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Adenosylcobinamide Plus Exogenous, Sterically Hindered, Putative Axial Bases: A Reinvestigation into the Cause of Record Levels of Co–C Heterolysis

Kenneth M. Doll and Richard G. Finke*

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

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A reinvestigation of an earlier Ph.D. thesis (Sirovatka, J. M. Ph.D. Thesis, Colorado State University, Fort Collins, CO, 1999) is reported herein. That thesis examined the thermolysis reaction of $AdoCbi^+BF_4^-$ in ethylene glycol solution with exogenous bases, *N*-methylimidazole (N-Me-Im) and the sterically hindered 1,2-dimethylimidazole, (1,2-Me_2-Im), 2-methylpyridine (2-Me-py), and 2,6-dimethylpyridine (2,6-Me_2-py). In the present work, multiple purities of each base have been utilized as a check to see if impurities in the nitrogenous bases are causing the observed homolysis and heterolysis product distributions as others have implied (Trommel, J. S.; Warncke, K.; Marzilli, L. G. *J. Am. Chem. Soc.* **2001**, *123*, 3358). The "impurity hypothesis" is disproven by a series of results, including the following: N-Me-Im displays an *invariant* 52 ± 10% heterolysis and the 1,2-Me_2-Im system displays an *invariant* 83 ± 7% heterolysis as a function of different base purification methods. Moreover, 2-Me-py and 2,6-Me_2-py also display an invariant ∼16 ± 5% heterolysis as a function of different purification methods. What is responsible for the high levels of Co–C heterolysis in the $AdoCbi^+$ plus sterically bulky base thermolyses was uncovered via a revisitation of our four, earlier alternative hypotheses for the enhanced Co–C heterolysis (Sirovatka, J. M.; Finke, R. G. *Inorg. Chem.* **1999**, *38*, 1697). Our prior number one alternative hypothesis is shown to be correct: the added bases simply deprotonate the ethylene glycol solvent, forming ethylene glycolate anion and base-H^+ as the key agents behind the previously ill-understood Co–C heterolyses. Also reported are Co(II)Cbi^+ titrations with five bases (1,2-Me_2-Im, N-Me-Im, pyridine, 2-MePy, and 2,6-Me_2-py). These experiments confirm Marzilli and co-workers' (*op. cit.*) results by showing that sterically hindered bases do *not* bind to Co(II)Cbi^+; therefore, Co(II)Cbi^+ EPR literature showing binding of bulky pyridines is erroneous as is the previously reported binding of bulky pyridine bases to Co(II)Cbi^+ by UV–vis spectroscopy (Sirovatka, J. Ph.D. Thesis, *op. cit.*). Also reported is our current best synthesis and purification of $AdoCbi^+BF_4^-$, work that builds off our 1987 synthesis of $AdoCbi^+BF_4^-$ (Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 8012). Finally, the multiple, compounding errors which have caused problems in this project are listed, notably errors in the protein X-ray crystallography literature, the EXAFS literature, the Co(II)Cbi^+ plus bulky-bases EPR literature, the misleading B_{12}-model literature, the erroneous experimental work (Sirovatka, *op. cit.*) and thus incorrect conclusions in one of our prior papers, as well as the erroneous implications in parts of the Marzilli and co-workers paper (*op. cit.*). It is hoped that a forthright reporting of these errors will help others avoid similar mistakes in the future when studying complex, bioinorganic systems.

Introduction

Adenosylcobalamin (AdoCbl) is an essential cofactor for at least 17 different enzymatic systems.[1−7] A key to the reactivity of AdoCbl is in the cleavage of the biologically rare Co−C bond. Comparison of solution studies of AdoCbl[6,8,9] data to enzymatic systems[10−12] reveals a remarkable ∼10^{12} fold acceleration of the cleavage of this bond. Exactly how AdoCbl-dependent enzymes accomplish this rate acceleration is still not well understood, however.[13,14]

Adenosylcobinamide (Figure 1) ($AdoCbi^+$) is an analogue of AdoCbl where the α-axial 5,6-Me_2-benzimidazole ligand (on the lower side of the corrin ring), has been removed. Studies of this molecule (and its binding to exogenous bases, vide infra)[1−3,5,15−18] have been shown to be biologically relevant via three crystal structures: two structures of

* To whom correspondence should be addressed. E-mail: rfinke@lamar.colostate.edu.

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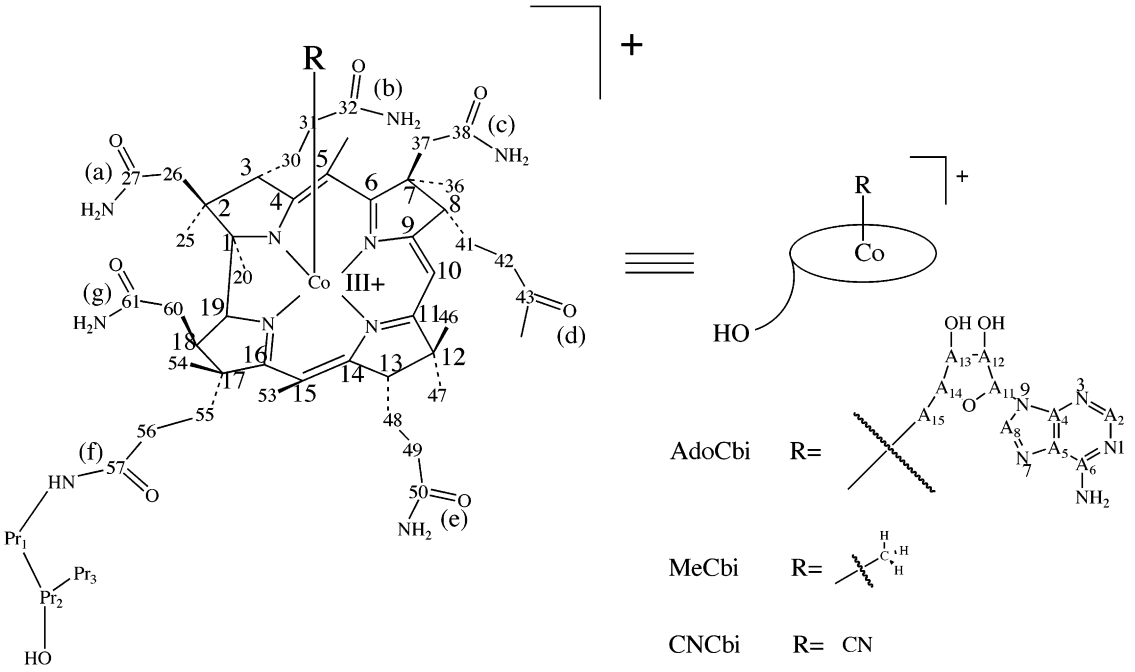

Figure 1. The structure of an alkyl cobinamide and IUPAC atom-numbering system.[15]

adenosylcobalamin-dependent enzymes, methylmalonyl CoA mutase[19,20] and glutamate mutase,[21] and one structure of the cobalamin binding domain of the methylcobalamin-dependent enzyme methionine synthase.[22] All three reveal that the appended 5,6-Me_2-benzimidazole is not coordinated to the Co, but instead has been replaced by the imidazole side chain of a histidine residue when the cobalamin cofactor is bound to these enzymes. The exact role(s) of the appended 5,6-Me_2-benzimidazole base-off, but protein side-chain histidine-imidazole base-on, form of AdoCbl naturally became one focal point of research in the B_{12} area following Ludwig and Matthew's seminal 1994 paper.[22]

A controversial, confusing, and historically very misleading aspect of some of the structural work was the exact value of the Co−N(histidine) axial bond length in the cobalamin·enzyme complexes. The structures were initially interpreted in terms of a relatively long Co−N bond length of 2.28−2.35 Å in glutamate mutase[21] and 2.53 Å in methylmalonyl-CoA mutase (MMCoA),[19] although as Marzilli correctly notes, "mixed redox and (we add β) ligand states in the crystals thwart clear conclusions"[23] about the true Co−N axial-bond length. The MMCoA system has also been studied by EPR[24] and EXAFS experiments.[25] The EXAFS data were initially suggested to be best fit by a Co−N(histidine) distance of 2.45 Å, although a poorer fit to a 2.13 Å bond is also found.[7,25] The crystal structure and "better fit" EXAFS distances are longer than the range of Co−N axial bond lengths found in free cobalamins and cobalamin analogues of 1.97−2.24 Å.[7,26] Randaccio and co-workers have since shown that Fourier filtering, possibly leading to a loss of part of the actual signal, and problems with performing only a "first-shell analysis", make such EXAFS results unreliable.[27] In this regard, R. Nuzzo has shown the impressive power of EXAFS performed with better data and out to the

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4th or 5th shells on other (non-B_{12}) systems.[28−30] EPR studies of the MMCoA·B_{12} holoenzyme find a hyperfine coupling constant of 108 G, consistent with a normal length Co−N bond,[24,31] and Marzilli and co-workers' most recent studies are quite important in also supporting a normal-length Co−N axial base bond.[23] EXAFS and protein X-ray structural work by Kratky and co-workers on B_{12}-dependent glutamate mutase shows that the experimental observation of apparently long Co−N(axial) bonds is a common, artifactual problem of mixed Co(III)/Co(II) ligand states.[32] That work illustrates the value of a histogram analysis of the Co−N lengths in the Cambridge Stuructural Database, Figure 9 elsewhere,[32a] showing that Co(III)−N distances in the 2.5 Å range are without precedent.

A lengthened Co−N bond could possibly, however, still be involved in transition state structure for Co−C cleavage. And, before the problems in the enzymic structural studies were clarified, a variable Co−N bond length was postulated to be important to Co−C bond cleavage[33,34] through both steric[35,36] and electronic[37,38] effects. Hence, an early hypothesis emanating from the *apparently* long Co−N(histidine) bond length is that the enzyme might be using the 5,6-Me_2-benzimidazole base-off/histidine base-on motif to activate or to control the mode of cleavage (homolysis vs heterolysis) of this key Co−C bond,[39] a hypothesis that now has to be amended to conceivably operate via a putatively long Co−N(axial) bond in the Co−C cleavage transition state.

Key Prior [AdoCbi·Axial-Base]$^+$ Chemical Precedent Studies

Because of the interest in axial-base effects on the mode and rate of Co−C bond cleavage, a comparison of the Co−C bond thermolysis reactions of AdoCbl[6,8,9] to [AdoCbi·solvent]$^+$ (i.e., without added axial base other than solvent) was carried out as early as 1987.[40] That work showed that the [AdoCbi·solvent]$^+$ system is only $\sim 10^2$ times less reactive than AdoCbl; hence, the axial ligand is *not* the source of the enzyme's 10^{12}-fold acceleration of the Co−C bond cleavage. This early, important result and conclusion has withstood the test of time.[36,41−48] Its significance has been underappreciated historically and perhaps even now: either the axial imidazole is not the source of the 10^{12} rate acceleration or the enzyme is doing something very different with the axial base than what can occur in enzyme-free solution. However, the effects of a possibly long Co−N(imidazole) bond on the Co−C cleavage process remained unexplored at the time. A chemical model study of Co−C bond homolysis of AdoCbi$^+$ with a series of exogenous axial bases, ideally with varying Co−N bond lengths, therefore became an important research goal.

In a series of papers,[1,5,16,17] the general mechanism for both the homolytic and heterolytic cleavage of the Co−C bond of AdoCbi$^+$, in the presence of exogenous bases, was uncovered. A comparison of *N*-methylimidazole (N-Me-Im)[17] versus the pyridine bases proved the most interesting of our studies, albeit with somewhat confounding results. Despite its aqueous pK_a of 7.3, and K_{assoc} 0.5 ± 0.1, N-Me-Im displayed as strong a bond, $\Delta H = -7.8 \pm 0.4$, and as much heterolysis, 48%, as the more basic 4-Me_2N-pyridine ($\Delta pK_a = 2.4$ units more basic than N-Me-Im; 4-Me_2N-pyridine, p$K_a = 9.7$, $\Delta H = -6.5 \pm 1.0$ kcal mol^{-1}, $K_{assoc} = 2.5 \pm 0.2$ M^{-1}, 45% heterolysis). Deconvolution of the kinetic data also revealed that AdoCbi$^+$ plus N-Me-Im undergoes Co−C *heterolysis* 30 700-fold faster than AdoCbi$^+$ and 350-fold faster than AdoCbl.

Because there was, and still is, no precedent for AdoCbl dependent enzymes utilizing Co−C heterolysis, it follows that the enzymes *must prevent it*[1,49,50]—even if "only" by selectively accelerating Co−C homolysis by $\sim 10^{12}$ with little to no acceleration of Co−C heterolysis. A long Co−N(axial) bond, predicted by MO calculations on B_{12}-models[39] to favor homolysis at a relatively long Co−N ~ 2.4 Å, seemed at the time to again be offering support for the "long Co−N(axial) bond" hypothesis—that is, a role for a variable length Co−N(axial) bond in helping control the mode and perhaps also the rate of Co−C homolytic versus heterolytic cleavage.

We previously tested the long Co−N hypothesis via molecular modeling and kinetic and product studies of axial bases of increasing steric hindrance (Figure 2) and a concomitantly longer Co−N bond.[1,51] Molecular modeling—in the end analysis somewhat deceiving molecular modeling—

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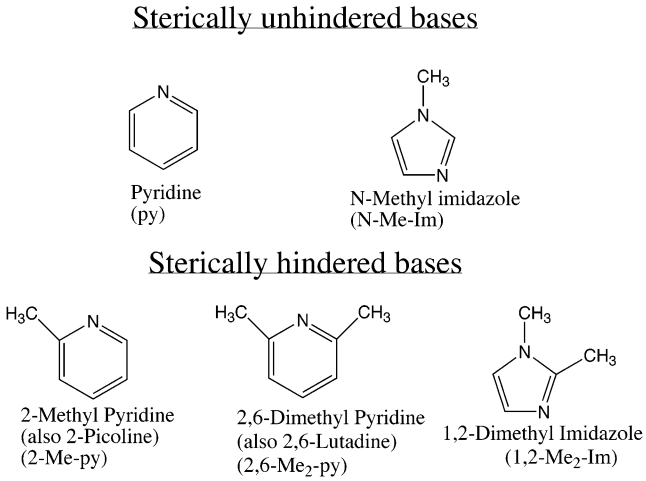

Figure 2. The structures and abbreviations of the exogenous bases that are employed in both the previous study[1] and in the present work.

shows that the axial Co−N bond of [AdoCbi·base]$^+$ increases from 2.090 Å with N-Me-Im to 2.129 Å with 1,2-Me_2-Im,[1] at least in the lowest energy conformers that were found, making the system of AdoCbi$^+$ plus 1,2-Me_2-Im and other sterically bulky bases seemingly ideal for further study at the time. However, we will now see herein that the (gas-phase) molecular modeling studies of *5-coordinate* AdoCbi$^+$ are misleading in that, in solution, 6-coordinate [AdoCbi·*solvent*]$^+$ shows no tendency to bind bulky bases at its axial coordination position, the steric effects of the base apparently lowering the Co−bulk-base bond energy to below the estimated 8 kcal/mol Co−solvent bond energy (i.e., that the binding of other axial bases must overcome).

The equilibrium binding constants (K_{assoc}) and the kinetic products (percent homolysis vs percent heterolysis) were studied with the sterically hindered bases in a 1996 study.[5] As expected, all three showed little or no binding in the ground state ($K_{assoc} \leq 0.03$). The most surprising result from all of our axial-base studies appeared next: the 1,2-Me_2-Im system exhibited a *record 91% heterolysis* (a result reproduced multiple times in our original paper,[1] and reproduced herein as well, vide infra). This experimental *increase* in Co−C heterolysis is contrary to the theoretically predicted decrease[39] of heterolysis with a longer Co−N bond (0.039 Å longer than an axial N-Me-Im by molecular modeling).[1,44] Hence, this result was of obvious interest for further study and a better understanding.

The above result requires that some effect beyond simple σ donation from the axial base to cobalt is occurring in these ill-understood AdoCbi$^+$ plus axial-base systems. In what will now prove to be an exemplary use of the scientific method, five possible, alternative hypotheses for the observed increase in Co−C heterolysis with 1,2-Me_2-Im were considered in 1999 as discussed elsewhere.[1] The leading alternative hypothesis considered at the time—which we will show herein turns out to be the correct answer—is that "(1) Deprotonation of ethylene glycol by 1,2-Me_2Im yields $HOCH_2CH_2O^-$, and that strongly σ-donating species is responsible for the observed Co−C heterolysis" (see p 1704 elsewhere[1]). However, a control experiment done at the time,[2] generating the expected amount of $HOCH_2CH_2O^-$ using Proton Sponge [1,8-bis(dimethylamino)naphthaline], showed only 5% Co-heterolysis (p 20 of the Supporting Information[1])—a far cry from the 91% heterolysis seen with added 1,2-Me_2-Im. That control experiment (which we will see is misleading) *appeared* to rule out this leading alternative hypothesis. Another possibility that *was* considered, albeit not in the detail of the other four listed on p 1704 elsewhere, is that a trace amount of impurity in the axial base could be causing the Co−C cleavage. We were aware that the thermolysis of 1×10^{-4} M AdoCbi$^+BF_4^-$ with, for example, high 0.3 M, 3000-fold excess amounts of bulky, very poorly coordinating axial bases requires that the axial base needs to be pure to the ≥99.9997% level to achieve even a ≤1:1 AdoCbi$^+$ to impurity level, assuming a single impurity was present and *assuming* that the putative impurity is problematic for Co−C thermolysis studies. We did check the purity of the 1,2-Me_2-Im by NMR (see p 1705 and 1706 elsewhere, top right-hand column) but did not see irreproducible kinetics or other evidence for the kinetic effects of impurities. The "insidious impurity issue"[52,53] eventually became de-emphasized[2] as we struggled to understand the puzzling 1,2-Me_2-Im results, which eventually focused us (correctly, as this work will show) on the other four alternative hypotheses presented on p 1704 elsewhere.[1] In the end, the only hypothesis of the four that *appeared* to explain all our data was a Co−N distant-dependent, competing σ and π effects[54,55] of the axial nitrogenous base[17] (a full discussion of this hypothesis is available in the original report).[1,17] However and as we will show herein, the nature of the base-H$^+$ countercation to the glycolate is crucial. A repeated control experiment, using the more basic and sterically bulky Proton Sponge to generate PS-H$^+$ and $HOCH_2CH_2O^-$, gives less

(51) Molecular modeling predicts Co−N bond lengths of 2.09 Å for *N*-methylimidazole, and 2.129 Å for 1,2-dimethylimidazole. Note that these are only zeroth-order estimates of these bond distances in the hypothetical gas-phase complexes.

(52) A valuable aspect of the work from Marzilli's group[23] and the present studies is that it draws attention to the rather common "insidious impurity problem": namely, that a trace impurity is causing problems in a reaction where one reagent is in large excess to the other reagents. Solvents are one common place where, for example, trace water or oxygen or other impurities can cause problems in reactions. Catalysis is a place where the substrate is in large excess vs the catalyst; the need to remove peroxides from olefins is a well know example.[53] Autoxidations catalyzed by trace radical initiators or other reactions that can have large chain lengths are another example. Useful to note here are the general ways that one has to deal with this problem: (i) studies testing the reproducibility of a system using multiple batches of reagents from multiple suppliers, or different lots from the same supplier; (ii) studies using reagents purified by multiple methods; and (iii) studies examining a large change in the ratio of reagents with a careful examination of the resultant.[23] Alternatively, (iv) the most powerful, but often most difficult, method of dealing with a trace impurity is to identify it directly, then either eliminate it, or alternatively to increase its concentration, that is, to decrease or increase its concentration and observe the effect.

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Co−C heterolysis than do the base-H^+ countercations (base = N-Me-imidazole or 1,2-Me_2-imidazole) or even Na^+.

Finally, in another set of control experiments done previously,[1,2] designed to provide evidence for or against sterically hindered base binding to a Co(II)Cbi^+-like transition state for AdoCbi$^+$ homolysis (and where even stronger binding might be expected for a Co(III)Cbi^+-like transition state for AdoCbi$^+$ heterolysis), the interaction of Co(II)Cbi^+ with sterically hindered bases was examined.[17] Figure 5 of our original report[1] appeared to show a reaction, but that was later determined to be an artifact in the experimental work[2] caused by oxygen contamination of air-sensitive Co(II)Cbi^+. We thank Prof. Marzilli and his students for originally bringing the problems in the Co(II)Cbi^+ titrations and the resultant Figure 5 elsewhere[1] to our attention.[56] Correction of those errors would not have occurred were it not for their experiments and insights of the Marzilli team. New titration experiments, performed with both purified and unpurified bases, are reported herein (Supporting Information Figures S1−S5) which confirm the findings of Marzilli and co-workers:[23] purified sterically hindered bases, including 2,6-Me_2py, do *not* detectably bind to Co(II)Cbi^+. Hence, there is no longer[1] evidence from these studies for the binding of bulky bases to a Co(II)-like transition-state for [Ado···Cbi^+]‡ homolysis. These Co(II)Cbi^+ plus sterically hindered base studies were initially only done *as extra control experiments* to see if we could obtain evidence for what appeared to be the kinetically detected effects of sterically bulky bases in the Co−C cleavage transition state. However, these seemingly innocent, "extra" control experiments proved very misleading when combined with the experimental error in their execution[2,57]—leading to results apparently showing that bulky bases could bind—as well as four other misleading items: (i) the incorrect EPR study reporting that Co(II)Cbi^+ could bind bulky pyridine bases[58,59] (a report now corrected by Marzilli's studies showing that impurities in unpurified Me-pyridines are what are actually being detected by EPR);[23] and (ii) misleading, claimed "B_{12} model" studies showing that *trans*-bis(dimethylglyoximato)isopropyl(2-aminopyridine)cobalt(III),[60] Me(CoDO(DOH)pn)(1,2-dimethylimidazole)-PF_6,[61] and (alkyl)bis(dimethylglyoximato)(1,2-dimethylimidazole)cobalt(III)[62] bind the bulky bases 2-NH_2-py and 1,2-Me_2-Im (bulky-base binding which is *not* found for AdoCbi$^+$ itself). Also misleading were (iii) the gas-phase molecular modeling studies of [AdoCbi·bulky-bases]$^+$ showing binding of the axial bases,[1] and (iv) the 5% heterolysis in the control experiment with [Proton Sponge−H^+][$HOCH_2CH_2O^-$] (an irreproducible[2] result; a reproducible 28 ± 8% is seen herein, vide infra). In short, the above combination of misleading/erroneous results meant that the correct answer to why 1,2-Me-imidazole causes record levels of Co−C heterolysis with AdoCbi$^+$ could not be uncovered until now and until the new experimental work, by another experimentalist (K. M. Doll), reported herein—despite the valuable report of the Marzilli team which has also been key to obtaining the correct answer.[23] The use of a correct scientific method where, as before, we consider all alternative hypotheses for the observed Co−C heterolysis,[63] has proved to be *the* key to uncovering the correct answer.

Results and Discussion

Purification of the Axial Bases. Since imidazole bases were the key to our earlier studies and conclusions, we began our studies here. Although other purification methods[64−66] have been used, the most common and also most practical literature purification[67−70] of 1,2-Me_2-Im is recrystallization from benzene.[71] Hence that was our method of choice as detailed in the Experimental Section.

Sterically hindered pyridines are usually synthesized commercially via the reaction of formaldehyde, ammonia, and an aldehyde or ketone at high temperature (>350 °C).[72] This synthesis often leaves unhindered pyridines as a contaminating byproduct. Literature on the purification of sterically hindered pyridines dates back to the 1950s.[73−75]

(56) (a) Marzilli, L. Private communication. We thank Prof. Marzilli and his student for this valuable input. (b) We, in turn, provided Professor Marzilli with a preprint of the present paper along with a request for his comments.

(57) A "correction" of these spectra was published as Figure 5′ elsewhere.[3] Unfortunately, this result has also proven to be unreliable and should be replaced by the repeatable results shown in Figure S5 in the Supporting Information herein.

(58) Bayston, J. H.; Looney, F. D.; Pilbrow, J. R.; Winfield, M. E. *Biochemistry* **1970**, *9*, 2164.

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(60) Summers, M. F.; Toscano, P. J.; Bresciani-Pahor, N.; Nardin, G.; Randaccio, L.; Marzilli, L. G. A Very Long Cobalt to Nitrogen Bond in a Coenzyme B_{12} Model. Relevance to the Role of the 5,6-Dimethylbenzimidazole in Co−C Bond Cleavage in Coenzyme B_{12}. *J. Am. Chem. Soc.* **1983**, *105*, 6259.

(61) Pahor, N. B.; Randaccio, L.; Zangrando, E. Vitamin B_{12} Model Compounds: Influence of Neutral Ligand Orientation on the Co−N Axial Bond Length. *Inorg. Chim. Acta* **1990**, *168*, 115.

(62) Bresciani Pahor, N.; Attia, W. M.; Geremia, S.; Randaccio, L.; Lopez, C. *Acta Crystallogr., Section C: Cryst. Struct. Commun.* **1989**, *C45*, 561.

(63) Platt, J. R. *Science* **1964**, *146*, 347.

(64) Imidazoles have also been purified by two patented methods reported in the Japanese literature. The first[65] involves distillation of a reaction solution, followed by cooling into a "wet cake" and centrifugal separation. The second[66] utilizes the dehydrogenation of the corresponding imidazoline in the presence of a nickel or platinum catalyst. Since the actual purity of the obtained product from either of these more involved methods is not available, these methods were not used.

(65) Kakimoto, T.; Ogawa, T. (Nippon Synthetic Chemical Industry Co., Ltd., Japan). *Jpn. Kokai Tokkyo Koho* JP 62164672 A2 19870721, 1987, p 3.

(66) Aoki, M.; Hara, Y. (Tosoh Corp., Japan). *Jpn. Kokai Tokkyo Koho* JP 2000319263 A2 20001121; 2000178256 A2 20000627, 2000, p 5.

(67) The literature reveals that although 1,2-dimethylimidazole is widely used (a structure search on Scifinder finds 526 references), it has been used without purification on studies of binding with metal porphyrins,[68] and with unspecified purification in the study of organometallic complexes.[69,70] This practice should not be continued.

(68) Inamo, M.; Nakajima, K. *Bull. Chem. Soc. Jpn.* **1998**, *71*, 883.

(69) Abuhijleh, A. L.; Woods, C. *J. Chem. Soc., Dalton Trans.* **1992**, 1249.

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(72) *Encyclopedia of Chemical Technology*; Kirk, R. E., Othmer, D. F., Eds.; John Wiley and Sons: New York, 1996.

(73) Brown, H. C.; Johnson, S.; Podall, H. *J. Am. Chem. Soc.* **1954**, *76*, 5556.

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Table 1. The Observed Percent Heterolysis and k_{obs} Data for the Thermolysis Reactions of AdoCbi$^+$ with Exogenous Base as Redetermined Herein and Compared to Those from Our Previous Report[1]

exogenous base	% heterolysis previous study[1]	% heterolysis this work[a]	relative ratio of heterolysis (this work)	k_{obs} (s^{-1}) previous study[1]	k_{obs} (s^{-1}) this work[a]	relative k_{obs} [b]
N-Me-Im[c]	48	52 ± 10	10 ± 2	3.4(±0.2) × 10^{-5}	4(±1) × 10^{-5}	13 ± 3
1,2-Me_2-Im[c]	91	83 ± 7	17 ± 2	4.3(±0.3) × 10^{-5}	4(±1) × 10^{-5}	13 ± 3
2-Me-py[c]	24	17 ± 5	3 ± 1	1.0(±0.1) × 10^{-5}	1.1(±0.1) × 10^{-5}	3.4 ± 0.3
2,6-Me_2-py[c]	6	16 ± 5	3 ± 1	0.89(±0.05) × 10^{-5}	1.0(±0.1) × 10^{-5}	3.1 ± 0.3
none	2	5	1	0.32(±0.10) × 10^{-5}	not examined	1

[a] Conditions: AdoCbi$^+BF_4^-$ (1×10^{-4} M) and sublimed TEMPO (2×10^{-2} M) in ethylene glycol plus 0.3 M (3000-fold excess) of the indicated axial base were mixed inside an inert atmosphere drybox in a Schlenk cuvette. That cuvette was then sealed, removed from the drybox, thermolyzed at 110 °C in a darkroom for 20 h (imidazole systems) or 156 h (pyridine systems), and cooled to room temperature, and then the Co−C bond cleavage products were analyzed by HPLC. [b] Relative k_{obs} using the well-established $k_{obs} = 0.32(\pm 0.10) \times 10^{-5}$ value from our earlier work[1] (as listed in column 5 above). [c] The following number of repeat experiments were performed for the averages and error bars given in columns 3 and 6 ("this work"): N-Me-Im (3 experiments); 1,2-Me_2−Im (4 experiments); 2-Me-py (7 experiments); and 2,6-Me_2-py (7 experiments).

Early methods relied on the distillation of azeotropes with water[74] or phenol. Newer purifications of 2,6-Me_2-py or 2-Me-py take advantage of the fact that common impurities will coordinate to inorganic compounds and the 2,6-Me-py or 2-Me-py can then be distilled. Compounds that have been used for this purpose are BF_3,[73] $AlCl_3$,[71] $CuCl_2$,[76] $ZnCl_2$,[76] $Ag(NO)_3$,[77] and recently Co((DO)(DOH)Me_2pn)Br_2.[23] This latter "affinity distillation" reagent is the one used to show that the purification method of 2-Me-py has an observable effect on whether (impurity) binding to Co(II)Cbi$^+$ can be detected by EPR and UV−vis spectroscopy.[23] For our studies herein of 2-Me-py and 2,6-Me_2-py, we chose two purification methods: traditional distillation utilizing a spinning-band-column, and affinity distillation utilizing Co(C_2(DO)(DOH))-Br_2.

We also attempted the direct detection of any impurities in the liquid bases by GC-MS (see the Experimental Section). However, in no case could we detect such impurites even though there are impurities in, for example, 2-Me-py, that the Marzilli team has shown do bind to Co(II)Cbi$^+$.[23]

[AdoCbi·Axial-Base]$^+$ Co−C Thermolysis Product Studies. Product studies were done on AdoCbi$^+$ thermolysis reactions with first the imidazoles (N-Me-Im; 1,2-Me_2-Im) and then with the hindered pyridine bases (2-Me-py; 2,6-Me_2-py). Different purities were tested by utilizing the as-received commercial bases as well as those purified by the methods cited above. Additional variations in purity (or at least potential variations in purity) were accomplished by utilizing freshly purchased as well as >2 year old bottles of the bases. In two cases, N-Me-Im and 1,2-Me_2-py, it was possible to use the *exact same bottles* of these two nitrogenous bases that were used in the previous report.[1]

As was done in the previous experiments,[1] ethylene glycol solutions of AdoCbi$^+BF_4^-$ (1×10^{-4} M) with each of the axial bases (0.3 M; 3000-fold excesses) were prepared inside an inert atmosphere drybox in a Schlenk cuvette. The cuvettes were sealed, removed from the drybox, and thermolyzed at 110 °C in a darkroom. The Co−C bond cleavage products were analyzed by HPLC with a focus on the key Co−C heterolysis product.

Comparisons of the original results to 9 different thermolysis solutions utilizing imidazoles, and 14 different thermolysis solutions utilizing pyridines, show that, *regardless of source or purity, the results did not change within experimental error from those we reported previously*,[1] Table 1. More specifically, the 52 ± 10% heterolysis with N-Me-Im as the added base, and 83 ± 7% heterolysis with Me_2-Im as the added base, are within experimental error of those in the earlier report (48% and 91%, respectively). The multiple repeats reveal that the error bars of the HPLC product method are in the ca. 5−10% range, a range consistent with the HPLC method utilized. A full table with each experiment is available in the Supporting Information, Tables S1 and S2.

The overall first-order rate constant of the production of Co(II) in the presence of N-Me-Im, calculated from the slope of a $\ln[(A_\infty/(A_\infty - A_t)]$ versus time plot at 474 nm, is 4(±1) × 10^{-5} s^{-1}, also within experimental error of the values in the original report, 3.4 (±0.2) × 10^{-5} s^{-1}. Note that although the k_{obs} rate constant contains both homolysis and heterolysis contributions [as Scheme 1 makes apparent, and as confirmed by the kinetic derivation elsewhere[1,16] and in the Supporting Information accompanying this paper (section S-1)], the relative increase in the % heterolysis (column 4, Table 1) matches the relative increase in the k_{obs} value (column 7, Table 1) within experimental error. This strongly suggests that all the increase in k_{obs} is due to Co−C heterolysis. It also means that our earlier deconvolution of k_{obs} into tentative "estimated"[1] homolysis ($k_{on,h}$) and heterolysis ($k_{on,het}$) components in Table 2 elsewhere is no longer justified so that those values should be discarded.

Experiments varying the concentration of the imidazoles were also performed. Plots of imidazole concentration versus percentage heterolysis showed the expected linear dependence over the concentration range studied (Figures 3 and 4).

The percent heterolysis for both of the hindered pyridines is, if anything, *increased*, not lowered, when the hindered pyridines are purified more. More likely, the percent heterolysis, 16 ± 5%, is the same within experimental error if one assumes the same level of error for the prior work, 6 ± 5%. The rate constants for the pyridine systems are also within experimental error of the original report, ~1.0 ± 0.1 × 10^{-5} s^{-1}. In short, the data do not support the hypothesis

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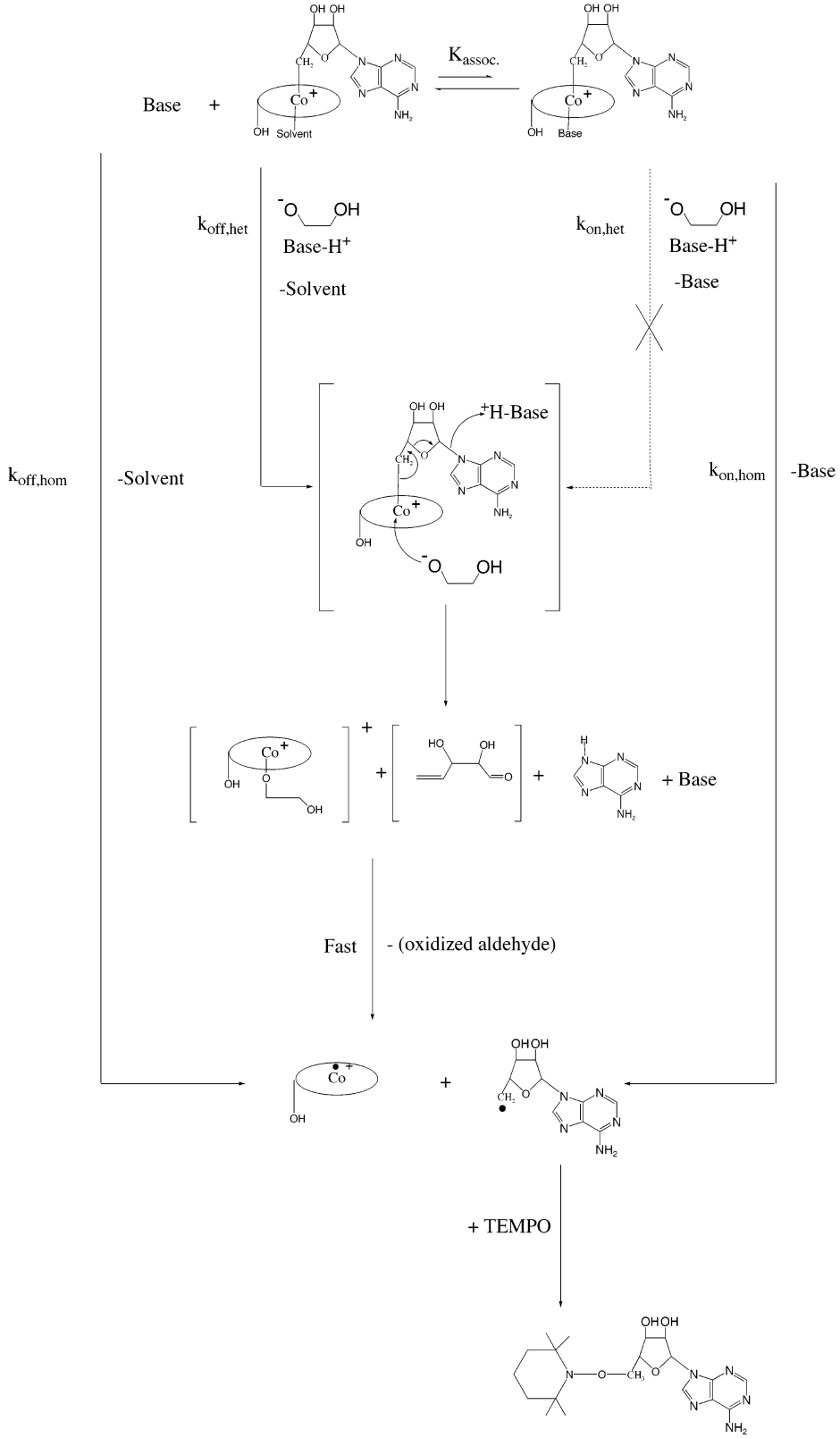
(76) Tomasik, P.; Woszczyk, A.; Kret, F. *Koks, Smola, Gaz* **1976**, *21*, 330.
(77) Bal, S. *Zesz. Nauk. Politech. Slask., Chem.* **1970**, *No. 50*, 309.

Scheme 1. The Established General Reaction Scheme[6,16] for the Homolysis and Heterolysis Reactions of AdoCbi$^+$ in Ethylene Glycol and in the Presence of TEMPO, but Now with the Added [Base-H$^+$][Glycolate$^-$] Co−C Heterolysis Pathway[a]



[a] The five key constants are defined by this scheme: K_{assoc}, $k_{off,hom}$, $k_{off,het}$, $k_{on,hom}$, and $k_{on,het}$. Note that, in the interest of simplicity and since we have no direct evidence for a prior equilibrium to a glycolate base-on species, [AdoCbi$^+$•glycolate$^-$], the [base-H$^+$][glycolate$^-$]-dependent pathway is depicted as a single step; that is, "$k_{off,het}$" may really be a composite with a separate $K_{assoc,glycolate}$, $k_{on,het,glycolate}$, and so on. It is not known for certain that the glycloate attacks at the "bottom", α-Cbi$^+$ position as shown, although this is the working mechanism suggested by the present studies. On the basis of literature precedent (Brown and other's seminal work cited in refs 41a−n elsewhere[16]), the kinetically important protonation step is actually at the β-oxygen of the Ado group (see Scheme 3 elsewhere[16]). We have deliberately simplified the base-H$^+$ protonation step in the above scheme by showing, as before (Scheme 1 elsewhere[16]), only the end-protonation of the adeninyl anion leaving group so as to keep this scheme as uncluttered as possible. The $k_{on,het}$ step is presumed to be slow as shown, but this is not known for certain.

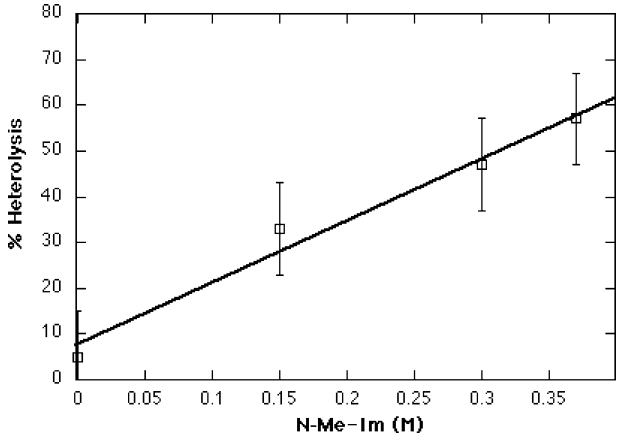


Figure 3. A plot of N-Me-Im concentration vs the observed percentage of AdoCbi$^+$ Co−C bond heterolysis. The slope and intercept of the line are 140 ± 15% M^{-1} and 8 ± 4%, respectively.

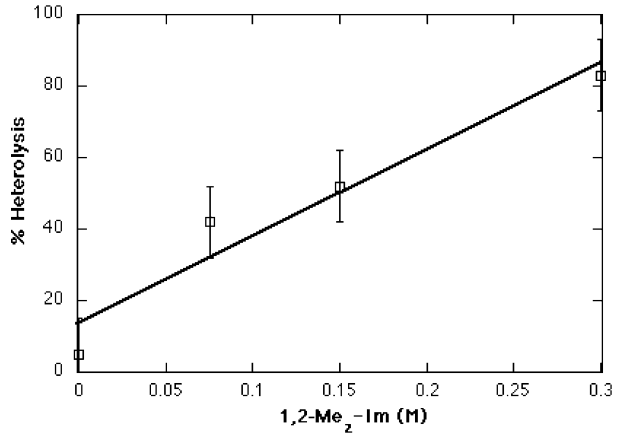


Figure 4. A plot of 1,2-Me_2-Im concentration vs the observed percentage of AdoCbi$^+$ Co−C bond heterolysis. The slope and intercept of the line are 240 ± 40% M^{-1} and 14 ± 8%, respectively.

strongly implied by others that an impurity in the exogenous bases is causing the Co−C observed heterolysis.[23] Also worth noting here is that the increase in k_{obs} upon adding bulky bases versus no added base is ~3-fold whereas coordinating bases such as 4-Me_2N-py cause a ≥100-fold increase in k_{obs} (see Table 2 elsewhere[16]).

Control Thermolysis of AdoCbl with Added Bases. Even though the results provide strong evidence against an impurity as the main, kinetically dominant additive when axial bases are added, we wished to try any other conceivable controls or other experiments that might prove informative on this point. We reasoned that if a trace impurity is present that can cause such large rate accelerations, then it might even be able to influence the Co−C cleavage products and kinetics of AdoCbl despite its appended 5,6-dimethylbenzimidazole. Hence, two separate control experiments were done determining the products and kinetics of a solution of AdoCbl with two different purities of N-Me-Im and 1,2-Me_2-Im and at 0.3 M (~3000 equiv vs AdoCbl). Low percentage heterolyses were observed, 5 ± 5% for N-Me-Im and 7 ± 5% for 1,2-Me_2-Im heterolysis, which did not change with different purities. An invariant first-order rate constant of 1.8 ± 0.2 × 10^{-4} s^{-1} was observed for each reaction, a value that was within experimental error of a

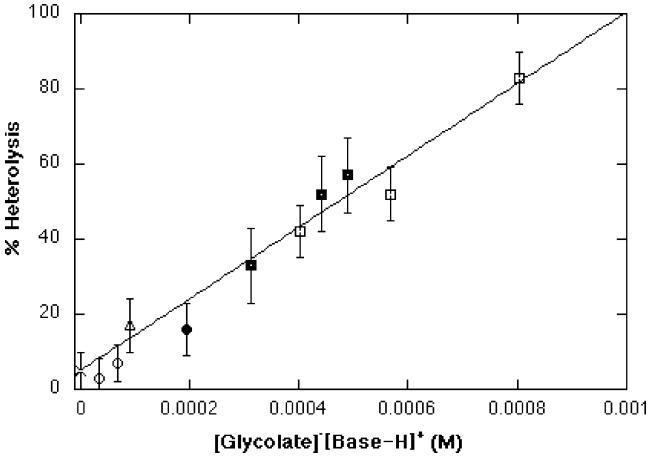


Figure 5. A plot of the percentage heterolysis of the Co−C bond vs the initial ethylene glycolate anion concentration calculated from pK_a values and concentration of the bases used. The data points are for the bases: 1,2-Me_2-Im (□), N-Me-Im (■), 2,6-Me_2-py (●), 2-Me-py (△), pyridine (○), and no added base (×). The slope and intercept of the line are 96000 ± 8000% M^{-1} and 5 ± 4%, respectively.

control thermolysis experiment performed concurrently using AdoCbl without these added bases. This is a value which is also within experimental error of literature values[6,9] (Table S3). Again, no evidence for any effect of trace impurities in the added axial base was found.

A Reinvestigation of Our Original, Leading Alternative Hypothesis That [Base-H$^+$][$HOCH_2CH_2O^-$] Is the Actual Cause of the Co−C Bond Heterolysis. We were led, by a consideration of the known mechanism of Co−C heterolysis,[6] to revisit this original, leading alternative hypothesis[1,3,5] (e.g., see p 1704 elsewhere[1]) for the record Co−C heterolysis when exogenous bases are added prior to the thermolysis of AdoCbi$^+$. We reasoned that if the general mechanism of cobamide Co−C cleavage,[1,5,6,16,17] Scheme 1, was as well established as we believed, then it should effectively predict what was going on with bulky base plus AdoCbi$^+$ thermolyses. Studying the mechanism,[6,16] especially the transition state for Co−C heterolysis shown, made it apparent that *both* the glycolate anion, [$HOCH_2CH_2O^-$], *and* the conjugate acid of the base, [base-H$^+$], should at least in principle be playing a role in accelerating Co−C heterolysis. This, in turn, led us to realize that the control we had done with Proton Sponge, as a sterically bulky base (and thus noncoordinating and, before, seemingly ideal base to generate the [$HOCH_2CH_2O^-$]), may well have misled us. Of course, the attraction of the "glycolate anion" hypothesis all along—why it was our number one explanation for the data previously[1]—is that (a) glycolate anion is sterically small, so it could bind readily to AdoCbi$^+$ when bulky bases did not (at least to a nonkinetically detectable level), and (b) [$HOCH_2CH_2O^-$] is also a strong σ donor and, hence, should promote the observed Co−C heterolysis.[4] A kinetic derivation and resultant rate law accompanying Scheme 1 is available in the Supporting Information (Section S-1).

We began our retest the "[base-H$^+$][$HOCH_2CH_2O^-$] mechanism" outlined in the above scheme by calculation of the expected initial concentration of [base-H$^+$][$HOCH_2CH_2O$-] from the pK_a's of all the bases studied.[78] Because

we now have data for three different concentrations of each imidazole, as well as data on three pyridine systems, our data span an initial [base-H^+][$HOCH_2CH_2O^-$] concentration range of nearly 10^5, from 1×10^{-4} M to 8×10^{-4} M. A plot of the percentage Co−C heterolysis versus [base-H^+][$HOCH_2CH_2O^-$] *shows a linear dependence*, Figure 5. *The fact that data from all of the bases fit the same line is a very important observation when combined with the fact that these bases differ completely in their ability to coordinate to AdoCbi*$^+$: pyridine and N-Me-Im have measurable association constants with $AdoCbi^+$, but 2-Me-py, 2,6-Me_2-py, and 1,2-Me_2-Im *show no detectable coordination*. The data in Figure 5 provide very strong evidence that the amount of [base-H^+][$HOCH_2CH_2O^-$] is the Co−C heterolysis-causing agent. Note that *both* the [$HOCH_2CH_2O^-$] *and* the [base-H^+] are important here.

We also repeated our earlier experiment[1] in which Proton Sponge was used to generate [Proton Sponge-H^+][$HOCH_2CH_2O^-$] at 8×10^{-4} M, the level expected from the pK_a of the most basic sterically bulky bases studied, 1,2-Me_2-Im. In significant contrast to the 5% value observed earlier,[2] we now reproducibly find a higher, 28 ± 8% $AdoCbi^+$ Co−C heterolysis. We speculate that the problem with this experiment in the earlier thesis work[2] was a failure to let the kinetically insoluble Proton Sponge dissolve completely before proceeding with the experiment. Experiments were also performed using multiple concentrations of [Proton Sponge-H^+][$HOCH_2CH_2O^-$], and a linear correlation of the % heterolysis versus the concentration of [Proton Sponge-H^+][$HOCH_2CH_2O^-$] is observed (Figure S8 of the Supporting Information). Importantly, these results confirm the validity of the 28 ± 8% Co−C heterolysis at 8×10^{-4} M [Proton Sponge-H^+][$HOCH_2CH_2O^-$]. Furthermore, independent data from our first, 1996, report[16] also using [Proton Sponge-H^+][$HOCH_2CH_2O^-$] fit nicely to the observed line, thereby providing additional confirmation of these now repeatable control experiments using Proton Sponge.

In comparison to the results for the other base-H^+ cations or Na^+ (vide infra), the [Proton Sponge-H^+][$HOCH_2CH_2O^-$] system demonstrates the importance of the specific [base-H^+] or other countercation in the Co−C heterolysis process. Noteworthy is that the observed 28 ± 8% for [Proton Sponge-H^+][$HOCH_2CH_2O^-$] *is significantly less than the* 83 ± 7% observed for the same initial concentration of [1,2-Me_2-Im-H^+][$HOCH_2CH_2O^-$]. These results can be understood by looking at the pK_a's of the [base-H^+] species involved. All of the bases plotted in Figure 5 have a pK_a value between 5.3 (pyridine) and 7.8 (1,2-Me_2-Im).[79] However, protonated Proton Sponge (conjugate acid pK_a of 12.4)[80] is considerably less acidic (i.e., Proton Sponge is considerably more basic), so that a slower Co−C bond heterolysis pathway leading to less Co−C heterolysis product[81−83] is expected *and observed*.

Further evidence for a countercation effect in the [cation$^+$][$HOCH_2CH_2O^-$] cleavage reaction was obtained by using a carefully weighed amount of fresh sodium metal added to ethylene glycol in the drybox to produce a known concentration of [Na^+][$HOCH_2CH_2O^-$]. A linear correlation was also established between [Na^+][$HOCH_2CH_2O^-$] and percentage heterolysis of the Co−C bond in this system (Figure S8 in the Supporting Information). A comparison of the slope of the line for Na^+ as the countercation (32000 ± 5000% M^{-1}) to the slopes of the lines where the countercations are base-H^+ (95000 ± 8000% M^{-1}; Figure 5) and Proton Sponge−H^+ (5000 ± 1000% M^{-1}; Figure S9 in the Supporting Information) reveals that, as now expected, (i) Na^+ facilitates Co−C heterolysis. However and more importantly, (ii) the Na^+ salt *is only 1/3 as effective as the available proton in base-H*$^+$. Note that a very important conclusion which follows from the plot in Figure 5 is that *all* the Co−C heterolysis above the intercept of 5 ± 4% *appears to be due to the [base-H*$^+$*][HOCH*$_2$*CH*$_2$*O*$^-$*]-assisted pathway*. This is an important, previously unappreciated finding.[84,85]

An important implication from the plot in Figure 5 follows from the fact that *N-MeIm also falls on the line in Figure 5*. This strongly suggests that N-MeIm induced Co−C heterolysis occurs primarily via the [base-H^+][$HOCH_2CH_2O^-$]-dependent pathway. (Note also here that the slope of the line in Figure 5 (i.e., the sensitivity of the Co−C cleavage to [base-H^+][$HOCH_2CH_2O^-$]) is ~96 000 M^{-1} while the slope of Figure 3 (i.e., the sensitivity of the Co−C cleavage to [N-MeIm]) is 140 M^{-1}, *a factor of 686 less*. This is strong kinetic evidence that even the coordinating N-MeIm gives its enhanced Co−C heterolysis (Figure 3 and Table 1 herein; also Table 2 elsewhere[1]) via the [base-H^+][$HOCH_2CH_2O^-$]-dependent pathway. Since the competing σ versus π effects of axial bases postulated earlier[1] followed previously only after the apparent (at that time; now known to be incorrect) ruling out of the [base-H^+][$HOCH_2CH_2O^-$] pathway as the number one explanation for the data (see p 1704[1]), it follows that *the hypothesis of competing* σ *vs* π *effects of axial bases no longer has experimental support and must be abandoned.*

(78) The pK_a's given in the text are aqueous values. However, values that are similar, and more importantly, of the same relative order, are observed in ethanol or methanol. For example, the pK_a's of py, 2-Me-py, and 2,6-Me_2-py are 5.2, 5.9, and 6.7 in water, and change to 4.4, 5.1, and 5.8 in 50% water/ethanol.[79] Hence, the pK_a values cited should follow the same relative order in the alcohol solvent, ethylene glycol.

(79) Schofield, K. *Hetero-Aromatic Nitrogen Compound, Pyrroles and Pyridines*; Plenum Press: New York, 1967.

(80) Gordon, A. J.; Ford, R. A. *The Chemist's Companion*; John Wiley and Sons: New York, 1972.

(81) The pK_a of the heterolysis penultimate product adeninyl should lie above the pK_a = 9.8 of adenine.[82,83]

(82) *Data for Biochemical Research*, 2nd ed.; Dawson, R. M. C., Elliot, D. C., Elliott, W. H., Jones, K. M., Eds.; Oxford University Press: Oxford, 1969.

(83) Ravindranathan, S.; Butcher, S. E.; Feigon, J. *Biochemistry* **2000**, *39*, 16026.

(84) (a) Interestingly, the *less hindered* base Me_2-N-py (conjugate acid pK_a[84b] = 9.7) studied in our 1996 report[16] with $AdoCbi^+$ appears to give a *constant % heterolysis* with increasing [Me_2-N-py]. (Note that the Co-binding site is presumably the same as the protonation, namely at the pyridine nitrogen,[79,85] Me_2-N-py-H^+.) This is consistent with heterolysis from the base-on [AdoCbi·py-NMe_2]$^+$ form, as detailed in our 1996 paper,[16] a situation different than the present studies involving bulky bases and their [BH^+][$HOCH_2CH_2O^-$]. (b) Christensen, J. J.; Hansen, L. D.; Izatt, R. M. *Handbook of Proton Ionization Heats*; John Wiley and Sons: New York, 1976.

(85) Forsythe, P.; Frampton, R.; Johnson, C. D.; Katritzky, A. R. *J. Chem. Soc., Perkin Trans. 2* **1972**, 671.

This factor of 686 also foretells why axial-base impurities that might be N-based ligands are not important in the Co−C cleavage reaction: they are kinetically incompetent versus the much faster, [base-H^+][$HOCH_2CH_2O^-$] pathway.

Summary and Conclusions

In summary, (i) all of our evidence strongly supports [base-H^+][$HOCH_2CH_2O^-$], and not bound, sterically bulky nitrogenous axial bases as it appeared previously,[1] nor some unspecified impurity in the axial bases as others had erroneously strongly implied,[23] as the key player in causing increased Co−C heterolysis with added bases. The prior competing σ vs π effects of axial bases has no experimental support at present and, therefore, must be abandoned as should the deconvolution of k_{obs} into $k_{on,h}$ and $k_{on,het}$ in Table 2 elsewhere. Attention to (ii) the much more common issue of the "insidious impurity problem",[52] and the summary of the ways to deal with this issue provided the Marzilli team's valuable contribution,[23] and in our footnote,[52] are noteworthy. The suggestion that (iii) the appended benzimidazole is present in AdoCbl to *prevent* Co−C heterolysis by OH^- (in water) or other good σ donors that might be present is an additional implication of this work.

There are a host of other, noteworthy take-home messages emanating from this work, including (iv) the need to be very cautious interpreting gas-phase molecular modeling studies if specific solvation or other, nonmodeled solvation phenomenon might be involved (i.e., the ca. 8 kcal/mol Co−ethylene glycol solvent bond dissociation energy in[17] [AdoCbi·solvent]$^+$ which must be overcome to make [AdoCbi·(bulky-base)]$^+$, a plausible reason stable [AdoCbi·(bulky-base)]$^+$ species are seen in (gas-phase) molecular mechanics simulations[1] but are not detectable in solution); and (v) the need to be very cautious—as we pointed out over 20 years ago[86]—in applying B_{12}-model studies to the interpretation of the much more complex and sterically encumbered *B_{12} itself*. The reports that the cobaloxime complexes bind 2-NH_2-py or 1,2-Me_2-Im are interesting results of general interest to inorganic chemists. They are also of interest is showing, in hindsight, *differences* compared to B_{12} and what is special about B_{12}. *But*, an intellectual mistake is made when the term of "coenzyme B_{12} model" is commonly used in the title and elsewhere in these papers. In point of fact, the X-ray structures of *trans*-bis(dimethylglyoximato)isopropyl(2-aminopyridine)cobalt(III),[60] *trans*-(alkyl)bis(dimethylglyoximato)-(1,2-dimethylimidazole)cobalt(III),[62] and Me(CoDO(DOH)-pn)(1,2-dimethylimidazole)PF_6[61] are more correctly termed "B_{12}-*anti*-models" in that they show bulky axial-base binding in their ground-state structures, results *not* seen in the sterically much more encumbered coenzyme B_{12}. The now 23 year-old lesson that one should use only the sterically more encumbered, different charge, electronically different, and less planar (than cobaloxime)[86] cobamides as B_{12} models wherever possible for the B_{12}·enzyme complex is once again emphasized.[86] The need to interpret other claimed "B_{12} model" data, as it *might* apply to coenzyme B_{12}·enzymes themselves, with extreme caution is also apparent. The (vi) need for caution in interpreting rate laws is another, albeit already well-known, take-home message emphasized by the present work. Our earlier conclusion that our observed kinetic dependence on bulky axial bases requires that the "bulky ligand must be involved *in* the rate-determining, Co−C bond cleavage step" has proven incorrect. The more precise conclusion at that time should have been that our kinetics required "that the bulky ligand is involved *prior to or in* the rate-determining step". We believed at the time that our controls ruling out impurities, as well as the glycolate anion and four other hypotheses, allowed us to make the first, textbook[87] statement; however, the present work shows that, in hindsight, the second, more cautious conclusion is the correct one. Also noteworthy is (vii) the need to approach mechanism with both kinetics as well as "all feasible spectroscopic methods" as Marzilli has noted.[23] However,

(86) Elliott, C. M.; Hershenhart, E.; Finke, R. G.; Smith, B. L. *J. Am. Chem. Soc.* **1981**, *103*, 5558.

(87) (a) The proper interpretation of rate laws merits some discussion, especially in light of Marzilli and co-workers incorrectly citing[23] as a "principle" (see p 3365 elsewhere[23]) our equating, *in this example*, the reaction order in each reagent with the composition of the transition state of the rate-determining step. All experienced kineticsts know that the above statement is not a *principle*, despite it being generally useful enough to be given as what Espenson calls "rule or really clue #1" for interpreting rate laws in his kinetic textbook (see p 127 elsewhere[87b]): "The concentration dependences in the rate law establish the elemental composition of the transition-state (of, we add, the rate-determining step, rds) and its charge". This valuable "clue" for interpreting rate laws works well in many cases and, therefore, is a valuable *heuristic device* for those just learning kinetics and how to interpret empirical rate laws. However, the reason this "clue" is not a "hard rule" or "principle" is that exceptions are known, albeit it somewhat obscure ones in most cases. The exception that the corresponding author teaches in his kinetics class is a hypothetical example from E. King[87c] as commented on elsewhere[87d] and taught to the corresponding author by his former colleague at Oregon, the expert kineticist Prof. R. M. Noyes. That example involves a enzyme mechanism with *parallel and catalytic* reactions of the enzyme (E) with reagents A and B reacting via reversible, parallel E·A and E·B adducts to give a common E·A·B intermediate, which then decomposes in a rds to E plus product. The derived rate law is $\alpha[Ez][A]^2[B]^{-1}$, which as Noyes aptly notes "does not obviously imply the stoichiometry of the transition-state of the rate-determining step". Noyes goes on to say: "Although this example provides a caveat against the uncritical equating of kinetics with transition state stoichiometry, a rather unusual combination of circumstances would be needed to create a situation where such difficulties would arise". The present example is perhaps a more common situation, one where a reagent in large, unchanging excess (e.g., solvent), as well as acid/base reactions, obscure the true rate law and make "clue #1" dangerous to apply. Note however and interestingly that if the [glycol] dependence of the rate law had been uncovered experimentally (i.e., in addition to the observed [bulky base] dependence), then interpretation of that resultant rate law via "clue #1" *would have yielded the generally correct interpretation*. Also meriting comment here is that it is very well-known that the empirical reaction *order* and the theoretical concept of *molecularity* are the same only for elementary steps.[87e] The above example, as well as common observation of, for example, fractional orders (e.g., in radical chain reactions), teaches that *only by doing the math (the kinetics derivation) for a proposed mechanism can one reveal the predicted rate law and overall order for a given mechanism under a specific set of experimental conditions.* Hence, this is the principle that the corresponding author teaches in his kinetics class, along with the useful heuristic device of "clue #1" which works in enough cases that Espenson also cites it in his textbook.[87b] (b) Espenson, J. H. *Chemical Kinetics and Reaction Mechanisms*, 2nd Ed.; McGraw-Hill: New York, 1995; p 127. (c) King, E. L. *J. Phys. Chem.* **1956**, *60*, 1378. (d) Noyes, R. M. In *Techniques of Chemistry, Vol 6, part 1*; Lewis, E. S., Ed.; J. Wiley: New York, 1974; p 489 (see pp 528−529). (e) Steinfeld, J. I.; Francisco, J. S.; Hase, W. L. *Chemcial Kinetics and Dynamics*; Prentice Hall: Englewood Cliffs, NJ, 1989; see p 5.

only kinetic studies can test whether impurities in sterically bulky bases are kinetically competent to cause the Co−C cleavage results seen in at least the present [base-H^+][$HOCH_2$-CH_2O^-] system. They are not.

Additional take-home messages are apparent as well, including the following: (viii) the main basis for the "transition-state mechanochemical triggering" hypothesis for the acceleration of Co−C homolysis discussed elsewhere[13,36] would hereby seem to now be taken away (i.e., no evidence for a [AdoCbi·bulky-base]‡ species exists at present); and (ix) the need for bioinorganic chemists to proceed with caution when beginning from hypotheses advanced by protein crystal structures of metalloenzyme active sites—a lesson that other examples,[88a] as well as textbooks in the area,[88b] also emphasize. Artifacts in early B_{12}-protein X-ray structures, cited in the Introduction, are the original source of the misleading "long Co−N(axial) bond" hypothesis. Caveat emptor! Noteworthy, however, is that these bioinorganic chemical precedent studies were able to probe the long Co−N(axial) bond hypothesis—and, in the end, to provide evidence against this hypothesis.

One of the most important take-home messages in our opinion is that (x) *only* by the use of a proper scientific method[63] both before[1] and herein, involving conceiving of all possible alternative hypotheses[63] (alternative mechanisms in this case), followed by attempts at their disproof, were we—and only we—able to reach an explanation supported by all the data. We find it heartening that what now at least appears to be the correct answer was obtained in a relatively short period of time, *despite* the 6 misleading pieces of literature data and experimental artifacts cited earlier which proved impossible for either our group[1] or Marzilli's[23] to navigate 100% correctly before. The importance of a proper scientific method is further emphasized by looking at the assertions of others that attributed "...the reported observations to impurities in the two (i.e., 2-Me-pyridine and 2,6-Me_2-pyridine) ligands".[23] Two errors here are that those authors did not consider any alternative hypotheses besides their "impurity hypothesis"[23] and they (over)extended their conclusions by strongly implying that impurities in the exogenous bulky bases were the source of the Co−C heterolysis versus homolysis kinetic and product results, a mistake of logic since those authors did not perform any kinetic studies.[23] (The need to do *both* kinetics and spectroscopy to correctly ascertain mechanism is again apparent.) Strongly supported by this work, then, is the case for the scientific method recommended by Platt 40 years ago[63] consisting of (a) a consideration of all possible alternative hypotheses, and (b) an emphasis on disproof ("for exploring the unknown, there is no faster method").[63]

Experimental Section

Materials. Each of the following was used as received: adenosylcobalamin (AdoCbl; Sigma, 98%), argon (General Air), ethylene glycol (Aldrich, 99.8% anhydrous), $Ce(NO_3)_3·6H_2O$ (Aldrich, 99.99% fresh bottle), sodium hydroxide (Fisher Scientific, ACS grade), ammonium hydroxide (Mallinckrodt AR-ACS grade, 29.3%), methanol (Fisher Scientific, HPLC grade), sodium chloride (Fisher Scientific, ACS grade), silver nitrate (Aldrich ACS reagent grade), phosphoric acid (Mallinckrodt AR-ACS grade), potassium phosphate dibasic (Mallinckrodt AR-ACS grade), sodium tetrafluoroborate (Aldrich), reagent alcohol (Fisher Scientific; anhydrous, ∼90% ethanol, ∼5% methanol, ∼5% isopropyl alcohol), sodium acetate (Mallinckrodt AR-ACS grade), acetic acid (Mallinckrodt AR-ACS grade), benzene (Aldrich 99.8% anhydrous), Proton Sponge (Aldrich), adenine (Sigma), and 5′-deoxy-adenosine (Aldrich). TEMPO (Aldrich, 99%) was sublimed before use. Distilled water was filtered through a Barnstead nanopure filtration system. The affinity distillation reagent, $Co(C_2(DO)(DOH))Br_2$, was synthesized by literature methods[89] with >90% purity as judged by ^{1}H NMR.

Adenosylcobinamide. $AdoCbi^+BF_4^-$ was synthesized according to a slightly modified literature synthesis reported by Hay.[40] The details of this updated synthesis are reported in the Supporting Information for the interested reader. The product was characterized by UV−vis spectroscopy, HPLC, and ^{1}H NMR. Purity was determined to be ∼96% by HPLC (isocratic 70% 0.9 M acetate buffer pH 4.5, 30% CH_3CN, at 5 mL/min) and ∼90% by ^{1}H NMR. The overall yield was 116.8 mg (37%, literature yield of the OH^- salt and using a phenol extraction step instead of the desalting column is 50%).[40]

Added Bases: Source and Purifications. Sterically hindered pyridines were both used as received or after being purified by 2 different methods. Method 1 was distillation on a spinning-band-column microdistillation still (ACE model 9595 ∼ 0.2 inches/theoretical plate with the ability to separate compounds with boiling points within 5−10 °C). Method 2 was affinity distillation, analogous to what was used by in the literature:[23] the base was stirred with the affinity distillation reagent, $Co(C_2(DO)(DOH))Br_2$, for 40 min and then distilled under vacuum. This procedure was repeated 3 times. After distillation by either method, the bases were stored in a −4 °C freezer and used within 8 h.

2-Methyl Pyridine (Picoline, Aldrich 98%). Method 1. 2-Me-py was distilled using a spinning-band-column at room temperature under reduced pressure. The collection flask was cooled in a dry ice/isopropyl alcohol bath.

Method 2. 2-Me-py (5.0 mL)was stirred with 290 mg of $Co(C_2(DO)(DOH))Br_2$ (∼0.1 M) for 40 min, and distilled at room temperature under reduced pressure. This process was repeated 3 times. It is of note that the affinity reagent makes a dark green solution when dissolved in the 2-Me-py. This solution turned brown when stirred for 40 min in the first distillation cycle, analogous to the color change from green to red observed by Marzilli and coworkers during their distillation of 2-Me-py.[23]

2,6-Dimethyl Pyridine (2,6-Lutadine, Aldrich 99+% Redistilled). Method 1. 1,2-Me_2-py was distilled at using a spinning-band-column at 35−40 °C under reduced pressure. The collection flask was cooled in a dry ice/isopropyl alcohol bath.

(88) (a) Several early X-ray structures of hemoprotein CO adducts which claimed an Fe−C−O angle of 120−140° were the initial source of the bent Fe−C−O controversy: Collman, J. P.; Brauman, J. I.; Halbert, T. R.; Suslick, K. S. *Proc. Natl. Acad. Sci.* **1976**, *73*, 3333. Later structural work showed that the error bars on these angles is ≥±25° as discussed in: Spiro, T. G.; Kozlowski, P. W. *Acc. Chem. Res.* **2001**, *34*, 137. (b) See p 120 of: Lippard, S. J.; Berg, J. M. *Principles of Bioinorganic Chemistry*, University Science Books: Mill Valley CA, 1994. In this text, the perhaps obvious yet noteworthy point is made that "One lesson for the student of bioinorganic chemistry is that protein crystal structures should not be considered as credible as small-molecule X-ray structures".

(89) Finke, R. G.; Smith, B. L.; McKenna, W. A.; Christian, P. A. *Inorg. Chem.* **1981**, *20*, 687.

1,2-Dimethyl Imidazole (Aldrich 98%). The bulky base was recrystallized by dissolving it in benzene, ~5 g/1 mL in a 25 mL scintillation vial with gentle heating in a 50 °C H_2O bath, and then putting the vial in a −4 °C freezer for ~2 h. Crystallization was aided with scratching of the glass vial, or by seeding with a crystal. After crystallization, the solution was filtered immediately through a cooled medium glass frit. If any residual color remained from the yellow-brown commercial 1,2-dimethyl imidazole, the process was repeated until the recrystallized solid was white. The crystals were then dried under vacuum at room temperature for 3 h. The white crystals showed a melting point of 35−37 °C where the commercial 1,2-Me_2-Im showed a melting point of 31−37 °C (Aldrich's reported melting point, 37−39 °C).

The nonsterically hindered bases pyridine (Aldrich, 99.8%, anhydrous) and 1-methyl imidazole (Aldrich, 99+% redistilled) were used as received or following purification using a spinning-band-column under reduced pressure. Pyridine was distilled at room temperature. In order to distill N-Me-Im at 90 °C, the spinning-band column had to be used in a nonspinning mode for sufficient N-Me-Im to be collected.

Instrumentation and Equipment. UV−vis absorption spectra (±1 nm) were recorded on a Hewlett-Packard model 8452A UV−vis diode array spectrophotometer equipped with a thermoelectric Hewlett-Packard 89090A Peltier cell block temperature controller operating at 25.0 ± 0.1 °C. HPLC was done with an HP 1050 HPLC with a 300 mm × 4.6 mm Alltech C-18 reverse phase column. ^{1}H NMR spectra were recorded on an Inova-300 spectrometer operating at room temperature and were referenced internally to the residual $CHCl_3$ peak ($CDCl_3$). GC-MS was performed on an Agilent 5973N/6890 with a 30 m Agilent HP-5 column. Centrifugation was done with an ICE model PR-2 centrifuge fitted with a 4-place rotor. A Corning 125 pH meter using a corning GP-combo electrode was used for pH measurements. Melting points were performed on a Mel-Temp II with a heating rate of 1 °C/min over the range of melting. All linear regressions were performed on a Power Macintosh 5400/120 using Kaleidagraph 3.51 and checked with Microsoft Excel 98.

All thermolysis (vide infra) samples were prepared in a Vacuum Atmospheres inert atmosphere drybox with an O_2 level <2 ppm, as monitored by a Vacuum Atmospheres model AO 316-C oxygen analyzer. Adenosylcobalamins and adeonsylcobinamides are photolabile; hence, all sample preparations done inside the drybox were shielded from light with aluminum foil. The thermolyses were carried out in a dark room with exposure only to photographic quality red light.

The thermolyses of AdoCbl and 8-MeOAdo were carried out in Schlenk cuvettes[9] prepared by fusing PTFE needle valves to 1 cm path length cuvettes onto 1 mL glass vials. The cuvettes' ability to maintain an oxygen free environment was tested with Co(II)Cbl$^\bullet$ (made from the photolysis of a drybox-prepared AdoCbl solution in ethylene glycol). No detectable decomposition was observed over the time scale used in our thermolyses (~1 week) in cuvettes taken outside the drybox.

Thermolysis temperatures were maintained by immersing the cuvettes in a 2 L oil bath equipped with a wound-wire heating element attached to a Barnant temperature controller and equipped with a magnetic stir bar. The temperature was verified (± 0.2 °C) using a mercury thermometer scaled to the appropriate temperature range.

Adenosylcobinamide$^+$ Plus Exogenous Bases Thermolyses and Analysis Procedure. First, ~3.3 mg (2.5×10^{-3} mmol) of AdoCbi$^+$$BF_4^-$ was weighed into a foil wrapped vial and taken into the drybox. Inside the drybox, ~31.2 mg (~2×10^{-4} mol) of solid TEMPO radical trap was added to the vial, and then, 10.0 mL of ethylene glycol (degassed 3 times by freeze/evacuate/refill with argon/thaw cycles) was added with a syringe, giving a ~2.5×10^{-4} M AdoCbi$^+$$BF_4^-$ (and ~2×10^{-2} M TEMPO) solution. Next, 1.5 mL aliquots of this solution were transferred into foil-covered Schlenk cuvettes, and 1.5 mL of the appropriate concentration solution of exogenous base (or sodium glycolate or Proton Sponge glycolate) in degassed ethylene glycol solution was added, resulting in a solution that was ~1.2×10^{-4} M AdoCbi$^+$$BF_4^-$ and the appropriate concentration (0.15−0.45 M) in exogenous base. The cuvettes were brought out of the drybox and into the darkroom for thermolysis at 110 °C. The UV−vis spectrum of each cell was followed by periodically removing it from the oil bath, taking a UV−vis spectrum, and then replacing in the oil bath. The results show, as expected,[6] conversion to Co(II)Cbi$^+$. Thermolyses were carried out at 110 °C for ≥20 h for the added N-Me-Im or 1,2-Me_2-Im, and ≥156 h for the added pyridine, 2-Me-py, or 2,6-Me_2-py. (This corresponds to ~4 half-lives for the imidazole systems and ≥7 half-lives for pyridine systems.) After thermolysis, samples were analyzed by HPLC (see Instrumentation and Equipment subsection) using the following elution program: flow 1 mL/min, isocratic 95% H_2O/5% CH_3CN for 20 min; ramp to 70% H_2O/30% CH_3CN over 10 min, isocratic 70% H_2O/30% CH_3CN for 30 min, ramp to 10% H_2O/90% CH_3CN over 10 min, isocratic 10% H_2O/90% CH_3CN for 10 min, return ramp to 95% H_2O /5% CH_3CN over 10 min. Using this method, the 3 homolysis and 1 heterolysis nucleoside products elute within 43 min in the order adenine, 8−5′-anhydrocyclicadenosine, 5′-deoxyadenosine, and Ado-TEMPO. Concentrations of these products in the reaction solutions were calculated by comparison to standard solutions,[40] and % heterolysis was calculated as [adenine]/[Ado$^\bullet$ derived products] × 100%,[5] or by [adenine]/[initial Co(II)Cbi$^+$] × 100%, which were within experimental error of each other. A control experiment was performed without the use of TEMPO, giving larger 8−5′-anhydrocyclicadenosine and 5′-deoxyadenosine homolysis peaks, but the same % heterolysis within experimental error.

Control Experiment Thermolyzing AdoCbl with Added Imidazoles. As a control experiment, AdoCbl was thermolyzed with imidazoles using the same experimental and analysis procedures that were used for AdoCbi$^+$ thermolysis reactions. Because AdoCbl thermolyzes faster than AdoCbi$^+$, a shorter thermolysis time of ~12 h was used.

Co(II)Cobinamide Titration with Axial Bases. Co(II)Cbi$^+$$BF_4^-$ titrations with axial bases were performed in a manner similar to the literature procedure, but with caution taken to avoid possible exposure to oxygen which it is believed to have caused an error in the original thesis[2] and resultant publication.[1] First, a solution of AdoCbi$^+$$BF_4^-$ in ethylene glycol (degassed by 3 freeze/pump/thaw under argon cycles) was prepared inside a drybox, sealed in a Schlenk cuvette, and placed 30 cm in front of a General Electric 275 W "Sun Lamp" for 20 h. The UV−vis spectrum was monitored and did not change with further exposure to the "Sun Lamp", indicating complete conversion to Co(II)Cbi$^+$$BF_4^-$. The cells were taken back into the drybox, and neat bases were added with a syringe (in the case of 1,2-Me_2-Im which is a solid at room temperature, a 7.25 M solution in ethylene glycol was used). The cells were taken back outside of the drybox, and the UV−vis spectra were taken. This process was repeated until the solutions were ~2 M in base concentration.

The titration results are available in the Supporting Information, with purified N-Me-Im (Figure S1); purified and commercial 1,2-Me_2-Im (Figure S1 and Figure S3); purified pyridine (Figure S4); purified and commercial 2,6-Me_2-py (Figure S5 and S6); and

commercial 2-Me-py (Figure S7). The titrations show that the unhindered bases, N-Me-Im and pyridine, bind with similar spectra, but hindered bases show no detectable binding, even up to 2 M base (∼20 000 equiv vs $AdoCbi^+$). As noted in the Introduction, these results correct the experimental work in an earlier thesis,[2] results which now agree with the published results of Marzilli and co-workers.[23] The incorrect Figures 5 (ref 1) and 5′ (ref 3) are hereby replaced by the correct Figure S6 in the Supporting Information of the present paper.

Attempted Check for Impurities in Bases by GC-MS. In an attempt to directly detect impurities in the bases, the solutions of axial bases (including the same bottle of 2,6-Me_2-py which was used previously in the erroneous Co(II)$Cbi^+BF_4^-$ titration results)[3] were analyzed by GC-MS (see Instrumentation and Equipment subsection). A 10 μL headspace injection was performed under the temperature program: 50−290 °C at 20 °C/min; source 180 °C; injector 280 °C. No significant impurities were detected,[90] indicating that any possible impurity either (i) is not present in quantities above our detection limit (≥0.5%); (ii) is not obtained by the sampling method employed; (iii) has the same retention time as the base being tested under the conditions employed; (iv) is retained by the GC column; or (v) is not detectable by the MS detector. In any case, the method did not prove useful and was not pursued further.

(90) There is an apparent ca. 0.4% impurity in the older bottle of 2,6-Me_2-py by GC-MS. The retention time of the "impurity" was very close to that of 2,6-Me_2-py obscuring its identification or even its unequivocal existence. The retention time of the "impurity" under these conditions was shown not to match either 2,6-Me_2-py or 2,3-Me_2-py.

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Supporting Information Available: Table S1, a comparison of the percent heterolysis of $AdoCbi^+$ thermolyses with various purities of added imidazoles; Table S2, a comparison of the percent heterolysis of $AdoCbi^+$ thermolyses with various purities of added sterically hindered pyridines; Table S3, a comparison of the percent heterolysis and rate of AdoCbl thermolyses with various purities of imidazoles; Figures S1−S7, titrations of Co(II)$Cbi^+BF_4^-$ with commercial or purified bases; Figure S8, a plot of % heterolysis of the Co−C bond vs the calculated concentration of [Proton Sponge-H^+glycolate$^-$]; Figure S9, a plot of % heterolysis of the Co−C bond vs the concentration of added [Na^+Glycolate$^-$]; section S-1, the derivation of the rate law accompanying Scheme 1; section S-2, adenosylcobinamide synthesis procedure. This material is available free of charge via the Internet at http://pubs.acs.org. This material is also available in the Ph.D. dissertation of K.M.D. (Colorado State University, spring 2003).

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