Listening to Reactive Intermediates: Application of Photoacoustic Calorimetry to Vitamin B$_{12}$ Compounds

**Rebecca R. Hung**[†] **and Joseph J. Grabowski***[,‡]

Contribution from the Departments of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and University of Pittsburgh, Pittsburgh, Pennsylvania, 15260

Received August 17, 1998

Abstract: The modern technique of photoacoustic calorimetry (PAC) has been critically evaluated for its utility and reliability in biophysical chemistry by applying it to reactive intermediates in the vitamin B$_{12}$ family of compounds. Measurement of the photoacoustic signals at 337 nm in 0.01 M phosphate buffer at pH 7.0, for methylcobalamin and methylcobinamide versus reference compounds from −2 to +40 °C, combined with measurement of the quantum yields for photodissociation (0.27 and 0.17, respectively), yields the bond dissociation energies of the corresponding cobalt−carbon bonds as BDE{Me−Cbl} = 36 and BDE{Me−Cbi} = 37 kcal mol^{-1}. Simultaneously, the reaction volumes for homolytic bond cleavage were determined to be 16 and 14 cm^3 mol^{-1}, respectively. These accurate measurements provide new paradigms for understanding the mechanisms by which the novel rearrangements catalyzed by coenzyme B$_{12}$ are achieved as well as establishing the validity of the PAC technique and data treatment. The further extension of PAC to the energetic analysis of the reactive intermediate methyl radical produced in the homolysis argues strongly for the broad utility of this systematic approach.

Introduction

From Minot and Murphy's initial recognition that liver extracts provided an effective treatment for pernicious anemia to Folkers and Smith's isolation of a crystalline factor, from Hodgkin's X-ray structure determination to Eschenmoser's and Woodward's total syntheses, vitamin B$_{12}$ has bridged the gap between disciplines, providing intriguing challenges to physicists, chemists, and biologists alike.[1,2] Two of its biologically active forms, coenzyme B$_{12}$ (5′-deoxyadenosylcobalamin, AdoCbl, Figure 1) and methylcobalamin (MeCbl) mediate carbon skeletal rearrangements by such enzymes as diol dehydrase, methylmalonyl CoA mutase, and ribonucleotide reductase as well as methyl group transfers essential for folic acid metabolism and nucleic acid biosynthesis. The cobalamin is composed of a cobalt(III) ion, coordinated equatorially by the corrin macrocycle and axially on its α (lower) face by 5,6-dimethylbenzimidazole, pendant from the corrin. A covalent metal−carbon bond on the β (upper) face of the complex completes the octahedral coordination sphere. The unique feature of cobalamins is the cobalt(III)−carbon bond,[3] whose homolytic cleavage to a 17-electron cobalt(II) radical (B$_{12r}$) and a highly reactive alkyl radical (eq 1) has been implicated as the initial step of coenzyme B$_{12}$-dependent reactions (eq 2). ESR evidence of substrate-dependent cobalt−carbon homolysis of the protein-bound coenzyme supports participation of these radicals in enzymatic catalysis.[4,5] A precise knowledge of the energetics of the cobalt−

$$RCH_2-B_{12} \rightleftharpoons RCH_2^{\bullet} + {}^{\bullet}B_{12r} \quad (1)$$
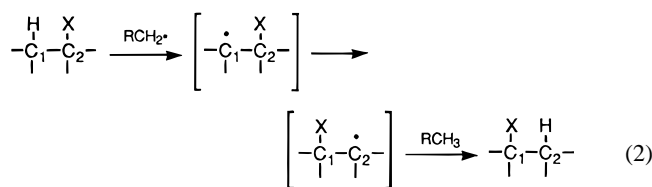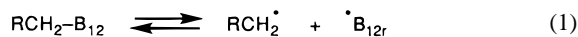
carbon bond in cobalamins is then essential for understanding the mechanism of B$_{12}$-mediated reactions.

Substantial efforts have been devoted toward dissection of the dynamics of homolysis and the remarkable rate acceleration, exceeding 10^{12}, achieved within the active site of the enzyme.[1] Traditional approaches to the determination of cobalt−alkyl bond dissociation energies (BDE) utilize equilibrium methods extrapolated from model systems undergoing thermolysis[6] and kinetic methods for systems in which homolysis proceeds at convenient rates.[7] Since the alkylcorrinoids are photolabile, the threshold wavelength of photolysis provides an estimate of an upper limit for the BDE.[8]

All previously described methods encounter limitations. Since thermal methods are limited to *model* systems in which equilibrium can be established, the natural coenzymes (AdoCbl and MeCbl) cannot be directly studied. Kinetic methods often require elevated temperatures (>80 °C) to achieve convenient rates of homolysis. At these high temperatures, substantial corrections must be made for competing heterolytic cleavage.[7] Also, the cobalamin exists as an equilibrium mixture of the two forms, one form with the axial benzimidazole ligand coordinated

---

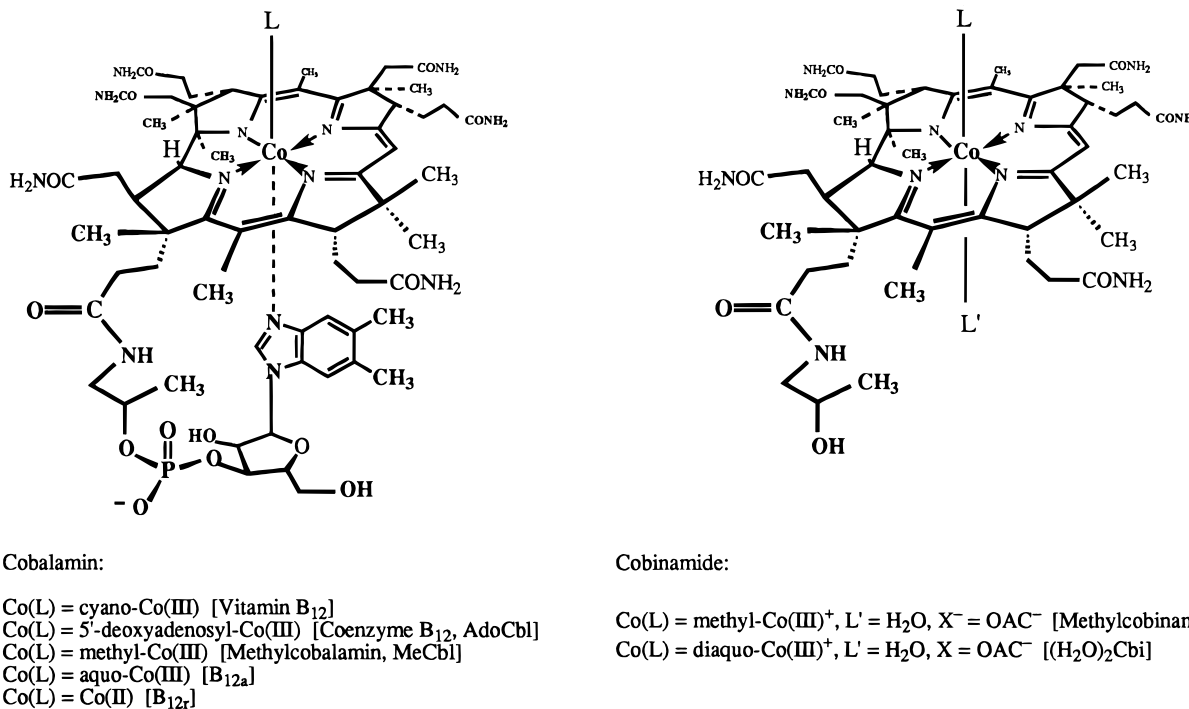[†] Harvard University.

[‡] University of Pittsburgh.

(1) *B$_{12}$*; Dolphin, D., Ed.; Wiley-Interscience: New York, 1982; Vol. 1.

(2) *Cobalamin: Biochemistry and Pathophysiology*; Babior, B. M., Ed.; Wiley-Interscience: New York, 1975.

(3) Halpern, J. *Science* **1985**, *227*, 869−875.

(4) Orme-Johnson, W. H.; Beinert, H.; Blakley, R. I. *J. Biol. Chem.* **1974**, *249*, 2338−2343.

(5) Zhao, Y.; Such, P.; Retey, J. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 215−216.

(6) Halpern, J.; Ng, F. T. T.; Rempel, G. L. *J. Am. Chem. Soc.* **1979**, *101*, 7125−7127.

(7) Halpern, J. *Polyhedron* **1988**, *7*, 1483−1490.

(8) Endicott, J. F.; Balakrishnan, K. P.; Wong, C.-L. *J. Am. Chem. Soc.* **1980**, *102*, 5519−5526.

Figure 1. Structures of the cobalamins and cobinamides.

Cobalamin:

Co(L) = cyano-Co(III) [Vitamin B$_{12}$]
Co(L) = 5'-deoxyadenosyl-Co(III) [Coenzyme B$_{12}$, AdoCbl]
Co(L) = methyl-Co(III) [Methylcobalamin, MeCbl]
Co(L) = aquo-Co(III) [B$_{12a}$]
Co(L) = Co(II) [B$_{12r}$]

Cobinamide:

Co(L) = methyl-Co(III)$^+$, L' = H$_2$O, X$^-$ = OAC$^-$ [Methylcobinamide, MeCbi]
Co(L) = diaquo-Co(III)$^+$, L' = H$_2$O, X = OAC$^-$ [(H$_2$O)$_2$Cbi]

to the cobalt (base-on) and the other with the benzimidazole free (base-off).[9] Over the range of temperatures required for the kinetic thermolysis method, the base-on/base-off equilibrium changes, necessitating additional corrections to disentangle the behavior of the two forms.

In light of the inherent limitations of conventional techniques and the resultant controversies, we now describe the application of PAC[10−12] as a method for the determination of the biologically important BDE of the cobalt−carbon bond in the prototype alkylcobalamin, methylcobalamin. This technique exploits the clean photohomolysis of organocobalamins and takes advantage of the extensive photochemical literature available for this family of compounds.[13−15] In contrast to other methods, PAC is conducted at or near ambient temperatures on the natural cofactor and in physiologically relevant aqueous buffers. Under the mild conditions of the photoacoustic experiment, heterolytic processes do not compete with photohomolysis. Over the limited temperature range of the experiment, the base-on/base-off equilibrium of the axial benzimidazole ligand remains effectively constant, further simplifying the analysis.[16]

Biological processes have been the subjects of previous photoacoustic studies, including the dissociation of carbon monoxide from myoglobin[17−20] and hemoglobin,[21] the photoi-

somerization of rhodopsin[22,23] and biliverdin,[24] and ion-pair formation following photoexcitation of reaction centers.[25] While the authors of these reports correctly recognized that the reaction volume of the photoinitiated process may significantly contribute to the photoacoustic signal in aqueous solutions, they examined complex biological systems that were not amenable to a straightforward evaluation of the photoacoustic method itself. No previous work using an independent method has been able to verify any conclusions from these PAC studies despite the recognized need "for careful interpretation of the photoacoustic data, particularly in regard to volume changes".[26] Our study examines the photoacoustic behavior of methylcobalamin (MeCbl) and the corresponding cobinamide (MeCbi, lacking the axial benzimidazole ligand, Figure 1) and in so doing, provides compelling evidence for the reliability of PAC to recover accurate enthalpies and volumes of reaction in aqueous solution.

Absorption of a photon by methylcobalamin results in an excited state that partitions within 8 ps between two channels, one resulting in homolytic cleavage of the cobalt−carbon bond to form a caged radical pair of the Co(II) species B$_{12r}$ and CH$_3$ and one resulting in heterolytic cleavage to a metastable Co(III) species that recombines to methylcobalamin by 10 ns.[27−29] In the PAC experiment, the formation and decay of this metastable

(9) Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1980**, 2267−2273.

(10) Rothberg, L. J.; Simon, J. D.; Bernstein, M.; Peters, K. S. *J. Am. Chem. Soc.* **1983**, *105*, 3464−3468.

(11) Braslavsky, S. E.; Ellul, R. M.; Weiss, R. G.; Al-Ekabi, H.; Schaffner, K. *Tetrahedron* **1983**, *39*, 1909−1913.

(12) Hung, R. R.; Grabowski, J. J. *J. Am. Chem. Soc.* **1992**, *114*, 351−353.

(13) Endicott, J. F.; Ferraudi, G. J. *J. Am. Chem. Soc.* **1977**, *99*, 243−245.

(14) Hogenkamp, H. P. C. *Biochemistry* **1966**, *5*, 417−422.

(15) Pratt, J. M. *J. Chem. Soc.* **1964**, 5154−5160.

(16) Koenig, T. W.; Hay, B. P.; Finker, R. G. *Polyhedron* **1988**, *7*, 1499−1481.

(17) Westrick, J. A.; Goodman, J. L.; Peters, K. S. *Biochemistry* **1987**, *26*, 8313−8318.

(18) Westrick, J. A.; Peters, K. S.; Ropp, J. D.; Sligar, S. E. *Biochemistry* **1990**, *29*, 6741−6746.

(19) Westrick, J. A.; Peters, K. S. *Biophys. J.* **1990**, *37*, 73−79.

(20) Leung, W. P.; Cho, K. C.; Chau, S. K.; Choy, C. L. *Chem. Phys. Lett.* **1987**, *141*, 220−224.

(21) Peters, K. S.; Watson, T.; Marr, K. *J. Am. Chem. Soc.* **1992**, *114*, 4276−4278.

(22) Marr, K.; Peters, K. S. *Biochemistry* **1991**, *30*, 1254−1258.

(23) Schulenberg, P. J.; Rohr, M.; Gärtner, W.; Braslavsky, S. E. *Biophys. J.* **1994**, *66*, 838−843.

(24) Ellul, R. M.; Braslavsky, S. E.; Weiss, R. G.; Al-Elkabi, H.; Schaffner, K. *Tetrahedron* **1983**, *39*, 1909−1913.
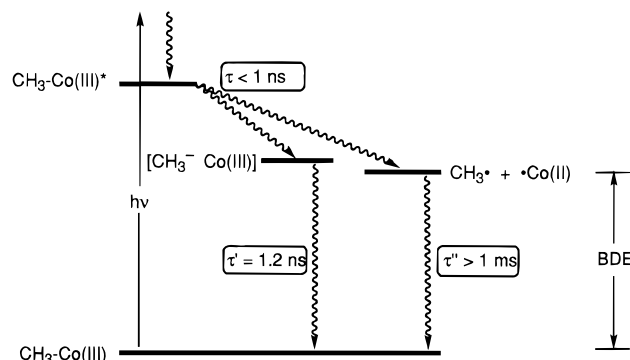
(25) Malink, S.; Churio, M. S.; Shochat, S.; Braslavsky, S. E. *J. Photochem. Photobiol. B: Biol.* **1994**, *23*, 79−85.

(26) Peters, K. S.; Snyder, G. J. *Science* **1988**, *241*, 1053−1057.

(27) Netzel, T. L.; Endicott, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 4000−4002.

(28) Lott, W. B.; Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1995**, *117*, 12194−12201.

(29) Walker, L. A., II; Jarrett, J. T.; Anderson, N. A.; Pullen, S. H.; Matthews, R. G.; Sension, R. J. *J. Am. Chem. Soc.* **1998**, *120*, 3597−3603.

Figure 2. Energy level diagram for the CH$_3$−Co(III) photodissociation. The values of τ and τ' are found in refs 27−29. The value of τ'' was calculated for the conditions of the PAC experiment assuming free radical recombination to be diffusion limited. The relative energy of the "metastable cob(III)alamin photoproduct", represented here as the heterolytic bond cleavage pair,[29] is completely unknown with respect to the homolytic bond cleavage to free radicals.

intermediate cannot be distinguished from internal conversion. The caged radical pair dissociates to yield free B$_{12r}$ and CH$_3^\bullet$ within 10 ns following flash photolysis as summarized in Figure 2. The radicals have several potential fates after escaping from the cage. CH$_3^\bullet$ can recombine with B$_{12r}$ to regenerate the cobalamin or can dimerize to yield ethane. If molecular oxygen is present, CH$_3^\bullet$ can be scavenged at the diffusion rate to yield the methylperoxy radical, which subsequently oxidizes the cobalamin to aquocob(III)alamin B$_{12a}$. Since all the secondary processes are bimolecular, they cannot proceed faster than the diffusion limit. When the steady-state concentration of radicals is maintained below 10^{-7} M in an oxygen-free environment, as in the PAC experiment, the lifetimes of CH$_3^\bullet$ and B$_{12r}$ extend well beyond 10 ms. Therefore, recombination of the radicals is effectively absent on the PAC time scale. Since homolysis and diffusion from the cage yield free radicals within 10 ns, this system is ideally suited for study by nanosecond photoacoustic calorimetry. In the absence of radical scavengers (such as O$_2$), the observed PAC signal is solely the consequence of the homolytic cleavage of the cobalamin to yield B$_{12r}$ and CH$_3^\bullet$.

Theory of Photoacoustic Analysis. The photoacoustic signal S_{obsd} (eq 3) is defined by the instrumental constant κ', the

$$S_{obsd} = \kappa' E_p (1 - 10^{-A}) f_h^{obsd} \chi_s(T) \qquad (3)$$

incident photon energy E_p, the optical absorbance of the sample A, a fractional parameter f_h^{obsd} characteristic of the photoacoustic properties of the sample, and the thermal expansivity of the solvent $\chi_s(T)$.[30] S_{obsd} arises from the volume expansion of the solvent following absorption of a photon with energy $h\nu$. As has been shown,[12,31] S_{obsd} can be divided into a thermal contribution, due to the enthalpy of the reaction and the thermal relaxation of excited states, and a reaction volume contribution, due to the differences between the partial molar volumes of the reactants and products (eq 4).

$$S_{obsd} = S_{thermal} + S_{volume} \qquad (4a)$$

$$= \kappa' E_p (1 - 10^{-A}) f_h \chi_s(T) + \kappa' E_p (1 - 10^{-A}) \frac{\Delta V_{chem}}{h\nu} \qquad (4b)$$

The thermal term is characterized by the parameter f_h, the fraction of the absorbed photon energy that is redeposited into

the solvent as heat. The volume term is characterized by ΔV_{chem}, the observed nonthermal volume change per absorbed photon.

The ratio of the observed photoacoustic signals for the sample of interest and a reference compound of known photoacoustic properties, each normalized to E_p and $(1 - 10^{-A})$, returns the experimental quantity f_h^{obsd} for the sample.[32] Note that $\chi_s(T) = (\alpha MW)/(C_p\rho)$, where α is the adiabatic coefficient of thermal expansion, MW is the molecular weight, C_p is the molar heat capacity, and ρ is the density (all of the solvent). Since α, C_p, and ρ of water all vary with temperature, $\chi_s(T)$ for water exhibits a strong temperature dependence.[33] This temperature dependence allows χ_s in aqueous solution to be conveniently varied when one acquires the photoacoustic signals of interest. By measurement of f_h^{obsd} over a suitable range of $1/\chi_s(T)$, f_h and ΔV_{chem} can be deduced respectively from the intercept and slope of eq 5, itself derived from combining eqs 3 and 4b.

$$f_h^{obsd} h\nu = f_h h\nu + \Delta V_{chem} \frac{1}{\chi_s(T)} \qquad (5)$$

The reaction enthalpy and volume change for homolytic cleavage of the cobalt−methyl bond can be defined (eqs 6 and 7) in terms of the experimental parameters f_h and ΔV_{chem} and

$$\Delta H_{reacn} = BDE\{Me-Cbl\} = (h\nu - f_h h\nu)/\Phi_{diss} \qquad (6)$$

$$\Delta V_{reacn} = \Delta V_{chem}/\Phi_{diss} \qquad (7)$$

the quantum yield for free radical production Φ_{diss}. Thus, the determination of the BDE of the cobalamin depends on two independent measurements, one involving photoacoustic calorimetry to determine f_h and ΔV_{chem} and the other involving actinometry to determine Φ_{diss}.

Experimental Section

Our photoacoustic calorimeter[34] is similar to instruments described previously and is illustrated in Figure 3.[35] Photoexcitation was accomplished with the direct output of a nitrogen laser (Laser Photonics model LN-1000, fwhm ~1 ns, 1.3 mJ/pulse) operated at ~ 1 Hz. A quartz attenuation cell containing variable concentrations of ferrocene in ethanol served to decrease the energy of the excitation pulse. Approximately 40% of each pulse was split off and focused onto the reflected cell containing either tetraphenylethylene or ferrocene in acetonitrile; this signal served as a relative measure of the photolysis energy (E_p, eq 3). Absolute photolysis energies were checked during each experiment by use of pyroelectric detector. The photoacoustic cell consisted of a standard UV quartz cuvette (1 × 1 cm) spring-loaded against the housing of the microphone. The microphones were custom built[36,37] and were based on a lead zirconate−lead titanate piezoceramic crystal (Transducer Products model LTZ-2) with a diameter of 4 mm and a resonant frequency of 500 kHz. After amplification, the acoustic signals from the three cells in our PAC apparatus were captured by

(30) Because all the photochemistry of the methylcorrinoids contributing to the observed photoacoustic signal is complete within 10 ns, the signals discussed here refer to integrated signals.

(31) Zimmt, M. B.; Vath, P. A. *Photchem. Photobiol.* **1997**, *65*, 10−14.

(32) The reference compound is chosen to have $f_h = 1.0$ and $\Delta V_{chem} = 0$ cm^3 mol^{-1}.

(33) The literature values of α, C_p, and ρ were separately fit to polynomial expressions that were used to interpolate the value of $\chi_s(T)$ at a specified temperature: *CRC Handbook of Chemistry and Physics,* 62nd ed.; CRC Press: Boca Raton, FL, 1981.

(34) Grabowski, J. J.; Bertozzi, C. R.; Jacobsen, J. R.; Jain, A.; Marzluff, E. M.; Suh, A. Y. *Anal. Biochem.* **1992**, *207*, 214−226.

(35) Rudzki, J. E.; Goodman, J. L.; Peters, K. S. *J. Am. Chem. Soc.* **1985**, *107*, 7849−7854.

(36) Patel, K. K. N.; Tam, A. C. *Rev. Mod. Phys.* **1981**, *53*, 517−550.

(37) Bernstein, M. Ph.D. Thesis, Harvard University, 1983.
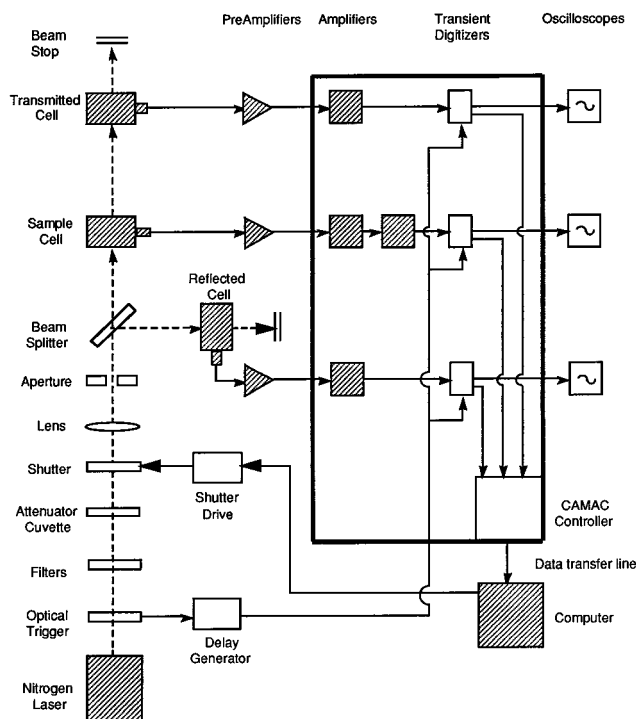
Figure 3. Schematic diagram of the photoacoustic calorimeter.

transient digitizers (10 or 50 ns resolution) and transferred to a computer for averaging and processing. The temperature was maintained by keeping the sample cell assembly in thermal contact with a copper block whose temperature was regulated by a variable-temperature bath (Neslab model RTE-110). Because turbulent flow of the cooling fluid within the block was detected by the microphone, the temperature bath was switched off briefly (<1 min) during actual data collection.

Methylcobalamin and methylcobinamide were gifts from Prof. Paul Dowd's research group, where they were synthesized and purified. Sets of photoacoustic waves of methylcobalamin (MeCbl) and methylcobinamide (MeCbi) and the reference compounds 8-anilinonaphthalene-1-sulfonate, ammonium salt hydrate (ANS) and 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) in sodium phosphate (10 mM, pH 7.0) for photoexcitation at 337.1 nm were collected at a series of temperatures from −2 to +40 °C (regulated to within ±0.1 °C). Because the cobalt−carbon bond is photolabile under ambient light, solutions of MeCbl and MeCbi were maintained in darkness by wrapping in aluminum foil and handled under a low-intensity red photographic safelight (≤25 W) during sample preparation and for the duration of the experiment. Samples were routinely deoxygenated with water-saturated argon for 30 min prior to data collection and maintained under argon atmosphere throughout. Typically 30−50 laser pulses were absorbed per sample to yield one photoacoustic wave. Incident energies were maintained such that less than 2% of MeCbl and MeCbi in the sample was photolyzed during the acquisition of one photoacoustic wave. Following acquisition, each photoacoustc wave was integrated and normalized for its respective absorbance $(1 − 10^{-A})$ and incident energy (E_p).

The photodissociation quantum yields of MeCbl and MeCbi upon irradiation at 337.1 nm were determined in air-saturated solution (10 mM sodium phosphate, pH 7.0) at 25 °C. The incident photon flux was calculated using potassium ferrioxalate[38−41] (0.006 M in 0.05 M H_2SO_4) as the actinometer. To determine the concentrations of MeCbl,

(38) Parker, C. A. *Photoluminescence of Solutions, with Applications to Photochemistry and Analytical Chemistry*; Elsevier: Amsterdam, 1968; p 207.

(39) Nicodem, D. E.; Aquilera, O. M. V. *J. Photochem.* **1983**, *21*, 189−193.

(40) Kuhn, H. J.; Braslavsky, S. E.; Schmidt, R. *Pure Appl. Chem.* **1989**, *61*, 187−210.

(41) Bunce, N. J. In *Handbook of Organic Photochemistry*; Scaiano, J., Ed.; CRC Press: Boca Raton, FL, 1989; Vol. 1, p 327.
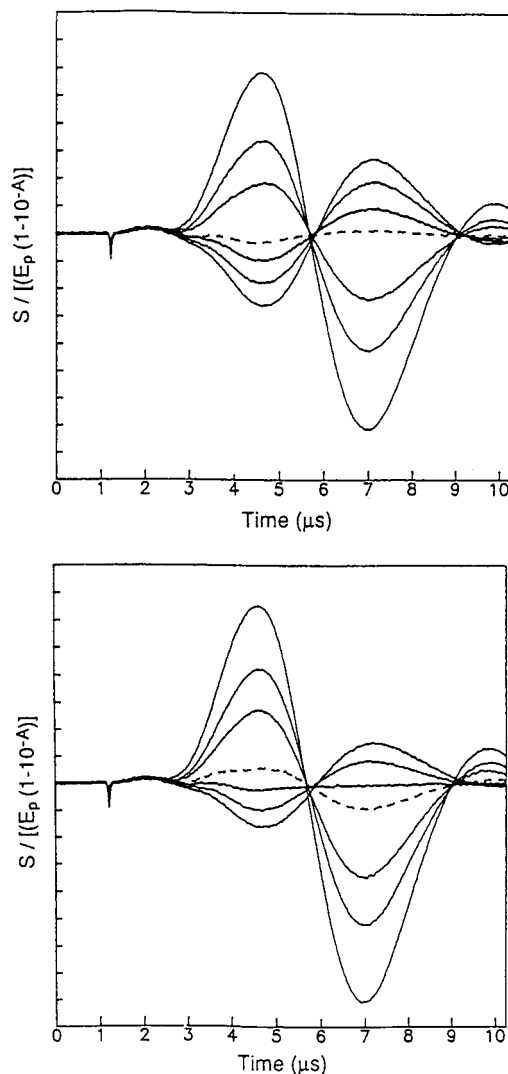
Figure 4. Normalized photoacoustic waves for 8-anilinonaphthalene-1-sulfonate (ANS) (top) and MeCbl (bottom) as a function of temperature (−2 to +25 °C). At 4 °C (- - -), the amplitude of the reference wave (ANS) approaches zero, while the amplitude of the MeCbl wave remains positive.

MeCbi, and the corresponding products, B_{12a} and $(H_2O)_2$Cbi, and to correct the incident photon flux for the inner-filter effect of the absorbance by the products at the excitation wavelength, we used extinction coefficients obtained from the literature.[42,43]

Results

In deoxygenated solution, a single photoacoustic transient for MeCbl was observed (Figure 4). Under these conditions, no secondary bimolecular reactions contribute to the observed signal. At a given temperature, the signal of MeCbl varied linearly with incident energy E_p as expected in the absence of multiphoton absorption. Note that the magnitude of the signal for MeCbl exceeds that of the reference compound ANS throughout the range of temperatures examined. Thus, if we were to mistakenly assign the observed photoacoustic signal of MeCbl exclusively to enthalpy (i.e., assume $f_h^{obsd} = f_h$), not only would we err in our estimation of the BDE, but the endothermic

(42) Brown, K. L.; Zour, X.; Salmon, L. *Inorg. Chem.* **1991**, *30*, 1949−1953.

(43) Autissier, D.; Bsarthelemy, P.; Penasse, L. *Bull. Soc. Chim. Fr.* **1980**, II92−II194.
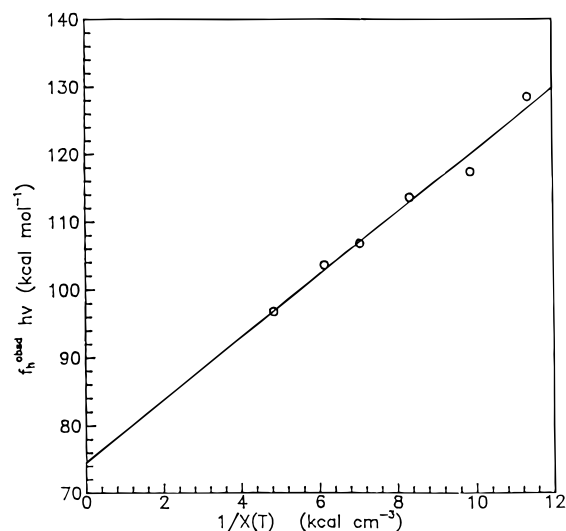
Figure 5. Typical plot of $(f_h^{obsd}h\nu)$ versus $1/\chi_s(T)$ as designated by eq 5 for MeCbl.

cleavage of the cobalt–carbon bond would be incorrectly interpreted as an exothermic process.

The thermal expansivity $\chi_s(T)$ governs the behavior of the enthalpic component of the signal. The reference compound ANS in dilute phosphate buffer yields a purely thermal photoacoustic signal that correlates well with the trend predicted by the values of χ_s in water (data not shown). In contrast, the signal for MeCbl cannot be directly correlated to $\chi_s(T)$ for water since MeCbl undergoes a temperature-independent molecular volume change upon photodissociation. The contribution of this volume change is readily apparent by comparing the signals of the reference compound ANS and MeCbl at 4 °C. Because the adiabatic coefficient of thermal expansion α approaches zero at 4 °C, $\chi_s(T)$ also approaches zero; at this temperature, heat deposition into the solvent, represented by the reference signal, does not contribute to the photoacoustic signal. The residual positive signal observed for MeCbl is due entirely to ΔV_{reacn}. When α (and hence $\chi_s(T)$) changes sign below 4 °C, the reference signal is inverted, while the signal of MeCbl remains positive until the temperature is further lowered. Analysis of the temperature-dependent photoacoustic behavior of MeCbl according to eq 6 yields the quantities $(f_h h\nu)$ and ΔV_{chem} from the intercept and slope, respectively (Figure 5). For MeCbl, from five independent experiments, we recover $(f_h h\nu) = 9.7 \pm 1.1$ kcal mol^{-1} and $\Delta V_{chem} = 4.4 \pm 0.3$ cm^3 mol^{-1}. MeCbi exhibits photoacoustic behavior similar to that of MeCbl (data not shown). The analysis of MeCbi data for three experiments yields $(f_h h\nu) = 6.3 \pm 0.5$ kcal mol^{-1} and $\Delta V_{chem} = 2.4 \pm 0.2$ cm^3 mol^{-1}.

These values are converted to the bond dissociation energy and the reaction volume by dividing the observed heat and volume by the appropriate photodissociation quantum yield Φ_{diss}. We applied the analysis of Taylor et al.[44] to determine Φ_{diss} for MeCbl and MeCbi upon irradiation at 337.1 nm. Potassium ferrioxalate ($\phi = 1.25$)[38–41] was utilized to determine the incident photon flux. Because oxygen has been established as an efficient scavenger of the alkyl radical, in air-saturated solutions primary homolyis is the rate-determining step.[44–46] The resulting reactive methylperoxy intermediate rapidly oxi-

dizes the 17-electron Co(II) corrinoid to the corresponding Co-(III) aquo species. Thus, in air-saturated solutions, MeCbl and MeCbi are converted to B$_{12a}$ and (H$_2$O)$_2$Cbi, respectively. The UV spectra of the reaction mixtures over the course of the photolyses show no evidence of the intermediate Co(II) species. We observe isosbestic points in both sets of spectra indicative of the clean conversion of the methyl corrinoid to the corresponding Co(III) species. After corrections were performed for the inner filter effect due to the absorbance of accumulating Co(III)–OH product, Φ_{diss} was calculated by comparing the conversion of the corrinoid with the calibration curve determined for the ferrixoalate actinometer. For excitation at 337.1 nm, we determined Φ_{diss}(MeCbl) as 0.27 ± 0.01 (three experiments) and Φ_{diss}(MeCbi) as 0.17 ± 0.02 (two experiments). These values of Φ_{diss} are in accord with the values of $\sim$0.3 and 0.25 determined for MeCbl at pH 7.0 (base-on form) and MeCbl at pH 1.0 (base-off form) under continuous-wave irradiation at 350 nm[44] and 0.27 ± 0.03 (for MeCbl) via ultrafast transient absorption spectroscopy at 400 nm.[29]

Using $\Phi_{diss} = 0.27 \pm 0.01$ for MeCbl, we calculate BDE-{Me–Cbl} $= 36 \pm 4$ kcal mol^{-1} and $\Delta V_{reacn} = 16 \pm 1$ cm^3 mol^{-1}. Using $\Phi_{diss} = 0.17 \pm 0.02$, we calculate BDE{Me–Cbi} $= 37 \pm 3$ kcal mol^{-1} and $\Delta V_{reacn} = 14 \pm 1$ cm^3 mol^{-1}. The errors reported here reflect the uncertainties of both the photoacoustic parameters and the quantum yield.

Discussion

Our photoacoustic determination of the BDE{Me–Cbl} of 36 kcal mol^{-1} in aqueous solution is the first direct measurement of this important property at nearly physiological conditions. It corroborates an estimate of 37 ± 3 kcal mol^{-1} elegantly measured in ethylene glycol at 120–141 °C by Martin and Finke[47] using a kinetic-based methodology. It is important to note that the photoacoustic method reported here and the kinetic method take advantage of completely independent assumptions. The concurrence of the bond dissociation energies by orthogonal approaches provides mutual validity to the two techniques. The kinetic method assumes with variable success that the radical trap operates with unit efficiency so that homolysis is the rate-determining step.[48–51] Furthermore, the kinetic method must assume a value for $\Delta H^{\ddagger}$ for the diffusion-controlled radical recombination of the back-reaction and must estimate cage effects.[7,16] In addition to the intrinsic uncertainties of the kinetic method,[52] complications unique to the cobalamins exist, requiring corrections for competing heterolytic reactions, and because a substantial fraction of the cobalamin exists in the base-off form (with the axial benzimidazole uncoordinated to the cobalt) at the operating temperatures of the kinetic experiment, the analysis requires an additional correction factor.[9]

PAC provides an independent approach to the accurate determination of the cobalt–carbon BDE, taking advantage of the photolability of the bond while directly monitoring the enthalpy of the cleavage. Photolysis induces exclusive homolysis of the methyl–cobalt systems at or near ambient temperature, obviating the need to make corrections for heterolysis. In contrast to the kinetic methodology, the photoacoustic method

(44) Taylor, R. T.; Smucker, L.; Hanna, M. L.; Gill, J. *Arch. Biochem. Biophys.* **1973**, *156*, 521–533.

(45) Blau, R. J.; Espenson, J. H. *J. Am. Chem. Soc.* **1985**, *107*, 3530–3533.

(46) Brown, K. L.; Zou, X. *Inorg. Chem.* **1992**, *31*, 2541–2547.

(47) Martin, B. D.; Finke, R. G. *J. Am. Chem. Soc.* **1990**, *112*, 2419–2420.

(48) Halpern, J.; Kim, S.-K.; Leung, T. W. *J. Am. Chem. Soc.* **1984**, *106*, 8317–8319.

(49) Finke, R. G.; Hay, B. P. *Inorg. Chem.* **1984**, *23*, 3041–3043.

(50) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 4820–4829.

(51) Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1469–1481.

(52) Berkowitz, J.; Ellison, G. B.; Gutman, D. *J. Phys. Chem.* **1994**, *98*, 2744–2765.

does not depend on the efficiency of a radical trap. For MeCbl, throughout the range of temperatures required in PAC, the cobalamin exists in the base-on form in greater than 97%, eliminating any error arising from the variable base-on/base-off equilibrium.[53]

The similarity of bond strengths found for MeCbl and MeCbi unequivocally demonstrates that coordination of the trans benzimidazole has no net influence on the strength of the cobalt−methyl bond. Any trans influence can be divided into an electronic effect and a steric effect. The electronic effect of axial base coordination on the Co−C BDE has been examined in model systems where the basicity of the donor ligand stabilizes Co(III) in the higher oxidation state relative to the Co(II) product.[42,54] This electronic effect is also reflected in the crystal structures of alkylcobalamins as a positive correlation between the Co−C and Co−ligand bond lengths.[55−58] In the cobalamin/cobinamide pairs previously examined, however, coordination of the cobalt by the axial benzimidazole was found to destabilize the cobalt−carbon bond toward cleavage, suggesting that the steric influence of the benzimidazole weakening the Co−C bond outweighs its stabilizing electronic influence.[59,60] These observations support the "butterfly" distortion theory for enzyme-promoted Co−C homolysis in which coordination of the axial base induces an upward conformational distortion of the corrin that sterically interacts to weaken the Co−C bond. In contrast to the enthalpic differences between bulkier alkylcobalamins and -cobinamides, we do not observe the ∼4−5 kcal mol^{-1} stabilization in MeCbi relative to MeCbl.[59] Instead, our experimental BDEs for MeCbl and MeCbi are nearly identical, consistent with the prediction of minimal energetic differences in the methyl derivatives made from previous methyl transfer equilibration experiments[61] and the observation of only minor perturbations of the cobalt−methyl bond stretch frequency in Raman studies.[62] While the presence of the corrin is essential for binding to the protein, it appears that direct steric interactions between the protein and the alkyl group are the dominant contributor to the labilization of the Co−C bond.

The homolysis of the cobalt−methyl bond results in ΔV_{reacn} of 16 ± 1 and 14 ± 1 cm^3 mol^{-1} for MeCbl and MeCbi, respectively. These values may be compared with ΔV_{reacn} of 16.4 ± 1.6 cm^3 mol^{-1} associated with the homolysis of the Co−C bond in the model compound [Co(N(CH$_2$CO$_2$)$_3$(H$_2$O)-(CH$_3$)] determined by variable-pressure pulse radiolysis.[63] Two sources of volume expansion are common to MeCbl, MeCbi,

and the model compound: that which accompanies bond cleavage/resolvation of the radicals and that from relaxation of electrostriction upon the reduction of the formal charge from Co(III) to Co(II). The similarity between the volumes of the unstrained model system, MeCbl, and MeCbi suggests that dissociation/resolvation, rather than the accompanying conformational change of the remaining ligands, is the predominant source of the reaction volume. This result provides homogeneous solution confirmation of the minor conformational differences found in the crystal structures of MeCbl and B$_{12r}$. The small volume difference accompanying homolysis of MeCbi compared with MeCbl may be attributed to the reduction in the butterfly distortion of the Co(III)−methyl species in the absence of the axial base. The conformation of MeCbi is relaxed toward the conformation of the pentacoordinate Co(II) radical as opposed to the more strained conformation of MeCbl. Although insufficient data are presently available on values of ΔV_{reacn} for cobalt−alkyl cleavage, the data presented here suggest that ΔV_{reacn} may serve as a sensitive measure of steric distortion in the corrinoid systems.

Conclusion

Given a reliable measure of the photodissociation quantum yield and the essential inclusion of the reaction volume in the analysis, photoacoustic calorimetry has been demonstrated to provide a direct method for the accurate determination of bond dissociation energies in a complex, biologically important molecule. PAC, in addition, provides a measure of ΔV_{reacn}, a quantity that may prove to be a sensitive indicator of conformational distortions and mechanistic detail as biological molecules undergo reactions. The unprecedented agreement between our PAC results and the BDEs and volumes estimated by independent methods provides compelling evidence for the reliability of the variable-temperature analysis of photoacoustic data. While the specific role of the protein in initiating cobalt−carbon cleavage currently remains unclear, photoacoustic calorimetry may be applied to elucidate the energetics of the coenzyme within the active site of the enzyme and thus provide thermodynamic absolutes for the evaluation of mechanistic proposals. It should also be noted that our results have provided a needed evaluation of photoacoustic calorimetry as was suggested earlier.[26] Finally, when we carry out a similar photolysis of MeCbl in the presence of oxygen, we are able to observe the enthalpic and volume contributions to the photoacoustic signal of the initial bond cleavage and the subsequent capture of the methyl radical by O$_2$. These results point to new directions for the application of time-resolved photoacoustic calorimetry to stepwise biological processes.

(53) Brown, K. L.; Peck-Siler *Inorg. Chem.* **1988**, *27*, 3548−3555.
(54) Ng, F. T. T.; Rempel, G. L.; Halpern, J. *J. Am. Chem. Soc.* **1982**, *104*, 621−623.
(55) Sagi, I.; Chance, M. R. *J. Am. Chem. Soc.* **1992**, *114*, 8061−8066.
(56) Sagi, I.; Wirt, D. M.; Chen, E.; Frisbie, S.; Chance, M. R. *J. Am. Chem. Soc.* **1990**, *112*, 8639−8644.
(57) Savage, H.; Lindley, P.; Finney, J.; Timmins, P. *Acta Crystallogr.* **1987**, *B43*, 280−295.
(58) Hodgkin, K. L.; Lindsey, J.; Sparks, R.; Truebloom, K.; Ehite, J. *Proc. R. Soc. London, Ser. A* **1962**, *266*, 494−517.
(59) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 8012−8018.
(60) Brown, K. L.; Brooks, H. *Inorg. Chem.* **1991**, *30*, 3420−3430.
(61) Krautler, B. *Helv. Chim. Acta* **1987**, *70*, 1268−1278.
(62) Nie, S.; Marzilli, P. A. *J. Am. Chem. Soc.* **1990**, *112*, 6084−6091.
(63) van Eldik, R.; Cohen, H.; Meyerstein, D. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1158−1159.