Acid–base chemistry of α-alkylcobalamins. Determination of additional formation constants for the 'tuck-in' species of base-off cobalamins

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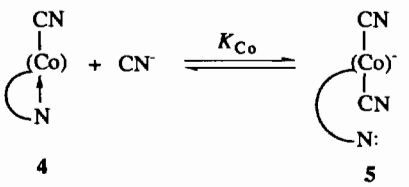
Abstract

Potentiometric titration of five α-alkylcobalamins, in which the axial organic ligand is in the 'lower' (α) axial position preventing coordination of the axial nucleotide, has provided the first values of the pK_a of the conjugate acid of the pendent, but uncoordinated, benzimidazole nucleotide in cobalamins. In every case, these values are lower than the pK_a of the conjugate acid of the free nucleoside, α-ribazole, at the same temperature. This suggests that, as is the case with base-off dicyanocobalamin and base-off but benzimidazole deprotonated β-alkylcobalamins, the free base nucleotide in the α-alkylcobalamins is associated with a corrin ring side chain to form what is known as the 'tuck-in' species. The data permit calculation of apparent equilibrium constants for formation of the 'tuck-in' species of the α-alkylcobalamins which vary from about 0.3 to 1.3, but are essentially temperature independent for a given complex. The isoenthalpic nature of this equilibrium is consistent with the principle interaction in the 'tuck-in' species being a hydrogen bond between the nucleotide B3 nitrogen and a side chain amide N-H, as is the case for dicyanocobalamin and the base-off β-alkylcobalamins. Further evidence for formation of 'tuck-in' species in α-alkylcobalamins has been obtained from comparison of the ^{13}C NMR resonances of the pendent nucleotide of these complexes with those of the free nucleotide, α-ribazole-3'-phosphate, and with those of dicyanocobalamin, in which the 'tuck-in' species is well characterized. These spectral comparisons suggest that formation of the 'tuck-in' species may be accompanied by a change in conformation about the nucleotide *N*-glycosidic bond, and that the conformation of the nucleotide ribose moiety in the 'tuck-in' species of the α-alkylcobalamins may be different from that in dicyanocobalamin.

Introduction

There has long been interest in the thermodynamics of the base-on/base-off reaction [1] of cobalamins (Fig. 1) in which the uncoordinated axial nucleotide may be protonated and trapped in acid media to produce the base-off species (eqn. (1)) [2–6]. The simplest thermo-



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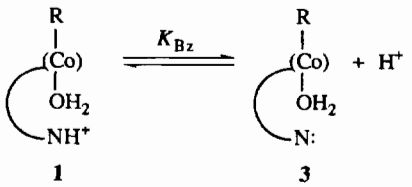
dynamic description of this process involves two consecutive equilibria, the proton dissociation from the protonated, base-off species shown in eqn. (2), and the axial ligand exchange process depicted in eqn. (3) [2,



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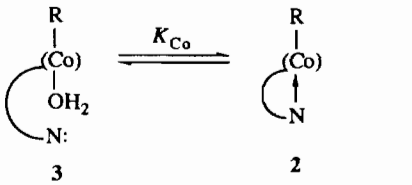

(3)

6–8]. The availability of the pK_a for the proton dissociation of the conjugate acid of the free nucleotide (α-ribazole) [2] should then permit calculation of the value of K_{Co} (eqn. (3)), the intrinsic equilibrium constant for substitution of the pendent nucleotide for water in

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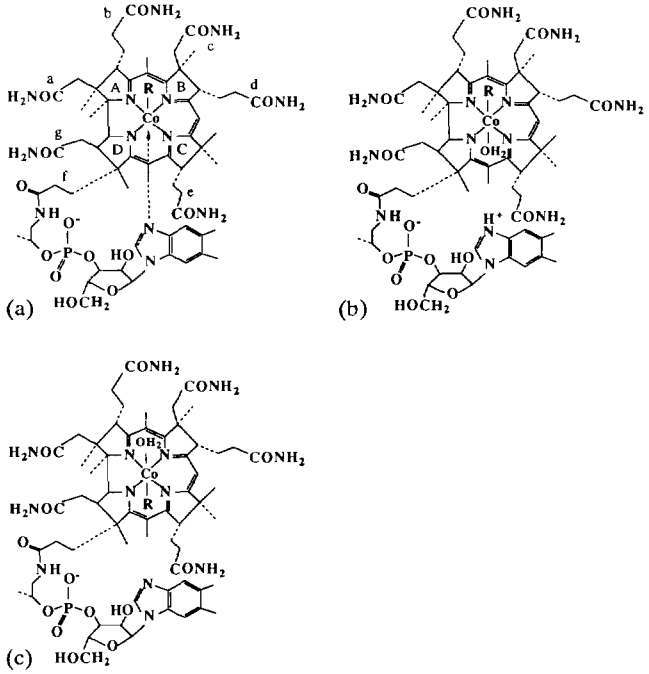
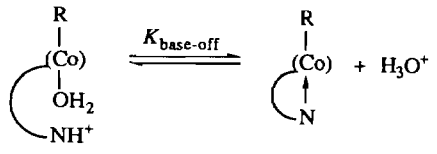


Fig. 1. (a) Structure of a base-on β-alkylcobalamin (β-RCbl) showing the standard lettering scheme for designation of the corrin rings and the amide side chains. (b) Structure of a base-off β-RCbl, in which the axial nucleotide is uncoordinated and protonated. (c) Structure of an α-alkylcobalamin (α-RCbl) in which the organic ligand occupies the 'lower' (α) axial ligand position and the nucleotide is prevented from coordinating.

the base-off but nucleotide deprotonated species [2, 4, 5].

However, the actual situation is more complicated than this. Cyanocobalamin (CNCbl) is well known to add cyanide to form the relatively stable ($K_{CN} = 3.0 \times 10^3$ M^{-1} [9]) dicyanocobalamin ($(CN)_2Cbl$), in which the pendent axial nucleotide is uncoordinated, but also unprotonated (eqn. (4)) [9, 10]. Thus, the base-off



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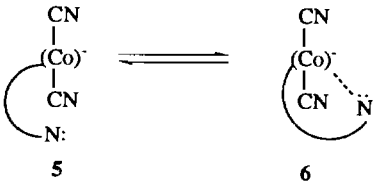
$(CN)_2Cbl$ (**5**) can be used as a model for the base-off but axial nucleotide deprotonated cobalamin species, **3**. A detailed comparison [8] of the ^{13}C NMR resonances of the pendent nucleotide of $(CN)_2Cbl$ with the ^{13}C NMR spectrum of the free base of the detached nucleotide (α-ribazole-3'-phosphate dianion) revealed a number of differences indicative of the formation of a complex between the pendent free base nucleotide and another part of the structure. These spectral differences, along with a comparison of the ^{13}C NMR spectrum of $(CN)_2Cbl$ to that of dicyanocobinamide ($(CN)_2Cbi$), a derivative in which the nucleotide has been removed chemically [11], suggested that $(CN)_2Cbl$ existed largely as a species in which the coordinating nitrogen of the axial nucleotide (B3, Fig. 2) is hydrogen bonded to a side chain amide N-H (eqn. (5)) [6]. This



(5)

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interaction was subsequently confirmed by ^{15}N NMR observations of the axial nucleotide and side chain amide nitrogens of $(CN)_2Cbl$ and $(CN)_2Cbi$ [12]. Complete assignment of the ^1H, ^{13}C and amide ^{15}N NMR spectra of $(CN)_2Cbl$ and $(CN)_2Cbi$ permitted location of this interaction to a hydrogen bond between the nucleotide B3 nitrogen and a g side chain amide N-H (Fig. 1) [13]. This hydrogen-bonded species of base-off cobalamin has become known as the 'tuck-in' species.

Since the enthalpies of formation of the base-on species of cobalamins, ΔH_{Co} (eqn. (3)), are negative [2, 6], the equilibrium is shifted towards the base-off species **3** at higher temperatures. Thus, for certain β-alkylcobalamins (β-RCbls) which are stable to thermolysis, detectable amounts of the base-off but nucleotide deprotonated species may be generated at higher temperatures. A detailed study of the temperature dependence of the α-carbon ^{13}C NMR resonance of β-$^{13}CH_3Cbl$ and of β-$^{-}OOC^{13}CH_2Cbl$ [8] permitted a dissection of the thermodynamics of the on/off process (eqns. (2), (3), and (6)). The value of K_H (eqn. (6))

(6)

3 7

thus determined for β-$^{13}CH_3Cbl$ ($K_H = 4.08 \pm 0.19$ [8]) was independent of temperature as anticipated for formation of a hydrogen bond in water [14, 15]. A similar value of $K_H = 3.29$ was obtained for β-$^{-}OOC^{13}CH_2Cbl$, and a value of $K_H = 2.6$ can be cal-

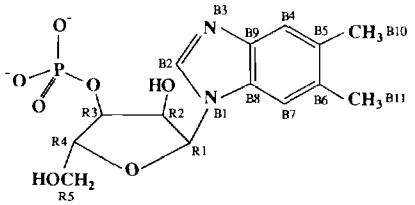


Fig. 2. Standard numbering scheme for the cobalamin axial nucleotide, 1-α-D-ribofuranosyl-r,6-dimethylbenzimidalole-3'-phosphate (α-ribazole-3'-phosphate) shown as the dianion.

culated for $(CN)_2$Cbl (eqn. (5)) from the estimate of Reenstra and Jencks of the pK_a of the conjugate acid of $(CN)_2$Cbl [9].

Because of the thermal lability of many RCbls, the high value of K_{Co} (eqn. (3)) for many RCbls [2, 6], the small number of β-RCbls that bind cyanide sufficiently strongly [8], and the acid lability of R(CN)Cbls, the opportunities to measure values of K_H have been very limited. However, methods for the synthesis of reasonable amounts of α-alkylcobalamins (α-RCbls), in which the organic ligand occupies the lower (α) axial ligand position (Fig. 1) [16–23], have recently been devised [24–29]. As coordination of the axial nucleotide is prevented in such derivatives, they represent a unique opportunity to directly titrate the pendent axial nucleotide of a cobalamin in the absence of coordination of the free base species. We now report a study of the acid–base properties of five α-RCbls (R = CH_3, CH_3CH_2, $NCCH_2$, CF_3CH_2 and CF_3) along with the ^{13}C and ^1H NMR characteristics of their pendent nucleotides.

Experimental

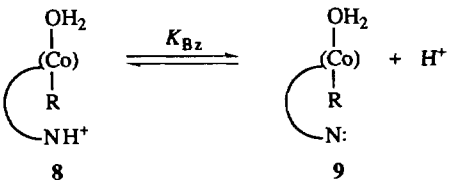
α-RCbls were obtained, as mixtures with the diastereomeric β-RCbls, by reductive alkylation of H_2OCbl (Roussell) with alkyl halides in zinc/10% acetic acid or in zinc/2% phosphoric acid [24–27]. In cases where little or no α diastereomer is obtained by this method (i.e. R = CH_3 and CH_3CH_2), the α-RCbl was obtained by anaerobic photolysis of the β-RCbl at pH 1.0 as recently described [28]. The α-RCbls were separated from the β-RCbls by cation exchange chromatography on SP-Sephadex [24], by flash chromotography on Amberlite XAD-2 [27], or by semipreparative HPLC [25–27, 30]. The α-RCbls were characterized by ^{13}C and ^{19}F NMR, UV–Vis spectroscopy, FAB-MS, and GC/MS determination of the organic products derived from the alkyl ligand upon anaerobic pyrolysis [24, 25, 27, 28].

Apparent pK_as for the pendent nucleotide of the α-RCbls were determined by potentiometric titration using a Radiometer PHM 84 pH meter equipped with a type C combined electrode. Aqueous samples containing 10–20 mM α-RCbl (ionic strength 1.0 M, KCl) in 1.5 ml, were titrated with HCl in a thermostatted sample cup (Radiometer). Standards and electrode rinse water were incubated at the titration temperature in a circulating water bath used to thermostat the samples ($\pm$0.2 °C). Titration data were corrected for titration of an identical blank which did not contain α-RCbl.

^1H and ^{13}C NMR spectra were obtained on a Bruker AMX 300 NMR spectrometer at 25 °C. Samples (D_2O, 2.0 ml) were 10–20 mM in α-RCbl, which had been pre-exchanged with D_2O to minimize the residual solvent peak in the ^1H NMR spectra, and contained TSP as an internal reference. Chemical shifts are reported in ppm downfield from TSP.

Results and discussion

If a 'tuck-in' species is formed in the α-RCBls, the relevant equilibria affecting the acid–base behavior of the axial nucleotide are as shown in eqns. (7) and (8).



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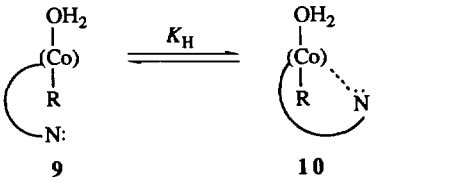

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The observed pK_a of an α-RCbl (eqn. (9)) is then related to K_{Bz} (eqn. (7)) and K_H (eqn. (8)) by eqn. (10). It is reasonable to anticipate that the value of

$$K_a = ([\mathbf{9}] + [\mathbf{10}])[H^+]/[\mathbf{8}] \quad (9)$$

$$K_a = K_{Bz}(K_H + 1) \quad (10)$$

pK_{Bz} (eqn. (7)) will be adequately represented by the pK_a of the conjugate acid of α-ribazole [2] as the value of the latter at 25 °C has been shown to be essentially identical to the microscopic pK_a for ionization from B3 (Fig. 2) of the zwitterion of the detached axial nucleotide, α-ribazole-3′-phosphate [8]. Further substitution of the phosphate of the nucleotide (i.e. by the isopropanolamine moiety to the nucleotide loop, Fig. 1) is not expected to significantly influence the acid–base properties of the benzimidazole moiety. Thus, eqn. (10) demonstrates that formation of a 'tuck-in' species in α-RCbls will lower the observed pK_a of the α-RCbl conjugate acid below the value of the pK_a of α-ribazole, and permit the calculation of the value of K_H (eqn. (8)) for formation of this species.

Observed values of the pK_a (eqn. (9)) for five α-RCbls (R = CH_3, CH_3CH_2, CF_3, CF_3CH_2 and $NCCH_2$) at various temperatures are shown in Table 1, along with values of pK_{Bz} at the same temperatures, taken as the pK_a of the conjugate acid of α-ribazole [2]. In every case, the value of pK_a at a given temperature is lower than the value of pK_{Bz} at the same temperature as anticipated (eqns. (7)–(10)) if the free base α-RCbl is in equilibrium with its 'tuck-in' species. The values of K_H (eqn. (8)) calculated from these data and eqn.

TABLE 1. Apparent pK_as and values of pK_{Bz} and K_H for the α-RCbls[a]

α-RCbl	T (°C)[b]					ΔH_H[c] (kcal mol^{-1})	ΔS_H[c] (e.u.)
	5.0	15.0	25.0	35.0	45.0		
pK_{Bz}[d]	5.89	5.71	5.56	5.40	5.25		
α-$NCCH_2$Cbl							
pK_a[e]	5.53 ± 0.01	5.38 ± 0.01	5.22 ± 0.01	5.07 ± 0.03	4.92 ± 0.02		
K_H[f]	1.29	1.14	1.14	1.14	1.14	−0.45 ± 0.24	−1.2 ± 0.8
α-CF_3Cbl							
pK_a[e]	5.69 ± 0.04	5.53 ± 0.02	5.34 ± 0.01	5.18 ± 0.01			
K_H[f]	0.585	0.514	0.622	0.660		0.91 ± 0.76	2.1 ± 2.6
α-CH_3Cbl							
pK_a[e]	5.63 ± 0.01	5.48 ± 0.02	5.35 ± 0.01	5.16 ± 0.01			
K_H[f]	0.820	0.698	0.622	0.738		−0.77 ± 0.93	−3.3 ± 3.2
α-CH_3CH_2Cbl							
pK_a[e]	5.78 ± 0.01	5.58 ± 0.01	5.43 ± 0.01	5.28 ± 0.01			
K_H[f]	0.288	0.349	0.349	0.318		0.54 ± 0.76	0.4 ± 2.6
α-CF_3CH_2Cbl							
pK_a[e]	5.70 ± 0.01	5.51 ± 0.01	5.41 ± 0.03[g]	5.22 ± 0.01	5.22 ± 0.01		
K_H[f]	0.549	0.585	0.413	0.514	0.622	0.22 ± 0.69	−0.3 ± 2.3

[a]Ionic strength 1.0 M, KCl. [b]± 0.02 °C. [c]From the slopes and intercepts of plots of ln K_H vs. $1/T$ (Fig. 3). [d]pK_a of the conjugate acid of the detached nucleoside, α-ribazole [2]. When necessary, values were interpolated or extrapolated from a plot of ln K_{Bz} vs. $1/T$. [e]Equation (9). From potentiometric titration of the α-RCbl. [f]Calculated from pK_a and pK_{Bz}, using eqn. (10). [g]An earlier report [24] of $pK_a = 5.54$ at 25 °C was evidently in error.

(10) are also listed in Table 1. These values can be seen to be relatively temperature independent for a given α-RCbl, and vary from about 0.3 to 1.3. This suggests that the α-RCbls exist 23–56% as the 'tuck-in' species at neutral pH. Values for the enthalpies (ΔH_H) and entropies (ΔS_H) for formation of the 'tuck-in' species (eqn. (8)) were obtained from plots of ln K_H versus $1/T$ (Fig. 3) and are also listed in Table 1. As previously found for ΔH_H for β-CH_3Cbl [6], the values of ΔH_H for the α-RCbls are extremely small, and probably statistically indistinguishable from zero. This is expected if the principle interaction in the 'tuck-in' species is a hydrogen bond, since formation of a hydrogen bond between a solvated donor and acceptor in water [14, 15] is isoenthalpic due to the enthalpic contributions of the solvating water molecules.

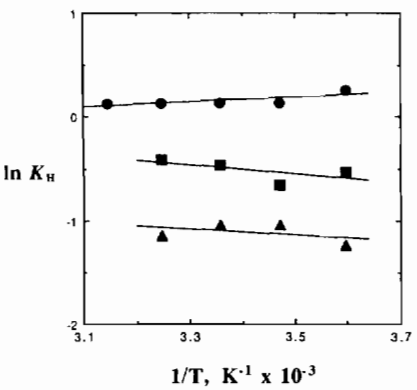

Fig. 3. Representative plots of ln K_H (eqn. (8)) vs. $1/T$. The solid lines are linear regression lines. (●), α-$NCCH_2$Cbl, slope = 227 ± 122 K, intercept = −0.61 ± 0.41; (■), α-CF_3Cbl, slope = −460 ± 383 K, intercept = 1.05 ± 1.31; (▲), α-CH_3CH_2Cbl, slope = −271 ± 385 K, intercept = −0.20 ± 1.32.

Further evidence for formation of 'tuck-in' species in the α-RCbls, and further characterization of these species, can be obtained by comparison of the ^{13}C NMR chemical shifts of the nucleotide of the α-RCbls to those of the detached nucleotide, α-ribazole-3′-phosphate (as the dianion), and to those of $(CN)_2$Cbl (Table 2). Such a comparison between the latter two species was the original observation which revealed the presence of the 'tuck-in' species [8]. Differences in chemical shift exceeding those attributable to experimental error ($\geq c.$ 0.25 ppm) must be considered to be significant, as the ^{13}C chemical shifts of the nucleotide of dicyano-3,5,6-trimethyl-benzimidazolylcobamide, in which N-methylation of the benzimidazole at B3 (Fig. 2) prevents formation of the 'tuck-in' species via hydrogen bonding, have been demonstrated to be essentially identical to those of *N*-methyl-α-ribazole-3′-phosphate methyl ester [13].

The comparison between $(CN)_2$Cbl and α-ribazole-3′-phosphate (Table 2) shows that the largest differences in the benzimidazole moiety occur at B2, B5, B6 and B8 (Fig. 2). This pattern is repeated in the α-RCbls, although there are clearly significant differences among the individual resonances across the series of α-RCbls and between individual α-RCbls and $(CN)_2$Cbl. These differences presumably reflect differences in the relative proportions of the 'tuck-in' and free base base-off species among these complexes. The very significant differences

TABLE 2. ^{13}C NMR chemical shifts for the α-ribazole-3'-phosphate dianion, $(CN)_2Cbl$, and the α-RCbls[a]

Atom[b]	$\delta^{13}C$ (ppm)						
	α-ribazole-3'-P[c]	$(CN)_2Cbl$[d]	α-CH_3Cbl	α-CH_3CH_2Cbl	α-CF_3Cbl	α-$NCCH_2Cbl$	α-CF_3CH_2Cbl
B2	145.65	145.22	144.98	144.97	145.00[f]	145.20[f]	145.03
B4	121.26	121.59	121.56	121.34	121.32	121.34	121.41
B5	134.23	135.07	134.66	134.96	134.80	134.69	134.77
B6	135.10	135.92	135.54	135.82	135.79	135.81	135.91
B7	113.70	113.63	113.23	113.55	113.35	113.65	113.52
B8	136.06	134.15	134.48	134.36	134.08	n.o.[g]	134.35
B9	142.87	142.96	142.61	142.39	n.o.[g]	n.o.[g]	142.79[f]
B10	21.92	22.08	22.01	22.24	22.19	22.33	21.97
B11	22.20	22.60	22.53	22.48	22.19	23.10	22.49
R1	88.51	88.38	87.67	88.04	87.99	87.96	87.98
R2	74.09	73.78	73.78	73.93	73.78	73.72	74.10
R3	75.75[e]	76.64	77.24	77.21	76.76	77.10	77.30
R4	85.93	85.66	86.59	86.84	86.34	86.40	86.89
R5	63.88	63.46	63.77	63.77	63.49	63.68	63.81

[a]25 ± 1 °C in D_2O. Chemical shifts are in ppm downfield from internal TSP. [b]Figure 2. [c]Ref. 7. In this earlier work, the assignments of the B5 and B6 resonances were incorrectly interchanged as were the assignments of B10 and B11. [d]Ref. 13. [e]The chemical shift of R3 is not directly comparable to those of the Cbls since in the detached nucleotide the phosphate moiety is dianionic. [f]Very broad. [g]Not observed, apparently due to excessive broadening.

in chemical shift between the free nucleotide and the α-RCbls and $(CN)_2Cbl$ at B6 and particularly at B8, suggest that there may be a difference in conformation about the B1–R1 *N*-glycosidic bond in the free nucleotide and in the 'tuck-in' cobalamins. Strict comparisons between the ribose ^{13}C resonances of the free nucleotide and of the 'tuck-in' cobalamins cannot be made since in the former, the phosphate moiety is dianionic, while in the latter, it is monoanionic. However, there are clearly significant differences between the α-RCbls and $(CN)_2Cbl$ at R1 and especially at R4. This suggests that the conformation of the ribose moiety in the 'tuck-in' species of the α-RCbls is different from that in the 'tuck-in' species of $(CN)_2Cbl$, presumably due to an effect of the more bulky α axial ligand in the former species.

We conclude that formation of 'tuck-in' species of base-off but benzimidazole deprotonated cobalamins is not prevented by the presence of the α axial organic ligand in α-RCbls, even when this ligand is fairly large as in α-CF_3CH_2Cbl and α-CH_3CH_2Cbl. Thermodynamic evidence for formation of 'tuck-in' species of α-RCbls has been obtained by observation of the lowering of the pK_a of the pendent nucleotide due to competition for the free base nucleotide (Table 1, eqns. (7) and (8)) and spectroscopic evidence has been obtained by comparisons of the ^{13}C NMR chemical shifts of the nucleotide of the α-RCbls with those of the free nucleotide. As is the case with the free base, base-off species of β-RCbls, the 'tuck-in' species of the α-RCbls is a major contributor at neutral pH.

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