Formation of an Association Complex without a Substitution Reaction Occurring between Methylcobalamin and Cyanide†

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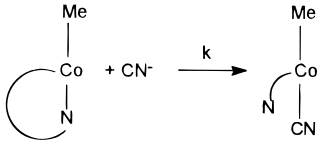
Introduction

Vitamin B_{12} has two forms (adenosylcobalamin (=AdoCbl); methylcobalamin (=MeCbl)) which are essential for a number of enzyme reactions. Recently we re-investigated the reaction between adenosylcobalamin and cyanide[1] and found, contrary to earlier literature,[2] that the reaction proceeds in only one kinetically observable step, with rate-determining attack of the first cyanide. This reaction is of particular interest as it provides a convenient means of examining factors which influence the rate of heterolytic cobalt−carbon cleavage, an important reaction in the methylcobalamin-dependent methyltransferase reactions.[3] Two key pieces of evidence led us to postulate that the attack of the first cyanide occurs at the β-adenosyl site rather than at the α-5,6-dimethylbenzimidazole (α-DMBI) site, as proposed by other authors:[4] (i) the large negative values for $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ for the rate-determining step (-127 ± 3 J mol^{-1} K^{-1} and -10.0 ± 0.4 cm^3 mol^{-1}, respectively) and (ii) the observation that there was no evidence for a reaction between adenosylcobalamin and other strong nucleophiles.

In the 1960's Dolphin et al. reported that no reaction occurs between MeCbl and CN^-.[5] More recently, however, it was reported in the literature that CN^- can displace the α-DMBI of MeCbl



with $k = 2.8 \times 10^{-2}$ s^{-1} for 0.10 M KCN, 0.1 M buffer ($NaHCO_3$), pH 10.5, and 25 °C.[2] Further information on the mechanism of the reaction between methylcobalamin and cyanide could in principle be obtained by examining the CN^- concentration, pH, temperature, and pressure dependences. In addition, measurement of activation parameters for this reaction could further confirm that associative attack of the first cyanide occurs at the β-adenosyl site of AdoCbl during cyanation, since cyanation of MeCbl would be expected to be dissociative in nature. There is a substantial amount of literature which demonstrates that ligand substitution reactions for cobalamins (not involving Co−C cleavage) occur dissociatively,[6] and displacement of the bulky α-DMBI by CN^- would not be expected to be an exception. This has led us to re-examine the reaction between methylcobalamin and cyanide. We were surprised to find that no substitution reaction occurs. We did, however, find evidence for association between the two species. Our findings are reported in this note.

Experimental Section

Methylcobalamin (MeCbl, >99%) was obtained from Sigma. All other chemicals were of AR grade and supplied by either Merck (NaCN, $NaClO_4$), Aldrich (CAPS buffer, CD_3OD, D_2O), or Fluka (NaI).

Solution pH was measured at 25.0 °C using a pH 537 WTW microprocessor pH meter equipped with an Ingold 402.M657 electrode. A saturated NaCl solution was used for the salt bridge solution, and the electrode was standardized using standard pH 6.86 and 9.18 buffer solutions. Measurement in alkaline solution was carried out under a N_2 atmosphere.

UV−vis spectra were measured using a Cary 1 spectrophotometer equipped with a thermostated cell compartment (±0.1 °C). Preliminary experiments demonstrated that decomposition of MeCbl by the light beam of the spectrophotometer was negligible. Stopped-flow experiments were performed using an Applied Photophysics SX-18MV stopped-flow spectrophotometer.

^{1}H-NMR spectra were obtained on a Bruker Avance DPX 300 spectrometer equipped with a BC-70/52 magnet system and a B-VT 3300 temperature control unit using a 5 mm QNP (^{1}H, ^{19}F, ^{31}P, ^{13}C) probe. For this probe the ^{1}H-π/2 pulse width is 10.4 μs. The data were acquired with 128 scans, a spectral width of 5995 Hz, 32 K data points, and a corresponding acquisition time of 2.73 s using the digital quadrature detection acquisition mode. For the acquisition we used a relaxation delay of 2 s and a π/6 pulse width. All solutions were prepared in either D_2O or CD_3OD, and TSP was added as an internal reference (0 ppm).

MeCbl solutions were prepared in a dark room using red light and were always protected from light during transportation. All solutions were aerobic. Experimental errors are given as one standard deviation of the mean value.

Results and Discussion

We first tried to repeat the experiment performed by Rudakova et al.[2] A MeCbl solution (ca. 2×10^{-4} M MeCbl, pH 11.0 ± 0.1) was mixed with equal volumes of a NaCN solution (0.20 M NaCN, pH 11.0 ± 0.1) in a tandem cuvette to give $[CN^-]_f = 0.10$ M (where f = final concentration of CN^- after mixing) and the absorbance change of the mixture monitored spectrophotometrically (300−600 nm). No reaction was observable for up to a period of 24 h after mixing.[7] A comparison of the spectrum before and after mixing (ca. 10 s later), however, showed that a *very* small absorbance decrease had occurred ($\Delta A_{max} < 0.05$; there were no shifts in wavelength maxima). Further experiments showed that this absorbance change was reproducible and not associated with the properties of the tandem cuvette employed. Similar experiments performed at higher

† Dedicated to Prof. Dr. Wolfgang Beck on the occasion of his 65th birthday.

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(7) An experiment (using a tandem cuvette) was also performed at exactly the same conditions as those used by Rudakova et al.; i.e. 0.10 M KCN, 0.10 M $NaHCO_3$ (adjusted to pH 10.5 with NaOH), 25 °C. Once again no reaction was observable directly after mixing and up to 10 h later.

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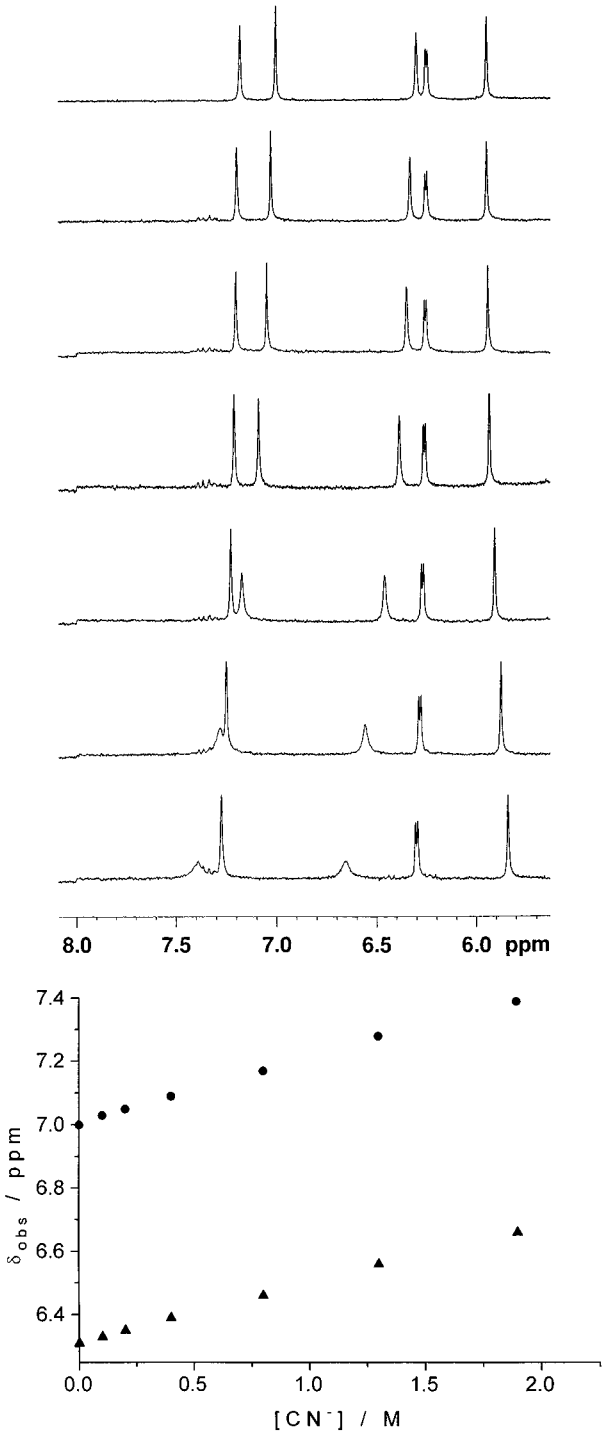

Figure 1. (a) Top: ^{1}H-NMR spectra for solutions of MeCbl in NaCN: [CN^-] = (from top to bottom) 0, 0.10, 0.20, 0.40, 0.80, 1.30, 1.90 M; [MeCbl] = ca. 1.5−2 mM, pD = 12.0 ± 0.2, D_2O, 25 °C, I = 2.0 M ($NaClO_4$)). (b) Bottom: Plot of chemical shift versus cyanide concentration for the B4 (▲) and B2 (●) α-DMBI signals of MeCbl in D_2O for the spectra given in (a).

[CN^-]$_f$ (0.50 M, 1.00 M) and varying pH conditions (pH 9, 7, 5, and 3) gave identical results.

Stopped-flow experiments with the same solutions showed that the observed absorbance change was "instantaneous" on the stopped-flow time scale; i.e. no reaction could be monitored.[8] We therefore turned to ^{1}H-NMR spectroscopy to further investigate the reason for the small absorbance change seen by UV−vis spectroscopy.

Chart 1. Labeling for α-DMBI of MeCbl

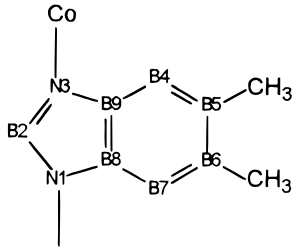

Figure 1 gives ^{1}H-NMR spectra of the aromatic region for solutions of MeCbl in NaCN ([NaCN] = 0−1.90 M, pD 12.0 ± 0.2, I = 2.0 M ($NaClO_4$), where I is the total ionic strength of the solution). The uppermost spectrum in Figure 1 is for MeCbl and the α-DMBI B4, B2, and B7 ^{1}H signals (see Chart 1) are clearly visible at 6.31, 7.00, and 7.20 ppm, respectively, and are in excellent agreement with literature values.[9] The C10 corrin ring and R1 ribose signals are at 5.97 and 6.25(6) (doublet), respectively. From Figure 1a it can be seen that the B2 and B4 signals move downfield and progressively broaden as the [CN^-] increases ($\Delta\delta$ = 0.36 and 0.33 ppm, respectively), while the B7, C10, and R1 signals are much less affected by a change in [CN^-] ($\Delta\delta$ = 0.08, 0.10, and 0.04 ppm, respectively). Plots of chemical shift for the B2 and B4 signals versus [CN^-] are given in Figure 1b and are essentially linear.

It has been demonstrated in the literature that substitution of the α-DMBI of cobalamins by CN^- (or any other ligand) results in large absorbance changes and shifts in the absorbance maxima; however the UV−vis spectra showed that practically no absorbance change occurred upon addition of cyanide to MeCbl. Such a small absorbance change could be due to some form of association between MeCbl and CN^-. The ^{1}H-NMR measurements of Figure 1a show that MeCbl is in fast exchange with another species on the NMR time scale (k ca. 10^2−10^3 s^{-1}), and since the UV−vis spectrum of the other species is practically identical to that for MeCbl, we propose that the new species is MeCbl·CN^-; that is

$$\text{MeCbl} + CN^- \rightleftharpoons \text{MeCbl}\cdot CN^- \qquad K_{assoc} \qquad (1)$$

If association occurs between MeCbl and CN^-, then a plot of chemical shift versus [CN^-] should exhibit curvature and the chemical shift should reach a limiting value at high [CN^-]. From Figure 1b is obvious that, even at high [CN^-], there is still a considerable amount of the cobalamin present as MeCbl rather than MeCbl·CN^-.

To further demonstrate that association indeed occurs, the same experiment was repeated in CD_3OD. Since CD_3OD is less protic compared to D_2O, it would be expected that the association between MeCbl and CN^- would be stronger in this solvent. The results are summarized in Figure 2, which shows the chemical shift of the B2 and B4 signals of the α-DMBI of the cobalamin as a function of [CN^-] in CD_3OD.[10] (It was not possible to work at [NaCN] > 1.12 M due to the limited solubility of NaCN in CD_3OD.) Once again the C10 and R1

(8) (a) There was a very small amount of photolysis of MeCbl by the light source, which could be kept to a minimum by reducing the slit width of the spectrophotometer. (b) The deadtime of the employed stopped-flow is 2 ms.

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(10) Signals attributable to the amide ^{1}H's of the corrin ring of MeCbl were also observed for MeCbl in CD_3OD (2 M $NaClO_4$). These signals disappeared on addition of 1.5×10^{-3} M NaOH (6 μL of 1 M NaOH added to 4 mL of solution) to give similar pH conditions to the solutions which contained CN^-.

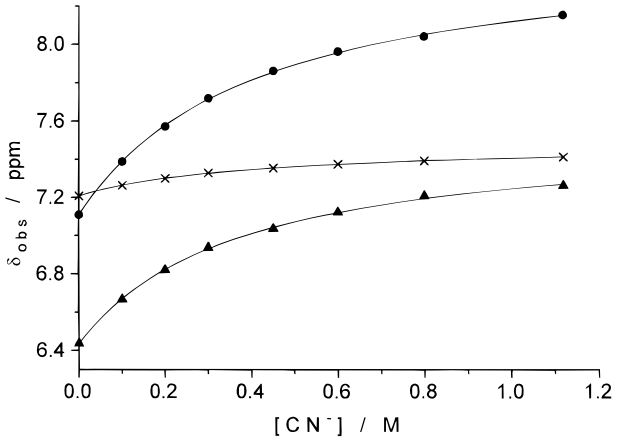

Figure 2. Plots of chemical shifts versus cyanide concentration for the B4 (▲), B2 (●), and B7 (×) α-DMBI signals of MeCbl in CD_3OD ([MeCbl] = ca. 1.5−2 mM, [NaCN] = 0−1.12 M, 25 °C, I = 2.0 M ($NaClO_4$). The data are fitted to (2) in the text (solid lines), fixing δ_{MeCbl} = 6.43 ppm (B4), 7.11 ppm (B2), or 7.21 ppm (B7) and giving $K_{assoc} = 2.6 \pm 0.1$ M^{-1}, $\delta_{MeCbl\cdot CN^-} = 7.55 \pm 0.02$ ppm, $K_{assoc} = 2.49 \pm 0.06$ M^{-1}, $\delta_{MeCb\cdot CN^-} = 8.53 \pm 0.02$ ppm, and $K_{assoc} = 2.51 \pm 0.03$ M^{-1}, $\delta_{MeCb\cdot CN^-} = 7.49 \pm 0.01$ ppm for the B4, B2, and B7 data, respectively.

signals were much less affected by the addition of CN^-. Now the expected curvature to a limiting chemical shift value typical of association is visible. It can easily be shown that

$$\delta_{obs} = (K_{assoc}\delta_{MeCbl\cdot CN^-}[CN^-] + \delta_{MeCbl})/(1 + K_{assoc}[CN^-]) \quad (2)$$

where δ_{obs}, $\delta_{MeCbl\cdot CN^-}$ and δ_{MeCbl} represent the observed chemical shift and the chemical shifts of MeCbl·CN^- and MeCbl, respectively, and K_{assoc} is defined by eq 1. Best fit of the B4, B2, and B7 data to eq 2 gave $K_{assoc} = 2.6 \pm 0.1$, 2.49 $\pm$ 0.06, and 2.51 $\pm$ 0.03 M^{-1}, respectively (see figure caption for further details) for the association in CD_3OD.

In principle it should be possible to observe separate signals for the MeCbl and MeCbl·CN^- species at low temperatures. The ^{1}H-NMR of a solution of MeCbl in 0.50 M NaCN (CD_3OD, I = 2.0 M ($NaClO_4$)) was therefore measured at −40 °C, and twice the number of signals (at 5.69, 5.94, 6.38, 6.46, 6.97, 7.23, 7.44, 7.49, 7.61, and 8.37 ppm) were observed as for the same solution at 25 °C. A spectrum of MeCbl in CD_3OD (2.0 M $NaClO_4$) at −40 °C was also measured (5.94, 6.38, 6.98, 7.22, and 7.61 ppm), and a comparison of these two spectra allowed assignment of the signals at 5.69, 6.46, 7.44, 7.49, and 8.37 ppm in the former spectrum to MeCBl·CN^-. In addition, $K_{assoc} = 10 \pm 2$ M^{-1} (−40 °C, CD_3OD, I = 2.0 M ($NaClO_4$)) could be estimated, since the peak areas of the MeCbl·CN^- signals were ca. 4.8 times larger than the MeCbl signals (the large error is a consequence of broad signals). The association between MeCbl and CN^- in CD_3OD is therefore exothermic (since $K_{assoc} = 2.5 \pm 0.1$ M^{-1} at 25.0 °C).

We have no explanation as to why Rudakova et al.[2] observed a reaction between MeCbl and CN^-, other than to suggest, as in the case of the "fast" reaction observed between AdoCbl and CN^- (of which we could find no evidence for its existence[1]), that the authors may have contaminated their reactant by exposing it to light (as for AdoCbl, MeCbl may have been converted to H_2OCbl^+ by the spectrophotometer light source; for $H_2OCbl^+ + CN^- \rightarrow CNCbl + CN^- \leftrightarrow (CN)_2Cbl^-$, $k_{obs(1)} = 6.3 \times 10^{-3}$ s^{-1} and $k_{obs(2)} = 3.5 \times 10^{-2}$ s^{-1} for [KCN] = 0.10 M, pH 11.7, 25 °C[11]). Alternatively, a reactant may have contained an impurity. It is rather "suspicious" that the rate constant for the "fast" reaction between AdoCbl and CN^- and their rate constant for the reaction between MeCbl and CN^- are identical within experimental error ($(2.9 \pm 0.1) \times 10^{-2}$ s^{-1} and $(2.8 \pm 0.3) \times 10^{-2}$ s^{-1}, respectively[2]).

MeCbl can be regarded as a zwitterion, with a localized negative (−1) charge located on the phosphate residue and a localized positive (+1) charge located in the vicinity of the Me−Co(III). Crystal structures of cobalamins have shown that the α-DMBI is always located perpendicular to the corrin ring, with the B7 proton pointing away (α) from the corrin ring.[12] Only the B2 and B4 signals of the α-DMBI of MeCbl·CN^- are significantly different from those of MeCbl. This suggests that the potentially π-interacting CN^- could be located in the vicinity of the corrin ring, so that the MeCbl·CN^- species could be regarded as an ion-pair (or solvent-separated ion-pair), rather than just an association complex. Examination of the effect of CN^- on all of the ^{1}H-NMR signals of MeCbl could probably pinpoint the exact location of the CN^- with respect to MeCbl.

There are a few examples of association between MeCbl and charged species in the literature. Fanchiang et al. found ion-pairing occurs between MeCbl and Pt(II) salts[13a−c] from kinetic measurements, UV−vis and ^{1}H-NMR spectroscopy (their observations using UV−vis spectroscopy for ion-pair formation between MeCbl and $Pt(CN)_4^{2-}$ are very similar to ours[13b]). It is also proposed that pre-association occurs between MeCbl and $AuCl_4^-$,[13c] $FeCl^{2+}$,[13c] and tetracyanoethylene.[13f] Ion-pair formation prior to substitution is also well established in the substitution reactions of the β-H_2O ligand of H_2OCbl^+.[14] Interestingly, the ^{1}H-NMR spectrum of MeCbl in 2.00 M NaI (in D_2O) gave much smaller and almost identical chemical shift changes for all the aromatic ^{1}H signals (spectrum not given), unlike MeCbl in 1.90 M NaCN (B4, −0.08 vs 0.33; B2, −0.07 vs 0.36; B7, −0.07 vs 0.08; C10, −0.04 vs 0.10; R1, −0.07 vs 0.04 ppm). This further supports the idea that there is a specific localized interaction (which could involve π bonding) between the MeCbl and CN^- in MeCbl−CN^-. Further NMR studies and a crystal structure of MeCbl in CN^- solution could in principle substantiate this.

As mentioned in the Introduction, in an earlier paper[1] we re-investigated the reaction between AdoCbl and CN^- and proposed that the first CN^- attacks the β-adenosyl site in the rate-determining step. No reaction was observed between MeCbl and CN^- under the same conditions, however. One possible explanation is the differences in the polarization of the Co−C bond.[15] In MeCbl the Co−C bond is polarized toward the Co atom, resulting in an electron-rich Co, and therefore, nucleophilic attack is unfavorable. For AdoCbl, however, the electron-withdrawing adenosyl group polarizes the Co−C bond toward the C and, unlike MeCbl, a concerted cleavage which does not involve a carbanion leaving group is possible. The observation of no reaction between MeCbl and CN^- also

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supports our suggestion that rate-determining attack of the first CN^- occurs at the β rather than the α site of AdoCbl, since it is postulated[1,4] that α attack would occur via the base-off AdoCbl (1.3% at 25.0 °C[16]) and a small fraction of MeCbl also exists in the base-off form (0.22%, 25.0 °C[17]). Thus, if CN^- can displace the α-DMBI of AdoCbl, it should also occur for MeCbl, but this is not what we found experimentally.

To summarize, the reaction between methylcobalamin and cyanide has been re-investigated. We have found no evidence for a substitution reaction by UV−vis spectroscopy. ^{1}H-NMR spectroscopy showed that the methylcobalamin is in fast exchange with a new species (25 °C, D_2O), which we propose to be the association complex MeCbl·CN^-.

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