Studies on the Mechanism of the Reaction between Coenzyme B_{12} and Cyanide: Direct ^{1}H NMR Spectroscopic Evidence for a (β-5′-Deoxyadenosyl)(α-cyano)cobalamin Intermediate

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The reaction between coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl) and tetrabutylammonium cyanide to give dicyanocobalamin, adenine, and 1-cyano-D-*erythro*-2,3-dihydroxy-4-pentenol has been examined in 92% *N*,*N*-dimethylformamide (DMF)/8% D_2O. Under these conditions rate-determining Co−C heterolytic cleavage is preceded by rapid addition of cyanide to AdoCbl to form an intermediate, (β-5′-deoxyadenosyl)(α-cyano)cobalamin ((β-Ado)(α-CN)Cbl$^-$), identified by ^{1}H NMR spectroscopy. Rate constants have been determined by both ^{1}H NMR and visible spectroscopies, with the latter showing saturation kinetics. The observed rate constant is pH-independent in the pH region studied, and replacing D_2O by H_2O increases it by ca. 10%. Increasing the percentage of D_2O in the DMF/D_2O solvent mixture also increases the reaction rate, and for $D_2O \geq 50\%$ there is a change in the rate-determining step, with formation of the (β-Ado)(α-CN)Cbl$^-$ intermediate becoming rate-determining. A mechanism in 92% DMF/8% D_2O is proposed which involves rapid reversible formation of (β-Ado)(α-CN)-Cbl$^-$ from base-off AdoCbl plus cyanide, followed by rate-determining solvent-assisted cleavage of the Co−C bond of the intermediate and subsequent rapid addition of a second cyanide to give the products.

Introduction

A recent paper of ours examined the kinetics and mechanism of the reaction between coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl) and cyanide in aqueous solution.[1] This study was carried out for a number of reasons: (1) The reaction between AdoCbl and cyanide results in quantitative heterolytic (rather than homolytic, or a mixture of both) Co−C bond cleavage and is therefore, broadly speaking, in the same general class of reactions as the methylcobalamin-dependent methyltransferases, in which a key step involves heterolytic Co−C bond cleavage. Factors which control the rate of Co−C heterolytic cleavage of alkylcobalamins are poorly understood, although a 1982 paper by Jencks and co-workers[2a] stands out in the prior alkylcobalamin plus cyanide literature, a paper we will return to later. (2) There is considerable discussion in the literature as to whether cyanide first substitutes at the α (lower) or β (upper) axial site of AdoCbl (Figure 1).[3−7] (3) Apart from cyanide, there are very few nucleophiles known which are capable of cleanly heterolytically cleaving the Co−C bond of AdoCbl, thiols[3] and imidazolates[8] being two of note. (4) Cyanation of cobalamins and other B_{12} corrinoids played a major part in the early characterization methods of these complexes prior to X-ray crystallography and NMR spectroscopy becoming widely available,[9] and understanding the mechanism of cyanide addition to alkyl corrinoids is therefore of fundamental interest. Interestingly, the other B_{12} coenzyme methylcobalamin is inert to cyanide (aqueous solution, $\leq$1.2 M CN$^-$).[10]

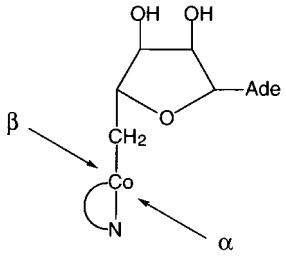

Figure 1. Schematic representation of AdoCbl, showing the two possibilities for approach (upper = β, lower = α with respect to the corrin ring of the cobalamin) of the CN$^-$ ligand.

In our earlier work we examined the reaction between AdoCbl and cyanide in aqueous solution,[1] as all prior studies have done.[3−6,9,11] The findings can be summarized as follows.

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(1) Addition of the first cyanide was rate-determining under all conditions, so that all other subsequent steps to give the reaction products dicyanocobalamin ($(CN)_2Cbl^-$), adenine, and the cyanohydrin 1-cyano-D-*erythro*-2,3-dihydroxy-4-pentenol are rapid. (2) The observed rate constant is acid-independent (unlike for acid-catalyzed heterolytic cleavage of AdoCbl[12]). (3) Activation parameters ($\Delta S^{\ddagger}$, $\Delta V^{\ddagger}$) typical for an associative rate-determining step were obtained. Since the reactants were converted directly to products without the build up of a monocyano intermediate, it was impossible to ascertain whether cyanide first substitutes at the α or β axial site of AdoCbl. However, it was postulated on the basis of the activation parameters that cyanide was probably first substituting at the β site.[1]

Further investigations on the AdoCbl plus cyanide reaction, but now in DMF (*N*,*N*-dimethylformamide)/D_2O solvent mixtures, have resulted in the direct observation of the monocyano intermediate and are the subject of this note. The reasoning behind examining the reaction in a less protic solvent mixture is quite simple. Displacement of the adenosyl ligand by cyanide is accompanied by protonation of probably the ribosyl oxygen of the adenosyl ligand.[13] This process is obviously rapid in aqueous solution (since the observed rate constant was found to be acid-independent[1]); however in a less protic solvent mixture it was hoped that this process may now be slow enough to the extent that if cyanide first substitutes at the β site of AdoCbl, this step may now become acid-dependent. Alternatively, if cyanide first substitutes at the α site of AdoCbl, then subsequent displacement of the Ado ligand by the second cyanide could be so slow that a (β-Ado)(α-CN)Cbl^- intermediate could be observed. This latter case has, in fact, been found to occur.

Results and Discussion

The reaction between AdoCbl and (TBA)CN (tetrabutylammonium cyanide) was examined in 92% DMF/8% D_2O solutions using (TBA)ClO_4 (tetrabutylammonium perchlorate) to maintain constant ionic strength (0.50 M). ^{1}H NMR spectroscopy proved to be a very informative way to follow the reaction.[14] The ^{1}H NMR spectrum of AdoCbl in 92% DMF-d_7/8% D_2O exhibits eight peaks at 8.26, 8.18, 7.33, 7.23, 6.56, 6.33 (d), 6.14, and 5.70 (d) ppm; three from the adenosyl ligand (A2, A8, A11 (d)), one from the corrin ring (C10), and four from the nucleotide (B2, B4, B7, R1 (d)).[15] The chemical shifts are substantially different from those found for AdoCbl in D_2O.[15] Importantly, addition of a small amount of D_2O to the DMF not only made the reaction between AdoCbl and (TBA)CN convenient to follow (see later in discussion; the observed reaction is faster when more D_2O is present), but greatly simplified interpretation of all ^{1}H NMR spectra by significantly reducing (>95%) the intensity of the amide side chain proton signals of the cobalamin, since proton−deuterium exchange between the amide protons and solvent D_2O occurs within minutes.

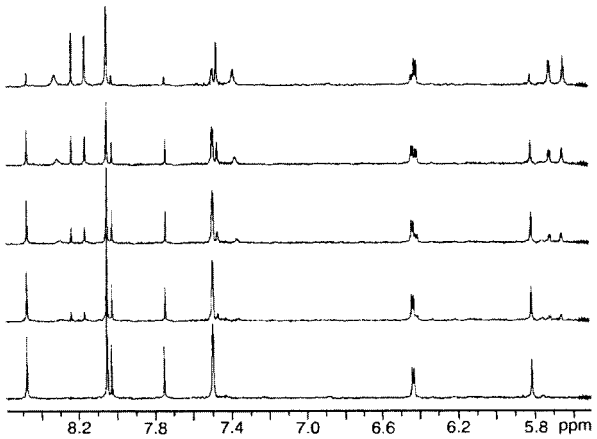

Figure 2. Selected ^{1}H NMR spectra of the aromatic region obtained upon dissolving AdoCbl in (TBA)CN (ca. 5×10^{-3} M AdoCbl, 5.00×10^{-2} M (TBA)CN, 92% DMF-d_7/8% D_2O, $I = 0.50$ M ((TBA)ClO_4), 25.0 °C). Spectra were taken (from top to bottom) immediately ($t_{rel} = 0$, ca. 2 min after mixing), at $t_{rel} = 1.55$, 3.10, and 4.65 h and at the completion of the reaction (22.5 h). All reported chemical shifts are relative to TSP (internal standard); a solvent (DMF) signal occurs at 8.05 ppm.

Figure 2 gives representative ^{1}H NMR spectra obtained immediately ($t_{rel} = 0$; ca. 2 min after mixing), at $t_{rel} = 1.55$, 3.10, 4.65, and 22.5 h after dissolving AdoCbl in a solution of tetrabutylammonium cyanide, (TBA)CN in 92% DMF-d_7/8% D_2O in red-light-only conditions. An immediate (<2 s) color change from red to blue-black occurs upon the addition of (TBA)CN to a solution of AdoCbl in 92% DMF-d_7/8% D_2O. The intensity of the signals of the intermediate at 8.34, 8.25, 8.18, 7.49, 7.40, 6.42 (d), 5.72 (d), and 5.65 ppm decrease with time until they are no longer observable. Product peaks at 8.49, 8.04, 7.76, 7.50, 7.49, 6.45 (d), and 5.82 ppm increase at a rate comparable to the rate of decrease of the signals of the intermediate and can be assigned to adenine (8.04, 7.76 ppm; confirmed by spiking the solution with additional adenine[16]) and $(CN)_2Cbl^-$ (the remaining peaks (B2, B4, B7, R1 (d), C10), confirmed by running the spectrum of authentic cyanocobalamin, CNCbl in ca. 7.0×10^{-2} M (TBA)CN in 92% DMF-d_7/8% D_2O). No other signals were observed in the aromatic region of the ^{1}H NMR spectrum; hence ⩽2% Co−C homolysis occurs.[17] The reaction intermediate is (β-Ado)(α-CN)Cbl^- rather than CNCbl on the basis of the following: (1) The chemical shifts of CNCbl are substantially different from those of the intermediate (7.47, 7.16, 6.64, 6.41 (d), and 6.12 ppm (0.50 M (TBA)ClO_4, 92% DMF-d_7/8% D_2O)). (2) Eight peaks (A2, A8, A11 (d), C10, B2, B4, B7, R1 (d)) are observed, which includes two doublets as would be expected for (β-Ado)(α-CN)Cbl^-. (Only seven major signals *and one doublet* would be observed if the adenosyl ligand had already been displaced from the cobalamin (ignoring the much less intense signals from the cyanohydrin); i.e., five for CNCbl and two for adenine.) Thus the monocyano intermediate is (β-Ado)(α-CN)Cbl^- rather than CNCbl. A first-order rate constant for the reaction between (β-Ado)(α-CN)Cbl^- and cyanide (in the absence of light) can be

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(16) The chemical shifts of the adenine signals were found to be concentration dependent: addition of ca. 1.5 equiv of authentic adenine to the reaction product solution caused the ^{1}H NMR aromatic adenine signals to shift slightly downfield (8.06 and 7.83 ppm, respectively).

(17) ^{1}H NMR spectra of the Co−C homolytic cleavage products 5′-deoxyadenosine and 8,5′-anhydroadenosine in 92% DMF-d_7/8% D_2O have signals in the aromatic region at 8.41, 8.25, 6.04 (d), and 8.19 and 6.12 ppm, respectively (0.50 M (TBA)ClO_4, 25.0 °C). Addition of ca. 5.0×10^{-2} M (TBA)CN to these solutions changed the chemical shifts by ⩽0.02 ppm.

obtained by plotting ln(area of the (β-Ado)(α-CN)Cbl$^-$ signal at δ = 8.25 ppm) versus time for the experiment shown in Figure 2, giving k_{obs} = (8.5 ± 0.4) × 10^{-5} s^{-1} (Figure A, Supporting Information).[18] Additional HMQC and HMBC experiments made the assignment of the majority of the (β-Ado)(α-CN)Cbl$^-$ ^{1}H NMR signals in the aromatic region possible (ppm): 8.34, B2; 8.25 and 8.18, A2 and A8; 7.49 and 7.40, B4 and B7; 6.42 (d), R1; 5.72 (d), A11; 5.65, C10.

The rate of conversion of (β-Ado)(α-CN)Cbl$^-$ to $(CN)_2$Cbl$^-$ in the presence of (TBA)CN could be considerably enhanced by exposing the solution to light, thus demonstrating that the intermediate contains a Co−C bond, prone to homolytic cleavage in the presence of light as is typical for adenosylcobinamides.[3] A ^{1}H NMR spectrum of a freshly prepared solution of AdoCbl in 5.0 × 10^{-2} M (TBA)CN which had been exposed to indirect sunlight for 1 h revealed that all cobalamin had been converted to $(CN)_2$Cbl$^-$ within this time. In the absence of light, the decomposition of the Ado ligand resulted in the Co−C heterolytic cleavage product adenine (and the cyanohydrin). However, in the presence of light some adenine and also additional Co−C homolytic products with ^{1}H NMR signals in the aromatic region were formed. (No attempt was made to determine the percentage heterolysis versus homolysis by identifying and quantitating these additional products from Ado composition in the presence of light.[19])

Alternatively, the formation of the (β-Ado)(α-CN)Cbl$^-$ intermediate and its subsequent conversion to $(CN)_2$Cbl$^-$ can be followed by visible spectroscopy. Figure 3a gives visible spectra before and ca. 20 s after mixing an AdoCbl solution (λ_{max} = 305, 337 ± 2 (s), 375, and 519 nm) with a (TBA)CN solution in a tandem (split-cell) cuvette (5.00 × 10^{-2} M (TBA)CN, 92% DMF/8% D_2O, I = 0.50 M ((TBA)ClO_4)). A substantial change occurs before the first spectrum is obtained after mixing, indicating that a reaction has already occurred, namely, the rapid formation of the (β-Ado)(α-CN)Cbl$^-$ intermediate (λ_{max} = 329, 392, 468, and 578 nm). The γ−band for the cobalamin moves to longer wavelengths (from 375 to 392 nm) as would be expected if the α-DMBI of AdoCbl was replaced by a cyanide ligand,[3] confirming evidence that an α-cyano species is formed. By following the visible spectrum of the reaction mixture over time, one can monitor the conversion of (β-Ado)(α-CN)Cbl$^-$ to $(CN)_2$Cbl$^-$ (and adenine and cyanohydrin) (see Figure 3b); λ_{max} for $(CN)_2$Cbl$^-$ = 314, 368, 420, 509 (s), 544, and 583 nm. The absorbance versus time data at a specific wavelength can be fitted to a first-order rate equation; for the experiment shown in Figure 3, k_{obs} = (8.86 ± 0.02) × 10^{-5} s^{-1} at λ = 625 nm (see Figure B, Supporting Information).[20] Note that this value is in excellent agreement with the ^{1}H NMR-determined value, (8.5 ± 0.4) × 10^{-5} s^{-1} (Figure A, Supporting Information).

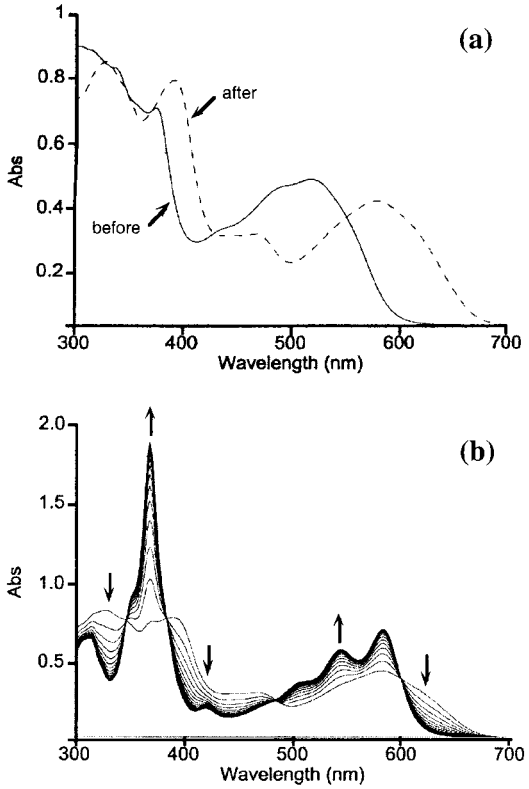


Figure 3. (a) Visible spectra measured before and directly after mixing (ca. 20 s later, t_{rel} = 0 min, tandem cuvette) AdoCbl with (TBA)CN (5.00 × 10^{-2} M (TBA)CN, 92% DMF/8% D_2O, I = 0.50 M ((TBA)ClO_4), 25.0 °C). (b) Selected visible spectra for the experiment shown in (a) at longer times (t_{rel} = 10, 60, 110, 160... min).

Experiments examining the rate of the reaction between AdoCbl and cyanide at different cyanide concentrations were carried out in order to deconvolute the observed rate constants. The observed rate constant for the reaction between AdoCbl and cyanide obtained from visible spectroscopy measurements was found to be independent of cyanide concentration for [CN^-] ≥ 2.00 × 10^{-2} M (Figure 4), but decreased with decreasing cyanide concentration for concentrations lower than this. Such curvature is typical of saturation kinetics, with a rate equation

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

where the equilibrium constant K corresponds to rapid formation of the (β-Ado)(α-CN)Cbl$^-$ intermediate prior to rate-determining cleavage of the Co−C bond of the intermediate. Fitting the data in Figure 4 to eq 1 gives k_2 = (9.3 ± 0.3) × 10^{-5} s^{-1} and K = (2.2 ± 0.4) × 10^2 M^{-1}.

The concentration dependence of the observed rate constant for the reaction between AdoCbl and cyanide was also examined by ^{1}H NMR spectroscopy. Importantly, AdoCbl and (β-Ado)-(α-CN)Cbl$^-$ are in fast exchange by ^{1}H NMR spectroscopy, since only 8, and not 16, signals were observed for a solution of ca. 1.4:1 (β-Ado)(α-CN)Cbl$^-$:AdoCbl.[21] The observed rate constant was found to be independent of CN^- concentration

(18) Identical results within experimental error were obtained by examining the increase of the peak area of one of the $(CN)_2$Cbl$^-$ signals (8.49 ppm) over time. It was felt, however, that rate constants obtained by following the disappearance of an intermediate signal would be more accurate than those obtained from following the appearance of a product, since the inevitably small signal-to-noise ratio due to the limited solubility of the cobalamin in the DMF/D_2O solvent mixture meant that more accurate and reliable data could be obtained over the first two half-lives of the reaction if the signal was decreasing rather than increasing. In addition, signals corresponding to the intermediate have an Abs_∞ = 0 and, therefore, have no associated experimental error with this value. This is not the case for product signals, however. Hence the reported rate constants have been obtained from following the decrease in the area of intermediate signals only.

(19) The aim of this experiment was simply to demonstrate that the intermediate contains a light-sensitive Co−C bond.

(20) (TBA)CN was found to decompose very slowly in DMF. It is suspected that this apparent decomposition actually arises from the reaction of an impurity in (TBA)CN reacting with DMF rather than (TBA)CN itself. Indeed, a signal at δ = 8.64 ppm was observed immediately by ^{1}H NMR spectroscopy after dissolving (TBA)CN in 92% DMF-d_7/D_2O (25.0 °C) The decomposition could be detected at wavelengths less than 600 nm by visible spectroscopy. All visible spectroscopy data were therefore analysed at λ > 600 nm.

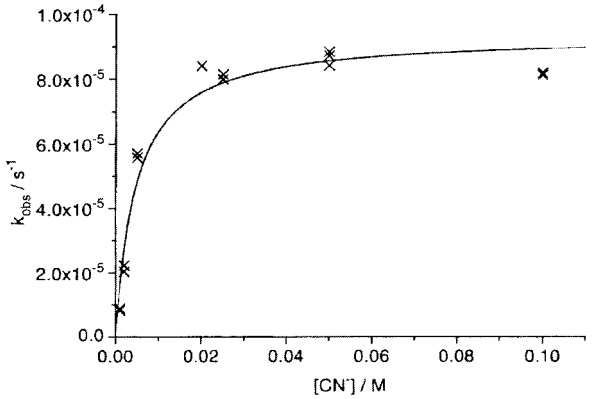


Figure 4. Plot of observed rate constant (k_{obs}) versus cyanide concentration for the reaction of AdoCbl with (TBA)CN (1.00×10^{-3}−0.100 M (TBA)CN, 92% DMF/8% D_2O, $I = 0.50$ M ((TBA)ClO_4), 25.0 °C). The solid line represents the best fit of the data to eq 1 in the text, giving $k_2 = (9.3 \pm 0.3) \times 10^{-5}$ s^{-1} and $K = (2.2 \pm 0.4) \times 10^2$ M^{-1}.

for $CN^- = 2.00 \times 10^{-2}$−0.120 M [22] (Table A, Supporting Information), $k_{obs} = (7.6 \pm 0.6) \times 10^{-5}$ s^{-1}. This is not unexpected, since under these conditions (i.e., $[CN^-] \geq 2.00 \times 10^{-2}$ M), only (β-Ado)(α-CN)Cbl^- is present, and $k_{obs} = k_2$ (eq 1). The value of k_2 obtained by ^1H NMR spectroscopy is in acceptable agreement (within 20%) with the k_2 value obtained by visible spectroscopy ($(9.3 \pm 0.3) \times 10^{-5}$ s^{-1}), especially if one keeps in mind the slight differences when comparing results in deuterated and unlabeled solvents (i.e., DMF-d_7 versus DMF). Note that it is likely that cyanide exists 100% in the CN^- form in 92% DMF/8% D_2O (i.e., no HCN), since control experiments which monitored the reaction between AdoCbl and (TBA)CN (5.00×10^{-2} M) in either 92% DMF/8% H_2O, 92% DMF/8% (0.0125 M $HClO_4$ in H_2O), or 92% DMF/8% (0.0125 M NaOH in H_2O) gave identical rate constants (visible spectroscopy) within experimental error ($k_{obs} = 7.92 \times 10^{-5}$, 8.09×10^{-5}, and 7.99×10^{-5} s^{-1}, respectively). If some of the cyanide had been present as HCN in 92% DMF/8% H_2O, then k_{obs} would have been expected to have become smaller or larger when H_2O was substituted by 0.0125 M $HClO_4$ or NaOH, respectively, since it has been shown that only CN^- (not HCN) can react with AdoCbl.[1] Importantly, this also means that k_2 must be acid-independent in the pH range studied.

Activation parameters for the cleavage of the Co−C bond of (β-Ado)(α-CN)Cbl^- were also determined. The temperature dependence of the reaction between AdoCbl and CN^- in 92% DMF/8% D_2O was therefore examined under conditions where the rate constant is independent of CN^-; that is, $k_{obs} = k_2$. $\Delta H^‡$ and $\Delta S^‡$ were found to be 90.0 ± 0.5 kJ mol^{-1} and -20.5 ± 0.2 J mol^{-1} K^{-1}, respectively.

A question which arises is does the present work, with its direct ^1H NMR spectroscopic detection of an (β-Ado)(α-CN)-Cbl^- intermediate using 92% DMF/8% D_2O, have any relevance to the AdoCbl plus cyanide mechanism in aqueous solution?

Table 1. Dependence of the Observed Rate Constant for the Reaction between AdoCbl and Cyanide on the Percentage of D_2O in DMF/D_2O Solvent Mixtures (5.00×10^{-2} M (TBA)CN, 25.0 °C)

% D_2O	$10^4 k_{obs}/s^{-1}$	"isosbestic pts"[24]/nm
4.0	0.33	349, 383, 475, 595
8.0	0.66	348, 384, 482, 598
15	1.5	347, 386, 486, 603
25	3.5	347, 386, 499, 607
50	5.9	347, 390, 528
100	5.9	347, 389, 531

In 92% DMF/8% D_2O cleavage of the Co−C bond of the α-cyano intermediate is the rate-determining step ($k_2 = 9.3 \times 10^{-5}$ s^{-1} (visible spectroscopy), 25.0 °C, $I = 0.50$ M ((TBA)-CN)); however in aqueous solution it was previously found that irreversible formation of the α-cyano intermediate was rate-determining ($k_1 = 0.57$ M^{-1} s^{-1}, 25.0 °C, $I = 1.0$ M ($NaClO_4$)[23]). To test this, we examined the dependence of the observed rate constant on the percentage of D_2O in the DMF/D_2O solvent mixture. From Table 1 it can be seen that the "isosbestic points"[24] change from four corresponding to the reaction of (β-CN)(α-Ado)Cbl^- and cyanide to give $(CN)_2Cbl^-$ at low amounts of D_2O to three corresponding to the direct conversion of AdoCbl and CN^- to give $(CN)_2Cbl^-$ at high ($\geq$50% D_2O) percentages of D_2O. Hence there is a changeover in the rate-determining step from rate-determining Co−C cleavage at the β site of the (β-Ado)(α-CN)Cbl^- intermediate to rate-determining substitution of CN^- at the α site of AdoCbl in solutions containing more D_2O than DMF.

From Table 1 it can also be seen that the rate of Co−C bond cleavage of the intermediate is more than two orders of magnitude smaller in 96% DMF/4% D_2O (3.3×10^{-5} s^{-1}) compared with 100% D_2O ($\geq 5.9 \times 10^{-3}$ s^{-1}; i.e., $\geq 10 k_{obs}$, since this step is not rate controlling in 100% D_2O). In addition, the rate constant for the reaction between AdoCbl and (TBA)CN (5.00×10^{-2} M) in 92% DMF/8% H_2O was approximately 10% larger (visible spectroscopy, four separate experiments gave $10^5 k_{obs} = 7.69$, 7.28, 7.66, and 7.65 s^{-1}, respectively) than the rate constant obtained in 92% DMF/8% D_2O (visible spectroscopy, four separate experiments gave $10^5 k_{obs} = 6.56$, 6.53, 6.64, and 6.76 s^{-1}, respectively). Both these results suggest that solvent is involved in the transition state preceding rate-determining cleavage of the Co−C bond.[25] A mechanism involving solvent-assisted heterolytic Co−C cleavage (and labilization of the Co−C bond by the nucleophile trans to the alkyl group) for alkylcobalamins in alkaline solution was recently proposed by Finke and co-workers.[26] An acid-independent path has also been found for Co−C heterolytic cleavage of alkylcobaloximes under mildly acidic conditions (pH 2.7−5.5) by Halpern and co-workers, although the authors propose that the mechanism involves protonation of the ribosyl oxygen rather than the solvent supplying the proton (no

(21) The ^1H NMR spectrum of a freshly prepared solution of AdoCbl (5.1×10^{-3} M) in ca. 9.3×10^{-3} M (TBA)CN (92% DMF-d_7/8% D_2O, 25.0 °C, $I = 0.50$ M ((TBA)ClO_4)) was recorded. Since $K = 2.2 \times 10^2$ M^{-1}, then under these conditions (β-Ado)(α-CN)Cbl^-/AdoCbl is ca. 1.4:1. The chemical shifts were found to be between those expected for AdoCbl and (β-Ado)(α-CN)Cbl^-; e.g., C10 5.84 (cf. C10(AdoCbl) = 6.14, C10((β-Ado)(α-CN)Cbl^-) = 5.65).

(22) It was not possible to carry out ^1H NMR experiments at $[CN^-] < 2.00 \times 10^{-2}$ M, since at least 2×10^{-3} M AdoCbl is required in solution to obtain an acceptable signal-to-noise ratio. CN^- concentrations were at least 10 times greater than this to ensure pseudo-first-order conditions.

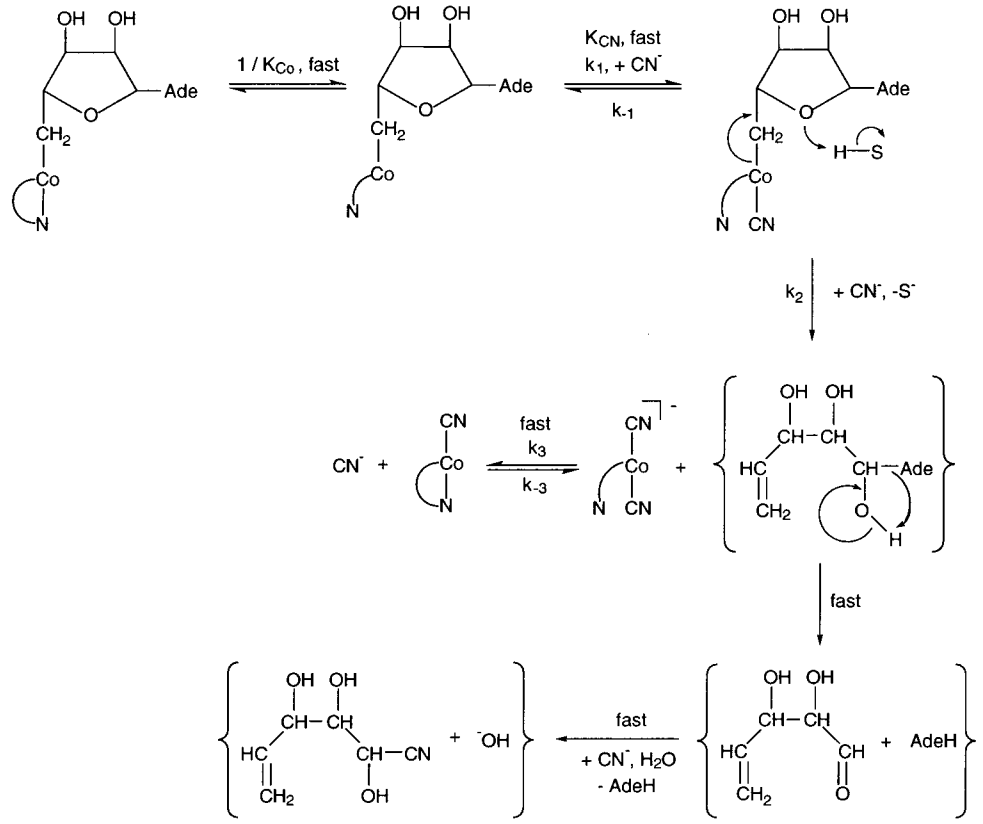
(23) (a) From ref 1, $k_1 = k(1 + K_{Co}) = 0.57$ M^{-1} s^{-1} using $K_{Co} = 76.6$ in H_2O, 25.0 °C.[23b] (b) Brown, K. L.; Hakimi, J. M.; Jacobsen, D. W. *J. Am. Chem. Soc.* **1984**, *106*, 7894.

(24) Upon close examination of the spectra it could be seen that the "isosbestic points" were not 100% clean due to the decomposition of (TBA)CN (or an impurity in (TBA)CN) in DMF.[20]

(25) The proton inventory technique[25b] can give information on the number of solvent molecules involved in the transition state and involves determining k_H/k_D for a wide range of H_2O (or D_2O):dipolar aprotic solvent ratios. The reaction between AdoCbl and CN^- is not ideal for such a study, however, since Co−C cleavage is only rate-determining for H_2O (or D_2O):DMF < 50%. (b) Espenson, J. H. In *Chemical Kinetics and Reaction Mechanisms*, 2nd ed.; McGraw-Hill: New York, 1995; p 219.

(26) Garr, C. D.; Sirovatka, J. M.; Finke, R. G. *J. Am. Chem. Soc.* **1996**, *118*, 11142.

Scheme 1



experiments were done to investigate this further).[27] Interestingly, an acid-independent path was not found for the heterolytic cleavage of AdoCbl in mildly acidic solutions (pH 2−5).[28]

The hereby modified[1] proposed mechanism for the reaction between AdoCbl and cyanide (in the absence of light) is given in Scheme 1. In 92% DMF/8% D_2O rapid formation of an α-cyano intermediate (K_{CN}) via a base-off AdoCbl ($1/K_{Co}$) is followed by rate-determining heterolytic Co−C cleavage (k_2); thus the equilibrium constant K, obtained by visible spectroscopy experiments, $= K_{CN}/K_{Co}$.[29] Addition of the second cyanide occurs after rate-determining Co−C cleavage and the β-ribosyl oxygen of AdoCbl is not protonated prior to Co−C cleavage, since k_2 was found to be independent of cyanide concentration and [H^+] concentration in the pH region studied, respectively. Rate-determining cleavage of the Co−C bond is proposed to be solvent-assisted, on the basis that k_2 decreases significantly as the dipolar aprotic component of the solvent mixture is increased, in addition to being larger (ca. 10%) when D_2O is replaced by H_2O.

The formation of (β-alkyl)(α-cyano)cobalamins from the reaction of alkylcobalamins with cyanide in aqueous solution has been reported in the literature;[2,30] however no values of K_{CN}/K_{Co} have been reported in other solvents. In their detailed mechanistic study of the reaction between cyanide and β-(oxocarbonyl)cobalamins (((methoxycarbonyl)methyl)cobalamin and (carboxymethyl)cobalamin), Jencks and co-workers found that fast, reversible formation of a (β-oxocarbonyl)(α-CN)cobalamin intermediate occurs prior to rate-determining heterolytic Co−C bond cleavage.[2] In addition, Jencks et al. demonstrated that stereospecific protonation occurs in experiments using labeled, chiral cobalamin reactants; thus protonation of the β-oxocarbonyl leaving groups occurs after the rate-determining step but prior to separation of the β group from the cobalamin.

It was found that only eight averaged ^1H NMR signals were observed in the aromatic region for a ca. 1.4:1 mixture of (β-Ado)(α-CN)Cbl^-/AdoCbl; that is, the two species are in fast exchange. This means that the observed rate constant, $k_{obs(1)}$, for the formation of the intermediate from AdoCbl and cyanide must be >100 s^{-1}. The observed rate constant $k_{obs(1)}$ for the same reaction in 100% D_2O is 5.9×10^{-4} s^{-1} ([(TBA)CN] $= 5.00 \times 10^{-2}$ M; see Table 1); therefore, in 92% DMF/8% D_2O the rate of cyanation is enhanced by over five orders of magnitude compared to that in D_2O. For biomolecular reactions involving small anions, rate enhancements of many orders of magnitude can occur when changing from a protic to a diprotic apolar solvent due to the increased activity of the anion (in this case, cyanide) as a result of its greatly reduced hydrogen-bonding interactions with the solvent.[31] Some of the rate enhancement may also be attributable to more AdoCbl existing in its base-off form in 92% DMF/8% D_2O.

There has been much discussion in the literature over factors which are important for Co−C heterolytic bond cleavage in alkylcobalamins, as opposed to those which lead to efficient Co−C homolytic bond cleavage. Our study confirms that the α-bonded ligand trans to the alkyl group plays a key role in Co−C heterolytic bond cleavage. In addition, whereas protonation of the ribosyl oxygen of AdoCbl is crucial prior to Co−C heterolytic cleavage in acidic solution,[12] in more alkaline conditions in the presence of cyanide the rate of Co−C heterolytic cleavage of the (β-Ado)(α-CN)Cbl^- intermediate is

(27) Jensen, M. P.; Zinkl, D. M.; Halpern, J. *Inorg. Chem.* **1999**, *38*, 2386.
(28) Jensen, M. P.; Halpern, J. *J. Am. Chem. Soc.* **1999**, *121*, 2181.
(29) K_{Co} would need to be determined in 92% DMF/8% D_2O to allow the calculation of K_{CN}. ($K_{Co} = 76.6$ in H_2O, 25.0 °C.[23b])
(30) (a) Nome, F.; Rezende, M. C.; Sabóia, C. M.; Clemente da Silva, A. *Can. J. Chem.* **1987**, *65*, 2095. (b) Brown, K. L. *J. Am. Chem. Soc.* **1987**, *109*, 2277.
(31) Parker, A. J. *Q. Rev., Chem. Soc.* **1962**, *16*, 163.

acid-independent and the solvent supplies the proton. The reason why trans-bound cyanide and imidazolate promote Co−C heterolysis would appear to be due to their strong σ electron donor abilities, leading to stabilization of the Co−C heterolytic cleavage transition state.[8,32] Kräutler has also proposed that a Co−C_α−C_β−O trans antiperiplanar conformation in the alkyl group is extremely favorable for Co−C heterolysis.[33]

To summarize, the reaction between AdoCbl and cyanide has been examined by ^{1}H and visible spectroscopies in 92% DMF/8% D_2O solutions and shown to involve a (β-Ado)(α-CN)Cbl$^-$ intermediate. Under these conditions Co−C bond cleavage of the α-cyano intermediate is the rate-determining step rather than addition of the first cyanide as found in aqueous solution. The kinetics of the reaction have been examined as a function of cyanide concentration by both ^{1}H and visible spectroscopies, and the latter shows saturation kinetics demonstrating that the formation of the α-cyano intermediate from AdoCbl and cyanide is a reversible process. Additional experiments have also been carried out which show that Co−C cleavage is solvent-assisted. There is a changeover in the rate-determining step when more D_2O is used in the DMF/D_2O solvent mixture, until for $D_2O \geq$ 50% addition of the first cyanide is rate-determining. These results, then, unify the mechanism of Co−C heterolytic cleavage of alkylcobalamins by cyanide (and presumably other nucleophiles such as imidazolates[8]), implicating both the trans effect and solvent-assisted Co−C heterolysis as important factors involved in the Co−C heterolytic cleavage process of alkylcobalamins in alkaline solution.

Experimental Section

(TBA)CN (96%) and 5′-deoxyadenosine (97%) were purchased from Aldrich, and (TBA)ClO_4 (≥99%) and adenine (≥99%) from Fluka. [*Caution: (TBA)CN is highly toxic*.] AdoCbl (98%) and CNCbl (99%) were obtained from Sigma. 8,5′-Anhydroadenosine was prepared by a modified published procedure,[34] in which 5 mol equiv of KCN was added and the pH adjusted to ca. 8.5, instead of the reported addition of both KCN and HCN. It was found unnecessary to wash the column with 1% HCN.

Visible spectra were measured using a Cary 1E spectrophotometer equipped with a thermostated cell compartment (25.0 ± 0.1 °C). ^{1}H NMR spectra were obtained on a Varian Inova 500 MHz spectrometer equipped with a 5 mm thermostated (25.0 ± 0.2 °C) probe. TSP was added as an internal reference.

(TBA)CN was stored under nitrogen and handled in a glovebag under positive nitrogen pressure. All solutions prepared for ^{1}H NMR measurements were made up in DMF-d_7. Unlabeled DMF was used for visible spectroscopy experiments. D_2O was used for both ^{1}H NMR and visible spectroscopy measurements unless explicitly stated otherwise. All solutions were filtered through a Millipore filter (0.45 μm).

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Supporting Information Available: A plot of ln(peak area, δ = 8.25 ppm) versus time corresponding to the experiment shown in Figure 2 (Figure A), plot of absorbance at 625 nm versus time for the experiment shown in Figure 3 (Figure B), and table giving observed rate constants for the reaction between AdoCbl and cyanide determined by ^{1}H NMR spectroscopy (Table A). This material is available free of charge via the Internet at htpp://pubs.acs.org.

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