Heteronuclear NMR Studies of Cobalt Corrinoids. 14. Amide ^{1}H and ^{15}N NMR Studies of Base-On Cobalamins[1]

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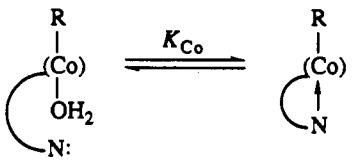
The amide ^{15}N and ^{1}H NMR resonances of a series of nine base-on cobalamins in DMSO-d_6 have been observed by inverse-detected ^{1}H, ^{15}N HMQC spectroscopy, in order to evaluate the possibility of the use of ^{15}N NMR as a probe of axial nucleotide ligation in macromolecular complexes of cobalt corrinoids with proteins. While the chemical shifts of the nucleotide loop (f side chain) amide nitrogen atoms fail to correlate with the strength of axial nucleotide coordination, the chemical shifts of the nucleotide loop amide proton show a smooth, if nonlinear, dependence on the free energy of formation of the base-on species. Amide proton chemical shift thermal gradients for the f amide proton clearly indicate that this proton is involved in an intramolecular hydrogen bond in solution. In addition, the proton chemical shift thermal gradients of the more downfield (*anti*) amide proton of the d side chain is dependent on the free energy of formation of the base-on species in a manner that suggests that the d amide is intramolecularly hydrogen bonded only in those cobalamins in which the axial nucleotide is relatively weakly coordinated. This is consistent with the idea (previously suggested for base-on 5′-deoxyadenosylcobalamin, i. e., coenzyme B_{12}) that, in those derivatives, the d amide is hydrogen bonded to the axial nucleotide glycoside nitrogen.

Introduction

In earlier work[2,3] the ^{31}P NMR chemical shift of the phosphodiester moiety of the nucleotide loop of base-on cobalamins (Cbl's)[4] was found to be a sensitive indicator of the strength of coordination of the axial 5,6-dimethylbenzimidazole ligand to the central cobalt atom. In fact, if the latter was expressed as the free energy of formation of the base-on species from the base-off, but 5,6-dimethylbenzimidazole free base, species (eq 1) (values



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readily calculable from the measured values of the base-on/base-off pK_a's,[3,5,6]), the ^{31}P chemical shifts were found to be linearly related to the values of ΔG_{Co}.[3] In addition, for the three base-on β-RCbl's whose crystal structures were known (β-AdoCbl,[7,8] β-CNCbl,[9] and CH_3Cbl[10]), the ^{31}P chemical shift was found to be linearly related to the axial Co–N bond distance.[3] This sensitivity of the phosphodiester ^{31}P resonance to the strength of axial nucleotide coordination was attributed to the known dependence of the ^{31}P chemical shifts of phosphate esters and phosphodiesters to O–P–O bond angles[11–14] and to a progressive increase in strain in the nucleotide loop with the shortening of the axial Co–N bond as ΔG_{Co} (eq 1) becomes more negative. Thus, the use of ^{31}P NMR as a probe of axial Co–N bond length, for instance for complexes of cobalt corrinoids with proteins, became a possibility.

However, while the use of ^{31}P NMR as such a probe for the complexes of H_2OCbl and CNCbl with a haptocorrin from chicken serum provided useful and interesting information,[15] it also demonstrated the shortcomings of ^{31}P NMR for the interrogation of such macromolecular species. First, the lack of any protons directly attached to the phosphorus atom prevents the use of polarization transfer or inverse detection methodologies for sensitivity enhancement. In addition, since the natural abundance of ^{31}P is 100%, the sensitivity cannot be enhanced by enrichment. Most importantly, the natural line widths of the ^{31}P resonances of RCbl's have been calculated to be about 0.4 Hz,[15] and values of 0.25–0.76 s have been measured for T_2.[16] This means that the increase in transverse relaxation rate that accompanies the slower rate of molecular tumbling in the macromolecular complexes leads to unacceptably broad ^{31}P resonances. To make matters worse, the transverse relaxation of the phosphorus nucleus of phosphodiesters is dominated by chemical shift anisotropy (CSA) since the ^{31}P nucleus has a small magnetic moment and the nearest proton is three bonds away, leading to inefficient dipolar relaxation.[15,17] Since CSA relaxation increases as the square of the field strength, the use of higher field to increase sensitivity is prohibited by excessive line broadening. Thus, for haptocorrin–cobalamin complexes, even at 4.7 T (i.e., 81 MHz) ^{31}P line widths of about 20 Hz were observed at 25 °C,[15] while at 11.75 T (202 MHz) the ^{31}P resonance is broadened to about 50 Hz.[16] The net result is that observation of the ^{31}P NMR resonance of such

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(4) Abbreviations: Cbl = cobalamin, $(CN)_2$Cbl = dicyanocobalamin, β-RCbl = β-alkylcobalamin (the alkyl ligand is in the "upper" axial ligand position), α-RCbl = α-alkylcobalamin (the alkyl ligand is in the "lower" axial ligand position), β-RCbi = β-alkylcobinamide (the axial nucleotide has been chemically removed), α-RCbi = α-alkylcobinamide, Ado = 5′-deoxyadenosyl, Bzm = 5,6-dimethylbenzimidazole. See Figure 1 for the structure and numbering scheme of the β-RCbl's.
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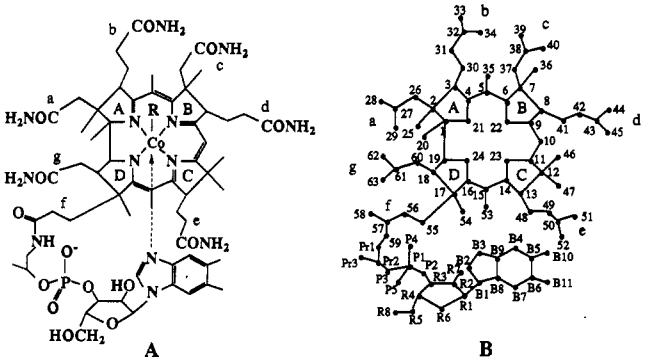

Figure 1. Structure (A) and numbering scheme (B) of a base-on β-alkylcobalamin (β-RCbl).

complexes requires large amounts of protein (0.2–1.3 mM in ~2.0-mL volume), the accumulation of tens to hundreds of thousands of scans, and thus long acquisition times.

We have consequently attempted to determine if the ^{15}N resonance of the cobalamin f amide (i.e., the nucleotide loop) side chain (Figure 1) or the ^{1}H resonance of the f amide proton can function as a probe of axial Co–N bond strength. Despite the inherent insensitivity of ^{15}N NMR, it has several advantages over ^{31}P NMR for observation of the nucleotide loop of Cbl–protein complexes. Both tranverse and longitudinal relaxations of the ^{15}N nucleus are quite slow in small molecules,[18] leading to exceedingly sharp resonances (i.e., observed line widths are dominated by inhomogeniety broadening) but also to the necessity of long delays before pulse repetition. Thus, the increase in relaxation rates accompanying macromolecular complexation[18] is unlikely to produce significant broadening, and it also permits more rapid pulsing. In addition, enrichment (the natural abundance is 0.365%), polarization transfer,[18,19] and inverse detection[1,20] can all be used to increase sensitivity, and the latter two methods also ensure that rapid pulsing will be possible. It thus seems likely that ^{15}N NMR of protein-bound cobalt corrinoids may actually be easier to accomplish than ^{31}P NMR.

In addition, we are most interested in studying the interactions of cobalt corrinoids with the proteins that bind them and have recently speculated that hydrogen-bonding interactions involving the corrin side chain amides are important in stabilizing the complexes of cobalamins with the haptocorrin from chicken serum.[21] In fact, studies of the activity of AdoCbl analogs in which the b, d, or e side chain amides have been hydrolyzed or N-methylated with several AdoCbl-requiring enzymes[22–25] are consistent with the idea that side chain amide hydrogen bonds are important in the binding of AdoCbl to enzymes. Since both amide ^{15}N[26–30] and ^{1}H[31–33] chemical shifts, as well as amide proton chemical shift thermal gradients,[31,34] are known to be sensitive to intramolecular hydrogen bonding, such measurements are ideally suited to detect hydrogen-bond interactions between Cbl's and proteins. Indeed, recent studies of 5'-deoxyadenosylcobalt corrinoids[1] have shown the utility of such observations for detecting hydrogen bonding in cobalt corrinoids. Most interestingly, a hydrogen bonding interaction involving one of the d side chain amide protons was found to occur only in the base-on species of β-AdoCbl, prompting speculation that the acceptor was the glycoside nitrogen of the axial nucleotide (B1, Figure 1).[1] We have consequently undertaken the following study of the ^{15}N and ^{1}H NMR characteristics of a series of nine base-on β-RCbl's in order to assess the suitability of such measurements as probes of Cbl structure and Cbl–protein interactions in Cbl–protein complexes, as well as to further characterize the d amide hydrogen-bonding bonding interaction previously identified in β-AdoCbl.

Experimental Section

$H_2OCbl{\cdot}OAc$ was from Roussel. β-Alkylcobalamins were synthesized by reductive alkylation of $(H_2OCbl)OAc$, purified, and characterized as described previously.[5,35–37] Two-dimensional ^{1}H, ^{15}N HMQC NMR spectra were obtained at 30 °C on a Bruker AMX300 NMR spectrometer using a 5-mm inverse broad-band probe at 300.136 MHz (^{1}H) and 30.415 MHz (^{15}N). Samples were 40–60 mM in β-RCbl in DMSO-d_6 (0.5 mL) and contained TSP as an internal ^{1}H reference. ^{15}N chemical shifts were referenced to external CH_3NO_2, but are reported relative to $NH_3(l)$ using $\delta_{CH_3NO_2}$ = 380.23 ppm.[38] The ^{1}H, ^{15}N HMQC spectra[1,20,39] were collected into 512 × 256 data matrices using 2623- and 3042-Hz sweep widths in the ^{1}H and ^{15}N dimensions, respectively. Totals of 512 and 1026 scans were collected per t_1 increment (138 μs). The data were processed with Gaussian multiplication using −5-Hz line broadening in both dimensions. Amide ^{1}H chemical shift gradients were measured on a GE QE300 NMR spectrometer operating at 300.669 MHz using the same samples. One-dimensional proton spectra were acquired at 5 °C temperature increments between 20 and 55 °C, and thermal gradients (reported as $-(\Delta\delta/\Delta T) \times 10^3$ ppm/°C) were determined from least-squares fits of chemical shift vs temperature data sets.

Results and Discussion

Amide ^{1}H and ^{15}N Chemical Shifts. As was the case with all other cobalt corrinoids observed to date,[1,20] the ^{1}H, ^{15}N HMQC spectra of the β-RCbl's in DMSO (Figure 2) consisted of 13 crosspeaks in the amide chemical shift region of both spectral dimensions. All but one of the nitrogen resonances had two crosspeaks corresponding to the *anti* (downfield) and *syn* (upfield)[40] protons of the six unsubstituted side chain amides (a, b, c, d, e, and g, Figure 1). The single nitrogen response that had only one crosspeak is therefore readily assignable to the f side chain substituted amide. The spectrum shown in Figure 2 for H_2OCbl was quite typical of all of the β-RCbl's investigated (R = CH_3CH_2, $CH_3CH_2CH_2$, $NC(CH_2)_3$, CH_3, CF_3CH_2, CF_3, CN),

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(40) As is the case for formamide,[41] acetamide,[42,43] NAD^+, and a number of substituted acetamides,[45] we assume that the downfield proton resonances of the side chain primary amides represent the *anti* (or *E*) amide protons and the upfield resonances represent the *syn* (or *Z*) protons.
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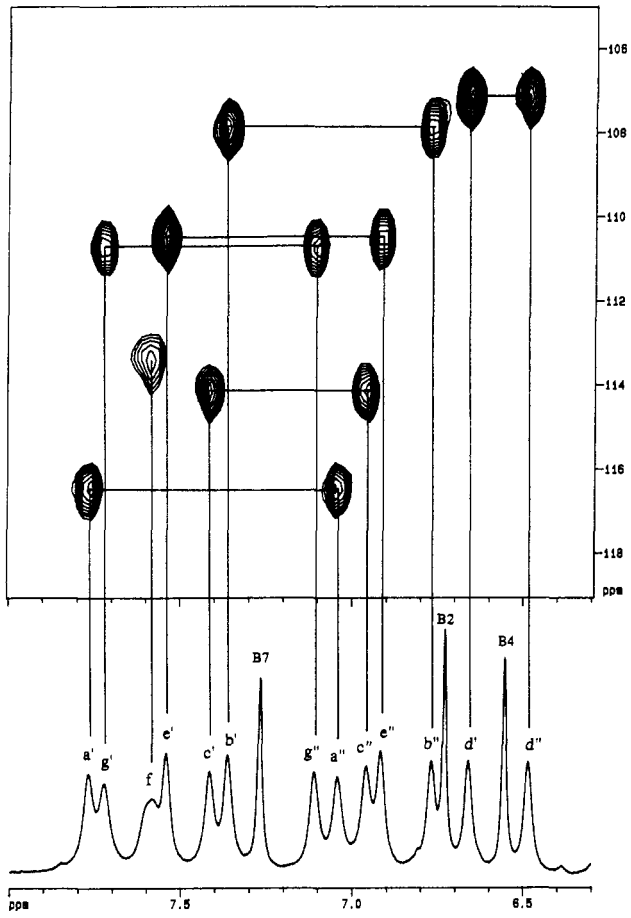

Figure 2. ^1H, ^{15}N HMQC spectrum of H_2OCbl (50 mM) in DMSO-d_6 showing the connectivities between the amide nitrogens and the amide *syn* and *anti* protons.

the HMQC spectra of which are shown in Figures S1–S7, respectively (available as supplementary material).

Complete assignments of the amide ^{15}N resonances of cobalt corrinoids have previously been made by observation of the through-space connectivities between the amide protons and those of the side chain methylenes, the corrin protons, and the corrin methyl groups.[1,46] Thus, when the complete assignments of the proton NMR spectrum have been available ($(CN)_2Cbl$,[46] base-on β-AdoCbl,[47] base-off β-AdoCbl,[48] β-AdoCbi,[49] α-AdoCbl,[37] and α-AdoCbi[37]), unambiguous assignments of the unsubstituted amide proton resonances, and hence of the amide nitrogen resonances from the HMQC connectivities, have been made possible by observation of the two-dimensional NOESY spectra in DMSO-d_6. In all such cases (i.e., six cobalt corrinoids in which the "upper" (or β) axial ligand is varied from H_2O, to CN^-, to Ado and the "lower" (or α) axial ligand is varied from H_2O, to CN^-, to Ado, to Bzm), the unsubstituted amide ^{15}N resonances have been found to fall in the order d, b, e, g, c, a, from high field to low. Only the position of the f amide ^{15}N resonance varies, falling between the g and c amide resonances in β-5′-deoxyadenosylcobalt corrinoids but between the c and a ^{15}N resonances in the α diastereomers. It thus seems likely that the same order of unsubstituted amide ^{15}N resonances will occur for the β-RCbl's, and these tentative assignments are shown in Figure 2 for H_2OCbl and summarized for all of the β-RCbl's in Table I. The corresponding amide ^1H chemical shift assignments are given in Table II.

Inspection of the chemical shifts in Tables I and II show that all of the β-RCbl's have ^1H and ^{15}N chemical shifts remarkably similar to those of β-AdoCbl;[1] i.e., the nature of the "upper" axial ligand has only minor effects on the amide ^1H and ^{15}N resonances. The unsubstituted amide ^1H chemical shifts are so similar across the series of nine β-RCbl's (the variation across the series ranges from 0.04 ppm for the *syn* protons of the b and c amides to 0.18 ppm for the *anti* proton of the d amide) that the e and g proton resonances are readily assigned even for β-CF_3CH_2Cbl's for which the e and g amide ^{15}N resonances overlap (at least at the ^{15}N resolution of the HMQC spectra obtained here).

By far the widest variation in both the amide ^{15}N and ^1H chemical shifts across the series of β-RCbl's occurs for the f amide, i.e., the nucleotide loop amide, suggesting that these chemical shifts may, indeed, be sensitive to the strength of coordination of the axial nucleotide. However, the f amide ^{15}N chemical shifts do not vary in any regular manner with the strength of axial ligation, as shown by the plot of $\delta_{^{15}N}$ vs $-\Delta G_{Co}$ (eq 1) in Figure 3A. In contrast, the f amide ^1H chemical shifts vary smoothly, if nonlinearly, with $-\Delta G_{Co}$, as shown in Figure 3B. Combined with our earlier observation that $\delta_{^1H} = 8.01$ ppm for the *f* amide proton of β-5′-deoxyadenosyl(3,5,6-trimethylbemzimidazolyl)cobamide, an analog of β-AdoCbl in which the axial Bzm is N-methylated at B3 (Figure 1) and hence unable to coordinate, these data suggest that progressive strain in the nucleotide loop due to increased strength of Bzm coordination causes a progressive downfield shift of the f amide proton resonance until a limiting value ($\delta_{^1H} \sim 7.58$ ppm) is reached for derivatives in which the axial Bzm is very tightly bound ($-\Delta G_{Co} > 10$ kcal mol^{-1}). Thus, the f amide ^1H chemical shift may well serve as a useful probe of axial ligation in protein complexes of cobalamins enriched in ^{15}N in which appropriate isotope-edited experiments should allow selective observation of the cobalamin amide protons, and the f amide proton resonance is readily assignable from an isotope-edited ^1H, ^{15}N HMQC map.

Amide ^1H Chemical Shift Thermal Gradients and Hydrogen Bonding in β-RCbl's. In our recent work with the 5′-deoxyadenosylcobalt corrinoids,[1] we noted with surprise that the chemical shifts of the d amide *syn* and *anti* protons of β-AdoCbl were identical at 30 °C. Since in all other cobalt corrinoids which had been studied up to that time the chemical shifts of the d amide protons differed by 0.4–0.6 ppm, this suggested that one of the d amide protons of β-AdoCbl might be involved in an intramolecular hydrogen bond since amide ^1H chemical shifts are known to be sensitive to hydrogen bonding.[31-33] This possibility was investigated by a study of the chemical shift thermal gradients, $-(\Delta\delta/\Delta T)$, of the 5′-deoxyadenosylcobalt corrinoids, since intramolecular hydrogen bonding is known to significantly reduce such gradients. The results strongly suggest that both the f amide proton and one of the d amide protons were hydrogen bonded in base-on β-AdoCbl, since their chemical shift thermal gradients were -1.44×10^{-3} and -2.45×10^{-3} ppm °C^{-1}, respectively, while those of all of the other amide protons studied ranged from 3.35 $\times 10^{-3}$ to 5.88 $\times 10^{-3}$ ppm °C^{-1}. The results shown in Table II show that none of the base-on β-RCbl's have chemical shift equivalent d amide protons except for β-AdoCbl. However, the difference in chemical shift between the d amide *anti* and *syn* protons ($\Delta\delta = 0.06$–0.18 ppm, not including those of β-AdoCbl) is much smaller than that for any other amide proton pair ($\Delta\delta = 0.41$–0.72 ppm). Thus, while the chemical shift difference between the d amide *anti* and *syn* protons of the newly studied β-RCbl's is not quite so striking as that of β-AdoCbl, these values of $\Delta\delta$ do suggest the possibility of hydrogen bonding.

We have consequently studied the temperature dependence of the β-RCbl amide proton chemical shifts, and the thermal

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Table I. Tentative Amide ^{15}N NMR Chemical Shift Assignments for the β-RCbl's[a]

R	$\delta_{^{15}N}$, ppm d	b	e	g	f	c	a	$-\Delta G_{Co}$,[b] kcal mol^{-1}
CH_3CH_2	107.3	108.2	110.4	110.8	112.9	113.8	116.0	1.77
$CH_3CH_2CH_2$	107.2	107.9	110.0	110.5	112.7	113.7	115.9	1.87
Ado[c]	106.7	108.0	110.0	110.4	112.3	114.3	116.1	2.57
$NC(CH_2)_3$	107.2	108.0	110.5	110.6	112.7	113.8	116.4	2.78
CH_3	107.1	108.1	110.4	110.7	113.3	113.7	116.0	3.64
CF_3CH_2	106.5	107.6	110.3	110.3	112.4	113.6	115.7	4.04
CF_3	107.1	107.9	110.6	110.8	113.3	114.1	116.1	5.62
CN	107.2	107.9	110.6	111.0	113.4	114.4	116.6	7.44
H_2O	107.1	107.9	110.4	110.7	113.4	114.1	116.5	10.48

[a] DMSO-d_6, 30 °C. [b] Calculated from values of K_{Co} (eq 1) at 25 °C. [c] Reference 1.

Table II. Tentative Amide ^1H NMR Chemical Shift Assignments and Chemical Shift Thermal Gradients for the β-RCbl's[a]

R	$\delta_{^1H}$, ppm ($-(\Delta\delta/\Delta T) \times 10^3$, ppm °C^{-1}) d	b	e	g	f	c	a
CH_3CH_2	6.60 (3.55 ± 0.08)	6.78 (4.68 ± 0.07)	6.89 (6.32 ± 0.06)	7.16 (3.46 ± 0.07)	7.90 (−1.89 ± 0.11)	6.97 (5.13 ± 0.05)	7.08 (4.42 ± 0.06)
	6.76 (−1.49 ± 0.20)	7.36 (3.87 ± 0.24)	7.58 (5.32 ± 0.20)	7.63 (2.67 ± 0.09)		7.56 (4.21 ± 0.04)	7.70 (4.61 ± 0.03)
$CH_3CH_2CH_2$	6.61 (3.37 ± 0.04)	6.80 (4.40 ± 0.04)	6.91 (6.24 ± 0.09)	7.15 (3.01 ± 0.09)	7.89 (−1.34 ± 0.12)	7.01 (5.00 ± 0.02)	7.10 (4.29 ± 0.02)
	6.74 (−1.13 ± 0.08)	7.39 (3.38 ± 0.07)	7.59 (5.15 ± 0.06)	7.74 (4.01 ± 0.14)		7.62 (4.14 ± 0.05)	7.82 (5.69 ± 0.08)
Ado[b]	6.58 (3.56 ± 0.10)	6.77 (4.56 ± 0.05)	6.86 (5.88 ± 0.04)	7.18 (5.10 ± 0.04)	7.83 (−1.44 ± 0.12)	6.95 (4.73 ± 0.05)	7.15 (5.10 ± 0.04)
	6.58 (−2.45 ± 0.21)	7.36 (3.35 ± 0.18)	7.52 (3.77 ± 0.19)	7.59 (4.96 ± 0.05)		7.54 (4.83 ± 0.14)	7.76 (4.96 ± 0.05)
$NC(CH_2)_3$	6.60 (4.33 ± 0.06)	6.78 (5.21 ± 0.08)	6.93 (6.21 ± 0.02)	7.13 (4.12 ± 0.09)	7.81 (−0.41 ± 0.10)	6.97 (5.09 ± 0.04)	7.12 (4.71 ± 0.04)
	6.70 (1.84 ± 0.02)	7.38 (3.55 ± 0.04)	7.62 (4.30 ± 0.07)	7.74 (4.72 ± 0.09)		7.57 (4.71 ± 0.04)	7.80 (5.74 ± 0.16)
CH_3	6.55 (4.71 ± 0.10)	6.76 (5.17 ± 0.11)	6.89 (5.82 ± 0.06)	7.11 (5.03 ± 0.08)	7.72 (−0.02 ± 0.24)	6.96 (5.31 ± 0.05)	7.08 (4.71 ± 0.05)
	6.62 (4.06 ± 0.04)	7.34 (3.81 ± 0.03)	7.57 (3.30 ± 0.04)	7.62 (4.61 ± 0.07)		7.55 (4.29 ± 0.04)	7.73 (4.98 ± 0.07)
CF_3CH_2	6.56 (4.23 ± 0.05)	6.79 (4.82 ± 0.02)	6.95 (5.60 ± 0.02)	7.11 (4.87 ± 0.09)	7.71 (1.28 ± 0.26)	7.01 (5.12 ± 0.07)	7.09 (4.39 ± 0.04)
	6.62 (3.13 ± 0.04)	7.36 (3.51 ± 0.02)	7.62 (4.43 ± 0.11)	7.74 (4.90 ± 0.05)		7.62 (3.31 ± 0.03)	7.80 (5.00 ± 0.06)
CF_3	6.53 (4.47 ± 0.03)	6.79 (4.47 ± 0.03)	6.94 (4.97 ± 0.05)	7.14 (5.10 ± 0.04)	7.65 (1.89 ± 0.19)	7.00 (4.98 ± 0.03)	7.07 (4.28 ± 0.06)
	6.65 (4.23 ± 0.03)	7.37 (3.57 ± 0.05)	7.59 (3.44 ± 0.03)	7.69 (4.37 ± 0.09)		7.59 (3.44 ± 0.03)	7.75 (4.83 ± 0.04)
CN	6.50 (4.49 ± 0.26)	6.76 (5.14 ± 0.07)	6.91 (5.49 ± 0.08)	7.14 (5.50 ± 0.08)	7.60 (0.99 ± 0.25)	7.01 (5.29 ± 0.09)	7.11 (4.91 ± 0.08)
	6.68 (4.43 ± 0.20)	7.34 (3.76 ± 0.11)	7.54 (4.41 ± 0.07)	7.64 (4.54 ± 0.12)		7.54 (4.41 ± 0.07)	7.76 (5.14 ± 0.07)
H_2O	6.51 (4.53 ± 0.06)	6.80 (4.71 ± 0.04)	6.95 (5.08 ± 0.03)	7.15 (5.21 ± 0.02)	7.59 (1.07 ± 0.11)	7.00 (5.17 ± 0.03)	7.08 (4.52 ± 0.02)
	6.69 (3.98 ± 0.04)	7.39 (3.47 ± 0.02)	7.57 (3.80 ± 0.03)	7.75 (4.11 ± 0.04)		7.45 (4.89 ± 0.03)	7.80 (4.83 ± 0.02)

[a] DMSO-d_6, 30 °C. [b] Reference 1.

gradients observed are listed in Table II. With the exception of the d amide *anti* proton and the f amide proton, all of the proton thermal gradients fall in the range (2.67–5.88) × 10^{-3} ppm °C^{-1}, and all save one fall in the range (3.01–5.88) × 10^{-3} ppm °C^{-1}. This is quite similar to the range (3.35–5.88) × 10^{-3} ppm °C^{-1} previously observed for the non-hydrogen-bonded amide protons of the 5′-deoxyadenosylcobalt corrinoids[1] and the range (3.39–6.16) × 10^{-3} ppm °C^{-1} previously observed for dicyanocobalt corrinoids.[20] However, all of the chemical shift thermal gradients for the f amide protons of the β-RCbl's are substantially smaller, ranging from −1.89 × 10^{-3} ppm °C^{-1} (for β-CH_3CH_2Cbl) to 1.89 × 10^{-3} ppm °C^{-1} (for β-CF_3Cbl). It thus seems likely that, as previously concluded for β-AdoCbl,[1] the f amide proton is involved in an intramolecular hydrogen bond,[50] despite the fact that, in the neutron diffraction structure of β-AdoCbl,[8] the f amide proton is hydrogen bonded to a water of crystallization. As discussed previously,[1] inspection of models suggests that the f amide could be hydrogen bonded to either the g or e amide carbonyl.

The situation for the d amide *anti* proton is more complex. For four of the β-RCbl's (R = CH_3CH_2, $CH_3(CH_2)_2$, Ado, and $NC(CH_2)_3$) the d amide *anti* proton chemical shift gradient falls between −2.45 × 10^{-3} and 1.84 × 10^{-3} ppm °C^{-1}. However, for the other five β-RCbl's, the thermal gradients for this proton fall in the "normal" range of (3.13–4.43) × 10^{-3} ppm °C^{-1}. For base-on β-AdoCbl, the neutron diffraction structure indicates a hydrogen bond between one of the d amide protons and the a amide carbonyl. However, the d amide proton with the low chemical shift thermal gradient ($-(\Delta\delta/\Delta T)$ = −2.45 × 10^{-3} ppm

(50) In a diamide compound designed to have two symmetrical hydrogen bonds between identical primary hydrogens,[51] the intramolecularly hydrogen bonded protons have proton chemical shift thermal gradients of 2.35 × 10^{-3} ppm °C^{-1} while for the solvent exposed protons the value is 4.5 × 10^{-3} ppm °C^{-1}.

(51) Rebek, J., Jr.; Marshall, L.; Wolak, R.; Parris, K.; Killovan, M.; Askew, B.; Nemeth, D.; Islam, N. *J. Am. Chem. Soc.* **1985**, *107*, 7476.

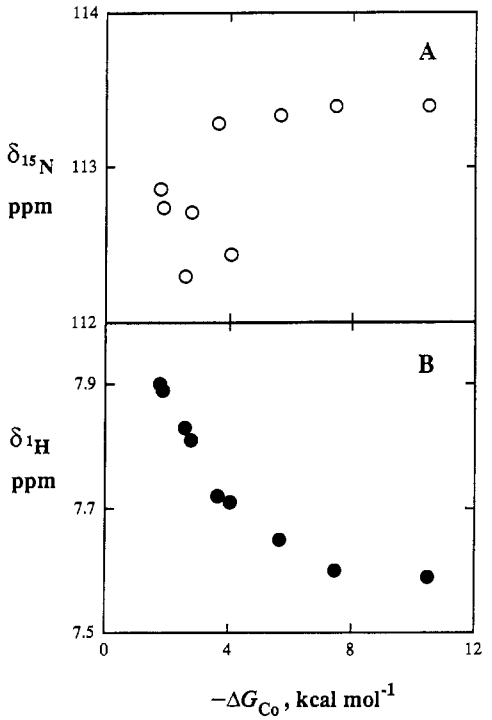

Figure 3. (A) Plot of $\delta_{^{15}N}$ for the f amide of the β-RCbl's vs $-\Delta G_{Co}$ (eq 1). The values of $-\Delta G_{Co}$ were calculated from the values of K_{Co} at 25 °C in ref 6. (B) Plot of $\delta_{^{1}H}$ for the f amide of the β-RCbl's vs $-\Delta G_{Co}$ (eq 1). The values of $-\Delta G_{Co}$ were calculated from the values of K_{Co} at 25 °C in ref 6.

°C^{-1}), had normal chemical shift thermal gradients in base-off β-AdoCbl, in α-AdoCbl, in which the organic ligand is in the "lower" axial ligand position and the Bzm ligand is uncoordinated, and in the 5′-deoxyadenosylcobinamides, in which the Bzm ligand has been chemically removed.[1] Since the hydrogen-bonded interaction of the d amide proton depended absolutely on the presence of a coordinated Bzm ligand, we postulated that the d amide proton with the low chemical shift thermal gradient could be participating in a hydrogen bond with the axial nucleotide glycoside nitrogen (B1, Figure 1), on the basis of the observation of a seemingly unstrained conformation in models in which the d amide and B1 are brought into close proximity. The current study seems to support this hydrogen bond assignment. The four compounds for which the d amide *anti* proton has a low thermal gradient are characterized by relatively weak coordination of the Bzm ligand to the metal atom ($-\Delta G_{Co} < 3.0$ kcal mol^{-1}, $K_{Co} < 1.60 \times 10^2$, at 25 °C). In these compounds, the relatively small amount of forward donation of electron density from the Bzm ligand to the metal atom would be expected to leave the Bzm relatively electron rich and make the B1 nitrogen a reasonably good hydrogen bond acceptor. For the remainder of the compounds for which $-(\Delta\delta/\Delta T) > 3.0 \times 10^{-3}$ ppm °C^{-1}, the Bzm ligand is more tightly bound ($-\Delta G_{Co}$ and K_{Co} range from 3.64 kcal mol^{-1} and 4.67×10^2, respectively, for R = CH_3 to 10.48 kcal mol^{-1} and 4.90×10^7, respectively, for R = H_2O), suggesting that in these compounds the Bzm is sufficiently electron depleted by coordination that B1 is no longer a satisfactory hydrogen bond acceptor. In fact, a plot of $-(\Delta\delta/\Delta T)$ vs $-\Delta G_{Co}$ for the d amide *anti* proton (Figure 4) suggests that progressive increases in forward donation from the Bzm ligand to the cobalt center are accompanied by progressive weakening of the d amide *anti* proton–B1 hydrogen bond by electron depletion of the acceptor and that this results in an increase of the amide proton chemical shift

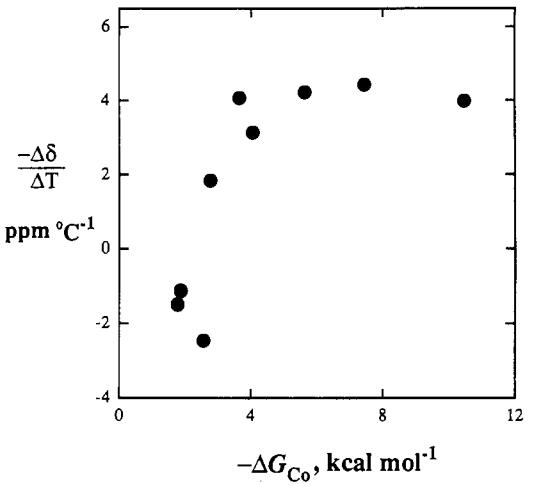

Figure 4. Plot of the proton chemical shift gradient, $-(\Delta\delta/\Delta T)$, for the d amide *anti* proton of the β-RCbl's vs $-\Delta G_{Co}$ (eq 1), the free energy (25 °C) of coordination of the axial 5,6-dimethylbenzimidazole ligand to the central cobalt atom.

thermal gradient from about -2×10^{-3} ppm °C^{-1} to leveling off at about 4×10^{-3} ppm °C^{-1}, a value presumably indicative of a fully solvent-exposed d amide *anti* proton. We conclude that, for those β-RCbl's for which $-\Delta G_{Co} < 3.0$ kcal mol^{-1}, the d amide nitrogen is hydrogen bonded via its *anti* proton to the axial nucleotide glycoside nitrogen in solution.

Interestingly, the proton chemical shift thermal gradients of the f amide protons show a similar dependence on $-\Delta G_{Co}$ (not shown), although it is not as regular as in the case of the d amide *anti* proton, and it levels off at a value of $-(\Delta\delta/\Delta T) < 2.0 \times 10^{-3}$ ppm °C^{-1}. This suggests that the strength of the hydrogen bond to the neighboring carbonyl is influenced by the nucleotide loop conformation, which is known to be dependent on $-\Delta G_{Co}$.[2,3] However, since the limiting value of $-(\Delta\delta/\Delta T)$ suggests that the f amide is still hydrogen bonded in the compounds with the largest values of $-\Delta G_{Co}$, the leveling off of the thermal gradient implies that nucleotide loop conformation may cease to depend significantly on the strength of coordination beyond a limiting value of ΔG_{Co}.

The data in Table II together with our earlier data[1,20] also permit the estimation of criteria which can be used for discriminating cobalt corrinoid intramolecular hydrogen bonding on the basis of on amide proton chemical shift thermal gradients. From these data it seems reasonable to conclude that, for cobalt corrinoids, intramolecularly hydrogen-bonded amide protons will have $-(\Delta\delta/\Delta T) \leq 2.0 \times 10^{-3}$ ppm °C^{-1}, while those which are not hydrogen bonded and presumably solvent exposed will have $-(\Delta\delta/\Delta T) \geq 3.0 \times 10^{-3}$ ppm °C^{-1}. Such criteria should be useful in discerning hydrogen-bonded interactions in the complexes between cobalt corrinoids and proteins.

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Supplementary Material Available: Figures S1–S7, showing the assigned ^{1}H, ^{15}N HMQC spectra of the β-RCbl's, R = CH_3CH_2, $CH_3CH_2CH_2$, $NC(CH_2)_3$, CH_3, CF_3CH_2, CF_3, and CN, respectively (8 pages). Ordering information is given on any current masthead page.