^{31}P NMR Observation of Phosphodiester Protonation of the Nucleotide Loop of Methylcobalamin

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Introduction

Recently Satterlee [1, 2] and Mishra and coworkers [3] have reported studies of the intrinsic ^{31}P nuclear magnetic resonance of cobalamins due to the phosphorus atom of the nucleotide loop. We became interested in this work because of the reported [1, 2] upfield shift of the phosphorus resonance of cyanocobalamin upon displacement of the axial ligand by cyanide (*i.e.*, formation of dicyanocobalamin). We hoped to be able to use the ^{31}P chemical shift of methylcobalamin as a probe for displacement of the axial benzimidazole by exogenous ligands, an equilibrium difficult to observe by uv-visible spectroscopy [4, 5]. To our surprise such a shift is absent in methylcobalamin but its ^{31}P-resonance does show a pH-dependence due to protonation of the phosphordiester moiety as described below.

Experimental

^{31}P-NMR spectra were obtained at 25 ± 1 °C on a Nicolet NT200 wide-bore superconducting spectrometer operating at 4.7 T (observe frequency 80.988 MHz). 8192 data points were collected over a 400 Hz sweep width. Generally 200–2000 transients were collected on samples 2 mM in cobalamins with 7.5 μ sec 30° pulses and a cycle time of 1.02 seconds. Proton decoupled spectra were obtained by irradiation of samples with a low level (0.2–0.4 watts) noise-modulated R_f field centered at 200.067 MHz which caused no change in chemical shift relative to proton coupled spectra of the same samples. Chemical shifts of proton decoupled resonances (relative to extenal 85% H_3PO_4) and line widths were determined by Gaussian linefits to the singlet resonances. All cobalamins were commercially obtained and used as received. Samples were prepared in 12 mm sample tubes in the dark in glass distilled water and contained buffers and inert electrolyte (KCl) as appropriate. A deuterium lock signal was provided by a concentric insert (Wilmad) containing D_2O.

The apparent pK_a for the base-on–base-off transition of methylcobalamin was determined by spectrophotometric titration of 1.0×10^{-4} *M* solutions at 535 nm, 25.0 ± 0.1 °C, ionic strength 1.0 *M* (KCl), on a Gilford Model 250 spectrophotometer.

Results and Discussion

Our observations of the ^{31}P-nmr spectra of cyano-aquo-, and hydroxo-cobalamin (Table I) essentially confirm the conclusions of Satterlee [1, 2], *i.e.*, i) the ^{31}P-nmr resonances of 1–2 m*M* solutions of cobalamins are readily observed either as an apparently symmetrical triplet (J_{H-P} *ca.* 6–8 Hz) due to spin coupling to the two structurally dissimilar but evidently magnetically equivalent hydrogens on either side of the phosphate moiety or as relatively sharp ($w_{1/2}$ *ca.* 2–3 Hz) singlets with proton noise decoupling, ii) the chemical shifts of the base-on species of these cobalamins are relatively independent of the upper axial ligand and occur within a few Hz of the reference and, iii) displace-

TABLE I. ^{31}P-NMR Data for Cobalamins in Aqueous Solution, 25 ± 1 °C.[a]

Compound	pH	$\delta_{^{31}P}$ (Hz)[b]	J_{H-P} (Hz)[c]	$w_{1/2}$ (Hz)[d]
CNCbl	9.60	−6.22	8.05	2.49
H_2OCbl	5.15	1.02	8.30	2.33
HOCbl	9.66	−7.32	7.80	3.43
$(CN)_2$Cbl[e]	9.90	−37.59	6.83	2.48
CH_3Cbl	5.00	−51.27	6.84	2.85
CH_3(CN)Cbl[e]	8.96	−51.40	6.33	2.88

[a]All cobalamins were 2×10^{-3} *M*. [b]Chemical shift in Hz from external 85% H_3PO_4. Negative chemical shifts are upfield from the reference. [c]Apparent coupling constant, in Hz, from the proton coupled resonances. [d]Width at half-height, in Hz, obtained by Gaussian linefits to the proton decoupled spectra (without exponential multiplication). [e]Sample made 0.10 *M* in KCN.

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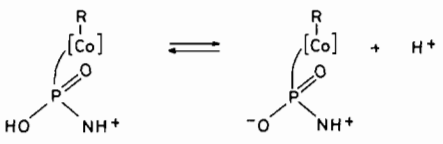
TABLE II. ^{31}P-NMR Data for Methylcobalamin in Aqueous Solution, 25 ± 1 °C.[a]

pH	$\delta_{^{31}P}$ (Hz)[b]	J_{H-P} (Hz)[c]	$w_{1/2}$ (Hz)[d]
5.00	−51.27	6.84	2.85
3.44	−52.56	7.33	3.06
3.05	−52.21	7.33	3.71
2.17	−53.39	7.33	2.84
1.25	−59.07	7.08	1.75
0.71	−74.25	7.33	2.84
0.49	−82.14	7.33	2.56
0.33	−92.74	7.81	3.10
0.29	−93.21	6.35	1.94
0.14	−98.82	6.85	2.04
0.04	−108.18	7.81	2.83

[a]Ionic strength maintained at 1.0 *M* with KCl. All samples were 2 × 10^{-3} *M* in methylcobalamin. [b]Chemical shift relative to external 85% H_3PO_4. Negative chemical shifts are upfield from the reference. [c]Apparent coupling constant, in Hz, from the proton coupled resonance. [d]Width at half-height, in Hz, obtained by Gaussian linefits to the proton coupled spectra (taken without exponential multiplication).

ment of the axial benzimidazole of cyanocobalamin by excess cyanide (*i.e.*, formation of dicyanocobalamin) causes about a 30 Hz upfield shift of the phosphorus resonance. However, we have found that ^{31}P resonance of base-on methylcobalamin occurs at relatively high field and that incubation with a 50-fold excess of KCN causes no change in the chemical shift (Table I). In addition, lowering the pH from 5.0 (base-on) to 2.17 (Table II), at which point the base-on–base-off equilibrium is displaced some 84% to the base-off form (pK_a = 2.89 ± 0.01) also causes little or no change in the ^{31}P chemical shift of methylcobalamin.

However, further lowering the pH below 2.17 does cause a substantial upfield shift of the ^{31}P resonance (Table II, Fig. 1A), which must be assigned to protonation of the phosphodiester group of the nucleotide loop (Equation 1).



(1)

This pK_a has been evaluated by application of Equation 2, for titrations with unknown acid end-points,

$$[HA] = P - K_{HA}[HA]/[H^+] \quad (2)$$

which can be recast as Equation 3 in which

TABLE III. ^{31}P-NMR Data for Methylcobalamin in Aqueous Sulfuric Acid, 25 ± 1 °C.[a]

H_o	$\delta_{^{31}P}$ (Hz)[b]	J_{H-P} (Hz)[c]	$w_{1/2}$ (Hz)[d]
2.48	−36.97	7.33	1.88
1.09	−50.79	7.52	1.11
0.68	−64.50	7.81	1.81
0.13	−87.38	7.57	1.10
−0.05	−93.76	7.32	1.27
−0.27	−106.98	7.58	1.48
−0.48	−116.62	7.54	2.26
−2.82	−126.35	–	3.30

[a]All samples were 2 × 10^{-3} *M* in methylcobalamin. [b]Chemical shift relative to external 85% H_3PO_4 Negative chemical shifts are upfield from the reference. [c]Apparent coupling constant, in Hz, from the proton coupled resonance. [d]Width at half-height, in Hz, obtained by Gaussian linefits to the proton coupled spectra (taken without exponential multiplication).

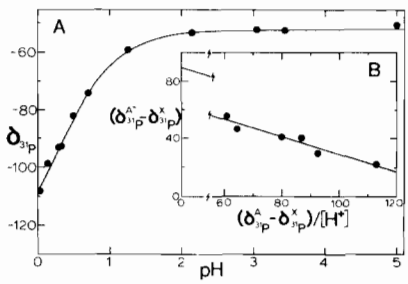


Fig. 1. A. Plot of observed ^{31}P chemical shifts (in Hz) of methylcobalamin *vs* pH. The solid line is calculated based on pK_{AH} = 0.22 ± 0.06, $\delta^{AH}_{^{31}P}$ = −141.64 Hz, $\delta^{A^-}_{^{31}P}$ = −52.21 Hz. B. Plot of $(\delta^{A^-}_{^{31}P} - \delta^{X}_{^{31}P})$ *vs.* $(\delta^{A^-}_{^{31}P} - \delta^{X}_{^{31}P})/[H^+]$ (*i.e.*, Equation 3) for methylcobalamin. The solid line is a linear regression line, intercept = 89.43 ± 7.32 Hz, slope = −0.604 ± 0.086 M^{-1}.

$$(\delta^{A^-}_{^{31}P} - \delta^{X}_{^{31}P}) =$$

$$= (\delta^{A^-}_{^{31}P} - \delta^{AH}_{^{31}P}) - K_{AH}(\delta^{A^-}_{^{31}P} - \delta^{X}_{^{31}P})/[H^+]_X \quad (3)$$

K_{AH} is the acid dissociation constant, $\delta^{A^-}_{^{31}P}$ is the chemical shift of the fully deprotonated species, $\delta^{AH}_{^{31}P}$ is the unknown chemical shift of the fully protonated species, and $\delta^{X}_{^{31}P}$ is the observed chemical shift at pH_x. A plot of the data in Table II according to Equation 3 using $\delta^{A^-}_{^{31}P}$ the average chemical shift observed at pH s 2.17–5.00 (−52.21 ± 1.13 Hz) is shown in Fig. 1B from which the values pK_{AH} = 0.22 ± 0.06 and $\delta^{AH}_{^{31}P}$ = −141.64 Hz were obtained. This protonation, which presumably causes only a minor perturbation of the electronic spectrum of methylcobalamin and occurs with a pK_a too low for accurate potentiometric titration of dilute solutions, has never been observed before.

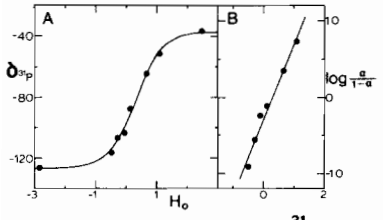


Fig. 2. A. Plot of observed ^{31}P chemical shifts (in Hz) of methylcobalamin *vs* H_o. The solid line is calculated based on $pK_{AH} = 0.30 \pm 0.04$, $\delta^{AH}_{^{31}P} = -126.35$ Hz, $\delta^{A^-}_{^{31}P} = -36.97$ Hz. B. Plot of log $\alpha/(1-\alpha)$ *vs.* H_o (*i.e.*, Equation 4) for methylcobalamin in sulfuric acid–water mixtures. The solid line is a linear regression line, intercept = -0.304 ± 0.041, slope = 0.997 ± 0.072.

In order to directly observe the fully protonated species and to confirm the assignment of the pH dependence seen in Fig. 1 as being due to titration of a protonatable group, we have titrated the ^{31}P resonance of methylcobalamin in H_2SO_4–H_2O mixtures using the Hammett acidity function, H_o [6–8]. These data (Table III and Fig. 2A) were fit to Equation 4, where

$$H_o = pK_{AH} + \log[\alpha(1-\alpha)] \quad (4)$$

α is the fraction of deprotonated species present at any H_o, and the term $\alpha/(1-\alpha)$ has been evaluated by Equation 5. The plot of Equation 4

$$\alpha/(1-\alpha) = (\delta^{AH}_{^{31}P} - \delta^{X}_{^{31}P})/(\delta^{X}_{^{31}P} - \delta^{A^-}_{^{31}P}) \quad (5)$$

using $\delta^{AH}_{^{31}P} = -126.35$ (at $H_o = -2.82$) and $\delta^{A^-}_{^{31}P} = -36.97$ (at $H_o = 2.48$) gave the value $pK_{AH} = 0.30 \pm 0.04$ (Fig. 2B). While the chemical shifts of both endpoint species occurs at about 15 Hz lower field than in the pH titration, presumably due to medium effects, the upfield shift upon protonation is identical in both media (89.43 Hz and 89.38 Hz).

The cause of the upfield shift of the phosphorus resonance of cyanocobalamin upon displacement of the axial base in cyanide (Table I) is at present unknown. However, the fact that methylcobalamin does not show such a shift implies that there is a substantial difference in the magnetic environment of the phosphorus atom in these two base-on cobalamins. Presumably, in base-on cyanocobalamin (in which the benzimidazole nucleotide is much more strongly bonded to cobalt ($pK_a = 0.1$ [9]) than in methylcobalamin) the phosphorus atom feels a magnetic influence from some other part of the structure but is removed from this influence when the benzimidazole is displaced. Evidently the average conformation of the nucleotide loop in base-on methylcobalamin is substantially different so that the phosphorus atom is free of this influence and there is little or no change in its magnetic environment when the benzimidazole is displaced. These interesting phenomena are currently under further investigation.

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