Inorg. Chem. 1988, 27, 3548-3555

Contribution from the Department of Chemistry,
The University of Texas at Arlington, Arlington, Texas 76019

Heteronuclear NMR Studies of Cobalamins. 9. Temperature-Dependent NMR of Organocobalt Corrins Enriched in ^{13}C in the Organic Ligand and the Thermodynamics of the Base-On/Base-Off Reaction[1]

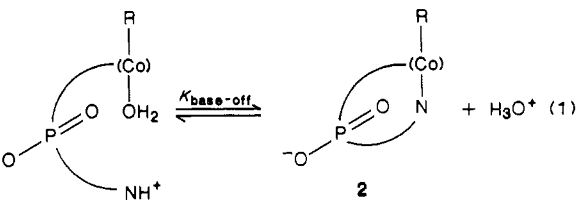
Kenneth L. Brown* and Sherri Peck-Siler

Received March 11, 1988

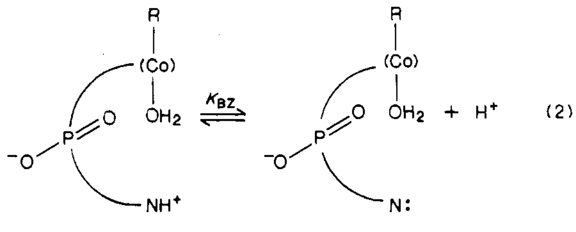
The synthesis of alkylcobinamides (RCbi) enriched in ^{13}C in the organic axial ligand by standard reductive alkylation procedures from ^{13}C-enriched alkyl halides has been shown to lead essentially exclusively to β-alkylcobamides (i.e., the organic ligand in the "upper" axial ligand position) by UV-visible and NMR spectroscopy and HPLC coupled with direct synthesis of $^{13}CH_3Cbi$ of known stereochemistry from ^{13}C-enriched methylcobalamin ($^{13}CH_3Cbl$). The ionic strength dependence of the on/off pK_a of CH_3Cbl, CH_3CH_2Cbl, and α-ribazole has also been investigated. Measurements of the relative ^{13}C resonance frequency of $^{13}CH_3Cbl$ and $^{13}CH_3Cbi$ as a function of temperature at several ionic strengths have been used to investigate the thermodynamics of the base-on/base-off reaction of cobalamins. Additional evidence has thus been obtained for a previously detected species of base-off, but benzimidazole-deprotonated, cobalamin in which the benzimidazole B3 nitrogen atom is hydrogen-bonded to an e side chain N-H, and the thermodynamics of formation of this species have been evaluated. These results have been confirmed by similar measurements on mixtures of $^{-}OOC^{13}CH_2Cbl$ and $^{-}OOC^{13}CH_2Cbi$. Although the new species is the predominant form (80-90%) of the base-off but benzimidazole-deprotonated cobalamins in solution at all ionic strengths, its presence has only a minor effect on the primary on/off equilibrium constants previously calculated for RCbl's.

Introduction

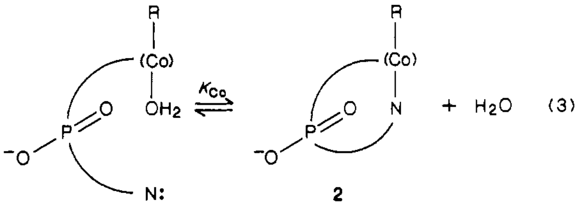
The well-known base-on/base-off reaction of cobalamins (eq 1)[2] is most simply viewed as the sum of the component equilibria in eq 2 and 3,[3-8] for which eq 4 relates $K_{\text{base-off}}$ (eq 1) to the equilibrium constants of eq 2 and 3. This reaction is so basic a chemical property of cobalamins that a thorough understanding of it is a necessary prerequisite to any attempt to master the chemistry and biochemistry of cobalamins. To date such an understanding of the base-on/base-off reaction is not at hand. For example, trends in the values of p$K_{\text{base-off}}$ for cobalamins with various upper axial ligands are not well-understood, and attempts to correlate these values with electronic and steric parameters of the upper axial ligand invariably fail.[8]



1 2



1 3



3 2

$$K_{\text{base-off}} = (1 + K_{\text{Co}})K_{\text{Bz}} \tag{4}$$

In a recent publication[9] one of us discussed the available evidence that eq 2-4 represent an oversimplified view of the base-on/base-off reaction, in that one or more species in addition to those shown in eq 2 and 3 are involved in a thermodynamically significant way. Such species would be those in which the pendant, but uncoordinated, axial nucleotide in either the protonated (**1**) or deprotonated (**3**) base-off species, or in both, is somehow associated noncovalently with the remainder of the structure; i.e., the nucleotide loop in the base-off species does not dangle freely. The occurrence of such species would, of course, alter the apparent equilibrium constants for the processes depicted in eq 2 and 3.

In the previous publication, methods for detecting and/or quantitating such additional species were also discussed.[9] Basically, two types of methods were suggested; spectroscopic methods and thermodynamic methods. Spectroscopic methods involve direct comparison of the spectral properties of the detached axial nucleoside (α-ribazole) or nucleotide (α-ribazole 3'-phosphate) with those of the appropriate base-off cobalamin species in order to detect the spectral consequences of any such noncovalent interactions on the pendant axial nucleotide. The converse experiment, in which the spectra of base-off cobalamins are directly compared with those of the relevant cobinamides (from which the axial nucleotide has been chemically removed), is capable of detecting the effect of such interactions on the spectra of the remainder of the molecule.

The thermodynamic method for detecting such additional species is in many ways complementary to the spectroscopic method. While the spectroscopic method can detect such species and possibly provide information regarding their structure, the thermodynamic method can detect the presence of such species and evaluate the thermodynamic parameters governing their formation. Basically, any measurement, direct or indirect, of K_{Co} (eq 3) permits calculation of an apparent value of K_{Bz} (eq 2) via eq 4. If the simplified scheme (eq 2 and 3) is adequate, such a value of K_{Bz} *must* be equivalent to the K_a value of α-ribazole (pK_a = 5.56 at 25 °C and ionic strength 1.0 M)[8] since substitution of phosphate at the nucleoside R3 carbon has been shown to have a negligible effect on the pK_a for benzimidazolium ionization (the

---

(1) Part 8: Brown, K. L.; Marques, H. M.; Jacobsen, D. W. *J. Biol. Chem.* **1988**, *263*, 1872-1877.

(2) Ladd, J. N.; Hogenkamp, H. P. C.; Barker, H. A. *J. Biol. Chem.* **1961**, *236*, 2114-2118.

(3) Pratt, J. M. *Inorganic Chemistry of Vitamin B_{12}*; Academic: New York, 1972; pp 151-152.

(4) Lexa, D.; Saveant, J. M. *J. Am. Chem. Soc.* **1976**, *98*, 2652-2658.

(5) Fanchiang, Y.-T.; Ridley, W. R.; Wood, J. M. *J. Am. Chem. Soc.* **1979**, *101*, 1442-1447.

(6) Grate, G. H.; Schrauzer, G. N. *J. Am. Chem. Soc.* **1979**, *101*, 4601-4611.

(7) Hogenkamp, H. P. C.; Kohlmiller, N. A.; Howsinger, R.; Walker, T. E.; Matwiyoff, N. A. *J. Chem. Soc., Dalton Trans.* **1980**, 1668-1673.

(8) Brown, K. L.; Hakimi, J. M.; Nuss, D. M.; Montejano, Y. D.; Jacobsen, D. W. *Inorg. Chem.* **1984**, *23*, 1463-1471.

(9) Brown, K. L. *J. Am. Chem. Soc.* **1987**, *109*, 2277-2284.

0020-1669/88/1327-3548$01.50/0

microscopic pK_a for proton dissociation from N3 of the zwitterion of α-ribazole 3′-phosphate is 5.54 at 25 °C and ionic strength 1.0 M).[9] If the simplified scheme is incorrect and a thermodynamically significant species exists in which the nucleotide of the protonated, base-off species (**1**) is associated with the remainder of the molecule, the apparent value of pK_{Bz} thus calculated will exceed the pK_a of α-ribazole. Conversely, if a thermodynamically significant species exists in which the nucleotide of the deprotonated, base-off species (**3**) is associated with the remainder of the molecule, the apparent value of pK_{Bz} will be lower than the pK_a of α-ribazole. If two additional species exist that are directly formed from the protonated and deprotonated base-off species, respectively, the calculated value of pK_{Bz} can be either higher or lower than that of the pK_a of α-ribazole depending on the value of the equilibrium constant for formation of each species. It is unlikely, however, that the equilibrium constants governing the formation of each species would be identical, thus leading to an apparent pK_{Bz} value fortuitously equal to the pK_a of α-ribazole and misinterpretation of the result.

In the previous publication referred to above,[9] the spectroscopic method was exploited by utilizing natural-abundance ^{13}C NMR since, in addition to providing evidence for the existence of such additional species, it is the spectroscopic method most able to provide structural information about any such species. A careful comparison of the ^{13}C NMR spectrum of the zwitterion of α-ribazole 3′-phosphate with the nucleotide resonances of five protonated, base-off cobalamins (i.e., **1**) failed to provide any firm evidence of an association of the protonated nucleotide with the remainder of the structure. However, comparison of the ^{13}C NMR spectrum of free-base α-ribazole with those of two base-off, but deprotonated, alkylcyanocobalamins did reveal evidence of an association. The converse experiment, in which the ^{13}C NMR spectrum of base-off, deprotonated dicyanocobalamin was compared to that of dicyanocobinamide, also revealed the interaction and pinpointed its site to the e side chain of the corrin ring. The interaction was thus characterized as a hydrogen bond between an e-side-chain amide N–H and the benzimidazole nitrogen (B3) with a possible additional hydrophobic interaction between the e-side-chain methylene groups and the top edge of the benzimidazole heterocycle.

There are several reports in the literature that are relevant to the thermodynamic method outlined above. For instance, Chemaly and Pratt[10] measured the temperature-dependent changes in the UV–visible spectrum of several alkylcobalamins in neutral, unbuffered aqueous solution. As the electronic spectral change accompanying the conversion of base-on to base-off cobalamins is quite large,[2] the formation of even relatively small equilibrium amounts of the base-off species (**3**) at high temperature is detectable by this method. From these measurements values of the enthalpy (ΔH_{Co}) and entropy change (ΔS_{Co}) associated with eq 3 were derived, which permit calculation of K_{Co} and hence pK_{Bz} (via eq 4) at 25 °C. The values of pK_{Bz} thus obtained ranged from 4.8 to 5.0, significantly below the pK_a of α-ribazole. Similar measurements have been made on 5′-deoxyadenosylcobalamin in aqueous solution by Halpern et al.[11] and Hay and Finke[12] and in ethylene glycol by Finke and Hay.[13] These measurements result in estimates of pK_{Bz} of 4.6–5.0, also significantly below the pK_a of α-ribazole. In addition, Reenstra and Jencks used their data on the kinetics and equilibria of formation of dicyanocobalamin from cyanocobalamin to calculate, via a thermodynamic cycle, a pK_{Bz} value for base-off, protonated dicyanocobalamin of 5.0.[14]

There are several reasons to believe that temperature-dependent UV–visible measurements may not provide an accurate picture of the thermodynamics of the base-on/base-off reaction. First, molar absorptivities of chromophores are well-known to be temperature-dependent due to the effects of temperature on the relative populations of vibronic states of both ground and excited electronic states. Such temperature dependence of the intensity of electronic transitions is very difficult to correct for without detailed knowledge of the vibronic structure of both states for the transitions utilized. Second, alkylcobinamides are well-known to undergo relatively large UV–visible spectral changes with temperature that display isosbestic points and may, or may not, be due to reversible formation of pentacoordinate species.[15] Presumably, base-off alkylcobalamins undergo similar temperature-dependent spectral changes. With the exception of the measurements on adenosylcobalamin in water by Hay and Finke,[12,16] who worked at a wavelength isosbestic for the temperature-dependent spectral changes of adenosylcobinamide, existing measurements do not take this effect into account. Finally, depending on the exact nature of the interactions between the pendant axial nucleotide and the remainder of the structure in any significant additional base-off species, and depending on the measurement wavelength, molar absorptivities of such additional species may be different from those of species **3**, further complicating the interpretation of results. This is particularly a problem since the electronic spectra of cobalamins are complicated and it is difficult to determine the effects of even minor structural alterations on the energies of ground and excited states and transition probabilities.

The use of NMR measurements of alkylcobalamins enriched in ^{13}C in the α-carbon of the organic ligand both obviates these problems and offers some attractive advantages. First, the ^{13}C chemical shift of such compounds is quite sensitive to the nature of the trans axial ligand. For instance, the base-on and base-off forms of ^{13}C-enriched methylcobalamin differ in chemical shift by about 7.7 ppm (or 385 Hz at 50 MHz).[17-19] Thus, ^{13}C NMR provides a high-resolution system for measuring the formation of even small amounts of base-off species. In addition, it is possible to control for any temperature dependence of the ^{13}C resonance of the base-off form by directly determining the *relative* resonance frequency of ^{13}C-enriched methylcobalamin and methylcobinamide in the same sample tube. This procedure had the added advantage of providing very high precision measurements since, by digital line-fitting methods, the relative frequencies of two resonances in the same sample can be determined with very high accuracy. Finally, the types of additional species anticipated, in which the axial nucleotide interacts with a side chain, for instance, but the nature of the trans axial ligand is unchanged, are very unlikely to have chemical shifts significantly different from those of the base-off species **3**, thus simplifying the interpretation of the dependence of the relative resonance frequency on temperatue. We now report the results of such measurements, which clearly provide both further evidence for formation of an additional species from the deprotonated, base-off species **3** and quantitation of the thermodynamics of its formation.

Experimental Section

Alkylcobalamins (RCbl) enriched in ^{13}C in the upper axial ligand were prepared by standard reductive alkylation procedures[8,9,20] using the appropriate alkylating agents enriched 90–99% in ^{13}C ($^{13}CH_3I$, $CH_3{}^{13}CH_2Br$, and $HOOC^{13}CH_2Br$). Yields of $HOOC^{13}CH_2Cbl$ were improved by using zinc wool in 10% acetic acid as the reducing system in place of sodium borohydride.[20] Factor B (cyanoaquacobinamide)[21]

---

(10) Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1980**, 2267–2273.

(11) Halpern, J.; Kim, S.-H.; Leung, T. W. *J. Am. Chem. Soc.* **1984**, *106*, 8317–8319.

(12) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 4820–4829.

(13) Finke, R. G.; Hay, B. P. *Inorg. Chem.* **1984**, *23*, 3041–3043.

(14) Reenstra, W. W.; Jencks, W. P. *J. Am. Chem. Soc.* **1979**, *101*, 5780–5791.

(15) Firth, R. A.; Hill, H. A. O.; Mann, B. E.; Pratt, J. M.; Thorp, R. G.; Williams, R. J. P. *J. Chem. Soc. A* **1968**, 2419–2428.

(16) Hay, B. P. Doctoral Dissertation, University of Oregon, 1986.

(17) Needham, T. E.; Matwiyoff, N. A.; Walker, T. E.; Hogenkamp, H. P. C. *J. Am. Chem. Soc.* **1973**, *95*, 5019–5024.

(18) Hogenkamp, H. P. C.; Vergamini, P. J.; Matwiyoff, N. A. *J. Chem. Soc., Dalton Trans.* **1975**, 2628–2634.

(19) Fanchiang, Y.-T.; Bratt, G. T.; Hogenkamp, H. P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2698–2702.

(20) Brown, K. L.; Peck, S. In *Organometallic Synthesis*; King, R. B., Eisch, J. J., Eds.; Elsevier: Amsterdam, in press; Vol. 4.

(21) Factor B is a mixture of the two stereoisomers of cyanoaquacobinamide, i.e. α-(CN)-β-(H_2O)Cbi and α-(H_2O)-β-(CN)Cbi. For convenience, this mixture will be referred to as factor B throughout.

was prepared by a slight modification[8] of the procedure of Renz.[22] Alkylcobinamides (RCbi) were obtained by reductive alkylation of factor B[20] again employing the appropriate ^{13}C-enriched alkylating agents. Again, the yield of $HOOC^{13}CH_2Cbi$ was somewhat improved by using the zinc/acetic acid reducing agent in place of borohydride. $^{13}CH_3Cbi$ was also prepared directly from $^{13}CH_3Cbi$ via cerrous hydroxide catalyzed phosphodiester cleavage[20] following the basic procedure of Hay.[16] α-Ribazole was obtained as described previously.[8]

Alkylcobamides were characterized by their UV–visible spectra and the characteristic changes thereof upon photolysis, acidification (for the RCbl's), and photolysis in excess cyanide (to form dicyanocobamides). The last reaction was used for quantitation[23] as previously described.[8] The purity of all preparations was assessed by high-performance liquid chromatography.[24]

pK_a's of α-ribazole, CH_3Cbl, and CH_3CH_2Cbl at various ionic strengths (KCl) and of CH_3CH_2Cbl at various temperatures (I = 1.0 M (KCl)) were determined spectrophotometrically on a Cary 219 recording spectrophotometer as previously described.[8] pH measurements were made on a Radiometer PHM 64 pH meter with electrode, samples, standards, and rinse water incubated at the measurement temperature.

NMR measurements were made on a Nicolet NT 200 wide-bore superconducting NMR spectrometer (4.7 T) operating at 50.311 MHz for ^{13}C. Samples contained ca. 100 mg of ^{13}C-enriched RCbl and ca. 35 mg of ^{13}C-enriched RCbi, dissolved in 4.0 mL of water 0.1 M KCl, or 1.0 M KCl (for R = $^{13}CH_3$) or 4.0 mL of 0.05 M CAPS buffer, pH 10.7, with the ionic strength adjusted to 1.0 M with KCl (for R = $^{-}OOC^{13}CH_2$). A concentric insert (Wilmad) contained D_2O for a lock signal and either sodium (trimethylsilyl)propionate (TSP, saturated, for R = $^{13}CH_3$) or *p*-dioxane (10% v/v, for R = $^{-}OOC^{13}CH_2$) for a reference. All chemical shifts are reported relative to TSP. In order to ensure temperature equilibration, samples (in 12-mm tubes) were incubated for 30 min in the NMR probe prior to ca. 20 min of data acquisition. Temperature measurements were made directly in the NMR probe with a thermistor device (Yellow Springs Instruments) immersed in a dummy sample containing the solvent, supporting electrolyte, and lock/reference solution but without the cobalt corrins. The dummy sample was allowed to incubate for 30 min in the probe before taking a series of 8–14 temperature measurements at 1-min intervals. In order to include the effects of sample heating due to the decoupling field, the decoupler was left on during the temperature measurement sequence but switched off momentarily for each measurement in order to avoid interference from the rf field. The thermistor device had been previously calibrated (every 5 °C in its linear range, every 2 °C outside the linear range) against a NBS-calibrated thermometer. The total variation in temperature during each measurement period (approximately equal in length to each data accumulation period) was less than 0.2 °C. Line widths and resonance frequencies were determined by digital line fits of each resonance in each spectrum. As it was found that the values obtained were sensitive to phasing, each set of transients was transformed and rephased four times and the resultant frequencies and line widths averaged.

Table I. ^{13}C NMR Properties of ^{13}C-Enriched Methylcobalt Corrins[a]

species	medium	δ	$\Delta\nu_{1/2}$, Hz[b]	J_{H-C}, Hz
$^{13}CH_3Cbl$, base-on	0.1 M $NaClO_4$	9.54	11.6	138.6
$^{13}CH_3Cbl$, base-off	0.19 M H_2SO_4[c]	1.75	3.7	143.7
$^{13}CH_3Cbl$, base-off	0.32 M HCl[d]	1.76	4.2	143.9
$^{13}CH_3Cbi$[e]	water	1.69	4.2	143.5
$^{13}CH_3Cbi$[e]	0.1 M KCl	1.70	4.0	143.2
$^{13}CH_3Cbi$[e]	1.0 M KCl	1.64	4.3	143.5
$^{13}CH_3Cbi$[f]	water	1.68	3.8	143.6
$^{13}CH_3Cbi$[f]	0.1 M KCl	1.72	4.1	143.3
$^{13}CH_3Cbi$[f]	1.0 M KCl	1.66	4.4	142.9

[a] 25 °C; all chemical shifts reported relative to TSP. [b] Width at half-height of fully ^{1}H-decoupled resonance. [c] pH 0.58. [d] pH 0.56. [e] From reductive alkylation of factor B with $^{13}CH_3I$. [f] From $Ce(OH)_3$ cleavage of $^{13}CH_3Cbl$.

Table II. ^{13}C NMR Properties of α-^{13}C-Enriched Ethylcobalt Corrins[a]

species	medium	δ	$\Delta\nu_{1/2}$, Hz[b]	J_{H-C}, Hz	J_{H-C-C}, Hz
$CH_3{}^{13}CH_2Cbl$, base-on	water	25.73	8.4	139.0	*c*
$CH_3{}^{13}CH_2Cbl$, base-on	1.0 M KCl	25.63	9.4	138.7	*c*
$CH_3{}^{13}CH_2Cbl$, base-off	0.11 M HCl[d]	23.39	4.4	144.9	8.4
$CH_3{}^{13}CH_2Cbl$, base-off	0.08 M H_2SO_4[e]	23.37	4.1	144.5	8.3
$CH_3{}^{13}CH_2Cbi$	water	23.09	4.4	144.6	8.1
$CH_3{}^{13}CH_2Cbi$	0.1 M KCl	23.12	4.6	144.6	8.3
$CH_3{}^{13}CH_2Cbi$	1.0 M KCl	23.02	4.4	144.5	8.4

[a] 25 °C; all chemical shifts reported relative to TSP. [b] Width at half-height of fully ^{1}H-decoupled resonance. [c] Two-bond H–C–C coupling not observed. [d] pH 1.07. [e] pH 1.08.

Table III. ^{13}C NMR Properties of α-^{13}C-Enriched (Carboxymethyl)cobalt Corrins[a]

species	pH[b,c]	δ	$\Delta\nu_{1/2}$, Hz[d]	J_{H-C}, Hz
$HOOC^{13}CH_2Cbl$, base-on	5.07[e]	14.84	9.4	143.1
$^{-}OOC^{13}CH_2Cbl$, base-on	10.34[f]	22.67	8.8	140.5
$^{-}OOC^{13}CH_2Cbl$, base-on	10.36[g]	22.71	8.1	140.2
$HOOC^{13}CH_2Cbl$, base-off	0.34[h]	2.43	7.9	147.3
$HOOC^{13}CH_2Cbl$, base-off	0.01[i]	2.40	8.7	147.5
$HOOC^{13}CH_2Cbl$, base-off	0.00[j]	2.41	7.8	147.5
$HOOC^{13}CH_2Cbi$	3.78[k]	2.64	5.0	147.2
$HOOC^{13}CH_2Cbi$	3.78[l]	2.50	6.1	147.2
$^{-}OOC^{13}CH_2Cbi$	10.18[m]	12.73	4.9	143.3
$^{-}OOC^{13}CH_2Cbi$	10.18[n]	12.57	5.5	143.9

[a] 25 °C; all chemical shifts reported relative to TSP. [b] The macroscopic pK_a's for base-off $HOOCCH_2Cbl$ are 2.34 and 7.24.[28] [c] The pK_a of $HOOCCH_2Cbi$ is 6.09.[28] [d] Width at half-height of fully ^{1}H-decoupled resonance. [e] 0.05 M acetate buffer, I = 1.0 M (KCl). [f] 0.05 M CAPS, I = 1.0 M (KCl). [g] 0.05 M CAPS, I = 0.025 M. [h] 0.5 M HCl, I = 1.0 M (KCl). [i] 0.65 M H_2SO_4. [j] 1.0 M HCl. [k] 0.05 M acetate buffer, I = 0.005 M. [l] 0.05 M acetate buffer, I = 1.0 M (KCl). [m] 0.05 M CAPS, I = 0.02 M. [n] 0.05 M CAPS, I = 1.0 M (KCl).

Results and Discussion

Stereochemical Nature of the Alkylcobinamides. There is considerable confusion in the literature regarding the stereochemical course of the reductive alkylation of cobinamides. In their original synthesis of ^{13}C-enriched methylcobinamide ($^{13}CH_3Cbi$), Needham et al.[17] obtained a product that displayed two ^{13}C resonances of approximately equal intensity differing in chemical shift by about 2 ppm. This was interpreted as indicating that the synthesis (reductive alkylation of diaquacobinamide ($(H_2O)_2Cbi$) with $^{13}CH_3I$) gave rise to the two diasteromeric methylcobalt corrinoids ($(\beta\text{-}^{13}CH_3)(\alpha\text{-}H_2O)Cbi$ and $(\beta\text{-}H_2O)(\alpha\text{-}^{13}CH_3)Cbi$, in which the methyl group is in the "upper" and "lower" axial ligand positions, respectively. This result was in direct contrast to the earlier, very careful studies of Müller and Müller[25] and Zagalak,[26] who found only a single isomer upon reductive methylation of cobinamides and identified it as the β-methyl isomer. In addition, the subsequent synthesis of $^{13}CH_3Cbi$ via reductive alkylation by Frick et al.[27] produced material with only a single ^{13}C resonance.

Under the relatively mild synthetic conditions used here (5-min alkylation time with relatively small molar excesses of alkylating

---

(22) Renz, P. *Methods Enzymol.* **1971**, *18*, 82–92.

(23) Barker, H. A.; Smyth, R. D.; Weissbach, H.; Toohey, J. I.; Ladd, J. N.; Volcani, B. E. *J. Biol. Chem.* **1960**, *235*, 480–488.

(24) Jacobsen, D. W.; Green, R.; Brown, K. L. *Methods Enzymol.* **1986**, *123*, 14–22.

(25) Müller, O.; Müller, G. *Biochem. Z.* **1963**, *337*, 179–194.

(26) Zagalak, B. *Acta Biochim. Pol.* **1963**, *10*, 387–398.

(27) Frick, T.; Francia, M. D.; Wood, J. M. *Biochim. Biophys. Acta* **1974**, *428*, 808–817.

(28) Brown, K. L.; Hakimi, J. M.; Huang, Y.-J. *Inorg. Chim. Acta* **1985**, *106*, 123–128.

agents), all reductive alkylations of factor B[21] with ^{13}C-enriched alkyl halides produced alkylcobinamide products that had a single, detectable ^{13}C resonance (Tables I–III). Since the two isomeric ^{13}C-enriched cyanoaquocobinamides (i.e., (β-^{13}CN)(α-H_2O)Cbi and (β-H_2O)(α-^{13}CN)Cbi) are known to have ^{13}C chemical shifts that differ by about 1 ppm,[17,29] it is clear that the magnetic environments of the two axial ligand positions are different. Hence, failure to observe two ^{13}C resonances for any of the alkylcobinamides enriched in ^{13}C in the α-carbon suggests that only a single stereoisomer is obtained. In addition, HPLC traces of all the alkylcobinamides indicated substantial homogeneity in that each consisted of a large peak (representing at least 95% of the material) and several very minor peaks, none of which accounted for more than 1 or 2% of the total material. We conclude that only a single stereoisomer of the alkylcobinamides is obtained by reductive alkylation of factor B, at least under the conditions used here.[30]

That the single stereoisomer of the alkylcobinamides obtained in this work is indeed the β-alkyl isomer is strongly indicated by the fact that the ^{13}C chemical shifts of the ^{13}C-enriched alkylcobinamides are nearly identical with those of the base-off species of the analogous ^{13}C-enriched alkylcobalamins (Tables I–III). In addition, the UV–visible spectrum of each alkylcobinamide was identical (above 280 nm) with that of the base-off species of the analogous alkylcobalamin. Considering the large differences in electronic spectra between the known stereoisomers of methylcobalt corrinoids,[30,36] this is excellent evidence that the β-alkyl isomers have been obtained. In order to be absolutely certain, $^{13}CH_3Cbi$ was synthesized directly from $^{13}CH_3Cbl$ via cerrous hydroxide catalyzed phosphodiester hydrolysis.[16,20,37] The material obtained was identical with that obtained by reductive alkylation of factor B with $^{13}CH_3I$ by HPLC mobility, and by UV–visible and ^{13}C NMR spectroscopy (Table I). It thus appears that not only does reductive alkylation of factor B under these conditions[30] lead to a predominance of one stereoisomer (by at least 20:1) but also the predominant isomer formed is the β-alkyl isomer.

This is a particularly striking observation since addition of $^{13}CN^-$ to $(H_2O)_2Cbi$ is known to lead to formation of a pair of stereoisomeric cyanoaquacobinamides[17,29] in which the α-cyano isomer predominates by about 2:1.[29] It is quite clear that in this case the products are under thermodynamic control (apparently due to labilization of trans cyanide by formation of trace $(CN)_2Cbi$), as Reenstra and Jencks[14] have measured equilibrium constants of 2.1×10^8 M^{-1} for addition of an α-cyano ligand to base-off β-cyano-α-aquacobalamin and 0.8×10^8 M^{-1} for addition of a β-cyano ligand to base-off β-aqua-α-cyanocobalamin, indicating a thermodynamic preference of CN^- for the lower (α) ligand position of 2.6:1. It thus seems logical to conclude that in the current case of reductive alkylation of factor B the products are under kinetic control and that there is an astonishing difference of a factor of at least 40 in preference for the two axial ligand positions under kinetic vs thermodynamic control. Since Pratt and co-workers[38] have observed that addition of $HC\equiv C^-$ to $(H_2O)_2Cbi$ leads to a roughly equimolar mixture of β- and α-ethynylcobinamide, it is tempting to conclude that the isomeric preference of the kinetically controlled products is due to steric factors and that only strictly linear ligands can add to both axial ligand positions. However, it is premature to draw this conclusion without further experimental evidence, which we are currently attempting to obtain.

Table IV. Effect of Ionic Strength on $pK_{\text{base-off}}$ for CH_3Cbl and CH_3CH_2Cbl and the pK_a of α-Ribazole[a]

I, M[b]	pK_a (α-ribazole)	$pK_{\text{base-off}}$ (CH_3Cbl)	$pK_{\text{base-off}}$ (CH_3CH_2Cbl)
1.0	5.56	2.90	4.16
0.64	5.55	2.87	4.15
0.36	5.51	2.84	4.08
0.10	5.47	2.76	4.02
0.05	5.41		
0.04	5.40	2.72	4.02

[a] All measurements at 25 ± 0.1 °C. [b] Ionic strength of buffered solutions adjusted with KCl.

It should be pointed out that the data in Tables I–III and our previous data on ^{13}CN-labeled cobalt corrinoids[29] indicate that the chemical shifts of alkylcobinamides and base-off alkylcobalamins enriched in ^{13}C in the α-carbon of the organic ligand are not identical although there are very similar. The signed differences in chemical shift, $\Delta\delta$, between the base-off cobalamins and the analogous cobinamides are 0.30, 0.08, −0.09, and −0.20 ppm for R = $CH_3{}^{13}CH_2$, $^{13}CH_3$, $HOOC^{13}CH_2$, and ^{13}CN, respectively. These differences appear to be significant and are clearly trended: $\Delta\delta$ becomes more negative as the donor power of the organic ligand decreases. This implies that the presence or absence of a pendant, but uncoordinated, nucleotide loop has a discernable influence on the magnetic environment of the α-carbon. As there is no evidence for an interaction of the nucleotide with the remainder of the structure in protonated, base-off cobalamins[9] and the nucleotide is far too remote from the macrocycle to cause a discernable inductive effect on the central cobalt atom, this influence on the magnetic environment of the α-carbon must be due to an effect on the conformation of the macrocycle as a whole. However, the basis for this conformational effect, as well as for the trend with donor power of the organic ligand, remain to be understood.

Effects of Ionic Strength. It has been stated that both K_{Co} (eq 3) and K_{Bz} (eq 2) are ionic strength dependent[39] and that K_{Co} is also dependent on the nature of the electrolyte anion.[39,40] The latter observation implies that electrolyte anions can bind to cobalt in base-off cobalamins, which is undoubtedly correct at least for some anions. However, the former assertion is surprising in that K_{Bz}, representing the dissociation of a cationic Brønsted acid, would be expected to show minimal ionic strength dependence due to the anticipated similarity of the effect of ionic strength on the activity coefficients for such cationic acids and hydronium ion. K_{Co}, which represents the exchange of two neutral axial ligands on a zwitterionic metal complex, would be expected to show little or no dependence on ionic strength. In order to clarify these issues, we have investigated the ionic strength dependence of the pK_a of α-ribazole and the values of $pK_{\text{base-off}}$ for CH_3Cbl and CH_3CH_2Cbl (Table IV). As ionic strength was maintained with KCl in these measurements, it is first necessary to determine if chloride ion can bind to the cobalt atom of base-off cobalamins. This question was examined by measurement of the ^{13}C chemical shifts of base-off $^{13}CH_3Cbl$ and $CH_3{}^{13}CH_2Cbl$ in HCl and H_2SO_4 and of $^{13}CH_3Cbi$ and $CH_3{}^{13}CH_2Cbi$ at several ionic strengths in KCl solution. As shown in Tables I and II, there is no experimentally significant difference in the chemical shift of the base-off ^{13}C-enriched alkylcobalamins in HCl or H_2SO_4 solution nor is there any significant trend of chemical shift with KCl concentration for the ^{13}C-enriched alkylcobinamides. Similar data are presented in Table III for the (carboxymethyl)cobalt corrinoids.

---

(29) Brown, K. L.; Hakimi, J. M. *Inorg. Chem.* **1984**, *23*, 1756–1764.

(30) More vigorous synthetic conditions can apparently lead to the production of stereoisomers.[31-36]

(31) Friedrich, W.; Nordmeyer, J. P. *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biol.* **1968**, *23B*, 1119–1120.

(32) Friedrich, W.; Meserschmidt, R. *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol.* **1969**, *24B*, 465–467.

(33) Friedrich, W.; Nordmeyer, J. P. *Z. Naturforsch, B: Anord. Chem., Org. Chem., Biochem., Biophys., Biol.* **1969**, *24B*, 588–596.

(34) Friedrich, W.; Moskophidis, M. *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys. Biol.* **1970**, *25B*, 979–983.

(35) Moskophidis, M.; Klotz, C. M.; Friedrich, W. *Z. Naturforsch., C: Biosci.* **1976**, *31C*, 255–262.

(36) Moskophidis, M. In *Vitamin B_{12}*; Zagalak, B., Friedrich, W., Eds.; deGruyter: Berlin, FRG, 1979; pp 189–192.

(37) Attempts to obtain $^{13}CH_3Cbi$ from $^{13}CH_3Cbl$ via $HClO_4$-catalyzed phosphodiester hydrolysis (following Zagalak[26]) were unsuccessful in that no alkylcobalt corrinoids having the correct mobility on ion-exchange chromatography could be obtained.

(38) Baldwin, D. A.; Betterton, E. A.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1983**, 225–229.

(39) Fanchiang, Y.-T.; Ridley, W. P.; Wood, J. M. *J. Am. Chem. Soc.* **1979**, *101*, 1442–1447.

(40) Fanchiang, Y.-T. *Inorg. Chem.* **1982**, *21*, 2344–2348.

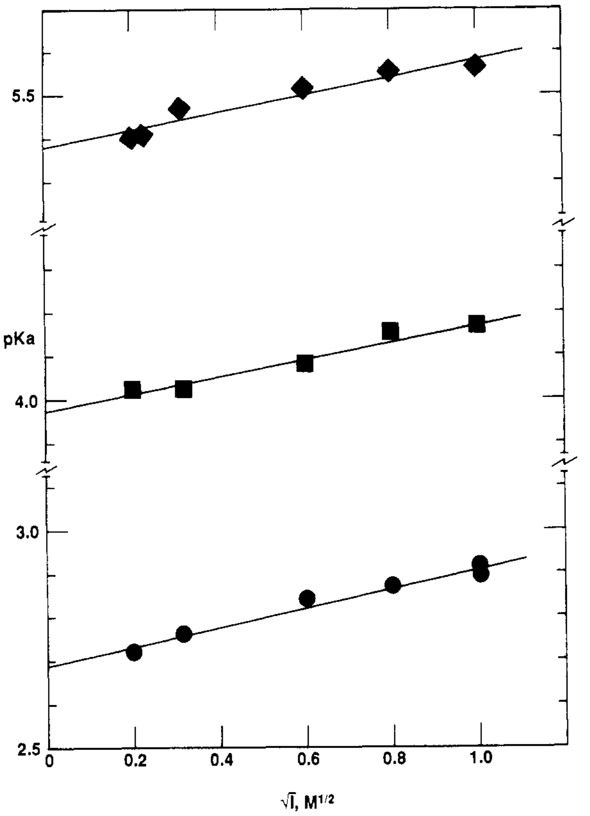


Figure 1. Plots of pK_a for α-ribazole (♦) and p$K_{\text{base-off}}$ for CH_3CH_2Cbl (■) and CH_3Cbl (●) vs the square root of the ionic strength, at 25.0 ± 0.1 °C (ionic strength maintained with KCl). The solid lines are least-squares regression lines: slope 0.199 ± 0.030, intercept 5.38 ± 0.02, r^2 = 0.918 (α-ribazole); slope 0.194 ± 0.020, intercept 3.97 ± 0.01, r^2 = 0.960 (CH_3CH_2Cbl); slope 0.218 ± 0.020, intercept 2.69 ± 0.01, r^2 = 0.968 (CH_3Cbl).

As it is unlikely that sulfate or bisulfate ions bind to cobalt in base-off alkylcobalamins, the similarity of the chemical shifts of the base-off ^{13}C-enriched alkylcobalamins in HCl and H_2SO_4 strongly suggests that chloride ion also does not bind. Additionally, the lack of any dependence of the chemical shift of the ^{13}C-enriched alkylcobinamides on chloride ion concentration over the range 0–1.0 M strongly argues against any significant binding of chloride ion.

Inspection of the data in Table IV shows that both the pK_a for α-ribazole and p$K_{\text{base-off}}$ for the representative RCbl's do indeed show a significant, if small, ionic strength effect. In each case the pK_a values correlate well with the square root of the ionic strength, as shown in Figure 1. Those correlations provide values of the pK_a's at zero ionic strength of 5.38, 3.97, and 2.69 for α-ribazole, CH_3CH_2Cbl, and CH_3Cbl, respectively. However, application of eq 4 for the simplified scheme for the base-on/base-off equilibria show that calculated values of K_{Co} for CH_3CH_2Cbl (24.8 ± 1.7) and CH_3Cbl (478 ± 21) are not significantly ionic strength dependent.

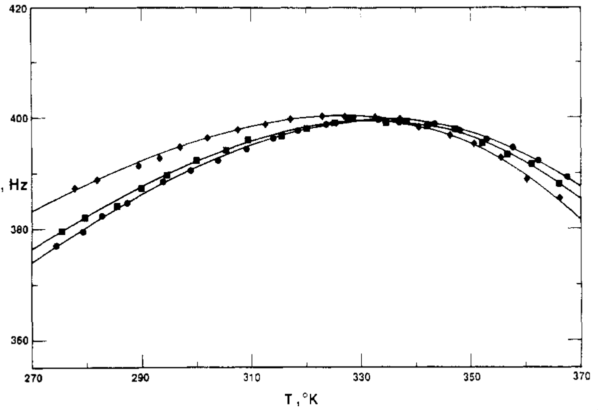


Figure 2. Plots of $\Delta\nu_T$, the difference in resonance frequency between $^{13}CH_3Cbl$ and $^{13}CH_3Cbi$ at 50.311 MHz, vs temperature at ionic strength 0 M (●), 0.1 M (■), and 1.0 M (♦). The solid lines are calculated from eq 5, and the parameter values are listed in Table V.

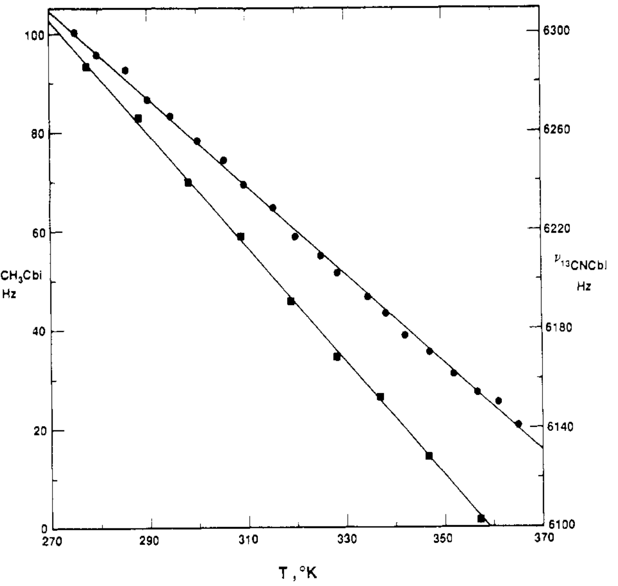


Figure 3. Plots of the ^{13}C resonance frequency (at 50.311 MHz) relative to TSP vs temperature at ionic strength 0.1 M (KCl) for $^{13}CH_3Cbi$ (●, left ordinate) and $^{13}CN(Cbl)$ (■, right ordinate). The solid lines are least-squares regression lines: slope −0.891 ± 0.010 Hz K^{-1}, intercept 345 ± 3 Hz, r^2 = 0.998 ($^{13}CH_3Cbi$); slope −2.31 ± 0.03 Hz K^{-1}, intercept 6930 ± 10 Hz, r^2 = 0.999 ($^{13}CN(Cbl)$).

Temperature-Dependent NMR Measurements. The temperature dependences of the relative resonance frequency $\Delta\nu_T$ of $^{13}CH_3Cbl$ and $^{13}CH_3Cbi$ at ionic strengths 1.0, 0.1, and 0.0 M are shown in Figure 2. Somewhat surprisingly the data do not show the anticipated monotonic decrease in $\Delta\nu_T$ with temperature but instead show an increase in $\Delta\nu_T$, which appears to be nearly linear at the lowest temperatures, followed by a concave downward dependence eventually leading to a net decrease in $\Delta\nu_T$ at the highest temperatures. This behavior suggests that the resonance frequencies of base-on $^{13}CH_3Cbl$, $^{13}CH_3Cbi$, or both show a temperature dependence, which may be linear, in addition to the temperature dependence of the resonance frequency of $^{13}CH_3Cbl$ due to perturbation of the base-on/base-off equilibrium. Indeed, plots of the resonance frequency of $^{13}CH_3Cbi$ (relative to TSP) give excellent linear correlations as shown for example at ionic strength 0.1 M in Figure 3 (r^2 = 0.998, slope −0.891 ± 0.010 Hz K^{-1}). It must, however, be pointed out that most of the change in resonance frequency of $^{13}CH_3Cbi$ over this temperature range is due to temperature dependence of the resonance frequency of the reference (TSP) relative to the transmitter frequency, as the resonance frequency of $^{13}CH_3Cbi$ changes by only 3–15 Hz (depending on the ionic strength) relative to the transmitter frequency over this temperature range. Unfortunately, it is not possible to directly determine if there is a linear temperature dependence of the resonance frequency of the base-on form of $^{13}CH_3Cbl$ as the base-on/base-off equilibrium is clearly significantly perturbed over this temperature range. However, the situation may be modeled by use of $^{13}CN(Cbl)$. Since the p$K_{\text{base-off}}$ value for CN(Cbl) is 0.10,[29] K_{Co} must be quite large (certainly in excess of 10^5 at 25 °C) for this compound, regardless of how the base-on/base-off equilibria are formualted. It is thus extremely unlikely that any significant amounts of base-off CN(Cbl) will be formed in the accessible temperature range. The resonance frequency of $^{13}CN(Cbl)$ (relative to TSP) at ionic strength 0.1 M was found to be significantly affected by temperature (Figure 3). The dependence is clearly linear (r^2 = 0.999) with a slope of −2.31 ± 0.03 Hz K^{-1}, i.e., significantly larger than the temperature-dependent change in resonance frequency of the reference relative to the transmitter frequency. Although the basis for the

Table V. Thermodynamics of the Base-On/Base-Off Equilibrium of $^{13}CH_3Cbl$ from the Simplified Scheme[a]

I, M	$\nu_0^{on} - \nu_0^{off}$, Hz	$m^{on} - m^{off}$, Hz K^{-1}	ΔH_{Co}, kcal mol^{-1}	ΔS_{Co}, cal mol^{-1} K^{-1}	K_{Co}[b]	pK_{Bz}[c]
0	166.6	0.777	−6.49	−14.0	50.1	4.39
0.1	192.6	0.687	−7.11	−15.6	63.8	4.57
1.0	237.0	0.546	−7.65	−16.9	82.6	4.82
1.0	240.7	0.530	−7.72	−16.9	93.0	4.87

[a] Equations 2–4. [b] At 25 °C; calculated from ΔH_{Co} and ΔS_{Co}. [c] At 25 °C; calculated from K_{Co} and $K_{base\text{-}off}$ (Table IV, Figure 1) via eq 4.

Table VI. Component Equilibria of the Base-On/Base-Off Reaction of $^{13}CH_3Cbl$ from the Complete Scheme[a]

I, M	K_{measd}[b]	K_H[c]	K_{Co}[c]
0	50.1	8.58	480
0.1	63.8	6.91	505
1.0	82.6	4.47	452
1.0	93.0	3.86	452

[a] Equations 2, 3, and 6. [b] Calculated at 25 °C from the enthalpies and entropies given in Table V. [c] Calculated from K_{measd} via eq 8 and 9 by using the values of the pK_a of α-ribazole (Table IV and Figure 2) as pK_{Bz}.

Table VII. Thermodynamics of the Base-On/Base-Off Equilibrium of $^{13}CH_3Cbl$ from the Complete Scheme (Ionic Strength 1.0 M)[a]

T, °C	K_{measd}[b]	pK_{Bz}[c]	p$K_{base\text{-}off}$[d]	K_H[e]	K_{Co}[e]
5.2	221	5.90	2.84	4.18	1140
15.1	137	5.68	2.86	3.79	656
25.0	87.8	5.56	2.90	4.16	452
34.9	57.8	5.40	2.92	4.14	297

[a] Equations 2, 3, and 6. [b] Calculated from the enthalpy and entropy in Table V at I = 1.0 M. [c] Taken as the pK_a of α-ribazole at I = 1.0 M.[8] [d] Reference 8. [e] Calculated from K_{measd} via eq 8 and 9.

temperature dependence of the resonance frequency of $^{13}CN(Cbl)$ is not clear, the linearity of the effect is what is important here.

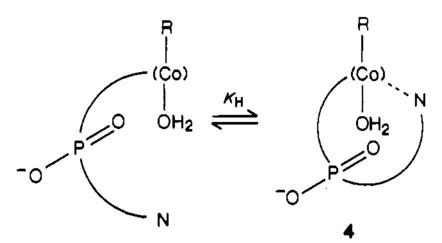
It is thus clear that the temperature dependence of the relative resonance frequency, $\Delta\nu_T$, of $^{13}CH_3Cbl$ and $^{13}CH_3Cbi$ that is not attributable to the perturbation of the base-on/base-off equilibrium may be legitimately attributed to linear dependencies of the resonance frequencies of the base-on and base-off species on temperature. This permits a complete analysis of the temperature dependence of $\Delta\nu_T$ (Figure 2) by eq 5, where ν_0^{on} and m^{on} are the

$$\Delta\nu_T = [(\nu_0^{on} - \nu_0^{off}) + (m^{on} - m^{off})T]e^{[(\Delta S_{Co}/R)-(\Delta H_{Co}/RT)]}/(1 + e^{[(\Delta S_{Co}/R)-(\Delta H_{Co}/RT)]}) \quad (5)$$

intercept and slope, respectively, of the linear dependence of the resonance frequency of the base-on species with temperature, ν_0^{off} and m^{off} are the analogous values for the base-off species, and ΔS_{Co} and ΔH_{Co} are the entropy and enthalpy associated with the on/off equilibrium (eq 3). The data in Figure 2 were fit to eq 5 by a nonlinear least-squares routine utilizing an iterative simplex minimization algorithm to obtain the parameter values listed in Table V. In order to gain some idea of the precision of the obtained values, the entire set of rather tedious measurements was repeated on a fresh sample at ionic strength 1.0 M. The remarkable agreement between the two sets of values (last two entries in Table V) generates significant confidence in the overall method and indicates that, despite the limited temperature range over which measurements could be obtained, the parameters in eq 5 are quite well determined by data sets of this size (20–24 data points).

The values of ΔH_{Co} and ΔS_{Co} (eq 3) so obtained may be used to calculate values of K_{Co}, for example at 25 °C as shown in Table V. These values show a very substantial ionic strength dependence, the calculated value of K_{Co} increasing by nearly 90% from ionic strength zero to 1.0 M. As discussed above, this is a highly unlikely result considering the simple, nonionic equilibrium represented by K_{Co}. In addition, these values of K_{Co} may be used in conjunction with the values of p$K_{base\text{-}off}$ (Table IV and Figure 1) to calculate values of pK_{Bz} via eq 4. These results show strong thermodynamic evidence for the inadequacy of the simplified scheme for the base-on/base-off equilibrium (eq 2 and 3). The calculated values of pK_{Bz} are substantially below the measured values of the pK_a of α-ribazole (Table IV and Figure 1), the difference varying from 0.99 to 0.69 with increasing ionic strength. As pointed out in the Introduction, calculated values of pK_{Bz} that show an increase in apparent acidity relative to that of the detached nucleoside suggest the existence of a thermodynamically significant species formed from the base-off, but benzimidazole deprotonated, species (i.e., **3** in eq 2 and 3). Such a species was recently detected by NMR spectroscopy and characterized as a base-off species in which the benzimidazole nitrogen (B3) is hydrogen-bonded to an amide N–H of the e side chain.[9]

The simplified scheme for the base-on/base-off equilibrium can thus be modified by inclusion of the equilibrium (eq 6) for formation of the hydrogen-bonded species (**4**) characterized by K_H.



3 **4** (6)

$$K_{measd} = \frac{[2]}{[3] + [4]} \quad (7)$$

$$K_{measd} = K_{Co}/(1 + K_H) \quad (8)$$

$$K_{base\text{-}off} = (1 + K_H + K_{Co})K_{Bz} \quad (9)$$

The thermodynamic parameters measured via the data in Figure 2 and obtained via eq 5 are thus relevant to the composite equilibrium represented by eq 7, which is related to K_{Co} (eq 3) and K_H (eq 6) via eq 8. Thus for the complete scheme, $K_{base\text{-}off}$ is given by eq 9. The only assumption inherent in this analysis is that the ^{13}C resonance frequencies at 25 °C of all species in which the axial nucleotide is not coordinated (i.e., **1**, **3**, and **4**) are not significantly different from one another and that these values are adequately represented by the resonance frequency of $^{13}CH_3Cbi$.

Table VI shows an analysis of the data in Table V by use of the complete scheme (eq 2, 3, and 6) in which K_{measd} at 25 °C (eq 7) is calculated from the measured enthalpies and entropies and K_H (eq 6) and K_{Co} (eq 3) are calculated from eq 8 and 9 by using the measured pK_a's of α-ribazole as values of pK_{Bz}. These results show that when species **4** is included in the equilibria the resultant values of K_{Co} are, in fact, ionic strength independent (average value 472 ± 25) as anticipated. K_H, however, is substantially ionic strength dependent, the derived values giving an excellent ($r^2 = 1.00$) linear plot of ln K_H vs $I^{1/2}$ (not shown, slope −0.724 ± 0.013 and intercept 2.15 ± 0.01, i.e., K_H = 8.62 at I = 0). This observation is not at all surprising considering the importance of solvent in the thermodynamics of hydrogen bond formation in water.[41,42] We note that while the value of K_H significantly exceeds 1.0 at all ionic strengths, so that **4** is in fact the predominant form of the base-off but benzimidazole-depro-

---

(41) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969.

(42) Fersht, A. R.; Shi, J.-P.; Knill-Jones, J.; Lowe, D. M.; Wilkinson, A. J.; Blow, D. M.; Brick, P.; Carter, P.; Waye, M. M. Y.; Winter, G. *Nature (London)* **1985**, *314*, 235–238.

Table VIII. Thermodynamics of the Base-On/Base-Off Reaction of Cobalamins, RCbl[a]

R	$pK_{base\text{-}off}$	K_{Co}	ΔH_{Co}, kcal mol^{-1}	ΔS_{Co}, cal mol^{-1} K^{-1}
CH_3CH_2[b]	4.16	2.00×10	-8.77 ± 0.73	-23.7 ± 2.5
$CH_3(CH_2)_2$[c]	4.10	2.37×10	-8.25 ± 0.81	-21.5 ± 2.8
$NC(CH_2)_3$[d]	3.50	1.10×10^2	-7.88 ± 1.75	-17.3 ± 1.7
$CH_3OOC(CH_2)_2$[e]	3.33	1.65×10^2	-7.94 ± 0.44	-16.6 ± 1.5
CH_3[f]	2.90	4.52×10^2	-7.56 ± 0.39	-13.2 ± 1.3
CF_3CH_2[g]	2.60	9.23×10^2	-8.02 ± 0.70	-13.5 ± 2.4
CF_2H[g]	2.15	2.60×10^3	-6.82 ± 0.45	-7.32 ± 1.53
$NCCH_2$[h]	1.81	5.62×10^3		
CF_3[g]	1.44	1.32×10^4	-7.04 ± 0.34	-4.68 ± 1.15
CN[i]	0.10	2.88×10^5		
H_2O[h]	−2.13	4.90×10^7		

[a] Ionic strength 1.0 M; equilibrium constants at 25 °C. [b] This work. $pK_{base\text{-}off}$ values at various temperatures were 4.16 (5.2 °C), 4.14 (15.1 °C), 4.16 (25.0 °C), and 4.21 (34.9 °C). From the simplified scheme $K_{Co} = 24.1$, $\Delta H_{Co} = -7.34 \pm 0.46$ kcal mol^{-1}, and $\Delta S_{Co} = -18.5 \pm 1.6$ cal mol^{-1} K^{-1}. [c] Reference 8. The simple scheme gives $K_{Co} = 28.0$, $\Delta H_{Co} = -7.12 \pm 0.78$ kcal mol^{-1}, and $\Delta S_{Co} = -17.4 \pm 2.7$ cal mol^{-1} K^{-1}. [d] Reference 8. The simple scheme gives $K_{Co} = 115$, $\Delta H_{Co} = -7.55 \pm 0.51$ kcal mol^{-1}, and $\Delta S_{Co} = -16.1 \pm 1.7$ cal mol^{-1} K^{-1}. [e] Reference 8. The simple scheme gives $K_{Co} = 168$, $\Delta H_{Co} = -7.75 \pm 0.41$ kcal mol^{-1}, $\Delta S_{Co} = -16.9 \pm 1.4$ cal mol^{-1} K^{-1}. [f] This work. [g] Reference 8. [h] Reference 45. [i] Reference 29.

tonated species in solution, its existence has very little effect on K_{Co}, the average value obtained here (472 ± 25) being indistinguishable from the value previously obtained (467)[8] at ionic strength 1.0 M by simple application of eq 4. This is due to the functional form of eq 9 and the relatively small value of K_H compared to K_{Co} (at least when R = CH_3).

The existence of previously obtained data on the temperature dependence of $pK_{base\text{-}off}$ for CH_3Cbl and the pK_a of α-ribazole at ionic strength 1.0 M[8] now allows an analysis of the thermodynamics of formation of the hydrogen-bonded species (**4**) and a reanalysis of the thermodynamics of the primary on/off equilibrium (eq 3). The relevant data are shown in Table VII, in which K_{measd} is again calculated from the enthalpy and entropy changes (at I = 1.0 M) in Table V and K_H and K_{Co} are calculated by use of eq 8 and 9. From the calculated values of K_{Co}, ΔH_{Co} (−7.56 ± 0.39 kcal mol^{-1}) and ΔS_{Co} (−13.2 ± 1.3 cal mol^{-1} K^{-1}) were obtained. These values are not significantly different from those previously obtained (−7.49 ± 0.43 kcal mol^{-1} and −13.0 ± 1.5 cal mol^{-1} K^{-1})[8] by simple application of eq 4, for the reasons stated above. Most importantly, however, the thermodynamics of formation of the hydrogen-bonded species **4** (eq 6) are precisely as anticipated. The free energy of formation of **4** at 25 °,c is −0.83 kcal mol^{-1}, almost at the midpoint of the range of −0.5 to −1.5 kcal mol^{-1} previously measured for formation of a hydrogen bond between two uncharged partners in water.[42] Similarly, K_H is seen to be isoenthalpic ($\Delta H_H = 0.10 \pm 0.43$ kcal mol^{-1}): the formation of **4** is driven by entropy ($\Delta S_H = 3.13 \pm 1.47$ cal mol^{-1} K^{-1}). This is exactly what is expected from the standard formulation of hydrogen bond formation in water.[41,42]

It must now be pointed out that the use of eq 5 in conjunction with the complete scheme (eq 2, 3, and 6) is only an approximation. Rederivation of the equation for the dependence of $\Delta\nu_T$ on T for the complete scheme leads to eq 10, in which an additional

$$\Delta\nu_T = [(\nu_0^{on} - \nu_0^{off}) + (m^{on} - m^{off})T]e^{[(\Delta S_{Co}/R)-(\Delta H_{Co}/RT)]}/(1 + e^{[(\Delta S_H/R)-(\Delta H_H/RT)]} + e^{[(\Delta S_{Co}/R)-(\Delta H_{Co}/RT)]}) \quad (10)$$

term involving the thermodynamics of formation of the hydrogen-bonded species occurs in the denominator. No attempt was made to fit the data to this equation as inclusion of two additional parameters (ΔH_H and ΔS_H) must surely lead to a statistically hopeless curve-fitting situation. However, it is clear that eq 5 represents an excellent approximation of eq 10 because the values of K_H are so small relative to K_{Co}. Thus, calculations based on the values of the thermodynamic parameters derived above (i.e., by use of eq 5) show that the additional term in the denominator of eq 10 accounts for less than 0.3% of the denominator at the lower end of our temperature range (5 °C). This value rises to 2% at 50 °C and 9% at 95 °C. Consequently, although eq 5 is only an approximation, its use cannot be causing a very significant perturbation of the result.

However, because of the approximation inherent in the use of eq 5, the limitations to the temperature range over which measurements could be made, and the relative complexity of the data acquisition and treatment, it is crucial to obtain confirmation of these results regarding the thermodynamics of formation of the hydrogen-bonded species (**4** in eq 6). Attempts to make similar NMR measurements on mixtures of $CH_3{}^{13}CH_2Cbl$ and $CH_3{}^{13}CH_2Cbi$ were unsuccessful due to the thermal lability of these species.[18] Even at temperatures as low as 60 °C irreversible changes in the NMR spectra of such mixtures indicated substantial decomposition. Attempts to analyze (by eq 5) data sets truncated at 60 °C from the $^{13}CH_3Cbl$/$^{13}CH_3Cbi$ samples readily showed that restriction of the data to this narrower temperature range led to inconsistent and unreliable results. However, (carboxymethyl)cobamides were found to be sufficiently temperature stable to permit NMR measurements over the necessary temperature range.

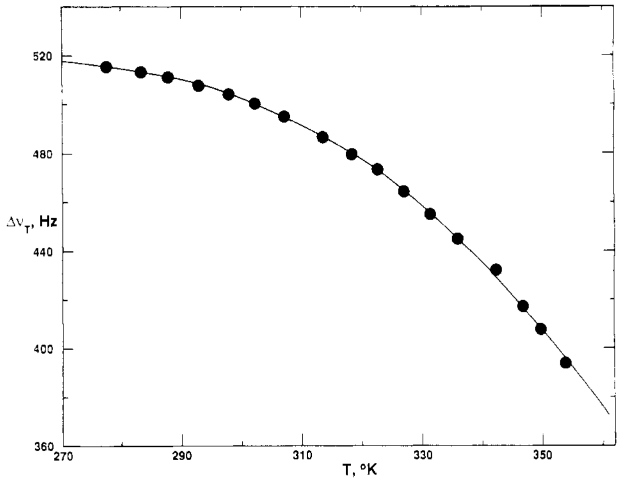


Figure 4. Plot of $\Delta\nu_T$, the difference in resonance frequency between $^-OOC^{13}CH_2Cbl$ and $^-OOC^{13}CH_2Cbi$ at 50.311 MHz, vs temperature at ionic strength 1.0 M and pH 10.7 (25 °C) in 3-(cyclohexylamino)propanesulfonate buffer (CAPS). The solid line is the least-squares fit of the data to eq 5.

The acid–base behavior of $HOOCCH_2Cbl$ is complicated by the ionization of the carboxymethyl organic ligand.[28,43] We have recently resolved the microscopic pK_a's for this complex to obtain (at ionic strength 1.0 M and 25 °C) values of 2.34 and 3.49 for $pK_{base\text{-}off}$ for the carboxyl-protonated and -deprotonated species, respectively.[28] The former value leads to an estimated value of K_{Co} of over 1600 for $HOOCCH_2Cbl$, suggesting that too little base-off species would be obtained at the highest attainable temperatures to reliably determine the relevant thermodynamic parameters. However, $pK_{base\text{-}off}$ for $^-OOCCH_2Cbl$ (3.49) predicts a value of K_{Co} of just over 100 and coupled with the relatively

---

(43) Walker, T. E.; Hogenkamp, H. P. C.; Needham, T. E.; Matwiyoff, N. A. *J. Chem. Soc., Chem. Commun.* **1974**, 85–86.

large difference in resonance frequency between base-on $^{-}OOC^{13}CH_2Cbl$ and $^{-}OOC^{13}CH_2Cbi$ at 25 °C (ca. 500 Hz, Table III) suggests this is an ideal species for such studies.

Measurements of the temperature dependence of the resonance frequency of $^{-}OOC^{13}CH_2Cbl$ relative to that of $^{-}OOC^{13}CH_2Cbi$ at ionic strength 1.0 M (Figure 4) were made at pH 10.7. The pH value was chosen to avoid any significant protonation (<1.0%) of the carboxylate of either $^{-}OOC^{13}CH_2Cbi$ ($pK_a = 6.09$ at 25 °C)[28] or $^{-}OOC^{13}CH_2Cbl$ ($pK_a = 7.24$ at 25 °C),[28] taking into account both the temperature dependence of the pK_a of the buffer and the anticipated temperature dependence of the pK_a's of the (carboxymethyl)cobalt species from that of model compounds.[44] Application of the complete scheme (eq 2, 3, and 6) by a nonlinear least-squares analysis according to eq 5 leads to values of −8.04 kcal mol^{-1} and −20.5 cal mol^{-1} K^{-1} for ΔH_{measd} and ΔS_{measd}, respectively, and 524.22 Hz and −0.003 69 Hz K^{-1} for $\nu_0^{\text{on}} - \nu_0^{\text{off}}$ and $m^{\text{on}} - m^{\text{off}}$, respectively. Thus, K_{measd} was 26.1, and values of 113 and 3.29 for K_{Co} and K_{H}, respectively, could be calculated. The last value is in adequate agreement with the average value of K_{H} (4.16) obtained for $^{13}CH_3Cbl$ at ionic strength 1.0 M.

Conclusions. Additional evidence for the formation of a base-off benzimidazole-deprotonated species of cobalamin in which benzimidazole N3 is hydrogen-bonded to the corrin e side chain (**4** in eq 6), previously characterized by ^{13}C NMR spectroscopy, has been obtained, and the thermodynamics of its formation have been evaluated. Although the equilibrium constant for formation of this species, K_{H}, is small (Table VI), it is the predominant form (80–90%) of the base-off, but benzimidazole deprotonated, species in solution at all ionic strengths. $pK_{\text{base-off}}$, the apparent pK_a describing the overall, protonic on/off equilibrium, is thus a composite depending upon both K_{Co} (eq 3) and K_{H} (eq 6) as well as pK_{Bz}. However, due to the functional form of this dependence (eq 9) and the small value of K_{H}, values of K_{Co}, the intrinsic affinity of the free-base pendant nucleotide for the cobalt atom, previously calculated without regard for the hydrogen-bonded species, are not significantly altered except for those complexes for which $pK_{\text{base-off}}$ exceeds 3.5. Table VIII summarizes the revised values for the thermodynamics of the base-on/base-off reaction with the hydrogen-bonded species taken into account. Clearly, only the values of ΔH_{Co} and ΔS_{Co} for CH_3CH_2Cbl and $CH_3(CH_2)_2Cbl$ are significantly altered by the new treatment. Most importantly the conclusions previously reached[8] regarding the thermodynamics of the on/off reaction of cobalamins are not altered. The enthalpy of formation of the base-on species, ΔH_{Co}, is essentially independent of the nature of the upper axial ligand despite variation in K_{Co} of 4 orders of magnitude, the average value being −7.88 ± 0.65 kcal mol^{-1}. All nine of the available values fall within 90% confidence limits (−7.88 ± 1.21 kcal mol^{-1}). Thus, all of the variation in K_{Co}, and hence $pK_{\text{base-off}}$, is due to variation in the entropy change upon coordination of the axial nucleotide. As previously discussed,[8] neither the origin of these variations in ΔS_{Co} nor the trend in ΔS_{Co} (and hence K_{Co} and $pK_{\text{base-off}}$) is understood. Attempts to gain further insight into this very fundamental chemical property of cobalamins are currently in progress.

Acknowledgment. This research was supported by the Robert A. Welch Foundation, Houston, TX (Grant Y-749). We are grateful to Dr. D. W. Jacobsen, Cleveland Clinic Foundation, for HPLC analysis.

Appendix

Derivation of Eq 5. The observed resonance frequency of an organocobalamin enriched in ^{13}C in the α-carbon, at neutral pH, is the weighted sum of the resonance frequencies of the base-on (**2**) and base-off (**3**) species present at equilibrium at the measurement temperature (eq 11), where ν_{on} and ν_{off} are the resonance

$$\nu_{\text{obsd}} = (1 - \alpha_{\text{on}})\nu_{\text{off}} + \alpha_{\text{on}}\nu_{\text{on}} \tag{11}$$

frequencies of the base-on and base-off species, respectively, and α_{on} is the fraction of base-on species at equilibrium. Substituting eq 12, from the mass-action definition of K_{Co} (eq 3), gives eq 13.

$$\alpha_{\text{on}} = K_{\text{Co}}/(1 + K_{\text{Co}}) \tag{12}$$

$$\nu_{\text{obsd}} = (\nu_{\text{off}} + \nu_{\text{on}}K_{\text{Co}})/(1 + K_{\text{Co}}) \tag{13}$$

Assuming that the resonance frequency of the relevant ^{13}C-enriched alkylcobinamide is identical with that of the base-off species, the measured quantity, $\Delta\nu_T$, the resonance frequency of the cobalamin relative to that of the cobinamide, is $\nu_{\text{obsd}} - \nu_{\text{off}}$ and is given by eq 14. Substituting the temperature dependence of K_{Co}

$$\Delta\nu_T = K_{\text{Co}}(\nu_{\text{on}} - \nu_{\text{off}})/(1 + K_{\text{Co}}) \tag{14}$$

$$K_{\text{Co}} = e^{[(\Delta S_{\text{Co}}/R) - (\Delta H_{\text{Co}}/RT)]} \tag{15}$$

(eq 15), along with the linear temperature dependencies of ν_{on} and ν_{off} (eq 16 and 17; see Figure 3), leads to eq 5 directly.

$$\nu_{\text{on}} = \nu_0^{\text{on}} + m_{\text{on}}T \tag{16}$$

$$\nu_{\text{off}} = \nu_0^{\text{off}} + m_{\text{off}}T \tag{17}$$

Derivation of Eq 10. For the complete scheme (eq 2, 3, and 6) the observed resonance frequency of an organocobalamin enriched in ^{13}C in the α-carbon, at neutral pH, is the weighted sum of the resonance frequencies of species **2**, **3**, and **4** (eq 18), where

$$\nu_{\text{obsd}} = \alpha_2\nu_2 + \alpha_3\nu_3 + \alpha_4\nu_4 \tag{18}$$

ν_2, ν_3, and ν_4 are those resonance frequencies, α_2, α_3, and α_4 are the fractions of species **2**, **3**, and **4** present at equilibrium, and

$$\alpha_2 + \alpha_3 + \alpha_4 = 1 \tag{19}$$

If it is assumed that the resonance frequencies of species **3** and **4** are the same, then eq 18 may be replaced by eq 20. From the

$$\nu_{\text{obsd}} = \alpha_2\nu_2 + \alpha_3\nu_3 + \alpha_4\nu_3 \tag{20}$$

mass-action definitions of K_{Co} (eq 3) and K_{H} (eq 6) and eq 19, α_2, α_3, and α_4 may be represented as in eq 21–23. The measured

$$\alpha_2 = K_{\text{Co}}/(K_{\text{Co}} + K_{\text{H}} + 1) \tag{21}$$

$$\alpha_3 = 1/(K_{\text{Co}} + K_{\text{H}} + 1) \tag{22}$$

$$\alpha_4 = K_{\text{H}}/(K_{\text{Co}} + K_{\text{H}} + 1) \tag{23}$$

quantity, $\Delta\nu_T$, the resonance frequency of the cobalamin relative to that of the cobinamide, is $\nu_{\text{obsd}} - \nu_3$, assuming that the resonance frequency of the relevant ^{13}C-enriched alkylcobinamide is identical with that of species **3**. Hence

$$\Delta\nu_T = K_{\text{Co}}(\nu_2 - \nu_3)/(K_{\text{Co}} + K_{\text{H}} + 1) \tag{24}$$

Substituting the temperature dependencies of K_{Co} (eq 15), K_{H} (eq 25), and ν_2 and ν_3 (eq 26 and 27) leads directly to eq 10.

$$K_{\text{H}} = e^{[(\Delta S_{\text{H}}/R) - (\Delta H_{\text{H}}/RT)]} \tag{25}$$

$$\nu_2 = \nu_0^{\text{on}} + m_{\text{on}}T \tag{26}$$

$$\nu_3 = \nu_0^{\text{off}} + m_{\text{off}}T \tag{27}$$

Registry No. $^{13}CH_3Cbl$ (base on), 43184-67-4; $^{13}CH_3Cbl$ (base off), 111266-92-3; $^{13}CH_3Cbi$, 115826-45-4; $CH_3{}^{13}CH_2Cbl$ (base on), 115826-48-7; $CH_3{}^{13}CH_2Cbl$ (base off), 111290-85-8; $CH_3{}^{13}CH_2Cbi$, 115826-46-5; $HOOC^{13}CH_2Cbl$ (base on), 115826-49-8; $^{-}OOC^{13}CH_2Cbl$ (base on), 115826-50-1; $HOOC^{13}CH_2Cbl$ (base off), 115826-51-2; $HOOC^{13}CH_2Cbi$, 115826-47-6; $^{-}OOC^{13}CH_2Cbi$, 115936-16-8; CH_3CH_2Cbl, 13422-56-5; CH_3Cbl, 13422-55-4.

---

[44] Brown, K. L.; Awtrey, A. W.; LeGates, R. *J. Am. Chem. Soc.* **1978**, *100*, 823–828.

[45] Brown, K. L.; Hakimi, J. M.; Jacobsen, D. W. *J. Am. Chem. Soc.* **1984**, *106*, 7894–7899.