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Class II Ribonucleotide Reductases Catalyze Carbon–Cobalt Bond Reformation on Every Turnover

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Abstract: Ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* catalyzes the reduction of nucleotides to deoxynucleotides with concomitant oxidation of two cysteines within the active site to a disulfide. RTPR requires adenosylcobalamin (AdoCbl) as a cofactor and as a radical chain initiator. Catalysis is initiated by homolysis of the carbon–cobalt bond of AdoCbl to yield cob(II)alamin, 5′-deoxyadenosine, and a protein-based thiyl radical. The turnover numbers for ATP with its allosteric effector dGTP, and for CTP with its allosteric effector dATP, are both 2 s^{-1}. The rate-limiting step for turnover in the steady state is re-reduction of the oxidized form of the protein, a conformational change, or both. Under conditions where [RTPR] ≫ [AdoCbl], the rates of ATP and CTP reduction do not vary linearly with [AdoCbl] but instead exhibit saturation behavior with turnover numbers of 10 s^{-1} (ATP) and 8.5 s^{-1} (CTP). This result suggests that dissociation of AdoCbl, which requires carbon–cobalt bond reformation, follows nucleotide reduction, but precedes the rate-limiting step in catalysis. A presteady-state analysis of the ATP reduction (using rapid chemical quench methods) in the presence of [5′-^{3}H]-AdoCbl reveals formation of product dATP at a k_{obs} of 55 ± 10 s^{-1} and tritium washout from [5′-^{3}H]-AdoCbl at 0.6 s^{-1}. The rate of washout is approximately equivalent to the rate of washout of ^{3}H in the absence of substrate. Measurement of the ratio of ^{3}H$_2$O:dATP over time reveals that washout of ^{3}H occurs at the end of each turnover. Production of ^{3}H$_2$O requires reformation of the carbon–cobalt bond. These steady-state and presteady-state data suggest that carbon–cobalt bond reformation and dissociation of AdoCbl into solution accompany each turnover and that the radical chain length of the RTPR-catalyzed nucleotide reduction is approximately one.

Introduction

There are an increasing number of enzymes involved in primary steps in metabolism in which metallo-cofactors act as radical chain initiators.[1] One important mechanistic question is whether one initiation event results in multiple conversions of substrates to products or whether an initiation event must occur on every turnover. In chemical terms, one would like to determine the chain length of the reaction. We have long been interested in the enzymes that catalyze an essential step in DNA biosynthesis, the ribonucleotide reductases (RNRs). These enzymes utilize tyrosyl radicals, glycyl radicals, or adenosylcobalamin (AdoCbl), depending on the source from which they are isolated, to generate transient thiyl radicals that are essential in catalysis.[1] Studies on the class II, AdoCbl-dependent, ribonucleoside triphosphate reductases (RTPRs) are now reported that provide the first direct evidence that these enzymes catalyze, on each turnover of substrate to product, carbon–cobalt bond homolysis and reformation. The chain-length of the reaction is one.

Class II RNRs catalyze the conversion of nucleoside triph-

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(1) Stubbe, J.; van der Donk, W. A. *Chem. Rev.* **1998**, *98*, 705−762.

Scheme 1
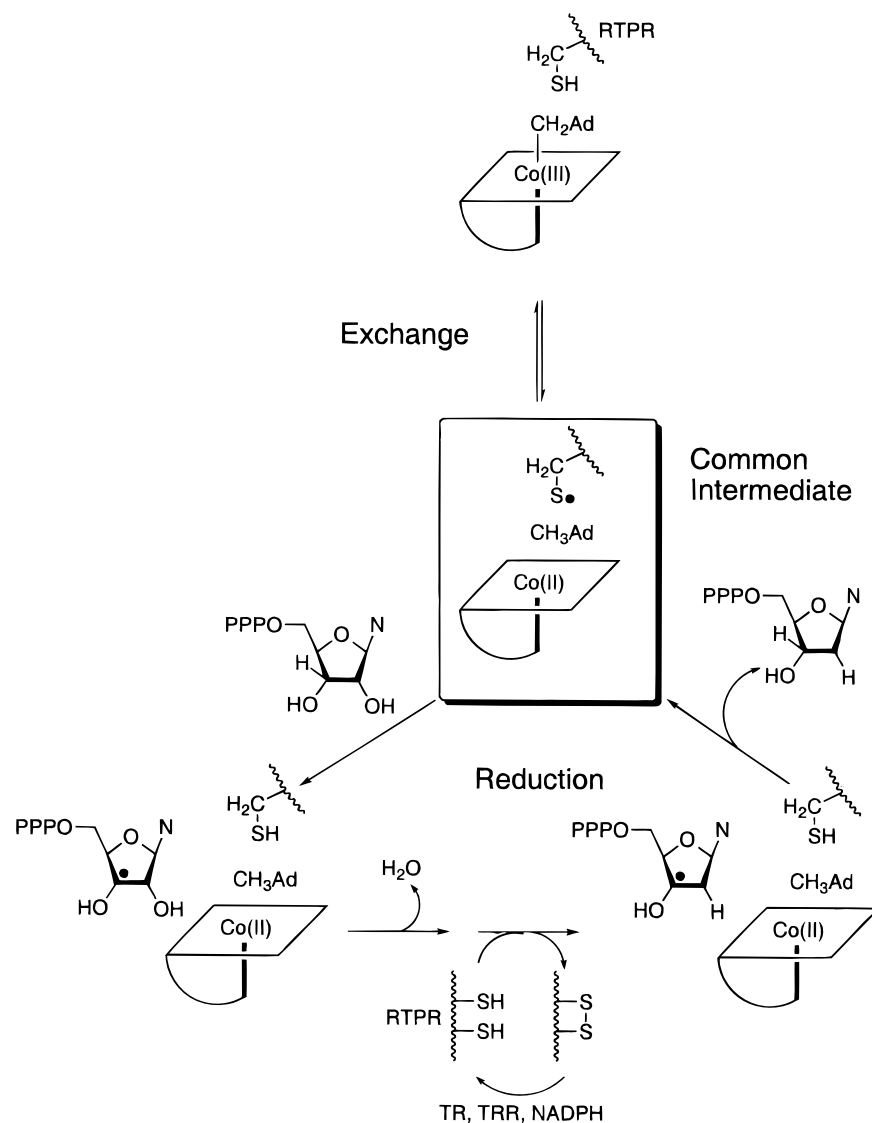
is the axial, dimethylbenzimidazole ligand of AdoCbl

osphates (NTPs) to deoxynucleoside triphosphates (dNTPs). In addition, these enzymes also catalyze the exchange of hydrogens from the 5′ position of AdoCbl with solvent.[2,3] This exchange reaction occurs in the absence of substrate, and its rate is maximized when a dNTP allosteric effector is present and the enzyme is in the reduced state.[4] Seminal experiments of Tamao and Blakley, Orme-Johnson et al., and more recent studies from our laboratories using stopped flow (SF) UV−vis spectroscopy, rapid chemical quench, and rapid freeze quench (RFQ) EPR spectroscopy, have provided much insight into the mechanism of the exchange reaction as well as the reduction reaction.[4−8]

(2) Beck, W. S.; Abeles, R. H.; Robinson, W. G. *Biochem. Biophys. Res. Commun.* **1966**, *25*, 421−425.

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

(4) Licht, S. S.; Booker, S.; Stubbe, J. *Biochemistry* **1999**, *38*, 1225−1233.

(5) Tamao, Y.; Blakley, R. L. *Biochemistry* **1973**, *12*, 24−34.

(6) Orme-Johnson, W. H.; Beinert, H.; Blakley, R. L. *J. Biol. Chem.* **1974**, *249*, 2338−2343.

(7) Licht, S.; Gerfen, G. J.; Stubbe, J. *Science* **1996**, *271*, 477−481.

(8) Licht, S. S. Ph.D. Thesis, Chapter 5. Massachusetts Institute of Technology, 1998.

Scheme 1 provides an overview of our current working model for these reactions.

The function of AdoCbl is to generate a thiyl radical, 5′-deoxyadenosine (5′-dA) and cob(II)alamin: the initiation event in both reactions. Studies of Tamao and Blakley[5] and Licht and Stubbe[8] have shown that the kinetics of cob(II)alamin formation and disappearance in the presence of a NTP substrate are biphasic: there is an initial rapid phase in which as much as 0.4 equiv of cob(II)alamin are formed with a k_{obs} of >200 s^{-1}, followed by a slower phase in which cob(II)alamin is consumed to a steady state level of 0.1 equiv with a rate constant of 20−50 s^{-1}. The steady-state rate of dNTP formation is 2 s^{-1}, and thus carbon−cobalt bond cleavage and reformation are much faster than steady-state turnover. At present, our studies[8] and earlier studies of Tamao and Blakley suggest that the rate-limiting step is re-reduction of the disulfide bond generated during dNTP formation and or a conformational change associated with this process.

Our recent studies on the exchange reaction in the absence of substrate suggest that this reaction is concerted (Scheme 1)[4]

C−Co Bond Reformation Catalyzed by RTPR

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or contains a high energy 5′-deoxyadenosyl radical intermediate, and that 5′-hydrogens from the cofactor appear in the solvent with a rate constant of >10 s^{-1}.[7] However, when substrate is present, the thiyl radical is thought to abstract a hydrogen atom from the 3′-position of the nucleotide to initiate the reduction concomitant with formation of an active site disulfide. The thiyl radical is then regenerated when the product 3′-deoxynucleotide radical is reduced. At this stage, the thiyl radical could initiate multiple rounds of nucleotide reduction without reformation of the carbon−cobalt bond of AdoCbl. Alternatively, AdoCbl could be reformed at the end of each turnover. Exchange of tritium into solvent when [5′-³H]-AdoCbl is the cofactor requires reformation of the carbon−cobalt bond and hence can be used as an indicator of this process.

Two types of experiments are presented that indicate that the chain length of the reduction reaction is approximately one. In one set of experiments, the concentration of RTPR is in large excess over AdoCbl. The k_{cat} for deoxynucleotide (dNTP) formation is measured by monitoring NADPH oxidation in a coupled assay using thioredoxin (TR) and thioredoxin reductase (TRR) (Scheme 1). These studies show that the rate of re-reduction of the disulfide (measured by a TR/TRR/NADPH coupled assay) generated during NTP reduction exceeds the product of k_{cat} and the concentration of the limiting reagent, AdoCbl. These observations require that a single AdoCbl can catalyze formation of multiple dNTPs and hence multiple disulfides. AdoCbl therefore must dissociate from the oxidized RTPR, a process which requires carbon−cobalt bond reformation. The rate of this process provides an upper limit for the chain length of this reaction.

In a second set of experiments carried out in the presteady state in the presence of substrate, the exchange of tritium from [5′-³H]-AdoCbl with solvent is used to monitor reformation of the carbon−cobalt bond. Comparison of the rate of tritium washout from the cofactor to the rate of dNTP formation provides an additional measure of the frequency of carbon−cobalt bond reformation. The ramifications of Nature's choice of a chain length of approximately one will be discussed.

Results

Steady State Analysis Indicating Carbon−Cobalt Bond Reformation on Each Turnover. The chain length of radical dependent reactions in biological systems has yet to be unambiguously established in any reaction. We have developed a method that could be generally useful in measuring chain lengths for AdoCbl-requiring enzymes. The method is based on the ability to use the steady-state rate of substrate turnover to measure AdoCbl dissociation from the enzyme at the end of each turnover. This process requires carbon−cobalt bond reformation. We have thus measured the rate of dNTP formation under two sets of conditions: Michaelis−Menten conditions where [AdoCbl] $\gg$ [RTPR] and conditions in which [RTPR] $\gg$ [AdoCbl] $\gg K_{m,AdoCbl}$. In the latter case, the [RTPR] that will turn over substrate (holoenzyme) is equal to the concentration of AdoCbl, the limiting reagent, if AdoCbl does not dissociate from RTPR. The rate of turnover (ν) would thus vary directly with [AdoCbl], and thus ν/[AdoCbl] would be constant. On the other hand, if AdoCbl dissociates more rapidly than the rate of dNTP production in the steady state, a single AdoCbl could effect conversion of multiple NTPs to dNTPs. It is possible that saturation kinetics would be observed, limited by the rate at which AdoCbl dissociates from RTPR.

Kinetic Parameters Under Conditions in which [AdoCbl] $\gg$ [RTPR]. Under standard Michaelis−Menten conditions using
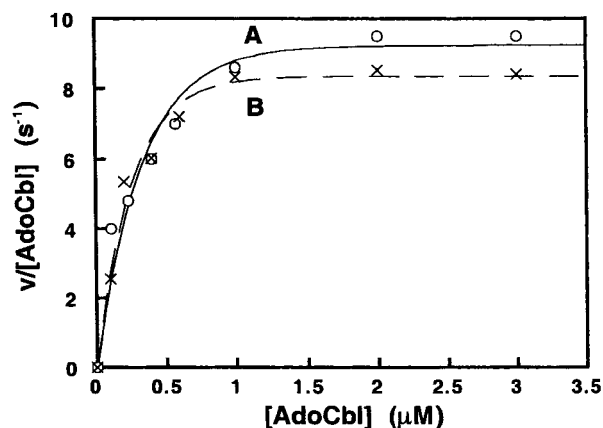
Figure 1. Dependence on [AdoCbl] of the rate of the turnover of (A) ATP (○, solid line) and (B) CTP (×, dashed line) under conditions in which [RTPR] $\gg$ [AdoCbl]. Each assay contained [RTPR] = 10 × [AdoCbl], 1 mM ATP (A) or 1 mM CTP (B), 1 mM dGTP (A) or 1 mM dATP (B), 85 μM TR, 1.5 μM TRR, 0.25 mM NADPH, 200 mM sodium dimethylglutarate (NaDMG) pH 7.3. Product formation was determined by monitoring NADPH consumption at 340 nm.

a coupled assay with TR, TRR, NADPH, the $K_{m,AdoCbl}$ and k_{cat} were determined for reduction of ATP and CTP. With both nucleotides, $K_{m,AdoCbl}$ is 0.2 μM, and k_{cat} is 2 s^{-1}. These numbers are similar to those previously observed.[9]

Kinetic Parameters Under Conditions in which [RTPR] $\gg$ [AdoCbl] $\gg K_{m,AdoCbl}$. Experiments were also carried out under conditions in which the ratio of [RTPR] to [AdoCbl] was 10:1, (where [RTPR] varied from 1 to 30 μM). Under these conditions, given the $K_{m,AdoCbl}$ determined above, all AdoCbl is bound to RTPR. The rate of dNTP formation was measured spectrophotometrically using TR/TRR/NADPH as described above. Above 0.8 μM AdoCbl, the rates were measured by SF UV−vis spectroscopy. The results with ATP as substrate and dGTP as allosteric effector are shown in Figure 1A. Interestingly, as the [AdoCbl] is increased, the rate of dNTP formation becomes independent of [AdoCbl]. The limiting value of ν/[AdoCbl) is 10 s^{-1} and is 5-fold greater than the turnover number of 2 s^{-1} observed under standard assay conditions.

A similar experiment was carried out in the presence of CTP with dATP as an allosteric effector. The results are shown in Figure 1B. Once again, the rate constant for dCTP formation is 8.5 s^{-1}, 4.5-fold higher than the turnover number observed under standard conditions. Given the experimental design, the only way one can observe turnover numbers greater than those observed when enzyme is saturated with substrate is if each AdoCbl is capable of participating in several turnovers of nucleotide to deoxynucleotide prior to the rate-determining step in nucleotide reduction. Previous studies of Tamao and Blakley[5] and more recent studies from our laboratory[8] suggest that either re-reduction of the active-site disulfide to two cysteines, a conformational change, or both are rate-limiting in the steady state. The simplest interpretation of these data is that the observed rate constant of RTPR re-reduction of ∼10 s^{-1} reflects the rate of AdoCbl dissociation from RTPR, which allows accumulation of a pool of oxidized RTPR that is greater than [AdoCbl].

³H₂O Release from [5′-³H]-AdoCbl in the Presteady State as an Indicator of C−Co Bond Reformation. Recently, we have studied in detail the RTPR-catalyzed rate of exchange of ³H from [5′-³H]-AdoCbl with solvent in the presence of

(9) Booker, S.; Licht, S.; Broderick, J.; Stubbe, J. *Biochemistry* **1994**, *33*, 12676−12685.

allosteric effector (dGTP) and in the absence of substrate. We have shown that this exchange reaction requires homolysis and reformation of the carbon–cobalt bond and occurs with a rate constant of 0.3 s^{-1} (Scheme 1). ^3H_2O formation thus places a lower limit on the rate of hydrogen washout from cofactor, and its measurement provides information about the timing and frequency of carbon–cobalt bond reformation. A similar experiment in the presence of substrate ATP, examining the ratio of ^3H_2O to dATP as a function of time, could be informative as to the timing and frequency of carbon–cobalt bond reformation under these conditions. If most of the release occurs prior to dATP formation, one would expect a burst of ^3H_2O. In addition, the ratio would be expected to decrease as a function of time. If ^3H_2O release occurs subsequent to dATP formation one would expect a burst of dATP and the ratio to increase as a function of time.
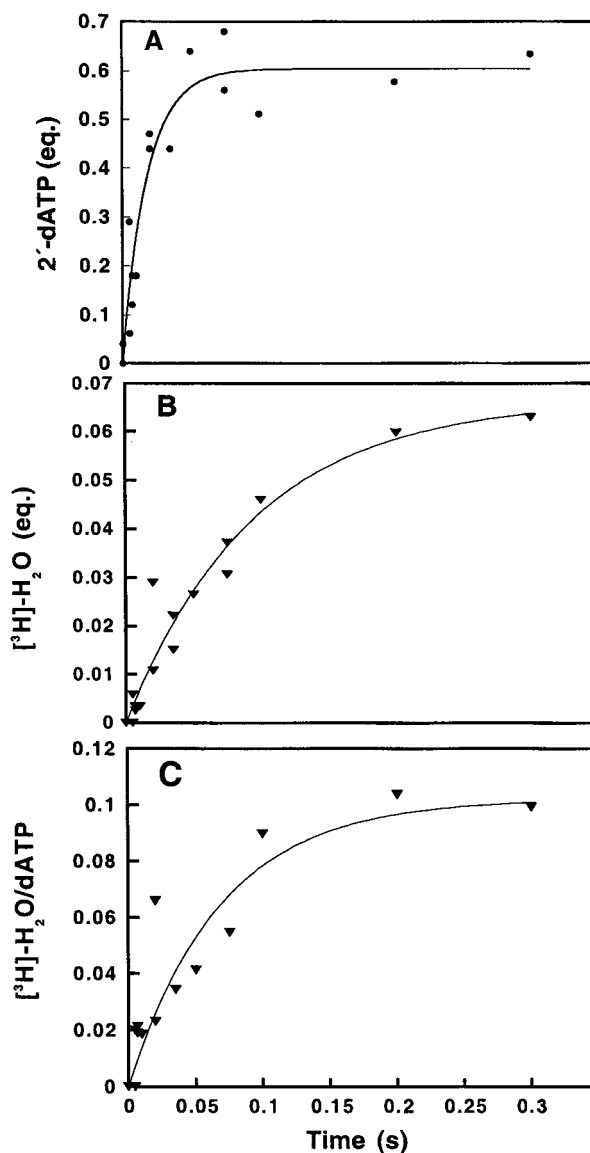
In order to obtain insight into carbon–cobalt bond homolysis and reformation, [14C]-ATP and [5′-3H]-AdoCbl were incubated with RTPR, and the reaction was stopped by rapid acid quench methods.[8] The amounts of [14C]-dATP and ^3H_2O were monitored using HPLC and bulb-to-bulb distillation, respectively. The results are shown in Figure 2A, B, and C. The rate of dATP formation (Figure 2A) yields a k_{app} of 55 ± 10 s^{-1}. In the first turnover 0.6 to 0.7 equiv of product are generated.[9,10] These results provide the first direct evidence that nucleotide reduction is not rate-limiting in the steady state.

The reaction mixture was also monitored for ^3H_2O formation. The results are shown in Figure 2B. The k_{app} is 0.6 s^{-1}, comparable to the number of 0.3 s^{-1} previously measured in the presence of allosteric effector dGTP and in the absence of substrate. Under the previous conditions of the exchange reaction (that is, no substrate), ^3H_2O release proceeded linearly as a function of time, in contrast with the behavior shown in Figure 2B. An explanation for the greatly reduced rate of ^3H_2O formation at later times in the presence of substrate is that the exchange rate is diminished when RTPR is oxidized. These results are compatible with our previous exchange studies with oxidized RTPR and dGTP.[4]

Finally, the ratio of ^3H_2O to dATP over time is shown in Figure 2C. The data are most consistent with ^3H_2O release occurring subsequent to dATP formation. This conclusion is supported by the similarities in the exchange rates in the presence and absence of substrate. If one C–Co bond cleavage event effected multiple turnovers of NTPs to dNTPs, then in the presence of substrates one would expect to observe substantially reduced rates of ^3H_2O formation, relative to experiments carried out in the absence of substrate. Given that there must be a selection effect on the process of tritium washout of at least a factor of 10 and a statistical factor of 2 associated with the 3 hydrogens of 5′-deoxyadenosine, the rate of hydrogen washout from the cofactor is at least 12 s^{-1} and is faster than the steady-state rate of formation of dATP, at 2 s^{-1}. These data thus suggest that C–Co bond reformation occurs faster than turnover.

Discussion

In the past five years many protein-based radicals have been identified in enzymes playing primary roles in metabolism.[1] Their mechanisms are being examined in detail. A mechanistic question common to all of these reactions is the chain length of the reaction. Does a single initiation event result in production of multiple products, or is an initiation event required for each
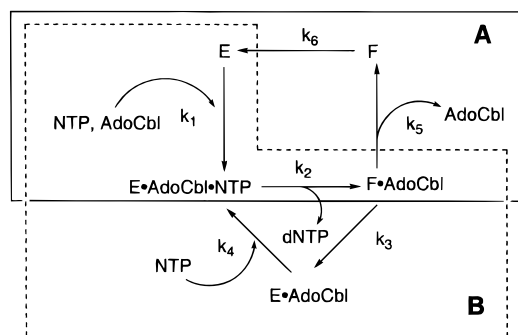
(10) The inability to generate 1 equiv of dNTP has also been previously noted. The reasons for this stoichiometry are unclear.

Figure 2. Formation of [14C]-dATP (A) and [3H]-H_2O (B) under single turnover conditions. RTPR (120 μM) in one syringe also containing 2 mM [U-14C]-ATP, 2 mM dGTP, 20 μM TR, 1 μM TRR, 2 mM NADPH, and 100 mM NaDMG pH 7.3, was mixed with 100 μM [5′-3H]-AdoCbl in a second syringe which also contained 2 mM [U-14C]-ATP, 2 mM dGTP, and 100 mM NaDMG, pH 7.3, followed by quenching with acid. The reaction mixture was worked up as described in the Experimental Section. Each data point represents one trial. The data are fitted to a single exponential (solid line). C, The ratio of [3H]-H_2O formed to [14C]-dATP as a function of time, taken from the data in A and B. The data are fitted to a single exponential.

turnover? This question has now been answered in the case of RTPR. Results utilizing steady state kinetics with [RTPR] ≫ [AdoCbl] and results using a presteady state analysis examining washout of radiolabel from the 5′-position of AdoCbl in the presence of substrate, both suggest that the chain length of the reaction is approximately one.

A demonstration that AdoCbl dissociates from RTPR after each turnover requires carbon–cobalt bond reformation and would establish a chain length of one. Experiments under steady state conditions in which [RTPR] ≫ [AdoCbl] were designed to address this question. Two unusual features of the kinetics of dNTP formation (measured as disulfide reduction (Scheme 1)) under these conditions suggest that turnover is limited by the rate of AdoCbl dissociation. First, the rate does not increase

Figure 3. Simplified kinetic models for RTPR turnover. In path A, AdoCbl dissociates from RTPR on each turnover, and in path B AdoCbl remains bound to RTPR between successive turnovers. E is the reduced form of RTPR, and F is the oxidized form of RTPR.

linearly with [AdoCbl] as it should if AdoCbl is acting as a limiting reagent. Instead, in the case of both purine and pyrimidine nucleotide substrates, saturation kinetics are observed (Figures 1A and 1B). Second, the turnover numbers V_{max}/[AdoCbl]$_0$ measured under these conditions are larger than the turnover number V_{max}/[RTPR]$_0$ measured under standard Michaelis−Menten conditions. The system behaves as if there is more enzyme turning over substrate than there is AdoCbl available. This kinetic behavior is consistent with a mechanism in which AdoCbl dissociates from oxidized RTPR after turnover, binds to a reduced RTPR and initiates a second turnover faster than the first molecule of RTPR can be re-reduced.

Figure 3 shows a simplified model considering the two extreme cases: in path A, the AdoCbl dissociates from RTPR on each turnover, requiring a chain length of one. In path B, the AdoCbl remains bound under multiple turnovers, and the chain length would depend upon the frequency with which the carbon−cobalt bond is reformed. Given the available experimental data, the steady-state assumption, and a few chemically reasonable assumptions, it is possible to make a crude estimate of the rate (v) at which dNTPs are generated as a function of [AdoCbl] and of the flux through path A: $v = k_2(k_3 + k_5)$[AdoCbl]$_0/(k_2 + k_3 + k_5)$ and $k_5/(k_3 + k_5)$, respectively. The derivation of this equation is shown in Supporting Information. The rate constants k_1 and k_4, binding of NTP and AdoCbl, are assumed to be fast. The rate constant k_2 encompasses many steps and is known not to be limited by either dNTP formation (55 s^{-1}, Figure 2A) or cob(II)alamin formation and disappearance (>200 s^{-1} and 20−50 s^{-1}, respectively).[5,8] The value for k_2 is 20−50 s^{-1} and k_3 and/or k_6 is 2 s^{-1}, limited by rereduction of the disulfide and or a conformational change accompanying this process.[5,8] The rate constant k_5 is 8−10 s^{-1} as measured in Figure 1A and B. The reduced form of RTPR is designated E, and the oxidized form is designated F. With this information, it is clear that the flux through path A predominates ($k_5/(k_3 + k_5) = 0.8$). The dissociation of AdoCbl on each turnover requires that the carbon−cobalt bond is reformed on every turnover, establishing a chain length for the reaction of approximately one. This simplified model also predicts quite well the rate of dNTP formation as a function of [AdoCbl] (data not shown).

Further support for a chain length of one is obtained from ^{3}H$_2$O release experiments using [5′-^{3}H]-AdoCbl under turnover conditions. If a single C−Co bond cleavage event resulted in formation of multiple dNTPs, then one would expect the amount of ^{3}H$_2$O released to be very low relative to the amount of dNTP generated, since the carbon−cobalt bond is not reformed and hence there is no opportunity for washout of ^{3}H from the cofactor. Instead, what is observed is that the apparent rate

constant for ^{3}H$_2$O formation in the presence of substrate is very similar to the rate constant observed in the absence of substrate. Furthermore, the rate constant of hydrogen exchange is similar to the rate constant of dNTP formation in the steady state. The observation that the ratio of ^{3}H$_2$O release to dATP formation increases with time during the first turnover suggests that most of the ^{3}H$_2$O release is associated with reformation of the C−Co bond <u>after</u> dATP formation. Thus, the amount and rate of ^{3}H released suggest that carbon−cobalt bond reformation follows each turnover.

The ability of RTPR to release AdoCbl after each turnover could have important physiological implications and may explain the seemingly paradoxical observation that when *Lactobacillus leichmannii* cells are grown in the presence of subsaturating amounts of AdoCbl, the amount of RTPR biosynthesized increases.[11,12] Calculations suggest that under these conditions, *L. leichmannii* cells produce RTPR in molar excess over AdoCbl molecules taken up by the cell. A turnover mechanism involving C−Co bond reformation and AdoCbl dissociation between turnovers would increase the effective concentration of holoenzyme, enabling a higher rate of DNA biosynthesis to be maintained.

Experimental Section

Nucleotides, nucleosides, and NADPH were obtained from Sigma. Alkaline phosphatase (calf intestine) was purchased from Boehringer-Mannheim. RTPR (specific activity of 1.4 U/mg), TR (specific activity of 300−700 U/mg), and TRR (specific activity of 3000−7000 U/mg) were isolated as previously described.[13−15] C$_{18}$ Sep-Pak cartridges were obtained from Whatman. Concentrations of RTPR were measured spectrophotometrically ($\epsilon_{280} = 101\,000$ M^{-1} cm^{-1}), and the enzyme was pre-reduced as previously described.[16] [5′-^{3}H] AdoCbl was prepared as previously described.[4] All operations involving AdoCbl were carried out under dim light or red light.

UV−vis spectroscopy was performed on a Cary 3 spectrophotometer at 37 °C. SF studies were carried out using an Applied Photophysics DX.17MV stopped-flow spectrophotometer. Data was collected at either 340 nm to monitor consumption of NADPH ($\Delta\epsilon_{340} = 6220$ M^{-1} cm^{-1}), or at 525 nm to monitor conversion of AdoCbl to cob(II)alamin ($\Delta\epsilon_{525} = 4800$ M^{-1} cm^{-1}). A minimum of five traces was used to obtain an average kinetic trace. Linear and nonlinear least-squares fits to SF data were carried out using either the Applied Photophysics system software or KaleidaGraph. Rapid quench experiments were performed on a Kin-Tek model RFQ-3 apparatus. To allow reproducible loading in dim light, the sample loops were loaded using Luer-tip gas-tight syringes that had been calibrated so that the displacement required to fill each sample loop was marked on the barrel of the syringe. Reverse phase HPLC was performed on an Altex HPLC system with an Econosil C$_{18}$ 10 μm column (4.6 × 250 mm). The flow rate was 1 mL/min, the elution profile was monitored by A_{260}, and fractions of 1 mL were collected. Scintillation counting was performed on a Beckman LS 6500 scintillation counter using Scint-A scintillation fluid at a ratio of 8.5 mL Scint-A per mL of eluate.

[AdoCbl]-Dependence of the Rate of Turnover when [RTPR] ≫ [AdoCbl]: Measurement of the Rate of AdoCbl Dissociation from RTPR. Assays contained in a volume of 500 μL: 200 mM sodium dimethylglutarate (NaDMG) pH 7.3, 1 mM ATP, 1 mM dGTP, 0.25 mM NADPH, AdoCbl (0.1−0.8 μM), 85 μM TR, 1.5 μM TRR, and RTPR (1−8 μM). RTPR was present in 10-fold excess over AdoCbl.

(11) Davis, R. L.; Layton, L. L.; Chow, B. F. *Proc. Soc. Exptl. Biol. Med.* **1952**, *79*, 273−276.
(12) Goulian, M.; Beck, W. S. *J. Biol. Chem.* **1966**, *241*, 4233−4242.
(13) Booker, S.; Stubbe, J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8352−8356.
(14) Lunn, C. A.; Kathju, S.; Wallace, C.; Kushner, S.; Pigiet, V. *J. Biol. Chem.* **1984**, *259*, 10469−10474.
(15) Russel, M.; Model, P. *J. Bacteriol.* **1985**, *163*, 238−242.
(16) Booker, S. Ph.D. Thesis. Massachusetts Institute of Technology, 1994.

All components except AdoCbl were mixed in a 500 μL quartz cuvette and incubated at 37 °C for 1−2 min. A background rate of NADPH consumption was measured by monitoring A_{340}. AdoCbl was added to the cuvette, and the rate of NADPH consumption was measured. Similar experiments were carried out using 1 mM CTP and 1 mM dATP. Under the conditions above where [AdoCbl] > 0.8 μM, SF UV−vis methods were required to measure the rate of NADPH consumption. RTPR (10−30 μM), 1 mM ATP (1 mM CTP), 1 mM dGTP (1 mM dATP), 175 μM TR, 3 μM TRR, and 0.5 mM NADPH in 200 mM NaDMG, pH 7.3, were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 1−3 μM AdoCbl, 1 mM ATP (1 mM CTP), 1 mM dGTP (1 mM dATP), and consumption of NADPH was monitored. Experiments were also carried out with [RTPR] < [AdoCbl]. Assays contained in a volume of 500 μL: NaDMG pH 7.3, 1 mM ATP (1 mM CTP), 1 mM dGTP (1 mM dATP), 0.25 mM NADPH, AdoCbl (0.6−4 μM), and RTPR (0.1−0.75 μM). AdoCbl was present in 5-fold excess over RTPR.

Rapid Acid Quench Experiments Under Single Turnover Conditions: Fate of the 5′-Hydrogens of [5′-³H]-AdoCbl and Rate of dATP Formation. Pre-reduced RTPR (120 μM), 20 μM TR, 1 μM TRR, 2 mM NADPH, 2 mM [U-¹⁴C]-ATP (5.2×10^6 cpm/μmol), 2 mM dGTP, and 100 mM NaDMG pH 7.3, were placed in one syringe and rapidly mixed at 37 °C with an equal volume from a second syringe of [5′-³H]-AdoCbl (100 μM, 1.2×10^6 cpm/μmol), 2 mM ATP, and 2 mM dGTP in the same reaction buffer. After the specified time, the reaction was quenched with 2% (v/v) perchloric acid (60−220 μL), collected in tubes containing 5′-dA (55 nmol) and 5′,8-cycloadenosine (15 nmol). Immediately after being quenched, samples were neutralized with equal volumes of 0.4 M KOH and 0.5 M NaDMG pH 7.3 (50−200 μL each). Neutralized samples were immediately quick-frozen in liquid nitrogen and stored on dry ice. The neutralized reaction mixtures were stable at −20 °C for up to a week. A zero time point was generated by omitting [5′-³H]-AdoCbl from the second syringe and putting it instead in the sample collection tube, so that the protein would be acid-precipitated before encountering it. Tritiated water was removed from the quenched samples by bulb-to-bulb distillation. All operations were carried out in a fume hood. Samples (∼1 mL) were transferred to silanized 50-mL pear-shaped flasks and shell-frozen in dry ice/acetone. The bulb-to-bulb distillation apparatus (two pear-shaped flasks connected by a Y-shaped adapter equipped with a stopcock) was evacuated and kept under static vacuum. The flask containing the sample was removed from the dry ice/acetone bath, and the empty flask was placed in the dry ice/acetone bath. Lyophilization of the sample was usually complete in 45 min or less. The distillate was transferred to a scintillation vial,

which was immediately capped. The flask containing the distillate was washed with 500 μL of water, which was also transferred to the scintillation vial. Scintillation fluid (8.5 mL) was added to the vial, and the distillate was counted.

The lyophilized material was redissolved in 1 mL of water, frozen on dry ice, and stored at −20 °C. It was then thawed and loaded onto reverse phase C_{18} Sep-Paks. Nucleotides were eluted with 10 mL of water. Nucleosides and AdoCbl were eluted with 50% (v/v) CH_3OH (aqueous) (10 mL), and the solvent was removed by lyophilization. The nucleotide-containing samples were lyophilized, then redissolved in 50 mM Tris, 0.1 mM EDTA (970 μL) to which 30 U of alkaline phosphatase was added. The reaction was incubated at 37 °C for 3 h. The reaction mixture was analyzed by HPLC with a linear gradient. Solvent A was H_2O and solvent B was MeOH: 0−2 min, isocratic elution with A; 2−7 min, 0−20% B; 7−30 min, 20% B. Adenine, adenosine, and 2′-deoxyadenosine (2′-dA) eluted at 12, 16.5, and 18 min, respectively. Fractions containing adenosine and 2′-dA were pooled and lyophilized. Because this HPLC method effected only partial separation of adenosine and 2′-dA, the 2′-dA containing fractions were further chromatographed by the method of Cory et al.[17] Borate columns (Dowex AG 1 × 2, exchanged into the borate form using sodium tetraborate) were poured into glass wool-stoppered 5.75 in. Pasteur pipettes, washed with 20 mL of water, and then equilibrated by washing with 1 mM sodium tetraborate (2 mL). The lyophilized samples were redissolved in 1 mL of water and loaded onto the borate columns. The columns were washed with 2 mL of 1 mM sodium tetraborate, and 2′-dA was eluted with 12 mL of 1 mM sodium tetraborate. UV−vis spectroscopy ($\epsilon_{260} = 15\ 200\ M^{-1}\ cm^{-1}$ for 2′-dA) was used to measure the recovery. The sample was lyophilized and redissolved in 1 mL water, and radioactivity was analyzed by scintillation counting.

Supporting Information Available: Derivation of the rate equation for dNTP formation according to the model in Figure 3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

(17) Cory, J. G.; Russell, F. A.; Mansell, M. M. *Anal. Biochem.* **1973**, *55*, 449−456.