Enzymatic Activity of Coenzyme B_{12} Derivatives with Altered Axial Nucleotides: Probing the Mechanochemical Triggering Hypothesis in Ribonucleotide Reductase

Kenneth L. Brown,* Xiang Zou, Jing Li, and Guodong Chen

Department of Chemistry and Biochemistry, Ohio University, Athens, Ohio 45701

Received July 26, 2001

Theoretical studies (*J. Inorg. Biochem.* **2001**, *83*, 121) of the involvement of the bulky 5,6-dimethylbenzimidazole (Dmbz) ligand of coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl) in the mechanism of activation of the carbon−cobalt bond of the coenzyme for homolytic cleavage by AdoCbl-dependent enzymes (the "mechanochemical triggering" mechanisms) have shown that a purely steric, ground-state mechanism can supply only a few kilocalories per mole (of the observed 13−16 kcal mol^{-1}) of activation, but that an electronic mechanism, operating to stabilize the transition state, can explain all of the observed catalytic effect. To address these mechanisms experimentally, analogues of AdoCbl in which the Dmbz ligand is replaced by benzimidazole (Ado(Bzim)Cbl) or by imidazole (Ado(Im)Cbl) have been prepared and characterized. Both of these analogues support turnover in the AdoCbl-dependent ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* at 100% of the activity of AdoCbl itself, but the Ado(Im)Cbl analogue has a significantly higher K_m. 5′-Deoxyadenosylcobinamide, the analogue in which the axial nucleotide has been chemically removed, in contrast, is inactive in the spectrophotometric assay, which indicates that it has at most 1% of the activity of AdoCbl. Stopped-flow spectrophotometric measurements of the formation of cob(II)alamin at the enzyme active site show that RTPR binds Ado(Bzim)Cbl slightly more weakly than it does AdoCbl, but binds Ado(Im)Cbl 8-fold more weakly. While the equilibrium constant for cob(II)alamin formation is nearly the same for Ado(Bzim)Cbl and AdoCbl, it is 5-fold smaller for Ado(Im)Cbl. Finally, the forward rate constant for enzyme-induced Co−C bond homolysis was about the same for Ado(Bzim)Cbl and for AdoCbl but was 17-fold smaller for Ado(Im)Cbl. These results are consistent with a small contribution from ground-state mechanochemical triggering, but they do not in themselves rule out transition-state mechanical triggering.

The enormous catalytic efficiency of the 5′-deoxyadenosylcobalamin- (AdoCbl, coenzyme B_{12}, Figure 1) dependent enzymes[1−3] for homolysis of the carbon−cobalt bond of the coenzyme remains unexplained for any such enzyme. For those enzymes in which the axial nucleotide remains coordinated to the metal upon coenzyme binding[4−10] (the class II[11,12] eliminases and the ribonucleoside triphosphate reductases), mechanisms prominently involving the axial 5,6-dimethylbenzimidazole (Dmbz) ligand (the so-called "mechanochemical triggering" mechanisms) have long been of interest, since they might provide a rationale both for the steric bulkiness of the naturally occurring axial nucleotide and for the fact that the cobalamin system is pentadentate.

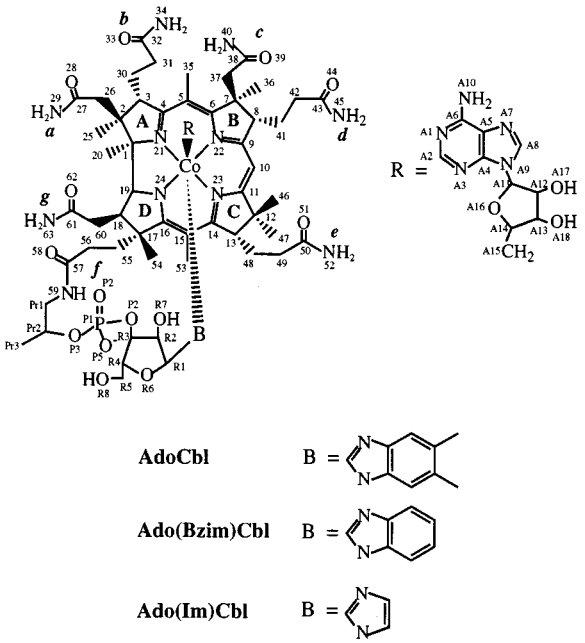


Figure 1. Structure and numbering scheme for 5′-deoxyadenosylcobalamin (AdoCbl) and the analogues with altered axial nucleotides utilized in this study.

* To whom correspondence should be addressed. Phone: 740-593-9465. Fax: 740-593-0148. E-mail: brownk3@ohiou.edu.

(1) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 4820.
(2) Brown, K. L.; Li, J. *J. Am. Chem. Soc.* **1998**, *120*, 9466.
(3) Brown, K. L.; Zou, X. *J. Inorg. Biochem.* **1999**, *77*, 185.
(4) Yamanishi, M.; Yamada, S.; Muguruma, H.; Murakami, Y.; Tobimatsu, T.; Ishida, A.; Yamauchi, J.; Toraya, T. *Biochemistry* **1998**, *37*, 4799.
(5) Abend, A.; Nitsche, R.; Bandarian, V.; Stupperich, E.; Rétey, J. *Angew. Chem., Int. Ed.* **1998**, *37*, 625.
(6) Ke, S.-C.; Torrent, M.; Museav, D. G.; Morokuma, K.; Warnke, K. *Biochemistry* **1999**, *38*, 12681.
(7) Lawrence, C. C.; Gerfen, G. J.; Samano, V.; Nitsche, M. J.; Robins, M. J.; Rétey, J.; Stubbe, J. *J. Biol. Chem.* **1999**, *274*, 7039.
(8) Abend, A.; Bandarian, V.; Nitsche, R.; Stupperich, E.; Rétey, J.; Reed, G. H. *Arch. Biochem. Biophys.* **1999**, *370*, 138.
(9) Shibata, N.; Masuda, J.; Tobimatsu, T.; Toraya, T.; Sato, K.; Morimoto, Y.; Yasuoka, N. *Structure* **1999**, *7*, 997.
(10) Masuda, J.; Shibata, N.; Morimoto, Y.; Toraya, T.; Yasuoka, N. *Structure* **2000**, *8*, 775.
(11) Bückel, W.; Golding, B. T. *Chem. Soc. Rev.* **1996**, 329.
(12) Golding, B. T.; Bückel, W. In *Comprehensive Biochemical Catalysis*; Sinnott, M. L., Ed.; Academic Press: London, 1997; Vol. 3, pp 239−259.

10.1021/ic010796i CCC: $20.00
Published on Web 09/21/2001

Recent molecular modeling studies[13,14] have investigated the feasibility of classical (i.e., ground state) mechanochemical triggering, in which enzyme-mediated steric compression of the long (2.24 Å[15,16]) axial Co−N bond of AdoCbl potentially labilizes the carbon−cobalt bond of the coenzyme. Such compression is indeed found to increase the upward fold of the corrin ring,[17] increase the Co−C bond length, and increase the Co−C−C bond angle, and all three effects are considerably smaller when the bulky Dmbz ligand is replaced by imidazole (Coβ-5′-deoxyadenosylimidazolylcobamide, Ado(Im)Cbl, Figure 1) demonstrating the steric nature of the mechanochemical effect. However, the net weakening of the carbon−cobalt bond by this effect is estimated to be only a few kilocalories per mole, far short of the 13−16 kcal mol^{-1} observed for enzymatic catalysis of Co−C bond homolysis.[1−3] This result is in essential agreement with recent density functional theory calculations,[19] which suggest that steric mechanisms involving the axial nucleotide are not particularly important in activating the carbon−cobalt bond for homolysis.[20]

Intriguing experiments by Sirovatka and Finke[22] have shown that exogenous bulky ligands increase the rate of thermal carbon−cobalt bond homolysis in AdoCbi$^+$, the coenzyme analogue in which the axial nucleotide has been chemically removed, but they do so without detectable binding to the ground state of AdoCbi$^+$. This means that they must exert their effects on the homolysis transition state and suggests another possibility whereby carbon−cobalt bond homolysis could be catalyzed by manipulation of the axial Co−N bond length. In this scenario (dubbed transition-state mechanochemical triggering[13]), the transition state is stabilized electronically by increased orbital overlap between the axial ligand and the metal as a result of compression of the axial Co−N bond. Molecular modeling and MO calculations show that the homolysis transition state can be stabilized by ca. 14 kcal mol^{-1} for AdoCbl and by as much as 15.5 kcal mol^{-1} for Ado(Im)Cbl by this mechanism, enough to completely explain the observed enzymatic catalysis.[13] Importantly, the differential effect of the steric bulk of the axial ligand on the energetics of the two mechanochemical triggering mechanisms suggests that they may be distinguished experimentally by studies of the coenzyme analogue Ado(Im)Cbl.[13] Since the ground-state mechanism is a steric mechanism, an enzyme utilizing this kind of mechanochemical triggering would be expected to be less efficient at catalyzing carbon−cobalt bond homolysis in Ado(Im)Cbl than in AdoCbl. In contrast, because the transition-state mechanochemical triggering effect is electronic, and because the energy cost for compressing the axial Co−N bond in Ado(Im)Cbl is smaller than that for AdoCbl, an enzyme utilizing this mechanism would be expected to be at least as efficient, if not more efficient, at catalyzing carbon−cobalt homolysis in Ado(Im)Cbl. To begin to address these questions, we now report the first synthesis and characterization of the AdoCbl analogues, Ado(Bzim)Cbl (Coβ-5′-deoxyadenosylbenzimidazolylcobamide) and Ado(Im)Cbl, in which the Dmbz ligand of AdoCbl is replaced by benzimidazole or imidazole, respectively (Figure 1), and we report the coenzymic activity of these analogues with the AdoCbl-dependent ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii*.

Experimental Section

L. leichmannii RTPR was obtained from transformed *Eschericia coli* HB101/pSQUIRE[23] (a gift from J. Stubbe, Massachusetts Institute of Technology) and was purified as described by Booker and Stubbe.[23] Auxiliary proteins for the coupled spectrophotometric assay, *E. coli* thioredoxin and thioredoxin reductase, were obtained from overproducing strains SK 3981[24] and K91/pMR14[25] as described.

The cyano derivatives of the Cbl analogues, Coβ-cyanobenzimidazolylcobamide (CN(Bzim)Cbl) and Coβ-cyanoimidazolylcobamide (CN(Im)Cbl), were obtained by "guided biosynthesis"[26−29] using fermentation of *Propionibacterium shermanii* on media supplemented with benzimidazole or imidazole, respectively, employing a modification of the method of Renz.[28,29] *P. shermanii* (ATC 13673) was grown on solid micro assay culture agar (DIFCO) in a test tube and was made anaerobic by overlaying the culture with unsolidified, sterilized agar solution. After 5 d of growth at 30 °C, the culture was stored at 4 °C until use.

For fermentation, a bacterial colony from the culture storage tube was grown in 4 mL of micro inoculum broth (DIFCO) for 4 h at 30 °C, diluted with 8 mL of micro inoculum broth, and grown for another 16 h at 30 °C. Subcultures were grown in a medium of the following composition. Corn steep (Sigma), 5.5 g, was suspended in 100 mL of water, brought to pH 7.1 with 50% NaOH, and autoclaved at 121−124 °C for 15 min. After cooling, 1.1 mL of phosphate buffer (2 g of $K_3PO_4 \cdot H_2O$ and 2 g of $NaH_2PO_4 \cdot 2H_2O$ per 10 mL), 1.1 mL of yeast extract (Sigma, 5 g/10 mL), and 2.2 mL of 50% aqueous glucose were added, followed by the 12 mL of broth culture which had incubated for 20 h. Magnesium chloride, 0.11 mL (0.4 g of $MgCl_2 \cdot 6H_2O$ per mL) and 0.11 mL of cobalt sulfate solution (12 mg of $CoSO_4 \cdot 7H_2O$ per mL) were then added, and the pH was adjusted to 7.0 with a sodium carbonate solution (240 g/L). The culture was incubated without shaking for 2 d at 30 °C, during which time the pH was readjusted to 7.0 with sodium carbonate solution, and 2.2 mL of 50% aqueous glucose was added every 12 h. This culture was then used to inoculate (at 10%) four 200-mL batches of corn steep media (prepared proportionally to the above), and these cultures were grown for 2 d and treated similarly as above. These final subcultures were then used to inoculate (at 10%) four 500-mL cultures of the same media in 4 L flasks which were incubated at 30 °C for 10 d without shaking. The pH was adjusted, as above, twice a day for the first 2 d and once a day thereafter. Glucose, 44 mL of 50% aqueous solution, was added to each flask every 12 h for the first 2 d, every 24 h from day 3 to day 6, and 60 mL was added daily from day 7 to day 9. Benzimidazole or imidazole (20 mg in ethanol) was added to each flask at the end of the second day, and 40 mg (in ethanol) was added at the end of the third and fifth days. At the end of the tenth day, the cells were harvested by centrifugation at 9000*g* for 30 min to yield 750 g of wet cell paste.

The cell paste was suspended in 4 L of 0.066 M phosphate buffer with a pH 7.0 and containing 4 g of KCN and was autoclaved at 121−

(13) Brown, K. L.; Marques, H. M. *J. Inorg. Biochem.* **2001**, *83*, 121.
(14) Sirovatka, J. M.; Rappé, A. K.; Finke, R. G. *Inorg. Chim. Acta* **2000**, *300*, 545.
(15) Lenhert, P. G. *Proc. R. Soc. London, Ser. A* **1968**, *A303*, 45.
(16) Savage, H. F. J.; Leadley, P. F.; Finney, J. L.; Timmons, P. A. *Acta Crystallogr., Sect. B* **1987**, *43*, 947.
(17) Defined as the angle between the normals to the least-squares planes through N21, C4, C5, C6, N22, C9, and C10, and through N24, C16, C15, C14, N23, C11, and C10.[18]
(18) Glusker, J. P. In *B_{12}*; Dolphin, D., Ed; Wiley-Interscience: New York, 1982; Vol. 1, p 23.
(19) Andruniow, T.; Zgierski, M. Z.; Kozlowski, P. M. *Chem. Phys. Lett.* **2000**, *331*, 509.
(20) Interestingly, more recent DFT calculations[21] show a small but consistent increase in R(Im)Cbl's Co−C bond dissociation energy relative to RCbl's bond dissociation energy, suggesting that there is at least a small steric effect of the axial base on the homolytic lability of the carbon−cobalt bond.
(21) Andruniow, T.; Zgierski, M. Z.; Kozlowski, P. M. *J. Am. Chem. Soc.* **2001**, *123*, 2679.
(22) Sirovatka, J. M.; Finke, R. G. *Inorg. Chem.* **1999**, *38*, 1697.
(23) Booker, S.; Stubbe, J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8352.
(24) ATCC 67286m. U.S. Patent 4,795,699.
(25) Russel, R.; Model, P. *J. Bacteriol.* **1985**, *163*, 238.
(26) Kräutler, B.; Konrat, R.; Stupperich, E.; Färber, G.; Gruber, K.; Kratky, C. *Inorg. Chem.* **1994**, *33*, 4128.
(27) Stupperich, E.; Steiner, I.; Rühlemann, M. *Anal. Biochem.* **1986**, *155*, 365−370.
(28) Hörig, J. A.; Renz, P.; Heckmann, G. *J. Biol. Chem.* **1978**, *253*, 7410.
(29) Renz, P. *Methods Enzymol.* **1971**, *18C*, 82.

124 °C for 20 min. After cooling, a clear, reddish supernatant was obtained by centrifugation at 15 000*g* for 20 min. The supernatant was loaded onto a 4 × 15 cm column of GC-161C (TosoHaas) absorbent and was washed with 2 L of water and 2 L of 5% aqueous acetonitrile. The corrinoids were eluted with 15% aqueous acetonitrile, and the eluate was reduced to a small volume by rotary evaporation. This preparation was further purified by semipreparative HPLC using a 10 × 250 mm C-8 column as previously described.[30] To obtain high purity CN(B)-Cbl, it was necessary to change the HPLC retention time of the product to effect separation from unidentified impurities. This was accomplished by conversion of the CN(B)Cbl to its aqua form by reduction with zinc in dilute acid followed by reoxidation in aerobic dilute HCl. Final purification by HPLC then permitted separation from the impurities that had coeluted with the cyano derivative. The purified aqua corrinoid was converted to its cyano derivative by reaction with KCN and desalting on a CG-161C column. The yield of purified CN(B)Cbl is approximately 15 mg. CN(Bzim)Cbl and CN(Im)Cbl were converted to their 5′-deoxyadenosyl derivatives by reduction with zinc in acetic acid followed by reaction with 5′-chloro-5′-deoxyadenosine[31] as described previously.[32]

Steady-state enzyme assays were performed using the coupled spectrophotometric assay[33] in which RTPR is reduced by thioredoxin which in turn is reduced by NADPH using thioredoxin reductase, as previously described.[2] UV−visible spectra and single wavelength measurements were made using a Cary 3 spectrophotometer, the sample compartment of which was thermostated to 37 °C when necessary using an electronic temperature controller.

Stopped-flow kinetic measurements of the RTPR-induced formation of the Co(II) corrinoid from Ado(Bzim)Cbl and Ado(Im)Cbl were made at the wavelength of maximal spectral change using an Applied Photophysics SX.17MV stopped-flow spectrophotometer equipped with an AN1 anaerobic accessory, as described previously.[2] One- and two-dimensional NMR spectra were obtained using a Varian Unity Inova 500 MHz NMR spectrometer, as previously described.[34]

Results and Discussion

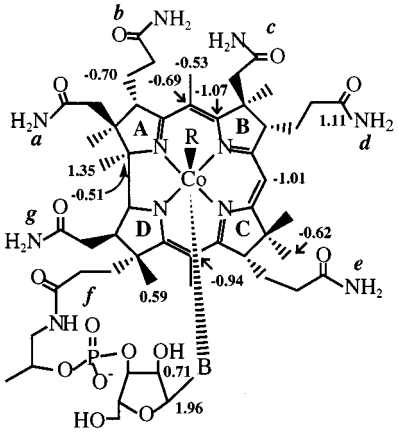
Preparation and Characterization of Ado(Bzim)Cbl and Ado(Im)Cbl. Although the preparation and characterization of the vitamin B_{12} analogue containing imidazole as the axial base has been described,[26] the coenzyme analogues with imidazole or benzimidazole, important compounds for understanding the role of the axial base in the activation of AdoCbl, have never been reported. These are, in fact, readily obtainable by reductive adenosylation of the cyano derivatives which can be obtained in small amounts from the admittedly tedious fermentation of *P. shermanii* on media supplemented with benzimidazole or imidazole.

The UV−visible spectra of Ado(Bzim)Cbl and Ado(Im)Cbl (available as Supporting Information) are quite similar to that of AdoCbl itself. There are significant differences in the molar absorbtivity at the UV maximum of 260 nm, and there are small differences in the region from 290 to 350 nm. More importantly, the position of the α band is shifted from 550 nm in AdoCbl to 540 nm in Ado(Bzim)Cbl and to 532 nm in Ado(Im)Cbl. In the spectra of the Co(II) derivatives, a shoulder near 310 nm in cob(II)alamin becomes a more pronounced band in the spectra of the Co(II)−Bzim and −Im derivatives, and a shift of the α band of the Co(II) derivative is observed (476 nm in cob(II)-alamin, 474 nm in the Bzim derivative, and 458 nm in the Im derivative). This means that the molar absorbtivity change upon homolysis of the carbon−cobalt bond is different for all three complexes, and the wavelength of maximal spectral change is different for Ado(Im)Cbl.

Complete assignments of the ^{13}C and ^{1}H NMR spectra of Ado(Bzim)Cbl and Ado(Im)Cbl were made using the now standard battery of two-dimensional NMR spectra and assignment strategies.[34] Spectral assignments and correlation tables for both analogues are available as Supporting Information.

With the exception of the resonances for the axial base, the ^{13}C NMR spectra of Ado(Bzim)Cbl and AdoCbl are nearly identical. The only significant differences occur for C30, C19, and A15, the resonances for which are shifted upfield by 0.53, 0.87, and 0.90 ppm, respectively, in Ado(Bzim)Cbl relative to AdoCbl. The upfield shift of A15, the cobalt-bound carbon, is somewhat surprising since the basicity of benzimidazole ($pK_a = 5.53$[35]) is nearly identical to that of α-ribazole-3′-phosphate ($pK_a = 5.56$[36]), Cbl's detached axial nucleotide, so that the effect is very unlikely to be due to a ground-state electronic trans influence. In the absence of an X-ray crystal structure (neither Ado(Bzim)Cbl nor Ado(Im)Cbl has yet yielded to crystallization), we cannot determine if this shift of the cobalt-bound carbon resonance reflects a significant difference in the Co−C bond length between Ado(Bzim)Cbl and AdoCbl.

The situation for Ado(Im)Cbl is quite different as there are numerous changes in chemical shift in the corrin ring and peripheral substituents compared to AdoCbl. These are shown in the structure below as the signed difference in the ^{13}C chemical shift between Ado(Im)Cbl and AdoCbl.



In addition to the changes shown, the A15 resonance is shifted 2.61 ppm downfield in Ado(Im)Cbl. In this case, the perturbation of the cobalt-bound carbon resonance could well be the result of a ground-state electronic trans influence since imidazole ($pK_a = 7.24$[37]) is considerably more basic than α-ribazole-3′-phosphate, and the resonance is shifted in the direction expected for such a trans effect.[38] Similarly, the chemical shift differences in the corrin ring at C1, C5, C6, C10, and C15, even though several of them are four bonds removed from the liganding imidazole nitrogen, could well be due to the electronic effect of coordination by the more basic axial base. However, it is difficult to see how the chemical shift differences at the C47, C54, and C20 methyls, or the difference at C30, could be due

(30) (a) Jacobsen, D. W.; Green, R.; Brown, K. L. *Methods Enzymol*. **1986**, *123*, 14. (b) Brown, K. L.; Zou, X.; Salmon, L. *Inorg. Chem*. **1991**, *30*, 1949. (c) Brown, K. L.; Salmon, L.; Kirby, J. A. *Organometallics* **1992**, *11*, 422.
(31) Kikugawa, K.; Ichino, M. *Tetrahedron Lett*. **1971**, 87.
(32) Brown, K. L.; Zou, X. *J. Am. Chem. Soc*. **1992**, *114*, 9643.
(33) Blakeley, R. L. *Methods Enzymol*. **1978**, *51*, 246.
(34) Brown, K. L. In *The Chemistry and Biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley and Sons: New York, 1999; p 197.
(35) Albert, A.; Goldacre, R.; Phillips, J. *J. Chem. Soc*. **1948**, 2492.
(36) Brown, K. L. *J. Am. Chem. Soc*. **1987**, *109*, 2277.
(37) Marques, H. M.; Egan, T. J.; Marsh, J. H.; Mellow, J. R.; Munro, O. Q. *Inorg. Chim. Acta* **1989**, *166*, 249.
(38) Bied-Charreton, C.; Septe, B.; Gaudemer, A. *Org. Magn. Reson*. **1975**, *7*, 116.

Table 1. Steady-State Kinetics for RTPR with AdoCbl and the Analogues Ado(Bzim)Cbl and Ado(Im)Cbl

coenzyme	V_{max} (%)	K_m (μM)	K_i (μM)
AdoCbl[a]	100	0.18 ± 0.01	
Ado(Bzim)Cbl	104 ± 3	0.14 ± 0.01	
Ado(Im)Cbl	105 ± 9	13 ± 1	
$AdoCbi^+$	$\leq 1\%$		30 ± 4[b]

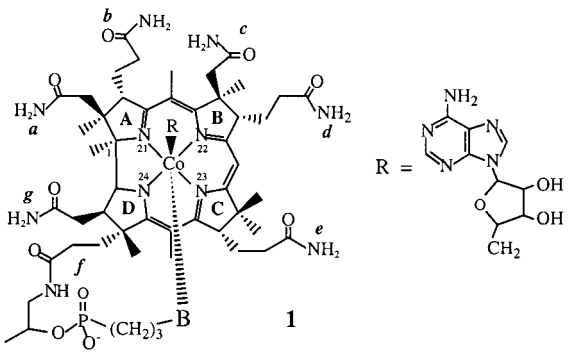
[a] Reference 2. [b] Pure uncompetitive inhibition (see Figure 2).

to such an electronic effect. It is quite possible that these differences result from a change in the corrin ring fold angle, since this fold angle in CN(Im)Cbl is reduced by 6.7° compared to that in CNCbl,[26] and molecular mechanics calculations[13] predict that Ado(Im)Cbl will also have a smaller fold angle than AdoCbl. The chemical shift changes at R1 and R2 in the ribose of the axial nucleotide are anticipated due to the change in the nitrogenous base.

However, the rather large chemical shift change at C43, the *d* side chain carbonyl carbon, is quite interesting. This change is accompanied by large changes in the ^1H chemical shift of the *d* amide N−H protons (0.53 and 0.76 ppm downfield for the anti and syn protons, respectively). We have previously shown[39] that for RCbl's in which the axial Dmbz ligand is relatively weakly bound (including AdoCbl[40]), there is a hydrogen-bonded interaction between the *d* side chain amide and the glycoside nitrogen of the axial nucleotide. Evidently in Ado(Im)Cbl, this hydrogen bond either is altered in strength or does not occur. In fact, since the chemical shifts of the *d* amide hydrogens in Ado(Im)Cbl resemble those of $AdoCbi^+$ (the analogue in which the axial nucleotide is missing) quite closely,[40] it seems most likely that this hydrogen-bonding interaction is absent in Ado(Im)Cbl.

Steady-State Enzyme Kinetics. The ability of the new coenzyme analogues to support steady-state enzyme turnover in the class II ribonucleoside triphosphate reductase from *L. leichmannii* was tested using the coupled spectrophotometric assay,[33] in which the natural reductant, thioredoxin, was used as the reducing agent with ATP as the substrate. The results are shown in Table 1. Both Ado(Bzim)Cbl and Ado(Im)Cbl supported steady-state turnover at the same level as AdoCbl itself. However, while the apparent K_m for Ado(Bzim)Cbl was essentially the same as that for AdoCbl itself, the K_m for Ado(Im)Cbl was some 70-fold higher.

These results are actually quite surprising. Toraya and co-workers[41−44] have studied the coenzymic activity of semisynthetic AdoCbl analogues in which the ribose of the axial nucleotide is substituted by a trimethylene group (structure **1**).



The analogue with Dmbz as the axial base had 59% of the activity of AdoCbl with diol dehydratase and had a K_m value (0.82 μM) essentially the same as that of AdoCbl (0.80 μM), indicating that the substitution provides an analogue with excellent binding and substantial coenzymic activity. When the axial base was Bzim, however, the activity fell to 45% of that of AdoCbl (with $K_m = 2.5$ μM), and for the imidazole analogue, the activity was only 8% (with $K_m = 0.99$ μM). We thus anticipated that the Ado(Bzim)Cbl and Ado(Im)Cbl analogues would have decreased coenzymic activity with RTPR which is evidently not the case.

However, the situation with diol dehydratase is more complicated in that this enzyme undergoes a mechanism-based inhibition[45] during normal turnover, in which the carbon−cobalt bond of the coenzyme is irreversibly cleaved, and the enzyme is irreversibly inhibited. The ratio of k_{cat} to the rate constant for inactivation (k_{inact}) is very high for AdoCbl (1.44×10^6), indicating that over one million turnovers occur before an inactivation event when the normal coenzyme is present. In the trimethylene analogues, k_{cat}/k_{inact} progressively decreases and reaches 3.3×10^3 for the imidazole complex. These results (and others with axial nucleotide-free analogues, vide infra) have been interpreted to mean that the axial base is important in stabilizing the highly reactive radical intermediates involved in the enzymatic reaction (implying that cob(II)alamin plays a direct role in such stabilization) and that the bulk of the axial base is a more important determinant of the efficacy of such stabilization than the basicity.[43] As no such mechanism-based inhibition occurs for RTPR, the situation with respect to AdoCbl analogues with altered axial bases may not be comparable for the two enzymes. Of course, it also remains possible that the mechanism of activation of the coenzyme by RTPR and diol dehydratase is different.

We also tested the activity of the axial nucleotide-free coenzyme analogue, $AdoCbi^+$, with RTPR and found no activity detectable by the coupled spectrophotometric assay (Table 1). Based on the sensitivity of the assay, an upper limit of 1% could be placed on the activity of $AdoCbi^+$. This seems to indicate that the axial Bzim contributes at least 3 kcal mol^{-1} to the enzymatic activation of AdoCbl for homolysis by RTPR.

$AdoCbi^+$ did, however, act as an effective inhibitor of RTPR albeit with fairly weak binding (Table 1). Interestingly, the inhibition pattern was pure uncompetitive (Figure 2), the classical interpretation of which involves the simultaneous binding of inhibitor and substrate (or coenzyme, as in this case) to the enzyme. This observation, to our knowledge the first example of uncompetitive inhibition for a class II AdoCbl-dependent enzyme, suggests the interesting possibility that there are two coenzyme binding sites per catalytically active unit of RTPR.

Toraya and co-workers,[46,47] and others,[48−50] have also investigated the activity of $AdoCbi^+$ and other axial nucleotide-free

(39) Brown, K. L.; Evans, D. R. *Inorg. Chem.* **1993**, *32*, 2544.
(40) Brown, K. L.; Zou, X. *J. Am. Chem. Soc.* **1993**, *115*, 1478.
(41) Toraya, T.; Ishida, A. *J. Biol. Chem.* **1991**, *266*, 5430.
(42) Toraya, T.; Miyoshi, S.; Mori, M.; Wada, K. *Biochim. Biophys. Acta* **1994**, *1204*, 169.
(43) Toraya, T. In *Vitamin B_{12} and B_{12}-Proteins*; Kräutler, B., Arigoni, D., Golding, B. T., Eds.; Wiley-VCH: Weinheim, 1998; p 303.
(44) Toraya, T. In *The Chemistry and Biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley and Sons: New York, 1999; p 783.
(45) Yamanishi, M.; Yamada, S.; Ishida, A.; Yamauchi, J.; Toraya, T. *J. Biochem.* **1998**, *124*, 598.
(46) Ishida, A.; Ichikawa, M.; Kobayashi, K.; Hitomi, T.; Kojima, S.; Toraya, T. *J. Nutr. Sci. Vitaminol.* **1993**, *39*, 115.
(47) Ishida, A.; Toraya, T. *Biochemistry* **1993**, *32*, 1535.
(48) Zagalac, B.; Pawelkiewicz, J. *Acta Biochim. Pol.* **1964**, *11*, 49.
(49) Yakusheva, M. I.; Poznanskaya, T. A.; Pospelova, I. P.; Rudakova, I. P.; Yurkevich, A. M.; Yakovlev, V. A. *Biochim. Biophys. Acta* **1977**, *484*, 216.

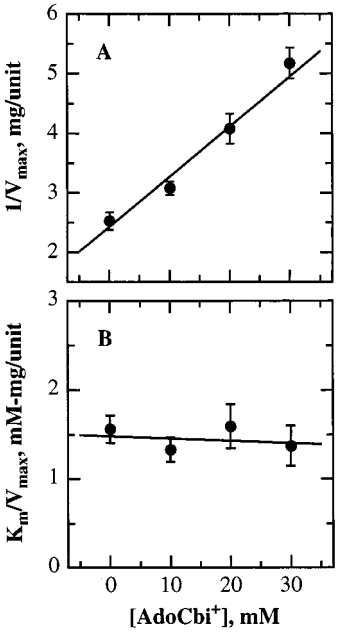


Figure 2. Secondary plots of the kinetics of inhibition of RTPR by $AdoCbi^+$. The solid lines are weighted linear regressions. (A) Plot of $1/V_{max}$ vs [$AdoCbi^+$] (slope = $(8.4 \pm 0.9) \times 10^{-2}$ mg $unit^{-1}$ μM^{-1}, intercept = 2.4 ± 0.1 mg $unit^{-1}$). (B) Plot of K_m/V_{max} vs [$AdoCbi^+$] (slope = $(-2.9 \pm 9) \times 10^{-3}$ mg $unit^{-1}$, intercept = 1.5 ± 0.1 μM mg $unit^{-1}$).

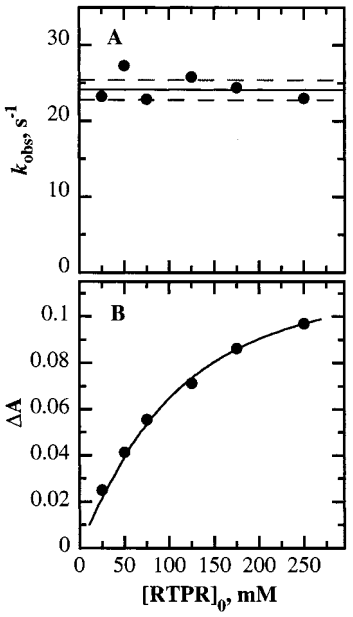
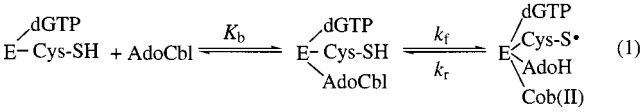


Figure 3. (A) Dependence of the observed rate constant for enzymatic formation of cob(II)alamin from Ado(Bzim)Cbl on the initial concentration of RTPR at 37 °C. The solid line is the average value (24.2 ± 1.4 s^{-1}), and the dashed lines are this value plus or minus one standard deviation. (B) Dependence of the absorbance change, ΔA, accompanying the reversible formation of cob(II)alamin from Ado(Bzim)Cbl on the initial concentration of RTPR. The solid line is a least-squares fit to a binding isotherm,[2] from which the values $\Delta A_\infty = 0.126 \pm 0.004$ and $K_b = (1.72 \pm 0.20) \times 10^4$ M^{-1} were obtained.

analogues with diol dehydratase. $AdoCbi^+$ and its phosphate were inactive as coenzymes but were weakly bound inhibitors. Interestingly, the methyl phosphate of $AdoCbi^+$, which was inactive as a coenzyme but a good competitive inhibitor ($K_i = 2.5$ μM[47]), undergoes slow, stoichiometric carbon−cobalt bond homolysis in the presence of substrate to yield an enzyme-bound cob(II)alamin species.

RTPR-Induced Carbon−Cobalt Bond Homolysis. The kinetics of enzymatic cleavage of the carbon−cobalt bond of Ado(Bzim)Cbl and Ado(Im)Cbl were studied using a dithiothreitol reductant, the allosteric activator dGTP, and no NTP substrate,[51,52] as described previously. The results (shown in Figure 3 for Ado(Bzim)Cbl) were qualitatively similar to those previously obtained with AdoCbl.[2,53] The observed first-order rate constant for the rapid, reversible formation of the Co(II) species at the enzyme active site was independent of the enzyme concentration, but the extent of its formation, as indicated by the absorbance change, ΔA (Figure 3B), increased hyperbolically with [RTPR]. As we have discussed,[2] the *simplest* interpretation of these results is that rapid, but fairly weak, coenzyme binding (K_b) is followed by the observable cleavage of the Co−C bond (k_f and k_r, eq 1). Equation 1 reflects the fact that the species formed at the active site is now known to contain a thiyl radical derived from the active site Cys408 residue via H-atom transfer to the Ado• radical formed from carbon−cobalt bond homolysis.[52]



(1)

The rate and equilibrium constants obtained from the analysis thus represent those for the coupled processes of Co−C bond homolysis and the follow-up H-atom transfer. For convenience, these processes are depicted as being concerted in the equation, but it is not yet known if they are in fact concerted or stepwise.

From the dependence of ΔA on [RTPR] (Figure 3B), the binding constant K_b and the maximal absorbance change when the coenzyme is "saturated" with enzyme can be obtained.[2] From the latter and the measured molar spectral change for stoichiometric formation of the Co(II) species from the starting AdoCbl analogue, the equilibrium constant ($K_{eq} = k_f/k_r$) for the bond cleavage step can be obtained,[52] and, hence, k_f and k_r can be calculated since $k_{obs} = k_f + k_r$. The results for both Ado(Bzim)-Cbl and Ado(Im)Cbl are summarized in Table 2, along with our previous results for AdoCbl.[2]

In a similar experiment with $AdoCbi^+$, no evidence of enzyme-induced carbon−cobalt bond cleavage could be detected. Interestingly, when $AdoCbi^+$ was mixed with reduced RTPR, there was a small absorbance decrease at 380 and 528 nm associated with a rate constant of about 4 s^{-1}. This is in contrast to the spectral changes observed when $AdoCbi^+$ is converted to cob(II)inamide by anaerobic photolysis (Supporting Information) which display isosbestic points at 309, 353, 407, 437, and 456 nm with increasing absorbance at 380 nm but decreasing absorbance at 528 nm. Thus, it is clear that the enzyme does not induce carbon−cobalt bond homolysis in

(50) Eberhard, G.; Schlayer, H.; Joseph, H.; Fridrich, E.; Utz, B.; Müller, O. *Biol. Chem. Hoppe−Seyler* **1988**, *369*, 1091.
(51) Tamao, T.; Blakley, R. L. *Biochemistry* **1973**, *12*, 24.
(52) Licht, S.; Gerfen, G. J.; Stubbe, J. *Science* **1996**, *271*, 477.
(53) It must be pointed out that our results for the temperature dependence of the RTPR-induced homolysis of AdoCbl lead to activation parameters that differ greatly from those obtained by the Stubbe group.[54] However, at 37 °C, our results for AdoCbl ($k_{obs} = 37 \pm 3$ s^{-1}, $K_b = (2.7 \pm 0.5) \times 10^4$ M^{-1}, $K_{eq} = 0.59 \pm 0.03$)[2] agree precisely with those of the Stubbe group ($k_{obs} = 42$ s^{-1},[52] Tamao and Blakley,[51] 48 s^{-1}; $K_b = (2.1 \pm 0.5) \times 10^4$ M^{-1},[54] Singh et al.,[55] $(2.1 \pm 0.5) \times 10^4$ M^{-1}); $K_{eq} = 0.65 \pm 0.05$ using the spectrophotometric method, 2.0 ± 0.3 using the kinetic method).[54]
(54) Licht, S. S.; Lawrence, C. C.; Stubbe, J. *Biochemistry* **1999**, *38*, 1234.
(55) Singh, D.; Tamao, Y.; Blakley, R. L. *Adv. Enzyme Regul.* **1977**, *15*, 81.

Table 2. Observed and Calculated Absorbance, Kinetic, and Equilibrium Data for the Reaction of AdoCbl, Ado(Bzim)Cbl, and Ado(Im)Cbl with RTPR at 37 °C

	AdoCbl[a]	Ado(Bzim)Cbl	Ado(Im)Cbl
λ, nm	525	525	538
$\Delta\epsilon_\lambda$, M^{-1} cm^{-1}	4800	4110	5220
K_b, M^{-1}	$(2.7 \pm 0.5) \times 10^4$	$(1.7 \pm 0.2) \times 10^4$	$(3.3 \pm 0.5) \times 10^3$
k_{obs}, s^{-1}	37.2 ± 2.5	24.1 ± 1.4	7.9 ± 0.8
K_{eq}	0.585 ± 0.033	0.691 ± 0.028	0.116 ± 0.009
k_f, s^{-1}	13.7 ± 1.2	9.8 ± 0.8	0.83 ± 0.12
k_r, s^{-1}	23.5 ± 2.1	14.2 ± 1.0	7.1 ± 0.9

[a] Reference 2.

$AdoCbi^+$, although the significance of the small spectral changes upon mixing $AdoCbi^+$ with RTPR is unclear.

The results in Table 2 show that RTPR binds Ado(Bzim)Cbl slightly more weakly than it does AdoCbl but binds Ado(Im)-Cbl about 8-fold more weakly. While the equilibrium constant for enzymatic formation of cob(II)alamin is about the same for Ado(Bzim)Cbl and for AdoCbl, it is about 5-fold smaller for Ado(Im)Cbl. The forward rate constant, k_f, for enzymatic formation of cob(II)alamin is nearly the same for Ado(Bzim)-Cbl as that for AdoCbl, but it is reduced by 17-fold for Ado-(Im)Cbl.

It is interesting to note that Finke and co-workers[22,56,57] have found that nonenzymatic thermal homolysis of $AdoCbi^+$ in the presence of exogenous ligands including pyridines and imidazoles leads to substantially more carbon−cobalt bond heterolysis than is observed for $AdoCbi^+$ itself or for AdoCbl. In particular, the *N*-methylimidazole complex, [AdoCbl·*N*-MeIm]$^+$, undergoes roughly 50% homolysis at 110 °C compared to ≤2% heterolysis for AdoCbl at this temperature.[1] This situation is complicated by the fact that for AdoCbl, the activation parameters for carbon−cobalt homolysis and heterolysis are strikingly different, so that the relative importance of the two pathways is strongly temperature dependent (8.5% heterolysis at 85 °C, 56.6% heterolysis at 45 °C).[3] However, the ratio of the two processes is evidently temperature independent in the range 85−110 °C for the *N*-methylimidazole and pyridine complexes.[56,57] The actual products and temperature dependence of carbon−cobalt bond cleavage for thermolysis of Ado(Im)Cbl itself have not yet been determined. Nonetheless, at the RTPR active site, Ado-(Im)Cbl is homolytically cleaved as evidenced by its ability to support turnover at the same rate as AdoCbl and by the direct observation of its Co(II) derivative in the stopped-flow kinetic experiments.

Our results seem to suggest that Co−C bond cleavage is not rate determining for enzymatic turnover, since the coenzyme analogues support turnover at the same rate as AdoCbl (Table 1), but the Co−C bond of Ado(Im)Cbl is cleaved by the enzyme 17 times more slowly (Table 2). However, it is important to point out that the kinetic measurements of RTPR-induced homolysis are carried out in the absence of substrate, while the steady-state measurements, of course, include a reducible NTP substrate. The kinetics of RTPR-induced carbon−cobalt bond homolysis in the presence of NTP substrates are more complicated and have not been studied in any detail.[51,52] It is consequently unclear whether carbon−cobalt bond homolysis is rate determining for RTPR under normal steady-state turnover or not.

Since the steady-state coenzymic activity of the axial nucleotide-free $AdoCbi^+$ is at best 1% of the activity of AdoCbl, the Dmbz axial nucleotide would seem to contribute at least 3 kcal mol^{-1} to the enzymatic activation of the AdoCbl for homolysis. However, we note that the axial nucleotide seems to be a significant binding determinant (K_b, Table 2), suggesting that $AdoCbi^+$ and other nucleotide-free analogues (which, in general, bind weakly to diol dehydratase as well)[46,47] may not be the best models for estimating the contribution of the axial Dmbz to enzymatic catalysis of carbon−cobalt bond homolysis. Further study of this question is in progress.

Nonetheless, the low coenzymic activity of $AdoCbi^+$ and the reduced rate constant for enzymatic carbon−cobalt bond cleavage in Ado(Im)Cbl are not inconsistent with ground-state mechanochemical triggering, in which the natural Dmbz ligand is expected to contribute only a few kilocalories per mole of steric activation for AdoCbl homolysis. To distinguish this mechanism from transition-state mechanochemical triggering, it is essential to know the real level of activity (if any) of axial base-free analogues such as $AdoCbi^+$ and other models for axially uncoordinated AdoCbl as well as the catalytic *efficiency* of the enzyme with the Ado(Im)Cbl analogue (i.e., the extent to which the enzyme catalyzes Co−C bond homolysis relative to the nonenzymatic, thermal homolysis of Ado(Im)Cbl, since the intrinsic reactivity of the Co−C bond seems to be influenced by the bulk of the axial ligand[13,21]). Both of these questions are currently under intensive study.

Acknowledgment. This research was supported by the National Institute of General Medical Sciences, grant GM 48858.

Supporting Information Available: Tables of ^1H and ^{13}C chemical shifts, 2-D NMR correlation tables for Ado(Bzim)Cbl and Ado(Im)-Cbl, UV−visible spectra of AdoCbl, Ado(Bzim)Cbl, and Ado(Im)Cbl and their Co(II) homolysis cleavage products, and the UV−visible spectra of $AdoCbi^+$ and cob(II)inamide. This material is available free of charge via the Internet at http://pubs.acs.org.

IC010796I

(56) Garr, C. D.; Sirovatka, J. M.; Finke, R. G. *J. Am. Chem. Soc*. **1996**, *118*, 11124.
(57) Sirovatka, J. M.; Finke, R. G. *J. Am. Chem. Soc*. **1997**, *119*, 3057.