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Heteronuclear NMR Studies of Cobalt Corrinoids Part 19—Amide ^{1}H and ^{15}N NMR Studies of Cobalamins and Cobinamides in Water

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Absolute assignments were made of the side-chain amide proton and nitrogen resonances of a series of cobalamins (Cbls, derivatives of vitamin B_{12}) and two cobinamides [Cbis, analogs in which the axial 5,6-dimethylbenzimidazole (Bzm) ligand has been chemically removed] in water. The *syn* and *anti* amide protons could always be distinguished based on the magnitudes of their NOEs to the neighboring methylene protons in ROESY spectra. The chemical shifts of the *c* amide nitrogen and *anti* proton of the two aqua complexes, aquacobalamin and 10-chloroaquacobalamin, are significantly perturbed from those of the other complexes and demonstrate that a hydrogen bond between the *c* carbonyl and the coordinated water molecule, previously seen in the x-ray crystal structures of these complexes, persists in aqueous solution. The ^{15}N chemical shift of the secondary *f* amide in the nucleotide loop of the Cbl's correlates linearly ($r^2 = 0.997$) with the solid-state axial Co—N bond distance, providing an excellent NMR probe of this bond length. Removal of the axial Bzm to form the Cbi's results in changes in the amide nitrogen and proton chemical shifts of the 'downwardly' projecting side-chains, characteristic of the influence of the magnetic anisotropy of the Bzm moiety on these amides in the Cbl complexes. Amide proton chemical shift thermal gradients and ^{15}N solvent-dependent chemical shifts are interpreted as being due to complex patterns of intramolecular hydrogen-bonded side-chain amides in equilibrium with forms fully hydrogen bonded to solvent. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Heteronuclear NMR has been extensively and increasingly used over the past 20 years to study the solution properties of vitamin B_{12} and its derivatives (Fig. 1). ^{15}N NMR of this class of compounds, originally hampered by low natural abundance and low sensitivity,[1–3] has benefited greatly from polarization transfer[4,5] and inverse detection methodologies.[5–11] We have been interested in the side-chain amide ^{1}H and ^{15}N NMR of cobalt corrinoids primarily because of the sensitivity of such amide resonances to hydrogen bonding effects.[12–19] Hydrogen bonding to cobalamin (Cbl) side-chain amides is now known to be important in the association of Cbls with enzymes [including the binding of methylcobalamin (CH_3Cbl) to methionine synthase,[20] and the binding of 5′-deoxyadenosylcobalamin (AdoCbl, coenzyme B_{12}) to methylmalonyl-coenzyme A mutase[21]] and is suspected to contribute significantly to the very tight binding of Cbl's to vitamin B_{12} binding proteins.[22] Because of the rapid exchange of Cbl amide protons with solvent in D_2O, almost all of the previous amide ^{15}N NMR work on these compounds has been done in DMSO-d_6. However, owing to the sensitivity of amide ^{1}H and ^{15}N to hydrogen bonding and the differences in hydrogen bond donor acidity and acceptor basicity between DMSO and H_2O,[23] Cbl amide ^{1}H and ^{15}N chemical shifts are expected to be significantly solvent dependent, and patterns of intramolecular hydrogen bonding previously revealed in DMSO-d_6 [5,7,8] may be substantially different in H_2O. Indeed, the ^{15}N NMR chemical shifts of simple amides are well known to be highly solvent dependent.[24] Current pulsed field gradient methodology for solvent suppression and modern hardware now permit the absolute assignment of Cbl amide ^{1}H NMR resonances in water. We now report the first thorough study of cobalt corrinoid amide ^{1}H and ^{15}N NMR in aqueous solution. A previous report of the amide chemical shifts of a single derivative (aquacobalamin (H_2OCbl)) in water has appeared.[10]

EXPERIMENTAL

Cyanocobalamin (CNCbl, vitamin B_{12}) and H_2OCbl were obtained from Roussel. CH_3Cbl,[25] AdoCbl,[26] trifluoromethylcobalamin (CF_3Cbl),[25] 10-chlorocyanocobalamin (10-Cl-CNCbl)[27] and 10-chloroaquacobalamin (10-Cl-H_2OCbl)[27] were prepared and characterized as described previously. Methylcobinamide (CH_3Cbi) and 5′-deoxyadenosylcobinamide (AdoCbi), the analogs

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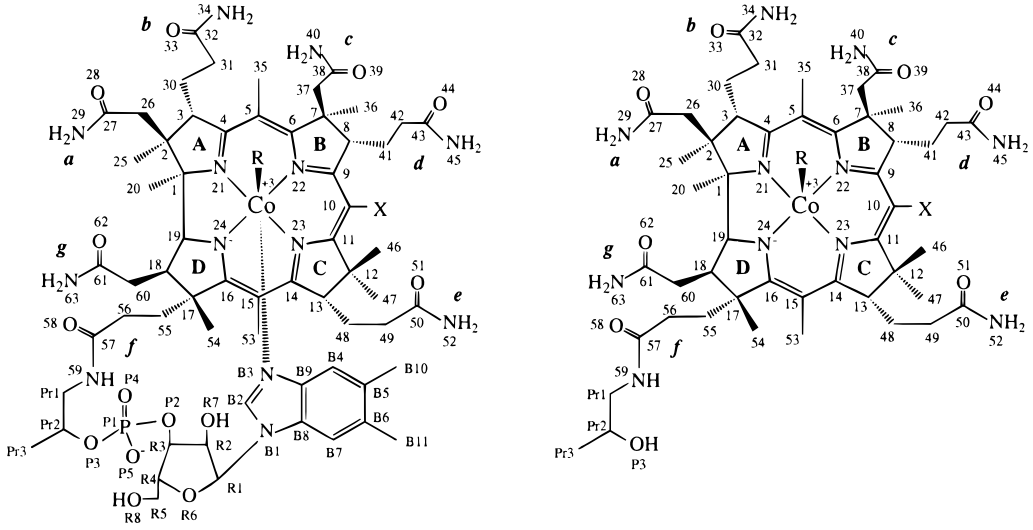
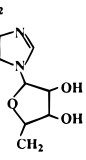


H_2OCbl	X = H	$R = H_2O$		CH_3Cbi	$R = CH_3$
10-Cl-H_2OCbl	X = Cl	$R = H_2O$			
CNCbl	X = H	R = CN		AdoCbi	R =
10-Cl-CNCbl	X = Cl	R = CN			
CF_3Cbl	X = H	$R = CF_3$			
CH_3Cbl	X = H	$R = CH_3$			
AdoCbl	X = H	R =			



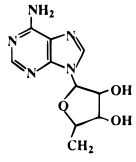


Figure 1. Structure of the cobalamins (Cbls) and cobinamides (Cbis).

of CH_3Cbl and AdoCbl lacking the axial nucleotide (Fig. 1), were obtained by reductive alkylation of aquacyanocobinamide (Factor B)[28] as described previously.[25,26] NMR samples *ca.* 0.7 ml, were prepared in H_2O–D_2O (90:10) and contained TSP as an internal 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.[29]

The ^1H, ^{15}N HMQC spectra were obtained at 300 K on a Bruker AMX 300 NMR spectrometer operating at 300.136 MHz (^1H) and 30.415 MHz (^{15}N). When necessary, spectra were obtained at other temperatures to remove ambiguities in cases of proton resonance overlap. Data were collected into 2048 × 256 data matrices over sweep widths of 15 ppm (^1H) and 225 ppm (^{15}N). The coupling delay was 5.27 ms and 128 scans, preceded by four dummy scans, were collected for each t_1 increment with a 1.2 s pulse delay and a 1.2–2.0 s presaturation pulse. Garp-Temp x-nucleus decoupling was used. The data were processed as 1024 × 512 data matrices with Gaussian multiplication (Gaussian factor 0.1) using − 5 Hz line broadening. The truncation in F_2 improved the signal-to-noise ratio, albeit with a slight sacrifice of resolution.

Rotating frame Overhauser enhancement (ROESY) spectra were obtained on a Bruker AMX 600 NMR spectrometer operating at 600.130 MHz. Data were collected into 2048 × 1024 matrices over sweep widths of 15 ppm in both dimensions with pulsed field gradient solvent suppression. The total spin-lock period was 200 ms, and 32–64 scans, as needed, preceded by two dummy scans, were collected for each t_1 increment. The data were processed as 2048 × 2048 data matrices with either shifted sine or Gaussian apodization. TOCSY spectra were collected similarly, except that the total spin-lock period was 70 ms including the 2.5 ms trim pulse at the beginning and end of the MLEV pulse train. These data were processed as 1024 × 1024 matrices with $\pi/2$ shifted Q-sine apodization.

When needed, ^1H, ^{13}C HMQC spectra were obtained on a Bruker AMX 600 spectrometer using Garp-Temp x-nucleus decoupling. Data were collected into 2048 × 256 data matrices over sweep widths of 12 ppm (^1H) and 225 ppm (^{13}C). A coupling delay of 2.37 ms was used, and 128 scans, preceded by two dummy scans, were collected for each t_1 delay. The data were processed as 1024 × 512 data matrices with $\pi/3$ shifted Q-sine apodization.

RESULTS

For those complexes for which absolute ^1H NMR assignments are known (H_2OCbl,[30] 10-Cl-CNCbl,[27] CNCbl,[31,32] CH_3Cbl,[11] AdoCbl[33] and AdoCbi[34]) the

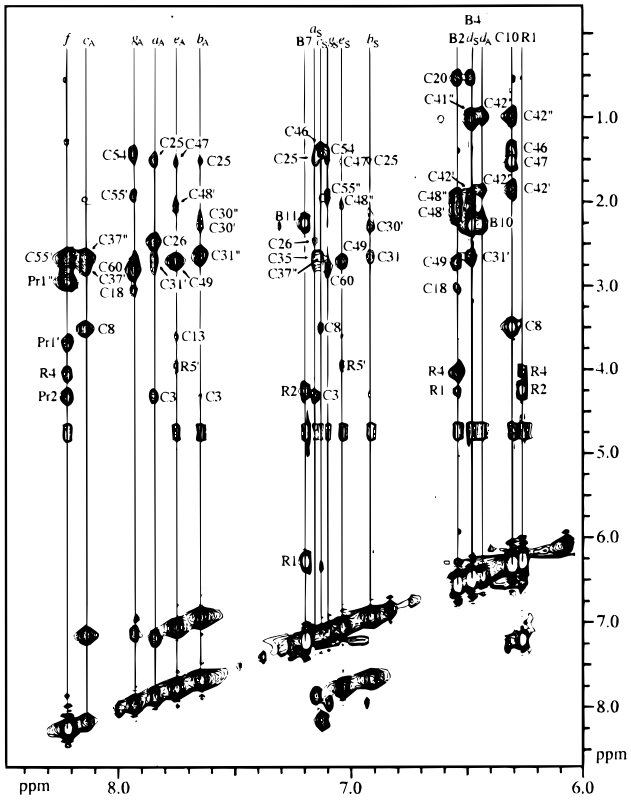


Figure 2. Downfield portion of the ROESY spectrum of H_2OCbl in H_2O–D_2O (90:10) showing the assignments of the amide proton resonances. Prime and double prime designations indicate the downfield and upfield signals, respectively, of diastereotopic methylene groups.

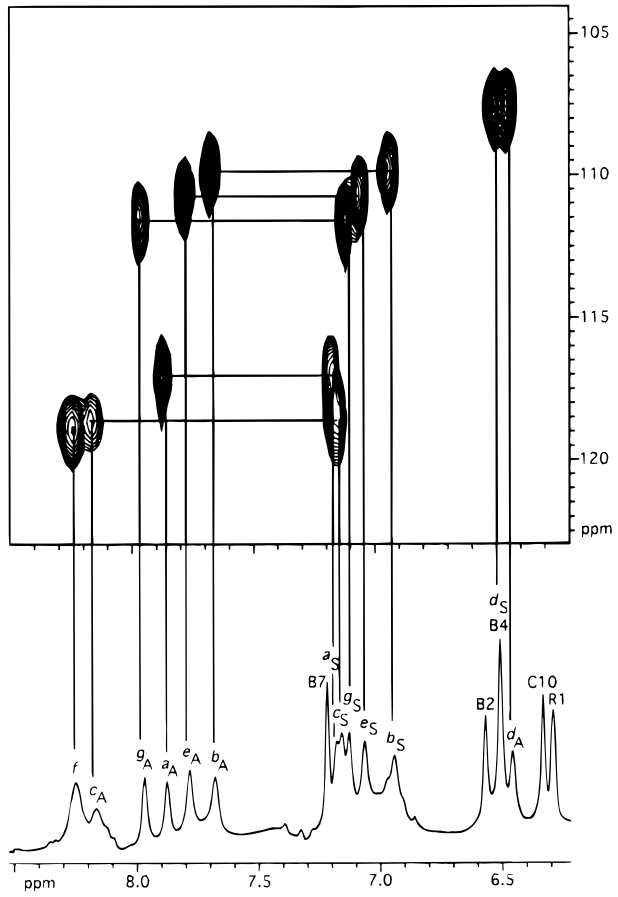


Figure 3. ^1H, ^{15}N HMQC spectrum of H_2OCbl in H_2O–D_2O (90:10) showing the assignments of the amide ^{15}N resonances.

corrinoid amide protons could be assigned from their proximity to side-chain methylene groups, peripheral methyl groups and corrin ring hydrogens as determined from the ROESY spectra in water at a 200 ms mixing time. An example is shown in Fig. 2 for H_2OCbl. For CH_3Cbi, the ^1H (and ^{13}C) spectrum in H_2O was assigned using a standard battery of 2D NMR experiments and assignment strategies described previously.[35,36] The

Table 1. Amide ^{15}N chemical shift assignments for cobalamins and cobinamides in H_2O

	$\delta_{^{15}N}$ (ppm)[a]							
Corrinoid	*d*	*b*	*e*	*g*	*c*	*a*	*f*	d_{Co-Nax}[b] (Å)
H_2OCbl[c]	107.6	109.9	110.7	111.6	118.8	117.0	119.0	1.925 (2)[d]
10-Cl-H_2OCbl	107.0	109.7	110.3	111.5	118.4	116.4	118.6	1.967 (4)[e]
CNCbl	107.3	109.7	110.5	111.6	115.0	116.2	118.3	2.011 (10)[f]
10-Cl-CNCbl	106.7	109.5	110.0	111.4	114.8	115.9	118.0	2.043 (14)[e]
CF_3Cbl	106.9	109.7	110.4	111.3	114.3	115.9	117.9	2.047 (10)[g]
CH_3Cbl	106.7	109.3	110.4	111.4	114.8	116.2	116.9	2.19 (2)[h]
AdoCbl	107.1	110.2	110.7	111.4	114.5	116.0	116.4	2.24[i]
Average	107.0 ± 0.3	109.7 ± 0.3	110.4 ± 0.2	111.4 ± 0.1	114.7 ± 0.3[j]	116.2 ± 0.4		
Average (DMSO)[k]	107.0 ± 0.3	107.9 ± 0.2	110.4 ± 0.2	110.6 ± 0.2	113.9 ± 0.3	116.1 ± 0.3	112.9 ± 0.4	
CH_3Cbi	108.7	110.2	109.2	111.9	114.2	116.0	117.0	
CH_3Cbi	108.2	110.2	109.0	111.5	114.5	115.7	116.9	

[a] Reported relative to NH_3 (l).
[b] The ^{15}N chemical shifts reported here for H_2OCbl differ by 5.4 ± 0.1 ppm from those reported in Ref. 10, apparently owing to an external referencing discrepancy.
[c] Axial Co—N_{B3} bond distance in the solid state.
[d] Ref. 10.
[e] Ref. 27.
[f] Ref. 37.
[g] Ref. 38.
[h] Ref. 39.
[i] Ref. 40.
[j] Chemical shifts for H_2OCbl and 10-Cl-H_2OCbl omitted from the average (see text).
[k] Ref. 8.

amide ^{15}N resonances could then be assigned from their connectivities to the amide ^{1}H resonances in the ^{1}H and ^{15}N HMQC spectra (Fig. 3). For CF_3Cbl and 10-Cl-H_2OCbl, amide assignments were made by analogy with those of the other RCbl's. The amide ^{15}N assignments are given in Table 1. For the RCbl complexes, there is little change in chemical shift across the series for each amide except for the *c* and *f* amides. The latter clearly shows a distinct trend across the series of compounds. For the other amides, the average chemical shift and standard deviation for the series of RCbl complexes in water are given, except for the *c* amide for which the chemical shifts of the two aqua complexes (H_2OCbl and 10-Cl-H_2OCbl) clearly deviate significantly from the chemical shifts of the others, and these have been omitted from the calculation of the average shift. For comparison, the average chemical shifts of the amide nitrogens of a series of nine RCbl's in DMSO-d_6 are also given.[8]

For all of the primary amides, it was always possible to distinguish the *syn* and *anti* protons based on the magnitudes of the NOEs between the two amide protons of a given side-chain and the methylene protons adjacent to the amide carbonyl. In all cases except for the *d* amide of the RCbl complexes, the more downfield of each pair of amide ^{1}H resonances showed a stronger NOE to the adjacent methylene protons and was consequently assigned to the *anti* proton. These assignments are in agreement with those previously made for simpler amides.[41–44] For the *d* amide of the RCbl's, these assignments were reversed, the more upfield ^{1}H resonance having the stronger NOE to the adjacent methylene. For the two RCbi complexes, however, the *d* amide protons behaved like those of the other amides, the more downfield resonance being the *anti* proton. The amide proton chemical shift assignments are summarized in Table 2. Again, with the exception of the *c* amide *anti* hydrogens of H_2OCbl and 10-Cl-H_2OCbl,

Table 2. Amide ^{1}H chemical shift assignments and chemical shift thermal gradients for cobalamins and cobinamides in water[a]

	$\delta_{^1H}$ (ppm) [$-(\Delta\delta/\Delta T) \times 10^3$ (ppm °C^{-1})][b]						
Compound	*d*	*b*	*e*	*g*	*c*	*a*	*f*
H_2OCbl	6.46[c]	6.94	7.06	7.13	7.16	7.18	8.25
	(5.35 ± 0.35)	(6.48 ± 0.11)	(7.58 ± 0.11)	(6.25 ± 0.11)	(8.28 ± 0.13)	(7.03 ± 0.30)	(10.23 ± 0.24)
	6.51[d]	7.68	7.78	7.97	8.16	7.88	
	(5.69 ± 0.37)	(7.64 ± 0.15)	(7.79 ± 0.15)	(8.10 ± 0.15)	(8.95 ± 0.17)	(6.22 ± 0.10)	
10-Cl-H_2OCbl	6.35[c]	6.93	7.04	7.11	7.11	7.14	8.21
	6.56[d]	7.65	7.77	7.94	8.08	7.86	
CNCbl	6.40[c]	6.88	7.01	7.08	7.05	7.12	8.25
	(5.14 ± 0.09)	(5.92 ± 0.05)	(6.70 ± 0.06)	(5.86 ± 0.08)	(6.98 ± 0.12)	(7.79 ± 0.07)	(8.62 ± 0.16)
	6.40[d]	7.61	7.74	7.93	7.48	7.84	
	(5.14 ± 0.09)	(7.40 ± 0.15)	(7.24 ± 0.10)	(7.75 ± 0.07)	(5.91 ± 0.05)	(7.29 ± 0.07)	
10-Cl-CNCbl	6.35[c]	6.92	7.03	7.10	7.03	7.14	8.20
	6.56[d]	7.64	7.75	7.96	7.54	7.86	
CF_3Cbl	6.36[c]	6.89	7.02	7.08	7.05	7.09	8.18
	6.41[d]	7.61	7.76	7.91	7.54	7.73	
CH_3Cbl	6.11[c]	6.81	7.04	6.99	6.97	7.05	8.17
	(4.23 ± 0.07)	(5.35 ± 0.13)	(10.14 ± 0.46)	(6.04 ± 0.23)	(8.94 ± 0.09)	(5.81 ± 0.09)	(7.79 ± 0.16)
	6.32[d]	7.54	7.77	7.87	7.78	7.76	
	(5.04 ± 0.16)	(6.62 ± 0.15)	(6.89 ± 0.25)	(8.02 ± 0.19)	(14.54 ± 0.43)	(6.89 ± 0.25)	
AdoCbl	6.27[c]	6.88	6.98	7.05	6.84	7.12	8.22
	(5.77 ± 0.07)	(6.46 ± 0.32)	(6.52 ± 0.07)	(6.16 ± 0.06)	(3.36 ± 0.20)	(6.54 ± 0.15)	(8.27 ± 0.05)
	6.41[d]	7.61	7.76	7.88	7.52	7.84	
	(5.06 ± 0.07)	(7.00 ± 0.05)	(8.49 ± 0.06)	(8.62 ± 0.08)	(5.94 ± 0.02)	(7.69 ± 0.06)	
Cbl average	6.33 ± 0.11[c]	6.89 ± 0.04	7.03 ± 0.03	7.08 ± 0.05	7.01 ± 0.13	7.11 ± 0.07	8.20 ± 0.05
	6.45 ± 0.09[d]	7.62 ± 0.04	7.76 ± 0.01	7.92 ± 0.04	7.53 ± 0.04[e]	7.81 ± 0.08	
DMSO average[f]	6.56 ± 0.04	6.78 ± 0.02	6.91 ± 0.03	7.14 ± 0.02	6.99 ± 0.02	7.10 ± 0.03	[g]
	6.67 ± 0.06	7.37 ± 0.02	7.58 ± 0.03	7.68 ± 0.06	7.56 ± 0.02	7.77 ± 0.04	
CH_3Cbi	6.70	6.97	6.65	7.17	6.89	7.14	8.01
	(3.93 ± 0.09)	(5.31 ± 0.05)	(4.96 ± 0.08)	(5.59 ± 0.07)	(6.62 ± 0.17)	(6.01 ± 0.06)	(6.58 ± 0.11)
	7.47	7.67	7.28	8.02	7.60	7.83	
	(6.13 ± 0.04)	(6.19 ± 0.06)	(5.54 ± 0.06)	(7.07 ± 0.09)	(8.85 ± 0.25)	(6.58 ± 0.04)	
AdoCbi	6.59	6.94	6.56	7.15	6.93	7.11	7.95
	(2.56 ± 0.07)	(4.83 ± 0.03)	(3.21 ± 0.05)	(5.54 ± 0.02)	(2.94 ± 0.23)	(4.68 ± 0.20)	(8.25 ± 0.11)
	7.73	7.64	7.18	7.86	7.65	7.92	
	(5.50 ± 0.03)	(6.40 ± 0.03)	(3.33 ± 0.11)	(6.50 ± 0.03)	(6.40 ± 0.03)	(6.00 ± 0.04)	

[a] Chemical shifts relative to internal TSP. The more upfield resonance of each amide pair is the *syn* proton and the more downfield is the *anti* proton except as noted.
[b] Values in parentheses.
[c] *Anti* proton.
[d] *Syn* proton.
[e] Chemical shifts for H_2OCbl and 10-Cl-H_2OCbl omitted from the average. See text.
[f] Ref. 8.
[g] Chemical shift trends across the series.

there is little variation of the individual amide proton chemical shifts across the series of RCbl complexes, and the average values are given in Table 2 along with the average values for these chemical shifts for nine RCbl's in DMSO-d_6.[8]

Amide chemical shift thermal gradients $[-(\Delta\delta/\Delta T) \times 10^3]$ have been widely used to study intramolecular hydrogen bonding in peptides[17,18,45,46] and have been used previously to infer hydrogen bonding patterns in Cbl amides in DMSO-d_6.[8] We consequently measured the amide proton chemical shift thermal gradients for H_2OCbl, CNCbl, CH_3Cbl, AdoCbl, CH_3Cbi and AdoCbi and these results are also summarized in Table 2.

DISCUSSION

With the exception of the *f* secondary amide (see below), the only significant deviations of amide ^{15}N chemical shifts across the series of RCbl complexes are for the *c* amide nitrogen of H_2OCbl and 10-Cl-H_2OCbl, which are shifted downfield about 4 ppm relative to the other RCbl *c* amide nitrogens (Table 1). X-ray diffraction studies of both complexes[10,27] show that in the solid state there is a hydrogen bond between the *c* amide carbonyl and one of the hydrogens of the coordinated water molecule. Such intramolecular hydrogen bonds in which an amide carbonyl acts as an acceptor are well known to cause downfield shifting of the amine nitrogen resonance.[13–16] In addition, the *c* amide *anti* protons in H_2OCbl and in 10-Cl H_2OCbl are shifted downfield by *ca.* 0.5 ppm relative to their positions in the other RCbl's (Table 2). These results provide compelling evidence that this hydrogen bond persists in aqueous solution and, taken together with ^{1}H[10] and ^{13}C[11] solution NMR evidence for this hydrogen bond in H_2OCbl, must be considered as conclusive proof. Importantly, no such downfield shift of the *c* amide ^{15}N resonance of H_2OCbl relative to other RCbl's is seen in DMSO-d_6.[8] Evidently in this solvent, competition among hydrogen bonding between the *c* amide carbonyl and the coordinated water, and between these partners and solvent, substantially favors hydrogen bonding to the solvent, and the intramolecular hydrogen bond seen in the solid state is disrupted, a critically important solvent effect. As pointed out previously,[11] the persistence of the intramolecular hydrogen bond to the coordinated water ligand in aqueous solution must be expected to influence significantly the energetics of ligand substitution in H_2OCbl, since these reactions are known to be dissociatively controlled.[47–49] The importance of this hydrogen bond in H_2OCbl ligand dissociation energetics is under study.

The *f* side-chain secondary amide nitrogen is clearly exceptional. Its chemical shift varies significantly (by nearly 3 ppm) across the series of RCbl's, and, in fact, varies as a function of the axial Co—N bond length (Table 1). The ^{31}P NMR chemical shift of the phosphodiester of the nucleotide loop of RCbl's is known to vary linearly with the axial Co—C bond length,[11,38,50] evidently as a result of the sensitivity of ^{31}P chemical shifts of phosphate esters to O—P—O angles[51–54] and a progressive change in the strain of the nucleotide loop as the Co—N_{ax} bond length changes from 1.925 Å in H_2OCbl[10] to 2.24 Å in AdoCbl.[40] An astonishingly good linear correlation ($r^2 = 0.997$, $n = 7$) between the ^{15}N chemical shift of the nucleotide loop *f* amide and the axial Co—C bond length is now established as well (Fig. 4). This suggests that nucleotide loop strain is also felt at the amide nitrogen and that this nitrogen's chemical shift is extraordinarily sensitive to the attendant changes in nitrogen orbital hybridization. This linear correlation (slope $= -7.93 \pm 0.19$ ppm Å^{-1}) shows substantially greater sensitivity of the *f* amide ^{15}N resonance to the axial Co—C bond length than that of the phosphodiester ^{31}P resonance (slope $= -2.26 \pm 0.08$ ppm Å^{-1}), which varies by little more than 1 ppm across the series of compounds.[38] The *f* amide ^{15}N resonance thus emerges as the most sensitive NMR indicator of axial Co—C bond length with great potential for interrogating this bond in protein–Cbl complexes.

This relationship between the *f* amide ^{15}N chemical shift and the axial bond length was not observed in DMSO.[8] In DMSO, the *f* amide ^{15}N chemical shift varied by only 1 ppm across a series of nine RCbl's and did not correlate with the axial bond length. A possible reason for this is the complication of intramolecular hydrogen bonding involving the *f* amide in DMSO, as evidenced by severely retarded amide proton chemical shift thermal gradients in this solvent. This is evidently not the case in water (see below) and so the dependence of the *f* amide ^{15}N chemical shift on nucleotide loop strain is not confounded by hydrogen bonding effects on chemical shift.

Removal of the axial 5,6-dimethylbenzimidazole (Bzm) ligand to form the RCbi's causes a *ca.* 1.4 ppm downfield shift, a *ca.* 1.4 ppm upfield shift and a *ca.* 0.5 ppm downfield shift of the amide ^{15}N resonances of the

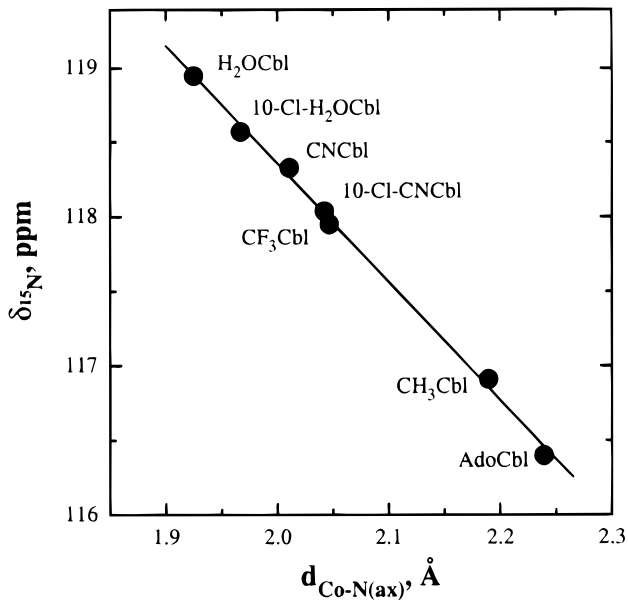


Figure 4. Plot of the ^{15}N chemical shift of the *f* amide of the RCbl's *vs.* the axial Co—N bond length from their x-ray crystal structures. The solid line is a linear regression, slope = −7.93 ± 0.19 ppm Å^{-1}, intercept = 134.2 ± 0.4 ppm (r^2 = 0.997).

'downwardly' projecting *d*, *e* and *b* propionamide side-chains, respectively, but much smaller (and probably not significant) changes in the amide ^{15}N chemical shifts of the 'upwardly' projecting *a*, *c* and *g* acetamide side-chains. These chemical shift changes evidently reflect the influence of the magnetic anisotropy of the Bzm ligand in the Cbl complexes on the amide nitrogens of the 'downwardly' projecting side-chains. Evidently, in the Cbl's, the *d* amide is in the shielding region of the Bzm and the *e* amide is in the deshielding region, and both of these amides remain, on average, sufficiently close to the Bzm, despite their thermal motional freedom, for their nitrogens to be significantly shifted by the Bzm magnetic anisotropy. The *b* amide is also evidently in the Bzm shielding region, but is much less affected by the Bzm anisotropy. These conclusions are in accord with the disposition of these side-chains in the solid-state x-ray diffraction structures of Cbl's.[10,27,38–40,55] In addition, the differences in amide proton chemical shifts between the RCbl's and RCbi's are in agreement with these conclusions. Thus, the *syn* and *anti* protons of the *d* amide are shifted downfield by *ca.* 0.3 and 1.2 ppm, respectively, in the Cbi's whereas the *syn* and *anti* protons of the *e* amide are shifted upfield by about 0.5 ppm. None of the other amide proton chemical shifts are significantly different between the Cbl's and the Cbi's.

The chemical shifts of the *d* amide protons of the RCbl complexes are clearly anomalous in water, as they also were in DMSO.[8] The average difference in chemical shift between the *syn* and *anti* protons of the *d* amide is only 0.13 ± 0.14 ppm in the RCbl's, while the average difference in chemical shift between the *syn* and *anti* protons of all of the other amides is 0.70 ± 0.12 ppm. Importantly, this anomaly does not appear in the *d* amides of the RCbi complexes ($\Delta\delta = \delta_{anti} - \delta_{syn} = 0.78$ ppm), demonstrating that it depends on the presence of the Bzm lower axial ligand. In DMSO, the anomalous *d* amide chemical shifts were attributed to hydrogen bonding of the *anti* proton, probably to the *N*-glycosidic nitrogen of the Bzm ligand, as evidenced by depressed proton thermal gradients for the d_{anti} proton, particularly in RCbl's with strongly electron-donating R groups.[8] However, there is no evidence for such hydrogen bonding in water. More likely, the anomalous *d* amide proton shifts are the result of the Bzm magnetic anisotropy, with one of the two *d* amide protons spending significantly more time in a more strongly shielding region of the Bzm field than the other.

The solvent-dependent changes in Cbl amide ^{15}N chemical shift (Table 1) are of considerable interest and potentially instructive of intramolecular hydrogen bonding patterns. Application of the Taft linear solvation energy equation[23] to the solvent-dependent ^{15}N chemical shifts of formamide[24] predicts that a solvent transfer from DMSO to water will shift an amide ^{15}N resonance downfield by about 2.4 ppm owing to differences in solvent hydrogen bond donor acidity and acceptor basicity. For the RCbl's, the *b* amide ^{15}N resonance is shifted downfield by 1.8 ± 0.3 ppm, the *g* amide resonance by 0.8 ± 0.3 ppm and the *c* amide resonance by 0.7 ± 0.4 ppm, none of the other amide ^{15}N resonances being significantly changed. One possible interpretation is that the *b* amide is completely solvent hydrogen bonded in both solvents, the *a*, *d* and *e* amides are completely intramolecularly hydrogen bonded in both solvents and the *c* and *g* amides are somewhere in between. However, this is probably much too simple a picture. In highly compact structures like proteins, where amides involved in intramolecular hydrogen bonding may well be completely shielded from the solvent, permanent intramolecular hydrogen bonds may well occur. In cobalt corrinoids, on the other hand, where the side-chain amides are necessarily more exposed to solvent, a more likely pattern is intramolecular hydrogen bonding in equilibrium with solvent hydrogen bonding.[56] The strength of the latter will clearly be dependent on the hydrogen bond donor and acceptor ability of the solvent, and so the extent of equilibrium intramolecular hydrogen bonding will vary from solvent to solvent. It thus seems likely that the solvent dependence of the Cbl amide ^{15}N chemical shifts represents subtle solvent-dependent changes in the equilibria between intramolecular hydrogen-bonded and solvent hydrogen-bonded amides.

Amide proton chemical shift thermal gradients have long been used to probe intramolecular hydrogen bonding in proteins, with amide protons displaying small thermal gradients assigned to intramolecular hydrogen bonds.[17,18,57,58] For the cobalt corrinoids in water, the amide proton chemical shift thermal gradients vary from 2.56 to 14.54 with most (82%) of the observations falling in the intermediate range 5–9 (Table 2). For perspective, the thermal gradients for the amide protons of acetamide in water were 25.3 ± 0.9 (*anti*) and 25.0 ± 0.9 (*syn*), but were only 7.15 ± 0.15 (*anti*) and 6.73 ± 0.18 (*syn*) in adipamide, a diamide which may well be in equilibrium between an intramolecularly hydrogen-bonded form and a fully solvent hydrogen-bonded form.[56] It therefore seems likely that the thermal gradients of the cobalt corrin amide protons represent multiple equilibria between intramolecularly hydrogen-bonded and solvent hydrogen-bonded forms, with the equilibrium for those amide protons displaying higher thermal gradients ($\gtrsim 9$) being displaced more towards solvent hydrogen-bonded forms and those with lower thermal gradients ($\lesssim 5$) being displaced more towards intramolecularly hydrogen-bonded forms.

Finally, the chemical shift thermal gradient of the c_{syn} amide proton of the 5′-deoxyadenosylcobalt corrins displays an interesting anomaly. Whereas the average thermal gradient of the c_{syn} proton of the other three RCbl's (H_2OCbl, CNCbl and CH_3Cbl) is 8.1 ± 1.0, in AdoCbl this thermal gradient is reduced to less than half that value (3.36 ± 0.20). A similar retardation of the c_{syn} thermal gradient occurs in AdoCbi compared with CH_3Cbi (Table 2). This observation suggests that the *syn* proton of the *c* amide may be involved in a hydrogen bond interaction with the adenosyl moiety of AdoCbl and AdoCbi, an interaction for which some evidence has previously been found in DMSO.[7]

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