Adenosylcobinamide, the Base-Free Analog of Coenzyme B_{12} (Adenosylcobalamin). 1.[1] Probing the Role of the Axial 5,6-Dimethylbenzimidazole Base in Coenzyme B_{12} via Exogenous Axial Base $K_{association}$, ΔH, and ΔS Measurements plus a Critical Review of the Relevant Biochemical Literature

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Adenosylcobinamide ($AdoCbi^+BF_4^-$), the base-free form of adenosylcobalamin (AdoCbl or coenzyme B_{12}), has been studied with a series of 14 exogenous α-axial bases. Specifically, equilibrium association constants, K_{assoc}, as a function of temperature were measured, and thus their associated ΔH and ΔS were obtained. Bases studied include the following: (i) exogenous 1,5,6-trimethylbenzimidazole [analogous to adenosylcobalamin's intramolecularly appended 5,6-dimethylbenzimidazole base]; (ii) sterically encumbered phosphine bases (none of which showed detectable binding in dramatic contrast to studies of, for example, cobaloxime B_{12} "models"); and (iii) electronically increasingly donating, but isosteric, 4-substituted pyridine axial bases. The general trends from the present K_{assoc} studies are 2-fold: the more electron donating the base, the greater the K_{assoc}, and bulky bases bind weakly if at all. This paper also contains a tabular summary of the existing, *non-Ado* $RCbi^+$ axial-base K_{assoc} literature plus the relatively few associated ΔH and ΔS values that are available. Selected B_{12} model axial-base K_{assoc} literature is also summarized as Supporting Information. In addition, the Discussion contains a critical analysis of the prior, B_{12} enzymic biochemical literature relevant to the role of AdoCbl's appended 5,6-dimethylbenzimidazole axial base.

Introduction

The Axial-Base Problem.[1] In 1968, the X-ray diffraction analysis[2] of coenzyme B_{12} (AdoCbl)[3] established four key structural features for this complex metallo-cofactor, Figure 1: the axial 5′-deoxyadenosyl alkyl group and its celebrated Co−C bond, the flexible corrin ring macrocycle, the six amide side chains attached to the corrin (three up and three down, Figure 1), and the intramolecularly appended 5,6-dimethylbenzimidazole axial base.[4]

Despite intense prior effort, the exact biological role of the appended axial base in AdoCbl has remained unclear.[4−8] Indeed, Marzilli noted in 1993 that "the role of the (5,6-dimethyl)benzimidazole ligand is the most uncertain aspect of the involvement of the Cbl component in the Co−C bond homolysis".[4f] The main reason for this uncertainty had been the absence, until recently, of an X-ray crystallographic structure

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(1) (a) Part 1, this paper, as well as Part 2[1b] are based on the following dissertation: Garr, C. D. Adocobinamide (Axial Base-off Coenzyme B_{12}) Equilibria and Co−C Bond Cleavage Kinetic Studies: Mechanistic Probes into the Function of Coenzyme B_{12}'s Axial Base. Ph.D. Dissertation, February 1993. (b) Part 2, Garr, C. D.; Sirovatka, J. M.; Finke, R. G. *J. Am. Chem. Soc.*, in press. (c) Sirovatka, J. M.; Finke, R. G. manuscript in preparation (on $[AdoCbi\cdot(N\text{-}MeIm)]^+$ K_{assoc}, ΔH, ΔS, and Co−C cleavage product and kinetic studies; to be submitted for publication in *J. Am. Chem. Soc.*).

(2) (a) Lenhert, P. G. *Proc. R. Soc. London, Ser. A* **1968**, *303*, 45. (b) More recent, high-resolution neutron diffraction studies of $AdoB_{12}$ are available: Bouquiere, J. P.; Finney, J. L.; Lehmann, M. S.; Lindley, P. F.; Savage, H. F. J. *Acta Crystallogr.* **1993**, *B49*, 79−89 (a 15 K structure). See also: Savage, H. F. J.; Lindley, P. F.; Finney, J. L.; Timmins, P. A. *Acta Crystallogr.* **1987**, *B43*, 280−295 (a 279 K structure).

(3) Abbreviations used herein include the following: AdoCbl or $AdoB_{12}$ (adenosylcobalamin or, equivalently, coenzyme B_{12} or, equivalently, 5′-deoxy-5′-adenosylcobalamin); $AdoCbi^+$ ($AdoCbi^+BF_4^-$, adenosylcobinamide or, equivalently, 5′-deoxy-5′-adenosylcobinamide); Co^{II}-Cbl (B_{12r}; cob(II)alamin, the d^7 Co(II) cobalamin product formed by homolytic cleavage of the AdoCbl's Co−C bond); $Co^{II}Cbi^+$ (cob(^{II}-)inamide, the axial-base-free analog of Co^{II}Cbl); AdoCbl-Me^+ (AdoCbl alkylated at the benzimidazole N3 nitrogen with a methyl group in order to render the benzimidazole incapable of coordinating to cobalt); AdoCbi-P-Me^+ (adenosylcobinamide methyl phosphate; that is, the methylated phosphate (but 5,6-dimethylbenzimidazole free) form of AdoCbl); TEMPO (2,2,6,6-tetramethylpiperidinyl-1-oxy).

(4) Lead general reviews of B_{12} chemistry include the following: (a) *B_{12}*; Dolphin, D., Ed.; Wiley-Interscience: New York, 1982; Vols. I and II. (b) *Vitamin B_{12}, Proceedings of the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor*; Zagalak, B., Friedrich, W., Eds.; Walter de Gruyter: New York, 1979. (c) Babior, B. M.; Krouwer, J. S. *CRC Crit. Rev. Biochem.* **1979**, *6*, 35. (d) Abeles, R. H.; Dolphin, D. *Acc. Chem. Res.* **1976**, *9*, 114. (e) Golding, B. T.; Rao, D. N. R. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987. (f) For a concise discussion of the possibility of an "ideal" Co−N bond to the axial 5,6-dimethylbenzimidazole ligand, see pp 248−249 of: Marzilli, L. In *Bioinorganic Catalysis;* Reedijk, J. Ed.; Marcel Dekker, Inc.: New York, 1993; pp 227−259. (g) Finke, R. G. In *Molecular Mechanisms in Bioorganic Processes*; Bleasdale, C., Golding, B. T., Eds.; The Royal Society of Chemistry: Cambridge, England, 1990. (h) Finke, R. G.; Schiraldi, D. A.; Mayer, B. J. *Coord. Chem. Rev.* **1984**, *54*, 1.

(5) (a) The bulk of mechanistic studies pertaining to the α-axial-base have been performed on cobaloxime "B_{12}-model" compounds.[5b−e,6] Even though these studies have made great strides in understanding the axial-base interactions in *cobaloxime*s (and thus valued contributions to inorganic chemistry), logic demands that all extensions to the α-axial-base effect in *coenzyme B_{12}* must be purely speculative, rigorously speaking. Restated, the B_{12} cofactor is a complicated and sterically encumbered molecule in comparison to any of the so-called "B_{12} models". For example, only B_{12} contains a flexible and formally −1 corrin ring and six important amide side chains and also contains an intramolecularly appended axial 5,6-dimethylbenzimidazole base. For lead references see: (b) Schrauzer, G. N.; Windgassen, R. J. *J. Am. Chem. Soc.* **1966**, *88*, 3738. (c) Toscano, P. J.; Marzilli, L. G. *Prog. Inorg. Chem.* **1984**, *31*, 105. (d) Bresciani-Pahor, N.; Forcolin, M.; Marzilli, L. G.; Randaccio, L.; Summers, M. F.; Toscano, P. J. *Coord. Chem. Rev.* **1985**, *63*, 1. (e) Randaccio, L; Bresciani-Pahor, N.; Zangrando, E.; Marzilli, L. G. *Chem. Soc. Rev.* **1989**, *18*, 225. (f) See also ref 1a−r provided as part of the Supporting Information.

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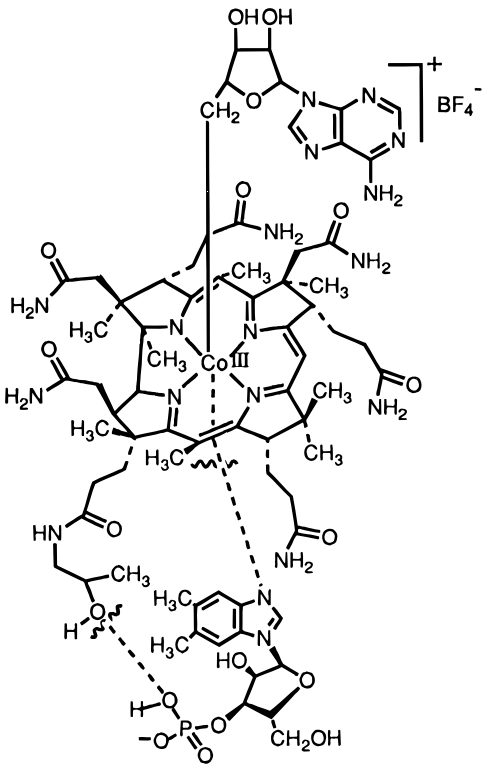


Figure 1. A composite representation of 5′-deoxy,-5′-adenosylcobinamide (AdoCbi$^+$ BF_4^-) plus, in the lower part of the figure, the α-ribazole (1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole) fragment produced by $Ce(OH)_3$-catalyzed H_2O addition across the phosphodiester bond in the adocobalamin (AdoCbl or, equivalently, coenzyme B_{12}) synthetic precursor. The two wavy lines locate the two chemical cleavage sites of the P−O and Co−N bonds present in the AdoCbl starting material. The 5,6-dimethylbenzimidazole nucleotide is shown with an exaggerated displacement from its normal, closer-to-cobalt orientation in base-on AdoCbl since it is, of course, completely absent in the isolated AdoCbi$^+BF_4^-$ used in the present studies.

determination for any of the *Ado*B_{12}-dependent enzymes. The recent description of the structure of methylmalonyl-CoA (MMCoA) mutase at 2.0 Å resolution reveals that, as first discovered for *Me*B_{12}-dependent methionine synthase,[9ab] the AdoB_{12} cofactor is bound in a dimethylbenzimidazole *base-off* form[9c] (in MMCoA mutase and probably in several other, *but not in all*, AdoB_{12}-dependent enzymes;[9b,d] *vide infra*). Instead, the nucleotide-loop appended 5,6-dimethylbenzimidazole serves to "anchor" the B_{12} cofactor to MMCoA mutase, and a protein side-chain-histidine imidazole serves as cobalt's axial base. Rather clearly, the modern era of X-ray crystallography-based B_{12} bioinorganic chemistry is now able to unfold, one in which virtually all previous thinking about the role of the 5,6-dimethylbenzimidazole axial base needs to be reanalyzed.

A second reason for the past uncertainty about the biological role of AdoCbl's axial nucleotide loop and associated, intramolecularly appended 5,6-dimethylbenzimidazole has been the absence, until more recently, of *reliable* studies of B_{12} enzymes using *authentic, pure* AdoCbi$^+$ with pure enzyme (AdoCbi$^+$ is the nucleotide loop and associated 5,6-demethylbenzimidazole-free derivative[3] of AdoCbl). An important series of four recent papers by Toraya and co-workers,[10] studying AdoCbi$^+$ (and other AdoCbl derivatives in which the nucleotide loop or axial-base has been modified) in combination with B_{12}-dependent diol dehydratase, fills this second void (see the Discussion section). There is also a recent, important study of AdoCbi$^+$ and Co^{II}-Cbi$^+$ binding to methylmalonyl-CoA mutase to yield *inactive* protein that does not exhibit Co−C cleavage,[10f] a discussion of which is postponed until later in the paper. A third reason for the past uncertainty about the role of B_{12}'s appended 5,6-dimethylbenzimidazole has been the absence of a critical analysis of the prior biochemical and bioinorganic literature related to the coenzyme B_{12} axial-base problem. However, the needed critical analysis is presented as part of the Discussion section herein (see the section, A Critical Analysis of Key Biochemical Literature), the first version of which was available in 1993,[1a] a year prior to first X-ray crystallographic evidence for the enzymic base-off form of AdoB_{12}. This latter fact is mentioned here only for the perspective it provides since, even then (i.e., in the absence of any enzyme structural information), the available enzymic data argued that the 5,6-dimethylbenzimidazole nucleotide might be less involved in the initial Co−C cleavage than previously assumed (see pp 168−170 elsewhere[1a]). A fourth reason for the past uncertainty about the biological role of AdoCbl's axial base was, prior to the present contribution, the absence of a study of *AdoCbi*$^+$ *itself* with a variety of axial bases, Scheme 1. Such studies are necessary in order to provide appropriate chemical precedent, and *a non-enzymic reference point*, for the chemically plausible roles for AdoCbl's 5,6-dimethylbenzimidazole axial base. The result of now being able to overcome all four of the deficiencies noted above is a more consistent picture, from both the B_{12} enzyme/biological and the bioinorganic perspectives, of the role of AdoCbl's appended axial-base (*vide infra*).

(6) (a) Marzilli, L. G.; Toscano, P. J.; Randaccio, L.; Bresciani-Pahor, N.; Calligaris, M. *J. Am. Chem. Soc.* **1979**, *101*, 6754. (b) Randaccio, L.; Bresciani-Pahor, N.; Toscano, P. J.; Marzilli, L. G. *J. Am. Chem. Soc.* **1980**, *102*, 7372. (c) Randaccio, L.; Bresciani-Pahor, N.; Toscano, P. J.; Marzilli, L. G. *J. Am. Chem. Soc.* **1981**, *103*, 6347. (d) Bresciani-Pahor, N.; Randaccio, L.; Toscano, P. J.; Marzilli, L. G. *J. Chem. Soc., Dalton Trans.* **1982**, 567. (e) Summers, M. F.; Toscano, P. J.; Bresciani-Pahor, N.; Nardin, G.; Randaccio, L.; Marzilli, L. G. *J. Am. Chem. Soc.* **1983**, *105*, 6259. (f) Summers, M. F.; Marzilli, L. G.; Bresciani-Pahor, N. Randaccio, L. *J. Am. Chem. Soc.* **1984**, *106*, 4478. (g) Bresciani-Pahor, N.; Randaccio, L.; Zangrando, E.; Summers, M. F.; Ramsden, J. H., Jr.; Marzilli, P. A.; Marzilli, L. G. *Organometallics* **1985**, *4*, 2086. (h) Parker, W. O., Jr.; Bresciani-Pahor, N.; Zangrando, E. Randaccio, L.; Marzilli, L. G. *Inorg. Chem.* **1985**, *24*, 3908. (i) Bresciani-Pahor, N.; Randaccio, L.; Zangrando, E.; Toscano, P. J. *Inorg. Chim. Acta* **1985**, *96*, 193. (j) Marzilli, L. G.; Summers, M. F.; Bresciani-Pahor, N.; Zangrando, E.; Charland, J.-P.; Randaccio, L. *J. Am. Chem. Soc.* **1985**, *107*, 6880. (k) Mealli, C.; Sabat, M.; Marzilli, L. G. *J. Am. Chem. Soc.* **1987**, *109*, 1593.

(7) (a) Grate, J. H.; Schrauzer, G. N. *J. Am. Chem. Soc.* **1979**, *101*, 4601. (b) Schrauzer, G. N.; Grate, J. H. *J. Am. Chem. Soc.* **1981**, *103*, 541.

(8) Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1980**, 2274.

(9) (a) Luschinsky-Drennan, C.; Huang, S.; Drummond, J. T.; Matthews, R. G.;Ludwig, M. L. *Science* **1994**, *266*, 1669−1674. (See also the introductory comments about this landmark paper: Stubbe, J. *Science* **1994**, 1663−1664.) (b) Drennan, L. C.; Matthews, R. G.; Ludwig, M. L. *Curr. Opin. Struct. Biol.* ***1994***, *4*, 919−929. (c) Mancia, F.; Keep, N. H.; Nakagawa, A.; Leadlay, P. F.; McSweeney, S.; Rasmussen, B.; Böscke, P.; Diat, O.; Evans, P. R. *Structure* **1996**, *4*, 339. We thank Professor Leadlay for sharing this important development with us prior to publication. (d) Ludwig, M. L.; Drenan, C. L.; Matthews, R. G. *Structure* **1996**, *4*, 505. (e) For the suggestion of two distinct subclasses of AdoB_{12}-dependent enzymes, depending upon whether or not a Ado$^\bullet$ or protein side-chain −S$^\bullet$ (or other −X$^\bullet$) substrate C−H bond, H$^\bullet$ atom-abstractor is present, see: Marsh, E. G. N. *Biochemistry* **1995**, *34*, 7542.

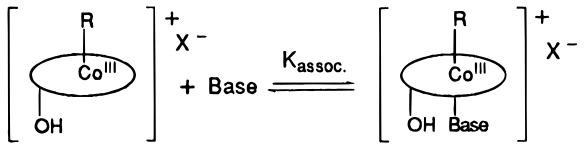
(10) (a) Ishida, A.; Ichikawa, M.; Kobayashi, K.; Hitomi, T.; Kojima, S.; Toraya, T. *J. Nutr. Sci. Vitaminol.* **1993**, *39*, 115. (b) Toraya, T.; Ishida, A. *J. Biol. Chem.* **1991**, *266*, 5430. (c) Ishida, A.; Toraya, T. *Biochemistry*, **1993**, *32*, 1535. (d) Studies of the pyridyl appended-base analog of coenzyme B_{12}: Toraya, T.; Miyoshi, S.; Mori, M.; Wada, K. *Biochem. Biophys. Acta* **1994**, *1204*, 169−175. (e) It is, of course, not surprising that a better picture of the role of AdoCbl's nucleotide loop and appended axial base required reliable studies of (i) *nucleotide loop and axial-base free* AdoCbi$^+$ with a B_{12} enzyme (Toraya's studies[10a−c]) and (ii) enzyme-free AdoCbi$^+$ with exogeneous axial bases (the present work and an accompanying paper1b). (f) See ref 50 for the interesting study from the Banerjee laboatories of AdoCbi$^+$, Co^{II}Cbi$^+$, and Co^{II}Cbl with methylmalonyl-CoA.

Table 1. Literature K_{assoc} (25 °C; H_2O) for Non-Ado Alkylcobinamides plus Various Bases

entry	R	base	pK_a of base[a]	K_{assoc} (M^{-1})	ref
1	CH_3	azide	4.4	4.17 (2.09)[b] [3.5 ± 0.2][c]	12c [12e][c]
2	CH_3	pyridine	5.2	6	12a
3	CH_3	pyridine	5.2	9	12d
4	CH_3	pyridine	5.2	7.32	12c
5	CH_3	4-CH_3−pyridine	6.0	11.5	12c
6	CH_3	imidazole	7.0	8.0	12c
7	CH_3	imidazole	7.0	11	12a
8	CH_3	imidazole	7.0	7.9	12b
9	CH_3	1-CH_3−imidazole	7.2	5	12a
10	CH_3	cyanide	9.1	230 [84 ± 3][c]	12a [12e][c]
11	CH_3	ammonia	9.2	0.1	12a
12	CH_3	ammonia	9.2	0.2	12d
13	CH_3	4-NH_2-pyridine	9.2	24.0	12c
14	CH_3	ethanolamine	9.4	0.03	12a
15	CH_3	piperidine	11.2	<0.01	12a
16	$CH_3CH_2CH_2$	imidazole	7.0	0.1	12a
17	CH_3CH_2	imidazole	7.0	0.32	12b
18	benzyl	azide	4.4	0.63 (0.32)[b]	12c
19	benzyl	pyridine	5.2	0.13	12c
20	benzyl	4-CH_3−pyridine	6.0	0.75	12c
21	benzyl	imidazole	7.0	0.06	12c
22	benzyl	4-NH_2−pyridine	9.2	1.8	12c
23	neopentyl	pyridine	5.2	0.11[d]	12c
24	neopentyl	4-NH_2−pyridine	9.2	0.53[d]	12c
25	neopentyl	imidazole	7.0	0.04	12b
26	isopropyl	imidazole	7.0	<0.01	12b
27	cyclohexyl	imidazole	7.0	<0.01	12b

[a] Aqueous pK_a values are taken from: Christensen, J. J.; Hansen, L. D.; Izatt, R. M. *Handbook of Proton Ionization Heats;* Wiley & Sons: New York, 1976. Since the same relative order of pK_a values is seen in MeOH and EtOH, *op. cit.*, these pK_a values should follow the same *relative order* in the closely related solvent, ethylene glycol. [b] The reported (observed) literature value is given first, with the value in parentheses having had the 2-fold statistical factor removed from this entry so that it can be compared directly to the rest of the entries in the table and in Table 3. [c] Brown's recent data in 1.0 M KCl ionic strength.[12e] [d] These two entries are 50 °C data.

Scheme 1. The $RCbi^+X^-$ plus Exogenous Axial Base Equilibrium



$$K_{assoc.} \equiv \frac{[RCbi^+ \bullet Base]}{[RCbi^+]\,[Base]}$$

It may, at first, seem surprising that there is no previous study of axial base K_{assoc} or associated thermodynamic parameters for the biologically relevant Ado-alkyl coenzyme B_{12} cofactor derivative, $AdoCbi^+$. But, there is one identifiable reason for this: prior to the report of an improved synthesis[11a] and the unequivocal characterization[11b] of $AdoCbi^+BF_4^-$, the required quantity of pure, well-characterized $AdoCbi^+$ starting material was simply not available for the present studies.

Previous Non-Ado $RCbi^+$ K_{assoc} Studies. There are a number of earlier reports of *non-Ado* alkylcobinamide axial base K_{assoc} measurements,[12] Table 1, plus a few axial-base-binding ΔH and ΔS measurements, the latter all being due to the efforts of Professor Ken Brown's laboratories,[12c] Table 2. Since these data have never been compiled in a single place before (some of the data are fairly recent[12c,e]) and also because they provide the needed reference point for the $AdoCbi^+$ values reported herein, the relevant K_{assoc} and ΔH and ΔS data (i.e., those which describe nitrogeneous-base binding , plus a few others) are assembled in Tables 1 and 2, respectively. Highlights of the trends in the data are also briefly summarized. A recent study, by Brown and Satyanarayana, of K_{assoc} constants for CN^- and N_3^- binding to a series of six β-$RCbi^+$ and four α-$RCbi^+$, is also available to the interested reader.[12e] Again for the convenience of the interested reader, we have also summarized, as Supporting Information, some of the key highlights of a much larger number of "B_{12} model" axial-base K_{assoc} values, even though an analysis of that data reveals that such "model" K_{assoc} values are, not unexpectedly, of little direct relevance to the biologically relevant *adocobalamin* axial-base problem.

Table 2. Literature ΔH and ΔS for Alkylcobinamide Axial-Base Equilibria (H_2O, Ionic Strength, 1.0 M)

entry	R	base	pK_a of base[a]	ΔH (kcal/ mol)[b]	ΔS (eu)[b]	ref
1	CH_3	pyridine	5.2	−3.8 ± 0.2	−8.8 ± 0.5	12c
2	CH_3	4-NH_2−pyridine	9.2	−5.3 ± 0.5	−11.4 ± 1.8	12c
3	benzyl	pyridine	5.2	−0.3 ± 0.5	−5.2 ±2.0	12c
4	benzyl	4-NH_2−pyridine	9.2	−2.1 ± 0.5	−5.7 ±1.6	12c
5	neopentyl	pyridine	5.2	−0.0 ± 0.6	−4.4 ± 1.8	12c
6	neopentyl	4-NH_2−pyridine	9.2	−1.6 ±0.6	−6.2 ± 4.0	12c

[a] Aqueous pK_a values are taken from: Christensen, J. J.; Hansen, L. D.; Izatt, R. M. *Handbook of Proton Ionization Heats;* Wiley & Sons: New York, 1976. [b] Literature error estimates.12c

(11) (a) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 8012. (b) For a 2D NMR study of AdoCbi+ see: Pagano, T. G.; Yohannes, P. G.; Hay, B. P.; Scott, J. R.; Finke, R. G.; Marzilli, L. G. *J. Am. Chem. Soc.* **1989**, *111*, 1484.

(12) (a) Pailes, W. H.; Hogenkamp, H. P. C. *Biochemistry* **1968**, *7*, 4160. (b) Baldwin, D. A.; Betterson, E. A.; Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1985**, 1613. (c) Brown, K. L.; Brooks, H. B. *Inorg. Chem.* **1991**, *30*, 3420. (d) Brodie, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *62*, 461. (e) Brown, K. L.; Satyanarayana, S. *Inorg. Chim. Acta* **1992**, *201*, 113−119. (f) Pratt has established, at least for non-alkyl cobamides, a linear free energy relationship of increasing log K with axial-base pK_a, log $K = a(pK_a) + b$ (where a = positive): Hamza, M. S. A.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1994**, 1377 (and earlier papers in this series). Note, however, that there are unresolved issues of composition and α,β-structural isomerism in the resulting $[(H_2O, CN)Cbi{\cdot}Base]^+$ product complexes in this work.

Several general trends and other findings emerge from a perusal of the $RCbi^+$ K_{assoc} values listed in Table 1. First, the K_{assoc} values are <1 for most alkyls and bases examined. (An exception is the relatively small alkyl, methyl, where K_{assoc} > 1 is generally the case.) Second, K_{assoc} values generally decrease as the steric bulk of the trans alkyl group is increased, as expected.[12] For example, CH_3Cbi^+ binds imidazole >1000-fold better than does isopropyl- or cyclohexylcobalamin, entry 8 vs entries 26 and 27, Table 1. Third, the more electron-donating the base, the greater the K_{assoc}, in general, again as expected.[12] (The relative electron-donating ability of each base is indicated by its solution pK_a in Table 1. Noteworthy here is Pratt's demonstration, at least for non-alkyl cobamides, of a linear log $K = a(pK_a) + b$ relationship, where a = positive.[12f])

An interesting counterexample to the third generalization above is provided by a comparison of CH_3Cbi's respective ca. 2300 and 10-fold stronger binding of CN^- vs the nearly equal pK_a ligands NH_3 and 4-NH_2-pyridine (entries 10, 11, 12, and 13, Table 1). This can be rationalized, as Brown first noted,[13] by involking a π-back-bonding contribution for CN^- binding, $CH_3CoC{\equiv}N \leftrightarrow CH_3Co^+{=}C{=}N^-$.

Table 2 compiles the available, again all non-Ado, alkylcobinamide axial-base thermodynamic parameters, ΔH and ΔS. A couple of insights can be gleaned from this table: first, such measurements are relatively rare in comparison to single-temperature K_{assoc} measurements, with Brown and Brooks providing the only prior data;[12c] second, while the observed values are of course dependent on the axial-base chosen and the specific trans-axial alkyl, the data at least to date all fall within the range of $\Delta H = 0$ to -5.3 kcal/mol and $\Delta S = -4$ to -11 eu.

Finally, it is worth mentioning that there is one aspect of AdoCbl's appended 5,6-dimethylbenzimidazole axial base that is well understood: it undergoes a facile α-axial base-on, base-off equilibrium, AdoCbl having been called "astonishingly labile for a diagmagnetic, d^6 Co(III) complex".[14] Hence, a slow approach to equilibrium is not a problem in the K_{assoc} equilibrium studies undertaken herein.

Present Studies of $AdoCbi^+$·Base Axial-Base Binding Constants. Herein we report a study of *Ado*$Cbi^+BF_4^-$ axial-base K_{assoc} constants and thermodynamic parameters (ΔH and ΔS, where measurable) for a series of 14 exogenous axial bases. The bases examined include the following: (i) 1,5,6-trimethylbenzimidazole (analogous to the intramoleculary appended 5,6-dimethylbenzimidazole base of adenosylcobalamin); (ii) sterically encumbered (but electronically similar)[15] phosphine bases; (iii) electronically increasingly donating, but isosteric,[16] 4-substituted pyridine bases. Studies of the (non) binding of RSH and RS^- are also reported, along with a tabulation of the upper limits to their K_{assoc} values, Table B, Supporting Information.[17] The results of these K_{assoc}, ΔH, and ΔS studies are then analyzed and discussed in terms of the key, B_{12}-enzymic biochemical literature. Studies of nitrogeneous and RSH/RS^- axial-base K_{assoc} and ΔH and ΔS for coordination to $Co^{II}Cbi^+$, the $AdoCbi^+$ Co−C bond homolyis corrin product, are in progress and will be reported separately in due course.[18]

The axial-base data reported herein are also a necessary prerequisite to the product, kinetic and mechanistic study of Co−C cleavage from these $AdoCbi^+$·Base complexes that is reported in an accompanying publication,[1b] work required to provide the *first firm chemical precedent and reference point* for the role of *Ado*Cbl's axial 5,6-dimethylbenzimidazole. The very interesting case of the *N*-methyl imidazole complex of $AdoCbi^+$, $[AdoCbi{\cdot}N\text{-MeIm}]^+$, is nearing completion and its K_{assoc}, ΔH, ΔS, and associated Co−C cleavage product and kinetic studies will be reported elsewhere.[1c] Also available[1b] are kinetic studies of Co−C cleavage of $AdoCbi^+$ in the presence of RS^-/RSH.[1b]

Experimental Section

Chemicals. $AdoCbi^+BF_4^-$ was prepared according to our literature procedure (98% pure by HPLC)[11a] and stored below 0 °C; solutions of $AdoCbi^+$ were protected from light at all times. 1,5,6-Trimethylbenzimidazole was prepared as previously described[19] (mp 143−144 °C; lit.[19] 142−143 °C). Reagent grade pyridine (MCB) and ethylene glycol (Baker) were freshly vacuum distilled from 4 Å molecular sieves. 4-Cyanopyridine (Aldrich) was recrystallized from CH_2Cl_2/ether. All other materials were Aldrich reagent grade (or better) unless otherwise indicated and were used as received, including: 4-methylpyridine, 4-aminopyridine, 4-(*N*,*N*-dimethylamino)pyridine, aniline, *N*,*N*-dimethylaniline, tricyclohexylphosphine (Strem), triphenylphosphine, triisopropylphosphine (Strem), tri-*n*-butylphosphine, triethylphosphine, *n*-butyldiphenylphosphine (Strem), and trimethylphosphine (Alfa). Glutathione (98−100%, Sigma), β-mercaptoethanol (99$^+$%, Aldrich), and dithioerythritol (99$^+$%, Aldrich) were used as received.

Instrumentation. UV−visible spectra were recorded on a Beckman DU-7 spectrophotometer equipped with a thermoelectric Peltier cell-block temperature controller. Sample temperatures were measured using a Princo Instruments Hg thermometer (NBS certified; ±0.2 °C) immersed in a cuvette filled with ethylene glycol.

Sample Preparation, Data Collection, and K_{assoc} Calculations. Like all alkylcobinamides, $AdoCbi^+$ exhibits a yellow to red shift upon α-axial base binding, providing a spectroscopic handle from which the desired K_{assoc} values were obtained. $AdoCbi^+$ samples (ca. 2×10^{-4} M) were prepared in Pyrex cuvettes (Spectrocell; 1 cm pathlength) and sealed with rubber septa; preselected base concentrations ranged from 2×10^{-2} to 2.0 M (10^2-10^4 equiv). UV−visible spectra were recorded from 600 to 350 nm vs a reference cell containing the same amount of base in ethylene glycol; data were collected at 380, 415, 455, and 520 nm as a function of temperature (10−50 °C)[20] and for the preselected base concentration. Isosbestic points were observed at 475 and 390 nm throughout these experiments for each of the axial bases whose K_{assoc} values are reported in Table 3 (see the sample spectra provided as Supporting Information). Association constants and their error bars were calculated by linear regression (eq 1; for [base] ≫ [$AdoCbi^+$·Base]). The results were independently checked using Drago's triangular method, eq 2,[21a] which gives a visual picture of the precision of the data and, hence, tends to give a more realistic estimate of the associated, and often larger, error bars. The error bars reported herein are *the larger* of those provided by the two methods. Thermodynamic

(13) (a) Brown K. L.; Gupta, B. D. *Inorg. Chem.* **1990**, *29*, 3854. (b) Brown, K. L.; Hakimi, J. M. *J. Am. Chem. Soc.* **1986**, *108*, 496. (c) Brown K. L.; Satyanarayana, S. *Inorg. Chem.* **1992**, *31*, 1366.
(14) Thusius, D. *J. Am. Chem. Soc.* **1971**, *93*, 2629.
(15) (a) Streuli, C. A. *Anal. Chem.* **1960**, *32*, 985. (b) Henderson, W. A.; Streuli, C. A. *J. Am. Chem. Soc.* **1960**, *82*, 5791.
(16) Ng, F. T. T.; Rempel, G. L.; Halpern, J. *J. Am. Chem. Soc.* **1982**, *104*, 621.
(17) The interested reader is also directed to the discussion of the prior literature of RSH/RS^- binding to alkycobamides that is provided as part of the Supplementary Materials.
(18) Garr, C. D.; Sirovatka, J. M.; Finke, R. G. Unpublished results and experiments in progress.
(19) Simonov, A. M.; Pozharskii, A. E.; Marianovskii, V. M. *Ind. J. Chem.* **1967**, *5*, 81.
(20) (a) Early attempts to measure K_{assoc} values by varying the temperature at constant axial-base and $AdoCbi^+BF_4^-$ concentrations gave non-linear ln K_{assoc} vs $1/T$ plots and thus proved useless. Such curved plots are almost surely due to the temperature dependence of the extinction coefficients involved, a now well-established point.[20b-d] (b) Firth, R. A.; Hill, H. A. O.; Mann, B. E.; Pratt, J. M.; Thorp, R. G.; Williams, R. J. P. *J. Chem. Soc. A* **1968**, 2419. (c) Brown, K. L.; Peck-Siler, S. *Inorg. Chem.* **1988**, *27*, 3548. (d) Waddington, M.; Finke, R. G. *J. Am. Chem. Soc.* **1993**, *115*, 4629 (see the section therein on the axial-base on-off K_{eq} studies).
(21) (a) Drago, R. S. *Physical Methods in Chemistry*; W. B. Saunders: Philadelphia, PA 1977; p 90. (b) Alelyunas, Y. A.; Fleming, P. F.; Finke, R. G.; Pagano, T. G.; Marzilli, L. G. *J. Am. Chem. Soc.* **1991**, *113*, 3781.

Table 3. Association Constants and Thermodynamic Parameters for $AdoCbi^+$ plus Selected Bases (Ethylene Glycol, 25 °C)

nitrogenous base	pK_a[a]	$K_{assoc(25\,°C)}$ (M^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
1,5,6-$(CH_3)_3$-benzimidazole	5.6	<0.2		
4-CN-pyridine	1.9	≤ 0.2		
pyridine	5.2	1.0 ± 0.2	−3.3 ± 0.4	−11 ± 1
4-CH_3−pyridine	6.0	1.0 ± 0.2	−3.2 ± 0.1	−10.6 ± 0.3
4-NH_2−pyridine[b]	9.2	2.4 ± 0.3	−4.5 ± 0.5	−14 ± 1
4-Me_2N−pyridine	9.7	2.5 ± 0.1	−6.5 ± 1.0	−20 ± 3
aniline (control)[b]	4.6	<0.06		
N,*N*-Me_2−aniline (control)[b]	5.1	<0.05		

[a] Aqueous pK_a values are taken from: Christensen, J. J.; Hansen, L. D.; Izatt, R. M. *Handbook of Proton Ionization Heats;* Wiley & Sons: New York, 1976. (Since the same relative order is seen in MeOH and EtOH, *op. cit.*, these pK_a values should follow the same relative order in the closely related solvent, ethylene glycol.) [b] These control experiments (i.e., with aniline and dimethylaminoaniline) were done to verify that the pyridine nitrogen of NH_2−pyridine, and not the amine nitrogen, coordinates to cobalt; see the Experimental Section for further details.

parameters (ΔH and ΔS) were derived in the usual fashion from ln K_{assoc} vs $1/T$ plots. Representative spectra overlays, data plots, and ln K_{assoc} vs $1/T$ plots are provided as Supporting Information.

$$\text{Abs} = \frac{-(\text{Abs} - \text{Abs}_o)}{[B_o]}\left(\frac{1}{K_{assoc}}\right) + \text{Abs}_\infty \qquad (1)$$

Equation 1 has been derived (and used) previously elsewhere[21b]; it is applicable to an equilibria of the form A + B ⇌ C, where A = $AdoCbi^+$, B = base, and C = $AdoCbi^+$·base. Additional definitions or conditions needed for the derivation[21b] are as follows: $[A_o]$ is $[AdoCbi^+]_{initial}$, $[B_o] = [B]_{initial} = [B]_{final}$ (since $[B_o]$ is ≥ $100[A_o]$ by design in these experiments), $[C_o] = 0$; the mass balance equation $[A_o] = [A] + [C]$ is obeyed and $[C_\infty] = [A_o]$; only two species are absorbing so that Abs (total; at the chosen λ) = $Abs_A + Abs_C$ (since B is largely nonabsorbing in the region scanned and since the reference cell contains the identical [B] used in the sample cell); Beer's law is obeyed, for example $Abs_o = b\epsilon_A[A_o]$ and $Abs_\infty = b\epsilon_C[C_\infty]$ $(=b\epsilon_C[A_o])$.

Drago's Equation and Method.[21a]

$$K^{-1} = \frac{(\text{Abs} - \text{Abs}_o)}{(\epsilon_c - \epsilon_a)} - [A_o] - [B_o] + \frac{([A_o][B_o](\epsilon_c - \epsilon_a))}{(\text{Abs} - \text{Abs}_o)} \qquad (2)$$

In cases where no apparent equilibrium was detectable (i.e., the visible spectrum showed no change upon addition of excess base) the maximum K_{assoc} was calculated using eq 3 which assumes that >5% of any base-on form would have been readily detectable by UV−visible.

Cases Where No (<5%) Base-on Form Could Be Detected.

$$K_{assoc} < \frac{0.05[A_o]}{([A_o] - 0.05[A_o])([B_o] - 0.05[A_o])} \approx \frac{0.05}{[B_o]} \qquad (3)$$

Control Experiments Showing That 4-Aminopyridine Binds through the Pyridine Nitrogen. The sample preparation and data collection for these two control experiments, using aniline and *N*,*N*-dimethylaniline, were done identically to the other K_{assoc} experiments described above. The results are presented in Table 3 (and demonstrate that 4-aminopyridine binds to $AdoCbi^+$ through the pyridine−and not the amino−nitrogen).

Control Experiment Measuring K_{assoc} for 4-(Dimethylamino)-pyridine Binding to $AdoCbi^+$ at 110 °C by Independent Kinetic Studies. During the course of our separate kinetic studies (reported elsewhere[1b]) of Co−C cleavage in these $[AdoCbi\cdot base]^+$ complexes, it was possible to obtain, from reciprocal plots done as part of our kinetic studies,[1b] an independent K_{assoc} at 110 °C for 4-(dimethylamino)-pyridine binding. The value so obtained, $K_{assoc} = 0.6 \pm 0.10\ M^{-1}$ at 110 °C, is in good agreement with the calculated value, $K_{assoc} = 0.3 \pm 0.2\ M^{-1}$ at 110 °C, obtained from the ΔH and ΔS measured herein for *p*-dimethylaminopyridine binding. The agreement within experimental error provides excellent, independent evidence for the validity of at least the 4-(dimethylamino)pyridine data (and, by inference, the other equilibrium data presented herein obtained and analyzed by identical methods).

Attempted $AdoCbi^+$ K_{assoc} Studies with RSH/RS^-. In a manner strictly analogous to the procedure described above for the nitrogeneous axial-base studies, the 25 °C K_{assoc} of ca. 1×10^{-4} M $AdoCbi^+$ with several thiols and thiolates in ethylene glycol were studied. Any pH adjustments necessary to reach the desired pH (i.e., as reported along with the data, Table B, Supporting Information), were made by the dropwise addition of 10% NaOH. K_{assoc} limits were calculated using eq 3, and are also tabulated in Table B, Supporting Information, since at each initial RSH concentration tested, from 1.0 to 1.4×10^5 equiv (1.0×10^{-4} M to 14.3 M), there is no detectable change in the visible spectrum (and hence no evidence for any thiol or thiolate binding to $AdoCbi^+$ in ethylene glycol under the conditions studied).

Results and Discussion

$AdoCbi^+$ K_{assoc} Measurements. Association constants for $AdoCbi^+$ (in ethylene glycol)[22] in the presence of exogenous base were measured according to the standard methods detailed in the Experimental section. For all bases displaying a measurable binding constant at 25 °C, concentration-dependent K_{assoc} measurements were made as a function of temperature in the range from 10 to 50 °C, Table 3. Standard ln K_{assoc} vs $1/T$ plots of the data yielded the associated ΔH and ΔS values summarized in Table 3.

Attempts were also made to measure association constants for $AdoCbi^+$ with six different tertiary phosphines (exhibiting cone angles[23] from 118 to >170°, Table A, Supporting Information). Although the relatively low solubility of phosphines in ethylene glycol proved to be a limiting factor, in each case the maximum possible phosphine concentration was used. *None of the phosphines tested, PR_3* (R_3 = Me_3, Et_3, *i*-Pr_3, Bu_3, $Cyclohexyl_3$, or Ph_2Bu), *promoted any change in the UV−visible spectrum of $AdoCbi^+$*, not even the relatively unencumbered, strong σ-donor trimethylphosphine ($K_{assoc} < 0.2$). This finding stands in dramatic contrast to the generally tight binding reported for phosphines with alkylcobaloximes.[24a] On the other hand, our observations are fully consistent with an early−but little cited−report that even 1 M PPh_3 did not bind to $RCbi^+$ (R = Me, Et, CH_2=CH−, and CN) nor to $Co^{II}Cbl$.[24b] Hence, the alkylcobaloxime B_{12}-models again[25] appear to be very poor quantitative mimics of the biologically relevant and much more complicated, side-chain containing and thus sterically encumbered $AdoCbi^+$.

Two distinct subsets of nitrogeneous base to $AdoCbi^+$ K_{assoc} values are summarized in Table 3; each is discussed in turn below. First, 1,5,6-trimethylbenzimidazole was tested for comparison of its *inter*molecular equilibrium (K_{assoc}, M^{-1}) to the *intra*molecular equilibrium (K'_{assoc}) of AdoCbl with its

(22) The choice of ethylene glycol as solvent was based on four considerations: (i) the B_{12}-binding pocket of B_{12}-dependent enzymes is almost surely not water-like and thus closer in its properties to ethylene glycol (i.e., than to water; this is also one reason that our Co−C thermolysis studies have emphasized ethylene glycol[1b,5,27]); (ii) the bases of interest have an increased solubility in ethylene glycol compared to water; (iii) most of the previous AdoCbl equilibria and kinetic studies have been performed in ethylene glycol;[1b,5,27] (iv) the nitroxide radical trapping method using TEMPO to study Co−C homolyses is cleanest in ethylene glycol (due to the greater stability of $Co^{II}B_{12}$ in the presence of TEMPO in ethylene glycol compared to water).[27]

(23) Tolman, C. A. *Chem. Rev.* **1977**, *77*, 313.

(24) (a) Ng, F. T. T.; Rempel, G. L.; Halpern, J. *Inorg. Chim. Acta* **1983**, *77*, L165; Halpern, J. *Chem Soc. Jpn.* **1988**, *61*, *13* [see Table 1 therein for studies of RCo(cobaloxime)(PR_3) complexes in toluene, acetone, and ethylene glycol (although K_{assoc} constants for phosphines are not reported)]. (b) Firth, R A.; Hill, H. A. O.; Pratt, J. M.; Thorp, R. G.; Williams, R. J. P. *J. Chem. Soc. A* **1969**, 381.

natural, appended 5,6-dimethylbenzimidazole base,[26] $K'_{assoc(25\ °C)}$ = 14.3 in ethylene glycol[27] (i.e., 93.5% base-on at 25 °C). Consistent with this expected weaker binding in the intermolecular case, exogenous 1,5,6-trimethylbenzimidazole showed no detectable binding to AdoCbi$^+$ ($K_{assoc} < 0.2\ M^{-1}$). Even in the hypothetical case of neat 1,5,6-trimethylbenzimidazole (ca. 9.4 M), the upper limit to K'_{assoc} ($=K_{assoc} \times 9.4$ M base) is ≤ 1.9, that is, ≥ 7.5-fold less than the intramolecular $K'_{assoc(25\ °C)} = 14.3$ for the AdoCbl cofactor. [Neat axial base is the obvious—although rigorously arbitrary—reference point, one that will be used throughout the paper, since the intramolecularly appended 5,6-dimethylbenzimidazole axial-base in B_{12} is effectively "neat" (and also enjoys any additional enthalpic or entropic advantages confered by being intramolecular).] These results provide the first quantitative evidence in support of the long-held suspicion that evolution necessarily led to the *intramolecularly* appended 5,6-dimethylimidazole base in order to overcome the otherwise intrinsically weak 5,6-dimethylbenzimidazole-to-cobalt binding in the AdoCbl cofactor, thereby ensuring that the nucleotide loop, the axial base, and the Ado-Co(corrin) cannot become separated. This finding is, we note, fully consistent with the MMCoA mutase X-ray crystallographic results,[9c] showing the the nucleotide loop and appended 5,6-dimethylbenzimidazole serve as a "anchor" for the B_{12} cofactor. It is also consistent with the existence of a class of AdoB$_{12}$-dependent enzymes in which the 5,6-dimethylbenzimidazole apparently remains coordinated to cobalt,[9b,d] *vide infra*.

The second subset of data in Table 3 employs a series of isosteric but increasingly basic, and thus increasingly more electron-donating, 4-substituted pyridines. These studies, chosen to probe the electronic effect of these axial bases on the K_{assoc}, ΔH and ΔS values, are modeled after Kallen, Brown and co-worker's original use of 4-substituted pyridines with methylcobaloxime,[28] as well as Halpern and co-worker's alkylcobaloxime Co−C homolysis B_{12} model studies using these same pyridine bases.[16] The results, Table 3, are unexceptional in that the observed K_{assoc} values increase as the basicity of the 4-substituted pyridine employed increases (i.e., the same trend seen for other RCbi$^+$ complexes,[12] Table 1, *vide supra*, as well as non-alkyl Cbi$^+$ complexes[12f]). Picking neat (12.4 M) pyridine as the reference point, and recalling that pyridine's pK_a (5.2) is approximately similar to that of 5,6-dimethylbenzimidazole (pK_a = 5.6), one finds that the *intra*molecular $K'_{assoc} = 14.3$ for the AdoCbl is essentially the same within experimental error as the K'_{assoc} for neat pyridine binding, K'_{assoc} ($=K_{assoc} \times 12.4$ M neat pyridine) = 12.4 (±2.5) at 25 °C. This comparison again suggests that the appended 5,6-dimethylbenzimidazole base in AdoCbl has been selected for some property *other* than its ability to bind tightly to the adocorrin cofactor. This finding is fully consistent with Toraya's B_{12}-enzyme studies[10] (see the section that follows titled Biological Implications) and, of course, also with the 5,6-dimethylbenzimidazole base-off finding highlighting the MMCoA mutase X-ray crystal structure.[9c]

The general trends in the [AdoCbi·base]$^+$ ΔH and ΔS data are similar in kind, but not size, to literature parameters derived for benzyl- and neopentylcobinamide with pyridine bases,[12c] Table 2. For example, benzylCbi$^+$ plus axial-base binding enthalpies range from -0.3 ± 0.5 to -2.1 ± 0.5 kcal/mol, and the entropies from -5.2 ± 2.0 to -5.7 ± 1.6 eu, for pyridine and aminopyridine, respectively.[12c] However, the binding parameters for MeCbi$^+$ plus pyridine and aminopyridine ($\Delta H = -3.82 \pm 0.15$ to -5.27 ± 0.5 kcal/mol and $\Delta S = -8.8 \pm 0.5$ to -11.4 ± 1.8 e.u) *do* fall in roughly the same range as those obtained herein for AdoCbi$^+$ (which, for pyridine and aminopyridine, respectively, are $\Delta H = -3.3$ and -4.5 kcal/mol and $\Delta S = -11.1$ and -13.7 eu).[12c]

Further scrutiny of the [AdoCbi·base]$^+$ axial-base binding ΔH and ΔS data reveals that some compensation in the net free energy function, ΔG, appears to be taking place (i.e., compensation between the enthalpy and the entropy).[29−31] Specifically, as the enthalpy becomes more favorable for the more basic pyridines (ΔH goes from -3.2 ± 0.2 to -6.5 ± 1.0 kcal mol^{-1}), the entropy shows the opposite, compensating trend toward less favorable values (ΔS goes from -10.6 ± 0.3 to -20 ± 3 eu), a trend which is larger than the experimental error.

Attempted AdoCbi$^+$ K_{assoc} Studies with RSH/RS$^-$. In a manner analogous to that for the other exogeneous bases tested, an attempt was made to measure the 25 °C K_{assoc} values for ca. 1×10^{-4} M AdoCbi$^+$ with several thiols and thiolates in ethylene glycol. At initial RSH concentrations from 1.0×10^{-4} to 14.3 M (i.e., 1.0 to 1.4×10^5 equiv, at least for β-mercaptoethanol) *there is no UV−visible evidence for any thiol or thiolate binding to AdoCbi$^+$ in ethylene glycol*, $K_{assoc} < 0.5\ M^{-1}$ for all cases tested (RSH = β-mercaptoethanol, dithioerythritol, glutathione; many of the thiols were examined at pH 7.2 and also at pH 10, the latter pH ensuring that a significant concentration of RS$^-$ is present, see Table B, Supporting Information). The results of these particular experiments show that, at least for each specific case and under the 25 °C temperature and other conditions tested, no thiol or thiolate α-axial binding could be detected.

Consistent with our findings for AdoCbi$^+$, Pailes and Hogenkamp could not detect any spectral changes when 1−2 M

(25) For further discussions of the limitations, and the strengths, of B_{12} model complexes see: (a) Elliott, C. M.; Hershenhart, E.; Finke, R. G.; Smith, B. L. *J. Am. Chem. Soc.* **1981**, *103*, 5558. (b) Finke, R. G.; Smith, B. L.; Droege, M. W.; Elliott, C. M.; Hershenhart, E. *J. Organomet. Chem.* **1980**, *202*, C25. (c) Finke, R. G.; Smith, B. L.; Mayer, B. J.; Molinero, A. A. *Inorg. Chem.* **1983**, *22*, 3677. (d) Milton, P. A.; Brown, T. L. *J. Am. Chem. Soc.* **1977**, *99*, 1390. (e) Halpern's work.[24a] (f) Rossi, M.; Glusker, J. P.; Randaccio, L.; Summers, M. F.; Toscano, P. J.; Marzilli, L. G. *J. Am. Chem. Soc.* **1985**, *107*, 1729. (g) Bresciani-Pahor, N.; Forcolin, M.; Marzilli, L. G.; Randaccio, L. Summers, M. F.; Toscano, P. J. *Coord. Chem. Rev.* **1985**, *63*, 1. (h) We note, however, that quantitative K_{assoc}, ΔH, and ΔS measurement in ethylene glycol for the following Ado−alkyl complexes would be of value and would permit a more quantitative comparison to the AdoCbi$^+$ studies provided in Table 3: adocobaloxime(solvent), adoCo(SALEN/SALOPH), and adoCo(tropocoronands).

(26) The free base (i.e. the detached nucleotide α-ribazole-3′-phosphate which contains a 5,6-dimethylbenzimidazole moiety) has a pK_a of 5.6,[26a] whereas the appended base has a pK_a of 3.5,[26bd] due to the extra driving force provided by coordination of the deprotonated 5,6-dimethylbenzimidazole base to Co(III).[26b−d] (a) Brown K. L. *J. Am. Chem. Soc.* **1987**, *109*, 2277. (b) Hayward, G. C.; Hill, H. A. O.; Pratt, J. M.; Vanston, N. J.; Williams, R. J. P. *J. Chem. Soc.* **1965**, 6485. (c) Dolphin, D. H.; Johnson, A. W.; Rodrigo, R. *Ann. N. Y. Acad. Sci.* **1964**, *112*, 590. (d) Broadie, J. D.; Poe, M. *Biochemistry* **1972**, *11*, 2534.

(27) (a) Finke, R. G.; Hay, B. P. *Inorg. Chem.* **1984**, *23*, 3041. (b) Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1469. (c) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 4820.

(28) Brown, K. L.; Chernoff, D.; Keljo, D. J.; Kallen, R. G. *J. Am. Chem. Soc.* **1972**, *94*, 6697.

(29) (a) A highly linear compensation[30] between ΔH and ΔS (i.e., an isoequilibrium relationship) has been reported for the binding of *p*-substituted pyridines to [Co^{II}heptamethylcobrinate]$^+$ClO$_4^-$.[29b] (b) For axial-base K_{assoc} and ΔH and ΔS studies of [Co^{II}heptamethylcobrinate]$^+$ ClO$_4^-$ in CH_2Cl_2 or toluene with a series of *p*-subsituted and other axial bases, see: Murakami, Y.; Hisaeda, Y.; Kajihara, A. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3642.

(30) For a brief but insightful discussion of compensation effects see: Benson, S. W. *Thermochemical Kinetics*; Wiley-Interscience: New York, 1976. See also refs 12−15 provided elsewhere.[31a]

(31) The role of compensation in the *kinetics* of Co−C homolysis is somewhat controversial:[31a,b] (a) Halpern, J. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 13. (b) Wolowiec, S.; Balt, S.; De Bolster, M. W. G. *Inorg. Chim. Acta* **1991**, *181*, 131.

cysteine was added to $MeCbi^+$ in H_2O at room temperature.[12a] Similarly, Hogenkamp found no evidence for 2-mercaptoethanol coordination to MeCbl by ^{13}C NMR at ambient temperature.[32] However, it initially appeared that the above three sets of *consistent results* were apparently in conflict with work reported by Law and Wood.[33] These authors initially interpreted the red to yellow spectral change, observed upon the addition of glutathione to AdoCbl (and at a pH claimed at first to be 7.4), as evidence for glutathione RSH coordination to Ado*Cbl*. But, these same authors subsequently corrected themselves by showing that a base-on to base-off change was all that was occurring due, simply, to an inadequate phosphate buffer capacity[34] and thus due to protonation of the 5,6-dimethylbenzimidazole of AdoCbl (i.e., recall that glutathione contains a second $-CO_2H$ group, over and above the one that is part of the amino acid moiety, and thus will protonate bases such as the 5,6-dimethylbenzimidazole in $AdoB_{12}$ in the absence of sufficient buffer). Hence, our results (i.e., demonstrating RSH/RS^- nonbinding) are actually fully consistent with all of the available literature[12a,32−34] examining RSH/RS^- plus $RCbi^+$ complexes.

We note, however, that thiols and thiolates *do bind* to *non-alkyl* cobalamins such as aquoCo(III)Cbl[35] —for example, glutathione does react with $HOCo^{III}Cbl$.[36] Thiols and thiolate binding to the cobaloxime[28,37] (or Costa-type[38]) B_{12} model compounds is also well established, but these results, when compared to the data for $AdoCbi^+$ summarized in Table B in the Supporting Information, once again demonstrate that the cobaloximes bind axial bases much too tightly to be reliable semiquantitative model complexes for this particular physical property of alkylcobamides.

Three Limiting Hypotheses for the Role of the Axial 5,6-Dimethylbenzimidazole Base. Three limiting hypotheses have appeared in the literature for the biological role(s) of the nucleotide loop and appended 5,6-dimethylbenzimidazole base in AdoCbl. First, there is the *base-on* hypothesis, in which the appended 5,6-dimethylbenzimidazole is crucial for the key initial Co−C homolysis step (the bioinorganic chemistry-based "corrin butterfly conformational distortion" theory[39]). Second, there is the *benzimidazole base-off, protein side-chain base-on* hypothesis in which the sixth ligand for AdoCbl is provided by a protein side-chain base (e.g., the suggestion long ago of histidine,[40] a suggestion now unequivocally established by X-ray crystallography for the case of MMCoA mutase[9c]). In this case, the appended 5,6-dimethylbenzimidazole serves to help bind and otherwise anchor the AdoCbl cofactor to the enzyme. Third, there is Pratt's *base-off* hypothesis for AdoCbl, derived from Pratt's interpretation[41] of an absorbance at 440 nm (ca. indigo absorption/yellow-orange transmittance) that is *present during (only) the catalytic steady state*, as suggesting that the cobalt to 5,6-dimethylbenzimidazole bond *is broken* during catalysis, at least in the B_{12}-dependent enzymes ribonucleotide reductase and ethanolamine ammonia-lyase. It should be emphasized that sequence alignment and other data indicate that there are very likely *two distinct subclasses* of $AdoB_{12}$ binding enzymes: the MMCoA mutase-like, benzimidazole-base-*off* class (MMCoA mutase, methyleneglutarate mutase, and glutamate mutase), and a second class that appears to be benzimidazole-base-*on* (diol dehydratase, glycerol dehydratase, ethanolamine ammonia lyase, and ribonucleotide triphosphate reductase).[9b,d]

Key Findings and Their Biological Implications. The most interesting finding from these studies is that, in comparison to other, equal pK_a bases, the appended 5,6-dimethylbenzimidazole base in AdoCbl *appears to have been selected for some reason other than its ability to bind especially tightly to the cobalt corrin*. In an accompanying paper[1b] we show that the appended 5,6-dimethylbenzimidazole is *not* unique in comparison to the other exogenous, intermolecularly binding bases tested in its ability to promote Co−C homolysis, although it is somewhat special in its ability to inhibit undesirable, nonbiological Co−C *heterolysis* in AdoCbl. Our product-based (and thus unequivocal) finding that the 5,6-dimethylbenzimidazole base-on form favors Co−C homolysis over heterolysis[1b] is consistent with Marzilli and Randaccio's suggestion,[42] based on their extensive quantitative structural studies of Co−N bond distances, that the unusually long Co−N bond length in AdoCbl may well have been selected, via the "fine-tuning" of evolution, to stabilize $Co^{II}B_{12}$—that is, at least for the subclass of $AdoB_{12}$-dependent enzymes that appear to remain in the 5,6-dimethyl-

(32) Hogenkamp, H. P. C.; Bratt, G. T.; Kotchevar, A. T. *Biochemistry* **1987**, *26*, 4723.
(33) Law, P. Y.; Wood, J. M. *J. Am. Chem. Soc.* **1973**, *95*, 914. See also refs 1−14 therein to the early literature of B_{12} plus thiols and related topics.
(34) (a) Frick, T.; Francia, M. D.; Wood, J. M. *Biochim. Biophys. Acta* **1976**, *428*, 808−818. (b) Unfortunately, the correction[34a] to their earlier, 1973 work[33] investigating AdoCbl and glutathione is buried as a few lines in a 1976 paper that investigates a different system (MeCbl plus RSH).[34a] Initially unaware of this correction, we (as we suspect others before us) repeated the original, flawed experiment (i.e., as part of other work studying glutathionylB_{12}). Fortunately, however, we also measured the pH, found it to be 3.9 and not the initially reported[33] 7.4 (for a mixture of 2×10^{-5} M AdoCbl, 0.05 M KH_2PO_4 buffer and *0.12 M* glutathione), and thus quickly realized that the buffer capacity had been exceeded. This, in turn, led us to believe initially that we had discovered an uncorrected error in the 1973 paper.[33] But, a subsequent careful search of the literature revealed Law and Wood's 1976 correction of their own work[34a] (and thus that we had only confirmed their explanation), and we mention it here in the hopes of saving others from also wasting time repeating those original, flawed experiments.[33]
(35) Nome, F.; Fendler, J. H. *J. Chem. Soc., Dalton Trans* **1976**, 1212.
(36) Adler, N.; Medwick, T.; Poznanski, T. J. *J. Am. Chem. Soc.* **1966**, *88*, 5018.
(37) (a) Brown, K. L.; Chernoff, D.; Keljo, D. J.; Kallen, R. G. *J. Am. Chem. Soc.* **1972**, *94*, 1894. (b) A RS−Co(DMG)$_2$(pyridine) complex has been identified in the following work: Schrauzer, G. N.; Windgassen, R. J. *J. Am. Chem. Soc.* **1967**, *89*, 3607.
(38) (a) Pellizer, G.; Tauszik, G. R.; Costa, G. *J. Chem. Soc., Dalton Trans* ***1973***, 317. (b) Alexander, V. *Inorg. Chim. Acta* **1989**, *163*, 143.
(39) (a) The corrin ring "butterfly" conformation has been much discussed in the literature; lead refs include Glusker's chapter[39b] plus others (and references 3f, 6, 15, 18c therein). (b) Glusker, J. P. In *B_{12}*; Dolphin, D., Ed., Wiley-Interscience: New York, 1982; Vol. 1, Chapter 3. (c) See also: Glusker, J. P. *Vitamins Hormones* **1995**, *50*, 1−76.
(40) (a) Lien, E. L.; Ellenbogen, L.; Law, P. Y.; Wood, J. M. *Biochem. Biophys. Res. Commun.* **1973**, *55*, 730. (b) Lien, E. L.; Ellenbogen, L.; Law, P. Y.; Wood, J. M. *J. Biol. Chem.* **1974**, *249*, 890. (c) Histidine has been identified as the axial ligand to cobalt in the *p*-cresoyl cobamide in the acetogenic bacterium *Sporomusa ovata*: Stupperich, E.; Eisinger, H., J.; Albracht, S. P. J. *Eur. J. Biochem.* **1990**, *193*, 105.
(41) (a) Pratt, J. M. *Inorg. Chim. Acta* **1983**, *79*, 27. (b) However, Pratt's interpretations, published in an unrefereed meeting abstract,[41a] should be viewed with caution. Specifically, it is not at all clear that the 440 nm absorption can, as claimed,[41a] "... now fairly confidently be identified as a distorted 5-coordinate form of the coenzyme (Co−R)." Indeed base-off $Co^{II}B_{12}$ has an absorbance close to this same region (e.g. see Figure 3C″ in ref 13 cited by Pratt or the equivalent Figure 2C″ elsewhere;[41c] enzyme-bound $Co^{II}B_{12}$ is reported to have a λmax at 470−478 nm[8b]), although authentic base-off AdoCbl (λ_{max} = 455 nm) does as well. However, $Co^{II}B_{12}$ is expected (and is obviously present, by UV−visible) in each of the references (10−13) cited.[41a] Hence, it is not 100% clear that the expected absorbances due to base-off $Co^{II}B_{12}$ have been factored out. Note also that none of the original authors of the papers cited[41a] mention anything unusual about the spectra they see (or the 440 nm absorbance), *even though two of the three studies were specifically concerned with the identification of enzyme-bound intermediates.* (c) Hollaway, M. R.; White, H. A.; Joblin, K. N.; Johnson, A. W.; Lappert, M. F.; Wallis, O. C. *Eur. J. Biochem.* **1978**, *82*, 143. (d) Studies of NC−Cbi^+, plus α-ribazole or α-ribazole-phosphate, binding to Intrinsic Factor: Andrews, E. R.; Pratt, J. M.; Brown, K. L. *FEBS Lett.* **1991**, *281*, 90.

benzimidazole base-on form. (See also Bürgi's recent work on the "anomolous structural trans effect" seen in coenzyme B_{12} model complexes.[42b]) The alternative, hybrid interpretation we can now support, one first made in a qualitative MO theory paper by Mealli, Sabat, and Marzilli,[6k] is that a *long Co−N distance may be important primarily to inhibit Co−C heterolysis.*[43a] *If* anything like a fine-tuned Co−N axial-base distance is in fact the case, *then* it would be an impressive example of the power of evolution, operating via many perturbations on time scales beyond normal human comprehension,[43b] to achieve exquisite chemical selectivity. It has been stated that the nucleotide loop makes a relatively strain-free 19-membered ring,[44a] a fact again arguing for its fine-tuning via evolution (we note, however, that others have argued, based on NMR studies, that some strain is present in the nucleotide loop as the Co−N bond distance varies from 2.19 Å[44b,c]).

For the subclass of $AdoB_{12}$-dependent enzymes operating by a histidine imidazole side-chain base on form,[9,10f] it could be that a 5,6-dimethylbenzimidazole base-on form is important for preventing Co−C heterolysis *outside* the enzyme (i.e., during transport?) that would occur if a very basic axial ligand were to bind.[1b] In this sense, then, the appended 5,6-dimethylbenzimidazole may serve as a "protecting group" (i.e., in addition to its crystallographically characterized "anchor" function inside the MMCoA-subclass of $AdoB_{12}$-dependent enzymes), protecting the B_{12} cofactor from the coordination of unwanted bases prior to its binding to the protein and, then, to the desired protein side-chain imidazole.

A Critical Analysis of Key Biochemical Literature of the Axial-Base Problem. Earlier studies of B_{12}-dependent enzymes[41,45−48] have provided pertinent albeit previously confusing information regarding the possible function(s) of AdoCbl's 5,6-dimethylbenzimidazole axial base. The uncertainty surrounding the role(s) of the axial-base indicated to us that a critical analysis of the prior axial-base literature was needed. Hence, the highlights of just such a previously unavailable analysis are summarized below.

It is important to reemphasize up front the recent protein sequence homology evidence[9a,b] which strongly suggests the existence of two classes of B_{12}-dependent enzymes: a 5,6-dimethylbenzimidazole base-on class, and a protein side-chain histidine imidazole base-on class which have the conserved, fingerprint sequence homologous to that first identified in the MeB_{12} binding region of methionine synthase.[9a,b,d] What is equally fascinating here, is that, so far, there *appears* to be a 1:1 correspondence between the 5,6-dimethylbenzimidazole base-on or base-off form, and a protein cysteine side-chain −S• (or other −X•) radical-chain mechanism (i.e., which correlates with the 5,6-dimethylbenzimidazole base-on form) vs histidine imidazole base-on form (i.e., which correlates with the operation of the traditional, Ado• H-atom abstraction, nonchain mechanism).[9b,d,e] Marsh has pointed out that this subdivision may, in turn, be due to respectively higher vs lower substrate C−H bond strength that the Ado• vs the −S• has to abstract.[9e] It remains to be seen, however, if this correlation is truly general and what, if anything, it implies about the Co−C activation and cleavage process (or, possibly, its microscopic reverse—see the discussion of a lowered spin density at cobalt in Co^{II}(imidazole) complexes, *vide infra*).

EPR spectra of active *diol dehydratase* holoenzyme provided early, direct evidence that a nitrogenous axial base is bound to $Co^{II}Cbl$.[3,45] In apparent conflict with this, however, is Pratt's interpretation[41] of the absorbance at 440 nm as evidence that the *alkyl*-cobalt cofactor is *five-coordinate* during catalysis (i.e., with the cobalt to 5,6-dimethylbenzimidazole bond broken, and no other axial-base present, with the implication that the Co(II) resulting from Co−C homolysis is four coordinate), at least in the B_{12}-dependent enzymes ribonucleotide reductase and ethanolamine ammonia-lyase. Challenging Pratt's questionable[41b] visible spectrum interpretations, however, is a recent report stating that the nitrogenous-base-free form of AdoCbl, adocobinamide ($AdoCbi^+X^-$; Figure 1) is "...completely inactive ... in the *diol dehydratase* reaction" (less than 0.02% of the active of the native cofactor), and that the apoenzyme of diol dehydratase "...shows very little affinity for $AdoCbi^+$" (roughly 4×10^{-5} the binding affinity shown for AdoCbl).[10a] It should be noted that three previous studies employing "$AdoCbi^+$" as a possible enzymic cofactor for diol dehydratase reached conflicting and confusing conclusions: reports exist ranging

(42) (a) Their specific hypothesis is that the enzyme controls the benzimidazole-to-cobalt distance to achieve an ideal Co−N length, one optimim for stabilizing $Co^{II}Cbl$ (see pp 242, 245, and 249 elsewhere[4f]). This hypothesis is an outstanding example of where B_{12} model studies *have* proved quite valuable and *have provided* otherwise unavailable insights.[25] (b) Bürgi and co-workers recent work on the "anomolous structural trans effect" (i.e., where trans ligands in B_{12} model complexes simultaneously lengthen or shorten): De Ridder, D. J. A.; Zangrando, E.; Bürgi, H.-B. *J. Mol. Struct.* **1996**, *374*, 63−83.

(43) (a) That is, a relatively long Co−N distance is apparently important in avoiding the the Co−C heterolysis that is expected from a shorter Co−N distance *and its implied stronger* σ-donation to cobalt. (b) Dawkins, R. *The Blind Watchmaker*; Longman Scientific and Technical: Essex, England, 1986.

(44) Work addressing the presence or absence of strain in the intramolecularly appended necleotide loop and 5,6-dimethylbenzimidazole in B_{12}: (a) Eschenmosher, A. *Angew. Chem. Int. Ed.* **1988**, *27*, 5; *Angew. Chem.* **1988**, *100*, 5. (b) Brown, K. L. *Inorg. Chem.* **1986**, *25*, 3111 (see the statement on p 3113). (c) Brown, K. L.; Evans, D. R. *Inorg. Chem.* **1993**, *32*, 2544 (see the statement about the progressive strain in the nucleotide loop on p 2546).

(45) (a) Foster, M. A.; Hill, H. A. O.; Williams, R. J. P. *Biochem. Soc. Symp.* **1970**, *31*, 187. (b) Rudiger, H. *Eur. J. Biochem.* **1971**, *21*, 264.

(46) (a) Kato, T.; Shimizu, S.; Fukui, S. *J. Vitaminol. (Kyoto)* **1964**, *10*, 89. (b) Toraya, T.; Ohashi, K.; Ueno, H.; Fukui, S. *Bioinorg. Chem.* **1975**, *4*, 245. (c) Toraya, T.; Fukui, S. *Adv. Chem. Ser.* **1980**, *191*, 139. (d) $AdoCbi^+$ has been reported to be an active cofactor for diol dehydratase with propanediol as a substrate, but not for glycerol or ethylene glycol: Zagalak, B.; Pawelkiewicz, J. *Acta Biochim. Pol.* **1964**, *11*, 49. However, one must realize that the highly pure, fully characterized AdoCbi+ needed for use in completely reliable enzymic studies was not generally available until 1987.[48] (e) Eberhard, G.; Schlayer, H.; Joseph, H.; Fridrich, E.; Utz, B.; Müller, O. *Biol. Chem. Hoppe-Seyler* **1988**, *369*, 1091−1098. These authors found $AdoCbi^+$ to be *inactive* with diol dehydratase (as Toraya too found[10a]), but the source and purity of their $AdoCbi^+$ were not identified (the *other* modified-base adocobamides used in this study are reported as being characterized by IR, paper chromatography, and UV/visible). Also, these authors report as "remarkable"[46e] their finding that adocobamides, modified in the 2-position of the benzimidazole, are still active with methylmalonyl-CoA mutase, even though these same base-modified adocobamides were inactive for diol dehydratase. Note that a more recent report finds that $AdoCbi^+$ itself is a 97% *inactive* cofactor when tested with methylmalonyl-CoA mutase.[50]

(47) (a) Others report $AdoCbi^+$ as inactive[46e,47b] (or substrate-dependent active to inactive)[46d] but, again, pure and fully characterized[48] (and thus reliable) $AdoCbi^+$ was not generally available until a decade after the following studies[47b] were performed. (b) Yakusheva, M. I.; Poznanskaya, A. A.; Pospelova, T. A.; Rudakova, I. P.; Yurkevich, A. M.; Yakovlev, V. A. *Biochim. Biophys. Acta* **1977**, *484*, 216. The *N*-methyl- (or *N*-benzyl-) benzimidazole B_{12} analogs (compounds **XIII** and **IX**) in this paper used in the enzymic studies were characterized only by CD and UV−visible spectroscopy. Moreover, their $AdoCbi^+$ in water shows a shoulder at ca. 500 nm, suggestive of a contaminant of base-on AdoCbl (λ_{max} 524 nm in H_2O), and lacks the more pronounced peak at 378 nm (in H_2O) seen in the spectra of well-characterized[48] $AdoCbi^+$. Toraya and co-worker's recent work *with well characterized AdoCbl-Me*$^+$ finds this AdoCbl derivative to be *totally inactive.*[10a] (c) For related work see: Brown, K. L.; Wu, G.-Z. *Organometallics* **1993**, *12*, 496.

(48) (a) A discussion of the conflicting reports of the enzymic activity of "$AdoCbi^+$", and of a literature report suggesting that the nucleotide loop in B_{12} was more involved in *binding* the cofactor to the enzyme than in the Co−C homolysis step, are discussed in our 1987 paper[11a] (see p 8017 and ref 37).

from inactive, to partially active, to fully active for the diol dehydratase holoenzyme using "AdoCbi$^+$" as cofactor,[46,47] something that we noted in 1987.[48a] A critical analysis of these early papers reveals that they are not definitive, since they did not use *clean, fully characterized AdoCbi*$^+$; also desirable for such studies is the use of cloned, overproduced, and then highly purified enzyme. Toraya and co-workers agree with this analysis, and independently point out that one of the earlier studies[46a] used "...a microbial product tentatively identified as AdoCbi$^+$".[10a] Note that the most recent Toraya and co-worker's study *uses pure, well-characterized AdoCbi*$^+$ and, again, finds that AdoCbi$^+$ is *completely inactive* as a cofactor for diol dehydratase.[10a] Significantly, AdoCbi$^+$ is also reported to be completely inactive[49] for ribonucleotide reductase (an important fact since the results with this enzyme bear directly on Pratt's hypothesis that the 440 nm band *in ribonucleotide reductase* is due to five-coordinate, base-off AdoCbl). AdoCbi$^+$ is also 97% inactive in methylmalonyl-CoA mutase,[50] an intriguing fact that we will return to in a moment.

Another previously confusing study, one which superficially appears to offer good support for Pratt's base-off hypothesis, employed a sample claimed to be "AdoCbl-Me$^+$" as cofactor (i.e., AdoCbl in which putative alkylation at the benzimidazole N3 nitrogen was used to render the benzimidazole incapable of coordinating to cobalt). This study reports a partially, 52% active enzyme (for *glycerol* dehydratase, EC 4.2.1.30).[47b] But, once again the characterization (UV−visible; CD) and proof of purity of the "AdoCbl-Me$^+$" employed in these studies is not rigorous by modern standards (e.g., HPLC, NMR, FAB-MS). Toraya and co-worker's most recent paper[10a] provides the needed, seemingly definitive study: when authentic, pure, and well-characterized AdoCbl-Me$^+$ is used as a cofactor (albeit with *diol* dehydratase, EC 4.2.1.28, from the same organism; we thank a reviewer for emphasizing this point), *a totally inactive cofactor (<0.02% the activity of AdoCbl) results*, one that again binds very weakly. Moreover, the work from Toraya's group shows one other key point: the nucleotide loop and associated 5,6-dimethylbenzimidazole must be *intramolecularly appended* to be an active cofactor (i.e., AdoCbi$^+$ plus nucleotide loop fragments such as 5,6-dimethylbenzimidazole, α-D-ribazole, or α-D-ribazole-3′-phosphate did not confer either tight binding nor activity on AdoCbi$^+$,[10a] results which are of further interest because they contrast dramatically the findings for binding NC-CoCbi$^+$ plus nucleotide loop *fragments* to porcine Intrinsic Factor[41d]). While the different B_{12}-dependent (glycerol vs diol) dehydratase enzymes employed by the two research groups require one to qualify the above comparison of results, the available evidence still strongly suggests that the more reliable findings are those from Toraya's labs.

Three other, more recent papers by Toraya and Ishida are also especially relevant to the axial-base question.[10b,c,d] In a 1991 study,[10b] these authors have examined the axial-base question by employing AdoCbl, modified by replacing the α-D-ribofuranose ring of the nucleotide loop with a variable length $-(CH_2)_n-$ methylene chain ($n = 2, 3, 4, 6$), again in conjunction with the enzyme diol dehydratase. Their findings strongly support the idea that the nucleotide loop and its associated phosphodiester appended 5,6-dimethylbenzimidazole are important for tight enzyme binding of AdoCbl, but that the nucleotide loop and attached 5,6-dimethylbenzimidazole are not absolutely necessary for the Co−C homolysis step (e.g., the $-OCH_2CH_2CH_2-$imidazoyl analog of AdoCbl showed an initial high activity but was then rapidly deactivated). In addition, this work provides good evidence that the nucleotide loop plays an "...indispensable role in preventing reactive intermediates from undesirable side reactions, and thus in keeping the holoenzyme active during catalysis." Next, in a 1993 paper,[10c] Ishida and Toraya examined adenosylcobinamide methyl phosphate, AdoCbi-P-Me, that is, the methylated phosphate—but 5,6-dimethylbenzimidazole free—form of AdoCbl, once again with diol dehydratase. This study reaches essentially the same conclusions as their 1991 study: no enzyme activity is seen with AdoCbi-P-Me as a coenzyme B_{12} substitute, but the Co−C bond is still cleaved in this (5,6-dimethylbenzimidazole-free) form of the cofactor (albeit perhaps not as fast as if the 5,6-dimethylbenzimidazole were present, a question that remains). *Toraya's results make it very clear that the intramolecularly nucleotide loop: (i) is important for binding the B_{12} cofactor to diol dehydratase, and especially (ii) "plays a pivotal role in preventing the radical intermediate(s) from (undergoing) unfruitful side reactions"*,[10c] even though the latter effect is, by definition, distal. Finally, in a 1994 paper,[10d] Toraya and co-workers find that the intramolecularly appended pyridine analog of coenzyme B_{12} binds the pyridine more tightly than the natural 5,6-dimethylbenzimidazoyl or imidazoyl congeners—analogous to the findings presented herein (i.e., our findings that exogenous pyridine has an *at least* 5-fold higher K_{assoc} than exogenous 1,5,6-trimethylbenzimidazole, Table 3). In addition, Toraya and co-workers' important paper[10d] shows that the initial activity and k_{cat}/K_m value for the pyridine analog of coenzyme B_{12} are, respectively, 25% and 68% that seen for coenzyme B_{12} itself, and that the *inactivation rate, k_{inact}/k_{cat} is also 100 times higher than for coenzyme B_{12} itself*—again providing *prima facie* evidence that, while the 5,6-dimethylbenzimidazole base is at least somewhat involved—again distally—in the initial Co−C homolysis step, this appended nucleotide loop and associated axial-base is even more important in providing tight, controlled binding to the protein of the cobalt cofactor and the resultant radical intermediates, at least in diol dehydratase.

Together the above findings confirm for the diol dehydratase subclass of B_{12}-dependent enzymes what the recent X-ray crystal structure of MMCoA mutase implies for its subclass: that the *direct coordination* of 5,6-dimethylbenzimidazole itself is *not absolutely* required for the initial Co−C bond cleavage step in

(49) Jacobsen, D. W.; DiGirolamo, P. M.; Huennekens, F. M. *Mol. Pharmacol.* **1975**, *11*, 174−184. In addition to no activity, AdoCbi$^+$ is reported therein to be a "very weak inhibitor" for ribonucleotide reductase. The authors concluded (p 182) that "the lower axial ligand (and, we would now add, nucleotide loop) may play an important role in binding the coenzyme ... to the active site.

(50) (a) Padmakumar, R.; Taoka, S.; Padmakumar, R.; Banerjee, R. *J. Am. Chem. Soc.* **1995**, *117*, 7033. (b) Unfortunately, there is no compelling evidence, in at least these initial studies, addressing the difficult issue of whether or not the crucial [^{15}N]-Co^{II}Cbi$^+$ and [^{15}N]-Co^{II}Cbl detected by EPR are on the *catalytic reaction pathway*, since the experiments were performed with photolytically generated Co^{II}, and not with Co^{II} formed during the catalytically active steady state. However, *highly suggestive* evidence that AdoCbi$^+$ binds at the active site as expected was provided,[50a] specifically (i) the strong binding of AdoCbi$^+$ (7−8 μM), (ii) the fact that Ado*Cbl* (which should be bound at the active site) provides Co^{II}C*bl* upon photolysis which also shows ^{15}N−Co coupling (although it is unclear if α- or β-[^{15}N]-CoCbl isomers are involved), and (iii) the sequence homology between MMCoA and the crystallographically characterized, base-off MeB$_{12}$ binding, methionine synthase. But, overall and by themselves, these initial studies do not unequivocally demonstrate that the 5,6-dimethylbenzimidazole base-off form of AdoCbl is involved in the catalytic cycle of MMCoA mutase. *Note added in proof*: Professor Banerjee kindly informs us that they now have submitted for publication data indicating that the protein-derived nitrogen ligand is bonded to cobalt in a radical-pair directly observed by EPR. (c) A tightly bound (suggested to be histidine or lysine bound), inactive, pink form of the B_{12} cofactor is known, again in methylmalonyl-CoA from *Propionibacterium shermanii*, see: Marsh, E. N.; Harding, S. E. *Biochem. J.* **1993**, *290*, 551−555. (d) Banerjee, R. Private communication (i.e., that no detectable Co−C cleavage is seen in the AdoCbi$^+$·MMCoA holoenzyme). We thank Professor Banerjee for sharing this unpublished information.

either class of enzymes, counter to what has often been assumed in most, but not all, of the prior B_{12} bioinorganic literature. But, the presence of the nucleotide loop and asociated 5,6-dimethylbenzimidazole or other nitrogeneous base of some type do appear to be required for Co−C cleavage in both classes of B_{12}-dependent enzymes (e.g., recall that neither MMCoA mutase nor diol dehydratase nor ribonucleotide triphosphate reductase are active with $AdoCbi^+$ alone). In addition, direct coordination of the 5,6-dimethylbenzimidazole base does accelerate modestly Co−C bond homolysis in diol dehydratase.[10d] The 5,6-dimethylbenzimidazole may also be important in favoring homolysis over heterolysis in the diol dehydratase subclass of enzymes, *assuming* that our enzyme-free $AdoCbi^+$ chemical precedent studies[1b] extrapolate to the AdoCbl·enzyme complex. This same, putative favoring of homolysis over heterolysis may also apply to the *axial histidine imidazole* in the MMCoA class of enzymes.

It is worth noting that the proposed crucial role of the nucleotide loop and the appended axial 5,6-dimethylbenzimidazole in controlling the resultant protein-bound[4h] organic radical intermediates is fully consistent with the *stereospecificity* shown by the protein-bound radical intermediates of diol dehydratase.[4h] Relevant in this regard are Hamilton's EPR results showing that enzyme-bound $Co^{II}Cbl$ is apparently highly oriented (i.e., and thus probably tightly bound).[51] Also noteworthy is Pilbrow's suggestion, based on his EPR studies of Co(II)cobamides, that *the spin density at Co(II)cobamides—and thus at least part of their reactivity with other radicals—can be controlled by the axial ligands*, with 4 coordinate or 5 coordinate (OH_2, or another oxygen ligand) forms having more spin density at cobalt, and *imidazole-ligated cobalt demonstratably having less*.[52] This offers one possible, chemically precedented (but still unproved) role for an axial imidazole ligand: that of lowering the spin density at cobalt, thereby presumably reducing the rate of Ado• recombination with its concommitant increase in the net rate of H• abstraction of an unactivated C−H bond in this subclass of AdoCbl-dependent enzymes.

Of special interest in light of the recent X-ray crystal structure of MMCoA mutase are biochemical studies of the axial-base effects in this particular enzyme. One study nearly 15 years ago reached the conclusion that the 5,6-dimethylbenzimidazole nucleotide is more important in achieving strong binding of the cofactor to methylmalonyl-CoA mutase than in the initial Co−C cleavage step[53] (see other work as well[46e]). A very recent study of methylmalonyl-CoA mutase (MMCoA mutase) from *Propionibacterium shermanii* provided direct EPR and other evidence—prior to the X-ray crystallographic confirmation—that a protein-side-chain nitrogeneous base is coordinated to MMCoA reconstituted with $AdoCbi^+$.[50] The resultant $AdoCbi^+$·MMCoA mutase enzyme is *97% inactive*. If one assumes that the $AdoCbi^+$ is bound at the active site (a difficult issue in the absence of evidence for catalytic turnover, but one in all probability now resolved by the X-ray diffraction study of MMCoA[9c]), and with the knowledge that no Co−C cleavage is observed,[50d] then it seems inescapable that it must be, as others first noted,[9] the *distal effect* of the base-off, but protein-anchored, nucleotide loop and associated 5,6-dimethylbenzimidazole which is crucial for the Co−C cleavage step in the MMCoA subclass of AdoCbl-dependent enzymes.

Lastly, it needs to be emphasized that 12 different AdoCbl-dependent rearrangement enzymes, plus two types of B_{12}-dependent reductases, are currently known.[4] Hence, even though two classes of B_{12}-dependent enzymes now appear to be fairly well established, additional crystal structures and other studies may reveal new surprises about the function(s) of the nucleotide loop and appended 5,6-dimethylbenzimidazole portion of AdoCbl.[54]

The Importance of the Appended AdoCbl Corrin Side-Chain Groups. All the past and present focus on the appended axial base in AdoCbl makes it important to mention studies of several different B_{12}-dependent enzymes in conjunction with AdoCbl modified in one or more amide side chains (via their conversion to $-CO_2H$, $-CO_2R$, or $-C(O)NHEt$ groups, for example). These investigations show that changing *even the single b-side-chain amide* can partially to completely inactivate the cofactor·enzyme complex.[55] In other words, these studies strongly suggest that the proper anchoring of the AdoCbl side chains is also crucial to the Co−C activation step. The interested reader is also referred to Brown and co-workers recent alkylcobamide model studies[56] demonstrating the role of the corrin side-chain *entropy* in assisting Co−C homolysis, at least in non-Ado cobalamins and in solution (i.e., outside of any B_{12}-dependent enzyme). Their results, we would argue,[56] suggest an *enthalpy* controlled weakening of the Co−C bond in the AdoCbl·enzyme complex.

Literature Suggesting That the Enzymic 10^{12} Acceleration and $\Delta\Delta G^{\ddagger}$ Lowering of 16 kcal/mol of AdoCbl Co−C Bond Homolysis Is Achieved by a Lowering of $\Delta\Delta H^{\ddagger}$ by ≥ 16 kcal/mol. In 1984 we provided the first study of the thermal homolysis of AdoCbl's Co−C bond, from which the realization came that the AdoCbl dependent enzymes were accelerating

(51) Hamilton, J. A.; Blakley, R. L.; Looney, F. D.; Winfield, M. E. *Biochem. Biophys. Acta* **1969**, *177*, 374.

(52) (a) Pilbrow has reported ESR studies of Co(II) cobalamins and cobinimides (the latter in the presence of exogenous axial bases such as imidazole, histidine, pyridine, PPh_3, SCN^-, and others), Bayston, J. H.; Looney, F. D.; Pilbrow, J. R.; Winfield, M. E. *Biochemistry* **1970**, *9*, 2164−2172. The authors' key findings include the following: (i) that only pyridine binds tightly enough (when present as solvent) to displace the appended 5,6-dimethylbenzimidazole of Co^{II}-Cbl; (ii) that, of the biological amino acids tested, only histidine binds tightly enough to remain coordinated to $Co^{II}Cbi^+$ at physiological pH (and $\geq 6 \times 10^{-3}$ M histidine); (iii) that only a trace of (what is interpreted as) 19 electron py-Co^{II}Cbl can be formed; (iv) that two isomers of $Co^{II}Cbi(PPh_3)^+$ are formed (possible α, *β* isomers?); (v) that the resultant $Co^{II}Cbi(PPh_3)$+ *is sterically distorted*. Hence, Co^{II}-$Cbi(PPh_3)^+$ is *an excellent place to initiate needed studies of structurally distorted* Co^{II}*Cbi*, and these and other studies of Co^{II}-Cbi^+ are among our current research goals.[18]

(53) Retey, J. In *B_{12}* ; Dolphin, D., Ed.; Wiley-Interscience: New York, 1982; Vol. 2, Chapter 13.

(54) (a) A case where this statement *may* be especially relevant is in the methylmalonyl-CoA rearrangement. Here, chemical model evidence for a carbanion in the rearrangement step is available,[54b] although it is still not definitive and, hence, controversial (others favor a radical[54c]). Any putative carbanion would presumably have to be formed via electron transfer from *base-on* B_{12} (base-on due to its more favorable Co(II)/Co(III) oxidation potential); that is, a benzimidazole or other base-on form would presumably be necessary *if* this putative e^- transfer step from the Co(II) cofactor to the substrate radical is actually occurring. (b) Choi, G.; Choi, S.-C.; Galan, A.; Wilk, B.; Dowd, P. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3174. However, in our opinion this paper is not yet definitive evidence for *rearrangement* at the carbanion vs the radical stage of the substrate intermediate. There is no doubt that carbanions are formed as part of the overall process in this interesting system, since D^+ is incorporated into the product. However, definitive evidence for kinetically preferred rearrangement at the carbanion stage must await further mechanistic study. (c) Wollowitz, S.; Halpern, J. *J. Am. Chem. Soc.* **1988**, *110*, 3112−3120. (d) He, M; Dowd, P. *J. Am. Chem. Soc.* **1996**, *118*, 711. There is an unnecessary assumption in this paper: the possible cyclopropyl ring-opened rearrangement product, around which the logic in this paper centers (i.e., the ring-opened product analogous to the cyclopropyl ring intact product **2** isolated in 62% yield), is (apparently) *assumed* to be completely absent. A simple control experiment, testing whether or not the ring-opened product is detectable (i.e., among the 38% of unaccounted for starting material), is needed to test this assumption.

(55) For lead references, see: Toraya, T.; Krodel, E.; Mildvan, A. S.; Abeles, R. H. *Biochem*istry **1979**, *18*, 417 and references therein.

Co−C cleavage by $10^{12\pm1}$, a $\Delta\Delta G^{\ddagger}$ lowering of 16 kcal/mol at 25 °C.[27a] That finding has done much to propagate interest in the axial base's possible role in the Co−C cleavage process; hence it merits brief dicussion herein. In the time since our initial paper, we have shown that only the formation of a "half Co−C bond", by 1 e^- reduction of AdoCbl (which places the added electron in the Co−C antibond[57]), gives a Co−C cleavage non-enzymic rate enhancement anywheres close to the 10^{12}. For the "half Co−C bonded" Ado---$Cbl^{-\bullet}$, the resultant lowering of $\Delta\Delta H^{\ddagger}$ is ca. 19 kcal/mol, from 31 kcal/mol[27,58] to ca. 12 kcal/mol (i.e., using the analogous Me---$Cbl^{-\bullet}$ data[59] to obtain the 12 kcal/mol estimate). Note however, that the 1 e^- reductive activation of AdoCbl is surely *not* occurring *in vivo* for reasons detailed elsewhere,[57] most notably the lack of a biological reductant at the negative −1.2 to −1.6 V vs SCE required to reduce AdoCbl,[57] as well as the fact that such a reduction yielded the wrong product, Co(I)Cbl.[57]

However, and while speculative, we would like to suggest that the AdoCbl enzymes operate by a mechanism involving a sizable $\Delta\Delta H^{\ddagger}$ lowering. Specifically, we would like to suggest that the AdoCbl binding enzymes convert ≥16 kcal/mol of the intrinsic H-bond and other binding energy[60,61] available for a large cofactor such as AdoCbl (i.e., with its now identified >16 surface H-bonds[9c]) into Co−C weaking and Co−C−C angular distortion, for a $\Delta\Delta H^{\ddagger}$ lowering of ≥16 kcal/mol. As excellent enzymic precedent for this hypothesis, we cite the case of β-galactosidase[62] (see also ref 35 elsewhere[11a]), the closest chemical precedent and only other case that we have been able to find of a quantitated 10^{10-12} enzymic rate enhancement *on the cleavage of a single bond*. In the case of β-galactosidase, this large rate acceleration is achieved by a $\Delta\Delta H^{\ddagger}$ lowering of −21 kcal/mol *working against* a less favorable, compensating $\Delta\Delta S^{\ddagger}$ of −25 eu, for a net $\Delta\Delta G^{\ddagger}$ lowering of ca. 13.5 kcal/mol at 25 °C (vs the reference point of the analogous S_N1 mechanism in solution).[63] This β-galactosidase example is fully consistent with the emerging picture developed above of AdoCbl "placed on the rack"[64] by the enzyme via H-bonding and other interactions to *its corrin side chains, its appended axial-base, and its Ado group*. A greatly weakened Co−C bond for a $\Delta\Delta H^{\ddagger}$ lowering of ≥16 kcal/mol is what one expects at this point, and experimental verification or disproof of this picture[64b] is an important goal of future research.

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Supporting Information Available: Text giving a discussion of axial base K_{assoc} values for Coenzyme B_{12} "Models", Table A of K_{assoc} Upper Limits for $AdoCbi^+$ plus phosphines in ethylene glycol at 25 °C, figures showing representative UV−visible spectra overlays of $AdoCbi^+$ as a function of [base], representative plots used to derive the K_{assoc} values, and all ln K_{assoc} vs $1/T$ plots, and Table B of data and upper limits to $AdoCbi^+$ plus RSH/RS^- K_{assoc} studies (11 pages). Ordering information is given on any current masthead page.

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(56) The importance of the amide side chains *in the solution chemistry of AdoCbl* is also emphasized by Brown's studies concerning the importance of the side-chain degrees of freedom, and thus entropy, in base-off vs base-on forms of AdoCbl. Note, however, that the greater side-chain freedom following homolysis *in solution* is not directly applicable to the protein-bound cofactor since, in the limit of this explanation, the protein would have to "let go completely" of the Co^{II}-Cbl cofactor. Hence, we suggest that Brown's valuable studies actually argue for an *enthalpy* controlled weakening, *within the protein·cofactor complex*, of the Co−C bond (i.e., over and above the cost of an entropically unfavorable tigher binding of Co^{II}Cbl). (a) Brown, K. L.; Hakimi, J. M.; Nuss, D. M.; Montejano, Y. D.; Jacobsen, D. W. *Inorg. Chem.* **1984**, *23*, 1463. (b) Brown, K. L.; Zou, X.; Evans, D. R. *Inorg. Chem.* **1994**, *33*, 5713. (c) Brown, K. L.; Cheng, S.; Marques, H. M. *Inorg. Chem.* **1995**, *34*, 3038. (d) Brown, K. L.; Evans, D. R.; Cheng, S.; Jacobsen, D. W. *Inorg. Chem.* **1996**, *35*, 217. In this work, neopentylcobalamin derivatives are found to be stabilized by a $\Delta G^{\ddagger}$ (25 °C) of 2.8 ± 1.0 kcal/mol to Co−C homolysis upon complexation to haptocorrin, a net effect that is 67 (±38)% enthalpic (the expected radical-cage effect by the haptocorrin) and 33 (±10)% entropic at 25 °C.

(57) Finke, R. G.; Martin, B. M. *J. Inorg. Biochem.* **1990**, *40*, 19.

(58) Garr, C. D.; Finke, R. G. *Inorg. Chem.* **1993**, *32*, 4414.

(59) (a) Martin, B. D.; Finke, R. G. *J. Am. Chem. Soc.* **1990**, *112*, 2419. (b) Martin, B. D.; Finke, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 585.

(60) For additional discussions of this point, see pp 8016−8018 elsewhere.[11a]

(61) Fully consistent with and supportive of this concept is Jacobsen, Brown, and co-worker's findings that haptocorrin binds vitamin B_{12} (i.e., NC−Cbl) with a *record* 5×10^{16} M^{-1} *binding constant*, one that involves a $\Delta H_{binding} = -28$ *kcal/mol* operating against a $\Delta S_{binding} = -18$ eu, see: Marchaj, A.; Jacobsen, D. W.; Savon, S. R.; Brown, K. L. *J. Am. Chem. Soc.* **1995**, *117*, 11640. The observation of much smaller binding constants for the AdoCbl-dependent enzymes is very strong evidence for the labilization of AdoCbl's Co−C bond via the expression of a considerable amount of intrinsic binding energy toward catalysis (e.g., toward Co−C lengthening and Co−C−C angular distortion).

(62) Jones, C. C.; Sinnott, M. L.; Souchard, I. J. L. *J. Chem. Soc. Perkin Trans. 2* ***1977***, 1191.

(63) If this picture is correct, then AdoCbl enzymes emerge as prototype examples of "enthalpy machines", that is highly entropically preorganized from the reference point of free amino acids (i.e., having lost considerable translational and other entropy), but in turn being able to perform considerable enthalpic work along the Co−C homolysis reaction coordinate.

(64) (a) Eyring, H.; Lumry, R.; Spikes, J. D. In *A Symposium on "The Mechanism of Enzyme Action"*; McElroy, W. D., Glass, B., Eds., Johns Hopkins Press: Baltimore, MD, 1954; pp 123−140. (b) See R. J. P. Williams' discussion[64c] for the definitions and distinctions of "rack, induced fit, entatic and induced matrix" cofactor plus enzyme combinations, depending upon whether the structural changes from their uncomplexed state are, respectively: in the cofactor and globally within the enzyme ("rack"); in the cofactor and locally within the enzyme ("induced fit"); just in the cofactor ("entatic"), or just in the enzyme ("induced matrix"; this latter case is ruled out for AdoCbl· enzyme complexes if one assumes that the Co−C in the AdoCbl cofactor is lengthened or otherwise significantly distorted). See also William's specific discussion on p 379 of AdoCbl as possibly either a rack or locally induced entatic state.[64c] Note that a comparison, by X-ray crystallography or other structural methods, of the structures of the cofactor and enzyme *alone* to the structure of the cofactor−enzyme *complex*, will in principle unequivocally distinguish these different Co−C activation mechanisms as defined above. (c) Williams, R. J. P. *Eur. J. Biochem.* **1995**, *234*, 363−381.