Thermodynamic and Kinetic Studies on the Reaction between the Vitamin B_{12} Derivative β-(*N*-Methylimidazolyl)cobalamin and *N*-Methylimidazole: Ligand Displacement at the α Axial Site of Cobalamins

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The equilibria and kinetics of substitution of the 5,6-dimethylbenzimidazole at the α site of β-(*N*-methylimidazolyl)-cobalamin by *N*-methylimidazole have been investigated, and the product, bis(*N*-methylimidazolyl)cobalamin, has been characterized by visible and ^{1}H NMR spectroscopies. The equilibrium constant for (N-MeIm)Cbl$^+$ + N-MeIm ⇌ (N-MeIm)$_2$Cbl$^+$ was determined by ^{1}H NMR spectroscopy (9.6 ± 0.1 M^{-1}, 25.0 °C, I = 1.5 M ($NaClO_4$)). The observed rate constant for this reaction exhibits an unusual inverse dependence on *N*-methylimidazole concentration, and it is proposed that substitution occurs via a base-off solvent-bound intermediate. Activation parameters typical for a dissociative ligand substitution mechanism are reported at two different N-MeIm$_T$ concentrations, 5.00 × 10^{-3} M ($\Delta H^‡$ = 99 ± 2 kJ mol^{-1}, $\Delta S^‡$ = 39 ± 5 J mol^{-1} K^{-1}, $\Delta V^‡$ = 15.0 ± 0.7 cm^3 mol^{-1}, and 1.00 M ($\Delta H^‡$ = 109.4 ± 0.8 kJ mol^{-1}, $\Delta S^‡$ = 70 ± 3 J mol^{-1} K^{-1}, $\Delta V^‡$ = 16.8 ± 1.1 cm^3 mol^{-1}). According to the proposed mechanism, these parameters correspond to the aquation of (N-MeIm)$_2$Cbl$^+$ and the ring-opening reaction of the α-DMBI of (N-MeIm)Cbl$^+$ to give the solvent-bound intermediate in both cases, respectively.

Introduction

Vitamin B_{12} and its derivatives are the most biologically important cobalt(III) compounds known and are essential in the nutrition of animals and humans.[1] The equatorial sites of vitamin B_{12} are occupied by a structurally unique corrin ring which is responsible for the exceptionally high lability of the axial sites of vitamin B_{12} compared to other Co(III) complexes with N-donor equatorial ligands. Typical substitution rate constants for the β site of vitamin B_{12} are ≅ 10^3 M^{-1} s^{-1}, compared to Co(III) porphyrins ≅ 2 M^{-1} s^{-1}, bis(ethylenediamine) ≅ 10^{-2} M^{-1} s^{-1}, bis(dimethylglyoximate) ≅ 10^{-4} M^{-1} s^{-1}, $(NH_3)_4$ ≅ 10^{-5} M^{-1} s^{-1}.[2] In the vitamin B_{12} derivatives, the α axial site is occupied by an intramolecularly bound 5,6-dimethylbenzimidazole (henceforth referred to as DMBI) and the β axial site is typically occupied by a water (aquacobalamin), cyanide (cyanocobalamin = vitamin B_{12}), 5′-deoxyadenosine (adenosylcobalamin = coenzyme B_{12}), or methyl group (methylcobalamin). The latter two forms are required for a number of enzyme reactions for which a key step involves homolytic or heterolytic cleavage of the β Co−C bond for the adenosylcobalamin-dependent isomerases and for the methylcobalamin-dependent methyltransferases.[3]

Co−C bond cleavage occurs considerably faster in the presence of the enzyme (e.g., for adenosylcobalamin a ca. 10^{12} rate enhancement;[4] for methylcobalamin a ca. 10^5 rate enhancement[5]) in comparison to the enzyme-free cofactors in solution. There has been much speculation as to what is responsible for this rate enhancement, and it now appears that a major contribution to Co−C homolysis arises from conformational changes in the enzyme which occur upon substrate binding, leading to a sterically strained adenosyl group and ultimately to cleavage of the Co−C bond.[6] Factors that determine the rate of Co−C heterolysis are less well understood, although it has been suggested that electronic factors are important.[3] Studies using model complexes of vitamin B_{12} have shown that the nature of the ligand trans to the Co−C bond can play a role in the kinetic and thermodynamic stability of this bond,[7] although until recently this has been regarded as unimportant as it was assumed that the DMBI remained coordinated at the α position during the enzyme reaction. However, the discovery using EPR spectroscopy and X-ray structure analysis that the α-DMBI can be displaced by a histidine residue from the enzyme for both

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adenosylcobalamin-dependent isomerases and methylcobalamin-dependent methyltransferases has opened up a completely new area for consideration,[3,8,9] namely the substitution chemistry of the α axial site of cobalamins.

There has been considerable interest in the substitution behavior of the β water ligand of aquacobalamin, which is readily substituted by a wide range of nucleophiles (e.g., L = SCN^-, CN^-, NO_2^-, Br^-, I^-, NCO^-, SO_3^{2-}, pyridine, imidazole, $S_2O_3^{2-}$, N_3^-, thiourea, glycine, NH_2R, $Fe(CN)_6^{4-}$).[2,10−13] It is generally agreed that this occurs via a dissociative interchange mechanism; the experimental evidence for this includes the observed rate constant being essentially independent of the nature of the entering ligand for a wide range of ligands, kinetic evidence for ion-pair formation in a few special cases[11] and positive volumes of activation.[11a,b,12] However, as far as we are aware, the only ligand known to be capable of substituting the DMBI at the α axial site of cobalamins (apart from (α-DMBI)-Co cleavage resulting from protonation of the N1 nitrogen of α-DMBI) is cyanide (adenosylcobalamin,[14a−c] cyanocobalamin,[14d] (oxocarbonyl)cobalamins,[14e,f] benzylcobalamin,[14g] other cobalamins[14h]), with the majority of authors probably regarding this site as being inert in the absence of cyanide and acid.[7,10b] During their experiments to determine formation constants for imidazolylcobalamins by visible spectroscopy, Marques et al.[15] observed the presence of a new species at higher imidazole concentrations which they were able to separate by HPLC and suggested that an imidazole had displaced the α-DMBI of the imidazolylcobalamins. In light of the evidence demonstrating that histidine can displace the α-DMBI ligand during the B_{12}-dependent enzyme reactions, it seems reasonable that this position could also be labile in the absence of the enzyme and that substitution studies on this reaction could be used as a means to increase our understanding of factors accompanying substitution of the α-DMBI by a histidine-type ligand.

The paper presents visible and ^{1}H NMR spectroscopic data that show that the α-DMBI ligand of β-(*N*-methylimidazolyl)-cobalamin (henceforth referred to as (*N*-methylimidazolyl)-cobalamin) can be readily displaced by *N*-methylimidazole under biological pH conditions, thus demonstrating that substitution of the α-DMBI of a cobalamin by a histidine type ligand can also occur in the absence of the enzyme. Equilibria and kinetic data for this reaction have been obtained for a range of pH conditions (pD 6.5−9.5 and pH 5.5−9.5, respectively). Kinetic studies reveal a rather interesting mechanism for this substitution process.

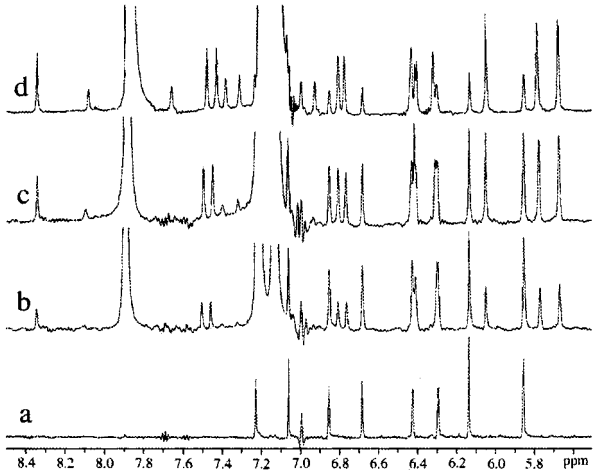

Figure 1. ^{1}H NMR spectra of the aromatic region for equilibrated solutions of HOCbl·HCl (ca. 3.0×10^{-3} M) and varying concentrations of N-MeIm$_T$: 3.00×10^{-3} (a), 5.00×10^{-2} (b), 0.100 (c), or 0.250 M (d) (25.0 °C, in D_2O, 0.100 M TAPS buffer, pD 8.51). The signals at 5.85, 6.14, 6.30(d) 6.43, 6.68, 6.85, 7.06, and 7.23 ppm in spectrum a can be attributed to (N-MeIm)Cbl$^+$. Increasing the N-MeIm$_T$ concentration (b → d) results in the decrease in the intensity of the (N-MeIm)-Cbl$^+$ signals and the concurrent appearance and increase in intensity of signals attributable to (N-MeIm)$_2$Cbl$^+$. Signal assignments are given in Table 1.

Results

Characterization of (N-MeIm)Cbl$^+$ and (N-MeIm)$_2$Cbl$^+$. Figure 1a gives the ^{1}H NMR spectrum of the aromatic region[16] of a ca. 1:1 mixture of HOCbl·HCl (H_2OCbl^+ and HOCbl; pK_a(H_2OCbl^+) = 8.13 (see below)) and N-MeIm$_T$ (N-MeIm$_T$ = N-MeIm + N-MeImH$^+$) at pD 8.51 ca. 2 h after mixing. The spectrum is very different from that of the starting materials (HOCbl/H_2OCbl^+ and N-MeIm/N-MeImH$^+$ have signals in the aromatic region at 6.19, 6.23(d), 6.44, 6.58, and 7.16 ppm and 7.13, 7.21, and 7.89 ppm, respectively (25.0 °C, pD 8.51, I = 1.5 M ($NaClO_4$)), and corresponds to that of (N-MeIm)Cbl$^+$. Eight signals at 5.85, 6.14, 6.30(d), 6.43, 6.68, 6.85, 7.06, and

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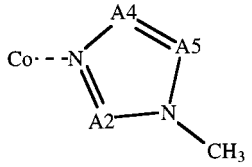
Table 1. ^{1}H NMR Assignments of the Protons in the Aromatic Region for (N-MeIm)Cbl$^+$ and (N-MeIm)$_2$Cbl$^+$.

(N-MeIm)Cbl$^+$ [a,b]		(N-MeIm)$_2$Cbl$^+$ [a–c]	
signal (ppm)	assignment	signal (ppm)[d]	assignment
5.85	β-A5	5.67	A5[e]
6.14	C10	5.78	A5[f]
6.30 (d)	R1	6.05	C10
6.43	β-A2	6.32	A2[f]
6.68	B4	6.41 (d)	R1
6.85	β-A4	6.43	A2[e]
7.06	B2	6.77	A4[f]
7.23	B7	6.81	A4[e]
		7.43	B4 or B7
		7.48	B4 or B7
		8.35	B2

[a] See Scheme 1 for coordinated N-MeIm labeling scheme. [b] See ref 17 for cobalamin labeling scheme. [c] Free (uncoordinated) N-MeIm$_T$ signals observed at 7.07, 7.20, and 7.85 ppm. [d] From spectrum 1b. [e] These signals belong to the same N-MeIm ligand (α or β). [f] These signals belong to the same N-MeIm ligand (α or β).

Scheme 1. Labeling Scheme for Coordinated N-MeIm Ligands



7.23 ppm are observed in the aromatic region, as expected for (N-MeIm)Cbl$^+$, corresponding to the B2, B4, B7, and R1 signals of the nucleotide, the C10 signal of the corrin ring and the three aromatic signals of the β-coordinated N-MeIm (A2, A4, A5 (see Table 1 for labeling schemes)). The signal assignments are given in Table 1; assignment was possible with the additional information provided by HMQC and HMBC experiments. At longer times the intensity of the β-A2 proton of (N-MeIm)-Cbl$^+$ gradually decreases due to relatively rapid proton exchange between this proton and solvent.[18] Both equilibria and rate constants have been previously reported for the reaction between H_2OCbl^+ and N-MeIm to form (N-MeIm)Cbl$^+$.[13,15,19]

Figure 1b gives the ^{1}H NMR spectrum of HOCbl·HCl in 5.00×10^{-2} M N-MeIm$_T$ (pD 8.51, 25.0 °C, I = 1.5 M ($NaClO_4$)). A whole set of new signals are observed and correspond to the 11 expected signals of (N-MeIm)$_2$Cbl$^+$; five from the nucleotide and corrin ring, three from the β-coordinated N-MeIm, and three from the α-coordinated N-MeIm, the latter having displaced the intramolecularly coordinated DMBI from the α axial site. Signals attributable to (N-MeIm)Cbl$^+$ are also visible. From Figure 1b–d it can be seen that the (N-MeIm)$_2$Cbl$^+$ signals in the aromatic region increase in intensity as the N-MeIm$_T$ concentration increases (with a corresponding decrease in the (N-MeIm)Cbl$^+$ signals), since more (N-MeIm)$_2$Cbl$^+$ is formed at higher N-MeIm$_T$ concentrations. Signal assignments for (N-MeIm)$_2$Cbl$^+$ in the aromatic region are summarized in Table 1.

Figure 2 gives visible spectra for (N-MeIm)Cbl$^+$ (λ_{max} ($\pm$1 nm) = 359, 414, 512 (shoulder) and 540 nm; [Cbl]$_T$ = 4.40×10^{-5} M, 0.100 M TAPS buffer, pH 8.50, 25.0 °C) and (N-MeIm)$_2$Cbl$^+$ (λ_{max} ($\pm$1 nm) = 358, 411, 511 (shoulder) and 541 nm; [Cbl]$_T$ = 4.40×10^{-5} M, 0.100 M TAPS buffer, pH 8.50, 25.0 °C). Both spectra are significantly different from that of HOCbl/H_2OCbl^+ at the same conditions (λ_{max} = 356, 414, 515(s) and 532 nm; [Cbl]$_T$ = 4.40×10^{-5} M, 0.100 M TAPS buffer, pH 8.50, 25.0 °C, I = 1.5 M ($NaClO_4$)). Wavelength maxima of (N-MeIm)Cbl$^+$ and (N-MeIm)$_2$Cbl$^+$ are very similar, which is to be expected since the complexes essentially only differ by the type of imidazole ligand they have coordinated to the α axial site. Similar visible spectra were observed by Marques and co-workers.[15]

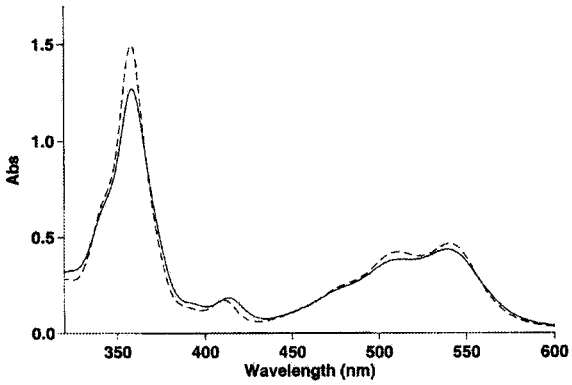


Figure 2. Visible absorbance spectra of (N-MeIm)Cbl$^+$ (—, [Cbl]$_T$ = 4.40×10^{-5} M, N-MeIm$_T$ = 1.50×10^{-3} M, λ_{max} (ϵ, M^{-1} cm^{-1}) = 359 (2.89×10^4), 414 (4.20×10^3), 512 (shoulder, 8.77×10^3), 540 (9.95×10^3) nm) and (N-MeIm)$_2$Cbl$^+$ (- - -, [Cbl]$_T$ = 4.40×10^{-5} M, N-MeIm$_T$ = 2.20 M, λ_{max} (ϵ, M^{-1} cm^{-1}) = 358 (3.40×10^4), 411 (3.96×10^3), 511 (shoulder, 9.66×10^3), 541 (1.06×10^4) nm). Errors in the extinction coefficients are estimated to be $\pm$4%. Both spectra are recorded at 25.0 °C in 0.100 M TAPS buffer, pH 8.50, I = 1.5 M ($NaClO_4$).

A FAB-MS spectrum (positive ion mode) was also obtained for a sample of (N-MeIm)$_2$Cbl$^+$·Cl$^-$ precipitated from a solution of HOCbl·HCl in ca. 2 M N-MeIm$_T$ (pH 8.5). A small peak was observed at m/z = 1492.9 (calculated m/z for (N-MeIm)$_2$Cbl$^+$ ($C_{70}H_{100}O_{14}N_{17}PCo$) = 1492.7), confirming that (N-MeIm)$_2$Cbl$^+$ is formed at high N-MeIm$_T$ concentrations.

Acid Dissociation Constants. The acid dissociation constants of H_2OCbl^+ and N-MeImH$^+$ at 25.0 °C and I = 1.5 M ($NaClO_4$) were determined by potentiometric titration to be (7.38 ± 0.16) $\times 10^{-9}$ M (pK_a = 8.13 $\pm$ 0.01) and (2.91 ± 0.10) $\times 10^{-8}$ M (pK_a = 7.54 $\pm$ 0.02), respectively. Both values are the mean of two separate determinations. The value for H_2OCbl^+ is in good agreement with previous values (at 25 °C): 8.10[20a] (I = 1.0 M, KCl), 8.1[14d] (I = 1.0 M, KCl), 8.09[20b] (I = 0.5 M, $NaNO_3$), 7.93[12a] (I = 1.0 M, KCl), 7.8[20c] (0.05 M phosphate buffer) and 7.62[20d] (I = 0.1 M, KNO_3). Interestingly, the presence of $NaClO_4$ (I = 1.5 M) appears to significantly increase the pK_a of N-MeImH$^+$; reported literature values (at 25 °C) include 7.21[13] (I = 1.0 M (KCl)), 7.2[21a] (I = 0.1 M, phosphate buffer), 7.25[21b] (I = 0.1 M, KCl), 7.20[21c] (I = 0.15 M, KCl), 7.19[21d] (I = 0.5 M, KNO_3), 7.20[21e] (I = 1.0 M, $(CH_3)_4N^+Cl^-$), 7.05[19] (I = 0.1 M, KNO_3) and 7.20[21f] (I = 0.15 M, KNO_3).

Equilibrium Constants. As shown in Scheme 2, when N-MeIm is reacted with H_2OCbl^+, both (N-MeIm)Cbl$^+$ and

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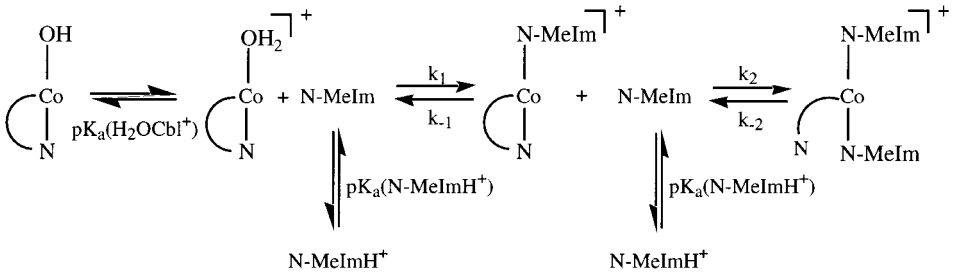
Scheme 2



$(N\text{-MeIm})_2Cbl^+$ form, the proportions of each cobalamin depending on the pH of the solution and the concentration of N-MeIm. It was therefore necessary to have a complete understanding of the equilibria (K_1) and kinetics (k_1, k_{-1}) for the formation of $(N\text{-MeIm})Cbl^+$ from H_2OCbl^+ and N-MeIm prior to investigating the reaction between $(N\text{-MeIm})Cbl^+$ and N-MeIm, so that the latter reaction could be studied under conditions where there would be no interference from the former reaction.

The equilibrium constant, $K_{obs(1)}$ for the equilibrium

$$HOCbl/H_2OCbl^+ + N\text{-MeIm}_T \overset{K_{obs(1)}}{\rightleftharpoons} (N\text{-MeIm})Cbl^+ \quad (1)$$

($N\text{-MeIm}_T$ = N-MeIm + $N\text{-MeImH}^+$) was determined at four pH conditions (pH 6.51, 6.99, 7.52, 7.98) by measuring the absorbance of equilibrated solutions containing a fixed amount of HOCbl·HCl (ca. 5×10^{-5} M, exact concentration known) and a varying amount of $N\text{-MeIm}_T$ ($0-2.34 \times 10^{-3}$ M) by visible spectroscopy (25.0 °C, I = 1.5 M ($NaClO_4$)). Figure 3 gives a plot of observed absorbance (A_{obs}) at 358 nm versus total N-MeIm concentration for the data obtained at pH 6.51. Similar plots at the other pH conditions are given in Figures A−C, Supporting Information. The fitted curve represents the best fit of the data to eq 2, which is derived in the Supporting Information.

$$A_{obs} = [A_{(N-MeIm)Cbl+}(X - Y)/2K_{obs(1)}[Cbl]_T] + [([Cbl]_T - (X - Y)/2K_{obs(1)})A_{H2OCbl+}/[Cbl]_T] \quad (2)$$

where $X = K_{obs(1)}[Cbl]_T + K_{obs(1)}[N\text{-MeIm}]_C + 1$, $Y = [X^2 - 4(K_{obs(1)})^2[Cbl]_T[N\text{-MeIm}]_C]^{1/2}$, $[N\text{-MeIm}]_C = [(N\text{-MeIm})Cbl^+] + [N\text{-MeIm}] + [N\text{-MeImH}^+]$, $[Cbl]_T = [H_2OCbl^+] + [HOCbl] + [(N\text{-MeIm})Cbl^+]$, $A_{(N-MeIm)Cbl+}$ = absorbance of $(N\text{-MeIm})Cbl^+$, and $A_{H2OCbl+}$ = absorbance of H_2OCbl^+. The equilibrium constant $K_{obs(1)}$ was found to be $(1.28 \pm 0.06) \times 10^4$, $(3.08 \pm 0.14) \times 10^4$, $(5.41 \pm 0.35) \times 10^4$, and $(6.26 \pm 0.35) \times 10^4$ M^{-1} at pH 6.51, 6.99, 7.52, and 7.98, respectively.

Assuming that only H_2OCbl^+ and N-MeIm react to give $(N\text{-MeIm})Cbl^+$ (i.e., that HOCbl and $N\text{-MeImH}^+$ do not react), corresponding K_1 values can be calculated, where K_1 is defined by

$$H_2OCbl^+ + N\text{-MeIm} \overset{K_1}{\rightleftharpoons} (N\text{-MeIm})Cbl^+ \quad (3)$$

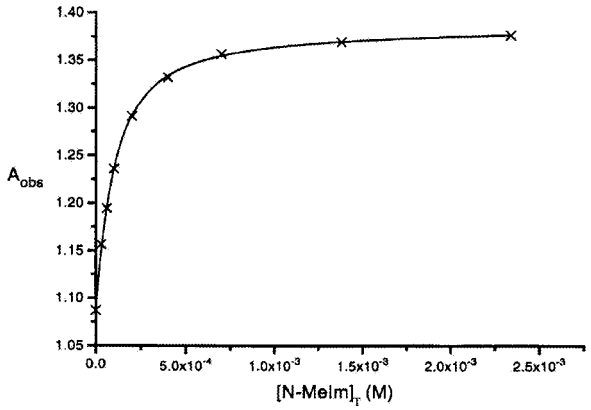


Figure 3. Plot of observed absorbance (A_{obs}) at 358 nm versus total N-MeIm concentration for equilibrated solutions of HOCbl·HCl (4.60×10^{-5} M) and varying concentrations of $N\text{-MeIm}_T$ ($0-2.34 \times 10^{-3}$ M); 0.100 M BIS-TRIS buffer, pH 6.51, I = 1.5 M ($NaClO_4$). The fitted curve represents the best fit of the data to eq 2; fixing $[Cbl_T] = 4.60 \times 10^{-5}$ M gives $K_{obs(1)} = (1.28 \pm 0.06) \times 10^4$ M^{-1}, $A_{H2OCbl+} = 1.089 \pm 0.002$, and $A_{(N-MeIm)Cbl+} = 1.386 \pm 0.002$.

Using potentiometrically determined values of $pK_a(H_2OCbl^+) = 8.13 \pm 0.01$ (25.0 °C, I = 1.5 M ($NaClO_4$)) and $pK_a(N\text{-MeImH}^+) = 7.54 \pm 0.02$ (25.0 °C, I = 1.5 M ($NaClO_4$)), a mean value of $K_1 = (1.47 \pm 0.07) \times 10^5$ M^{-1} is obtained. The excellent agreement between the four K_1 values obtained at different pH conditions demonstrates that the assumptions that HOCbl and $N\text{-MeImH}^+$ do not react are valid for this system.

Attempts were made to determine the equilibrium constant $K_{obs(2)}$ for

$$(N\text{-MeIm})Cbl^+ + N\text{-MeIm}_T \overset{K_{obs(2)}}{\rightleftharpoons} (N\text{-MeIm})_2Cbl^+ \quad (4)$$

by visible spectroscopy. There was, however, considerable scatter in the plots of observed absorbance versus N-MeIm concentration, since the visible spectra of $(N\text{-MeIm})Cbl^+$ and $(N\text{-MeIm})_2Cbl^+$ are very similar (Figure 2). Values of $K_{obs(2)}$ were therefore determined in D_2O at seven pD conditions by ^1H NMR spectroscopy by comparing the signal area of one signal from each of the two complexes (5.88 ± 0.04 ppm for $(N\text{-MeIm})Cbl^+$, 5.71 ± 0.05 ppm for $(N\text{-MeIm})_2Cbl^+$) at each pD condition for a series of solutions with varying $N\text{-MeIm}_T$ concentrations ($5.00 \times 10^{-2} - 1.20$ M); see Table A in the Supporting Information. The data are summarized in Figure 4. Note that under all conditions formation of $(N\text{-MeIm})Cbl^+$ from (H_2OCbl^+ + N-MeIm) is complete; that is, no H_2OCbl^+ (or HOCbl) is present. Assuming only N-MeIm reacts with $(N\text{-MeIm})Cbl^+$ to form $(N\text{-MeIm})_2Cbl^+$ (i.e., $N\text{-MeImH}^+$ cannot coordinate to $(N\text{-MeIm})Cbl^+$), K_2 can be calculated, where K_2 corresponds to the equilibrium

$$(N\text{-MeIm})Cbl^+ + N-MeIm \overset{K_2}{\rightleftharpoons} (N\text{-MeIm})_2Cbl^+ \quad (5)$$

(21) (a) Hamza, M. S. A.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1994**, 1367. (b) Catalán, J.; Claramont, R. M.; Elguero, J.; Laynez, J.; Menéndez, M.; Anvia, F.; Quian, J. H.; Taagepera, M.; Taft, R. W. *J. Am. Chem. Soc.* **1988**, *110*, 4105. (c) Paiva, A. C. M.; Juliano, L.; Boschcov, P. *J. Am. Chem. Soc.* **1976**, *98*, 7645. (d) Lenarcik, B.; Barszcz, B. *Rocz. Chem.* **1977**, *51*, 1849. (e) Oakenfull, D. G.; Jencks, W. P. *J. Am. Chem. Soc.* **1971**, *93*, 178. (f) Li, N. C.; White, J. M.; Doody, E. *J. Am. Chem. Soc.* **1954**, *76*, 6219.

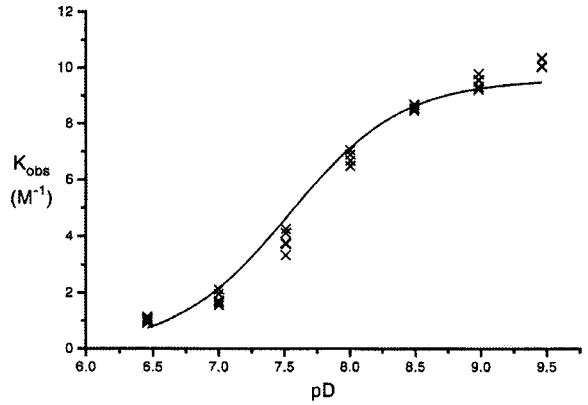


Figure 4. Plot of the observed equilibrium constant $K_{obs(2)}$ versus pD, where $K_{obs(2)}$ refers to the equilibrium given in eq 4. The fitted curve represents the best fit of the data to eq 6; fixing $K_{a(N-MeImH+)} = 2.91 \times 10^{-8}$ M gives $K_2 = 9.6 \pm 0.1$ M^{-1}.

The fitted curve in Figure 4 represents the best fit of the data to the equation

$$K_{obs(2)} = (K_a(\text{N-MeImH}^+)K_2)/(K_a(\text{N-MeImH}^+) + \log10^{-1}(-\text{pD})) \quad (6)$$

Fixing K_a(N-MeImH$^+$) to 2.91×10^{-8} M (p$K_a = 7.54$) gives $K_2 = 9.6 \pm 0.1$ M^{-1}. The excellent fit of the data shows that the assumption that only N-MeIm can react with (N-MeIm)Cbl$^+$ is valid.

Kinetics. The concentration dependence of the observed rate constant for the reaction

$$\text{HOCbl/H}_2\text{OCbl}^+ + \text{N-MeIm}_T \xrightarrow{k_{obs(1)}} \text{(N-MeIm)Cbl}^+ \quad (7)$$

was examined by visible spectroscopy (358 nm) at six different pH conditions, pH 6.01, 7.01, 7.30, 8.00, 8.50, and 9.00 (25.0 °C, $I = 1.5$ M ($NaClO_4$)). Pseudo-first-order conditions with respect to N-MeIm$_T$ were used, and N-MeIm concentrations were low enough ($< 5.00 \times 10^{-3}$ M) so very little (N-MeIm)$_2$Cbl$^+$ was formed, usually much less than 4%. For each pH condition the plot of the observed rate constant, $k_{obs(1)}$, versus [N-MeIm]$_T$ was linear, and intercepted the origin (Figures D−I, Supporting Information), meaning that the reaction is irreversible under all experimental conditions. Values for $k_{obs(1)}$ were obtained from the slopes of the plots. Since it has previously been shown that only H_2OCbl^+ and N-MeIm react to form (N-MeIm)Cbl$^+$ [13]

$$\text{H}_2\text{OCbl}^+ + \text{N-MeIm} \xrightarrow{k_1} \text{(N-MeIm)Cbl}^+ \quad (8)$$

the pH-independent rate constant k_1 can be obtained by allowing for the fraction of H_2OCbl^+ and N-MeIm present at each pH condition. Values of $k_1 = 68.9$, 80.2, 74.4, 79.5, 85.1, and 85.1 M^{-1} s^{-1} were obtained at pH 6.01, 7.01, 7.30, 8.00, 8.50, and 9.00, respectively, giving a mean $k_1 = 79 \pm 6$ M^{-1} s^{-1}.

The concentration dependence of the observed rate constant, $k_{obs(2)}$ for the reaction

$$\text{(N-MeIm)Cbl}^+ + \text{N-MeIm} \rightleftharpoons^{k_{obs(2)}} \text{(N-MeIm)}_2\text{Cbl}^+ \quad (9)$$

was measured at pH 8.50 by visible spectroscopy (358 nm, 25.0 °C, $I = 1.5$ M ($NaClO_4$)).The results are summarized in Figure 5 and show that $k_{obs(2)}$ decreases with increasing N-MeIm.

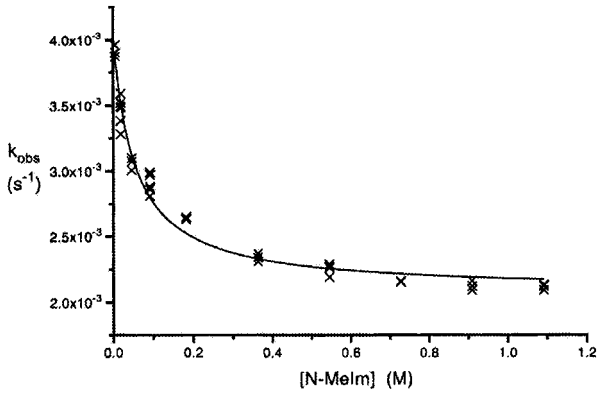


Figure 5. Plot of observed rate constant, $k_{obs(2)}$, versus [N-MeIm] for the reaction (N-MeIm)Cbl$^+$ + N-MeIm ⇄ (N-MeIm)$_2$Cbl$^+$ at pH 8.50 (0.100 M TAPS buffer, 25.0 °C, $I = 1.5$ M ($NaClO_4$)). The curve represents the best fit of the data to eq 11 in the text. Fixing $K_2 = 9.6$ M^{-1} gives $k_3 = (2.08 \pm 0.02) \times 10^{-3}$ s^{-1} and $k_{-4} = (4.00 \pm 0.04) \times 10^{-3}$ s^{-1}.

Results of kinetic scanning experiments (from 300 to 600 nm) confirmed that the same reaction is being followed over the entire concentration range, since the reaction isosbestic points are independent of concentration. For example, at pH 8.50, isosbestic points were observed at 335 ± 1, 366 ± 1, 404 ± 1, 410 ± 1, 448 ± 3 and 563 ± 1 nm for both 0.800 and 5.00×10^{-3} M N-MeIm$_T$ (25.0 °C, $I = 1.5$ M ($NaClO_4$)).

The concentration dependence of $k_{obs(2)}$ was also examined at pH 7.00 ([N-MeIm]$_T = 5.00 \times 10^{-3} - 1.20$ M ([N-MeIm] $= 1.12 \times 10^{-3} - 0.269$ M). The results are summarized in Figure J, Supporting Information. Much smaller changes (≤ 15%) in $k_{obs(2)}$ were observed at this pH condition, although once again it is apparent that $k_{obs(2)}$ decreases with increasing N-MeIm concentration for [N-MeIm] < 0.18 M. For 0.18−0.27 M N-MeIm, $k_{obs(2)}$ was found to increase ca. 5%. It is likely that this small increase arises from medium effects,[22] since above 0.18 M N-MeIm, N-MeImH$^+$ inevitably contributes significantly (> 40%; [N-MeImH$^+$] > 0.62 M) to the cationic component of the ionic strength. For the experiments shown in Figure 5 (pH 8.50), N-MeImH$^+$ always contributes ≤8% to the cationic component of the ionic strength.

Figure 6a shows the behavior of $k_{obs(2)}$ as a function of pH (0.800 M N-MeIm$_T$, 25.0 °C, $I = 1.5$ M ($NaClO_4$)). The pH dependence of the observed rate constant at 5.00×10^{-3} M N-MeIm$_T$ (25.0 °C, $I = 1.5$ M ($NaClO_4$)) was also examined at pH 5.50, 7.00, and 8.50 and found to be pH-independent ($k_{obs(2)} = (3.93 \pm 0.06) \times 10^{-3}$ s^{-1}; see Table B in the Supporting Information).

Activation Parameters. The temperature dependence of k_{obs} was examined at pH 8.50 for two N-MeIm$_T$ conditions, 5.00×10^{-3} (18−40 °C) and 1.00 M (10−40 °C) in 0.100 M TAPS buffer at $I = 1.5$ M ($NaClO_4$). The reason for choosing these two conditions will become clearer in the Discussion, but basically, conditions have been chosen where the rate constants either correspond to aquation of (N-MeIm)$_2$Cbl$^+$ or ring-opening in (N-MeIm)Cbl$^+$ to give an intermediate. Activation parameters were obtained from Eyring plots, giving $\Delta H^{\ddagger} = 99 \pm 2$ kJ mol^{-1}, $\Delta S^{\ddagger} = 39 \pm 5$ J mol^{-1} K^{-1} and $\Delta H^{\ddagger} = 109.4 \pm 0.8$ kJ mol^{-1}, $\Delta S^{\ddagger} = 70 \pm 3$ J mol^{-1} K^{-1} for 5.00×10^{-3} and 1.00 M N-MeIm$_T$, respectively. The pressure dependence of the observed rate constant at 15.0 °C was also investigated and plots

(22) Wilkins, R. G. *Kinetics and Mechanism of Reactions of Transition Metal Complexes*, 2nd ed.; VCH: Weinheim, Germany, 1991; p 116.

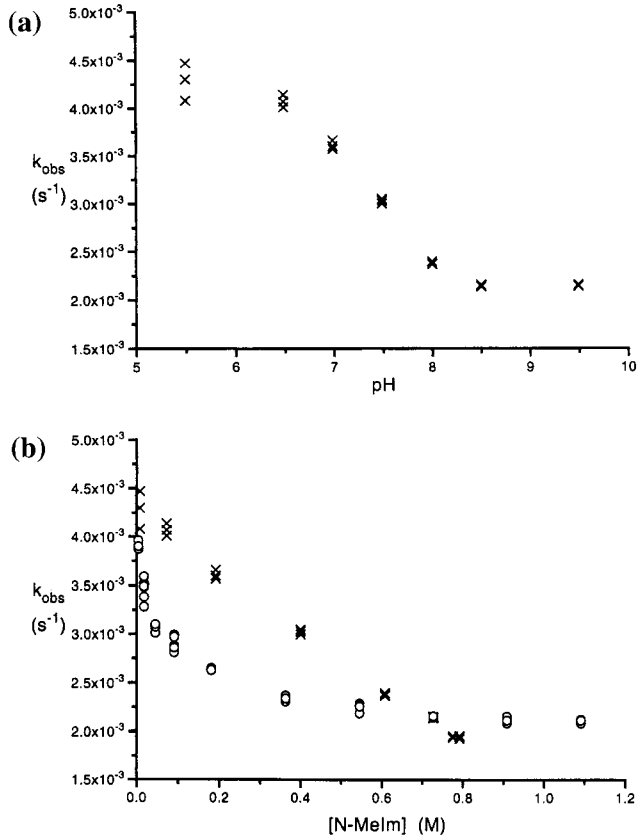


Figure 6. (a) Plot of observed rate, $k_{obs(2)}$ versus pH for the reaction (N-MeIm)Cbl^+ + N-MeIm $\rightleftarrows$ (N-MeIm)$_2Cbl^+$ (0.800 M N-MeIm$_T$, 0.100 M buffer (except for the data at pH 9.49), 25.0 °C, I = 1.5 M ($NaClO_4$)). (b) Plot of $k_{obs(2)}$ versus [N-MeIm] (X) for the data given in (a). The data from Figure 5 are also shown on the plot (O).

of ln $k_{obs(2)}$ versus pressure gave $\Delta V^{\ddagger}$ = 15.0 ± 0.7 and 16.8 ± 1.1 cm^3 mol^{-1} at 5.00 × 10^{-3} and 1.00 M N-MeIm$_T$, respectively (pH 8.50, 0.100 M TAPS buffer, I = 1.5 M ($NaClO_4$); Figures K and L, Supporting Information).

Discussion

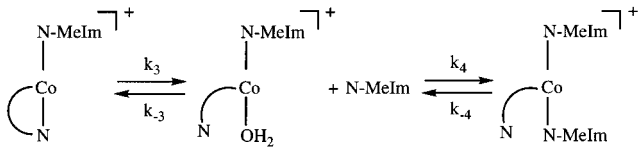
Two things were required prior to studying the reaction between (N-MeIm)Cbl^+ and N-MeIm to form (N-MeIm)$_2Cbl^+$. First, the existence of (N-MeIm)$_2Cbl^+$ in solutions containing high concentrations of N-MeIm needed to be demonstrated, which was achieved by ^{1}H NMR spectroscopy (Figure 1) and FAB-MS. In addition, both the equilibrium (K_1) and rate constant (k_1) for the formation of (N-MeIm)Cbl^+ from H_2OCbl^+ and N-MeIm were determined, so that the reaction of interest could be studied under conditions where the former reaction would not interfere. Recently, both K_1 and k_1 were determined using KCl as the supporting electrolyte.[13,15] It has since been found, however, that chloride binds to H_2OCbl^+,[10f,12c] making these data questionable. In addition, it is useful to re-determine these values at a total ionic strength of 1.5 M using $NaClO_4$ as the supporting electrolyte to allow comparison with other data reported in this paper.

K_1 was determined by visible spectroscopy as the prior study in KCl had done.[15] Our value of K_1 = (1.47 ± 0.07) × 10^5 M^{-1} (25.0 °C, I = 1.5 M ($NaClO_4$)) is larger than the values determined using either KCl or KNO_3 as a supporting electrolyte (K_1 = (4.3 ± 0.5) × 10^4 M^{-1}, 25.0 °C, I = 1.0 M (KCl);[15] K_1 = (2.5 ± 0.50) × 10^4 M^{-1}, 25 °C, I = 0.10 M (KNO_3)[19]). Since K_1 is so large, a simple calculation shows that eq 1 is always irreversible under all experimental conditions in which the equilibria or kinetics of the reaction of (N-MeIm)Cbl^+ with N-MeIm to give (N-MeIm)$_2Cbl^+$ were studied; that is, no H_2OCbl^+ or HOCbl are present at equilibrium.

Our value of k_1 = 79 ± 6 M^{-1} s^{-1}, 25.0 °C, I = 1.5 M ($NaClO_4$)) differs significantly from that reported in KCl (k_1 = 16.6 ± 0.3 M^{-1} s^{-1}, 25.0 °C, I = 1.0 M[13]); hence, it appears that the formation of chlorocobalamin hinders the rate of formation of (N-MeIm)Cbl^+ from H_2OCbl^+ and N-MeIm, as might be expected.[10f,12c]

The equilibrium constant K_2 (eq 5) was determined by ^{1}H NMR spectroscopy to be 9.6 ± 0.1 M^{-1}, meaning that significant concentrations (>5%) of (N-MeIm)$_2Cbl^+$ exist in solution for N-MeIm ≥ 6 mM. Marques and co-workers estimated K_2 to be 1.6 M^{-1} using HPLC;[15] however, this value is less reliable since once again KCl was used to maintain ionic strength (I = 1.0 M).

The most interesting result is the decrease in the observed rate constant, $k_{obs(2)}$ for the reaction (N-MeIm)Cbl^+ + N-MeIm $\rightleftharpoons$ (N-MeIm)$_2Cbl^+$ at pH 8.50 (Figure 5) with increasing N-MeIm concentration. While rather unusual, there are several cases of this reported in the literature.[23] The results are consistent with a mechanism of the type



(10)

with the associated rate equation

$$k_{obs(2)} = (k_3k_4[\text{N-MeIm}] + k_{-3}k_{-4})/(k_{-3} + k_4[\text{N-MeIm}]) \quad (11)$$

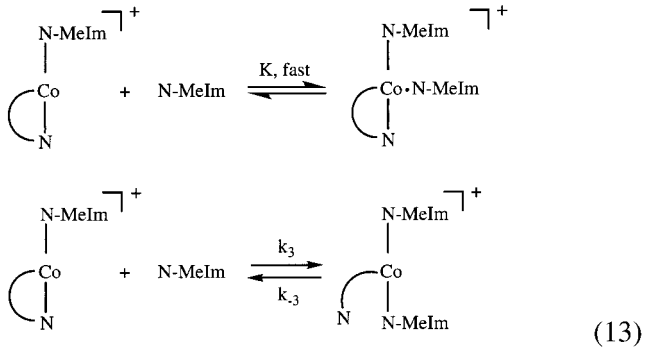
$$= K_2k_{-4}[\text{N-MeIm}] + k_{-4}/(1 + K_2k_{-4}[\text{N-MeIm}]/k_3) \quad (12)$$

It can be seen from eq 11 that at high N-MeIm concentrations $k_{obs(2)}$ will reach a limiting value (= k_3). The observed rate constant also approaches a limiting value at low N-MeIm concentrations (= k_{-4}). Hence, $k_{obs(2)}$ decreases with increasing N-MeIm concentration simply because k_{-4} is larger than k_3. The curve superimposed on the data in Figure 5 represents the fit of the data to eq 11, fixing K_2 = 9.6 M^{-1}.[24] The best fit gives k_3 = (2.08 ± 0.02) × 10^{-3} s^{-1} and k_{-4} = (4.00 ± 0.04) × 10^{-3} s^{-1}. Additional experiments were carried out to investigate the pH dependence of k_{-4} from measurements of $k_{obs(2)}$ (= k_{-4}) for solutions containing 5.00 × 10^{-3} M N-MeIm$_T$ at various pH conditions (pH 5.50, 7.00 and 8.50). The results are summarized in Table B of the Supporting Information and demonstrate that k_{-4} is pH-independent (= (3.93 ± 0.06) × 10^{-3} s^{-1}).

A second possible mechanism which also fits the data given in Figure 5 is a "dead end" type of mechanism

(23) (a) Toma, H. E.; Malin, J. M.; Giesbrecht, E. *Inorg. Chem.* **1973**, *12*, 2084. (b) Malin, J. M.; Toma, H. E.; Giesbrecht, E. *J. Chem. Ed.* **1977**, *54*, 385. (c) Byers, W.; Cossham, J. A.; Edwards, J. O.; Gordon, A. T.; Jones, J. G.; Kenny, E. T. P.; Mahmood, A.; McKnight, J.; Sweigart, D. A.; Tondreau, G. A.; Wright, T. *Inorg. Chem.* **1986**, *25*, 4767. (d) Byrd, J. E.; Wilmarth, W. K. *Inorg. Chim. Act. Rev.* **1971**, 7.

(24) This assumes that K_2 is the same in H_2O and D_2O.



(13)

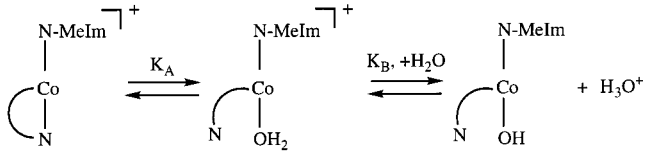
with the corresponding rate equation

$$k_{obs(2)} = k_3/(1 + K[\text{N-MeIm}]) + k_{-3} \qquad (14)$$

Fitting the data gives $K = 15 \pm 2\ M^{-1}$ ($k_3 = (1.89 \pm 0.05) \times 10^{-3}\ M^{-1}\ s^{-1}$, $k_{-3} = (2.03 \pm 0.03) \times 10^{-3}\ s^{-1}$), which is larger than that expected for association between (N-MeIm)-Cbl^+ and neutral N-MeIm. It is therefore unlikely the reaction is occurring via a mechanism of this type.

The pH profile, Figure 6a, also qualitatively supports the concentration profile at pH 8.50 in that once again $k_{obs(2)}$ decreases with increasing pH; increasing pH corresponding to an increase in N-MeIm concentration. However, if eq (11) (or (12)) could explain both the concentration profile data at pH 8.50 and the pH profile data at 0.800 M N-$MeIm_T$, then both sets of data should superimpose upon one another in a plot of $k_{obs(2)}$ versus N-MeIm concentration, which is clearly not the case as seen in Figure 6b. It therefore initially appears that one or more additional pH dependent equilibria must be added to the mechanism given in eq 10.

Several possibilities were considered to explain the discrepancy between the concentration profile data at pH 8.50 and the pH profile data at 0.800 M N-$MeIm_T$. One possibility is that an α-hydroxo intermediate could form in alkaline solution; that is



(15)

The pK_a for the α-aqua intermediate should be in the pH 6−10 range (since $pK_a(H_2OCbl^+) = 7.6-8.1$[12a,14d,20] and pK_a's(diaquacobinamide) = 5.9 and 10.3[25]). However, if the deprotonation of the α-aqua group of the intermediate was important in the pD range 6.5−9.5, since increasingly more of the α-hydroxo intermediate would be formed with increasing pD, less (N-MeIm)$_2$$Cbl^+$ would be formed. This would result in $K_{obs(2)}$ decreasing with increasing pD in alkaline solution. From Figure 4, it can be seen that this does not occur; hence the formation of an α-hydroxo intermediate in the pH range of this study can be ruled out. On further thought, it becomes apparent that significant proportions of the α-hydroxo intermediate should only occur at much higher pH conditions, since the formation of the extremely stable base-on (N-MeIm)Cbl^+ chelate ensures that the amounts of base-off α-aqua intermediate, and hence base-off α-hydroxo intermediate, are minimal under the experimental conditions of this study. Similarly, the α-DMBI of the base-off aqua intermediate would be expected to protonate with a pK_a ca. 5.5,[14h] but the formation of (N-MeIm)Cbl^+ once again ensures that much more acidic pH conditions are required before a base-off $DMBIH^+$ species is present in any significant amounts. The section entitled "Possible Intermediates" in the Supporting Information discusses the latter two points in more detail.

The ^1H NMR spectrum of HOCbl (0.100 M CAPS buffer, D_2O, pD 10.5) was also recorded to see if there was any evidence for the existence of a base-off α-hydroxo complex for this cobalamin, HOCbl, in alkaline solution, pD 10.5. Five signals were observed in the aromatic region, consistent with the literature.[17] No additional signals were observed.

The possibility that the small discrepancies in the results are due to changes in the concentration of the cationic species contributing to the total ionic strength was also considered. Further experiments demonstrated that this cannot by itself be responsible for the deviation seen in Figure 6b, however, and are also discussed in more detail in the Supporting Information.

The discrepancies may be due to a change in the mode of coordination of N-MeIm to the cobalamin upon acidification of the solution; that is, from nitrogen (N3) to carbon (C2) coordination.[26] However, ^1H NMR chemical shifts in the aromatic region for (N-MeIm)Cbl^+ and (N-MeIm)$_2$$Cbl^+$ change by ≤0.03 and ≤0.10 ppm, respectively, upon going from pD 9.5 to pD 6.5. It was therefore considered unlikely that a change in the coordination mode of N-MeIm is occurring, though this cannot be totally ruled out.

One explanation for the apparent inconsistencies seen in Figure 6b is that the effective concentration of N-MeIm available for binding to the α-aqua intermediate is pH-dependent, and decreases with decreasing pH. Perhaps an unproductive (i.e., unproductive in that it does not lead to product) ion-pair involving N-$MeImH^+$ occurs, which leads to a decrease in the effective concentration of N-$MeImH^+$ and hence N-MeIm also. The deviation between the data is very small; the difference between the two sets of results in Figure 6b is ≤30%. Alternatively, π stacking interactions between N-$MeImH^+$ could be responsible for decreasing the effective concentration of N-MeIm available for binding.[27]

Activation parameters were obtained for the reaction (N-MeIm)Cbl^+ + N-MeIm ⇄ (N-MeIm)$_2$$Cbl^+$ at pH 8.50 at two

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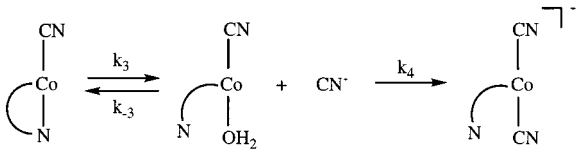
different N-MeIm$_T$ conditions, 5.00×10^{-3} and 1.00 M N-MeIm$_T$. According to the mechanism given in eq 10, the rate constants obtained under these conditions correspond to the aquation of $(N\text{-MeIm})_2Cbl^+$ and ring-opening in $(N\text{-MeIm})Cbl^+$, respectively. Large $\Delta H^{\ddagger}$ values are obtained for both reactions (99 ± 2 and 109.4 ± 0.8 kJ mol^{-1}, respectively), corresponding to the breakage of the Co−N bond. The significantly positive values of $\Delta S^{\ddagger}$ (39 ± 5 and 70 ± 3 J mol^{-1} K^{-1}, respectively) and $\Delta V^{\ddagger}$ (15.0 ± 0.7 and 16.8 ± 1.1 cm^3 mol^{-1}, respectively) clearly support the operation of a dissociative or dissociative interchange type of mechanism for both reactions. By way of comparison, $\Delta V^{\ddagger}$ values of 16.9 ± 0.8, 12.2 ± 0.5, and 10.0 ± 0.8 cm^3 mol^{-1} were reported for the dissociation of the ligands (L) pyridine, 3-acetylpyridine and *N*,*N*′-dimethylthiourea from LCbl, respectively.[12c] The latter data were all interpreted in terms of a dissociatively activated substitution mechanism.

Jencks and co-workers have examined the reaction between cyanocobalamin and cyanide in alkaline solution to form dicyanocobalamin, and on the basis of their results proposed the following mechanism:[14d]



(16)

Studies at two pH conditions (pH 9.0, 11.7) showed that the individual rate constants were independent of pH in this pH range; thus, even up to pH 11.7 insignificant amounts of an α-hydroxo base-off species are formed in this system. This mechanism is the same as our proposed mechanism, except that the reaction of the base-off aqua intermediate with cyanide to give cyanocobalamin is irreversible. In this study the rate constant for ring-opening of the intramolecularly coordinated α-DMBI of CNCbl to give the base-off α-aqua intermediate was found to be 4.2×10^{-2} s^{-1}, which is 20 times larger than the corresponding rate constant for ring-opening of $(N\text{-MeIm})Cbl^+$ (2.08×10^{-3} s^{-1}). This most likely arises from the known enhanced trans ligand effect of cyanide compared with N-MeIm.[7] Jencks points out that the ratio k_{-3}/k_4 represents the "effective molarity" for intramolecular addition of DMBI relative to the entering ligand. This ratio is small for both the $CNCbl/(CN)_2Cbl^-$ (2.1×10^{-2} M) and $(N\text{-MeIm})Cbl^+/(N\text{-MeIm})_2Cbl^+$ (5.4×10^{-2} M) systems; thus the substitution of DMBI at the α axial site of the base-off α-aqua intermediate is not significantly enhanced by it being intramolecularly coordinated to the cobalamin.

To summarize, the equilibria and kinetics of the reaction between $(N\text{-MeIm})Cbl^+$ and N-MeIm have been studied using ^{1}H NMR and visible spectroscopies. The equilibria and kinetics of the reaction between H_2OCbl^+ and N-MeIm have also been reexamined. An unusual decrease in observed rate constant with increasing ligand concentration was observed for the reaction between $(N\text{-MeIm})Cbl^+$ and N-MeIm. The proposed mechanism involves formation of a base-off α-aqua intermediate prior to substitution of N-MeIm at the α site to form $(N\text{-MeIm})_2Cbl^+$. While the concentration dependence data at pH 8.50 fit the proposed mechanism well, this is not the case for the pH-dependent data at 0.800 M N-MeIm$_T$. The formation of an α-hydroxo intermediate or a base-off DMBI-protonated species cannot account for this discrepancy, since insignificant amounts of these species exist in the pH range studied. π stacking interactions between $N\text{-MeImH}^+$ may account for the deviations, however.

Experimental Section

Hydroxycobalamin hydrochloride (HOCbl·HCl, >96%) was purchased from Fluka and *N*-methylimidazole (N-MeIm, ≥99%) from Aldrich. All solutions were made up in 0.100 M buffer (CAPS, CHES, TAPS, TES, BES, BIS-TRIS;[28] Sigma) unless specifically stated otherwise and at a total ionic strength of 1.5 M ($NaClO_4$, ≥98%, BDH). Distilled water was purified through a Milli-Q ultrapure water system.

All pH measurements were carried out at 25.0 °C using an Orion Model 710A pH meter in conjunction with an Orion 9101 BN glass electrode and an Orion 900200 double junction reference electrode. The outer chamber of the reference electrode was filled with 1.60 M NH_4NO_3/0.200 M $NaNO_3$, pH 7.0, which has been shown to have the same performance characteristics as the usual KCl filling solution. This was necessary since the solutions contained ClO_4^-. The electrodes were standardized using BDH 4.01, 6.98 and 10.00 buffer solutions; the latter buffer was freshly made up from a BDH pH 10 buffer capsule prior to use. Measurements in alkaline solution were carried out under a nitrogen or argon atmosphere. The error in individual pH measurements is no more than ±0.01.

^{1}H NMR spectra were recorded on an Inova 500 MHz NMR spectrometer equipped with a 5 mm thermostated (25.0 ± 0.2 °C) probe. All solutions were prepared in D_2O and TSP was used as an internal standard.

Visible spectra were recorded on a Cary 1E spectrophotometer equipped with a thermostated 8 × 6 cell changer and operated with WinUV Bio software (version 2.00). Unless stated specifically otherwise, all measurements were recorded at 25.0 ± 0.1 °C. Kinetic data at elevated pressures were recorded at 15.0 °C using a thermostated self-built high-pressure unit in conjunction with a Shimadzu UV-2101PC spectrophotometer.[29] An excellent fit to a single first-order rate equation was found for all kinetic data for at least 6 half-lives (OLIS−KINFIT fitting program[30]).

All reported errors represent one standard deviation of the mean value.

Acidity Constants. Acid Dissociation Constant of H_2OCbl^+. 40.00 mL of a HOCbl·HCl solution (ca. 5×10^{-3} M (exact concentration known[31]), $I = 1.5$ M ($NaClO_4$)) was titrated with aliquots (0.100, 0.200, or 0.250 mL) of a NaOH solution (9.65×10^{-2} M, $I = 1.5$ M ($NaClO_4$)) under nitrogen at 25.0 °C. The experiment was done in duplicate. **Acid Dissociation Constant of $N\text{-MeImH}^+$.** 25.00 mL of an N-MeIm solution (ca. 5×10^{-3} M (exact concentration known[31]), $I = 1.5$ M ($NaClO_4$)) was titrated with aliquots (0.100 or 0.200 mL) of a $HClO_4$ solution (0.115 M, $I = 1.5$ M ($NaClO_4$)) under argon, 25.0 °C. The experiment was done in duplicate, with the N-MeIm used in the second titration being freshly vacuum distilled.

Equilibrium Constant Determinations. $H_2OCbl^+ + N\text{-MeIm} \rightleftharpoons (N\text{-MeIm})Cbl^+$. Four separate sets of experiments were carried out at different pH conditions (pH 6.51, 6.99, 7.52, 7.98; 0.100 M buffer,

(28) Abbreviations: CAPS = (3-cyclohexylamino)-1-propanesulfonic acid, CHES = (2-*N*-cyclohexylamino)ethanesulfonic acid, TAPS = ((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)-1-propanesulfonic acid, TES = 2-((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulfonic acid, BES = *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, BIS-TRIS = 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol.

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(31) (a) The commercially available HOCbl·HCl purchased from Fluka was found to contain $17 \pm 2\%$ H_2O (by conversion to $(CN)_2Cbl^-$ (0.10 M KCN, 0.10 M phosphate buffer, pH 10.3; $\epsilon_{(CN)2Cbl-}$ at 367 nm = 3.04×10^4 mol^{-1} cm^{-1} [31b]). All HOCbl·HCl concentrations were therefore corrected to allow for this. (b) Barker, H. A.; Smyth, R. D.; Weissbach, H.; Toohey, J. I.; Ladd, J. N.; Volcani, B. E. *J. Biol. Chem.* **1960**, *235*, 480.

I = 1.5 M ($NaClO_4$)). Solution mixtures containing a constant concentration of HOCbl·HCl (ca. 5×10^{-5} M, exact concentration known[31]) and varying concentrations of N-MeIm$_T$ ($0-2.34 \times 10^{-3}$ M, N-MeIm$_T$ = N-MeIm + N-MeImH$^+$) were prepared in vials, capped and left to equilibrate overnight in a thermostated bath (25.0 ± 0.1 °C). The following day the absorbance of each solution was measured at 358 nm using the same cuvette (1 cm) for all measurements.

(N-MeIm)Cbl$^+$ + N-MeIm ⇌ (N-MeIm)$_2$Cbl$^+$. Seven separate sets of experiments were carried out at different pD conditions (pD 6.46, 7.00, 7.51, 8.00, 8.49, 8.98, 9.46; 0.100 M buffer, I = 1.5 M ($NaClO_4$)). Solution mixtures containing a known amount of HOCbl·HCl (ca. 4.5×10^{-3} M, exact concentration known[31]) and varying concentrations of N-MeIm$_T$ ($5.00 \times 10^{-2}-1.20$ M) were prepared in D_2O in NMR tubes, capped and left to equilibrate overnight in a thermostated bath (25.0 ± 0.1 °C). The ^{1}H NMR spectrum of each sample was recorded the following day and the relative proportions of (N-MeIm)Cbl$^+$ versus (N-MeIm)$_2$Cbl$^+$ calculated from the areas of the (N-MeIm)Cbl$^+$ and (N-MeIm)$_2$Cbl$^+$ signals at δ = 5.88 ± 0.04 (β-A5) and 5.71 ± 0.05 ppm (β or α-A5), respectively. These signals were chosen since they are well separated from all other signals including those of the free (uncoordinated) N-MeIm(H$^+$).

Kinetic Measurements. H$_2$OCbl$^+$ + N-MeIm → (N-MeIm)Cbl$^+$. Experiments were carried out at six different pH conditions for a range of N-MeIm$_T$ concentrations (pH 6.01: [N-MeIm]$_T$ = $8.00 \times 10^{-4}-1.50 \times 10^{-2}$ M; pH 7.01: [N-MeIm]$_T$ = $2.50 \times 10^{-4}-2.00 \times 10^{-3}$ M; pH 7.30: [N-MeIm]$_T$ = $3.13 \times 10^{-4}-1.80 \times 10^{-3}$ M; pH 8.00: [N-MeIm]$_T$ = $2.50 \times 10^{-4}-1.50 \times 10^{-3}$ M; pH 8.50: [N-MeIm]$_T$ = $3.13 \times 10^{-4}-1.80 \times 10^{-3}$ M; pH 9.00: [N-MeIm]$_T$ = $2.50 \times 10^{-4}-5.00 \times 10^{-3}$ M; all in 0.100 M buffer, I = 1.5 M ($NaClO_4$)). In a typical experiment, 0.900 mL of a N-MeIm$_T$ solution ($1.38 \times 10^{-3}-3.00 \times 10^{-2}$ M N-MeIm$_T$) and 0.900 mL of a ca. 1.00×10^{-4} M HOCbl·HCl solution were mixed in a tandem cuvette and the absorbance monitored at 358 nm. For N-MeIm$_T$ ≤ 1.20×10^{-3} M, 0.450 mL of ca. 1.00×10^{-4} M HOCbl·HCl solution and 0.450 mL of 0.100 M buffer solution were used instead of 0.900 mL of ca. 1.00×10^{-4} M HOCbl·HCl solution.

(N-MeIm)Cbl$^+$ + N-MeIm ⇌ (N-MeIm)$_2$Cbl$^+$: Concentration Dependence Studies at pH 7.00 and 8.50. It was established early on that the observed rate constant, $k_{obs(2)}$, was independent of the cobalamin reactant used (solid HOCbl·HCl or a solution of H$_2$OCbl$^+$ or (N-MeIm)$_2$Cbl$^+$). For example, studying the rate of the reaction in 0.100 M N-MeIm$_T$, pH 8.50 using solid HOCbl·HCl reactant gave $10^3 k_{obs(2)}$ = 2.81, 2.88 and 2.99 s^{-1} (three separate experiments). Alternatively, when a solution of HOCbl·HCl in 0.200 M N-MeIm was used (i.e., predominantly have (N-MeIm)$_2$Cbl$^+$), identical rate constants within experimental error were obtained (two separate experiments gave $10^3 k_{obs(2)}$ = 2.86 and 2.97 s^{-1}). This N-MeIm$_T$ concentration (0.100 M N-MeIm$_T$) and pH condition (pH 8.50) were specifically chosen to minimize the experimental error in the results (i.e., maximize the absorbance change for both reactants), since under these conditions the equilibrium mixture consists of a ca. 1:1 mixture of (N-MeIm)Cbl$^+$ and (N-MeIm)$_2$Cbl$^+$.

The fact that the observed rate constant is independent of the cobalamin reactant used is not unexpected, since the reaction was always studied under pseudo-first-order conditions and the formation of (N-MeIm)Cbl$^+$ from H$_2$OCbl$^+$ and N-MeIm is rapid and irreversible under all conditions chosen to determine $k_{obs(2)}$. Solutions were therefore prepared so as to obtain the maximum absorbance change under the reaction conditions. Hence if (N-MeIm)$_2$Cbl$^+$ was the main cobalamin species at equilibrium, solid HOCbl·HCl (or a solution of H$_2$OCbl$^+$) was used as the cobalamin reactant, whereas if (N-MeIm)Cbl$^+$ was the main species, a solution of (N-MeIm)$_2$Cbl$^+$ (in an aqueous solution of N-MeIm) was used.

Attempts were also made to isolate solid (N-MeIm)$_2$Cbl$^+$·Cl$^-$ for use in kinetic studies. In a typical experiment 50 mg of HOCbl·HCl was dissolved in a N-MeIm solution (0.40 mL, ca. 2 M, pH 8.5) and left to react at room temperature for ca. 2 h. Cold acetone was added (ca. 10 mL, 0 °C) to the solution and the solution left standing in a freezer (−20 °C) for 1 h. The resulting red product was collected and dried in a vacuum desiccator. The product was ca. 80% pure (i.e., 20% unwanted (N-MeIm)Cbl$^+$ observed by ^{1}H NMR spectroscopy), and it was suspected the product contained other impurities or even a small amount of N-MeIm since $k_{obs(2)}$ obtained using this material (0.100 M N-MeIm$_T$, pH 8.50) were substantially (ca. 20% larger) different from those obtained using either solid HOCbl·HCl or a solution of HOCbl·HCl in 0.200 M N-MeIm.

pH Dependence Studies. Seven pH conditions were investigated (pH 5.50, 6.49, 6.99, 7.49, 8.00, 8.50, 9.49). Ca. 4 μL of an H$_2$OCbl$^+$ solution (ca. 3.6×10^{-2} M) was added to a 1 cm cuvette that contained 3.00 mL of the appropriate solution (0.800 M N-MeIm$_T$, 0.100 M buffer (with the exception of the data collected at pH 9.49 in which no buffer was used), I = 1.5 M ($NaClO_4$)). Control experiments in the absence of buffer (CHES, TAPS, TES, BES or BIS-TRIS) gave identical results within experimental error (± 2%) with the exception of those obtained at pH 9.49 using CHES buffer. The latter results were found to be slightly dependent on the buffer concentration ($10^3 k_{obs}$ (each value is the mean of 3 determinations) = 2.16, 1.95, and 1.75 for 0, 0.100, and 0.200 M CHES buffer, respectively). The rate constants reported at pH 9.49 were therefore obtained in buffer-free solution. The absorbance was monitored at 358 nm.

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Supporting Information Available: Derivation of eq 2; $K_{obs(2)}$ values at different pD conditions (Table A); $k_{obs(2)}$ values for [N-MeIm]$_T$ = 5.00×10^{-3} M at pH 5.50, 7.00, and 8.50 (Table B); plots of A_{obs} versus [N-MeIm]$_T$ for equilibrated solutions of HOCbl·HCl and N-MeIm$_T$ at pH 6.99, 7.52, and 7.98 (Figures A−C, respectively); plots of $k_{obs(1)}$ versus [N-MeIm]$_T$ at pH 6.01, 7.01, 7.30, 8.00, 8.50, and 9.00 (Figure D−I, respectively); plot of $k_{obs(2)}$ versus N-MeIm at pH 7.00 (Figure J), plots of ln$k_{obs(2)}$ versus pressure at [N-MeIm]$_T$ = 5.00×10^{-3} and 1.00 M (Figures K and L, respectively). This material is available free of charge via the Internet at http://pubs.acs.org.

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