Activation Parameters for the Carbon−Cobalt Bond Homolysis of Coenzyme B_{12} Induced by the B_{12}-Dependent Ribonucleotide Reductase from *Lactobacillus leichmannii*

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Received May 18, 1998

Abstract: The temperature dependence of the kinetics of the rapid, reversible cleavage of the carbon−cobalt bond of 5′-deoxyadenosylcobalamin (AdoCbl, coenzyme B_{12}) catalyzed by the ribonucleotide triphosphate reductase (RTPR) of *Lactobacillus leichmannii* has been studied by stopped-flow spectrophotometry. At a given temperature and constant concentration of AdoCbl, the observed rate constants, k_{obs}, are essentially independent of the initial concentration of RTPR, but the spectral change, and hence the amount of cob(II)alamin formed at equilibrium, shows a hyperbolic dependence on $[RTPR]_0$. This is interpreted as being a consequence of the known relatively weak binding of AdoCbl to the enzyme so that increasing enzyme concentration drives the binding equilibrium toward enzyme−coenzyme complex. Fitting the absorbance change data to a binding isotherm gives values of the binding equilibrium constant, K_b, and the maximal absorbance change obtainable when the coenzyme is "saturated" with enzyme. From the latter value and the difference in molar absorptivity of AdoCbl and cob(II)alamin, the concentration of enzyme-bound cob(II)alamin, and consequently the equilibrium constant, K_{eq}, for its formation from enzyme-bound AdoCbl, can be calculated. These values of K_{eq} have been used to deconvolute the forward, k_f, and reverse, k_r, rate constants from the measured k_{obs} values for cob(II)alamin formation at the active site. The enzyme is found to catalyze the Co−C bond homolysis of AdoCbl by a factor of 1.6×10^9 ($\Delta\Delta G^{\ddagger} = 13$ kcal mol^{-1}) at 37 °C. Eyring plots of k_f and van't Hoff plots of K_{eq} both show distinct discontinuities at about 32 °C which may indicate the formation of an inactive, or less active, conformer at lower temperature or a change in mechanism. The activation parameters for the forward rate constant for cob(II)alamin in the upper temperature regime ($\Delta H_f^{\ddagger} = 20 \pm 1$ kcal mol^{-1}, $\Delta S_f^{\ddagger} = 13 \pm 4$ cal mol^{-1} K^{-1}) show that the entropy of activation is essentially the same as that for the nonenzymatic thermal homolysis of AdoCbl, but the enthalpy of activation is 13 ± 2 kcal mol^{-1} lower. The enzyme thus appears to catalyze the Co−C bond cleavage reaction entirely enthalpically. Several possibilities for the enthalpic catalysis of AdoCbl homolysis by RTPR are discussed.

Introduction

The means by which coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl[1])-requiring enzymes catalyze the cleavage of the carbon−cobalt bond of the coenzyme by as much as 12 orders of magnitude[2−5] remains one of the most important unsolved problems in this area of bioinorganic chemistry. While many mechanisms have been proposed, including enzyme-induced distortion of the corrin ring to sterically labilize the Co−C bond,[6−13] enzymatic compression of the axial Co−N bond causing transmission of steric compression to the upper axial ligand,[10,14] lengthening of the axial Co−N bond to stabilize the emerging Co^{II} oxidation state,[11,14,15] corrin ring distortion by twisting the axial Co−N bond to rotate the 5,6-dimethylbenzimidazole nucleotide,[11,14] and direct "pulling" or bending of the Co−C bond by interaction of the Ado ligand with the protein,[10,11,14,16] little is actually known about how these enzymes activate AdoCbl for Co−C bond homolysis.

In contrast, the nonenzymatic thermal homolysis of AdoCbl has been thoroughly studied by Finke and Hay.[2−4] By working in the temperature range of 85−110 °C and correcting for the occurrence of competing heterolysis and the small amount of base-off species present, these workers obtained values of 33 $\pm$ 2 kcal mol^{-1} and 11 $\pm$ 3 cal mol^{-1} K^{-1} for the enthalpy and entropy of activation, respectively, for Co−C bond homolysis.

(1) Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); RTPR, ribonucleotide triphosphate reductase; DTT, dithiothreitol; TR, thioredoxin; TRR, thioredoxin reductase; AdoH, 5′-deoxyadenosine; Bzm, 5,6-dimethylbenzimidazole; CH_3Cbl, methylcobalamin; CNCbl, cyanocobalamin (vitamin B_{12}); CH_3Cbi^+, methylcobinamide; $CH_3Me_3BzmCba^+$, methyl-3,5,6-trimethylbenzimidazolylcobamide.

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S0002-7863(98)01729-6 CCC: $15.00
Published on Web 09/02/1998

However, while reports of kinetic observations of enzyme-induced AdoCbl homolysis have appeared,[5,17−19] the temperature dependence of the enzyme-catalyzed process is not known.[20] Knowledge of how an AdoCbl-dependent enzyme affects the enthalpy and entropy of activation for Co−C bond homolysis would provide an important clue to guide the search for mechanisms.

In many ways, the ribonucleotide triphosphate reductase (RTPR,[1] EC 1.17.4.2) from *Lactobacillus leichmannii* is the ideal AdoCbl-dependent enzyme for such a study. It is the only AdoCbl-dependent enzyme known that can catalyze Co−C bond cleavage in an incomplete system lacking substrate, thus avoiding the complications of turnover. In addition, simple dithiols such as dihydrolipoate and dithiothreitol (DTT[1]) can substitute for the physiological reducing system thioredoxin(TR[1])/thioredoxin reductase (TRR[1])/NADPH. Tamao and Blakely[17] have shown, and Licht et al.[18] have confirmed, that mixing a solution containing the enzyme, the allosteric effector dGTP, and a suitable reductant with a solution containing AdoCbl and dGTP leads to rapid ($k_{obs} \sim 38\ s^{-1}$) reversible formation of about 0.2 equiv of cob(II)alamin. This kinetically competent reversible formation of cob(II)alamin has also been shown to generate an EPR active species with the same rate constant.[18,23] Licht et al.[18] have recently shown that this EPR-active species consists of enzyme-bound cob(II)alamin and a thiyl radical derived from the active site Cys408 residue, although it is not yet known whether formation of this thiyl radical occurs in a concerted process with Co−C bond cleavage or in a stepwise process with subsequent, fast hydrogen atom transfer from the −SH functionality to the Ado$^{\bullet}$ radical following Co−C bond cleavage.

We now report confirmation and extension to various temperatures of the original kinetic measurements of the RTPR-induced cleavage of AdoCbl by Tamao and Blakley.[17] These measurements permit an analysis of the activation parameters for enzyme-induced AdoCbl homolysis and of the thermodynamics of cob(II)alamin formation at the active site, providing new insight to the catalytic process and new guideposts for the search for the mechanism of enzymatic activation of AdoCbl.

Experimental Section

Materials. *Lactobacillus leichmannii* RTPR was obtained from transformed *Eschericia coli* HB101/PSQUIRE,[24] the generous gift of J. Stubbe, Massachusetts Institute of Technology, and was purified as described by Booker and Stubbe.[24] *E. coli* TR was obtained from overproducing strain SK 3981,[25] and *E. coli* TRR was from overproducing strain K91/pMR14.[26]

Methods. Enzyme assays were performed on a Cary 3 UV−visible spectrophotometer, the cell compartment of which was thermostated at 37 °C. The assay[27] mixture (500 μL) contained the following: 25 mM HEPES (pH 7.5), 4.0 mM EDTA, 1.0 mM dGTP, 2.0 mM ATP, 200 μM NADPH, 20 μM TR, 0.15 μM TRR, and 0.1−0.5 μM RTPR. The reaction was initiated by addition of AdoCbl (8 μM) and monitored by the decrease in 340-nm absorbance of NADPH using $\Delta\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$ after correction for the background (<10% of the observed activity). Using this assay, the enzyme had a specific activity of 0.31 μmol min^{-1} mg^{-1}. While this is significantly lower than that reported by Booker and Stubbe (1.5 μmol min^{-1} mg^{-1}),[24] in our hands, enzyme provided by these workers also had a specific activity of 0.31 mmol min^{-1} mg^{-1}.

The RTPR-induced formation of cob(II)alamin from AdoCbl was monitored by following the absorbance change at 525 nm, the wavelength of maximal spectral change, in an Applied Photophysics SX.17MV stopped-flow spectrophotometer equipped with an AN1 anaerobic accessory. The light path was 1.0 cm and the dead time was about 1.8 ms. The driving syringes and sample lines were filled with 50 mM sodium dithionite 12 h before each experiment and were thoroughly washed with deoxygenated 0.2 M sodium dimethylglutarate buffer, pH 7.3, immediately before the samples were introduced. The sample unit was thermostated with a circulating water bath, and the temperature was monitored continuously using a sensor built into the sample handling unit. In the temperature range used (23−40 °C), the variation in temperature during an experiment was about ±0.1 °C. One gram of sodium dithionite was dissolved in the water bath fluid shortly before the experiment, and argon was bubbled continuously through the bath fluid during the experiments. The enzyme solutions were deaerated by stirring under an argon atmosphere for 1 h. All other working solutions were deaerated by bubbling with argon for 1 h.

Enzyme, dGTP, DTT (or TR, TRR, and NADPH), and buffer (0.2 M sodium dimethylglutarate, pH 7.3) were placed in one syringe and coenzyme, dGTP, and the same buffer were placed in the other. The sample solutions were thermally equilibrated in the driving syringes for at least 20 min before each run. The final reaction mixture (after mixing) generally consisted of 50 μM AdoCbl, 2 mM dGTP, 25 mM DTT (or 10 μM TR, 0.5 μM TRR, and 2 mM NADPH), 0.2 M sodium dimethylglutarate buffer (pH 7.3), and 25−200 μM RTPR. For each reaction, five or six traces were averaged and the average was used for parameter fitting, employing the least-squares kinetic analysis program supplied with the Applied Photophysics operating program.

UV−visible spectra were measured on a Cary 3 UV−visible spectrophotometer, the sample compartment of which was thermostated with an electronic temperature controller. Samples were placed in 1.0 cm path length airtight screw-capped cuvettes. Air-sensitive samples were treated as described above and transferred into the cells with gastight syringes.

Results

A typical stopped flow absorbance trace is shown in Figure 1. As described by Tamao and Blakley,[17] a rapid, first-order decrease in 525-nm absorbance, representing the reversible formation of cob(II)alamin, was complete in about 100 ms. This was followed by the much slower decrease in absorbance representing the irreversible cleavage to cob(II)alamin and AdoH.[17] The first-order rate constant for the slower change, $6.1 \times 10^{-3}\ s^{-1}$, was about 6000-fold slower than that for the faster change. Consequently, the faster spectral change could be independently observed in a 200-ms time window (i.e., the slower change has proceeded only 0.1% to completion after 200 ms) and successfully fitted to a single-exponential decay to obtain the observed rate constant and the absorbance change, ΔA.

The first-order rate constant for the rapid formation of cob(II)-alamin, k_{obs}, was essentially independent of the concentration of RTPR in the range 25−200 μM, as seen in Figures 2 and 3A, in agreement with Tamao and Blakley.[17,28] At 37 °C using

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(28) In some experiments, there was a tendency for k_{obs} to increase slightly with enzyme concentration. However, in all experiments, the average value of k_{obs} was taken as the best estimate.

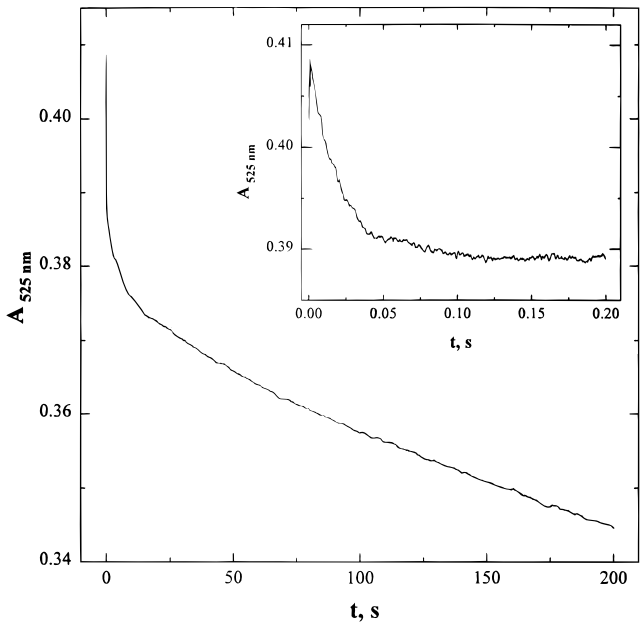

Figure 1. Absorbance changes at 525 nm for the reaction of RTPR with AdoCbl in the presence of dGTP and DTT observed by stopped-flow spectrophotometry at 37 °C. The final reaction mixture contained 50 μM AdoCbl, 50 μM RTPR, 25 mM DTT, and 2 mM dGTP in 0.2 M sodium dimethylglutarate buffer, pH 7.3.

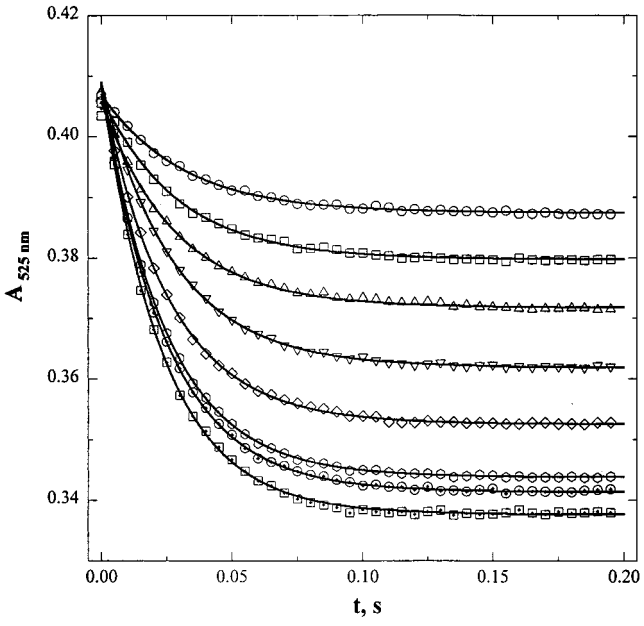

Figure 2. Absorbance changes at 525 nm for the rapid, reversible formation of cob(II)alamin by reaction of various concentrations of RTPR with AdoCbl in the presence of dGTP and DTT at 37 °C. The symbols are the experimental points, and the solid lines are nonlinear least-squares fits to a single-exponential decay. The final concentrations (after mixing) were 50 μM AdoCbl, 25 mM DTT, 2 mM dGTP, and 25, 37.5, 50, 75, 100, 125, 150, or 200 μM RTPR (from top to bottom).

DTT as the reductant, the average value of k_{obs} was 37.2 ± 2.5 s^{-1} (Figure 3A) in good agreement with Tamao and Blakley's value of 38 s^{-1} with a dihydrolipoate reductant, and the value of 42 s^{-1} obtained by Licht et al.[18] using the TR/TRR/NADPH reductant. This suggests that the results are not significantly affected by the choice of reductant.

Also in agreement with Tamao and Blakley,[17] we find the amplitude of the rapid absorbance change to depend significantly on the concentration of RTPR, as seen in Figures 2 and 3B.

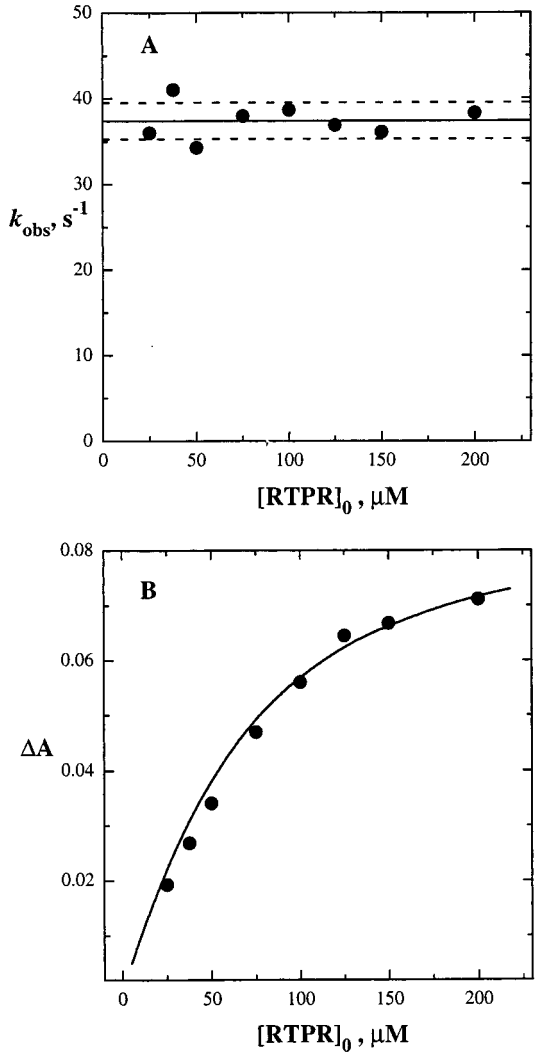

Figure 3. (A) Dependence of the observed rate constant for the rapid, reversible formation of cob(II)alamin (k_{obs}, from the data in Figure 2) on the initial concentration of RTPR. The solid line is the average value (37.2 ± 2.5 s^{-1}), and the dashed lines are this value plus or minus one standard deviation. (B) Dependence of the absorbance change, ΔA, accompanying the fast, reversible formation of cob(II)alamin (from the data in Figure 2) on the initial concentration of RTPR. The solid line is a least-squares fit to eq 3 from which the values $\Delta A_\infty = 0.0886 \pm 0.0035$ and $K_b = (2.65 \pm 0.49) \times 10^4$ M^{-1} were obtained.

The simplest interpretation of this phenomenon uses the classical[17] and widely accepted[18] reaction scheme shown in eq 1. In this scheme, the relatively weak binding of AdoCbl to

$$\mathrm{E{-}SH(dGTP)} + \mathrm{AdoCbl} \overset{K_b}{\rightleftharpoons} \mathrm{E{-}SH(dGTP)(AdoCbl)} \overset{k_f}{\underset{k_r}{\rightleftharpoons}} \mathrm{E{-}S^{\bullet}(dGTP)(AdoH)(cob(II))} \quad (1)$$

the enzyme-dGTP complex ($K_b \sim 2 \times 10^4$ M^{-1})[29] is followed by homolytic cleavage of the Co−C bond of AdoCbl and transfer of the thiol hydrogen from Cys408 to the Ado$^\bullet$ radical to form the observed products. In the scheme, the latter two processes are depicted as occurring in the same step for simplicity, but it is not yet known if they occur in concerted or stepwise fashion (vide infra). The increase in the absorbance change with increasing $[RTPR]_0$ can then be interpreted as a perturbation of the weak binding equilibrium and described as

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Table 1. Observed and Calculated Absorbance, Kinetic, and Equilibrium Data for the Reaction of AdoCbl with RTPR in the Presence of dGTP and DTT

T, °C	k_{obs}, s^{-1} [a]	ΔA_∞ [b]	K_b, M^{-1} [c]	K_{eq} [d]	k_f, s^{-1} [e]	k_r, s^{-1} [e]
23	7.26 ± 0.98	0.0320 ± 0.0018	(2.34 ± 0.54) × 10^4	0.154 ± 0.012	1.0 ± 0.2	6.3 ± 1.1
26	8.80 ± 1.13	0.0484 ± 0.0035	(2.05 ± 0.45) × 10^4	0.253 ± 0.026	1.8 ± 0.3	7.0 ± 1.2
29	14.1 ± 1.4	0.0689 ± 0.0023	(2.46 ± 0.28) × 10^4	0.403 ± 0.019	4.0 ± 0.5	10.1 ± 1.1
32	22.5 ± 1.7	0.0845 ± 0.0039	(2.99 ± 0.49) × 10^4	0.543 ± 0.035	7.9 ± 0.8	14.6 ± 1.5
33.5	25.9 ± 3.2	0.0871 ± 0.0066	(2.09 ± 0.70) × 10^4	0.570 ± 0.092	9.4 ± 2.1	16.5 ± 2.5
35	28.0 ± 2.0	0.0878 ± 0.0042	(2.68 ± 0.47) × 10^4	0.577 ± 0.039	10.2 ± 1.0	17.7 ± 1.7
37	37.2 ± 2.5	0.0886 ± 0.0035	(2.65 ± 0.49) × 10^4	0.585 ± 0.033	13.7 ± 1.2	23.5 ± 2.1
38.5	39.4 ± 2.3	0.0948 ± 0.0025	(2.09 ± 0.19) × 10^4	0.653 ± 0.024	15.6 ± 1.1	23.8 ± 1.6
40	49.9 ± 6.8	0.0953 ± 0.0049	(2.33 ± 0.61) × 10^4	0.658 ± 0.048	19.8 ± 2.9	30.1 ± 4.8

[a] Average of six to eight observed rate constants at $[RTPR]_0 = 25-200$ μM. [b] Limiting absorbance change at 525 nm from fits of ΔA vs $[RTPR]_0$ according to eq 3. [c] Binding constant for AdoCbl to the RTPR−dGTP complex (eq 1) from fits of ΔA vs $[RTPR]_0$ to eq 3. [d] Calculated from the maximal absorbance change, ΔA_∞, and the difference in molar absorbtivity of AdoCbl and cob(II)alamin at 525 nm. [e] Calculated from $K_{eq} = k_f/k_r$ (eq 1) and $k_{obs} = k_f + k_r$.

a classical binding isotherm, eq 2, where ΔA is the absorbance

$$\Delta A = \frac{\Delta A_\infty [RTPR]_{eq}}{[RTPR]_{eq} + K_d} \quad (2)$$

change, ΔA_∞ is the limiting value of ΔA when the coenzyme is "saturated" with enzyme, $[RTPR]_{eq}$ is the equilibrium concentration of RTPR, and $K_d = 1/K_b$. The data were actually fitted to eq 3, which is derived from eq 2 by substituting eq 4 and rearranging.

$$[RTPR]_0 = \left\{K_d + \left(1 - \frac{\Delta A}{\Delta A_\infty}\right)[AdoCbl]_0\right\} \Big/ \left(\frac{\Delta A_\infty}{\Delta A} - 1\right) \quad (3)$$

$$[RTPR]_{eq} = [RTPR]_0 - \left(\frac{\Delta A}{\Delta A_\infty}\right)[AdoCbl]_0 \quad (4)$$

Fitting the data at 37 °C (Figure 3B) gave $\Delta A_\infty = 0.0886 \pm 0.0035$ and $K_b = (2.65 \pm 0.49) \times 10^4$ M^{-1}. Note that the method provides a much more precise value of ΔA_∞ ($\sigma = 4.0\%$) than of K_b ($\sigma = 18.5\%$).

The value of ΔA_∞ can be used to estimate the number of equivalents of cob(II)alamin formed at the active site and hence the equilibrium constant, $K_{eq} = k_f/k_r$ (eq 1), using the change in molar absorptivity for the conversion of AdoCbl to cob(II)alamin ($\Delta\epsilon_{525} = 4800$ M^{-1} cm^{-1}),[18] as well as any perturbation of the molar absorptivities by binding to the protein. The latter possibility was investigated by recording the visible spectra of AdoCbl (50 μM) alone and in the presence of RTPR (200 μM) and dGTP (2 mM) and of cob(II)alamin (50 μM) alone and in the presence of RTPR (200 μM), dGTP (2 mM), and AdoH (0.2 mM). Under these conditions, assuming $K_b = 2 \times 10^4$ M^{-1},[29] ca. 80% of the AdoCbl is bound to RTPR and ca. 94% of the cob(II)alamin ($K_b = 9.5 \times 10^4$ M^{-1})[30] is bound to RTPR. The reference cell had the same components except for the cobalamins, and the spectra were recorded at various temperatures from 20 to 40 °C. There was no significant difference in absorbance at $\lambda > 400$ nm of AdoCbl or of cob(II)alamin when they were complexed with RTPR. Consequently, the value of $\Delta\epsilon_{525} = 4800$ M^{-1} cm^{-1} was used in conjunction with ΔA_∞ to calculate that 0.369 ± 0.015 equiv of cob(II)alamin was formed at saturating enzyme concentration at 37 °C and that $K_{eq} = k_f/k_r = 0.585 \pm 0.033$.

From this value for K_{eq} and the fact that for the reversible formation of cob(II)alamin $k_{obs} = k_f + k_r$, values of $k_f = 13.7 \pm 1.2$ s^{-1} and $k_r = 23.5 \pm 2.1$ s^{-1} could be calculated. Thus, assuming that Hay and Finke's data[3] for the nonenzymatic thermal homolysis of the Co−C bond of AdoCbl can be legitimately extrapolated to 37 °C, RTPR accelerates this bond cleavage by a factor of 1.6×10^9.

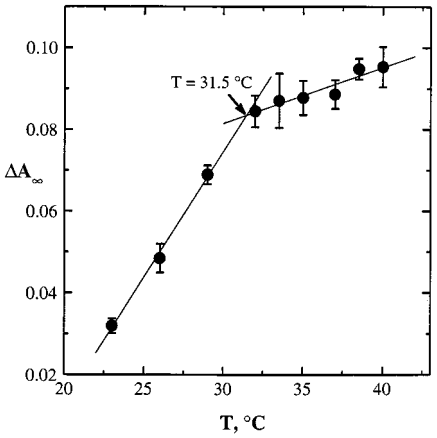

Figure 4. Plot of the maximal absorbance change, ΔA_∞ (eqs 2 and 3), for the rapid, reversible formation of cob(II)alamin vs temperature, showing the sharp break at 31.5 °C.

These experiments and calculations were repeated at a total of nine temperatures between 23 and 40 °C, using six to eight concentrations of RTPR between 25 and 200 μM at each temperature. The resulting kinetic and equilibrium parameters are collected in Table 1. At 38.5 °C, the entire experiment was repeated using the TR/TRR/NADPH reductant, and the results ($k_{obs} = 43.6 \pm 1.7$ s^{-1}, $k_f = 14.3 \pm 0.9$ s^{-1}) were essentially the same as those with the DTT reductant ($k_{obs} = 39.4 \pm 2.3$ s^{-1}, $k_f = 15.6 \pm 1.1$ s^{-1}). The temperature dependencies of ΔA_∞, k_f, and K_{eq} are shown in Figure 4, a plot of ΔA_∞ vs T, Figure 5, an Eyring plot of k_f, and Figure 6, a van't Hoff plot for K_{eq}. For each of these three parameters, there is a pronounced break in the temperature dependence at about 31−32 °C. These breaks are reminiscent of the breaks in temperature dependence at ca. 28−31 °C previously observed by Tamao and Blakley[17] for the amplitude of the rapid formation of cob(II)alamin and for the UV temperature difference spectra of RTPR alone, in the presence of dGTP, and in the presence of dGTP and dihydrolipoate. Because of the pronounced break in the temperature data for these parameters, it is inappropriate to use all of the k_f data together to calculate activation parameters for cob(II)alamin formation. Consequently, the data above 30 °C were used to calculate the values $\Delta H_f^\ddagger = 20 \pm 1$ kcal mol^{-1} and $\Delta S_f^\ddagger = 13 \pm 4$ cal mol^{-1} K^{-1}, while the data below 30 °C were independently correlated to provide the values $\Delta H_f^\ddagger = 42 \pm 4$ kcal mol^{-1} and $\Delta S_f^\ddagger = 83 \pm 14$ cal mol^{-1} K^{-1}. Similarly, in the van't Hoff plot for K_{eq} (Figure 6), the data at $T > 30$ °C provided the values $\Delta H° = 4.6 \pm 0.8$ kcal mol^{-1} and $\Delta S° =$

(30) Yamada, R.; Tamao, Y.; Blakley, R. L. *Biochemistry* **1971**, *10*, 3959.

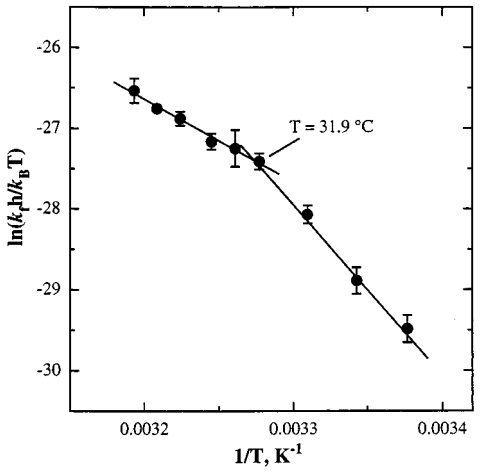

Figure 5. Eyring plot for the forward rate constant, k_f, for formation of cob(II)alamin at the RTPR active site (eq 1), where h is Planck's constant and k_B is Boltzmann's constant. The solid lines are linear least-squares fits to the data above and below 30 °C, from which the activation parameters in Table 2 were derived.

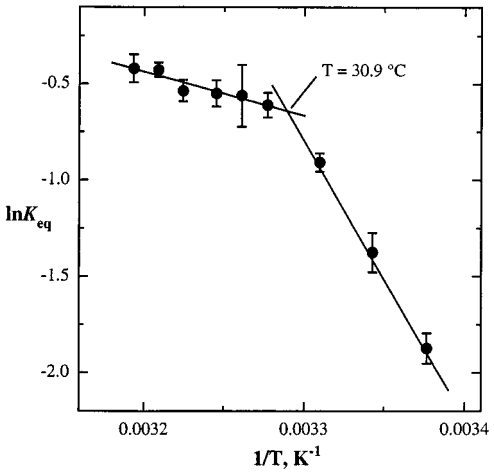

Figure 6. Van't Hoff plot for the equilibrium constant for formation of cob(II)alamin at the active site of RTPR (eq 1). The solid lines are linear least-squares fits, from which the thermodynamic data in Table 2 were derived.

Table 2. Summary of Thermodynamic and Activation Parameters for the Reaction of AdoCbl with RTPR in the Presence of dGTP and DTT

	T > 30 °C	T < 30 °C
$\Delta H_f^{\ddagger}$, kcal mol^{-1} [a]	20 ± 1	42 ± 4
$\Delta S_f^{\ddagger}$, cal mol^{-1} K^{-1} [a]	13 ± 4	83 ± 14
$\Delta H°$, kcal mol^{-1} [b]	4.6 ± 0.8	28.5 ± 0.4
$\Delta S°$, cal mol^{-1} K^{-1} [b]	14 ± 3	93 ± 2

[a] From Eyring plots for k_f (eq 1), Figure 5. [b] From van't Hoff plots for K_{eq} (eq 1), Figure 6.

14 ± 3 cal mol^{-1} K^{-1} while the data at T < 30 °C gave $\Delta H°$ = 28.5 ± 0.4 kcal mol^{-1} and $\Delta S°$ = 93 ± 2 cal mol^{-1} K^{-1}. These thermodynamic and activation parameters are summarized in Table 2.

The temperature dependence of the less precisely determined values of K_b, the equilibrium constant for AdoCbl binding to the RTPR−dGTP complex (eq 1) is shown in Figure 7 in the form of a van't Hoff plot. There is no discernible temperature dependence, but significantly, the average value, K_b = (2.4 ± 0.3) × 10^4 M^{-1}, agrees well with the value of (2.1 ± 0.4) × 10^4 M^{-1} determined by Singh et al.[29] by ultrafiltration.

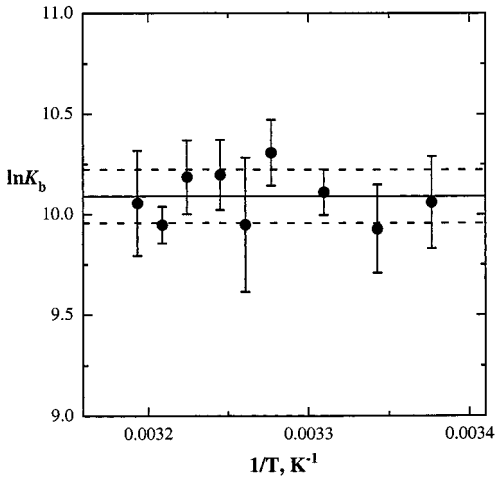

Figure 7. Plot of ln K_b, the binding constant for AdoCbl to the RTPR−dGTP complex (eq 1) vs 1/T. The solid line is the average value of ln K_b (10.09 ± 0.13), and the dashed lines are this value plus or minus one standard deviation.

Discussion

It is important to note that the thermodynamic and activation parameters measured here are for the RTPR-induced homolysis of AdoCbl in the *absence* of substrate, a reaction whose physiological significance is unknown. In the presence of an NTP substrate, the kinetics of cob(II)alamin formation are more complex, with biphasic, "overshoot" kinetic traces representing an initial formation of cob(II)alamin followed by a decay to a lower steady-state value.[17] While Tamao and Blakley[17] observed that the initial rate of formation of cob(II)alamin (i.e., the rate constant for the first phase) was approximately the same as that (ca. 40 s^{-1}) observed in the absence of substrate, Licht et al.[18] report that cob(II)alamin formation in the presence of substrate is some five times faster. Clearly, further work is needed to clarify the kinetics of cob(II)alamin formation in the presence of substrate.

The temperature discontinuities originally observed by Tamao and Blakley[17] were attributed by these authors to a change in conformation of the protein at temperatures below ca. 30 °C. The classical interpretation of the breaks seen in the Eyring plot for RTPR-induced AdoCbl cleavage (Figure 5) and the van't Hoff plot for the equilibrium formation of cob(II)alamin at the active site (Figure 6) would be a change of mechanism or a change of rate-determining step. It is certainly possible that a different conformation of the enzyme, stabilized at lower temperatures, could actually catalyze the reaction via a different mechanism.[31,32] However, a more likely scenario would be that a conformation stabilized at lower temperature is in fact inactive (or much less active) and that efficient catalysis requires isomerization to the active conformer, thus imposing the thermodynamics of this isomerization on the observed rate and equilibrium constants. We choose to focus on the results from the data obtained in the higher, physiological temperature regime since it seems likely that the activation parameters observed in this temperature range are relevant to the physiologically significant catalytic process. We note that the activation parameters we observe in the lower temperature regime are, within experimental error, the same as those reported by Stubbe et al.[21] in a preliminary report of the temperature dependence of RTPR-induced AdoCbl homolysis, evidently conducted under

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different conditions. This seems highly unlikely to be coincidental and, therefore, suggests that one focus on finding the cause of the temperature breaks in this and other[17] studies. The circumstances which bring about the very different results in the lower temperature regime are currently under vigorous collaborative investigation.[22]

The magnitude of the equilibrium constant, $K_{eq} \sim 0.5$ (eq 1), for the formation of cob(II)alamin, AdoH, and thiyl radical at the enzyme active site at $T > 30$ °C is quite extraordinary. Equilibrium constants for Co−C bond homolysis, eq 5, are

$$\mathrm{R{-}Co(Chel)} \underset{k_{recomb}}{\overset{k_{homo}}{\rightleftharpoons}} \mathrm{R^{\bullet} + Co^{II}(Chel)} \qquad (5)$$

known to be extremely small (ca. 5×10^{-9} in this temperature range for alkylcobalt porphyrins[33,34]). Part of the extraordinary effect of the enzyme on this equilibrium is due to the fact that it is coupled to the exergonic follow-up hydrogen atom transfer from the active site thiol to the Ado• radical (eq 6).[35] The

$$\mathrm{RCH_2^{\bullet} + R'SH} \overset{K_H}{\rightleftharpoons} \mathrm{RCH_3 + R'S^{\bullet}} \qquad (6)$$

magnitude of this thermodynamic pull on the Co−C bond homolysis equilibrium can be estimated from literature values for the rate constants for the forward and reverse processes of eq 6.[36−45] Measured rate constants for the formation of the thiyl radical in eq 6 range from 1.8×10^7 to 5.1×10^8 M^{-1} s^{-1},[39−45] while those for the reverse process range from 1.3×10^3 to 1.4×10^4 M^{-1} s^{-1}.[36−39] The best models for the H-atom transfer occurring on the enzyme would seem to be the reaction of 2-mercaptoethanol ($k = 4.7 \times 10^7$ M^{-1} s^{-1})[39] or glutathione ($k = 1.3 \times 10^8$ M^{-1} s^{-1})[38] with the ethanol radical, $HOCH_2CH_2^{\bullet}$, in the forward direction and the reaction of the cysteine thiyl radical with 2-propanol ($k = 2.0 \times 10^4$ M^{-1} s^{-1})[38] or the dithiothreitol thiyl radical with 2,5-dimethyltetrahydrofuran (5×10^3 M^{-1} s^{-1})[36] in the reverse direction. Hence, the equilibrium constant for this hydrogen atom transfer would be expected to be about 10^4. If this is approximately correct, then the equilibrium constant for Co−C bond homolysis (eq 5) at the enzyme active site is about 5×10^{-5}.

The equilibrium constant for the nonenzymatic homolysis of AdoCbl (eq 5) can be estimated from the kinetics for this process as well. Assuming, again, that Hay and Finke's data[3] can be appropriately extrapolated to 37 °C, the forward rate constant for Co−C bond homolysis, k_{homo} (eq 5) for AdoCbl is 8.7×10^{-9} s^{-1} at this temperature. The reverse process, k_{recomb}, is known to be diffusion controlled, and its rate constant has been measured by two different groups[46−48] by flash photolysis and found to be 1×10^9 s^{-1}. Thus, the equilibrium constant for Co−C bond homolysis of AdoCbl in the absence of enzyme is about 1×10^{-19}. This means that the enzyme must displace this equilibrium toward Ado• and cob(II)alamin by an astonishing 5×10^{14}, in addition to catalyzing the bond cleavage by 1.6×10^9.[49] The combined effect of the enzymatic displacement of the Co−C bond homolysis equilibrium and the follow-up H-atom transfer permits formation of a stoichiometrically significant concentration (ca. 0.35 equiv) of radical at the active site, evidently a necessity for efficient catalysis of the ensuing chemistry, despite the fact that it is initiated by an otherwise enormously unfavorable bond homolysis.

If it is correct that our values for the activation parameters for RTPR-induced AdoCbl homolysis in the 30−40 °C temperature range (Table 2) represent the physiologically significant process, they may be directly compared to the activation parameters for the nonenzymatic thermal homolysis of AdoCbl reported by Hay and Finke ($\Delta H^{\ddagger} = 33 \pm 2$ kcal mol^{-1}, $\Delta S^{\ddagger} = 11 \pm 3$ cal mol^{-1} K^{-1}),[3] *assuming* that these are not significantly temperature dependent in the range 30−85 °C,[53] a necessary assumption at this point but a proposition which is currently under investigation.[54] This comparison shows that the entropy of activation is essentially the same for the enzyme-induced and nonenzymatic processes ($\Delta\Delta S^{\ddagger} = -2 \pm 5$ cal mol^{-1} K^{-1}) but that the enthalpy of activation is lowered by the enzyme by 13 kcal mol^{-1} ($\Delta\Delta H^{\ddagger} = 13 \pm 2$ kcal mol^{-1}). This in turn leads to the conclusion that the enzymatic catalysis of AdoCbl Co−C bond homolysis by RTPR is *entirely* enthalpic in nature (in agreement with a suggestion originally made by Finke and coworkers),[55,58] and this enthalpy difference leads to a rate enhancement of 1.4×10^9, completely accounting for the observed enhancement of 1.6×10^9.

Although, as pointed out above, it is not yet known if the coupled processes of Co−C bond homolysis and H-atom transfer from the active site thiol to the Ado• radical occur as concerted or stepwise processes at the enzyme active site, if they are stepwise, then the first step, Co−C bond homolysis, is surely the rate-determining step. This must be the case since the observed rate constant, k_f, for formation of cob(II)alamin at the active site is 13.7 s^{-1} at 37 °C, and the follow-up H atom transfer must be much faster than this. Measured second-order rate constants for the reaction of small molecular weight thiols with carbon-centered radicals are on the order of 5×10^7 to 1×10^8 M^{-1} s^{-1}.[37,39−45] This is slightly below the diffusion-controlled limit, suggesting that there is a real, but small, chemical barrier. Such a large rate would be expected to persist at the enzyme

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(49) Note that this means that the enzyme−dGTP complex, in its thiyl radical state, must bind cob(II)alamin and AdoH enormously tightly. However, as discussed below, there is ample precedent for extremely tight binding of cobalamins to proteins.[50−52]
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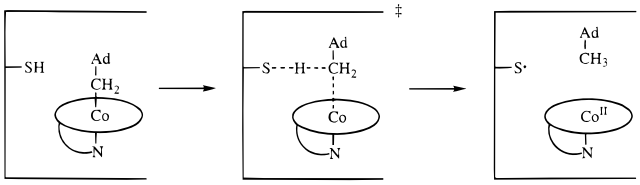
active site, providing a very high rate constant for follow-up H atom transfer.[56]

Given the current state of knowledge in this area, the following three mechanisms seem to be the most likely possibilities to contribute to the enthalpic catalysis of AdoCbl homolysis by RTPR.

1. Transition State Stabilization by Partial Hydrogen Atom Transfer from Cys408. If cleavage of the Co−C bond and hydrogen atom transfer from the active site −SH to the Ado• radical are concerted, then the active site thiol participates in the transition state for Co−C bond cleavage and can stabilize it as shown in the scheme.[13,58] The emerging unpaired electron in the transition state is thus delocalized onto the sulfur atom providing stabilization of the evolving carbon-centered radical. As pointed out above, the thiyl radical is about 6 kcal mol^{-1} (1×10^4-fold) more stable than an alkyl radical so that significant stabilization of the transition state could occur through such a mechanism. However, the discussion of the thermodynamics of the H atom transfer reaction above suggests that transition-state stabilization by this mechanism should be limited to <6 kcal mol^{-1}. Hence, if this mechanism does contribute to the catalysis of Co−C bond homolysis, it cannot be the only mechanism in play, since RTPR lowers the enthalpy of activation for Co−C homolysis by 13 kcal mol^{-1} and catalyzes the cleavage by 1.6×10^9-fold.



This is clearly an attractive mechanism for RTPR catalysis of AdoCbl homolysis, since it is known that there is a thiol function at the active site and that the products of AdoCbl homolysis are in fact cob(II)alamin, AdoH, and a thiyl radical. However, there is no experimental evidence to support such a mechanism to date. In fact, recent work by Sirovatka and Finke[59] shows that the enthalpy of activation for the nonenzymatic thermal homolysis of AdoCbl in ethylene glycol is unaffected by the presence of exogenous 2-mercaptoethanol (0.02−1.0 M). Moreover, the nucleoside product observed is AdoH, instead of 8,5′-anhydroadenosine, the latter being known to be rapidly formed ($k \sim 5 \times 10^5$ s^{-1} at 110 °C)[60] from Ado• in the absence of a radical trap or quench.[2−4,60,61] Hence, in this exogenous case at least, the thiol is capable of rapidly quenching the Ado• radical by H atom transfer but does not appear to participate in the transition state, suggesting that the coupled processes occur in a stepwise manner in this nonenzymatic, chemical precedent case. Hence, if such catalysis of Co−C bond cleavage by partial H-atom transfer does occur at the enzyme active site, it likely requires precise positioning of the −SH function relative to the bound AdoCbl.

2. Ground-State Co−C Bond Distortion. Molecular orbital[16,62] and molecular mechanics calculations[63] show that the strength of the Co−C bond is sensitive to angular distortion. Thus, ground-state distortion of the already large (121−124°[64]) Co−C−C bond angle in AdoCbl could represent an effective mechanism for catalyzing Co−C bond cleavage. Studies of the enzymatic activity of analogues of AdoCbl altered in the structure of the Ado ligand, some of which are partially active coenzymes and some of which are inactive inhibitors,[65−68] provide some experimental support for this hypothesis. The AdoCbl analogue N^1-deaza-AdoCbl is 56% as active as AdoCbl and N^3-deaza-AdoCbl is 46% as active as AdoCbl with diol dehydratase, while N^{10}-monomethyl-AdoCbl has 12% of the activity of AdoCbl with the glycerol dehydratase of *Aerobacter aerogenes*. In contrast, N^7-deaza-AdoCbl and N^{10},N^{10}-dimethylAdoCbl are completely inactive inhibitors with diol dehydrase. One way to interpret these results is that the N7 and N10 nitrogens participate in essential hydrogen-bonding contacts with the active site, the former participating in one hydrogen bond and the latter in two. While the purpose of such hydrogen bonds might only be to position the Ado ligand properly for catalysis, they might also exert an upward "pull" on the adenine ring, which lies parallel to the corrin ring over the "southern" quadrant of the molecule in all of the known crystal structures of AdoCbl.[64,69−73] Such an upward pull would increase the Co−C−C bond angle and provide bond bending strain to lower the Co−C bond energy.[63] The possibility of such catalysis by RTPR is currently under experimental examination.

3. Corrin Ring "Butterfly" Distortion/Mechanochemical Triggering Hypothesis. Many authors have suggested that the flexibility of the corrin ring may be exploited by B_{12}-dependent enzymes to labilize the Co−C bond of AdoCbl by increasing the upward folding[74] of the corrin to sterically strain this bond.[4,6−14,75−80] One version of this mechanism, referred to as "mechanochemical triggering," envisions enzymatic compression of the long (2.24 Å)[64] axial Co−N bond and a consequent increase in the steric interactions of the bulky 5,6-dimethylbenzimidazole (Bzm[1]) axial nucleotide with the underside of the corrin as the means by which an enzyme could engender an increase in the upward corrin fold. It is now known from spectroscopic measurements[81] and X-ray crystallography[82] that

(56) Medium effects at the active site could affect the rate of H atom transfer, but solvent effect studies[57] suggest that such effects would not lower the rate constant by more than a factor of 2.

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class I AdoCbl-dependent enzymes,[83] typified by methylmalonylCoA mutase and the other mutases, bind AdoCbl in its base-off form, with a histidine residue becoming the axial ligand, as is the case with CH_3Cbl[1] binding to the B_{12}-dependent methionine synthase.[84] It seems unlikely that these enzymes would substitute a less bulky histidine ligand for the pendent Bzm if such mechanochemical triggering were involved in catalysis. Indeed, both the X-ray crystal structure of methylmalonylCoA mutase[82] and EXAFS results[85] show that the histidine-ligated coenzyme at the mutase active site has an extraordinarily long axial Co−N bond. However, it is also now clear that the class II enzymes, including diol dehydratase[86,87] and evidently RTPR,[87] bind AdoCbl in its normal base-on form and are consequently candidates for this kind of catalysis.[88]

Several lines of recent work provide evidence supporting the viability of this kind of mechanochemical triggering as a catalytic mechanism for these enzymes. Kräutler et al.[96] prepared an analogue of CNCbl[1] in which the axial Bzm is replaced by the less bulky imidazole ligand (i.e., *Coβ*-cyanoimidazolylcobamide) by fermentation of *Propionibacterium shermanii* supplemented with imidazole. A comparison of the X-ray crystal structure with that of CNCbl itself showed that the corrin ring fold angle[97] was reduced from 18.0° in CNCbl to 11.3° in the imidazole derivative. This result implies steric interactions between the normal Bzm axial ligand and the corrin ring do indeed contribute significantly to the upward folding of the corrin ring.

More recently, Marques and co-workers[99] used NMR-restrained molecular modeling techniques to study the solution structure of CH_3Cbl, methylcobinamide ($CH_3Cbi^{+\ 1}$), the axial nucleotide-free analogue of CH_3Cbl in which the Bzm nucleotide has been chemically removed, and methyl-3,5,6-trimethylbenzimidazolylcobamide ($CH_3Me_3BzmCba^{+\ 1}$), the base-off analogue of CH_3Cbl in which the coordinating nitrogen (B3) of the axial nucleotide is blocked by a methyl group. This methodology permits structural characterization of the cobinamide and the trimethylbenzimidazolyl species which have never been successfully crystallized and consequently have not been characterized by X-ray crystallography. The corrin ring fold angle in CH_3Cbl was found to be 16.4° ± 1.9° (compared to the solid-state value of 15.8°),[100] but was reduced to 12.3° ± 1.8° in CH_3Cbi^+ and to 8.5° ± 0.4° in $CH_3Me_3BzmCba^+$. This result shows that the absence of a base in the lower axial ligand position allows the corrin to adopt a more planar structure and directly implies that, in complete cobalamins with Bzm lower axial ligands, the corrin ring is under strain.

Kratky and co-workers[101] have recently shown that the corrin ring fold angle in a series of 16 complete cobalamins for which the most accurate X-ray crystal structures are available is inversely related to the axial Co−N bond distance, with the fold angle decreasing from about 20° to about 10° as the axial Co−N bond length increases from 1.93 Å to about 2.27 Å. While this does not necessarily mean that such an inverse relationship would hold within a single cobalamin complex, it does suggest that the fold angle is coupled to the axial Co−N bond length and clearly shows the substantial flexibility of the corrin ring.

Finally, it must be pointed out that such steric compression of the axial Co−N bond of AdoCbl would come at a substantial energy cost. However, it is very clear from studies of vitamin B_{12} binding proteins that at least some proteins are capable of binding cobalamins very tightly, generating large amounts of binding energy.[50−52] In the extreme case, the haptocorrin from chicken serum binds CNCbl with a binding constant, K_b, of 9.3×10^{15} M^{-1} at 37 °C,[52] representing the release of 22.7 kcal mol^{-1} of binding free energy. Should the intrinsic affinity of AdoCbl for RTPR be this high, the observed rather weak binding ($K_b = 2.4 \times 10^4$, $\Delta G_b = -6.2$ kcal mol^{-1}) suggests that a large amount of binding free energy (as much as 16.5 kcal mol^{-1}) is potentially available for distortion of the coenzyme by compression of its axial Co−N bond. The fact that the enzyme must bind cob(II)alamin + AdoH very much more tightly[49] than it binds AdoCbl is consistent with the idea that the intrinsic affinity of RTPR for cobalamins is very high but that a substantial amount of the energy released on binding AdoCbl is used to distort the coenzyme, possibly by compressing the axial Co−C bond.

The mechanochemical triggering mechanism is thus a viable and attractive possibility for enzymatic catalysis of Co−C bond homolysis. However, to date, most of the available evidence supporting this mechanism is structural and all of it is non-

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enzymatic. Clearly, studies with AdoCbl-dependent enzymes will be necessary to validate this mechanism.

Acknowledgment. This research was supported by the National Institute of General Medical Sciences, Grant GM 48858. The authors are grateful to Prof. J. Stubbe, Massachusetts Institute of Technology, for the generous gift of *E. coli* HB101/PSQUIRE and for sharing preprints of her own studies.[20,21] The authors are also grateful to Prof. R. G. Finke, Colorado State University, for numerous helpful discussions of these results and their interpretation.

JA981729Z