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NMR Observations of ^{13}C-Enriched Coenzyme B_{12} Bound to the Ribonucleotide Reductase from *Lactobacillus Leichmannii*

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The ^{13}C NMR resonance and one-bond ^{1}H–^{13}C coupling constants of coenzyme B_{12} enriched in ^{13}C in the cobalt-bound carbon have been observed in the complex of the coenzyme with the B_{12}-dependent ribonucleotide reductase from *Lactobacillus leichmannii*. Neither the ^{13}C NMR chemical shift nor the ^{1}H–^{13}C coupling constants are significantly altered by binding of the coenzyme to the enzyme. The results suggest that ground-state Co–C bond distortion is not utilized by this enzyme to activate coenzyme B_{12} for C–Co bond homolysis.

Coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl; Figure 1) is a cofactor in the enzymatic catalysis of about a dozen reactions involving 1,2-intramolecular rearrangements or the reduction of ribonucleotides. The catalytic cycle of all AdoCbl-dependent enzymes is initiated by the enzyme-induced homolysis of the C−Co bond of the coenzyme (the "activation" of AdoCbl) to form the Co^{II} species [cob(II)-alamin] and a radical, a reaction that is catalyzed by 10^{9}−10^{14}-fold.[1−5] Despite many years of intensive research on this coenzyme and the enzymes that utilize it, the mechanism of enzymatic activation of AdoCbl is not yet known for any AdoCbl-dependent enzyme, and this remains one of the outstanding problems in bioinorganic chemistry.[6,7] For example, while structural studies of these enzymes have led to mechanistic proposals (see, for example, refs 8−10), there remain disagreements (see, for example, refs 11 and 12), and the mechanistic question is not settled for any such case (see ref 13 for a recent review).

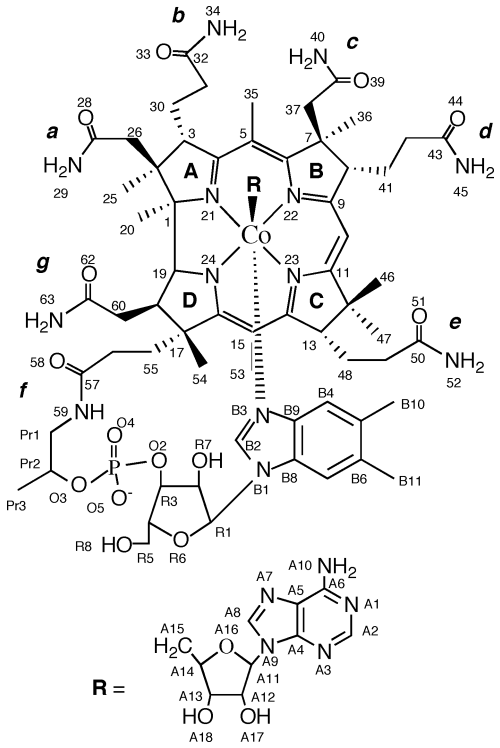

Figure 1. Structure and numbering scheme of 5′-deoxyadenosylcobalamin (AdoCbl, coenzyme B_{12}).

The AdoCbl-dependent enzymes fall into three classes depending on the nature of the migrating group and the substituent on the C atom to which this group migrates.[14,15] The class I enzymes are mutases, which catalyze carbon skeleton rearrangements, the class II enzymes include the

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eliminases and the AdoCbl-dependent ribonucleotide reductases, and the class III enzymes are aminomutases, which catalyze the migration of an amino group and require pyridoxal phosphate as well.[13] Major differences between the class I and class II enzymes, including the binding mode for the coenzyme,[13] the EPR spectra of the biradical that can be trapped during turnover,[16] and the tolerance for structural alterations in the coenzyme, suggest that the mechanism of enzymatic activation may be different for different classes of enzymes.[17]

The catalytic power of these enzymes for AdoCbl activation is astonishing given the fact that the reaction is most likely a simple bond dissociation lacking a true transition state.[17−19] As such, the reaction can only be catalyzed by enzymatic destabilization of the AdoCbl reactant, stabilization of the products of homolysis, or a combination of the two. Destabilization of the coenzyme would involve stretching of the Co−C bond in the ground state and could be engendered by selective interaction of the Ado ligand with active site residues[20−23] or possibly manipulation of the axial Co−N bond.[21,22,24−27] Banerjee, Spiro, and co-workers[28,29] have used cryogenic resonance Raman measurements to determine that the Co−C bond stretching frequency of AdoCbl is hardly affected by binding to the class I enzyme methylmalonylCoA (MMCoA) mutase and concluded that the Co−C bond is only slightly weakened (~0.5 kcal/mol) by the enzyme, in agreement with EXAFS measurements, which showed no change in the Co−C bond distance upon binding to this enzyme.[30] More recently, Brunold and coworkers have used electronic absorption, circular dichroism, and magnetic circular dichroism spectroscopy to study the interaction of AdoCbl and cob(II)alamin with the class I enzymes MMCoA mutase[31,32] and glutamate mutase.[33] These results confirm a lack of ground-state distortion of AdoCbl by these class I enzymes and suggest instead that these enzymes stabilize the cob(II)alamin homolysis product. We now report ^{13}C and ^{13}C spectral-edited ^{1}H NMR observations of AdoCbl enriched in ^{13}C in the cobalt-bound carbon ([A15-^{13}C]AdoCbl)[34] bound to the AdoCbl-dependent ribonucleoside triphosphate reductase (RTPR) of *Lactobacillus leichmannii* (the X-ray crystal structure of which is known[35]), which address the question of ground-state reactant destabilization in a class II enzyme.

Because the binding of AdoCbl to RTPR is relatively weak ($K_b = 2.7 \times 10^4\ M^{-1}$),[2] the ^{13}C NMR resonance of [A15-^{13}C]AdoCbl (1.0 mM in 0.05 M phosphate buffer, pH 7.2) has been observed in the presence of the allosteric effector dGTP (6.0 mM) and increasing concentrations of recombinant RTPR[36] at 37 °C to force the binding equilibrium by mass action. Representative ^{13}C NMR signals are shown in Figure 2A, and the results are given in Table 1.

As [A15-^{13}C]AdoCbl is titrated with RTPR, the line width of its ^{13}C NMR resonance increases substantially because of the increased rotational correlation time of the cobalamin in the complex with RTPR, indicating that free and bound [A15-^{13}C]AdoCbl are in fast exchange on the NMR time scale. The observed line broadening is quite close to that expected for a protein of this size.[37] The chemical shift can be seen to be independent of complexation to RTPR (average $\delta = 26.5 \pm 0.2$ ppm, compared to 26.5 ppm for free [A15-^{13}C]AdoCbl). Similar results were obtained in the presence of the reducible substrate, ATP (6.5 mM, average $\delta = 26.6 \pm 0.1$ ppm).

The diastereomeric A15 methylene protons[41] of [A15-^{13}C]-AdoCbl were also observed in the presence of dGTP and increasing concentrations of RTPR using the one-bond heteronuclear multiple quantum coherence experiment without decoupling. Representative spectra are shown in Figure

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(37) $M = 82$ kDa[36] and $D = 6.4 \times 10^{-7}$ cm^2/s[38] lead to $r = 38$ Å (Stokes−Einstein relation, lit.[39] $r = 34$ Å by light scattering) and $\tau_R = 50$ ns.[40] Assuming the transverse relaxation is dominated by the dipolar relaxation and the "nonextreme narrowing limit" ($\omega_C\tau_R = 248 \gg 1$), the predicted line width for the fully immobilized ^{13}C nucleus is 212 Hz and that for 70% bound 163 Hz. If chemical shift anisotropy contributes 20% to the transverse relaxation, the expected line width at 70% binding would be 130 Hz compared to the 133 Hz observed.
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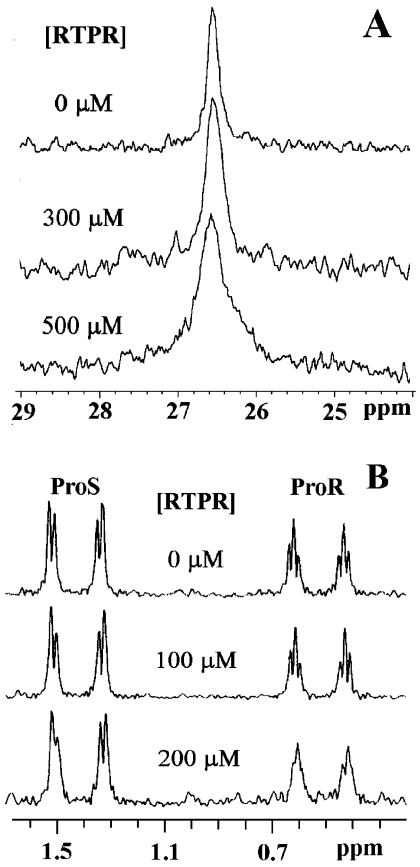

Figure 2. (A) Directly observed ^{13}C NMR signals for [A15-^{13}C]AdoCbl (1.0 mM) in the presence of dGTP (6.0 mM) and an increasing concentration of RTPR, as indicated, in a 50 mM phosphate buffer, pH 7.2, 37 °C. (B) Slices through the ^{1}H-coupled ^{1}H−^{13}C HMQC spectra of [A15-^{13}C]AdoCbl (1.0 mM) in the presence of dGTP (6.0 mM) and an increasing concentration of RTPR, as indicated, in a 50 mM phosphate buffer, pH 7.2, 37 °C.

Table 1. ^{13}C NMR Parameters of [A15-^{13}C]Cbl Bound to RTPR

[RTPR] (mM)	% bound[a]	$\delta_{^{13}C}$ (ppm)	$\Delta\nu_{1/2}$ (Hz)
0	0	26.5	14.8
146	13.9	26.7	28.5
300	28.4	26.4	33.4
437	40.8	26.6	76.9
500	46.4	26.3	79.8
800	70.2	26.3	133.2

[a] Calculated from $K_b = 2.7 \times 10^4$ M^{-1} (ref 2).

2B, and the results are collected in Table 2. Neither the chemical shift nor the ^{1}H−^{13}C coupling constants of the pro-*R* or pro-*S* A15 H atoms are significantly altered by binding to the enzyme.

Progress along the reaction coordinate for the enzyme-induced Co−C bond homolysis of AdoCbl must be accompanied by a rehybridization of the A15 carbon from sp^3 to sp^2 as the Co−C bond is stretched. This, in turn, would cause a large downfield shift of the A15 ^{13}C NMR resonance. Thus, ground-state destabilization of AdoCbl by stretching of the Co−C bond would be expected to give rise to a downfield shift of this resonance. Moreover, the chemical

Table 2. ^{1}H NMR Chemical Shifts and ^{13}C−^{1}H Coupling Constants for the A15 Methylene Protons of [A15-^{13}C]AdoCbl Bound to RTPR

		pro-*S*		pro-*R*	
[RTPR] (mM)	% bound[a]	$\delta_{^{1}H}$ (ppm)	$^{1}J_{HC}$ (Hz)	$\delta_{^{1}H}$ (ppm)	$^{1}J_{HC}$ (Hz)
0	0	1.44	134.8	0.53	140.3
100	9.6	1.43	136.8	0.52	139.5
146	13.9	1.43	136.9	0.50	141.2
200	19.0	1.43	135.0	0.53	138.7
291	27.5	1.45	137.4	0.50	143.9
408	40.8	1.42	135.2	0.49	142.0

[a] Calculated from $K_b = 2.7 \times 10^4$ M^{-1} (ref 2).

shifts of atoms in cobalamins are affected by the magnetic anisotropy of the cobalt dipole to varying extents depending on the distance of the atom from the metal and the spatial position of the atom relative to the dipole symmetry axis.[42,43] Because the magnetic anisotropy of the induced dipole in AdoCbl is rather large (-14.3×10^{-29} cm^3/molecule[43]) and the A15 C atom is close to the metal ($d_{Co-C} = 2.24$ Å[44,45]), the effect of the cobalt dipole on the A15 chemical shift is very large. Using McConnell's equation,[42] we can estimate from these values that stretching of the Co−C bond of AdoCbl by 0.1 Å would cause about a 1 ppm downfield shift of the A15 resonance in addition to the effect of rehybridization at A15.

Similarly, rehybridization of the A15 carbon due to distortion of the Co−C bond should have a significant effect on the one-bond C−H coupling constant. In fact, Marzilli has argued that one-bond ^{1}H−^{13}C coupling constants are a more sensitive indicator of changes in carbon hybridization than ^{13}C chemical shifts.[46] This is because one bond spin−spin coupling is usually dominated by the Fermi contact term,[47] which depends on the electron density at the nucleus. Because s orbitals alone have electron density at the nucleus, $^{1}J_{CH}$ values are sensitive probes of the s orbital character at the carbon nucleus and, hence, of hybridization. As a result, our inability to detect any measurable change in the ^{13}C NMR chemical shift or the one-bond C−H coupling constants of the A15 carbon of AdoCbl upon binding to RTPR strongly suggests that ground-state destabilization of the Co−C bond does not occur for this class II enzyme.

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