Toward an Understanding of the Activation of Organocobalt Corrinoids for Co-C Bond Homolysis in Aqueous Solution: Temperature Control in the Determination of Kinetic Constants for the Thermal Decomposition of Neopentylcobalamin

Kenneth L. Brown* and Daniel R. Evans

Department of Chemistry, Box CH, Mississippi State University, Mississippi State, Mississippi 39762

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Examination of literature values for the thermolysis of neopentylcobalamin (NpCbl) in *aqueous* solution shows significant variation in the observed activation parameters. Re-evaluation of the reaction conditions and the techniques employed reveals that this variation is due to errors in temperature maintenance and measurement. Thus, the thermolysis of the carbon-cobalt bond of NpCbl in aqueous solution has been reexamined (T = 14.8-39.6 °C) taking particular care to carefully maintain and measure the temperature. The resulting observed activation parameters, $\Delta H_{obs}^{\ddagger}$ = 26.5 ± 0.1 kcal mol^{-1} and $\Delta S_{obs}^{\ddagger}$ = 12.5 ± 0.3 cal mol^{-1} K^{-1}, are larger than values previously reported which we believe in part to be attributable to this small but significant systematic error. The thermodynamic parameters for the base-off/base-on equilibrium of NpCbl have been determined by an NMR method which is also shown to be susceptible to a potential systematic error in temperature measurement. The thermodynamic parameters for the base-off/on equilibrium were found to be ΔH_{meas} = −4.1 ± 0.1 kcal mol^{-1} and ΔS_{meas} = −12.5 ± 0.3 cal mol^{-1} K^{-1}. Using these values, the observed rate constant for NpCbl thermolysis can be corrected for the substantial amount of base-off species present in neutral aqueous solution. The corrected activation parameters for the base-on species are $\Delta H_{on}^{\ddagger}$ = 28.3 ± 0.2 kcal mol^{-1} and $\Delta S_{on}^{\ddagger}$ = 19.3 ± 0.6 cal mol^{-1} K^{-1}. Methods for data analysis and the effects or errors in temperature measurement on the final results are discussed in detail.

Introduction

5′-Deoxyadenosylcobalamin (AdoCbl,[1] coenzyme B_{12}, Figure 1) is known to participate in a number of important enzymatic reactions in which 1,2-intramolecular rearrangements of substrates occur.[3-6] There is general agreement that the initial step in the catalytic cycle involves reversible carbon-cobalt bond homolysis to give a 5′-deoxyadenosyl radical and cob(II)alamin, the so-called "activation" of AdoCbl. Though many mechanisms have been proposed to explain this activation,[4e,8-21] little is actually known about this important process. Finke and Hay[7] have shown that the lability of the Co-C bond of AdoCbl is increased approximately 10^{12}-fold by the enzyme diol dehydratase. These authors' data[7b] show that AdoCbl is extremely stable (the half-time for thermolysis of AdoCbl at 37°C is 1.9 years). In addition, studies of the thermolysis of AdoCbl are complicated by the fact that there is competing Co-C bond heterolysis due to presence of the β-oxygen atom in its organic ligand.[7,22-24] Due to the extraordinary stability of the C-Co bond of AdoCbl and the complicating side reaction, investiga-

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(1) (a) IUPAC-IUB nomenclature[2] is used throughout. Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); NpCbl, neopentylcobalamin; $NpCbi^{+}$, neopentylcobinamide; CNCbl, cyanocobalamin (vitamin B_{12}); Bzm, 5,6-dimethylbenzimidazole; NpBr, 1-bromo-2,2-dimethylpropane (neopentyl bromide); NBS, National Bureau of Standards. (b) All cobalamins discussed in this paper have the organic ligand coordinated to the cobalt at the upper (or β) face of the complex unless explicitly stated otherwise.
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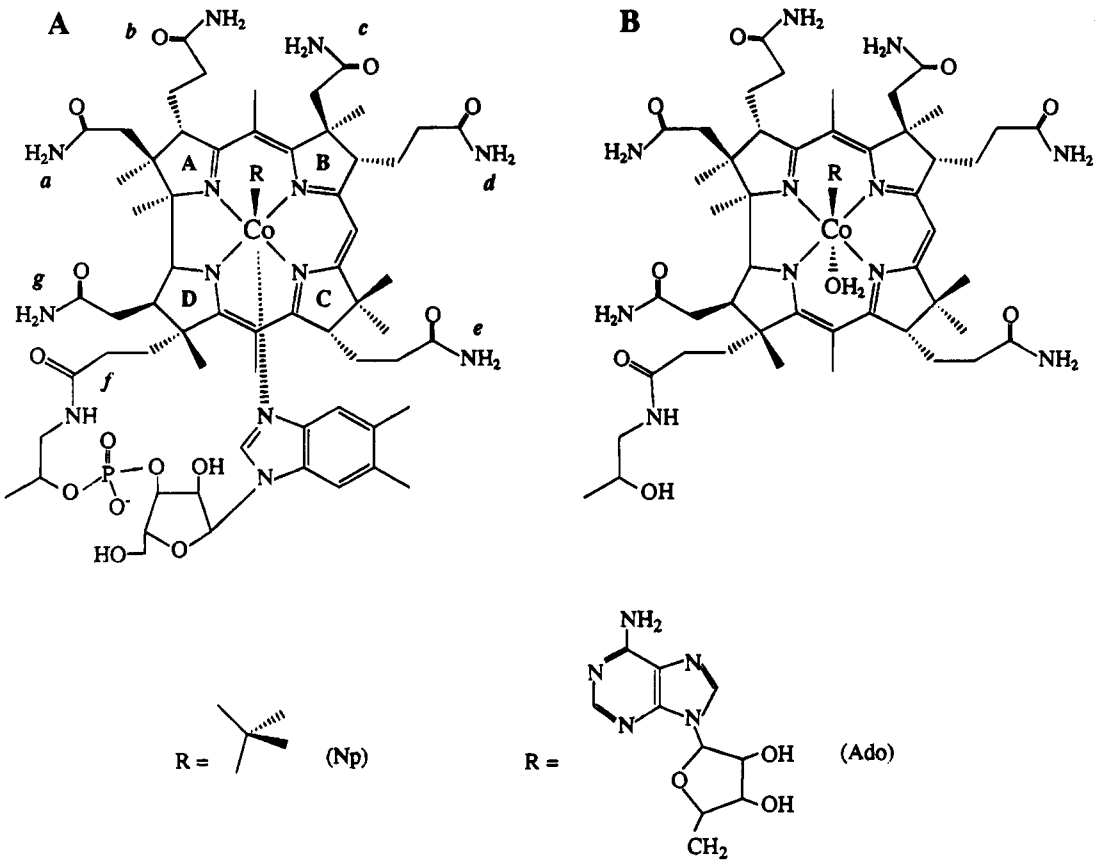

Figure 1. Structure of the 5′-deoxyadenosyl (Ado) and neopentyl (Np) derivatives of (A) cobalamin and (B) cobinamide.

tions of the thermal C−Co cleavage of AdoCbl and its derivatives are technically difficult.

These difficulties may be circumvented by the use of model compounds, such as, NpCbl,[25−29] which decomposes far more rapidly than AdoCbl and does so exclusively by a homolytic pathway.[26−29] Due to the intramolecular coordination equilibrium of the pendent nucleotide, NpCbl exists as two species, the so-called base-off and base-on species, at neutral pH (Figure 1). It is well known that the base-off species of NpCbl, and the analogous cobinamide species in which the axial nucleotide has been removed by phosphodiester hydrolysis (Figure 1), are some 2−3 orders of magnitude less reactive than the base-on species,[7c,9,10,26,28,29] a phenomenon which has been shown to be due entirely to entropic effects.[28] We are consequently interested in exploring the structural aspects of corrinoids which affect the entropy of activation for Co−C bond homolysis, in an attempt to try to understand how AdoCbl activation might be entropically catalyzed. As a result, we wish to study the thermolysis of NpCbl analogs in which the corrinoid structure has been chemically altered, for instance by altering the number and/or structure of the upwardly projecting side chains. This requires that the activation parameters for the thermolysis of the parent compound, NpCbl, be known accurately in order to permit precise comparisons to those for the NpCbl analogs. Unfortunately, the activation parameters currently in the literature for NpCbl are quite diverse, leading to significant questions as to what the correct values actually are.

Five sets of values are currently available in the literature for the activation parameters for NpCbl thermolysis.[26−29] Of these, two sets were determined in ethylene glycol[27,29] and three (including one from this laboratory) were determined in aqueous solution.[26−28] Since there is a well known solvent effect[30] that has been quantitatively explained in terms of the expected solvent cage effect,[31] data obtained in these two solvents are not directly comparable until the effect of the solvent cage on the thermolysis reaction of NpCbl has been deconvoluted. Of the three sets of values determined in *aqueous* solution, the observed enthalpy of activation varies from 23.1 to 25.1 kcal mol^{-1} while the observed entropy of activation varies from 2 to 7.9 cal mol^{-1} K^{-1}.

In light of this variability, we have carefully re-examined the experimental conditions, techniques, and methods of data analysis we previously employed in our study of NpCbl thermolysis in aqueous solution.[28] In the process of so doing, we discovered a small, but significant systematic error in temperature control which we now believe to be responsible for the variation in the literature values for NpCbl thermolysis in aqueous solution.[32−33] We now describe the effect of this temperature error on the observed rate constants and activation parameters for the thermolysis of NpCbl. We also present a

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detailed statistical analysis which results in the proper propagation of all sources of uncertainty in the final values of the activation parameters.

Experimental Section

Materials. 1-Bromo-2,2-dimethylpropane (neopentyl bromide, NpBr[1]) was from Aldrich and CNCbl was from Roussel. NpCbl was prepared in a manner similar to that originally described by Schrauzer and Grate.[26] $NpCbi^+$ was prepared as described previously.[28] Glass-distilled water was used throughout, and all work with organocobalt corrinoids was carried out in the dark (with the aid of flashlights) to prevent photolytic decomposition. Measurements of pH were made using a Radiometer PHM 84 pH meter and a Radiometer type C combination glass electrode with electrode, samples, standards, and rinse water incubated at the appropriate temperature.

UV−visible spectra were obtained on a Cary 219 recording spectrophotometer equipped with a thermostated five-cell sample turret, and temperature was maintained by the use of a Neslab RTE-220 circulating water bath.

Purity of samples was checked periodically using analytical HPLC which was carried out using a 0.46 × 25 cm Beckman Ultrasphere Octyl column with 25 mM ammonium phosphate buffer (pH 3.0, solvent A) and CH_3CN (solvent B)[35] using a flow rate of 2.0 mL/min. The gradient used was 5% B for 0.5 min, followed by a linear increase to 30% B over 9 min. After a 1.5 min hold at 30% B, a 1 min decrease to 5% B re-established initial conditions.

Temperature was measured using a YSI 702A thermistor probe and a YSI 740AX signal conditioner or a Cole Parmer 93-100 thermistor device.[36] Each thermistor probe−signal conditioner combination was carefully calibrated against NBS calibrated thermometers using stem corrections. Samples for spectrophotometric kinetic measurements (total volume 3.0 mL, in 1.000 cm path length quartz cuvettes) contained $(1.0-2.0) \times 10^{-5}$ M NpCbl, 0.1 M phosphate buffer, and sufficient KCl to adjust the ionic strength to 1.0 M. The NpCbl stock solution was stored in a mildly acidic HCl solution (pH 3) at −20 °C in containers covered with aluminum foil to prevent decomposition. Reactions were initiated by addition of a small volume of NpCbl stock solution to the sample cuvette which had been incubated in the spectrophotometer cell block for at least 30 min. Sample temperature was measured by thermistor measurement in a dummy sample cuvette before and after each kinetic run, and the kinetic results were rejected unless the two measurements agreed to within <0.2 °C.[37] Absorbance was measured as a function of time at 352 nm for at least 5 half-times, and the data were fitted to eq 1, where A_t is the absorbance at time t,

$$A_t = \Delta A[(1 - \exp(-k_{obs}t))] + A_0 \quad (1)$$

A_0 is the initial absorbance, and ΔA is the difference between the final absorbance and initial absorbance, using a non-linear least squares algorithm. For thermolysis of the neutral species of NpCbl, k_{neut} ($=k_{obs}$), was determined as the weighted-average of four or five kinetic runs at pH ≥ 7.5. Since $pK_{base-off}$, the pK_a for the base-off/on reaction of NpCbl, is ≤5.3 in the temperature range employed here,[28] this condition insures the absence of any protonated, base-off NpCbl. For temperatures >30°C, for which $t_{1/2}$ < 40 min, the decrease in temperature upon initiation of the reaction was minimized by pre-incubating the NpCbl stock solution at the measurement temperature, adding the stock solution directly to the cuvette in the cell block with a digital pipet, and mixing the sample with a plastic rod which had also been pre−incubated at the measurement temperature.

Anaerobic NMR samples of NpCbl and $NpCbi^+$ (23−35 mM) in 0.7 mL of "100%" D_2O, containing 0.1 M potassium phosphate buffer (pD = 8.2) and at ionic strength 1.0 M (KCl) were prepared as follows. The complex (in the protonated, base-off form for NpCbl) was dissolved in 1.0 mL of 99.9% D_2O and evaporated to dryness. This was repeated twice to remove exchangeable hydrogens and minimize the solvent residual peak. Separately, the appropriate amount of potassium phosphate salts and KCl were similarly exchanged with D_2O. Under an argon atmosphere, the phosphate/KCl mixture was dissolved in 0.7 mL of "100%" D_2O and this solution was used to dissolve the sample. The solution was immediately transferred to a 9 in. 5 mm NMR tube and frozen. The sample was then evacuated and allowed to thaw. The freeze−pump−thaw cycle was repeated twice more and the sample was sealed under vacuum.

^1H NMR spectra were recorded on a GE QE-300 NMR spectrometer operating at 300.669 MHz. Typically, 160 scans were collected into 16K data sets using a spectral width of 3610 Hz. The data were processed using exponential multiplication of 0.5 Hz. Resonance frequencies were determined by digital fitting of the peaks of interest to Lorenzian line shapes. At least 12 min of temperature equilibration was allowed before data acquisition. The actual temperature of the sample in the probe was measured in a manner similar to that described by van Geet.[38] The calibrated thermistor probe was placed in a 5 mm NMR tube which was placed in the probe cavity for temperature measurement. The measured temperature equilibration half-time was about 1 min.

Results

Thermolysis Kinetics. As mentioned above, the thermolysis reaction for NpCbl has been extensively examined.[26-29] The mode of cleavage of the Co−C bond has been determined to be homolytic,[26-29] where the products obtained upon cleavage of the Co−C bond are Np• and cob(II)alamin. The neopentyl radical is scavenged quite readily in presence of "$Co(D_2H_2)$",[27] O_2,[26,28] or H-TEMPO.[29] Under aerobic conditions the neopentyl radical reacts with molecular oxygen with a second-order rate constant of 2.65×10^9 M^{-1} s^{-1}.[39] The competence of molecular oxygen as a radical trap for this thermolysis reaction has been demonstrated quite conclusively,[28] as the rate of thermolysis of NpCbl in anaerboic aqueous solution in the presence of excess H-TEMPO has been shown to be identical (within experimental error) to the rate obtained for the thermolysis of the same complex in air-saturated aqueous solution.

The results of our re-examination of the kinetics of thermal decomposition of NpCbl in aerobic aqueous solution are summarized in Table 1. The reaction of interest can be seen to be quite sensitive to temperature as an increase in the temperature of 5 deg results in about a 2-fold increase in the observed rate constant. The tabulated values of k_{neut}, the observed first-order rate constant for thermolysis of the neutral species of NpCbl, represent weighted averages, eq 2,[40] where W_i is a

$$k_{neut} = (\sum W_i k_i)/N \quad (2)$$

normalized weight, k_i is the ith observed rate constant, and N is the number of observations. The normalized weights, W_i (eq 3), were obtained from the specific weights, w_i, which were

$$W_i = N w_i / \sum w_i \quad (3)$$

determined from the standard deviation, s_{ki}, for each individual determination as $w_i = (1/s_{ki})^2$. As pointed out by Benson,[38a] the sum of the normalized weights must be equal to the number of observations, N, in order to calculate the weighted standard deviation of the weighted mean. Clearly, some rate data fit eq 1 better than others, and thus this procedure takes this variability

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Table 1. Rate Constants for the Thermolysis and Equilibrium Constants for the Base-Off/On Reaction of NpCbl in Neutral Aqueous Solution[a]

T/°C[b]	k_{neut}/s^{-1} [c]	K_{meas}[d]	f_{on}[e]	k_{on}/s^{-1} [f]
14.8	$(2.36 \pm 0.08) \times 10^{-5}$	2.213 ± 0.038	0.689 ± 0.010	$(3.43 \pm 0.13) \times 10^{-5}$
20.0	$(5.61 \pm 0.09) \times 10^{-5}$	1.925 ± 0.030	0.658 ± 0.010	$(8.53 \pm 0.19) \times 10^{-5}$
25.1	$(1.30 \pm 0.01) \times 10^{-4}$	1.697 ± 0.025	0.629 ± 0.009	$(2.07 \pm 0.03) \times 10^{-4}$
30.0	$(2.70 \pm 0.02) \times 10^{-4}$	1.535 ± 0.021	0.605 ± 0.008	$(4.46 \pm 0.07) \times 10^{-4}$
35.0	$(5.50 \pm 0.09) \times 10^{-4}$	1.376 ± 0.018	0.579 ± 0.008	$(9.50 \pm 0.20) \times 10^{-4}$
39.6	$(1.064 \pm 0.006) \times 10^{-3}$	1.260 ± 0.016	0.558 ± 0.007	$(1.91 \pm 0.03) \times 10^{-3}$
45.2[g]	$(2.06 \pm 0.06) \times 10^{-3}$	1.146 ± 0.014	0.534 ± 0.006	$(3.86 \pm 0.12) \times 10^{-3}$
55.1[g]	$(6.33 \pm 0.07) \times 10^{-3}$	0.954 ± 0.010	0.488 ± 0.005	$(1.30 \pm 0.02) \times 10^{-2}$

[a] Ionic strength 1.0 M (KCl), pH 7.69−7.84 (depending on temperature). [b] All temperature measurements maintained within ±0.1 °C. [c] Weighted averages of the observed rate constant for the aerobic thermolysis of the neutral species of NpCbl unless stated otherwise. The uncertainty represents the weighted standard deviation about the weighted mean of four or five determinations unless explicitly stated otherwise. [d] Measured equilibrium constant for the conversion of the base-off species to the base-on species at neutral pH. Calculated from $\Delta\nu_{obs}$ and $\Delta\nu_0$ for temperatures employed for the NMR experiment and interpolated as necessary (see Figure 4). [e] Fraction of NpCbl present in the base-on form (where the fraction of base-on was determined from $K_{meas}/(1 + K_{meas})$). [f] Corrected rate constant as corrected from k_{neut} for the percentage of base-on present. [g] Unweighted averages and standard deviations which were not used in Eyring plots (Figure 3). See text.

into account. The normalized weights (eq 3) were also used to determine the weighted standard deviation by eq 4. The effect

$$s_{k_{neut}} = [(\sum W_i^2(k_i - k_{neut})^2)/(N-1)]^{1/2} \quad (4)$$

of this procedure is to put more emphasis on those fits that gave inherently smaller standard deviations for the observed rate constants. By doing so, the imprecision is decreased for some but not all of the values. For the rate constants obtained at the temperatures of 25.1, 30.0, and 39.6 °C, the weighted averages and standard deviations were identical to those obtained arithmetically, but this was not the case for the remaining determinations.[41]

Great pains were taken to minimize any change in sample temperature upon initiation of the reaction. For temperatures significantly above ambient (T > 30 °C), this included preincubation of the NpCbl stock solution, addition of the preincubated NpCbl while the sample remained in the spectrophotometer cell block, and sample mixing with a plastic mixing device which had also been pre-incubated at the sample temperature. Even this methodology was insufficient to prevent a significant, measurable sample temperature drop at T > 40 °C, simply due to removing the sample compartment cover from the spectrophotometer. This can be seen in Figure 2, where ΔT_{obs}, the difference between the equilibrium temperature (here, 55.0 °C) and the observed temperature, is plotted against time for a dummy sample after the sample compartment was opened for the length of time needed to initiate a reaction. These data fit a simple exponential from which the time constant for temperature re-equilibration was determined to be $(3.36 \pm 0.04) \times 10^{-3}$ s^{-1} ($t_{1/2}$ = 3.4 min) and the maximum temperature deviation due to opening the sample compartment and "initiation" of the mock reaction was 0.56 ± 0.1 °C. Since the observed halftime for NpCbl thermolysis at this temperature was 1.82 min (Table 1), it is clear that temperature re-equilibration (to within the required tolerance of ±0.1 °C) will not occur until the reaction is nearly complete. Thus, accurate kinetic data for this reaction cannot be obtained at temperatures this far above ambient using conventional methodology.

The effect of this temperature perturbation is clearly seen in an Erying plot (Figure 3) in which the data at T > 40 °C (open symbols) clearly fall below the line determined by the six data points at $T \leq 40$ °C. As a result, the data at T > 40 °C were not used in calculating the activation parameters for NpCbl

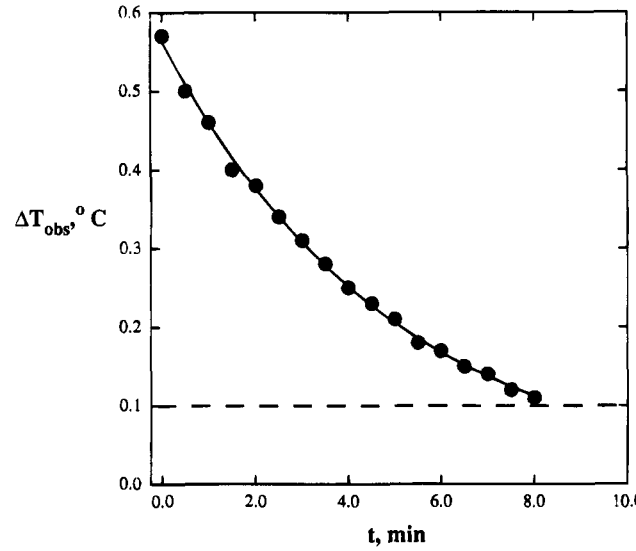

Figure 2. Plot of ΔT_{obs}, the difference between the equilibrium temperature (55.0 °C) and the observed temperature, vs time for a mock kinetic sample arfter reaction "initiation". Data were fit to a single decreasing exponential using a non-linear least-squares algorithm to give 0.561 ± 0.004 °C for the maximum temperature deviation and $(3.36 \pm 0.04) \times 10^{-3}$ s^{-1} for the time constant for temperature re-equilibration. The dashed line indicates the tolerated limit for the temperature variation.

thermolysis. For this analysis, the uncertainties in k_{neut} and T were propagated into the standard deviation of $\ln(k_{neut}h/k_BT)$, s_Y, via eq 5, and a weighted least squares regression[42] was used,

$$s_Y = [(s_{k_{neut}}/k_{neut})^2 + (s_T/T)^2]^{1/2} \quad (5)$$

where the specific and normalized weights were determined from s_Y values as described above. The resulting values for $\Delta H_{obs}^{\ddagger}$ and $\Delta S_{obs}^{\ddagger}$ (Table 2) were 26.5 ± 0.1 kcal mol^{-1} and 12.5 ± 0.3 cal mol^{-1} K^{-1}, respectively. Inclusion of the rate constant determined at 45.2 °C lowers the intercept of the Eyring plot (and hence $\Delta S_{obs}^{\ddagger}$) by 2.4%, while inclusion of both data points at T > 40 °C lowers the intercept by 31%.

Axial Nucleotide Coordination Equilibrium. It is now abundantly clear NpCbl exists significantly as the base-off species, even in neutral solution where the axial nucleotide is completely unprotonated.[26-29] Since the protonated base-off species is at least 2 orders of magnitude less reactive than the neutral species,[26,28,29] rate constants for the thermolysis of the

(41) The data for the remaining temperatures were as follows. 14.8 °C: $k_{neut} = (2.36 \pm 0.08) \times 10^{-5}$ s^{-1}, $k_{uw} = (2.46 \pm 0.12) \times 10^{-5}$ s^{-1}. 20.0 °C: $k_{neut} = (5.61 \pm 0.09) \times 10^{-5}$ s^{-1}, $k_{uw} = 5.59 \pm 0.17 \times 10^{-5}$ s^{-1}. 35.0 °C: $k_{neut} = (5.50 \pm 0.09) \times 10^{-4}$ s^{-1}, $k_{uw} = (5.51 \pm 0.13) \times 10^{-4}$ s^{-1}. k_{uw} is the unweighted average of the k_{obs}'s.

(42) Mortimer, R. G. *Mathematics for Physical Chemistry*; Macmillan Publishing Co., Inc.: New York, 1981; pp 266−296.

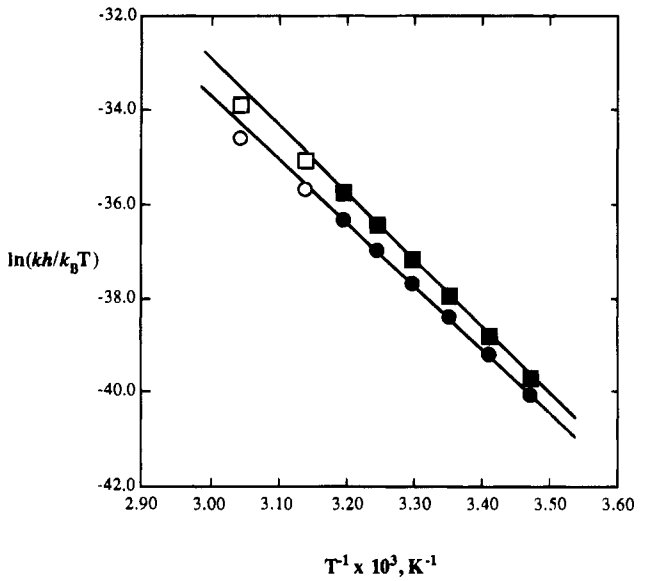

Figure 3. Eyring plot of the observed (●, ○) rate constants of the thermolysis of the neutral species of NpCbl and the corrected (■, □) rate constants for the thermolysis of the base-on species of NpCbl. The solid lines are weighted linear regression lines for which the data at $T > 40$ °C (open symbols) were *not* used (see text): observed, slope = $(-1.334 \pm 0.005) \times 10^4$ K, intercept = 6.29 ± 0.16, $r^2 = 0.9998$; corrected, slope = $(-1.422 \pm 0.009) \times 10^4$ K, intercept = 9.70 ± 0.30, $r^2 = 0.9998$.

Table 2. Observed and Corrected Activation Parameters for the Thermolysis of NpCbl

T/°C	$\Delta H_{obs}^{\ddagger}$/ kcal mol^{-1}	$\Delta S_{obs}^{\ddagger}$/cal mol^{-1} K^{-1}	$\Delta H_{on}^{\ddagger}$/ kcal mol^{-1}	$\Delta S_{on}^{\ddagger}$/cal mol^{-1} K^{-1}	ref
25−60[a]	23.4 ± 0.2	2.6 ± 0.1			26
25.4−45.6[b]	23.1 ± 0.7	2 ± 2	26.7 ± 1.2	15 ± 5	27
25−55[c]	25.1 ± 0.5	7.9 ± 1.5	28 ± 1	21 ± 1	28
14.8−39.6[d]	26.5 ± 0.1	12.5 ± 0.3	28.3 ± 0.2	19.3 ± 0.6	*e*

[a] pH 7.0 using sodium phosphate buffer (ionic strength unspecified). [b] pH 6.8 using potassium phosphate buffer (ionic strength unspecified). [c] pH 7.5 using potassium phosphate buffer (ionic strength 1.0 M (KCl)). [d] pH 7.69−7.84 (depending upon temperature) using potassium phosphate buffer (ionic strength 1.0 M (KCl)). [e] This work.

base-on species, k_{on}, can be calculated from the observed rate constants for the neutral species, k_{neut}, from $k_{on} = k_{neut}/f_{on}$, where f_{on} is the fraction of NpCbl as the base-on species at neutral pH. Estimation of the relative amounts of base-on and base-off NpCbl at neutral pH is the second major source of error in evaluation of the activation parameters for thermal homolysis of NpCbl.

We have previously discussed[43] what we see as the shortcomings of the spectrophotometric method[7b,10a27] for estimating the effect of temperature on the base-off/on equilibrium of RCbls, and at that time developed a new method based on measurement of the temperature dependence of the ^{13}C chemical shift difference between $^{13}CH_3Cbl$ and $^{13}CH_3Cbi^+$.[43] Waddington and Finke[29] have now greatly extended the utility of this method by use of the ^1H NMR resonance of the C10 hydrogen (Figure 1), which permits its use with RCbls in which the organic ligand cannot be easily labeled. We have consequently employed the same method using sealed anaerobic NRM samples of NpCbl and $NpCbi^+$ at neutral pH, which proved to be extraordinarily stable even at $T > 80$ °C.

The difference in resonance frequency, $\Delta\nu_{obs}$, between the C10 proton of $NpCbi^+$ and that of NpCbl was determined at

(43) Brown, K. L.; Peck-Siler, S. *Inorg. Chem.* **1988**, *27*, 3548.

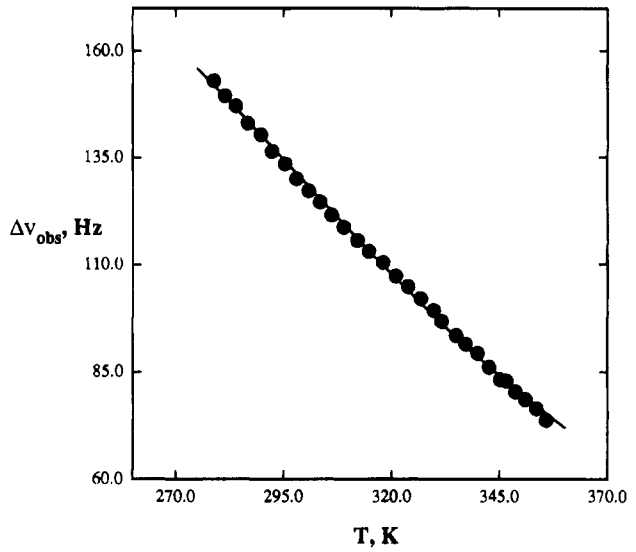

Figure 4. Plot of $\Delta\nu_{obs}$, the difference in ^1H NMR resonance frequency between the C10 hydrogen of $NpCbi^+$ and NpCbl, vs temperature. The solid line is a non-linear least squares curve fit (see eq 6) from which the values $\Delta H_{meas} = -4.1 \pm 0.1$ kcal mol^{-1}, $\Delta S_{meas} = -12.5 \pm 0.3$ cal mol^{-1} K^{-1}, and $\Delta\nu_0 = 206 \pm 1$ Hz were obtained.

approximately 3 °C intervals between about 5 and 83 °C and the data were fitted to eq 6 where ΔH_{meas} and ΔS_{meas} refer to

$$\Delta\nu_{obs} = \{\Delta\nu_0 \exp[(\Delta S_{meas}/R) - (\Delta H_{meas}/RT)]\}/\{1 + \exp[(\Delta S_{meas}/R) - (\Delta H_{meas}/RT)]\} \quad (6)$$

the equilibrium constant, K_{meas},[44] for the conversion of all base-off species to the base-on form, and $\Delta\nu_0$ is the difference in resonance frequency between the base-on species and the base-off species. The data (Figure 4) were treated as previously described[29,43] to obtain the values of $\Delta\nu_0 = 206 \pm 1$ Hz, $\Delta H_{meas} = -4.06 \pm 0.1$ kcal mol^{-1} and $\Delta S_{meas} = -12.5 \pm 0.3$ cal mol^{-1} K^{-1} for the apparent equilibrium between all base-off species and base-on NpCbl. We note that these thermodynamic parameters are significantly different from those determined by Waddington and Finke[29] for the off/on equilibrium of NpCbl in ethylene glycol, particularly the entropy term ($\Delta\Delta S = \Delta S_{ethylene\ glycol} - \Delta S_{H_2O} = -5.3 \pm 1.0$ cal mol^{-1} K^{-1}). This mimics the solvent effect previously seen be Finke and Hay[7b,34] for AdoCbl ($\Delta\Delta S = -7.2 \pm 3.7$ cal mol^{-1} K^{-1}) and confirms the conclusion[29] that the base-off/on equilibrium of RCbls is highly solvent dependent. Thus, while NpCbl is 63% base-on in neutral aqueous solution at 25 °C, it is only 26% base-on in ethylene glycol at this temperature.[29] With these values, the rate constants for thermolysis of the base-on species of NpCbl, k_{on}, can be calculated at neutral pH and any temperature (Table 1). However, in order to account for the uncertainty inherent in the measurement of the difference between the two frequencies, K_{meas} was calculated from eq 7,[45] which permits the ready

$$K_{meas} = \Delta\nu_{obs}/(\Delta\nu_0 - \Delta\nu_{obs}) \quad (7)$$

propagation of the uncertainties in $\Delta\nu_{obs}$ and $\Delta\nu_0$ into K_{meas} by eq 8.[46] The standard deviations of both f_{on} (=$K_{meas}/(K_{meas}$ +

(44) This analysis includes the implicit assumption that the "tuck-in" species[43] of base-off, but benzimidazole-deprotonated NpCbl, if indeed it occurs to any significant extent,[29] is as kinetically inert as base-off NpCbl.

(45) The values obtained for K_{meas} from eq 7 are identical to those obtained from ΔH_{meas} and ΔS_{meas}.

(46) The standard deviations of the observables $\Delta\nu_{obs}$ and $\Delta\nu_0$ employed were 0.5 Hz and 1.0 Hz, respectively.

$$s_{K_{meas}} = \{[(\Delta\nu_{obs}s_{\Delta\nu_0})^2 + (\Delta\nu_0 s_{\Delta\nu_{obs}})^2]/(\Delta\nu_0 - \Delta\nu_{obs})^4\}^{1/2} \quad (8)$$

1))[47] and k_{neut} were propagated from these values using standard techniuqes,[42] into the standard deviation of k_{on} as shown in eq 9. The Eyring plot for the corrected rate constants is shown in

$$s_{k_{on}} = [(s_{k_{neut}}/k_{neut})^2 + (s_{f_{on}}/f_{on})^2]^{1/2} \quad (9)$$

Figure 3. From the final values of k_{on} and their standard deviations, the activation parameters (and their standard deviations) for the thermolysis of base-on NpCbl were determined by a weighted least squares regression as described above. The specific weights were obtained in a manner identical to that for the observed rate constants (eq 5) except that $s_{k_{neut}}$ and k_{neut} are replaced by $s_{k_{on}}$ and k_{on}. This gave (Table 2) $\Delta H_{on}^{\ddagger} = 28.3 \pm 0.2$ kcal mol^{-1} and $\Delta S_{on}^{\ddagger} = 19.3 \pm 0.6$ cal mol^{-1} K^{-1}. These values are significantly different from those obtained in ethylene glycol by Waddington and Finke,[29] due to the expected solvent cage effect.[31]

In should be noted here that an additional source of error can be made in such NMR measurements if the probe temperature is not directly monitored using an external device. Temperatures directly measured in the sample probe using a thermistor device generally agreed quite well with those given by the NMR spectrometer's VT pyrometer at near ambient temperatures. However, at temperatures far from ambient, temperature differences as large as 5−6 °C can occur between the VT thermocouple (positioned just below the sample in the VT gas stream) and in the sample itself. This potential source of error is depicted in Figure 5, (where the ordinate is the observed temperature while the abscissa is the designated VT temperature.

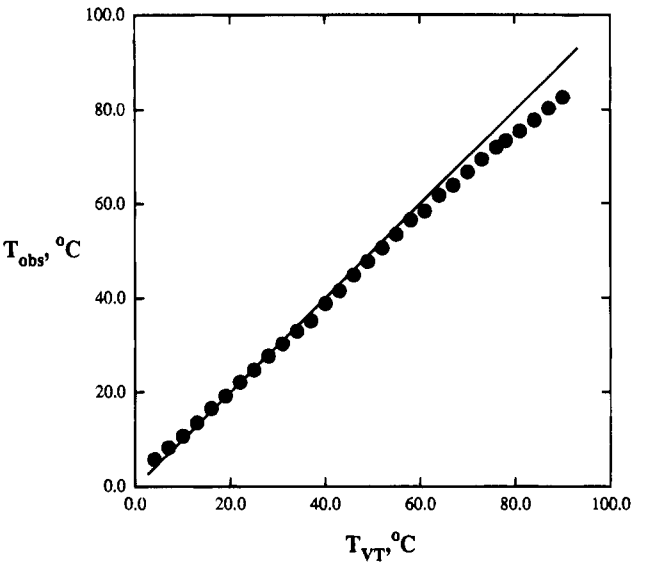

Figure 5. Plot of T_{obs}, the measured NMR sample probe temperature, vs T_{VT}, the temperature indicated by the spectrophotometer's VT pyrometer. The solid line is a plot of $T_{obs} = T_{VT}$.

Discussion

The problem of temperature maintenance during a kinetic experiment was treated some time ago by Horton and this treatment is quite applicable for the discussion at hand.[48] It must be stressed that the errors caused by such temperature maintenance problems are not, in general, observed in the standard deviation of the observed rate constants, except in the most extreme cases. Our experience with the maintenance and measurement of temperature during kinetic measurements of the thermolysis of NpCbl strongly suggests that problems in this area are a major source of error and contribute significantly to the discrepancies among the literature values *in aqueous solution* for the activation parameters for this process. Because of the high sensitivity of this reaction to temperature (a 10 deg change in temperature results in an approximate 4-fold increase in the rate constant), a temperature measurement error of as little as 0.5 °C can cause an 8% error in the observed rate constant. This is particularly problematic for this reaction at above-ambient temperatures due to the unavoidable decrease in sample temperature upon reaction initiation and the fact that at $T > 40$ °C, the half-time for the reaction is comparable to the half-time for temperature equilibration. Even when extreme care is exercised to minimize such temperature deviations, measurable decreases in temperature occur when these reactions are initiated. For example (see Figure 2, $T_{equil} = 55.0$ °C), simply removing the sample compartment cover for the length of time necessary to add the NpCbl and mix the reaction solution (*without* removing the sample from the cell block) causes a measured temperature drop of 0.6 °C. Since the calculated half-time for thermolysis at this temperature is 1.8 min, and the temperature equilibration half-time (in our apparatus) is 3.4 min, it is clear that rate constants of sufficient accuracy for determination of these activation parameters cannot be obtained at these temperatures using this methodology (i.e., the time required for the sample temperature to retun to within 0.1 °C of the equilibrium temperature is equal to 4.7 times the reaction half-time). It is very important to note, though, that the standard deviations that were obtained for the two rate constants at 45.2 and 55.1 °C in no way reflect the variation that is occurring due to this temperature perturbation. Since these temperature deviations must result in an under estimation of the rate constants at the upper end of the temperature range of measurements, and since the problem becomes more severe with increasing temperature, the result of inclusion of such data is to decrease both the slope, and more severely, the intercept of an Eyring plot, leading to significant underestimation of both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. This effect is clearly evident from the data in Figure 3. Inclusion of the data point at 45.2 °C (Table 1) causes a 0.1 kcal mol^{-1} decrease in $\Delta H_{obs}^{\ddagger}$ and a 0.3 cal mol^{-1} K^{-1} decrease in $\Delta S_{obs}^{\ddagger}$. However, inclusion of the data point at 55.1 °C as well, causes a 1.2 kcal mol^{-1} decrease in $\Delta H_{obs}^{\ddagger}$ and a 3.9 cal mol^{-1} K^{-1} (31%!) decrease in $\Delta S_{obs}^{\ddagger}$, despite the fact that the correlation coefficient for this Eyring plot is 0.999. Comparison of our redetermined values (Table 2) with those we previously determined[29] clearly shows that our past determination led to an under estimation for both parameters.

Our experience with this reaction allows the establishment of certain guidelines for accurate kinetic measurements using conventional techniques. Whenever the temperature deviation upon reaction initiation exceeds 0.2 °C, accurate kinetic data cannot be obtained if the half-time for the reaction under study is smaller than the half-time for temperature reequilibration. However, in circumstances where the temperature deviation is not too extreme, it is possible to obtain accurate kinetic data in cases where the reaction and temperature reequilibration half-times are comparable. In such cases, it is necessary to exclude data from the beginning of the reaction, during the period in which the sample temperature has not yet reached the equilibration temperature (within the usual limits of ±0.1 °C). In order to do so reliably, it is absolutely necessary to know both the

(47) The standard deviation in f_{on} may be readily determined by the following equation: $s_{f_{on}} = s_{K_{meas}}/(1 + K_{meas})$.
(48) Horton, W. S. *J. Phys. Colloid Chem.* **1948**, *52*, 1129.

temperature deviation upon reaction initiation and the time constant for temperature equilibration in the equipment being used. We have measured a temperature equilibration half-time of 3.4 min for unstirred samples in quartz cuvettes in a well thermostate cell block. In Pyrex glass cuvettes, the substantially lower thermal conductivity of the cuvette material should give rise to a longer temperature equilibration half-time.[48]

The second area of concern in evaluation of the activation parameters for NpCbl and similarly labile complexes is the method used to estimate the thermodynamics of the base-off/on reaction to permit correction of the observed rate constants for the occurrence of the base-off species at neutral pH. It is particularly important to obtain accurate estimates for these parameters for Np−cobalt corrinoids since the existence of a substantial amount of the base-off species at neutral pH and ambient temperature makes this correction rather large for these compounds compared to that required for other RCbls.[7b,d,49] We have previously discussed[43] the shortcomings of the use of temperature-dependent UV−visible measurements as a means of assessing base-off/on thermodynamics, including the known temperature sensitivity of cobalt corrinoid molar absorptivities. To these problems, we must add the instability of the base-on species of NpCbl, and the difficulty in making sufficiently accurate UV−visible measurements under the strictly anaerobic conditions which would be needed to stabilize the complex at spectrophotometric concentration. This leads to a severely restricted temperature range over which temperature-dependent spectrophotometric measurements can be made.[27] For the temperature range used by Kim *et al.*[27] (10.2−30.6 °C) the value of f_{on} for NpCbl decreases only from 0.708 to 0.600. We estimate that the difference in molar absorptivity between fully base-on NpCbl and the base-off species at 522 nm is 2.18×10^3 M^{-1} cm^{-1}, so that under the conditions used by Kim *et al.*[27] the maximum temperature dependent absorbance change measurable is only 0.047. This provides an extremely narrow scale for determining the temperature-dependence of the off/on equilibrium constant.

In contrast, these problems are neatly circumvented using the NMR method described above. While any trace of O_2 remaining in the NMR samples after seal-off induces the decomposition of a similar trace of NpCbl, the relatively high concentration of the samples (23−35 mM) ensures that the resulting small amount of H_2OCbl will not interfere with location of the C10H resonance of the Np−cobalt corrinoid. In addition, as Waddington and Finke[29] have pointed out, the C10H resonance of cobalt corrinoids, in the absence of any base-off/on equilibrium, is quite temperature insensitive. Finally, the measured difference in resonance frequency between NpCbl and $NpCbi^+$ changed by nearly 80 Hz (at 300 MHz) over the temperature range 5−80 °C. Since for NMR spectra of sufficiently high digital resolution (0.22 Hz per point, here) the difference between two NMR resonances can easily be located to better than ±0.5 Hz by line shape analysis, this method provides an excellent means of quantitating the temperature dependence of the base-off/on reaction of NpCbl.

A potential area of complication in such NMR measurements is the accurate determination of sample temperature in the NMR probe. As shown in Figure 5, the temperature indicated by the NMR spectrometer VT pyrometer can differ substantially from the temperature measured in the sample using a suitable temperature probe apparatus. This is apparently due to the small distance between the VT thermocouple and the sample, since below ambient temperature are consistently overestimated and above ambient temperatures are consistently underestimated. Fitting the $\Delta\nu_{obs}$ data to the indicated VT pyrometer temperatures rather than the measured sample temperatures gave values of ΔH_{meas} and ΔS_{meas} of −3.6 kcal mol^{-1} and −11.2 cal mol^{-1} K^{-1}. These erroneous values give rise to a global decrease in the measured base-on/off equilibrium constant which serves to underestimate the fractional amount of base-on species present under neutral conditions. This makes the base-on species appear to be more reactive and seriously overestimates the activation parameters $\Delta H_{on}^{\ddagger} = 30.2 \pm 1.2$ kcal mol^{-1} and $\Delta S_{on}^{\ddagger} - 26.1$ cal mol^{-1} K^{-1}.

The values obtained by the NMR method, $\Delta H_{meas} = -4.1 \pm 0.1$ kcal mol^{-1} and $\Delta S_{meas} = -12.5 \pm 0.3$ cal mol^{-1} K^{-1}, differ substantially from those reported by Kim *et al.*[27] ($\Delta H_{meas} = -5.9$ kcal mol^{-1} and $\Delta S_{meas} = -20$ cal mol^{-1} K^{-1}). On the basis of the discussion above, we believe the new values to be the best available estimates of these important thermodynamic parameters. In our earlier work,[28] the thermodynamic parameters for the base-off/on equilibrium of NpCbl were estimated by an entirely different method, based on observation of the spectral difference between the neutral species of NpCbl and its complex with a vitamin B_{12} binding protein (haptocorrin) from chicken serum (which binds only the base-on species of Cbls[50]), and the temperature dependence of the measured values of $pK_{base-off}$. The values of ΔH_{meas} and ΔS_{meas} obtained by that method, -3.6 ± 0.3 kcal mol^{-1} and -14 ± 1 cal mol^{-1} K^{-1}, respectively, are in fact, in reasonably good agreement with those now determined by the NMR method.

Careful propagation of the uncertainties in all measured quantities ($\Delta\nu_{obs}$, k_{neut}, and T) into the final activation parameters show that the intercept of an Eyring plot (i.e., $\Delta S^{\ddagger}/R$) is 3.2-fold more sensitive to the relative standard deviation of the measured quantities than is the slope ($-\Delta H^{\ddagger}/R$). Thus, the determination of precise entropies of activation requires that the utmost care be taken in the measurement of each observable.

Conclusion

Two sources of errors have been detected in the experiments used to determine the activation parameters for NpCbl thermolysis by spectrophotometric analysis. The first error results from the fact that the conventional techniques employed for the initiation of the reaction cause an unavoidable drop in the temperature. The half-time for temperature equilibration in 3.0 mL quartz cuvettes (3.4 min) is fairly invariant over the temperature range of 40−60 °C. Reestablishment of the equilibration temperature (within the accepted tolerance of ±0.1 °C) depends not only on this half-time but also on the initial temperature change. If the sample temperature is approximtely 20 °C above ambient, then this temperature error becomes significant only if the reaction half-time is of comparable size to the half-time for temperature re-equilibration. This problem may be minimized by incubation of the sample solution at the appropriate temperature for at least 30 min, as well as incubation of the stock solution and a mixing device prior to the reaction initiation. For temperatures in which the reaction half-time is larger than the temperauture equilibration half-time, decisions must be made regarding the exclusion of data points that are collected at the incorrect temperature. These decisions cannot be made without an intimate knowledge of (1) temperature changes incurred upon reaction initiation and (2) the time constant for temperature re-equilibration. Data that are analyzed without taking this methodological problem into account, result in rate constants that are underestimations of the true rate

(49) Martin, B. D.; Finke, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 585.

(50) Brown, K. L.; Brook, H. B.; Behnke, D.; Jacobsen, D. W. *J. Biol. Chem.* **1991**, *266*, 6737.

constants. Similarly, inclusion of this erroneous data into Eyring plots results in a significant underestimation of the activation parameters. Potential errors also exist in the measurement of NMR sample temperature. It must be stressed that accurate temperature measurements cannot be made without the use of an external device. This is due to the fact that the temperature displayed by the spectrometer's VT unit is not the temperature of the sample, but rather of the VT thermocouple which is some distance below the sample itself. In the present example, failure to account for this error results in a global decrease in the measured equilibrium constant for the base-off/on equilibrium. Since this thermodynamic data is necessary to correct the observed rate constants, the potential error would then be propagated into these corrected rate constants, which gives rise to overestimations with respect to the true values. These overestimations, in turn, make the base-on species appear to be more reactive. Introduction of this data into an Eyring plot erroneously overestimates the calculated activation parameters.

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