The Kinetics of Benzimidazole Dissociation in Methylcobalamin[1]

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Abstract: The kinetics of dissociation of 5,6-dimethylbenzimidazole (dmbz) from methylcobalamin (CH_3Cbl), in methanol, have been evaluated using variable temperature ^1H or ^{13}C NMR spectra. The signals due to the axial methyl group were observed. From the detailed fitting of the NMR line shapes the activation parameters for the exchange were determined to be $\Delta H^{\ddagger} = 10.9 \pm 0.9$ kcal/mol, $\Delta G^{\ddagger} = 12.7 \pm 0.1$ kcal/mol, and $\Delta S^{\ddagger} = -6.7 \pm 3.3$ cal/(K mol) from the ^1H spectra and $\Delta H^{\ddagger} = 11.4 \pm 1.1$ kcal/mol, $\Delta G^{\ddagger} = 12.7 \pm 0.2$ kcal/mol, and $\Delta S^{\ddagger} = -4.9 \pm 4.4$ cal/(K mol) from the ^{13}C spectra. An Arrhenius plot based on the combined data, which were in good agreement, yielded $\Delta H^{\ddagger} = 11.1 \pm 0.6$ kcal/mol, $\Delta G^{\ddagger} = 12.7 \pm 0.1$ kcal/mol, and $\Delta S^{\ddagger} = -5.9 \pm 2.4$ cal/(K mol). The rate of dmbz dissociation in 50% methanol–water was found to be essentially the same as in neat methanol. The $\Delta H^{\ddagger}$ for dmbz dissociation is significantly lower than expected, based on comparison with $\Delta H^{\ddagger}$ for dissociation of 1-(2-trifluoromethylphenyl)imidazole from the heptamethyl ester of cobyrinic acid. The lower value may be ascribed to repulsive steric interactions that impede approach of dmbz to the metal. The comparative strengths of interaction of nitrogen bases, as mediated by steric effects, may play an important role in determining the reactivity of the cobalt–carbon bond in methylcobalamin or coenzyme B_{12} bound to proteins.

The substitution reactions of cobalt(III) complexes involving a planar or nearly planar chelate ring coordination have been extensively studied.[3-14] The six-coordinate alkylatocobalt^{III}(chelate)L complexes, RCo(chelate)L, in which L is a sixth ligand bound to cobalt in the position trans to the alkyl, have been of special interest for their relationship to alkylcobalamin systems.[9-14] When the leaving group is small, e.g., H_2O, and the entering group also has a small steric requirement, ligand substitution appears to occur via a dissociative interchange (I_d) mechanism.[6,8,10,11]

However, it has been demonstrated for several RCo^{III}(chelate)L compounds that substitution of L occurs via a purely dissociative (D) pathway, with no evident participation from entering ligand.[9b,12,13-15] For such a process the rate of the forward step in eq 1 is not strongly dependent on solvent nucleophilicity or polarity.

$$\text{RCo(chelate)L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{RCo(chelate)} + \text{L} \qquad (1)$$

Although a large body of kinetics and equilibrium data exists for base exchanges and substitutions in alkylatocobalt^{III}(chelate)L complexes, there has not been a direct measurement of the kinetics of 5,6-dimethylbenzimidazole (dmbz) dissociation from either methylcobalamin (CH_3Cbl) or adenosylcobalamin. In fact, the only quantitative kinetics data for ligand dissociation from an alkyl corrinoid system are those for dissociation of 1-(2-trifluoromethylphenyl)imidazole ($ImCF_3$) from the heptamethyl ester of cobyrinic acid, I.[14]

It has been recognized that the nature of the coordination in the position trans to the Co–C bond exerts a powerful influence on the reactivity[16-31] and ease of photolysis[32-35] of that bond. Various lines of evidence have been offered relating to the displacement of dmbz in cobalamin systems bound to proteins,[29,37-41] and in active enzyme systems,[42-53] but definitive data regarding the binding to cobalt in these situations are lacking. The work reported here was carried out with the aim of learning something of the energetics and rates of dissociation of dmbz from methylcobalamin under solution conditions comparable to those in systems of biochemical interest. As a secondary goal we desired to obtain additional data as a basis for comparing the alkylato cobalt(III) corrinoids with other RCo(III) chelate systems.

Experimental Section

Materials. [90% ^{13}C]Methylcobalamin was prepared according to a published procedure[54] using cyanocobalamin purchased from Sigma Chemical Co. and [90% ^{13}C]CH_3I obtained from Koch Isotopes, Inc. The purity of the [^{13}C]methylcobalamin was ascertained using UV-visible and NMR spectroscopy and thin-layer chromatography on cellulose using four solvent systems.[55]

Absolute methanol was obtained by overnight reflux over magnesium metal and subsequent distillation under nitrogen. A 1 M solution of HCl in absolute methanol was prepared by dissolving a measured volume of HCl gas into a known volume of absolute methanol on a vacuum line.

The potentiometric titration[56] to the half-equivalence point (pH 2.72)[57] of 2 ml of a 0.005 M solution of [90% ^{13}C]methylcobalamin in absolute methanol-d_4 was carried out in a water-free drybox with the addition of incremental amounts of the 1 M HCl solution in methanol. A buffer solution of 0.01 *m* oxalic acid and 0.01 *m* ammonium oxalate in absolute methanol with a determined pH value[56] of 3.45 was used to standardize the pH meter.

The [90% ^{13}C]methylcobalamin was dried at 41 °C under vacuum over P_2O_5 before being taken into the drybox. All solutions containing methylcobalamin were handled exclusively under a red photographic safe light.

The solutions of [90% ^{13}C]methylcobalamin in a 50:50 methanol:D_2O solvent system were prepared analogously to the above solutions; however, they were made using the mixed solvent in all cases and were not manipulated in the drybox.

NMR samples were degassed by successive freeze–thaw cycles and sealed under vacuum. They were stored at 5 °C in the dark.

The heptamethyl ester of cobyrinic acid, I, was prepared as described previously.[14] For preparation of [90% ^{13}C] compounds, the preparation was conducted on a small scale using [90% ^{13}C]CH_3I in the alkylation step.

1-(2-Trifluoromethylphenyl)imidazole, $ImCF_3$, was prepared as described previously,[14] and purified by vacuum sublimation at 40 °C.

Solutions of I and $ImCF_3$ in methanol were prepared with a 2:1 ratio of base to I, to achieve a measurable signal for the coordinated base. The concentration of I was about 0.2 M.

NMR Spectra. The quality of the data obtainable for CH_3Cbl solutions, particularly in the ^{13}C spectra, was severely limited by the low solubility of CH_3Cbl. Proton NMR spectra were recorded on a Varian HR-220 spectrometer equipped with a Nicolet TT-220 Fourier transform accessory and computer-magnetic disc system and a Varian B-4540 temperature controller. The transients obtained from the application of 20-μs pulses were accumulated as 8192 points in the time domain and transformed into a 4096 point spectrum in the frequency domain. Data acquisition time was 1.64 s; the number of pulses collected ranged from 1000 to 4000. Peak positions were determined relative to the internal methyl resonance of solvent methanol by computerized examination of the Fourier transformed spectrum. Temperature measurements were made at the beginning and end of

data collection at each temperature, using a sealed tube of absolute methanol with added HCl for calibration.[58]

Proton-decoupled ^{13}C NMR spectra were recorded on a JEOL FX-60 (15.00 MHz) spectrometer. When using methanol-d_4 as solvent, the solvent CD_3 group signal was used for lock. When methanol-D_2O was used as solvent, the solvent deuterium resonance was used as lock. Transients resulting from 20-μs pulses were collected as 8192 points in the time domain. The number of pulses accumulated varied from 1500 to 17 000. Temperature measurements were made using the JEOL NM5471 temperature controller which provides a direct probe temperature reading using an inlaid copper-constantan thermocouple. These temperature measurements were checked by switching the probe to ^1H and obtaining the spectrum of absolute methanol plus a small amount of HCl in a sealed tube.

The ^{19}F NMR spectra were obtained for the I-ImCF_3 solutions as described previously.[14]

Line Shape Analyses. A two-site computer program, modified for graphics display, was used for analysis of the NMR exchange spectra. Input parameters consisted of ordered pairs of shift-intensity data describing the observed NMR line shapes. Typically about 200 such pairs for the ^1H data and 200 for the ^{13}C data were used as input to describe the line shape. Additional input parameters are P_A, the fractional population of the downfield site of exchange; T_{2A} and T_{2B}, the transverse relaxation times of the two sites of exchange; and $\Delta\nu$, the difference in resonance frequencies for sites A and B.

P_A was obtained via integration of the areas of a number of spectra in the stopped exchange region. From measurements of relative areas in the ^{13}C spectra in the stopped exchange region, between -52 and -22 °C, there was no evidence of a significant variation in relative populations of the base-on and base-off forms as a function of temperature. We therefore assumed that these relative populations remain unchanged throughout the temperature interval over which line shapes are fitted. As a check on this assumption the weighted average chemical shift in the spectrum at 25 °C was calculated from a knowledge of the chemical shifts of the base-on and base-off forms, assuming no change in P_A and P_B from the lower temperatures. The agreement obtained was within the accuracy of the measured chemical shifts.

In analyzing the ^1H spectra P_A and P_B were initially estimated from the relative peak areas in the slow exchange limit. However, the presence of an extraneous absorption in the vicinity of the downfield peak rendered the estimates somewhat uncertain. As a result, P_A was treated as a variable in fitting spectra at several temperatures in the temperature range below coalescence, and a value chosen that gave best overall fit for all the spectra. The value ($P_A = 0.46$ for the spectra of Figure 4) compares reasonably well with the estimate of 0.44 based on relative areas. P_A was held constant for all temperatures. The weighted average chemical shift of the line at 25 °C was consistent with this assumption.

It was possible to obtain the value of P_A and P_B for the I-ImCF_3 solutions from spectra in the stopped exchange region, at -8, 25, 29, and 34 °C. At higher temperatures, the equilibrium shifts in the direction of more coordinated base. It is not possible under these circumstances to determine a reliable value for the enthalpy of activation, but it was possible to extract from the spectra a reasonably good value for $\Delta G^{\ddagger}$ (Table I). A graph of $\Delta\nu_{1/2}$ for the dominant (free base) peak as a function of temperature showed a well-defined maximum at 61 °C. From an analysis of the equation for the line shape in the region of intermediate and slow exchange rate,[59] the variation in $\Delta\nu_{1/2}$ provides a good basis for estimating τ. The value of $\Delta G^{\ddagger}$ at coalescence estimated from the variation in $\Delta\nu_{1/2}$ with temperature was in accord with the value estimated from the line shape analyses of spectra at 50, 55, 60, and 66 °C.

In analyzing the CH_3Cbl spectra T_{2A} and T_{2B} were calculated from the line widths at half height, $\Delta\nu_{1/2}$. In the ^1H spectra the measured values of $\Delta\nu_{1/2}$ were measured for CH_3Cbl in solutions of pH adjusted so that the samples were entirely in the base-on or base-off forms. The $\Delta\nu_{1/2}$ values for the base-on form were found to be 4.1 Hz from 25 °C to about -18 °C. At -33 °C the lines broadened slightly to 4.6 Hz. For the base-off form $\Delta\nu_{1/2}$ was found to be 4.0 Hz at 5 °C.

In the ^{13}C spectra the values of $\Delta\nu_{1/2}$ were obtained from the stopped exchange spectra -42 and -52 °C. The base-on form yielded $\Delta\nu_{1/2}$ of 11 Hz, the protonated, base-off form yielded 8.2 Hz. We assumed that $\Delta\nu_{1/2}$ values are constant through the temperature interval studied. The values of $\Delta\nu_{1/2}$ for CH_3Cbl in the 50:50 methanol-water mixed solvent at 25 °C were found to be 12 Hz for both the base-on and base-off forms. It is noteworthy that somewhat wider lines, 20 Hz, were reported for CH_3Cbl in the base-on form in water[60] than we observe here for CH_3Cbl in methanol.

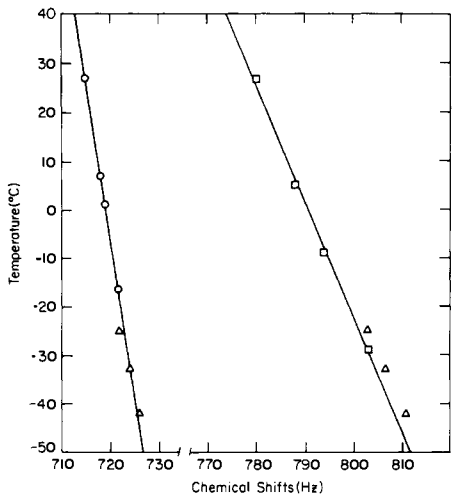


Figure 1. Chemical shifts as a function of temperature of the ^1H resonances of the axial methyl group in methylcobalamin in the base-on (○) and base-off (□) forms. The points represented by △ correspond to observations of spectra in the stopped exchange region for solutions containing comparable amounts of the base-on and base-off forms.

In the CH_3Cbl solutions, the ^1H chemical shift separations, $\Delta\nu$, between resonances due to the base-on and base-off forms were determined at 25 °C from separate measurements of the chemical shifts in solutions of pH adjusted so that the base was completely on or completely off. In the stopped exchange region at low temperature the chemical shift separation could be measured directly as a function of temperature.

In the ^{13}C NMR, no change in chemical shift separation as a function of temperature was noted; $\Delta\nu$ was 106 Hz at both the high and low temperatures. Our results for methanol solutions are thus consistent with the observations of Hogenkamp et al.[35] for the ^{13}C methyl resonances of CH_3Cbl and methylaquocobinamide in H_2O in the range from 0 to 80 °C. The temperature dependence of the ^{13}C methyl resonance chemical shift was found to be the same in the two cases.

In the proton spectra, $\Delta\nu$ was found to vary with temperature, from 65 Hz at 27 °C to 84 Hz at -42 °C. We were able to measure chemical shifts for the separate resonances at several temperatures, Figure 1. The chemical shifts appeared to vary linearly with temperature. However, slightly larger separations were observed in the slow exchange region for solutions in which both the base-on and base-off forms were present in comparable amounts. This small discrepancy may arise from ionic strength effects. To obtain an optimal fit between calculated and observed spectra in the intermediate exchange region, $\Delta\nu$ was allowed to increase to as much as 3 Hz from the value taken from the graph. In practice an optimal fit in terms of the least sum of squares was arrived at with $\Delta\nu$ values no more than 1 or 2 Hz larger than predicted from Figure 1. Tests of alternative choices for $\Delta\nu$ showed that this level of uncertainty produces a negligible effect on calculated activation parameters.

The parameters obtained as described were used as input for an iterative program that calculates the best average exchange lifetime, τ_e, between calculated and experimental spectra, at each temperature. Both root mean square deviations and visual comparisons of plots of calculated and experimental spectra were used in making the choice of optimal values of the exchange lifetime, τ_e, and any other quantities allowed to vary.

Results

Figure 2 shows the observed ^{13}C NMR spectra in the axial $^{13}CH_3$ region at various temperatures of [^{13}C]methylcobalamin in absolute methanol, with HCl added to achieve comparable concentrations of the base-on and base-off forms. The calculated spectra providing an optimal fit to the observed spectra are superimposed on the observed spectra. An Arrhenius plot of ln $(0.48/\tau_e)$ vs. $1000/T$ is shown in Figure 3.

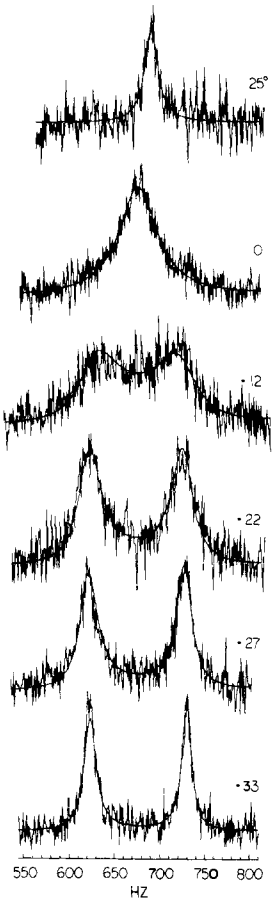


Figure 2. ^{13}C NMR spectra of the axial methyl resonance for methylcobalamin in methanol (20% CD_3OD) as a function of temperature, with pH adjusted to produce comparable concentrations of base-on and base-off forms. The chemical shifts (Hz) are given relative (upfield) to the CH_3 resonance of solvent. The lines through the spectra represent the best fit calculated spectra.

The linear least-squares fit to the results yields the activation parameters and 90% confidence limits listed in Table I.

The ^{13}C spectrum of a solution of [^{13}C]methylcobalamin in 50% methanol-D_2O mixed solvent, with pH appropriately adjusted, was also examined at several temperatures. The chemical shift separation between the ^{13}C resonances due to the base-on and base-off forms is 114.8 Hz in the mixed solvent system as compared with 106 Hz in absolute methanol. A complete fitting of line shapes over a wide range of temperatures was not attempted; the 50% CH_3OH:D_2O solvent becomes so viscous at lower temperatures that lock can no longer be maintained. However, from fitting of spectra in the region of coalescence, at −5 and −12 °C, a $\Delta G^{\ddagger}$ value of 12.7 ± 0.3 kcal/mol was obtained. This is to be compared with the value of 12.7 ± 0.1 kcal/mol obtained in absolute methanol, Table I.

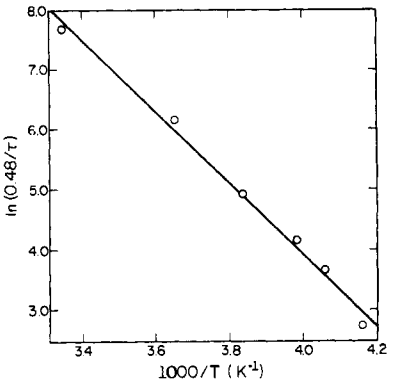


Figure 3. Arrhenius plot based on ^{13}C NMR data of the log mean time for dissociation of dmbz from methylcobalamin as a function of temperature. The line is described by the equation $(\ln (1/\tau_A) = (578/T) + 2.225$.

Figure 4 shows ^1H spectra at various temperatures for methanol solutions of methylcobalamin, with comparable concentrations of base-on and base-off. (Note the presence of an extraneous absorption. This absorption was added to the calculated spectra as a third, nonexchanging site to optimize the fit of calculated and observed spectra. It has no effect on the calculated results.) The calculated spectra based on line shape analysis are superimposed. The Arrhenius plot of ln $(0.54/\tau_e)$ vs. $1000/T$ from the ^1H results are shown in Figure 5. The activation parameters and 90% confidence limits obtained from a linear least-squares fit of the data are listed in Table I.

A best estimate of the activation parameters for benzimidazole dissociation was obtained by combining the ^1H and ^{13}C data, as shown in Figure 6. It is gratifying that the two sets of data are in excellent accord. The activation parameters and 90% confidence limits obtained from a least-squares fitting of the combined data are $\Delta H^{\ddagger}$ = 11.1 ± 0.6 kcal/mol, $\Delta G^{\ddagger}$ = 12.7 ± 0.1 kcal/mol, and $\Delta S^{\ddagger}$ = 5.9 ± 2.4 cal/(K mol) (Table I).

The ^{19}F spectra of solutions of the heptamethyl ester of cobyrinic acid, I, with excess $ImCF_3$ were obtained in methanol at various temperatures. Two signals were observed; the lower field resonance is ascribed to the coordinated base, the more intense higher field resonance to the free base. Assuming that the equilibrium constant for coordination of $ImCF_3$ to I in methanol is on the order of magnitude of that reported for coordination of imidazoles to methylcobinamide in water, ~10 M^{-1},[32,61] it is not unreasonable that only about 10% of $ImCF_3$ will be bound to I under the concentration conditions of the present experiment. This is approximately what we observe. As indicated above, it was not possible to carry out line shape fittings with sufficient reliability to furnish meaningful values of $\Delta H^{\ddagger}$ or $\Delta S^{\ddagger}$. However, it was possible to estimate $\Delta G^{\ddagger}$; the value of 16.4 ± 0.3 kcal/mol obtained for the exchange is very close to the value for $\Delta G^{\ddagger}$ obtained for the same exchange in CH_2Cl_2 solvent, Table I.

Table I. NMR Base Exchange Results for Cobalt Corrinoid Systems

Compound	Base	Nucleus obsd	Solvent	T_c[a]	$\Delta H^{\ddagger}$[b]	$\Delta G^{\ddagger}$[c]	$\Delta S^{\ddagger}$[b]	E_a	k_1 (s^{-1}) (25 °C)
[^{13}C]Methylcobalamin	dmbz	^{13}C	CD_3OD	−6	11.4 ± 1.1	12.7 ± 0.2	−4.9 ± 4.4	12.0 ± 1.1	2090
	dmbz	^{13}C	CD_3OD/D_2O	−3	...	12.7 ± 0.3	...	...	...
Methylcobalamin	dmbz	^1H	CD_3OD	−8	10.9 ± 0.9	12.7 ± 0.1	−6.7 ± 3.3	11.5 ± 0.9	2030
Methylcobalamin	dmbz	^1H, ^{13}C	CD_3OD		11.1 ± 0.6	12.7 ± 0.1	−5.9 ± 2.4	11.7 ± 0.6	...
I[d]	$ImCF_3$	^{19}F	CH_2Cl_2	49	18.7 ± 3	16.7 ± 0.2	6.0 ± 3	19.3 ± 3	3
I	$ImCF_3$	^{19}F	CD_3OD	61	...	16.4 ± 0.3	...	...	...

[a] Coalescence temperature, °C. [b] At 25 °C. [c] At coalescence temperature. [d] Data from ref 14.

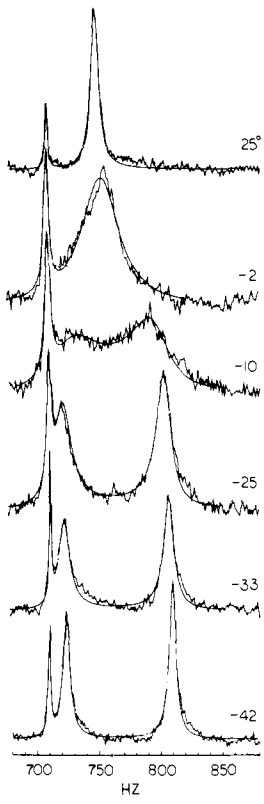


Figure 4. ^{1}H NMR spectra of the axial methyl resonance for methylcobalamin in CD_3OD, as a function of temperature, with pH adjusted to produce comparable concentrations of base-on and base-off forms. Chemical shifts are relative to the CH_3 resonance of solvent. The best fit calculated spectra are superimposed on the observed spectra.

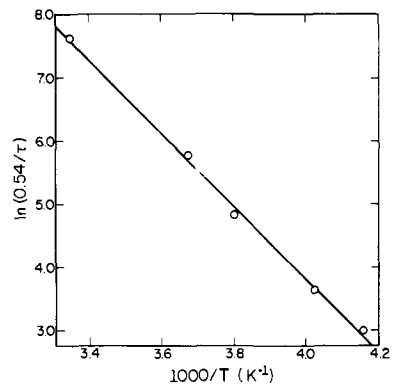


Figure 5. Arrhenius plot based on ^{1}H NMR data of log mean time for dissociation of dmbz from methylcobalamin as a function of temperature. The line is described by the equation $\ln(1/\tau_A) = 438/T + 1.674$.

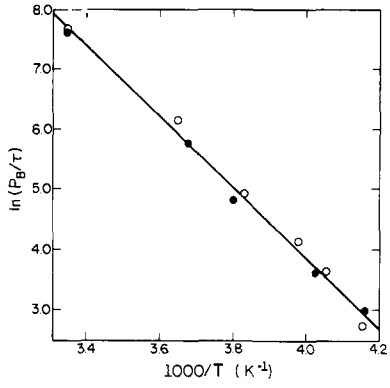


Figure 6. A combined Arrhenius plot based on both ^{1}H (●) and ^{13}C (○) data of log mean time for dissociation of dmbz from methylcobalamin as a function of temperature. The line is described by the equation $\ln(1/\tau_A) = 309/T + 1.186$.

Discussion

Qualitative indications of the dissociation rate of dmbz from CH_3Cbl have been reported prior to this work. Brodie and Poe showed that benzimidazole dissociation resulting from pH change produced a marked change in the axial CH_3 group chemical shift.[62] The ^{13}C NMR spectra of cobalamins with various axial alkyl ligands show only a single ^{13}C resonance in water at 25 °C at pH values that correspond to comparable quantities of the base-on and base-off forms.[63] These results require that the mean time between exchanges of the two forms, τ_e, is short in comparison with $1/\Delta\nu$. However, the observation that the observed line is exceptionally wide in some instances suggests that the fast exchange limit has not been reached, i.e., that coalescence lies not far below room temperature. By using methanol as solvent, it becomes possible to observe spectra corresponding to the stopped exchange and intermediate exchange region, and thus obtain quantitative kinetics data.

In a spectrophotometric kinetics study of the reaction of CN^- with alkyl cobalamins a small spectral change at short times was associated with a rate-determining dissociation of dmbz, followed by rapid binding of CN^- to the vacant position.[64] However, as noted below these observed rates are much too slow to correspond to dmbz dissociation.

Evaluation of Kinetics Data. The accuracy and precision of kinetics parameters calculated from NMR line shape data are limited by several factors.[65] The variable that most acutely affects calculated values of $\Delta H^{\ddagger}$ in most cases is the temperature dependence of the chemical shift separation, $\Delta\nu$. For both the ^{13}C and ^{1}H spectra we were able to determine the value of this variable by independent measurements with sufficient accuracy so that it is not an important uncertainty in our results.

A second factor of importance is the inherent line width of the resonances at each site in the absence of exchange. This is a potentially serious problem in the present case because of the possibility of a significant contribution from scalar relaxation of the second kind,[66,67] due to scalar coupling of either ^{1}H or ^{13}C with ^{59}Co. However, in solutions with pH values such that the cobalamin is totally in the base-on or base-off form, the ^{1}H line widths showed essentially no variation with temperature in the interval from −42 to 25 °C.

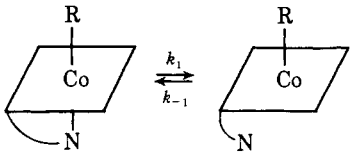
A third factor that might cause errors in the computed kinetics parameters is unaccounted for variation in the relative populations of the base-on and base-off forms as a function of temperature. It is possible to observe variations in P_A and P_B by observations of relative peak areas in the slow and stopped exchange region at various temperatures. However, in the intermediate exchange region, the only feasible means of probing this variable is to allow P_A and P_B to vary, seeking an optimal fit between calculated and observed spectra. In the CH_3Cbl solutions, there is no evidence in the stopped exchange region of a temperature variation in populations. Near the fast exchange limit, at 25 °C, the chemical shift of the single resonance occurs at a chemical shift that is a properly weighted average of the shifts for base-on and base-off forms, assuming no change in populations from the slow exchange region. The values of relative populations derived from best fits with spectra were within about 0.02 of the values obtained from the stopped exchange spectra. Variations of relative populations with ±0.02 resulted in alterations in values of the exchange parameter τ_e by about 5 to 10%.

All of these considerations suggest that the values of $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ for the dmbz dissociation from CH_3Cbl are determined by the NMR data within uncertainties at the 90%

confidence limits as shown in Table I. It is gratifying that the kinetics parameters based on the ^1H data are in good agreement with the values based on ^{13}C spectra. As a basis for subsequent discussion we employ values of $\Delta G^{\ddagger} = 12.7$ kcal/mol, $\Delta H^{\ddagger} = 11.1$ kcal/mol, and $\Delta S^{\ddagger} = -5.9$ cal/(mol K) for the benzimidazole dissociation.

Nature of the Dissociative Process. The substitutions of bases from six-coordinate alkylatocobalt(III) chelate complexes have been extensively studied. The alkyl groups are strongly trans-labilizing; thus ligand substitutions are generally quite rapid. In aprotic solvents the reactions are observed to follow a first-order rate law, with no contribution from entering ligand.[13,14] A purely dissociative (D) process is presumably involved. The activation enthalpy for base dissociation in such a case may be expected to reflect fairly well the energetics of the cobalt-ligand bond. In a protic solvent such as water some contribution might be expected from an interchange pathway. However, the importance of solvent participation in the transition state is limited by steric factors; that is, by the access solvent may have to either the coordination sphere of the metal or to some appropriate point of attachment to the departing ligand. The coordination environment about cobalt in methylcobalamin or in I is very crowded. It is thus very unlikely that solvent participates in dissociation of $ImCF_3$ from I, or of dmbz from methylcobalamin. This conclusion is supported by the observation that the rate of dissociation of $ImCF_3$ from I is essentially the same in methanol and CH_2Cl_2 solutions. The coordination about cobalt is even more crowded in methylcobalamin. We may thus assume that in methylcobalamin the dissociation of coordinated dmbz occurs as a purely D process. The observation that the rate of base exchange is essentially the same in absolute methanol and methanol-water solvent is consistent with this interpretation.

The exchange observed in the NMR experiments with methylcobalamin is that between the base-on form of methylcobalamin and a base-off form in which methanol or water occupies the sixth coordination position. Although there have at various times been claims that methylcobalamin exists in a polar solvent such as water partially in the form of a five-coordinate complex,[68] more recent evidence supports the conclusion that this species is essentially completely in the six-coordinate form.[35,70] The overall process observed in the NMR experiment can be formulated as the net result of three distinct processes (S represents coordinating solvent), eq 2-4.



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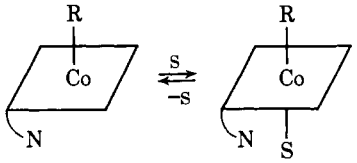

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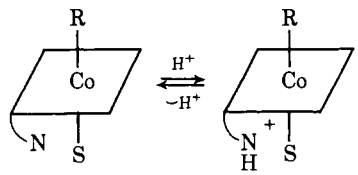

(4)

Processes 3 and 4 may of course occur concurrently. For both processes the activation barrier should be quite small. Thus the overall rate of the forward reaction is governed by $\Delta G^{\ddagger}$ for reaction 2, the base dissociation from the metal. Assuming that the rates of the forward processes in (3) and (4) are faster than for the reverse reactions, the average lifetime between exchanges for a nucleus in the base-on form, τ_A, is simply $1/k_1$. Since the mean time between exchanges determined from the line shape fitting, τ_e, is given by $1/\tau_e = 1/\tau_A + 1/\tau_B$, and since we have that $\tau_A = (P_A/P_B)\tau_B$, $k_1 = P_B/\tau_e$.

Estimated values of the first-order dissociation constants for base dissociation, k_1, at 25 °C are listed in Table I. It should be noted that k_1 for dmbz dissociation from CH_3Cbl is much larger than the estimate based on spectrophotometric kinetics data.[64] The process observed in that work, if indeed a legitimate kinetics process was being followed, is most certainly not a measure of dmbz dissociation.

To compare the activation parameters for dissociation of dmbz from methylcobalamin with data for dissociation processes involving other bases coordinated to cobalt centers, some basis for comparison of the bases themselves is needed. We have shown by comparison of data for $ImCF_3$ exchanges that the cobalt center in I is a substantially weaker Lewis acid site toward this base than the cobalt of methylatobis(dimethylglyoximato)cobalt(III).[14] A precise comparison of the basicities of $ImCF_3$ and dmbz is not available. The pK_a value for imidazole is 7.0,[71] which is to be compared with a pK_a of 6.0 for dmbz[72] and 4.7 for 1-β-D-ribo-dmbz.[72] It thus appears likely that dmbz is a slightly weaker base *toward proton* than $ImCF_3$. However, the substantially smaller value of $\Delta H^{\ddagger}$ for dissociation of dmbz from methylcobalamin as compared with dissociation of $ImCF_3$ from I cannot be accounted for by the difference in basicities as measured by pK_a values.

It is evident from examination of molecular models that the base-on form of methylcobalamin is sterically crowded in the region surrounding coordinated dmbz. ^{13}C chemical shift changes accompanying replacement of dmbz by a less bulky ligand have been interpreted[63] in terms of steric interactions. The crystal structure for adenosylcobalamin[73] shows the dmbz to be tilted from its expected orientation relative to the Co-N bond axis. These steric effects should be considerably less pronounced in the coordination of $ImCF_3$ to I. The coordinating ligand itself is considerably more compact. While the presence of methyl ester functions on the side chains adds some additional bulk to the side chains, these can be turned away from the coordinating ligand. Finally the base ligand is not constrained by its attachment to the side chain emanating from the D ring, as in the cobalamins. In contrast to the much altered steric environment at the coordination site, the electronic character of the cobalt in I should be very similar to that in methylcobalamin. Thus a substantial portion of the 7-8 kcal/mol difference in $\Delta H^{\ddagger}$ values for $ImCF_3$ dissociation from I and dmbz dissociation from methylcobalamin can be ascribed to steric factors. Pyridine and 1-methylimidazole differ in base strengths toward aqueous proton by 1.7 pK units. Empirical acid-base correlations suggest that the enthalpy of complex formation of the imidazole with methylcobaloxime should be only about 1.8 kcal/mol larger than for the more weakly basic pyridine.[74] Thus it is reasonable to suppose that the difference in base strengths between $ImCF_3$ and dmbz will contribute *at most* perhaps 2.5 kcal/mol; we thus estimate that repulsive steric interactions cause a lowering of about 5 kcal/mol in $\Delta H^{\ddagger}$ for dmbz coordination in methylcobalamin. Since the exchange process measured in the NMR experiment is purely dissociative in character, these relative $\Delta H^{\ddagger}$ values are probably good measures of the relative enthalpies of interaction of the bases with the cobalt centers. Our admittedly rather crude comparison raises several interesting considerations in connection with binding of cobalamins to proteins, and activation of the Co-C bond, as discussed in the following section.

The comparatively low value of $\Delta S^{\ddagger}$ for dmbz dissociation from methylcobalamin reflects the fact that the dissociating base is attached to the corrinoid molecule. The factors that contribute to $\Delta S^{\ddagger}$ include solvent reordering, hydrogen bonding interactions with basic sites on the side chain, and so forth. Thus the observed $\Delta S^{\ddagger}$ value does not reveal much of chemical interest about the base dissociation process.

Base Coordination in Protein Binding. There is widespread evidence that the reactivity of the Co-C bond in both methylcobalamin and adenosylcobalamin is significantly affected by the nature of the base coordinated in the trans position. Efforts have been made to identify the base bound to cobalt in the binding of methylcobalamin to proteins, in nonenzymatic as well as enzymatic cases. It has been demonstrated that the dmbz moiety is involved in binding of CH_3Cbl to hog intrinsic factor.[37,39] These studies also suggest a strongly hydrophobic binding pocket surrounding the sixth coordination position. It is not clear, however, whether dmbz is displaced in the complex. As an example of protein-bound cobalamin in an enzymatic system, Hamilton and co-workers have observed the ESR spectrum of the radical centered at adenosylcobalamin in the course of the nucleotide reductase enzymatic reaction.[47] The Co(II) species observed in this case has the appearance of five- or six-coordinate cobalt(II) with a nitrogen ligand bound to cobalt. Thus, the conclusion in this and related studies is that benzimidazole *or a similar nitrogen base* is bound to cobalt in the paramagnetic cobalt(II) form of the coenzyme.

The NMR studies presented here are relevant in several respects to the modeling of cobalamin binding to proteins and to its coenzyme activity. Binding of cobalamin to protein in any particular case could involve the amide side chains, a portion of the corrin system, and the ribose, phosphate, or dmbz portions of the chain attached to the D ring. It is not generally thought that coordination at cobalt contributes in an essential way to the energetics of binding. However, conditions in the region of the lower coordination position may be drastically altered by coordination. It is useful to consider two distinctly different hypothetical situations.

(a) If the pocket created by attachment to the protein is entirely water-free, the environment below the corrin ring provides no effective competitors with cobalt for binding to the nitrogen of dmbz. Thus, displacement of dmbz occurs only when there is available at the site of binding a base that binds more strongly to cobalt than dmbz. We have noted that the enthalpy of interaction of dmbz with cobalt, as estimated from $\Delta H^{\ddagger}$, is smaller than expected because of steric interactions. Thus a base with a smaller steric requirement, particularly an imidazole residue, might preferentially bind to cobalt. From model system studies sulfur bases appear to bind to alkylatocobalt(III) chelate complexes less strongly than imidazole or pyridine.[13] However, a cysteinyl or methionyl residue might, because of a smaller steric requirement, compete with dmbz. Additional experiments are needed to test this possibility.

(b) If water molecules are present in the coordination environment they can shift the equilibrium for base dissociation in the direction of the base-off form, by competing with cobalt for coordination at nitrogen through hydrogen bonding. The effective acidity of the medium in the binding pocket could be quite high if a small number of water molecules are involved in hydrogen bonding not only to the dmbz but to basic sites on the protein. Although some differential degree of hydrogen bond interaction with dmbz and nitrogen of the protein, especially imidazole, may occur, as a rough approximation it will be true that the effective basicities of all candidate nitrogens in the binding pocket toward coordination at cobalt will be substantially reduced. As a result coordination at cobalt by water, or by a more polarizable center that is a relatively poor base toward hydrogen bonding acids, is strongly favored.

At this point there are too few data available to permit relating these possible situations to specific protein binding and coenzyme properties of either CH_3Cbl or adenosylcobalamin. It is, however, reasonable to speculate that model (a) applies to binding in enzymatic systems, in which activation of the cobalt-carbon bond might occur by replacement of dmbz by a nitrogen ligand of lower steric requirement and higher basicity, e.g., imidazole.

On the other hand, a binding arrangement in which dmbz remains bound to the cobalt, whether or not water is present in the binding pocket, or in which a "softer" base is bound to cobalt, might apply to binding of the alkylcobalamins to transport proteins. It is also possible to consider that the reactivity of the Co-C bond might be turned off or on by a protein conformation change or an alteration in binding of the coenzyme that causes water to be admitted to or excluded from the binding pocket.

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Stereochemistry of Intermediates in Thiamine Catalysis. 2. Crystal Structure of DL-2-(α-Hydroxybenzyl)thiamine Chloride Hydrochloride Trihydrate

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Abstract: The structure of 2-(α-hydroxybenzyl)thiamine, an intermediate in a thiamine catalyzed reaction, has been determined by single crystal x-ray diffraction techniques. In this compound the thiamine conformation is similar to that found for the 2-(α-hydroxyethyl) adduct[1] and the minor modifications which do exist account very well for differences that are observed in the NMR spectra[2] of the two compounds. The intramolecular S···O interaction which was first characterized in the previous adduct structures is found in this compound as well. The close intramolecular contact between the overlapped parallel phenyl and pyrimidinium rings is a structural feature that provides additional conformational stability as well as offering some interesting mechanistic implications. The crystal structure was determined using diffractometer data obtained by the θ:2θ scan technique with Cu radiation from a crystal having space group symmetry $C2/c$ and unit cell parameters a = 27.82 (4), b = 7.478 (8), c = 24.11 (3) Å, and β = 110.00 (7)°. The structure was solved by direct methods and refined by least squares to an R = 0.080 for all 3871 independent reflections and an R = 0.065 for the 2340 observed reflections.

Thiamine, in the form of the pyrophosphate ester, is a coenzyme in a number of enzyme systems that catalyze the decarboxylation of α- keto acids and the transfer of aldehyde or acyl groups.[3] Thiamine C(2) adducts are intermediates in the reaction mechanism.[4] Many of these intermediates are sufficiently stable under mildly acidic conditions to be isolated. It has previously been shown with 2-(α-hydroxyethyl)thiamine, HET, that when substitution occurs on C(2) the molecular conformation with respect to the C(3,5′) bridge carbon atom undergoes a substantial change from that which characterizes the free thiamine molecule.[1] Besides having a different molecular conformation, the adduct compounds display an apparent conformational stability that is imparted through an intramolecular S···O interaction with the O(2α1) oxygen.