observed after prolonged incubation (45 min). This suggests that the enzyme functions in a reduced state, although the process of transient cleavage and reformation of the Co−C5′ bond itself does not consume reducing equivalents from an external source. The results clearly show that C408A-RTPR catalyzes the transient cleavage of the Co−C5′ bond and that cleavage does not require thiyl radical formation.

Catalysis of Hydrogen Exchange by Wild-Type RTPR. To compare epimerization with 5′-hydrogen exchange with solvent, the rate of the 5′-hydrogen exchange of adenosylcobalamin with D$_2$O catalyzed by RTPR was examined under the same conditions as the epimerization reactions with mutated RTPRs. The results are given in Figure 4. The pro-*R* and pro-*S* 5′-hydrogens of adenosylcobalamin undergo exchange with deuterium of D$_2$O at the same rate. This indicates the two 5′-hydrogens become equivalent in the intermediate, which is consistent with rotation of the deuteriomethylene group upon transient cleavage of the Co−C5′ bond. No exchange could be observed when C408A-RTPR was used in the reaction, in agreement with previous studies that set a lower limit of exchange of $<10^{-5}\ \mathrm{s}^{-1}$ and the proposed mechanism, in which the thiyl radical on Cys 408 is an intermediate in the 5′-hydrogen exchange (*3, 7, 8*).

The simplest kinetic mechanism for the exchange reaction is that of Scheme 2. In this mechanism, the parameter K has the same meaning as in Scheme 1. While exchange is reversible, the data collected describe an irreversible process when exchange of unlabeled adenosylcobalamin takes place in pure D$_2$O, so the central step is treated as irreversible. On the basis of this mechanism, the pseudo-first-order rate constant for exchange can be obtained by fitting data to eq 7

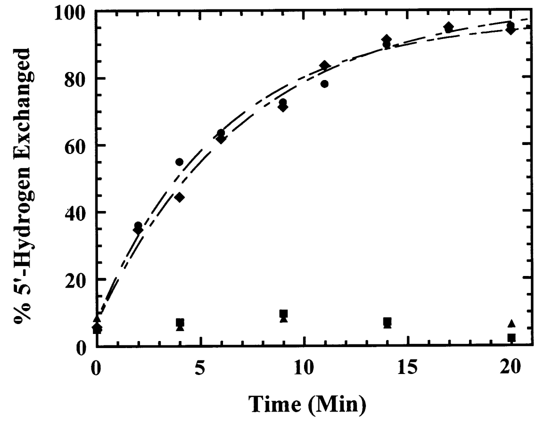


FIGURE 4: Exchange of 5′-hydrogens of adenosylcobalamin with solvent deuterium catalyzed by RTPR and C408A-RTPR. The reactions were carried out using buffer and reagents made with D$_2$O. The reaction conditions are 50 mM HEPES (pD 7.4), 320 μM dGTP, 20 mM DTT, and 0.6 mM adenosylcobalamin at 37 °C. The pro-*R* and pro-*S* 5′-hydrogens (circles and diamonds) exchange rapidly and at the same rate in the RTPR catalyzed reaction. The data were fitted to single exponentials, and an observed rate constant of $0.15 \pm 0.02\ \mathrm{min}^{-1}$ was determined. No apparent 5′-hydrogen exchanges (squares and triangles) could be observed in reactions catalyzed by C408A-RTPR.

Scheme 2

$$\mathrm{E} + \mathrm{A} \overset{K}{\rightleftharpoons} \mathrm{EA} \xrightarrow{k_{\mathrm{cat}}{}^{\mathrm{ex}}} \mathrm{EA}^{\mathrm{x}} \overset{K}{\rightleftharpoons} \mathrm{E} + \mathrm{A}^{\mathrm{x}}$$

A = adenosylcobalamin
A$^{\mathrm{x}}$ = [5'-^{2}H$_{2}$]adenosylcobalamin

$$R = 1 - \exp(-\,k_{\mathrm{obs}}t) \tag{7}$$

where R is the percentage of 5′-hydrogens exchanged, and k_{obs} is the observed rate constant. In this kinetic mechanism (Scheme 2), the exchange rate constant ($k_{\mathrm{cat}}{}^{\mathrm{ex}}$) is given by eq 8

$$k_{\mathrm{cat}}{}^{\mathrm{ex}} = \frac{k_{\mathrm{obs}}(K_{\mathrm{d}} + [\text{5}'\text{-hydrogen}])}{[\mathrm{E}]_{\mathrm{T}}} \tag{8}$$

where k_{obs} is the observed rate constant, $[\mathrm{E}]_{\mathrm{T}}$ is the total enzyme concentration, and K_{d} is the dissociation constant of adenosylcobalamin from RTPR. The value of $k_{\mathrm{cat}}{}^{\mathrm{ex}} = 1.5 \pm 0.3\ \mathrm{s}^{-1}$ was calculated for the 5′-hydrogen exchange using eq 8 and assuming the value of 200 ± 67 μM for the dissociation constant of adenosylcobalamin from wt-RTPR (*17*). The value of 200 ± 67 μM for K_{d} is within error of our value for C408A-RTPR.

Epimerization of (5′R)-[5′-^{2}H$_{1}$]Adenosylcobalamin Catalyzed by Wild-Type RTPR. The ability to measure a k_{obs} for the exchange of the hydrogens, which destroys the chirality of the adenosylcobalamin, suggests that it might be possible to measure the rate constant for epimerization $k_{\mathrm{cat}}{}^{\mathrm{ep}}$ by wt-RTPR. Because the exhange of the 5′-hydrogen requires carbon−cobalt bond homolysis, the rate of the Co−C5′ bond cleavage must be greater than or equal to that of hydrogen

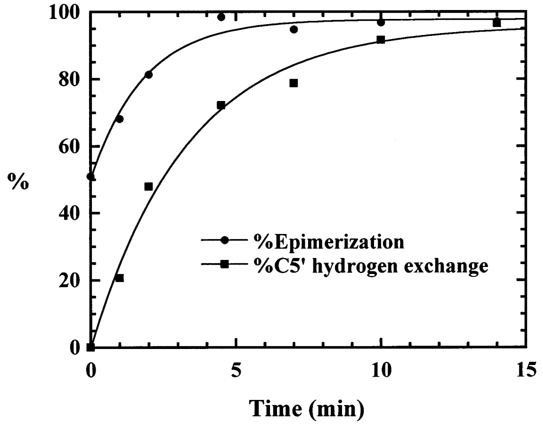


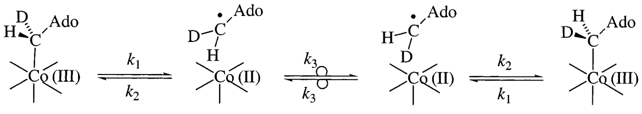
FIGURE 5: Rates of epimerization and 5′-hydrogen exchange catalyzed by RTPR. The reactions were carried out using buffer and reagents made with D_2O. The reaction conditions are 50 mM HEPES (pD 7.4), 320 μM dGTP, 20 mM DTT, 0.5 mM (5′*R*)-[5′-$^2H_1$]adenosylcobalamin [(5′*R*)/(5′*S*) = 3:1], and 2.5 μM of RTPR at 37 °C. The Co−C5′ bond cleavage was measured by monitoring the epimerization of the two chirally deuterated isomers. The hydrogen exchange was measured by monitoring the total loss of signals of the C-5′ hydrogens because of exchange with solvent deuterium. Data were fitted to single exponentials as described in the text. The observed rate constants were 0.55 ± 0.09 and 0.30 ± 0.04 min^{-1} for epimerization and 5′-hydrogen exchange, respectively.

exchange. To compare the rates of these two processes, the stereoselectively labeled (5′*R*)-[5′-$^2H_1$]adenosylcobalamin was incubated with RTPR in buffer prepared with D_2O and in the presence of dGTP and DTT. The rate of epimerization was measured by monitoring the relative signal intensity change of the C-5′*S* and C-5′*R* protons. The rate of C-5′ hydrogen exchange was measured by monitoring the total loss of signals of the C-5′ protons. The data for both epimerization and hydrogen exchange are displayed in Figure 5. Corrections for washout are not necessary because the C-5′*S* and C-5′*R* protons undergo exchange at the same rate, and only the change in relative signal intensities of the C-5′*S* and C-5′*R* protons are measured for the epimerization reaction. The observed rate constants for epimerization and the 5′-hydrogen exchange were obtained from least-squares fittings of the data to eqs 5 and 7, respectively. The rate constants for epimerization and 5′-hydrogen exchange were calculated using eqs 6 and 8, respectively. It should be mentioned that the total concentration of 5′-hydrogen was the same as that of the coenzyme when (5′*R*)-[5′-$^2H_1$]-adenosylcobalamin was used, whereas the total concentration of 5′-hydrogen was two times higher than that of the coenzyme when the undeuterated adenosylcobalamin was used. This was because of the presence of two 5′-hydrogens to be exchanged in the undeuterated coenzyme. The catalytic rate constant (k_{cat}^{ex}) for the 5′-hydrogen exchange is 1.4 ± 0.2 s^{-1}, which is within experimental error the same as the value of 1.5 ± 0.3 s^{-1} measured for exchange of unlabeled adenosylcobalamin in D_2O. The rate constant for epimerization (k_{cat}^{ep}) by wt-RTPR is 5.1 ± 1 s^{-1}. Faster epimerization than hydrogen exchange and substrate reduction is consistent with the proposed mechanism (*7, 8*).

DISCUSSION

Mechanism of C5′-Epimerization. The present work shows that the thiol group of Cys 408 is not required for the cleavage of the Co−C5′ bond of adenosylcobalamin at the active site of the B_{12}-dependent RTPR. This follows from the fact that both C408S-RTPR and C408A-RTPR and wt-RTPR catalyze the 5′-epimerization of (5′*R*)-[5′-$^2H_1$]adenosylcobalamin. Epimerization at C-5′ is a newly discovered reaction of RTPR, and the only one catalyzed by both the wild-type enzyme and its variants mutated at Cys 408. Epimerization at C-5′ must proceed through Co−C5′ bond cleavage, and it is observed under the conditions of the present experiments when the allosteric activator dGTP is present. These are the same requirements as for the overall reduction reaction (eq 1) and of 5′-hydrogen exchange by wild-type RTPR. Because the C408S-RTPR and C408A-RTPR cannot form thiyl radicals, they do not catalyze either the 5′-hydrogen exchange of adenosylcobalamin or the reduction of NTPs.

Scheme 3



The simplest mechanism by which C408A-RTPR and C408S-RTPR can catalyze C5′-epimerization is that shown in Scheme 3. The Co−C5′ bond undergoes cleavage to cob(II)alamin and the 5′-deoxy[5′-$^2H_1$]adenosyl radical. The deuteriomethylene group of the radical can then either rapidly recombine (k_2) or rotate about its bond to C4′ and then recombine with cob(II)alamin to generate the epimer. Because no cob(II)alamin was detected in the course of epimerization by the Cys 408 variants, the equilibrium for cleavage of the Co−C5′ bond must be unfavorable, that is $k_2 > 50k_1$, in the presence of the allosteric activator dGTP. The value of k_2 may be in the range of the recombination rate constant after flash photolytic cleavage of adenosylcobalamin, approximately 10^9 s^{-1} (*20*). It follows that the value of $k_1 < 2 \times 10^7$ s^{-1}.

Epimerization arises from torsion of the deuteriomethylene group in the cleaved state of the coenzyme. The rate constant for torsion in Scheme 3 is k_3. If deuteriomethylene rotation is not hindered in the active site, an estimate of 10^{12} s^{-1} for this rate constant can be obtained from the energy barrier of 0.15 kcal mol^{-1} for rotation about a C−C bond (*22, 23*). In this case, torsion is expected to be 2 or 3 orders of magnitude faster than recombination. Then, Co−C5′ bond cleavage governed by k_1 should be rate limiting in epimerization. In the mechanism of Scheme 1, the rate constant *k* is half the value of k_{cat}^{ep}, and it represents the summation of processes governed by k_1, k_2, and k_3 in Scheme 3. Therefore, it may be that k_1 in Scheme 3 is approximately equal to *k* in Scheme 1, that is, 0.14 s^{-1} for C408A-RTPR, 0.21 s^{-1} for C408S-RTPT, and 2.4 s^{-1} for wt-RTPR. If there is an unusualy high barrier to torsion of the deuteriomethylene group in the [5′-^2H]deoxyadenosyl radical, these values will be lower limits for the rate constants governing Co−C5′ bond cleavage. We note that dGTP is much more effective in promoting the exchange reaction than other effectors, and we do not know whether it is more effective in promoting epimerization.

The values of k_{cat}^{ep} can be used to calculate the activation energy for each epimerization reaction. The differential free energies of activation ($\Delta\Delta G^{\ddagger}$) between the wild-type and Cys 408 variants for catalysis of epimerization are 1.4 and

Table 1: Rates of Nucleotide Reduction, Co−C5′ Bond Cleavage, and 5′-Hydrogen Exchange Catalyzed by RTPR and Cys 408 Variants

	Rate constants		
reaction	C408A-RTPR (s^{-1})	C408S-RTPR (s^{-1})	RTPR (s^{-1})
nucleotide reduction[a]	0	0	0.55 ± 0.07[b]
epimerization (k_{cat}^{ep})[c]	0.28 ± 0.04	0.42 ± 0.10	5.1 ± 1.0
5′-H exchange (k_{cat}^{ex})[d]	0	0	1.5 ± 0.3
5′-D exchange (k_{cat}^{ex})[e]	0	0	1.4 ± 0.2

[a] Nucleotide reaction was measured in 50 mM HEPES buffer (pH 7.4) using the coupled assay method as described ref *1*. [b] Standard errors were determined from least-squares fittings of the data using KaleidaGraph software. [c] Epimerization was measured by monitoring the stereochemical scrambling of the C-5′ deuterium on (5′*R*)-[5′-$^2H_1$]adenosylcobalamin. [d] 5′-Hydrogen exchange was measured using buffer and reagents made with D_2O (pD 7.4) by monitoring the loss of the 5′-hydrogens from adenosylcobalamin. [e] 5′-Exchange was measured by observing the loss of 5′-deuterium from (5′*R*)-[5′-^2H]adenosylcobalamin.

1.6 kcal mol^{-1} for C408S-RTPR and C408A-RTPR, respectively. The similarities in these $\Delta G^{\ddagger}$ values support similar epimerization mechanisms for the wild-type and Cys 408 variants.

We do not know the basis for the modest differences in epimerization rates between the wild-type and the mutated RTPRs. However, if the rates are controlled by the dissociation rates of adenosylcobalamin, it could be that the off rates differ for the wild-type and mutated enzymes. We do not know the dissociation rate constants for adenosylcobalamin, which is rather weakly bound to RTPR.

Enhancement of Cobalt−Carbon Bond Cleavage in Adenosylcobalamin. In the presence of dGTP, wt-RTPR enhances the rate of Co−C5′ bond cleavage by a factor of 4×10^{10} based on the rate constant of 40 s^{-1} in the spectrophotometric observation of cob(II)alamin formation (*7*, *12*, *21*). The present data can be used to set a minimum value on the enhancements of Co−C5′ bond cleavage by dGTP in the cases of C408A- and C408S-RTPR. On the basis of the epimerization rate constants in Table 1, the rate enhancements would be 2.8×10^8 and 4.2×10^8, respectively. If as in the wild-type enzyme the rates of Co−C5′ cleavage are 10 times the minima estimated from epimerization, the rate enhancements would be 2.8 and 4.2×10^9, respectively. In any case, the replacement of Cys 408 by alanine or serine preserves most of the capacity of dGTP to promote cobalt−carbon bond cleavage, without forming the Cys 408−thiyl radical.

Mechanism of Thiyl Radical Formation. By demonstating that cobalt−carbon bond cleavage occurs rapidly in the active sites of C408A- and C408S-RTPR, the present results implicate the stepwise mechanism in Scheme 1 for epimerization. Inasmuch as Co−C5′ bond cleavage is a fast equilibrium step in Scheme 1, and epimerizations by the mutated species of RTPR occur at 10% of the rate by the wild-type enzyme, there is reason to consider that epimerization and thiyl radical formation share common mechanistic features. The stepwise mechanism for thiyl radical formation in eq 3 shares its first step with the mechanism for epimerization. The values of k_{cat}^{ep} for epimerization by Cys 408 variants are within factors of 3 or 4 of k_{cat}^{ex} for solvent hydrogen exchange by the wild-type enzyme (Table 1). Solvent exchange requires the intermediate formation of the Cys 408-thiyl radical. The stepwise mechanism of eq 3 for thiyl radical formation should thus be regarded as a viable and possible mechanism.

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REFERENCES

1. Blakeley, R. L. (1978) *Methods Enzymol. 51*, 246−259.
2. Lin, A. I., Ashley, G. W., and Stubbe, J. (1987) *Biochemistry 26*, 6905−6909.
3. Booker, S., Licht, S., Broderick, J., and Stubbe, J. (1994) *Biochemistry 33*, 12679−12685.
4. Singh, D., Tamao, Y., and Blakley, R. L. (1977) *Adv. Enzymol. Reg. 15*, 81−100.
5. Abeles, R. H., and Beck, W. S. (1967) *J. Biol. Chem. 242*, 3589.
6. Hogenkamp, H. P. C., Ghambeer, R. K., Brownson, C., Blakley, R. L., and Vitols, E. (1968) *J. Biol. Chem 243*, 799.
7. Licht, S. S., Booker, S., and Stubbe, J. (1999) *Biochemistry 38*, 1221−1233.
8. Licht, S. S., Gerfen, G. J., and Stubbe, J. (1996) *Science 271*, 477−481.
9. Gerfen, G. J., Licht, S., Willems, J.-P., Hoffman, B. M., and Stubbe, J. (1996) *J. Am. Chem. Soc. 118*, 8192−8197.
10. Booker, S., and Stubbe, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8352−8356.
11. Sintchak, M. D., Arjara, G., Kellog, B. A., Stubbe, J., and Drennan, C. L. (2002) *Nat. Struct. Biol. 9*, 293−300.
12. Tamao, Y., and Blakley, R. L. (1973) *Biochemistry 12*, 24−34.
13. Gaudemer, A., Zylber, J., Zylber, N., Baran-Marszac, M., Hull, W. E., Fountoulakis, M., König, A., Wölfle, K., and Rétey, J. (1981) *Eur. J. Biochem. 119*, 279−285.
14. Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., and
15. Volcani, B. E. (1960) *J. Biol. Chem. 235*, 480−488.
16. Bock, R. M., Ling, N. S., Morell, S. A., and Lipton, S. H. (1956) *Arch. Biochem. Biophys.*
17. Clarke, A. R. (1996) in *Enzymology* (Engel, P. C., Ed.) pp 199−221, Academic Press, San Diego, CA.
18. Licht, S. S., Lawrence, C. C., and Stubbe, J. (1999) *Biochemistry 38*, 1234−1242.
19. Cheung, A., Parry, R., and Abeles, R. H. (1980) *J. Am. Chem. Soc. 102*, 384−385. Cheung, A., et al. *J. Am. Chem. Soc. 62*, 253−264.
20. Summers, M. F., Marzilli, L. G., and Bax, A. (1986) *J. Am. Chem. Soc. 108*, 4285−4294.
21. Cole, A. G., Yoder, L. M., Shiang, J. J., Anderson, N. A., Walker, L. A., II, Banaszak Holl, M. M., and Sension, R. (2001) *J. Am. Chem. Soc. 124*, 434−441.
22. Brown, K. L., and Li, J. (1998) *J. Am. Chem. Soc. 120*, 9466−9474.
23. Pacansky, J., and Dupuis, M. (1982) *J. Am. Chem. Soc. 104*, 415−421.
24. Suter, H. U., and Ha, T.-K. (1991) *Chem. Phys. 154*, 227−236.

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