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Detailed kinetic and thermodynamic studies on the cyanation of alkylcobalamins. A generalized mechanistic description †

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Received 10th July 2002, Accepted 30th July 2002
First published as an Advance Article on the web 18th September 2002

Ligand substitution equilibria of different cobalamins (XCbl, X = Ado, CF_3CH_2, n-Pr, $NCCH_2$ and CN^-) with cyanide have been studied. It was found that CN^- substitutes the 5,6-dimethylbenzimidazole (DMBz) moiety in the α-position in all cases. A reinvestigation of the reactions of coenzyme B_{12} (X = Ado) and CF_3CH_2Cbl with CN^- and an investigation of the same reaction for X = n-Pr, demonstrate that the unfavorable formation constants in these cases require very high cyanide concentrations to produce the 1 : 1 complex, which causes the kinetics of the displacement of DMBz by cyanide to be too fast to follow. The kinetics of the displacement of DMBz by CN^- could be followed for X = β-$NCCH_2$ and CN^- to form $NCCH_2(CN)Cbl$ and $(CN)_2Cbl$, respectively. Both reactions show saturation kinetics at high cyanide concentration and the limiting rate constants are characterized by the activation parameters: X = $NCCH_2$, $\Delta H^{\neq} = 85 \pm 2$ kJ mol^{-1}, $\Delta S^{\neq} = +97 \pm 6$ J K^{-1} mol^{-1}, $\Delta V^{\neq} = +12.7 \pm 0.5$ cm^3 mol^{-1}; X = CN^-, $\Delta H^{\neq} = 105 \pm 2$ kJ mol^{-1}, $\Delta S^{\neq} = +81 \pm 6$ J K^{-1} mol^{-1} and $\Delta V^{\neq} = +13.1 \pm 0.3$ cm^3 mol^{-1}. These parameters are interpreted in terms of a limiting D mechanism. A complete analysis of the *trans* effect order of the substituent X is presented. The results enable the formulation of a general mechanism that can account for the substitution behavior of all the investigated alkylcobalamins.

Introduction

The coenzymatic forms of vitamin B_{12} are involved in the catalysis of about 15 enzymatic reactions in various organisms, including the 1,2-intramolecular rearrangements catalyzed by 5′-deoxyadenosylcobalamin (AdoCbl) requiring enzymes[1–3] and the methyl transfer reactions catalyzed by methylcobalamin requiring enzymes.[1,4,5] The thermolysis of AdoCbl indicates that the AdoCbl-dependent enzymes can increase the rate of Co–C homolysis by a factor of at least 10^9 at 25 °C.[6,7] It is now thought that steric distortion and crowding at the β-site containing the alkyl group may play a crucial role. It has also been suggested that electronic factors are important for this process.[8] It seems clear that the nature of the ligand in the position *trans* to the Co–C bond plays a significant role in the kinetic and thermodynamic stability of this bond.

Methylcobalamin (CH_3Cbl) and coenzyme B_{12} (AdoCbl) undergo substitution of their axial benzimidazole ligand by a protein histidine residue during complexation to the CH_3-Cbl-dependent methionine synthase,[9] the class I[10] AdoCbl-dependent mutases (methylmalonyl-coenzyme A mutase and glutamate mutase),[11] and the class III AdoCbl-dependent D-lysine-5,6-aminomutase.[12] Comparison of the ligand substitution reactions *trans* to the axial alkyl ligand in coenzyme B_{12} and other β-alkylcobalamins to the work performed on substitution reactions *trans* to a non-alkyl ligand is required to further our understanding of the mechanisms of these reactions.

In general, ligand substitution reactions of vitamin B_{12} follow a dissociative (I_d or D) mechanism.[13,14] In the case of AdoCbl, however, evidence for an associative substitution mechanism was reported for the reaction with cyanide.[15] It was postulated that attack of the first cyanide occurred at the β-(5′-deoxy-5′-adenosyl) site rather than at the α-5,6-dimethylbenzimidazole site.[15] More recently it was found that when the reaction between AdoCbl and cyanide is carried out in 92% DMF/8% D_2O, an intermediate (β-Ado)(α-cyano)-cobalamin species can be identified by ^{1}H NMR spectroscopy.[16] It follows that the first nucleophile attacks the α-position and not the β-position as postulated before.[15] The rate-determining heterolytic cleavage of the Co–C bond is thus preceded by the rapid addition of cyanide to the α-position in AdoCbl. The associative mechanism obtained for the reaction of AdoCbl and CN^- remains surprising since it is generally expected that the introduction of a metal–carbon bond will induce a dissociative substitution reaction in the *trans* position and not an associative reaction as reported for the reaction of the coenzyme with cyanide.[15] We recently reported that the reactions of β-CF_3Cbl and β-CF_3CH_2Cbl with CN^- follow limiting D and I_d mechanisms, respectively.[17] Reenstra and Jencks[18] found that the rate of CN^- addition to CNCbl reaches a limiting value at high [CN^-], also indicating a dissociative mechanism.

In order to improve our understanding of the reasons for the unexpected mechanistic changeover referred to above, we reinvestigated the reactions of AdoCbl, CF_3CH_2Cbl and CNCbl with CN^- and have studied the cyanation reactions of two more alkylcobalamins, in which the nature of the alkyl group was varied, *viz.* β-n-Pr and β-$NCCH_2$, to determine the activation parameters for comparison with those determined for other alkylcobalamins.[17] For this purpose the kinetics of the substitution reactions with cyanide was studied as a function of nucleophile concentration, temperature and pressure.

† UV-Vis spectra of β-$NCCH_2Cbl$, (β-$NCCH_2$)(α-CN)Cbl and $(CN)_2Cbl$ (Fig. S1). UV-Vis spectra of β-CF_3CH_2Cbl, (β-CF_3CH_2)-(α-CN)Cbl and $(CN)_2Cbl$ (Fig. S2). See http://www.rsc.org/suppdata/dt/b2/b206706d/

 DOI: 10.1039/b206706d

Experimental

Materials

All chemicals were P.A. grade and used as received. CAPS buffer was purchased from Sigma. $NaClO_4$ and NaCN were purchased from Merck. Ultra pure water was used in the kinetic and thermodynamic measurements. The preparations and measurements were carried out in diffuse light since all the alkylcobalamins are known to be very light sensitive.[1,19]

Cyanocobalamin and AdoCbl were supplied by Sigma. H_2OCbl was from Roussel. The other alkylCbl's were prepared as described in the literature[20,21] by reacting Co(II) cobalamin with suitable alkylating agents. In a typical reductive alkylation, H_2OCbl (10 mg, *ca*. 0.01 mmol) in 5.0 mL of 10% acetic acid or 5% NH_4Cl was purged with argon for 1 h, zinc wool (0.01 mol), quickly freshened with 1.0 M HCl, was added, and the reduction was allowed to proceed for 30 min. Alkyl halide (*ca*. 1 mmol, R = n-Pr, CF_3CH_2 and $NCCH_2$ for the preparation of n-PrCbl, CF_3CH_2Cbl and $NCCH_2$Cbl, respectively) was introduced, and the reaction was allowed to proceed for 30 min. The reaction mixtures were desalted by chromatography on Amberlite XAD-2[22] and the β-isomers were separated by HPLC.[20,23,24]

Instrumentation and measurements

The pH of the solutions was measured using a Mettler Delta 350 pH meter with a combined glass electrode. It was calibrated with standard buffer solutions at pH 7.0 and 10.0. UV-Vis spectra were recorded on Shimadzu UV-2101 and Hewlett Packard spectrophotometers.

Analytical HPLC was performed on a 4.6 × 250 mm Beckman C_8 ultrasphere column while semipreparative HPLC was performed on a 10 × 250 mm Beckman C_8 ultrasphere column, using 50 mM aqueous ammonium phosphate buffer (pH 3.0) and acetonitrile as described previously.[20,23,24]

Kinetic measurements were carried out on an Applied Photophysics SX 18MV stopped-flow instrument coupled to an online data acquisition system. At least eight kinetic runs were recorded under all conditions, and the reported rate constants represent the mean values. All kinetic measurements were carried out under pseudo-first order conditions, *i.e*. the nucleophile concentration was at least in ten fold excess. Measurements under high pressure were carried out using a home-made high pressure stopped-flow instrument.[25] Kinetic traces were analysed with the OLIS KINFIT (Bogart, GA) program.

The UV-Vis spectrophotometers and stopped-flow instruments were thermostated to the desired temperature ±0.1 °C. Values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were calculated from the slopes and intercepts, respectively, of plots of $\ln(k/T)$ *versus* $1/T$, and values of $\Delta V^{\neq}$ were calculated from the slope of plots of $\ln(k)$ *versus* pressure.

Results and discussion

Kinetics of the reaction of XCbl with cyanide

We have investigated ligand substitution reactions between different XCbls (X = n-Pr, $NCCH_2$, CN^-) and cyanide. In addition, we reinvestigated[15,17] the reaction of AdoCbl and CF_3CH_2Cbl with cyanide, using higher cyanide concentrations to study the kinetic and thermodynamic *trans* effect of the different X groups in more detail. The systems investigated can be represented by reaction (1), in which the first displacement of DMBz by cyanide (K_{CN1}) to give X(CN)Cbl is followed in some cases by substitution of X by cyanide (K_{CN2}) to produce $(CN)_2$Cbl and X^-, where X^- represents the product(s) formed from X. In other cases, X(CN)Cbl is stable in the dark and is then the final product.

Preliminary experiments at pH 9–11, in which the UV-Vis spectrum was scanned in the range 300 to 700 nm, showed that CN^- reacts rapidly with the XCbl complexes studied and that the equilibria are established within the mixing and measurement time.

We first investigated the kinetics of the reaction between β-n-PrCbl and CN^- at pH 11 and 5 °C. This reaction was found to be too fast to be followed on the stopped-flow instrument (deadtime of *ca*. 2–4 ms). The product of the reaction, n-Pr(CN)Cbl, shows a significant absorbance increase in the range 580–600 nm and is very stable in the dark, and the value of K_{CN1} was previously reported to be 1.3 M^{-1}.[26] This low value of K_{CN1} requires high cyanide concentrations to form detectable amounts of the β-n-Pr(CN)Cbl product, which, along with the anticipated inductive effect of the n-Pr ligand, makes the reaction too fast to be monitored. It was reported previously that the reactions of MeCbl and CH_2BrCbl with CN^- are also too fast to be monitored by stopped-flow techniques.[17]

Kinetics of the reaction of β-$NCCH_2$Cbl with cyanide

Fig. 1 shows a plot of k_{obs} *versus* $[CN^-]$ for the reaction of 3×10^{-5} M β-$NCCH_2$Cbl with excess CN^- ($[CN^-] = 0.01$ to 0.3 M) at pH 11, $I = 0.5$ M ($NaClO_4$) and 5 °C. This plot shows typical saturation kinetics and a limiting value of k_{obs} (= k_1 in reaction (2)) is reached at high $[CN^-]$. The intercept can be assigned to a contribution of the back reaction, whereas the observed curvature can be considered as evidence in favor of a limiting D or an I_d mechanism. The kinetic data are for the first substitution reaction that involves displacement of α-DMBz by CN^- as shown in reaction (1). The product spectrum obtained in this case suggests the formation of the intermediate,

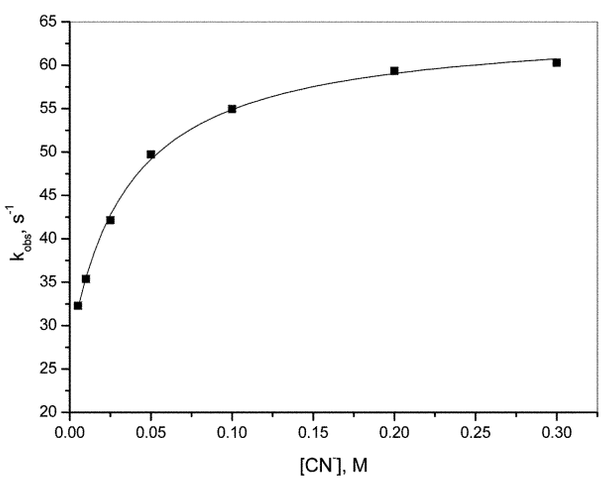
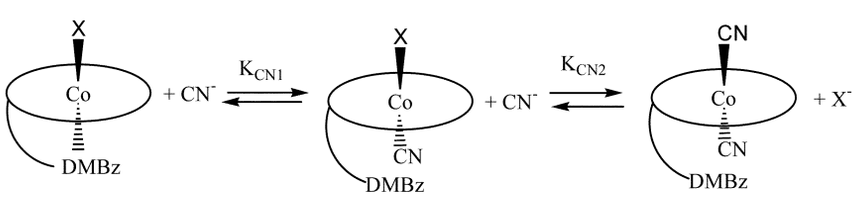
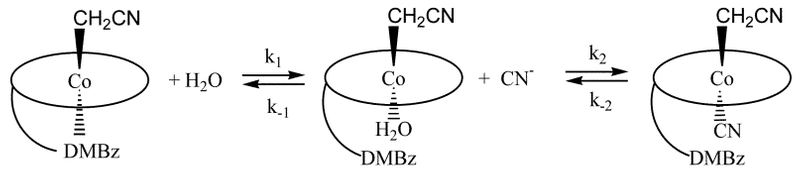


Fig. 1 Plot of k_{obs} *versus* $[CN^-]$ for the reaction between β-$NCCH_2$Cbl and CN^- at pH 11, 5.0 °C and $I = 0.5$ M ($NaClO_4$); the solid line is a fit to eqn. (3) in the text.



(1)



(2)

$NCCH_2(CN)Cbl$, since new bands appeared at 380, 560 and 610 nm, and this complex is light sensitive, but indefinitely stable in the dark (see ESI Fig. S1 †).

On the basis of all the available data, the suggested mechanism for the reaction between β-$NCCH_2Cbl$ and CN^- can be represented by reaction (2), which involves dechelation of DMBz to form a six-coordinate aqua intermediate.[27] If k_{-2} has a significant value as seen in Fig. 1, then the observed rate law is given by eqn. (3). The data in Fig. 1 were fitted to eqn. (3) and resulted in $k_1 = 64.7 \pm 0.7\ s^{-1}$, $k_{-2} = 27.4 \pm 0.8\ s^{-1}$ and $k_2/k_{-1} = 27.9 \pm 2.7\ M^{-1}$ at 5 °C, from which an overall equilibrium constant $k_1k_2/k_{-1}k_{-2} = 65.9 \pm 2.9\ M^{-1}$ can be calculated. This value is in very good agreement with the spectrophotometric value of $63.8 \pm 1.4\ M^{-1}$ reported previously.[26] The value of k_2/k_{-1} represents the efficiency for cyanide compared to DMBz of the nucleotide loop to scavenge the six-coordinate intermediate, which will depend on the selected cyanide concentration. These results are consistent with our direct measurement of the dechelation rate constant of DMBz (k_1), by acidification of β-$NCCH_2Cbl$ to produce the protonated base-off species.[28] The value of k_1 obtained here is in good agreement with that obtained from the pH-jump experiments[28] ($k_1 = 83 \pm 13\ s^{-1}$ at 5 °C), substantiating the assignment of the D mechanism in eqn. (2).

$$k_{obs} = \frac{k_1k_2[CN^-] + k_{-1}k_{-2}}{k_{-1} + k_2[CN^-]} \quad (3)$$

The reaction between β-$NCCH_2Cbl$ and CN^- was studied as a function of temperature and pressure at a high cyanide concentration (0.3 M), *i.e.* where $k_{obs} = k_1$, and the results are reported in Table 1 and Fig. 2, respectively. Fig. 2 demonstrates

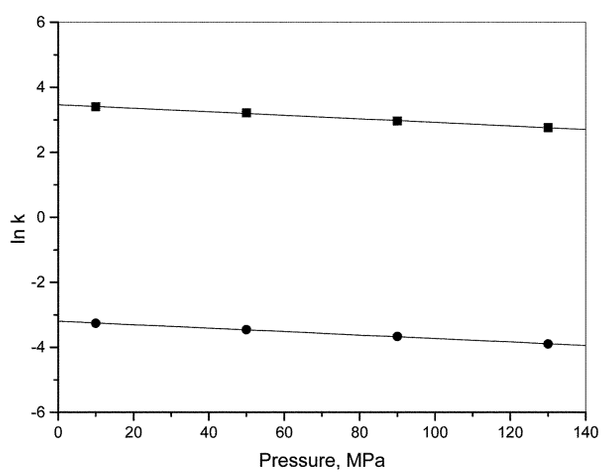


Fig. 2 Plot of ln k_{obs} *versus* pressure for the reaction between β-$NCCH_2Cbl$ and CN^- (■), and for the reaction between CNCbl and CN^- (●) measured at 0.3 and 0.4 M CN^-, respectively; the best fit of the data (solid line) gives $\Delta V^{\neq} = +12.7 \pm 0.5$ and $+13.1 \pm 0.3\ cm^3\ mol^{-1}$ at 0 and 25.0 °C, respectively.

a good linear correlation between ln(k) and pressure. The activation parameters $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were found to be $85 \pm 2\ kJ\ mol^{-1}$ and $+97 \pm 6\ J\ K^{-1}\ mol^{-1}$, respectively, and the activation volume $\Delta V^{\neq} = +12.7 \pm 0.5\ cm^3\ mol^{-1}$ at 0 °C. These data, along with the observed rate law and the agreement between the values of k_1 obtained from the pH-jump experiments[28] and from the saturation kinetics for cyanide substitution, suggest that the first step of the reaction of β-$NCCH_2Cbl$ with CN^- indeed follows a limiting D mechanism, *i.e.* a dissociative dechelation of DMBz. The volume of activation is significantly positive and supports this suggestion. We have recently reported a value for $\Delta V^{\neq}$ of $+14.8\ cm^3\ mol^{-1}$ for the reaction of β-CF_3Cbl and CN^-.[17] Furthermore, the volume of activation for the reaction of β-(*N*-methylimidazolyl)cobalamin with *N*-methylimidazole was also reported to be significantly positive, *viz.* $+15.0 \pm 0.7$ and $+16.8 \pm 1.1\ cm^3\ mol^{-1}$ at 5×10^{-3} and 1 M *N*-methylimidazole, respectively, which correspond to the aquation of $(N\text{-MeIm})_2Cbl^+$ and the dechelation reaction of the α-DMBz of $(N\text{-MeIm})Cbl^+$, respectively.[29] In addition, $\Delta V^{\neq}$ for ligand substitution on $[Co(TMPP)(H_2O)_2]^{5+}$ and $[Co(TPPS)(H_2O)_2]^{3-}$, where TMPP = 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphine and TPPS = 5,10,15,20-tetrakis(*p*-sulfonatophenyl)porphine, was found to be +14.4 and +15.4 $cm^3\ mol^{-1}$, respectively,[30,31] which is very close to the theoretical value of $+13\ cm^3\ mol^{-1}$ expected for a limiting D substitution mechanism for an octahedral complex involving the displacement of a water molecule.[31,32]

The operation of a limiting D mechanism requires the intermediacy of a five-coordinate species or transition state.[27] These species are known for the R–Co(III) complexes of bis(salicylaldehyde)ethylenediamine and bis(acetylacetone)ethylenediamine prepared by Costa and coworkers,[33–35] as confirmed by the X-ray crystal structure of the methylcobalt derivatives of these chelates.[36–38] There is also evidence for penta-coordinate alkylcobalt species in alkyl cobaloximes[39,40] and in alkylcobaltoctaethylporphyrins.[41] The temperature and pressure dependence of the UV-Vis spectra of alkylcobinamides and alkylcobalamins (in acidic medium) is also consistent with the existence of five- and six-coordinate species among the alkylcobalt corrinoids,[42,43] as we have discussed elsewhere.[28]

Table 1 Kinetic data for the reaction of $NCCH_2Cbl$ and CNCbl with CN^- as a function of temperature[a]

T/°C	k_{obs}[b]/s^{-1}	
	R = CN	R = $NCCH_2$
2.5		43.4 ± 0.2
5.0		60.3 ± 2.5
7.5		80.2 ± 3.1
10.0		119.4 ± 2.2
12.5		159.7 ± 1.1
15.0	$(8.4 \pm 0.6) \times 10^{-3}$	223.1 ± 2.4
20.0	$(17.3 \pm 0.9) \times 10^{-3}$	
25.0	$(39 \pm 2) \times 10^{-3}$	
30.0	$(78 \pm 2) \times 10^{-3}$	
35.0	$(152 \pm 4) \times 10^{-3}$	
$\Delta H^{\neq}$/kJ mol^{-1}	105 ± 2	85 ± 2
$\Delta S^{\neq}$/J K^{-1} mol^{-1}	81 ± 6	97 ± 6

[a] Experimental conditions. For $NCCH_2Cbl$: $[NCCH_2Cbl] = (2–4) \times 10^{-5}$ M, $[CN^-] = 0.3$ M, pH 11.0, $I = 0.5$ M ($NaClO_4$). For CNCbl: $[CNCbl] = 5 \times 10^{-5}$ M, $[CN^-] = 0.4$ M, pH 11.0, $I = 0.5$ M ($NaClO_4$). [b] Under the selected experimental conditions, $k_{obs} = k_1$.

Kinetics of the reaction of CNCbl with CN^-

Fig. 3 shows a plot of k_{obs} *versus* $[CN^-]$ for the reaction of 5×10^{-5} M CNCbl with excess CN^- ($[CN^-] = 0.005$ to 0.4 M) at pH 11, $I = 0.5$ M ($NaClO_4$) and 25 °C. This plot shows saturation kinetics and a limiting value of k_{obs} is reached at high $[CN^-]$.

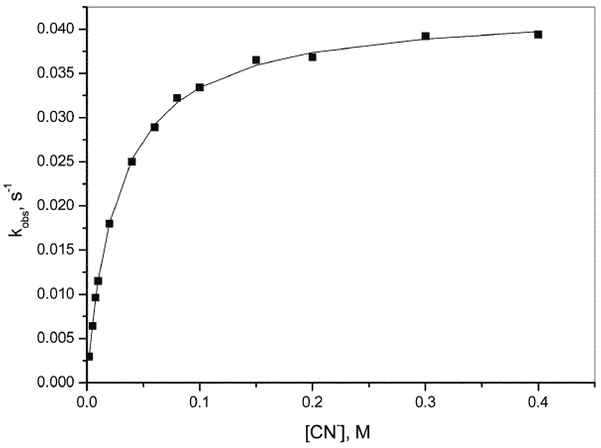


Fig. 3 Plot of k_{obs} *versus* [CN^-] for the reaction between CNCbl and CN^- at pH 11, 25.0 °C and $I = 0.5$ M ($NaClO_4$); the solid line is a fit to eqn. (3) in the text.

The limiting rate constant was found to be 0.042 s^{-1}, in excellent agreement with that found before (0.042 s^{-1}).[18] The intercept and observed curvature can be interpreted in the same way as above. The kinetic data are for the substitution of α-DMBz by CN^-, since the new bands observed at 367, 540 and 580 nm are characteristic for $(CN)_2Cbl$.[44]

On the basis of the available data, the suggested mechanism for the reaction between CNCbl and CN^- is similar to that for the reaction of β-$NCCH_2Cbl$ and CN^- shown in reaction (2). This reaction involves dissociative dechelation of DMBz to form a six-coordinate intermediate aqua complex.[27] If k_{-2} has a significant value as seen in Fig. 3, then the observed rate law is expressed by eqn. (3). The data in Fig. 3 were fitted to eqn. (3) and resulted in $k_1 = 0.042 \pm 0.001$ s^{-1}, $k_{-2} = (7 \pm 3) \times 10^{-5}$ s^{-1} and $k_2/k_{-1} = 36.9 \pm 1.5$ M^{-1} at 25 °C, from which an overall equilibrium constant $k_1k_2/k_{-1}k_{-2} = (2.2 \pm 0.1) \times 10^4$ M^{-1} was calculated. This value is in agreement with the spectrophotometric value of about 10^4 M^{-1} reported in the literature.[18,45] The value of k_2/k_{-1} represents the efficiency of cyanide compared to DMBz of the nucleotide loop to scavenge the six-coordinate intermediate, which will depend on the selected cyanide concentration. This mechanism is in agreement to that suggested by Reenstra and Jencks[18] where they also found that the rate of CN^- addition to CNCbl reaches a limiting value at high [CN^-]. Further evidence for the suggested mechanism came from the direct measurement of the dechelation rate constant of DMBz (k_1) through acidification of CNCbl to produce the protonated base-off species, as described elsewhere.[28]

The reaction between CNCbl and CN^- was studied as a function of temperature and pressure at a high cyanide concentration (0.4 M), *i.e.* where $k_{obs} = k_1$, and the results are reported in Table 1 and Fig. 2, respectively. Fig. 2 demonstrates a good linear correlation between ln(k) and pressure. The activation parameters $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were found to be 105 ± 2 kJ mol^{-1} and +81 ± 6 J K^{-1} mol^{-1}, respectively, and the activation volume $\Delta V^{\neq} = +13.1 \pm 0.3$ cm^3 mol^{-1} at 25 °C. These data along with the observed rate law suggest that the reaction of CNCbl with CN^- indeed follows a limiting D mechanism.

By way of comparison, the limiting rate constant (k_1 in reaction (2)) for the reactions of the different XCbl's with CN^- were found to be 0.042 (25 °C), 8.8 (10 °C)[17] and 64.7 s^{-1} (5 °C) for X = CN^-, CF_3 and $NCCH_2$, respectively. Also the overall second order rate constants calculated from the initial slope of the curvature obtained for the reactions of XCbl's with CN^-, *i.e.* $k_1k_2/k_{-1} = K_1k_2$ from eqn. (3), were found to be 1.1 (25 °C), 217 (10 °C), 483 (5 °C) and 1×10^3 M^{-1} s^{-1} (5 °C), for X = CN^-, CF_3, $NCCH_2$, and CF_2H, respectively. Unfortunately, this reaction was found to be too fast to be followed by stopped-flow techniques (dead time 2–4 ms) for X = CH_3, CH_2Br and n-Pr. We conclude that the overall second order rate constants for the reaction of XCbl's with CN^-, which represent a composite value for K_1 and k_2 in reaction (2), follow the kinetic *trans* effect and decrease in the order n-Pr ≥ Me > CF_2H > $NCCH_2$ > CF_3 > CN^-. A similar *trans* effect trend is observed in the values of k_{-2} (reaction (2)), *viz.* 7×10^{-5} (25 °C), 2.6 (10 °C), and 27.4 s^{-1} (5 °C) for X = CN^-, CF_3 and $NCCH_2$, respectively. In comparison however, the observed second order rate constants for the reaction of CF_3CH_2Cbl and AdoCbl with CN^- were reported to be 0.24 and 7.4×10^{-3} M^{-1} s^{-1} at 25 °C, respectively.[15,17] These two values are not in agreement with the kinetic *trans* effect order given above, suggesting that the first substitution reaction in (1) may not be the rate-determining step observed for these complexes. Furthermore, these complexes are the only two of the XCbl's series that react further in the presence of CN^- to give $(CN)_2Cbl$. This led us to reinvestigate the reactions of CF_3CH_2Cbl and AdoCbl with CN^- in order to resolve the apparent discrepancy observed in the *trans* effect order of these groups (X = Ado and CF_3CH_2).

Reaction of AdoCbl with cyanide

The reaction of AdoCbl with CN^- has been known for several decades. This reaction proceeds *via* heterolytic cleavage of the Co–C bond and the reaction products were isolated and characterized as $(CN)_2Cbl$, adenine and the cyanohydrin of D-erythro-2,3-dihydroxy-4-pentanol.[46] The mechanism of this reaction in aqueous solution and whether CN^- attacks the α- or the β-position of AdoCbl first, remained controversial in the literature.[15,19a,46–48] Recently, it was suggested that the rate-determining heterolytic cleavage of the Co–C bond for this reaction in 92% DMF/8% D_2O is preceded by the rapid addition of cyanide to the α-position. The formation of an intermediate (β-Ado)(α-cyano)cobalamin species could be identified by ^1H NMR spectroscopy.[16]

We have previously reported that AdoCbl reacts with CN^- in a single kinetically observable step, with no evidence for the displacement of DMBz in the α-position by CN^- in the first step. The rate-determining step was suggested to involve the displacement of the Ado group in the β-position by CN^- since no intermediate was detected.[15] This study was carried out at [CN^-] between 0.005 and 0.5 M. Under the selected conditions (pH = 11, $I = 1$ M, [CN^-] = 0.005–0.5 M and 25 °C), a linear CN^- concentration dependence with negligible intercept was obtained and the second order rate constant was found to be 7.4×10^{-3} M^{-1} s^{-1}.[15] It now turns out that the selected concentration range was not high enough to observe any change in the UV-Vis spectrum that would suggest rapid substitution of α-DMBz by CN^- prior to rate determining Co–C bond cleavage, since the binding constant for CN^- to AdoCbl is expected to be very low, and a high [CN^-] will be required to observe any significant change in the UV-Vis spectrum.

UV-Vis spectra recorded before and after mixing (*ca.* 3 s) of AdoCbl with 2 M CN^- showed a significant increase in absorbance in the range 560 to 640 nm and a decrease in absorbance in the range 470 to 530 nm as shown in Fig. 4. The difference between the spectrum before and after adding CN^- to AdoCbl is not pronounced at low cyanide concentration, since significantly higher concentrations of CN^- are required to displace the DMBz group from the α-position in a rapid step to form Ado(CN)Cbl. Ado(CN)Cbl then reacts further with a second CN^- to give $(CN)_2Cbl$. Formation of $(CN)_2Cbl$ from Ado(CN)Cbl and CN^- is accompanied by good isosbestic points at 347, 380, 524 and 608 nm, a decrease in absorbance at 620 to 650 nm, and the appearance of new bands at 367, 540 and 580 nm as shown in Fig. 4. It is known that substitution of α-DMBz by CN^- is accompanied by an increase in absorbance at 580 to 620 nm[26] due to a new d–d transition common to all complexes of this type. Interestingly, the UV-Vis spectrum obtained directly after mixing AdoCbl with 2 M CN^-, or higher concentrations, matches that obtained for the reaction of

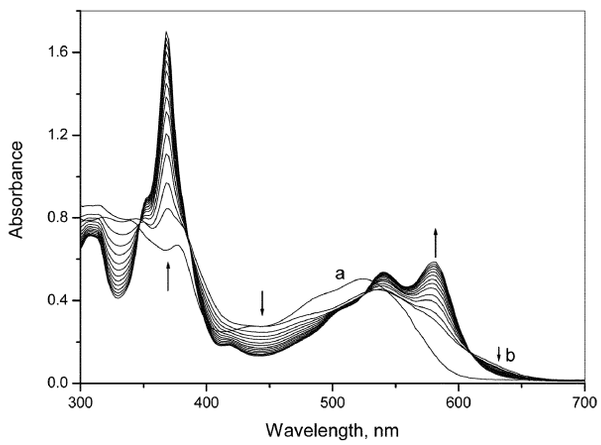


Fig. 4 (a) UV-Vis spectra of AdoCbl before mixing with 2 M CN^-. (b) Selected visible spectra for the reaction of AdoCbl and 2 M CN^-, pH 11.0 and 25.0 °C directly after mixing (*ca.* 3 s) and then recorded every 20 s.

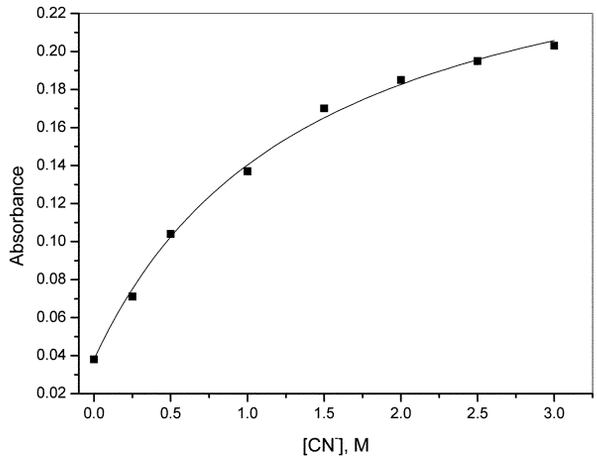


Fig. 5 Change in absorbance at 620 nm on addition of CN^- to AdoCbl; the solid line is a fit to eqn. (4) in the text and results in K_{CN1} = 0.6 ± 0.1 M^{-1}.

AdoCbl and tetrabutylammonium cyanide in a less protic solvent (92% DMF/8% D_2O).[16] Also, the UV-Vis spectral changes that accompany the conversion of Ado(CN)Cbl to $(CN)_2$Cbl are almost identical in both cases (*i.e.* in aqueous and less protic solvents). The conversion of (β-Ado)(α-CN)Cbl to $(CN)_2$Cbl can be monitored by following the decrease in absorbance at 620 to 650 nm, or the increase in absorbance at 367 or 580 nm. In the case of less protic solvents, the rate-determining heterolytic Co–C cleavage was preceded by rapid addition of CN^- to AdoCbl to form Ado(CN)Cbl.[16] This confirms that the first step in the reaction of AdoCbl and CN^- is indeed the substitution of α-DMBz by CN^- to form (β-Ado)-(α-CN)Cbl.

The value of K_{CN1} is thus difficult to determine spectrophotometrically using the normal method (titrating AdoCbl with different concentrations of CN^-) because the Ado(CN)Cbl formed undergoes heterolytic Co–C cleavage and reacts with CN^- to give $(CN)_2$Cbl. Consequently, the spectrophotometric titration was carried out by injecting a small volume of a concentrated AdoCbl solution (to minimize dilution effects) into 3 ml of a buffer at pH 11 containing different concentrations of CN^-. The UV-Vis spectrum was recorded directly after mixing and then compared with those recorded at different [CN^-]. This allowed us to follow the change in absorbance upon increasing the cyanide concentration. In another approach to determine K_{CN1}, a tandem cuvette containing AdoCbl and CN^- was used and then the change in absorbance upon mixing different concentrations of CN^- with the same concentration of AdoCbl was followed.

The spectrophotometric titrations were monitored by following the increase in absorbance at 600 to 620 nm, where the largest change in absorbance occurred. Selected data are shown in Fig. 5, where the solid line represents a fit of the data to eqn. (4).

$$A_x = A_o + (A_\infty - A_o)K_{CN1}[CN^-]/(1 + K_{CN1}[CN^-]) \quad (4)$$

The values of A_o and A_∞ represent the absorbances of AdoCbl and (β-Ado)(α-CN)Cbl, respectively, and A_x is the absorbance at any cyanide concentration. The values of K_{CN1} and A_∞ were calculated from eqn. (4), and K_{CN1} was found to be 0.6 ± 0.1 M^{-1}. The analysis of these data by plotting log $(A_x - A_o)/(A_\infty - A_x)$ *versus* log[CN^-] gave a good linear plot with a slope of 1.13 ± 0.03, which indicates one CN^- ligand is coordinated to the cobalt atom. The intercept of this linear plot gives the value of log *K*, which is in a good agreement with that obtained from eqn. (4).

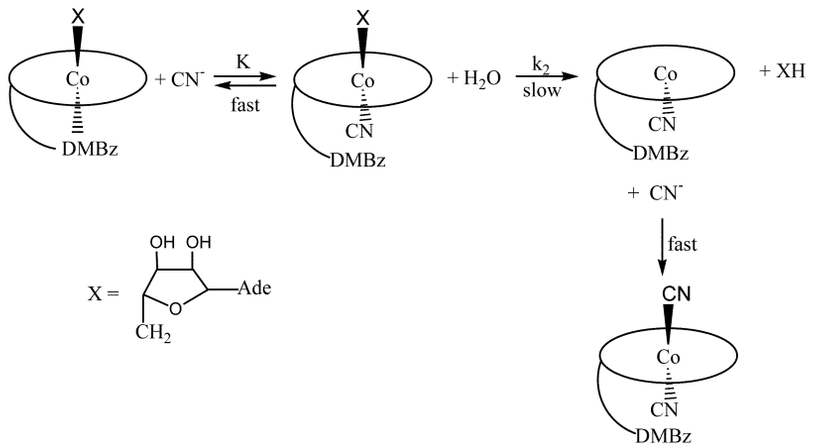
We were not able to measure the kinetics of the first reaction step between 8 × 10^{-5} M AdoCbl and 1 M CN^- at pH 11 and 5 °C, since the reaction in aqueous solution was found to be too fast to be followed by stopped-flow technique (dead time 2–4 ms). The reactions between CH_3Cbl, CH_2BrCbl and n-PrCbl with CN^- were also too fast to be followed in this way.[17] However, recently the reaction of AdoCbl with CN^- was carried out in a less protic solvent (92% DMF/8% D_2O) to slow down the rate of Co–C heterolysis of the intermediate (Ado)(CN)Cbl, since the displacement of the adenosyl ligand by CN^- is accompanied by protonation, presumably at the ribosyl oxygen of the adenosyl ligand.[16] Brasch and Haupt suggested that the rate determining heterolytic Co–C cleavage is preceded by a rapid addition of CN^- to AdoCbl to form the intermediate (β-Ado)(α-CN)Cbl. The mechanism in this case involves rapid reversible formation of (β-Ado)(α-CN)Cbl from base-off AdoCbl and CN^-, followed by rate determining solvent assisted heterolytic cleavage of the Co–C bond of the intermediate and the subsequent rapid formation of $(CN)_2$Cbl (see Scheme 1). The observed rate constant for this reaction was found to be independent of [CN^-] above 0.02 M, but decreased with decreasing CN^- concentration. The experimental data were fitted to the rate law given in eqn. (5).[16]

$$k_{obs} = k_2K[CN^-]/(1 + K[CN^-]) \quad (5)$$

In aqueous solution, solvent-assisted rate-determining Co–C heterolysis of the intermediate is much faster due to the protic nature of the solvent. Under such conditions no curvature was observed and eqn. (5) simplifies to $k_{obs} = k_2K[CN^-]$. Values of $\Delta S^\#$ and $\Delta V^\#$ for the reaction of AdoCbl with CN^- in aqueous solution at pH 11 were reported to be -127 ± 3 J K^{-1} mol^{-1} and -10 ± 0.4 cm^3 mol^{-1}, respectively, and an associative substitution mechanism was suggested.[15] However, in terms of the proposed mechanism in Scheme 1,[16] the reported activation parameters present a composite value for the formation of the intermediate (β-Ado)(α-CN)Cbl complex (*K*) and the subsequent heterolysis step (k_2). For the rate-determining heterolysis reaction, it has been suggested[16] that a solvent molecule is involved in the transition state preceding rate-determining cleavage of the Co–C bond. Thus, it is quite reasonable to expect $\Delta S^\#$ and $\Delta V^\#$ to be both significantly negative for the suggested mechanism in Scheme 1, which can now account for the apparent associative nature of the substitution process.[15]

Pratt[19] has also proposed that the attack of CN^- proceeds first through the displacement of α-DMBz, and then in a second, slower step, substitution of the Ado group by CN^- takes place to form $(CN)_2$Cbl, which is in line with our present interpretation of the data.

In addition, the reaction between 5′-deoxyadenosylcobinamide (AdoCbi) and CN^- in aqueous solution at pH 11 and in



Scheme 1

92% DMF/8% D_2O was recently studied.[49] Saturation kinetics were obtained and it was suggested that the mechanism involves rapid formation of (β-Ado)(α-CN)Cbi, followed by solvent-assisted, rate determining cleavage of the Co–Ado bond. Evidence for the rapid formation of (β-Ado)(α-CN)Cbi was also obtained from ^{1}H NMR spectroscopy.[49] The value of K_{CN1} for the reaction of AdoCbi with CN^- (5.6 M^{-1}) was found to be 9 times higher than that obtained in the present study for the reaction of AdoCbl with CN^- (0.6 M^{-1}). The difference between the two equilibrium constants is due to the base-on/base-off reaction of AdoCbl, which preceeds the ligand substitution step, but is absent for AdoCbi.

Reaction of β-CF_3CH_2Cbl with cyanide

We have recently reported that β-CF_3CH_2Cbl reacts with CN^- in a single kinetic step with no evidence for displacement of DMBz from the α-site prior to the rate-determining substitution of the CF_3CH_2 group by CN^-.[17] The data were obtained at low CN^- and did not show significant changes in the UV-Vis spectrum on mixing the reactants; *i.e.*, there was no evidence for rapid formation of a CF_3CH_2(CN)Cbl intermediate *via* the displacement of α-DMBz by cyanide. Under these conditions, a non-linear dependence on the CN^- concentration was observed and the second order rate constant calculated from the initial slope was found to be 0.24 M^{-1} s^{-1} at 10 °C. The kinetic data were fitted to a rate law similar to that given in eqn. (5) and values of k_2 and K obtained were reported to be 0.036 ± 0.001 s^{-1} and 9.8 ± 0.5 M^{-1}, respectively (Fig. 6 in ref. 17). $\Delta S^{\#}$ and $\Delta V^{\#}$ were found to be −25 ± 4 J K^{-1} mol^{-1} and +8.9 ± 1.0 cm^3 mol^{-1}, respectively, on which basis an I_d mechanism was suggested for this reaction.

The similarities between β-CF_3CH_2Cbl and AdoCbl, as well as the apparent discrepancy in the kinetic *trans* effect order led us to reinvestigate the reaction of β-CF_3CH_2Cbl at higher concentrations of CN^-. UV-Vis spectra recorded before and directly after mixing (*ca.* 3 s) 5 × 10^{-5} M β-CF_3CH_2Cbl with 0.8 M CN^- at pH 11 and 25 °C, showed a significant increase in absorbance in the range 580 to 620 nm (see ESI, Figure S2†). This demonstrates that higher concentrations of CN^- are required to displace α-DMBz in a rapid pre-equilibration step to form CF_3CH_2(CN)Cbl, which then reacts further to give $(CN)_2$Cbl. Formation of $(CN)_2$Cbl is accompanied by a decrease in absorbance at 600 to 640 nm and the occurrence of new bands at 367, 540 and 580 nm.

As with AdoCbl (see above), the spectrophotometric titration to determine K_{CN1} for CF_3CH_2Cbl was carried out by injecting a small volume of a concentrated solution of CF_3CH_2Cbl (to minimize dilution effects) into different CN^- concentrations and following the change in absorbance as a function of the cyanide concentration. The tandem cuvette method (6 × 10^{-5} M CF_3CH_2Cbl and varying [CN^-]) was also used to follow the absorbance at 600 to 620 nm upon mixing, where the largest change in absorbance occurred. The experimental data were fitted to eqn. (4) and the value of K_{CN1} was found to be 13.0 ± 1.5 M^{-1}, which is in good agreement with that determined previously from the kinetic studies, *viz.* 9.8 ± 0.5 M^{-1}.[17]

The kinetics of the reaction between 5 × 10^{-5} M β-CF_3CH_2Cbl and 0.4 M CN^- was followed at pH 11 and 3 °C, and the second order rate constant was found to be 1.25 × 10^3 M^{-1} s^{-1}. This value fits in nicely with the order presented in Table 2 for the effect of the axial group X on the rate of the reaction. The kinetic *trans* effect was found to decrease in the order n-Pr ≥ Ado ≥ Me ≥ CF_3CH_2 > CF_2H > $NCCH_2$ > CF_3 > CN^-.

We conclude on the basis of the agreement between the value of K_{CN1} determined thermodynamically during the course of this work (13.0 ± 1.5 M^{-1}) and that obtained previously based on kinetic data (9.8 ± 0.5 M^{-1}),[17] as well as the consistent *trans* effect order now observed for the reactions of different XCbl's with CN^-, that the reaction between β-CF_3CH_2Cbl and CN^- proceeds in a similar fashion to the reaction of AdoCbl with CN^- according to the mechanism given in Scheme 1. The rate determining step is the cleavage of the Co–C bond, which is preceded by a rapid formation of CF_3CH_2(CN)Cbl (K_{CN1} = 13 M^{-1} and k_{CN} = 1.25 × 10^3 M^{-1} s^{-1} at 5 °C) and followed by the reaction with CN^- to produce $(CN)_2$Cbl as the final product.

Overall discussion

The substitution reactions of β-$NCCH_2$Cbl and CNCbl with CN^- that involve rate-determining displacement of α-DMBz studied here, proceed through a limiting D mechanism. Recently we reported that the reactions of β-CF_3Cbl and β-CF_3CH_2Cbl with CN^- proceed through a limiting D and I_d mechanism, respectively.[17] However, for the reaction between AdoCbl and CN^-, it was previously suggested[15] that the reaction proceeds through an associative mechanism. The kinetic, thermodynamic and activation parameters obtained during the course of this work and those reported before for substitution reactions of different XCbl's are summarized in Table 2. Table 2 shows that the value of K_{CN1} increases significantly from 0.67 (X = Et) to 10^4 M^{-1} (X = CN^-) as the upper axial ligand becomes more electron withdrawing in nature. K_{ImH} for substituting imidazole for H_2O *trans* to the axial (X) group similarly increases significantly from 0.5 (X = Ado) to 1.6 × 10^5 M^{-1} (X = H_2O). It is also known that the complex-formation constants for the substitution of water by another ligand decrease and approach zero as the *trans* ligand is varied in the order H_2O > cyanide > CF_3 > $NCCH_2$ > CH_2CF_3 > methyl > Ado > ethyl.[19,50,51] This weakening of the bond between cobalt and all other ligands (X) only makes sense if the bond to H_2O is also being weakened in the same way as in the above *trans* effect order. One would therefore expect that the Co–OH_2 bond could

Table 2 Thermodynamic, kinetics and activation parameters for the cyanation and base-on/base-off reactions for a series of cobalamins (XCbl's)

X	$pK_{base\text{-}off}$ [a]	k^{H} [b]/M^{-1} s^{-1}	k_1 [c]/s^{-1}	k_{CN} [d]/M^{-1} s^{-1}	K_{CN1} [e]/M^{-1}	K_{ImH} [g]/M^{-1}	$\Delta H^{\#}_{CN}$/kJ mol^{-1}	$\Delta S^{\#}_{CN}$/J K^{-1} mol^{-1}	$\Delta V^{\#}_{CN}$/cm^3 mol^{-1}
Et	4.16				0.67				
n-Pr	4.1				1.3				
Ado	3.67			$\geq 10^4$	0.6 [f]	0.5	53.0 ± 0.6 [h]	−127 ± 3 [h]	−10.0 ± 0.4 [h]
CH_3	2.89			$\geq 10^4$	0.4, 1.2	8			
CH_2CF_3	2.6			1250 (3 °C) [f]	13 [f]		71 ± 1 [i]	−25 ± 4 [h]	+8.9 ± 1.0 [i]
CF_2H	2.15			1000 (10 °C) [i]	3.27				
$NCCH_2$	1.81	913	64.6 [f]	483 (5 °C) [f]	63.8	301	85 ± 2 [f]	+97 ± 6 [f]	+12.7 ± 0.5 [f]
CF_3	1.44	31.8	8.8 [i]	217 (10 °C) [i]	123 [i]	700	77 ± 3 [i]	+44 ± 11 [i]	+14.8 ± 0.8 [i]
CN	0.1	0.28	0.042 [f]	1.1 (25 °C) [f]	3×10^3, 10^4	1.4×10^4	105 ± 2 [f]	+81 ± 6 [f]	+13.1 ± 0.3 [f]
H_2O	−2.13					1.6×10^5			

[a] Refs. 28 and 60. [b] Rate constant for the acid catalyzed reaction see ref. 28. [c] Limiting rate constant (k_1), see eqn. (2) in the text. [d] Overall second order rate constant (K_1k_2) for the substitution of α-DMBz by CN^- (see text). [e] Data from refs. 17, 18, 26 and 45. [f] This work. [g] Refs. 51, 57 to 59. [h] Ref. 15. [i] Ref. 17.

be weakened to such an extent that a five-coordinate complex could be formed.[28,43]

The overall second order rate constant (k_{CN}), which represents the composite quantity K_1k_2 according to reaction (2) and eqn. (3), decreases according to the kinetic *trans* effect in the order n-Pr ≥ Ado ≥ Me > CF_3CH_2 (1.25×10^3 M^{-1} s^{-1}) > CF_2H > $NCCH_2$ > CF_3 > CN^- (1.1 M^{-1} s^{-1}). A similar trend is also observed for the reverse aquation rate constant k_{-2}. These results are in agreement with those of Pratt *et al.*[52] who showed that the kinetic *trans* effect order for the substitution of H_2O by CN^- for different cobinamides (XCbi) increases in the order X = H_2O ≤ DMBz < OH^- < CN^- < CH_2=CH–, Me and Et, with corresponding rate constants ranging from ≤ 10^3 to ≥ 10^8 M^{-1} s^{-1}.

The stretching frequency ν_{CN} (for cyanide *trans* to X), when X = H_2O was found to be 2133 cm^{-1}. However, this value was found to be 2082 cm^{-1} when X = Et, which is close to the stretching frequency for free CN^- (2079 cm^{-1}),[19,52] *i.e.*, the coordinated cyanide becomes more ionic in character. It is clear from this argument that the positive charge on cobalt decreases from X = H_2O to X = Et. It was also found that the Co–N_{DMBz} and Co–C bond lengths in RCbl's increase in the order H_2O < CN < CF_3 < $NCCH_2$ < CF_2H < CF_3CH_2 < CH_3 < Ado.[53–55] This suggests that AdoCbl is a labile complex for ligand substitution reactions. Furthermore, in the case of AdoCbl, steric effects may also play an important role in controlling its substitution behaviour.[56]

A decrease in the labilization effect of X in going from adenosyl to CH_2CF_3 and CF_3, causes a drastic decrease in the fraction of the base-off species and as a result in the fraction of the five-coordinate species. We have recently demonstrated on the basis of UV-Vis spectra recorded in acidic medium under pressure, that the base-off species of the first two compounds (AdoCbl and n-PrCbl) of the series of alkylcobalamins (see Table 2 for a list of these XCbl's) are mainly five-coordinate species.[28] For some of the XCbl's, X = Me, CF_3CH_2, $NCCH_2$ and vinyl, we suggest that the base-off species of these complexes exist in solution as an equilibrium between five- and six-coordinate species. However, for the last three complexes in the XCbl series (X = CF_3, CN and H_2O), the base-off forms of these compounds are mainly six-coordinate, aqua species. It seems clear that the X group in the upper, β-axial position has a significant influence on this equilibrium and by increasing the electron withdrawing ability of the alkyl group, the equilibrium is shifted towards the six-coordinate complexes.[28]

The values of $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta V^{\#}$ for the reaction of XCbl with CN^- are also included in Table 2. The values for $\Delta S^{\#}$ and $\Delta V^{\#}$ show the same mechanistic trends and support the suggested reaction mechanisms. It should be noted that in the case of AdoCbl, the reported activation parameters are for the combined rapid formation of (β-Ado)(α-CN)Cbl and the subsequent rate-determining, solvent assisted heterolysis reaction as outlined in Scheme 1. In the case of CF_3CH_2Cbl, the activation parameters are for the solvent assisted cleavage of the Co–C bond, since these were determined under conditions where heterolysis is rate-determining.

Thus in terms of our goal to study the kinetic *trans* effect and the role of the axial alkyl ligand in controlling the mechanism of the substitution reactions *trans* to the alkyl ligand, this study has revealed how the alkyl ligand controls the kinetics and thermodynamics of the reaction of XCbl's with CN^-, and has clarified the apparent discrepancies observed in the kinetic *trans* effect order. In addition, for the reactions of AdoCbl and β-CF_3CH_2Cbl with cyanide, we now have demonstrated that the earlier observed kinetics are not due to the displacement of α-DMBz by CN^-, but rather due to the rate-determining solvent assisted heterolysis of the Co–C bond following the rapid formation of (α-CN)(β-Ado)Cbl and (α-CN)(β-CF_3CH_2)Cbl, respectively. In this way we have now reached a generalized mechanistic description for all alkylcobalamins studied to date.

Acknowledgements

The authors gratefully acknowledge financial support from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie (to R. v. E.), the Alexander von Humboldt Foundation (fellowship to M. S. A. H.), and the National Institute of General Medical Sciences, USA (Grant GM 48858 to K. L. B.). M. S. A. H. thanks the Ain Shams University for sabbatical leave. The authors kindly thank Dr Nicola Brasch (Australian National University, Canberra) for helpful comments.

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