Solution Behavior and Complete ^1H and ^{13}C NMR Assignments of the Coenzyme B_{12} Derivative (5'-Deoxyadenosyl)cobinamide Using Modern 2D NMR Experiments, Including 600-MHz ^1H NMR Data

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Abstract: Two-dimensional (2D) NMR methods have been used to assign completely the ^1H and ^{13}C NMR spectra of the (5'-deoxyadenosyl)cobinamide cation ($AdoCbi^+$) in D_2O. Most of the ^1H spectral assignments were made by using 2D homonuclear shift correlation spectroscopy (COSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA), absorption-mode (phase sensitive) 2D nuclear Overhauser effect (NOE) spectroscopy, and spin-locked NOE spectroscopy (also called ROESY, for rotating-frame Overhauser enhancement spectroscopy). Most of the protonated carbon resonances were assigned by using ^1H-detected heteronuclear multiple-quantum coherence (HMQC) spectroscopy. The nonprotonated carbon resonances, as well as the remaining unassigned ^1H and ^{13}C NMR signals, were assigned from long-range ^1H-^{13}C connectivities determined from ^1H-detected multiple-bond heteronuclear multiple-quantum coherence spectroscopy (HMBC). Comparison of the ^{13}C chemical shifts and ^1H NOEs of $AdoCbi^+$ with those of coenzyme B_{12} ((5'-deoxyadenosyl)cobalamin) and its benzimidazole-protonated, base-off form indicates that the electronic properties and structure of $AdoCbi^+$ are similar to that of coenzyme B_{12} in the protonated, base-off form. The ^{13}C chemical shifts of most of the carbons of $AdoCbi^+$ do not vary significantly from those of base-off, benzimidazole-protonated coenzyme B_{12}, indicating that the electronic environment of the corrin ring is also similar in both compounds. However, significant differences in the chemical shifts of some of the corresponding carbons of the b, d, e, and f corrin side chains (namely, C30, C42, C49, and C55, respectively) in $AdoCbi^+$ and in base-off, benzimidazole-protonated coenzyme B_{12} indicate that the positions of these side chains may be different in $AdoCbi^+$ compared to base-off coenzyme B_{12}, possibly due to the lack of the dimethylbenzimidazole nucleotide in $AdoCbi^+$. These differences, however, are relatively minor and do not suggest any important interactions between the protonated, base-off 5,6-dimethylbenzimidazole and the e propionamide side chain. Thus, a consistent view is emerging that in unprotonated, base-off cobalamins the benzimidazole moiety interacts with the e side chain whereas in protonated, base-off species this interaction is absent. Significantly, the adenosyl moiety has the same major conformation in $AdoCbi^+$ and in both forms of coenzyme B_{12}.

Homolysis of the Co-C bond of coenzyme B_{12}((5'-deoxyadenosyl)cobalamin) is generally accepted to be the essential first step in the enzymatic reactions for which coenzyme B_{12} is a cofactor.[1-4] Recent studies document a $\geq 10^{13}$ acceleration of the coenzyme B_{12} Co-C bond cleavage in the holoenzyme in comparison to cleavage of this bond in the absence of enzyme in solution.[5] However, the question of exactly how the enzyme accomplishes this rate enhancement remains unanswered. Although it seems very likely that the enzyme must use its cofactor·enzyme intrinsic binding energy[5] to weaken the Co-C bond or otherwise "trigger" the Co-C bond homolysis step, the molecular-level details of this process are unknown but of considerable interest. Specifically, B_{12}-cofactor-localized mechanisms that have been postulated to account for the enzyme-assisted Co-C bond homolysis include[3] (a) a distortion in the corrin ring increasing the steric interaction with the adenosyl moiety (the "butterfly" or upward conformational theory),[3,5] (b) a direct lengthening or angular distortion of the Co-C bond by the protein, and (c) mechanisms that focus on the position or Co-N bond length[6,7] of the axial 5,6-dimethylbenzimidazole (DMBz) ligand. UV spectral data have been used to suggest that the Co-DMBz bond is broken during catalysis, giving the so-called base-off (benzimidazole-unprotonated) form of the coenzyme,[8] and several studies exist showing that axial base-free (5'-deoxyadenosyl)cobinamide ($AdoCbi^+$, Scheme I) is still a partially active cofactor.[5] However, the lack of an improved synthesis and isolation procedure until recently[5] for $AdoCbi^+$, in which the dimethylbenzimidazole nucleotide has been *chemically* cleaved and thus completely removed from the coenzyme, inhibited investigations of this derivative of coenzyme B_{12}.

The adenosyl-cobalt bond is very stable when compared to other alkyl-cobalt bonds; e.g., coenzyme B_{12}[9] and $AdoCbi^+$[5] exhibit the largest $\Delta H_h^{\ddagger}$ (Co-C bond homolysis activation enthalpy) when compared to other alkyl-B_{12}'s (20-27 kcal/mol)[10] or other alkyl-Cbi^+'s (27-32 kcal/mol).[10a] $AdoCbi^+$ also exhibits the largest $\Delta H_h^{\ddagger}$ value measured to date, 37.5 kcal/mol,[5] when compared to all literature values for Co-C bond homolysis activation enthalpies (17-37.5 kcal/mol).[5,9-11] Recent thermolysis data on $AdoCbi^+$ demonstrate that the Co-C bond homolysis rate constant is 10^{-2} times that of the coenzyme.[5] These results (as well as other data)[5] suggest that the axial base effect probably accounts for only 10^2 of the $\geq 10^{13}$ rate enhancement of the Co-C bond homolysis upon going from "base-off" $AdoCbi^+$ in solution to the active

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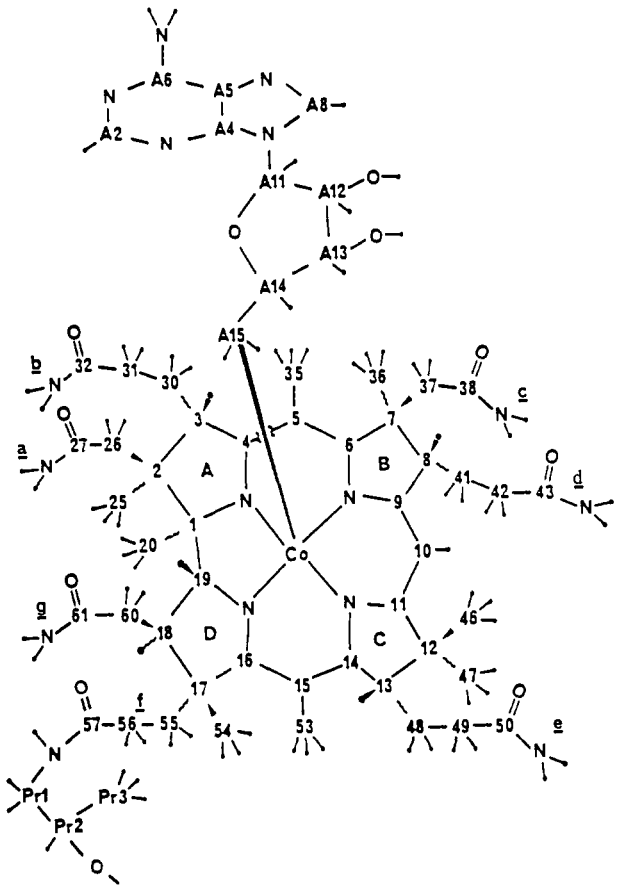
Scheme I



form of coenzyme B_{12} in the holoenzyme.[5] However, the interpretation of such a comparison depends on how electronically and structurally similar AdoCbi$^+$ is to the enzyme-bound, base-off form of coenzyme B_{12}. Restated, the $\geq 10^{13}$ difference is probably telling us that the enzyme-bound coenzyme B_{12} is structurally or electronically much different from axial base-free and enzyme-free AdoCbi$^+$. It follows, then, that a necessary comparison is of the electronic and structural properties of AdoCbi$^+$ to those of coenzyme B_{12} bound to the enzyme. Such a comparison begins with a study of AdoCbi$^+$, the focus of the present work. In addition, it is essential to determine whether any isomerization has occurred in the cobinamide or 5'-deoxyadenosyl moiety during the chemical transformation of coenzyme B_{12} to AdoCbi$^+$.

One means to examine the electronic and structural properties of AdoCbi$^+$ is through NMR spectroscopy.[12] The complexity of the molecule requires that two-dimensional (2D) NMR methods be used. For example, 2D NOE experiments can give structural information about the molecule. Assignment of the ^{13}C NMR spectrum of AdoCbi$^+$ will allow a comparison of the electronic and structural properties of AdoCbi$^+$ with those of coenzyme B_{12}. Modern 2D NMR techniques, which were used previously to assign completely the ^1H and ^{13}C NMR spectra of coenzyme B_{12},[13] have now been used to make complete assignments of the ^1H and ^{13}C NMR spectra of AdoCbi$^+$. In particular, herein we report the following NMR studies of AdoCbi$^+$: 2D COSY (homonuclear shift correlation spectroscopy),[14] HOHAHA (homonuclear Hartmann–Hahn) spectroscopy,[15] absorption-mode (phase sensitive) 2D NOE (nuclear Overhauser effect) spectroscopy,[16] spin-locked NOE spectroscopy (also called ROESY, for rotating-frame Overhauser enhancement spectroscopy),[17] ^1H-detected heteronuclear multiple-quantum coherence (HMQC) spectroscopy,[18] and ^1H-detected multiple-bond heteronuclear multiple-quantum coherence spectroscopy (HMBC).[19]

Experimental Section

A solution of AdoCbi$^+$ (42 mg), prepared as previously described[5] and >99% pure by HPLC, was lyophilized and dissolved in 0.5 mL of 99.8% D_2O (Aldrich) to give a pD of 4.8. When necessary the pD was adjusted with 35% DCl and/or NaOD. All of the 2D NMR experiments, except the HOHAHA and ROESY experiments, were performed on a Nicolet NT-360 spectrometer equipped with a Cryomagnet Systems (Indianapolis, IN) broad-band probe (for the HMQC and HMBC experiments). All of the 2D NMR experiments on the NT-360 spectrometer were carried out at room temperature (20 °C) without sample spinning. The HOHAHA and ROESY experiments were performed on a Bruker AM-600 instrument in Karlsruhe, West Germany. Proton and carbon chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$ (TSP). The exact carbon shifts were measured from a 1D spectrum obtained on a General Electric QE-300 instrument. The INEPT experiment was also performed on the QE-300 instrument.

COSY. The COSY spectrum resulted from a 512 × 1024 data matrix size with 16 scans per t_1 value. The delay time between scans was 1.8 s. The spectrum was processed by using the NMR2 program (New Methods Research Inc., Syracuse, NY) with an exponential line narrowing of 2 Hz, a Gaussian line broadening of 6 Hz, and a sine bell filter used prior to Fourier transformation in both the t_2 and t_1 dimensions.

HOHAHA Spectroscopy. The spectrum in Figure 1 resulted from a 2048 × 2048 data matrix size with 32 scans per t_1 value. The delay time between scans was 3.0 s. The mixing time was 80 ms. One watt of power provided a 48-μs 90° ^1H pulse width. A squared sine bell filter was used prior to Fourier transformation in both the t_2 and t_1 dimensions.

Spin-Locked NOE Spectroscopy (ROESY). The spectrum in Figure 2 resulted from a 2048 × 2048 data matrix size with 4 scans per t_1 value. The delay time between scans was 3.0 s and the mixing time was 200 ms. A 5.7-kHz rf field strength (44-μs 90° pulse width) was used. A shifted sine bell filter (shifted 45° and 22.5° in the t_2 and t_1 dimensions, respectively) was used before Fourier transformation in both dimensions.

Absorption-Mode NOE Spectroscopy. The absorption-mode NOE spectrum resulted from a 512 × 2048 data matrix size with 32 scans per t_1 value. The delay time between scans was 1.8 s and the mixing time was 200 ms. Gaussian line broadening (6 and 9 Hz) was used in the t_2 and t_1 dimensions, respectively.

HMQC Spectroscopy. The one-bond ^1H–^{13}C shift correlation spectrum of Figure 3 resulted from a 512 × 1024 data matrix size with 128 scans per t_1 value (preceded by four dummy scans). The delay time between scans was 0.5 s. Thirty watts of ^{13}C rf power and a 43-μs 90° pulse width were used. A double-exponential multiplication function was used prior to Fourier transformation in the t_2 and t_1 dimensions.

HMBC Spectroscopy. The multiple-bond ^1H–^{13}C shift correlation spectra (Figures 4 and 5) resulted from a 128 × 1024 data matrix size with 256 scans (preceded by four dummy scans) per t_1 value and a delay time between scans of 1.0 s. Thirty watts of power and a 50-μs 90° ^{13}C pulse width were used. The values of Δ_1 (the delay between the first 90° proton pulse and the first 90° ^{13}C pulse) and Δ_2 (the delay between the first and second 90° ^{13}C pulses) were 3.5 and 50 ms, respectively. In the t_2 dimension a double-exponential multiplication was used prior to

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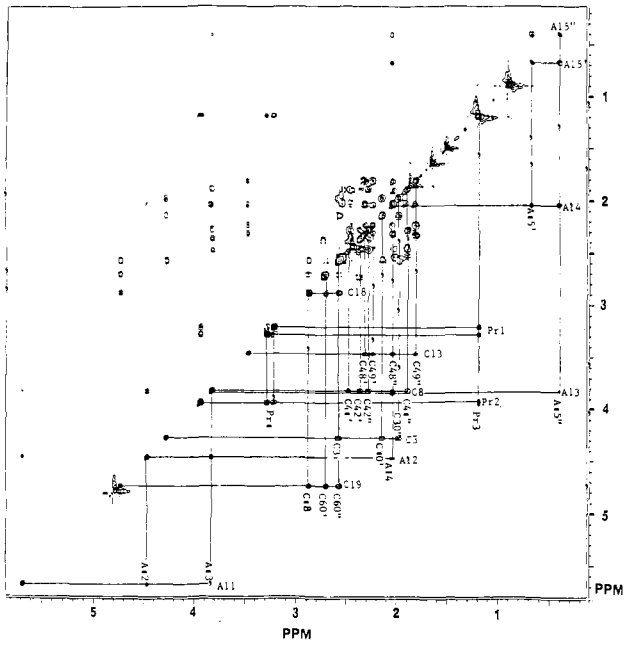

Figure 1. 2D HOHAHA spectrum of AdoCbi$^+$ at 600 MHz. The connectivity patterns for the adenosyl ribose ring and the propanolamine protons are indicated in the figure by drawn lines. Connectivity between the corrin ring methine protons and the methylene protons of the corresponding side chains is also indicated by drawn lines.

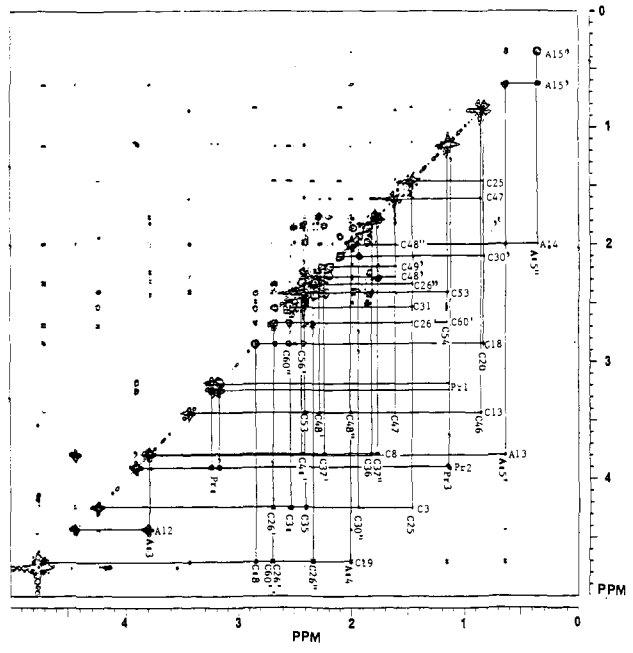

Figure 2. Part of the 2D ROESY spectrum of AdoCbi$^+$ at 600 MHz obtained with a 200-ms spin lock period. Both positive and negative peaks are shown. NOE connectivities are shown by drawn lines to those cross peaks that are negative relative to the diagonal.

Fourier transformation. In the t_1 dimension a sine bell filter was used and the data were zero-filled once prior to Fourier transformation.

Results

Assignment of the ^{1}H NMR Spectrum. Most of the proton signals of AdoCbi$^+$ have been assigned by using two-dimensional COSY, HOHAHA, and NOESY experiments. We identify the ^{1}H nuclei by the carbon atom to which they are attached. For nonequivalent geminal protons, H′ and H″ refer to protons with the downfield and upfield signals, respectively. We designate the ^{13}C nuclei by using the numbers in the scheme following C for carbons of the corrin ring and its side chains, A for carbons of the adenosyl moiety, and Pr for carbons of the propanolamine groups (see Scheme I).

In presenting the results, we emphasize where difficulties were encountered with previous assignment strategies[13] and how we overcame these difficulties. The general approach to the assignments followed that described previously.[13]

We will first discuss the COSY spectrum (supplementary Figure S1). All the protons from the adenosyl ribose ring were assigned from the COSY spectrum. The most upfield signal of a single proton is assigned to A15H″. It has been shown in a study of ([5′,5′-^{2}H$_2$]adenosyl)cobalamin[20] that the A15 protons give a doublet and a triplet in the ^{1}H NMR spectrum, with the triplet being the most upfield signal of a single proton in the spectrum. The same splitting pattern is seen in the ^{1}H NMR spectrum of AdoCbi$^+$, as well as in the spectrum of protonated, base-off coenzyme B_{12}, where the most upfield signal of a single proton was assigned to A15H″.[13b] The A15 protons are shifted upfield due to anisotropy of cobalt and of the corrin ring. A15H″ is tentatively assigned as the proton on A15 that is farther away from A14H and closer to the Co(III) center and the corrin ring. Neutron diffraction data[21] of coenzyme B_{12} show that one of the A15 protons is about 0.4 Å closer to A14H than the other. With the assumption that a similar orientation exists in AdoCbi$^+$, A15H′ can be assigned as the proton closest to A14H from the ROESY and absorption-mode NOE spectra (see below) since it has a stronger NOE to A14H. The A15H″ signal shows correlation to two signals, A15H′ and A14H. A14H in turn shows a correlation to A13H, which shows a correlation to A12H. A12H shows a correlation to A11H, completing the assignment of the adenosyl ribose protons.

In the COSY spectrum there are also some correlations for the side chains. There is a correlation between a doublet at 1.21 ppm corresponding to three protons and a signal of a single proton at 3.98 ppm. The only methyl group in the molecule attached to a protonated carbon is Pr3H$_3$. Pr3H$_3$ shows connectivity to a signal that is assigned to Pr2H, which in turn shows connectivity to Pr1H$_2$.

For the corrin ring, there are correlations between C19H and C18H, C8H and C41H″, C13H and the C48 methylene protons, and C3H and C30H′. The assignment of at least one signal in each of these pairs was made from NOESY spectra (see below).

Since the COSY experiment does not show relayed connectivities, the COSY spectrum does not show the connectivity networks for the corrin side chains. HOHAHA spectroscopy allows the connectivity networks for the corrin side chains to be seen, as well as the other connectivities seen in the COSY spectrum, since it does show relayed connectivities (Figures 1 and S2). The relayed connectivities allow the protons to be assigned to a specific propionamide side chain for side chains b, d, e, and f, but not necessarily to specific methylene groups. These assignments are based on the corrin methine assignments from NOE spectra (see below). Sometimes it is extremely difficult to distinguish between direct and relayed connectivities in the HOHAHA experiment[13a] and such is the case in the spectra of Figures 1 and S2. Therefore, other experiments (see below) were needed for more specific assignments.

Two types of two-dimensional NOE experiments, ROESY and absorption-mode (phase sensitive) NOE, were used to establish through-space connectivities between the various networks of coupled protons (Figures 2, S3, and S4). Since there were several cases in which one of the experiments gave an NOE peak that was not seen in the other, a more complete assignment of the proton signals of AdoCbi$^+$ was made by using the NOEs found from both of these experiments. NOE connectivities were used to complete the assignment of most of the ^{1}H NMR spectrum starting with the downfield proton signals.

The three most downfield proton signals do not have any cross peaks in the COSY spectrum, but only one does not show any

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Table I. Summary of Observed NMR Connectivities in (5′-Deoxyadenosyl)cobinamide with the Homonuclear Hartmann–Hahn (HOHAHA), Absorption-Mode NOE, Spin-Locked NOE (ROESY), and Heteronuclear Multiple-Bond Correlation (HMBC) Methods[a]

signal	HOHAHA (^1H)	NOE (^1H) absorption-mode NOE	NOE (^1H) ROESY	HMBC
C20		C25, C18	C25, C18, C30′	C1, C2, C19
C25		C3, C26′, C20, C30″, C31	C3, C26, C20, C30′, C31	C1, C2, C3, C26
C26′	C26″	C19, C3, C25, C26″	C19, C3, C25, C26″	C2, C25, C27
C26″	C26′	C19, C26′	C19, C26′, C25	C2, C27
C3	C30, C31	C25, C26′, C30, C31, C35	C25, C26′, C30″, C31, C35	C1, C2, C4, C26, C30, C31
C30′	C3, C30″, C31	C3, C31[b]	C20, C25, C30″, C31	C2, C3, C31, C32
C30″	C3, C30′, C31	C3, C25[b]	C3, C30′	C2, C3, C31, C32
C31	C3, C30	C3, C25, C30′	C3, C25, C30′	C30
C35		C3	C3, C36	C4, C5, C6
C36		C8	C8, C35, C37′, C41′	C6, C7, C8, C37
C37′	C37″	C8	C8, C36	C7, C36, C38
C37″	C37′		C8	C7, C36, C38
C8	C41, C42	C10, C36, C37′, C41″	C10, C36, C37, C41′	
C41′	C41″, C42, C8	C10	C8, C36, C41″	C42
C41″	C41′, C42, C8	C8	C41′	C9, C42, C43
C42′	C42″, C41, C8			C8, C43
C42″	C42′, C41, C8			C8
C10		C8, C41′, C46, C47	C8, C46, C47	C8, C9, C11, C12
C46		A11, C10, C13, C47	C10, C13, C47	C11, C12, C13, C47
C47		C10, C13, C46	C10, C13, C46, C49′, C48	C11, C12, C13, C46
C13	C48, C49	C46, C47, C48, C53	C46, C47, C48, C53	C14, C49
C48′	C48″, C49, C13	C13	C13, C47, C48″, C49″	
C48″	C48′, C49, C13	C13	C13, C47, C48′, C49	C49, C50
C49′	C49″, C48, C13		C47, C48″, C49″	C13, C48, C50
C49″	C49′, C48, C13		C49′, C48	C13, C50
C53		C13, C54	C13, C54	C14, C15, C16
C54		A11, C19, C53, C60″	C53, C60′	C16, C17, C18, C55
C55′	C55″, C56			C56, C57
C55″	C55′, C56	C56′	C56′	
C56′	C56″, C55	C18, C55″	C18, C55″, C56″	
C56″	C56′, C55		C56′	C55, C57
C18	C19, C60	C20, C56′, C60′	C20, C56′, C60	C1, C17, C19, C54, C60, C61
C60′	C60″, C18, C19	C18, C19	C18, C19, C60″	C17, C18, C61
C60″	C60′, C18, C19	C54	C18, C54, C60′	C17
C19	C18, C60	A14, A15′, C18, C26, C54, C60	A14, A15′, C18, C26, C60′	C1, C18, C20, C60
Pr1	Pr2, Pr3	Pr2	Pr2, Pr3	C57, Pr2, Pr3
Pr2	Pr1, Pr3	Pr1, Pr3	Pr1, Pr3	
Pr3	Pr1, Pr2	Pr2	Pr1, Pr2	Pr1, Pr2
A2				A4, A6
A8		A11, A12, A13	A11, A12, A13	A4, A5, A11
A11	A12, A13	A8, A12, A14, C46, C54	A8, A12, A14	A4, A8, A12
A12	A11, A13, A14	A8, A11, A13	A8, A11, A13	A11
A13	A11, A12, A14, A15″	A8, A12, A14, A15	A8, A12, A15′	A14, A15
A14	A12, A13, A15	A11, A13, A15′, C19	A11, A15, C19	A13, A15
A15′	A15″, A14	A13, A14, A15″, C19	A13, A14, A15″	A14
A15″	A13, A14, A15′	A13, A15′	A14, A15′	A14

[a] Primes and double primes denote downfield and upfield ^1H signals, respectively, of geminal methylene protons; if absent, cross peaks include both proton signals. [b] Cross peak between C30′ and C30″ is obscured by noise close to the diagonal in the absorption-mode NOE spectrum.

NOEs. Based on the HMBC spectrum (see below) and the absence of NOEs to sugar protons, this signal is assigned to A2H. Another signal that shows NOEs to A11H, A12H, and A13H is assigned to A8H. The remaining peak is assigned to C10H, the only remaining uncoupled, single proton at a conjugated site. Also, the intensity of this peak was found to decrease with time in a more acidic D_2O solution (pH 2.1). In coenzyme B_{12} and other cobalamins, C10H readily undergoes exchange with D_2O in acid.[22,23] Furthermore, the shift position at 7.06 ppm is distinctive.

In the ROESY spectrum (Figure S3), C10H shows three NOE cross peaks, two of which correspond to methyl protons and the other to a single proton. The latter is assigned to C8H as described previously.[13a] The two methyl groups close to C10H are $C46H_3$ and $C47H_3$. One of the methyl groups has a more intense cross peak in the NOESY spectrum and corresponds to the methyl group that is closer to C10H. There are similar C18H–C19H coupling constants for AdoCbi$^+$ (10.8 Hz) and coenzyme B_{12} (10.5 and 10.2 Hz in the base-on and base-off forms, respectively[13b]), *suggesting that the conformations of the corrin ring are very similar in these B_{12} derivatives.* X-ray crystal structure data of coenzyme B_{12} show that $C47H_3$ is closer to C10H.[24] Therefore, we assume that $C47H_3$ is closer to C10H in AdoCbi$^+$ and assign the peak at 1.66 ppm to $C47H_3$. This assignment is supported by NOEs in the ROESY spectrum (Figure 2) from C48H′ and C48H″ to $C47H_3$ and the absence of such NOEs from the C48 methylene groups to $C46H_3$. The C48 methylene groups are on the same side of the corrin ring and are therefore closer to $C47H_3$ and $C46H_3$. From this starting point one can progress around the corrin ring and assign most of the resonances following previously described strategies.[13a] Ambiguities in the assignments of the C30, C31, C55, and C56 methylene protons can be removed by using ^1H–^{13}C connectivities. All the observed NOE and HOHAHA connectivities are given in Table I.

Assignment of the ^{13}C NMR Spectrum. The assignments of the protonated carbons were made from a HMQC[18] spectrum (Figures 3 and S5). The assignment of the carbons whose protons are downfield of 3 ppm and the methyl group carbons is fairly

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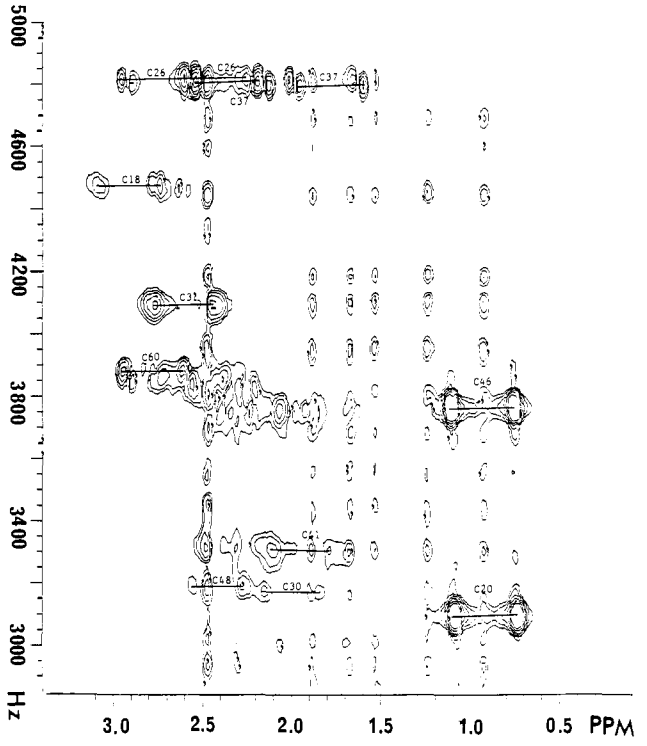

Figure 3. Part of the ^{1}H-detected $^{1}H-^{13}C$ shift correlation (HMQC) spectrum of AdoCbi^{+}. The center of each pair of cross peaks connected with a drawn line corresponds to the proton chemical shift. The cross peaks in a given pair are separated by a distance equal to the $^{1}H-^{13}C$ coupling constant. In the ^{13}C scale 0 Hz = 102.9 ppm (^{13}C frequency is 90.8 MHz on the NT-360 spectrometer). The ridges of noise in the spectrum are caused by incomplete cancellation of the protons attached to the ^{12}C nuclei. All peaks shown are folded over in the ^{13}C dimension. The more upfield peaks in the spectrum are also the more upfield peaks in the ^{13}C spectrum.

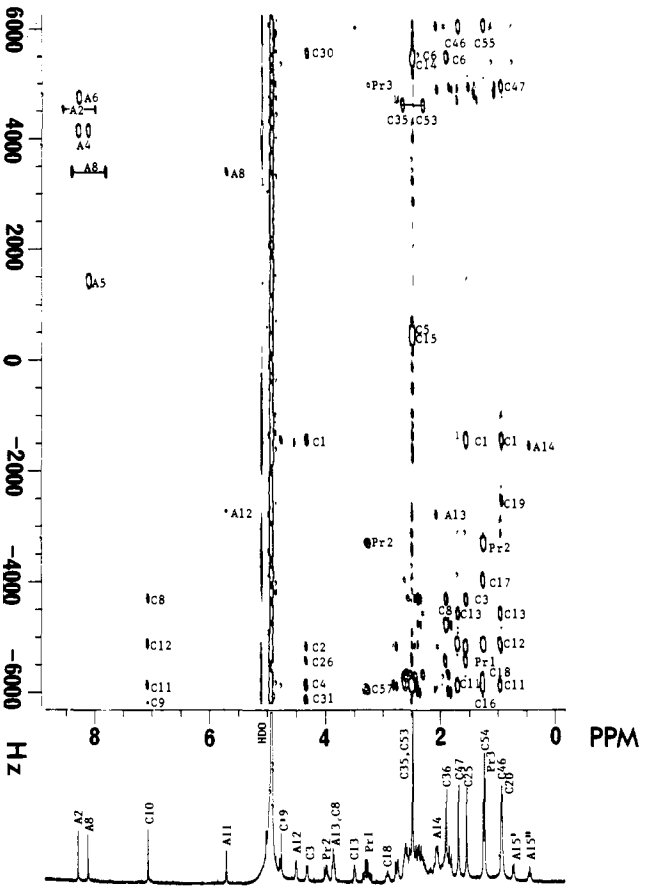

Figure 4. ^{1}H-detected $^{1}H-^{13}C$ multiple-bond shift correlation (HMBC) spectrum of AdoCbi^{+} (360 MHz ^{1}H, 90.8 MHz ^{13}C). The conventional 1D ^{1}H spectrum is shown at the bottom of the figure. Pairs of correlations with a drawn line connecting them (i.e., A2, A8, and C35/C53) are incompletely suppressed one-bond correlations. There is foldover in the far upfield (<36 ppm) and far downfield (>159 ppm) regions of the ^{13}C spectrum. In both foldover regions, the most upfield signal in the region is also the most upfield in that region of the ^{13}C spectrum.

straightforward. The methyl groups, as expected, give the strongest signals in the HMQC spectrum. The A13 and C8 proton signals exactly overlap. By comparison with coenzyme B_{12}, A13 should be the more downfield resonance. This assignment can be confirmed from $^{1}H-^{13}C$ multiple-bond correlations. The assignments of the methylene carbons of the corrin side chains were more difficult due to the fact that they are in a crowded region in the proton dimension and in some cases a crowded region in the ^{13}C dimension (Figure 3). In some cases, only one of a pair of nonequivalent methylene protons gave a correlation in the HMQC spectrum, specifically C30, C41, C48, and C60. This causes some uncertainty in assigning the ^{13}C resonances of C30 and C31, whose ^{1}H resonances have not been assigned unambiguously. The resonance of C30H′, which was assigned from the COSY spectrum, does not show a correlation in the HMQC spectrum. However, the ^{13}C shifts of C30 and C31 differ by almost 10 ppm. Since none of the other protonated carbon resonances were found to differ by more than about 1 ppm from the shifts found for coenzyme B_{12}, these signals were assigned by analogy and by long-range $^{1}H-^{13}C$ connectivities (see below). The assignments of C42, C49, C55, and C56 are complicated due to the fact that the ^{13}C and ^{1}H resonances are in a crowded and difficult to interpret section of the HMQC spectrum (Figure 3). The ^{13}C resonance of C46 also occurs in this crowded region of the ^{13}C spectrum and, although its ^{1}H resonance is well separated from the others, an exact ^{13}C chemical shift cannot be determined from the HMQC spectrum. The ^{13}C resonance of C46 was assigned by using an INEPT experiment. The other carbon resonances in this region can be assigned by long-range $^{1}H-^{13}C$ connectivities.

Since the HMQC experiment does not provide assignment information for nonprotonated carbons, it was necessary to use HMBC[19] to complete the ^{13}C assignments. This experiment shows connectivity between protons and carbons that are two to three bonds away. The nonprotonated adenine carbons were readily assigned. A6 shows correlation to A2H only, A5 shows correlation to A8H only, and A4 shows correlation to both (Figure 4). A correlation between A14H and A13 removes the ambiguity of the assignment for C8 and A13 from the HMQC spectrum. Connectivity around the corrin ring can also be established from the HMBC spectrum (Figures 4 and S6), as was described previously.[13a] All the observed multiple-bond $^{1}H-^{13}C$ connectivities are given in Table I.

It is impossible to give unambiguous assignments for C5 and C15 from the HMBC experiment because C5 and C15 show no connectivities to protons other than the overlapping $C35H_3$ and $C53H_3$. However, it was found that the proton chemical shifts of $C35H_3$ and $C53H_3$ change slightly and separate on lowering the pH from 4.8 to at least 3.8, a pH where N1, the nitrogen between A2 and A6 in the adenine ring, is partially protonated (as found by a pH titration followed by ^{1}H NMR spectroscopy (data not shown)). The assignments of these two methyl signals at pH 3.8 were made from a NOESY spectrum in which NOEs from C3H and C13H to the methyl groups showed the positions of $C35H_3$ and $C53H_3$, respectively. The positions of C3H and C13H were followed as a function of pH, removing any doubt of their positions at pH 3.8. An HMBC spectrum was recorded at pH 3.8. ^{13}C spectra were recorded at pH 4.07 as well as pH 3.8. The peaks for C5 and C15 moved slightly farther apart as the pH decreased. Therefore, the more upfield of these two signals in the HMBC spectrum at pH 3.8 is also the more upfield peak in the HMBC spectrum at pH 4.8. The HMBC spectrum at pH 3.8 (Figure S7) shows that C15 is upfield of C5, thus assigning the resonances at 110.1 and 111.0 ppm at pH 4.8 to C15 and C5, respectively.

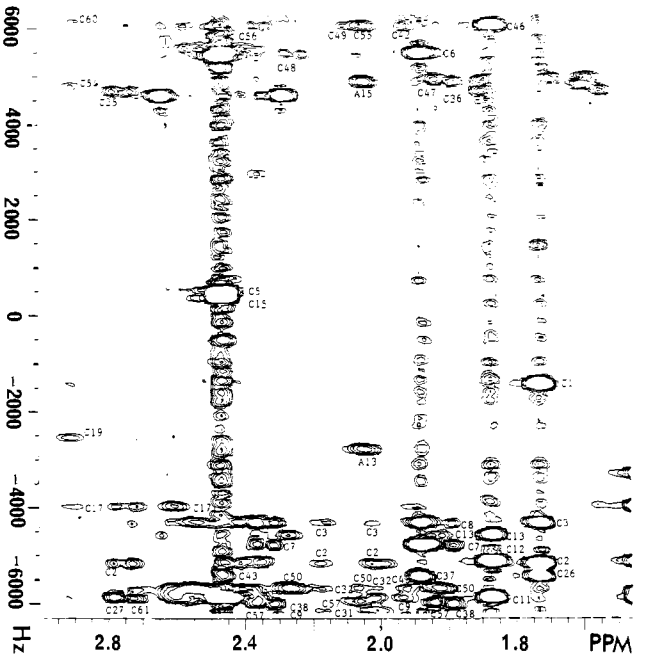

Figure 5. Expanded region of Figure 4 displaying the multiple-bond ^1H–^{13}C correlations of the methylene protons. The lowest contour level in this figure is 2.5 times lower than that of Figure 4.

The assignment of the carbonyl resonances was difficult since the protons that show connectivity to them are in a very crowded region (Figure 5). The only protons that show connectivity to these carbonyl carbons are methylene protons. Although the proton assignments for some methylene groups (i.e., C30, C31, C55, and C56) were not determined unambiguously, they have been assigned to a specific side chain and would show connectivity to a particular carbonyl in any case. In this way, all the carbonyl carbons were assigned.

Verification of the proton and ^{13}C assignments of C30 and C31 can be made from the HMBC spectrum (Figure 5). C30H′, which was assigned from the COSY spectrum, shows a correlation to C31, confirming the ^{13}C assignments made from the HMQC spectrum. Another proton resonance at 2.03 ppm also shows a correlation to C31, thus assigning this resonance to C30H″.

The proton and ^{13}C assignments of C55 and C56 can also be made from the HMBC spectrum by comparing the intensity of their correlations to C57, which was assigned from the correlation between this carbonyl carbon and Pr1H_2. In Figure 5, three correlations to C57 can be seen. Two are of greater intensity than the third. The less intense one can be assigned to C56H″ in the following way: One would expect protons on the same carbon to show correlations of similar intensity to a given carbon. For example, in Figure 5 both C37 methylene protons show correlations of similar intensity to C38. It can also be seen in Figure 5 that the two C49 methylene protons show correlations with similar intensities to C50, while C48H″ shows a correlation to C50 with a different (weaker) intensity. The proton signal (C56H″) with the weaker correlation to C57 also shows a correlation to a carbon that has been unambiguously assigned to C55 from a correlation to C54H_3. This carbon signal (C55) cannot possibly be assigned to C56 because C54H_3 is too many bonds away from C56 to show any correlation in the HMBC spectrum. Therefore, the proton signal that shows weaker correlation to C57 must be C56H″. The other two proton signals that show a stronger correlation to C57 were then assigned to C55H′ and C55H″. The remaining methylene proton in this side chain (identified from the HOHAHA spectrum; see above), C56H′, is buried under the noise from C35H_3/C53H_3 in the HMBC spectrum. The ^{13}C shift of C56 was determined from the correlation of this carbon to C55H′.

Assignment of the ^{13}C chemical shifts of C42 and C49, which was impossible from the HMQC spectrum (Figure 3), is readily done from the HMBC spectrum (Figure 5). C41H″ shows a correlation to C42. C48H″ and C13H both show correlations to C49. These resonances and those of C55 and C56 were most easily assigned by comparing the relative positions of C42, C46, C49, C55, and C56 in the HMBC spectrum. These resonances occur in a very crowded region of the ^{13}C spectrum and exact chemical shifts cannot be determined precisely. The HMBC spectrum shows that correlations for C42 and C49 (from C41H″ and C48H″, respectively) have the same ^{13}C chemical shift and are upfield of a correlation from C47H_3 to C46 (whose exact ^{13}C chemical shift was determined from an INEPT experiment; see above), assigning them to the peak at 34.0 ppm in the one-dimensional ^{13}C spectrum. The correlation from C54H_3 to C55 is downfield of the correlation to C46 and upfield of the correlation to C56 (Figures 4 and 5), assigning the peaks at 34.3 and 35.0 ppm to C55 and C56, respectively, and completing the ^{13}C assignments.

The ^1H and ^{13}C assignments of AdoCbi$^+$ are now complete. The corresponding ^1H and ^{13}C resonances of AdoCbi$^+$ and coenzyme B_{12} at pH 2.1 and 7.0 are shown in Table II.

Discussion

The absence of signals for the dimethylbenzimidazole nucleotide in the ^1H NMR spectrum of AdoCbi$^+$ confirms the finding[5] that the dimethylbenzimidazole nucleotide loop has been chemically removed. The ^1H and ^{13}C resonances whose chemical shifts show the greatest difference when comparing AdoCbi$^+$ to protonated, base-off coenzyme B_{12} are those of Pr2. This is consistent with cleavage occurring at the O–P bond, leaving Pr2 adjacent to a hydroxyl group in AdoCbi$^+$ instead of a phosphate group as it is in coenzyme B_{12}. There is no evidence for cleavage at a second site or for isomerization at any site in the cobinamide since the ^1H and ^{13}C chemical shifts of Pr1, which is only one bond removed from Pr2, hardly differ when comparing AdoCbi$^+$ to protonated, base-off coenzyme B_{12}. The small differences in shifts for other carbons in this part of the base-off species (Pr3 and C55) suggest some difference in the f side chain conformation results from cleavage of the nucleotide loop.

The sugar on the adenosyl moiety appears to have the same major conformation in both AdoCbi$^+$ and coenzyme B_{12} as judged by ^1H NOE data. The evidence for very similar conformations of the adenosyl moiety in AdoCbi$^+$ and coenzyme B_{12} is the NOE cross peaks A11H–C54H_3, A11H–C46H_3, A14H–C19H, and A15H′–C19H, which are found for AdoCbi$^+$ and both base-on and protonated, base-off coenzyme B_{12}. However, in AdoCbi$^+$ there is no NOE between A14H and C46H_3, an NOE that was used as evidence for an equilibrium between two conformations of the adenosyl moiety in both base-on and protonated, base-off coenzyme B_{12}.[13b]

Most of the adenine carbons of AdoCbi$^+$ have ^{13}C NMR chemical shifts that are closer to those found for base-on coenzyme B_{12} than the protonated, base-off form. The greatest shift differences are the A2 and A6 signals, probably due to the protonation of N1, which occurs at pH 2.1, conditions necessary to form base-off coenzyme B_{12}. Since N1 of adenosine has a pK_a of 3.6,[25] this nitrogen would be protonated at pH 2.1 (protonated, base-off coenzyme B_{12}) but not at pH 7.0 (base-on coenzyme B_{12}). The ^{13}C NMR shifts of A2 and A6 in AdoCbi$^+$ are much closer to those of coenzyme B_{12} in the base-on form than the protonated, base-off form, most likely due to the fact that at pH 4.8 N1 of the adenosyl moiety has not been protonated.

Except for the differences noted above, the ^1H and ^{13}C chemical shifts of AdoCbi$^+$ are generally closer to those of the benzimidazole-protonated, base-off form than the base-on form of the coenzyme, which is expected since AdoCbi$^+$ is serving as a "base-off" analogue for coenzyme B_{12}. The A15 ^{13}C resonances of both AdoCbi$^+$ and protonated, base-off coenzyme B_{12} are shifted substantially upfield in comparison to base-on coenzyme B_{12} by 5.7 and 5.0 ppm, respectively, indicating that whatever is in the trans axial position in protonated, base-off coenzyme B_{12} is also

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Table II. ^{1}H and ^{13}C NMR Chemical Shift and Signal Assignments for (5′-Deoxyadenosyl)cobinamide at pH 4.8 and Comparisons to the Corresponding Signals in (5′-Deoxyadenosyl)cobalamin (Coenzyme B_{12}) at pH 2.1 and pH 7.0[a]

	^{13}C NMR			^{1}H NMR		
	cobinamide	coenzyme		cobinamide	coenzyme	
assignment	pH 4.8	pH 2.1[b]	pH 7.0[c]	pH 4.8	pH 2.1[b]	pH 7.0[c]
C35	18.2	18.4	18.3	2.46	2.43	2.45
C53	18.2	18.4	18.8	2.46	2.46	2.43
C25	19.2	19.4	19.9	1.52	1.48	1.36
C54	20.6	20.8	19.6	1.24	1.40	1.36
A15	21.6	22.3	27.3	0.74	1.46	1.55
				0.43	0.38	0.57
C36	22.0	21.8	21.7	1.87	1.82	1.70
C47	22.3	22.6	23.9	1.66	1.67	1.32
Pr3	22.3	21.5	21.7	1.21	1.23	1.21
C20	26.7	26.7	23.5	0.91	0.81	0.47
C30	27.9	29.0	29.2	2.18	2.11	2.06
				2.03	1.97	1.96
C48	28.7	28.9	30.3	2.42	2.21	2.22
				2.07	1.92	2.00
C41	29.1	29.4	28.7	2.54	2.21	1.75
				1.92	1.75	0.81
C42	34.0	35.2	34.8	2.43	2.35	0.88
				2.33		1.73
C49	34.0	35.0	38.1	2.26	2.21	2.54
				1.83	1.86	
C46	34.1	34.4	34.2	0.92	1.00	0.87
C55	34.3	35.2	34.6	2.37	2.51	2.45
				1.84	1.85	2.06
C56	35.0	34.4	34.4	2.54	2.31	1.78
				2.06	1.85	
C60	35.2	35.2	34.8	2.73	2.78	2.65
				2.63		
C31	37.8	37.9	38.3	2.62	2.55	2.50
C18	42.0	42.2	42.5	2.92	2.85	2.65
C37	45.7	45.6	45.3	2.32	2.61	2.19
				1.79	2.14	1.72
C26	45.7	46.2	46.2	2.78	2.60	2.41
				2.42	2.46	
C2	48.5	47.4	49.5			
Pr1	49.0	48.9	47.8	3.30	3.38	3.54
				3.28	3.27	3.16
C12	49.1	49.5	49.5			
C7	53.0	53.2	53.1			
C13	55.1	55.4	55.8	3.50	3.43	2.89
C3	58.0	58.1	58.5	4.32	4.23	4.10
C8	58.1	58.1	57.5	3.87	3.73	3.29
C17	61.7	61.8	60.8			
Pr2	69.1	75.2	76.0	3.98	4.36	4.33
A13	74.9	74.9	76.6	3.87	3.90	3.74
A12	75.4	76.0	75.6	4.50	4.34	4.54
C19	77.6	77.6	76.8	4.77	4.70	4.24
A14	88.6	88.9	88.6	2.05	1.98	2.54
C1	89.8	89.8	88.5			
A11	90.2	91.1	91.0	5.71	5.61	5.56
C10	100.2	100.4	97.7	7.06	6.97	5.93
C15	110.1	109.8	106.9			
C5	111.0	111.2	108.4			
A5	121.5	121.5	121.8			
A8	143.2	145.5	143.8	8.13	8.21	8.00
A4	151.6	151.0	151.8			
A2	155.4	148.4	156.0	8.31	8.43	8.19
A6	158.1	153.5	158.7			
C14	165.7	166.7	167.2			
C6	166.2	166.2	166.6			
C9	174.7	175.1	173.1			
C38	177.6	177.4	177.9			
C57	178.2	178.3	178.2			
C61	178.7	179.1	179.0			
C16	178.87	179.4	178.7			
C11	178.93	179.0	177.6			
C27	178.99	179.1	179.3			
C4	179.07	179.4	178.7			
C43	180.9	180.9	180.3			
C32	181.0	181.1	181.1			
C50	181.1	181.0	181.1			

[a] Shifts relative to internal trimethylsilyl propionate. [b] Reference 13b. [c] Reference 13a.

in the trans axial position in AdoCbi$^+$. It has been observed in ^{13}C-enriched alkyl corrinoids[26] that substitution of a strong-field ligand (e.g., dimethylbenzimidazole) by a weak one (e.g., water) leads to substantial upfield shifts of the carbon attached to cobalt. The ^{13}C shifts of the methine bridge carbons C5, C10, and C15 differ by at most 0.3 ppm between AdoCbi$^+$ and protonated, base-off coenzyme B_{12}, indicating that the electronic environments of the corrin ring are similar in both compounds. Downfield shifts of these signals on substitution of a strong-field ligand by a weak one in either axial position[27] have been attributed to a decreased charge density at the methine bridge carbons caused by increased electron demand by cobalt centers bearing weak-field axial ligands.[28] Downfield shifts of >2.5 ppm in C5, C10, and C15 are seen upon going from base-on coenzyme B_{12} to the protonated, base-off form (Table II).

Another chemical shift related to the electronic environment of the corrin ring is the ^1H chemical shift of C10H, which has been shown to correlate with the energy of the first π–π^* electronic transition in the corrin.[22] As the chemical shift of C10H moves upfield, the electronic absorption bands move to lower energy, indicating an increase in the charge density at C10. The ^1H chemical shift of C10H differs by <0.1 ppm between AdoCbi$^+$ and protonated, base-off coenzyme B_{12}, while C10H is shifted >1 ppm upfield in base-on coenzyme B_{12}. Since there is little difference in the ^{13}C chemical shifts of the methine bridge carbons and in the ^1H chemical shift of C10H between AdoCbi$^+$ and protonated, base-off coenzyme B_{12}, the cobalt must have a similar coordination environment in both compounds.

The ^{13}C shifts of the other corrin ring carbons of AdoCbi$^+$ are very similar (in most cases <0.5 ppm different) to those of coenzyme B_{12} at pH 2.1, indicating that the conformations of the corrin ring in both compounds are very similar. Two exceptions are the ^{13}C shifts of C2 and C14, which are 1.1 ppm downfield and 1.0 ppm upfield, respectively, of the values found for these carbons in coenzyme B_{12} at pH 2.1. The diamagnetic anisotropy of the carbonyl groups of the b and e side chains could be the cause of the differences in shifts for C2 and C14 between AdoCbi$^+$ and coenzyme B_{12} at pH 2.1 since they may be in different positions in the coenzyme and cobinamide. Analysis of the X-ray crystal structures of several corrinoids in which the dimethylbenzimidazole nucleotide is intact has indicated that the c, d, and e side chains exhibit conformational diversity.[29] The absence of the dimethylbenzimidazole nucleotide in AdoCbi$^+$ could increase the conformational mobility of these side chains.

In support of this conformational diversity, a change in the positions of the b, d, and e propionamide side chains is indicated by ^{13}C shifts that are 1.1, 1.2, and 1.0 ppm upfield for C30, C42, and C49, respectively, in AdoCbi$^+$ compared to coenzyme B_{12} at pH 2.1. There are also substantial differences (>0.2 ppm) in the ^1H chemical shifts of at least one of the protons of the c, d, and e side chains between AdoCbi$^+$ and protonated, base-off coenzyme B_{12}. Theses differences are most likely caused by the absence of the dimethylbenzimidazole nucleotide, which allows the side chains to be in slightly different positions. Therefore, we have no strong evidence from the corrin or side-chain signals for any specific interactions between these moieties and the protonated benzimidazole group.

In contrast, a comparison of the ^{13}C NMR spectrum of the dianion of α-ribazole 3′-phosphate with the corresponding resonances of seven *unprotonated*, base-off alkylcobalamins, formed by the displacement of the benzimidazole of base-on cobalamins by cyanide, shows some evidence of an association of the dimethylbenzimidazole with the remainder of the structure.[30] The unprotonated, base-off form of coenzyme B_{12} was not included in the study because it undergoes cyanolysis of the upper Co–C bond in excess cyanide.[31] Evidence for the point of the interaction of the pendant dimethylbenzimidazole in unprotonated, base-off cobalamins with the remainder of the structure was found by comparing the ^{13}C NMR spectra of unprotonated, base-off dicyanocobalamin and dicyanocobinamide.[30] Hydrogen bonding between an e side chain N–H and the benzimidazole nitrogen (B3) was indicated by significant differences in the ^{13}C chemical shifts of C48, C49, and C50 between the two compounds. Our comparison of the ^{13}C NMR spectra of AdoCbi$^+$ and protonated, base-off coenzyme B_{12} provides no firm evidence for any association between the e side chain and the dimethylbenzimidazole in protonated, base-off coenzyme B_{12}. Only one of the three e side chain carbons, C49, shows a significant (>0.2 ppm) difference in ^{13}C chemical between AdoCbi$^+$ and protonated, base-off coenzyme B_{12}. Likewise, a comparison of the ^{13}C NMR spectrum of the zwitterion of α-ribazole 3′-phosphate with the corresponding nucleotide resonances of five protonated, base-off cobalamins (including coenzyme B_{12})[30] also failed to provide any evidence of association of the protonated dimethylbenzimidazole with the remainder of the structure.

In *summary*, all the NMR results confirm the chemical nature of AdoCbi$^+$ and reveal no evidence for isomerization. The corrin ring conformations in the cobinamide and in coenzyme B_{12} are similar, justifying the comparison of Co–C bond dissociation energies. Chemical shift differences do reveal electronic differences between the ring systems, consistent with the absence of a coordinated dimethylbenzimidazole group in the cobinamide. Although the adenosyl moiety has the same major conformation in AdoCbi$^+$ and both base-on and protonated, base-off coenzyme B_{12}, no firm evidence was obtained for an equilibrium between two conformations of the adenosyl moiety in AdoCbi$^+$, as found previously[13b] in coenzyme B_{12}. In previous studies of protonated, base-off coenzyme B_{12} the adenosyl moiety was protonated, as confirmed by ^{13}C NMR shifts in this study. The interaction between unprotonated, base-off dimethylbenzimidazole and the e side chain[30] cannot be detected in protonated species,[13b,30] and comparisons between ^{13}C shifts of AdoCbi$^+$ and protonated, base-off coenzyme B_{12} are consistent with this interpretation. This difference could result from preferential solvation of the B3H$^+$ moiety of protonated base-off 5,6-dimethylbenzimidazole as suggested by 2D NOE spectra of coenzyme B_{12} at pH 2.1.[13b] Thus, significant differences in the conformation of the nucleotide loop are likely between unprotonated and protonated, base-off cobalamins.

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Registry No. AdoCbi$^+$, 81670-23-7.

Supplementary Material Available: The COSY spectrum, part of the HOHAHA spectrum showing connectivities between the methylene protons, the ROESY spectrum showing the NOE connectivities of the downfield protons, the absorption-mode NOE spectrum, the HMQC spectrum, part of the HMBC spectrum showing the methine proton region, and part of the HMBC spectrum of AdoCbi$^+$ at pH 3.8 (10 pages). Ordering information is given on any current masthead page.

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