Thermolysis of Neopentylcobalamin Analogs Complexed to Haptocorrin: Side Chain Entropy and Activation of Organocobalamins for Carbon−Cobalt Bond Homolysis

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The kinetics of the thermal Co−C bond homolysis of the complexes of a vitamin B_{12} binding protein (haptocorrin) with a series of analogs of neopentylcobalamin modified in side chain structure have been studied. The analogs include the C13 epimer in which the *e* propionamide side chain adopts an "upwardly" axial conformation and a series of *c* side chain-modified analogs, including the *c*-monocarboxylate, the *c*-*N*-methylamide, the *c*-*N*,*N*-dimethylamide, and the *c*-*N*-isopropylamide. Activation parameters for the thermal homolysis of these complexes show that the previously observed stabilization of alkylcobalamins by haptocorrin is due to both enthalpic and entropic factors. With the exception of that for the analog having the bulkiest *c* side chain substituent, neopentylcobalamin-*c*-*N*-isopropylamide, the enthalpies of activation are independent of analog structure, but the entropies of activation increase with the steric bulk of the *c* side chain and with the number of "upwardly" projecting side chains, as previously observed for protein-free neopentylcobalamin and its analogs. The results are discussed in terms of the solvent cage effect on Co−C bond homolysis and the importance of corrin ring side chain thermal motions to the entropy of activation for this reaction.

Introduction

Coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl;[1] Figure 1) is involved in the catalysis of a number of enzymatic reactions most of which involve 1,2-intramolecular substrate rearrangements. Direct ESR observations of intermediates with unpaired spins[2−6] have produced general agreement that the first step in the catalytic cycle of such enzymes is the homolytic cleavage of the carbon−cobalt bond of AdoCbl to produce a 5′-deoxyadenosyl radical and paramagnetic cob(II)alamin, the-so called "activation" of coenzyme B_{12}.[5] Studies of the nonenzymatic thermolysis of AdoCbl at elevated temperatures[7−9] suggest that the AdoCbl-dependent enzymes, ribonucleotide reductase[10] and ethanolamine deaminase,[11] are capable of catalyzing the thermal homolysis of AdoCbl by some 9−12 orders of magnitude[7,8,12] While details of the mechanism(s) by which these enzymes achieve this level of catalysis remain obscure, there is general agreement that the flexibility of the corrin ring is important and that enzymes exploit this property by inducing an upward flex in the corrin ring to sterically accelerate Co−C bond cleavage.[13−20]

AdoCbl itself is only slowly thermolyzed at elevated temperatures,[7−9] and its thermolysis results in competing Co−C bond homolysis and heterolysis,[7−9] the latter resulting from the presence of a β oxygen atom in its organic ligand, which permits elimination of cob(III)alamin.[21−24] Consequently, simpler model compounds, particularly benzyl- and neopentylcobalamin (NpCbl[1]), have been widely studied, as these complexes decompose strictly by Co−C bond homolysis since they lack both a β heteroatom and β hydrogens, thus preventing elimination of either cob(III)alamin or cob(I)alamin.[20,25−32] Recent

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(1) Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); NpCbl, neopentylcobalamin; HC, chicken serum haptocorrin; H_2OCbl$^+$, aquacobalamin; CNCbl, cyanocobalamin (vitamin B_{12}); CH_3-Cbl, methylcobalamin; Np-13-epiCbl, neopentyl-13-epicobalamin; NpCbl-*c*-COO$^-$, neopentylcobalamin-*c*-monocarboxylate; NpCbl-*c*-NHMe, neopentylcobalamin-*c*-*N*-methylamide; NpCbl-*c*-NMe_2, neopentylcobalamin-*c*-*N*,*N*-dimethylamide; NpCbl-*c*-NHiPr, neopentylcobalamin-*c*-*N*-isopropylamide.

(2) Valinsky, J. E.; Abeles, R. H.; Mildvan, A. S. *J. Biol. Chem*. **1974**, *249*, 2751.
(3) Valinsky, J. E.; Abeles, R. H.; Fee, J. A. *J. Am. Chem. Soc*. **1974**, *96*, 4709.
(4) Babior, B. M.; Moss, J. H.; Gould, D. C. *J. Biol. Chem*. **1972**, *247*, 4389.
(5) Orme-Johnson, W. H.; Beinert, H.; Blakley, R. L. *J. Biol. Chem*. **1974**, *249*, 2338.
(6) Zhao, Y.; Such, P.; Rétey, J. *Angew. Chem., Int. Ed. Engl*. **1992**, *31*, 215.
(7) Finke, R. G.; Hay, B. P. *Inorg. Chem*. **1984**, *23*, 3041. Correction published: Finke, R. G.; Hay, B. P. *Inorg. Chem.* **1985**, *24*, 1278.
(8) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc*. **1986**, *108*, 4820.
(9) Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1469.
(10) Tamao, T.; Blakley, R. L. *Biochemistry* **1973**, *12*, 24.
(11) Hollaway, M. R.; White, H. A.; Joblin, K. N.; Johnson, A. W.; Lappert, M. F.; Wallis, O. C. *Eur. J. Biochem*. **1978**, *82*, 143.
(12) Brown, K. L.; Cheng, S.; Marques, H. M. *Inorg. Chem.* **1995**, *34*, 3038.
(13) Toraya, T.; Krodel, E.; Mildvan, A. S.; Abeles, R. H. *Biochemistry* **1979**, *18*, 417.
(14) Halpern, J. *Pure Appl. Chem*. **1983**, *55*, 1059.
(15) Pett, V. B.; Liebman, M. N.; Murray-Rust, P.; Prasad, K.; Glusker, J. P. *J. Am. Chem. Soc.* **1987**, *109*, 3207.
(16) Toraya, T.; Ishida, A. *Biochemistry* **1988**, *27*, 7677.
(17) Halpern, J. *Bull. Soc. Chim. Fr*. **1988**, 187.
(18) Marzilli, L. G. In *Bioinorganic Catalysis*; Reedijk, J., Ed.; Marcel Dekker: New York, 1993; pp 227−258.
(19) Pratt, J. M. *Pure Appl. Chem*. **1993**, *65*, 1513.
(20) Waddington, M. D.; Finke, R. G. *J. Am. Chem. Soc*. **1993**, *115*, 4629.
(21) Hogenkamp, H. P. C.; Oikawa, T. G. *J. Biol. Chem*. **1964**, *239*, 1911.
(22) Schrauzer, G. N.; Sibert, J. W. *J. Am. Chem. Soc*. **1970**, *92*, 1022.
(23) Gerards, L. E. H.; Balt, S. *Recl. Trav. Chim. Pays-Bas* **1992**, *111*, 411.
(24) Gerards, L. E. H.; Balt, S. *Recl. Trav. Chim. Pays-Bas* **1994**, *113*, 137.
(25) Grate, J. H.; Schrauzer, G. N. *J. Am. Chem. Soc.* **1979**, *101*, 4601.
(26) Schrauzer, G. N.; Grate, J. H. *J. Am. Chem. Soc*. **1981**, *103*, 541.
(27) Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1980**, 2274.
(28) Blau, R. J.; Espenson, J. A. *J. Am. Chem. Soc.* **1985**, *107*, 3530.
(29) Kim, S.-H.; Chen, H. L.; Feilchenfeld, N.; Halpern, J. *J. Am. Chem. Soc*. **1988**, *110*, 3120.
(30) Brown, K. L.; Brooks, H. B. *Inorg. Chem.* **1991**, *30*, 3420.

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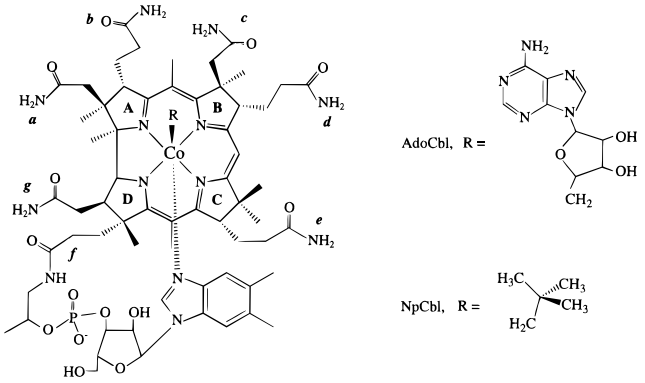

Figure 1. Structure of 5′-deoxyadenosylcobalamin (AdoCbl, coenzyme B_{12}) and neopentylcobalamin (NpCbl).

results from our laboratory[12,30,31,33] suggested that corrin ring side chain thermal motions might be an important source of entropic activation for the homolysis of such complexes. The importance of side chains in the entropy of activation for Co−C bond homolysis is apparent from a comparison of the RCbls to alkylcobaloximes, simple bis(dimethylglyoximato)cobalt model complexes which lack any side chains, and to alkylcobaltoctaethylporphyrins. The entropy of activation for homolysis of (1-phenylethyl)cobaloximes with substituted pyridine axial ligands is characterized by entropies of activation in the range −3.9 to +3.9 cal mol^{-1} K^{-1},[34] while homolysis of benzylcobaltoctaethylporphyrin complexes with phosphine axial ligands occurs with activation entropies of 2−7 cal mol^{-1} K^{-1}.[35] In contrast, the entropies of activation of benzylcobalamin[30] and NpCbl[32] are about 19 cal mol^{-1} K^{-1}.

Recently, we studied the thermolysis of NpCbl analogs in which the number and/or structure of "upwardly" projecting side chains is altered, in order to explore the importance of side chain thermal motions in the activation of Co−C bond homolysis. These have included Np-13-epiCbl, the NpCbl epimer in which the *e* propionamide side chain (Figure 1) adopts an upwardly axial position,[36] and a series of NpCbl analogs in which the upwardly projecting *c* acetamide was systematically altered in order to vary its steric bulk, including the *c*-monocarboxylate (NpCbl-*c*-COO^-), the *c*-*N*-methylamide (NpCbl-*c*-NHMe), the *c*-*N*,*N*-dimethylamide (NpCbl-*c*-NMe_2), and the *c*-*N*-isopropylamide (NpCbl-*c*-NHiPr).[12] The enthalpies of activation for these analogs were essentially unaltered from that of NpCbl itself,[32] suggesting that alteration of the number of side chains at the upper corrin face or substitution at the *c* side chain, even with very bulky groups, does not induce any ground state strain in the Co−C bond. For the series of *c* side chain substituted analogs, NpCbl-*c*-COX, the entropy of activation increases in the order X = O^-, NH_2, NHMe, NMe_2, NHiPr, the same order as the van der Waals volume of X, from 16.4 ± 0.4 to 24.9 ± 0.3 cal mol^{-1} K^{-1}, although the last two compounds have essentially the same $\Delta S^{\ddagger}$ value.

These effects on the activation entropy for homolysis of NpCbl analogs can be interpreted in terms of the importance of side chain rotational motions to the entropy of the complex. In this view, steric interference of the rotational mobility of the upwardly projecting side chains in the ground state by the bulky Np ligand decreases the ground state entropy of the complex. In the homolysis transition state, the increased separation of the Np ligand from the cobalt corrinoid decreases this steric suppression of side chain motion so that reaction progress increases the net entropy of the system. In the case of the *c* side chain substituted analogs, increasing the steric bulk of the substituent progressively increases the steric resistance to side chain rotation, progressively decreasing the ground state entropy. Molecular mechanics calculations[12] have shown that the steric barrier to *c* side chain rotation does indeed increase with increasing size of the side chain substituent and that the barrier to rotation decreases substantially as the Co−C bond is lengthened, approaching the putative transition state. These values for activation entropies were then used to estimate[12] that as much as 30−40% of the catalytic power of an AdoCbl-dependent enzyme for catalysis of AdoCbl homolysis could come from maximal restriction of ground state side chain entropy by the enzyme. Such catalysis might by effected by an enzymatically induced upward flex of the corrin ring, which would increase the steric interactions between the acetamide side chains and the bulky Ado ligand.

However, the thermal mobility of Cbl side chains could be significantly different at an enzyme active site from that in solution. Clearly, in solution, when the *c* side chain is sterically prevented from inwardly projecting conformations, it may still adopt many outwardly projecting conformations. This may not be the case in a protein Cbl-binding pocket, where outward rotation of the side chains may be prevented by nearby amino acid residues. Presumably, increased Co−C separation as the transition state is reached would still provide new conformations for the side chains in the interior space, providing entropic activation for the reaction, but it is not clear how the additional motional restrictions in the enzyme-bound ground state would affect the activation entropy in such circumstances.

In order to address this question, we have now studied the thermal Co−C bond homolysis of the side chain-altered NpCbl analogs bound to a vitamin B_{12} binding protein, a haptocorrin (HC^1) from chicken serum. We have previously used this protein as a model for the interactions between Cbls and enzymes.[31,37,38] This haptocorrin is known to tightly bind Cbls with the upper axial ligand position open to the solution[38] and to stabilize RCbls toward thermal homolysis by some 2−3 orders of magnitude, an effect which has not yet been explained and is also addressed by these studies.

Experimental Section

Fresh blood obtained from chickens at slaughter was centrifuged for 20 min at 9000*g*, and the serum was stored at −20 °C. A modification of the method of Jacobsen et al.[39] was used to purify haptocorrin from chicken serum. Serum was thawed and filtered by suction through cotton gauze and Whatman No. 4 filter paper. Under dim red illumination, 50 mL (settled volume) of Sephacryl beads (S-200-HR; Sigma Chemical Co., St. Louis, MO) containing 1.94 μmol/mL of covalently attached (aminopropyl)cobalamin[39] were added to 2.9 L of filtered chicken serum, and the mixture was stirred for 16.5 h at 4 °C. The unsaturated CNCbl binding capacity of the serum before treatment with the cobalamin affinity beads was 511 ng of CNCbl/mL. After treatment, the binding capacity was 0.24 ng/mL, signifying that essentially all of the apo-HC had been adsorbed by the beads. The serum was then aspirated from the settled beads, and the beads were

(31) Brown, K. L.; Brooks, H. B.; Behnke, D.; Jacobsen, D. W. *J. Biol. Chem*. **1991**, *266*, 6737.
(32) Brown, K. L.; Evans, D. R. *Inorg. Chem*. **1994**, *33*, 6380.
(33) Brown, K. L.; Zou, X.; Evans, D. R. *Inorg. Chem*. **1994**, *33*, 5713.
(34) Ng, F. T. T.; Rempel, G. L.; Halpern, J. *J. Am. Chem. Soc*. **1982**, *104*, 621.
(35) Geno, M. K.; Halpern, J. *J. Am. Chem. Soc*. **1987**, *109*, 1238.
(36) Stoeckli-Evans, H.; Edmond, E.; Hodgkin, D. C. *J. Chem. Soc., Perkin Trans. 2* **1972**, 605.
(37) Brown, K. L.; Marques, H. M.; Jacobsen, D. W. *J. Biol. Chem*. **1988**, *263*, 1872.
(38) Marques, H. M.; Brown, K. L.; Jacobsen, D. W. *J. Biol. Chem*. **1988**, *263*, 12378.
(39) Jacobsen, D. W.; Montejano, Y. D.; Huennekens, F. M. *Anal. Biochem*. **1981**, *113*, 164.

packed into a 3 cm diameter column. The column was washed sequentially with 350 mL of 0.05 M sodium phosphate (pH = 7.0) containing 0.5 M NaCl, 400 mL of 0.05 M sodium acetate (pH = 4.5) containing 0.5 M NaCl, 150 mL of 0.05 M sodium phosphate (pH = 7.0) containing 0.5 M NaCl, 400 mL of 0.05 M sodium glycinate (pH = 9.0) containing 0.5 M NaCl, 150 mL of 0.05 M sodium phosphate (pH = 7.0) containing 0.5 M NaCl, and 100 mL of 1.0 M guanidine hydrochloride (ultrapure; GIBCO BRL, Gaithersburg, MD) (pH = 7.0). The column was then treated with 45 mL of 7.5 M guanidine hydrochloride (pH = 7.0) (fraction A) for 15 min to release apo-HC. This was followed by two additional treatments with 50 mL of 7.5 M guanidine hydrochloride (pH = 7.0) (fractions B and C) for 30 min each which contained the bulk of the released apo-HC. The three fractions were dialyzed exhaustively against 0.05 M sodium phosphate (pH = 7.0) containing 0.5 M NaCl to remove guanidine hydrochloride, Fractions A, B, and C, contained total unsaturated CNCbl binding capacities of 11.9, 404.4, and 214.5 μg, respectively. The overall yield of apo-HC based on the initial CNCbl binding capacity and the recovered binding capacity in fractions B and C was 42%. The specific activities of fractions B and C were 12.8 and 34.6 mg of CNCbl bound/mg of protein. Protein concentration was determined by the BCA method (Pierce, Rockford, IL) using bovine serum albumin as a standard.

NpCbl, Np-13-epiCbl, and the *c* side chain-altered NpCbl-*c*-COX complexes[12] were obtained as described previously[30,33] and stored in 10^{-3} M HCl as their protonated, base-off forms at −20 °C.

UV−visible spectra and single-wavelength kinetic measurements were made on a Cary 219 recording spectrophotometer equipped with a five-cell thermostated cell turret. Temperature was controlled and measured as described previously.[32] Spectrophotometric samples (0.4 mL total volume in 1.0 cm path length quartz microcuvettes) contained (5−9) × 10^{-6} M NpCbl derivative and a slight molar excess (3%) of apo-HC, 0.1 M potassium phosphate buffer, pH 7.5, and KCl to adjust the ionic strength to 1.0 M. Reactions were run aerobically, as dissolved O_2 has been shown to be a kinetically competent trap for the Np$^\bullet$ and cob(II)alamin radicals.[12,30,32,33] Kinetics were monitored at the wavelength of maximum absorbance increase (350−356 nm) and at the wavelength of maximum absorbance decrease (320−324 nm). First-order rate constants were obtained from nonlinear least-squares regressions of the absorbance vs time data to the appropriate exponential function. For each derivative, kinetic measurements were made at 6−10 temperatures over the range 35−60.5 °C, except for NpCbl-*c*-NHiPr, for which the temperature range 5−30 °C was used. The rate constants were fitted to the Eyring equation by a weighted least-squares procedure[28] to obtain the enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation.

Results

Binding of NpCbl and its analogs to HC results in the spectral changes shown in Figure 2 for NpCbl-*c*-NHiPr as an example. These spectra were recorded at 5 °C in order to prevent any significant decomposition of the NpCbl-*c*-NHiPr during the experiment. As is now well documented, NpCbl and its analogs exist at neutral pH as a mixture of the base-on and base-off species, despite the fact that the axial nucleotide is not protonated under these conditions.[12,20,26,30,32,33] This is clearly seen in Figure 2 where the spectrum at pH 7.5 (dashed line) displays some of the spectral features of the base-off species at pH 1.0 (solid line). Upon addition of apo-HC to the neutral sample of NpCbl-*c*-NHiPr, a large spectral change occurs immediately, and the spectrum of the complex (Figure 2, dotted line) strongly resembles that of a base-on RCbl. This confirms our earlier conclusions that Cbls are bound to HC in the base-on form.[31,37]

When the HC-NpCbl-*c*-NHiPr complex was warmed to 25.5 °C, thermal decomposition of the protein-bound NpCbl analog ensued, as shown in Figure 3. First-order spectral changes were observed (Figure 3, inset) with isosbestic points at 336, 369, 409, 454, and 527 nm. The final spectrum strongly resembled that of HC-bound H_2OCbl^+,[37] the anticipated product of Co−C bond homolysis in aerobic solution where cob(II)alamin is rapidly oxidized to H_2OCbl^+. The kinetics of the thermolysis of the complexes of each of the NpCbl analogs with HC were studied at the wavelengths of maximal absorbance decrease (near 322 nm) and increase (near 353 nm). For the HC-NpCbl complex, dissolved oxygen in air-saturated solution was shown to be kinetically competent to trap the products since the observed rate constant (at 55 °C) was unchanged when the reaction was run in O_2-saturated solution. In another control experiment, the rate constant for HC-NpCbl thermolysis at 55

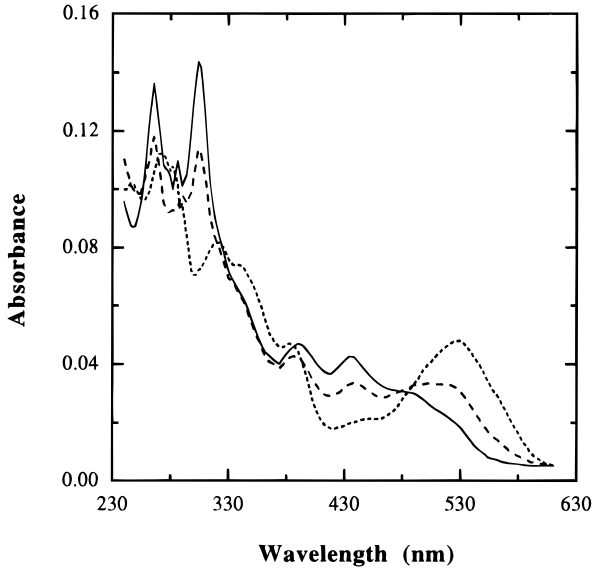

Figure 2. UV−visible spectra of NpCbl-c-NHiPr and its complex with haptocorrin at 5 °C: solid line, base-off, pH 1.0 in 0.1 M HCl; dashed line, neutral species, pH 7.5 in 0.1 M potassium phosphate buffer; dotted line, haptocorrin complex, 5.0 × 10^{-6} M haptocorrin, pH 7.5 in 0.1 M potassium phosphate buffer. The NpCbl-*c*-NHiPr concentration was 4.95 × 10^{-6} M for each spectrum, and ionic strength was adjusted to 1.0 M with KCl.

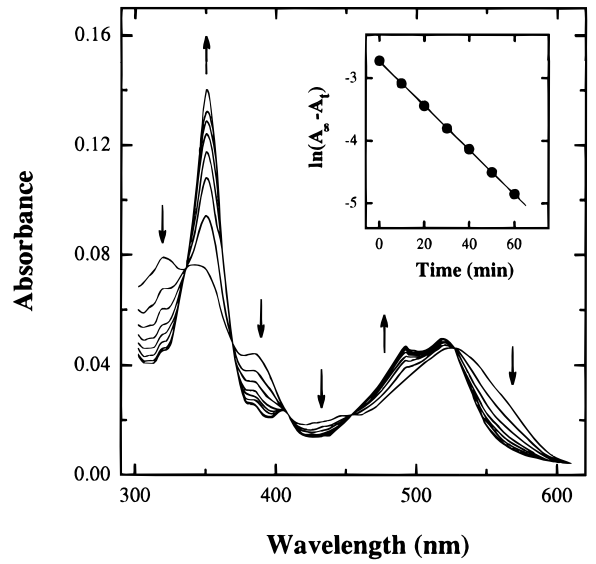

Figure 3. Time-dependent spectral changes due to the thermal Co−C bond homolysis of the haptocorrin complex of NpCbl-c-NHiPr at 25.5 °C. The sample was 4.95 × 10^{-6} M in NpCbl-*c*-NHiPr and 5.0 × 10^{-6} M in haptocorrin, pH 7.5 in 0.1 M potassium phosphate buffer, ionic strength 1.0 M (KCl). The arrows show the direction of the spectral changes at t = 0, 10, 20, 30, 40, 50, 60, and 110 min. Inset: Semilogarithmic plot of the absorbance changes at 351 nm. The solid line is a least-squares regression line, slope = −0.0355 ± 0.0002 min^{-1}, intercept = −2.73 ± 0.006, r^2 = 0.999, and k_{obs} = (5.91 ± 0.03) × 10^{-4} s^{-1}.

Table 1. Activation Parameters for the Thermal Homolysis of NpCbl and Its Side Chain-Altered Analogs and of Their Complexes with Chicken Serum Haptocorrin[a]

	NpCbl-*c*-COO$^-$	NpCbl	NpCbl-*c*-NHMe	NpCbl-*c*-NMe$_2$	NpCbl-*c*-NHiPr	Np-13-epiCbl
			HC Complexes			
$\Delta H^{\ddagger}$, kcal mol^{-1}	30.2 ± 0.1	30.2 ± 0.1	30.9 ± 0.1	31.2 ± 0.4	28.3 ± 0.1	31.6 ± 0.2
$\Delta S^{\ddagger}$, cal mol^{-1} K^{-1}	14.8 ± 0.3	15.0 ± 0.2	17.9 ± 0.5	22.1 ± 0.7	21.4 ± 0.4	20.5 ± 0.5
$\Delta\Delta S^{\ddagger}_{mod}$,[b] cal mol^{-1} K^{-1}	−0.2 ± 0.4	0	2.9 ± 0.5	7.1 ± 0.7	6.4 ± 0.4	5.5 ± 0.5
			Protein-Free Complexes			
$\Delta H^{\ddagger}$,[c] kcal mol^{-1}	27.5 ± 0.1	28.3 ± 0.2[d]	28.7 ± 0.2	29.4 ± 0.2	29.1 ± 0.1	29.7 ± 0.2[e]
$\Delta S^{\ddagger}$,[c] cal mol^{-1} K^{-1}	16.4 ± 0.4	19.3 ± 0.6[d]	21.1 ± 0.7	24.8 ± 0.6	24.9 ± 0.3	24.0 ± 0.6[e]
$\Delta\Delta S^{\ddagger}_{mod}$,[b] cal mol^{-1} K^{-1}	−2.9 ± 0.7	0	1.8 ± 0.9	5.5 ± 0.8	5.6 ± 0.7	4.7 ± 0.8
$\Delta\Delta S^{\ddagger}_{HC}$,[f] cal mol^{-1} K^{-1}	1.6 ± 0.5	4.3 ± 0.6	3.2 ± 0.9	2.7 ± 0.9	3.5 ± 0.5	3.5 ± 0.8

[a] pH 7.5 in 0.1 M potassium phosphate buffer, ionic strength 1.0 M (KCl). [b] Difference in entropy of activation between the NpCbl analog and NpCbl itself. [c] Reference 12 except as noted. [d] Reference 32. [e] Reference 33. [f] Difference in entropy of activation between the protein-free and haptocorrin-bound NpCbl derivative.

°C was also unchanged (within experimental error) by addition of a 2-fold excess of apo-HC. Rate constants at various temperatures were used to construct Eyring plots (not shown) from which activation parameters for the thermal homolysis were extracted. These results are collected in Table 1, which also contains the activation parameters for the thermolysis of the protein-free NpCbl analogs. The latter have been carefully corrected for the presence of the base-off species and so represent the activation parameters for the base-on species only.[12,32,33] In order to allow a precise comparison of the activation parameters for protein-bound NpCbl analogs to those of the HC-NpCbl complex itself, kinetics for the latter complex were reexamined. The resulting activation parameters ($\Delta H^{\ddagger}$ = 30.2 ± 0.1 kcal mol^{-1}, $\Delta S^{\ddagger}$ = 15.0 ± 0.2 cal mol^{-1} K^{-1}) were not significantly different from those previously obtained ($\Delta H^{\ddagger}$ = 29.9 ± 0.5 kcal mol^{-1}, $\Delta S^{\ddagger}$ = 14.1 ± 1.5 cal mol^{-1} K^{-1})[31] but are more precise.

As seen in Table 1, there is little or no variation in the enthalpy of activation for the protein-free NpCbl analogs, the average value being 28.9 ± 0.8 kcal mol^{-1}. As discussed elsewhere,[12] this strongly suggests that the structural modifications considered here do not cause any ground state Co−C bond strain which would be expected to significantly affect $\Delta H^{\ddagger}$. However, the entropies of activation vary quite significantly with structure. For the NpCbl-*c*-COX series, $\Delta S^{\ddagger}$ increases with increasing steric bulk of the X substituent but levels off at about 24.8 cal mol^{-1} K^{-1} for the two bulkiest substituents (X = NMe$_2$, and NHiPr). This represents an increase in activation entropy, $\Delta\Delta S^{\ddagger}_{mod}$ (Table 1), of about 5.5 cal mol^{-1} K^{-1} (or about 25%) above that for NpCbl itself. A similar increase (4.7 cal mol^{-1} K^{-1}) occurs upon epimerization of the *e* side chain in Np-13-epiCbl. These results have been interpreted[12,33] to mean that increasing the steric bulk of the *c* side chain, or placing a new side chain in an upwardly axial position, decreases the ground state entropy of the complex due to increased steric restriction of upwardly projecting side chain rotation by the bulky Np ligand. In the case of the *c* side chain-modified analogs, the increased steric hindrance to *c* side chain rotation with increased bulk of the side chain is supported by molecular mechanics calculations.[12] The partial relief of this steric restriction to side chain rotation as the Co−C bond is stretched during the reaction progress, also supported by molecular mechanics calculations,[12] leads to the observed effects on $\Delta S^{\ddagger}$. The leveling off of $\Delta S^{\ddagger}$ seen in this series is interpreted to mean that once the *c*-COX moiety has reached a certain size, the *c* side chain is effectively excluded from any inwardly pointing conformations and further increases in bulk have little or no additional effect on *c* side chain rotational mobility.

For the HC-bound NpCbl analogs, the data in Table 1 show that, with the exception of that for the analog with the bulkiest *c* side chain substituent, NpCbl-*c*-NHiPr, the enthalpies of activation are again essentially independent of structure, the average value being 30.8 ± 0.6 kcal mol^{-1}. This value is 1.9 ± 0.9 kcal mol^{-1} ($p < 0.1$) greater than that of the protein-free analogs, demonstrating that the complexes experience some enthalpic stabilization of Co−C bond homolysis upon complexation to the protein. This could be a microenvironment effect of the protein binding pocket, as it is similar in magnitude to the difference in $\Delta H^{\ddagger}$ for NpCbl in ethylene glycol[20] and in water,[32] although other explanations are possible as discussed below. Nonetheless, with the exception of NpCbl-*c*-NHiPr, the effect of side chain modification on the homolysis of protein-bound NpCbls is entirely entropic, as is the case for the protein-free NpCbl analogs. Most importantly, the trends in $\Delta S^{\ddagger}$ for the HC-bound NpCbl analogs are quite similar to those for the protein-free complexes. Thus, with the exception of that for the HC-NpCbl-*c*-COO$^-$ complex, the entropy of activation increases with increasing bulk of the *c* side chain but shows a leveling off for the most bulky side chain substituents, as also observed for the protein-free analogs. While the differential entropies of activation due to structural modification, $\Delta\Delta S^{\ddagger}_{mod}$, are larger for each analog when complexed to the protein than when free in solution, in all cases but one the increases are not statistically significant. We conclude that additional restrictions of side chain thermal mobility due to complexation to a protein Cbl-binding pocket, if indeed such occur here, do not significantly affect the entropic participation of side chain mobility in Co−C bond homolysis.

The HC complex of NpCbl-*c*-NHiPr is clearly exceptional. In addition to an increase in $\Delta S^{\ddagger}$ relative to HC-NpCbl ($\Delta\Delta S^{\ddagger}_{mod}$ = 6.4 ± 0.4 cal mol^{-1} K^{-1}), there is a significant decrease in the enthalpy of activation relative to the HC complexes of all of the other analogs ($\Delta\Delta H^{\ddagger}$ = 2.5 ± 0.6 kcal mol^{-1} ($p < 0.02$)). These effects combine to increase the rate of homolysis of HC-NpCbl-*c*-NHiPr by a factor of 700 (at 25 °C) relative to that of HC-NpCbl, the effect being nearly equally entropic (43%) and enthalpic (57%) at this temperature. Here it seems likely that, for the very bulky NHiPr substituent, restriction of side chain mobility by enclosure in the binding pocket does cause steric strain in the Co−C bond since the *c* side chain may be unable to adopt any conformations which do not cause steric contact between the *c* side chain substituent and the Np ligand. The resultant increase in the ground state Co−C bond enthalpy presumably generates the observed effect on $\Delta H^{\ddagger}$.

Discussion

With the exception of NpCbl-*c*-NHiPr, NpCbl and its analogs are stabilized toward thermal Co−C bond homolysis by an average of 380-fold (at 25 °C) upon complexation to haptocorrin. Since it is clear that the upper axial ligand position of HC-

bound Cbls is readily available to solution,[37−39] this effect has not yet been adequately explained. The remote possibility that the HC complexes are totally inert and the observed reactivity is due to a very small amount of free Cbl in rapid equilibrium with the complex is eliminated by the observation that the rate constant for thermolysis of the HC-NpCbl complex was not altered by addition of a 2-fold excess of Apo-HC. In our earlier work on the thermolysis of HC-bound NpCbl and benzylCbl,[31] we concluded that the stabilization of these complexes by complexation to HC was entirely an entropic effect. However, in the case of NpCbl, this conclusion rested on values of the activation parameters for free NpCbl which have subsequently been redetermined with greater accuracy and precision.[32] The results in Table 1 clearly show that NpCbl and its analogs are stabilized upon complexation to HC by both an increase in the enthalpy of activation and a decrease in the entropy of activation. The average increase in $\Delta H^{\ddagger}$ is 1.9 ± 1.0 kcal mol^{-1} (with the exception of that for NpCbl-*c*-NHiPr), while the average decrease in $\Delta S^{\ddagger}$ ($\Delta\Delta S^{\ddagger}_{HC}$ in Table 1) is 3.1 ± 0.9 cal mol^{-1} K^{-1}, so that, at 25 °C, the stabilization is 67% enthalpic and 33% entropic.

It is now clear that metal-carbon bond dissociation reactions proceed through solvent-caged radical pairs[20,40−44] and that cage effects can contribute significantly to the observed kinetics of these reactions. Using the formalism of Koenig et al.[41] (eq 1),

$$\mathrm{M{-}L} \underset{k_c}{\overset{k_1}{\rightleftharpoons}} [\overline{\mathrm{M^{\bullet}L^{\bullet}}}] \underset{k_{-1}}{\overset{k_d}{\rightleftharpoons}} \mathrm{M^{\bullet} + L^{\bullet}} \qquad (1)$$

for the case where one or both of the free radical products are rapidly trapped (as here, by O_2), the observed rate constant for homolysis, k_{obs}, is given by eq 2, where F_c, the fractional cage

$$k_{obs} = (1 - F_c)k_1 \qquad (2)$$

efficiency, is given by eq 3. Frisbie and Chance[45] have reported

$$F_c = k_c/(k_c + k_d) \qquad (3)$$

a large decrease in the quantum yield for CH_3Cbl photolysis upon complexation of CH_3Cbl to human HC and attributed this effect to an increase in the rate constant, k_c, for geminate recombination within the solvent cage. While such an effect is possible, it occurs to us that complexation of an RCbl to a protein the size of HC should also result in a reduction of the rate constant, k_d, for diffusional separation of the caged radical pair. Although the diffusion constants for cob(II)alamin and the Np$^{\bullet}$ radical are not known, it seems quite likely that the diffusional motion of both of these species contributes to k_d for NpCbl homolysis despite the fact that their mass ratio is 18.7:1. The situation for HC-complexed NpCbl is, however, quite different, since the mass ratio for HC-cob(II)alamin and Np$^{\bullet}$ is 938:1. Clearly, in this case, virtually all of the motion involved in the diffusional separation of the caged pair is due to Np$^{\bullet}$ diffusion. Thus, on complexation to the protein, the contribution of cob(II)alamin motion to the diffusional separation of the caged pair is lost and k_d must decrease, leading to an increase in F_c.

Other than Garr and Finke's[43] value of F_c for 5′-deoxyadenosylcobinamide in ethylene glycol at 110 °C ($0.94 \leq F_c \leq 1.0$), values for fractional cage efficiencies are not readily available. However, the cage efficiency for AdoCbl in water at ordinary temperature can be estimated[20] to be 0.7 ± 0.8 from the original flash photolysis study of Endicott and Netzel.[46] The precision of this estimate can be increased substantially by using a more recent value of k_c for AdoCbl,[47] which gives $F_c = 0.7 \pm 0.2$. If this value is approximately correct for NpCbl, then complexation to HC would have to increase F_c to just over 0.999 in order to explain the 380-fold decrease in the observed thermolysis rate constants. This would require a 1220-fold reduction in k_d, which clearly cannot be the case, since the loss of the contribution to diffusional separation by cob(II)alamin motion must have a much smaller effect on k_d than this. Thus, if the cage effect is solely responsible for the stabilization of RCbls by HC, F_c must be increased by both a decrease in k_d and an increase in the rate of geminate recombination (k_c).

Further insight into the importance of cage effects here can be obtained by examining the cage effect on the observed activation parameters. As shown by Koenig et al.,[41] the activation parameters for homolysis are altered by the cage effect as in eqs 4 and 5.[48] As discussed previously,[12,41] the term ($\Delta S^{\ddagger}_d$

$$\Delta H^{\ddagger}_{obs} = \Delta H^{\ddagger}_1 + F_c(\Delta H^{\ddagger}_d - \Delta H^{\ddagger}_c) \qquad (4)$$

$$\Delta S^{\ddagger}_{obs} = \Delta S^{\ddagger}_1 + F_c(\Delta S^{\ddagger}_d - \Delta S^{\ddagger}_c) \qquad (5)$$

$- \Delta S^{\ddagger}_c$) must be positive, since $\Delta S^{\ddagger}_d$ is the entropy of activation due to diffusional separation of the caged pair, while $\Delta S^{\ddagger}_c$ must be negative, since it is the entropy of activation for recombination within the cage. This has led to the conclusion that the cage is in fact an enthalpic phenomenon,[41] which in turn implies that ($\Delta H^{\ddagger}_d - \Delta H^{\ddagger}_c$) must also be positive. This must be true anyway, since $\Delta H^{\ddagger}_d$ is surely positive[41] while $\Delta H^{\ddagger}_c$ must be zero (or negative[49]). Thus, if the fractional cage efficiency is indeed increased when NpCbl or its analogs complex to HC, eqs 4 and 5 predict that both $\Delta H^{\ddagger}_{obs}$ and $\Delta S^{\ddagger}_{obs}$ should increase. As discussed above, $\Delta H^{\ddagger}_{obs}$ does indeed increase (with the exception of that for NpCbl-*c*-NHiPr) upon complexation, and the cage effect may well be responsible for this increase. However, $\Delta S^{\ddagger}_{obs}$ decreases upon complexation to HC, and so, if there is an increase in the cage efficiency upon complexation, which seems quite likely, some other factor must counter the expected increase in $\Delta S^{\ddagger}_{obs}$.

We previously suggested[31] that the entropic stabilization of NpCbl and benzylcobalamin upon complexation to HC might be due to hydrogen-bonding interactions between the corrin side chains and the protein binding pocket. Such an interaction would decrease side chain thermal mobility and thus lower the entropy of activation by decreasing the extent of relief of the steric restriction to side chain rotation in the transition state due to the departing Np ligand. The current data can be brought to bear on this question since the structural alterations of the NpCbl analogs alter the ability of the side chains to act as hydrogen bond donors. Thus, the decrease in the entropy of activation upon complexation to the protein ($\Delta\Delta S^{\ddagger}_{HC}$, Table 1) should reflect the alteration of hydrogen-bonding capabilities of the analogs if such a mechanism is occurring. As a result, NpCbl itself would be expected to have the largest value of $\Delta\Delta S^{\ddagger}_{HC}$, those analogs which have lost one N−H donor (NpCbl-*c*-NHMe and NpCbl-*c*-NHiPr) to have somewhat lower values of $\Delta\Delta S^{\ddagger}_{HC}$,

(40) Koenig, T.; Finke, R. G. *J. Am. Chem. Soc.* **1988**, *110*, 2657.
(41) Koenig, T. W.; Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1499.
(42) Daikh, B.; Finke, R. G. *J. Am. Chem. Soc*. **1992**, *114*, 2938.
(43) Garr, C. D.; Finke, R. G. *J. Am. Chem. Soc*. **1992**, *114*, 10440.
(44) Wayland, B. B.; Gridnev, A. A.; Ittel, S. D.; Fryd, M. *Inorg. Chem.* **1994**, *33*, 3830.
(45) Frisbie, S. M.; Chance, M. R. *Biochemistry* **1993**, *32*, 13886.
(46) Endicott, J. F.; Netzel, T. L. *J. Am. Chem. Soc*. **1979**, *101*, 4000.
(47) Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1993**, *115*, 12152.
(48) The cage interpretation requires the implicit assumption that $\Delta H^{\ddagger}_1$ and $\Delta S^{\ddagger}_1$ are gas phase values unaffected by differential solvation.
(49) McMillen, D. F.; Golden, D. M. *Annu. Rev. Phys. Chem*. **1982**, *33*, 493.

and those that have lost both N−H donors (NpCbl-*c*-NMe_2, NpCbl-*c*-COO^-, and, presumably, Np-13-epiCbl) to have the lowest values of $\Delta\Delta S^{\ddagger}_{HC}$. Inspection of the data in Table 1 shows that there is at least a suggestion of such a pattern in the $\Delta\Delta S^{\ddagger}_{HC}$ values for the NpCbl-*c*-COX series in which the values decrease in the order NpCbl > NpCbl-*c*-NHMe, NpCbl-*c*-NHiPr > NpCbl-*c*-NMe_2 > NpCbl-*c*-COO^-. However, the differences between the $\Delta\Delta S^{\ddagger}_{HC}$ values are small and are not, in general, statistically significant for adjacent members of the order given above. In addition, the $\Delta\Delta S^{\ddagger}_{HC}$ value for Np-13-epiCbl would seem to be too large, unless there is a fortuitous hydrogen bond acceptor in the binding pocket which can interact with this misplaced side chain. We conclude that the present data cannot distinguish between the presence or absence of hydrogen-bonding interactions with the corrin side chains in the Cbl-HC complexes. This question is currently being addressed by attempts to measure the thermodynamics of the binding of side chain-altered Cbls to HC and by attempts to observe the amide ^1H and ^{15}N NMR resonances of HC-bound Cbls.

Conclusion

NpCbl and its side chain-altered analogs (with the exception of NpCbl-*c*-NHiPr) are stabilized toward thermal homolysis bysome 380-fold (or 3.5 kcal mol^{-1}) by complexation to haptocorrin due to an average increase in $\Delta H^{\ddagger}_{obs}$ of ∼1.9 kcal mol^{-1} and a decrease in $\Delta S^{\ddagger}_{obs}$ of ∼3.1 cal mol^{-1} K^{-1}. Consideration of the expected effect of complexation to the protein on the solvent cage effect leads to the conclusion that the cage efficiency must increase due to the reduced rate of diffusional separation of the caged pair. However, it is clear that the increased cage efficiency alone cannot account for the observed stabilization of NpCbl and its analogs. This increased cage efficiency should lead to increases in both the observed enthalpy and entropy of activation. In contrast, the entropy of activation is decreased upon complexation to the haptocorrin. This observation is consistent with our earlier suggestion that entropic stabilization of NpCbl by haptocorrin results from restriction of the motion of the acetamide side chains by hydrogen-bonding interactions with the protein binding pocket. However, the actual changes in $\Delta S^{\ddagger}_{obs}$ observed here among the side chain-altered analogs of NpCbl are not sufficiently different to provide direct support for this hypothesis.

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