Inorganica Chimica Acta, 46 (1980) 157–166

Cobalamin Ligation and Interaction with Sodium Dodecyl Sulfate and Bovine Serum Albumin Studied by ^{31}P and ^{13}C NMR

JAMES D. SATTERLEE

Department of Chemistry, Northern Illinois University, DeKalb, Ill. 60115, U.S.A.

Received January 26, 1980

Carbon-13 nuclear magnetic resonance has been employed to study the ligation modes of hydroxycobalamin by $K^{13}CN$ and $NaS^{13}CN$. The pH and temperature dependence of di(^{13}C)cyanocobalamin spectra reveal the presence of a kinetic trans *effect which labilizes the coordinated cyanides in this complex. The carbon spectra are utilized in conjunction with phosphorous-31 nmr of the phosphodiester in the benzimidazole side arm to establish correlations between the spectra and the coordination state of the cobalamin. It is found that this phosphorous resonance indicates the benzimidazole coordination state by virtue of its resonance position and serves as a probe of pH dependent properties of cobalamin. ^{31}P nmr is further demonstrated to be capable of reporting upon the coordination state of cobalamins in their interaction with the detergent sodium dodecyl sulfate and the cobalamin binding protein, bovine serum albumin, indicating its potential for reporting upon enzyme intermediates of vitamin B_{12} dependent enzymes.*

Introduction

Cobalamins are thought to serve as the active site prosthetic groups for the coenzyme B_{12} dependent enzymes and because of this much effort has been expended to define the chemistry of cobalamins, corrins and related cobalt complexes [1–6]. Studies on these model systems have been carried out for the purpose of discovering the kinetic, thermodynamic, electronic and spectroscopic properties and reactivities which characterize such complexes and the results [1–17] serve as a chemical foundation for interpreting enzyme data. Of particular interest in defining the enzymic mechanism for various B_{12} dependent enzymes is the nature of the mode of coenzyme association with specific proteins as well as the identification of coenzyme intermediates. These problems have been approached spectroscopically through the use of optical absorption data [18, 19], from kinetic studies [20, 21], and electron paramagnetic resonance data [22, 23].

One of the more powerful techniques for observing events at protein active sites is nuclear magnetic resonance (nmr) spectroscopy and despite its repeated application to cobalamins, corrins and coenzymes its potential for studying B_{12} dependent enzymes has not yet been realized [12–14, 24–30]. Although advances have been made in understanding the proton, carbon and fluorine nmr spectra of various B_{12} model systems no significant nmr study involving either native enzymes, or transporting proteins, such as the transcobalamins, has yet been published. It is likely that this is due, at least in part, to the large molecular weights [2] encountered for most coenzyme B_{12} enzymes. The commonly studied enzymes such as Diol dehydrase (molecular weight 250,000 Daltons), Ethanolamine ammonia-lyase (520,000) and Glycerol dehydrase (192,000) are so large that they preclude establishing solution concentrations necessary for carbon and even proton nmr. One means of circumventing this problem would be to study the coenzyme binding subunits of the dissociated proteins, but, while such information may prove interesting there is no guarantee that the data gathered would bear relevance to the active enzyme. Moreover, in the native resting enzyme diamagnetic cobalt(III) is the normal coenzyme oxidation state and as such proton resonances of the coenzyme are not expected to be observed outside the normal diamagnetic chemical shift range of 1 part per million (ppm) upfield to 12 ppm downfield from tetramethylsilane (tms), based upon results from model studies [24–28]. Since this is precisely the region in which the protein resonances absorb, such studies will undoubtedly be complicated by overlap with protein peaks and a general lack of resolution even for enzyme subunits with molecular weights as low as 40,000 Daltons.

As spectroscopic probes of the cobalamin active site, carbon and phosphorous nuclear magnetic resonance appear to offer several advantages. In the former the increased spectral resolution due to the wide chemical shift range (~250 ppm) and the ability to synthesize isotopically (^{13}C) enriched corrin macrocycles makes it an attractive technique and

advances in this direction are being made by Hogenkamp and Matwiyoff [12, 14]. Recent work from this laboratory [32] indicated that the phosphorous nucleus of the naturally occurring benzimidazole 'side arm' (Fig. 1) reflects changes in cobalamin ligation and oxidation states, thus suggesting the possibility of its use in protein studies. One important benefit of this reporter nucleus is that spectra will be free from the complicating protein background associated with carbon and proton nmr.

Work is presented here which further demonstrates the utility of ^{31}P and ^{13}C nmr in studies of cobalamins by extending previous carbon magnetic resonance studies to encompass some dynamic aspects of cobalamin ligation which depend upon temperature and pH. In addition, the phosphorous resonance is found to reflect changes in ligation caused by the pH dependent cobalamin interaction with sodium dodecyl sulfate (SDS), indicating its ability to report on at least one intermediate form (benzimidazole dissociated) suspected to occur during enzymic catalysis.

Experimental

Hydroxycobalamin and coenzyme B_{12} were purchased from Sigma. All sample manipulations were carried out under conditions of controlled lighting. Hydroxycobalamin samples were in general handled under reduced lighting whereas coenzyme B_{12} solutions were handled strictly in the dark. Samples of hydroxycobalamin were checked for purity by column chromatography on Biogel and the migration of a single band indicated a pure sample. Coenzyme B_{12} samples were used without further purification. $K^{13}CN$ and $NaS^{13}CN$, each 90 atom percent enriched, were purchased from Merck and KOCN and NaN_3 were obtained from J. T. Baker. All ligand salts and hydroxycobalamin were dried *in vacuo* at elevated temperatures prior to use.

The bis(ethylenediamine)cobalt(III) complexes, $Co(en_2XB)$, were prepared by literature methods [35, 36]. These complexes were isolated, dried *in vacuo* and analyzed: $Co(en_2SO_3{}^{13}CN)^0$, $C_5H_{16}N_5SO_3Co$, calcd. C 21.33, H 5.59, N 24.48; found C 21.06, H 5.65, N 24.41%; $Co(en_2Cl^{13}CN)Cl$, $C_5H_{16}N_5Cl_2Co$, calcd. C 22.03, H 5.78, N 25.28; found C 22.11, H 5.80, N 25.32%.

NMR spectra were recorded on various instruments. Carbon spectra were obtained on a Jeol PS100 spectrometer operating at 25.14 MHz. In general 500 to 50,000 transients were acquired employing 8191 data points taken over spectral widths of either 4000 or 6250 Hz. Phosphorous nmr spectra were obtained on an NTC 150 spectrometer operating at 60.44 MHz as well as a Jeol PS100 operating at 40 MHz. The NTC spectrometer was operated in the quadrature phase detection mode, while the Jeol spectrometer was operated in the normal pulse-Fourier transform mode. Variable temperature studies were carried out with ^{13}C and ^{31}P nuclei on the Jeol instrument employing the standard variable temperature controller. Temperatures were regulated to within ±1 °C over the required data accumulation time. Probe temperatures were calibrated with a copper-constantin thermocouple and were measured before and after each data accumulation. Sample pH's were measured before and after data accumulation, employing a Corning Digital 112 research pH meter and an Ingold pH electrode, and were found to be invariant in a given sample. Nmr samples were run in 99% D_2O with and without buffers present and the pH values reported in this work are the uncorrected meter readings.

Cobalamin association with Bovine Serum Albumin was carried out in accord with the procedures previously published [47]. BSA was purchased from Sigma and used without further purification. TRIS [tris(hydroxymethyl)aminomethane; Sigma] and MES [4-morpholineethane sulfonic acid; Aldrich] were the buffers of choice. The nmr solutions contained internal *p*-dioxane as a reference for carbon chemical shifts. The shifts were then referenced to external tetramethylsilane (tms) and reported as such in all Figures and Tables. Phosphorous chemical shifts are reported relative to external 85% phosphoric acid (pa).

Results and Discussion

Structural Aspects of Hydroxycobalamin Ligation

The structure of hydroxycobalamin indicated from crystallography [3, 33] and the abbreviations for it which are employed in this text are illustrated in Fig. 1. Cobalamins offer two potential axial coordination positions; one is occupied by –OH in hydroxycobalamin, H_2O in aquocobalamin and a ribonucleotidyl group in coenzyme B_{12}, while the other is occupied by the benzymidazole moiety of the phosphodiester containing 'side arm'. The former is often referred to as the 'upper' coordination position, the latter as the 'lower' coordination position. Two conformations are accessible to the side arm. The one in which the benzimidazole remains coordinated to cobalt is termed 'base-on' or 'arm-on' while dissociation of the benzimidazole is labelled 'base-off' or 'arm-off'.

The process of benzimidazole dissociation appears to be important in the operation of several coenzyme B_{12} dependent enzymes. Several results [6] indicate that the side arm is required for proper enzymic activity and suggestions have even been made that base dissociation plays an integral role in the catalytic

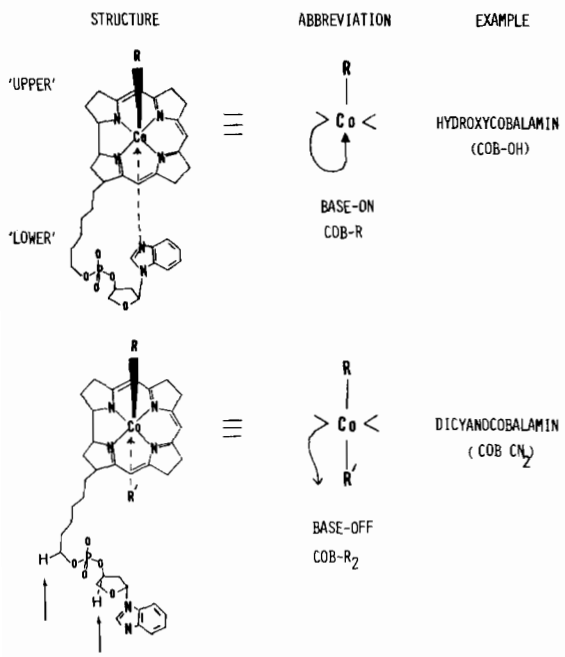


Fig. 1. The two forms of cobalamin ligation are demonstrated in this figure. The top figure, with benzimidazole coordinated, is typical of hydroxycobalamin(R=OH), aquocobalamin ($R=H_2O$), or Vitamin B_{12} (R=CN). This also illustrates the difference between "upper" and "lower" coordination sites. In the bottom figure the benzimidazole has been displaced (by R'), showing the base-off configuration. The two arrows mark the protons which couple to the phosphorous nucleus. The abbreviations shown here will be used throughout the text.

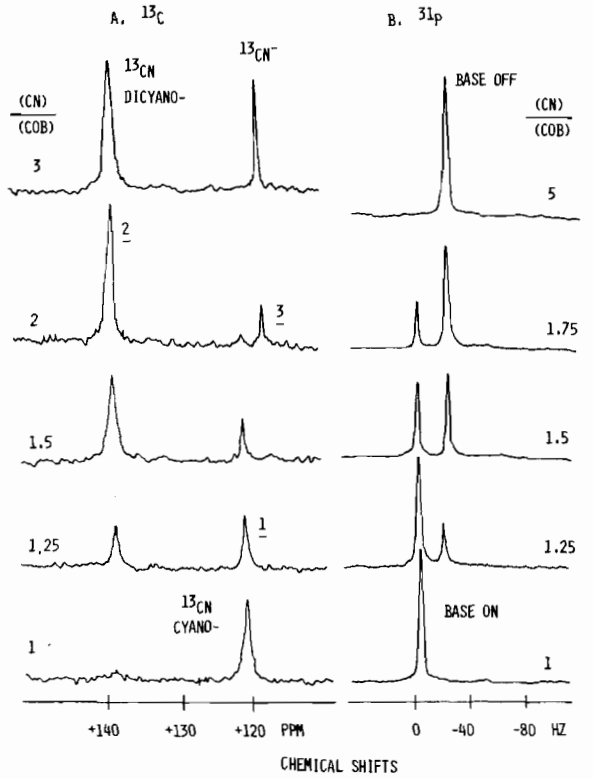


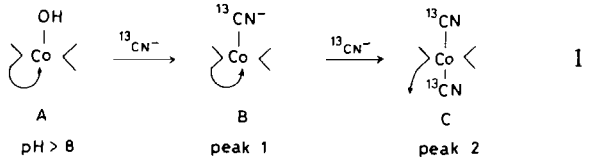
Fig. 2 A. The C-13 spectrum of hydroxycobalamin solutions (0.10 *M*) containing increasing amounts (bottom to top) of $K^{13}CN$. It is obvious that as the amount of cyanide increases a sequential appearance of peaks occurs. Peak 1 is due to cyanocobalamin, peak 2, which appears at concentration ratios greater than 1:1 is assignable to dicyanocobalamin and peak 3 to free cyanide ion in solution. B. A corresponding P-31 nmr spectrum showing the gradual transformation from the base-on to the base-off forms with increasing cyanide ion concentration. These spectra are from unbuffered solutions at pH 9.0.

mechanism. Moreover, the generally accepted view of the catalytic mechanism of some B_{12} enzymes invokes axial ligand exchange [3, 4, 6].

Carbon Resonance Assignments in the Cyanide Complexes

The carbon nmr spectrum of (^{13}C) dicyanocobalamin [Cob-($^{13}CN)_2$] has been previously reported [12, 14], although no mention has been made of the (^{13}C)cyanocobalamin, (Cob-^{13}CN; vitamin B_{12}), nor of the sequence of ligation events encountered during formation of the bis ligand complexes. By varying the relative solution concentration ratios of $K^{13}CN$ and hydroxycobalamin at pH 9.0 it has been possible to unambiguously make resonance assignments in both the carbon and phosphorous spectra of these compounds as shown in Fig. 2. In the absence of $K^{13}CN$ the carbon spectrum (Fig. 2A) consists only of peaks attributable to the cobalamin and the internal reference, *p*-dioxane. This is not shown in Fig. 2A because these resonances are at natural abundance and have such low intensity that they are not complicating features when $^{13}CN^-$ is employed. Increasing the $K^{13}CN$ concentration relative to that of hydroxycobalamin results in the sequential appearance of three peaks, labelled 1, 2, 3 in Fig. 2A. The first peak to appear, peak 1, displays increasing intensity up to a $K^{13}CN$: cobalamin ratio of 1:1 and thereafter decreases in intensity with increasing $K^{13}CN$ concentration. Simultaneously with peak 1's decrease is the increasing intensity of peak 2. At a $K^{13}CN$: cobalamin ratio of 1.5:1 the relative areas of peaks 1 and 2 are 1:2 indicating that the peaks 1 intensity is attributable to one half the nuclei compared to peak 2. At the cyanide to cobalamin ratio of 2:1 peak 1 has disappeared completely leaving peak 2. In spectra at even higher cyanide concentrations a third peak (3) grows in intensity relative to the now constant intensity of peak 2.

This pattern of behavior is consistent with the following ligation process caused by titrating hydroxycobalamin solutions with $K^{13}CN$ (Equation 1). In this process hydroxycobalamin (A) is abbreviated



as indicated in Fig. 1. Accordingly, peak 1 is assigned to the monocyano complex (^{13}C)cyanocobalamin, vitamin B_{12} (B) peak 2 comes from the dicyano complex (^{13}C)dicyanocobalamin (C) and peak 3 originated from excess $K^{13}CN$ alone. The identity of peaks one and two as originating from cobalt coordinated cyanide is further indicated by their larger linewidths relative to free $^{13}CN^-$, which is obvious in Fig. 2 and has been reported by Matwiyoff *et al.* [12] as well. This increased linewidth is due to the broadening effect caused by the rapid quadrupolar relaxation of the cobalt ion ($S = 7/2$; electric quadruple moment 0.5×10^{-24} cm^2) among its nuclear spin levels and is a characteristic of spin 1/2 carbon nucleus bound to quadrupolar nuclei [34].

The assignment of peak 2 as the dicyano complex is indicated not only by its larger intensity relative to peak 1, but by the dramatic trans effect it exhibits. When a second cyanide ion is added to the cobalt primary coordination sphere (process B → C, Eqn. 1) the result is that the coordinated ^{13}CN resonance no longer possesses benzimidazole as its *trans* ligand, but each coordinated cyanide possesses the other as a *trans* ligand. The result of replacing benizimidazole by the $^{13}CN^-$ group is to shift the coordinated cyanide resonance downfield. This can be understood solely on the basis of the much greater electron withdrawing inductive effect of a *trans* cyanide resulting in deshielding the oppositive carbon nucleus. The result, as expected, is that the dicyano complex exhibits a single resonance shifted downfield from the positions of both free $^{13}CN^-$ (or $K^{13}CN$) and the monocyano complex. A single resonance is observed for the dicyano complex due to the magnetically similar environments of both cyanide coordination positions and the lowered spectral resolution caused by the cyanide linewidth, as previously discussed [12].

Phosphorous Resonance Assignments in the Cyanide Complexes

In the fully proton decoupled ^{31}P nmr spectrum of hydroxycobalamin, aquocobalamin and coenzyme B_{12}, single phosphorous resonances from each compound are observed. These resonances originate from the phosphodiester moiety in the benzimidazole containing side arm (Fig. 2B, Table I). Considering the cyanide ligation process depicted in eqn. 1 only the addition of the second cyanide ion (process B → C) should be expected to affect the ^{31}P nmr spectrum because it is only this process which affects the phosphodiester environment. The transition from base-on to base-off forms proceeds smoothly with increasing ratios of $K^{13}CN$:cobalamin as shown in Fig. 2B, where the resonance at −6 Hz is due to the base-on form, while the resonance which appears in solutions of the dicyano complex and is attributable to the base-off form appears at −30 Hz.

TABLE I. The Carbon-13 and Phosphorous-31 Chemical Shifts for Many Cobalamin Complexes.

Compound[a]	pH[b]	Chemical Shift[c]	
		^{13}C (PPM)	^{31}P (Hz)
Cob–OH	9.0		−6.1
Cob–OH	5.0		+2.3
Cob–*CN	9.0	+121.7	−5.1
Cob–*CN_2	9.0	+138.6	−29.9
Cob–CN + SDS	7.5	+120.2	−5.3
	1.5	+107.4	−30.1
Cob–S*CN	7.0	+136.0	−6.0
Cob–N_3	9.0		−4.9
Cob–Bzm	9.0		−6.1
Cob–Im	9.0		−6.0
Cob–OCN	9.0		−5.5
Vit B_{12}	9.0	+121.7	
Coen–B_{12} + excess CN^-	9.0	+139.1	−39.1
($Coen_2Cl$*CN)	9.0	+144.3	
($Coen_2SO_3$*CN)	9.0	+159.1	

[a]Abbreviations employed are: Cob = cobalamin; SDS = sodium dodecyl sulfate; Bzm = benzimidazole; Im = imidazole. [b]PH values reported are the meter reading of the deuterium oxide solutions. [c]Chemical shifts are reported in parts per million (ppm) downfield (+) from external TMS for carbon and Hz upfield (−) and downfield (+) from external 85% phosphoric acid for phosphorous. Solution concentrations in this study ranged from 0.07 *M* to 0.005 *M*, although for the complexes reported in this table all data were uniformly obtained at cobalamin concentrations of 0.010 *M*.

The phosphorous resonance of cobalamins, by virtue of its shift position, is capable of reporting the benzimidazole coordination state in cobalamins. Its potential usefulness in protein studies is enhanced by the finding that, comparing proton coupled versus fully proton decoupled phosphorous spectra, a nuclear Overhauser enhancement of 0.5 occurs upon proton decoupling, indicating that mechanisms other than dipole–dipole coupling contribute to the phosphorous relaxation, thus reducing the *enhancement* from the theoretical value of 1.24. This decoupled resonance will display an intensity 1.5 times the magnitude of the coupled spectrum. In reality the practical advantage of this enhancement in terms of accumulating data is much greater, because, whereas the proton decoupled spectrum is a single sharp signal, the coupled spectrum is a triplet (*c.f.* Fig. 5) which means its intensity is distributed over three peaks. In so far as can be ascertained this triplet is

first order, implying that the two protons to which phosphorous is coupled (see arrows, Fig. 1) exhibit magnetic equivalence despite their structural dissimilarity. From the coupled spectrum of variously ligated cobalamins the coupling constant J_{P-H} is determined to be 7.5 ± 0.1 Hz (Table I) and is independent of the benzimidazole coordination state as well as the *trans* ligand.

Carbon and Phosphorous Spectral pH Dependencies

Two pH dependent processes affecting the axial ligation behavior of cobalamins can be detected by nmr over the biochemically relevant range of pH 10 to pH 4. The first of these is quite dramatic and involves dicyanocobalamin. The nmr spectra presented in Fig. 3 reveal this process to be the pH dependent replacement of one of the two $^{13}CN^-$ ligands in this complex by benzimidazole, the reverse

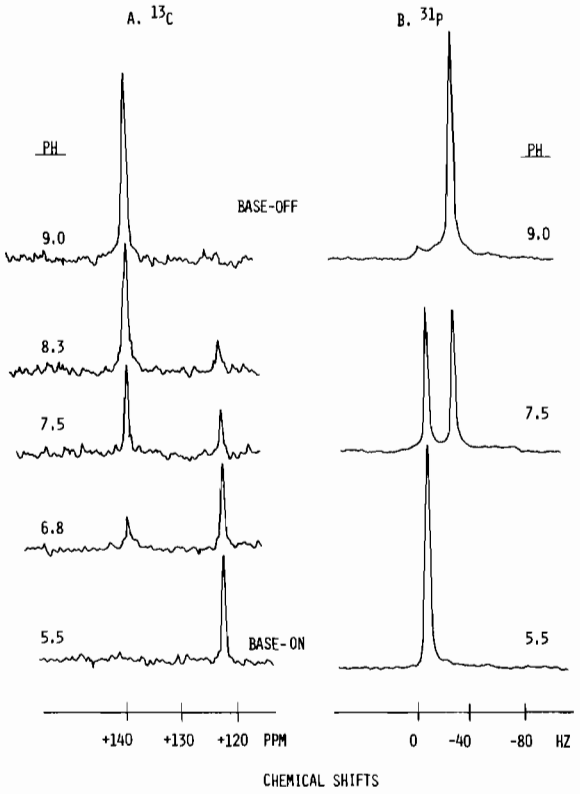


Fig. 3. PH dependence of the carbon and phosphorous spectra of dicyanocobalamin. The variation in cyanide ligation is shown fully in the C-13 spectra (A). In this case the peak characteristic of the dicyanocomplex (+138 ppm) decreases in intensity while a peak at the appropriate shift for the monocyanocomplex (+122 ppm) increases in intensity, with decreasing pH. The replacement of a single coordinated cyanide is corroborated by the change of the P-31 spectrum from the base-off form to the base-on form at lower pH, indicating the favored coordination of benzimidazole at lower pH.

process (C → B) depicted in eqn. 1. In detail, Fig. 3A shows that at pH's between 12 and 8 ^{13}C nmr reveals the presence of only a resonance attributable to (^{13}C)dicyanocobalamin. Over this same region the phosphorous nmr (proton decoupled, Fig. 3B) reveals a resonance characteristic of dissociated benzimidazole (base-off form). However, titrating from pH 8 to lower pH leads to the progressive disappearance of the dicyano ^{13}CN resonance and the simultaneous appearance of a ^{13}CN resonance at 121 ppm, which is characteristic of (^{13}C)cyanocobalamin. Accompanying these changes in the carbon spectrum were changes in the phosphorous spectrum in which the ^{31}P resonance characteristic of the base-off form lost intensity and was replaced by a ^{31}P resonance characteristic of the base-on form. Below pH 7, down to pH 4 no further pH dependent shifts could be detected in either the phosphorous, or carbon spectrum. Repeated study has shown that this reaction is reversible.

A molecular view of the process most consistent with the data at hand is that one of the coordinated cyanide ligands is titrated off the cobalt ion with a pK of approximately 7.5. This data indicates a pH dependent labilization of coordinated cyanide, but whether both cyanides become increasingly labile and only the proximity of a nearby nucleophile (the benzimidazole) which can effectively compete for a cobalt coordination position results in replacement of one of the cyanides is unknown at this time, although this is considered likely. Work is currently in progress to determine this point unambiguously, but several results which have appeared in print are relevant to this process.

Consider the formation of dicyanocobalamin from vitamin B_{12} (process B → C, eqn. 1). The formation constant for this process (10^4) [11], which is the substitution of coordinated benzimidazole by cyanide has been shown to be small in comparison to the following reactions of a closely related cobalamin model system [1] in which benzimidazole is not a nucleophile competing for the same coordination position as cyanide ion. In eqns. 2 and 3 it is cobalt

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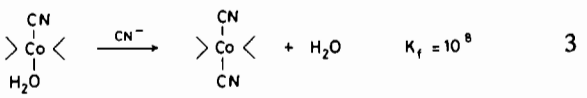

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corrinoids, rather than cobalamins, which are undergoing reaction and, so, a cautious approach to interpretation is understood, but what this data, together with the observations contained herein, seems to indicate is that there are at least two effects which modify axial ligand reactivity in these systems. One is

that the presence of an external nucleophile such as benzimidazole may lower the stability of the dicyano complex significantly. Another is that, other conditions being equal, coordinated cyanide exerts a significant *trans* effect toward coordination of another cyanide, as witnessed by the large decrease in the formation constant of the process in eqn. 3 relative to that in eqn. 2.

Pratt [11] has pointed out that a kinetic *trans* effect may be a consequence of the thermodynamic *trans* effect observed in corrins and cobalamins. Moreover, the order of ligand replacement reactions should increase as the *trans* ligand of a complex is changed from benzimidazole to cyanide. To test whether such lability of the Co–^{13}CN bond exists in cyanocobalamins the temperature dependent linebroadening of both ^{13}CN and ^{31}P resonances were determined at 25° and 95 °C for both (^{13}C)vitamin B_{12} and (^{13}C)-dicyanocobalamin. This was carried out at pH 10 to minimize any pH dependent contribution to ligand exchange. The results from the carbon spectrum show a linewidth for coordinated ^{13}CN which is 50 Hz larger at 95° compared to 25 °C. For the vitamin a broadening of less than 10 Hz at 95 °C was detected. The difference noted for two such similar compounds suggests that the dynamic line broadening is due to cyanide exchange between free (in solution) and coordinated sites. Assuming chemical exchange broadening as its source, this linewidth difference leads to a cyanide dissociation rate of 160 sec^{-1} at 95 °C for dicyanocobalamin, suggests a kinetic *trans* effect is present in these complexes, and supports the previous suggestion that cyanide is a better *trans* effector than benzimidazole [1].

What then is the mechanism of the pH dependent cyanide substitution? Based upon a previous kinetic study [42] a reasonable proposal, which is consistent with all of the experimental data presented so far is as follows. Formation of the dicyano complex results in kinetic labilization of the axial cyanides relative to the monocyanide. This leads to a small, but apparently non-zero, concentration of free cyanide in solution. At pH values above pH 8 the dissociated cyanides, which may presumably originate either from the upper or lower cobalamin coordination positions, exist in solution as CN^- with vanishingly small amounts of HCN, the hydrolysis product, present. Because the overall formation constants (eqns. 1, 2, 3) are so large no ligand replacement of cyanide by water occurs. However, below pH 8 significant hydrolysis of the dissociated cyanide ion occurs such that competition for the cobalt ligation site is between benzimidazole and HCN on the lower side and H_2O and HCN on the upper. Although HCN is well known to be less reactive towards cobalt ligation than cyanide ion [42], it must compete quite favorably compared to water, leaving cyanide coordinated in the upper cobalamin position. However, in the lower position HCN apparently does not compete effectively with benzimidazole, thus allowing ligand replacement of cyanide by benzimidazole in the lower cobalamin position.

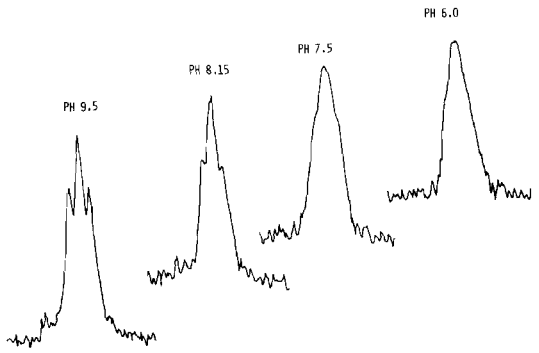
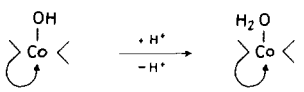


Fig. 4. PH dependence of the hydrogen–phosphorous coupling as detected, in this figure, by observation of the proton coupled P-31 resonance of the phosphodiester moiety. The coupling is lost simultaneously with the peak experiencing broadening. Qualitatively the pK of 7.5 estimated from this type of data agrees well with the pK observed (7.65) for the conversion of hydroxycobalamin to aquocobalamin.

The second pH dependent process which is detectable by ^{31}P nmr is shown in Fig. 4. Here, the well defined triplet in the proton coupled spectrum of hydroxycobalamin is seen to become less well resolved as the pH is decreased. This effect is observed only for hydroxycobalamin, not for either the vitamin or dicyanocobalamin and is attributable to broadening of the phosphorous resonance. That this affect is observed only for hydroxycobalamin and not for the other forms suggests that the dynamic linebroadening is not due to protonation of the phosphodiester moiety, nor of any other functional group in the vicinity of the phosphorous nucleus because this should cause the loss of resolution similarly in the two cyanide ligated forms. Since this is not observed it suggests that the process that phosphorous is detecting is the protonation of coordinated hydroxide. This process (eqn. 4) results in the replacement of the inert hydroxide ligand with the more labile water molecule. It seems likely that the



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triplet collapse is caused by the equilibrium between the hydroxo and aquo complex and the observed pK of 7.65 [1] for this process agrees well with the disappearance of the triplet structure. The only other process capable of effecting such line broadening could be the protonation of the phosphodiester, but that process occurs at pH values near 1.

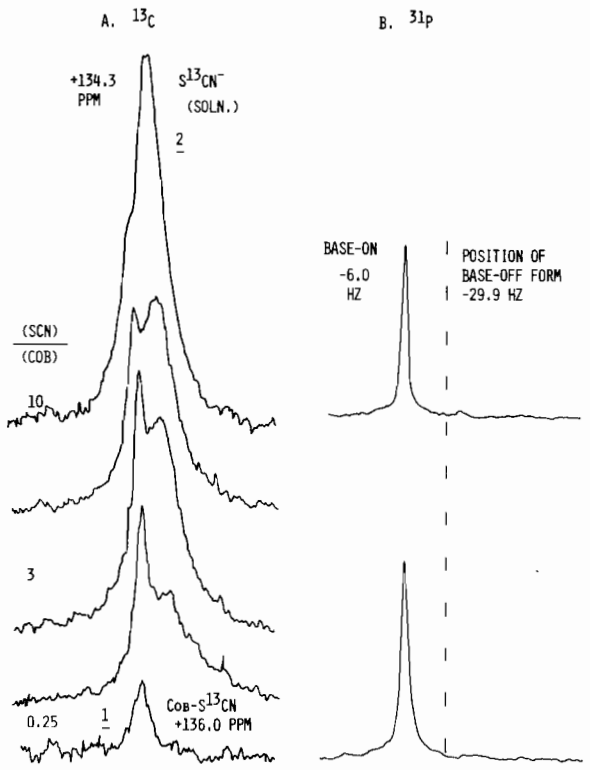

Fig. 5. Carbon-13 (A) and phosphorous-31 (B) spectra of the ligation of hydroxycobalamin (aquocobalamin) by $KS^{13}CN$. In the carbon spectrum only a single coordinated thiocyanate peak is detected (1) and as the concentration of thiocyanate increases (bottom to top) the only other detectable resonance is attributable to free thiocyanate in solution. The phosphorous resonance confirms the presence of only the monothiocyanato complex by revealing only a base-on P-31 spectrum even at very high thiocyanate ion concentrations.

Carbon and Phosphorous NMR of $S^{13}CN^-$ Complexes

The effect of increasing the concentration of $NaS^{13}CN$ relative to hydroxycobalamin on the carbon spectrum is shown in Fig. 5. In solutions at pH 7.5 increasing the $NaS^{13}CN$:hydroxycobalamin ratio results first in increasing area for peak 1, which reaches a maximum. At $NaS^{13}CN$:cobalamin ratios greater than 1:1 a second peak (peak 2) increases in intensity as additional thiocyanate is added to the solution. Throughout this process the phosphorous spectrum exhibits a chemical shift (proton decoupled) 6 Hz upfield from phosphoric acid, indicating that no base-off form is present. Both the carbon and phosphorous spectra concur that only the monothiocyanatocobalamin is formed at room temperature as well as at all temperatures down to +5 °C.

Such behavior, although dissimilar to cyanide ligation, is expected on the basis of the small formation constant for dithiocyanatocorrins [20] and the fact that even at mole ratios of thiocyanate in ten fold excess of cobalamin only monothiocyanatocobalamin could be crystallized [44].

Cobalamin Interaction with Other Ligands

The data presented in Table I show that besides thiocyanate several potential ligands which have been shown to interact with hydroxocobalamin do not form bis complexes. Neither imidazole, benzimidazole, cyanate, nor azide ions affect the base-on form of hydroxocobalamin at pH 9 as detected by ^{31}P nmr.

Spectroscopic trans *Effect in the NMR of Coordinated Cyanide*

Spectroscopic *trans* effects have been detected in cobalamins, corrins and cyano–cobalt complexes from studies of the cyanide infrared stretching frequencies [1]. Although the work presented here does not comprise a thorough, exhaustive, systematic study, four complexes presented in Table I serve to demonstrate the existence of a spectroscopic *trans* effect which influences the chemical shift of coordinated cyanide. In the case of vitamin B_{12} where benzimidazole is the ligand *trans* to cyanide, compared to dicyanocobalamin, the chemical shift of coordinated cyanide shifts 17 ppm downfield upon substitution of cyanide for benzimidazole. This shift is 14.3 ppm upfield when the benzimidazole is replaced by a water molecule (or remains without a *trans* ligand) such as at pH 1.5 in the presence of the detergent SDS (*vide infra*). Similarly, in two other cobalt complexes, the *trans* substituted cyano-bis-ethylenediamines, $[Coen_2{}^{13}CN(SO_3)]^0$ and $[Coen_2{}^{13}CN(Cl)]^+$, the cyanide chemical shift moves downfield when chloride ion is replaced by sulfite ion.

Even though the data presented here are not complete they do indicate the presence of an nmr detectable *trans* effect similar to that which has already been observed for several cobinamides and methyl-cobalamins [12, 14], and which parallels infrared observations of similar cobalt complexes [45, 46]. The limited observations presented here indicate that the spectroscopic *trans* effect observed in the carbon nmr spectrum follows the pattern that the more electron withdrawing the *trans* substituent is, the further downfield the observed resonance lies.

Cobalamin Interaction with Sodium Dodecyl Sulfate (SDS) and Bovine Serum Albumin (BSA)

SDS and BSA represent two systems which are known through previous work to interact with cobalamin derivatives. The influence of SDS on hydroxo- and cyanocobalamin is to raise the pK for benzimidazole dissociation from the cobalt ion. In detergent free solutions of aquocobalamin the pK for this process is approximately zero. In the presence of SDS Beckmann and Brown [41] observed that for various cobalamins the pK for benzimidazole

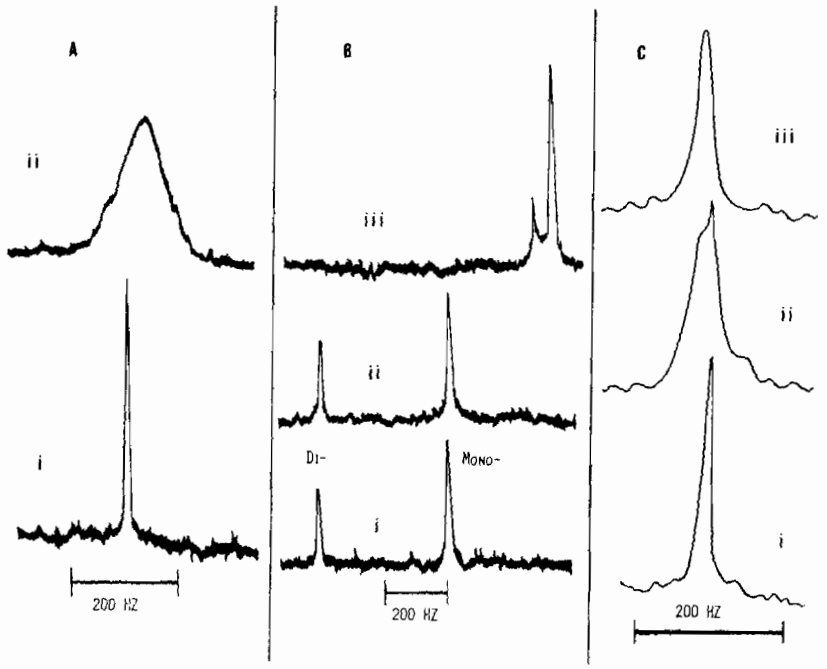

Fig. 6. (A) ^{31}P nmr spectra of hydroxycobalamin in the presence of 0.50 *M* SDS at (i) pH = 8.5; (ii) pH = 1.5. (B) ^{13}C nmr spectra of dicyano- and cyanocobalamin in the presence of 0.50 *M* SDS: (i) prior to adding SDS; (ii) SDS added, pH = 8.5; (iii) SDS added, pH = 1.5. (C) ^{31}P nmr spectra of hydroxycobalamin titrated with bovine serum albumin: (i) CobOH–no BSA, pH = 7.4; (ii) CobOH:BSA concentration is 1.0:0.5; (iii) CobOH:BSA concentration ratio is 1.0:1.2.

dissociation was raised to between pH 2 and 3. For cyanocobalamin this pK shifts to pH 3.2 when the SDS concentration is above the critical micellar level. This is probably the result of the base-off form's larger association constant (10^3 larger) with the aqueous micelles. That observation suggests that the specific micelle-cobalamin interaction is through the lower side of the cobalamin, or with the dissociated, protonated benzimidazole.

The process just described is detectable both by phosphorous and carbon nmr as shown in Fig. 6. In Fig. 6A the effect on the ^{31}P resonance of a Cob–^{13}CN solution containing 0.5 *M* SDS. Between pH 8.5 and 4.6 only a proton decoupled ^{31}P resonance due to base-on cobalamin is observed. Below pH 4.6 this resonance broadens and shifts position. By pH 1.5 the spectrum consists of a broad resonance centered at the position of base-off cobalamin (–30 Hz) with a width at half maximum height of approximately 70 Hz.

In Fig. 6B the effect caused by altering the pH of a solution containing both Cob–^{13}CN and Cob–$(^{13}CN)_2$ as well as 0.5 *M* SDS is demonstrated. Upon the addition of SDS to an aqueous solution of the two cobalamins at pH 8.5 the cyanide chemical shifts of both cobalamin species move upfield. For the dicyanocobalamin the shift is 14 Hz while for the cyanocobalamin the shift is 37 Hz. Upon lowering the pH the dicyano peak gradually disappears paralleled by a simultaneous increase of intensity in the cyano resonance. Throughout this process both resonances shift upfield until, a pH 1.5, the spectrum reveals only two resonances. The major peak has shifted 360 Hz (14.3 ppm) upfield as a result of the loss of benzimidazole as a *trans* ligand. The minor peak was determined to be free cyanide ion in solution because further addition of hydroxocobalamin caused it to disappear along with increasing the intensity of the major peak.

The most important conclusion which may be drawn from these results is that in this model system for protein–cobalamin association the ^{31}P nmr resonance has proven that it can detect the base-on and base-off interconversion of a cobalamin. It is, therefore, capable of differentiating at least one potential enzyme intermediate state.

The association of cyanocobalamin with SDS represents one manner of association available for cobalamins with macromolecules; association through the lower, or benzimidazole end of the molecule. A completely different mode of association was inferred from optical data for the interaction of cobalamins with BSA [47]. The suggested mode of cobalamin binding to BSA is through histidine coordination to the cobalt in the upper position, *trans* to coordinated benzimidazole. We have confirmed that cobalamin binding to BSA does not radically alter the ^{31}P nmr spectrum, indicating that the lower part of the

cobalamin molecule does not participate in binding, although BSA bound cobalamin does exhibit a phosphorous resonance shifted 5 Hz closer to phosphoric acid (*i.e.* downfield). In Fig. 6C proceeding from the lower trace (i) to the upper trace (iii) demonstrates the effect upon the phosphorous spectrum of adding BSA to a hydroxocobalamin solution maintained at pH 7.4 with TRIS buffer. After each addition of BSA the solution was incubated at one hour at 37 °C. The only change reflected in these spectra is that the resonance at −6.1 Hz loses intensity while a peak at −1.0 Hz gains intensity and becomes the sole peak in the spectrum at BSA:cobalamin ratios above 1.0. This peak may thus be assigned to the cobalamin–BSA complex. The resonance of this complex is broadened by only 5 Hz relative to the uncomplexed species, has a chemical shift indicative of the base on form and indicates that the benzimidazole side arm is not intimately involved in cobalamin–BSA binding.

Summary of Conclusions

Hydroxocobalamin ligation has been studied by ^{31}P and ^{13}C nmr as a means of defining the ligation processes available to this molecule and as a model for interpreting data from studies incorporating macromolecules. This technique offers an advantage over optical spectroscopy for detecting changes in cobalamin ligation, namely, observation of the phosphodiester resonance indicates *by direct observation* the cobalt's ligation state. Besides establishing spectroscopic correlations derived from direct observation of ligand binding a unique pH dependence of axial ligation has been observed. The labilization of coordinated cyanide is interpreted as evidence of a dynamic *trans* effect in dicyanocobalamin.

Ultimately the goal of this research is to employ the phosphodiester resonance to study vitamin B_{12}–enzyme–inhibitor complexes and as a model for such studies this work reveals that phosphorous nmr is capable of determining the mode of cobalamin interaction with sodium dodecyl sulfate micelles and bovine serum albumin. This work demonstrates that the phosphodiester resonance as well as the cyanide carbon resonance can be of utility in axial ligation assignments of trapped enzyme intermediates which are of relevance for interpreting the catalytic mechanism of B_{12} dependent enzymes.

Acknowledgements

The author wishes to thank the donors of the Petroleum Research Fund, administered by the American Chemical Society, the Research Corporation and the Northern Illinois University Graduate School for their support of this research. This investigation was also supported, in part, by the National Institutes of Health Research Grant No. RR 01077 from the division of Research Resources. The author wishes to further thank the staff of the Purdue University Biological Magnetic Resonance Laboratory for their professional hospitality.

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